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APPLICATIONS OF MONOCLONAL ANTIBODIES TO STUDIES OF STRUCTURE/FUNCTION RELATIONSHIPS IN *ESCHERICHIA COLI* F_1-ATPASE

by

Richard G. Tozer

Department of Biochemistry

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

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
July, 1987

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ABSTRACT

Twenty-one monoclonal antibodies to *Escherichia coli* F_1-ATPase were characterized and used in structural studies of the enzyme. Solid phase competition assays placed antibodies recognizing the same or overlapping epitopes into competition subgroups. Immunoprecipitation and membrane-binding studies identified those antibodies recognizing external epitopes on soluble and membrane-bound forms of the holoenzyme and demonstrated that the δ and ϵ subunits are substantially exposed in F_1F_0.

Analysis of partially cleaved α subunit using Western blots mapped antibody epitopes to its carboxyl-half. Analysis of CNBr/weak acid cleaved β located the non-inhibitory B-II antibody epitopes between Asp-345 and Met-380 and the inhibitory B-I antibody epitopes between Asp-381 and the carboxyl terminus. The ϵ-binding site on β was mapped to the same region as the B-I antibodies. The Fab fragment of a B-I antibody and ϵ were cross-linked indicating the proximity of their binding sites in native F_1.

Cross-linking studies revealed neither differences in quaternary structure between soluble and membrane-bound F_1 nor any association between δ or ϵ and any F_0 subunit. Cross-linking studies of F_1 in the presence of the ATPase activators ethylene glycol and antibody ϵ-4 examined the mechanism of ϵ-inhibition. Ethylene glycol displaced ϵ only from its site of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) cross-linkage to β demonstrating that ϵ-inhibition is mediated through β. In contrast, antibody ϵ-4 did not alter EDC cross-linking of ϵ to β showing that this interaction is not always inhibitory. ϵ

could also be cross-linked to β by EDC in F_1F_0 where it is not inhibitory. The inhibitory effect of ϵ and the B-I antibodies binding to the carboxyl-terminal region of β region suggested a function for this region.

The antibodies were used to characterize an α-δ cross-link produced during column centrifugation of F_1 by oxidation of sulfhydryls. This cross-link affected neither the enzyme's activity nor susceptibility to ϵ-inhibition, casting doubt on rotational models of F_1 catalysis. The α-δ cross-link also resulted in a loss of binding affinity between F_1 and F_0.

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FOR CHRISTINE
AND MY PARENTS

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NOMENCLATURE

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
BS^3	Bis(sulfosuccinimidyl)suberate
DCCD	N,N'-Dicyclohexylcarbodiimide
dpm	Decays per minute
DTT	Dithiothreitol
DSP	2,2'-Dithiobis(succinimidyl) propionate
EDC	1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGS	Ethyleneglycol bis(succinimidyl succinate)
ELISA	Enzyme-linked immunosorbent assay
F_1 or F_1-ATPase	The peripheral portion of the proton-translocating ATPase
F_0	The integral membrane portion of the proton-translocating ATPase
HPLC	High performance liquid chromatography
IF_1	Inhibitory polypeptide of mitochondrial F_1
K_d	Dissociation constant

LDAO	Lauryldimethylamine N-oxide
Nbf	4-chloro-7-nitrobenzofurazan
NEM	N-ethylmaleimide
OSCP	Oligomycin sensitivity conferring protein
P_i	Inorganic phosphate
PMSF	Phenylmethylsulfonyl-fluoride
p.s.i	Pounds per square inch
SDS	Sodium dodecyl sulfate
sulfo-SANPAH	6-(4'-azido-2'-nitrophenylamine)-hexanoate
sulfo-SMCC	4-(N-maleimidomethyl)-cyclohexane-1-carboxylate
sulfo-SMPB	4-(P-maleimidophenyl)butyrate

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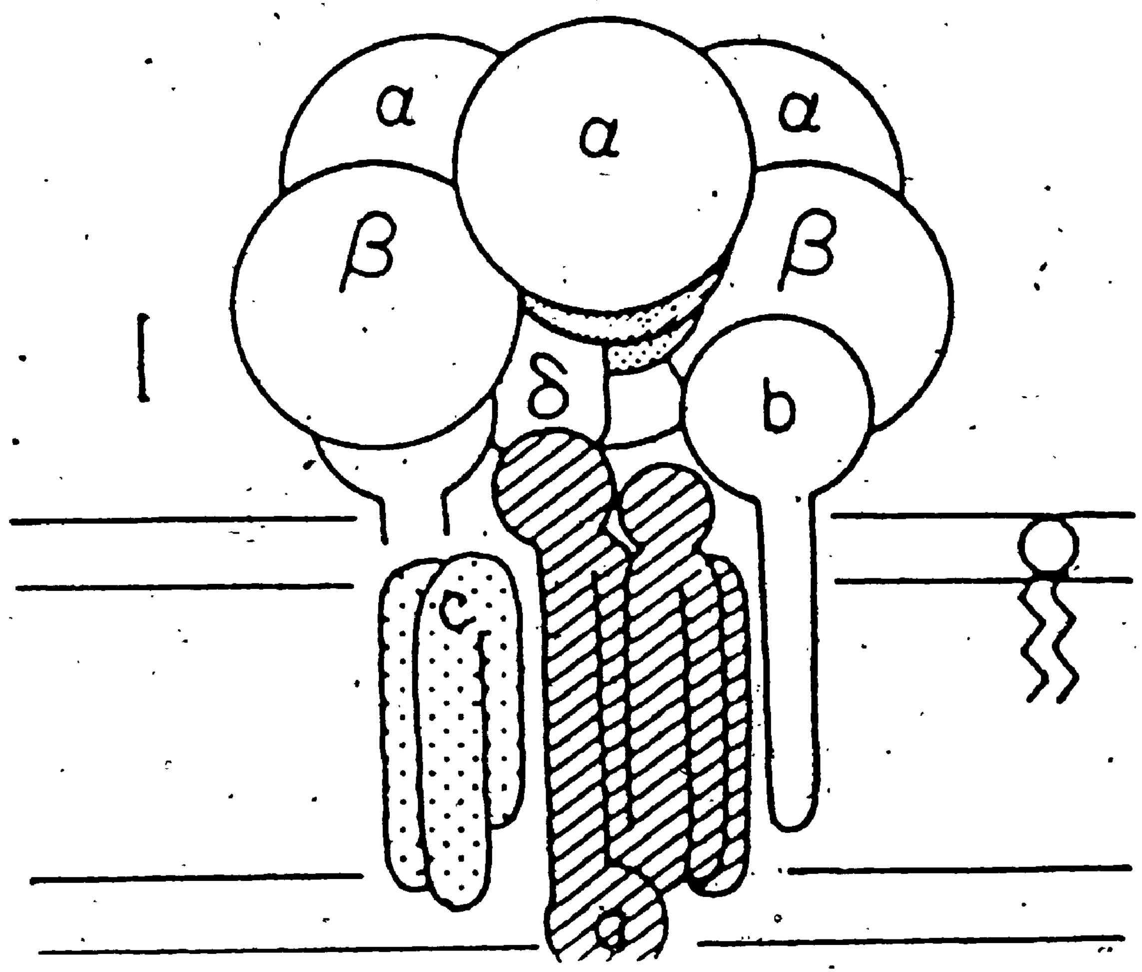
1.0 INTRODUCTION

1.1 Overview

The proton-translocating ATPase is a ubiquitous enzyme found in inner mitochondrial membranes, chloroplast thylakoid membranes and bacterial cytoplasmic membranes. In mitochondria, chloroplasts and some bacteria, the respiratory chain or photosystem enzymes pump protons across a membrane forming a proton gradient. The ATPase is capable of coupling this source of potential energy to the synthesis of ATP from ADP and inorganic phosphate (P_i). In some bacteria, the ATPase functions in the reverse mode. Instead of synthesizing ATP as described previously, ATP is hydrolysed and the energy is used to pump protons out of the cell thereby generating a proton gradient. This source of potential energy is harnessed by cellular processes such as the active transport of some nutrients. For current reviews see Senior and Wise, 1983; Bragg, 1984; Vignais and Satre, 1984; and Senior, 1985.

The ATPases of mitochondria, chloroplasts and bacteria are similar in structure consisting of two sections named F_1 and F_0 (Figure 1.1). F_1 is a peripheral protein that can be removed from the membrane using low ionic strength buffers that either lack magnesium or contain EDTA. This section contains the sites of phosphoryl bond synthesis and hydrolysis. The other section, F_0, is an integral membrane protein that can be solubilized only through the use of detergents. It serves as a proton specific pore. When the two sections are separated, F_1 is only capable of ATP hydrolysis and F_0 facilitates free passage of

Figure 1.1. Model of quaternary structure of F_1F_0 of *E. coli*. This structure is a simplified model of F_1F_0 taken from Aris and Simoni, 1983. The γ subunit is the dense, cross-hatched sphere and the ε subunit is the dense, stippled sphere. The sizes of the spheres are proportional to the molecular weights of the subunits. Note that only a single subunit c is shown. The bar is 1.2 nm long.


α
α
α
β
β
δ
b
c
a

protons through the membrane. All the subunits of *Escherichia coli* F_1 and F_0 are coded by genes in the *unc* operon which maps at approximately 83 minutes in the *E. coli* chromosome. The entire nucleotide sequence for this operon is known and mutations in some of the genes have been characterized (Walker *et al.*, 1984; Senior, 1985; Parsonage *et al.*, 1987).

1.2 Subunit Structure and Function

The subunit structures of mitochondrial, chloroplast and bacterial F_1's are similar. *E. coli* F_1 consists of five types of subunits named α through ϵ in order of decreasing molecular weight with a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Senior and Wise, 1983). The minimum requirement for catalytic activity is an $\alpha_3\beta_3\gamma$ complex; no single subunit has catalytic activity (Futai, 1977; Dunn and Futai, 1980).

The α subunit has a molecular weight of 55,282 and is coded by the *uncA* gene (Walker *et al.*, 1984). Isolated α has a site capable of binding one molecule of ATP or ADP with K_d's of 0.1 and 0.9 μM respectively (Dunn and Futai, 1980). This is referred to as a "tight" nucleotide-binding site and exhibits an extremely slow dissociation rate of 0.21 min^{-1} (Dunn, 1980). Upon binding ATP, the α subunit undergoes a conformational change resulting in a smaller molecular weight value as determined by sedimentation equilibrium and a change in susceptibility to digestion by trypsin (Dunn, 1980; Senda *et al.*, 1983). There is fairly conclusive evidence that this site is not catalytic (Gresser *et al.*, 1979; Rosen *et al.*, 1979) and its function in the holoenzyme is not yet known, though structural and

regulatory roles have been suggested. Binding sites for Mg^{2+} have also been detected on the α subunit (Smith *et al.*, 1985).

The β subunit has a molecular weight of 50,286 and is coded by the *uncD* gene (Walker *et al.*, 1984). Through equilibrium dialysis, a nucleotide-binding site has been detected with K_d's of approximately 25 μM for ADP and 50-100 μM for ATP (Issartel and Vignais, 1984). Both the genes of the α and β subunits exhibit sequence homology to other nucleotide-binding proteins (including each other) and both exhibit sequences homologous to the Rossman fold, a structure that binds nucleotides (Duncan *et al.*, 1986). The nucleotide-binding site on β is currently thought to be the catalytic site for a number of reasons. Certain chemical modification reagents including N,N'-dicyclohexylcarbodiimide (DCCD) and 4-chloro-7-nitrobenzofurazan (Nbf-Cl) abolish ATPase activity after reaction with specific sites on the β subunit (Satre *et al.*, 1979; Lunardi *et al.*, 1979). Photoaffinity analogues of phosphate and the mitochondrial natural ATPase inhibitor, IF_1, inhibit ATPase activity with the concurrent labelling of the β subunit (Vignais and Satre 1984). The β subunit also has a binding site for the fluorescent antibiotic aurovertin, an inhibitor of most bacterial and mitochondrial F_1's (Vershoor *et al.*, 1977; Dunn and Futai, 1980). Binding sites for Mg^{2+} have recently been detected on this subunit (Futai *et al.*, 1987).

The γ subunit has a molecular weight of 31,559 and is coded by the *uncG* gene (Walker *et al.*, 1984). As it is essential for the reconstitution of an active complex, it is thought to be important in organizing the α and β subunits into their proper positions (Dunn and Heppel, 1981). It is possible that the γ subunit may have a role in

the passage of protons through the complex and may in some instances regulate the activity of the complex. In the chloroplast enzyme, disulfide exchange reactions within the γ subunit result in either latent or active forms of the enzyme (McCarty, 1979). To this end it is also notable that antibodies raised against the γ subunit often inhibit enzyme activity (Smith and Sternweis, 1982).

The δ and ε subunits are coded by the *uncH* and *uncC* genes and have molecular weights of 19,328 and 15,051 respectively (Walker *et al.*, 1984). The δ subunit has been shown to bind to the amino terminus of an α subunit (Dunn *et al.*, 1980) and has an elongated shape (Sternweis and Smith, 1977). Cross-linking experiments have shown that the ε subunit is in close proximity to both a β subunit and the γ subunit (Lötscher *et al.*, 1984). The ε subunit can also bind to purified γ subunit (Dunn, 1982). Both the δ and ε subunits bind rapidly, reversibly and independently to the $\alpha_3\beta_3\gamma$ complex and both appear to be required for the binding of F_1 to F_0 (Sternweis, 1978; Futai *et al.*, 1974; Smith and Sternweis, 1977). For this reason these subunits have often been depicted at the F_1/F_0 interface, often forming a stalk between the $\alpha_3\beta_3\gamma$ complex and F_0 (Aris and Simoni, 1983; Futai and Kanazawa, 1983; Senior and Wise, 1983; see Figure 1.1). The ε subunit is also a partial non-competitive inhibitor of soluble *E. coli* F_1 (Laget and Smith 1979; Sternweis and Smith, 1980).

The subunit structures of other bacterial F_1's appear to be homologous to that of *E. coli*, although in some cases there appears to be functional reversal of the α and β subunits (Dunn *et al.*, 1985). The *E. coli* F_1 subunit structure is also homologous to that of chloroplast F_1. Some differences are observed between the minor

subunits of *E. coli* F_1 and those of mitochondrial F_1 (Senior and Wise, 1983). The *E. coli* F_1 subunits δ and ϵ appear to be homologous to the mitochondrial oligomycin sensitivity conferring protein (OSCP) and the δ subunit respectively. The mitochondrial ϵ subunit has no *E. coli* equivalent. The mitochondrial F_1 also has an inhibitor protein called IF_1 that is not present in *E. coli* F_1 (Senior and Wise, 1983).

Less is known about the subunits of F_0 (for a review see Hoppe and Sebald, 1984). In *E. coli*, the F_0 subunits are named a, b and c, have molecular weights of 30,285, 17,230 and 8,264 respectively and are present in a subunit stoichiometry of ab_2c_{10-12}. Subunits a, b and c are coded by the *uncB*, *uncF* and *uncE* genes respectively (Walker *et al.*, 1984). From studies involving chemical labelling and susceptibility to digestion by proteases, subunits a and c appear to be largely buried in the membrane (Senior, 1983). In contrast subunit b appears to consist of a small hydrophobic amino-terminal domain that traverses the membrane with the bulk of the molecule protruding into the cytoplasm perhaps in the form of a long hairpin (Senior, 1983). Subunits a and c appear to be equivalent to the mitochondrial ATPase 6 subunit and DCCD-binding protein respectively (Senior and Wise, 1983). There does not appear to be a mitochondrial equivalent to subunit b.

The function of each F_0 subunit is not yet entirely understood but all three appear to be required for the formation of an active proton pore (Friedl *et al.*, 1983; Schneider and Altendorf, 1984). Subunit b may be important for binding F_1 to F_0 (Hoppe *et al.*, 1983; Perlin *et al.*, 1983; Bragg, 1984). Cross-linking experiments have shown

that it is in close proximity to a β subunit (Aris and Simoni, 1983). Subunit c, a chloroform/methanol soluble proteolipid, is found in other species of bacteria with its primary structure largely conserved (Senior and Wise, 1983). One highly conserved region contains a membrane-buried aspartic acid residue (Asp-61) that is specifically labelled by DCCD (Senior and Wise, 1983). Modification of Asp-61 by DCCD or mutation leads to blockage of proton conductance through F_0 (Negrin *et al.*, 1980; Sebald and Hoppe, 1981, Hoppe *et al.*, 1982) and the concommittant inhibition of F_1F_0 (Hoppe and Sebald, 1981; Sebald *et al.*, 1980).

1.3 Catalytic Mechanism

Intact F_1 contains six nucleotide-binding sites. Three do not exchange with medium nucleotide and may be the sites observed on the α subunits through the use of equilibrium dialysis. The remaining three sites do exchange with medium nucleotide and have been postulated to be the catalytic sites (Cross and Nalin, 1982). These sites appear to lie either on the β subunits or at the α/β interfaces (Senior and Wise, 1983). Within each class of binding site, differences between individual sites are apparent. For example among the exchangeable sites, only a single high affinity site is detected during catalysis; among the non-exchangeable sites, one site is observed to be vacant when catalysis is not occurring (Kironde and Cross, 1986). The significance of this asymmetry is not yet known, but asymmetry has also been shown through differences in reactivity of each of the three β subunits to various chemical modification reagents (Stan-Lotter and

Bragg, 1986a, 1986b).

A great deal of evidence has accumulated to indicate that subunit/subunit interactions are very important for catalytic activity. For example, no isolated subunit exhibits catalytic activity (Dunn and Futai, 1980) and mutations in either the α or β subunits can alter activity (Senior, 1985). Three point mutations in the α subunit which lead to low levels of ATPase activity also lead to changes in the pattern of aurovertin fluorescence in the presence and absence of ADP (Note that aurovertin binds exclusively to the β subunit). The pattern seen is identical to that observed when aurovertin binds to dissociated β subunit. This observation has been interpreted to mean that α/β subunit interactions are necessary for normal enzyme activity (Wise *et al.*, 1981). Additionally, certain chemical reagents such as DCCD and Nbf-Cl lead to inactivation of the enzyme after modification of a residue on a single β subunit (Lunardi *et al.*, 1979; Satre *et al.*, 1980). These subunit/subunit interactions appear to manifest themselves as the positive cooperativity which is observed between catalytic sites. Several workers have shown that when only a single catalytic site on F_1 is occupied by nucleotide, the rate of catalysis is very slow (known as "unisite catalysis"). However the binding of nucleotide to a second site appears to promote catalysis at the first site with a rate enhancement of approximately 10^6-fold (Cross *et al.*, 1982; Grubmeyer *et al.*, 1982). There is some evidence that nucleotide binding to a third site may further promote activity (Wong *et al.*, 1984; Dunn *et al.*, 1987).

Energy is not required for the formation or hydrolysis of the β-γ phosphate anhydride bond at the active site. Under conditions in which

F_1 is in molar excess over ATP so that promotion of catalysis does not occur, release of product is very slow and free interconversion between ATP and ADP·P_i occurs at the catalytic site (Choate *et al.*, 1979). A very small change in free energy occurs during bond formation or hydrolysis and the equilibrium constant for the hydrolysis reaction has been estimated to be 0.5 (Grubmeyer *et al.*, 1982). Energy appears to be required for binding and release of nucleotide from the catalytic sites (Cross, 1981). Study of the stereochemistry of the reaction shows that it is unlikely that the hydrolytic/synthetic reactions proceed via a covalent F_1-phosphate intermediate and that ADP is the primary phosphate acceptor in the synthetic mode. During hydrolysis there is an inversion of the γ-phosphorous atom which is consistent with the occurrence of a direct in-line transfer of the phosphoric residue between ADP and water. Consequently the reaction mechanism of F_1 may resemble that of myosin rather than that of ATPases such as the Na^+,K^+-ATPase (Webb *et al.*, 1980).

Using these observations, Paul Boyer postulated a model for the mechanism of F_1 called the "binding change" or "alternating sites" mechanism (Cross *et al.*, 1984). He proposed that two or three sites alternate between catalytic and promotional roles during the catalytic cycle. Energy derived from the proton gradient is used to drive conformational changes that affect the binding affinities for nucleotides at the various sites. These conformational changes lead to binding of substrate at one site and promotion of release of product at another. It is noteworthy that in this model, proton flow is coupled to catalysis indirectly. Other workers have proposed that protons passing through F_1F_0 are actually involved in the reaction (Mitchell,

1979). Recently a model utilizing aspects of both theories has appeared (Scarborough, 1986).

Models have also been proposed detailing the nature of the conformational changes that occur during catalysis. These share the common feature of involving rotational displacement of the α/β hexamer in relation to the γ, δ and ϵ subunits and subunit b of F_0. The first such model was proposed by Gresser and coworkers (1982). More detailed models have been outlined by Cox and coworkers (1984) and Mitchell (1985). These models will be more fully discussed in chapter 5.

1.4 Immunological Studies of F_1F_0

F_1-ATPase has been the focal point of a large number of studies involving the use of antibodies raised against the whole enzyme or particular subunits. Sources of F_1 have included mammalian and yeast mitochondria, chloroplasts, *Rhodospirillum rubrum* chromatophores and a variety of bacteria including *E. coli*, *Micrococcus lysodeikticus*, and the thermophilic bacterium PS3. These studies have investigated various structural and mechanistic aspects of the enzyme.

Antibodies raised against F_1 have been used to determine the orientation of F_1 in membranes and that of the membrane preparations themselves. Berzborn and coworkers (1975) used immunodiffusion studies to demonstrate that F_1 was located on the external surface of chromatophores prepared from *R. rubrum*. Oppenheim and Salton (1973) used ferritin-conjugated antibodies to show that F_1 was located on the external surface of isolated membrane vesicles from *M.*

lysodeikticus and was not present on its mesosomes. Christiansen and coworkers (1969) used anti-F_1 antibodies as non-permeant probes to demonstrate that submitochondrial particles prepared from beef heart mitochondria were everted by showing that the antibodies could inhibit enzymatic activity.

Antibodies have also been used to immunoprecipitate F_1 or the F_1F_0 complex from complex mixtures. Ludwig and Capaldi (1979) used this approach to demonstrate that the F_1F_0 complex isolated from chloroform treated beef heart submitochondrial particles has the same constituent subunits as the complex immunoprecipitated from a Triton X-100 extract. Brusilow and coworkers (1981) and Klionsky and Simoni (1985) used subunit specific antisera to show that the F_1 subunits produced by *in vitro* transcription/translation systems were being correctly assembled.

Antibodies have been used to demonstrate the homology in structure between F_1-ATPases from various sources through the property of immunologic cross-reactivity. Whiteside and Salton (1970) showed that antisera raised against F_1 from *M. lysodeikticus* were able to inhibit to varying degrees the activity of F_1-ATPases from *Sarcina lutea*, *S. flava*, and *M. varians* but not those from *Sporosarcina ureae* or *Bacillus subtilis*. Philosoph and Gromet-Elhanan (1981) demonstrated that antibodies raised against F_1 from *R. rubrum* were able to inhibit the activity of spinach chloroplast F_1. Rott and Nelson (1981) used subunit specific antisera raised against yeast, rat liver, swiss chard and *E. coli* F_1 subunits and the technique of immunoblotting to demonstrate which subunits in F_1 are most closely conserved. They found that each anti-β antiserum

recognized every organism's β subunit. The yeast anti-α antiserum recognized all the other α subunits weakly and the swiss chard anti-α antiserum only recognized the α subunit of rat liver. The yeast anti-γ antiserum only recognized the *E. coli* γ subunit. Hicks and coworkers (1986) demonstrated that antibodies raised against chloroplast α and γ subunits and the *E. coli* β subunit could recognize the analogous subunits from the F_1 of the cyanobacterium *Spirulina platensis*. These results are consistent with sequence homologies observed among F_1 subunits from different sources (Walker *et al.*, 1985).

