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THE CYTOPLASMIC MEMBRANE C_4-DICARBOXYLATE TRANSPORT COMPONENTS IN *ESCHERICHIA COLI* K12: IDENTIFICATION AND PURIFICATION STUDIES

by

Katherine E. A. Walker

Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
June 1986

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ISBN 0-315-33049-X

THE UNIVERSITY OF WESTERN ONTARIO
FACULTY OF GRADUATE STUDIES

CERTIFICATE OF EXAMINATION

Chief Advisor	Examining Board

Advisory Committee

The thesis by
Katherine E. A. Walker

entitled
The Cytoplasmic Membrane C_4-Dicarboxylate
Transport Components in Escherichia coli K12:
Identification and Purification Studies

is accepted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Date 24 July 1986

Chairman of Examining Board

ABSTRACT

The C_4-dicarboxylate transport system of Escherichia coli K12 is responsible for the active uptake of succinate, fumarate and malate. Genetic and biochemical evidence has indicated that at least three distinct components appear to be involved in this transport process. Two of these transport components are the cytoplasmic membrane-bound succinate binding proteins, SBP1 and SBP2, which are coded for by the dctB (16 minutes) and dctA (78 minutes) genes respectively. Early work on these two transport proteins involved their isolation via aspartate-Sepharose chromatography from detergent-solubilized cytoplasmic membrane vesicles. The impurity of the isolated dicarboxylate transport proteins necessitated the development of techniques for their identification and purification. Three affinity techniques were developed that utilized the recognition of the transport components' substrate binding sites in order to identify and enrich the proteins prior to further purification steps. These techniques included aspartate-Sepharose affinity chromatography, immunoblotting of cytoplasmic membrane proteins with succinate-specific antiidiotypic antibodies and photoaffinity labelling of proteins in isolated membranes and whole cells. All three techniques involved a comparison of a wild-type strain (CBT43) with various dicarboxylate transport-mutant strains. The original aspartate-Sepharose protocol was optimized in terms of the isolation, solubilization and affinity chromatography of the cytoplasmic membrane proteins. The results of [^{14}C]-succinate binding studies with affinity-purified proteins from strain CBT43 and affinity chromatography of transport-mutant cytoplasmic membrane proteins suggested that a 53K protein was a good

candidate for the SBP2 transport component. The immunoblotting studies employed antiidiotypic antibodies raised against the antigen binding site of succinate-specific IgG molecules and again implicated a 53K protein in the transport process. Finally, photoaffinity labelling of whole cells with N-(4-azido-2-nitrophenyl)aspartic acid suggested that a 47K protein might be involved in dicarboxylate transport. Further studies with dctA and dctB transformants are necessary to confirm the identity of the 53K protein as a succinate transport component and to establish the relationship, if any, between the 47K and 53K proteins.

ACKNOWLEDGEMENTS

I wish to acknowledge my advisor, Dr. Ted Lo, and all of the members of his lab for their help and support over the years. In particular, I'd like to thank Doug Kuntz and Tony D'Amore for many useful discussions and advice, and especially for their friendship and laughter. Finally, I want to thank my husband, David, and the rest of my family and friends for their unfailing support and confidence in me. Thanks, Dave, for many hours of waiting patiently in the lab while I finished "just fifteen more minutes of work"!

The Medical Research Council of Canada is also gratefully acknowledged for their financial support.

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NOMENCLATURE

AA - aspartyl azide
ATP - adenosine 5'-triphosphate
BSA - Bovine serum albumin
cbt - carboxylate transport
CCCP - carbonyl cyanide m-chlorophenylhydrazone
CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC - critical micellar concentration
CPM - counts per minute
DBP - dicarboxylate binding protein
dct - dicarboxylate transport
DEAE - diethylaminoethyl
DFS - difluorosuccinate
DPM - decays per minute
EDCD - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA - ethylenediaminetetraacetic acid
IgG - immunoglobulin G
IPTG - isopropylthiogalactoside
K_a - association constant
K_d - dissociation constant
K_i - inhibition constant
K_m - Michaelis-Menten constant
mCi - milliCuries
PBS - phosphate-buffered saline
P6DG - polyacrylamide 6000 molecular weight desalting gel
RAA - rabbit anti-aspartate
RARAA - rabbit anti-rabbit anti-aspartate
SBP - succinate binding protein
SDS - sodium dodecyl sulphate
TBS - Tris-buffered saline
TCA - trichloroacetic acid
TSF - tosylsulphonyl fluoride
uCi - microCuries

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CHAPTER 1

INTRODUCTION

The Structure and Function of Biological Membranes - The study of cellular substrate transport in living organisms involves, in part, the study of the structure and function of biological membranes. Biological membranes in both procaryotes and eucaryotes are composed mainly of lipid and protein molecules that form a selective permeability barrier which controls the cytoplasmic composition of the living cell (1,2). Membranes may also contain a small amount of carbohydrate in the form of glycolipids and glycoproteins. The proportion of protein to lipid varies in different membranes, ranging from 1:4 in the myelin membrane of the mammalian peripheral nervous system to 4:1 in the inner mitochondrial membrane. Phospholipids constitute the major type of membrane lipid and membranes differ in the types of phospholipids that they contain. In general, the proteins are responsible for the various membrane functions while the lipids provide the structural framework in which the proteins can function (1). There is also evidence that the activity of some membrane proteins is dependent upon or regulated by an association with specific phospholipids (3). Included among the types of functions mediated by membrane proteins are communication between the cell and its environment by interaction of substances with specific receptors, energy conversion through processes such as respiration and photosynthesis, biosynthesis of cell constituents by various membrane-bound enzymes and maintenance of the intracellular environment by the selective transport of specific substrates and ions (1).

The study of biological membranes was revolutionized a decade

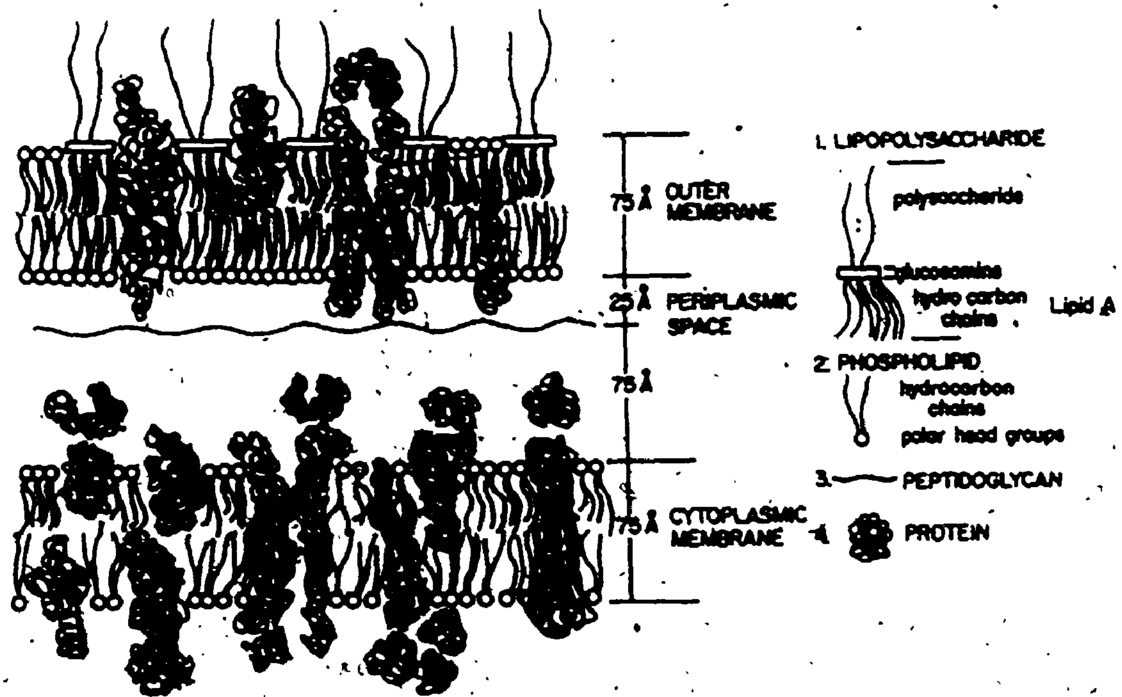
ago by the Fluid Mosaic Model proposed by Singer and Nicolson to explain the biological and physical properties of membranes (4). They envisioned the membrane as a lipid bilayer that has various proteins associated with or embedded in it. The arrangement of the amphipathic lipids in this bilayer is such that their hydrophobic regions interact with each other in the membrane interior while their hydrophilic head groups are exposed to the aqueous environment on either side of the membrane. The fluidity of this lipid bilayer is controlled by the types and amounts of its constituent lipids. This fluidity, in turn, allows for the lateral mobility and/or conformational changes of the associated membrane proteins which are necessary for their many functions (1). Membrane proteins can be divided into two broad groups that differ in their degree of association with the lipid bilayer. Integral or intrinsic membrane proteins are embedded in the membrane via their hydrophobic domains and they either span the entire thickness of the membrane or they are partially embedded in it. These proteins can only be released from the membrane by disrupting the hydrophobic interactions of the lipid bilayer with organic solvents, chaotropic agents or detergents (5). In contrast, peripheral or extrinsic membrane proteins are not embedded in the membrane matrix. The association of most peripheral proteins with the membrane appears to involve electrostatic and hydrogen bonding with the exposed hydrophilic regions of the integral membrane proteins and perhaps glycolipids (1,5). These proteins can be released from the membrane by a change in the ionic strength or pH of their environment.

<u>The Gram-Negative Bacterial Cell Envelope</u> - The permeability barrier of gram-negative bacteria, such as <u>Escherichia coli</u>, is such

more complex than that of other procaryotic and eucaryotic organisms. The cell envelope of these organisms consists of three distinct layers, an outer membrane, a peptidoglycan layer and an inner cytoplasmic membrane, all of which are visible in electron microscopic studies (6). The structure of this cell envelope is shown schematically in Figure 1.

(i) The Outer Membrane and Peptidoglycan Layer - The outer membrane is composed chiefly of lipopolysaccharides, phospholipids and large amounts of a few species of proteins. The lipopolysaccharides are found exclusively in the outer leaflet of this membrane and they comprise the somatic O antigens responsible for the endotoxic activity of E. coli (7). The two major proteins in the outer membrane are the porins (OmpF and OmpC) and the TuII* or OmpA protein (8,9). The former proteins are arranged as trimers that span the membrane and act as channels for the transport of small molecular weight, hydrophilic substances. The TuII* protein is a receptor for a number of bacteriophages. Two of the other proteins in the outer membrane include the major lipoprotein, a structural protein, and the LamB protein which serves as a component of the maltose transport system and as the lambda bacteriophage receptor (10, 11). This latter protein is induced by growth of the bacteria on maltose. Directly underneath the outer membrane and covalently attached to some of its proteins is the peptidoglycan layer. This layer, which is composed of repeating units of N-acetylglucosamine and N-acetylmuramic acid cross-linked by peptides, maintains the shape of the bacterium and plays a role, along with the cytoplasmic membrane, in the osmotic regulation of the cell (7). It also acts as a barrier to substances with molecular weights above 1000 Daltons. The compartment of the cell in which this peptidoglycan layer resides is the periplasmic

Figure I-1. The gram-negative bacterial cell envelope. The macromolecular constituents (lipopolysaccharide, phospholipid, peptidoglycan and protein) of the outer membrane, periplasmic space and cytoplasmic membrane are indicated in this diagram. This figure was reproduced with permission from T. Lo (59).


75 Å OUTER MEMBRANE
25 Å PERIPLASMIC SPACE
75 Å
75 Å CYTOPLASMIC MEMBRANE
1. LIPOPOLYSACCHARIDE
polysaccharide
hydro carbon chains
Lipid A
2. PHOSPHOLIPID
hydrocarbon chains
polar head groups
3. PEPTIDOGLYCAN
4. PROTEIN

space. The periplasm contains many soluble, hydrolytic enzymes and membrane-derived oligosaccharides, as well as the binding protein components of various transport systems. The overlying outer membrane serves to keep these periplasmic constituents within the cell (7).

(ii)The Cytoplasmic Membrane - Finally, the innermost layer of the gram-negative cell envelope is the cytoplasmic membrane. This membrane is analogous to the phospholipid bilayer plasma membrane of eucaryotes, although it contains considerably more protein (70 to 80% versus 50%) (6). This membrane acts as the true selective permeability barrier of the bacterial cell (6,7). Since there are no intracellular membranes in bacteria, the cytoplasmic membrane contains the cytochromes and enzymes involved in respiration and oxidative phosphorylation, as well as many integral membrane "permeases" or transport proteins, enzymes and chemotaxis receptors. These cytoplasmic membrane proteins account for 10 to 20% of the total cellular protein (5). The cytoplasmic and outer membranes are associated with each other at a number of adhesion zones known as Bayer's junctions that are thought to be important in membrane biogenesis (12). In order to study their respective structures and functions, these two membranes must be separated from each other. Techniques for their separation include spheroplast formation, in which the outer membrane and peptidoglycan layer are removed from the osmotically-protected cytoplasmic membrane by a Tris-EDTA-lysozyme treatment, selective detergent solubilization of the cytoplasmic membrane from a total envelope preparation and sucrose density centrifugation of the crude envelope fraction (13,14). The procedures differ in the increasing efficiency of outer and cytoplasmic membrane separation in the above order of presentation.

Types of Membrane Transport - The selective transport of substrates into gram-negative bacteria would be expected to require mechanisms for translocation across all three layers of their cell envelope. Some substances are able to enter the cell by passive diffusion across the membranes and periplasmic space in response to their concentration gradients. However, the transport of the majority of metabolites is mediated by specific soluble and/or membrane-bound protein carriers, which combine reversibly with their substrates (7). The three types of carrier-mediated transport processes include facilitated diffusion, active transport and group translocation (2,15). Facilitated diffusion involves the thermodynamic movement of a substrate down its concentration gradient and, as such, it does not require additional metabolic energy. In contrast, both active transport and group translocation require metabolic energy since they result in the intracellular accumulation of a substrate against its electrochemical or osmotic concentration gradient. Group translocation further involves a chemical modification of the substrate during transport. An example of this type of transport system is the phosphoenolpyruvate-dependent:sugar phosphotransferase system of E. coli. This system involves three soluble proteins and one membrane-bound protein that participate in the translocation and concomitant phosphorylation of a number of different sugars across the cytoplasmic membrane (16).

Active Transport Systems in E. coli - Active transport processes are responsible for the accumulation of many sugars, amino acids and ions in E. coli (7). In general, the active transport systems can be divided into two main groups, namely, periplasmic binding protein-dependent systems and systems which require only cytoplasmic membrane-

bound transport components (17,18). The binding protein-dependent systems are sensitive to cold, osmotic shock procedures which disrupt the integrity of the outer membrane and release the periplasmic binding proteins from the cell. In contrast, the binding protein-independent systems are osmotic shock-resistant and their activity can be demonstrated in energized cytoplasmic membrane vesicles isolated from spheroplasts of E. coli (15,19). The method of energy coupling to the transport process also appears to be different in both types of systems. The binding protein-dependent systems utilize ATP or another phospho-compound to drive transport while binding protein-independent systems rely on the transmembrane protonmotive force generated by the cytoplasmic membrane respiratory chain (18). The exact nature of the energy coupling processes is not entirely clear for either type of transport system. In the case of the three transport systems for lactose, proline and melibiose that are dependent upon the membrane potential for activity, transport of their substrates seems to involve the symport of hydrogen ions or another cation (sodium, lithium) in response to the transmembrane, electrochemical proton gradient formed during respiration (20,21,22). This type of energy coupling is analogous to the symport of sodium ions with sugars and amino acids observed in mammalian cells (1).

(i) Binding Protein-Dependent Transport Systems - Some examples of binding protein-dependent, active transport systems in gram-negative bacteria are the systems specific for maltose, glutamate, leucine-isoleucine-valine, sulphate, inorganic phosphate, histidine, arabinose, galactose and D-ribose (17). The substrate-binding site of the periplasmic binding proteins is responsible for the specificity of these transport systems and the binding proteins are thought to interact with

one or more specific membrane-bound components for delivery of the substrate across the cytoplasmic membrane (23). Three of these systems that have been most extensively studied are the histidine and maltose transport systems of E. coli and the oligopeptide transport system of Salmonella typhimurium. All three of these systems appear to require three cytoplasmic membrane transport components in addition to a periplasmic binding protein (18,24). In addition, the maltose transport system also includes the outer membrane LamB protein, which has been shown to interact specifically with the periplasmic maltose binding protein (25). It has recently been shown by genetic and biochemical studies that extensive amino acid sequence homology exists between the cytoplasmic membrane-bound HisP, MalK and OppD proteins of the above three transport systems. This homology includes a binding site for ATP or another nucleotide and therefore may shed some light on the mechanism of energy coupling in these systems (18,25). These and other binding protein-dependent systems have been genetically characterized to varying degrees and many of the periplasmic binding proteins have been purified in an active form and sequenced. These include the binding proteins specific for maltose, phosphate, galactose, arabinose, D-ribose and leucine-isoleucine-valine (27-32). Purified periplasmic binding proteins have also been used in some systems to reconstitute transport in spheroplasts, membrane vesicles and permeabilized whole cells of transport mutant strains (33). In addition, some of these binding proteins have been crystallized and have been shown by X-ray crystallographic analyses to be remarkably similar in three-dimensional structure and substrate-binding conformations (29,30,32).

(ii) Binding Protein-Independent Transport Systems - In contrast to

the periplasmic binding proteins, much less is known about the membrane-bound transport components of the binding protein-independent active transport systems. These transport systems do not rely on a soluble binding protein for activity; rather, their membrane-bound components contain specific substrate binding sites and they are able to actively transport their substrates in isolated cytoplasmic membrane vesicles. Substrates that are transported by these membrane carriers include lactose, melibiose, proline, glycine, aspartic acid and many other amino acids (15,17,19). As mentioned earlier, the energy source for these transport systems appears to be the protonmotive force generated across the cytoplasmic membrane by the electron transport chain. Accumulation of substrate in the cytoplasm seems to involve cation-substrate symport in response to this protonmotive force, which has been demonstrated in the lactose, proline and melibiose systems (20-22). The active, uphill transport of substrate is coupled to the passive, downhill flow of protons or another cation across the membrane (1). It has generally been accepted that translocation of the substrates across the cytoplasmic membrane involves reversible, conformational changes in the carrier proteins that alternately make the substrate accessible to the periplasmic and cytoplasmic sides of the membrane. The binding of substrate to the carrier may also involve the induced-fit mechanism postulated for enzyme-substrate binding, in which the substrate-binding site is not fully formed until the substrate binds to the protein (34).

The Lactose Permease of E. coli - The lactose transport system of E. coli has been intensively investigated for thirty years and is by far the best-studied of all of the bacterial cytoplasmic membrane active transport systems. Even so, the membrane carrier has only recently been

purified and reconstituted in an active form in proteoliposomes. In addition, amino acid sequence data and monoclonal antibody studies are now being used to reveal its three-dimensional configuration in the cytoplasmic membrane in an effort to elucidate the molecular events in lactose transport. The techniques used to identify, purify and reconstitute the lactose carrier may well be applicable to other cytoplasmic membrane transporters in E. coli and other organisms. These techniques have successfully addressed the problems of membrane carrier levels and activity and will now be focused on in some detail.

The lactose permease of E. coli is a 46,500 Dalton integral membrane polypeptide that is encoded for by the lac y gene of the lactose operon (35). Its synthesis is induced by β-galactosides, such as isopropylthiogalactoside (IPTG), and it is responsible for the active transport of a wide number of α and β galactosides. The properties of the lactose transport system have been studied in detail by many investigators in both whole cells and cytoplasmic membrane vesicles since its discovery in 1956 (35). Studies with membrane vesicles and various radioactive or fluorescent substrate analogues indicated that both binding and transport of substrate by the lac carrier occurred with a ratio of one substrate molecule per transporter (36,37). Active transport of radioactive lactose with an apparent K_m of 0.2 mM was demonstrated in membrane vesicles that were "energized" by an electron-donating compound that interacted with the respiratory chain to impose a proton gradient across the membrane (38). In addition, appreciable binding of a non-transportable substrate analogue to the membrane-bound carrier was also only observed if the membrane vesicles were first incubated with an electron donor (36). Therefore, both the initial

binding of substrate to the carrier and the transport of substrate across the membrane involved the protonmotive force, suggesting that energy-mediated, conformational changes in the protein carrier were necessary for both processes (37). The ratio of protons bound or translocated along with the substrate by the lac permease was also 1:1 with respect to the carrier (38). Finally, membrane vesicles were covalently labelled with a radioactive photoaffinity label specific for the lactose permease and SDS polyacrylamide gel electrophoresis was used to identify the transport protein as a single polypeptide of 30K Daltons molecular weight (35).

The next step in the study of the lactose carrier was the purification of this 30K Dalton polypeptide and the subsequent demonstration that it alone was responsible for the binding and translocation of lactose across the cytoplasmic membrane. Amplification of the levels of the lac carrier protein in E. coli by a plasmid containing the lac y gene aided in the identification and purification of the transporter (35). The lac permease accounted for 10 to 15% of the total cytoplasmic membrane protein in the plasmid-bearing strain, compared to only 0.9% in the haploid, wild-type strain (35,39). The amino acid sequence of the purified protein matched that predicted by the DNA sequence of the lac y gene, although a discrepancy was evident in the predicted and observed molecular weights (46,500 versus 30,000 Daltons). Moreover, a 30K polypeptide was the major protein synthesized in a cell-free translations of the lac y plasmid (35). It was found that the activity of the purified lactose carrier was sensitive to the detergent used for its membrane extraction. In particular, extraction of affinity-labelled lac carrier with Triton X-100 completely abolished its binding abilities

(40). The most successful solubilization of active lac carrier protein was accomplished with the non-ionic detergent, octylglucoside. Lactose binding and transport activity was demonstrated by reconstituting either solubilized total membrane extracts or purified lac permease into liposomes prepared from _E. coli_ phospholipids (41,42). Purification of the lac permease from the octylglucoside-solubilized membrane extract was accomplished with DEAE-Sepharose chromatography (42). Reconstitution of the carrier protein into a lipid bilayer environment that mimicked its _in vivo_ environment allowed for the observation of both lactose counterflow in proteoliposomes preloaded with lactose and active transport of lactose into normal proteoliposomes. The kinetic characteristics of the reconstituted lactose transport system were very similar to that of the _in vivo_ system and the transport mechanism also appeared to involve lactose-proton symport (20,43).

Studies are now being carried out with membrane vesicles and antibodies raised against the purified protein in order to determine the three-dimensional structure of the lac carrier in the cytoplasmic membrane (44,45). Other studies have indicated that the purified protein has about 85% helical content, consistent with it being a membrane protein, and that its functional unit in the membrane appears to be a dimer (46,47). These studies should yield more information about the molecular mechanisms of lactose transport. The melibiose and proline transport systems of _E. coli_ have also recently been reconstituted in an active form in proteoliposomes using methods similar to those employed for the lac permease (22,48).

The C_4 Dicarboxylate Transport System in E. coli - The dicarboxylic acid transport system of _E. coli_, in particular its

cytoplasmic membrane transport components, is the focus of study in this thesis. This system is responsible for the high-affinity, active transport of the dicarboxylic acids, succinate, fumarate and malate, and the low-affinity transport of aspartate in E. coli. The apparent K_m for whole cell transport of these various substrates is 14 uM for succinate, 20 to 30 uM for fumarate and malate and 30 uM for aspartate (49,50). Like the lactose transport system, the dicarboxylate system involves membrane-bound components that actively transport the substrates in energized cytoplasmic membrane vesicles (49,51). Similarily, the mechanism of this active transport process appears to involve the symport of two protons with each substrate molecule (52). However, unlike the other membrane-bound systems in gram-negative bacteria, the dicarboxylate transport system is also osmotic shock sensitive and therefore appears to involve a periplasmic binding protein as well (49).

(i)General Properties - Early studies on this transport system focused on describing it biochemically and genetically in both whole cells and cytoplasmic membrane vesicles. These studies showed that only one transport system was involved in the uptake of the dicarboxylic acids in E. coli and that this system was inducible by succinate, the preferred substrate, and repressible by glucose (49). By using a strain that was defective in the metabolism of succinate, it was possible to show a concentrative, active uptake of this substrate in both whole cells and membrane vesicles (49,51). The energy source for the latter process was supplied by either a physiological (D-lactate) or artificial (reduced phenazine methosulphate) electron doner that feeds electrons into the respiratory chain (51). Compounds that either interrupted the flow of electrons through the respiratory chain or

abolished the proton gradient established during respiration were able to inhibit succinate transport in both whole cells and membrane vesicles (49,51,53). However, the results were complicated by the fact that compounds that inhibit oxidative phosphorylation also inhibited succinate transport in whole cells, but not in membrane vesicles (49, 51). In addition, reagents that directly inhibit the cytoplasmic membrane Ca^{+2}, Mg^{+2}-activated ATPase were able to inhibit membrane vesicle succinate transport and mutants defective in this ATPase were also defective in succinate transport in both whole cells and membrane vesicles (53). These anomalous results left open the question of the involvement of the ATPase in E. coli dicarboxylate transport.

(ii) Transport Component Characterization - The involvement of at least three distinct components in E. coli dicarboxylate transport was suggested by both genetic and biochemical studies. Three different types of mutants were isolated that were defective in some aspect of succinate transport. These mutants could be separated into two broad phenotypic classes. One class of mutants (cbt) was defective only in whole cell transport, but was able to transport succinate normally in membrane vesicles (49,51). Conjugation studies along with P1 phage transduction studies mapped this mutant allele at 16 minutes on the E. coli chromosome (49,54). The second class of mutants (dct) was defective in both whole cell and membrane vesicle succinate transport and these mutants mapped at either 78 minutes (dctA) or 16 minutes (dctB) on the E. coli chromosome. These results suggested that the former class of mutants was defective in a transport component exterior to the cytoplasmic membrane, while the latter class was defective in a membrane-bound transport component. All of the mutants were unable to

grow on media containing succinate, fumarate or malate as the sole carbon source, but their ability to grow on acetate showed that their failure to grow on the dicarboxylic acids was not due to a defect in any of the TCA cycle or glyoxylate shunt enzymes (49). In addition, the cbt mutants were unable to utilize the monocarboxylic acid, D-lactate, as a carbon source (49).

(iii) Periplasmic Dicarboxylate Binding Protein - Preliminary studies on the purification of the three genetically-identified dicarboxylate transport components were carried out using the technique of affinity chromatography (55). This technique involves the immobilization of the substrate or a substrate analogue on a suitable column support and the subsequent passage of sample containing the protein through the affinity column. Only proteins that have a specificity for the column ligand should bind to the column and elution of these bound components results in a total or partial purification of the protein of interest. In the case of the dicarboxylate transport system, aspartate was covalently coupled via its amino group to succinic anhydride-diaminodipropylamine-substituted Sepharose 4B (56). When osmotic shock fluid from wild-type cells was passed through this column, a single protein peak was eluted from the column with 0.2 M succinate (56). Examination of this peak on SDS polyacrylamide gels showed a single polypeptide of 15.4K Daltons molecular weight. The fact that cbt mutants were deficient in this protein and the demonstration by equilibrium dialysis that it bound radioactive succinate with an apparent binding constant (K_d) of 40 uM led to the identification of this protein as the periplasmic dicarboxylate binding protein or DBP. This DBP was also shown to bind fumarate, malate and D-lactate with binding constants of 55 uM, 34 uM and 1.3 mM

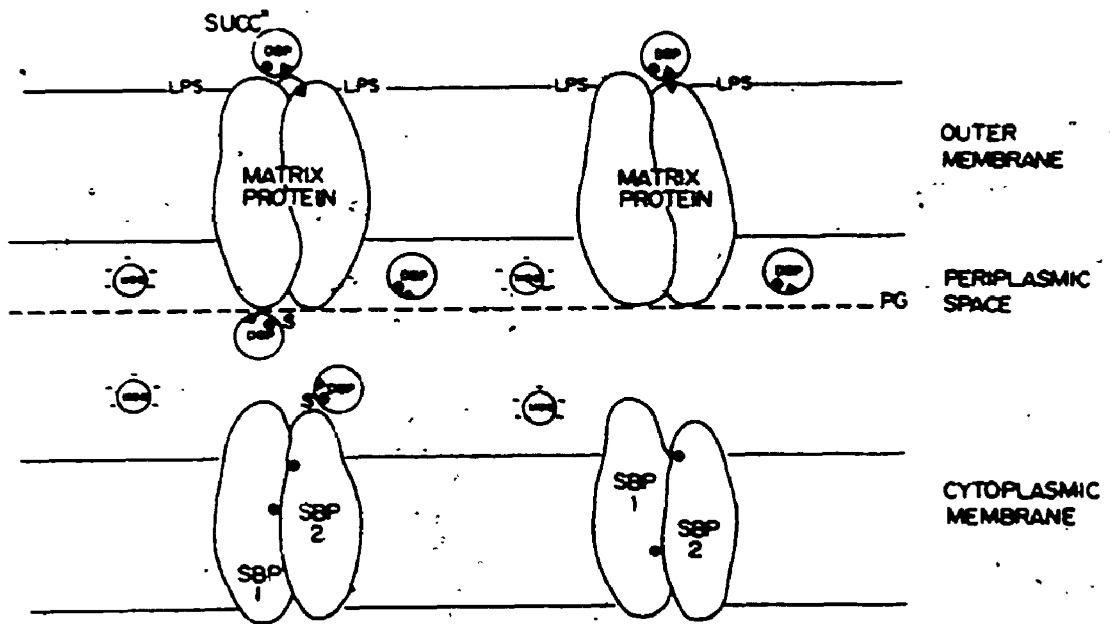
respectively. Selective disruption of the cell envelope of E. coli further revealed that DBP was present on the outer surface of the outer membrane and on both sides of the peptidoglycan layer in the periplasmic space (57). Finally, addition of purified DBP to whole cells of cbt mutants or to wild-type strains from which the external DBP was removed was shown to restore or increase transport of succinate in these strains (58).

On the basis of the above evidence, it was postulated that DBP was responsible for the translocation of dicarboxylic acids across the outer membrane of E. coli, possibly through interactions with the outer membrane matrix protein or porin trimers. The fact that cbt mutants defective in the DBP component were unable to grow on low malate media (0.025%), but were able to grow on high malate media (0.1%), further suggested that DBP was only necessary for high affinity dicarboxylate transport at low concentrations of substrate (49,58). In contrast, both types of dct mutants were unable to grow on either concentration of malate (49). It has been postulated that the role of periplasmic binding proteins in bacterial transport is to maintain a high concentration of substrate in the periplasmic space and to make this substrate accessible to the membrane-bound transport components (59). In the case of the majority of binding protein transport systems, transport of substrate is absolutely dependent upon the presence of a functional periplasmic binding protein. The ability of the membrane-bound components to transport substrate in isolated membrane vesicles suggests that the dct system is a binding protein-stimulated as opposed to a binding protein-dependent transport system.

(iv) Cytoplasmic Membrane Succinate Binding Proteins - Solubilized

cytoplasmic membrane vesicles were also examined by aspartate-Sepharose affinity chromatography. The non-ionic detergent Lubrol 17A-10 was used to solubilize the vesicle proteins and two distinct protein peaks were eluted with 0.2 M succinate when solubilized wild-type membranes were run on the affinity column (60). These peaks were designated as SBP1 (succinate binding protein 1) and SBP2 respectively due to their elution order from the aspartate-Sepharose column and the demonstration that they bound the dicarboxylic acids succinate, fumarate and malate in equilibrium binding studies (60,61). The apparent binding constants for succinate were found to be 23 uM and 2.3 uM for SBP1 and SBP2 respectively and this correlated well with the apparent K_m of 20 uM for succinate transport in wild-type membrane vesicles (60). The apparent binding constants of the isolated proteins for malate and fumarate were somewhat higher (47 uM and 7 uM). A comparison of these values with the binding affinities of normal (right-side-out) and French-pressed (inside-out) membrane vesicles for malate (4 and 30 uM respectively) suggested that the substrate recognition sites of SBP1 and SBP2 were oriented towards to the cytoplasm and periplasm of the cell respectively (61). Finally, it was shown that solubilized membrane vesicles from <u>dctA</u> mutants lacked the SBP2 component and were defective in malate binding to right-side-out membrane vesicles, while <u>dctB</u> mutants were deficient in the SBP1 component and were defective in inside-out vesicle malate binding (61). Taken together, these results suggested that both membrane-bound transport components were necessary for cytoplasmic membrane succinate transport. Surface-labelling studies of wild-type membrane vesicles further showed that both SBP1 and SBP2 could be labelled in both right-side-out and inside-out vesicle preparations and

Figure I-2. Schematic model of C_4-dicarboxylate transport in *E. coli* K12. In this model, the dicarboxylate binding protein (DBP) which can be released by EDTA-sucrose treatment is thought to bind onto the matrix protein (porin) on the outer surface of the outer membrane. Dicarboxylate binding protein (DBP), which is released from the cell envelope by osmotic shock or lysozyme treatment, is depicted as the one located in the periplasmic space. SBP1 and SBP2 represent the two dicarboxylate membrane transport components.
LPS - lipopolysaccharide; PG - peptidoglycan; SUCC or S. - succinate; MDO - membrane-derived oligosaccharides.
● - substrate recognition sites on various transport components;
▶ - protein-protein recognition sites.
This figure was reproduced with permission from T. Lo (59).


SUCC
LPS
LPS
LPS
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MATRIX PROTEIN
MATRIX PROTEIN
OUTER MEMBRANE
PERIPLASMIC SPACE
PG
SBP 1
SBP 2
SBP 1
SBP 2
CYTOPLASMIC MEMBRANE

that the two components could be covalently joined with a non-penetrating cross-linking reagent (62). This suggested that both SBP1 and SBP2 were transmembrane proteins that were in close proximity to each other in wild-type cytoplasmic membranes. Presumably, translocation of dicarboxylic acids across this membrane could then occur via a hydrophilic transport channel formed by SBP1 and SBP2 (59,62). The model shown in Figure 2 summarizes the proposed interactions of the three dicarboxylate transport components of *E. coli* K12.

Technical Difficulties Encountered in Membrane-Bound Transport Component Identification - The membrane-bound, active transport systems in *E. coli* have been biochemically well-characterized *in situ* in both whole cells and cytoplasmic membrane vesicles, as was discussed previously for both the lactose and dicarboxylate transport systems. However, difficulties have been encountered when attempts were made to purify the carrier proteins in an active form for further studies. It is desirable to study these carriers in an active, purified form in order to obtain additional structural and functional information about the transport process. Purification of the water-soluble, periplasmic binding proteins merely involves the fractionation of osmotic shock fluid or periplasm on suitable molecular sieve or ion exchange columns (27-32). In contrast, membrane proteins must first be removed from their hydrophobic, membrane environment before conventional purification techniques can be employed. Therefore, the first step in the purification of membrane proteins involves the use of a reagent that can dis-dissolve into the membrane and disrupt the protein-lipid interactions to effect solubilization of the membrane components. Non-ionic detergents, such as Triton X-100 or Lubrol PX, are favoured over ionic detergents

and organic solvents for the solubilization of biological membranes, because the latter reagents cause extensive denaturation of proteins (5,63). The solubilized membrane proteins are insoluble in an aqueous environment in the absence of detergent and, even in the presence of detergent, problems are often encountered with precipitation and aggregation of membrane proteins during the course of their purification (5).

Non-ionic detergents are amphipathic molecules which partition into the membrane such that their hydrophobic domains interact with the hydrophobic interior of the lipid bilayer while their hydrophilic domains remain in contact with the aqueous environment (5,63). At a high enough concentration of detergent, protein-detergent micelles are formed in which the detergent has substituted for the membrane lipid (5). However, the extent to which the detergent can actually substitute for the membrane lipid in terms of preserving the conformation and activity of the extracted protein depends upon both the particular membrane protein and the detergent (63,64). This is why it is often difficult, if not impossible to follow the purification of a membrane protein, be it an enzyme or transport protein, by assaying for its biological activity. This becomes even more difficult when the protein is present in very low levels in the membrane and when it also has not yet been identified as a specific polypeptide of a certain molecular weight prior to purification. All of the above technical considerations have, therefore, hampered progress in the identification and purification of the membrane-bound transport components. The one exception so far to these obstacles is the β-galactoside or lactose transport protein of E. coli, which was discussed earlier. In addition, both the melibiose and proline carriers of E. coli have recently been reconstituted in an active

form in liposomes and further efforts are being directed towards the purification of these two membrane carriers (22,65).

Identification and Purification Techniques Utilized in This Study of the Cytoplasmic Membrane Dicarboxylate Transport Components - The purpose of this thesis is to extend the preliminary studies carried out with the cytoplasmic membrane components of the dicarboxylate transport system of E. coli. When the aspartate-Sepharose column fractions from Lubrol-solubilized wild-type vesicles were initially examined on SDS polyacrylamide gels at the outset of this study, it became evident that they were highly impure. Therefore, this study examined three different techniques for the identification and purification of the transport components. The problem of low levels of membrane transporter was solved in the lactose transport system by introducing a plasmid carrying the lac Y gene into a suitable E. coli strain (35). Since the dctA and dctB genes had not been cloned when this study was initiated, all of the purification and identification techniques that were employed for the dicarboxylate transport components involved reagents that were designed to have a specificity or affinity for the succinate binding proteins. These affinity techniques utilized the recognition of the transport components' substrate binding sites as the first step in the isolation of the transport proteins in order to enrich for these proteins prior to further purification steps. The three techniques that were examined in this study were aspartate-Sepharose affinity chromatography of solubilized cytoplasmic membrane proteins, immunoblotting of cytoplasmic membrane proteins with succinate-specific antiidiotypic antibodies and photoaffinity labelling of proteins in isolated membranes and whole cells.

The first technique involved optimization of both the solubilization and affinity chromatography of cytoplasmic membrane proteins in order to address the problem of succinate transport component purity. The affinity-purified proteins were then tested for succinate binding ability with four different binding assays. Transport mutants were also examined with the same affinity column system in an attempt to identify the cytoplasmic membrane dicarboxylate transport components.

The second technique utilized antibodies that were raised in rabbits against the variable region or idiotype of injected IgG molecules that were specific for the succinate analogue, aspartate. The rationale behind this technique was that some of these antiidiotypic antibodies should be specific for the antigen binding site of the first IgG molecule and, thus, the antigen binding site of these anti-antibodies would constitute an internal image of the first antigen, aspartate (66). By virtue of their similarity to the dicarboxylate transport substrate analogue, aspartate, the antigen binding sites of the antiidiotypic antibodies might then also display a binding specificity for the succinate transport components. These antibodies could then be used to identify the transport components without their prior purification. Identification of the membrane-bound dicarboxylate transport components involved binding of the antiidiotypic antibodies to Western Blots of total membrane vesicle or cytoplasmic membrane proteins from wild-type and transport-mutant strains.

Finally, the third affinity technique employed an aryl azide dicarboxylate substrate analogue that became a highly-reactive, covalent label when irradiated with the appropriate wavelength of light (67). The photoreactive derivative of aspartate, N-(4-azido-2-nitrophenyl)-

aspartic acid, was synthesized, purified and characterized with respect to its ability to specifically inhibit whole cell succinate transport. This reagent was then utilized in photoaffinity labelling studies with cytoplasmic membranes and whole cells of wild-type and transport-mutant strains in order to covalently label and identify the dicarboxylate transport components.

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CHAPTER II

ISOLATION OF CYTOPLASMIC MEMBRANE TRANSPORT COMPONENTS

Classical methods of protein purification usually involve the initial separation of the appropriate subcellular fraction on an ion exchange or molecular sieve column. If the protein of interest is an enzyme, column fractions are tested for enzyme activity and the relevant fractions are then pooled and subjected to further purification steps. The purification of the protein can be easily followed by measuring the fold increase in enzyme specific activity at each step. However, if a membrane receptor or binding protein with no enzymatic activity is involved, the above approach can be difficult, especially if the protein is only present in small amounts in the cell. The presence of a small amount of enzyme can be amplified many times as it converts substrates into products. In contrast, the same amount of a non-enzymatic protein may be difficult to detect, since the binding of substrate to a protein or membrane receptor is a single, reversible event. Successive rounds of binding are not detectable since the substrate is not chemically altered.

To circumvent this problem, tissues or organisms that contain increased levels of the protein of interest are used and/or the first step of the purification involves specifically labelling or enriching the desired protein. An example of the former approach is the purification of the lactose transport component from an E. coli strain that overproduces the protein due to the presence of a recombinant plasmid containing the lac Y gene. In addition, a specific photolabel was used to follow the purification of the lac permease (1). Many proteins have

also been purified by affinity chromatography (2,3). In this technique, the substrate or a substrate analogue is covalently attached to a suitable column support. When the sample is passed through the column, only proteins that have a specific "affinity" for the immobilized substrate will bind to the column; all other nonspecific proteins can be washed through the column (4). The bound protein can then be eluted by one of a number of methods, such as the passage of excess free substrate through the column. While the protein may not be pure after affinity chromatography, this step increases the proportion of the desired protein in the sample prior to further classical purification steps.

For this reason, the technique of affinity chromatography was used as the first step in the isolation of the cytoplasmic membrane components of the dicarboxylate transport system in E. coli K12. In previous studies, the non-ionic detergent Lubrol 17A-10 was used to solubilize cytoplasmic membrane vesicles of wild-type cells. The solubilized extract was chromatographed on an aspartate-Sepharose affinity column and two protein peaks, designated SBP1 and SBP2, were eluted with succinate from the column. Equilibrium dialysis showed that both of these protein peaks exhibited [^{14}C-2,3]-succinic acid binding activity (5,6). This purification scheme and subsequent binding studies were carried out over the span of several days. To avoid the problem of possible protein degradation, the time of isolation was reduced to one day when the studies described in this chapter were initiated. However, when the protein eluted from the aspartate-Sepharose column was examined on an SDS polyacrylamide gel, it was still found to be highly impure.

In this chapter, an alternative method for the preparation and solubilization of E. coli cytoplasmic membranes was investigated. As

well, the aspartate-Sepharose affinity column protocol was optimized. The parameters that were examined included the growth media of the cells, the composition of the column buffer, the solubilizing detergent and the addition of exogenous phospholipids during the solubilization step. Several different binding assays were used to characterize the proteins isolated from the affinity column as succinate binding proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media - The *E. coli* K12 strain that was used in all of the experiments in this chapter was CBT43. Its relevant genetic markers are: *sdh*, *frd*, *thi* (5). In addition, strains JW301 (*ompF*$^-$, *ompC*$^-$) and PL6 (*ompA*$^-$) were used in one experiment. Bacteria were grown in either LB or minimal M9 medium (7). The LB medium consisted of 1% Bactotryptone (10 gm/l), 0.5% yeast extract (5 gm/l), 1% sodium chloride (10 gm/l) and 1 mM sodium hydroxide. The composition of one litre of M9 medium was 6 gm dibasic sodium phosphate, 3 gm monobasic potassium phosphate, 2 gm ammonium chloride and 0.223 gm magnesium chloride. This was supplemented after autoclaving with 0.2% glycerol, 0.001% thiamine hydrochloride, 100 uM calcium chloride, 1 mM magnesium chloride and 1% LB for better growth. Sodium [^{35}S]-sulphate (0.5 mCi/l) was included for studies that required labelled cells. Both LB and M9 media were also supplemented with 85 mM (0.2%) potassium succinate in order to induce the dicarboxylate transport system (8).

Preparation of Cytoplasmic Membrane Vesicles - Right-side-out cytoplasmic membrane vesicles were prepared by a modification of the method of Kaback *et al* (5,9). An overnight stationary-phase culture of

LB-grown bacteria was inoculated 1:11 into fresh LB and grown to mid to late-log phase (2 - 3 hours) at 37°C and 140 rpm in a New Brunswick rotary shaker. The absorbance of the culture at 600 nm was determined with a Beckman DU-8 spectrophotometer. The cells were harvested and washed twice with 40 volumes (ie. 40 ml/gm wet weight of cells) of 10 mM Tris-HCl, pH 8.0. The outer membrane and peptidoglycan layer were dissociated by incubating for 1 hour at 25°C in 30 mM Tris-HCl (pH 8.0) containing 20% sucrose, 10 mM potassium EDTA (pH 7.0) and 5 mg hen egg white lysozyme per gram of cells. The resulting spheroplasts were then broken open by gentle homogenization with a Fisher Dyna-Mix in 100 mM potassium phosphate (pH 6.6) containing 20% sucrose and 20 mM magnesium sulphate. The released nucleic acids were degraded with deoxyribonuclease (896 Kunitz units/gram cells) and ribonuclease (1332 Kunitz units/gram cells) at 37°C in 50 mM potassium phosphate, pH 6.6. Whole cells and cell wall debris were removed from the membrane vesicles by two low speed centrifugations (500 x g) in 15 volumes of 100 mM phosphate (pH 6.6) containing 10 mM potassium EDTA (pH 7.0). The vesicles were pelleted at 15,000 rpm (27,000 x g) and were washed once with the phosphate-EDTA buffer. They were then washed with 50 mM potassium phosphate, pH 6.6. A Lowry protein assay (10) with a BSA standard was used to determine the vesicle protein concentration, which was adjusted to 5 or 10 mg/ml with phosphate buffer. The vesicles were stored in 2.5 - 5 mg aliquots at -80°C.

<u>Preparation of Cytoplasmic Membranes</u> - Cytoplasmic membranes were prepared by a modification of the method of Schnaitmann <u>et al</u> (11). Bacteria were grown in LB medium overnight and were then inoculated into fresh LB or M9 medium if labelled cells were desired. For LB-LB pre-

parations, the bacteria were diluted 1:11 and were grown for 3 hours (late-log) as described previously. The LB-M9 preparations were first spun down and then the cells were resuspended in a 1:10 dilution of their former volume into M9 medium containing 0.5 mCi [^{35}S]-sulphate per litre. They were grown for 5 hours at 37°C and 140 rpm to late-log phase.

The cells were harvested and washed with approximately 40 volumes of 50 mM potassium phosphate, pH 6.6. The cell pellet was weighed and resuspended in 4 volumes of phosphate buffer, containing 0.002% tosyl sulphonyl fluoride (TSF). All subsequent steps included 0.002% TSF. The cells were broken at 4°C with the French Press, 3 times at 9000 psi. Unbroken cells and debris were removed by spinning the preparation at 5000 x g for 15 minutes. Total membranes were isolated from the supernatant at 100,000 x g for 30 minutes using a T150 (37K rpm) or T160 (35K rpm) rotor. The membranes were washed once with the phosphate buffer and were spun again at 100,000 x g. The cytoplasmic membranes were then selectively solubilized with 1% Triton X-100 (or another non-ionic detergent) in 10 mM Tris-HCl (pH 8.0), 5 mM magnesium chloride at 37°C for 30 minutes. This was spun at 100,000 x g and the solubilization step was repeated on the pellet. The supernatants, containing the solubilized cytoplasmic membranes, were pooled and quick-frozen in dry ice-acetone. The outer membrane pellet was overlaid with a minimal volume of phosphate buffer and frozen. All fractions were stored at -20°C.

Aspartate-Sepharose Preparation - Aspartate-coupled Sepharose was prepared by the method of Cuatrecasas (5,12). Sepharose 4B (200 ml) was washed extensively in a Buchner funnel with deionized water and then was resuspended in an equal volume of water. The Sepharose was activated

with 50 gm of finely-divided cyanogen bromide in 150 ml of water for 12 minutes. During the course of the reaction, the temperature was kept at 20°C by adding ice directly to the mixture and the pH was kept above 11 with 8 N sodium hydroxide. Excess ice was added to stop the reaction and the Sepharose was quickly filtered and washed with 20 volumes of cold water. The activated Sepharose was then resuspended in a 150 ml solution of 52.48 gm 3,3'-iminobispropylamine in cold water previously adjusted to pH 10 with cold 6 N hydrochloric acid. This mixture was slowly stirred for 16 hours at 4°C. The Sepharose was washed with 20 volumes of water and resuspended in 200 ml of cold water. Solid succinic anhydride (30 gm) was added with stirring to the Sepharose and the pH was kept close to 6 with 8 N sodium hydroxide until no further drop in pH occurred (about 15 minutes). The Sepharose was then stirred for 5 hours at 4°C. It was washed again with 20 volumes of water and resuspended in 200 ml of water. Potassium aspartate (54.8 gm) was added with stirring to the Sepharose and the pH was adjusted to 5 with 6 N hydrochloric acid. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (12 - 15 gm) in about 30 ml of water was added dropwise over a period of 15 minutes to the stirred Sepharose. The reaction was allowed to continue at 25°C for 20 hours. The aspartate-Sepharose was then washed extensively with water and nonspecific sites were blocked with 0.2 M glycine, pH 8.0, at 25°C for 2 hours.

Protein Fractionation by Aspartate-Sepharose Affinity Column - Both cytoplasmic membrane vesicles and solubilized cytoplasmic membranes were used as starting material for the affinity column. In a representative example of the former case, 3 mg of quickly-thawed membrane vesicles were solubilized with 0.5% Triton X-100 in a 10:1 detergent:pro-

tein ratio in 25 mM potassium phosphate, pH 6.6. The vesicles in detergent were homogenized by hand with several strokes and were then shaken or stirred gently at 25°C for 30 to 45 minutes. The mixture was then centrifuged at 2500 rpm (1300 x g) in a clinical table-top centrifuge for 20 minutes. The supernatant was diluted 10 times with deionized water and loaded onto a 10 ml aspartate-Sepharose column (1 cm ID) equilibrated with 10 mM potassium phosphate (pH 6.6), 0.05% Triton, 5 mM potassium EDTA (pH 7) and 0.002% TSF. The column was washed with 25 ml of the same buffer and then any bound protein was eluted with 50 ml of 0.2 M potassium succinate in buffer collected in 1.5 - 2 ml fractions. The absorbance at 280 nm (OD_{280}) of each fraction was determined, or 50 ul aliquots of each tube were counted if [^{35}S]-labelled membranes were used. The column peak was pooled and TCA precipitated for analysis with SDS polyacrylamide gel electrophoresis. The 10% TCA precipitation step was followed by an ether wash and acetone precipitation to remove detergent. Alternatively, the protein samples were desalted using one of several methods. The desalting methods included concentration and serial washing of the sample on a YM5 or YM10 Amicon ultrafiltration membrane or passage of the sample through a 30 ml (1.5 cm ID) Biogel P6DG column in 10 mM potassium phosphate (pH 6.6), 0.01% Triton buffer at 4°C. The desalted protein emerged in the column void volume and could either be examined further on an ion exchange column or be used in binding studies.

When solubilized cytoplasmic membranes were examined on the affinity column, they were thawed quickly in a 37°C water bath and were then diluted 1:1 with deionized water and 0.002% TSF was added. The solubilized membranes from 1 - 2 litres of cells (3 - 6 mg protein) were

loaded onto a 35 - 40 ml aspartate-Sepharose column (1.5 cm ID) equilibrated with 10 mM potassium phosphate (pH 6.6), 0.05 or 0.01% Triton, 5 mM potassium EDTA (pH 7) and 0.002% TSF. The column was washed with 75 ml of buffer and was then eluted in 2 ml fractions with first 100 mM potassium phosphate-Triton-EDTA buffer and then 0.2 M potassium succinate or 0.5 M sodium chloride in the same buffer. Each protein peak was pooled and then either TCA precipitated or desalted as described above.

Preparation of E. coli Phospholipids - E. coli phospholipids were extracted by a modification of the Bligh and Dyer procedure (13). Six litres of LB-grown (1:11 dilution) CBT43 cells (10 gm wet weight) were harvested after growing for 3 hours at 37°C. The cells were washed once with 400 ml of 50 mM phosphate buffer (pH 6.6) and were resuspended in 100 ml of deionized water. Four hundred millilitres of methanol: chloroform (2:1) were added and the mixture was vigorously agitated. It was incubated for 3 hours at 25°C with occasional agitation. The extract was then centrifuged for 10 minutes at 150 x g to remove the cell debris and aggregated protein. Chloroform and deionized water (125 ml each) were added to the supernatant, which was then centrifuged to break the solvent phases. The upper methanol/water phase and the protein debris at the interface were aspirated off and discarded. The lower chloroform phase was transferred to a separatory funnel where water and a small amount of 0.1 N hydrochloric acid was added to extract any residual protein. A few drops of benzene and later absolute ethanol were added to the chloroform phase as it was brought to dryness on a rotary evaporator. The remaining lipid residue was dissolved in 20 ml of chloroform:methanol (2:1) and was stored at -20°C. An inorganic phosphate determination (14) gave a value of 58.8 mg of phospholipid ex-

tracted.

The phospholipids were then acetone-ether washed according to the method of Newman and Wilson (15). The chloroform/methanol solution was evaporated under nitrogen gas to around 300 ul (200 mg/ml phospholipid) and this was resuspended in 6 ml of nitrogen-bubbled, anhydrous acetone containing 2 mM 2-mercaptoethanol. The suspension was stirred under nitrogen gas in the dark for 6 hours at 25°C to remove the neutral lipid. The acetone-insoluble phospholipid was collected on a Whatman #1 filter and was then dissolved in 10 ml of anhydrous ether containing 2 mM 2-mercaptoethanol. The ether solution was centrifuged for 15 minutes at 2500 x g and the supernatant was transferred to a weighed glass tube. It was evaporated to dryness under nitrogen gas. A small amount of chloroform was added to the residue, which was again evaporated to dryness under nitrogen to form a thin film of lipid on the walls of the tube. The phospholipid was then lyophilized for 3 hours to remove any residual water and organics. After acetone-ether washing, 62% of the phospholipid was recovered. The lipid was vortex-dispersed under nitrogen gas in 2 mM 2-mercaptoethanol at 50 mg/ml and was stored at -20°C.

SDS Polyacrylamide Gel Electrophoresis - Gel electrophoresis of the cytoplasmic membrane preparations and affinity column proteins was performed according to the method of Laemmli (16). Samples were solubilized in sample buffer of 3% SDS, 5% 2-mercaptoethanol, 0.0625 M Tris-HCl (pH 6.8) and 10% glycerol for 5 minutes at 100°C or 30 minutes at 70°C. The samples were then spun down and loaded onto a 1.5 mm slab gel. Routinely, separating gels of 11.25:0.3% acrylamide:bisacrylamide in 0.375 M Tris-HCl (pH 8.8), 0.1% SDS were used with stacking gels of 4.5:0.12% acrylamide:bisacrylamide in 0.125 M Tris-HCl (pH 6.8), 0.1%

SDS. A Tris-glycine-SDS (25 mM-192 mM-0.1%) running buffer was used. After electrophoresis, gels were stained with 0.125% Coomassie Brilliant Blue R-250 in 50% methanol, 10% acetic acid and they were destained in 10% methanol, 5% acetic acid. Gels with radioactive samples were dried and exposed to Kodak X-OMAT X-ray film for autoradiography.

Binding Assays - Four different binding assays were used to detect [^{14}C-2,3]-succinic acid binding to the proteins eluted from the aspartate-Sepharose column.