Some of the most interesting work done with antibodies to date has dealt with their effects upon the activity of the enzyme. Antisera raised against F_1 is inhibitory regardless of the source of the enzyme (Philosoph and Gromet-Elhañan, 1981; Nelson *et al.*, 1973; Mollinedo *et al.*, 1980; Yoshida *et al.*, 1979). Antibodies have been raised against various subunits of F_1 and their effects studied. Using the chloroplast enzyme, Nelson and coworkers (1973) raised antibodies to the α, β, γ and δ subunits. The anti-α and anti-γ antisera inhibited photophosphorylation, ATP-stimulated proton uptake and heat-activated ATPase activity. No effects were seen with either the anti-β or anti-δ antisera. The negative results obtained with the anti-β antiserum are thought to have arisen because the antiserum only recognized denatured β subunit. Yoshida and coworkers (1979) raised antisera against the α through ϵ subunits of F1 from the thermophilic bacterium PS3. The anti-α and anti-β antisera strongly inhibited ATP hydrolysis and $^{32}P_i$-ATP exchange on both soluble and membrane-bound F_1. The anti-δ antiserum slightly inhibited the exchange reaction while the

anti-γ and anti-ε antisera had no effect. The anti-γ and anti-ε antisera were unable to react with intact F_1F_0 in an Ouchterlony double diffusion assay suggesting that these subunits are buried in the membrane-bound form of the enzyme. Antibodies have also been raised against subunit c of F_0 and these antibodies have been able to bind to the surface of membranes and prevent proton leakage in everted, F_1-stripped, membranes (Loo *et al.*, 1983). These antibodies also prevented the binding of F_1 to membranes demonstrating that a region of subunit c is exposed at the membrane surface near the F_1-binding site (Loo and Bragg, 1981).

The most comprehensive study of antibody effects to date was performed by Smith and Sternweis (1982) using F_1 from *E. coli* and purified antibodies raised against each F_1 subunit. The antibodies raised against the α subunit recognized only denatured subunit and thus were not useful. Antibodies raised against the β and γ subunits inhibited ATP hydrolysis by both soluble and membrane-bound F_1. The anti-δ antibodies caused F_1 to dissociate from the membrane and inhibited respiration-dependant but not ATP-dependant transhydrogenase activity (*i.e.* protons leaked through F_0). The anti-ε antibodies inhibited membrane-bound F_1 but not respiration-dependant transhydrogenase activity. Thus the membrane was still capable of supporting the generation of a proton motive force. The antibodies led to a small but significant stimulation of soluble F_1 along with the dissociation of the ε subunit from the enzyme. This property was used by Sternweis (1978) to prepare ε-depleted F_1 by passing F_1 though an affinity column prepared by coupling anti-ε antibodies to CNBr-activated Sepharose. Smith and Sternweis also showed that the

catalytically active $\alpha_3\beta_3$ hexamer produced by trypsin treatment of F_1 contained a small fragment of the γ subunit emphasizing the importance of this subunit in the catalytic unit.

The very nature of polyclonal antisera limits their application in finer studies of enzyme structure and mechanism. This is due to polyclonal antisera consisting of vast assortments of antibodies that bind to different epitopes on the surface of an enzyme with different affinities. To overcome these limitations many researchers have turned to monoclonal antibodies. It is possible to fuse antibody producing B-cells with a myeloma tumour cell to create an immortal cell line that produces antibody characteristic of the original B-cell. By careful screening and cloning one can isolate and perpetuate a cell line that produces a homogeneous population of antibodies that recognizes a single epitope. By immunizing an animal with antigen of choice and then using that animal's spleen for the fusion procedure, one can produce monoclonal antibodies directed against any protein or other biological constituent of interest. This method also allows one to produce antibodies in large amounts. Monoclonal antibodies have been used for a variety of applications including use as affinity adsorbents for purification of hormones and interferons, as aids in the classification of microorganisms, as means to selectively kill tumour cells and as means to study antibody diversity and the mechanisms of the immune response (Nisonoff, 1982). Recently researchers are finding monoclonal antibodies useful in the study of enzymes.

In the study of F_1, four groups in addition to our own have begun to make use of monoclonal antibodies. One group raised a monoclonal antibody directed against the β subunit of *E. coli* F_1 (Brusilow

et al.; 1981; Klionsky and Simoni, 1985). This antibody was used in the analysis of the products of an *in vitro* transcription/translation system containing portions of the *unc* operon. It was necessary to determine whether the subunits produced were capable of correctly assembling into the holoenzyme. Immunoprecipitation of the entire complex by the anti-β monoclonal antibody would indicate whether correct assembly had occurred.

Another group raised monoclonal antibodies to subunits of the chloroplast F_1 (Lunsdorf *et al.*, 1984; Tiedge *et al.*, 1985; Ehrig *et al.*, 1986). They have 11 antibodies directed against α, 2 against β and 7 that recognize both α and β (of which 2 also recognize γ). The most interesting experiments performed consisted of decorating F_1 with antibodies and examining the complex with the electron microscope. Through these experiments, they were able to demonstrate that there are indeed three α and three β subunits in F_1 and that they are organized in an alternating manner.

Monoclonal antibodies have been raised against yeast F_1 (Hadikusumo *et al.*, 1984; Choo *et al.*, 1985; Hadikusumo *et al.*, 1986). They have been used to immunoprecipitate F_1 in studies with mutant subunits. Properties of these antibodies have also been carefully characterized. Binding affinities were determined through solid phase assays and antibodies recognizing closely placed epitopes were organized into groups. The antibodies' ability to immunoprecipitate F_1 and their effects upon the enzyme's activity were also determined. In their set of monoclonal antibodies, they have nine antibodies that recognize the β subunit as well as four antibodies that recognize a 25,000-dalton peptide that can be immunoprecipitated with

other F_1 subunits from a Triton X-100 extract. The nine anti-β antibodies appear to recognize four or five distinct epitopes. Interestingly, those antibodies recognizing the same epitope often exhibit very different effects upon the enzyme's activity as well as different abilities to immunoprecipitate the intact F_1 complex.

Another group has raised antibodies directed against the α and β subunits and the OSCP of pig heart mitochondrial F_1 (Moradi-Améli and Godinot, 1983; Archinard *et al.*, 1984; Godinot *et al.*, 1986; Moradi-Améli and Godinot, 1987). One of the anti-β monoclonal antibodies only inhibits the enzyme in the presence of P_i. Another anti-β antibody appears to prevent hysteretic inhibition induced by preincubation of F_1 in the presence of Mg^{2+} and ADP. This antibody has no effect on ATP hydrolysis but does inhibit ATP synthesis and net P_i-ATP exchange. Both of these antibodies cross-react with F_1 from chloroplasts, rat liver, beef heart and yeast mitochondria as well as various species of bacteria. The anti-OSCP antibody is able to bind to everted inner mitochondrial vesicles (as seen by electron microscopy), does not cross-react with *E. coli* δ-subunit (the bacterial subunit most homologous to mitochondrial OSCP) and has no effect upon ATP synthesis or proton translocation. If the antibody is preincubated with OSCP, the activating effect of this subunit on the reconstitution of ATP synthesis with everted mitochondrial vesicles is lost suggesting that the antibody interferes in the interaction between OSCP and F_1 or F_0.

1.5 Purpose of Thesis

Monoclonal antibodies may be useful tools for the investigation of various structural/functional aspects of the *E. coli* F_1-ATPase. Before initiating such studies the antibodies must be extensively characterized. Chapter 2 outlines experiments in which immunoprecipitations and membrane-binding assays were performed to determine which antibodies recognize exposed or buried epitopes in F_1 and F_1F_0. Solid phase competition assays were used to place those antibodies recognizing overlapping epitopes into competition subgroups. Results obtained by other workers in the laboratory detailing antibody effects on enzyme activity and antibody cross-reactivity are also described.

The remainder of the thesis describes various experiments using the antibodies as tools for the study of structural/functional relationships in F_1. Chapter 3 describes experiments that mapped the locations of antibody epitopes within the primary structures of the α and β subunits. Difficulties were encountered in producing suitably sized epitope-containing fragments for purification and subsequent amino acid analysis. The significance of this problem and the means by which it was overcome are applicable to other studies involving monoclonal antibodies. The mapping of the anti-β antibody epitopes to a carboxyl-terminal segment of the β subunit led to the formulation of a model of structure and function of the domains of β.

Chapter 4 describes cross-linking experiments in which monoclonal antibodies were used for the direct detection of cross-linked products

on Western blots. One series of studies investigated the quaternary structure of F_1F_0 in membranes looking for evidence that the δ and ϵ subunits form a connecting stalk between F_1 and F_0. Another set of experiments studied mechanisms of activation of F_1 by antibody ϵ-4, ethylene glycol and lauryldimethylamine N-oxide (LDAO). These used comparisons of cross-linking patterns in the presence and absence of the activating agent to determine whether subunit interactions involving the inhibitory ϵ subunit had been altered. Finally, the location of the region of β that interacts with ϵ was found by partial cleavage of cross-linked β-ϵ. This, in conjunction with the results of the studies of ethylene glycol activation led to the proposal of a role for ϵ in F_1F_0.

Chapter 5 investigates the formation of a specific intersubunit cross-link by the preparative technique of column centrifugation. This cross-link's effects upon the activity of F_1 are significant in light of the rotational model of the enzyme's catalytic mechanism. The potential risks and utility of column centrifugation for studies involving other enzymes are also discussed.

2.0 CHARACTERIZATION OF MONOCLONAL ANTIBODIES

2.1 Introduction

Our laboratory has a bank of 21 monoclonal antibodies that recognize the subunits of the *E. coli* F_1-ATPase. The production of these hybridomas has been described in detail elsewhere (Dunn *et al.*, 1985) but is briefly summarized below. The antibodies recognizing the α, β and δ subunits, and one recognizing the γ subunit (γ-3) were produced from the fusion of the spleen cells of a rat immunized with purified F_1 and the cells of a non-antibody-secreting mouse myeloma line. The remaining monoclonal antibodies that recognize the γ and ε subunits were produced by the fusion of the spleen cells of a rat immunized with purified γ and ε subunits with a non-antibody-secreting rat myeloma line. Among the hybridomas, four produce antibodies directed against the α subunit, six against the β subunit, three against the γ subunit, four against the δ subunit and four against the ε subunit. Antibodies were named by adding an arbitrary arabic numeral to the Greek letter name of the subunit recognized. This information is summarized in Table I.

In order to make full use of the monoclonal antibodies as tools in studies of structure/function relationships of F_1 it was necessary to characterize various properties of the antibodies. Initial characterization of the antibodies by workers in the laboratory detailed the antibodies' effects upon ATPase activity and their ability to recognize ATPases from various organisms. The isotypes of the antibodies were also determined. The results of this work will be

TABLE I

Monoclonal antibodies to *E. coli* F_1-ATPase

Antibody designation	Immunogen	Rodents immunized	Myeloma Line	Isotype
α-1	F_1	PVG rat	SP-2/0 (mouse)	IgG2b
α-2	F_1	PVG rat	SP-2/0 (mouse)	IgG1
α-3	F_1	PVG rat	SP-2/0 (mouse)	IgG1
α-4	F_1	PVG rat	SP-2/0 (mouse)	IgG1
β-1	F_1	PVG rat	SP-2/0 (mouse)	IgG2a
β-2	F_1	PVG rat	SP-2/0 (mouse)	IgG2a
β-3	F_1	PVG rat	SP-2/0 (mouse)	IgG1
β-4	F_1	PVG rat	SP-2/0 (mouse)	IgG2a
β-5	F_1	PVG rat	SP-2/0 (mouse)	IgG2a
β-6	F_1	PVG rat	SP-2/0 (mouse)	IgG2a
γ-1	γ and ε	AO rat	YB-2 (rat)	IgG2a
γ-2	γ and ε	AO rat	SP-2/0 (mouse)	IgG2a
γ-3	F_1	PVG rat	SP-2/0 (mouse)	IgG2a
δ-1	F_1	PVG rat	SP-2/0 (mouse)	IgM
δ-2	F_1	PVG rat	SP-2/0 (mouse)	IgG2b
δ-3	F_1	PVG rat	SP-2/0 (mouse)	IgG2b
δ-4	F_1	PVG rat	SP-2/0 (mouse)	IgM
ε-1	γ and ε	AO rat	YB-2 (rat)	IgG2a
ε-2	γ and ε	AO rat	SP-2/0 (mouse)	IgG1
ε-3	γ and ε	AO rat	YB-2 (rat)	IgG1
ε-4	γ and ε	AO rat	YB-2 (rat)	IgG1

Antibodies were named by adding an arabic numeral to the *E. coli* F_1 subunit which is recognized. Details about rodent immunization protocols, cell hybridizations and determination of antibody isotypes will be found in Dunn *et al.*, 1985.

summarized in the section 2.4.

The experiments that follow were performed to characterize a few properties of the epitopes recognized by the antibodies. It was important to determine which epitopes are located on the surface of the assembled holoenzyme or at subunit interfaces and which epitopes are exposed when F_1 is bound to membranes. Immunoprecipitations and the binding of radiolabelled antibody to membranes were used to accomplish these goals. It was also important to determine the proximity of the antibody epitopes relative to one another. For this to be accomplished, the ability of antibodies to bind simultaneously to the same subunit was determined. Antibodies that bind to nearby epitopes would be expected to inhibit one another's binding through steric hindrance. Information obtained from these experiments was important for formulating and interpreting further experiments utilizing the antibodies as tools for structural and mechanistic studies of F_1.

2.2 Materials and Methods

2.2.1 Materials

Tryptone, yeast extract and Freund's complete adjuvant were obtained from Difco (Detroit, Mich.). Sepharose CL4B and molecular weight markers were purchased from Pharmacia P-L Biochemicals (Uppsala, Sweden). Bovine intestinal alkaline phosphatase and p-nitrophenylphosphate were purchased from Boehringer Mannheim (Dorval, Qué.). IODO-GEN was purchased from Pierce (Rockford, Ill.). $Na^{125}I$ was obtained from New England Nuclear (Boston, Mass.). Chemicals for

electrophoresis and Bio-Gel P-10 were purchased from Bio-Rad (Mississauga, Ont.). Fraction V bovine serum albumin (BSA), defatted BSA, ATP, uracil, arginine, dithiothreitol (DTT) and N-ethylmaleimide (NEM) were purchased from Sigma (St. Louis, Mo.). CNBr was purchased from Fisher (Toronto, Ont.). The flat bottom, 96-well microtiter plates were obtained from Dynatech (Alexandria, Va.).

2.2.2 Preparation of F_1, F_1F_0, Subunits and Membranes

E. coli strains ML308-225 (*unc*$^+$) (Simoni and Shallenberger, 1972) and AN1460 (*unc*$^+$ overproducer) (Downie *et al.*, 1980) were grown to stationary phase at 37°C with vigorous aeration in a salts medium (Tanaka *et al.*, 1967) which was supplemented with 1% glycerol, 0.05% tryptone and 0.025% yeast extract. Growth medium for strain AN1460 was supplemented with 0.2 g/l arginine, 0.1 g/l uracil, 5% tryptone and 2.5% yeast extract. *E. coli* strains AN180 (*unc*$^+$) (Butlin *et al.*, 1971) and AN887 (*unc*$^-$ due to Mu insertion) (Butlin *et al.*, 1971) were grown in unsupplemented L-broth. F_1 was prepared by published methods (Smith and Sternweis, 1977; Futai *et al.*, 1974) with the following modification. Prior to the extraction of F_1, the membranes were washed an additional time with 1 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 10% glycerol. The α, β, γ and ϵ subunits were prepared from F_1 purified from strain AN1460 and the δ subunit was prepared from F_1 purified from strain ML308-225 using published methods (Dunn and Futai, 1980; Dunn, 1982; Smith and Sternweis, 1977). F_1F_0 was purified using the method of Foster and Fillingame (1979) except that the material was not

purified on sucrose gradients.

Membranes were prepared from *E. coli* strains AN180 and AN887 by suspending frozen cells in 4 volumes of 50 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$ and breaking them by two passes through a French Press at 10,000 p.s.i. The suspension was cleared of unbroken cells and cell wall debris by centrifugation at 10,000 rpm in a Beckman JA-20 rotor for 10 min. The supernatant was then centrifuged 2 h at 45,000 rpm in a Beckman Ti 50 rotor, the supernatant discarded and the membrane pellet resuspended in a minimum volume of 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$. Note that all procedures were performed at 4°C.

2.2.3 Enzyme-linked Immunosorbent Assays (ELISA)

A method similar to that of Engvall (1980) was used. Wells of flat bottom polyvinylchloride microtiter plates received 0.1-ml aliquots of protein dissolved in buffer A (100 mM sodium carbonate, pH 9.7, 0.2% sodium azide). The plates were covered and incubated at 37°C for 3 h. The protein solutions were removed and the plates were washed three times with buffer B (0.9% NaCl, 10 mM potassium phosphate, pH 7.4, 0.05% Tween 20, 0.02% sodium azide). The wells were then blocked by overnight incubation at 4°C with 0.2 ml of buffer C (buffer B with 5 mg/ml BSA). The next day, the plates were washed with buffer B and 0.1-ml aliquots of antibody solution (dilutions were made in buffer C) were added to the wells. The plates were incubated 2 h at room temperature, then the antibody solutions were removed and the plates washed with buffer B. The wells then received 0.1-ml aliquots of second antibody diluted into buffer C and were incubated 2 h at room

temperature. Second antibody, often antibody raised against the first, was labelled either by iodination or conjugation to bovine intestinal alkaline phosphatase and applied in 0.1-ml aliquots of a suitable dilution in buffer C to the wells and allowed to incubate at room temperature for 2 h. The second antibody was removed by aspiration and the plates were washed with buffer B. If iodinated second antibody was used, the wells were cut out and counted in a γ-counter. If alkaline phosphatase-conjugated antibody was used, the plates were developed by the addition of 0.1-ml aliquots of 1 mg/ml p-nitrophenylphosphate in 50 mM sodium carbonate, pH 9.6, 1 mM $MgCl_2$ at room temperature. The plates were read with a Dynatech MR 600 microplate reader (purchased through Fisher, Toronto, Ont.) at a wavelength of 410 nm for sample determination and a reference wavelength of 740 nm.

2.2.4 <u>Preparation, Purification and Labelling of Antibodies</u>

Antibody production was monitored through the use of ELISA using purified F_1 and the hybridomas were maintained in culture. Antibodies were obtained by harvesting the culture supernatants of the hybridomas when the buffering capacity of the medium was nearing exhaustion. They were stored at 4°C either aseptically or with the addition of 0.1% sodium azide. Due to problems associated with the use of rat/mouse hybrids, most of the antibodies were not maintained by passage *in vivo*.

Antibody α-1 was purified on a 1.5 x 3 cm column of ATPase-Sepharose (prepared as described in Dunn *et al.*, 1985). The α-1 hybridoma culture supernatant was adjusted to pH 7.5 and loaded onto

the column at a flow rate of 0.5 ml/min. The column was then washed with 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl until the absorbance at 280 nm of the flow through fractions was zero. This was repeated using 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl. The antibody was eluted with 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl and the eluate dropped into tubes containing an equal volume of 1 M Tris-HCl, pH 8.0. Fractions containing antibodies were identified by their absorbance at 280 nm and these were pooled, dialyzed into phosphate buffered saline, pH 7.4, filter sterilized, and stored aseptically at 4°C. The purification was monitored by ELISA. Other antibodies were purified using similar methods as described elsewhere (Dunn *et al.*, 1985).

Polyclonal antisera were prepared by injecting 0.1 to 0.5 mg of purified protein in complete Freund's adjuvant into New Zealand white rabbits. Antibody production was monitored through ELISA and the animal was immunised with additional antigen as required. Anti-rat IgG was prepared by injecting rabbits with purified rat IgG and purifying the antisera on a column of rat IgG-Sepharose (Dunn *et al.*, 1985).

Bovine intestinal alkaline phosphatase was coupled to antibodies using glutaraldehyde as described by Voller *et al.* (1976).
Antibodies were iodinated using the IODO-GEN (Pierce) method (Fraker and Speck, 1978) and the iodinated proteins were separated from free iodine using column centrifugation (Penefsky, 1979) on 1-ml columns of Bio-Gel P-10 equilibrated with phosphate buffered saline, pH 7.4. Anti-rat IgG-Sepharose was prepared using published methods (March *et al.*, 1974).

2.2.5 Immunoprecipitations

Twenty-µl aliquots of anti-rat IgG-Sepharose were incubated with 1-ml samples of hybridoma culture media in Eppendorf centrifuge tubes for 1 h at room temperature with mixing. The samples were centrifuged, the supernatant was removed by aspiration and the Sepharose was washed 3 times by repeated centrifugation with 1-ml portions of buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl. The anti-rat IgG-Sepharose with bound rat antibodies was then incubated with 60 µg of purified ATPase in 0.26 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM ATP, 5 mg/ml BSA and 10% glycerol. The samples were incubated for 1 h at room temperature with mixing and then the Sepharose was collected by centrifugation and washed as above. The bound rat IgG and ATPase were solubilized by the addition of 30 µl of SDS-sample buffer containing 10 mM NEM, boiling for 10 min followed by the addition of 20 µl of water and an additional 3 min of boiling. The Sepharose was centrifuged down and 50 µl of the supernatant was electrophoresed on a 10-20% gradient SDS-polyacrylamide slab gel which was stained with Coomassie Brilliant Blue R-250.

2.2.6 Membrane Binding Assays

Various amounts of iodinated monoclonal antibodies were incubated with 60 µg of membranes prepared from *E. coli* strains AN180 or AN887 in 0.25 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 2 mM ATP, 5 mg/ml BSA, 10% glycerol. They were incubated with gentle agitation for 1 h at 4°C and then centrifuged for

15 min in an Eppendorf centrifuge. The pellet was washed three times with 1 ml of buffer and the pellet and the tube were then counted in a γ-counter.

2.2.7 Antibody Binding Competition Assays

Buffers used were identical to those used in the ELISA's with concentrations of the ATPase subunit applied to the wells of microtiter plates ranging from 0.1 to 1 μg/ml. The plates were blocked by overnight incubation with BSA at 4°C. Solutions of purified monoclonal antibodies labelled by either iodination with ^{125}I or conjugation to alkaline phosphatase were applied to the wells with varying concentrations of culture supernatant and the plates were allowed to incubate overnight at room temperature. Concentrations of subunits applied to the wells and the specific activity of the labelled antibodies were chosen in order to saturate the binding sites and generate a signal sufficient for accurate determination. In some cases, particularly with the antibodies that recognized the smaller subunits, lower affinities of the antibodies necessitated the use of purified antibody rather than the dilute culture supernatant. The solutions were then removed by aspiration and the wells were washed as in ELISA's. The wells were cut out and counted in a γ-counter. Those experiments utilizing alkaline phosphatase-conjugated antibodies were developed as described for ELISA's using p-nitrophenylphosphate. All assays were performed in duplicate or triplicate.

2.2.8 Other Methods

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). SDS-sample buffer contained 125 mM Tris-HCl, pH 6.8, 2% SDS, 10% sucrose, 0.01% bromphenol blue and either 10 mM NEM or 50 mM DTT. Gels were stained in 0.05% Coomassie Brilliant Blue R-250, 45% methanol, 5% acetic acid and destained in 20% methanol, 5% acetic acid. Protein concentrations of F1 solutions were determined by the method of Bradford (1976) and those of membrane and antibody solutions were determined by the method of Lowry and coworkers (1951). BSA was used as the standard for both methods. Although the Bradford method of protein determination using this standard can underestimate the protein content with some proteins, results obtained for F_1 solutions using the Bradford and Lowry methods with this standard were identical to within 5%.

Distilled, deionized water was used in all experiments.