(i) P6DG Column - A desalted sample of affinity-purified protein from 6 litres of LB-grown cells was concentrated down to 1 - 2 ml with a YM5 Amicon ultrafiltration membrane. Binding was carried out at 25°C for 60 minutes in a 1.5 ml Eppendorf tube with 100 ul of sample and 10 ul of 2 mM or 200 uM carrier-free [^{14}C]-succinic acid. This was continuously mixed on a rotary mixer. The sample was then loaded onto a 5 ml Biogel P6DG column (1 cm ID) equilibrated in 50 mM potassium phosphate (pH 6.6 or 7.5), 0.01% Triton (or another detergent) and 3 drop fractions were collected with the same buffer using an LKB Redi-Rac fraction collector. The protein, along with any bound succinate, was eluted in the void volume followed by a large peak of free succinate. From calculations of protein concentration, recovery from the column (using [^{35}S]-labelled protein) and specific activity of the [^{14}C]-succinate, the molar ratio of succinate:protein was determined. A modified Lowry assay (17) containing 1% SDS was used for all detergent-containing samples.

(ii) Proteoliposomes - Liposomes were prepared by a modification of the procedure of Newman and Wilson (15). Purified phospholipids from Serdary were mixed in a ratio of 70:15:15 for phosphatidylethanolamine, phosphatidylglycerol and cardiolipin respectively to approximate the

ratio in the E. coli cytoplasmic membrane (18). All three phospholipids were dioleoyl and the sources were E. coli (PE), egg lecithin (PG) and beef heart (cardiolipin). The phospholipids (50 mg total) were dissolved in chloroform in a 16 x 100 mm test tube and the chloroform was evaporated under nitrogen gas with rotation to coat the sides of the tube with a film of lipid. This was then lyophilized for 3 hours to get rid of any traces of chloroform and water. The lipid was vortex-dispersed under nitrogen gas in 1 ml of 50 mM potassium phosphate (pH 7.5 or 7.0), 2 mM 2-mercaptoethanol. It was then sonicated in a Branson bath (117 V, 50 - 60 Hz, 125 Watts) for 15 minutes under nitrogen gas. Triton X-100 (0.2%) was added to the water bath and the level of the bath was adjusted to give maximum agitation of the lipid. The resulting liposomes were kept on ice until the protein samples were ready for reconstitution.

Proteoliposomes were made next using the octylglucoside dilution procedure described by Chen and Wilson (18). Desalted, concentrated protein (788 ul) from the affinity column was vortexed gently with 200 ul of liposomes and 90 ul of 15% octylglucoside (1.25% final) and this was kept on ice for 10 minutes. It was then quickly squirted with a Pasteur pipette into 30 ml of 50 mM potassium phosphate (pH 7.0 or 7.5), 1 mM dithiothreitol at 25°C and immediately stirred. The proteoliposomes were collected by centrifugation at 85,000 x g for 60 minutes at 4°C (29K rpm in Ti60 rotor). The pellet was gently resuspended in 100 to 200 ul of phosphate buffer using a glass rod and a Hamilton syringe.

The proteoliposomes were assayed for succinate binding activity with the method of Newman et al (1). An aliquot of proteoliposomes (10 ul) and 90 ul of phosphate buffer was pipetted directly onto a 25 mm,

0.22 um Millipore GS filter. The vacuum was turned off and the filter valve was closed and 200 ul of carrier-free 200 uM [^{14}C]-succinate was pipetted carefully onto the liposome spot. Incubations of 2 to 5 minutes were followed by filtration and a 5 ml wash of ice-cold phosphate buffer and the filters were counted in scintillation fluid. Alternatively, the proteoliposomes were directly incubated with the 200 uM [^{14}C]-succinate in an Eppendorf tube and then they were filtered and washed. Binding was also assayed by incubating 10 ul of proteoliposomes with 200 uM [^{14}C]-succinate in buffer (100 ul total sample volume) for 15 minutes, then spinning the samples for 10 minutes at 70,000 rpm in a Beckman airfuge. After a 100 ul buffer wash, the inside of the tubes were dried with a Kimwipe and the tubes were counted in scintillation fluid. In all cases, a blank value was obtained using sham "reconstituted" liposomes.

(iii)Nitrocellulose Filtration Method - A modification of the method of Lever (19) was used to assay succinate binding with Schleicher and Schuell nitrocellulose filters. Aliquots of concentrated, desalted protein (10 - 25 ul) were incubated with 20 uM [^{14}C]-succinate in 50 mM potassium phosphate buffer (pH 6.6; with or without added detergent) for 60 minutes at 25°C on a rotary mixer (100 ul total sample volume). The samples were pipetted directly onto 25 mm, 0.45 um filters with the vacuum off. The samples were filtered and 1 ml of 10 mM potassium phosphate (pH 6.6) was added with the vacuum off and then filtered immediately. This wash was repeated with a further 1 ml of buffer. The filters were counted in scintillation fluid. The blank consisted of [^{14}C]-succinate in phosphate buffer. The crude membrane detergent extract and the affinity column wash were also assayed for binding

ing activity. Binding in all fractions was also carried out in the presence of 2.5 mg/ml phospholipid (described in (ii)) or phospholipid plus 40% glycerol.

(iv)Equilibrium Dialysis - Equilibrium dialysis cells holding slightly more than 100 ul per side were used (5) with Spectrapor 3 dialysis membrane (molecular weight cut-off of 3500 Daltons). The membrane was pretreated by boiling successively in 1 mM potassium EDTA and deionized water and then soaking in phosphate buffer. Sample (100 ul) in phosphate-detergent buffer was added to one chamber with a Hamilton syringe and 100 ul of 100 uM [^{14}C]-succinate was added to the other chamber. The specific activity of the succinate was such that the amount of sample added could bind 5% of the total radioactivity if active. Binding was carried out for 24 hours at 4°C and the dialysis cells were agitated on a rotary mixer. An aliquot (80 ul) from each chamber was counted. Binding was also carried out in the presence of 2.5 mg/ml phospholipid and 40% glycerol.

Chemicals - All chemical used were obtained from commercial sources and were of the highest available purity. Bactotryptone and yeast extract were bought from Difco Laboratories (Detroit, MI). Radioactive [^{14}C-2,3]-succinic acid, [^{3}H-2,3]-aspartic acid and sodium [^{35}S]-sulphate were purchased from the New England Nuclear Company (Boston, MA). Molecular weight standards were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Nitrocellulose paper was bought from Schleicher and Schuell, Inc. (Keene, NH) and phospholipids were obtained from Serdary Research Laboratories (London, Ontario). Octylglucoside was purchased from Boehringer Mannheim (West Germany), while CHAPS, Lubrol PX and Polyoxyethylene Ether W1 were bought from the Sigma Chemical

Co. (St. Louis, MO). Triton X-100 was obtained from the Amersham Corporation (Arlington Heights, IL).

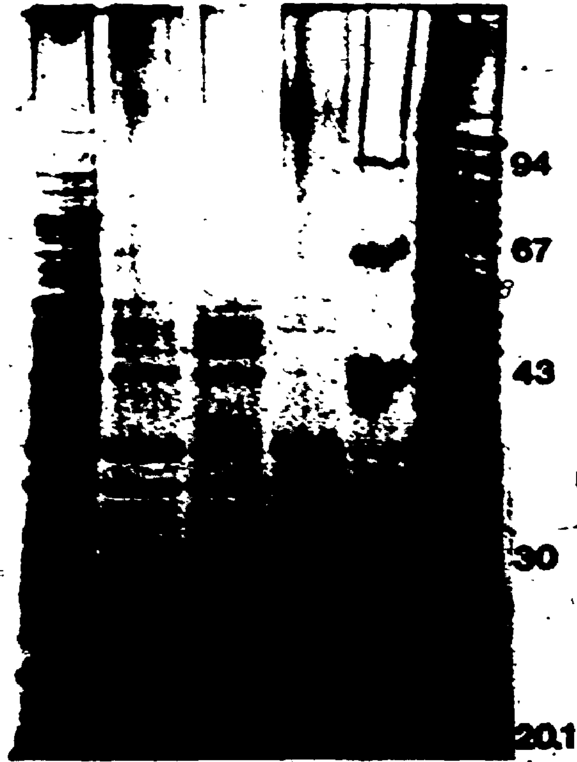
RESULTS

Comparison of Membrane Vesicles and Cytoplasmic Membranes - Kaback membrane vesicles (9) were prepared from the LB-grown wild-type CBT43 strain and their protein profile was compared to that of various outer membrane preparations on an SDS gel. Figure 1 shows an extensive contamination of the membrane vesicles with the two major outer membrane proteins, porin (OmpF,C) and TuII* (OmpA) (20,21). The membrane vesicles were compared to outer membranes prepared from CBT43 and outer membranes from strains JW301 and PL6 lacking the proteins porin and TuII* respectively. In contrast, the corresponding cytoplasmic membrane preparations from CBT43 (Figure 1) and the other two strains (data not shown) showed little or no evidence of contamination with outer membrane proteins. These membranes were prepared by selective Triton solubilization of sonicated total membranes, as described in Experimental Procedures. Figure 2 shows a similar pattern for outer and cytoplasmic membranes prepared from French-pressed CBT43 cells. Therefore, the method of cell disruption (sonication versus French pressure cell) did not appear to affect the purity of the solubilized cytoplasmic membranes.

Affinity Chromatography of Solubilized Membrane Vesicles - Two important parameters that influence a detergent's solubilization efficiency are the detergent's concentration in solution and the ratio (weight:weight) of detergent to protein (22). The ideal conditions for three non-ionic detergents were determined prior to affinity chromato-

Figure II-1. Outer membrane contamination of CBT43 membrane vesicles. Cytoplasmic membrane vesicles were isolated from LB-grown CBT43 cells and were prepared for SDS polyacrylamide gel electrophoresis as described in Experimental Procedures. Outer membranes and Triton-solubilized cytoplasmic membranes were prepared from sonicated whole cells of the bacterial strains shown in this figure. This and all subsequent gels shown in this chapter were 1.5 mm, 11.5% polyacrylamide slab gels. MV - membrane vesicle; OM - outer membrane; CM - cytoplasmic membrane. Lane 1 - CBT43 MV, 200 ug; 2 - CBT43 OM, 25 ug; 3 - JW301 OM, 25 ug; 4 - PL6 OM, 25 ug; 5 - CBT43 CM, 100 ug

Figure II-2. CBT43 outer and cytoplasmic membranes. Outer membranes and Triton-solubilized cytoplasmic membranes were prepared from LB-grown, French-pressed CBT43 cells as described in Experimental Procedures. OM - outer membrane, 50 ug; CM - cytoplasmic membrane, 100 ug.


94
67
43
30
20.1
1 MV
2 OM
3
4
5 CM

94
67
30
OM
CM

Table II-I. Optimization of membrane vesicle solubilization conditions. [^{35}S]-labelled CBT43 cytoplasmic membrane vesicles were prepared as described in Experimental Procedures. The vesicles were incubated with different detergents in 10 mM phosphate buffer (pH 6.6) for 45 minutes at 25°C with shaking. The total sample volume was kept constant at 100 ul and the amount of vesicle protein was adjusted inn each sample to yield the desired detergent:protein (wt:wt) ratio. Samples were spun at either 1300 x g or 100,000 x g. An aliquot of the supernatant (20 ul) was counted in scintillation fluid to determine the percent solubilization of each sample. Asterisks indicate the optimal solubilization condition for each detergent.

A - The detergent:protein ratio was held constant at 10:1 while the detergent concentration was changed as indicated in the table.

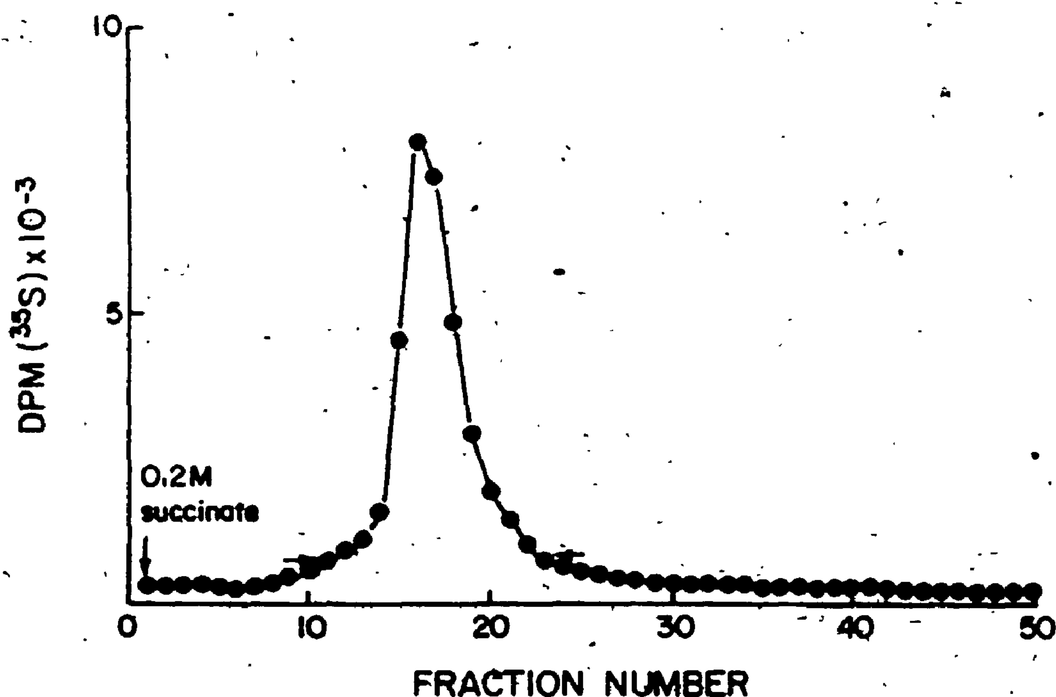
B - The detergent concentration was held constant while the detergent: protein ratio was changed as indicated in the table.

Figure II-3. Aspartate-Sepharose column profile for CBT43 membrane vesicles. [^{35}S]-labelled CBT43 cytoplasmic membrane vesicles (3 mg) were solubilized with 1% Lubrol PX (15:1 detergent:protein ratio) and were prepared for affinity chromatography as described in the text. The 10 ml aspartate-Sepharose column was washed with 25 ml of 10 mM phosphate buffer (pH 6.6) containing 0.1% Lubrol PX and 0.002% TSF. Bound protein was eluted with 50 ml of 0.2 M succinate in the same buffer, as indicated on the graph. Protein was detected by measuring the radioactivity ([^{35}S]-DPM) in each column fraction.

Optimization of Membrane Vesicle Solubilization Conditions

A Detergent	Concentration	Solubilization Efficiency 1300 x g	100,000 x g
Triton X-100	0.25%	44%	47%
	0.5%	68%	71% *
	1.0%	61%	62%
Lubrol PX	1%	34%	37% *
	2%	39%	37%
	4%	37%	31%
Octylglucoside	7.5 mM	27%	34%
	15 mM	42%	56% *
	30 mM	42%	44%

B Detergent	Detergent:Protein Ratio	Solubilization Efficiency 1300 x g	100,000 x g
0.5% Triton X-100	5:1	50%	52%
	10:1	65%	62% *
	15:1	63%	58%
	20:1	64%	61%
1% Lubrol PX	5:1	31%	33%
	10:1	37%	36%
	15:1	52%	44% *
	20:1	52%	48%
15 mM Octyl-glucoside	5:1	43%	27%
	10:1	58%	44% *
	15:1	55%	40%
	20:1	58%	39%



graphy of solubilized membrane vesicles. Table 1 shows that the best solubilization conditions were 0.5% Triton X-100 (10:1 ratio), 1% Lubrol PX (15:1 ratio) and 15 mM octylglucoside (10:1 ratio). These conditions solubilized 65%, 52% and 58% of membrane vesicle protein respectively after centrifugation of the extracts at 1300 x g. Solubilization efficiency was generally only slightly lower when the extracts were spun at 100,000 x g. Therefore, 1300 x g extracts were used in the subsequent experiments since the time of manipulation was much shorter when using the table-top centrifuge as compared to the ultra-centrifuge. This was the same centrifugation protocol that was used in the original work on the succinate transport components (5,6).

Aspartate-Sepharose chromatography of the detergent-solubilized membrane vesicles indicated that a wide range of proteins were binding to the affinity column. The column profile of M9-grown, Lubrol-solubilized vesicles is shown in Figure 3. The column was washed with 10 mM potassium phosphate, pH 6.6, 0.1% Lubrol, 0.002% TSF buffer to remove unbound proteins and then bound proteins were eluted with 0.2 M potassium succinate in the same buffer. Only one distinct peak of [^{35}S]-labelled protein was eluted from the column. Similar column profiles were obtained with a variety of non-ionic detergents, column lengths and column conditions. Occasionally, two protein peaks were eluted from the column as in the early work on this system, but this was not a reproducible observation. Figure 4 is an autoradiogram showing the proteins eluted from the affinity column after solubilization of [^{35}S]-labelled vesicles with 0.5% Triton X-100, 1% Lubrol PX or 15 mM octylglucoside. The protein profiles of the first experiment (left-hand lanes) were similar to that of a second experiment (right-hand lanes) run with

Figure II-4. Affinity-purified proteins from CBT43 membrane vesicles. [^{35}S]-labelled proteins that were eluted with 0.2M succinate from aspartate-Sepharose columns of solubilized membrane vesicles were examined by SDS polyacrylamide gel electrophoresis, as described in Experimental Procedures. Proteins were prepared for electrophoresis by acetone precipitation. The detergents used for vesicle solubilization and affinity chromatography were: T - Triton X-100; L - Lubrol PX; O - octylglucoside; +EDTA - 5 mM EDTA was included in the affinity column buffer.

Figure II-5. Affinity-purified CBT43 cytoplasmic membrane protein. Total membranes from sonicated, [^{35}S]-labelled CBT43 cells were solubilized with 1% Triton X-100 as described in Experimental Procedures. The cytoplasmic membrane extract was loaded onto 35 ml aspartate-Sepharose columns equilibrated with 100 mM phosphate buffer (pH 7.0) containing 0.1% Triton, 5 mM EDTA and 0.002%TSF. The columns were washed with 75 ml of column buffer and protein was eluted with 100 ml of 0.2 M succinate in the same buffer. The protein was prepared for electrophoresis by P6DG column desalting and acetone precipitation, as described in Experimental Procedures. S - succinate elution.

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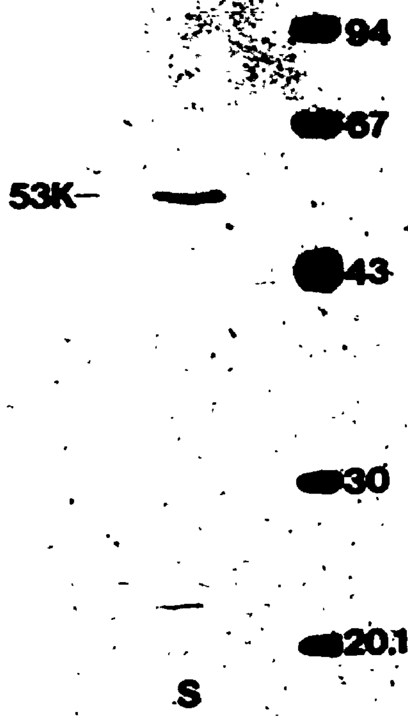
14.4
T L O
T L O
+ EDTA


94
67
53K
43
30
20.1
S

5 mM EDTA in the phosphate column buffer. Similar results were obtained when solubilized vesicle extracts from LB-grown cells were examined on the affinity column.

Affinity Chromatography of Solubilized Cytoplasmic Membranes - Due to the outer membrane contamination of the Kaback membrane vesicles and the impurity of the vesicle proteins eluted from the aspartate-Sepharose column, affinity chromatography of cytoplasmic membranes prepared by the method of Schnaitmann et al (11) was attempted. As outlined in Experimental Procedures, the major difference in this method was that the cells were harvested and their membranes were isolated and solubilized all in one day. The solubilized extracts were then quick-frozen in dry ice-acetone to prevent denaturation of the proteins. In contrast, membrane vesicles were prepared over a two day period and then were frozen prior to solubilization. In addition, the solubilized vesicle extracts were only spun at 1300 x g to remove unsolubilized material, as compared to 100,000 x g in the Schnaitmann method. It is conceivable that the impurity of the vesicle-solublized protein eluted from the affinity column might be due to the fact that membrane fragments were present along with solublized proteins in the "solublized" supernatant. This possibility was borne out by the results obtained from affinity chromatography of 100,000 x g solubilized cytoplasmic membranes, as described below.

The first experiment used 1% Triton-solubilized membranes from sonicated, [^{35}S]-labelled CBT43 cells. The affinity column was washed with 100 mM phosphate buffer (pH 7) containing 0.1% Triton, 5 mM EDTA and 0.002% TSF. Bound protein was then eluted with 0.2 M succinate in the same buffer. As with the membrane vesicles, only one peak of protein

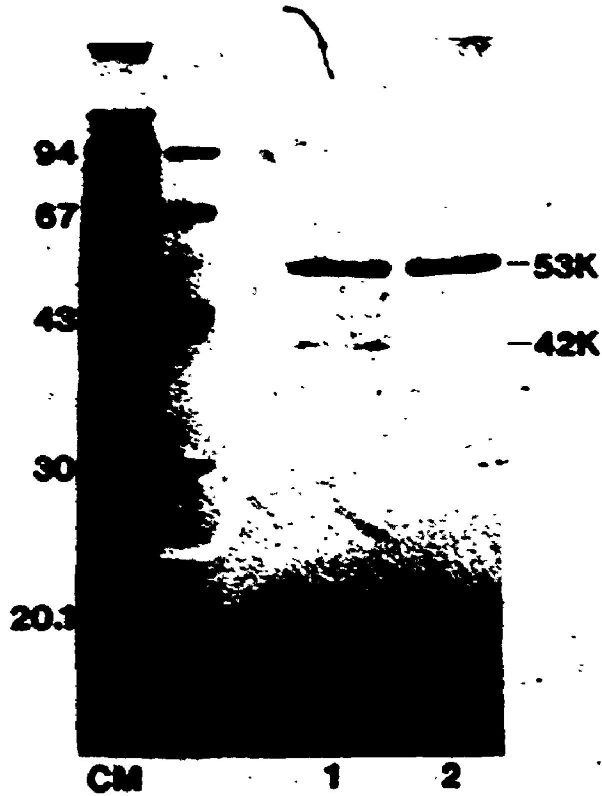
was eluted from the column. In this and all subsequent experiments, only one major protein peak was eluted with succinate from the aspartate-Sepharose column, whether absorbance at 280 nm or DPM of labelled protein was measured. This occurred regardless of the column length, type of detergent or buffer that was used. Figure 5 is an SDS gel of the protein eluted from the column. The purity of the protein was markedly better than in the solubilized vesicle experiments. Only one major protein of 53K Daltons molecular weight bound to the column.

Investigation of Affinity Column Conditions - In order to optimize the affinity column conditions and to assess the nature of the interaction of the bound proteins with the column, various parameters were investigated. These included the growth medium, buffer ionic strength and pH, type of detergent and presence or absence of exogenous phospholipids.

(1) Growth Medium - Affinity column profiles from cells grown in rich (LB) and minimal (succinate-glycerol M9) media were compared on SDS gels. In this and in all subsequent experiments, French-pressed cells were used instead of sonicated cells as in the first experiment. This was done to minimize any protein degradation caused by oxidation and the heat generated in sonication. As well, sonication sometimes causes a redistribution of proteins between the outer and cytoplasmic membranes in *E. coli* (11). Figures 6 and 7 compare the proteins from M9 and LB-grown cells respectively that bound to the affinity column. Lane 2 of each gel shows that one major band (53K) bound to the column whether the cells were M9 or LB-grown. The column was washed with 100 mM phosphate buffer and then eluted with succinate, as in the experiment with the sonicated M9-grown cells. Therefore, the method of cell disruption did

Figure II-6. Affinity-purified cytoplasmic membrane proteins from M9-grown cells. Proteins from Triton-solubilized, [^{35}S]-labelled, French-pressed cells were affinity-purified as described in the text. Samples were desalted by a P6DG column and acetone-precipitated prior to electrophoresis. All of the column buffers contained 0.05% Triton, 5 mM EDTA and 0.002% TSF.
Lane 1 - 10 mM phosphate (pH 7) buffer wash, 0.2 M succinate elution; 2 - 100 mM phosphate buffer wash, 0.2 M succinate elution.
CM - cytoplasmic membrane.

Figure II-7. Affinity-purified cytoplasmic membrane proteins from LB-grown cells. Cytoplasmic membrane proteins from Triton-solubilized, French-pressed cells were affinity-purified and prepared for electrophoresis as in Figure II-6. Protein was detected by measuring the absorbance at 280 nm of each column fraction.
Lane 1 - 10 mM phosphate buffer wash, 0.2 M succinate elution; 2 - 100 mM phosphate buffer wash, 0.2 M succinate elution.
CM - cytoplasmic membrane.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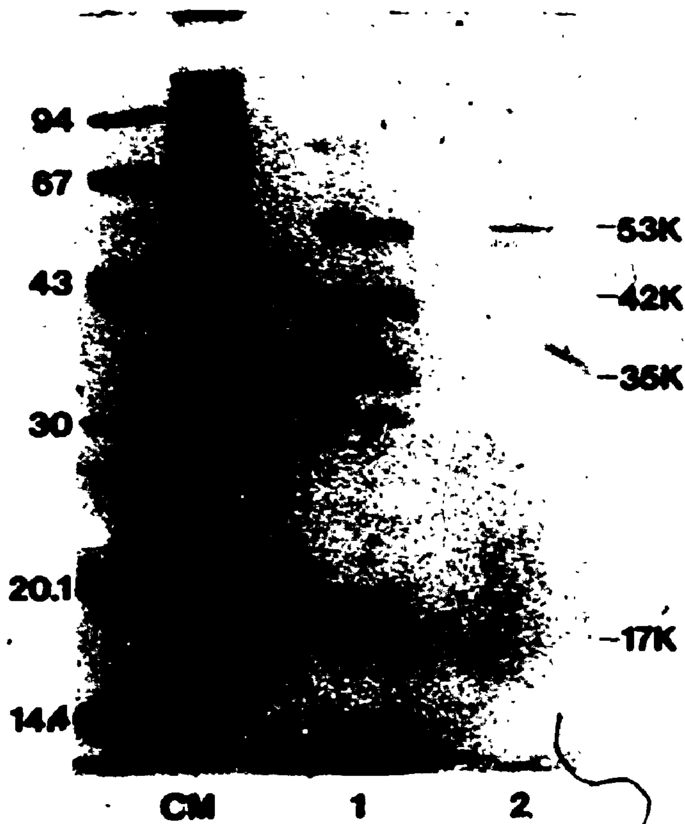
20.1
53K
42K
CM
1
2


94
67
43
30
20.1
14.4
53K
42K
35K
17K
CM
1
2

not seem to affect the binding of the 53K protein to the column.

Lane 1 of each gel shows the effect of decreased buffer ionic strength on the affinity column profile. In both cases, the columns were washed with 10 mM instead of 100 mM phosphate buffer and were then eluted with succinate. Two major proteins (53K and 42K) bound to the column in the case of M9 cells. The same two proteins, along with two other proteins (35K and 17K), bound to the column from the LB cells. Therefore, LB-grown cells had four major cytoplasmic membrane proteins that bound to the aspartate-Sepharose column in low ionic strength (10 mM) buffer, whereas M9-grown cells possessed only two major proteins. In high ionic strength (100 mM) buffer, only the 53K protein bound to the column regardless of the growth medium.

Since whole cell succinate transport in CBT43 was shown to be induced by succinate and repressed by glucose (8), the affinity column profiles from M9 cells grown in either glycerol-succinate or glucose were examined. The columns were run in high ionic strength (100 mM) buffer and were eluted with succinate. The 53K band was eluted from the column in both cases, so its synthesis did not appear to be induced by succinate and/or repressed by glucose. Even though the M9-grown cells had higher succinate transport activity and had a "cleaner" affinity column profile, LB-grown cells were used in subsequent experiments due to the higher yield of cells per litre of growth medium.

(ii) Ionic Strength of Column Buffer - The type of proteins that bound to the aspartate-Sepharose column depended on the ionic strength of the column buffer, for both M9 and LB-grown cells. Figure 8 illustrates this point in a slightly different manner. In this experiment Triton-solubilized proteins from LB-grown CBT43 cells were separated on

Figure II-8. Ionic strength effect on affinity column protein profile. Triton-solubilized, cytoplasmic membrane proteins from LB-grown cells were isolated on aspartate-Sepharose columns equilibrated with 10 mM phosphate (pH 7) buffer containing 0.05% Triton, 5 mM EDTA and 0.002% TSF. The columns were eluted sequentially with 100 mM phosphate buffer (lane 1) and then with 0.2 M succinate in the same buffer (lane 2). The samples were TCA precipitated prior to electrophoresis as described in the text.
CM - cytoplasmic membrane.

Figure II-9. Effect of pH on affinity column protein profile. Triton-solubilized, cytoplasmic membrane proteins from LB-grown cells were affinity-purified at different pH values of phosphate (pH 6.6, 7.0, 7.5) or Tris-HCl (pH 8.0) column buffer, as described in the text. All columns were washed with buffer of low ionic strength equivalent to that of 10 mM phosphate (pH 6.6) containing 0.01% Triton, 5 mM EDTA and 0.002% TSF. Lane 1 for each pH value represents proteins eluted by buffer with an ionic strength equivalent to that of 100 mM phosphate (pH 6.6) buffer. Lane 2 shows the proteins subsequently eluted from the same columns with 0.5 M sodium chloride.
* - solubilized cytoplasmic membrane proteins kept at 4°C overnight prior to affinity chromatography.

94
67
43
30
20.1
14.4

CM 1 2

-53K
-42K
-35K
-17K

94
67
43
30
20.1

1 2 1 2 1 2 1 2 1 2

pH 6.0 6.5 7.0 7.5 8.0

affinity columns equilibrated in 10 mM phosphate, pH 7 buffer. The columns were washed with the same buffer and then were eluted with 100 mM phosphate buffer. This increase in ionic strength eluted the 42K, 35K and 17K proteins from the column. When 0.2 M succinate was subsequently passed through the column, the 53K protein was eluted. Therefore, the 53K protein was able to bind more tightly to the aspartate-Sepharose column, for whatever reason, since it required a higher ionic strength buffer for elution than did the other three proteins. As such, it was a good candidate for a cytoplasmic membrane succinate binding protein.

This relationship between column protein binding and buffer ionic strength was investigated further. In one experiment, Triton-solubilized, LB-grown membranes were run on affinity columns equilibrated with 10 mM phosphate buffer (pH 7), containing 0.05% Triton, 5 mM EDTA and 0.002% TSF. The columns were washed with increasing concentrations of phosphate buffer (from 25 to 75 mM) and were then eluted with succinate. It was observed that the 42K, 35K and 17K proteins were washed off the column when the buffer concentration was raised to 50 mM, whereas the 53K protein remained bound to the column. It was reasoned that if the interaction of the 53K protein with the aspartate-Sepharose column was truly an affinity interaction and not just an ionic attraction, then a lower amount of succinate than 0.2 M should be able to remove the protein from the column. In another experiment, solubilized membranes were loaded onto an aspartate-Sepharose column under the conditions described above, and the column was washed successively with 20 and 50 mM succinate. The 53K protein remained bound to the column and was only eluted with 200 mM succinate. The implications of the tight

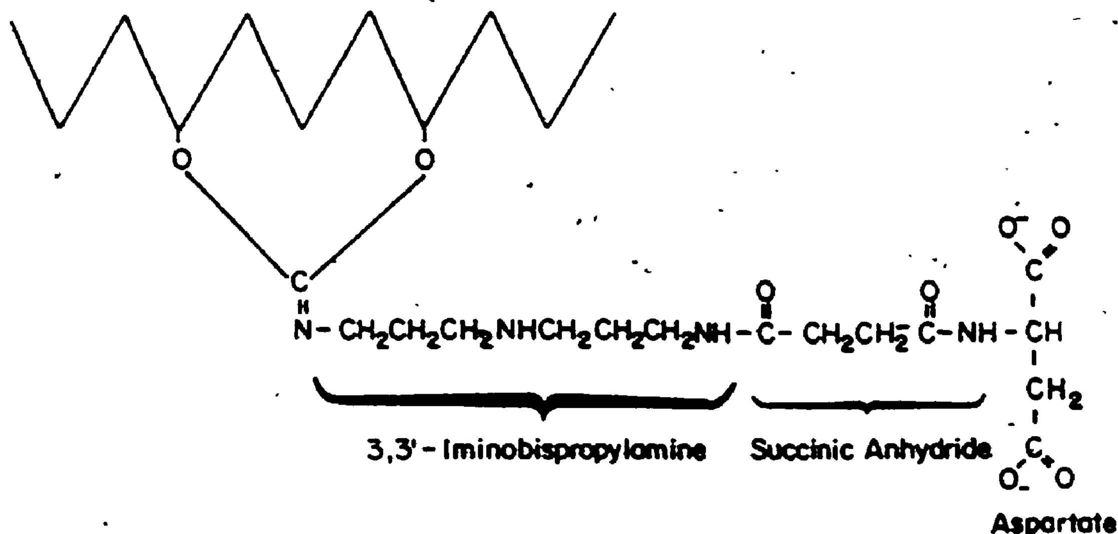
interaction of this protein with the affinity column will be addressed in a later section.

(iii)pH Effect on Column Binding - In order to further address the question of an affinity versus an ionic interaction between the aspartate-Sepharose column and the four isolated proteins, affinity columns of Triton-solubilized membrane proteins were run with buffers of different pH values. The results of this experiment are presented in Figure 9 and the rationale for the experiment is shown in Figure 10. The affinity column used in this work consists of aspartate residues covalently linked via a spacer group to Sepharose 4B, as described in Experimental Procedures (Figure 10). The aspartate molecule contains two carboxyl groups and one amino group which are ionized at physiological pH (23). Even though the amino group is tied up in a covalent linkage in the aspartate-Sepharose, the free carboxyl groups contribute a net negative charge to the affinity resin at the pH of the column buffer. Proteins also possess net charges dependent on their isoelectric points and the pH of their environment (23). It is possible that the proteins which bind to the affinity column have a net positive charge at the pH used in the experiments (ie. the proteins are below their isoelectric points) and they only bind to the column because of an ionic interaction.

To ensure that pH was the only parameter that was changed in the experiment, the ionic strength of the various buffers was held constant. A 10 mM, pH 6.6 phosphate buffer was used as the starting point. The Henderson-Hasselbach equation (23) was then used to calculate the concentration of pH 7.0 and 7.5 phosphate and pH 8.0 Tris buffers having the same ionic strength as the 10 mM, pH 6.6 phosphate buffer. The buffer concentrations used to wash the four columns were: (i)10 mM phos-

Figure II-10. Schematic representation of aspartate-Sepharose. This figure shows the structure of the aspartate-coupled Sepharose used in the affinity column studies. A 3,3'-iminobispropylamine-succinic anhydride spacer group was attached to cyanogen bromide-activated Sepharose 4B as described in Experimental Procedures. Aspartate was then covalently attached via its amino group to the substituted Sepharose. Both carboxyl groups of aspartate were ionized at the pH values used in these studies.

Table II-II. Physical characteristics of various detergents. The critical micellar concentrations (CMC) and micelle molecular weights (MMW) of the detergents used in this study are listed in this table. The detergent concentrations used for cytoplasmic membrane solubilization and affinity chromatography are also listed for each detergent.



Physical Characteristics of Various Detergents

Detergent	CMC	MMW +	Solubilization Concentration	Column Buffer
Triton X-100	0.02% ++	90,000	1%	0.05% or 0.01%
Lubrol PX	0.006% *	64,000	1%	0.01%
Polyoxyethylene Ether W1	ND	ND	1%	0.01%
Octylglucoside	0.73% **	68,000	0.44% (15 mM)	0.44%
CHAPS	0.49% +++	6150	1%	0.05% or 0.01%

+ (30)
++ (22)
* (29)
** (26)
+++ (28,30)

phate (pH 6.6), (ii)8 mM phosphate (pH 7.0), (iii)6.7 mM phosphate (pH 7.5) and (iv)26.7 mM Tris-HCl (pH 8.0). The columns were then eluted with ten times strength buffer and finally with 0.5 M sodium chloride. It was observed that 0.5 M sodium chloride eluted the same proteins from the column as did 0.2 M succinate in previous experiments. However, a 30K protein was also eluted along with the 53K and 42K proteins by sodium chloride (Figure 9). This result was also observed in several other experiments when either succinate or sodium chloride was used as the column eluant and will be discussed later. The use of sodium chloride prevented the need for removal of succinate from the column fractions prior to [^{14}C]-succinate binding studies to be described later.

The first lane in Figure 9 for each pH value shows the proteins eluted by ten times strength buffer and the second lane is the sodium chloride elution profile. For the pH 6.6, 7.0 and 7.5 buffers, the 35K and 17K bands were the major proteins eluted by the ten times strength buffer. The 17K protein no longer bound to the column at pH 8.0. These results suggest that the 17K protein may have approached its isoelectric point as the pH was raised to 8.0 and therefore its ionic interaction with the column was abolished. The 53K and 42K proteins were the major proteins that were eluted with sodium chloride at all pH values. Since these two proteins, along with the 35K protein, bound to the column at all the pH values, either they did have a true affinity for aspartate-Sepharose or their isoelectric points were above pH 8.0. Most proteins in E. coli have isoelectric points from pH 5.0 to 6.5 (24). As will be discussed in Chapter 3, all four of the proteins isolated from the aspartate-Sepharose column had isoelectric points below pH 8 and there-

fore their interaction with the column appeared to involve more than just an ionic attraction.

It should be noted that the protein profile of the first two lanes in Figure 9 was essentially the same as that of the second pair of lanes. The only difference between the two sets is that the membranes for the first set were kept at 4°C overnight prior to affinity chromatography, whereas the membranes for the latter set were frozen as per usual. This suggests that neither possible protein degradation in the 4°C sample nor freezing of the sample affected the interaction of the proteins with the column.

(iv)Detergent Effect on Protein Fractionation - It has been observed for some time that non-ionic or switterionic detergents are the agents of choice for gentle, non-denaturing solubilization of biological membranes (22). A survey of the literature reveals that the choice of detergent depends upon the particular enzyme or protein being studied. A detergent which efficiently extracts a certain enzyme in an active form may not extract another enzyme or it may inhibit its activity or denature it when extracted (25). Five different detergents were investigated in this work. Triton X-100, Lubrol PX, Polyoxyethylene Ether W1 and octylglucoside are all non-ionic detergents, whereas CHAPS is a zwitterionic detergent. All of the detergents, with the exception of octylglucoside, were used for membrane solubilization at concentrations well above their critical micellar concentration (CMC). This information is summarized in Table II. Octylglucoside has been shown to be effective at solubilizing membranes at concentrations very near to or even below its CMC (26,27). A concentration of 15 mM octylglucoside (its CMC is 25 mM) was shown to be effective in solubilizing cytoplasmic

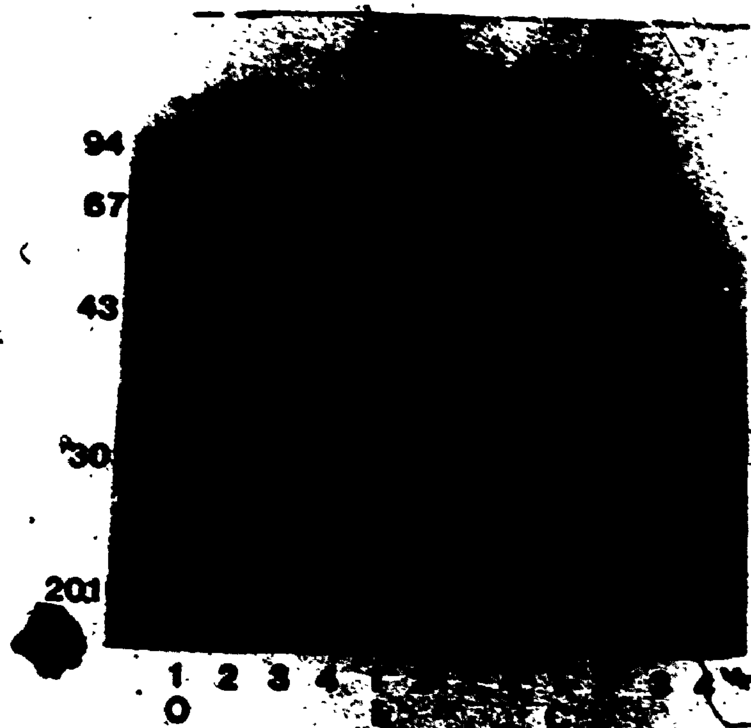
membrane vesicles, as discussed earlier in this chapter.

Figure 11 compares the results from cytoplasmic membranes solubilized and affinity-purified in the presence of octylglucoside, Lubrol PX or CHAPS. The columns were washed with 10 mM potassium phosphate (pH 6.6) and were then eluted successively with 100 mM phosphate and 0.5 M sodium chloride. Lanes 1 through 4 for each detergent show the outer membrane, cytoplasmic membrane and the proteins eluted from the affinity column with 100 mM phosphate and sodium chloride respectively. All three of the detergents had similar membrane protein profiles and showed good separation of outer and cytoplasmic membranes. The solubilization of the cytoplasmic membrane seemed to be more complete in this experiment than in the original experiments with the Kaback membrane vesicles (Table I). This could be due to the fact that solubilization was carried out at 37°C in this case as compared to 25°C for the vesicles. The insoluble material in the Kaback membrane vesicle preparations was likely due to the outer membrane contaminants.

Figure 11 shows that only the 35K and 17K proteins bound to the octylglucoside column; virtually no protein was eluted with sodium chloride. Although octylglucoside seemed to solubilize the cytoplasmic membrane effectively, it appeared to inhibit the binding of the 53K and 42K proteins to the affinity column. In contrast, the affinity column profile for Lubrol PX was very similar to that of Triton X-100 discussed earlier. The 42K, 35K and 17K proteins were eluted with 100 mM phosphate and the 53K protein was eluted with sodium chloride, although the level of this latter protein was relatively low. An earlier experiment with Lubrol PX also indicated some contamination with outer membrane proteins in its affinity column protein profile. Finally, the major

Figure II-11. Detergent effect on protein fractionation. LB-grown cytoplasmic membranes were solubilized with three different detergents and their affinity-purified proteins were compared. The detergent concentrations used for cytoplasmic membrane solubilization and affinity chromatography are listed in Table II-II. Each column was washed with 10 mM phosphate buffer (pH 6.6) containing 5 mM EDTA, 0.002% TSF and detergent and was then eluted successively with 100 mM phosphate buffer (pH 6.6) and then 0.5 M sodium chloride.
Lanes 1-4 for each detergent are: 1 - outer membrane, 20 ug; 2 - cytoplasmic membrane, 200 ug; 3 - 100 mM phosphate-eluted proteins; 4 - 0.5 M sodium chloride-eluted proteins.
O - octylglucoside; L - Lubrol PX; C - CHAPS.

Figure II-12. Phospholipid effect on membrane solubilization and protein fractionation. LB-grown cytoplasmic membranes were solubilized with 1% Triton X-100 in the absence (-PL) or presence (+PL) of 3 mg/ml of E. coli phospholipids as described in Experimental Procedures. All of the columns were equilibrated in 10 mM potassium phosphate buffer (pH 6.6) containing 0.01% Triton, 5 mM EDTA and 0.002% TSF, except where Na indicates sodium phosphate buffer and 0.05% indicates 0.05% Triton in the column buffer. Lanes 1 and 2 for each column indicate elution by 100 mM phosphate and 0.5 M sodium chloride respectively.
CM - cytoplasmic membrane.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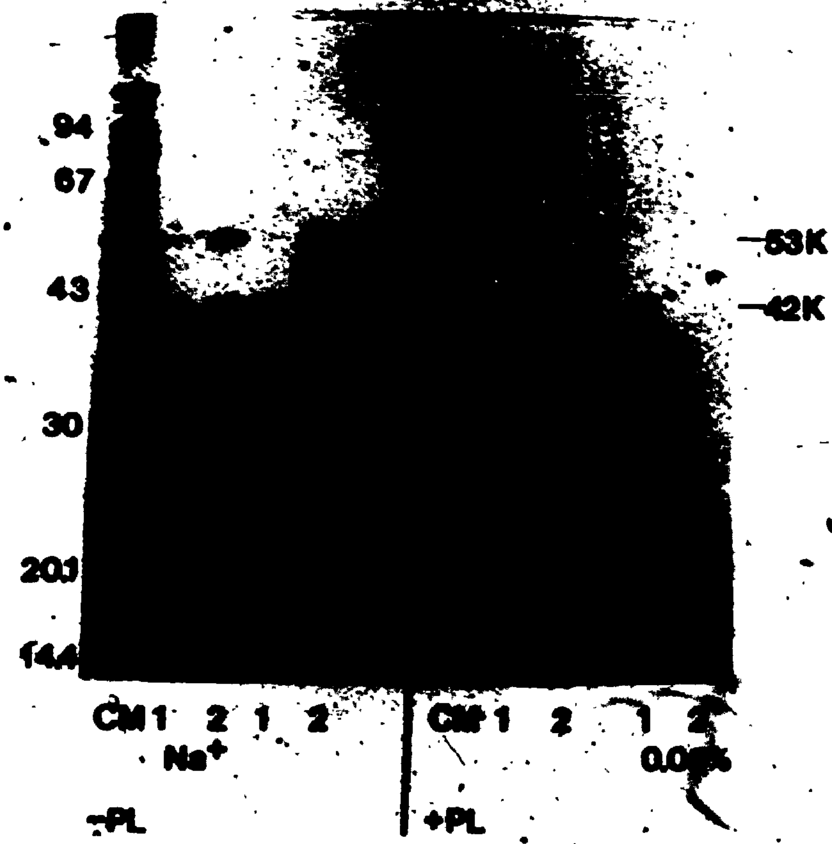
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43
30
20.1
14.4
53K
42K
CM 1
2
1
2
Na+
CM 1
2
1
2
-PL
+PL

protein eluted with 100 mM phosphate from the CHAPS column was the 17K band; the 35K band was very faint. Sodium chloride eluted both the 53K and 42K proteins from the CHAPS column and the yield of the 53K protein appeared to be higher than for the Lubrol column. A fourth detergent, Polyoxyethylene Ether W1, which is structurally similar to Lubrol PX, was investigated in a separate experiment (data not shown). Its affinity column protein profile, however, was very different from that of Lubrol; the 35K band was absent, while the 53K and 17K proteins were present in very low amounts.

It should be noted that the detergent concentrations of the column buffers used in this experiment and in Figure 9 were 0.01% (except for octylglucoside), whereas 0.05% detergent was used in all of the earlier experiments. In the case of Triton, when the detergent concentration was decreased from 0.05% to 0.01%, the 42K protein moved from the 100 mM phosphate elution lane to the sodium chloride lane (Figure 9). The Lubrol 42K protein remained in the 100 mM phosphate lane when 0.01% detergent was used (Figure 11), while the CHAPS-extracted 42K protein was eluted with sodium chloride regardless of the detergent concentration in the buffer. These results indicated that the affinity of the 42K protein for the aspartate-Sepharose column was influenced by the type and concentration of detergent used. In contrast, the 53K protein was only eluted with sodium chloride (or succinate) regardless of the concentration of Triton or CHAPS in the affinity column buffer.

(v)Phospholipid Effect on Membrane Solubilization and Protein Fractionation - When integral membrane proteins are extracted from their *in vivo* lipid environment, they often lose their native conformation even when mild, non-ionic detergents are used. This change in confor-

mation may result in a loss of biological activity as well. Therefore, purification of the protein by affinity methods and assays of its function might not be possible. For this reason, some membrane proteins have been extracted or assayed after extraction in the presence of exogenous phospholipids (18,30). The phospholipids that were used were those found *in vivo* with the protein of interest and they were thought to preserve the conformation and hence the activity of the extracted protein. Phospholipids were added during the solubilization of CBT43 cytoplasmic membranes in order to determine if any additional proteins would bind to the aspartate-Sepharose column.

Cytoplasmic membranes were solubilized with 1% Triton in the presence or absence of acetone/ether-washed *E. coli* phospholipids at a concentration of 3 to 8 mg phospholipid per milligram of membrane protein. The lipids were extracted as described in Experimental Procedures. The solubilized membranes were then run on affinity columns washed with 10 mM phosphate buffer containing 0.01% Triton and proteins were eluted first with 100 mM phosphate, then with 1 M sodium chloride. The SDS gel profiles of total cytoplasmic membrane proteins solubilized with or without added phospholipids were very similar (Figure 12). The OD_{280} profiles of the columns from the Triton plus phospholipid-solubilized proteins were similar to that of samples without phospholipids. However, very little Coomassie-stained protein was evident in the lanes from the phospholipid samples. This occurred whether the proteins were ether-washed, then acetone-precipitated or just acetone-precipitated following TCA precipitation. Either the proteins were more soluble in the ether or acetone due to the presence of the added phospholipids, or they did not stain with Coomassie because the phospho-

lipids were not completely removed prior to electrophoresis. In any case, Figure 12 indicates that both the 53K and 42K proteins bound to the affinity column when membranes were solubilized with added phospholipids and that the 42K protein was eluted with 100 mM phosphate when the concentration of Triton was raised to 0.05% in the column buffer. A 0.75 mm silver-stained gel confirmed the fact that the same set of proteins bound to the aspartate-Sepharose column for membranes solubilized in the presence or absence of phospholipids. Therefore, subsequent experiments did not use added phospholipids.

(vi)Effect of Potassium, Sodium and Magnesium Ions on Column Binding - In whole cell transport studies with both LB and M9-grown CBT43 cells, it was observed that succinate transport was much higher when the cells were assayed in potassium phosphate instead of sodium phosphate buffer (31). Therefore, the effect of sodium versus potassium ions on binding of solubilized cytoplasmic membrane proteins to the aspartate-Sepharose column was investigated. The left-hand lanes of Figure 12 (-PL) show the results of the experiment. Both columns were run with 10 mM phosphate buffer containing 0.01% Triton. The proteins eluted with 100 mM phosphate and 0.5 M sodium chloride were essentially the same, except for minor changes in band intensity, whether sodium or potassium phosphate buffer was used. The only noteworthy differences were a decreased level of the 35K protein and a slight increase in the amount of the 53K protein eluted from the potassium phosphate column. Subsequent experiments used potassium phosphate buffer in case the potassium ion had an activating effect on succinate binding similar to that seen in whole cell transport.

Most of the affinity column experiments included 5 mM EDTA and

0.002% TSF in the column buffers to inhibit any magnesium-dependent or serine proteases. To account for the possibility that divalent cations might be necessary for the activity of the succinate binding proteins, affinity columns of CHAPS-solubilized proteins were run with either 5 mM magnesium chloride or 5 mM potassium EDTA in the column buffers. An SDS gel from this experiment showed no difference in the proteins that bound to the column in magnesium or EDTA-containing buffers, so EDTA was included in further experiments as a precaution against proteolysis.

Desalting of Succinate Binding Proteins - The protein profiles presented in the preceding sections consisted mainly of proteins which were eluted from the affinity column and were then immediately TCA precipitated in preparation for SDS gel analysis. In order to determine if these proteins were indeed succinate binding proteins, it was necessary to do binding assays with [^{14}C-2,3]-succinic acid. The proteins were eluted from the affinity column with a high ionic strength buffer, either 100 mM phosphate, 0.2 M succinate or 0.5 M sodium chloride. High ionic strength buffers might interfere in the binding of succinate to the proteins, since succinate itself is a charged molecule and charge might play a role in the affinity of the binding proteins for their substrate. Therefore, the protein preparations had to be "desalted" prior to any binding assays. Several desalting methods were investigated. They included column desalting using Sephadex G25 and Biogel P6DG gels, Amicon ultrafiltration desalting and dialysis.

Sephadex G25 is a carbohydrate polymer in the form of porous beads which exclude molecules with molecular weights of roughly 25,000 Daltons and higher. It was tried in both columns and batchwise in a centrifugation method to desalt the protein samples. In the latter

case, it did not prove to be an effective means of desalting and in both cases the recovery of protein from the G25 was very low, presumably due to nonspecific binding to the gel.

Biogel P6DG is composed of polyacrylamide beads which exclude molecules above 6000 Daltons molecular weight. It was found to be a fast, effective means for desalting the protein samples. Although it also tended to bind the proteins nonspecifically, the yield from the P6DG was better than for the G25. It was also expected that the P6DG would allow for better recovery of the whole range of proteins eluted from the affinity column, since P6DG's molecular weight exclusion limit is much lower than that of the G25.

Figure 13 shows the elution profiles from both the aspartate-Sepharose and P6DG columns for M9-grown, Triton-solubilized CBT43 membranes. The cells were labelled with [^{35}S]-sulphate in order to follow the recovery of the proteins in the various steps. Typically, the 100 mM phosphate and 0.5 M sodium chloride peaks from the affinity column were pooled, then concentrated down to around 5 ml each with a YM5 or YM10 Amicon ultrafiltration membrane. Each sample was then loaded onto a 30 ml P6DG column (1.5 cm ID) equilibrated in 50 mM potassium phosphate, pH 6.6 and 0.01% Triton at 4°C. The columns were eluted with the same buffer and the protein-detergent micelles emerged in the void volume of the column while the salts were eluted later. Protein was followed by OD_{280} measurements or, in this case, [^{35}S]-DPM and salt was followed with a conductivity meter. The recoveries of the 100 mM peak from the Amicon and P6DG column were 69% and 75% respectively, while the NaCl peak recoveries were 79% and 51%.