2.3 Results

2.3.1 Immunoprecipitations

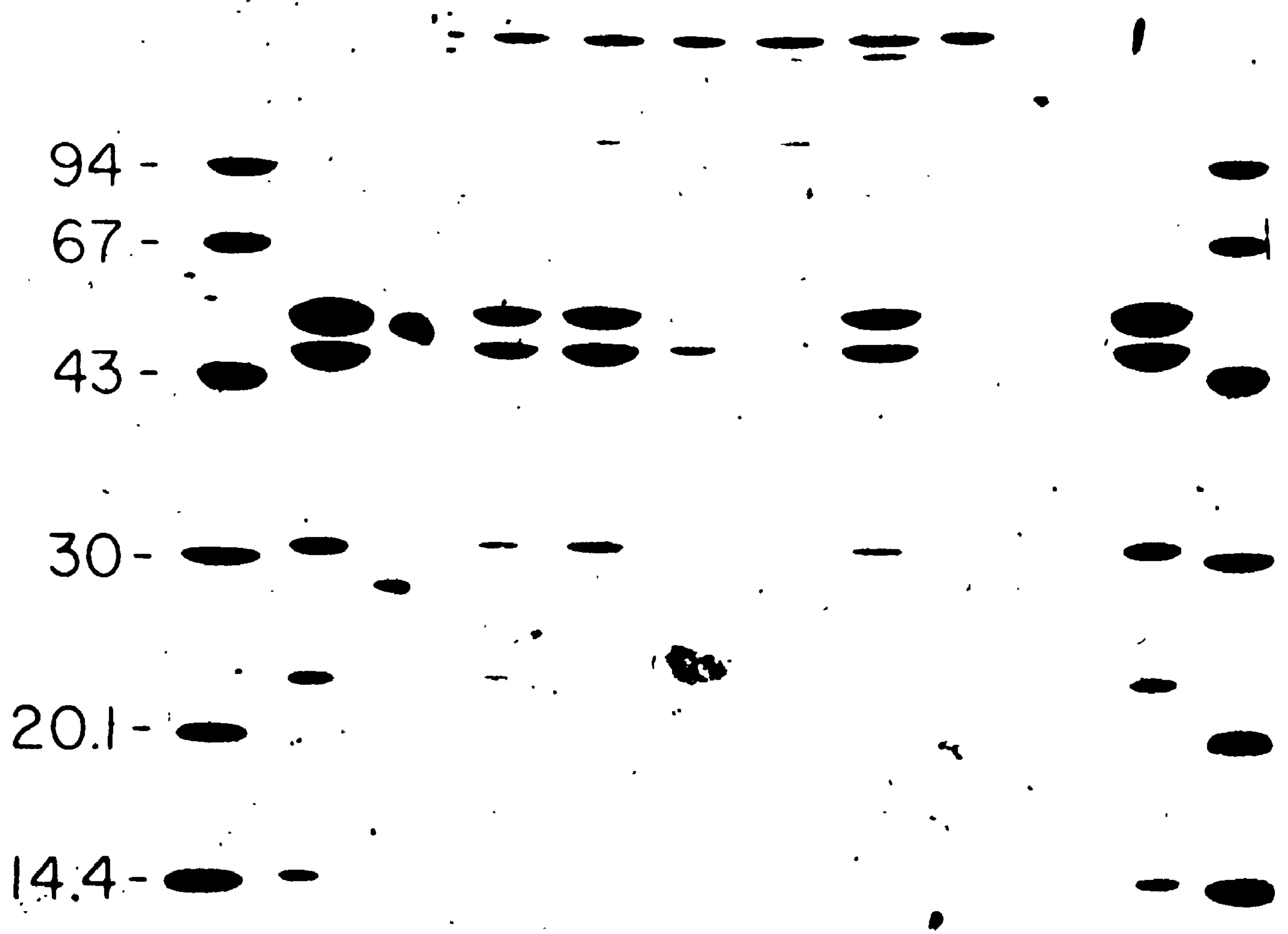
A large portion of the surface of each subunit of F_1 interacts with other subunits and consequently lies in the interior of the enzyme. As the antibodies were raised and screened using F_1 that would have been in a largely dissociated state, some antibodies would have been produced that recognize epitopes that are buried within intact F_1.

Such antibodies will not bind to the intact holoenzyme and thus should not be able to immunoprecipitate the entire complex. The converse will be true for those antibodies recognizing external epitopes. Sufficient quantities of F_1 were utilized in the immunoprecipitations so that after SDS-gel electrophoresis, the subunits of the enzyme could be visualized by staining the gel with Coomassie blue. DTT was omitted from the SDS-sample buffer to prevent the reduction of the antibody into its constituent light and heavy chains that would migrate to positions obscuring visualization of the α, β and γ subunits. To prevent disulfide exchange reactions, the SDS-sample buffer contained 10 mM NEM.

An experiment using a representative sample of antibodies is shown in Figure 2.1. Note that some DTT diffused from the F_1 standard (in lane 1) into the lane containing the α-1 experiment (lane 2) leading to the reduction of that antibody. The heavy chain of the antibody migrated with an apparent molecular weight of 53,000 and could be mistaken for the α or β subunit. This particular experiment was repeated (data not shown) and the α-1 antibody did not immunoprecipitate intact F_1 suggesting that the α-1 epitope lies buried within the holoenzyme. Antibody α-2 behaved the same way (data not shown). In contrast antibodies, α-3 (lane 3) and α-4 (data not shown) immunoprecipitated the intact complex suggesting that their epitopes are located on the exterior surface of F_1.

Antibodies β-1 (lane 4), β-4, β-5 and β-6 (data not shown) immunoprecipitated the intact complex whereas antibody β-2 (lane 5) and β-3 (data not shown) only immunoprecipitated free β subunit. The results obtained with the anti-γ antibodies were difficult to

Figure 2.1. Immunoprecipitation of F_1-ATPase by monoclonal antibodies and anti-rat IgG-Sepharose. Immunoprecipitations of F_1 by monoclonal antibodies and anti-rat IgG-Sepharose were performed using the method described in section 2.2.5. Fifty µL-samples containing SDS-solubilized/NEM alkylated protein were electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel which was stained with Coomassie Brilliant Blue R-250 as described in section 2.2.8. The lanes are as follows: lanes 1 and 10, *E. coli* F_1 standards; lane 2, α-1; lane 3, α-3; lane 4, β-1; lane 5, β-2; lane 6, γ-1; lane 7, δ-2; lane 8, ε-3; lane 9, control with no antibody.



94 -
67 -
43 -
30 -
20.1 -
14.4 -
$M_r \times 10^{-3}$
1 2 3 4 5 6 7 8 9 10

interpret. Antibody γ-1 (lane 6) immunoprecipitated only trace amounts of F_1 subunits and γ-2 (data not shown) immunoprecipitated smaller amounts. These low levels may have resulted from weak binding of the antibody to γ and subsequent dissociation during the experiment, or they may have resulted from a normally buried epitope on γ being exposed on partially dissociated F_1. Antibody γ-3 (data not shown) did not immunoprecipitate any F_1 subunits.

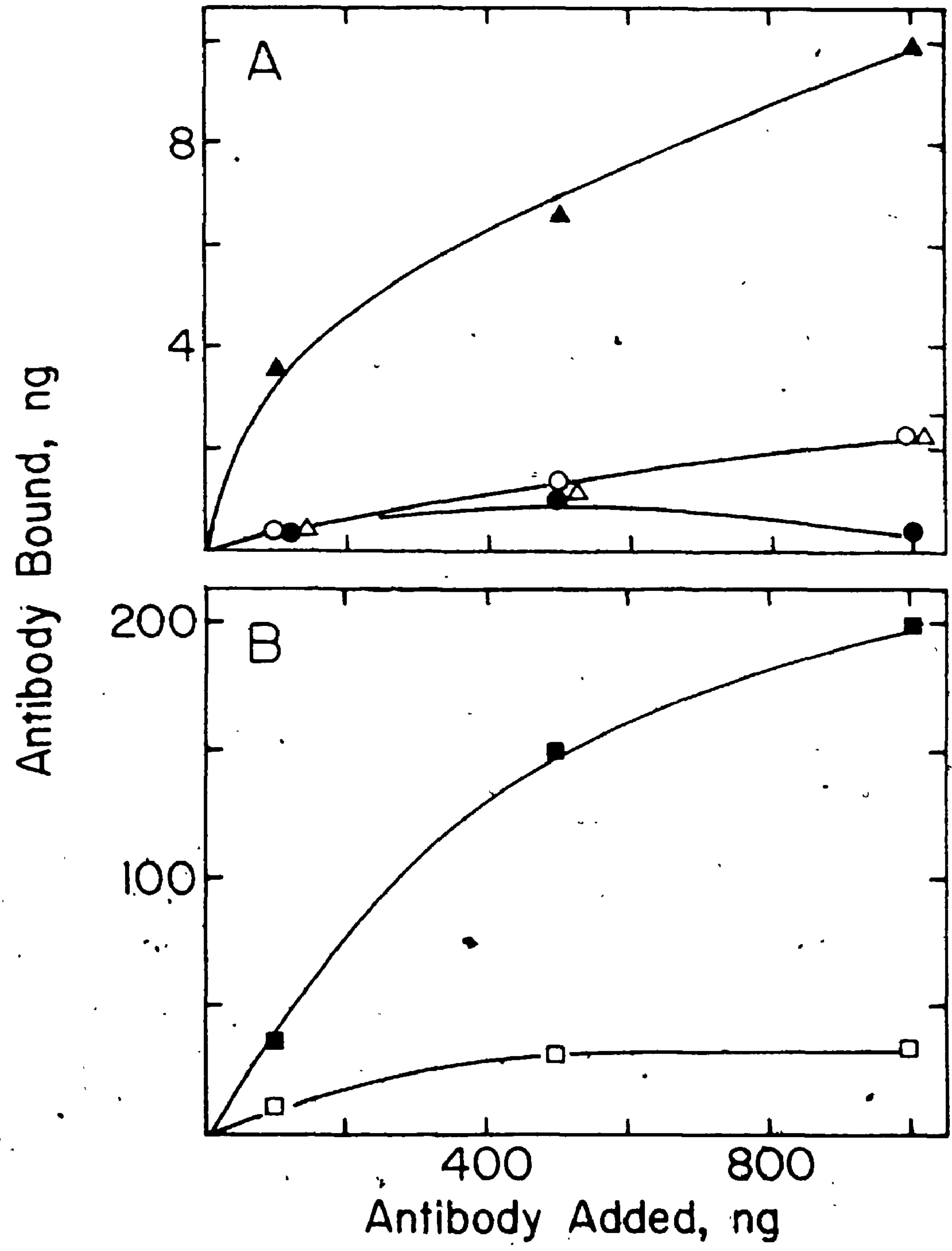
All the anti-δ antibodies, as demonstrated by antibody δ-2 (lane 7), immunoprecipitated F_1. The IgG antibodies (δ-2 and δ-3) immunoprecipitated significantly more F_1 than the IgM antibodies (δ-1 and δ-4) (data not shown). This may have resulted from the lower affinity of the anti-rat IgG-Sepharose for the IgM molecules. Antibody ε-3 (lane 8) and ε-1 (data not shown) only immunoprecipitated a trace amount of free ε subunit. Antibody ε-4 (data not shown) immunoprecipitated low amounts of F_1. Antibody ε-2 did not immunoprecipitate any subunits (data not shown).

It was important to determine whether the antibody epitopes were exposed in F_0-associated F_1. With the inhibitory or stimulatory anti-α and anti-β antibodies, this was easily determined by observing whether they affected the activity of membrane-bound F_1 (Dunn *et al.*, 1985 and summarized in section 2.4). However, for antibodies with no activity effects, this method was not applicable. Initially, attempts were made to determine which antibodies could immunoprecipitate F_1F_0 from a partially purified F_1F_0 preparation. Unfortunately these experiments were hampered by the instability of the F_1F_0 complex after purification and consequently the results were inconclusive. Thus another method was attempted.

2.3.2 Binding of Antibodies to Membrane-Bound F_1

The binding of ^{125}I-labelled antibodies to membrane-bound F_1 was measured to determine which antibodies' epitopes were exposed in membrane-bound F_1. Antibodies were incubated with inverted membrane vesicles prepared from either strain AN180, which is wild type with respect to F_1, or strain AN887, which produces very low amounts of F_1 due to a Mu phage insertion (Downie *et al.*, 1980). Binding of antibodies to strain AN887 vesicles was used as a measure of non-specific binding. Recovery of ATPase activity from AN180 membrane vesicles was 66%. In Figure 2.2A the results of an experiment testing two anti-ϵ antibodies, ϵ-1 and ϵ-4, are shown. It was observed that ϵ-4 bound only to AN120 membranes whereas ϵ-1 bound to neither. Figure 2.2B shows the results of a similar experiment using antibody δ-2. It shows that δ-2, like ϵ-4, bound only to AN120 membrane vesicles. Comparison of the amount of binding seen with antibody ϵ-4 seen with that of δ-2 indicates that ϵ-4 is only binding to a small amount of total membrane-bound F_1 present. This may be due to a number of factors such as lower affinity of ϵ-4 compared to δ-2 for their respective epitopes or blockage of the ϵ-4 epitope in some membrane-bound F_1. It is also possible that upon the binding of F_1 to membranes, a conformation change occurs in the ϵ-4 epitope decreasing the affinity of the antibody for it. Low but significant levels of binding to AN887 membranes by any of these antibodies was due to low-level production of F_1 which was detected and quantitated using Western blots (data not shown). This analysis showed that the AN887 membranes

Figure 2.2. Binding of ^{125}I-monoclonal antibodies to membranes from *E. coli* strains AN180 and AN887. Binding of ^{125}I-antibodies to membranes prepared from strains AN180 (unc^{+}) and AN887 (unc^{-}) was measured using the method described in section 2.2.6. Membrane pellets and the tubes were counted in a γ-counter. Recovery of ATPase activity in the pellet was 66%. The symbols in the panels are as follows: panel A; ●, ε-1 with AN180; ○, ε-1 with AN887; ▲, ε-4 with AN180; △, ε-4 with AN887; panel B; ■,δ-2 with AN180; □,δ-2 with AN887.


A
8
4
B
200
100
400
800
Antibody Bound, ng
Antibody Added, ng

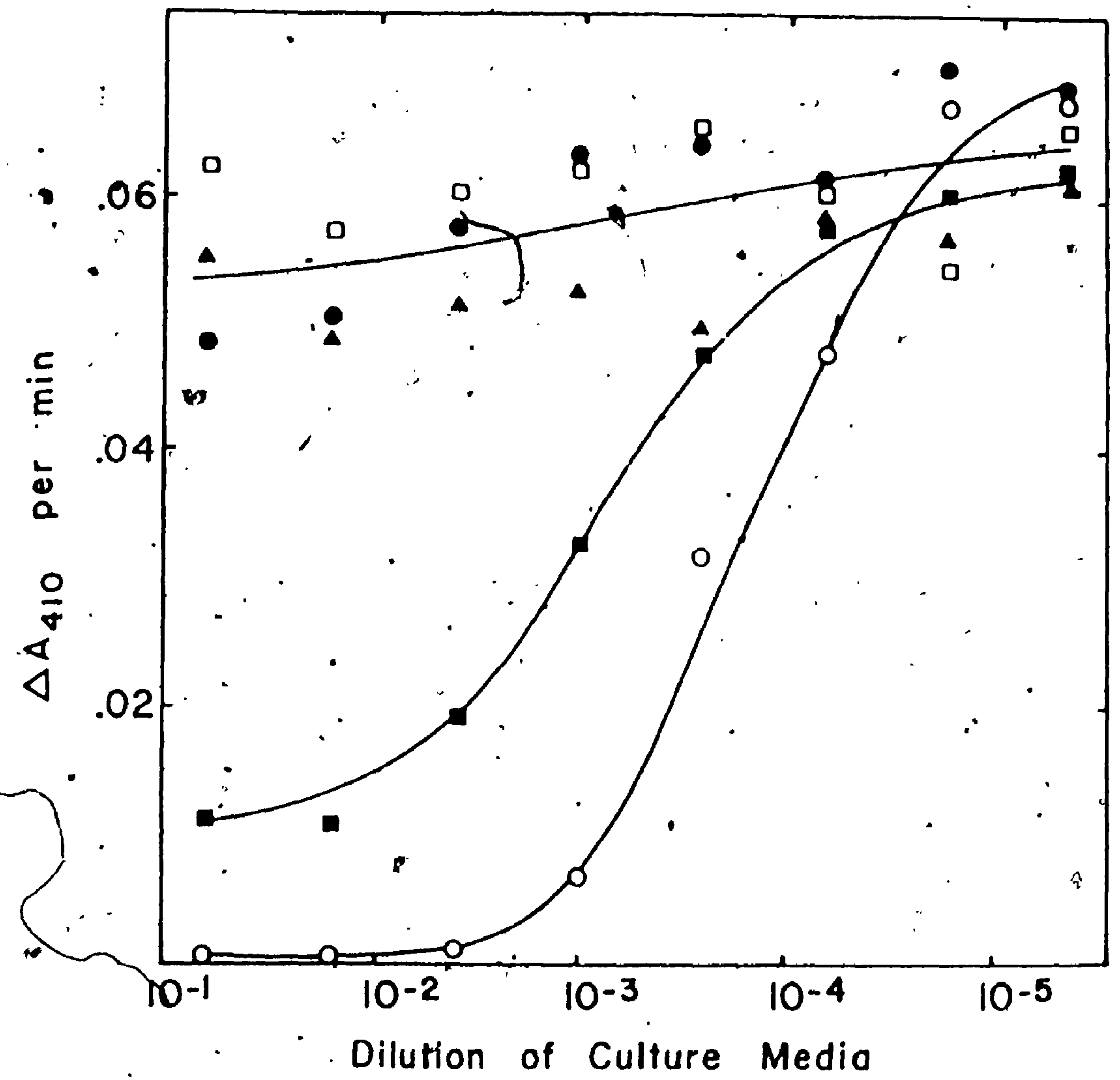
used in these experiments contained 12% as much F_1 as those from strain AN180. Similar experiments were performed using the γ-1, γ-2 and γ-3 antibodies and none of these bound to either AN887 or AN120 membranes.

2.3.3 Antibody Competition Studies

Experiments were performed to determine which groups of antibodies have epitopes spaced closely enough together to prevent simultaneous binding. The ability of purified antibody or hybridoma culture supernatant to inhibit the binding of labelled antibody to the appropriate purified subunit was therefore tested. Individual experiments are shown in Figures 2.3 to 2.7.

Figure 2.3 shows a typical experiment in which hybridoma culture supernatants of the anti-α antibodies and the anti-β antibody β-6 (as a control) were tested to see if they could inhibit the binding of alkaline phosphatase-conjugated α-3 antibody to purified α subunit. Antibody α-3 competed well against itself, and higher concentrations of antibody α-4 also competed with α-3 binding. Differences between antibodies' ability to compete with each other probably reflect a combination of varying antibody concentrations among the hybridoma supernatants and the different affinities of each antibody for its epitope. The remaining antibodies did not compete for binding. A similar experiment was performed using labelled α-1 (data not shown). Antibody α-1 and high concentrations of α-2 were able to completely abolish labelled α-1 binding. High concentrations of α-3 and α-4 prevented a maximum of 30% of the binding of α-1. On the basis of

Figure 2.3. Monoclonal antibody competition for α-3-binding site. A solid phase binding competition study was performed as described in section 2.2.7. Binding plates were prepared using pure α subunit at a concentration of 0.5 μg/ml. Each well received 0.3 μg of alkaline phosphatase-conjugated α-3 in various dilutions of hybridoma culture media. After overnight incubation, the plates were washed and the binding of labeled α-3 was determined by addition of p-nitrophenyl phosphate. The symbols are as follows: ●, α-1; □, α-2; ○, α-3; ■, α-4; ▲, β-6.



.06
.04
.02
ΔA410 per min
10-1
10-2
10-3
10-4
10-5
Dilution of Culture Media

these results, α-1 and α-2 were placed in the A-I competition subgroup and α-3 and α-4 were placed in the A-II competition subgroup. The small inhibition in binding observed between the two competition subgroups may reflect spatial proximity between the epitopes of the two groups.

The anti-β antibodies were placed into two competition subgroups as well. The first competition subgroup, B-I, contained antibodies β-1, β-4, β-5 and β-6. An experiment demonstrating competition against the binding of iodinated β-1 is shown in Figure 2.4. Note that β-6 was the strongest competitor in this group whereas β-5 was the weakest. Another experiment demonstrated that β-2 could compete for binding with itself and β-3, although β-3 was ineffectual in competing against β-2. Consequently β-2 and β-3 were placed in the same competition subgroup, B-II.

None of the anti-γ antibodies was able to inhibit one another's binding to the γ subunit (Figure 2.5) and thus each antibody was placed into a separate competition subgroup. Antibody γ-1 was placed into competition subgroup C-I, γ-2 into C-II and γ-3 into C-III. All the anti-δ antibodies inhibited the binding of iodinated δ-3 (Figure 2.6) and were thus placed into a single competition subgroup, D-I.

The anti-ε antibodies were placed into two competition subgroups: E-I contained ε-1 and E-II contained ε-2, ε-3 and ε-4. An experiment using iodinated ε-3 is shown in Figure 2.7. Antibody ε-4 competed more effectively with ε-3 than ε-3 did with itself probably reflecting differences in affinity for ε subunit as purified antibodies rather than hybridoma culture supernatants were used in this experiment. Antibody ε-2 did not compete against ε-3 in this experiment, but the

Figure 2.4. Monoclonal antibody competition for β-1-binding site. A solid phase binding competition study was performed as described in section 2.2.7. Binding plates were prepared using pure β subunit at a concentration of 0.1 μg/ml. Each well received 0.1 μg/ml ^{125}I-β-1 in various dilutions of hybridoma culture media. The plates were incubated overnight and then the wells were excised and counted in a γ-counter. The symbols are as follows: ▲, β-1; ●, β-2; ■, β-3; ○, β-4; □, β-5; △, β-6; ▽, γ-1.

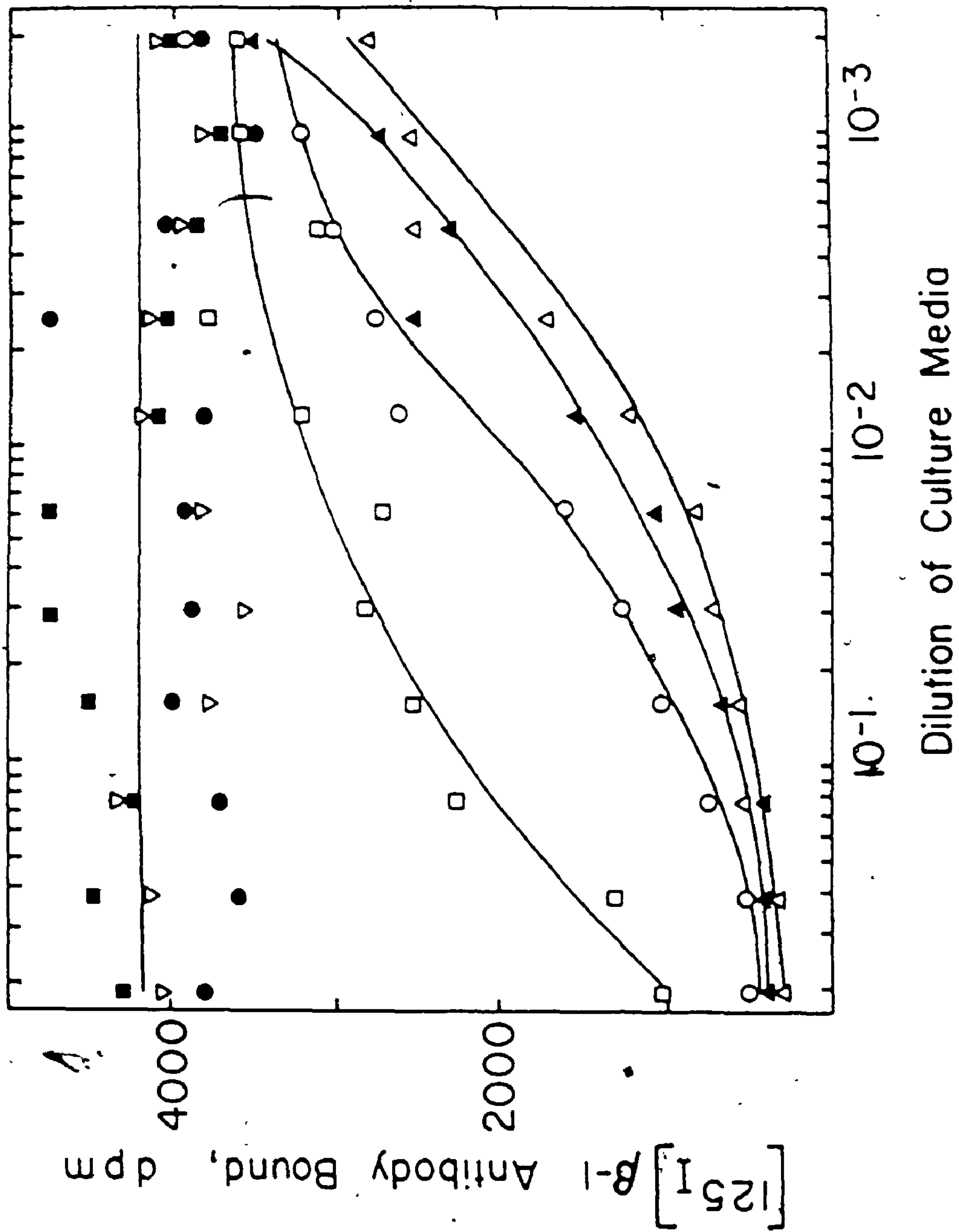

[125I] β-1 Antibody Bound, dpm
4000
2000
10-1
10-2
10-3
Dilution of Culture Media

Figure 2.5. Binding competition studies with anti-γ monoclonal antibodies. Solid phase binding competition studies were performed as described in section 2.2.7. Binding plates were prepared with 0.5 μg/ml of γ subunit. Each well received 2 μg/ml of labeled antibody and various concentrations of purified unlabeled antibodies. The plates were incubated overnight, then the wells were excised and counted in a γ-counter. Experiments in each panel received the following iodinated antibodies: panel A, ^{125}I-γ-1; panel B, ^{125}I-γ-2; panel C, ^{125}I-γ-3. The symbols are as follows: ●, γ-1; ▲, γ-2; ■, γ-3; O, β-1; Δ, β-2.

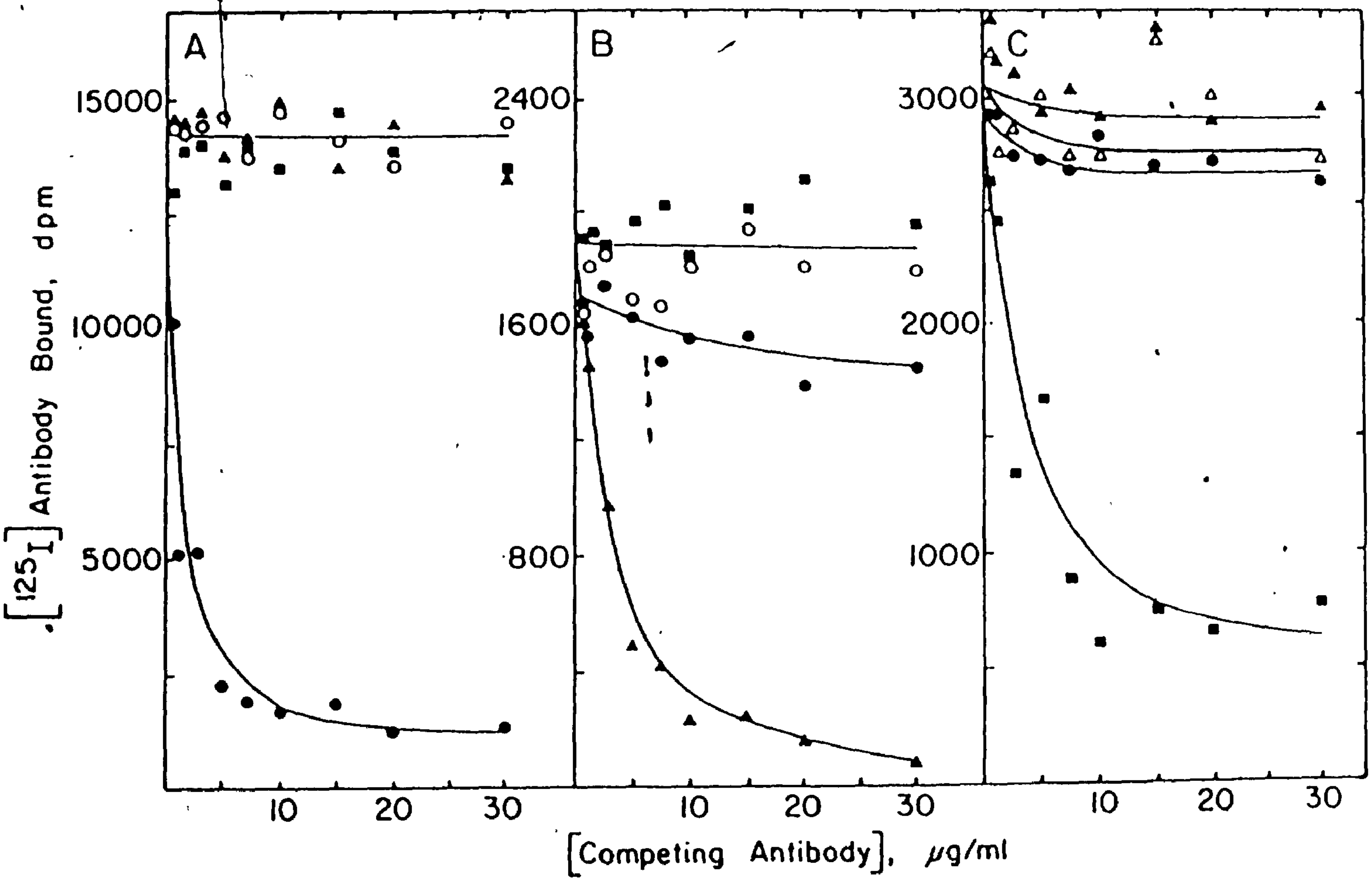
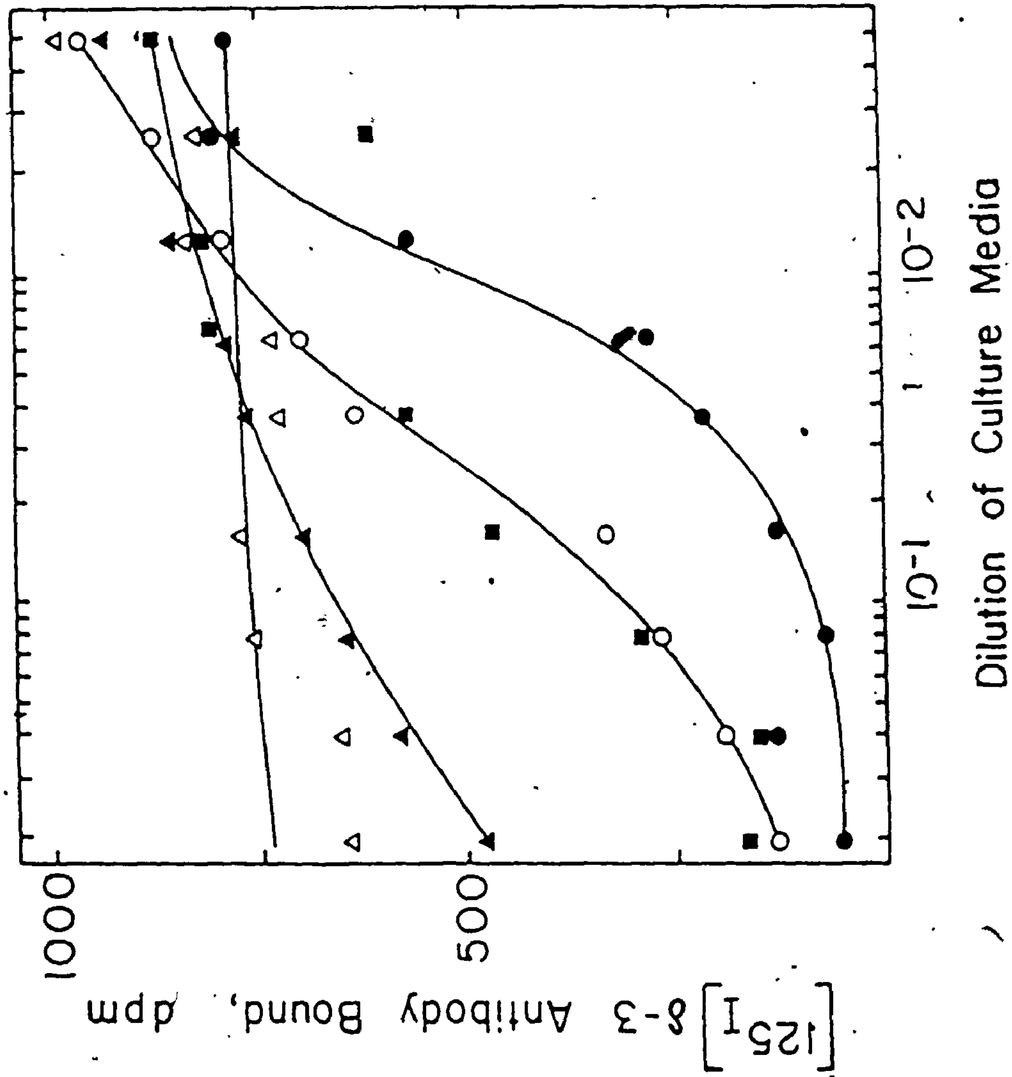
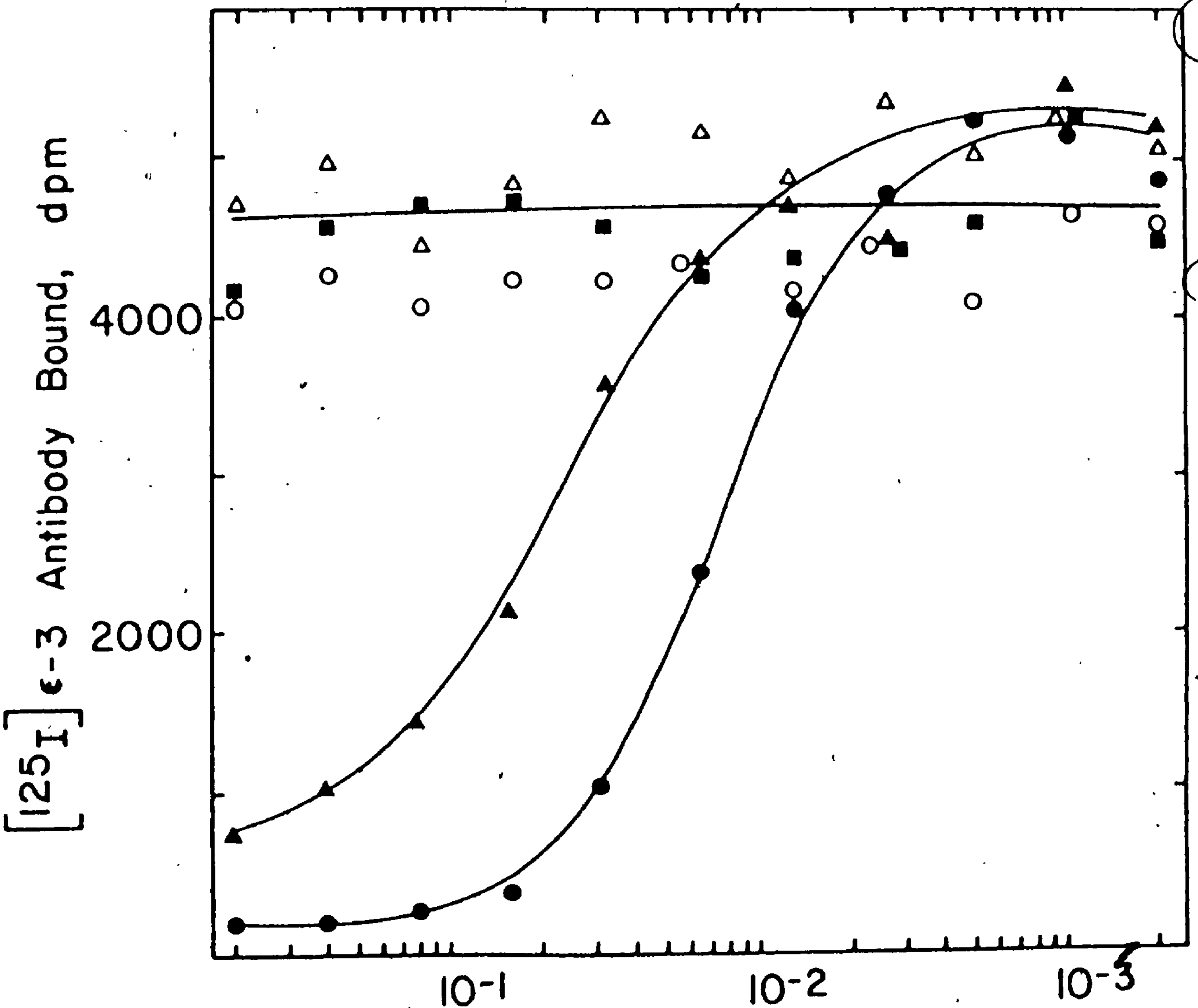

A
B
C
[125I] Antibody Bound, dpm
15000
10000
5000
2400
1600
800
3000
2000
1000
10
20
30
[Competing Antibody], μg/ml

Figure 2.6. Monoclonal antibody competition for δ-3-binding site. A solid phase binding competition study was performed as described in section 2.2.7. Binding plates were prepared using pure δ subunit at a concentration of 1.0 μg/ml. Each well received 2 μg/ml of ^{125}I-δ-3 in various dilutions of hybridoma culture supernatant. The plates were incubated overnight, then the wells were excised and counted in a γ-counter. The symbols are as follows: ●, δ-1; ■, δ-2; ▲, δ-3; ○, δ-4; △, α-3.



[125I] δ-3 Antibody Bound, dpm
1000
500
Dilution of Culture Media
10-1
1
10-2

Figure 2.7. Monoclonal antibody competition for ε-3-binding site. A solid phase binding competition study was performed as described in section 2.2.7. Binding plates were prepared using 1 μg/ml of ε subunit. Each well received 2 μg/ml of ^{125}I-ε-3 in various dilutions of hybridoma culture media. The plates were incubated overnight and the wells were excised and counted in a γ-counter. The symbols are as follows: ○, ε-1; ■, ε-2; ▲, ε-3; ●, ε-4; △, α-4.



[125I] ε-3 Antibody Bound, dpm
4000
2000
10-1
10-2
10-3
Dilution. of Culture Media

binding of iodinated ε-2 was inhibited strongly by ε-4 and less strongly by ε-3 (data not shown). Thus ε-2 may have the weakest affinity for ε in the E-II competition subgroup.

2.4 Discussion

In order to make fullest use of the monoclonal antibodies, extensive characterization of their properties was required. The ability of antibodies to bind to soluble and membrane-bound F_1 and the proximity of antibody epitopes relative to one another were determined. Other properties characterized by workers in the laboratory included the ability of antibodies to recognize ATPase subunits derived from other sources and the effects of certain antibodies upon F_1-hydrolytic activity (Dunn *et al.*, 1985). These results are summarized in Table II.

The unifying observation was that antibodies whose epitopes are located near one another (*i.e.* members of the same competition subgroup) tend to share similar properties. This does not imply that these antibodies are identical. Differences were observed among members of the same competition subgroup in regards to ease of elution from F_1-affinity columns, shape of inhibition curves and direction of activity effects. Thus antibodies in the same competition subgroup may recognize overlapping epitopes or may recognize and stabilize different conformations of the same epitope.

Competition subgroup A-I contains the anti-α antibodies α-1 and α-2. Both these antibodies recognize epitopes not exposed on the surface of intact F_1 and have no effect upon the enzyme's activity. Antibody

TABLE II

Summary of Antibodies

Competition subgroup	Clone	Able to precipitate ATPase	ATPase activity effects	Cross-reaction
A-I	α-1	No	ND[a]	Azotobacter
A-I	α-2	No	ND	ND
A-II	α-3	Yes	Partial (-)	ND
A-II	α-4	Yes	Stimulation	ND
B-I	β-1	Yes	Strong (-)	ND
B-I	β-4	Yes	Strong (-)	ND
B-I	β-5	Yes	Strong (-)	ND
B-I	β-6	Yes	Strong (-)	ND
B-II	β-2	No	ND	Widespread
B-II	β-3	No	ND	Widespread
C-I	γ-1	No	ND	Azotobacter
C-II	γ-2	No	ND	Mitochondrial, chloroplast F_1
C-III	γ-3	No	ND	ND
D-I	δ-1	Yes	ND	ND
D-I	δ-2	Yes	ND	ND
D-I	δ-3	Yes	ND	ND
D-I	δ-4	Yes	ND	ND
E-I	ε-1	No	ND	ND
E-II	ε-2	No	ND	ND
E-II	ε-3	No	ND	ND
E-II	ε-4	Yes	ND	ND

[a]ND, not detected.

Antibodies were placed in binding competition subgroups based on the results of solid-phase competiton studies such as those shown in Figures 2.3 through 2.7. Competition subgroups are designated by an upper case letter which corresponds to the Greek letter name of the subunit recognized, and a Roman numeral. Ability to precipitate ATPase was determined by immunoprecipitations such as the experiment shown in Figure 2.1. ATPase activity effects and cross-reactivity are described in section 2.4.

α-1 recognizes a 53,000-dalton protein found in whole cell SDS-extracts of *Azotobacter macrocytogenes* using Western blots. Weak cross-reactivity is also observed for proteins found in *Bacillus subtilis*, *Lactobacillus casei* and *Micrococcus luteus*. The other competition subgroup, A-II, contains antibodies α-3 and α-4, which recognize epitopes on the external surface of F_1. Antibody α-3 partially inhibits the activity of soluble F_1 by 30% and has no effect upon membrane-bound F_1, whereas α-4 stimulates the activity of soluble F_1 by 40% and that of membrane-bound F_1 by 10%. The stimulatory effect of α-4 may be due to partial relief of ADP inhibition (Dunn and Zadorozny, unpublished observations). The contrast in activity effects emphasizes the fact that members of each competiton subgroup are not identical. Neither of these antibodies cross-react with ATPase subunits from other sources.

The six anti-β antibodies belong to two different competition subgroups, B-I and B-II. The four B-I antibodies, β-1, β-4, β-5 and β-6 all recognize epitopes on the surface of F_1. They are all strong inhibitors of ATPase activity, reducing the activity of soluble F_1 between 85 to 90% and the activity of membrane-bound F_1 between 40 to 60%. Preliminary studies of their inhibitory effects seem to show that some antibodies inhibit with positive cooperativity while others inhibit with negative cooperativity (Dunn and Zadorozny, unpublished observations). The B-II antibodies, β-2 and β-3 recognize an epitope buried within the assembled holoenzyme and have no effect upon activity. These antibodies recognize ATPase subunits from spinach chloroplast and rat liver mitochondria using purified F_1 and ELISA. They also recognize proteins on Western blots of the whole cell SDS-

extracts described previously for the anti-α antibodies as well as extracts from *Norcardia rhodochrous* and *Streptococcus faecalis*.

Similarities appear in the properties of the A-I and B-II antibodies and the A-II and B-I antibodies. Both the A-I and B-II antibodies recognize internal epitopes that are conserved in F_1-ATPases from various sources. Perhaps these epitopes are located in regions important for the structural stability of the holoenzyme. Mutant *E. coli* strains exist that produce unstable F_1 complexes (Kanazawa *et al.*, 1980). As described in section 1.2, it is apparent that subunit interactions are essential for normal catalytic activity. Therefore subunit interfaces must play an important role in the catalytic cycle and thus it is not surprising that certain structural features of these regions should be conserved through evolution. The A-II and B-I antibodies recognize external epitopes and have effects upon catalytic activity. Their epitopes do not appear to be conserved in F_1-ATPases from other sources and thus it is not likely that they recognize a structure such as the catalytic site or other nucleotide-binding sites that have been tentatively identified using sequence homologies between *E. coli* F_1 and other nucleotide-binding proteins (Walker *et al.*, 1982). As conformational changes are postulated to occur during catalysis, it is possible that these antibodies could restrict necessary conformational freedom by binding anywhere on the surface of the enzyme.

The three anti-γ antibodies recognize distinct epitopes and are thus placed into three competition subgroups. Unfortunately the immunoprecipitation results were inconclusive as to whether any of

these antibodies recognize exposed epitopes on soluble F_1. More sensitive immunoprecipitations using a method described elsewhere (Dunn and Tozer, 1986) also failed to immunoprecipitate F_1 using the anti-γ monoclonal antibodies (Dunn and Zadorozny, unpublished data). These studies did indicate however that γ-1 immunoprecipitates ε-depleted F_1, suggesting that the γ-1 epitope might be located at the γ-ε interface. None of the anti-γ antibodies were able to bind to membrane-bound F_1, demonstrating that the epitopes are not exposed on F_1F_0 either. Polyclonal anti-γ antisera are able to inhibit *E. coli* F_1 in both the soluble and membrane-bound forms of the enzyme indicating that some surfaces of the γ subunit must be exposed to the medium (Smith and Sternweis, 1982). Antibody γ-1 recognizes a protein from *A. macrocytogenes* with an apparent molecular weight of 36,000 and antibody γ-2 recognizes the γ subunit from mitochondrial and chloroplast F_1.

The anti-δ antibodies all belong to a single competition subgroup, D-I and all recognize an epitope that is exposed on the surface of F_1 whether it is bound to the membrane or not. The δ subunit is postulated to serve in linking F_1 to F_0 (Sternweis, 1978), and consequently a great deal of its surface area should be involved in subunit/subunit interactions (see Figure 1.1). It is interesting however, that there is a sufficiently large region of the δ subunit exposed to allow the binding of an antibody molecule.

The anti-ε antibodies have been placed into two competition subgroups. Subgroup E-I contains ε-1 and subgroup E-II contains ε-3, ε-2 and ε-4. As with the anti-γ antibodies, the immunoprecipitation results were inconclusive. Modified immunoprecipitations showed that

ϵ-4 could immunoprecipitate the holoenzyme wherease ϵ-1 could only immunoprecipitate free ϵ (Dunn and Tozer 1987). Antibody ϵ-4 is also able to bind to membrane-bound F_1 and partially inhibit the activity of the complex (Dunn and Tozer, 1987). The ϵ subunit, like the δ subunit is postulated to have a role in binding F_1 to F_0 (Sternweis, 1978). Since ϵ is even smaller than δ and it is proposed to have a spherical shape rather than δ's elongated one (Sternweis and Smith, 1980; Sternweis and Smith, 1977), the finding that a large area of this subunit is exposed in membrane-bound F_1 is even more surprising. The anti-ϵ antibodies, ϵ-1 and ϵ-4, are also able to stimulate ATPase activity. These properties will be described in chapter 4.

Neither the anti-δ nor the anti-ϵ antibodies cross-react with ATPase from rat liver mitochondria or spinach chloroplast as determined using purified F_1 and ELISA. More conclusive results might be obtained using whole cell SDS-extracts and Western blots.

This extensive characterization of the antibodies has allowed their use in a variety of applications. The antibodies of the B-I competition subgroup are being used as tools in the study of the enzyme's catalytic mechanism. Immobilized antibody ϵ-4 has been used for the preparation of ϵ-depleted F_1 for studies of the mechanism of the inhibitory effects of that particular subunit (Dunn, 1986a; Dunn *et al.*, 1987). Colloidal gold-labelled ϵ subunit, anti-δ monoclonal antibodies and immunoelectronmicroscopy are being used as a potential means to map the location of the δ and ϵ subunits within F_1 quaternary structure. The antibodies have also been used in the development of an improved Western blot protocol (Dunn, 1986b). In subsequent chapters studies will be described in which the antibodies

served as tools for identification of subunit products, for determination of holoenzyme integrity after particular treatments, and as agents for perturbation of the enzyme.

3.0 MAPPING THE LOCATION OF THE ANTIBODY EPITOPES WITHIN THE PRIMARY STRUCTURES OF THE α AND β SUBUNITS

3.1 Introduction

The characterization of a bank of 21 monoclonal antibodies that recognize the subunits of the *E. coli* F_1-ATPase was described in the previous chapter. Two competition subgroups, A-II and B-I contain antibodies that recognize the α and β subunits respectively and affect the activity of the holoenzyme. Two other competition subgroups, A-I and B-II contain antibodies that recognize epitopes on the α and β subunits that are buried within the holoenzyme and have been conserved in ATPases from various sources. Knowledge of the location of the epitopes of the anti-α and anti-β antibodies within each subunit's primary structure may provide useful insights into the properties of the antibodies and their effects upon the enzyme.