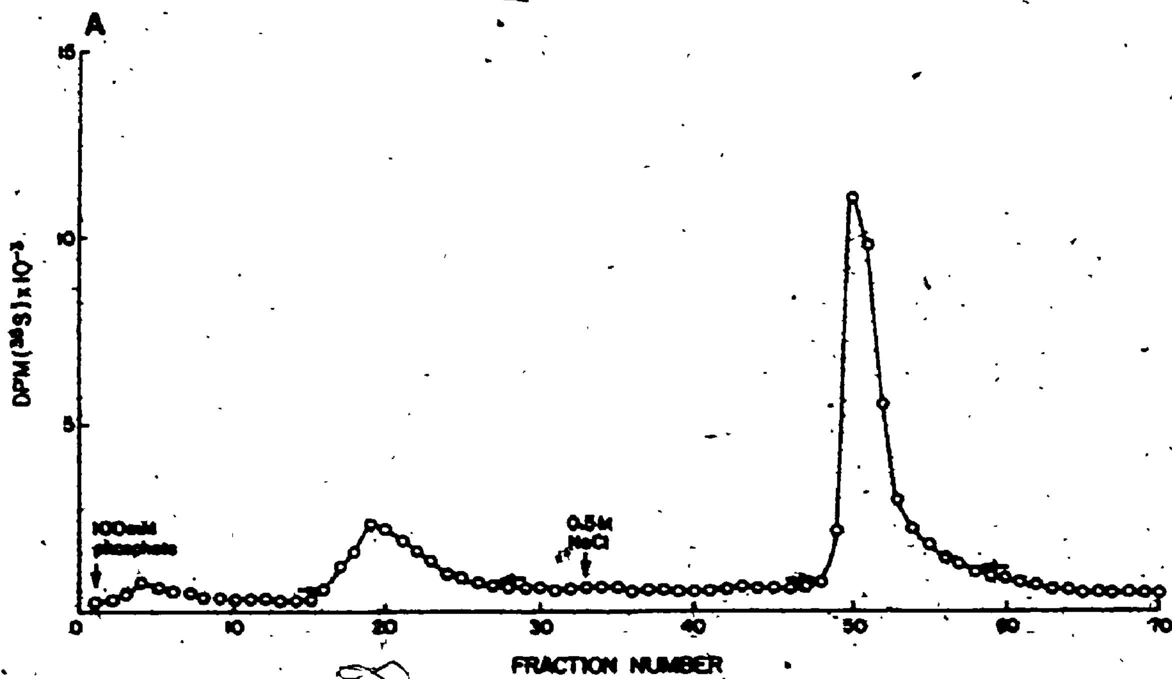
Although the recovery of the NaCl peak from the P6DG column was

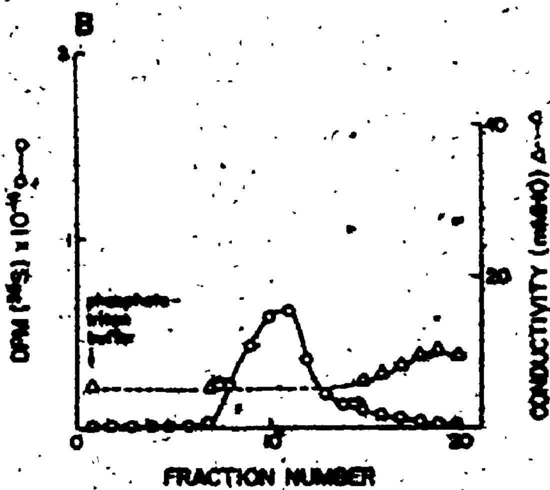
Figure II-13. Aspartate-Sepharose and Biogel P6DG column profiles.

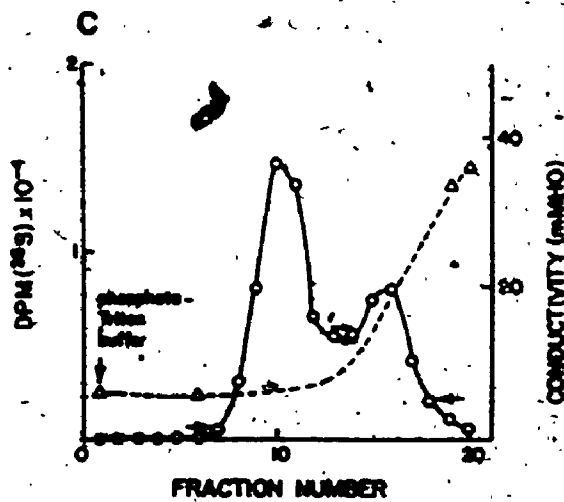
A - [^{35}S]-labelled, Triton-solubilized cytoplasmic membranes were loaded onto an aspartate-Sepharose column equilibrated with 10 mM potassium phosphate buffer (pH 6.6) containing 5 mM EDTA, 0.01% Triton and 0.002% TSP. Protein was eluted first with 100 mM phosphate buffer, then with 0.5 M sodium chloride in the same buffer and was detected by measuring the radioactivity ([^{35}S]-DPM) in each column fraction.

B - The 100 mM phosphate peak from A was concentrated on a YM10 Amicon ultrafiltration membrane and was desalted on a 30 ml Biogel P6DG column as described in Experimental Procedures. The column was washed with 50 mM potassium phosphate buffer (pH 6.6) containing 0.01% Triton. Protein was followed by [^{35}S]-radioactivity and salt was followed by conductivity.

C - The sodium chloride peak from A was also desalted on the Biogel P6DG column.


A
DPM (35S) x 10^-3
100mM phosphate
0.5M NaCl
FRACTION NUMBER

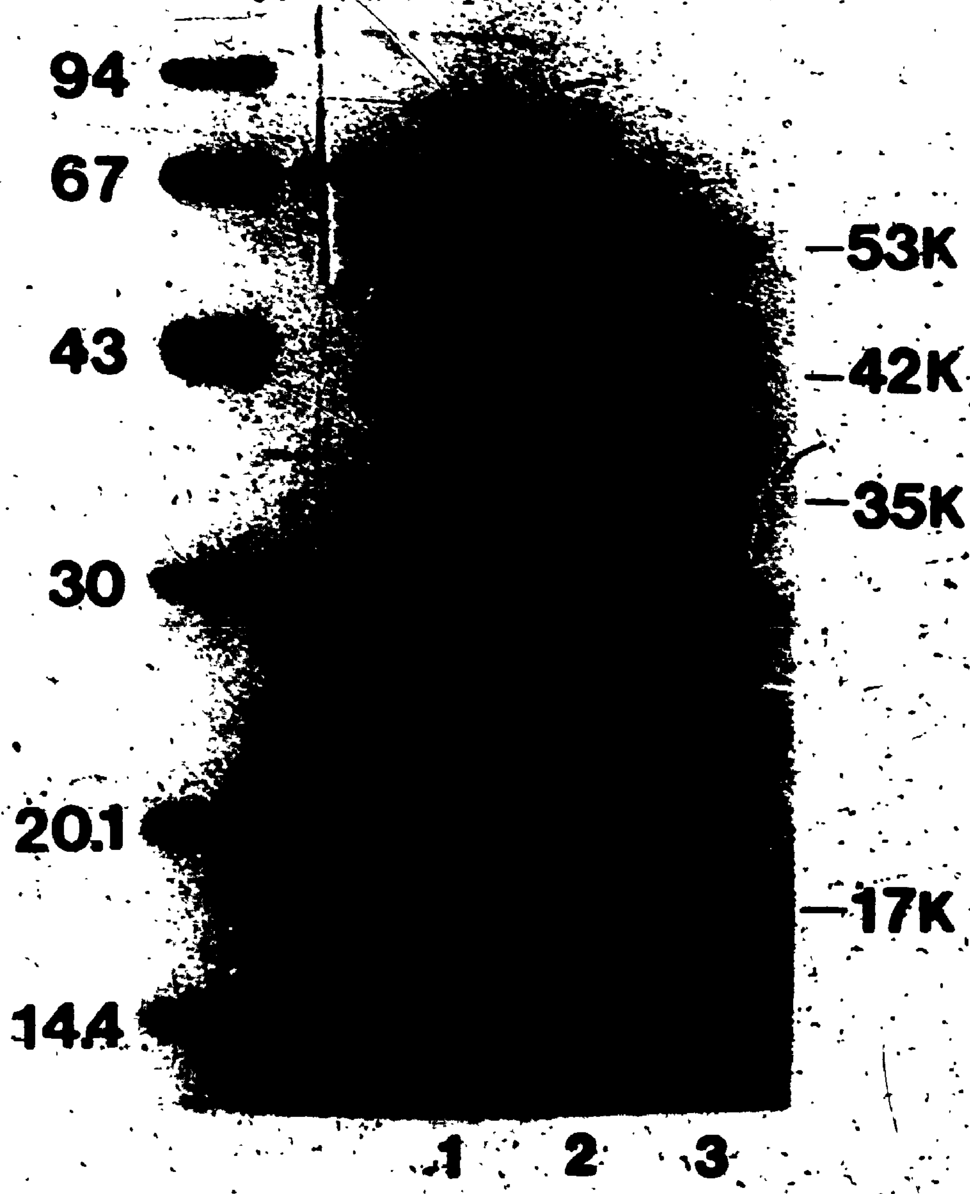

B
phosphate-triton buffer
FRACTION NUMBER
CONDUCTIVITY (mMHO)


C
phosphate-Triton buffer
FRACTION NUMBER
CONDUCTIVITY (mMHO)

respectable, Figure 13C shows that a second peak of protein emerged from the column directly underneath the salt peak. When the various protein peaks were examined on an SDS gel, it became apparent that one of the protein species was being preferentially bound to the column. Figure 14 shows the first and second NaCl peaks eluted from the P6DG column. Both the 53K and 42K proteins were retained by the column somewhat and were present in both the first and second peaks. The molecular weight of the "42K" protein that was retained by the P6DG column was slightly higher than that of the protein eluted in the first peak. This 42K doublet was observed in several other experiments as well, but more often it appeared as a single molecular weight species. However, the 17K protein was only present in the second peak and was therefore unavailable for binding studies carried out with the first peak. This result may explain the absence of the 17K protein in the early experiments (Figures 5 to 7) where P6DG desalting was routinely used prior to SDS gel electrophoresis. It should also be noted that the 17K protein was present in the sodium chloride peak in this particular experiment, instead of the 100 mM peak as in many of the previous figures. This "movement" of the 17K protein between the 100 mM and NaCl peak was observed in several other experiments. This suggested that it might be a degradation product of one of the other affinity-purified proteins and its presence in either fraction may have depended on whether proteolysis occurred before or after affinity chromatography.

Due to the loss of protein on the P6DG column, Amicon ultrafiltration membranes (YM5 or YM10) were used in further experiments to concentrate and desalt the protein samples. Pooled affinity column fractions were concentrated and diluted several times in the Amicon unit

Figure II-14. Desalted affinity column proteins. This gel shows the 100 mM phosphate and 0.5 M sodium chloride proteins after elution from the P6DG desalting columns described in Figure II-13.
Lane 1 - desalted 100 mM phosphate proteins; 2 - desalted NaCl proteins (first P6DG peak); 3 - desalted NaCl proteins (second P6DG peak)


94
67
43
30
20.1
14.4
53K
42K
35K
17K
1
2
3

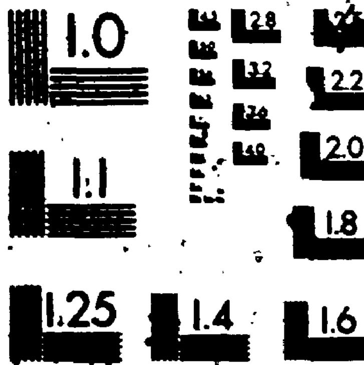
with buffer in order to lower the ionic strength of the samples prior to binding studies. When CHAPS was used instead of Triton X-100 in solubilization and purification, samples were concentrated via the Amicon and then were desalted by dialysis overnight at 4°C in the appropriate buffer. Dialysis removed excess detergent from the sample in addition to lowering its ionic strength. However, dialysis could not be used with Triton since it has a low CMC (0.015%) and forms very large micelles (90,000 Daltons molecular weight) (22). In contrast, CHAPS' CMC is much higher (0.49%) and its micelles are much smaller (6000 Daltons molecular weight) (28), so it can be readily removed by dialysis. To prevent any loss of the 17K protein, Spectrapor-3 dialysis tubing with a molecular weight cutoff of 3500 Daltons was used.

Succinate Binding Assays - Several different binding assays were used to determine if the proteins isolated by the aspartate-Sepharose column were succinate binding proteins. These included P6DG column binding, proteoliposomes, nitrocellulose-filtration and equilibrium dialysis. Routinely, solubilized cytoplasmic membranes (20 mg) from 6 litres of LB-grown cells (10 - 12 gm wet weight) were loaded onto four 40 ml aspartate-Sepharose columns (1.5 cm ID). The columns were washed with 10 mM potassium phosphate, pH 6.6 buffer and then 100 mM phosphate and 0.5 M sodium chloride peaks were eluted. The column buffers included 5 mM potassium EDTA and 0.002% TSF along with either 0.05% or 0.01% detergent. As mentioned earlier, succinate was avoided as an eluant for the affinity column if binding studies were to follow, since its removal via desalting might not be complete. Even a small residual amount of "cold" succinate might interfere with the binding of radioactive succinate to the proteins.

Either Triton X-100 or CHAPS-solubilized proteins were used for the binding studies. The average yield of protein eluted from the affinity column per gram wet weight of cells was 31 ug for the 100 mM phosphate-eluted peak (0.01% Triton of CHAPS) and 11 (CHAPS) to 20 ug (Triton) for the NaCl-eluted peak. Both detergents were shown to extract the same set of affinity column proteins (with the exception of the 35K protein with CHAPS), with little or no outer membrane contamination. However, the two detergents are chemically and physically very different (22,28). The proteins might then be expected to behave differently with respect to conformation and substrate binding ability in each detergent. The peaks eluted from the affinity column were concentrated and desalted as described above and were then stored on ice at 4°C prior to the binding assays. It should be mentioned that SDS gel profiles of the 100 mM and NaCl proteins kept on ice during the binding studies were not any different than those of samples that were TCA precipitated immediately after affinity chromatography. Therefore, proteolytic degradation did not appear to be a problem during the binding assays. The fractions tested for binding activity were the original membrane-detergent extracts, the proteins washed through the affinity column and the 100 mM phosphate and 0.5 M sodium chloride peaks eluted from the column.

(i)<u>P6DG Column Binding</u> - This binding assay was only used for the Triton-solubilized proteins because the CHAPS-solubilized proteins interacted too strongly with the Biogel P6DG, as indicated by column recovery studies with [^{35}S]-labelled, CHAPS-extracted proteins. This assay measures both bound and free substrate, in this case [^{14}C]-succinate. It makes use of the fact that the protein (or protein-

2



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

detergent micelles) and any bound substrate are eluted in the void volume of the column, whereas the free substrate is retarded by the column. The molar binding ratio of substrate to protein can be determined from the substrate's specific activity and the amount of protein in the assay. This assay is dependent upon the ability of the protein-substrate complex to remain intact during passage through the P6DG column. This ability is in turn dependent upon the K_d of binding of the complex. Therefore, a compromise has to be made between a column long enough to separate the bound and free substrate, but short enough to allow minimal disruption of the protein-substrate complex.

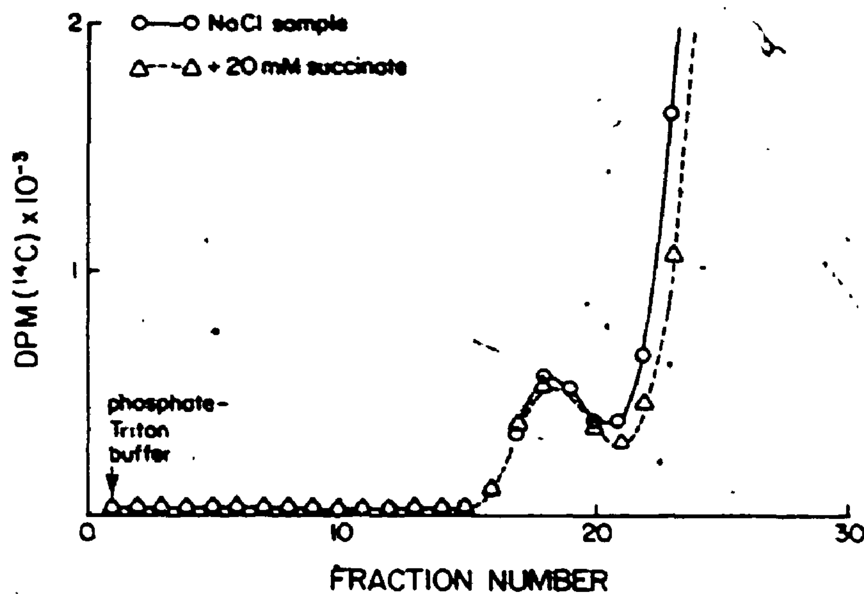
As discussed earlier, the recovery of the affinity-purified proteins from the P6DG column was not 100%. So, the first step was to determine the optimal conditions for recovery of the proteins from the P6DG. This was done with [^{35}S]-labelled affinity-purified proteins. The conditions that gave both the best recovery of protein and the best separation of protein and free succinate were a 5 ml P6DG (1 cm ID) column equilibrated with 100 mM potassium phosphate, pH 7.5 buffer containing 0.01% Triton. Fractions of 3 drops were collected with an LKB Redi-Rac fraction collector. These conditions gave 86% and 34% recoveries for the 100 mM and NaCl peaks respectively. The high ionic strength of the 100 mM phosphate buffer may have interfered with the binding of the succinate to the protein, so 50 mM phosphate was used instead. This resulted in a decrease in the recovery of the proteins from the column to 68% and 25% for the 100 mM and NaCl peaks respectively.

The binding assay was carried out as described in Experimental Procedures. Just prior to binding, the affinity-purified, desalted

proteins were concentrated down to a final volume of around 1 ml in a 10 ml Amicon unit. Binding was carried out for 60 minutes with both 200 uM and 20 uM carrier-free [^{14}C-2,3]-succinic acid. The specificity of binding was determined by the addition of "cold" or unlabelled 20 mM or 2 mM succinate to the samples 10 minutes prior to loading them onto the P6DG column. The columns were washed with 1 M sodium chloride in-between samples and then were equilibrated with column buffer in order to remove any bound proteins. The results of one such binding experiment are presented in Figure 15 and Table III. Figure 15 is a plot of the binding of 200 uM [^{14}C]-succinate to the NaCl protein sample with and without the addition of cold 20 mM succinate. The cold succinate did not compete out any of the binding even though it was 100 times in excess of the radioactive succinate. This was true for the 200 uM and 20 uM succinate binding of both the 100 mM and NaCl samples, as seen in Table III. Average molecular weights of 26K (35K and 17K) and 47.5K (53K and 42K) Daltons were used to calculate the molar ratios of protein:succinate for the 100 mM and NaCl samples respectively. Ideally, one would expect the molar ratio to be close to 1 in the case of a purified succinate binding protein, assuming one binding site per protein molecule. However, this does not take into account the fact that the protein may be inactivated or its conformation may be changed to a certain degree during the purification process, as will be discussed later. In any case, although the molar ratios were much greater than 1 for both the 100 mM and NaCl samples (Table IIIA), it should be noted that the NaCl sample had a much higher (5 to 6 times) succinate binding capacity, as indicated by a lower protein:succinate ratio, than did the 100 mM sample.

Figure II-15. Succinate binding assay: P6DG column profile. Triton-solubilized, affinity-purified proteins were desalted as shown in Figures 13 and 14 and were assayed for succinate binding activity as described in Experimental Procedures. This graph shows the co-elution of bound 200 uM [^{14}C]-succinate with the NaCl peak from a 5 ml P6DG column equilibrated in 50 mM potassium phosphate buffer (pH 7.5) containing 0.01% Triton X-100. Competition of binding by 20 mM "cold" succinate was also measured. The data from these binding experiments are summarized in Table III.

Table II-III. Succinate binding of affinity column fractions: P6DG column assay. Triton-solubilized, affinity-purified proteins were assayed for 200 and 20 uM [^{14}C]-succinate binding activity as described in Experimental Procedures and illustrated previously in Figure II-15. Table IIIA shows the amount of succinate bound (picomoles) by each protein fraction and the competition of that binding by 100 times excess succinate. The molar ratios of protein:succinate were calculated as described in the text. Table IIIB shows the binding of 200 and 20 uM [^{14}C]-succinate to 100 mM phosphate and 0.5 M sodium chloride control samples as described in the text. This binding was subtracted from that of the 100 mM and sodium chloride samples to yield the corrected protein:succinate ratios.



Succinate Binding of Affinity Column Fractions: PGDG Column Assay

A Sample	Succinate Bound (pmoles) 200 uM	20 uM	Molar Ratio of Protein:Succinate 200 uM	20 uM
100 mM peak (691 pmoles of protein)	27.6	18.9	25:1	37:1
+ 100x cold succinate	25.9	18.1	27:1	38:1
NaCl peak (83 pmoles of protein)	17.6	13.4	4.7:1	6.2:1
+ 100x cold succinate	20.1	13.8	4.1:1	6.0:1

B Sample	Succinate Bound (pmoles) 200 uM	20 uM	Ratio of 200/20 uM	Corrected Molar Ratios 200 uM	20 uM
100 mM peak (691 pmoles of protein)	27.6	18.9	1.46	25:1	37:1
100 mM blank	15.6	7.4	2.11	-	-
Corrected sample	12.0	11.5	1.04	58:1	60:1
NaCl peak (83 pmoles of protein)	17.6	13.4	1.31	4.7:1	6.2:1
NaCl blank	10.5	7.4	1.42	-	-
Corrected sample	7.1	6.0	1.18	12:1	14:1

It was noted that the concentrated samples were always opaque and their absorbance at 280 nm (OD_{280}) was always greater than 1, regardless of the original detergent concentration used or the method of desalting. The concentrated protein contributed to part of the OD_{280}, but the phenyl group of Triton X-100 also absorbs at 280 nm. Along with the protein, the Triton was concentrated by the Amicon because its micelle molecular weight is so large (22). To ensure that the succinate binding seen in the P6DG column assay was indeed due to the protein and not just nonspecific binding or trapping of succinate by the Triton micelles, a control experiment was carried out. Blank volumes of 100 mM phosphate and 0.5 M sodium chloride buffers were concentrated and desalted in exactly the same manner as the protein samples. Succinate binding to the concentrated, desalted buffers was then measured. Table IIIB shows the results of that experiment. When the binding due to the concentrated buffers was subtracted from the binding of the protein samples, the molar ratios of protein to succinate became larger. Clearly, concentrated Triton micelles contributed to the succinate binding of the protein samples. However, even when this control value was subtracted from the protein samples, the NaCl sample still showed a higher succinate binding capacity (4 to 5 times higher) than did the 100 mM sample. The amount of succinate binding by the 100 mM and NaCl blanks may in fact be an overestimation of the nonspecific binding by detergent micelles in the protein samples, since micelles form more readily in the absence of protein (22). It is also interesting to note that when the corrected values for the amount of 200 uM versus 20 uM succinate bound were compared, the ratio of 200 uM/20 uM succinate bound was very nearly 1 for both samples. This suggested that this corrected component of

binding did not reflect nonspecific binding or trapping of succinate by the protein-detergent micelles, since binding was already saturated at the 20 uM succinate concentration.

(ii)Proteoliposome Binding Assay - In order to compensate for the possible loss of conformation and biological activity in the extracted, affinity-purified proteins, attempts were next made to reconstitute the proteins into liposomes. This has been done successfully for the lactose permease of E. coli (1,15,18) and recently for two other bacterial membrane transporters (32,33). The yield of phospholipids extracted from E. coli was too low (60 mg from 6 litres of cells) to be used routinely, so a mixture of the three major phospholipids found in the cytoplasmic membrane was prepared from commercially-purified lipids. A ratio of 70:15:15 for phosphatidylethanolamine, phosphatidylglycerol and cardiolipin respectively was used to approximate in vivo conditions (15). Liposomes were prepared as described in Experimental Procedures (15) and then the affinity-purified proteins were used to prepare proteoliposomes by the octylglucoside dilution procedure (18). Both Triton and CHAPS-solubilized proteins were used in the reconstitution studies. The amount of 200 and 20 uM [^{14}C]-succinate bound to the proteoliposomes was measured using both membrane filtration (1) and centrifugation methods.

Several technical problems were encountered with the proteoliposome reconstitution and binding studies. Only a small fraction of the Triton-solubilized proteins were incorporated into the liposomes, as determined by a Lowry assay that indicated 3% and 18% incorporation of the 100 mM and NaCl samples respectively. An SDS gel showed that most of the 100 mM protein (35K, 17K) and a lot of the NaCl protein (53K,

42K) remained in the supernatant after centrifugation of the proteoliposomes. The results with the CHAPS proteins were somewhat better. Although a Lowry assay indicated that only 10% and 12% respectively of the 100 mM and NaCl proteins were incorporated into the liposomes, an SDS gel of the supernatants showed that the 17K protein seemed to be selectively incorporated into the 100 mM proteoliposomes. As well, no detectable protein remained in the supernatant from the NaCl proteoliposomes, indicating fairly complete incorporation of both the 53K and 42K proteins. The accuracy of the Lowry assay was in question since the liposomes were not completely digested, even with the addition of 1% SDS, and the samples had to be spun prior to measuring their absorbance. Finally, when the CHAPS proteoliposomes were assayed for succinate binding activity by either the centrifugation or filtration method, the background level of binding by blank liposomes was as high or higher than that of the proteoliposome samples. This background binding or trapping of succinate by the liposomes was high enough in some cases to obscure any expected binding due to the proteins. It was found that replicates of each assay condition were not reproducible for either binding method, although different combinations of incubation times and washings were tried. Therefore, the proteoliposome binding assay did not appear to be a useful approach for measuring succinate binding to the affinity-purified proteins and it was not investigated further.

(iii) Membrane Filtration Method - Another method that has been used successfully to demonstrate substrate binding for both periplasmic and membrane proteins is the membrane filtration method described in Experimental Procedures. This method was used for the HisJ binding protein of *Salmonella* sp. (19) and the aspartate chemotaxis receptor of *E. coli*

(30). It uses nitrocellulose filters to bind the protein-substrate complexes, so it is dependent upon the ability of the protein to adhere to the filter and on the ability of the complexes to survive filtration and washing.

For this system, only CHAPS-solubilized proteins were assayed due to the problems encountered earlier with nonspecific binding to the concentrated Triton micelles. Dialysis was used to remove excess detergent and salts from the concentrated CHAPS proteins. One problem that was encountered instead with these proteins was that the 100 mM and NaCl samples tended to precipitate out of solution after dialysis. Not much precipitation occurred when the original CHAPS extract or affinity column wash was dialyzed. This phenomenon was probably a result of the removal of excess detergent and/or phospholipid associated with the concentrated proteins before dialysis. However, the addition of exogenous phospholipids to the samples prior to dialysis did not correct the precipitation problem. After trying different phosphate and CHAPS concentrations and different dilutions of the samples in dialysis buffer, conditions were found that left a reasonable amount of protein in solution after dialysis. The affinity-purified proteins from 12 litres of LB-grown cells were concentrated to an OD_{280} of no more than 0.50 and then were dialyzed overnight at $4^{\circ}C$ against 100 volumes of 50 mM potassium phosphate, pH 6.6, 0.5% CHAPS buffer. The high concentration of CHAPS used in dialysis was diluted out with phosphate buffer in the binding studies to a final concentration of 0.1%. When the 100 mM and NaCl samples were centrifuged after dialysis, 33% and 88% respectively of the proteins remained in the supernatant. Even though the OD_{280} of the CHAPS extract and column wash were very high (2.7 and 1.5

respectively), about 90% of the proteins in these samples also remained in solution after dialysis. SDS gel analysis of the spun and unspun samples showed no qualitative difference in the protein pattern of each fraction.

Binding with 20 uM [^{14}C]-succinate was carried out with both the spun and unspun protein samples for 60 minutes at 25°C as described in Experimental Procedures. [^{35}S]-Labelled samples were used to determine the amount of protein bound to the nitrocellulose filters. It was found that 79% of the 100 mM protein and 77% of the NaCl protein bound to the filters in 50 mM potassium phosphate, pH 6.6 regardless of the detergent concentration. The results of the succinate binding studies are presented in Table IVA. The results, done in triplicate, were fairly reproducible. Molecular weights of 17K and 47.5K (42K, 53K average) were used to calculate the molar ratios for the 100 mM and NaCl samples respectively. Even though the majority of the protein remained in the supernatant for all of the samples, the binding of succinate by the spun samples was no higher than that of the blank. Combinations of different protein fractions were tried to see if a necessary cofactor in one fraction could stimulate binding in another fraction, but there was no difference in succinate binding in the combination samples. However, succinate binding was detected in the unspun samples, with the exception of the CHAPS extract. Two aspects of this binding were similar to that observed with the P6DG column assay. The NaCl sample again bound about 5 times more succinate than the 100 mM sample, as indicated by its lower protein:succinate ratio. In addition, this binding could not be competed out with the addition of 100 times excess cold succinate. This latter point will be discussed later.

Table II-IV. Succinate binding of affinity column fractions: Membrane filtration assay. CHAPS-solubilized, affinity-purified proteins were assayed for 20 uM [^{14}C]-succinate binding activity as described in the text. Binding was carried out for 60 minutes at 25°C. Table IVA compares the binding of succinate to both spun and unspun preparations of 100 mM and NaCl protein samples, as well as the 10 mM phosphate affinity column wash and the total CHAPS membrane extract. The data was corrected for the amount of protein bound to the nitrocellulose filters and the molar ratios of protein:succinate were calculated as described in the text. Table IVB shows the binding of succinate to the spun samples in the presence of 40% glycerol and 2.5 mg/ml *E. coli* phospholipids. The binding to the samples was corrected for the amount of succinate bound to the control.

Succinate Binding of Affinity Column Fractions:
Membrane Filtration Assay

A Sample	Succinate Bound (pmoles)				Molar Ratio of Protein: Succinate	
	protein	unspun	spun	protein	unspun	spun
CHAPS extract	-	1.4 ±0.3	1.4 ±0.4	-	-	-
Column wash	-	13.8 ±0.3	11.9 ±0.2	-	-	-
Corrected	-	12.0	0.1	-	-	-
100 mM peak	258 pmoles	5.9 ±0.8	1.6 ±0.2	85 pmoles	44:1	53:1
Corrected	-	4.1	-	-	63:1	
NaCl peak	45	5.5 ±0.8	2.0 ±0.1	40 pmoles	8.2:1	20:1
Corrected	-	3.7	0.2	"	12:1	200:1
Control	-	1.8 ±0.4	1.8 ±0.4	-	-	-

B Sample (+PL and glycerol)	Succinate Bound (pmoles)	Corrected (pmoles)	Molar Ratio of Protein: Succinate
CHAPS extract	6.8 ± 2.8	-	-
Column wash	46.4 ± 8.3	9.0	-
100 mM peak (85 pmoles of protein)	43.5 ± 4.2	6.1	14:1
NaCl peak (40 pmoles of protein)	40.8 ± 5.0	3.4	12:1
Control	37.4 ± 8.8	-	-

To ensure that the binding observed in the unspun samples was not due to uptake of succinate by contaminating bacteria, several control experiments were carried out. These included the use of bacterial growth inhibitors in the succinate binding assays and binding studies with several other radioactive substrates. In the early work on these transport components, equilibrium dialysis binding of succinate to the proteins was carried out in 50 mM phosphate, pH 6.6 buffer that contained 50 mM arsenate to inhibit bacterial growth (5). Arsenate acts as an analogue of inorganic phosphate and causes inhibition of oxidative phosphorylation (32). When a lower amount of arsenate (10 mM) was added to the protein samples in the membrane filtration method, it inhibited succinate binding to the 100 mM and NaCl samples to the extent of only 19% and 13% respectively, but it did not inhibit binding to the affinity column wash. This same concentration of arsenate inhibited whole cell 25 uM succinate uptake in CBT43 by 32% (31). The difficulty with using arsenate in this instance is that it was acting against a high background of phosphate buffer (50 mM) and therefore might not be able to effectively inhibit ATP synthesis. However, it was found that the addition of 50 mM sodium arsenate to the assay completely inhibited any binding of succinate to the unspun protein samples. More importantly complete inhibition of succinate binding to these samples was also observed when 50 mM sodium phosphate was added to the 50 mM phosphate assay buffer. This suggested that succinate binding can be affected by the total ionic strength (100 mM) of the added arsenate or phosphate in the assay buffer. Since 100 mM phosphate has no effect on succinate uptake by bacteria or membrane vesicles, this inhibitory effect showed that the observed association of succinate with the protein samples was

due to binding and not due to uptake by contaminating bacteria.

Another reagent the can inhibit bacterial succinate uptake is carbonyl cyanide m-chlorophenylhydrazone (CCCP), which acts to uncouple oxidative phosphorylation from the respiratory chain by collapsing the membrane proton gradient (33). CCCP (5 uM) completely inhibited binding to the unspun 100 mM and NaCl samples and inhibited binding to the column wash by 75%. It was demonstrated previously that 10 uM CCCP had no effect on binding of malate to the succinate transport components in intact cytoplasmic membrane vesicles (34). However, CCCP is also known to act as a sulfhydryl reagent and sulfhydryl groups were shown to be involved in succinate binding to the isolated transport components in earlier studies (5). It is conceivable that the inhibitory effect of CCCP on succinate binding could differ depending upon the conformation of the membrane proteins in their isolated versus in situ state.

Finally, incubation of the protein samples with radioactive 20 uM proline, glycine and aspartate showed no binding or transport of these substrates in any of the samples. This last observation suggested that the binding observed in the unspun samples was not due to succinate uptake into contaminating bacteria since neither of the three control substrates were bound or incorporated above their respective control values. One would expect that the above three amino acids would be taken up as readily or better than succinate if contaminating bacteria were responsible for the observed succinate binding.

When the E. coli aspartate chemotaxis receptor was tested for aspartate binding with the membrane filtration method, no binding was detected unless the binding was carried out in the presence of phospholipids and 40% glycerol (30). The K_d for aspartate binding with this

method was found to be 3 uM, the same order of magnitude as that of the dicarboxylate transport system (5). Succinate binding assays were carried out with the spun samples in 50 mM phosphate, pH 6.6 buffer containing 2.5 mg/ml E. coli phospholipids and 40% glycerol. All of the samples, with the exception of the CHAPS extract, bound succinate (Table IVB). Similar to the results observed in the proteoliposome binding assays, the results were not very reproducible and there was a high background of binding in the control. The same difficulty in filtering the samples was encountered as in the proteoliposome method. The molar ratios of protein:succinate were very similar for both the 100 mM and NaCl samples when the control value was subtracted. The addition of a 100 times excess of cold succinate did not compete out the succinate binding either. The inability to detect any succinate binding in the CHAPS detergent extract in either the presence or absence of phospholipids and glycerol may be explained by the low level of succinate binding proteins relative to other proteins in the extract. The fact that the apparent succinate binding was actually lower in the extract than in the phospholipid-glycerol control may be because of excess detergent in the extract that acted to "solubilize" the exogenous phospholipids and allowed the sample to filter.

(iv) Equilibrium Dialysis - The above-mentioned binding assays separate free and bound succinate and measure bound succinate directly. Equilibrium dialysis measures the change in free succinate due to binding by the sample and therefore measures bound succinate indirectly. It measures the equilibrium distribution over time of free substrate between two chambers separated by a dialysis membrane. Radioactive substrate is added to one chamber of the dialysis cell and the sample is

concentration of free substrate is changed and the equilibrium is shifted towards the chamber containing the sample. This was the method that was used in the early experiments with the succinate transport components (5). The buffer used in those experiments was 50 mM phosphate, pH 6.6 containing 50 mM arsenate and 0.1% Lubrol 17A-10. Binding of 50 uM [^{14}C]-succinate to the CHAPS-solubilized proteins was assayed in 50 mM potassium phosphate buffer (pH 6.6) containing 0.2% CHAPS at 4°C as described in Experimental Procedures. After 24 hours of incubation, a small amount of binding was observed in the affinity column wash, but neither the 100 mM nor NaCl samples bound succinate. An initial experiment showed that equilibration of succinate across the dialysis membrane was attained at 20 hours of incubation. When binding was carried out in the presence of 2.5 mg/ml E. coli phospholipids and 40% glycerol, equilibrium was not achieved even after 27 hours of incubation. Unfortunately, the phospholipids appeared to either trap or bind the free succinate, as was observed in both the proteoliposome and nitrocellulose filtration binding assays, and this interfered with its equilibration across the dialysis membrane.

DISCUSSION

The success of a protein purification scheme in part depends upon the initial preparation of the sample to be analyzed. Since the original protocol for the isolation of the cytoplasmic membrane dicarboxylate transport components did not yield a pure preparation, alternative methods for cytoplasmic membrane preparation and affinity chromatography were investigated.

It was observed that Kaback membrane vesicles contained a substantial amount of the two major outer membrane proteins, porin and TuII* (20). In contrast, membranes prepared by selective solubilization of the cytoplasmic membrane were essentially free of outer membrane contamination. In addition to yielding purer cytoplasmic membrane preparations, this latter method offered the advantage of isolating the membranes in one day in a solubilized form that could be frozen, ready for aspartate-Sepharose chromatography on another day. The freezing of the solubilized membranes did not seem to affect the binding of the proteins to the affinity column. The Kaback membrane vesicles were prepared, instead, over a two-day period and then had to be solubilized prior to chromatography. Finally, only four major proteins (53K, 42K, 35K and 17K) were eluted from the aspartate-Sepharose column when solubilized cytoplasmic membranes were used instead of membrane vesicles. An explanation of this observation could be the greater purity of the cytoplasmic membranes and the fact that the sample was really "solubilized" (26). After incubation with detergent, the cytoplasmic membranes were centrifuged at 100,000 x g, whereas the membrane vesicles were only spun at 1300 x g. It is conceivable that membrane fragments remained in the supernatant of the solubilized membrane vesicles and that the presence of these fragments resulted in many more proteins binding to the aspartate-Sepharose column.

The aspartate molecule was coupled via its amino group to the modified Sepharose gel (12). This made aspartate an analogue of the dicarboxylic acids and left its two carboxyl groups free to interact with their environment. At the buffer pH values used in these experiments, the carboxyl groups of the aspartate-Sepharose were fully ionized.

Therefore, the column may have acted as an ion exchanger as well as an affinity column (23). With the exception of the ribosomal proteins and DNA binding proteins, the majority of E. coli proteins possess isoelectric points between pH 5.0 and 6.5 (24) and would not bind to the aspartate-Sepharose column at and above pH 6.6. The studies in this chapter showed that only the 53K and 42K cytoplasmic membrane proteins remained bound to the aspartate-Sepharose column at pH 6.6 in high ionic strength buffer containing 0.01% Triton. Even when the pH of the column buffer was raised to 8.0 while the conductivity remained constant, both proteins were still able to bind to the column. An examination of their respective isoelectric points (see Chapter 3) indicated that both the 53K and 42K proteins should have been released from the aspartate-Sepharose column at pH 8.0 if it were just acting as an ion exchanger. Therefore, both proteins appeared to have a true affinity for the column. Even so, it is still possible that the affinity of the succinate binding proteins for their substrate depends in part upon an ionic interaction between the negatively-charged carboxyl groups of the succinate molecule and a substrate binding site containing several strategically-placed positive charges. Two carboxyl groups are, after all, the common structural feature of succinate, fumarate and malate, the three substrates of this transport system. This could explain why 0.5 M sodium chloride was just as effective as 0.2 M succinate in releasing the 53K and 42K proteins from the aspartate-Sepharose.

Non-ionic detergents solubilize biological membranes by dissolving into the hydrophobic bilayer and replacing the membrane phospholipids to a certain extent (22). Their advantage is that they do not tend to denature membrane proteins as do the ionic detergents and or-

ganic solvents. However, the conformation and hence activity of a membrane protein depends upon its hydrophobic environment. This active conformation may or may not change when a protein is solubilized, depending on the non-ionic detergent that is used (29,30). Since the aspartate-Sepharose column is dependent upon the activity of the solubilized membrane proteins, the effect of five different non-ionic detergents on solubilization and affinity chromatography was examined. All of the detergents solubilized basically the same set of membrane proteins. The affinity column protein profiles were very similar for membranes solubilized with Lubrol PX, Triton X-100 or CHAPS. Four major proteins (53K, 42K, 35K and 17K) bound to the Lubrol and Triton columns, while three of these proteins (53K, 42K and 17K) were isolated with CHAPS. The addition of E. coli phospholipids during Triton solubilization did not alter the pattern of proteins that bound to the column, indicating that additional phospholipids were not necessary for binding of the proteins to the affinity column. It was also noted that the behaviour of the 42K protein was dependent upon the type and amount of detergent that was used in the column buffer. In Lubrol or 0.05% Triton-containing buffer, the 42K protein was eluted with 100 mM phosphate, while sodium chloride or succinate was necessary for its elution in buffer that contained CHAPS or 0.01% Triton. In contrast, the 53K protein was always eluted with sodium chloride with all three detergents. Several of the major proteins were missing or present in lower amounts when the affinity columns were run with octylglucoside or Polyoxyethylene Ether W1-solubilized proteins. Presumably, these two detergents inhibited the binding of these proteins to the column. Only Triton or CHAPS-solubilized proteins were used in further succinate binding studies

because of the lower levels of the 53K protein isolated from Lubrol-solubilized membranes and the apparent outer membrane contamination of these proteins.

Substrate binding assays are routinely used to follow the purification of binding or transport proteins. Several criteria are useful in determining if the binding activity measured for the purified protein has physiological significance. First, the isolated protein should demonstrate similar substrate specificities and kinetics when compared to the *in situ* transport system in whole cells or isolated membrane vesicles. The purified protein should also respond in a similar manner to various inhibitors that interact with the intact system. Finally, the molar binding ratio of protein to substrate is expected to be close to 1, assuming one binding site per protein molecule. The above criteria, of course, assume that binding is assayed under ideal conditions that approximate the *in situ* environment of the protein. All of the criteria, in particular the last one, depend upon the protein remaining in a functional state after its isolation from the cell.

In the case of soluble binding proteins, such as the periplasmic transport proteins of gram-negative bacteria, the above three conditions are relatively easy to satisfy. Purification of these proteins generally does not involve manipulations that disturb the conformation, and hence activity, of the isolated proteins. Even so, the yield of active periplasmic binding protein is never 100%. In contrast to the situation with the soluble proteins, attempts to isolate functional membrane transport proteins have not met with the same success. As discussed in this chapter and in Chapter 1, these proteins must first be removed from their membrane environment prior to purification and bind-

ing studies. When these proteins are removed from their natural environment, their native conformation and, thus, biological activity may be partially or totally abolished, even if mild reagents such as non-ionic detergents are employed (25).

The conformation and activity of a membrane protein depends both directly and indirectly upon its association with other proteins in a membrane of a defined lipid composition (29). A change in protein conformation during isolation may expose some sites on the protein that were previously hidden and, conversely, may make inaccessible some sites that were exposed in the native state (37). Therefore, an isolated membrane transport protein may or may not display the same substrate specificities and kinetics is it does in vivo. In addition, its sensitivity or resistance to various inhibitory agents may be different. The native conformation of some membrane proteins is also dependent upon the membrane potential, so these proteins would not be expected to retain their biological activity when isolated. Even if the purified protein is reconstituted into a suitable lipid environment (ie. liposomes), a certain proportion of the protein may be irreversibly denatured during the course of the purification. Therefore, one can never expect to attain a 100% yield of active membrane protein and the molar binding ratio of protein to substrate will most likely be less than 1. Indeed, the best reported yields are 10% or less for reconstitution studies with various procaryotic and eucaryotic membrane transport proteins. These include the E. coli lactose permease, the human erythrocyte nucleoside transporter and the human erythrocyte monosaccharide transporter. Wright et al demonstrated reconstitution of galactoside binding and countertransport in proteoliposomes with a 7% yield of active lac car-

rier (38). A slightly better yield of 10% was achieved by Tse et al for the reconstitution of uridine transport in proteoliposomes containing the nucleoside transporter (39). Finally, Baldwin et al found that the yield of active monosaccharide transporter was dependent upon whether mixed-size (0.5% yield) or uniform, unilamellar (5% yield) liposomes were used (40).

Four different binding assays were used in this chapter in order to assess the succinate binding ability of the CBT43 cytoplasmic membrane proteins isolated by the aspartate-Sepharose column. Three of the binding methods measured the binding of substrate to protein directly since free and bound succinate were separated during the assay. These methods (P6DG column filtration, proteoliposome binding, nitrocellulose membrane filtration) depend upon the stability of the protein-substrate complex; the binding has to be tight enough (low K_d) that the complex won't be disrupted when it is separated from the free succinate. The fourth binding method (equilibrium dialysis) detected binding indirectly, since the difference between free and bound substrate was compared.

Two of the binding assays that were investigated in this chapter, namely proteoliposome binding and equilibrium dialysis, were not successful. The advantage of the former method is that it reconstitutes the extracted proteins into a lipid environment that mimics their original in vivo environment. Unfortunately, neither the Triton nor CHAPS-solubilized proteins used in this work were very amenable to the octylglucoside dilution reconstitution and assay procedures used successfully for the lactose permease and other bacterial transporters (15,32,33). Due to technical problems encountered with protein incorporation, nonspecific binding and reproducibility of results, reconstitution of

succinate binding activity into liposomes was not achieved. Another factor that must be considered for this binding assay is that the octylglucoside dilution procedure was used to form the proteoliposomes. This detergent was shown earlier in this chapter to inhibit the binding of both the 53K and 42K proteins to the aspartate-Sepharose column. Residual amounts of octylglucoside may also have interfered with binding to the reconstituted proteins. Equilibrium dialysis is the method routinely used to measure the binding of a protein and its substrate. The advantage of this method is that the interaction of the protein and its substrate is generally not disrupted. However, since this method only detects binding indirectly, it is dependent upon the activity of a significant fraction of the isolated protein. This becomes very difficult when only small amounts of a specific protein are available, as in this study. It is not surprising, then, that succinate binding to the affinity column fractions was not observed using this method.

Both the P6DG column asssay and the nitrocellulose membrane filtration methods gave similar succinate binding results for both the 100 mM and NaCl affinity column fractions. The corrected ratios for 20 uM [^{14}C]-succinate binding were: 100 mM sample - 60:1, NaCl sample - 14:1 and 100 mM sample - 63:1, NaCl sample - 12:1 for the P6DG column and nitrocellulose filtration binding assays respectively. The similarity of the results was particularily significant in light of the fact that Triton-solubilized proteins were used in the former assay, while CHAPS-solubilized proteins were used in the latter assay. In particular, the NaCl samples, which contained the 53K and 42K proteins, had a 4 to 5 fold higher succinate binding capacity than did the 100 mM samples. The greater binding capacity of the NaCl samples correlated well with the

greater affinity of the 53K and 42K proteins for the aspartate-Sepharose column in comparison to the 35K and 17K proteins in the 100 mM samples. The binding capacity of the NaCl samples (7 to 8%) was also comparable to that of the yields discussed earlier for other isolated transport proteins. Since the NaCl sample contained two distinct proteins, ideal binding of succinate would give protein:succinate ratios of 1:1 only if both proteins were active and capable of binding the substrate. This ideal ratio would drop to 2:1 if only one of the proteins could bind succinate and was present at 50% of the total protein content. The ratio would drop again to 4:1 if the protein was present in a random orientation in detergent micelles.

The P6DG column and nitrocellulose membrane filtration binding assays were not without their limitations. These included the nonspecific binding of succinate by the concentrated Triton micelles in the former assay and the fact that only the unspun samples showed detectable succinate binding activity in the latter assay. In addition, the succinate binding that was observed with both assays could not be competed out with "cold" succinate with either the 100 mM or NaCl samples. Considering the fact that the conformation of the isolated proteins, and thus their succinate binding kinetics, may have been altered in their artificial environment, this last observation may not be too surprising. It is possible that the association constants (K_a) of the isolated proteins for succinate may have been very much greater than their dissociation constants (K_d), and therefore the concentration of "cold" succinate that was used may not have been sufficient to compete out the bound radioactive succinate. In the case of the NaCl sample, this result can be correlated with the fact that the 53K and 42K proteins were

only removed from the affinity column with 0.2 M succinate or 0.5 M sodium chloride.

Although they are not conclusive, the results of this chapter suggest that one or both of the proteins in the NaCl sample may be good candidates for the cytoplasmic membrane succinate binding proteins. Both the 53K and 42K proteins bound tightly to the aspartate-Sepharose affinity column. Their interaction with this column appeared to involve more than just an ionic attraction since both proteins still bound to the column above their isoelectric points. Finally, one or both of the proteins was responsible for the higher succinate binding activity observed in the NaCl samples. In order to provide further evidence for the identification of the 53K and/or 42K protein as succinate binding proteins, the affinity column profiles of several succinate transport mutants were examined in the next chapter.

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CHAPTER III

THE ROLE OF MUTANTS IN TRANSPORT COMPONENT IDENTIFICATION

A mutation is a heritable change in the DNA sequence of a structural or regulatory gene (1). If the change involves the loss or addition of a large section of DNA within the gene (deletion and insertion mutations), the mutation will result in the loss of expression of its gene product and will be manifested phenotypically as the loss of a certain cellular function (1,3). Alternatively, if the mutation only involves a change in one or a few base pairs of the gene (point mutation), it may result in the expression of an altered gene product that leads to a phenotypic change of function (3). Several types of point or base substitution mutations exist. Missense mutations change an amino acid coding sequence in the gene and lead to the substitution of a different amino acid in the resulting mutant protein. Nonsense mutations change an amino acid coding sequence to a stop codon sequence and result in the synthesis of a truncated, non-functional protein or no protein at all. Finally, frameshift mutations involve the addition or deletion of one or a few bases to the gene sequence which then alters the gene's reading frame and thus leads to the synthesis of a highly-altered, non-functional mutant protein (1,4). A point mutation can also be silent if it does not affect the amino acid sequence of the protein or if the amino acid change does not affect the protein's structure or function (3).

Mutations occur spontaneously in bacterial genes at an average frequency of 10^{-6} per gene duplication as a consequence of errors in the fidelity of replication (3,4). Mutations can also be induced when chem-

ical or physical mutagens interact with the DNA and accelerate spontaneous mutation rates by several orders of magnitude (5). Single point mutations are identified by their ability to revert to the wild-type condition at a frequency close to that of the original mutation, with the exception of single base insertions or deletions (frameshift mutations) which usually revert at a much lower frequency (4). Reversion to the wild-type phenotype may involve either a back mutation at the same site of the gene or suppression of the original mutation by a secondary mutation in the same gene (intragenic) or in a different gene (intergenic), as in the case of suppressor tRNA molecules in E. coli (2,4).

Mutants have proven to be very useful in the identification and isolation of various proteins and in comparative structural and functional studies with the purified proteins. Deletion and insertion mutations provide "tight", generally non-revertable mutants (1) whose loss of a particular function may be correlated to the loss of a specific protein band on an SDS polyacrylamide gel. In contrast, point mutations result in varying degrees of functional loss due to the synthesis of different types of mutant proteins. These "leaky" mutants can usually be reverted to the wild-type phenotype. The mutant proteins may differ in molecular weight and/or isoelectric point from the wild-type protein and these differences may be detected on SDS and isoelectric focusing gels respectively.

A variety of E. coli succinate transport mutants were examined in this chapter in an attempt to identify the proteins isolated by aspartate-Sepharose chromatography of wild-type, CBT43 cytoplasmic membrane proteins. The 53K and 42K proteins were of particular interest since they were shown in Chapter 2 to bind succinate more effectively

than the 35K and 17K proteins. The mutants' affinity column protein profiles were examined for changes in protein species, molecular weights and isoelectric points. The mutant strains that were used in this study included two spontaneous mutants, LL3 and LL5, a spontaneous revertant of LL5 and various Mu phage-induced mutants. It was not possible to select a spontaneous or induced revertant from LL3. LL3 and LL5 were shown to be defective in earlier studies in the _dctA_ and _dctB_ genes and lacked the cytoplasmic membrane succinate transport components SBP2 and SBP1 respectively (6). The Mu-induced mutants were selected from CBT43 cells infected with the temperature-sensitive, ampicillin-resistant Mu bacteriophage, Mu_cts_62_amp_. This phage inserts randomly into the _E. coli_ genome and this insertion is stable or lysogenic at 32°C (7,8). Therefore, depending upon the site of insertion of the Mu phage, a particular gene can effectively be deleted and its gene product will no longer be expressed.

EXPERIMENTAL PROCEDURES

Bacterial Strains - The _E. coli_ K12 strains used in this chapter and their relevant genetic markers are listed in Table I. The isolation and properties of the first 3 strains were described in an earlier publication (6).

Isolation of Transport Revertants - Spontaneous revertants from strains LL3 (_dctA_) and LL5 (_dctB_) were isolated on minimal medium (medium A) plates containing either 0.2% succinate or malate as the carbon source and 0.001% thiamine hydrochloride. The composition of 20x strength medium A was 210 gm dibasic potassium phosphate, 90 gm monobasic potassium phosphate, 20 gm ammonium sulphate and 10 gm sodium

Table III-I. Genetic characteristics of E. coli K12 strains. The relevant genetic characteristics of the E. coli strains used in this chapter are listed in this table. The genetic markers are: sdh - succinate dehydrogenase; frd - fumarate reductase; thi - thiamine hydrochloride requirement; gal - galactose biosynthesis; dctA, dctB - dicarboxylate transport; argG, metB, his, leu - arginine, methionine, histidine and leucine requirements respectively.

Genetic Characteristics of E. coli K12 Strains

Bacterial Strain	Genetic Markers	Origin
CBT43	sdh,frd,thi	Laboratory stock
LL3	F' sdh^+,gal^+/ F sdh,frd,dctA,thi	Laboratory stock
LL5	F' $argG^+$/F^- argG,dctB, thi,(metB,his,leu)	Laboratory stock
LL5 Rev	F' $argG^+$/F^- argG,thi	This work
4-31	sdh,frd,thi,dctA or dctB	This work

citrate dihydrate in a total volume of 1 litre (9). The cells were grown overnight at 37°C in 10 ml of LB medium. They were then spun down and washed with 5 ml of M9 medium and their absorbance at 600 nm was determined. Their OD_{600} was adjusted to 4.0 (7 x 10^8 cells/ml) and 10^3 to 10^8 cells were plated on the succinate and malate media. After a week's incubation at 37°C, 50 large, distinct colonies were picked up and toothpicked onto a succinate plate. The colonies that grew on this plate were replica-plated onto succinate, malate, acetate and glycerol plates. Colonies that arose on both succinate and malate plates were checked for succinate transport reversion with a whole cell transport assay. No spontaneous transport revertants were selected from LL3, so nitrosoguanidine mutagenization was attempted (1,5). A lawn of 10^8 bacteria was spread onto succinate and malate plates and a single crystal of nitrosoguanidine was placed at the edge of each plate. Distinct colonies that grew after 7 days incubation at 37°C were assayed for transport activity.

Isolation of Mu Phage-Induced Mutants - Succinate transport deletion mutants were selected from CBT43 cells infected with a temperature-sensitive, ampicillin-resistant Mu phage (7). Stationary-phase CBT43 cells were grown for 3 hours at 37°C in LB medium (1:11 dilution) as described in Chapter 2. The cells were harvested and were resuspended in fresh LB medium to give an OD_{600} of 4.0 (5 - 7 x 10^8 cells/ml). An aliquot (50 ul) of isolated Mu phage (Mucts62amp) containing 5 x 10^9 phage particles was then added to 10 ml of CBT43 cells and the phage were preadsorbed onto the cells at the permissive temperature, 32°C, with shaking for 20 minutes. This gave a multiplicity of 1 for infection. The cells were then pelleted and were

vortexed in 5 ml of fresh LB containing 20 mM sodium citrate. Following growth overnight at 32°C, the cells were washed again with LB-citrate medium and were then grown for 3 hours at 32°C in 10 ml of LB-citrate containing 40 ug of ampicillin/ml. Finally, the cells were washed with 5 ml of M9 medium and were then resuspended in the same volume of M9 to give approximately 10^9 cells/ml. Dilutions from 10^{-2} to 10^{-6} were made in M9 medium and an aliquot (50 to 100 ul) of each dilution was plated out on minimal media plates containing 50 or 100 uM 2,2-difluorosuccinate and 0.2% pyruvate as a carbon source (6). The plates were incubated at 32°C and any colonies that arose were checked for the presence of a Mu insertion by replica plating onto ampicillin plates and by inhibition of growth on LB plates at the non-permissive temperature, 42°C. Colonies that still grew under these conditions were replica-plated onto malate plates and were then screened for succinate transport activity, as described in the following section.