Understanding the mode of action of the A-II and B-I antibodies may provide information about the catalytic mechanism of the enzyme. Kinetic studies are under way by other workers in the laboratory to define which step or steps in the catalytic cycle the antibodies perturb. Conformational changes in F_1 are thought to be crucial for catalysis (Cross, 1981). It is possible that these might be hindered by the binding of antibodies anywhere on the surface of the α and β subunits and thus the antibody effects would be somewhat non-specific in nature. A more interesting possibility is that the antibody effects result from specific interactions with regions of the subunits that are important for catalytic activity. The nucleotide-binding sites on the

α and β subunits have been tentatively identified and various amino acid residues have been shown to be important for catalytic activity through the use of mutants and chemical modification studies (Duncan *et al.*, 1986). Thus the possibility that the antibodies are binding at or near catalytic or regulatory sites can be determined by orienting the epitopes relative to the sites already determined. Such information in tandem with the kinetic studies might allow further definition of subunit regions important for catalytic activity.

Determination of the actual quaternary structure of F_1 is an ongoing goal of a number of workers. To this end studies have made use of cross-linking reagents (Bragg and Hou, 1982), florescent energy transfer measurements (Richter *et al.*, 1985), electron microscopy (Akey *et al.*, 1983), and most notably X-ray diffraction (Pedersen, 1982). The results, often inconclusive and sometimes conflicting, have led to the formulation of several models. None of these is entirely satisfactory. X-ray crystallography appears to be the most promising approach to ultimately define the structure of F_1. Studies of F_1 from rat liver mitochondria at 0.9 nm resolution show 6 large masses arranged in a hexagonal manner around a central space with a two fold axis of symmetry. The monoclonal antibodies may provide useful information for these studies. Knowledge of the location of the epitopes within subunit primary structure, whether the epitopes are exposed or buried within the holoenzyme and the proximity of the epitopes to neighbouring subunits could be used to orient subunit primary structure within F_1 quaternary structure.

In this chapter, the efforts to map the location of the epitopes of the anti-α and anti-β monoclonal antibodies will be described. One

common method of epitope mapping makes use of the complete cleavage of the protein, separation of the fragments by a method such as reversed phase high performance liquid chromatography (HPLC) and then assay of the fragments' ability to bind to the antibodies. The composition of the antibody-binding fragments is then determined by either amino acid analysis or the use of a sequenator, and by comparing this result to the known sequence of the protein, the origin of the fragment can be identified (for an example see Altschuh and Regenmortel, 1982). Problems associated with this technique because of the structural nature of some epitopes will be illustrated and methods to partially overcome these problems described.

3.2 Materials and Methods

3.2.1 Materials

The sources of most chemicals used in this section have been described in section 2.2.1. Worthington TPCK-Trypsin was purchased from Technicon (Mississauga, Ont.). Goat anti-rabbit IgG, clostripain, *Staphylococcus aureus* V8 protease, pronase, 2-nitro-5-thiocyanobenzoic acid and N-bromosuccinimide were purchased from Sigma (St. Louis, Mo.). Nitrocellulose (0.2 micron) was prepared by MSI Inc. and purchased from Fisher (Toronto, Ont.). HPLC-grade acetonitrile and isopropanol were also purchased from Fisher.

3.2.2 Preparation of F_1, Subunits, Membranes and Antibodies

E. coli strain AN1460 (*unc*$^+$ overproducer) (Downie *et al.*, 1980) was grown as previously described (Dunn *et al.*, 1985). *E. coli* strain KF20 (*uncD*$^-$), an amber mutant which produces a truncated β subunit (Noumi *et al.*, 1986), was obtained from Dr. M. Futai (Department of Microbiology, Okayama University, Okayama, Japan). F_1 and the α, β and γ subunits were purified using published methods (Futai *et al.*, 1974; Smith and Sternweis, 1977; Dunn and Futai, 1980). The production and characterization of the subunit-specific antibodies have been described previously (Dunn *et al.*, 1985 and chapter 2).

3.2.3 Cleavage of the α and β Subunits

CNBr cleavage (for cleavage at methioninyl residues)--Purified subunit was precipitated by the addition of 4 volumes of acetone and incubation on ice for 1 h. The solution was then centrifuged for 20 min at 15,000 rpm in a Beckman JA-20 rotor at 4°C. The supernatant was removed by aspiration and the pellet was washed with cold 80% acetone and centrifuged as before. The pellet was dried under nitrogen and then dissolved in 70% formic acid so that the protein concentration was 1 mg/ml. CNBr, dissolved at 5%(w/v) in 70% formic acid, was added to the protein solution to a final concentration of 0.5%. The solution was incubated for various periods of time at 37°C. The cleavage was terminated by the addition of 10 volumes of distilled water and

lyophilization.

S. aureus V8 protease digestion (for cleavage at glutamyl residues)--Purified subunit was precipitated by acetone as described previously. The protein was then resolubilized at a concentration of 1 mg/ml in 0.1 M NH_4HCO_3, 8 M urea. After a 30 min incubation at room temperature, the urea was diluted to 2 M with 0.1 M NH_4HCO_3. The protease, dissolved in distilled water, was added for a 20 µg/ml final concentration to the protein. The solution was incubated 16 h at 37°C. The reaction was terminated by boiling for 5 min.

Trypsin digestion (for cleavage at arginyl and lysyl residues)--Purified subunit was precipitated by acetone as described previously. The protein was dissolved in 50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 8 M urea and incubated at room temperature for 30 min. The urea was diluted to 2 M by the addition of 50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ and the resulting protein concentration was 1 mg/ml. TPCK-treated trypsin dissolved at 1 mg/ml in 1 mM HCl was added for a final concentration of 10 µg/ml to the denatured subunit and the cleavage was allowed to proceed 16 h at 37°C. The reaction was terminated by the addition of phenylmethylsulfonylfluoride, dissolved in a minimum volume of 95% ethanol, to a final concentration of 1 mM. Both removal of the amino-terminal fifteen residues of the α subunit and cleavage of the α subunit in half were performed using published procedures (Dunn *et al.*, 1980; Senda *et al.*, 1983).

Clostripain digestion (for cleavage at arginyl residues)--Purified subunit was precipitated by acetone as described previously. The protein was then resuspended in 50 mM sodium phosphate, pH 7.5, 1 mM DTT, 8 M urea and allowed to incubate at room temperature for 30 min.

DTT resulting in a 1 mg/ml protein concentration. Clostripain dissolved at 1 mg/ml in 50 mM N-ethylmorpholine-HCl, pH 8.0, 1 mM DTT, 10% glycerol was added for a final concentration of 10 μg/ml to the protein solution. The solution was incubated at room temperature for various periods of time and the digestion was terminated by boiling for 5 min.

Weak Acid Hydrolysis (for cleavage at aspartyl-prolyl bonds)--Purified subunit was dissolved in 20% acetic acid and incubated at 60°C for the specified period of time. The reaction was terminated by freezing the solution followed by lyophilization. For "in gel" weak acid hydrolysis, a lane containing protein of interest was excised from an SDS-polyacrylamide gel and incubated in 20% acetic acid, 50% methanol for 32 h at 60°C with two changes of buffer during the first hour. The gel slice was then incubated in three changes of 1% acetic acid for 1 h at room temperature to remove excess methanol and was then frozen and lyophilized. Prior to electrophoresis, the gel slab was incubated for 20 min at 60°C with 3 changes of DTT-containing SDS-sample buffer to resolubilize the protein fragments. The gel was then placed on top of the stacking gel of a 10 to 20% SDS-polyacrylamide gradient gel. Electrophoresis was carried out in the second dimension and the samples were electroblotted onto nitrocellulose for subsequent immunological probing.

Other Cleavages--Cyanylations (for cleavage at cysteinyl residues) were performed using the method of Jacobson *et al.* (1973).
Cleavage with N-bromosuccinimide (for cleavage at tryptophanyl and tyrosyl residues) was performed by dissolving the protein to a 2 mg/ml concentration in 8 M urea/acetate, pH 4.0 and adding N-bromosuccinimide

at a 10 M excess over protein. The reaction mix was incubated at 37°C in the dark for various lengths of time and the reaction was terminated by freezing and lyophilization. Weak base hydrolysis (non-specific cleavage method) was performed by dissolving acetone-precipitated subunit to 1 mg/ml in 0.5 M NaOH, incubating the protein at 37°C for various lengths of time and terminating the reaction by neutralizing the solution with the addition of an equal volume of 0.5 M HCl. Pronase (non-specific protease) was added to a 4 µg/ml final concentration to 0.44 mg/ml of acetone-precipitated subunit dissolved in 100 mM Tris-HCl, ph 7.5, 5 mM $MgCl_2$, 10 % glycerol. It was incubated for various lengths of time at 37°C and then the digestion was terminated by the addition of SDS-sample buffer and subsequent boiling. Subtilisin (non-specific protease) digestion was performed as for *S. aureus* V8 digestion using 30 µg/ml protease.

3.2.4 Reversed Phase HPLC

Reversed phase HPLC was performed on a Waters (Mississauga, Ont.) system utilizing an M680 gradient controller, two M510 pumps, an M481 variable wavelength UV-vis detector and a Rheodyne injector. Peptides were detected by measuring the absorbance of the eluate at 210 nm. Separations were performed using Waters phenyl, C_{18} or C_8 µBondapak reversed phase columns. Several gradients were applied using acetonitrile or isopropanol as solvents and trifluoroacetic acid as the counter ion and all were applied at room temperature. All buffers and samples were filtered before use.

3.2.5 Gel Electrophoresis, Western Blotting and Assays

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). SDS-sample buffer, gel stain and destain were described in section 2.2.8. Western blots were performed as follows. Proteins were electrophoretically transferred to nitrocellulose essentially as described by Towbin *et al.* (1979) with the modifications suggested by Dunn (1986b). Prior to electroblotting, the SDS-polyacrylamide gel was incubated for 1 h with four changes of a buffer consisting of 50 mM Tris-HCl, pH 7.4, 20% glycerol. Proteins were electroblotted for 1 h at 1 amp at 30°C using a Bio-Rad (Mississauga, Ont.) Transblot apparatus equipped with a glass cooling coil. The transfer buffer consisted of 10 mM sodium bicarbonate, 3.3 mM sodium carbonate, 20% methanol. The nitrocellulose was stained with 0.1% Amido Black in fixative (45% methanol, 9% acetic acid) and destained in fixative. The nitrocellulose was blocked by incubation in Blot Rinse Buffer (40 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 0.1% Tween 20, 0.04% NaN_3) with 4% BSA for 1 h at room temperature. The nitrocellulose was then incubated in a dilution of hybridoma culture supernatant in Blot Rinse Buffer containing 0.4% BSA for 4 h at room temperature with gentle agitation. The nitrocellulose was then rinsed with three changes of Blot Rinse Buffer over 30 min. Rat antibodies were detected by incubation of the nitrocellulose in Blot Rinse Buffer with 0.4% BSA containing rabbit ^{125}I-anti-rat IgG at an activity of 50,000 dpm/ml. When blots were probed with rabbit polyclonal sera, goat ^{125}I-anti-rabbit IgG was used for detection. In several instances, proteins on the nitrocellulose were detected with

iodinated monoclonal antibodies, thus omitting the need for labelled second antibody. The nitrocellulose was washed as before, dried and radioautographed. Antibodies were iodinated by the IODO-GEN (Pierce) method (Fraker and Speck, 1978). Protein determinations were performed as described in section 2.2.8.

Cleavage fragments were assayed for ability to bind antibodies using methods described in section 2.2.7. Fragments were added to a dilution of alkaline phosphatase or ^{125}I-labelled-antibody in ELISA buffer C and this mixture was added to wells of microtiter plates containing bound, whole subunit.

Distilled, deionized water was used in all experiments.

3.3 Results

3.3.1 Exhaustive Cleavages of the α, β and γ Subunits

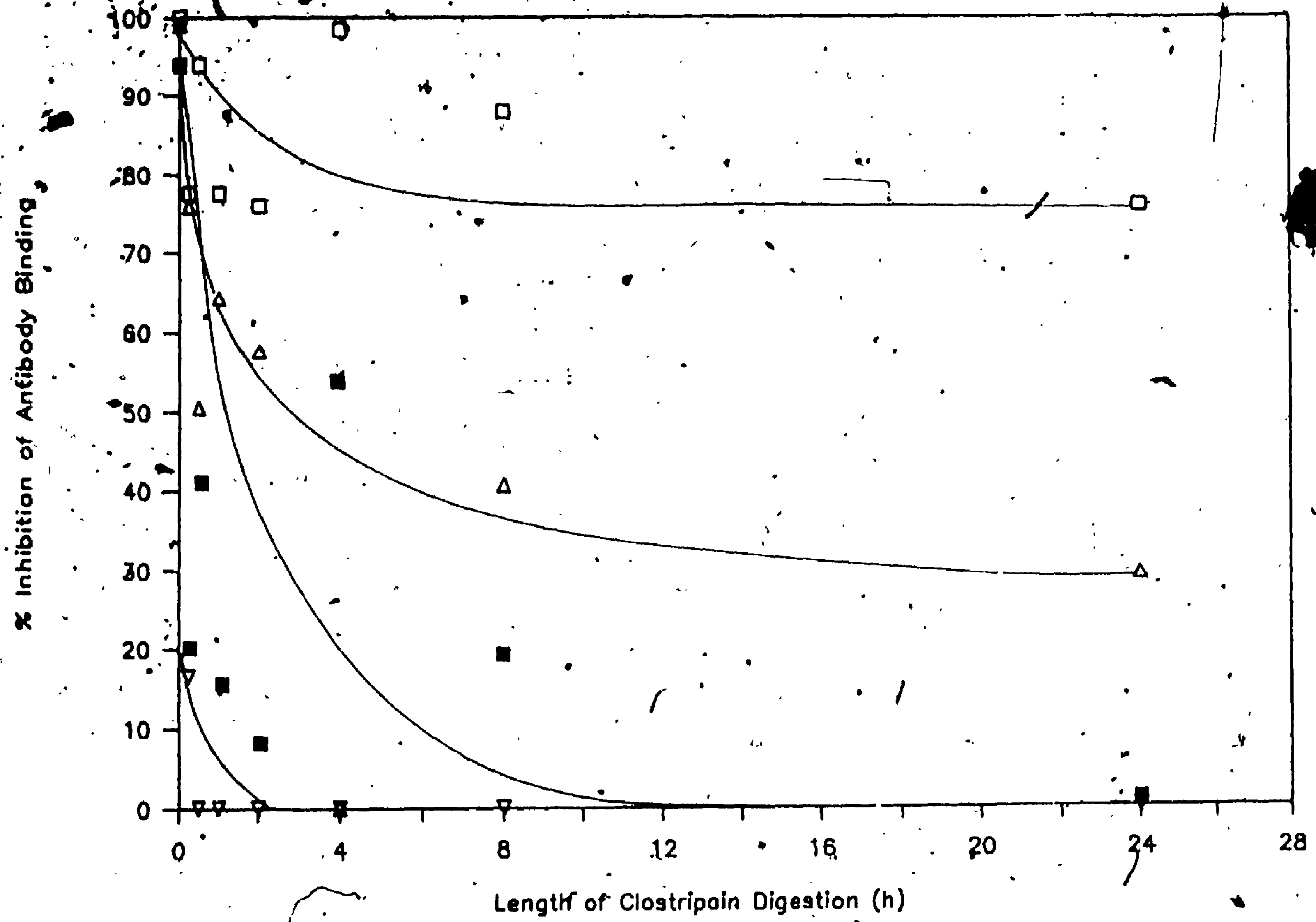
In order to produce small sized epitope-containing peptides suitable for purification using reversed phase HPLC and subsequent amino acid analysis, the α and β subunits were cleaved by a variety of chemical and enzymatic means. Cleavage methods were chosen on the basis of specificity for certain low frequency peptide bonds and efficiency of reaction. This was essential for production of peptides that could be identified by comparison with peptides predicted from the known amino acid sequence of the subunits. The resulting peptides were then assayed for their ability to bind to monoclonal antibodies by determining whether they could compete with whole subunit for binding of labelled antibody in a solid phase assay (similar to that described

in section 2.2.7).

An important initial experiment was performed to determine whether the anti-α and anti-β antibodies could recognize denatured subunit. Purified α or β subunit was denatured by performing an acetone precipitation and resuspending the protein in 8 M urea and incubating the solution for 30 min at room temperature. The denatured subunit was then added to the wells of the microtiter plates in an antibody-containing buffer which diluted the urea to 2 M. No loss of binding activity was observed with any of the anti-α or anti-β antibodies (data not shown). This suggested that either the antibodies were recognizing sequential determinants or that the epitopes were able to partially renature. These results encouraged proceeding with the peptide mapping through complete cleavages of the subunits.

The results of a typical epitope mapping experiment are shown in Figure 3.1. In this experiment different concentrations of α subunit, digested for various periods of time with clostripain, were added to 1000-fold dilutions of alkaline phosphatase-labelled α-1 or α-3 antibodies (representatives of the A-I and A-II competition subgroups respectively) in wells of microtiter plates coated with 10 ng of whole α. The longer α was digested with clostripain, the less effectively it inhibited the binding of α-3 antibodies to the α subunit bound to the plate. This indicated that clostripain rapidly destroyed the α-3 epitope. In contrast, the length of time of clostripain digestion had less effect upon the inhibition of α-1 binding. Although there was some loss of binding inhibition, a clostripain-resistant peptide appeared to be formed. This encouraged attempts to isolate the epitope-containing peptide by HPLC as will be described below.

Figure 3.1. Loss of binding inhibition by clostripain-digested α subunit. Purified α subunit was digested for various periods of time. Digested α was added to wells of a microtiter plate coated with 10 ng of undigested α subunit in a 1,000 fold dilution of alkaline phosphatase-labelled α-1 or α-3 monoclonal antibodies. Binding of labelled antibody was measured by a colorimetric assay. Details are described in section 3.2. Symbols represent the following: □, α-1 antibody with 1 μg digested α subunit; △, α-1 antibody with 0.1 μg digested α subunit; ■, α-3 antibody with 10 μg digested α subunit: ▽, α-3 antibody with 1 μg digested α subunit.



% Inhibition of Antibody Binding
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
Length of Clostripain Digestion (h)

Similar experiments were performed using clostripain-digested β subunit and trypsin, *S. aureus* V8 protease or CNBr-cleaved α and β subunits (data not shown). It was observed that exhaustive trypsin or *S. aureus* V8 protease digestion of either subunit abolished all inhibition of antibody binding. Exhaustively CNBr-cleaved α subunit was also unable to inhibit binding of the anti-α antibodies. Peptides produced from exhaustive CNBr cleavage of the β subunit did not inhibit binding of the anti-β antibodies of the B-I subgroup to whole β suggesting that the epitope was destroyed. However, binding of the B-II antibody, β-2, to whole β was inhibited nearly 100% by β cleaved by CNBr for 24 h. There was, however, a slow loss of binding activity over extremely prolonged CNBr cleavage.

Similar preliminary trials were attempted with the anti-γ antibodies γ-1 and γ-3 cleaving the γ subunit with CNBr and clostripain (data not shown). It was found that the γ-3 epitope nearly completely destroyed after either cleavage method but some residual activity remained (<5%) suggesting that neither of the cleavage methods was complete. The γ-1 epitope seemed to be resistant to cleavage by either method. Due to the difficulties in obtaining sufficient quantities of this subunit for purification of peptides and subsequent analysis, the project was not continued.

Attempts were made to purify the clostripain fragment of α that bound antibody α-1 using various reversed phase HPLC columns and solvent gradients. In a typical experiment, 50 μg of clostripain-digested α subunit was loaded onto a Waters C_{18} μBondapak column and a linear gradient from 0 to 60% acetonitrile with 0.1% trifluoroacetic acid as the counter ion, was applied over 60 min with a flow rate of 1

ml/min. 1-ml fractions were collected. All of the binding activity eluted between 55 and 60% acetonitrile (data not shown). The absorbance profile at 210 nm showed three unresolved peaks in this area but the activity did not coelute precisely with any of them. Further attempts to resolve the active peptide by modifying the chromatographic conditions were unsuccessful. That this peak eluted with such a high concentration of acetonitrile suggested that the fragment had a high molecular weight which would make its usefulness for epitope mapping doubtful. A large size fragment or a family of fragments would not be amenable to amino acid analysis geared to locate a small region of sequence. Similar problems also plagued purification of the β-2 epitope-containing CNBr fragment. SDS-polyacrylamide gels of exhaustively cleaved β revealed that a fragment with an apparent molecular weight of 13,700 was produced and Western blots showed that this fragment was recognized by β-2 (data not shown). Other approaches were used to identify this fragment.

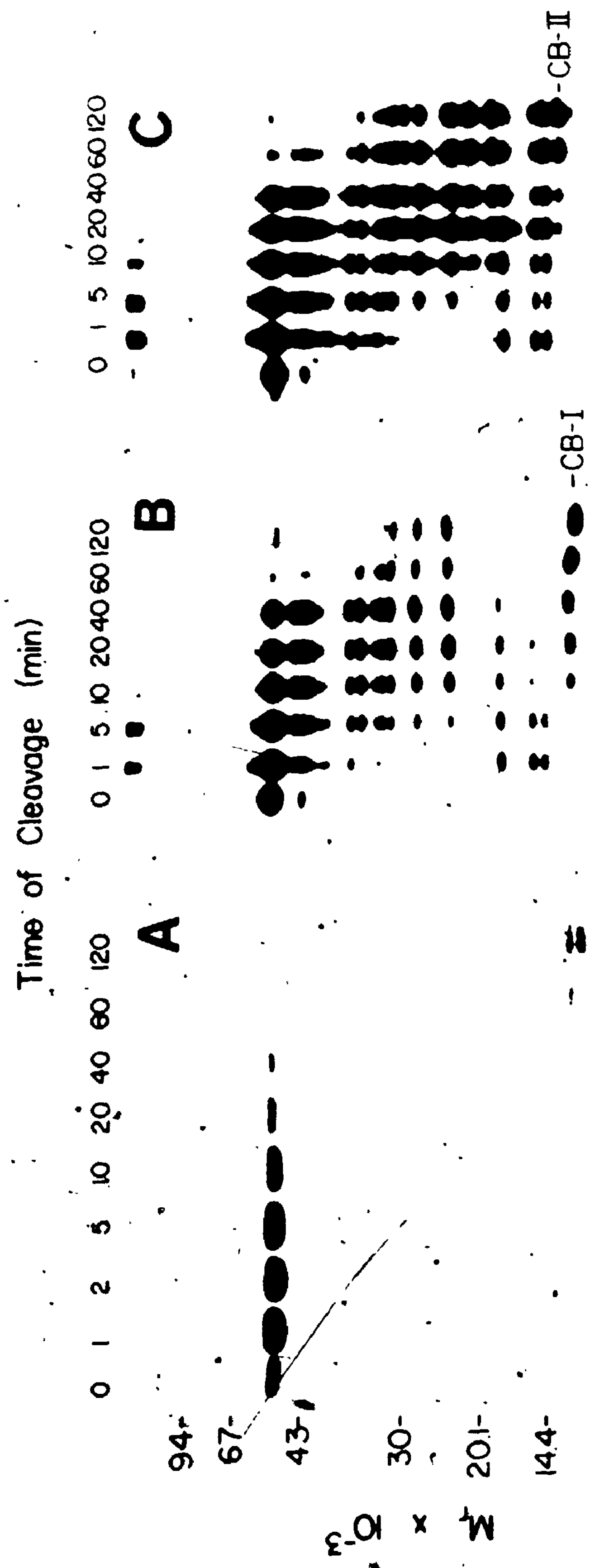
3.3.2 Partial CNBr Fragmentation of the β Subunit

Further attempts at locating the epitopes of the anti-β monoclonal antibodies made use of partial cleavages of the β subunit in an extension of the technique of Cleveland mapping (Cleveland *et al.*, 1977). Conditions were chosen such that cleavage occurred at only a fraction of susceptible sites yielding large fragments which could be analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. The patterns of total fragments obtained and those recognized by the antibodies provide information about the likely

locations of the epitopes within the amino acid sequence of β. Figure 3.2 shows a time course of CNBr cleavage of the β subunit in which the fragments were electrophoresed on an SDS-polyacrylamide slab gel and stained (panel A) or electroblotted onto nitrocellulose and probed using monoclonal antibodies β-1 (panel B) and β-2 (panel C). β-1 is an example of the inhibitory B-I antibodies; β-2 is an example of the non-inhibitory B-II antibodies. The smallest fragment recognized by β-1 (labelled CB-I in panel B) had an apparent molecular weight of 12,600, appeared early in the time course (5 min) and disappeared upon prolonged cleavage (data not shown). It was the only fragment recognized by β-1 but not by β-2, whereas there were several fragments recognized by β-2 alone. The smallest fragment recognized by β-2 (labelled CB-II in panel C) had an apparent molecular weight of 13,700, appeared relatively late in the time course (20 min) and was identical to the CNBr resistant peptide discussed earlier. Other antibodies in classes B-I and B-II had specificities identical to those of β-1 and β-2, respectively.