Whole Cell Succinate Transport Assay - Bacteria were grown overnight in 10 ml of LB medium at 37°C (32°C in the case of the Mu deletion mutants). A 1:10 dilution of each strain was made into fresh LB medium (0.5 ml culture into 4.5 ml LB) containing 0.2% succinate in several 50 ml Falcon culture tubes and these were incubated for 3 hours at 140 rpm in a New Brunswick rotary shaker. Individual tubes from each strain were pooled and the cells were harvested at 2000 x g for 15 minutes and were resuspended to one-half their original volume in cold, 50 mM potassium phosphate, pH 7.5. The absorbance at 600 nm was read and the cells were pelleted again and were resuspended in a volume of cold phosphate buffer that gave an OD_{600} equal to 4.0. The cells, buffer and radioactive succinate were all kept on ice during the course

of the assays.

Ten minutes prior to the start of each transport assay, the cells were taken out of the ice and 600 ul of 4, 8 or 100 uM carrier-free, [^{14}C-2,3]-succinate was mixed with 480 ul of 50 mM potassium phosphate, pH 7.5 buffer in a Sarstedt mini-vial containing a stirring flea. At the zero timepoint, 120 ul of cells were added to the stirring succinate-buffer vial. Aliquots (200 ul) were withdrawn and filtered through 25 mm, 0.45 um Nucleopore filters at one minute time intervals up to 5 minutes. The filters were counted for radioactivity after at least 60 minutes incubation in scintillation fluid.

Cytoplasmic Membrane Preparations and Affinity Chromatography - Cytoplasmic membrane isolation and asparate-Sepharose column chromatography of solubilized membrane preparations from the transport mutants were carried out as described in Chapter 2. Various solubilization and column conditions were investigated. In the case of the Mu phage-induced mutants, a quick-screening procedure of their total membrane proteins was utilized prior to affinity chromatography studies. SDS gels of total membranes (outer and cytoplasmic membranes) from sonicated cells were examined for any gross changes in membrane protein profiles.

Gel Electrophoresis - SDS polyacrylamide gel electrophoresis was used to detect any molecular weight changes in the affinity-purified proteins from the mutants. This method was previously described in Chapter 2.

In order to examine any isoelectric point differences in the proteins, isoelectric focusing polyacrylamide tube gels and O'Farrell two-dimensional gels were utilized (10). After a 10% TCA precipitation and two ether washes, samples were dissolved in sample buffer consisting

of 9.5 M urea, 2% Triton X-100, 2% ampholines (pH 3 - 10 or 5 - 7) and 5% 2-mercaptoethanol. The isoelectric focusing gels (11 cm x 2.5 mm) consisted of 3.77:0.22% acrylamide:bisacrylamide, 9 M urea, 2% Triton and 2% ampholines. The upper and lower chambers of the gel apparatus contained 20 mM sodium hydroxide and 10 mM phosphoric acid respectively. Samples were loaded at the cathode (basic) end of the gels and electrophoresis was carried out to equilibrium for roughly 6000 Volt-hours. The gels were either fixed in 30% ethanol, 10% acetic acid prior to Coomassie staining (0.5% Coomassie Blue R250 in 35% ethanol, 10% acetic acid) and densitometric scanning, or tube gels containing [^{35}S]-labelled proteins were cut into 3 mm slices which were dissolved with hydrogen peroxide prior to scintillation counting. A blank, 0.5 cm wide gel was also cut into 3 mm sections which were then incubated overnight in vials containing 2 ml of boiled, deionized water. The pH gradient was measured with a pH meter and the isoelectric points of the various protein bands were determined. An alternative method of isoelectric focusing utilized the LKB Multiphor System (11), which involves the simultaneous electrophoresis of different samples in a slab gel. A 0.3 mm thick, 7.3:0.2% acrylamide:bisacrylamide horizontal slab gel containing 9 M urea, 2% Triton X-100 and 2% ampholines was used. Electrophoresis was carried out to equilibrium and the pH gradient was measured immediately after focusing with an LKB surface pH electrode.

Some of the isoelectric focusing tube gels were incubated for 15 minutes in SDS sample buffer and were then frozen in dry ice-acetone and stored at -20°C. When needed, the tube gels were quickly thawed at 37°C and 1.5 mm, 11.5% polyacrylamide SDS slab gels were used for electrophoresis in the second dimension. The slab gels were fixed with 50%

methanol, 10% acetic acid and were washed with 10% methanol, 5% acetic acid prior to Coomassie staining.

Chemicals - Radioactive [^{14}C-2,3]-succinic acid was purchased from the New England Nuclear Co. (Boston, MA). Ampholytes used in isoelectric focusing were obtained from LKB (Bromma, Sweden). Urea was bought from the Sigma Chemical Company (St. Louis, MO).

RESULTS

Comparison of Wild-Type and Transport Mutant Strains - The aspartate-Sepharose column profiles of the *dctA* and *dctB* mutants (LL3 and LL5), isolated in earlier work on the succinate transport system (6), were examined and compared to the profile for the wild-type strain, CBT43, discussed in Chapter 2. Prior to this comparison, the growth characteristics of these strains on various media were checked and their succinate transport rates were determined in order to ensure that their phenotypes were accurate.

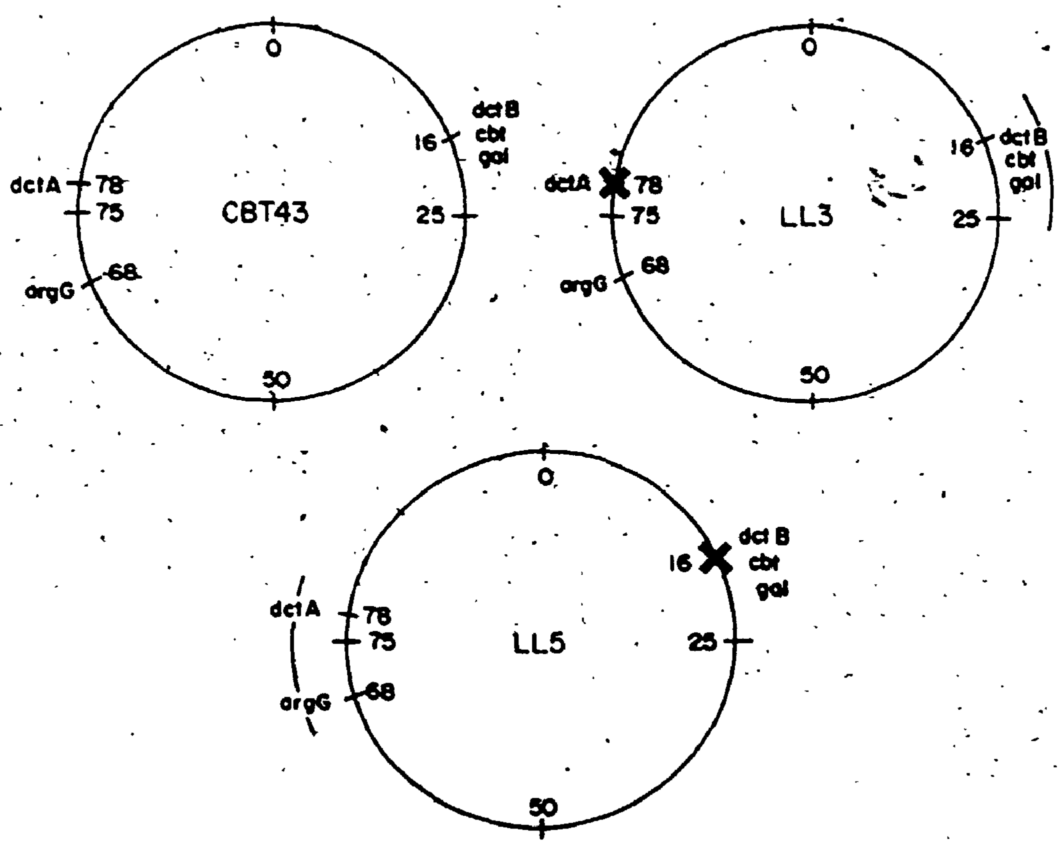
(i) Growth Characteristics - The genetic composition of the wild-type and transport-mutant strains which were studied in this chapter were presented in Table I of Experimental Procedures. Growth of CBT43, LL3 and LL5 on minimal media plates containing 0.2% succinate, malate, acetate, glycerol or D-lactate as the carbon sources is summarized in Table II. Strain CBT43 was defective in the cytoplasmic membrane enzymes succinate dehydrogenase and fumarate reductase (Table I). Therefore, it was unable to grow on media which contained either succinate or acetate as the sole carbon source. However, the fact that CBT43 could use malate as a carbon source indicated that its dicarboxylate transport system was intact. CBT43 was originally selected to be *sdh*$^-$ and

Table III-II. Growth characteristics of *E. coli* K12 strains. The growth of the bacterial strains on minimal medium A plates containing a 0.2% carbon source was checked after 48 hours at 37°C (*32°C in the case of strain 4-31). The carbon source was 0.2% pyruvate when growth in the presence of 2,2-difluorosuccinate (DFS) or β-chlorolactate (β-CL) was checked. The carbon sources were: SUC - succinate; MAL - malate; ACE - acetate; GLY - glycerol; D-LAC - D-lactate.

Figure III-I. Schematic representation of the *E. coli* genome. The relevant genetic loci of strains CBT43, LL3 and LL5 and their positions on the *E. coli* chromosome are shown in this diagram. The F-prime factors of strains LL3 and LL5 cover the regions of the *dctB* and *dctA* genes and the symbol "X" denotes a lesion in their respective genes.

<u>Growth Characteristics of E. coli K12 Strains</u>

Bacterial Strain	Growth on: SUC	MAL	ACE	GLY	D-LAC	DFS	B-CL
CBT43	-	+	-	+	+	-	+
LL3	-	-	+	+	+	+	+
LL5	-	-	+	+	+	+	+
LL5 Rev	+	+	+	+	+	+	+
4-31	-	-	-	+	+	+	+



frd$^-$ in order that its whole cell and membrane vesicle transport studies reflected only the uptake and not the metabolism of succinate (12). Due to its lack of succinate metabolism, it was possible to demonstrate concentrative, active uptake in CBT43 cytoplasmic membrane vesicles (13), which previous investigators had failed to do.

The spontaneous mutants LL3 and LL5 were selected from E. coli K12 strains that contained F' episomes covering the gal and argG regions of the E. coli chromosome respectively (6). The parental strain of LL3 was in fact constructed by transferring the F'gal episome to strain CBT43. The gal and argG episomes in turn encompassed the regions of the chromosome that contained the dctB (16 minutes) and dctA (78 minutes) respectively, as shown schematically in Figure 1. Therefore, the parental strains of the LL3 and LL5 mutants contained two copies of the dctB and dctA genes respectively. When spontaneous mutations in the succinate transport system were selected in these strains, the F'gal strain had a much higher probability of mutation in the dctA gene since it was present in only a single copy, as compared to a double copy for the dctB gene. A similar argument explains the preferential selection of dctB mutants from the F'argG strain. The frequency of occurrence of the LL3 and LL5 mutants was found to be about 2×10^{-6}, which is in agreement with a single spontaneous mutation in each strain (6).

Neither of the mutant strains were defective in succinate dehydrogenase (Table I), so they were able to grow on acetate media. Therefore, their failure to grow on either succinate or malate media and their method of selection suggested that they were succinate transport mutants. They were selected on minimal media containing 50 uM 2,2-difluorosuccinate and 0.5% acetate (6). It was observed earlier that

difluorosuccinate was transported into E. coli cells via the dicarboxylate transport system and that it noncompetitively inhibited the enzyme malate dehydrogenase. This in turn inhibited the growth of the cells on acetate, since both the TCA and glyoxylate cycles were blocked (6,14). However, cells that were unable to transport succinate would survive on acetate plates in the presence of difluorosuccinate. Confirmation of a defect in dicarboxylate transport in the LL3 and LL5 mutants selected in this way was made with whole cell succinate transport assays (Figure 2).

It should be noted that the dctB mutant, LL5, was also unable to grow on minimal media in the absence of the amino acids methionine, histidine and leucine when it was originally isolated (6). In this study, however, LL5 seemed to have lost its growth dependence on these amino acids (denoted by brackets in Table I). The reason for this apparent reversion was unknown. The three amino acids do not share any common biosynthetic enzymes (14). The only link that could be found between them was a gene (leuK) located at 18 minutes on the E. coli chromosome that controlled the regulation of biosynthetic enzymes for histidine and leucine (15). A mutation in this gene, however, would not explain the loss of methionine auxotrophy, since the original defect in LL5 occurred at the metB locus at 88 minutes on the genetic map (6,15). An alternative explanation is that all three auxotrophic mutations were originally the result of a missense or nonsense mutation in the same amino acid codon, which could then be reverted by a single intergenic suppressor mutation (4).

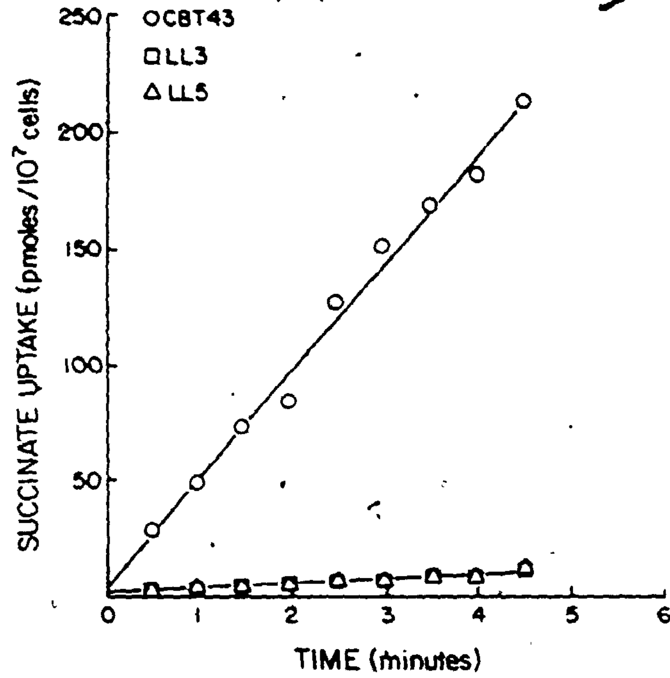
(ii) Whole Cell Succinate Transport - Whole cell transport of 50 uM [^{14}C]-succinate was carried out with LB-grown CBT43, LL3 and LL5 as described in Experimental Procedures. The growth conditions that were

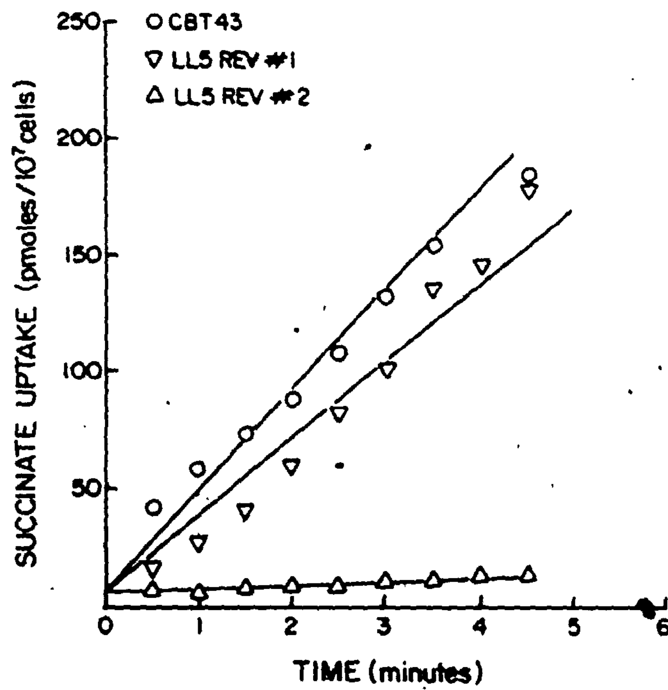
used gave the maximum activity of succinate uptake (16). These were the same growth conditions that were used for the preparation of the solubilized cytoplasmic membranes used for affinity chromatography. Figure 2 is a plot of the uptake of succinate over time in the three strains. Both LL3 and LL5 were essentially devoid of succinate transport activity (4.1% and 4.4% of control respectively), while CBT43 transported the substrate at a rate of 46.6 pmoles per minute per 10^7 cells.

As mentioned earlier, LL3 was selected from a strain that was derived from CBT43 and so its genetic background was very similar to that of CBT43. In contrast, LL5 was originally selected from a strain that had a different genetic background than CBT43. Therefore, in order to rule out any differences that these two mutants might have when compared to each other and strain CBT43 as a consequence of their different genetic backgrounds, an attempt was made to isolate spontaneous revertants from them. These revertants were selected on both succinate and malate plates as described in Experimental Procedures and then they were screened for succinate transport activity. If the defects in LL3 and LL5 were due to single point mutations, as suggested by their selection frequencies (10^{-6}) and method of selection (6), then it was theoretically possible to obtain spontaneous revertants that had regained the ability to transport succinate (1,4). From a total of 50 LL5 revertants that were tested, only one was able to grow well on both succinate and malate media (Table II) and it had also regained the ability to transport succinate. In fact, its transport rate of 32.9 pmoles per minute per 10^7 cells was about 76% of the rate of CBT43 cells assayed in the same experiment (43.2 pmoles/min/10^7 cells). Figure 3 shows the transport of 50 uM succinate by this revertant (#1), CBT43 and another

Figure III-2. Succinate transport in CBT43, LL3 and LL5. Whole cell transport of 50 uM [^{14}C]-succinate was assayed over 5 minutes in strains CBT43, LL3 and LL5 as described in Experimental Procedures. Late-log, LB-grown cells were resuspended in 50 mM potassium phosphate buffer (pH 7.5) to an optical density of 4.0 (600 nm). Cells (120 ul) were added at time zero to 600 ul of 100 uM [^{14}C]-succinate and 480 ul of phosphate buffer. Aliquots (200 ul) were removed at 1 minute intervals and were filtered, washed with phosphate buffer and counted for radioactivity. The relative rates of succinate transport in the three strains were: CBT43 - 46.6; LL3 - 1.9; LL5 - 2.1, expressed as picomoles of succinate/10^7 cells/minute.

Figure III-3. Succinate transport in LL5 revertants. Whole cell transport of 50 uM [^{14}C]-succinate in CBT43 was compared to two spontaneous revertants of strain LL5. Transport assays were carried out as in Figure 2. The relative rates of succinate uptake were: CBT43 - 43.2; LL5 Rev #1 - 32.9; LL5 Rev #2 - 1.9, expressed as picomoles of succinate/10^7 cells/minute.


250
200
150
100
50
0
1
2
3
4
5
6
O CBT43
□ LL3
Δ LL5
SUCCINATE UPTAKE (pmoles/10⁷ cells)
TIME (minutes)


250
200
150
100
50
0
1
2
3
4
5
6
O CBT43
∇ LL5 REV #1
Δ LL5 REV #2
SUCCINATE UPTAKE (pmoles/10⁷ cells)
TIME (minutes)

possible revertant (#2). Although LL5 Rev #2 grew on both succinate and malate, it was not a true transport revertant and it was not characterized further.

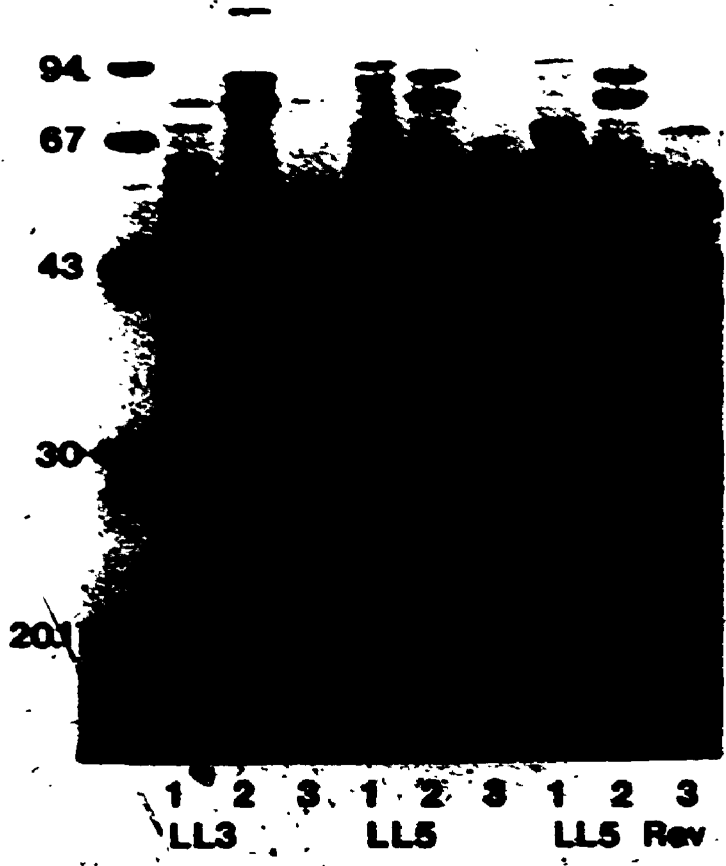
Even though LL3 was thought to be a single point mutant as well, no spontaneous revertants could be isolated from it. Any colonies that were isolated on succinate plates were unable to grow on malate media and their succinate transport activities were no different than that of LL3. Selection of LL3 revertants was attempted next in the presence of the potent mutagen, nitrosoguanidine. This is an alkylating agent that causes mainly GC to AT transitions and therefore it raises the mutation frequency for a particular gene by several orders of magnitude (1,5). However, putative LL3 revertants that were isolated on succinate plates in the presence of the mutagen were not able to transport succinate in whole cell assays. This suggested that the original mutation in LL3 involved either a frameshift mutation that only reverted at a very low frequency or a deletion or insertion that could not be reverted or suppressed (1,4).

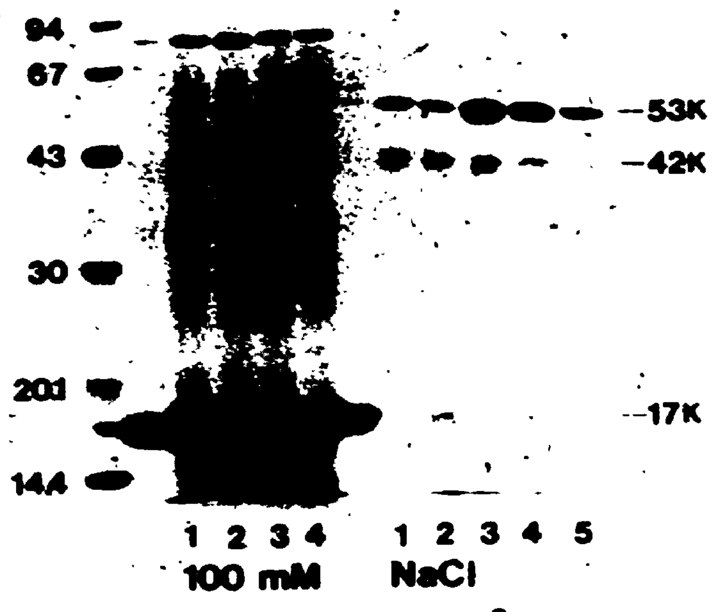
(iii)Affinity Chromatography - Solubilized cytoplasmic membranes were prepared from LB-grown CBT43, LL3, LL5 and LL5 Rev cells and their aspartate-Sepharose column profiles were examined on SDS polyacrylamide gels. The original designation of the cytoplasmic membrane transport components as SBP1 and SBP2 was made because of the elution of only the first or second protein peak from affinity columns of the dctA and dctB mutants LL3 and LL5 respectively (17). However, in the studies presented in this chapter the affinity column profiles of strains LL3, LL5 and LL5 Rev were similar to that of strain CBT43 (Chapter 2). Only one protein peak was consistently eluted with succinate (or sodium chloride)

during affinity chromatography of their cytoplasmic membranes. An explanation for this discrepancy could be the differences in cytoplasmic membrane preparation and solubilization between the earlier studies and this assay. The gel in Figure 4 shows the proteins eluted from the column for Triton-solubilized LL3, LL5 and LL5 Rev membranes. The total membrane extract, 100 mM phosphate and 0.2 M succinate fractions are shown for each strain. The same four major proteins were eluted from the aspartate-Sepharose columns of all three mutants and these proteins were identical in molecular weight to the proteins isolated from CBT43 membranes. The affinity columns were run in buffer containing 0.05% Triton, so the 42K, 35K and 17K proteins were eluted with 100 mM phosphate, while the 53K protein was eluted with 0.2 M succinate. However, the levels of both the 53K and 17K proteins appeared to be lower relative to the 42K and 35K proteins in LL3 when it was compared to the other two strains. Lower levels of these two proteins were evident as well in LL3 when its affinity-purified proteins were compared to those of its parental strain, $CBT43_c$ (see Figure 8 in Chapter 2). A difference in the levels of the 53K protein among the four strains was also observed when CHAPS-solubilized, affinity-purified proteins were examined on an SDS gel. Figure 5 shows that, apart from some differences in the minor bands, the 17K band was the major protein eluted by 100 mM phosphate in all of the strains. The 53K and 42K proteins were eluted with sodium chloride in all four strains. The low levels of the 42K protein may have been due to the fact that the membranes used in this experiment were only extracted once with CHAPS and a second extraction may have been necessary to more effectively solubilize the 42K protein. Again, the levels of the 53K protein appeared to be highest in

Figure III-4. Affinity column protein profiles of mutant strains LL3, LL5 and LL5 Rev. LB-grown, Triton-solubilized cytoplasmic membrane proteins were purified by aspartate-Sepharose chromatography as described in Experimental Procedures in Chapter II. The columns were washed with 10 mM phosphate buffer (pH 6.6) containing 0.05% Triton X-100, 5 mM EDTA and 0.002% TSF. Proteins were detected in the column fractions by absorbance at 280 nm. Proteins were prepared for SDS polyacrylamide gel electrophoresis by 10% TCA precipitation, followed by acetone precipitation. Electrophoresis was carried out on a 1.5 mm, 11.5% polyacrylamide slab gel.
Lanes 1, 2 and 3 for each strain are: 1 - cytoplasmic membrane proteins; 2 - 100 mM phosphate-eluted proteins; 3 - 0.2 M succinate-eluted proteins.

Figure III-5. Comparison of affinity column protein profiles of CBT43 and transport-mutant strains. LB-grown, CHAPS-solubilized cytoplasmic membrane proteins were purified by aspartate-Sepharose chromatography as in Figure 4. The columns were washed with 10 mM phosphate buffer (pH 6.6) containing 0.05% CHAPS, 5 mM EDTA and 0.002% TSF. Bound proteins were eluted successively with 100 mM phosphate buffer and 0.5 M sodium chloride buffer. The proteins were prepared for electrophoresis as in Figure 4.
100 mM - 100 mM phosphate-eluted proteins; NaCl - 0.5 M sodium chloride-eluted proteins.
Lane 1 - CBT43; 2 - LL3; 3 - LL5; 4 - LL5 Rev; 5 - 4-31.


94
67
43
30
20.1
1 2 3 1 2 3 1 2 3
LL3
LL5
LL5 Rev


94
67
43
30
20.1
14.4
—53K
—42K
--17K
1 2 3 4 1 2 3 4 5
100 mM NaCl

LL5 and its revertant and lowest in LL3.

The above results suggest that the 53K protein was present in lower quantities in strain LL3 when either Triton or CHAPS-solubilized membrane proteins were examined on the aspartate-Sepharose column. Previous binding studies with cytoplasmic membrane vesicles showed that strain LL3 was defective in the SBP2 transport component coded for by the dctA gene (6). Since both LL5 and its revertant harbour two copies of the dctA gene, the observed elevated levels of the 53K protein in both of these strains is also in agreement with the notion that the 53K protein may in fact be the SBP2 transport component. Therefore, a tentative identification of the 53K protein as SBP2, the defective protein in LL3, may be made at this point. An identification of the SBP1 transport component was not possible since it is not evident from these results what the corresponding defect is in the dctB mutant, LL5. Except for their respective succinate transport phenotypes, strains LL5 and LL5 Rev are isogenic. However, the affinity column protein profiles of these two strains did not reveal any differences in either the Triton or CHAPS-solubilized proteins.

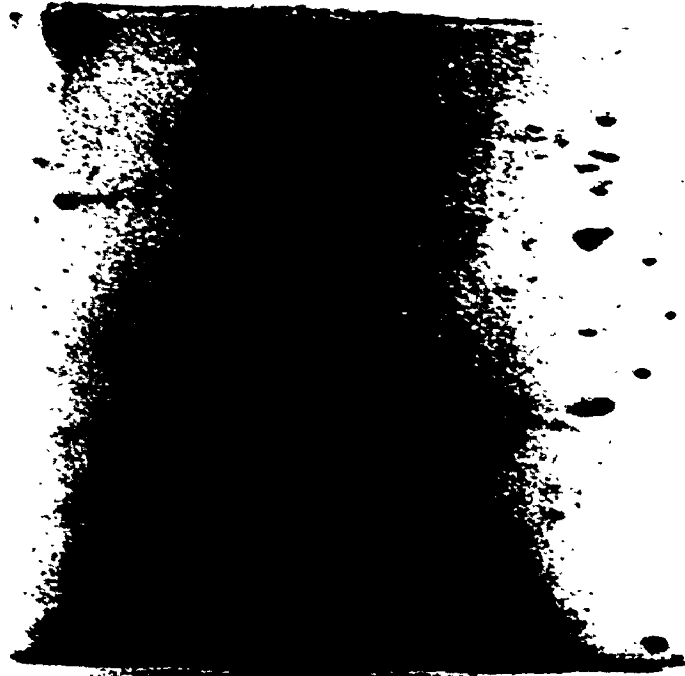
Isoelectric Focusing Analysis of Affinity Column Proteins - The results of the affinity chromatography studies with the spontaneous transport mutants, LL3 and LL5, led to a preliminary identification of the 53K protein as the SBP2 transport component. The lower levels of this protein in strain LL3 could have been due to either a decreased level of synthesis or a decreased affinity of the protein for the aspartate-Sepharose column. The defect in LL3 appeared to be due to a deletion or insertion mutation, since neither spontaneous or nitrosoguanidine-induced revertants were isolated from this strain. In contrast,

the genetic defect in strain LL5 did not appear to affect either the molecular weights or levels of the proteins isolated from the affinity column when compared to its revertant, LL5 Rev. The isolation of this spontaneous revertant from LL5 confirmed the suggestion that its defect might be a single, point mutation (1,6). It is possible that the mutation in LL5 did not affect the substrate binding ability of its defective transport component, directly or indirectly. Instead, it may have occurred at a site on the transport protein that only affected the translocation of the substrate across the cytoplasmic membrane. This would explain why the mutant transport proteins could still be isolated by affinity chromatography. In any case, if the mutations in LL3 and LL5 resulted in the substitution (or change) of an amino acid(s) that changed the net charge on their respective defective proteins, then this change might be detected on isoelectric focusing gels.

The affinity-purified cytoplasmic membrane proteins from CBT43 were first examined on O'Farrell two-dimensional gels, as described in Experimental Procedures (8,10). This allowed for a correlation of the isoelectric species separated in the first dimension with protein bands of a certain molecular weight in the second dimension. Proteins from the aspartate-Sepharose columns of LL3, LL5 and LL5 Rev membranes were then only separated in the first dimension and their isoelectric points were compared with the CBT43 proteins.

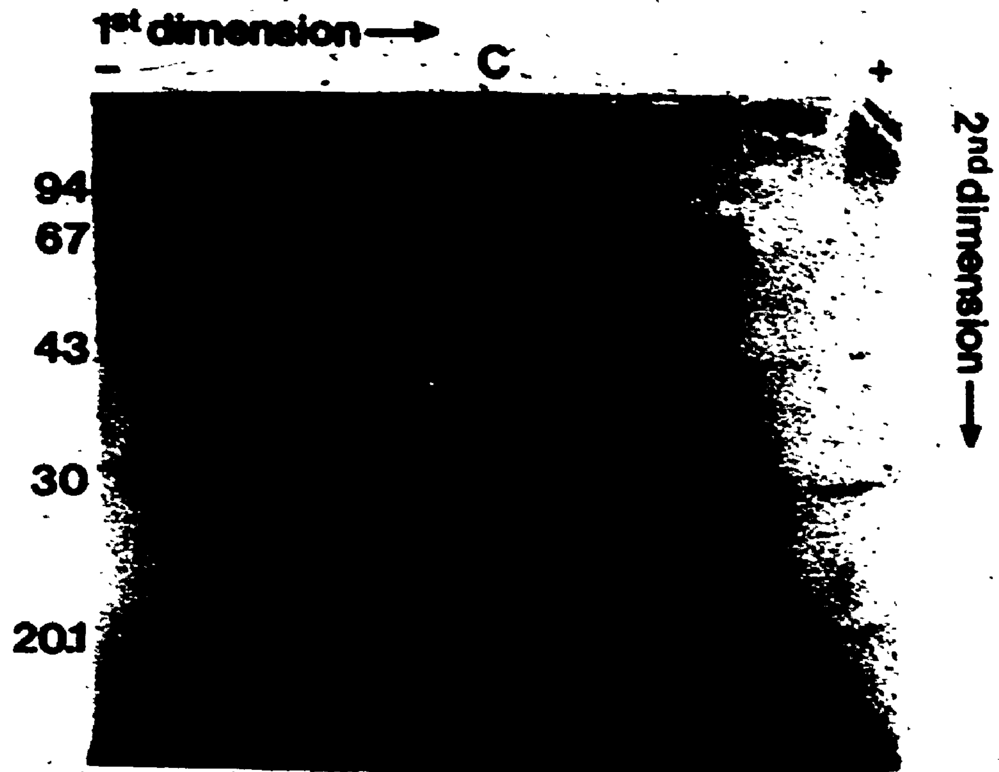
(i) <u>Two-Dimensional Gel Analysis of CBT43 Proteins</u> - Figures 6A, B and C are the O'Farrell gels from [^{35}S]-labelled, Triton-solubilized CBT43 cells, showing the protein species from the total cytoplasmic membrane extract, 100 mM phosphate and 0.2 M succinate affinity column peaks respectively. The affinity column buffer contained 0.05% Triton.

Figure III-6. Two-dimensional gel electrophoresis of CBT43 cytoplasmic membrane and affinity-purified proteins. Isoelectric focusing was carried out to equilibrium in the first dimension from pH 5 to 7, as described in Experimental Procedures. Tube gels (11 cm x 2.5 mm) of 4% polyacrylamide contained 9 M urea, 2% Triton X-100 and 2% ampholines. Separation in the second dimension involved 1.5 mm, 11.5% polyacrylamide SDS slab gels. The symbols "+" and "-" indicate the acidic and basic regions of the pH gradient respectively.
A - [^{35}S]-labelled, Triton-solubilized cytoplasmic membrane proteins.
B - 100 mM phosphate-eluted proteins from the aspartate-Sepharose column (run in 0.05% Triton).
C - 0.2 M succinate-eluted proteins (following page).

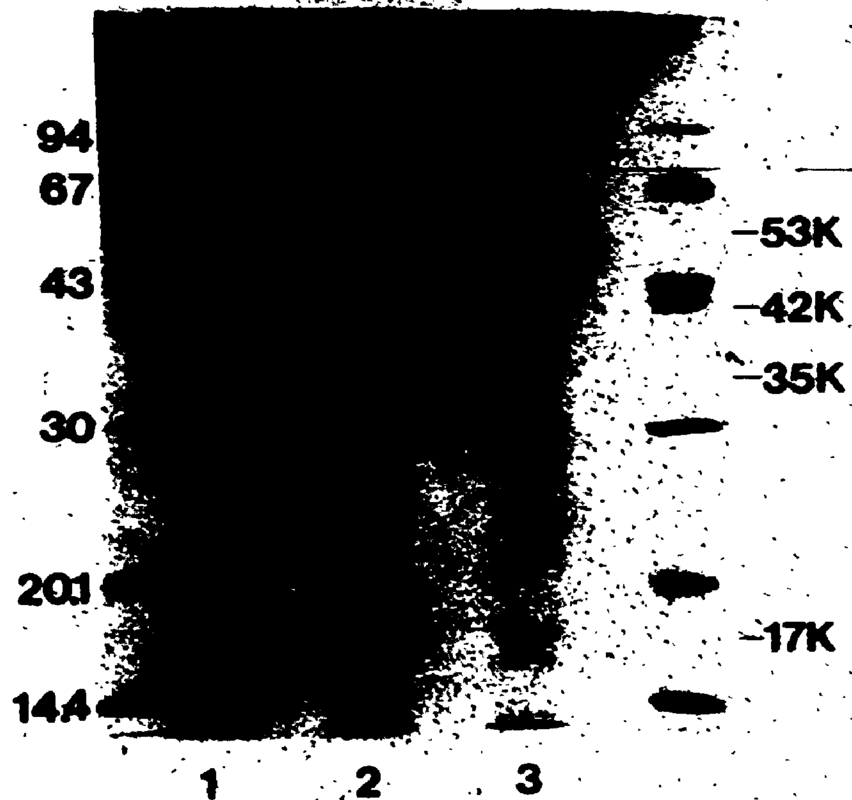

1st dimension
A
−
+
2nd dimension

−
B
+
94
67
43
30
20.1
14.4

Figure III-7. SDS gel of [^{35}S]-labelled CBT43 affinity column proteins. The corresponding 11.5% polyacrylamide, SDS gel is shown for the affinity-purified proteins separated by two-dimensional gel electrophoresis in Figure 6.
Lane 1 - cytoplasmic membrane proteins; 2 - 100 mM phosphate-eluted proteins; 3 - 0.2 M succinate-eluted proteins.



1st dimension
-
C
+
2nd dimension
94
67
43
30
20.1



94
67
43
30
20.1
14.4
-53K
-42K
-35K
-17K
1
2
3

The first dimension pH gradient was from pH 7 to 5, read from left to right in all of the figures. The major protein species were readily identified in the gel of the 100 mM peak (Figure 6B) as the 53K, 42K, 35K and 17K bands, along with an additional band of 56K molecular weight, and their positions on the gel corresponded well to the SDS gel in Figure 7. Both the 42K and 17K proteins appeared to be a mixture of two major proteins with slightly different isoelectric points. One major spot that corresponded to the 53K protein was present in the succinate peak (Figure 6C), as well as several minor species with different isoelectric points. The isoelectric points (pI) of the major affinity-purified proteins in CBT43 were found to be 7.2 to 7.3 (53K), 7.2 to 7.5 (42K), 6.8 (35K) and 6.5 to 6.7 (17K). These values were determined by densitometric scanning of the Coomassie-stained isoelectric focusing tube gels shown in Figure 8. These gels were run with a pH 5 to 7 gradient in the presence of 9 M urea and 2% Triton X-100.

The two-dimensional gels in this study were compared with the results of Neidhardt et al (18) for known E. coli proteins in order to see if any of the major proteins could be identified. The 53K, 42K and 17K proteins were not among the identified proteins on Neidhardt's reference gel. The 35K protein may have been the outer membrane protein TuII* (OmpA) (19), although the molecular weight (33K) and isoelectric point of TuII* (pI about 6) are different than those of the 35K protein in this study (pI of 6.8). It was originally thought that the 53K protein from the succinate peak (Figure 6C) might be the alpha subunit of the E. coli ATPase, a major cytoplasmic membrane enzyme (20). However, the isoelectric point of the 53K protein (7.2 - 7.3) was not as acidic as that of the ATPase alpha subunit (pI of 6.4) (21). In addi-

2

Figure III-8: Isoelectric focusing of CBT43 affinity column proteins. Affinity-purified 100 mM phosphate and 0.2 M succinate-eluted proteins from [^{35}S]-labelled CBT43 cytoplasmic membranes (Figure 7) were separated on 4% polyacrylamide isoelectric focusing tube gels containing 9 M urea, 2% Triton X-100 and 2% ampholines (1.6% pH 5-7, 0.4% pH 3-10). The "+" and "-" signs indicate the acidic and basic ends of the pH gradient respectively. The isoelectric points of the various bands are indicated in the figure.
Gel 1 - 100 mM phosphate-eluted proteins; 2 - 0.2 M succinate-eluted proteins.

−

7.5
7.3
7.2
6.8
6.7
6.5

7.3
7.2

1 2

+

tion, when monoclonal antibodies specific for the alpha subunit (22) were incubated with Western Blots of either total cytoplasmic membranes or proteins isolated with aspartate-Sepharose chromatography, the alpha polypeptide was only detected in the whole membranes. The isoelectric point of the 56K protein from the 100 mM peak (Figure 6B) was much closer to that of the alpha subunit, but this was not one of the major proteins that was reproducibly isolated from the affinity column.

(ii)Isoelectric Focusing Analysis of Mutant Proteins - A comparison of the isoelectric points of the affinity-purified proteins in CBT43 and the mutant strains LL3, LL5 and LL5 Rev was carried out next. Two different methods were used for the pI determinations. The protein samples were run on 4% polyacrylamide tube gels, as described previously for the CBT43 proteins, and on 7.5% polyacrylamide horizontal ultrathin slab gels. Despite the fact that the sample buffer contained 9 M urea and 2% Triton, sample solubility problems were encountered with both methods. This might be related to the problems of aggregation or precipitation of the concentrated membrane protein samples that were discussed in Chapter 2. This solubility problem was especially pronounced in the ultrathin slab gels, since larger amounts of protein had to be run in each lane (40 ug) in a very concentrated form (10 ul) in order that Coomassie-stained bands could be visualized. This was due to the fact that much of the protein apparently ran on top of the ultrathin gel and was therefore removed during the fixation step.

The data presented in Table III represent the best results obtained for the isoelectric points of the affinity-purified proteins in CBT43, LL3, LL5 and LL5 Rev. Part A shows the pI values for the Triton-solubilized 53K protein determined in tube gels with a pH gradient of 3

Table III-III. Isoelectric points of affinity-purified proteins. Proteins isolated via aspartate-Sepharose chromatography of CBT43, LL3, LL5 and LL5 Rev cytoplasmic membranes were separated on isoelectric focusing gels.
A - Triton-solubilized, [^{35}S]-labelled proteins (0.05% Triton in column buffer) were examined on 4% polyacrylamide tube gels (2.5 mm x 11 cm) as in Figure 8. All gels were stained with 0.5% Coomassie Blue.
* - gels which were also sliced and counted for radioactivity.
B - CHAPS-solubilized proteins were examined on 7.5% polyacrylamide horizontal slab gels (0.3 mm thick) containing 8 M urea, 2% Triton and 2% ampholines (pH 3-10) as described in Experimental Procedures.
* - results from a separate slab gel.

Isoelectric Points of Affinity-Purified Proteins

	Protein Species	IEF Conditions	Isoelectric Points CBT43	LL3	LL5	LL5 Rev
A	53K	pH 3-10 + SDS	7.6*	7.3*	7.5*	7.5*
	53K	pH 5-7 + SDS	7.1*	7.1*	7.0	7.0
B	53K	pH 3-10	7.8*	7.7	7.7	7.7
	42K++	"	8.0,7.6*	7.9,7.5	7.9,7.5	7.9,7.5
	17K	"	6.5*	6.4	6.4	6.4

++ two different isoelectric species

to 10 or 5 to 7. Both experiments included 1% SDS and excess Triton in the sample buffer in an attempt to achieve more complete solubilization of the sample proteins. Slight differences were observed in the isoelectric point of the 53K protein among the four strains in both experiments. The higher pI values in the pH 3 to 10 gels might reflect either experimental variation or the broader pH gradient. Due to the possible errors involved in comparing samples in different tube gels, the CHAPS-solubilized, affinity-purified proteins from the four strains were directly compared on ultrathin slab gels in Part B. It was evident that the isoelectric points of the 53K, 42K and 17K proteins were identical in strains LL3, LL5 and LL5 Rev.. The values for these three proteins in CBT43 were obtained from a separate slab gel and this might explain the difference of 0.1 pH units for each value. The higher pI values of the 53K protein in the ultrathin gel (B) in comparison to the tube gel analyses (A) could be related to the method of pH gradient determination. The pH gradient in the ultrathin gels was measured immediately after focusing with a surface pH electrode, whereas the tube gels were sliced and soaked in deionized water prior to the pH determination. This probably resulted in a more accurate determination of the isoelectric points in the ultrathin gels, due to the instability with time of the basic end of the pH gradient, which could have been a factor with the tube gels (23). In any case, the affinity-purified proteins from the wild-type and transport-mutant cytoplasmic membranes appeared to be identical with respect to molecular weight and isoelectric point.

<u>Mu Phage-Induced Mutants</u> - None of the bacterial strains that have been discussed so far showed a detectable difference in the molecular weights or isoelectric points of their affinity-purified proteins.

The lower levels of the 53K protein in LL3 suggested that it was the defective transport component, SBP2, in this strain. A corresponding identification of the defective transport component, SBP1, in strain LL5 was not made. Therefore, in order to obtain a clearer picture about the identity of the membrane proteins isolated by aspartate-Sepharose chromatography, it was desirable to construct succinate transport mutants that carried a deletion in either the dctA or dctB genes. These mutants were selected from CBT43 cells that were infected with a temperature-sensitive, ampicillin-resistant Mu phage, as described in Experimental Procedures. This bacteriophage is able to insert randomly into the E. coli chromosome at the permissive temperature (32°C) and this effectively results in the total or partial deletion of the gene into which it is inserted (7). Therefore, the gene product is either synthesized in a truncated form or not at all. Since the insertion of the phage is a random process, there is theoretically an equal probability of selecting mutants in any gene in E. coli (8).

(i)Selection of Mutants - Succinate transport deletion mutants were selected from Mu-infected CBT43 cells by plating them at different dilutions on minimal media containing 50 or 100 uM 2,2-difluorosuccinate and pyruvate as the carbon source. As discussed earlier, difluorosuccinate only inhibits the growth of cells that have an intact dicarboxylate transport system and therefore it selects for mutants that are defective in succinate transport (6). To distinguish them from spontaneous mutants, colonies that arose on the difluorosuccinate plates were replica-plated onto ampicillin-containing media; only mutants that contained a Mu phage insertion should be resistant to ampicillin since the parental strain, CBT43, was ampicillin-sensitive. In addition, ly-

sis of mutants at 42°C also confirmed the presence of the Mu phage due to the lytic activity of the phage at the non-permissive temperature (7). Parental CBT43 cells and any spontaneous transport mutants were able to survive at 42°C, although their growth was retarded. The frequency of occurrence for the Mu phage deletion mutants was found to be 1.5 +/- 0.4 x 10^{-4} (n=6) for both 50 and 100 uM difluorosuccinate selections. This also confirmed the fact that these mutants did not arise spontaneously, since spontaneous mutation frequencies for E. coli genes are several orders of magnitude lower than the above frequency (2).

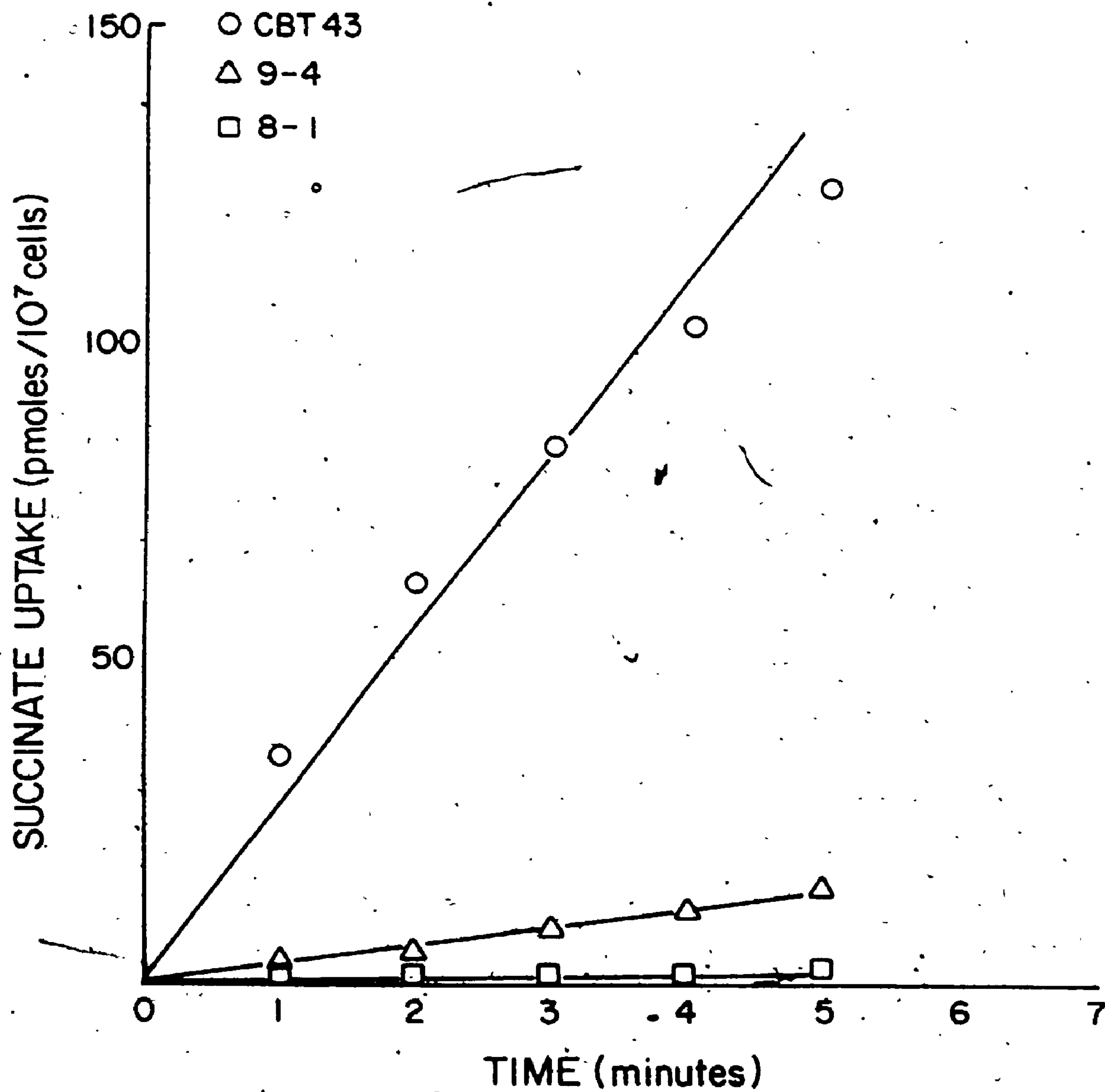
(ii) Succinate Transport Screening - The Mu phage-induced mutants were next screened for whole cell succinate transport activity, as described in Experimental Procedures. Prior to this screening procedure, the mutants were first tested for growth on 0.2% malate media; only those mutants that failed to grow on malate were tested further for succinate transport activity. The reason for this was that mutants that are selected with difluorosuccinate could potentially be defective in either the periplasmic binding protein, DBP, or one of the cytoplasmic membrane components, SBP1 or SBP2, of the dicarboxylate transport system. Earlier studies showed that mutants defective in the former component were not able to grow in low malate (0.04%) media, but were able to grow in high malate (0.2%) media, presumably because of diffusion of substrate at this concentration across the outer membrane and its subsequent transport via the intact cytoplasmic membrane components (24, 25). Therefore, mutants that failed to grow on high malate media should be defective in cytoplasmic membrane dicarboxylate transport. When the growth patterns of these mutants on various carbon sources were compared to that of the parental strain, CBT43, the mutants only differed from

CBT43 in their ability to grow on malate and their ability to grow in the presence of difluorosuccinate (Table II). Like CBT43, they were able to grow on glycerol, but were unable to grow on succinate or acetate since they were <u>sdh</u>$^-$ and <u>frd</u>$^-$.

A total of 68 mutants were tested for 2, 4 and 50 uM [^{14}C]-succinate transport activity and the majority of the strains had transport rates ranging from 0.5 to 15% of the control CBT43 value. Figure 9 compares the transport of 2 uM succinate in two of the Mu-induced mutants, 8-1 (2.2%) and 9-4 (11.1%) with the transport observed for CBT43 cells. In contrast, Mu-induced mutants that were selected with 2 mM β-chlorolactate had 2 uM succinate transport activities between 40 and 60% of the wild-type value (16). Earlier studies showed that mutants selected with β-chlorolactate were defective in the periplasmic dicarboxylate binding protein (24). This reason for this was that D-lactate shares this transport component with the dicarboxylic acids for translocation into the periplasm, but its transport across the cytoplasmic membrane involves another system (25). All of the strains used in this chapter were able to grow on D-lactate media (Table II). This fact, along with their extremely low succinate transport activities, suggested that they were not defective in periplasmic succinate transport.

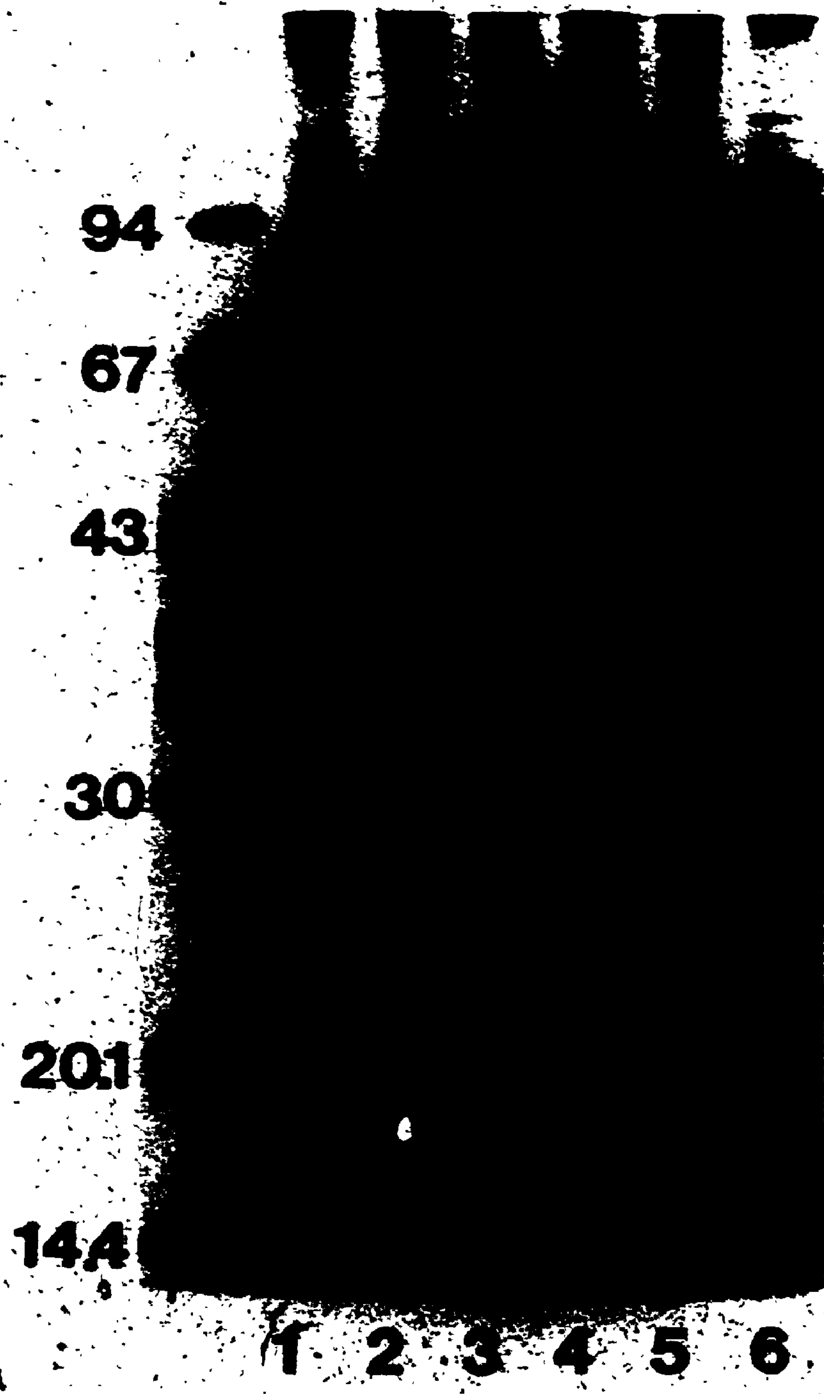
(iii) <u>Comparison of Membrane Proteins</u> - Total membranes (outer and cytoplasmic) were prepared as described in Experimental Procedures from the difluorosuccinate-selected mutants that had low succinate transport activities. These membranes were examined on SDS gels in order to determine if any gross differences were evident in their protein profiles. Figure 10 compares the total membrane protein patterns of CBT43 and five

Figure III-9. Comparison of succinate transport in CBT43 and Mu-induced mutants. Whole cell transport of 2 uM [^{14}C]-succinate was assayed in CBT43 and 2 Mu-induced mutants (9-4 and 8-1) as described in Figure 2. The relative rates of succinate uptake were: CBT43 - 27.9; 9-4 - 3.1; 8-1 - 0.6, expressed in picomoles of succinate/10^7 cells/minute.



150
100
50
0
O CBT 43
Δ 9-4
□ 8-1
SUCCINATE UPTAKE (pmoles/10⁷ cells)
0
1
2
3
4
5
6
7
TIME (minutes)

Figure III-10. Comparison of total membrane protein profiles in CBT43 and Mu-induced mutants. Total membranes (outer and cytoplasmic) were prepared from LB-grown sonicated cells as described in Experimental Procedures. Membranes (approx. 50 mg) were dissolved in SDS sample buffer and their protein profiles were examined on an 11.5% polyacrylamide SDS gel.
Lane 1 - CBT43; 2 - 8-1; 3 - 8-86; 4 - 9-34; 5 - 9-80; 6 - 10-47.



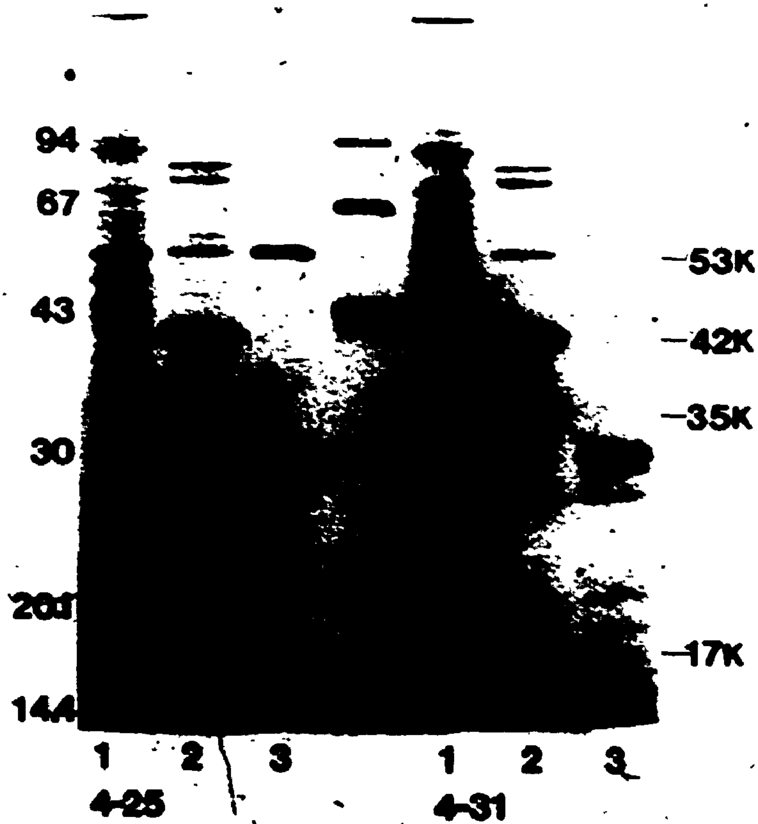
94
67
43
30
20.1
14.4
1
2
3
4
5
6

Mu-induced mutants. Mutants 9-34 and 10-47 showed striking differences in their protein patterns when compared to CBT43. However, mutants 8-1, 8-86 and 9-80, along with seven other mutants that were examined, were virtually identical with respect to total membrane proteins to CBT43. It was evident that an examination of the membrane proteins in the mutants could not reveal the deletion or disappearance of only a single protein species that could be related to succinate transport. The mutants had to be examined further with aspartate-Sepharose affinity chromatography in order to reveal if any of the proteins isolated from CBT43 membranes were missing.

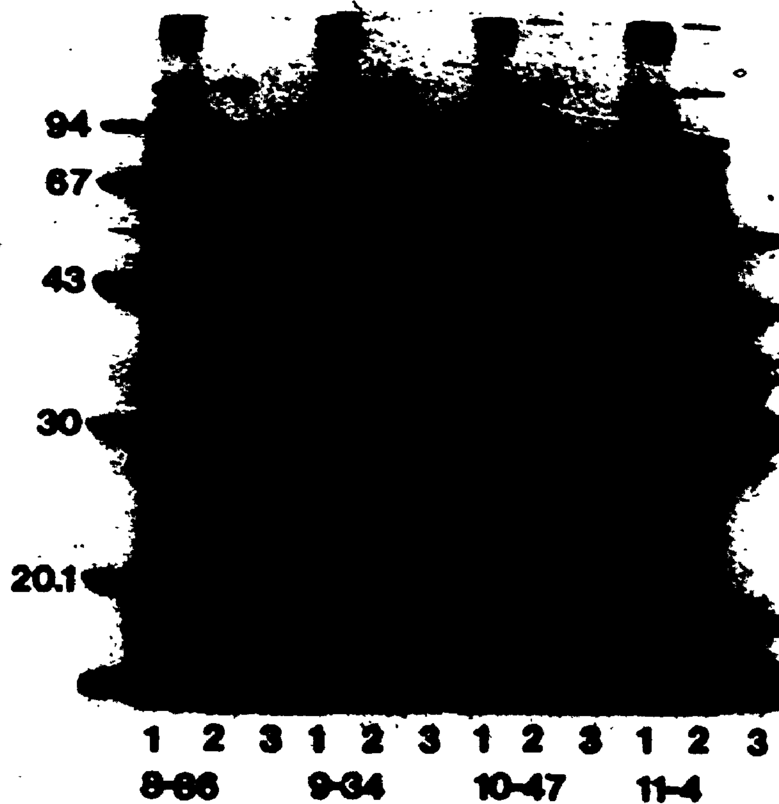
LB-grown, Triton-solubilized cytoplasmic membranes were prepared from thirteen Mu deletion mutants and the protein profiles of affinity columns run in 0.05% Triton were compared. Figure 11 shows the results from seven of these mutants. In six of the mutants, the 42K, 35K and 17K proteins were present in the 100 mM phosphate peak and the 53K protein was eluted with 0.2 M succinate. The molecular weights of these four proteins were identical to those observed earlier in CBT43 and the spontaneous transport mutants. The levels of two of these proteins, the 53K and 17K proteins, were also lower in these six mutants, similar to the results discussed earlier for the LL3 mutant. However, the most important observation that can be made from Figure 11 is that the sodium chloride peak of mutant 4-31 completely lacked the 53K protein that was present in CBT43 and all of the other mutants. Whole cell transport studies with 4 uM [^{14}C]-succinate indicated that strain 4-31 had only 1.4% of the transport activity relative to CBT43. Unfortunately, 4-31 did not appear to be a stable mutant since the absence of the 53K protein was not a reproducible observation. This protein was isolated in

Figure III-11. Affinity column profiles of Mu-induced mutants. Triton-solubilized, LB-grown cytoplasmic membrane proteins from 5 strains of Mu-induced mutants were fractionated by aspartate-Sepharose chromatography. The affinity columns were washed with 10 mM phosphate buffer (pH 6.6) containing 5 mM EDTA, 0.05% Triton and 0.002% TSF.
A - proteins isolated from strains 4-25 and 4-31 (selected with 50 uM 2,2-DFS);
B - proteins isolated from strains 8-86, 9-34, 10-47 and 11-4 (selected with 100 uM 2,2-DFS).
Lane 1 - cytoplasmic membrane proteins; 2 - 100 mM phosphate-eluted proteins; 3 - 0.2 M succinate-eluted proteins.

A



B



subsequent experiments from both Triton and CHAPS-solubilized 4-31 membranes (Figure 5). This mutant was checked for reversion to the wild-type phenotype, but it still had low succinate transport activity and was unable to grow on high malate media. The implications of the 53K protein's reappearance in strain 4-31 will be dealt with in the discussion.

DISCUSSION

The results of Chapter 2 showed that four major proteins were routinely isolated with the technique of aspartate-Sepharose chromatography from Triton-solubilized cytoplasmic membranes of CBT43, an *E. coli* strain that was wild-type with respect to succinate transport. Two of these proteins (17K and 35K) were only bound to the column in low ionic strength buffer containing 0.01% Triton, while the other two proteins (42K and 53K) were only eluted from the column with high ionic strength buffer containing succinate or sodium chloride. These latter two proteins were therefore considered to be good candidates for the cytoplasmic membrane dicarboxylate transport proteins. Succinate binding studies with both the P6DG column and nitrocellulose filtration methods also suggested that the 53K and 42K proteins exhibited a higher succinate binding capacity as compared with the proteins eluted by 100 mM phosphate. However, despite their ability to bind with succinate, the involvement of these two proteins in the dicarboxylate transport process can only be demonstrated through the use of appropriate transport mutants. The properties of a number of dicarboxylate transport mutants were examined in this chapter. The affinity-purified proteins from various types of mutants were analyzed on SDS polyacrylamide and

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isoelectric focusing gels in an attempt to correlate the loss or change in position of a particular protein with the genetic defect in each strain.

Two of the mutant strains that were examined, LL3 and LL5, were spontaneous mutants that were selected in earlier studies with 2,2-difluorosuccinate (6). Both of these mutants were unable to grow on succinate or malate media, and their ability to grow on acetate media suggested that their failure to use the dicarboxylic acids as carbon sources was not as a result of a defect in an enzyme of the citric acid cycle or glyoxylate shunt (6,12). Instead, whole cell succinate transport studies indicated that the inability of these two strains to grow on the dicarboxylic acids was due to a defect in the transport of these substrates. The frequency of occurrence of these two mutants (10^{-6}) and the merodiploid nature of each strain suggested that the phenotypes of LL3 and LL5 were due to single gene mutations in the dctA and dctB genes respectively. In addition, the isolation of a spontaneous revertant from LL5 that had regained both the abilities to grow on and transport succinate suggested that the defect in LL5 involved a single point mutation (1,12). The failure of LL3 to revert either spontaneously or in response to the mutagen nitrosoguanidine suggested that its defect might involve a deletion or insertion in the dctA gene that could only be reverted at a very low frequency, if at all (1,4). Finally, the inability of LL5 to grow on high malate along with its ability to grow on D-lactate further suggested that its succinate transport defect did not involve the nearby cbt gene for the periplasmic dicarboxylate binding protein (12,16,25).

This last argument can also be applied to the deletion mutants

that were selected with difluorosuccinate from Mu-infected CBT43 cells. These mutants were also unable to grow on succinate or malate and their whole cell succinate transport rates were comparable to those of the spontaneous mutants, LL3 and LL5. In the case of the deletion mutants, however, it was not determined whether an alteration of the *dctA* or *dctB* gene was specifically involved in their repective phenotypes. Nevertheless, these mutants were shown by several criteria to contain a Mu phage insertion that interfered with their ability to transport succinate. These criteria included their resistance to ampicillin, their inability to grow at the non-permissive temperature and their frequency of occurrence (10^{-4}). The only anomaly that was observed with all of the strains, both spontaneous and deletion mutants, was their ability to grow on media containing β-chlorolactate. Their ability to grow on D-lactate suggested that the dicarboxylate binding protein in all of the mutants was normal and therefore they should have been sensitive to growth inhibition by β-chlorolactate (12,25).

The initial isoelectric focusing and two-dimensional electrophoresis studies with the Triton-solubilized, CBT43 affinity-purified proteins indicated that the two proteins, 53K and 42K, that interacted most strongly with the aspartate-Sepharose column had isoelectric points of pH 7.3 and 7.2 to 7.5 respectively. The higher isoelectric points of these two proteins may partly explain the greater affinity that they had for the negatively-charged residues of the affinity column in comparison to the 100 mM phosphate-eluted 35K and 17K proteins. However, the fact that both the 53K and 42K proteins still bound to the affinity column when the pH of the column buffer was above their respective isoelectric points (see Chapter 2) ruled out the possibility that they only had an

ionic attraction for the aspartate-Sepharose. It should be noted that the determination of the pI values of the affinity-purified proteins in the presence of urea may not have reflected the true charge on these proteins either in vivo or during aspartate-Sepharose chromatography. Urea disrupts the non-covalent forces that govern the three-dimensional structure of polypeptide chains and therefore it may change the overall charge on the proteins by exposing previously-hidden ionic sites (26). The proteins in this study were insoluble in the sample buffer in the absence of urea. Therefore, while isoelectric focusing may not have given an accurate determination of the isoelectric points of the affinity-purified proteins, it allowed for a comparison of the pI profiles between the various strains.

When the solubilized cytoplasmic membrane proteins from mutants LL3, LL5 and LL5 Rev were examined after aspartate-Sepharose chromatography on SDS and isoelectric focusing gels, no detectable differences were evident in the molecular weights or isoelectric points of the isolated proteins. The 53K, 42K, 35K and 17K proteins were present in all of the mutants and all four proteins appeared to have isoelectric points that were similar to those of their counterparts in CBT43. However, the SDS gels (Figures 4 and 5) showed that the levels of the 53K protein were lower in both Triton and CHAPS-solubilized membranes of the dctA mutant, LL3. In fact, the levels of this protein in strains CBT43, LL3, LL5 and LL5 Rev roughly approximated the dctA gene dosage in these four strains (6). Therefore, this led to a preliminary identification of the 53K protein as the dctA gene product, SBP2. The sample solubility problems that were encountered in the isoelectric focusing studies did not allow for a reliable quantitation of the levels of the

various isoelectric species in these four strains. However, in the first experiment of Table III (pH 3 - 10) the 53K protein was again more evident in LL5 and LL5 Rev than in CBT43 and LL3. The reduced levels of the 17K protein in LL3 may or may not have been significant as well. This 17K protein was also present in lower amounts in most of the Mu-induced mutants, as was the 53K protein. However, as was discussed in Chapter 2, the levels and elution position (100 mM or NaCl peak) of this 17K protein also differed in CBT43 in some experiments. The same observations were made for a 30K protein that sometimes coeluted with the 53K protein in the NaCl (or succinate) peak of CBT43 affinity-purified proteins. This 30K protein was also present in lower amounts in LL3 (Figure 4) and in some of the Mu-induced mutants. The inconsistent results that were observed for both the 17K and 30K proteins led to the suggestion that they might be degradation products of one or more of the other affinity-purified proteins.

The disappearance of the 53K protein in the initial studies with the affinity-purified proteins of the Mu-induced mutant, 4-31, further strengthened the notion that this protein might be involved in dicarboxylate transport. Unfortunately, the apparent instability of this mutant with respect to the presence of the 53K protein precluded further studies with this mutant. It is possible that the original defect in 4-31 occurred when the Mu phage inserted into the dctA gene and thus effectively deleted its gene product. Since the insertion of this phage is temperature-sensitive, a slight rise in the incubation temperature (32°C permissive) during growth of strain 4-31 could have resulted in the liberation of the phage DNA from the E. coli chromosome. If the released phage carried along with it some of the genomic DNA, then the

gene into which it was originally inserted might still be defective. This could explain the reappearance of the 53K protein in mutant 4-31, which was still defective in succinate transport. The loss of a few amino acids from the mutant 53K protein would not be evident as a change in molecular weight on SDS gels. Unfortunately, the technical limitations of the isoelectric focusing studies did not allow for a reproducible determination of the isoelectric point of the 53K protein in 4-31 either.

The results of this chapter and Chapter 2 point to the 53K protein as a possible candidate for the ctyoplasmic membrane transport component SBP2. The evidence includes the 53K protein's tight interaction with the aspartate-Sepharose column, its ability to bind more succinate than the 100 mM phosphate-eluted proteins, its diminished levels in the dctA mutant, LL3, and its absence in initial experiments in the Mu-induced mutant, 4-31. The first two criteria also apply to the 42K protein, which was coeluted with the 53K protein from affinity columns run with 0.01% Triton (or CHAPS). However, the levels and physical characteristics (molecular weight, isoelectric point) of the 42K protein did not appear to be altered in any of the mutants in comparison to CBT43. Therefore, the involvement of this protein in the succinate transport process cannot be resolved at this point. As well, the genetic defect in the dctB mutant, LL5, is not evident from the results of Chapters 2 and 3. It is possible that LL5 could be defective in one of the other affinity-purified proteins, say the 42K protein. This defect might involve an amino acid change that did not affect the binding of the protein to the affinity column and could not be detected as a change in either molecular weight or isoelectric point with the methods

that were used. Alternatively, the defect in LL5 might involve a mutation in a regulatory gene involved in dicarboxylate transport. In order to confirm the identity of the 53K protein and elucidate the genetic defect in LL5, transformation studies are now being carried out with LL3 and LL5 and ColE1 plasmids (27) that harbour the dctA and dctB genes respectively. So far, the studies have yielded transformants that are able to grow on succinate and transport this substrate in whole cell assays as well (28). The transformants are now being subcloned in order to specifically isolate and identify the dctA and dctB genes and their corresponding gene products.

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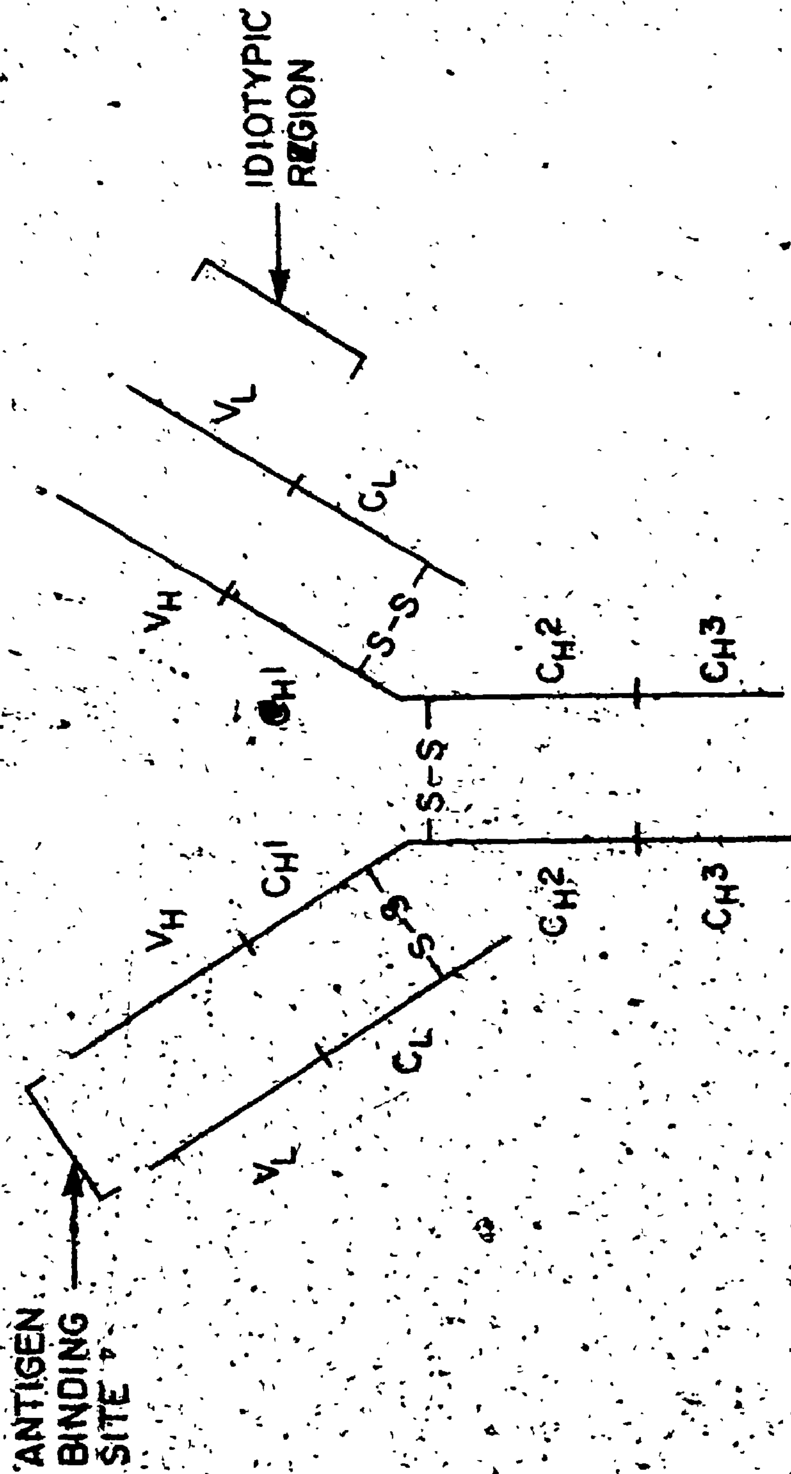
CHAPTER IV

ANTIIDIOTYPIC ANTIBODIES AS PROBES FOR TRANSPORT COMPONENTS

More than ten years ago Niels Jerne proposed the Network Theory to explain the regulation of the mammalian immune system (1). Essentially he proposed that in a normal immune response an antibody or IgG molecule not only recognized its specific antigen, but could also elicit the formation of other antibody molecules, and so on. This antibody network would provide an effective means of regulating and fine-tuning the immune response of an organism. Since this reaction network occurred within a single organism, anti-antibodies would only be raised against the epitopes of the variable region or idiotype of the IgG molecules (Figure 1). A certain proportion of these antibodies might be directed against an idiotope that encompassed the antigen-binding site of the original antibody. The antigen binding site of this latter set of antiidiotypic antibodies would then be similar in three-dimensional shape to the original antigen and as such would constitute an internal image of the antigen.

Jerne's theory has been borne out by several studies over the past few years. Auto-antibodies directed against the acetylcholine receptor and thyrotropin (TSH) receptor have been found in the sera of patients suffering from myasthenia gravis and Graves' disease respectively (2,3,4). It was postulated that these autoimmune diseases were a result of aberrant idiotype-antiidiotype network interactions. The basis for this speculation was the observation that the sera of some of these patients also contained antibodies that bound to experimentally-produced anti-receptor antibodies. Auto-antiidiotypic antibodies have

Figure IV-1. Schematic representation of the IgG molecule. The structure of the bivalent IgG molecule is illustrated in this figure. In particular, the idiotypic region of the molecule, comprised of the variable regions of the heavy and light chains, should be noted.
V - variable region; C - constant region; H - heavy chain; L - light chain.


IDIOTYPIC REGION
ANTIGEN BINDING SITE
V_H
V_L
C_L
C_H1
C_H2
C_H3
S-S
S-S
S-S
V_H
C_H1
C_H2
C_H3
C_L
V_L

also been implicated in the normal decrease in immune response seen in aged mice (5). Old animals showed a greater antiidiotypic response when presented with an antigen than did young animals, and thus their primary immune response was lowered.

As well as occurring naturally in the immune systems of both normal and diseased mammals, antiidiotypic antibodies have been produced and used experimentally. The production of antiidiotypic antibodies allows one to obtain an antibody against a specific protein without initially purifying the protein (4). The protein's substrate is used as an antigen to produce the first antibody, and then this antibody is injected into a second animal to raise antiidiotypic antibodies. These antibodies can then be used to study the biological function of the protein _in situ_ and/or they can be used to purify the protein. Antiidiotypic antibodies have been successfully raised with specificity for various neurotransmitter and hormone receptors, including the β-adrenergic, acetylcholine and insulin receptors (6,7,8). These antibodies acted as either receptor agonists or antagonists. Antiidiotypic antibodies specific for the acetylcholine receptor were even able to induce myasthenia gravis experimentally in rabbits (9). Finally, antiidiotypic antibodies are now being tested as vaccines to stimulate active immunity against various infectious diseases (4,10). The antiidiotypic antibody is injected in place of the causative agent into the susceptible host and the result is immunity without the harmful effects of the disease.

In view of the difficulties encountered thus far in the identification and isolation of the dicarboxylate transport components of _E. coli_, it was decided to attempt to raise antiidiotypic antibodies

specific for the succinate binding proteins. The transport substrate analogue, aspartate, was coupled to BSA and was used as the first antigen in rabbits. Anti-antibodies were then raised in a second set of rabbits. These antibodies were used in Western blots of wild-type and transport-mutant cytoplasmic membranes in an attempt to identify the sera which contained internal image or succinate-specific antiidiotypic antibodies. It was hoped that this would lead to the identification and possibly purification of the cytoplasmic membrane succinate transport proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains - All the strains used in this study were *Escherichia coli* K12 and were described earlier in Chapter 3. The strains were CBT43, LL3, LL5, LL5 Revertant and 4-31.

Preparation of Aspartate-BSA - Aspartate-BSA was prepared by a modification of the procedures for the linking of bradykinin to BSA and the linking of glycine methyl ester to lysozyme or ribonuclease (11,12). Bovine serum albumin (BSA, 100 mg) was dissolved in 5 ml of deionized water, along with 200 mg potassium L-aspartate and the pH was adjusted to 4.75 with 1 N hydrochloric acid. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCD, 1.5 gm) was added to the BSA solution. This mixture was incubated for 1 hour at 25°C on a rotary mixer. After removal of free aspartate by a 40 ml Sephadex G-50 (medium mesh) column equilibrated in 5 mM potassium phosphate, pH 6.6, the aspartate-BSA was stored in 2.5 mg aliquots at -20°C. Efficiency of coupling was determined by the inclusion of 1 uCi of L-[^{3}H]-aspartate in the reaction mixture.

Immunization Regime - New Zealand albino female rabbits were

injected subcutaneously at four sites with a total of 1 - 2 mg of aspartate-BSA mixed 1:1 (vol:vol, 1 ml total) with complete Freund's adjuvant. At two successive 10 day intervals after the first injection, the rabbits were boosted subcutaneously and intramuscularily at four sites with a total of 1 - 2.5 mg aspartate-BSA in 1 ml PBS (phosphate-buffered saline - 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM dibasic sodium phosphate, 1.5 mM monobasic potassium phosphate, pH 7.4) Seven to ten days after the first injection, the rabbits were bled from the peripheral ear vein. The immune sera were obtained after incubating the blood at 25°C for several hours, then overnight at 4°C. The supernatants were decanted and centrifuged at 15,000 rpm (27,000 x g) to remove any red blood cells. After sterilization by millipore-filtration and addition of sodium azide to prevent bacterial growth, the sera were stored at 4°C. Ten days to 2 weeks after the first bleed, the rabbits were again boosted with aspartate-BSA in PBS and were bled after a further 7 to 10 days. This regime was continued for a total of 3 to 4 bleeds.

A similar procedure was used to immunize a second set of rabbits with the anti-aspartate IgG isolated from the first rabbits' sera (described below). In this case, 25 to 500 ug of anti-aspartate IgG in PBS or 100 ug of IgG coupled to keyhole limpet hemocyanin were used per injection. These rabbits were boosted at 10 to 14 day intervals and were bled at 7 to 10 days after each injection. This regime was carried on for up to 6 bleeds.

Isolation of the First Antibody - (i) Protein A-Sepharose - Serum IgG was isolated with a Protein A-Sepharose column (13). Protein A-Sepharose was prepared by coupling protein A (5 mg) from *Staphylococcus*

aureus to 1.5 ml of cyanogen bromide-activated Sepharose 4B (14) in 3 ml of a 0.1 M borate buffer (pH 9.0) containing 0.5 M sodium chloride. Coupling occurred for 2 hours at 25°C or 16 hours at 4°C. Active sites on the Sepharose were then blocked with 0.2 M glycine, pH 8.0 at 25°C for 2 hours. Finally, alternate high and low pH washes (0.1 M borate, pH 9.0 and 0.1 M acetate, pH 4.0) removed any non-covalently bound protein A. Typically, 0.5 ml of serum was loaded onto a 1.5 ml column (0.5 cm ID) equilibrated in 100 mM sodium phosphate, pH 8. This column was then washed with 10 ml of buffer and IgG was eluted with 100 mM glycine, pH 3. Fractions (0.5 ml) were collected into an equal volume of 100 mM Tris-HCl, pH 8 to minimize denaturation of the IgG. The IgG was detected by absorbance at 280 nm. The Protein A-Sepharose column was stored at 4°C in 100 mM phosphate, pH 8 containing 0.02% azide.

Alternatively, serum IgG was isolated batchwise using 5 ml of protein A-Sepharose in a 60 ml coarse sintered glass funnel on a filter flask. Serum (5 - 6 ml) was mixed with the Sepharose and allowed to filter slowly by gravity. The Sepharose was then washed under vacuum 8 times with 7 ml aliquots of the phosphate buffer (pH 8). IgG was eluted with eight 5 ml aliquots of glycine collected serially into 5 ml aliquots of Tris buffer. This latter method allowed for much quicker processing of the serum IgG.

(ii) BSA-Sepharose - The affinity-purified IgG was desalted with several washes of 10 mM phosphate, pH 8 and then was concentrated to its original serum volume on a PM10 Amicon ultrafiltration membrane. The IgG (about 5 ml) was then loaded onto a 1.5 ml BSA-Sepharose 4B column equilibrated with the same buffer. BSA-Sepharose was prepared by a procedure similar to that for protein A-Sepharose except that 0.1 M

sodium bicarbonate, pH 8.3 containing 0.5 M sodium chloride was the coupling buffer. Ten milligrams of BSA were coupled per millilitre of Sepharose 4B. Rabbit anti-aspartate (RAA) IgG should not adsorb to the BSA-Sepharose and should have been in the 10 mM phosphate column wash. The anti-BSA IgG bound to the column and could be eluted with 0.1 M glycine, pH 3. This BSA-Sepharose step was only necessary if the aspartate-Sepharose used in the next step had BSA instead of glycine blocking its nonspecific sites.

(iii)Aspartate-Sepharose - Aspartate-Sepharose (see Chapter 2) was used to isolate the RAA IgG. IgG from 20 ml of serum was loaded onto two 20 ml aspartate-Sepharose columns (1.5 cm ID) equilibrated with 10 mM phosphate, pH 8. The column was washed with 50 ml of the same buffer and the bound RAA IgG was then eluted with 100 mM phosphate, pH 8 in 1.5 ml fractions. After washing with PBS on a PM10 Amicon membrane, the concentration of the RAA IgG was adjusted to around 1 mg/ml and 250 ul aliquots were frozen at -20°C. In some experiments pre-immune serum IgG was purified in the same way to serve as a control. All of the buffers in the above procedures (i - iii) contained 0.02% sodium azide, with the exception of the PBS.

Conjugation of IgG to Keyhole Limpet Hemocyanin - In later experiments, purified RAA IgG and control (pre-immune) IgG were covalently linked to Keyhole limpet hemocyanin (KLH) with glutaraldehyde prior to immunization of the second set of rabbits. The method described by Mishell and Shiigi (15) was used. After dialysis against 0.1 M sodium phosphate, pH 6.8 for several hours, control IgG (3.4 mg) and RAA IgG (5 mg) were concentrated to 1 ml via a PM10 Amicon membrane. KLH (5:1 weight:weight ratio of KLH:IgG) was added to each preparation

and then 100 ul of fresh, aqueous 0.5% glutaraldehyde was added slowly. After agitation for 60 minutes at 25°C on a rotary mixer, 100 ul of 0.1 M ammonium carbonate was added to stop the reaction. The samples were dialyzed for 4 hours at 4°C against the carbonate buffer and then overnight at 4°C against 50 mM potassium phosphate, pH 7.5. Each sample was spun in an Eppendorf centrifuge (15,000 x g) for 5 minutes to remove any precipitate and then was loaded onto a 30 ml Sepharose 2B column (1 cm ID) equilibrated in 50 mM phosphate, pH 7.5. The same buffer was used to elute 1 ml fractions. The first peak from each column contained the IgG-KLH conjugates. A Lowry assay determined the protein concentration and the IgG-KLH was stored in 250 ul aliquots at -20°C.

Isolation of the Second Antibody - (i)Protein A-Sepharose - Screening of the sera from the second set of rabbits for the antiidiotypic IgG was done with the Western Blot technique (described below). In some cases, the serum IgG from these rabbits was purified by protein A-Sepharose. This rabbit anti-rabbit anti-aspartate (RARAA) IgG was then desalted by washing several times with TBS (Tris-buffered saline - 20 mM Tris-HCl, pH 7.4, 146 mM sodium chloride) plus 0.02% azide on a PM10 Amicon membrane and was stored at 4°C.

(ii)RAA IgG-Sepharose - To achieve further purification of the RARAA IgG, it was passed through a 1 ml RAA IgG-Sepharose column. The IgG-Sepharose column was prepared in the same manner as BSA-Sepharose, except that the coupling buffer was 0.25 M bicarbonate, pH 8.8 containing 0.5 M sodium chloride. Two milligrams of RAA IgG were coupled to 1 ml of Sepharose 4B. The above coupling procedure would result in a random orientation of the coupled IgG with respect to the Sepharose 4B.

(iii)RAA IgG-Protein A-Sepharose - To obtain a more specific orienta-

tion of the RAA IgG with respect to the Sepharose 4B, a modification of the method of Schneider et al (16) was used. This method involved the covalent linkage of RAA IgG to protein A-Sepharose in order to direct the antigen binding site of the IgG molecule away from the Sepharose. RAA IgG (6 mg) was dialyzed overnight at 4°C against 0.1 M borate, pH 8.2. The IgG (6 ml) was then mixed with 0.5 ml of washed protein A-Sepharose and was allowed to bind for 30 minutes at 25°C. The Sepharose was washed with excess borate buffer and then with 10 ml of 0.2 M triethanolamine, pH 8.2. It was resuspended in 10 ml of the triethanolamine buffer containing 20 mM dimethylsuberimidate and was reacted for 45 minutes at 25°C. The Sepharose was filtered and resuspended in 20 mM ethanolamine, pH 8.2 for 5 minutes. This was followed with 3 borate washes. The above sequence of events was then repeated to react an excess of F_c fragments with the RAA IgG-protein A Sepharose. This would block any protein A sites not occupied by the RAA IgG. The enzyme papain was used to generate bivalent F_c fragments from control IgG (17). Concentrated IgG was dialyzed at 25°C against 0.1 M acetate, pH 5.5 and then was digested for 4 hours at 37°C with 1% papain in the acetate buffer containing 1 mM EDTA and 1 mM cysteine. This mixture was then dialyzed overnight at 25°C against 10 mM phosphate, pH 8 with azide. The F_c fragments were purified on a protein A-Sepharose column. They were concentrated to 10 ml and dialyzed against 0.1 M borate buffer (pH 8) prior to coupling with the RAA IgG-protein A-Sepharose.

Cytoplasmic Membrane Preparations - Kaback membrane vesicles and solubilized cytoplasmic membranes were prepared from the various bacterial strains as outlined in Chapter 2.

Electrophoresis and Western Blot Techniques - Membrane vesicles

were pelleted by centrifugation and resuspended in 3% SDS sample buffer containing 5% 2-mercaptoethanol. Solubilized cytoplasmic membranes were acetone-precipitated to remove detergent and were then resuspended in the SDS sample buffer. The samples were either boiled for 5 minutes or heated at 70°C for 30 minutes. Electrophoresis was carried out as described in Chapter 2. Samples of membrane proteins (50 - 200 ug per lane) from the wild-type CBT43 and the mutants LL3, LL5, LL5 Rev and 4-31 were loaded onto 11.5% polyacrylamide, 0.1% SDS, 1.5 mm slab gels.

After electrophoresis, proteins in the gel were transferred electrophoretically to 0.45 um nitrocellulose paper using a modification of the Western Blot method of Towbin et al (18). A sandwich of wetted 3 mm paper surrounded the polyacrylamide gel-nitrocellulose paper blot and Scotch-Brite pads on either side of the 3 mm paper ensured an even transfer of proteins. Transfer was carried out for 90 minutes at 40 volts and 0.5 amperes in an electrophoretic destaining chamber containing recirculated Laemmli running buffer (25 mM Trizma base, 192 mM glycine, 0.1% SDS) with 20% methanol. The blot was stained for 5 minutes in 0.05% Amido black, 50% methanol, 10% acetic acid and was destained for a few minutes in 10% methanol, 5% acetic acid. It was then rinsed in 3 changes of deionized water and was incubated for several hours in 2 to 3 changes of TBS containing 0.02% azide. The lanes of the blot were then cut out and incubated in 7 ml of 6% BSA (or 5% skim milk powder, "BLOTTO" (19)) in TBS on a rotary mixer for 2 hours at 25°C to eliminate nonspecific binding. Each strip was rinsed once with 10 ml of TBS and then was incubated in 5 ml of 6% BSA-TBS containing various amounts of RARAA or control serum or IgG for 4 hours at 25°C. The strips were rinsed 6 times with 10 ml of TBS and were incubated overnight at 25°C

with 50,000 to 100,000 CPM of [^{125}I]-protein A in 5 ml of 6% BSA-TBS. Finally, the strips were rinsed 6 times with 10 ml of TBS and then were air-dried and exposed to a Kodak X-OMAT X-ray film at -80°C for several days. Any protein bands that developed were scanned for intensity using a Beckman DU-8 spectrophotometer.

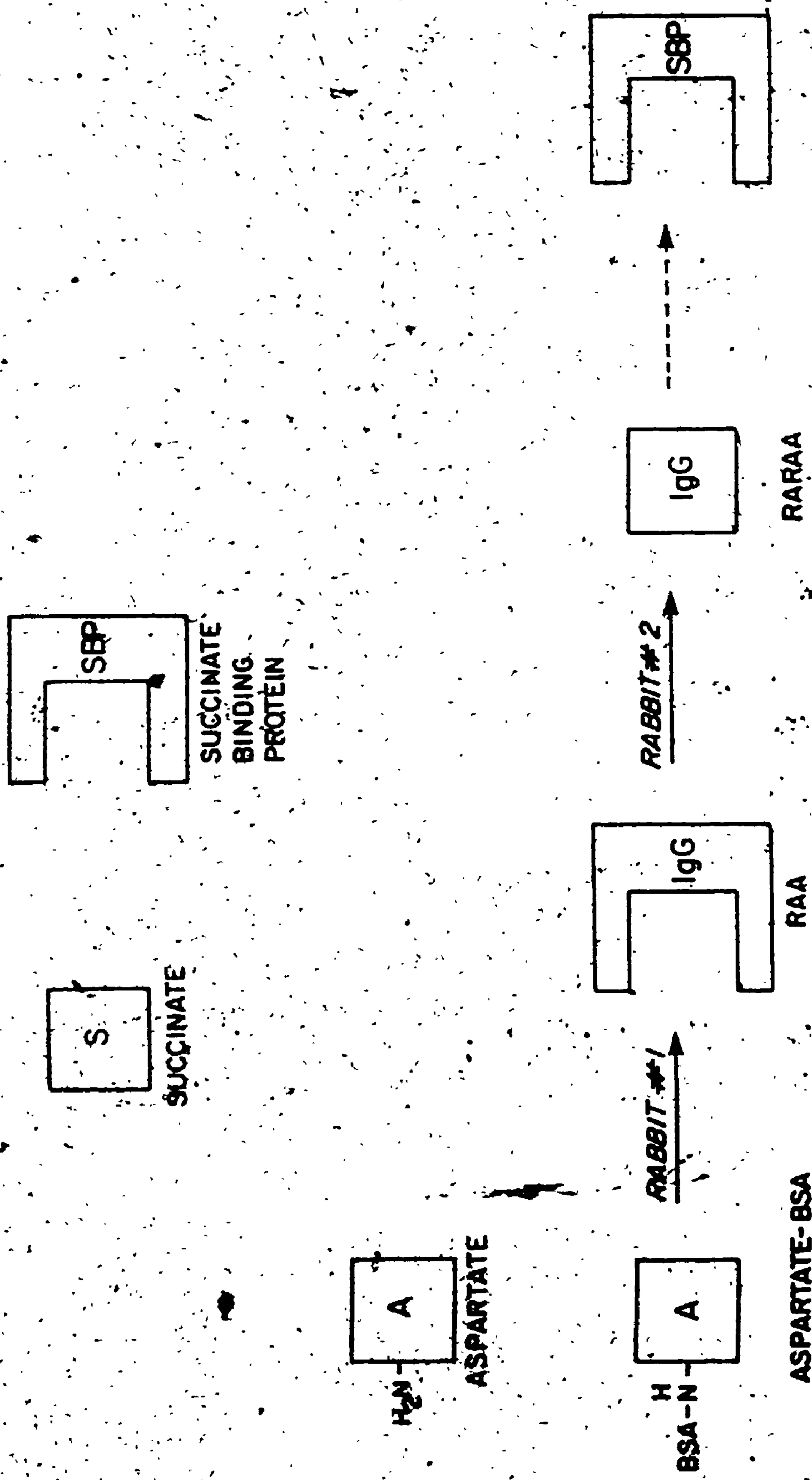
Iodination of Protein A - Protein A (12 ug) was iodinated in 50 mM potassium phosphate, pH 7.5 with 500 uCi of sodium [^{125}I]-iodide for 30 seconds using 1 ul of 2.25 mg/ml chloramine T and then 5 ul of 10 mg/ml sodium metabisulphite (20). The protein A was desalted on a 10 ml Biogel-P6DG column equilibrated with TBS containing 1 mg/ml BSA, 5% glycerol, 0.02% azide and it was stored at 4°C behind lead.

Chemicals - Radioactive chemicals were purchased from the New England Nuclear Co. (Boston, MA), with the exception of the sodium [^{125}I]-iodide from ICN Biomedicals Inc. (Irvine, CA). Staphylococcus aureus protein A and Fraction V Bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). Nitrocellulose paper was obtained from Schleicher and Schuell Inc. (Keene, NH).

RESULTS

Antiidiotypes as Probes for Transport Components - The rationale behind the use of antiidiotypic antibodies in the identification of the dicarboxylate transport components is shown in Figure 2. It suggests that the antigen binding site of an IgG molecule raised against the aspartate moiety of the transport substrate analogue, aspartate-BSA, may be similar in "shape" or specificity to the substrate binding site of proteins which bind succinate. When this first antibody, rabbit anti-aspartate (RAA) IgG, is injected into a second set of rabbits, only

Figure IV-2. Specificity of antiidiotypic antibodies for the dicarboxylate transport components. The succinate analogue, aspartate, was attached to bovine serum albumin (BSA) as described in Experimental Procedures. The aspartate-BSA was injected into rabbit #1. The IgG that was raised against the aspartate moiety of aspartate-BSA was isolated from the immune serum by aspartate-Sepharose chromatography as described in the text. This rabbit anti-aspartate (RAA) IgG was then injected into rabbit #2. The antiidiotypic (rabbit anti-rabbit anti-aspartate, RARAA) IgG that was raised may be able to bind to the transport components by virtue of the fact that its antigen binding site is similar in three-dimensional "shape" to succinate.



H2N
A
ASPARTATE
S
SUCCINATE
SBP
SUCCINATE
BINDING
PROTEIN
H
BSA-N
A
ASPARTATE-BSA
RABBIT #1
IgG
RAA
RABBIT # 2
IgG
RARAA
SBP

antibodies against the variable region or idiotype of the IgG molecule will be formed. Some of these antiidiotypic antibodies may be directed against the hypervariable region or antigen binding site of the RAA IgG and thus would be an internal image of the original antigen (1). This set of antiidiotypic (rabbit anti-rabbit anti-aspartate, RARAA) antibodies may then have the ability to recognize and bind to the substrate binding site of the transport components.

Aspartate-BSA - It was previously demonstrated that when aspartate was coupled to Dextran T-10 through its amino group it behaved essentially like succinate. The Dextran-aspartate competitively inhibited whole cell succinate transport in CBT43 with a K_i of around 0.5 uM (21). This substrate analogue seemed to bind to the transport components much more tightly than succinate itself, perhaps because of the high local concentration of aspartate surrounding the dextran molecule (10 molecules of aspartate per molecule of dextran). In this study, aspartate was coupled to bovine serum albumin (BSA) in an attempt to make the aspartate an analogue of succinate to which antibodies could be raised. The coupling procedure utilized a water-soluble carbo[illegible]ide to link free carboxyl and amino groups together (11,12). The amino group of aspartate would be expected to attach to the gamma-carboxyl groups of aspartate and glutamate residues in BSA, as well as to the carboxyl terminus. However, aspartate also contains two carboxyl groups and these could attach to the epsilon-amino groups of lysine and arginine in BSA. As well, aspartate molecules could be cross-linked to each other. This may explain the much higher coupling efficiency of aspartate-BSA (200 to 500 aspartate molecules per BSA molecule) in comparison to Dextran-aspartate. Dimethyl suberimidate was used in the latter case to

link only the amino group of aspartate to the amino group of 3,3'-iminobispropylamine already coupled to the Dextran T-10 (21).

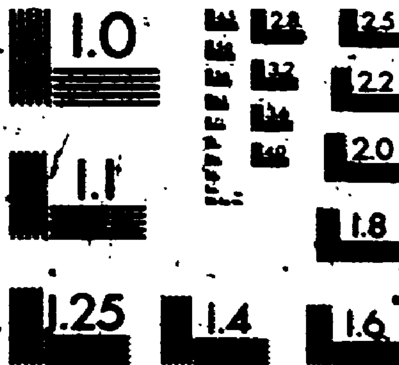
Figure 3 shows that the molecular weight of BSA (67K) was indeed greatly increased by the carbodiimide incorporation of aspartate residues. A molecular weight of greater than 90,000 Daltons indicated that a substantial amount of aspartate was coupled to the BSA. The aspartate-BSA might be envisioned as a BSA molecule with a three-dimensional polyaspartate network on its surface. In addition, some of the BSA molecules may also have been cross-linked to each other. This and other aspartate-BSA preparations were used for the production of the RAA IgG in the first set of rabbits.

Isolation of RAA IgG - Figure 4 indicates the various steps in the purification of the RAA IgG from the immune sera of rabbits injected with aspartate-BSA. A protein A-Sepharose column (13) was first used to affinity-purify total IgG from the rabbit serum (Figure 4A). About 6 to 8 milligrams of IgG were isolated per millilitre of serum. This IgG preparation contained IgG directed against BSA and many other antigens besides aspartate.

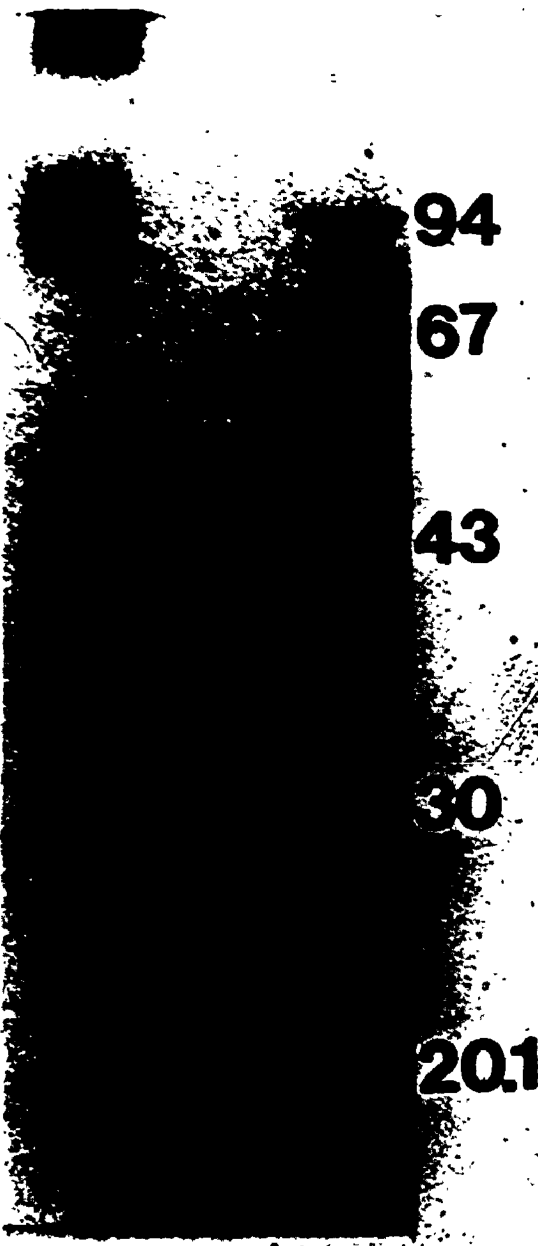
A partial purification of the RAA IgG was attempted next. Instead of just injecting total serum IgG into the second set of rabbits, an enriched fraction of RAA IgG was used. The increased proportion of specific IgG in the injection would hopefully increase the probability of raising antibodies directed against the RAA IgG. Therefore, the preparation was enriched for RAA IgG by sequential passages through BSA-Sepharose (Figure 4B) and then aspartate-Sepharose (Figure 4C) columns, as described in Experimental Procedures. The former column removed the anti-BSA IgG. This step was necessary because the aspartate-Sepharose

Figure IV-3. Molecular weight of aspartate-BSA. Electrophoresis of aspartate-BSA (50 ug) on an 11.5% polyacrylamide SDS gel indicated a molecular weight of greater than 90K Daltons. The molar ratio of aspartate to BSA in this preparation was 316:1.
A - aspartate-BSA.

3



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

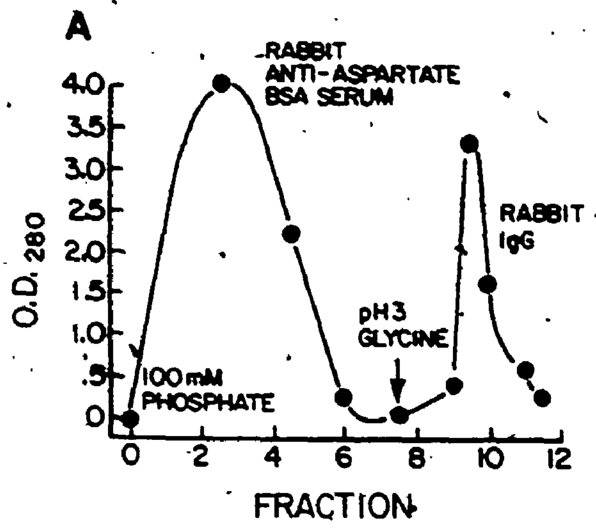


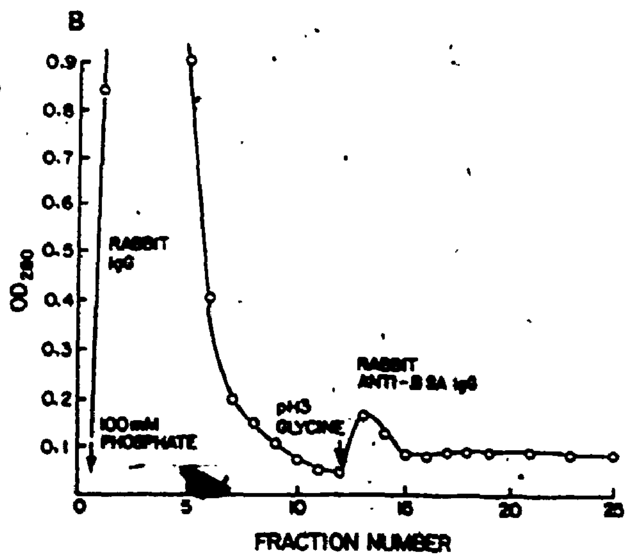
94
67
43
30
20.1
A

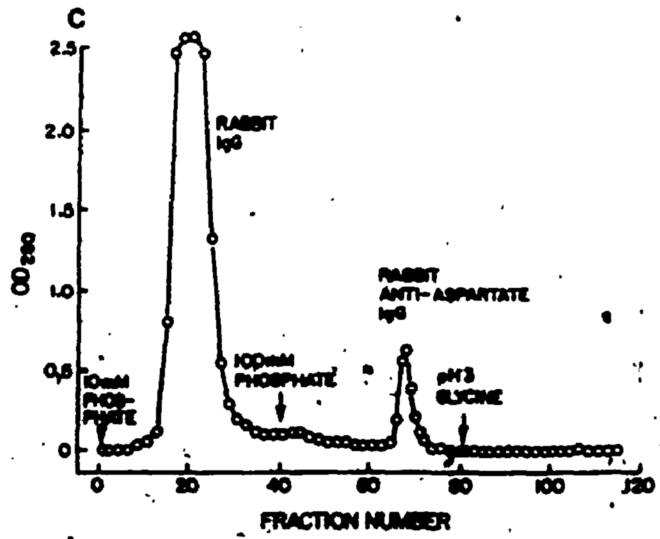
Figure IV-4. Purification of RAA IgG. A - Protein A-Sepharose purification of RAA IgG. Immune serum (0.5 ml) was loaded onto a 1.5 ml protein A-Sepharose column. Unbound serum proteins (1 ml fractions) were washed off the column with 100 mM phosphate buffer (pH 8.0). The bound rabbit IgG was eluted with 0.1 M glycine (pH 3.0) and 0.5 ml fractions were collected into an equal volume of 0.1 M Tris-HCl (pH 8.0). Optical density at 280 nm (OD_{280}) was used to detect protein in the column fractions.

B - Removal of anti-BSA IgG. A 1.5 ml BSA-Sepharose column was used to adsorb anti-BSA IgG from the total serum IgG prior to RAA IgG purification. Serum IgG (20-30 mg) was loaded onto the column and 1 ml fractions of the 10 mM phosphate wash (pH 8.0) were collected and saved for further purification on aspartate-Sepharose. Anti-BSA IgG was eluted with 0.1 M glycine (pH 3.0), as in Figure 4A.

C - Aspartate-Sepharose purification of rabbit anti-aspartate IgG. The 10 mM phosphate washes from two BSA-Sepharose columns (10 ml serum IgG) were loaded onto a 20 ml aspartate-Sepharose column. The column was washed with 10 mM phosphate (pH 8.0) and the rabbit anti-aspartate IgG was eluted in 1 ml fractions with 100 mM phosphate (pH 8.0). All of the buffers in the above procedures contained 0.02% azide.