The β subunit was cyanylated to cleave it at its single cysteinyl residue Cys-137, the products were electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel, electroblotted onto nitrocellulose and probed with the monoclonal antibodies. It was observed that both β-1 and β-2 recognized the carboxyl terminus-containing 35,000-dalton fragment (data not shown). In order to determine whether any of the recognized CNBr fragments contained sequences amino-terminal to Cys-137, a two dimensional cleavage method was used. The products of partially cyanylated β were separated on an SDS-polyacrylamide gel, excised and subjected to partial CNBr cleavage while still in the gel.

Figure 3.2. Time course of CNBr cleavage of β subunit. Purified β subunit was cleaved by CNBr for various times, electrophoresed on a 10 to 20% SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue R-250 (Panel A), or electroblotted onto nitrocellulose. The nitrocellulose sheets were blocked with BSA and exposed to hybridoma culture media and ^{125}I-anti-rat IgG and then radioautographed. Details are described in section 3.2. The blot shown in panel B was probed with β-1 hybridoma culture medium. The blot shown in panel C was probed with β-2 hybridoma culture medium. Digestion of β was carried out for the indicated times. CB-I and CB-II are the two smallest fragments recognized by antibodies β-1 and β-2 respectively.



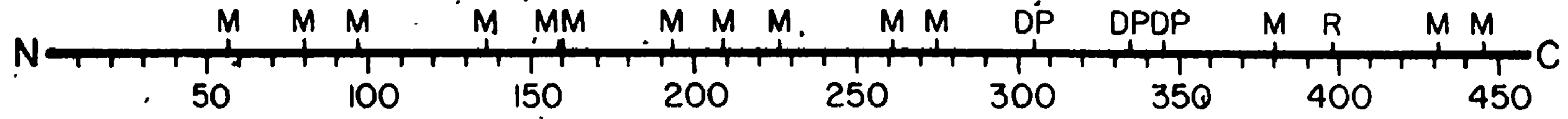
Time of Cleavage (min)
0 1 2 5 10 20 40 60 120
A
0 1 5 10 20 40 60 120
B
0 1 5 10 20 40 60 120
C
-CB-I
-CB-II
94-
67-
43-
30-
20.1-
14.4-
$M_r \times 10^{-3}$

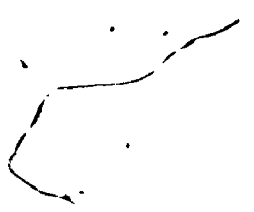
The pieces of gel were placed into the sample wells of a second gel which was then run and electroblotted onto nitrocellulose. The mobilities of the CNBr fragments recognized by the anti-β antibodies in this experiment were identical to those with molecular weights less than 35,000 obtained by the CNBr fragmentation of whole β (data not shown). This result implies that the epitopes are located close to the carboxyl terminus. To map the epitopes of the anti-β antibodies more precisely, another cleavage method was looked for that would take advantage of any unique peptide bonds present in the carboxyl-terminal region.

3.3.3 Weak Acid Hydrolysis of the β Subunit

The β subunit has three aspartyl-prolyl bonds involving aspartyl residues 305, 335 and 345 (Figure 3.3, panel A). The aspartyl-prolyl peptide bond is sensitive to hydrolysis by weak acid under mild conditions that do not hydrolyse most other peptide bonds involving aspartyl residues (Inglis, 1983). Treatment of β with 20% acetic acid at 60°C for 4 h produced fragments with apparent molecular weights, on SDS-polyacrylamide gels, of 33,000-35000, 17,500, 15,400 and 14,400 (data not shown), as expected if cleavage occurred at a fraction of the aspartyl-prolyl bonds. The three smaller fragments constitute a nested set, each arising from cleavage at one of these bonds, and all containing the carboxyl terminus of β (Figure 3.3, panel B). Analysis by Western blotting revealed that all three small fragments were recognized by both β-1 and β-2, indicating that both epitopes lie within the last 141 amino acid residues of the β subunit (data not

Figure 3.3. Cleavage sites within the β subunit and resulting fragments. In panel A the primary structure of the β subunit is represented by a line on which are shown CNBr and weak acid cleavage sites (Walker *et al.*, 1984). The termination site of the β subunit at R-398 in the mutant KF20 is also included (Noumi *et al.*, 1986). Panel B shows a schematic representation of the nested set of weak acid fragments recognized by the antibodies, their relative molecular weights and, in parentheses, their apparent molecular weights from SDS-polyacrylamide gels. Panel C shows schematic representations of the two smallest CNBr fragments recognized by β-1 (CB-I) and β-2 (CB-II), their relative molecular weights and, in parentheses, their apparent molecular weights from SDS-polyacrylamide gels. The N and C denote the amino and carboxyl termini respectively.


A
β Cleavage Sites
M M M M MM M M M. M M DP DPDP M R M M
N
C
50 100 150 200 250 300 350 400 450
Residue Number
B
Weak Acid Fragments
M_r = 18,400 (17,500)
M_r = 15,400 (15,400)
M_r = 14,200 (14,400)
C
Small Reçognized
CNBr Fragments
=10,400 (12,600)
CB-II M_r = 11,200 (13,700)

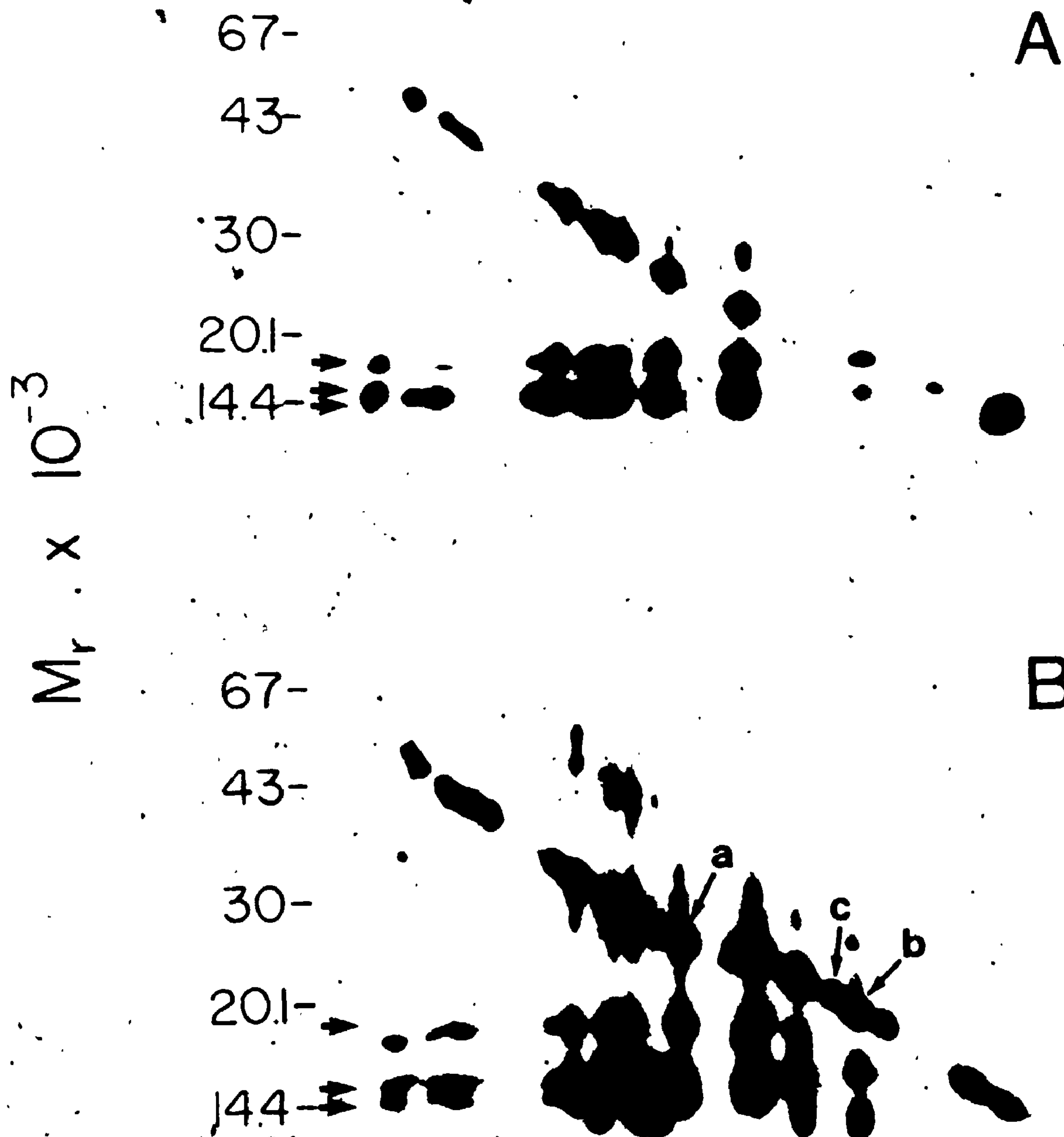


shown). The carboxyl-terminal weak acid fragments were also produced during the CNBr cleavage (note the presence of these recognized fragments at the shorter times shown in Figure 3.2, panels B and C), due to the acidic conditions present during this procedure.

Cleavage at the aspartyl-prolyl bonds was used for determining which CNBr fragments contained the carboxyl terminus of β by a two-dimensional method (Figure 3.4) The CNBr fragments were separated on an SDS-polyacrylamide gel, then lanes were treated with weak acid as described in section 3.2.3. The lanes were applied to the second gel, and the fragments separated by electrophoresis and then electroblotted onto nitrocellulose. This method was useful because the electrophoretic resolution of peptides produced by the first cleavage method was preserved during the weak acid hydrolysis step, facilitating comparison of the original fragments and their products. Diffusion of the polypeptides during the lengthy weak acid hydrolysis step was prevented by inclusion of 50% methanol, which precipitated the protein within the gel and shrank the polyacrylamide gel matrix. Resolubilization of the fragments after the methanol treatment was reasonably complete, but a small fraction was only slowly solubilized during electrophoresis in the second dimension. With very high affinity antibodies and very sensitive staining methods, this led to streaking, as seen with β-2 in Figure 3.4, panel B.

Figure 3.4, panel A shows an immunoblot probed with antibody β-1. The weak acid fragments lie below the original CNBr fragments which are on the diagonal. All of the CNBr fragments which were both larger than 17,500 daltons and recognized by β-1 were cleaved by weak acid to generate three recognized fragments which are identical to those

Figure 3.4. Two-dimensional CNBr-weak acid cleavage of β subunit. Ten μg of purified β subunit was cleaved with 0.5% CNBr in 70% formic acid for 20 min at 37°C and electrophoresed on a 12% SDS-polyacrylamide gel. It was subjected to an "in gel" weak acid hydrolysis, electrophoresed in the second dimension in a 10 to 20% SDS-polyacrylamide gradient gel, electroblotted onto nitrocellulose, blocked with BSA, exposed to hybridoma culture media and ^{125}I-anti-rat IgG and then radioautographed. Details are described in section 3.2. The blots were probed as follows: panel A, β-1; panel B, β-2. The arrows on the left hand sides of the figures point to the three weak acid fragments produced from intact β. In panel B, spots a, b and c represent three classes of fragments as discussed in section 3.3.3.


A
67-
43-
30-
20.1-
14.4-
B
67-
43-
30-
20.1-
14.4-
a
b
c
Mr x 10-3

derived from intact β subunit. Thus, all of the large (>17,500 daltons) recognized CNBr fragments must contain the aspartyl-prolyl bonds and the original carboxyl terminus. This result suggests that residues very near the carboxyl terminus form part of the B-1 epitope.

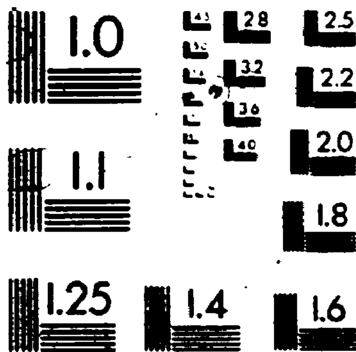
The immunoblot probed with β-2 (panel B) showed a very different pattern; one can discern three classes of recognized CNBr fragments. Some generated the same weak acid fragments that were produced from intact β subunit (such as the spot labelled "a"). These CNBr fragments contain the carboxyl terminus and correspond to those which were recognized by antibody β-1 in panel A. Others, such as the spot labelled "b", generated weak acid fragments that migrated with apparent molecular weights of about 2,000 less than the fragments derived from intact β. These CNBr fragments thus contain the aspartyl-prolyl bonds. They must also contain part of the sequence which lies to the carboxyl-terminal side of these bonds, as the epitope is located in this region. The smaller molecular weights of the recognized weak acid fragments imply that they arose from CNBr fragments which had been cleaved at a methionine residue near the carboxyl terminus. Two methionine residues, Met-431 and Met-445, are located in the appropriate region to give rise to fragments of the observed sizes. The only other methionine residue which is located to the carboxyl-terminal side of the aspartyl-prolyl bonds is Met-380. It is probable that a homoserine residue derived from cleavage at this latter methionine residue is the carboxyl terminus of the spot labelled "c", which gave no recognized weak acid fragments. CNBr fragments which have this carboxyl terminus would be expected to yield weak acid fragments with molecular weights

of 6,000 daltons and less, which are probably too small to bind to nitrocellulose. In order to account for these three classes of fragments, the β-2 epitope must lie between residues Asp-345 and Met-380 (Figure 3.3, panel A). The CNBr fragment CB-II observed in Figure 3.2, panel C thus resulted from cleavage at Met-275 and Met-380 (Figure 3.3, panel C).

The lack of recognition, by β-1 (Figure 3.4, panel A), of spots such as "b" which were recognized by β-2 indicates that residues lying to the carboxyl-terminal side of Met-431 must be an essential part of the B-I epitope. The CNBr fragment CB-I, recognized by β-1 (Figure 3.2, panel B) must therefore contain the carboxyl-terminus, and consist of residues Asp-381 (the amino terminus produced by cleavage at Met-380) to Leu-459 (Figure 3.3, panel C).

Further experiments supported this assignment of epitopes within the primary structure. Partially CNBr-cleaved β subunit was subjected to various periods of weak acid hydrolysis. The CNBr fragment, CB-I, recognized by β-1 was resistant to prolonged weak acid hydrolysis, as expected for the sequence from Asp-381 to Leu-459. In contrast, the CNBr fragment CB-II recognized by β-2 was sensitive to weak acid hydrolysis, as expected since the sequence between Met-275 and Met-380 contains the three aspartyl-prolyl bonds. Other supporting evidence was derived from analysis of *E. coli* strain KF20, which has an amber mutation in the *uncD* gene resulting in termination of β at residue Arg-398 (Figure 3.3, panel A) (Noumi *et al*., 1986). An SDS extract of whole cells was analyzed by Western blotting. Both polyclonal anti-β rabbit antibodies and monoclonal antibody β-2, but

2



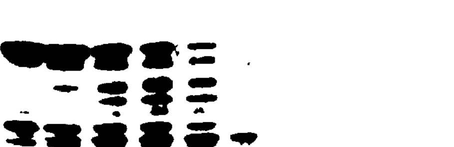
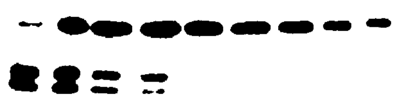
not antibody β-1, recognized a 45,000-dalton protein in this extract (data not shown).

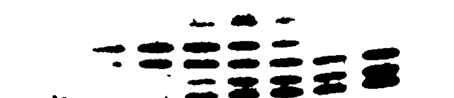
3.3.4 Preliminary Mapping of Epitopes Within the α Subunit

As with the β subunit, the α subunit could not be cleaved to generate small fragments that were able to bind to the anti-α monoclonal antibodies. A preliminary experiment made use of the observation that the α subunit in the presence of ATP is cleaved by trypsin to yield two fragments (Senda *et al.*, 1983). The larger 30,000-dalton fragment contains the amino terminus and the smaller, 25,000-dalton fragment contains the carboxyl terminus. The results of an experiment in which the α subunit was cleaved under these conditions, electroblotted onto nitrocellulose and probed with monoclonal antibodies is shown in Figure 3.6, panel A. The first lane shows a blot which was probed with polyclonal rabbit anti-α antiserum. The two termini-containing fragments can be distinguished. The antisera reacts primarily against the 30,000-dalton fragment, but the 25,000-dalton fragment is also recognized. Three representative anti-α antibodies, α-1, α-3 and α-4 all recognize the smaller, carboxyl terminus-containing fragment (lanes 2, 3 and 4).

The α subunit was then subjected to CNBr cleavage for various periods of time as shown in Figure 3.5. Panel A shows the stained gel and panels B and C show Western blots probed with the monoclonal antibodies α-1 and α-3 (representatives of the A-I and A-II competition subgroups, respectively). It can be seen that both antibodies seem to recognize the same CNBr fragments. Weaker recognition by α-3 of the

Figure 3.5. CNBr time course cleavage of the α subunit. Purified α subunit was cleaved by CNBr for various times, electrophoresed on a 10 to 20% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. The nitrocellulose sheets were blocked with BSA and exposed to hybridoma culture media and ^{125}I-anti-rat IgG and then radioautographed. Details are described in section 3.2. Panel A shows the stained gel; panels B and C show Western blots probed with α-1 and α-3 hybridoma culture supernatants respectively. Lanes are as follows: lane 1, 0 min; lane 2, 1 min; lane 3, 5 min; lane 4, 10 min; lane 5, 20 min; lane 6, 40 min; lane 7, 1 h; lane 8, 1 h 30 min; lane 9, 2 h; lane 10, 4 h.



$M_r \times 10^{-3}$
94 -
67 -
43 -
30 -
20.1 -
14.4 -
A
B
C
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

fragment which migrates with an apparent molecular weight of 10,000 is probably due to lower affinity for its epitope. It was important to determine if any of the three fragments migrating with apparent molecular weights in the range of 40,000 to 50,000 were primary fragments that contained the original amino terminus. Thus whole F_1 was cleaved with trypsin in the presence of 1 mM ATP which leads to removal of fifteen amino acids from the amino terminus of the α subunit (Dunn *et al.*, 1980). The material was then cleaved for various lengths of time with CNBr, the fragments electrophoresed on an SDS-polyacrylamide gradient gel and electroblotted onto nitrocellulose. This experiment is shown in Figure 3.6, panel B showing a Western blot probed with iodinated α-1 and panel C, a Western blot probed with α-3. The difference in mobilities of the trypsin-cleaved and uncleaved α can be seen very clearly by comparing non-CNBr cleaved-α subunit in lanes 1 and 2 of panel B. The change in migration appears to be due to a loss of between 2,000 to 3,000 daltons from the subunit. As can be seen by comparing lanes 3 with 4 and 5 with 6 in panels B and C, no difference is seen in the mobilities of any CNBr fragment. As no large fragment has an altered apparent molecular weight, none contains the original amino terminus. In conjunction with the pattern of fragments produced with the CNBr time course in Figure 3.5, panels B and C, it would appear that both epitopes are probably lying within carboxyl-terminal half of the molecule. If the 14,800-dalton fragment is a primary fragment then the epitopes are lying within the carboxyl-terminal-third of the molecule. The fragment that migrates with an apparent molecular weight of 25,000 in the trypsin cleaved lanes is the same cleavage product observed when purified α subunit is cleaved with trypsin in the

Figure 3.6. Tryptic digest of purified α subunit in presence of ATP and time course of CNBr cleavage of trypsin-treated F_1. For the experiment shown in panel A, purified α subunit was cleaved by trypsin in the presence of 1 mM ATP, the products electrophoresed on a 10 to 20% SDS-polyacrylamide gel and electroblotted onto nitrocellulose. The nitrocellulose sheets were blocked with BSA, exposed to hybridoma culture media followed by ^{125}I-anti-rat or ^{125}I-anti-rabbit antibodies and then radioautographed. Details are described in section 3.2. Lane 1 shows a nitrocellulose strip probed with rabbit anti-α antiserum; lanes 2, 3 and 4 show nitrocellulose strips probed with antibodies α-1, α-3 and α-4 respectively. Panels B and C show an experiment in which native F_1 was treated with trypsin in the presence of ATP to remove 15 amino acid residues from the amino terminus of the α subunit. Cleaved and uncleaved F_1 were then subject to CNBr cleavage for various lengths of time and the products were electrophoresed, electroblotted and probed as described above. The nitrocellulose was probed with either ^{125}I-α-1 (panel B) or ^{125}I-α-3 (panel C). Odd numbered lanes show CNBr cleaved F_1; even numbered lanes show CNBr cleaved, trypsin-treated F_1. Times of CNBr cleavage are as follows: lanes 1 and 2, zero time; lanes 3 and 4, 5 min; lanes 5 and 6, 10 min; lanes 7 and 8, 20 min; lanes 9 and 10, 1 h.

A
94-
67-
43-
30-
20.1-
14.4-
$M_r \times 10^{-3}$
1 2 3 4

B
C
94-
67-
43-
30-
20.1-
14.4-
$M_r \times 10^{-3}$
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

presence of ATP. Unfortunately the α subunit contains no useful cleavage sites in this region of the molecule to narrow down the actual locations of the two epitopes.

The result that there is no apparent difference between the CNBr fragments recognized by members of either the A-I or A-II competition subgroup is not surprising in view of the ability of the two competition subgroups to partially inhibit one another's binding to whole α. This reflects the proximity of the two epitopes in tertiary structure. Partial cleavages of the α subunit using agents such as N-bromosuccinimide, pronase, subtilisin and weak base (data not shown) also did not produce fragments that were recognized by antibodies of one competition subgroup only.

3.4 Discussion

Difficulty was encountered in precisely mapping the epitopes of the anti-α and anti-β monoclonal antibodies. Techniques relying upon exhaustive cleavage of the subunits and identification of epitope-containing peptides were unsuccessful as cleavage of the α and β subunits by several chemical and enzymatic methods did not yield peptides smaller than 10,000 to 11,000 daltons which were recognized by the antibodies. This suggests that the epitopes of the A-I, A-II, B-I and B-II classes of antibodies are assembled from amino acid residues which are widely separated in the primary structure. A similar situation has been documented for the epitope of an anti-lysozyme monoclonal antibody (Amit *et al.*, 1986). X-ray crystallography was used to determine the structure of epitope and antibody combining site.