A
RABBIT
ANTI-ASPARTATE
BSA SERUM
RABBIT
IgG
pH3
GLYCINE
100 mM
PHOSPHATE
O.D. 280
FRACTION


B
RABBIT
IgG
RABBIT
ANTI-BSA IgG
pH3
GLYCINE
100mM
PHOSPHATE
OD280
FRACTION NUMBER


C
RABBIT
IgG
RABBIT
ANTI-ASPARTATE
IgG
10mM
PHOS-
PHATE
100mM
PHOSPHATE
pH3
GLYCINE
OD280
FRACTION NUMBER

used in the original experiments was blocked with BSA instead of glycine to prevent nonspecific adsorption of proteins. In later experiments, the BSA-Sepharose column was omitted.

The aspartate-Sepharose column was used to select for RAA IgG. An average of 58 ug of RAA IgG was usually isolated from each milligram of unfractionated IgG with this technique. To check the specificity of the interaction between the IgG and affinity column, the 10 mM phosphate column wash was concentrated and passed through a second aspartate-Sepharose column. When this column was eluted with 100 mM phosphate, no protein peak emerged. This indicated that the aspartate-Sepharose did not appear to be nonspecifically binding a certain fraction of the serum IgG. After concentration and desalting with a PM10 Amicon ultrafiltration membrane, the RAA IgG was used to raise antibodies in a second set of rabbits.

A total of 5 rabbits were used to raise RAA IgG at various times. Relevant information about these rabbits is listed in Table I. The pre-immune (control) serum IgG from rabbits #4 and #5 was chromatographed on aspartate-Sepharose to determine the proportion, if any, of IgG that bound to the column nonspecifically. In both cases, IgG from the pre-immune serum was eluted with 100 mM phosphate from the affinity column. This "control" IgG was concentrated and washed and was reloaded onto two other aspartate-Sepharose columns. The columns were eluted with a 10 to 100 mM phosphate (pH 8) gradient. RAA IgG from the immune sera of the same two rabbits was treated in an identical manner. The elution profiles of these columns are presented in Figure 5. All of the IgG loaded onto the columns remained bound until the phosphate gradient was applied. Both the RAA and control IgG peaks were eluted from the

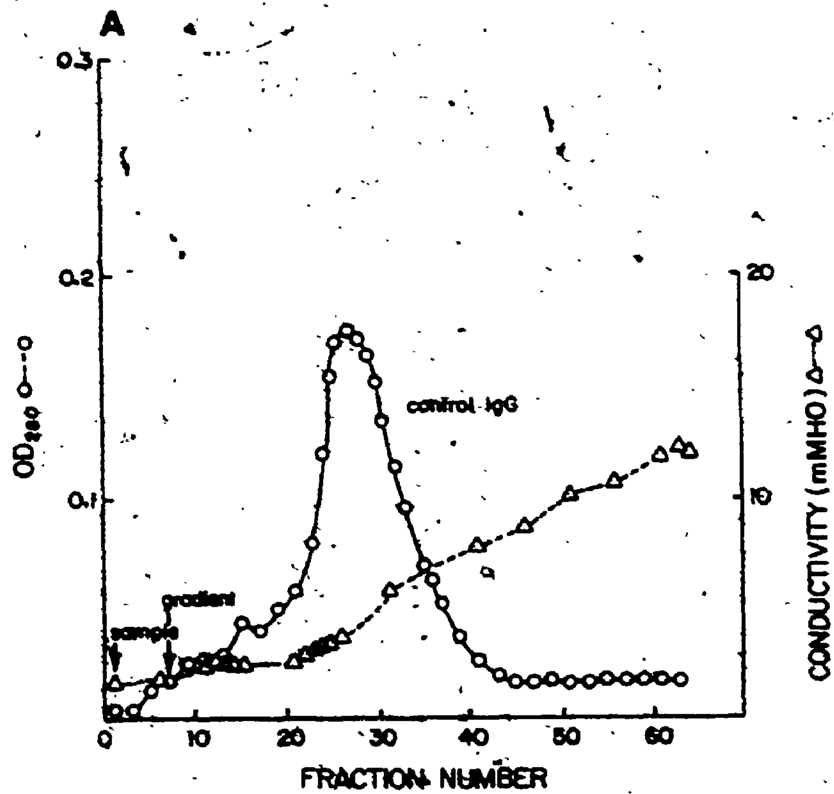
Table IV-1. RAA IgG rabbit statistics. The first set of rabbits was immunized with 1 to 2 mg of aspartate-BSA as described in Experimental Procedures. The amount of RAA IgG that was isolated from each rabbit's serum is expressed in micrograms of RAA IgG per milligram of total serum IgG. The RAA IgG isolation procedure is described in detail in the text and in Figure 4. The RAA IgG from successive bleeds of some of the rabbits was pooled and processed together where indicated. The control values for rabbits #4 and #5 indicate the amount of RAA IgG isolated from their pooled pre-immune sera.

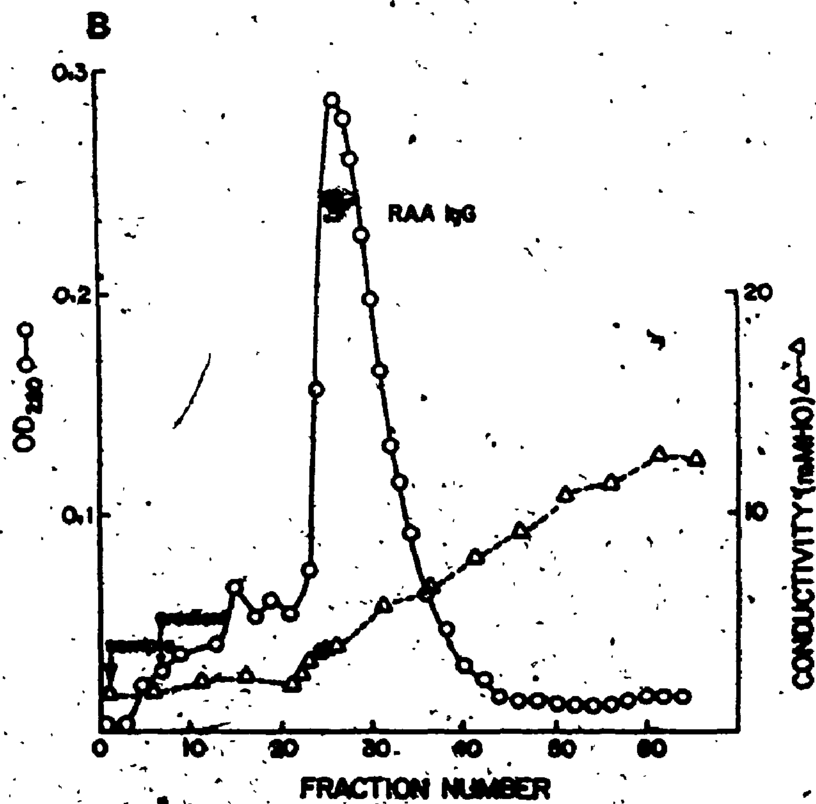
RAA IgG Rabbit Statistics

Rabbit #	Aspartate-BSA Injected (mg)	Bleed #	Yield of RAA IgG (ug/mg serum IgG)
1	2	1	90
		2	45
		3	ND
		4	50
		5	34
2	2	1	ND
		2	processed with rabbit #3 IgG
3	2	1	32
		2,3,4	115
4	1	1,2	processed with rabbit #5 IgG
5	1	1,2	41
4 (control)	-	1,2	processed with rabbit #5 IgG
5 (control)	-	1,2	25

Figure IV-5. Comparison of aspartate-Sepharose purification of RAA and control IgG. Pre-immune and immune IgG were isolated by Protein A-Sepharose from the sera of two of the aspartate-BSA-injected rabbits (#4 and #5, Table I) as shown in Figure 4A. This IgG was then purified further on aspartate-Sepharose columns as in Figure 4C. Both pre-immune and immune IgG that bound to this column was rechromatographed on a second aspartate-Sepharose column as described in the text. A 10 to 100 mM phosphate buffer (pH 8.0) gradient was used to elute the bound IgG. Both pre-immune and immune IgG were eluted at a phosphate conductivity of about 4 mMHO.

A - control IgG (pre-immune); B - RAA IgG (immune).


A
0.3
0.2
0.1
0
OD280 o—o
20
10
CONDUCTIVITY (mMHO) △—△
control IgG
gradient
sample
0
10
20
30
40
50
60
FRACTION NUMBER


B
0.3
0.2
0.1
OD280 o—o
20
10
CONDUCTIVITY (mMHO) △—△
RAA IgG
0
10
20
30
40
50
60
FRACTION NUMBER

column at the same phosphate conductivity (about 4 mMHO). The only difference between the control and RAA IgG isolated by the aspartate-Sepharose column was the yield. The combined control sera from both rabbits yielded only 25 ug of IgG for each milligram of unfractionated serum IgG. Almost double that amount (41 ug/mg) of RAA IgG was isolated from the combined immune sera. Obviously, a certain fraction of the IgG was interacting nonspecifically with the aspartate-Sepharose, presumably through an ionic interaction. This finding is actually not surprising, since the control serum may contain IgG directed against the aspartate moiety of other foreign proteins. In addition, the negatively-charged carboxyl groups of the aspartate-Sepharose (see Chapter 2) make it an ion exchange as well as an affinity column, which can bind with other IgG molecules besides the RAA IgG. Both the control and RAA IgG isolated from the column were used to raise antiidiotypic antibodies.

Isolation of RARAA IgG - Rabbit anti-rabbit anti-aspartate IgG (RARAA) was isolated from the sera of the second set of rabbits in the various ways described in Experimental Procedures. Whole serum was tested for the presence of antiidiotypic IgG by incubation with Western Blots of CBT43 cytoplasmic membrane proteins (to be described in detail below). Those preparations that appeared to contain RARAA IgG were affinity-purified on protein A-Sepharose. To specifically enrich for RARAA IgG, the total serum IgG was then treated in one of two ways. A RAA IgG-Sepharose column was used to select RARAA IgG from the total IgG preparations. This method suffered from the fact that the IgG was attached to the cyanogen bromide-activated Sepharose in a random orientation. Therefore, not all of the antigen-binding sites on the RAA IgG

molecules were accessible to the RARAA IgG. Alternatively, a protein A-Sepharose column to which RAA IgG was covalently linked was used. The IgG was bound to the protein A through its F_c domain. Therefore, this column had the IgG antigen binding sites oriented away from the column matrix and thus left them available for binding to the RARAA IgG. The concentration of RARAA IgG that was used gave approximately 50% saturation of the protein A sites. This was the concentration that Schneider et al (16) found gave maximal binding of antigen to the IgG on the column with minimal steric hindrance. The remaining sites on the protein A molecules were blocked with an excess of F_c fragments generated by papain digestion of control IgG.

A total of 16 rabbits were used to raise RARAA IgG and details about their immunization regimes are presented in Table II. Only one of the 16 rabbits that were tested for the production of succinate-specific antiidiotypic antibodies appeared to produce the desired internal image antibodies. The results from studies with this RARAA IgG preparation (RARAA #1) will be presented in detail in the next section.

Identification of Transport Components with RARAA IgG - For the purpose of identifying the cytoplasmic membrane succinate transport proteins with the antiidiotypic IgG, the Western Blot technique described in Experimental Procedures was used. This technique also yielded information on the specificity of the various RARAA IgG preparations described above. Membrane vesicles, cytoplasmic membrane and outer membrane protein preparations from wild-type and mutant strains were solubilized in SDS sample buffer and separated on 11.5% SDS polyacrylamide gels. The proteins were then transferred electrophoretically to nitrocellulose paper and individual lanes were cut out and in-

Table IV-II. RARAA IgG rabbit statistics. The immunization regimes of the 16 rabbits injected with RAA IgG (or control IgG) are summarized in this table and are detailed in Experimental Procedures. Freund's complete adjuvant was included in the initial injections of IgG, while the booster injections involved IgG in phosphate-buffered saline.

RARAA IgG Rabbit Statistics

Rabbit #	RAA IgG Injected (ug)	# of Bleeds
1	500 ug initial, 500 ug boost	7
2	500 ug initial, 250 ug boost	5
3,4	320 ug initial, 160 ug boost	2
5	25 ug initial, 25 ug boost	4
6	50 ug initial, 25 ug boost	4
7	75 ug initial, 25 ug boost	4
8,9,10	270 ug initial, 25 ug boost	5
11,12, 14,15	100 ug initial and boost RAA-KLH	2
13,16	100 ug initial and boost control-KLH	2

cubated with various amounts of RARAA or control serum or IgG. Any protein bands that were recognized by the IgG preparations were visualized with [^{125}I]-labelled protein A.

The first experiment compared the proteins labelled with RARAA #1 (fourth bleed) serum IgG in CBT43, LL3 (dctA) and LL5 (dctB) cytoplasmic membrane vesicles. The IgG used in this experiment was only purified with protein A-Sepharose. Figure 6 is an autoradiogram showing the particular protein bands recognized by the RARAA #1 IgG. It was evident that no proteins were recognized by the control IgG, whereas the RARAA IgG bound to 5 or 6 major bands in all three bacterial strains. The molecular weights of these bands were 73K, 53K, 37K, 29K, 19.2K and 17K (or 15.8K). To determine if any of the protein bands in the autoradiogram reflected a specific binding protein the intensity of each band was determined spectrophotometrically. The results are shown in Table III. The LL5/LL3 ratio is a normalization of the relative band intensities in the two mutant strains and it was used because these two strains were not isogenic. Therefore, one might expect to see differences in the levels of various membrane protein species characteristic of each strain. When the LL5/LL3 ratio was examined, the most striking differences were found for the 53K, 37K and 17K bands. The intensity of the 53K band was about 5.4 times greater in LL5 than in LL3, whereas the opposite results were evident for both the 37K and 17K bands. The intensities of both these bands were about 3 times greater in LL3 than in LL5. Both mutants were also compared to the wild-type strain, CBT43. Except for its F' factor, LL3 was isogenic with CBT43, as was discussed in Chapter 3. The intensities of the 53K and 17K bands were also greater in LL5 (3 times) and LL3 (2 times) respectively when compared to

Figure IV-6. Western blot of RARAA #1 IgG and cytoplasmic membrane vesicles. Membrane vesicle proteins (200 ug) of strains CBT43, LL3 and LL5 were separated on an 11.5% polyacrylamide SDS slab gel as detailed in Experimental Procedures. The proteins were then transferred electrophoretically to nitrocellulose paper and were incubated with various amounts of RARAA #1 IgG (protein A-Sepharose-purified). IgG-labelled bands were visualized with [^{125}I]-protein A and autoradiography. The incubation conditions were: 1 - 2.5 ug RARAA IgG; 2 - 5 ug RARAA IgG; 3 - 10 ug RARAA IgG; C - 10 ug control (pre-immune) IgG.

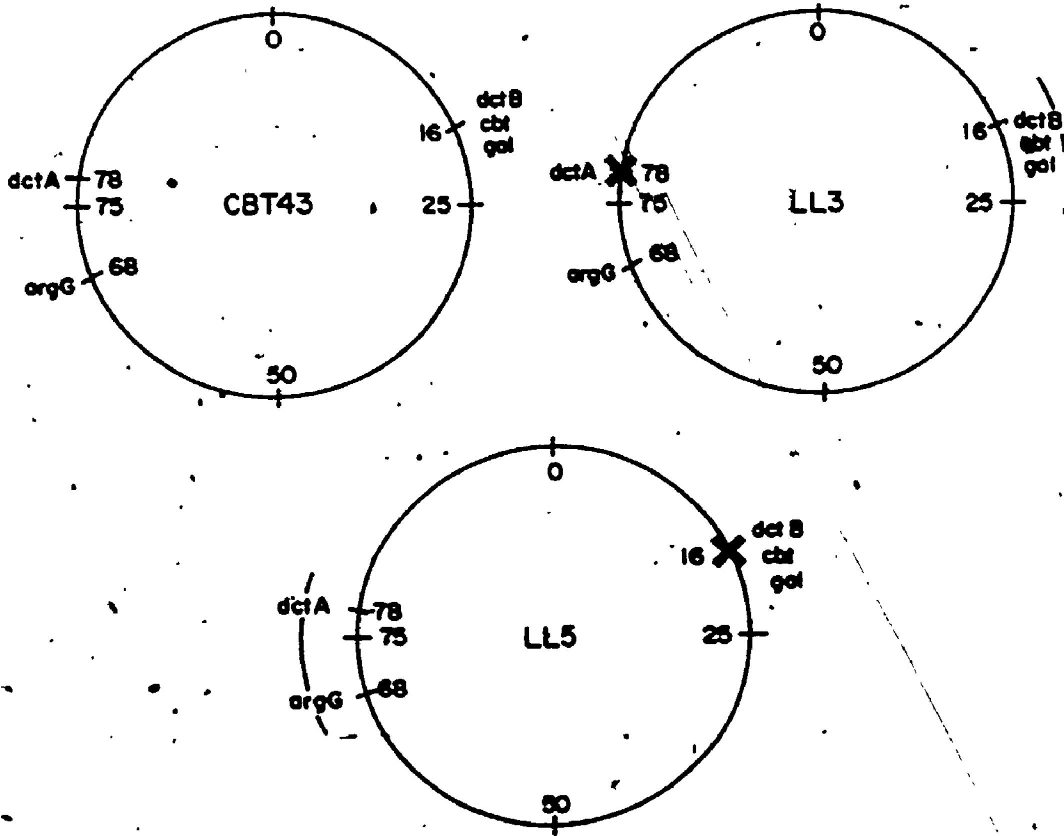
73K
53K
37K
29K
19.2K
17K
15.8K
94
67
43
30
20.1
14.4
1 2 3 C
CBT43
1 2 3 C
LL3
1 2 3 C
LL5

Table IV-III. Quantitation of RARAA #1 IgG binding to cytoplasmic membrane proteins. Individual lanes in Figure 7 were scanned for absorbance at 500 nm with a Beckman DU-8 spectrophotometer. The intensity of each protein band refers to the area under each scanned peak minus the background area. The LL5/LL3, LL3/CBT43 and LL5/CBT43 ratios are a normalization of the relative intensities of each band among the three bacterial strains. The 37K/53K ratio is a measure of the relative intensities of the 37K and 53K bands within each strain.

Figure IV-7. Genetic maps of wild-type and dicarboxylate transport mutant *E. coli* strains. The chromosome and F-prime factor of strains CBT43, LL3 and LL5 are depicted in this schematic diagram of the standard *E. coli* genetic map. The symbol "X" denotes a lesion in the *dctA* (78 minutes) and *dctB* (16 minutes) genes in LL3 and LL5 respectively.

Quantitation of RARAA#1 IgG Binding to Cytoplasmic Membrane Proteins

Molecular Weight	Band Intensity CBT43	LL3	LL5	LL5/LL3 Ratio	LL3/CBT43 Ratio	LL5/CBT43 Ratio
73K	2.19	0.94	1.54	1.64	0.42	0.70
53K	1.51	0.83	4.50	5.42	0.55	2.98
37K	3.63	3.43	1.16	0.34	0.94	0.32
29K	1.28	2.07	1.98	0.96	1.62	1.55
19.2K	1.44	1.91	2.53	1.32	1.33	1.76
17K	1.25	2.80	0.97	0.35	2.25	0.78
15.8K	-	-	0.70	-	-	-
37K/53K Ratio	2.40	4.12	0.26			



CBT43, but the levels of the 37K band were similar in both CBT43 and LL3.

An analysis of the genotypes of the three E. coli strains used was relevant to the interpretation of these different RARAA IgG binding patterns. The genotypes are schematically illustrated in Figure 7. The wild-type strain, CBT43, possessed one copy of each of the dctA and dctB genes. The dctA mutant, LL3, contained an episome covering the region of the chromosome around the gal gene (17 minutes) and therefore this mutant harboured two copies of the dctB gene. An episome covering the region around the argG gene (68 minutes) was present in the dctB mutant, LL5, and so it had two copies of the dctA gene (22). Analysis of the solubilized membrane proteins of strains LL3 and LL5 by aspartate-Sepharose chromatography originally suggested that they were defective in the SBP2 and SBP1 transport components respectively (23). Strain LL5 possessed two copies of the dctA gene. Therefore, if both copies of this gene were expressed and if its gene products (SBP2) were inserted into the cytoplasmic membrane, one would expect that LL5 should have an increased amount of SBP2 relative to that present in strains CBT43 and LL3. This alteration in the relative amounts of SBP1 and SBP2 present in this mutant might in turn be revealed by the amount of RARAA IgG bound to various bands in Western Blots of its membrane proteins. Since the intensity of the 53K band was much greater in LL5 than in either LL3 or CBT43 (Table III), this suggested that the 53K band might be SBP2. This result is also in agreement with the observation in Chapter 3 that the 53K protein was present in smaller quantities in the dctA mutant, LL3.

Strain LL3 possessed two copies of the dctB gene and by a similar

argument, it might be expected to have more SBP1 labelled by RARAA IgG. Since the intensity of the 37K band was about 3 times greater in LL3 than LL5, this suggested that the 37K band might be SBP1. However, the intensity of the 37K band in CBT43 was not any different than that of LL3, even though CBT43 possessed only one copy of the dctB gene. This might be due to the inability of the extra gene product to insert into the cytoplasmic membrane. Finally, the ratio of the 37K/53K intensities was highest (4.12) in LL3 and lowest (0.26) in LL5. It should be noted that the increased intensity of the 17K band in LL3 in comparison to LL5 also made it a candidate for the SBP1 transport component. However, the levels of this protein were very similar in both CBT43 and LL5, even though the latter strain is defective in the dctB gene coding for the SBP1 gene product. In addition, it may also be remembered from the results of Chapters 2 and 3 that a comparison of the levels and affinity column elution positions of this 17K protein among the bacterial strains varied in different experiments.

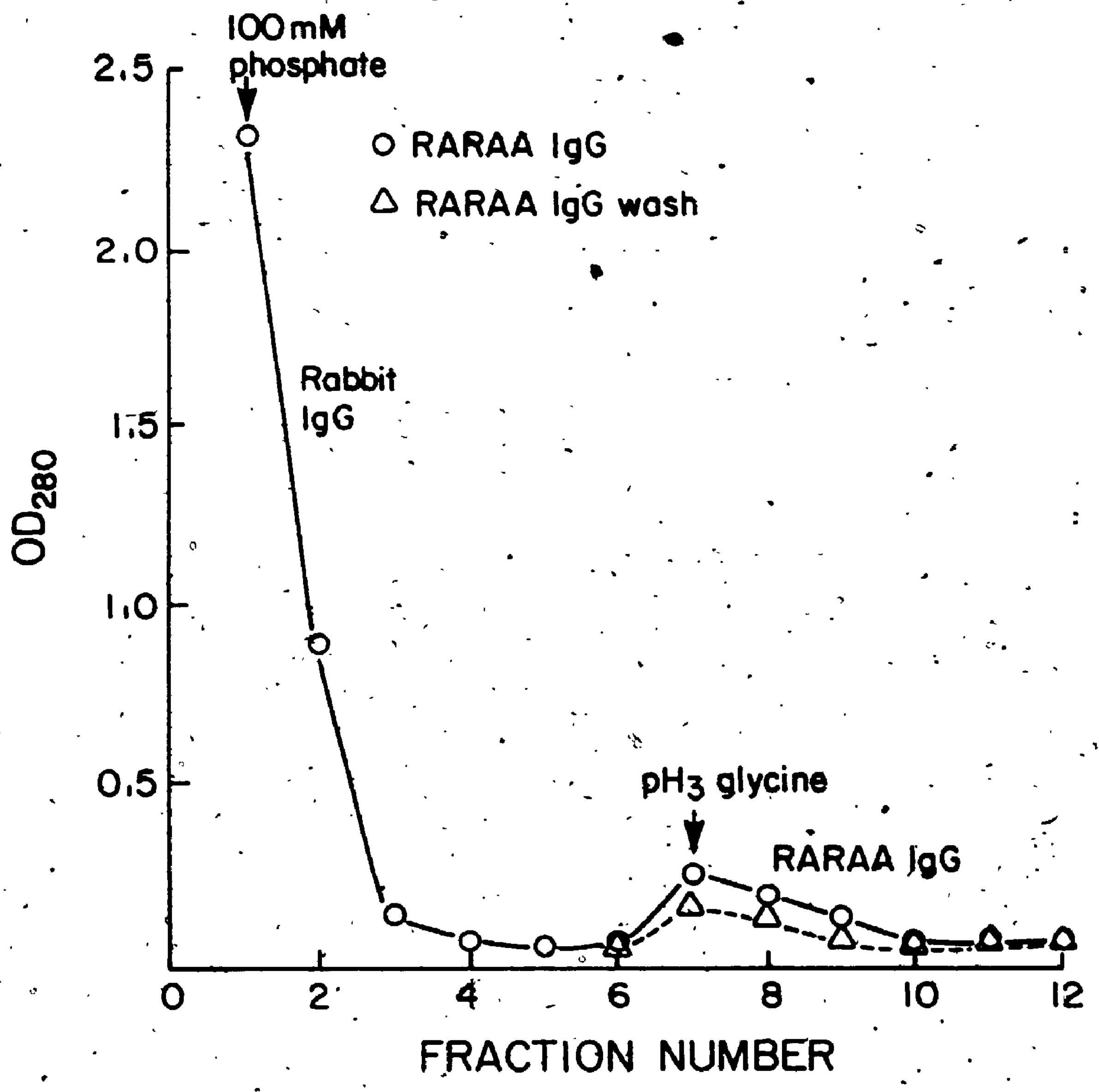
RARAA IgG Specificity - To answer the question of the specificity of the RARAA #1 IgG's interaction with the various protein bands, succinate was used to attempt to inhibit the IgG binding in the Western Blot assay. Succinate (10 mM) was included in the buffer during incubation of the blots with RARAA IgG. As a control, 10 mM glutamate and 20 mM sodium chloride were also used. Glutamate is similar in structure to succinate but should not have interfered with the binding if a succinate binding protein were involved, since it is not a substrate of the dicarboxylate transport system. Sodium chloride was used as an ionic strength control since the addition of succinate would raise the ionic strength of the blot buffer and this in itself might have interfered

with the binding of the RARAA IgG. The results of three separate succinate inhibition experiments yielded conflicting results. The results ranged from a decrease in binding of RARAA #1 IgG to the Western Blot proteins in the presence of succinate, glutamate or sodium chloride, to no inhibition at all and finally to an increase in binding in all cases. Since neither an inhibition nor enhancement of RARAA IgG binding occurred reproducibly and exclusively with succinate, this method made it impossible to decide whether succinate had any effect on the binding of antibody to the proteins in the Western Blot assay.

An alternative method that was used to demonstrate the specificity of the RARAA #1 IgG binding was the fractionation of this IgG on columns containing covalently-bound RAA IgG (the first antibody). Up to this point, total serum IgG purified by protein A-Sepharose was used in all of the Western Blots. The RARAA IgG was further fractionated as described previously on either a RAA IgG-Sepharose or RAA IgG-protein A-Sepharose column to see if the Western Blot protein patterns were altered. When the former column was used, most of the IgG did not bind to the column and only a small peak of presumably specific RARAA IgG was eluted. However, when the same amount of pre-immune serum IgG was loaded onto the column, a peak of similar magnitude was bound. In addition, when the RARAA IgG that was bound to the column was used in Western Blot assays, either no protein bands were illuminated or the pattern was so complex that it was not interpretable. In any case, the results were not reproducible. Apparently, the RAA IgG-Sepharose column was not very specific with respect to the IgG bound. This could be due to the fact that the RAA IgG was randomly attached to the Sepharose 4B.

Indeed, Figure 8 shows that the RAA IgG-protein A-Sepharose was

Figure IV-8. RAA IgG-Protein-A Sepharose purification of RARAA IgG. A RAA IgG-protein A-Sepharose column was constructed as described in Experimental Procedures. Serum IgG (1 ml) from RARAA rabbit #1 was loaded onto a 200 ul RAA IgG-protein A-Sepharose column (12 mg RAA IgG/ml Sepharose) equilibrated and washed with 100 mM phosphate (pH 8.0). Bound RARAA IgG was eluted with 0.1 M glycine (pH 3.0) and was collected in an equal volume of 0.1 M Tris-HCl (pH 8.0). The isolation of IgG from 1 ml of serum gave a peak with a 280 nm absorbance of 0.24. Subsequent isolations of IgG from the 100 mM phosphate column wash of the first peak gave peaks of 0.17 OD_{280}.


100mM
phosphate
O RARAA IgG
Δ RARAA IgG wash
Rabbit
IgG
pH3 glycine
RARAA IgG
OD_{280}
2.5
2.0
1.5
1.0
0.5
0
2
4
6
8
10
12
FRACTION NUMBER

much more efficient in binding RARAA IgG. Total serum IgG (1 ml) from RARAA #1 was loaded onto a 500 ul column equilibrated with 100 mM phosphate buffer (pH 8), as described in Experimental Procedures. Bound IgG was eluted from the column with glycine buffer (pH 3). The absorbance at 280 nm (OD_{280}) of the eluted peak was 0.24 as compared to only 0.04 for the RAA IgG-Sepharose column, even though 3 times as much serum IgG was loaded onto the latter column. When the RAA IgG-protein A-Sepharose column wash was reloaded onto a second column, a peak of 0.17 OD_{280} was eluted. A peak of this same magnitude was still eluted even after several consecutive passages through the column. This suggested that some IgG may have bound to nonspecific sites on the column and that the specific RARAA IgG might be represented by the difference in OD_{280} (0.07) between the first eluted peak (0.24) and subsequent passes (0.17) through the column.

The results of the Western Blot assay with the IgG eluted from the RAA IgG-protein A-Sepharose column are shown in Figure 9. It was evident that the same protein bands were recognized by the IgG that bound to or was washed through the column. This was true for all three bacterial strains. However, the 53K-specific IgG showed a significant enrichment on the column when the LL5 membranes were probed. This anti-53K IgG enrichment in LL5 correlated well with the higher levels of this protein that were observed in this strain relative to CBT43 and LL3 in the initial experiments with the RARAA #1 IgG. The amount of nonspecific IgG bound to the RAA IgG-protein A-Sepharose column unfortunately obscured the expected enrichment of anti-53K IgG in CBT43 or LL3. Two possibilities may explain this high background. One possibility is that all of the IgG species that were detected in Western Blots using

Figure IV-9. Western blot of RARAA #1 IgG after passage through the RAA IgG-Protein A-Sepharose column. Cytoplasmic membrane vesicle proteins (200 ug) from strains CBT43, LL3 and LL5 were separated on an SDS gel and were electrophoretically transferred to nitrocellulose paper as in Figure 6. Each lane was incubated with 20 ug of the following RARAA #1 IgG preparations:
Lane 1 - IgG washed through the RAA IgG-Protein A-Sepharose column (Figure 8); 2 - IgG eluted from the column; 3 - total IgG before passage through the column.

94
67
43
30
20.1
1 2 3
CBT43

1 2 3
LL3

73K
53K
37K
29K
1 2 3
LL5

the total serum IgG may have been specific for the RAA IgG and thus were able to bind to the column. A more likely alternative is that the column may have bound much of the IgG nonspecifically through protein A sites that were not successfully blocked with F_c fragments.

<u>Technical Difficulties with Antiidiotypic Antibody Production</u> - When the serum IgG from rabbits #2 through #15 (Table II) was examined in the same manner as that just described for RARAA #1, the presence of succinate-specific, internal image antibodies was not evident. Some of the problems that were encountered with antiidiotypic antibody production in these 15 rabbits will be outlined below.

Cytoplasmic membrane vesicles were routinely used in the Western Blots of RARAA IgG from rabbit #1. It may be remembered that the control or pre-immune IgG from this rabbit did not bind to any membrane vesicle proteins in any of the bacterial strains. However, most of the control sera from the other 15 rabbits lit up bands in the Western Blot assays. When fractionated outer and cytoplasmic membrane proteins were used instead of membrane vesicles, it was found that the control IgG from these rabbits recognized proteins in both membranes of CBT43. Some of the bands were common to both membranes, while others were unique to either the outer or cytoplasmic membrane. This recognition of <u>E. coli</u> proteins by the pre-immune sera might be explained by the fact that this bacterium is a normal resident of the large intestine of all mammals (24). Periodic infections in an animal would lead to the development of antibodies against various <u>E. coli</u> constituents. Porin and TuII* (25, 26) are two of the major outer membrane proteins in <u>E. coli</u>, so it was not surprising that many of the control sera in fact had IgG directed against these two proteins.

To circumvent the problem of existing antibodies to E. coli proteins in the rabbits' sera, outer membrane proteins were covalently coupled to Sepharose 4B. The idea was to preadsorb the serum IgG onto the outer membrane-Sepharose prior to Western Blot analysis in an attempt to reduce the complexity of the protein patterns in the blots. It was hoped that since most of the background IgG in the control and immune sera was directed against outer membrane constituents, the IgG that remained in the immune sera after passage through the column might be specific RARAA IgG. However, the use of this column met with only limited success when control sera were tested. The column was fairly efficient in removing the anti-TuII* IgG from the sera, but it was less efficient with the anti-porin IgG and it did not remove any of the other membrane-specific IgG species.

In any case, when the immune sera of rabbits #2 to #15 were subsequently examined in Western Blot assays, it was found that the proteins that were recognized by the majority of the sera were not much different from those initially recognized by their corresponding control sera. While each rabbit's serum IgG recognized a different set of membrane proteins, the proteins detected by the IgG of a particular rabbit remained relatively unchanged thoughout its successive bleeds. In addition, the protein patterns of wild-type and transport-mutant cytoplasmic membrane blots were not significantly different when probed with the same IgG preparation. The major protein that was illuminated by some of the control sera and many of the immune sera had a molecular weight of 73K Daltons. The 37K and 53K proteins which were tentatively identified in the succinate transport mutants by RARAA #1 IgG as SBP1 and SBP2 were either faint or entirely absent in blots probed with IgG

from rabbits #2 to #16. The 73K protein might be the large subunit of the cytoplasmic membrane enzyme succinate dehydrogenase. This enzyme is a particularily good antigen and is in fact a major membrane protein in E. coli grown aerobically on succinate (27).

The problems that are encountered in raising antiidiotypic antibodies are often due to the phenomenon of immunosuppression. The injection of an antibody into an animal would initially elicit the formation of anti-antibodies directed against the foreign IgG. However, if the level of the injected IgG is too high, immunosuppression might occur instead. The foreign IgG, through its F_c component, would shut down further synthesis of antibodies directed against it. In time, autoantiidiotypic antibodies would also be formed according to the Network Theory of the immune system. Both of these processes occur normally as a means of regulating antibody production and the immune response (1,5, 28). Rabbits #5 to #7 were injected with low amounts (25 to 75 ug) of RAA IgG in order to see if their immune response was better or different than that of rabbits #8 to #10, injected with higher amounts (270 ug) of IgG (see Table II). It was observed, however, that there was not much difference in the immune response of the two sets of rabbits. As discussed previously, the control sera of rabbits #5 to #10 already contained IgG directed against E. coli membrane proteins, and the pattern of proteins recognized by their corresponding immune sera was not substantially different. Further fractionation of the immune serum IgG on a RAA IgG-protein A-Sepharose column also did not result in the enrichment of any of the IgG species.

In an alternate approach, RAA IgG was covalently coupled with glutaraldehyde to Keyhole limpet hemocyanin (KLH) as described in Ex-

perimental Procedures and rabbits #11, #12, #14 and #15 (Table II) were immunized with the RAA IgG-KLH complex. It was hoped that the cross-linking of the IgG molecules to the large (8×10^6 Daltons) KLH molecule would sterically inhibit the immunosuppressive properties of the IgG's F_c domain. In addition, KLH is a good antigen and should stimulate the immune system (20). Control IgG that was retained on the aspartate-Sepharose column (Figure 5) was also linked to KLH and was injected into rabbits #13 and #16. However, the antibody response of the rabbits injected with the control IgG-KLH was no different than that of the RAA IgG-KLH-injected rabbits. In addition, the immune sera of rabbits #11 to #16 again recognized the same set of membrane proteins as did their respective control sera.

DISCUSSION

Antiidiotypic antibodies were originally used experimentally to study several different hormone and neurotransmitter membrane receptors (3,6,7,8,9). These receptors had already been identified by various means as discrete proteins of a certain molecular weight. The antibodies were used in the study of their biological functions and as an aid in their purification. The results of Chapters 2 and 3 tentatively identified a 53K protein as one of the succinate transport components of the *E. coli* cytoplasmic membrane. This chapter explored the possibility of raising antiidiotypic antibodies specific for the succinate binding proteins and the use of these antibodies in the identification of the transport proteins.

The advantages of using antiidiotypic antibodies in protein purification are two-fold. First, the protein need not be purified

before specific antibodies are raised against it (29,30). These antibodies could potentially bind much tighter to the protein than its actual substrate does, due to the complex nature of the antigen-antibody interaction (31). The tight, specific binding of the antibodies would then aid in the identification and purification of the proteins. This would be especially important in the purification of receptors that are only present in small amounts in the cell membrane. This is the case for the majority of receptor proteins (32), and it may be true for the succinate transport proteins of E. coli as well.

Several difficulties are encountered in the production of antiidiotypic antibodies. The first and foremost of these is that the right type of antiidiotypes must be produced by the test animals. Only antiidiotypes that are specific for the antigen binding site of the first antibody and therefore are the internal image of the first antigen will be useful. The ability of an animal to produce internal image antibodies depends upon its ability to recognize the specific antigen binding site idiotope of the injected IgG molecule. Not all animals will have the ability to respond to this idiotope within their immune repertoire and these animals will not make internal image antiidiotypes (1). Investigators have also observed that the antiidiotypic response of an animal is transient and the time course of the response is different in each animal (30). The initial synthesis of antiidiotypic antibodies is followed by a secondary synthesis of anti-antiidiotypic antibodies, and so on. It is possible for cyclical waves of antiidiotypic antibodies to be produced in the same animal: However, the fidelity of recognition for the original IgG molecule and thus for the protein of interest decreases with each successive round of antiidiotype production (33).

So it is important to monitor over time the antiidiotypic production of the animals.

In order to compensate for the above difficulties, a large number of rabbits, 16 in total (Table II), were used to raise succinate-specific antiidiotypic antibodies in this study. As well, successive bleedings were carried out with the animals, particularily with rabbits #5 through #10. The problem of possible immunosuppression was dealt with by injecting different amounts of RAA IgG into rabbits #5 to #10 and by using an IgG-KLH complex as the antigen in rabbits #11 to #16. The results of the Western Blot studies indicated that only one of the 16 rabbits (RARAA #1) may have produced the desired internal image antiidiotypes. Many of the pre-immune sera of the other 15 rabbits (#2 to #16) contained E. coli-specific antibodies that bound to the protein blots and in some cases their pattern of binding was identical to that of the corresponding immune sera. In addition, the IgG preparations from these rabbits did not detect any significant differences in binding to blots of wild-type and transport-mutant membrane proteins.

However, the control serum of rabbit #1 did not contain any E. coli-directed antibodies and its immune serum lit up 6 distinct bands (73K, 53K, 37K, 29K, 19.2K and 17K) in Western Blots of CBT43 membrane vesicles. When LL3 and LL5 membrane proteins were subsequently probed with this IgG preparation, differences in the levels of 3 of these proteins (53K, 37K and 17K) were evident between strains CBT43, LL3 and LL5 (Figure 6). Passage of RARAA #1 IgG through a column containing the first antibody (RAA IgG-protein A-Sepharose) also resulted in the enrichment of the anti-53K IgG species when LL5 membranes were examined. A high background of IgG binding to this column made it difficult to de-

termine if any of the other IgG species were also enriched. Unfortunately, reproducible results were not obtained for succinate-specific inhibition of RARAA #1 IgG binding to the membrane proteins. One might expect that succinate would inhibit any succinate-specific binding of the RARAA IgG to the membrane proteins. Alternatively, the binding of succinate to its specific binding proteins might induce a conformational change in these proteins that would facilitate the binding of RARAA IgG to them. The K_d for binding of an antigen-antibody complex is typically around 10^{-8} to 10^{-9} M (32), wheras the K_d for binding of succinate to the dicarboxylate binding proteins is about 10^{-5} M (22). Thus, one would expect that the IgG would easily displace the succinate in the Western Blot assays.

Of the 6 membrane proteins that were recognized by the RARAA #1 IgG, one was a good candidate for a succinate binding protein. This was the 53K protein, which was recognized strongly by the RARAA IgG of rabbit #1 and was present in lower amounts in some of the blots with other antiidiotype preparations. Cytoplasmic membranes of LL3, the *dctA* mutant, contained very little of this protein relative to CBT43 and LL5, the *dctB* mutant. As such, the 53K protein was tentatively identified as the *dctA* gene product, SBP2. This protein also had the same molecular weight as one of the cytoplasmic membrane proteins eluted by succinate or sodium chloride from aspartate-Sepharose columns (Chapters 2 and 3) of both wild-type and mutant strains. The 53K protein, along with a 42K protein, was also shown by two different binding assays in Chapter 2 to bind more succinate than the proteins eluted by 100 mM phosphate from the affinity column. The most interesting observation is that the results of both the RARAA IgG immunoblotting and aspartate-Sepharose

affinity chromatography of cytoplasmic membrane proteins indicated that the levels of this 53K protein approximated the dctA gene dosage in strains CBT43, LL3 and LL5. In other words, the 53K protein was present in larger quantities in the strain (LL5) that harboured two copies of the dctA gene and was present in reduced quantities in the strain (LL3) defective in this gene. The identification of the 37K and 17K proteins as succinate binding proteins was not as convincing. The studies in this chapter indicated that the 37K protein was present in reduced quantities in LL5 as compared to CBT43 and LL3. However, the concomitant increase in levels of this protein in LL3 due to its higher dctB gene dosage relative to CBT43 was not observed. The molecular weight of this 37K protein was also slightly higher than that of a protein (35K) eluted by 100 mM phosphate from the affinity column, while the levels and elution position of the 17K protein were not shown to be reproducible in the affinity chromatography studies.

In light of the difficulties encountered in raising succinate-specific antiidiotypes in 15 of the rabbits, one of the questions that might be considered is whether or not aspartate-BSA was the best choice for an antigenic substrate analogue. It was shown that when the aspartate was coupled to the BSA with a water-soluble carbodiimide, a cross-linked network of aspartate residues was formed. Therefore, the specificity of the RAA IgG that was isolated may not have been for a single aspartate residue, but instead for a polyaspartate oligopeptide. It should be noted that this same carbodiimide was used to prepare the aspartate-Sepharose as well, and this might explain the large amount of RAA IgG that bound to the column. An alternative method for creating an antigenic succinate analogue might be the use of glutaraldehyde to link

the amino group of aspartate to the epsilon-amino groups of lysine residues in a carrier protein such as BSA or hemoglobin. This method was used successfully to raise antibodies against the small neurotransmitter gamma-aminobutyric acid (34). The specificity of antigen binding usually encompasses the three-dimensional sequence or structure of several amino acids in a protein antigen (35). However, some antibodies are specific for only one group of a heterooligomeric antigen. In fact, antiidiotypic antibodies to the human erythrocyte glucose transporter have been raised using glucosamine-Sepharose 4B as the first antigen (36). Therefore, it is difficult to say whether an aspartate polymer linked to BSA would be a a better succinate analogue than aspartate linked via glutaraldehyde to BSA or vice versa.

Another fact that must be considered is that some proteins are extensively denatured when run on SDS gels and the extent of renaturation in the Western Blot assay depends upon the particular protein and the experimental conditions (36). A certain proportion of the succinate binding proteins may have been present in a denatured form in the Western Blots and this may have interfered with the binding of the antiidiotypic antibodies in some of the experiments. In any case, the observation that only one out 16 rabbits in this study appeared to produce succinate-specific, internal image antibodies is not too different from results obtained by other investigators. In particular, Homcy _et al_ found that only one out of 11 rabbits tested was positive for the production of antiidiotypic antibodies specific for the β-adrenergic receptor (6).

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CHAPTER V

A PHOTOAFFINITY LABEL FOR THE DICARBOXYLATE TRANSPORT SYSTEM

The technique of photoaffinity labelling has been used to specifically label and identify a wide number of membrane enzymes and receptors in various biological systems. The basic theory behind this technique involves the synthesis of a radioactively-labelled, photoreactive derivative from the substrate or substrate analogue of the protein of interest. The photolabel is incubated with the membrane or protein sample and then light of the appropriate wavelength and intensity causes the covalent attachment of the affinity label to specific sites in the sample. Separation of the sample proteins on an SDS polyacrylamide gel and subsequent autoradiography of the gel allows the identification of the polypeptide chains which are labelled by the radioactive, photoreactive compound. The photoaffinity label can then also be used to follow the purification of the specific protein (1). This is especially useful in the case of non-enzymatic membrane proteins, such as transport proteins, which are usually present in low amounts in the cell and are thus often difficult to detect and purify.

Several criteria must be met in order to ensure that a photoreactive compound is able to specifically label the protein of interest. One must be able to demonstrate that the affinity label either binds to the same site on the protein as does the substrate or that it is able to specifically inhibit the physiological function of the protein. During photolysis, if the concentration of the photolabel is in great excess of the number of specific sites in the sample, nonspecific labelling of other proteins can also occur. The ideal photolabel should have a K_d

for binding to the protein that is close to the number of protein sites in the membrane. However, under the appropriate conditions, a photolabel with a lower affinity for the specific sites can also be used successfully (1). Finally, a radioactive photolabel of very high specific activity is desirable in order to be able to detect even very small amounts of the specific protein. Iodinated labels have proven very useful in this instance, due to their ease of preparation, high carrier-free specific activity and ease of detection by autoradiography (1).

Some examples of biological systems that have been probed by synthetic photoaffinity labels are the rat neural potassium channel (2), the rabbit IgG molecule (3), the beef heart cytochrome c oxidase (4), the ovine lutropin receptor (5), the duck erythrocyte β-adrenergic receptor (6) and the human erythrocyte D-glucose transporter (7). The glucose transporter has also been photolabelled by cytochalasin B, a compound which contains an intrinsic photoreactive group (8). In addition, the B-adrenergic receptor in turkey erthrocytes has been successfully labelled with an affinity probe that is not photoreactive. This non-covalent labelling was possible only because the label had such a high affinity for the receptor (K_a of 10^{-10} M) (9).

An aryl azide derivative of the relevant substrate was used as the photolabel in all of the above examples, with the exception of cytochalasin B. Aryl azides are the reagents of choice in biological photoaffinity labelling because they can be activated at wavelengths above 300 nm, which greatly reduces the risk of photo-oxidation and aggregation of proteins (1). Their phenyl group also allows for the addition of an [^{125}I]-atom as a radioactive tag. During photolysis, the azido group forms a highly-reactive nitrene group which then covalently

inserts into CH and NH bonds of nearby molecules (1). Therefore, in biological systems reactions can occur with solvent molecules and non-specific amino acid residues, as well as with specific proteins. The probability of reaction with a particular protein increases if a specific constituent on the aryl nitrene has an affinity for that protein.

Therefore, an aryl azide was used as the starting material to design a photoaffinity label specific for the succinate transport system. Aspartate was chosen as the transport substrate analogue because it is also transported by the dicarboxylate system, albeit less efficiently than succinate (10), and because it possesses an amino group through which a photoreactive dicarboxylate derivative could be formed. The aspartate was coupled through its amino group to 4-fluoro-3-nitrophenyl azide to yield the photolabel N-(4-azido-2-nitrophenyl)aspartic acid. This photolabel was then used in both whole cells and membrane preparations of wild-type and transport-mutant strains in an attempt to identify the dicarboxylate transport components.

EXPERIMENTAL PROCEDURES

Bacterial Strains - The E. coli K12 strains used in these studies were described in Chapter 3. They are: CBT43, LL3, LL5, LL5 Rev and 4-31.

Synthesis of 4-Fluoro-3-nitrophenyl Azide - The first reaction in the synthesis of the photoaffinity label was a combination of the methods of Fleet et al (3) and Hagedorn et al (11). 4-Fluoro-3-nitroaniline (5.0 gm, 0.0320 moles) was dissolved in a mixture of warmed, concentrated hydrochloric acid (30 ml) and deionized water (5 ml). Sodium nitrite (2.4 gm, 0.0348 moles) in water (5 ml) was added dropwise

over 5 minutes while the temperature of the reaction was kept between -20°C and -15°C with a dry ice-acetone bath. The reaction mixture was then stirred for 10 minutes and was quickly filtered into a flask precooled to -20°C. The mixture was diluted with water (40 ml) and then diethyl ether (100 ml) was added. Sodium azide (2.2 gm, 0.0338 moles) in water (8 ml) was added while the temperature of the reaction was kept between -10°C and -5°C. The reaction was allowed to continue for 30 minutes. The ether layer was removed and the remaining aqueous layer was extracted with another 100 ml of ether. The ether extracts were pooled and dried on a rotary evaporator (37°C). Recrystallization from petroleum ether (30 - 60°C) resulted in 2.04 gm (35% yield) of 4-fluoro-3-nitrophenyl azide as orange needles, melting point 53 - 55°C, NMR ($CDCl_3$) δ 7.70 (m,1H), 7.25 (m,2H), characteristic of the product (11).

Synthesis of N-(4-Azido-2-nitrophenyl)aspartic Acid - The second reaction was a modification of the method of Ramirez et al for the synthesis of 4-(3'-aminopropyl)amino-3-nitrophenyl azide (12). 4-Fluoro-3-nitrophenyl azide (1.0 gm, 0.0055 moles) was dissolved in 95% ethanol (20 ml). L-Aspartic acid (0.70 gm, 0.0052 moles) and sodium carbonate (1.10 gm, 0.0103 moles) in deionized water (30 ml) was added to the solution, along with 50 uCi of L-[^{3}H-2,3]-aspartic acid. The mixture was stirred in a foil-covered flask and was heated to around 55 - 60°C for 1 hour, then allowed to cool slowly down to room temperature for several hours. The heating process caused the normally insoluble azide to go into solution. This heating-cooling sequence was repeated several times over the course of 4 days and 1 to 2 ml of ethanol was added to the mixture prior to each heating period. Unreacted azide (0.5 gm) was filtered from the solution and then the solution was acidified

with 25 ml of 0.1N hydrochloric acid in order to precipitate the orange-red product, N-(4-azido-2-nitrophenyl)aspartic acid.

Purification of N-(4-Azido-2-nitrophenyl)aspartic Acid - The product was adsorbed onto several grams of BioSil A silicic acid (200 - 400 mesh) with a rotary evaporator and this was loaded onto a 40 x 3 cm (ID) column of the same silicic acid. The column was eluted with a 1:1 mixture of methanol:ethyl acetate. A small amount of unreacted, orange azide was eluted first, followed by a broad band of the orange-red product. Salts remained at the top of the column. Purity of the column fractions was assessed with thin layer chromatography using Whatman Linear K silica gel plates (20 x 20 cm) and a 9:1 ethyl acetate:methanol solvent system. After drying on the rotary evaporator, the product was dissolved in 150 ml of water:methanol (40:1) and insoluble material was removed by filtration. N-(4-azido-2-nitrophenyl)aspartic acid (aspartyl azide) (425 mg, 52.4% yield) was recovered and it was stored at 4°C in the dark. NMR ($DMSO-d_5$) confirmed the identity of this product: δ 8.75 (d,1H), 6.8 - 8.0 (m,2H), 7.7 (d,1H), 7.4 (dd,1H), 7.1 (d,1H), 4.5 (m,1H) and 2.7 (d,2H).

Inhibition of Whole Cell Succinate Transport by Aspartyl Azide - Whole cell uptake of [^{14}C-2,3]-succinic acid by the wild-type strain, CBT43, was carried out as described in Chapter 3. During the 10 minute incubation period prior to addition of the cells to the transport assay, varying concentrations of aspartyl azide (500 uM to 4 mM) were mixed with the [^{14}C]-succinate and phosphate buffer. Transport studies at various concentrations of succinic acid (2 uM to 20 uM) were used to determine the apparent K_i of aspartyl azide inhibition of succinate uptake. Lineweaver-Burk (13) and Dixon (14) plots were used to deter-

mine the kinetic constants.

Iodination of Aspartyl Azide - A modification of the methods of Hunter and Greenwood (15) for labelling proteins and Brown and Aurbach (9) for labelling hydroxybenzylpindolol was used to iodinate and isolate labelled aspartyl azide. Aspartyl azide (11 nmol - 11.4 ul of 960 uM) was mixed with ferric chloride (0.09 nmol - 9 ul of 10 uM) and 2 mCi of sodium [^{125}I]-iodide (0.9 nmol - 19.4 ul of 46.4 uM) in 47.75 ul total of 0.1 M potassium phosphate, pH 7.5 buffer in an Eppendorf tube. Chloramine T (1.8 nmol - 2.25 ul of 225 ug/ml) was added and the tube was immediately closed and vortexed. It was sealed with Parafilm and was incubated for 7 days at 25°C in a lead container in the fume hood. At the end of this time, 11.25 ul of 1 mg/ml sodium metabisulfite were added to the reaction mixture.

The sample was then loaded onto a 1 ml disposable column of Whatman DE52 cellulose equilibrated with 1 mM Tris-HCl, pH 8.0. The following solutions were passed through the column and were collected batchwise in 15 ml disposable, plastic screw-cap tubes: 1) 5 ml of 1 mM Tris-HCl (pH 8.0), 2) 10 ml of 10 mM sodium chloride in 1 mM Tris-HCl, 3) 5 ml of 20 mM sodium chloride in 1 mM Tris-HCl and 4) 10 ml of 5 mM hydrochloric acid. Fractions (0.5 ml) were then collected when another 15 ml of 5 mM hydrochloric acid were passed through the column. Aliquots (5 ul) were taken from each tube and were counted for radioactivity. The bulk of the free sodium [^{125}I]-iodide was eluted with the 10 mM sodium chloride, whereas the [^{125}I]-aspartyl azide was eluted only after 17 ml of 5 mM hydrochloric acid (fraction 14 - 15) were passed through the column. The fractions containing the iodinated photolabel were pooled and were stored in the dark behind lead at 25°C.

Whole Cell Transport of [^{125}I]-Aspartyl Azide - Transport assays were carried out as previously described in Chapter 3. The uptake of [^{125}I]-aspartyl azide by the wild-type strain, CBT 43, was examined at a number of aspartyl azide concentrations (10 uM to 500 uM) and time intervals (15 seconds and 1 minute). The apparent K_d of transport was calculated from a Lineweaver-Burk plot (13).