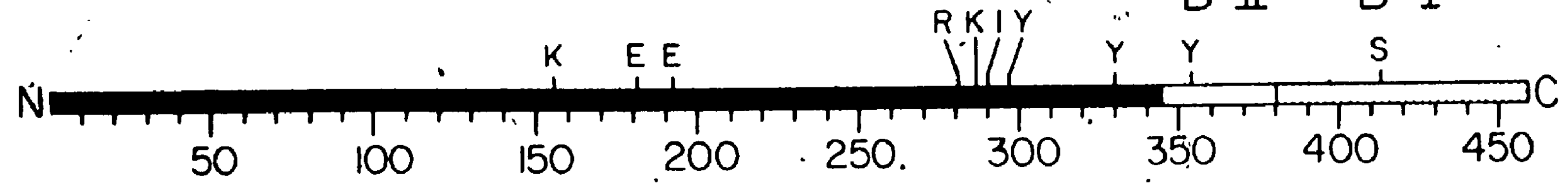
The epitope was found to be quite large with dimensions of approximately 2 by 3 nm and structurally to consist of a flat surface with some shallow depressions and elevations. More significantly, the amino acid residues that form the epitope are contributed by two stretches of the polypeptide chain separated by 89 residues in the primary structure. Cleavage in the intervening region would separate the two sections of polypeptide chain and destroy the epitope. Such topographically assembled epitopes may in fact be more common than sequential epitopes (Benjamin *et al.*, 1984).

The detection of the small (<14,000 dalton) peptides on Western blots has only been possible with the modified Western blot protocol in which SDS is largely removed from the proteins following electrophoresis allowing their renaturation. Analysis of peptides smaller then 10,000 daltons is not feasible in this system due to the limits of resolution of small proteins by SDS-gel electrophoresis. Finer mapping of the epitopes may be possible using other methods. One could synthesize peptides containing amino acid sequences found in the carboxyl-terminal regions of the α and β subunits and see if they inhibit binding. Synthetic peptides would be chosen containing amino acid sequences that span cleavage sites. Nevertheless, the previous failure to obtain inhibitory peptides from a wide variety of means of cleavage shows that this strategy would probably fail. One could also purify large epitope-containing fragments, such as those produced by clostripain with affinity columns utilizing immobilized monoclonal antibody of interest. These fragments can then be exhaustively cleaved with trypsin and these smaller fragments purified by reversed phase HPLC. Amino acid analysis would allow these fragments to be mapped

within subunit primary structure. High abundance fragments would be expected either to contain or to be near the actual epitope.

The epitopes of both groups of anti-β monoclonal antibodies are located near the carboxyl terminus of the β subunit of *E. coli* F_1-ATPase (Figure 3.7). Antibody β-1, a representative of the competition subgroup B-I which contains antibodies able to inhibit hydrolytic activity of the ATPase, binds to a region between Asp-381 and Leu-459. Residues around Met-431 or Met-445 may be involved in the epitope, as cleavage at either of these residues did not produce a polypeptide recognized by the antibodies. Recognition of an assembled epitope by the B-I antibodies implies that the carboxyl-terminal region of the β subunit is organized into a compact unit, *i.e.*, a domain. Chemical modification studies have demonstrated that one residue in the carboxyl-terminal region of the β subunit is closely associated with the catalytic site. Residue Ser-413 is equivalent to His-427 in the mitochondrial β subunit, a residue which is labelled by 5'-p-fluorosulfonylbenzoyl adenosine (Esch and Allison, 1978; Bullough and Allison, 1986a, 1986b) (Figure 3.7). It is noteworthy that the reactive group of this affinity label is situated in the position of the phosphoryl moiety of the nucleotide. Thus part of the postulated carboxyl-terminal domain is located in the region of the nucleotide-binding site where catalysis occurs. Affinity reagents which have the reactive group situated on the purine ring label β more toward the centre of the sequence (Vignais and Satre, 1984), suggesting that residues in this portion of the sequence form the site which binds the nucleoside moiety of ATP. One possible structure for a central nucleotide-binding domain has been formulated by Duncan and coworkers

Figure 3.7. Location of chemically modifiable residues and regions containing antibody epitopes within the β subunit. The primary structure of β is represented by the bar. Indicated residues are modified by Nbf-Cl (K-155, Y-297) (Andrews *et al.*, 1984a, b; Sutton and Ferguson, 1985), DCCD (E-181, E-192) (Esch *et al.*, 1981; Yoshida *et al.*, 1981, 1982), phenylglyoxal (R-281) (Viale and Vallejos, 1985), 8-azido-ATP (K-287, I-290, Y-297) (Hollemans *et al.*, 1983), 5'-p-fluorosulfonylbenzoyl adenosine (Y-354, S-413) (Esch and Allison, 1978; Bullough and Allison, 1986a) and 5'-p-fluorosulfonylbenzoyl inosine (Y-330) (Bullough and Allison, 1986b). The portion of the subunit containing the B-II epitope is shown in white; the portion containing the B-I epitope is stippled. The N and C denote the amino and carboxyl termini respectively


B-II
B-I
R K I Y
K
E E
Y
Y
S
N
C
50
100
150
200
250
300
350
400
450
Residue Number

(1986). It is possible that this postulated carboxyl-terminal domain, together with the central domain, provides the unique environment required at the catalytic site where the free energy of ATP hydrolysis is essentially nil. The carboxyl-terminal domain could also function as a gate which can close over a bound substrate molecule by moving relative to the central domain. Release of product should then require a substantial conformational change, in which the carboxyl-terminal domain would move away from the central domain.

Antibody β-2, a representative of the competition subgroup B-II which contains antibodies that recognize highly conserved epitopes not exposed on the surface of F_1-ATPase, binds to a region located between Asp-345 and Met-380. This region is thus located at a subunit interface and its conservation in other ATPases may suggest functional importance as discussed in chapter 2. Examination of β subunit sequences from organisms that did and did not cross-react with β-2 did not provide a means of further narrowing down the location of the epitope.

The aspartyl-prolyl linkages of the β subunit provided an important key in mapping the β epitopes. The acid lability of these bonds enabled development of a simple method for mapping the epitopes using two-dimensional SDS-polyacrylamide gel electrophoresis and partially CNBr-cleaved β. The three aspartyl-prolyl bonds are a conserved feature in many β subunits (Walker *et al.*, 1985; Ohta and Kagawa, 1986). All three bonds are found in *Rhodospirillum rubrum*, *Rhodopseudomonas blastica* and human mitochondrial β subunit. The first two bonds are found in bovine and yeast mitochondrial β subunit as well as the chloroplast β subunits from tobacco, spinach, barley,

wheat and maize. These residues and the unique pattern of fragments that result upon weak acid hydrolysis can serve as a marker of the β subunit. They may also serve as a means to localize the epitopes of other anti-β monoclonal antibodies currently being investigated by other workers.

The one salient conclusion about the epitopes of the two anti-α competition subgroups is that they are fairly close together in primary structure, separated by a distance of at most 90 amino acid residues. The fact that the competition subgroups also partially inhibit each other's binding suggests that they are close in subunit tertiary structure. As the two competition subgroups recognize internal and external epitopes respectively, the epitopes may be located very close to a subunit junction. Some workers have suggested that the catalytic sites of the enzymes are located at α/β junctions (Senior and Wise, 1983).

4.0 STRUCTURE/FUNCTION INVESTIGATIONS OF F_1-ATPASE USING CHEMICAL CROSS-LINKING

4.1 Introduction

Chemical cross-linking has been used to obtain structural information about the quaternary structure of proteins and large macromolecular complexes. The ability to cross-link two polypeptides by a relatively short cross-linking reagent indicates that they must be proximal to one another (for a review of applications see Peters and Richards, 1977). This chapter will describe a number of experiments using chemical cross-linking to investigate a number of structural aspects of F_1 and F_1F_0.

A number of workers have used cross-linking to study the quaternary structure of F_1 (reviewed by Klein *et al.*, 1977). Bragg and Hou have made extensive use of various bifunctional alkyl imidates to study *E. coli* F_1 (Bragg and Hou 1975, 1976, 1980 and 1986a). They demonstrated the formation of α_2, α-β, α-δ, β_2, β-γ, β-δ, β-ϵ and γ-ϵ cross-linked products. Similar cross-linked products have been obtained using chloroplast F_1 (Baird and Hammes, 1976).

Hermolin and coworkers (1983) studied subunit arrangement in *E. coli* F_0 by cross-linking F_1-stripped membranes and then purifying F_0. They detected a-b, a-b_2 and b_2 cross-linked products. Aris and Simoni (1983) cross-linked purified F_1F_0 and detected a-b, b_2, β-a, β-b and c_2 cross-linked products. Similar studies have also been performed on purified chloroplast F_1F_0 demonstrating close interactions between the α, β and γ subunits with F_0 subunits (Süss, 1986). All the previous

studies used reversible cross-linkers and identified the resulting cross-linked products by two-dimensional gel-electrophoresis, reversing the cross-link in the second dimension. Although this method is effective for purified proteins, it is cumbersome and impractical for very complex mixtures of proteins such as those that result from the cross-linking of membranes.

One disadvantage of performing cross-linking studies using purified F_1 or F_1F_0 is that the subunit arrangement of these complexes may differ from that of native F_1F_0 present in membranes. Aris and Simoni (1983) used antisera raised against the α, β and b subunits of *E. coli* F_1F_0 and Western blots for the analysis of the products resulting from cross-linking of membranes. They observed the formation of β-b cross-linked products. Unfortunately their limited bank of antisera precluded more detailed investigation of F_1F_0 subunit interactions.

Further investigation is needed to better define the proposed interactions of the δ and ϵ subunits with F_0. Although several models of F_1F_0 place the δ and ϵ subunits close to one another, at the interface between F_1 and F_0 (Figure 1.1) they have never been cross-linked either to each other or to any F_0 subunit. Data placing these subunits in this location have been obtained from reconstitution studies (Sternweis, 1978; Sternweis and Smith, 1977), subunit homologies (Senior and Wise, 1983) and fluoresence energy transfer measurements (Richter *et al.*, 1985). An unlikely explanation for the negative cross-linking results is that these subunits are located neither near each other nor F_0. A more likely explanation is that the previous studies used methods of cross-linking and analysis not

suitable for production or detection of such cross-links. This may result from lack of suitably placed subunit side chains or lack of sensitivity of previous methods of analysis.

Monoclonal antibodies and Western blots provide a powerful means to overcome the above problems. Monoclonal antibodies with their extreme specificity and high sensitivity facilitate detection of low frequency products. Used in conjunction with Western blots, cross-linked proteins can by analyzed by a one dimensional system circumventing the need for reversible cross-linkers. Thus a wider range of cross-linking reagents can be used including non-specific, photoactivatable cross-linkers. Some studies to be described in this chapter make use of these techniques to investigate the subunit arrangement of F_1F_0 in membranes.

Cross-linking studies may also be a useful means to detect changes in subunit interactions. Some agents which affect the activity of F_1 may do so by perturbation of F_1 quaternary structure. The ε subunit is a dissociable inhibitor of soluble F_1 (Sternweis and Smith, 1980; Laget and Smith, 1979) which binds tightly to the γ subunit (Dunn, 1982) and can be cross-linked to both the β and γ subunits (Lötscher *et al.*, 1984; Bragg and Hou, 1980). From the study of the activation of ATPase activity in the presence of the detergent LDAO it was postulated that ε-inhibition results from the interaction of ε with a β subunit rather than the γ subunit (Lötscher *et al.*, 1984). Bragg and Hou (1986b) showed that LDAO did not affect the formation of the zero-length cross-link between β and ε by the water soluble carbodiimide 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC). Thus the subunit through which ε-inhibition is mediated is still in doubt.

Other studies to be described in this chapter investigated two other activators of F_1 in addition to LDAO that may operate by altering subunit interactions involving ϵ. Antibody ϵ-4 can bind to ϵ and activate ATPase activity while ϵ is still associated with F_1 (Dunn and Tozer, 1987). Ethylene glycol increases medium viscosity and should inhibit ATPase activity by retarding conformational changes. Instead, at certain concentrations it actually activates ϵ-replete F_1 (Dunn and Zadorozny, unpublished observations). Study of the effects of these two agents upon the formation of cross-links involving the ϵ subunit may provide useful information concerning the mechanism of ϵ-inhibition. Partial cleavages using techniques described in chapter 3 were also applied to cross-linked β-ϵ to map the ϵ-binding site on β.

4.2 Materials and Methods

4.2.1 Materials

Sources of most chemicals have been described in previous Materials sections. LDAO was purchased from Calbiochem (San Diego, Ca.). Cross-linking chemicals were purchased from Pierce (Rockford, Ill.). Ethylene glycol was purchased from Baker (Phillipsburg, N.J.). Sephadex LH60 was purchased from Pharmacia P-L Biochemicals (Uppsala, Sweden). Cysteine was purchased from Sigma (St. Louis, Mo.). Papain and ADP were purchased from Boehringer Mannheim (Dorval, Qué.)

4.2.2 Preparation of Materials for Cross-linking

The methods for growing cells, purifying F_1 from *E. coli* strain AN1460 (*unc*$^+$ overproducer) (Downie *et al.*, 1980) and purifying and iodinating antibodies were described in section 2.2.4. Subunit b was purified by S. Dunn using the method of Perlin and Senior (1985) with the modification that the partially purified subunit was run on an SDS-polyacrylamide gel, electroblotted onto nitrocellulose and extracted with pyridine. Subunit c was purified using the method of Fillingame (1976) except that gel filtration was performed using Sephadex LH60. Polyclonal antisera were raised in rabbits with these subunits using methods described in section 2.2.4. Antibodies were purified on affinity columns made by conjugation of the appropriate subunit to CNBr-activated Sepharose using the carbonate method (March *et al.*, 1974). Purification followed methods described in section 2.2.4 but the antibodies were eluted with 0.25 M NH_4OH into tubes containing an equal volume of 1 M Tris-HCl, pH 6.8. The anti-δ antiserum was partially purified by incubating it with F_1-stripped membranes followed by the removal of the membranes by centrifugation.

Membranes were prepared for cross-linking studies as follows. All procedures were performed at 4°C. Frozen cells obtained from *E. coli* strains AN1460 (*unc*$^+$ overproducer) or ML308-225 (*unc*$^+$) (Downie *et al.*, 1980) were suspended as a 20%(w/v) solution in 50 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 10% glycerol, 1 mM PMSF. The cells were broken by two passes through a French Press at 10,000 p.s.i. To remove unbroken cells and debris, the cell suspension was centrifuged twice in a Beckman JA-20 rotor at 11,000 rpm for 10 min

saving the supernatant each time. The supernatant was then centrifuged for 2 h at 38,000 rpm in a Beckman Ti50 rotor. The supernatant was discarded and the pellet was resuspended in 10 mM triethanolamine-H_2SO_4, pH 7.5, 10 mM EDTA, 10% glycerol and centrifuged as before. The supernatant was discarded and the pellet was resuspended in a minimum volume of 50 mM triethanolamine-HCl, pH 7.5, 5 mM $MgCl_2$, 10% glycerol, frozen using dry ice/ethanol and stored at -80°C.

Fab fragments of antibody β-6 were prepared by digesting 0.5 mg/ml antibody with 10 µg/ml papain in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM cysteine, 2 mM EDTA for 2 h at room temperature and terminating the digestion with the addition of NEM to a concentration of 10 mM. The Fab fragments were purified on a Pharmacia P-L Biochemicals Mono-S fast protein liquid chromatography column connected to the Waters HPLC system described in section 3.2.4. The Fab fragments were eluted by running a linear gradient from 100% buffer A to 100% buffer B, over 30 min at room temperature. Buffer A consisted of 100 mM sodium acetate, pH 4.5, 2 mM EDTA and buffer B consisted of 1 M sodium acetate, pH 6.0, 2 mM EDTA.

When necessary, F_1 was changed into appropriate buffers using the column centrifugation method described in section 2.2.4.

4.2.3 Membrane Cross-linking

Dithiobis(succinimidyl propionate)--DSP was dissolved in a minimum volume of Me_2SO and added to a final concentration of 0.4 mM to 2 mg/ml membranes in 20 mM triethanolamine-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol. The reaction mix was incubated for 3 min at room

temperature and the reaction was terminated by the addition of Tris-HCl, pH 7.8 and NEM to final concentrations of 0.5 M and 10 mM respectively.

Ethyleneglycol bis(succinimidyl succinate) (EGS), Bis(sulfosuccinimidyl)suberate (BS^3), Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), Sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (sulfo-SMPB)--EGS, BS^3, sulfo-SMCC and sulfo-SMPB were dissolved in a minimum volume of Me_2SO and added to 5 mg/ml membranes in 20 mM triethanolamine-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol to a final concentration of 2 mM. The reaction mix was incubated for 1 h at room temperature (30 min for EGS) and the reaction was terminated by the addition of Tris-HCl, pH 7.8 and NEM to final concentrations of 0.5 M and 10 mM respectively.

EDC was dissolved in a minimum volume of 50 mM triethanolamine-HCl, pH 7.5, 5 mM $MgCl_2$, 10% glycerol and added to 5 mg/ml membranes in the same buffer for a final concentration of 1 mM. The reaction mix was incubated for 1 h at room temperature and the reaction was terminated by the addition of sodium acetate and NEM to final concentrations of 50 mM and 10 mM respectively.

Sulfo-succinimidyl 6-(4'-azido-2'-nitrophenylamine)hexanoate--Sulfo-SANPAH was dissolved in a minimum amount of Me_2SO and added to 2.5 mg/ml membranes in phosphate buffered saline, pH 8.0, for a final concentration of 2.5 mM. The reaction mix was incubated 1 h in the dark at room temperature. The reaction was terminated by the addition of ethanolamine-HCl, pH 9.0, NEM, EDTA and glycerol to final concentrations of 25 mM, 5 mM, 2.5 mM and 25% respectively. The reaction mix was then incubated for 30 min at room temperature at a

distance of 5 cm from an unfiltered short wave ultraviolet lamp (Mineral Light SL-2537, Ultra-violet Products, Inc. San Gabriel, Ca.). The reaction was terminated by the addition of DTT-containing SDS-sample buffer and subsequent boiling.

4.2.4 DSP and EDC Cross-linking of F_1 in the Presence of ε-4 Fab

Purified ε-4 Fab (Dunn and Tozer, 1987) was added to 1 mg/ml F_1 in the buffer appropriate for the cross-linker used, in a 4-fold molar excess over F_1 and incubated at room temperature for 15 min. DSP dissolved in a minimum amount of Me_2SO was added to a final concentration of 0.4 mM to protein in 20 mM triethanolamine-HCl, pH 7.5, 0.5 mM EDTA, 22.5% sucrose. The reaction mix was incubated for 30 sec at room temperature and the reaction was then terminated with the addition of Tris-HCl, pH 7.8 and NEM to final concentrations of 0.5 M and 10 mM respectively. EDC, dissolved in a minimum amount of 20 mM Mops-NaOH, pH 7.5, was added to a final concentration of 0.36 mM to protein present in the same buffer. The reaction mix was incubated for 30 min at room temperature and the reaction was then terminated by the addition of sodium acetate to a final concentration of 50 mM.

4.2.5 EDC and DSP Cross-linking of F_1 in the Presence of Ethylene Glycol and LDAO

F_1, at a 1 mg/ml concentration, was incubated for 10 min at room temperature in 20 mM Mops-NaOH, pH 7.5, 10 mM ADP, 5 mM $MgCl_2$ with 0%, 30% and 40% ethylene glycol. EDC, dissolved in a minimum volume of

distilled, deionized water, was then added to a 4 mM final concentration. Samples were removed at various time intervals after incubation at room temperature and sodium acetate and ethanolamine-HCl, pH 9.0 were each added to 50 mM final concentrations to terminate the reaction. A zero-time point consisted of F_1 to which sodium acetate and ethanolamine-HCl, pH 9.0 were added before the EDC. For DSP cross-linking, the reaction conditions were the same except that DSP was dissolved in a minimum amount of Me_2SO and added to the protein to a 0.1 mM final concentration. Samples were removed at various time intervals after incubation at room temperature and the reaction was then terminated by the addition of Tris-HCl, pH 7.8 to a 0.5 M final concentration. The zero-time point consisted of a sample withdrawn before addition of DSP. EDC cross-linking of F_1 in the presence of 0.1% and 0.3% LDAO was performed as described for EDC cross-linking in the presence of ethylene glycol.

4.2.6 Weak Acid Hydrolysis of EDC Cross-linked F_1

EDC cross-linked F_1 was prepared using the method described in section 4.2.5. Weak acid hydrolysis utilized the two-dimensional method described in section 3.2.3.

4.2.7 Chemical Cross-linking of β-6 Fab to F_1

Purified F_1 was cross-linked to β-6 Fab fragment with BS^3 or sulfo-SMCC through the following procedure. Reactions were performed in 50 mM sodium phosphate, pH 8.0, 150 mM NaCl, 2 mM EDTA, 2 mM ATP.

Purified F_1 was present at 0.2 mg/ml and β-6 Fab, when required, was present at 0.5 mg/ml, resulting in a 1:5 molar ratio of F_1 to Fab. BS^3 was added in cross-linking buffer to a final concentration of 0.05 mM and sulfo-SMCC to a final concentration of 0.2 mM. The reaction mixtures were incubated for 30 min at room temperature and then the reactions were terminated with the addition of Tris-HCl, pH 7.8 to a 0.5 M final concentration followed by a 10 min incubation at room temperature. For those experiments in which the proteins were alkylated, NEM was added to a final concentration of 10 mM and the samples were incubated a further 10 min at room temperature. The SDS-sample buffer did not contain DTT for these samples.

4.2.8 Gel Electrophoresis, Western Blotting and Assays

These procedures have been described in sections 2.2.8 and 3.2.5.

Distilled, deionized water was used in all experiments.

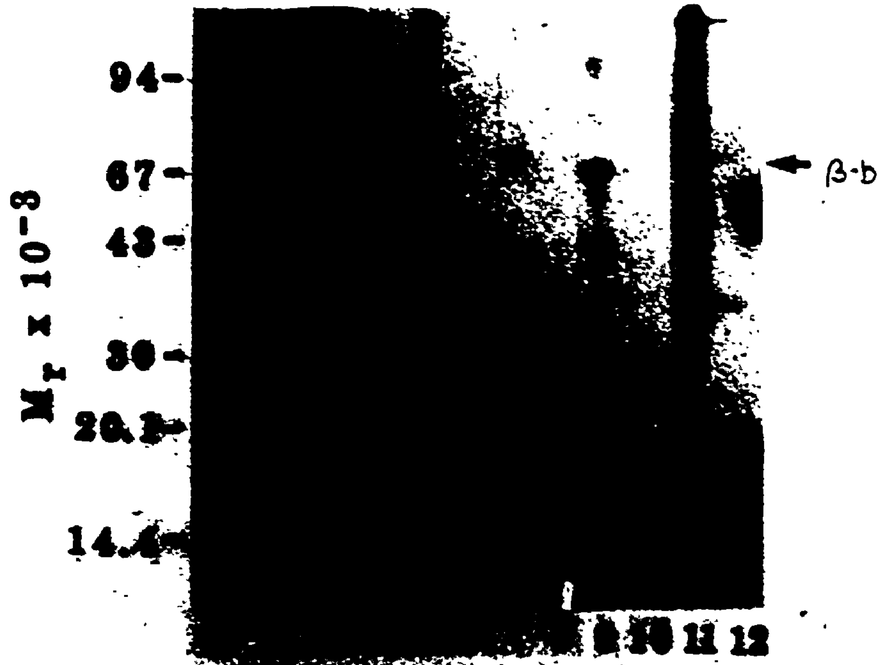
4.3 Results

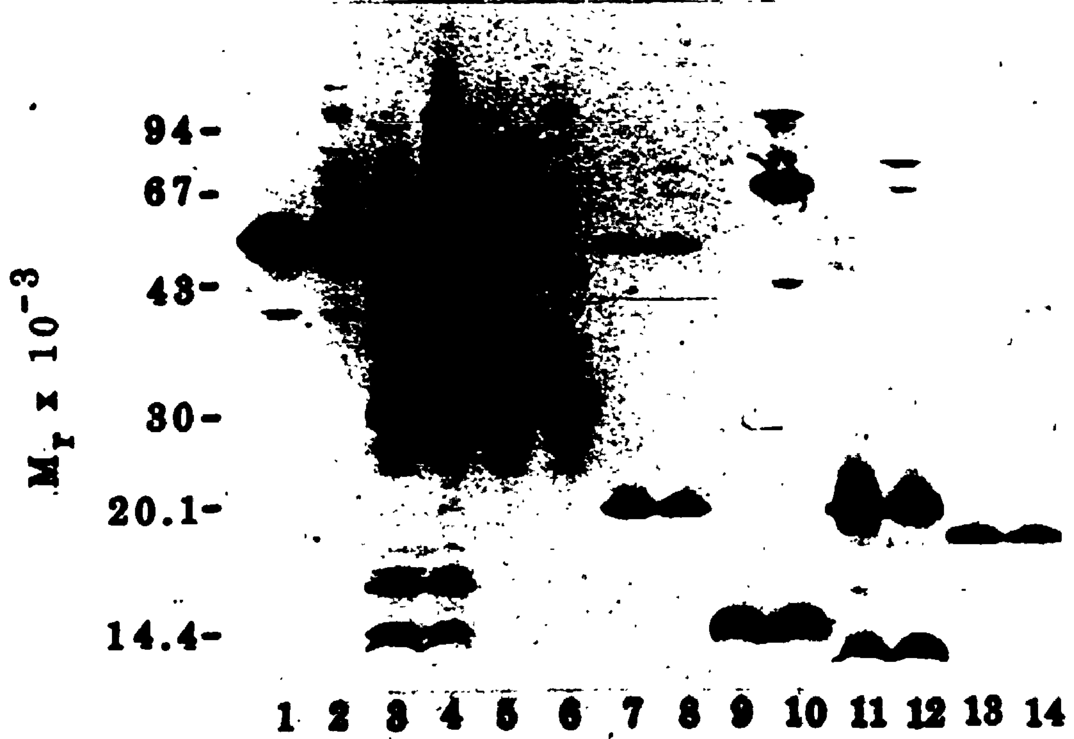
4.3.1 Cross-linking of Membranes

Membranes from *E. coli* strains AN1460 and ML308-225 were cross-linked with a variety of cross-linking reagents in order to investigate the subunit arrangement of F_1F_0. Figure 4.1 shows an experiment in which AN1460 membranes were cross-linked with DSP, a DTT-reversible, 8 carbon atom-long cross-linking reagent. It is a

Figure 4.1. *E. coli* membranes cross-linked with DSP. DSP was used to cross-link membranes from *E. coli* strain AN1460 as described in section 4.2.3. Samples were prepared for electrophoresis using NEM-containing SDS-sample buffer, electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel and electroblotted onto nitrocellulose as described in section 3.2.5. Lanes contained 50 µg of protein. The nitrocellulose was probed with the antibodies listed below followed by ^{125}I-anti-rat or ^{125}I-anti-rabbit antibodies and then radioautographed. Odd numbered lanes show DSP cross-linked membranes; Even numbered lanes show Me_2SO control membranes. Lanes show Western blots probed with the following antibodies: lanes 1 and 2, α-1; lanes 3 and 4, β-2; lanes 5 and 6, γ-1; lanes 7 and 8, δ-2; lanes 9 and 10, ε-1; lanes 11 and 12, affinity purified rabbit anti-b antibodies.