Photoaffinity Labelling of Cytoplasmic Membranes - Kaback cytoplasmic membrane vesicles of CBT43, as described in Chapter 2, were used in preliminary experiments to determine optimal photolysis conditions. Varying amounts of membrane vesicles (100 ug to 2 mg protein) were incubated for 30 minutes at 25°C on a rotary mixer with different concentrations of [^{125}I]-aspartyl azide (1 uM to 500 uM) in a total of 300 ul of 50 mM potassium phosphate, pH 6.6 buffer. The membranes were then irradiated for 4 seconds in shallow, polypropylene mini-vials at a distance of 11 cm from a 1000 Watt mercury lamp (Porta-Cure 1000F from the American Ultraviolet Company). The membranes were spun down in an Eppendorf centrifuge, were washed once with 300 ul of phosphate buffer (50 mM, pH 6.6) and then were solubilized in SDS sample buffer at 70°C for 30 minutes. Membrane proteins were separated on 1.5 mm, 11.5% polyacrylamide slab gels, as described in Chapter 2. The gel was stained, dried and exposed to a Kodak X-OMAT X-ray film for several weeks at -80°C. Further experiments routinely used 500 ug of membrane proteins and 2 uM [^{125}I]-aspartyl azide. As well, a 0.5 cm thick glass plate was placed on top of the samples in order to screen out damaging wavelengths of light below 300 nm. It was determined previously with a Beckman DU-8 wavelength scan that the aspartyl azide had absorbance maxima at 473 and 263 nm.

For comparison of membrane labelling patterns between CBT43 and the various transport mutants, crude cytoplasmic membranes (non-Kaback) were prepared. In a modification of the procedure for Kaback membrane vesicles described in Chapter 2, the nuclease step was omitted after the spheroplasts were homogenized. The membranes were then broken into smaller fragments with the French Press and further homogenization, were washed once with phosphate buffer and were frozen in buffer at -20°C. Membranes (500 ug) from these strains were then photolyzed with 2 uM [^{125}I]-aspartyl azide and examined on SDS gels as outlined above.

Photoaffinity Labelling of Cells - Cells were grown in LB medium under the conditions described for the transport assays in Chapter 3. After harvesting and washing with 50 mM potassium phosphate (pH 6.6) buffer, the cell pellets were weighed and were resuspended in phosphate buffer to a concentration of 50 mg/ml. An aliquot of cells (5 mg) was incubated for 30 minutes with [^{125}I]-aspartyl azide in a total of 300 to 400 ul of buffer and was then photolyzed under glass for 8 seconds. Cells were prepared for electrophoresis in the same manner as the membrane preparations. Some of the parameters that were investigated were [^{125}I]-aspartyl azide concentration (2 uM and 500 uM), time of incubation (2 to 30 minutes), inhibition of nonspecific binding with PABA (para-aminobenzoic acid) and inhibition of aspartyl azide binding with 2 and 20 mM succinate, aspartate or glutamate. These conditions were studied in CBT43 and the transport mutants.

Localization of [^{125}I]-Aspartyl Azide in Whole Cells - CBT43 cells that were photolyzed with 2 uM and 500 uM aspartyl azide as described in the above section were used in this study. After photolysis, the cells (5 mg each, duplicate) were pelleted and were selectively

disrupted according to the methods of Heppel and Kaback (16,17) in order to determine the fate of the photoaffinity label. The cells were washed once with 200 ul of 50 mM potassium phosphate, pH 6.6. Loosely-bound, surface proteins were removed with a Tris-EDTA-sucrose (33 mM (pH 7.2)-1 mM-20%; 300 ul) treatment for 10 minutes. Proteins associated with the outer membrane and underlying periplasm were then released by osmotic shock in cold 5 mM magnesium chloride for 10 minutes at 4°C. Then, incubation for 30 minutes in the Tris-EDTA-sucrose solution containing 125 ug/ml of lysozyme removed the peptidoglycan layer and the rest of the periplasm. The resulting spheroplasts were homogenized to release the cytoplasmic contents and the cytoplasmic membranes were then pelleted at 13,000 x g. All of the fractions were counted to determine the amount of [^{125}I]-aspartyl azide incorporated. Alternatively, the proteins in each fraction were precipitated with 10% TCA and were then separated on a 1.5 mm, 11.5% polyacrylamide SDS slab gel. The gel was stained, dried and exposed to an X-ray film in order to visualize the subcellular location of the photolabel.

<u>Chemicals</u> - 4-Fluoro-3-nitroaniline and para-aminobenzoic acid were purchased from the Sigma Chemical Company (St. Louis, MO). Sodium nitrite and sodium azide were both obtained from the Fisher Scientific Company (Fair Lawn, NJ). BioSil A silicic acid was bought from Bio-Rad Laboratories (Richmond, CA), while the silica gel plates were purchased from Whatman Limited (Maidstone, Kent, UK). Both [^{14}C-2,3]-succinic acid and L-[^{3}H-2,3]-aspartic acid were purchased from the New England Nuclear, Co. (Boston, MA), while sodium [^{125}I]-iodide was bought from ICN Biomedicals, Inc. (Irvine, CA).

RESULTS

Synthesis of N-(4-Azido-2-nitrophenyl)aspartic Acid - The photoaffinity label, N-(4-azido-2-nitrophenyl)aspartic acid, was synthesized in two steps (3,11,12), as detailed in Experimental Procedures and as shown diagramatically in Figure 1. 4-Fluoro-3-nitroaniline (I) was treated with nitrous acid (from the addition of sodium nitrite to dilute hydrochloric acid) and hydrochloric acid to form the unstable diazonium salt (II) (18). This salt was then treated *in situ* with sodium azide to yield 4-fluoro-3-nitrophenyl azide (III), a bright orange solid with a melting point of 53 to 55°C. Its NMR spectrum is shown in Figure 2. The overall yield after purification was 35%. Compound III was then reacted with L-aspartic acid, using sodium carbonate as an acid acceptor, to give the orange-red product N-(4-azido-2-nitrophenyl)aspartic acid (aspartyl azide) (IV) in 52% yield based on recovered 4-fluoro-3-nitrophenyl azide (50%).

Purification and Identification of N-(4-Azido-2-nitrophenyl)-aspartic Acid - The aspartyl azide was separated from the unreacted 4-fluoro-3-nitrophenyl azide and various contaminating salts by chromatography on a BioSil A silica gel column, as described in Experimental Procedures. Due to the low solubility of some sample components in the column solvent, the sample was initially adsorbed by drying onto a few grams of the silica gel. This was then loaded onto the column. A band of unreacted orange azide was eluted first with the methanol:ethyl acetate (1:1) solvent. This was followed by a broad band of orange-red aspartyl azide. Brown, insoluble salts remained at the top of the column. The purity of each column fraction was assessed by thin layer chromatography (TLC). Figure 3 shows the TLC results. Purified 4-

Figure V-1. Reactions in the synthesis of N-(4-azido-2-nitrophenyl)aspartic acid (aspartyl azide). The photolabel, aspartyl azide, was synthesized under the reaction conditions described in the text. The compounds involved in the reactions were: I - 4-fluoro-3-nitroaniline; II - diazonium salt intermediate; III - 4-fluoro-3-nitrophenyl azide; IV - N-(4-azido-2-nitrophenyl)aspartic acid (aspartyl azide)

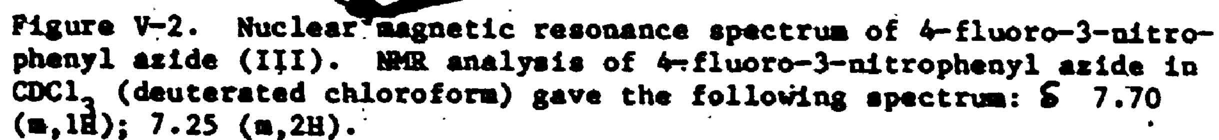

Figure V-2. Nuclear magnetic resonance spectrum of 4-fluoro-3-nitrophenyl azide (III). NMR analysis of 4-fluoro-3-nitrophenyl azide in $CDCl_3$ (deuterated chloroform) gave the following spectrum: δ 7.70 (m,1H); 7.25 (m,2H).

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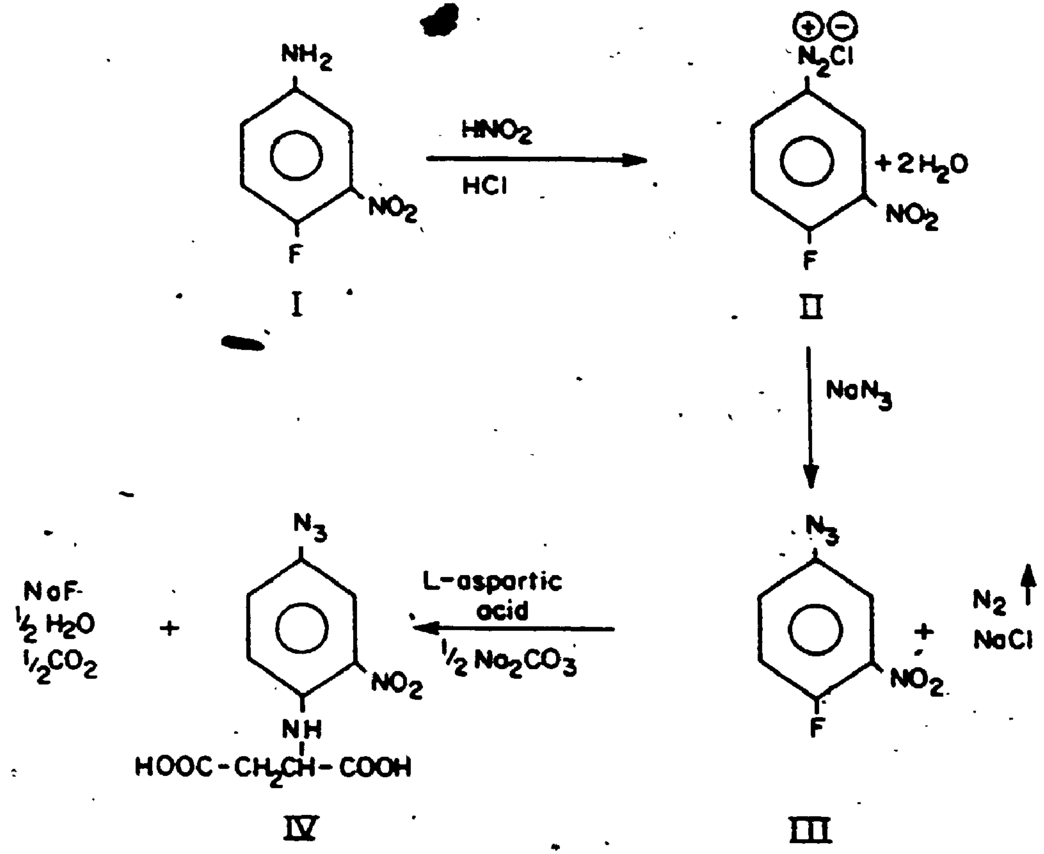
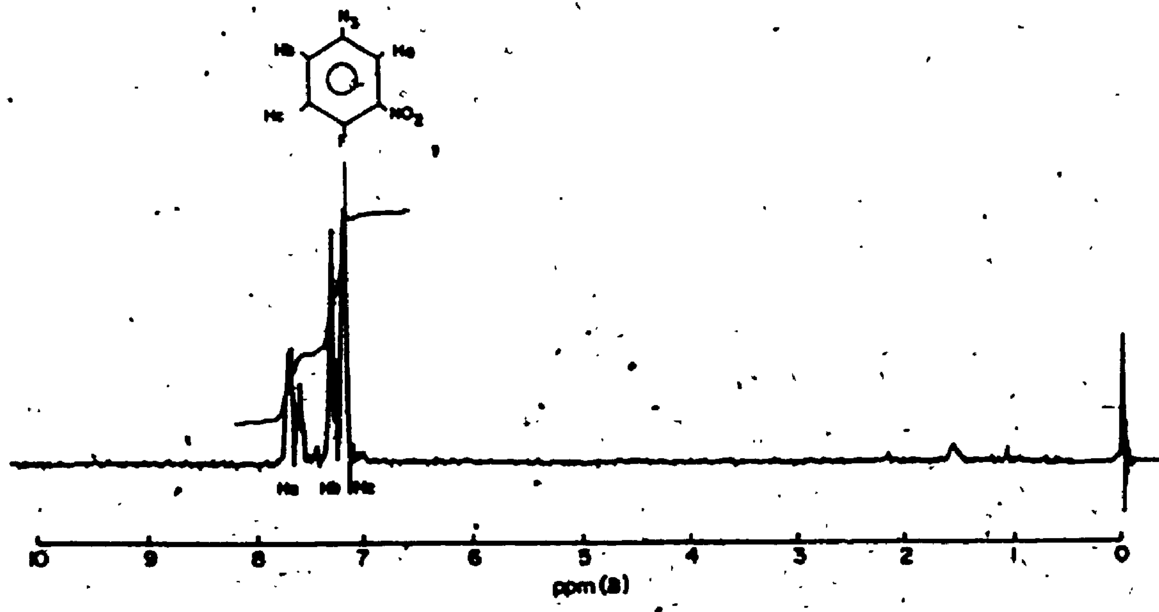




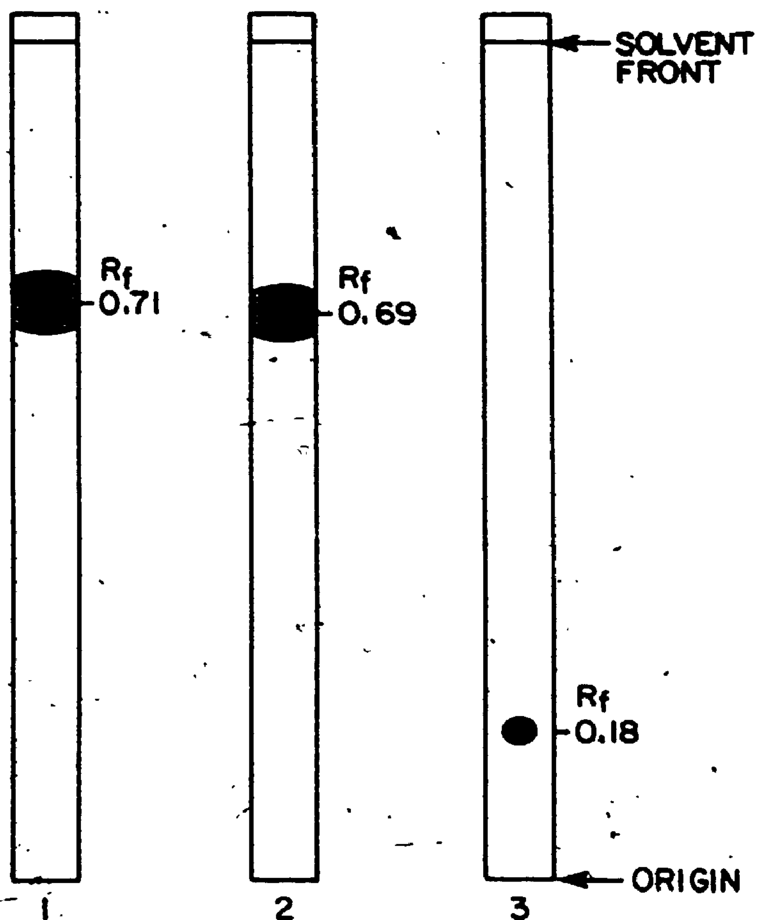
fluoro-3-nitrophenyl azide was run in lane 1 as a standard. Lane 2 corresponds to the unreacted 4-fluoro-3-nitrophenyl azide, the first band from the silica gel column. The second band, the aspartyl azide, was run in lane 3. It showed a small amount of contamination with unreacted azide. It should be noted that the silica gel column was run in 1:1 ethyl acetate:methanol in order to decrease the elution time for the aspartyl azide. The TLC plates, in contrast, were run in much lower methanol (9:1 ethyl acetate:methanol) to facilitate the separation and identification of the two compounds.

The identification of the major spot in lane 3 of the TLC plate as N-(4-azido-2-nitrophenyl)aspartic acid was confirmed by NMR. The peaks and their corresponding chemical groups are shown in Figure 4. The aspartyl azide was found to be very soluble in both water and methanol. After purification, it was dissolved to a concentration of 9.6 mM in deionized water containing 2.5% methanol and was stored in the dark at $4^{o}C$.

Inhibition of Whole Cell Succinate Transport - Prior to the use of the aspartyl azide as a photoaffinity label, it was necessary to determine whether it was specific for the succinate transport system. Transport studies were carried out with CBT43 cells as described in Chapter 3. Varying amounts of carrier-free [^{14}C-2,3]-succinic acid and unlabelled aspartyl azide were used to determine if the photolabel was able to inhibit whole cell succinate transport. In preliminary studies with 2 uM succinate, it was found that a concentration of 500 uM aspartyl azide was the lowest value that inhibited succinate uptake. Aspartyl azide concentrations from 500 uM to 4 mM were then used in transport assays with 2 to 20 uM [^{14}C]-succinate in order to determine the appar-

Figure V-3. Thin layer chromatography of aspartyl azide. The purity of the bands eluted with methanol:ethyl acetate (1:1) from the BioSil A silicic acid column was assessed on Whatman Linear K silica gel plates using a 9:1 ethyl acetate: methanol solvent system. The mobility of each compound relative to the solvent front is noted next to its spot. Lane 1 - 4-fluoro-3-nitrophenyl azide standard; 2 - unreacted 4-fluoro-3-nitrophenyl azide (band #1 from column); 3 - aspartyl azide (band #2 from column).

Figure V-4. Nuclear magnetic resonance spectrum of aspartyl azide. NMR analysis of aspartyl azide in DMSO-d_5 (deuterated) gave the following spectrum: δ 8.75 (d,1H); 6.8-8.0 (m,2H); 7.7 (d,1H); 7.4 (dd,1H); 7.1 (td,1H); 4.5 (m,1H); 2.7 (d,2H).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Rf 0.69
Rf 0.18
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1
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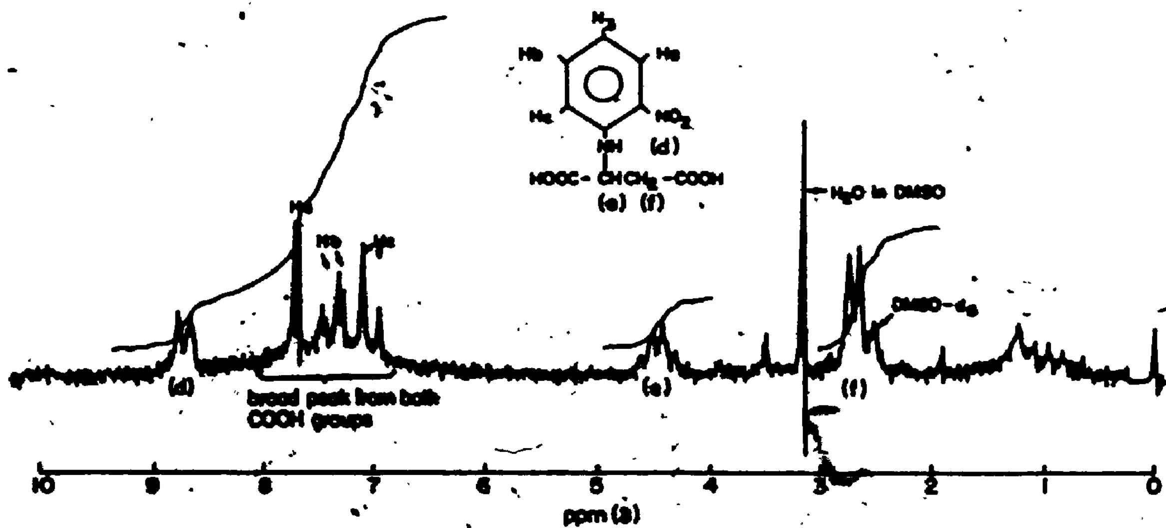
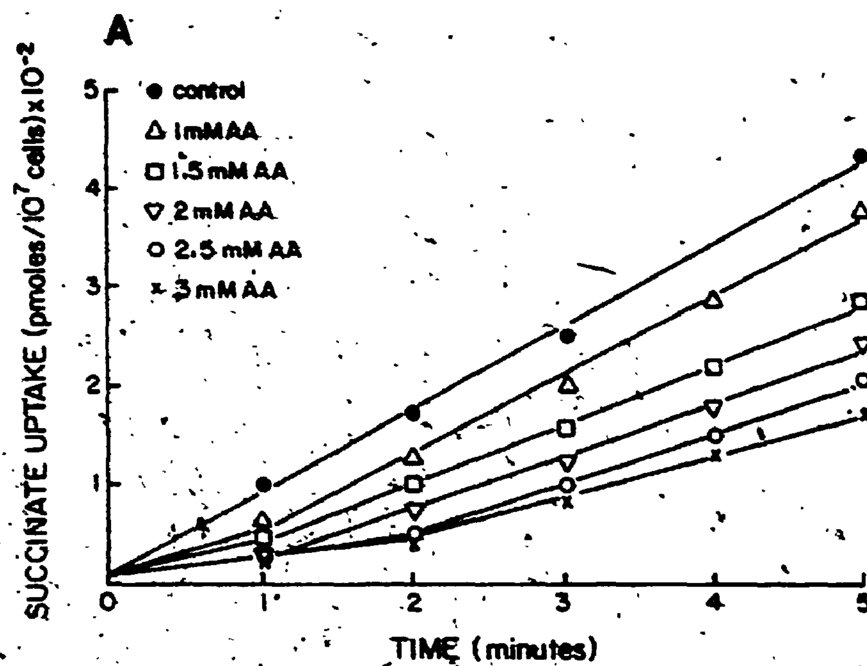
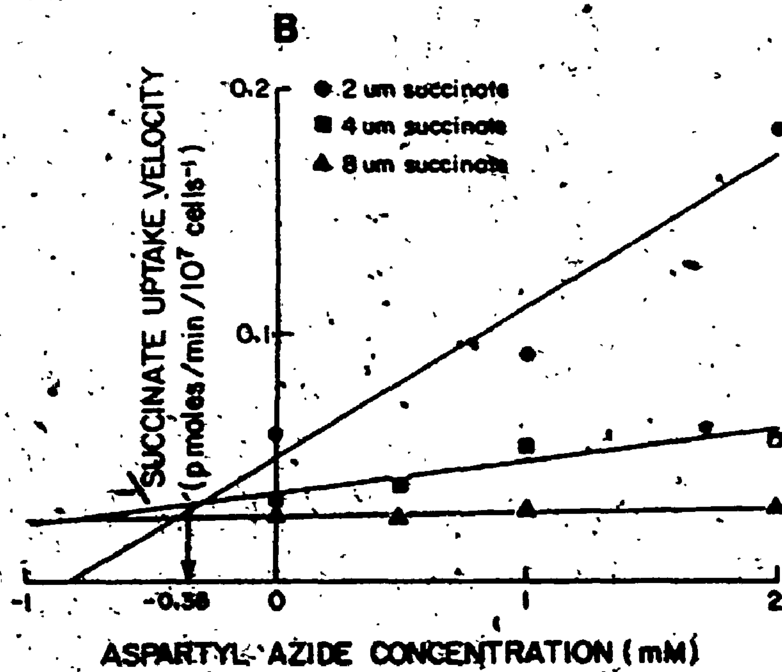

HOOC- CHCH2 -COOH
(e) (f)
NH (d)
NO2
H2O in DMSO
DMSO-d6
(d)
(e)
(f)
broad peak from both COOH groups
10
9
8
7
6
5
4
3
2
1
0
ppm (δ)

ent K_i of inhibition. As described in Experimental Procedures, an aliquot of cells was added at time zero to an incubation mixture of [^{14}C]-succinate and aspartyl azide in phosphate buffer. Figure 5A is a plot of succinate transport in LB-grown cells over 5 minutes in the presence of 1 to 3 mM aspartyl azide. It shows that the rate of succinate transport decreased as the aspartyl azide concentration was increased. Similar results were obtained for M9-grown cells, although the uptake velocity was higher in all cases, as expected from results discussed in Chapter 2.

The reciprocal of each line's slope in Figure 5A was derived by computer and these values were plotted against the aspartyl azide concentration, according to the methods of Dixon (14). This plot yielded information about the apparent K_i of inhibition and also about the type of inhibition, competitive or non-competitive, that occurred. The results from one such experiment are shown in Figure 5B. The point on or above the X-axis at which the various lines intersected gave the value of $-K_i$. If the lines intersected at any value of 1/V above the X-axis, this indicated competitive inhibition. However, if the lines intersected on the X-axis, then non-competitive inhibition was indicated. K_i values of 0.33, 0.43 and 0.35 mM were obtained for aspartyl azide inhibition of succinate transport in three separate experiments. This gave an apparent K_i of 0.37 +/- 0.04 mM aspartyl azide. It was evident in all three experiments that the lines on the Dixon plot intersected above the X-axis and, therefore, this suggested that the aspartyl azide competitively inhibited succinate transport. The apparent K_m for succinate transport was also calculated with a Lineweaver-Burk plot (13), which used the reciprocal of the control slope values (no inhibitor

Figure V-5. Aspartyl azide inhibition of CBT43 succinate transport. CBT43 cells were grown to late-log in LB medium at 37°C and were prepared for transport studies as described in Chapter 3. Briefly, the cells were washed with 50 mM potassium phosphate buffer (pH 7.5) and were resuspended to an OD_{600} of 4.0 in this same buffer. An aliquot of cells (120 ul) was added at time zero to a stirred mixture of [^{14}C]-succinic acid (2 to 20 uM) and aspartyl azide (0.5 to 4 mM) in phosphate buffer to give a total volume of 1.2 ml. At one minute intervals, 200 ul of the assay mixture were filtered and washed with two 5 ml aliquots of cold phosphate buffer.
A - The inhibition of uptake of 20 uM [^{14}C]-succinate (pmoles/10^7 cells) by various concentrations of aspartyl azide (1 to 3 mM) was plotted against time (minutes).
B - The reciprocal of the rates of uptake (pmoles/10^7 cells/minute^{-1}) for 2, 4 and 8 uM succinate was plotted against aspartyl azide concentration (mM). The intersection of the lines above the X axis at a value of approximately -0.35 mM indicated an apparent K_i of 0.35 mM for competitive aspartyl azide inhibition of succinate transport.


A
control
1mMAA
1.5 mM AA
2 mM AA
2.5 mM AA
3 mM AA
SUCCINATE UPTAKE (pmoles/10⁷ cells) x 10⁻²
TIME (minutes)


B
2 um succinate
4 um succinate
8 um succinate
1/SUCCINATE UPTAKE VELOCITY (pmoles/min/10⁷ cells⁻¹)
-0.38
ASPARTYL AZIDE CONCENTRATION (mM)

added) and the reciprocal of the succinate concentrations. A value of 6.44 +/- 0.65 uM was obtained in the three experiments for the K_m, and this was close to the value of 11 uM reported earlier for this transport system (19).

It should be noted that not only did the slope of the lines in Figure 5A decrease with increasing aspartyl azide concentration, but the absolute value of succinate taken up by the cells was decreased at the early timepoints, so much so that the lines for the higher azide concentrations no longer extrapolated to a positive value on the Y-axis. This might explain the variation obtained (11%) in the K_i measurements. It was also observed that two slopes could be calculated for some of the lines with the higher inhibitor concentrations. Succinate transport was inhibited to a greater extent during the first two minutes of uptake in the presence of 2.5 and 3 mM aspartyl azide, relative to the last 3 minutes of uptake. The cells seemed to partially "recover" from the aspartyl azide inhibition. When the initial rates of succinate uptake in the presence of the higher aspartyl azide concentrations (2.5, 3 mM) were used to determine the photolabel's inhibition constant, neither competitive nor non-competitive inhibition was indicated. Instead, complex kinetics that were interpreted as mixed inhibition were observed. An explanation for these mixed kinetics might be that aspartyl azide inhibition of succinate uptake by general mechanisms other than competitive inhibition were superimposed upon its competitive inhibition at the early timepoints in Figure 5A. In any case, this complex inhibition did not allow for a K_i determination from the initial rates of succinate uptake. Later photoaffinity experiments which involved an initial incubation of whole CBT43 cells with the aspartyl azide for

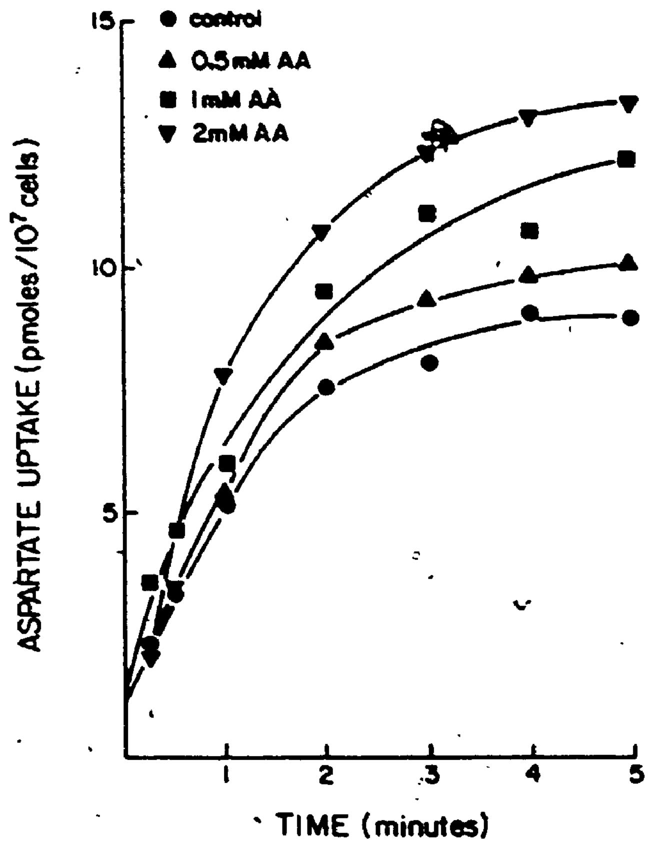
periods of 2 to 30 minutes indicated that the same proteins were labelled at all incubation times, suggesting that degradation of the photolabel was not the problem. In addition, the slopes of the lines calculated at the later timepoints in Figure 5A indicated the competitive inhibition kinetics seen in Figure 5B. Therefore, it was felt that these data reflected the competitive interaction of the photolabel with the transport system and they were used to calculate the apparent K_i for aspartyl azide inhibition of succinate transport.

Aspartyl Azide Inhibition of Aspartate Transport - Since the photoaffinity label that was constructed for the succinate transport system consisted of aspartate covalently linked to a phenylazide group, the effect of aspartyl azide on aspartate transport was also investigated. Kay showed that aspartate is transported by two different systems in *E. coli* (10). The dicarboxylic acid *dct* system of this study is also a low affinity transport system for aspartate (K_m of 30 uM in *ast*$^-$ strain), while a more specific *ast* system transports aspartate with a much higher affinity (K_m of 3.5 uM in *dct*$^-$ strain). The former system is inducible, whereas the latter system is constitutive. Aspartate is taken up very rapidly by *E. coli* and it is metabolized immediately upon entry into the cell. Therefore, 15 second as well as 5 minute timepoints were used in the transport assays and a strain that was defective in citrate synthetase (*gltA*$^-$) was used in order to see concentrative uptake (10).

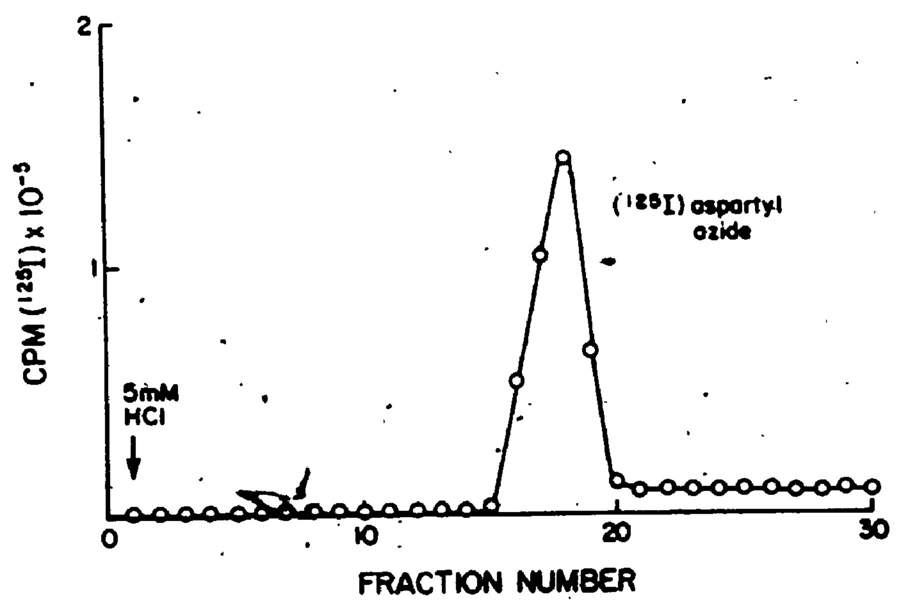
The *gltA*$^-$ strain that was used in this study was H30, the parental strain of CBT43. The cells were grown in LB medium with 0.2% succinate to mimic the growth conditions of the CBT43 cells used in the succinate transport studies. Figure 6 shows the results of 3.9 uM L-

Figure V-6. Aspartyl azide inhibition of H30 aspartate transport. The uptake of 3.9 uM [^{3}H]-aspartic acid was measured in strain H30 (gltA) in the presence of 0.5 to 3 mM aspartyl azide. Cells were grown in LB medium and were prepared and assayed for transport activity as in Figure 5. Uptake of 3.9 uM aspartate (pmoles/10^7 cells) was plotted against time (minutes).

Figure V-7. Purification of [^{125}I]-aspartyl azide on DEAE Cellulose. Aspartyl azide (11 nmol) was iodinated with sodium [^{125}I]-iodide (0.9 nmol) in the presence of ferric chloride and chloramine T as described in Experimental Procedures. After terminating the reaction with sodium metabisulphite, the sample (61 ul) was loaded onto a 1 ml column of DE52 Cellulose equilibrated in 1 mM Tris-HCl, pH 8.0. Successive washes with 1 mM Tris buffer (5 ml), 10 mM sodium chloride in Tris buffer (10 ml) and 20 mM sodium chloride in Tris buffer (5 ml) removed greater than 95% of the free iodine from the column. [^{125}I]-aspartyl azide was eluted from the column with 25 ml of 5 mM hydrochloric acid. The elution profile of the aspartyl azide by the last 15 ml of hydrochloric acid in 500 ul aliquots is shown in this figure.



control
0.5mM AA
1mM AA
2mM AA
ASPARTATE UPTAKE (pmoles/10⁷ cells)
TIME (minutes)



(¹²⁵I) aspartyl azide
5mM HCl
CPM (¹²⁵I) x 10⁻³
FRACTION NUMBER

$[^3H$-2,3]-aspartic acid uptake by strain H30. It was evident that the aspartate transport by these cells was rapid indeed, approaching a maximum value at 2 to 3 minutes. The results from an experiment that used 15 second timepoints to measure aspartate transport indicated that the uptake was still linear up to 75 seconds and no inhibition was observed in the presence of aspartyl azide. In fact, the 5 minute transport studies in Figure 6 indicated that the aspartyl azide appeared to stimulate aspartate uptake. However, as far as competitive inhibition by aspartyl azide was concerned, only the succinate transport system seemed to be involved. This was expected since the high affinity aspartate transport system has a specificity for alpha-amino groups (10) and this group was involved in a covalent bond in the aspartyl azide molecule.

Iodination of Aspartyl Azide - Many mammalian membrane receptors are present in very low amounts in the cell, specifically in the range of 10^{-10} to 10^{-13} moles of receptor per milligram of membrane protein (1). This may also be the case for the cytoplasmic membrane dicarboxylate transport components of *E. coli*, as discussed previously. For this reason, radioactive compounds with a very high specific activity are needed to do binding or photoaffinity labelling studies with these proteins. It was not possible to prepare $[^3H]$-aspartyl azide with a high enough specific activity to detect binding in CBT43 cytoplasmic membranes. When 50 uCi of L-$[^3H$-2,3]-aspartic acid were included during the synthesis of 1.4 millimoles of aspartyl azide, the product had a specific activity of only 5 uCi $[^3H]$ per millimole. It was obvious that this specific activity was too low to observe binding in membrane preparations, unless prohibitively large amounts of sample were used. Therefore, iodination of the photoaffinity label was attempted.

Iodinated derivatives of receptor substrates and substrate analogues have been prepared at very high specific activity by several investigators (1,7,9,20,21). Carrier-free sodium [^{125}I]-iodide with a specific activity of approximately 2000 Ci per millimole was used with chloramine T in the iodination procedure of Hunter and Greenwood (15). Virtually 100% incorporation of iodine was possible when approximately equimolar amounts of iodine and substrate were used in this method. The iodine was either incorporated into a tyrosine residue if the substrate was a protein (20), or into the phenyl ring of a benzyl substrate derivative (7,9,21). If the latter example also included an azido group on the phenyl ring, then the iodine atom was located on the same ring as the photoreactive group (7,21). The advantage of this type of compound was that the radioactive label remained with the covalently-linked azido group after photolysis, even if degradation of the remainder of the substrate molecule occurred (1).

After iodination of the affinity labels, it was necessary to remove any free [^{125}I]-iodide ion from the label so that further studies could be carried out with the purified compound. A molecular sieve column was useful if the iodinated label was a protein (20) and other iodinated labels were purified by organic extraction (9). The molecular weight of aspartyl azide was too low (295 gm/mole) for purification on a molecular sieve column and ethyl acetate extraction of the iodination mixture did not prove to be effective. Instead, a DEAE cellulose column was used for the selective removal of free iodide ion from the iodinated aspartyl azide, as outlined in Experimental Procedures. The column was initially equilibrated in 1 mM Tris-HCl (pH 8.0) and at this pH and ionic strength both the iodide ions and the aspartyl azide were bound to

the column. The azide was presumably bound to the DEAE through its two carboxyl groups which would be fully ionized at pH 8 (22). The ionic strength of the buffer was raised with sodium chloride and this resulted in the elution of better than 95% of the free iodine from the column. The pH was then lowered to near 2 by passing 5 mM hydrochloric acid through the column. This reduced the charge on the aspartyl azide molecule and it was eluted from the column. Figure 7 is the elution profile of the DEAE column. The elution of unlabelled aspartyl azide was detected by its orange colour and its elution position coincided exactly with that of the iodo-aspartyl azide.

By using a molar amount of sodium [^{125}I]-iodide slightly less than the amount of substrate, it is possible to synthesize an iodinated derivative of almost the same specific activity as that of the [^{125}I]-iodide (2000 Ci/mmole) (1). In the case of aspartyl azide, a molar ratio of approximately 10:1 for azide:iodide was used so that the orange colour of the photolabel could be used to follow any subsequent manipulations. Therefore, 1.94 mCi of sodium [^{125}I]-iodide (0.9 nmol) were used to iodinate 11 nmoles of aspartyl azide, as described in Experimental Procedures. The highest specific activity of iodinated aspartyl azide that was obtained in several experiments was 6.69 Ci per millimole, which was only 3.8% of the theoretical yield (176 Ci/mmole). This calculation assumed that 100% of the iodo-aspartyl azide was eluted from the DEAE column and, therefore, its actual specific activity may have been slightly higher. This low specific activity was only attained after a 7-day iodination period. Even the addition of a 1.9 uM ferric chloride catalyst (23) did not improve the iodination efficiency. In contrast, reaction times of only 1 to 30 minutes were necessary for

almost 100% incorporation of iodine into other affinity labels (7,9, 21).

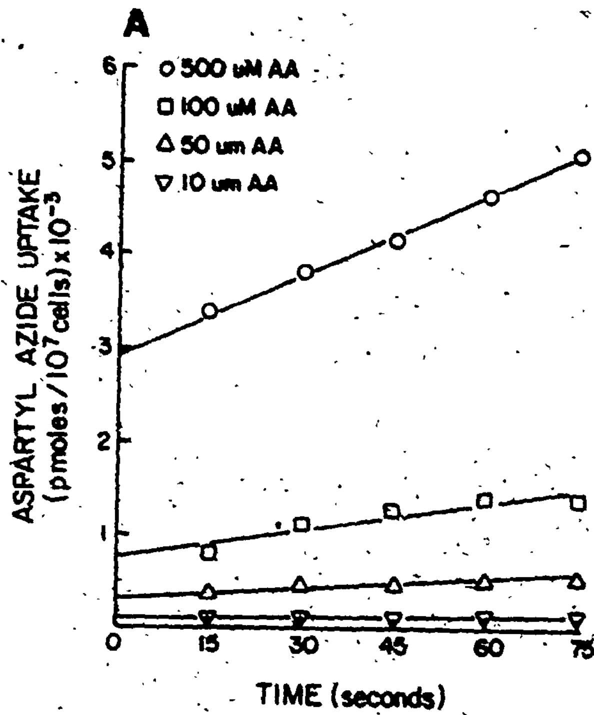
Whole Cell Transport of [^{125}I]-Aspartyl Azide - After a radioactive derivative of an affinity label has been prepared, the next step is to determine the binding constant of the label with the cells or membranes of interest. If the K_d for binding agrees with the K_i for the affinity label's inhibition of the receptor function, then it is fairly safe to say that the label is specific for the substrate binding site of the receptor (1). This complements the kinetic data which can be derived from Dixon plots, which indicate either competitive or non-competitive inhibition (14). As discussed earlier, the specific activity of the [^{3}H]-aspartyl azide was not high enough to be useful in either binding or photolabelling studies. Even though the specific activity of the [^{125}I]-aspartyl azide was several orders of magnitude higher than that of the tritiated label, it was still not a sensitive enough probe for binding studies. The apparent K_i for aspartyl azide inhibition of succinate transport was so high (0.37 mM) that this amount of cold azide would dilute out the iodinated azide (2 - 6 uM) so much that it would not be detected in binding studies. Therefore, the transport of iodinated aspartyl azide by CBT43 cells was investigated instead. It was hoped that this study would indicate the method of entry of the azide into the cells and would answer some of the questions raised in the succinate inhibition assays.

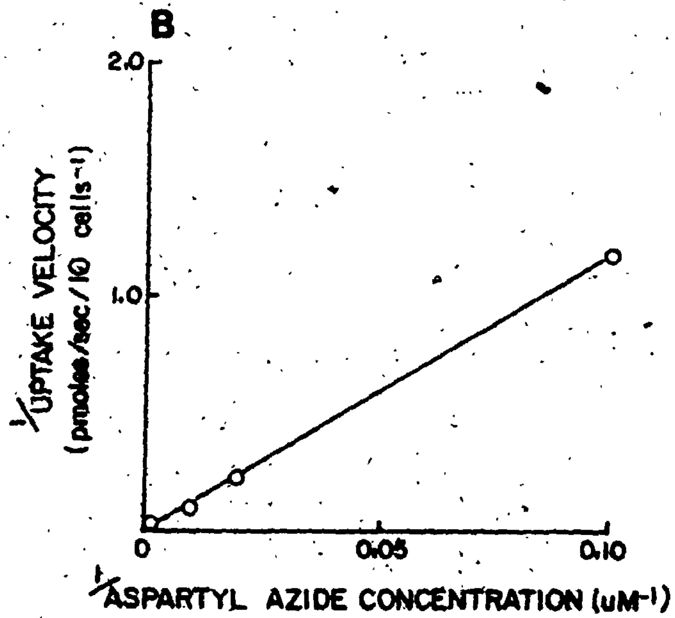
Concentrations of aspartyl azide from 10 to 500 uM were used in a 15 second timepoint transport assay with LB-grown CBT43 cells. Figure 8A is a plot of [^{125}I]-aspartyl azide transport over time and Figure 8B shows a Lineweaver-Burk plot of the same data (13). The increasing Y

Figure V-8. Uptake of [^{125}I]-aspartyl azide by CBT43 cells. LB-grown CBT43 cells were prepared for transport studies as in Figure 5 and were assayed for [^{125}I]-aspartyl azide uptake at 15 second time intervals as described in Experimental Procedures.

A - Uptake of 10 to 500 uM [^{125}I]-aspartyl azide (pmol/10^7 cells) is plotted against time (seconds).

B - The reciprocal of the uptake velocities (pmoles/10^7 cells/sec^{-1}) is plotted against the reciprocal of the aspartyl azide concentration. The x and y intercepts are 0.001 uM^{-1} and -0.011 pmoles/10^7 cells/sec^{-1} respectively.

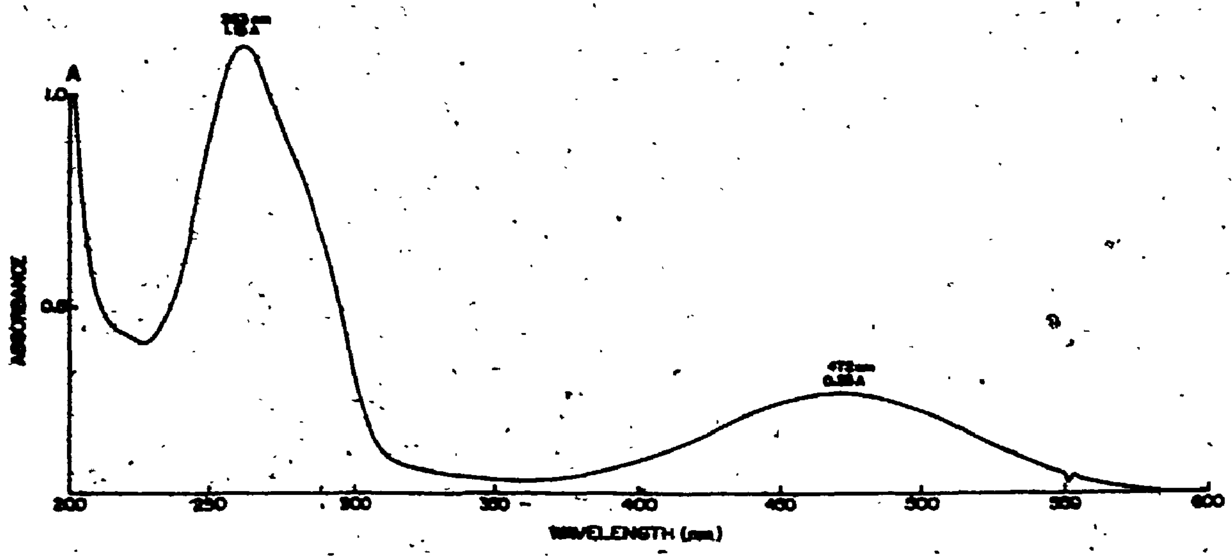

A
○ 500 uM AA
□ 100 uM AA
△ 50 um AA
▽ 10 um AA
ASPARTYL AZIDE UPTAKE
(pmoles / 10^7 cells) x 10^-3
0
1
2
3
4
5
6
15
30
45
60
75
TIME (seconds)

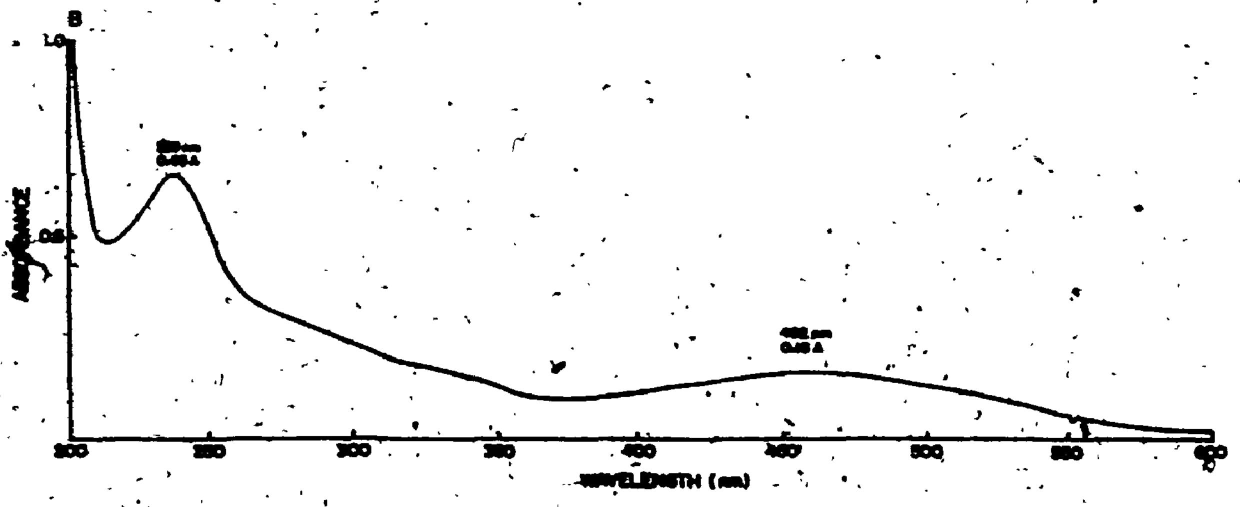

B
1/UPTAKE VELOCITY
(pmoles/sec/10 cells^-1)
2.0
1.0
0
0.05
0.10
1/ASPARTYL AZIDE CONCENTRATION (uM^-1)

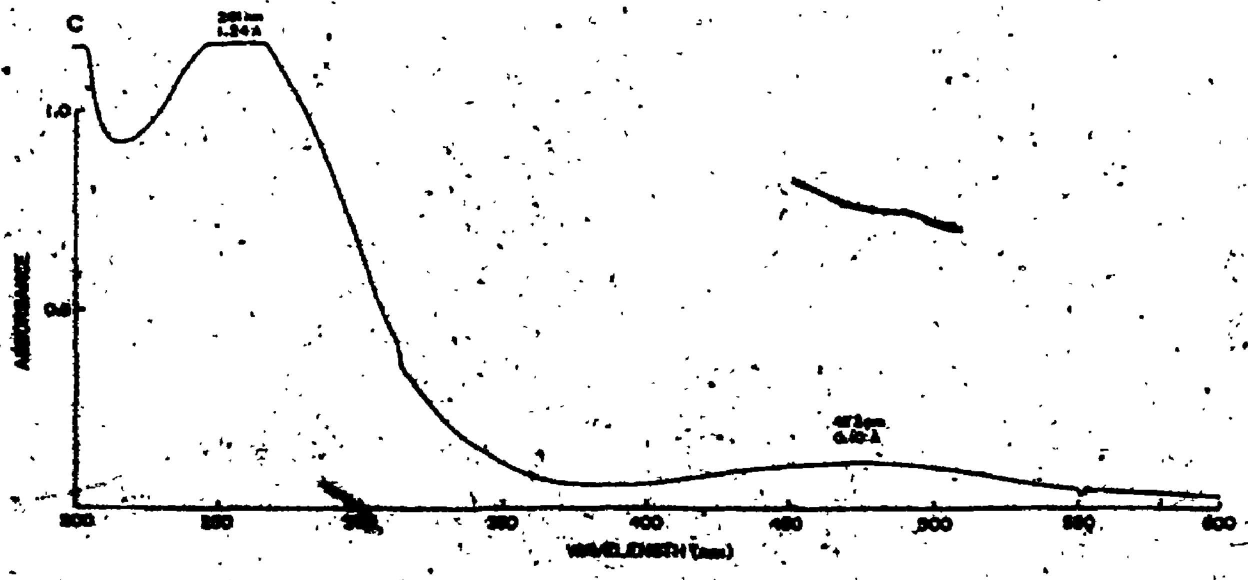
intercepts in Figure 8A presumably reflect an increased amount of [^{125}I]-aspartyl azide bound to either the bacterial cells or nitrocellulose filters at the higher concentrations of the photolabel. Both the X and Y intercepts (0.001 and -0.011 respectively) in the latter plot were very nearly zero. Therefore, it appeared that the aspartyl azide was transported passively into the cell since a value of 1/0 indicated an apparent K_m of infinity. Although the aspartyl azide was able to competitively inhibit whole cell succinate transport, it did not appear to be transported by this same active transport system.

Technical Considerations for Photoaffinity Labelling - Before photoaffinity labelling studies could commence, it was first necessary to determine the absorbance maxima of the aspartyl azide under the conditions to be used in photolysis. The aspartyl azide was diluted to a concentration of 100 uM with 50 mM potassium phosphate (pH 6.6) photolysis buffer and its wavelength maxima were determined before and after photolysis for 4 seconds with a 1000 Watt mercury lamp. The wavelength scans in Figures 9A and 9B were carried out from 600 down to 200 nm with a Beckman DU8 spectrophotometer. Two peaks of maximum absorbance occurred at 472 and 263 nm (Figure 9A). After photolysis, the 472 nm peak was shifted slightly to 462 nm and its absorbance was decreased (Figure 9B). The 263 nm peak was replaced with a new peak of lower absorbance at 239 nm. The shifts and decrease in absorbance of the peaks indicated that the mercury lamp was effective in activating the aspartyl azide under the conditions used. It is desirable, however, to carry out photolysis at wavelengths above 300 nm, if possible, to avoid inactivation of the proteins of interest by photo-oxidation or dimerization that often occurs at ultraviolet wavelengths (1). The aryl

Figure V-9. Wavelength scans of aspartyl azide. The wavelength maxima of aspartyl azide (100 uM) in 50 mM potassium phosphate buffer (pH 6.6) were determined before and after photolysis for 4 seconds with a 1000 Watt mercury lamp.
A - before photolysis, maxima of 472 nm, 263 nm.
B - after photolysis, maxima of 462 nm, 239 nm.
C - after photolysis (glass filter), maxima of 473 nm, 261 nm.
Photolysis with a glass filter (C) screened out wavelengths of light below 300 nm.


A
1.0
0.5
ABSORBANCE
200
250
300
400
500
600
WAVELENGTH (nm)


B
1.0
0.5
ABSORBANCE
200
300
400
500
600
WAVELENGTH (nm)


C
1.0
0.5
ABSORBANCE
200
400
600
WAVELENGTH (nm)

azides, such as aspartyl azide, are good candidates in this instance since they absorb light above 300 nm. Photolysis of aspartyl azide was carried out above 300 nm by using a glass plate to cover the samples and thus to filter out the ultraviolet wavelengths. Figure 9C shows that photolysis under these conditions only affected the 473 nm peak. Its absorbance was decreased, while the peak at 261 nm remained relatively unchanged in position and absorbance.

When an aryl azide is activated by light of the appropriate wavelength, the aryl nitrene that is formed either reacts with solvent molecules in the bulk solution, with nonspecific residues in the membranes or cells of interest or with specific sites for which the photolabel has an affinity. The most specific reaction occurs when the amount of photolabel that is used closely approximates the number of specific, affinity sites in the membranes or cells. That is, when the photolabel is used at a concentration that equals its K_d for binding to the specific sites, it should be able to saturate roughly 50% of the sites. If the K_d of the label that is used is much greater than the number of specific sites, a larger proportion of the label will be involved in nonspecific binding, unless the excess label is rapidly inactivated by the solvent. Most membrane receptors are present at a concentration of 10^{-10} to 10^{-13} moles per milligram of membrane protein and this translates to an average of 10^{-6} to 10^{-9} M at a concentration of 10 mg/ml of membrane protein. Therefore, a photolabel should have a K_d of 10^{-6} M or less to be useful (1).

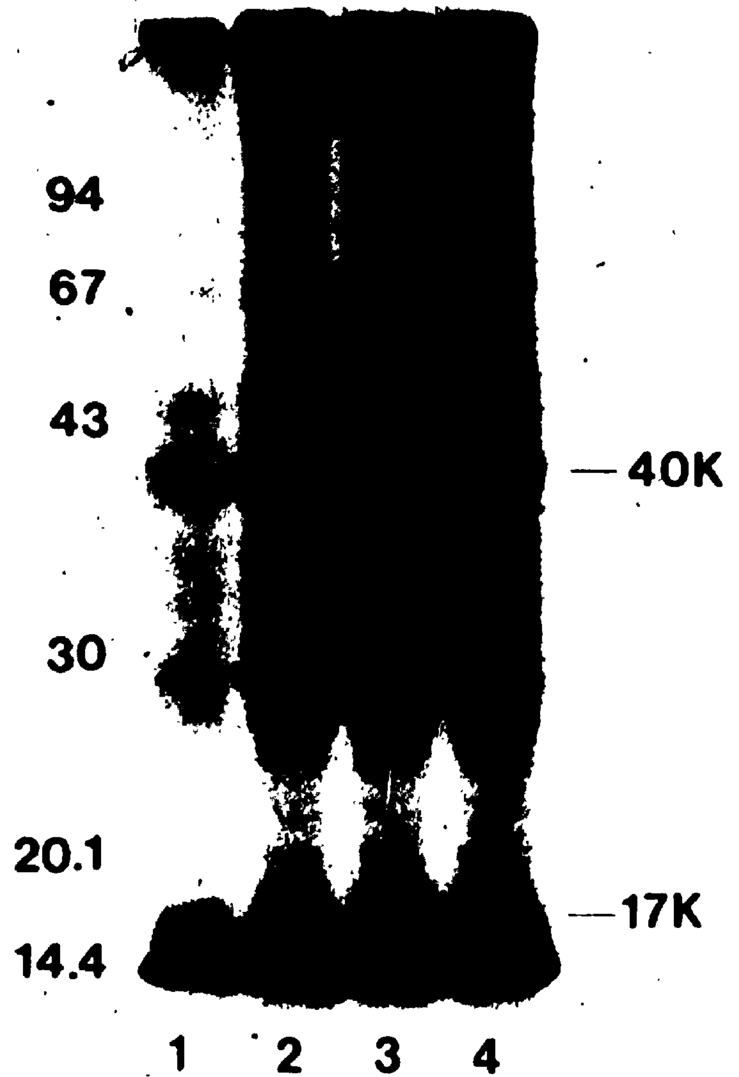
The apparent K_i for aspartyl azide inhibition of whole cell succinate transport was shown to be 0.37 mM. The Dixon plot also suggested that this inhibition was competitive. Due to the mechanical

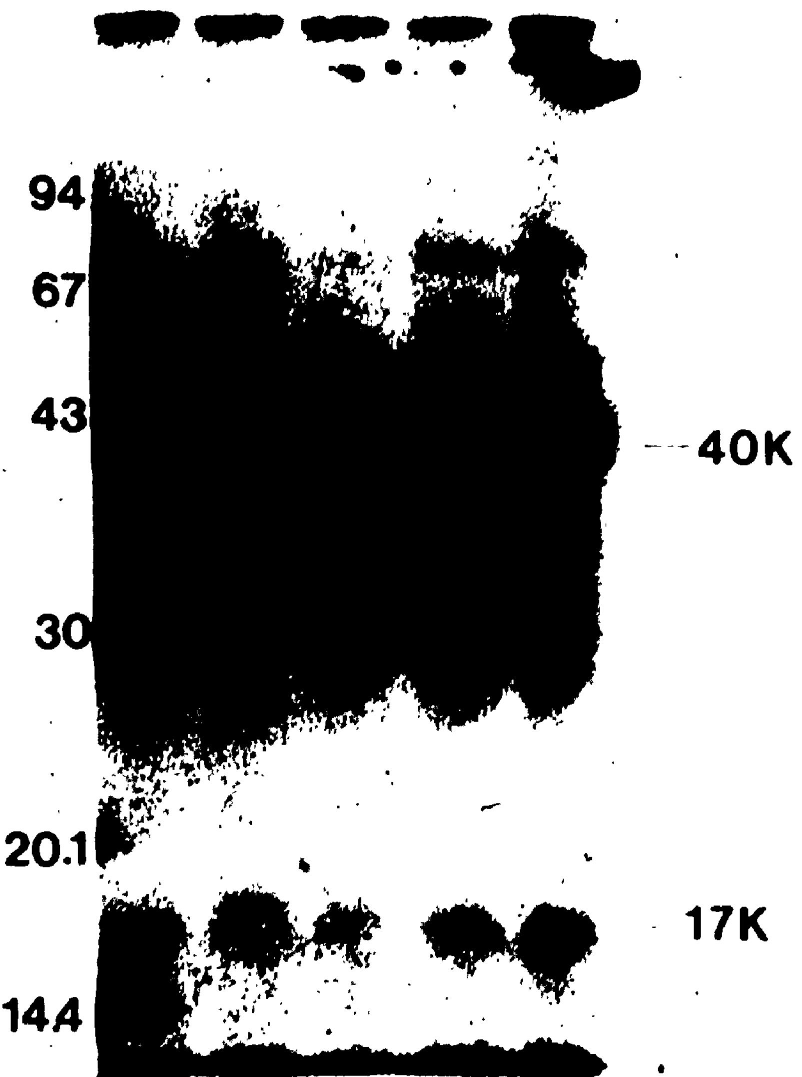
limitations of the binding studies discussed earlier, it was assumed that the K_i roughly approximated the K_d for binding of aspartyl azide to the transport components. This K_d value is much larger than the maximum value of 10^{-6} M for ideal site-specific labelling and the label would almost certainly be in excess of the specific sites at this concentration. However, specific labelling might still be possible if the excess photolabel was inactivated quickly enough by the solvent before it could attack nonspecific sites (1). Therefore, a range of aspartyl azide concentrations was used in the initial photolabelling experiments in order to determine the cut-off point for specific labelling.

Photoaffinity Labelling of Membrane Vesicles - Since the focus of this thesis is the membrane-bound dicarboxylate transport components, photoaffinity labelling studies were initially carried out with CBT43 cytoplasmic membrane vesicles that had been shown to contain active succinate transport components (24). Vesicles were incubated for 30 minutes in phosphate buffer (50 mM, pH 6.6) with concentrations of aspartyl azide ranging from 1 to 50 uM and photolysis was carried out as described in Experimental Procedures. The membrane proteins were then separated on an 11.5% polyacrylamide SDS gel and labelled proteins were detected by autoradiography. It was found that 500 ug of membrane vesicle protein was the maximum amount that could be run on each lane of the gel without major distortions of the bands. Coomassie Blue-stained, control lanes that contained membrane vesicles before and after photolysis without the addition of aspartyl azide did not show any apparent differences in their protein patterns and this indicated that at least no major protein aggregation occurred during photolysis. Figure 10 is the autoradiogram from this experiment. It was evident that a number of

Figure V-10. Photolabelling of CBT43 membrane vesicles. CBT43 Kaback membrane vesicles were prepared as described in Chapter 2. Membrane vesicles (500 ug) were incubated for 30 minutes with various concentrations of [^{125}I]-aspartyl azide (1 uM to 50 uM) in 50 mM potassium phosphate buffer (pH 6.6) in a total sample volume of 300 ul. Photolysis was carried out for 4 seconds with a 1000 Watt mercury lamp. The vesicles were pelleted, washed once with phosphate buffer and were solubilized in SDS sample buffer. The labelled proteins, in this and all subsequent experiments, were separated on a 1.5 mm, 11.5% polyacrylamide SDS slab gel. [^{125}I]-labelled proteins were detected by autoradiography.
Lane 1 - 50 uM aspartyl azide (AA); 2 - 10 uM AA; 3 - 2 uM AA; 4 - 1 uM AA.

Figure V-11. Succinate protection of CBT43 membrane vesicle photolabelling. Membrane vesicles (500 ug) were photolabelled with 2 uM [^{125}I]-aspartyl azide and were analyzed on an SDS gel as in Figure 10, except that a glass filter was used for photolysis above 300 nm. Succinate protection was assayed by pre-incubating the vesicles with 20 mM succinate (or aspartate) for 5 minutes prior to the addition of the photolabel. Para-aminobenzoic acid (PABA) was also used as a scavenger for excess reactive aspartyl azide.
Lane 1 - control; 2 - 20 mM succinate protection; 3 - 20 mM aspartate protection; 4 - 10^{-4} M PABA; 5 - 10^{-3} M PABA.


94
67
43
30
20.1
14.4
40K
17K
1
2
3
4
V-10


94
67
43
30
20.1
14.4
40K
17K
1
2
3
4
5
V-11

different proteins were labelled with aspartyl azide, in particular several bands of around 40K Daltons molecular weight as well as 73K, 47K, 30K and 17K bands. The protein pattern remained the same at all the aspartyl azide concentrations. Only the intensity of the bands changed since the specific activity of the photolabel was higher at the lower concentrations of aspartyl azide. The lowest concentration of photolabel that could be used (1 uM) was limited by the concentration of the [^{125}I]-aspartyl azide preparations (from 2.5 to 6 uM) and the necessity of using 1 to 2 x 10^6 CPM of label per lane in order to see any bands. Even so, periods of 5 to 7 days were necessary for the detection of labelled proteins. This experiment used all of the wavelengths of the mercury lamp, whereas only wavelengths above 300 nm were used in subsequent experiments. However, the pattern of proteins that were labelled did not appear to be dependent upon the wavelength of light used.

The next step was to determine if the proteins that were labelled by the aspartyl azide were succinate-specific proteins. Vesicles were preincubated for 5 minutes with either 2 or 20 mM succinate or aspartate prior to the addition of the photolabel (2 uM) and subsequent photolysis. Aspartate was included as a control since the photolabel did not inhibit its uptake by whole cells. Figure 11 shows the results of the protection studies with 20 mM succinate or aspartate. In both cases, the same set of proteins was labelled as in the control vesicles incubated only with aspartyl azide. There was no significant decrease in binding of the photolabel to any of the proteins in the presence of either 20 mM succinate or aspartate. In fact, 20 mM succinate seemed to increase the overall binding slightly. This lack of succinate protection may not be

too surprising in light of the fact that 700 mM glucose was shown to provide only 65% protection in 500 nM cytochalasin B photolabelling studies with the human erythrocyte D-glucose transporter (25). Higher succinate concentrations were not used in these protection studies due to the possibility that the increased ionic strength might interfere with aspartyl azide binding.

It should be noted that the compound para-aminobenzoic acid (PABA) was used in the last two lanes of Figure 11. This compound is an effective "scavenger" for excess reactive photolabel in the bulk solution and, thus, it reduces nonspecific labelling of proteins. It is especially useful as a scavenger for 4-azido-2-nitrophenyl derivatives, such as aspartyl azide, since PABA only absorbs light below 320 nm and therefore it does not interfere with the activation of the aspartyl azide at 470 nm (1). The addition of PABA to the photolysis mixture did not change the protein pattern that was observed. Therefore, it was assumed that the labelling that occurred in its absence was largely specific, perhaps because the rate of inactivation of the label by the solvent molecules was rapid enough.

An alternative method that was used to determine if the aspartyl azide was reacting specifically with the succinate transport components was the use of mutants in the photolysis studies. Crude cytoplasmic membrane vesicles (non-Kaback) were prepared by French-Pressing and homogenizing spheroplasts from CBT43, LL3, LL5, LL5 Rev and 4-31, as described in Experimental Procedures. Membranes (500 ug) were photolyzed in the presence of 2 uM [^{125}I]-aspartyl azide and inhibition of the photolabeling by 20 mM succinate, aspartate and glutamate was investigated. A much more complex pattern of labelling was evident in this

study than was observed for the Kaback membrane vesicle preparations and, as such, it was difficult to compare the various bacterial strains. A 40K band, similar to that observed in CBT43 membrane vesicles, was one of the major proteins labelled in the crude membranes and again succinate did not inhibit the binding of the photolabel. An interesting observation was that no proteins were labelled in the LL3 (dctA) control lane, but protein bands were evident in the lanes that contained succinate, aspartate or glutamate. A possible explanation for the increased complexity of the binding pattern observed in this experiment is that the method of preparation for these membranes did not guarantee that they remained as intact vesicles. Perhaps this method provided additional membrane sites for nonspecific binding of the photolabel.

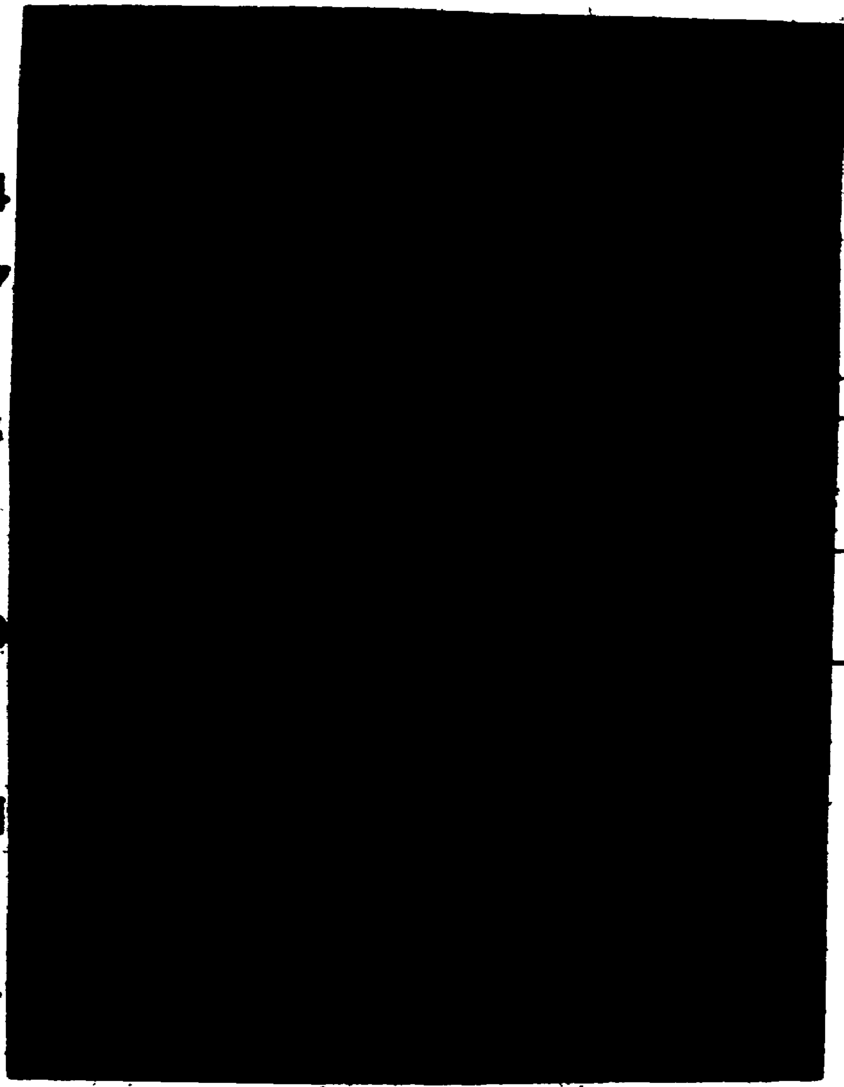
Photoaffinity Labelling of Whole Cells - Due to the complexity of the binding patterns that were observed for the photolyzed membranes of the various bacterial strains, whole cells were used in subsequent photolabelling studies. It was thought that these in vivo studies might provide more accurate information about the binding sites of the photolabel. In contrast to the in vitro labelling studies, inactivation or denaturation of the succinate transport components should not be a factor in whole cell studies. In addition, the original information about competitive inhibition of succinate transport by aspartyl azide was obtained from whole cell transport assays.

The five different bacterial strains were grown in LB medium under conditions identical to those used for the whole cell transport assays. Cells were prepared for photolysis as described in Experimental Procedures. Since the relative amounts of the succinate transport proteins would be lower in the whole cells than in the isolated mem-

branes, 5 milligrams (wet weight) of cells were used in all of the labelling studies. Figure 12 shows the results of an experiment that involved the photolysis of CBT43 cells after a 30 minute preincubation with either 2 uM or 500 uM [^{125}I]-aspartyl azide. The latter concentration was used as a control since the apparent K_i for aspartyl azide inhibition of succinate transport was 369 uM (0.37 mM). If this K_i truly reflected the K_d for binding of aspartyl azide to the transport components, then photolysis at 2 uM might not be able to detect the succinate binding proteins. However, Figure 12 shows that the same proteins were labelled by the photolabel at both the 2 and 500 uM aspartyl azide concentrations. The labelling was less intense in the latter case because of the lower specific activity of the 500 uM photolabel. One major protein of 47K Daltons molecular weight was labelled in the control lane (lane 1) for both aspartyl azide concentrations. The labelling of this 47K protein was considerably diminished in the presence of either 2 or 20 mM succinate. In addition, preincubation of the cells with succinate resulted in the labelling of two new proteins of 35K and 29K Daltons molecular weight. It should also be noted that lane 4 for each aspartyl azide concentration corresponds to a 15 minute preincubation of the cells with the aspartyl azide. The fact that the same proteins were labelled during preincubation of the cells from 2 up to 30 minutes with the photolabel indicated that metabolism of the aspartyl azide was not a problem in the whole cell studies. A preincubation time of 30 minutes was used in order to ensure for sufficient interaction between the photolabel and the cytoplasmic membrane transport components.

Further experiments involved the comparison of photolabelling

Figure V-12. Photolabelling of CBT43 Cells. CBT43 cells were grown to late-log in LB media, as described earlier for the succinate transport assays. The cells were washed once with 50 mM potassium phosphate, pH 6.6. An aliquot (5 mg wet weight) of cells was then incubated with [^{125}I]-aspartyl azide (2 or 500 uM) in phosphate buffer (total sample volume of 300 ul). Photolysis was carried out with a glass filter for 8 seconds with a 1000 Watt mercury lamp. Protection of photolabelling was assayed in the presence of 2 or 20 mM succinate as described previously. Unless otherwise indicated, the time of incubation of the cells with the aspartyl azide was 30 minutes and 10^{-3} M PABA was included in the samples. Cells were prepared for SDS polyacrylamide gel electrophoresis as in Figure 10. The photolysis conditions that were examined were: Lane 1 - 2 or 500 uM aspartyl azide (as indicated); 2 - 2 mM succinate protection; 3 - 20 mM succinate protection; 4 - 15 minute incubation of photolabel with cells; 5 - PABA not included in the sample.


94
67
43
30
20.1
14.4
47K
35K
29K
1 2 3 4 5 1 2 3 4 5
2 uM
500 uM

patterns between the wild-type and transport-mutant strains. Figures 13A and 13B show the results of 2 uM aspartyl azide photolysis in the absence or presence of 20 mM succinate, aspartate or glutamate. It can be seen that the 47K band was again labelled in the control lane (lane 1) for the CBT43 cells. A very interesting observation is that the mutant strains', LL5 and LL5 Rev, control lanes had the same labelling pattern as did CBT43 cells preincubated with 20 mM succinate (Figure 12). The intensity of the 47K band was decreased with a concomitant increase in labelling of the 35K and 29K proteins. In addition, the control lane of the LL3 mutant showed an almost complete absence of labelling of any protein bands. This was the same observation that was made earlier when LL3 cytoplasmic membranes were photolabelled with aspartyl azide. Finally, only the 47K band was labelled in the 4-31 Mu-induced mutant, and this labelling was decreased in the presence of succinate, aspartate or glutamate. The most interesting observation was that preincubation of all of the strains, with the exception of 4-31, with 20 mM succinate, aspartate or glutamate seemed to increase the intensity of the 35K and 29K bands. This effect was the most pronounced in the presence of glutamate. It was even evident in the LL3 mutant which did not show any labelling of these proteins in the absence of the dicarboxylic acids.

Localization of [^{125}I]-Aspartyl Azide in Whole Cells - Photoaffinity labelling studies with aspartyl azide in whole cells of E. coli K12 detected three major proteins in the various bacterial strains. However, the location of these proteins within the cell was not evident from these studies. The fact that the genetic defects in the mutant strains affected cytoplasmic membrane translocation of dicarboxylic

Figure V-13. Photolabelling of whole cells: comparison of different bacterial strains. Whole cells of strains CBT43, LL3, LL5, LL5 Rev and 4-31 were photolabelled with [^{125}I]-aspartyl azide, as in Figure 12. The time of incubation of the cells with the photolabel was 30 minutes. When succinate, aspartate or glutamate protection was assayed, 2 uM aspartyl azide was used for photolabelling. All of the samples contained 10^{-3} M PABA. The following conditions were examined:
Lane 1 - 2 uM aspartyl azide; 2 - 500 uM aspartyl azide; 3 - 20 mM succinate protection; 4 - 20 mM aspartate protection; 5 - 20 mM glutamate protection.
A - CBT43, LL3 and LL5; B - LL5, LL5 Rev and 4-31 (following page).

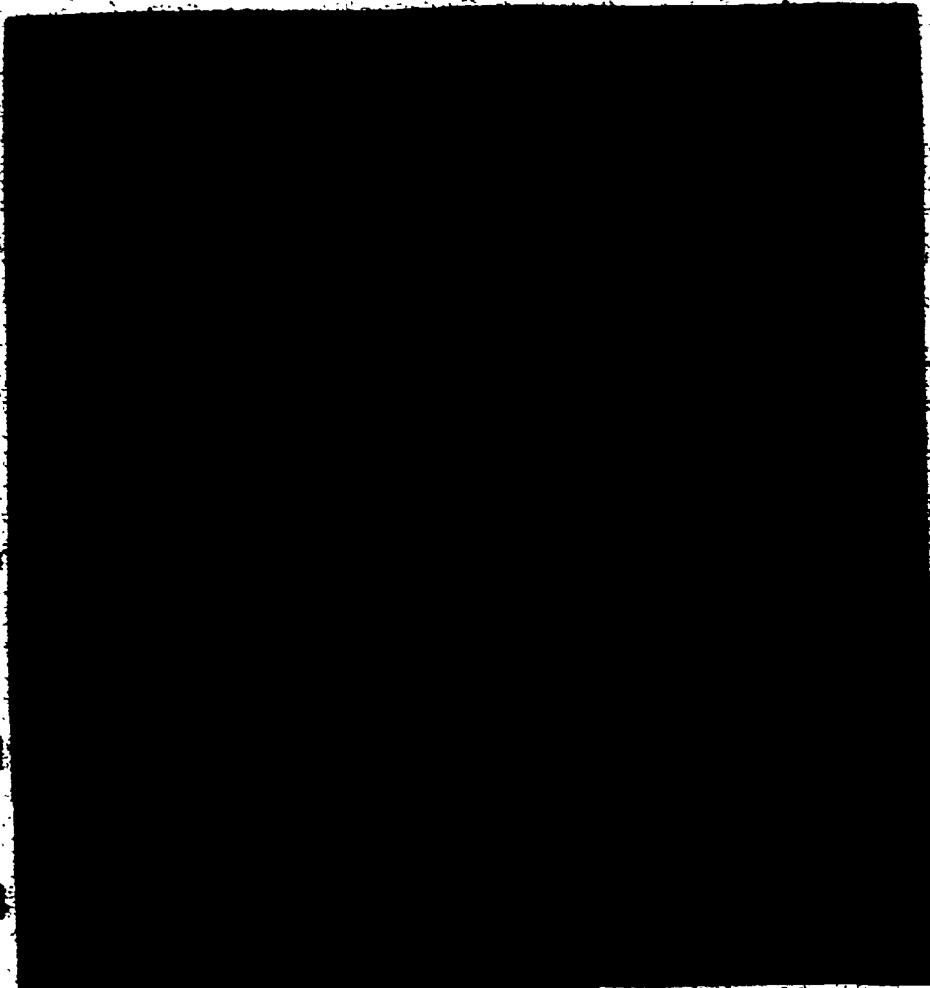
A

94
67
43
30
20.1
14.4

—47K
—35K
—29K

1 2 3 4 5 1 2 3 4 5 1 2

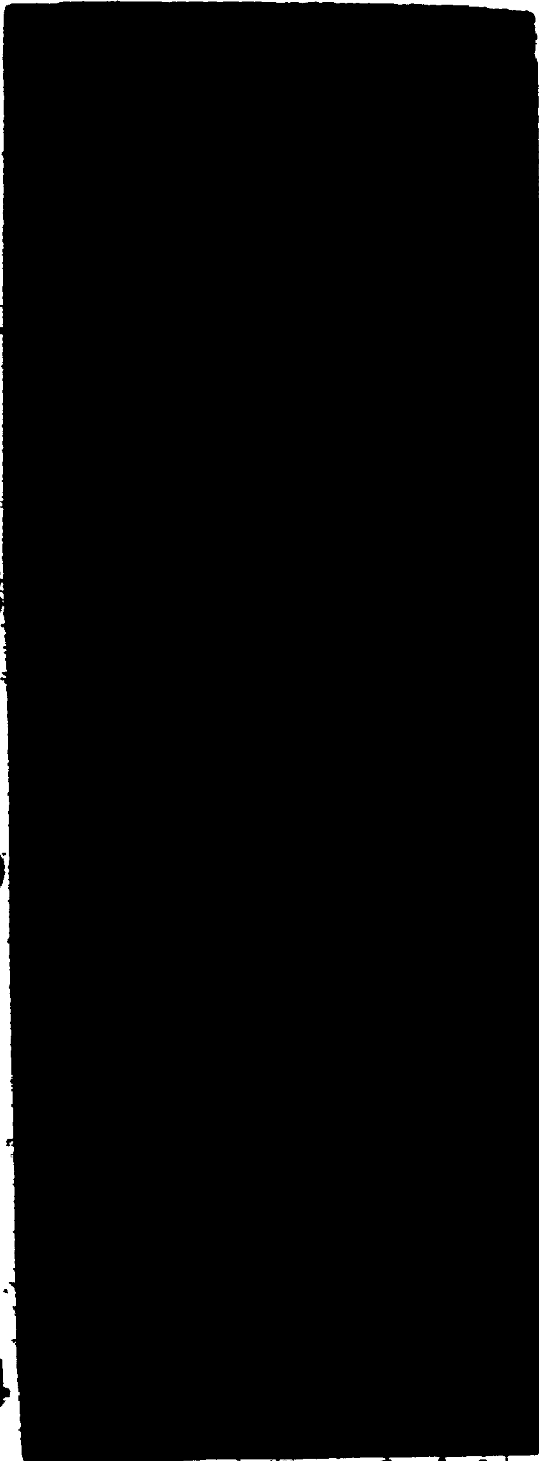
CBT43 LL3 LL5


B
94
67
43
30
20.1
14.4
47K
35K
29K
3 4 5 1 2 3 4 5 1 2 3 4 5
LL5
LL5 Rev
4-31

acids suggested that the 47K protein and perhaps the 35K and 29K proteins might be membrane proteins. This assumed, of course, that the photolabel had specifically detected succinate binding proteins. To resolve this question, CBT43 cells that were photolabelled with 2 uM [^{125}I]-aspartyl azide were selectively disrupted following photolysis in order to determine the location of the photolabel. The methods of Heppel and Kaback (16,17) were used as described in Experimental Procedures. The fractions that were examined included the photolysis buffer, the whole cell phosphate wash, the peripheral outer membrane proteins, the outer membrane and upper periplasm, the peptidoglycan and lower periplasm, the cytoplasm and the cytoplasmic membrane. The amount of radioactivity in each fraction was used to determine the proportion of [^{125}I]-aspartyl azide associated with it.

After separation of the whole cells from the photolysis buffer, it was evident that only 3% of the total photolabel added was associated with the cells. The majority of the photolabel (97%) remained in the photolysis supernatant and phosphate wash. This was consistent with the fact that only a small number of specific sites existed in the cells relative to the amount of aspartyl azide used in photolysis (1). After the cells were disrupted and the remaining subcellular fractions were counted, approximately equivalent amounts of [^{125}I]-aspartyl azide were found in each fraction. Therefore, it was not possible to localize the covalently-bound photolabel to any one cell compartment with this method. In an alternative approach, the proteins in each subcellular fraction were TCA precipitated and separated on an SDS gel. Figure 1A shows that the 47K protein was present to some extent in all of the fractions, including the cytoplasmic membrane pellet. However, the

Figure V-14. Localization of [^{125}I]-aspartyl azide in CBT43 cells. CBT43 cells (5 mg) were photolabelled with 2 uM [^{125}I]-aspartyl azide, as in Figure 12. After photolysis, the cells were washed once with 50 mM potassium phosphate (pH 6.6) and were selectively disrupted according to the methods of Heppel (16) and Kaback (17) as described in the text. The proteins that were released by each treatment were precipitated by 10% TCA and were examined on an SDS gel in order to localize the photolabel to a particular cellular compartment. The cellular fractions that were examined included:
Lane 1 - peripheral outer membrane proteins (Tris-EDTA-sucrose); 2 - outer membrane and upper periplasmic proteins (osmotic shock); 3 - peptidoglycan and lower periplasmic proteins (lysozyme); 4 - cytoplasmic proteins (13,000 x g homogenization supernatant); 5 - cytoplasmic membrane proteins, whole cells and debris (pellet).


94
67
43
30
20.1
14.4
47K
35K
29K
1
2
3
4
5

cytoplasm appeared to contain by far the majority of this protein. Both the 35K and 29K proteins were also evident in the cytoplasmic membrane pellet. The implications of these results will be addressed in the discussion.

DISCUSSION

The design of an appropriate photoaffinity label for a specific membrane enzyme or receptor involves several important considerations. The photolabel must contain a substrate or substrate analogue with a specific affinity for the protein of interest. It must also contain a photoreactive group that can be activated under conditions that preserve the native state of the biological system. It is also desirable to use a photolabel that has a radioactive tag in close proximity to the photosensitive group in order to follow the label in the event of metabolism or breakdown of the remainder of the molecule (1). Finally, the photolabel must be able to interact specifically with the enzyme or receptor of interest. This specificity is a function of several factors and is determined by either direct binding studies or indirect inhibition studies with the biological system. The photolabel has an inherent affinity (K_d or K_i) for the specific sites in the membrane or cell, but nonspecific reactions with other proteins can also occur if the label is in excess of the specific sites and/or if the label contains other groups that are reactive towards amino acid residues. These nonspecific reactions can be controlled if excess label is inactivated either by nearby solvent molecules or by added "scavenger" compounds (1).

The photoaffinity label, N-(4-azido-2-nitrophenyl)aspartic acid, that was synthesized in this study for the dicarboxylate transport

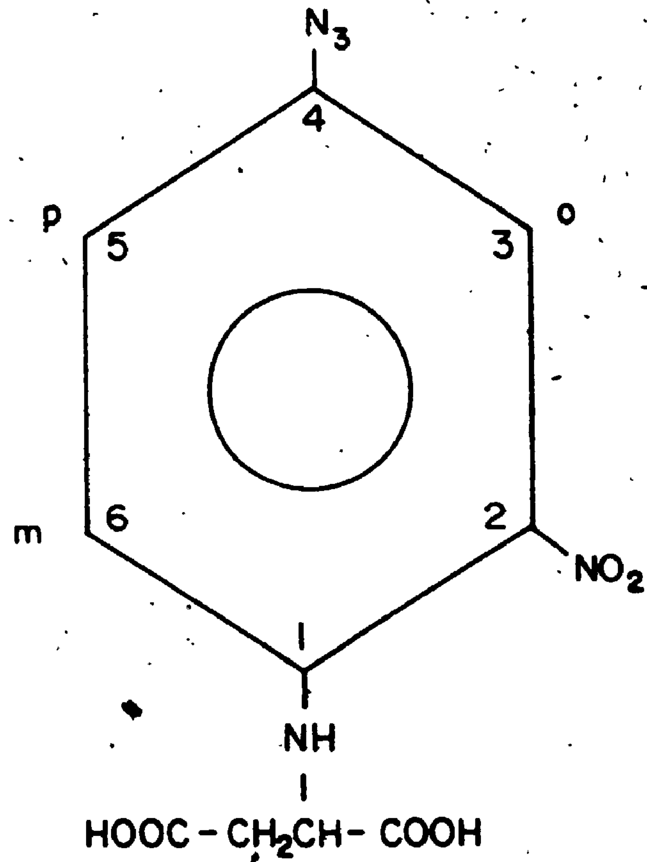
system of E. coli met most of the above requirements. Aspartate was chosen as the affinity portion of the molecule because it is also a substrate of the transport system (K_m of 30 uM). In addition, its amino group provided the means of derivatization with 4-fluoro-3-nitrophenyl azide in order to form an aryl azide (12). The aspartyl azide was photoreactive at non-denaturing wavelengths of light and its phenyl ring provided a site for the addition of a radioactive iodine atom. However, the chemistry of other groups on the ring interfered with the iodination of the photolabel (see Figure 15 below) (23). If 100% incorporation of iodine had occurred, as with other photolabels (7,9,20,21), the theoretical specific activity of the iodinated aspartyl azide would have been 176 Ci/mmole. This is considerably higher than the theoretical specific activity that could be attained by using the same quantity of [^{3}H]-aspartate in the synthesis of the photolabel. The problem was that large quantities of carrier-free [^{3}H]-aspartate were required if a reasonable amount of photolabel was to be synthesized. Also, the yield of aspartyl azide from the reaction, and thus the amount of [^{3}H] incorporation, would never be 100%. These studies showed that the highest specific activity [^{125}I]-aspartyl azide that could be prepared was 6.69 Ci/mmole. This low specific activity precluded its use in binding studies with whole cells or membrane preparations. However, its high-energy gamma-radiation readily allowed the detection of labelled proteins following photolysis by autoradiography, instead of by scintillation counting of gel slices. This ability to detect specific sites that occur in low levels in the sample is extremely important in light of the fact that a maximum of 10% of specific sites are labelled during one round of photolysis; due to decomposition of most of the label through intra-

molecular rearrangement or reaction with the solvent (1). A technique that has been used recently to successfully iodinate low-reactive aryl azides to high specific activity involves the use of thallium trifluoroacetate (26). A disadvantage of this method is that thallium compounds are highly toxic (23).

Figure 15 is relevant to the explanation for the low amount of iodine incorporation into aspartyl azide. The nitro group located on carbon 2 of the aspartyl azide molecule is a deactivating group that acts to destabilize the benzene ring and, therefore, makes an electrophilic substitution such as iodination very difficult, if not impossible (23). Any iodination that does occur will only be in a position on the ring meta to the nitro group (C-6). The azido group is also a deactivating, meta director and it would also direct the iodine atom to the C-6 atom of the ring. Amino groups, including aspartate, are strongly activating ortho, para directors and would thus favour iodination at C-4 or C-6. Since the C-4 position was already occupied by the azido group, the only position on the ring that was possible for iodination was the C-6 atom. The presence of two deactivating groups, but only one activating group, on the ring and the steric hindrance of the bulky iodine atom and the aspartate group may have been factors in the low yield of iodo-aspartyl azide. Other affinity labels contained either no group at the C-2 position of the phenyl ring (1) or had a strongly-activating hydroxyl group on the ring (7,9,21) and, therefore, iodination was strongly favoured. Unfortunately, the nitro group was necessary in 4-fluoro-3-nitrophenyl azide in order to make the fluorine atom a good leaving group during the synthesis of aspartyl azide (23).

The specificity of the aspartyl azide for the dicarboxylate

Figure V-15. Structural features of N-(4-azido-2-nitrophenyl)aspartic acid. The structure of the benzene ring of aspartyl azide is relevant to the low iodination efficiency of the photolabel (3%). Carbon atoms are numbered (1 - 6) and the ring positions that are ortho (o), para (p) and meta (m) with respect to the nitro group are shown. The chemistry of this benzene ring favours a low iodination yield at C-6.



N3
4
p
5
o
3
m
6
2
NO2
1
NH
HOOC - CH2CH - COOH

transport system was determined by whole cell inhibition studies. The complex kinetics of the aspartyl azide inhibition of succinate transport made it difficult to determine its K_i constant precisely. Several possibilities may explain the phenomenon of initial inhibition followed by partial recovery of succinate transport in the presence of aspartyl azide. One explanation is that the aspartyl azide was initially metabolized upon entry into the cells and thus its effective concentration was lowered. This would account for the initial large inhibition of succinate transport seen at the early timepoints for the higher aspartyl azide concentrations and the recovery of transport at the later timepoints when its concentration was lowered. Alternatively, there may have been a rapid turnover of the succinate transport components which effectively replaced the inactivated proteins with new transport components. A third possibility, that was discussed earlier, is that the higher concentrations of aspartyl azide inhibited succinate transport at the early timepoints through a general mechanism (ie. outer membrane perturbation or blockage) that did not directly involve the succinate transport components, as indicated by the mixed kinetics. Inhibition and recovery of uptake have also been observed for glucose inhibition of 4-fluoro-glucose transport in *Pseudomonas putida*, and metabolism of glucose was postulated as the cause of the phenomenon (27). However, recovery in this case occurred only after several hours of incubation with glucose and only a small fraction of the glucose was metabolized. The fact that the same proteins were photolabelled with aspartyl azide in whole cells when preincubation times ranging from 2 to 30 minutes were used suggested that metabolism of the photolabel was not a problem in this study. A final, but perhaps remote, explanation for the above

phenomenon is that the aspartyl azide was transported into the cells via a system other than the active dicarboxylate system. This possibility was investigated by whole cell transport studies with [^{125}I]-aspartyl azide that indicated the passive transport or diffusion of the photolabel into the cell. This passive transport of the photolabel may have lowered its concentration in the vicinity of the succinate transport components enough to partially reverse its inhibitory effects.

When the initial rates of succinate transport in the presence of aspartyl azide were used to calculate the photolabel's inhibition constant, complex kinetics that were interpreted as mixed inhibition were observed. However, when data from the later timepoints on the succinate transport rate graph (Figure 5A) were used for the higher aspartyl azide concentrations, competitive inhibition was indicated. The results from the Dixon plot (14) indicated that the aspartyl azide was a competitive inhibitor of succinate transport with an apparent K_i of 0.37 +/- 0.04 mM. The fact that the photolabel did not also inhibit the high affinity aspartate transport system can be explained by an absolute requirement of this system for a free alpha-amino group (10). The high K_i value for the succinate transport system necessitated the use of aspartyl azide concentrations in the photolabelling studies that were in excess of the number of specific, affinity sites in the sample. In addition, the aspartyl azide contained a highly-reactive, electron-withdrawing nitro group on its phenyl ring that could also contribute to nonspecific labelling (1). However, rapid inactivation of the excess photolabel by the solvent appeared to occur since the addition of para-aminobenzoic acid as a "scavenger" for activated aspartyl azide did not change the photolabelling patterns in cytoplasmic membranes or whole cells. There-

fore, nonspecific labelling did not appear to be a problem with this system.

Photolabelling studies with wild-type (CBT43) cytoplasmic membrane vesicles indicated that several proteins (73K,47K,40K,30K,17K) were labelled with [^{125}I]-aspartyl azide. This labelling was not inhibited by 2 or 20 mM succinate, perhaps because a higher concentration of succinate was needed for protection of photolabelling, similar to the results observed for glucose protection of cytochalasin B labelling of the human erythrocyte D-glucose transporter (25). Alternatively, the dicarboxylate membrane carriers may not have been accessible to the succinate in these "non-energized" membrane vesicle preparations. As such, they may not have been subject to the same photolabelling and succinate protection patterns that were observed in the energized whole cells discussed below. In addition, the complex pattern of labelling that was evident when photolyzed crude cytoplasmic membranes were compared among the different bacterial strains made it difficult to interpret the data. Several bands of around 40K molecular weight were again the major labelled proteins in all five bacterial strains (CBT43, LL3, LL5, LL5 Rev, 4-31). Photolabelling studies with whole cells were therefore carried out next. These studies offered the advantage of an <u>in vivo</u> system that contained the membrane carriers in an active form, less susceptible to artefacts than the membrane preparations, but the disadvantage of diluting out the number of specific, cytoplasmic membrane sites, if any, for the photolabel. Nevertheless, differences in photoaffinity labelling of whole cells were evident among the five bacterial strains. Both 2 and 500 uM aspartyl azide gave the same labelling patterns for a particular strain. This suggested that the K_d, and therefore the K_i, of the

photolabel might be lower than its apparent value of 0.37 mM.

The results from these in vivo studies indicated that two types of sites were available for aspartyl azide binding in whole cells. One type of site, the 47K protein, was detected in wild-type CBT43 cells and in the Mu-induced mutant, 4-31. Since labelling of this site was blocked by succinate, it was considered to be a high-affinity site for the photolabel with respect to succinate specificity. In addition, the 47K protein might be involved in succinate transport, since it was absent in strain LL3. This 47K protein was also detected in low levels in strains LL5 and LL5 Rev, especially in the presence of the dicarboxylic acids. The second type of site, the 35K and 29K proteins, was present in CBT43 and LL3 only after the addition of succinate, but it was present at all times in LL5 and its revertant and it was absent in 4-31. These proteins were thought to be low-affinity sites for the aspartyl azide that were unmasked only when the preferred high-affinity site (47K protein) was blocked with succinate or was absent due to mutation. However, this reasoning does not explain why succinate was able to enhance binding of the photolabel to these putative low-affinity sites in LL5 and LL5 Rev and especially in LL3, where the two proteins were not evident unless succinate was added. It would seem that succinate should have been able to inhibit binding of a succinate-specific photolabel to low-affinity sites more readily than to high-affinity sites. If these low-affinity sites are indeed involved in succinate transport, a possible explanation for their enhanced photolabelling in the presence of succinate could be the induced-fit model discussed in Chapter 1. This model postulates that the substrate recognition site of a protein or enzyme is not fully formed in the absence of its substrate (28). In other

words, addition of succinate to the dicarboxylate transport proteins might induce the formation of their substrate binding sites to make them more accessible for photolabelling. Both aspartate and glutamate also caused this enhancement of binding to the 35K and 29K proteins and inhibited binding of the aspartyl azide to the 47K protein in CBT43. The latter observation was not surprising in the case of aspartate, since it is also transported by the dicarboxylate system, albeit less efficiently than succinate (10). However, the dicarboxylate transport system has an absolute specificity for two carboxyl groups and chain length in its substrates, so glutamate should not have inhibited photolabelling if succinate binding proteins were involved (29).

A conclusive identification of any of the bands labelled by aspartyl azide in the whole cells as SBP1 or SBP2 was not possible from the above results. The 47K protein was a good candidate for SBP2 since it was absent in the *dctA* mutant, LL3, but present in its parental strain, CBT43. The labelling of this protein in CBT43 was also inhibited by succinate. However, the 47K protein was only present in low levels in LL5, even though this strain harboured two copies of the *dctA* gene thought to code for SBP2 (30). In addition, the same proteins were labelled in both LL5 and LL5 Rev, even though the former strain was unable to transport succinate, while the latter strain had regained this ability. The results of the affinity chromatography studies of Chapter 3 also did not indicate any difference between LL5 andd LL5 Rev, and a defect in a regulatory gene was postulated as one possible reason. Nevertheless, the absence of the 47K protein in LL3 leads to the suggestion that it may be the 53K protein that was tentatively identified by the studies in Chapters 2, 3 and 4 as the *dctA* gene product, SBP2. It

is conceivable that attachment of the aspartyl azide moiety to the 53K protein may have altered its mobility on SDS gels, as will be discussed in Chapter 6. One of the reasons that the 47K band was only detected in whole cells and was not intensively labelled in the cytoplasmic membrane preparations may be that the vesicle studies were not carried out under "energized" conditions, in contrast to the whole cell studies. Affinity-labelling studies of membrane vesicles containing the lac permease showed that optimal labelling of the carrier was not observed unless the vesicles were energized with an electron donor (31). These results suggested that binding of substrate to the carrier was dependent upon the conformation of the protein in response to the energy state of the membrane (32).

Finally, neither the whole cell [^{125}I]-aspartyl azide transport nor the photolabelling studies revealed the intracellular location of the covalently-bound label. Therefore, it was not certain if any or all of the labelled proteins were the membrane-bound transport components, SBP1 and SBP2, even though the genetic defects in the mutants affected translocation of succinate across the cytoplasmic membrane. Selective disruption of photolyzed CBT43 cells indicated that labelled proteins were present in all of the subcellular fractions (Figure 14). This observation was not surprising since the small molecular weight of the aspartyl azide would probably enable it to enter the cell via the porin channels of the outer membrane, and its hydrophobic phenyl group may have allowed it to cross the cytoplasmic membrane as well. The 47K protein was also present in all of the fractions, including the cytoplasmic membrane. Its presence in the periplasmic and outer membrane fractions may be explained by membrane damage that occurred during sphero-

plast formation. If the 47K protein is indeed the membrane-bound protein, SBP2, then its apparent presence in the cytoplasm could be due to contamination of the 13,000 x g cytoplasmic supernatant with micro-membrane vesicles that were not spun down. Alternatively, the cytoplasmic 47K protein could be a precursor of the membrane-bound form. Ongoing studies with this system involve the transformation of LL3 and LL5 mutants with ColEI plasmids (33) that contain chromosomal DNA from the regions of the <u>dctA</u> (78 minutes) and <u>dctB</u> (16 minutes) genes. Photoaffinity labelling studies with these transformants will hopefully answer some of the questions raised in this chapter and will aid in the identification and purification of the cytoplasmic membrane succinate transport components.

4 OF 4

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

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CHAPTER VI

SUMMARY AND CONCLUSIONS

This thesis investigated the use of three different affinity techniques for the isolation and identification of the cytoplasmic membrane dicarboxylate transport components in *E. coli* K12. Affinity reagents designed to interact with the substrate binding site of the succinate transport proteins were used for the preliminary identification and enrichment of the transport components prior to subsequent purification steps. The first method involved the affinity chromatography of detergent-solubilized cytoplasmic membranes on aspartate-Sepharose columns. This technique was used in the initial studies on this transport system to show that two separate membrane-bound components, SBP1 and SBP2, might be involved in cytoplasmic membrane succinate transport (1,2). Chapter 2 first dealt with optimizing the isolation, solubilization and affinity purification of the cytoplasmic membrane proteins. In addition, four different binding assays were employed to demonstrate the succinate binding activity of affinity-purified proteins from the wild-type strain, CBT43. The affinity chromatography approach was extended in Chapter 3 to include the selection and comparison of various succinate transport mutants with strain CBT43. Chapter 4 utilized a novel approach for the identification of the transport components, namely immunoblotting studies with antiidiotypic antibodies directed against the antigen binding site of succinate(aspartate)-specific IgG molecules. Finally, Chapter 5 investigated the synthesis and use of a photoaffinity label, N-(4-azido-2-nitrophenyl)aspartic acid, for transport component identification. The latter two techniques again involved the compara-

tive use of the affinity reagents in cytoplasmic membrane and/or whole cell preparations from wild-type and transport-mutant strains.

Table I summarizes the proteins that were identified or isolated by the various affinity techniques. In particular, the protein which was identified by each technique to be deficient or absent in the dctA mutant, LL3, is noted by an asterisk. A conclusive identification of any of the proteins in Table I as the cytoplasmic membrane transport components, SBP1 or SBP2, cannot be made by considering the results from any one affinity technique alone, since experimental and conceptual difficulties were encountered with each method. However, when the results of the affinity chromatography (Chapters 2 and 3) and antiidiotypic antibody immunoblotting (Chapter 4) studies are considered together, the 53K protein emerges as a good candidate for the dctA gene product, SBP2. The evidence from Chapter 2 supporting this conclusion includes the tight interaction of the 53K protein with the aspartate-Sepharose column and the use of two different binding assays to demonstrate that this protein (along with the 42K protein) bound 4 to 5 times more succinate than the other affinity-purified proteins from CBT43. The results of Chapter 2 are strengthened by the indication that the levels of this 53K protein appeared to approximate the dctA gene dosage in CBT43, LL3 and LL5 when membranes from these three strains were examined by either the affinity chromatography or immunoblotting techniques of Chapters 3 and 4 respectively. Some of the other proteins in Table I were also common to two of the affinity techniques, but the evidence to support the involvement of any of these proteins in succinate transport was not as convincing as for the 53K protein.

A comparison between the 53K protein and the best-studied bac-

Table VI-I. E. coli proteins isolated/identified by affinity techniques. The molecular weights of the E. coli proteins that were isolated or identified by affinity chromatography (Chapters 2 and 3), anti-idiotypic antibody immunoblotting (Chapter 4) and photoaffinity labelling (Chapter 5) respectively are summarized in this table.
* - indicates the protein in each technique that was decreased or absent in strain LL3 when compared to strain CBT43.

E. coli Proteins Isolated/Identified by Affinity Techniques

Technique	Molecular Weight of Proteins (Daltons)
Affinity Chromatography	53K*, 42K, 35K, 17K
Antiidiotypic Antibody Immunoblotting	73K, 53K*, 37K, 29K, 19.2K, 17K
Photoaffinity Labelling	47K*, 35K, 29K

terial transporter, the lactose permease, might be useful at this point. As discussed in Chapter 1, the lactose permease is a 46.5K Dalton polypeptide responsible for the active transport of α and β galactosides across the cytoplasmic membrane of E. coli (3). Discrepancies have been noted between the molecular weight of the transporter derived from DNA sequencing of the lac Y gene (46.5K) and the observed molecular weight of the isolated permease on SDS gels (30K). At the present time, there is no explanation for these disparate observations (3). Studies with isolated membrane vesicles have indicated that both the initial binding of substrate to the lac carrier and its transport across the membrane involve the protonmotive force, suggesting that energy-mediated conformational changes occur in the protein (4). The lac permease has been purified and reconstituted in an active form into E. coli liposomes using the non-ionic detergent octylglucoside (5,6). The proportion of active lac carrier that was reconstituted into the proteoliposomes was reported by Wright et al to be 7% (7).

The aspartate-Sepharose-purified 53K protein of this study was shown by two different binding studies (P6DG column, nitrocellulose filtration) in Chapter 2 to bind succinate with an activity (7 to 8%) similar to that of the lac permease. Since these binding studies were carried out with samples that contained a 42K protein in addition to the 53K protein, the binding efficiency that was observed may in fact have been higher if the 53K protein was the only active species. Like the lac permease, the activity of the 53K protein, in terms of its aspartate-Sepharose binding ability, was sensitive to the type of detergent used for its extraction. Its binding to the affinity column was inhibited by octylglucoside and was best when either Triton X-100 or CHAPS

were used. In contrast, octylglucoside extraction yielded the most active preparations of the lac carrier protein, whereas Triton completely abolished its activity (7). The conformation of the 53K protein in situ in the membrane vesicle preparations or in whole cells also appeared to affect the binding ability of the various affinity techniques that were employed. Whereas succinate-specific antiidiotypic antibodies were shown in Chapter 4 to bind to the 53K protein when Kaback membrane vesicles were used, binding to this protein was not detected if Triton-solubilized cytoplasmic membranes were employed instead, even though this latter type of preparation was used successfully as the starting material for the affinity column studies of Chapters 2 and 3. Clearly, the above similarities and differences between the 53K protein and the lac permease, namely low binding efficiency, sensitivity to detergents and dependence upon conformation and membrane energy state for activity, serve to emphasize the difficulties encountered in the study of membrane integral proteins.

How then do the results of the photoaffinity labelling studies of Chapter 5 fit in with the results from the other two affinity techniques? A 47K polypeptide was identified as the major protein labelled in CBT43 cells and photolabelling of this protein was inhibited by succinate. The fact that this protein was absent in the dctA mutant, LL3, suggested that it might be involved in succinate transport and, in fact, might be SBP2. However, this protein was present in relatively low amounts in LL5 and its spontaneous revertant, LL5 Rev, the two strains which were diploid for the dctA gene, and therefore did not reflect their dctA gene dosage when compared to CBT43. Unlike the affinity chromatography and immunoblotting studies of Chapters 2, 3 and 4 which

employed fractionated proteins, the 47K protein may not have been as accessible for binding with the photoaffinity label in the whole cells. It is conceivable that the genetic defect (dctB) in strains LL5 and LL5 Rev may have affected the appropriate membrane incorporation of the 47K protein such that its conformation was not amenable for photolabelling. Therefore, excess amounts of this protein may not have been detected in these two strains. It has been observed by other investigators that the conformation of some membrane proteins can be altered by various chemical or physical factors. This altered conformation may in turn be translated into an altered electrophoretic mobility when the proteins are examined on SDS polyacrylamide gels. For example, the covalent attachment of cytochalasin B to the human erythrocyte D-glucose transporter caused a change in protein conformation that resulted in an altered elution position during ion exchange chromatography (8). Another example is the E. coli outer membrane OmpA or TuII* protein. This protein has a molecular weight of 33K or 28K Daltons on SDS gels depending on whether or not it has been boiled in the presence of SDS prior to electrophoresis (9). Therefore, it is conceivable that the 47K protein might in fact be a covalent and/or heat-modified (during photolysis) form of the 53K protein.

If the 47K protein that was identified by the photolabelling studies of Chapter 5 is indeed the 53K protein, two other interesting comparisons with the lac carrier protein might be made. The first is that appreciable amounts of the 47K protein were only labelled in whole cells, but not in Kaback membrane vesicles. This observation might be due to the reliance of photolabel binding upon the energy state of the cytoplasmic membrane, similar to that seen for substrate binding to the

lactose carrier (4). Second, whereas photolabelling of the lac permease did not affect its mobility on SDS gels, attachment of aspartyl azide to the 53K protein may have shifted its apparent molecular weight to 47K Daltons, similar to the situations discussed above for other membrane proteins (8,9). However, without further supporting evidence, the co-identity of the 53K and 47K proteins is only a suggestion at this time. The identities of the 35K and 29K proteins that were detected in LL5 and LL5 Rev, and were only evident in CBT43 and LL3 in the presence of succinate are also unknown. Since these proteins were also identified by one of the other affinity techniques, a very cautious suggestion might be made that one or both of these proteins are also involved in succinate transport.

In conclusion, a firm identification of the 53K protein as the SBP2 dicarboxylate transport component and the identification of the SBP1 component await the results of cloning studies with the dctA and dctB genes. At the present time, LL3 and LL5 strains that have been transformed with ColE1 plasmids harbouring the dctA and dctB genes respectively are being subcloned in an effort to isolate and identify the genes (10). The advantages of these studies should be two-fold. An increased copy number of the genes will hopefully lead to increased expression of the gene products which will aid in the identification of the transport components in mini- or whole cells. In addition, amplification of the transport protein levels will aid in the isolation of these proteins from the transformants and will yield increased amounts of the transport components for binding and photolabelling studies. The genes for several other E. coli cytoplasmic membrane proteins have been cloned to high copy number with varying degrees of success (11,12,13).

Increased expression of both the lactose permease (11) and the *a* subunit of the F_o component of the *E. coli* ATPase (12) were found to have inhibitory effects on cell growth and survival. In the former case, the degree of growth inhibition was observed to be dependent upon the copy number of the gene (11). In contrast, elevation of the copy number of the genes coding for the *E. coli* fumarate reductase led to a greater than 30-fold amplification of the enzyme, apparently without any deleterious effects on the cells (13). Instead, the increased levels of the enzyme were accomodated in novel intracellular, membranous tubules. Preliminary studies with the succinate transport transformants have indicated that the growth of these strains is enhanced in comparison to LL3 and LL5, and that the transformants appear to contain intracellular structures similar to those observed for fumarate reductase overproduction (10). The affinity techniques that have been developed in this thesis will hopefully aid in the identification and isolation of the succinate transport components from the *dctA* and *dctB* transformants.

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