Figure 4.2. Cross-linking of *E. coli* membranes with EDC. *E. coli* AN1460 membranes were cross-linked with EDC as described in section 4.2.3. The samples were prepared for electrophoresis using DTT-containing SDS-sample buffer, electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel and electroblotted onto nitrocellulose as described in section 3.2.5. The lanes contained 50 µg of protein. The nitrocellulose was probed with the antibodies listed below followed by ^{125}I-anti-rat or ^{125}I-anti-rabbit antibodies and then radioautographed. Odd numbered lanes show H_2O control membranes; even numbered lanes show EDC cross-linked membranes. Lanes show Western blots probed with the following antibodies: lanes 1 and 2, α-1; lanes 3 and 4, β-2; lanes 5 and 6, γ-1; lanes 7 and 8, δ-2; lanes 9 and 10, ε-1; lanes 11 and 12, rabbit anti-δ antiserum; lanes 13 and 14, affinity purified rabbit anti-b antibodies.


94-
67-
43-
30-
20.1-
14.4-
$M_r \times 10^{-3}$
β-b
11 12


94-
67-
43-
30-
20.1-
14.4-
$M_r \times 10^{-3}$
1 2 3 4 5 6 7 8 9 10 11 12 13 14

homobifunctional imidoester that cross-links lysyl residues by reacting with their ε-amino groups. Cross-linked subunits were identified by comparing protein bands recognized by different monoclonal antibodies on Western blots in cross-linked and non-cross-linked membranes. As an example, note the protein migrating with an apparent molecular weight of 74,000 that was recognized by the anti-α antibody, α-3 in lane 1 and also by the anti-δ antibody, δ-2 in lane 7. As this band was not detected in non-cross-linked membranes (compare lane 1 with lane 2) and disappeared upon reduction with DTT (data not shown) it must contain cross-linked proteins. From this information the protein band must have resulted from cross-linkage of the α and δ subunits. Other predominant cross-linked products detected by this method included the 85-90,000-dalton β-γ product (lanes 3 and 5), the 66-69,000-dalton β-ε product (lanes 3 and 9), the 42-44,000-dalton γ-ε product (lanes 5 and 9). The α_2 (lane 1) and the α-β products (lanes 1 and 3) migrated with apparent molecular weights greater than 100,000 and are difficult to distinguish. All of these products have been reported for soluble F_1 (Bragg and Hou, 1980). The identity of the 85,000-dalton product recognized by the anti-α antibody in lane 1 is unknown. It may be an α_2 product which contains an intrasubunit cross-link leading to anomalous migration on the gel. It could also be an α-γ cross-linked product or a product involving an unknown membrane component. Nevertheless, these results indicate that at least at the level of resolution of the cross-linking reagent DSP, there is no large difference in subunit arrangement between soluble and membrane-bound F_1. These experiments were also repeated using membranes prepared from *E. coli* strain ML308-225 and the same cross-linked products were

observed (data not shown).

As stated in the introduction, a goal of these studies was to determine the nature of the subunit interactions between F_1 and F_0. The cross-link formed between β and F_0 subunit b is evident in lane 11, migrating with an apparent molecular weight of 82-84,000. The b_2 dimer was also formed, migrating with an apparent molecular weight of 35,000. Another protein band migrating with an apparent molecular weight of 28-31,000 may represent cross-linked b-c. These products have already been reported by Aris and Simoni (1983). No cross-linked product representing an ϵ-F_0 or δ-F_0 product was detected using the monoclonal antibodies (lanes 7 and 9) and either anti-b (lane 11) or anti-c antisera (data not shown).

EDC was also used to cross-link membranes from both *E. coli* strains AN1460 and ML308-225. EDC forms a zero-length cross-link between amino and carboxyl groups and its cross-linking of ϵ to a β subunit has been documented (Lötscher *et al.*, 1984). An experiment using AN1460 membranes is shown in Figure 4.2. The β-ϵ product was formed in membrane-bound F_1 migrating with an apparent molecular weight of 68,000 (lanes 4 and 10). This also demonstrates the similarity of quaternary structure of soluble and membrane-bound F_1 as the formation of this cross-link requires closely opposed reactive groups. Other cross-linked products formed included α-δ cross-linked products running with apparent molecular weights of 64-68,000 and 72,000 (lanes 2, 8 and 12), a γ-ϵ product migrating with an apparent molecular weight of approximately 46-48,000 (lanes 6 and 10), and a β-γ-ϵ product migrating with an apparent molecular weight of 86,000 (lanes 4, 6 and 10). A b_2 dimer with an apparent molecular weight of 35,000 was also formed (very

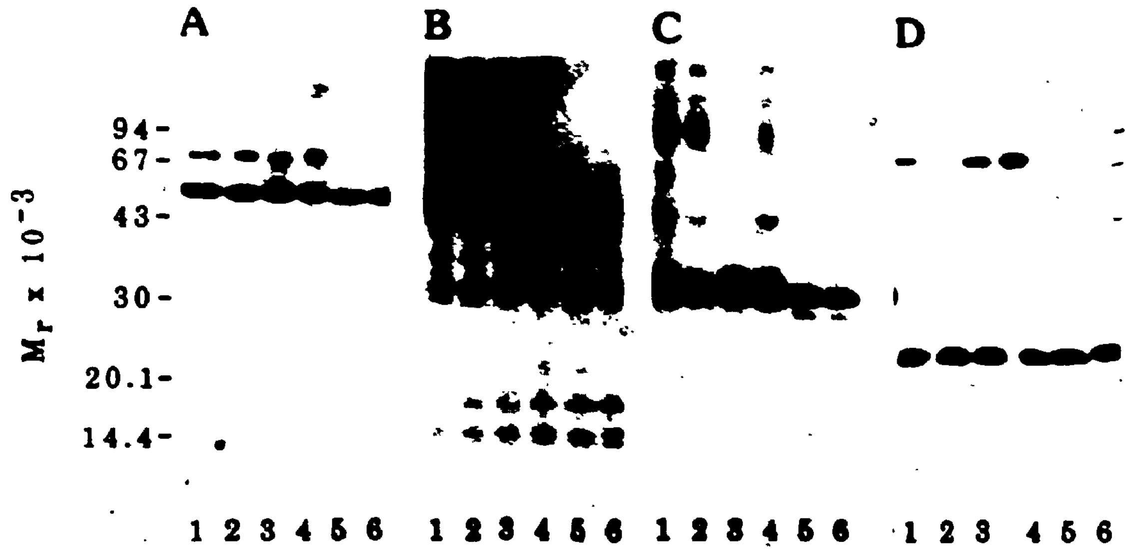
faint band in lane 14). The multiple α-δ products may result from intrasubunit cross-links or different sites of intersubunit cross-links. They may also result from different stages of proteolytic cleavage of the δ subunit. A cross-linked product recognized by the anti-δ antiserum and migrating with an apparent molecular weight of 36,000 is observed in lane 12. This protein was also observed in membranes cross-linked with DSP and probed with anti-δ antiserum (data not shown) and it disappeared when the cross-link was reversed with DTT. This product did not react with the anti-δ monoclonal antibody (lane 8), the anti-ϵ monoclonal antibody (lane 10) or with the anti-b (lane 14) or anti-c (data not shown) antisera. It is too small to contain δ cross-linked to subunit a. As it does not appear to contain any F_1 or F_0 subunits and it only reacts with the anti-δ antiserum not with the anti-δ monoclonal antibody, it is possible that it is an artifact resulting from lack of specificity of the antiserum. If it does involve δ it is possible that the formation of the cross-link destroys the δ-2 epitope accounting for its lack of recognition by the monoclonal antibody. The ϵ subunit also appeared to have been cross-linked to an unidentified protein yielding a product that migrated with an apparent molecular weight of 34,000 (lane 10). This product was not observed with other cross-linking reagents and was not observed when this experiment was repeated. Thus it may also be an artifact. The two cross-linked products did not react with either the anti-b or anti-c antisera (lane 14 and data not shown).

Note that in Figure 4.2, the anti-γ monoclonal antibody appears to recognize the α and β subunits (lanes 5 and 6), the anti-δ monoclonal antibody appears to recognize the α subunit (lanes 7 and 8) and the

anti-δ antiserum appears to recognize both α and β (lanes 11 and 12). This phenomenon occurs often when Western blots, in conjunction with the renaturation protocol, are used for analyses involving the F_1 minor subunits. As the subunits have largely regained their native conformations, if they desorb from the nitrocellulose they are capable of binding to their binding sites on the α and β subunits. This is particularly apparent with δ and ϵ because their small size leads to a weak interaction with the nitrocellulose and increases the probability of desorption.

Membranes from *E. coli* strain AN1460 were cross-linked with a variety of other cross-linking reagents in order to optimize the chance of cross-linking amino acid side chains with different chemical natures or distances of separation from those cross-linked by DSP, EDC or reagents used by other workers (Figure 4.3). EGS is a longer, non-reversible version of DSP with a length of 12 carbon atoms. BS^3 is the same length as DSP but is more hydrophilic. Sulfo-SMPB and sulfo-SMCC are hydrophilic, heterobifunctional reagents that cross-link lysyl and cysteinyl residues and have carbon atom-lengths of 8 and 6 respectively. None of these reagents are reversible by DTT. The same cross-linked products produced by DSP seem to have been also produced by most of the reagents with the following exceptions. Sulfo-SMCC and sulfo-SMPB did not produce the γ-ϵ product which migrated with an apparent molecular weight of 45-46,000 (panels C and F, lanes 4 and 5) and the β-ϵ cross-linked product which migrated with an apparent molecular weight of 74,000 (panels B and F, lanes 4 and 5). This probably reflects a lack of adjacent lysyl and sulfhydryl residues in the two subunits. Sulfo-SMCC also did not produce the β-γ product

Figure 4.3. *E. coli* membranes cross-linked with a variety of cross-linking reagents. *E. coli* AN1460 membranes were cross-linked with EGS, BS^3, sulfo-SMCC and sulfo-SMPB as described in section 4.2.3. Samples were prepared for electrophoresis with NEM-containing SDS-sample buffer, electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel and electroblotted onto nitrocellulose as described in section 3.2.5. Lanes contained 50 μg of protein. The nitrocellulose was probed with the antibodies listed below followed by ^{125}I-anti-rat or ^{125}I-anti-rabbit antibodies and then it was radioautographed. Panels show blots probed with the following antibodies: panel A, α-3; panel B, β-1; panel C, γ-1; panel D, δ-2; panel E, rabbit anti-δ antiserum; panel F, ε-1; panel G, affinity purified rabbit anti-b antibodies. Lanes contain membranes treated with the following reagents: lane 1, EGS; lane 2, BS^3; lane 3, sulfo-SMCC; lane 4, sulfo-SMPB; lane 5, Me_2SO control; lane 6, H_2O control.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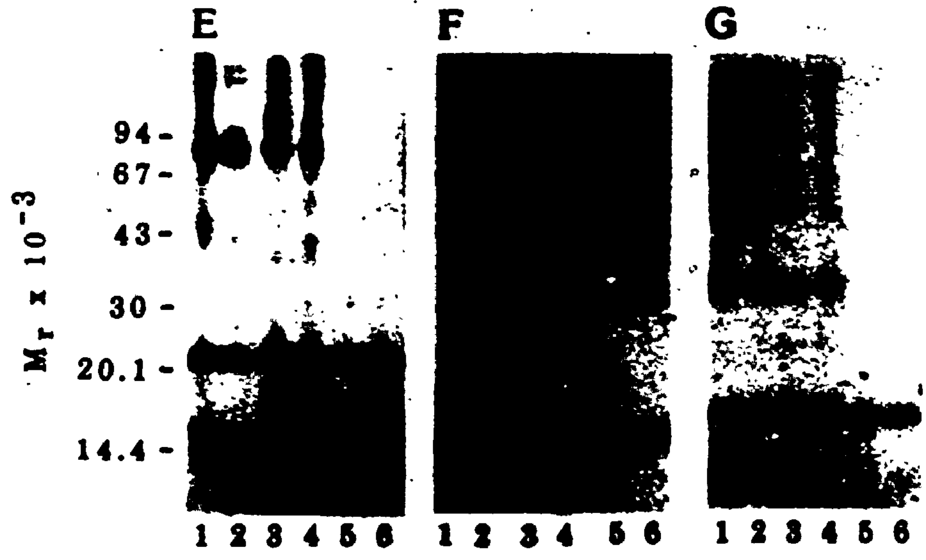
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which migrated elsewhere with an apparent molecular weight of approximately 94,000 (panels B and C, lane 4). This difference between sulfo-SMPB and sulfo-SMCC may reflect the shorter length sulfo-SMCC. As with DSP, all the cross-linked products observed with these reagents have been reported by other workers using different reagents and soluble F_1. The anti-δ antiserum recognized the same protein observed with the EDC in Figure 4.2 here migrating with an apparent molecular weight of approximately 36-40,000 (panel E, lanes 1 to 4). This protein band was not observed in non-cross-linked membranes (panel E, lanes 5 and 6). This protein band was not recognized by the anti-δ monoclonal antibody, δ-2 (panel D, lanes 1 to 4) thus exhibiting the same properties of the protein band seen with EDC.

Another cross-linking reagent, sulfo-SANPAH, was used on AN1460 membranes. This reagent is a non-reversible, heterobifunctional reagent that has an amino-reactive group at one end of the molecule and an aromatic azide at the other. Upon irradiation, the azido group is converted into a nitrene which can react with virtually any nearby covalent bond and form a new bond. Thus it is the least specific reagent used so far. It is 11 carbon atoms in length. This reagent was applied to AN1460 membranes (data not shown). The same products formed by the other reagents were also produced by sulfo-SANPAH. In particular it formed a 74,000-dalton α-δ product, a 68,000-dalton β-ε product, a 44,000-dalton γ-ε product, a 76,000-dalton β-b product and a 36-39,000-dalton b_2 dimer. The products observed in this experiment were produced with other cross-linking reagents and no interaction between either ε or δ with an F_0 subunit was demonstrated.

4.3.2 Cross-linking of F_1 in Presence of ε-4 Fab

As described in the section 4.1, ε-4 monoclonal antibody is capable of activating F_1 perhaps by altering the interactions between ε and the other subunits. Such alterations might lead to substantial changes in enzyme quaternary structure resulting in an altered cross-linking pattern. The cross-linking of ε to β and γ is well documented (see section 4.1) and quantitative changes in the formation of the β-ε and γ-ε cross-linked products would indicate alterations in subunit interactions.

Figure 4.4 shows an experiment in which F_1 was cross-linked with either EDC (panels A and B) or DSP (panels C and D) in the presence of ε-4 Fab. The β-ε cross-linked product which migrated with an apparent molecular weight of 68,000 was produced regardless of the presence of ε-4 Fab (panels A and C, lanes 3 and 5). The same was true for the β-γ product which migrated with an apparent molecular weight of 80,000 and is visible in panels B and D, lanes 3 and 5. The presence of ε-4 Fab also seemed to have little effect upon the amounts of cross-linked subunits produced. The γ-ε product, formed by DSP, migrated with an apparent molecular weight of 44,000 and thus unfortunately migrated with the same approximate molecular weight of the Fab fragment. ^{125}I-γ-1 detected γ-ε in panel D, lanes 3 and 5, but ^{125}I-ε-1 detected both ε-4 Fab and γ-ε (panels A and C, lanes 2, 3 and 5). Antibody ε-1 can detect ε-4 Fab because small amounts of ε dissociate from the nitrocellulose and bind to the Fab fragment. Since the Fab fragment and the iodinated antibody recognise distinct epitopes, they can bind simultaneously (see section 2.3.3). Consequently it is impossible to

Figure 4.4. Effect of ε-4 Fab upon EDC and DSP cross-linking of F_1. EDC and DSP cross-linking of F_1 in the presence or absence of ε-4 Fab was performed as described in section 4.2.4. Samples were prepared for electrophoresis using NEM-containing SDS-sample buffer, electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel and electroblotted onto nitrocellulose as described in section 3.2.5. Lanes contained 2.5 µg of F_1. The panels show blots probed with ^{125}I-ε-1 (panels A and C) and ^{125}I-γ-1 (panels B and D). The blots in panels A and B are an EDC cross-linking experiment and the blots in panels C and D are a DSP cross-linking experiment. The lanes are as follows: lane 1, F_1 standard; lane 2, control F_1 and ε-4 Fab; lane 3, cross-linked F_1 and ε-4 Fab; lane 4, control F_1; lane 5, cross-linked F_1.

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D
$M_r \times 10^{-3}$
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67
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1 2 3 4 5
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1 2 3 4 5
1 2 3 4 5
β-γ

quantitate differences in the formation of this cross-linked product using the anti-ε antibody. Nevertheless it is barely detectable by the anti-γ antibody and no change in amount of γ-ε seems to have occurred (panel D, lanes 3 and 5). This poor detection of γ-ε by the anti-γ antibody may result from the antibody epitope being destroyed by cross-linkage. Note that the γ-1 epitope is believed to be near the ε-binding site on γ (Section 2.5). It is also odd that the ε-4 Fab fragment was not cross-linked to ε (panels A and C). Nevertheless, ε-4 activation does not appear to be the result of a significant change in quaternary structure of the complex. This also shows that mere association of the ε subunit with F_1 and with β in particular, is not always inhibitory.

4.3.3 Cross-linking of F_1 in the Presence of Nucleotide and Ethylene Glycol

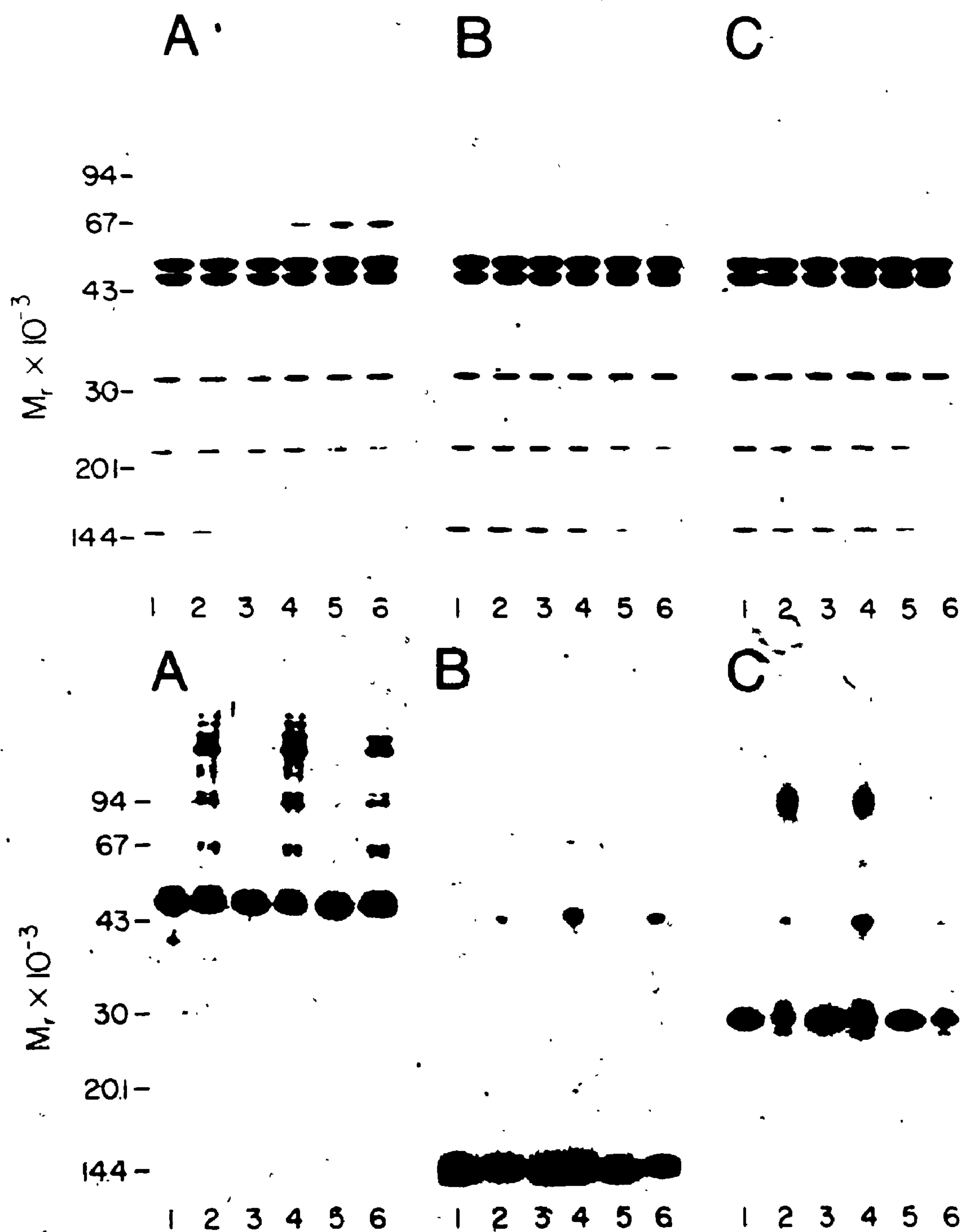
At concentrations of up to 30%, ethylene glycol activates the hydrolytic activity of ε-replete F_1 (Dunn and Zadorozny, unpublished results). In order to understand the mechanism of this effect, experiments similar to those using ε-4 Fab were performed. F_1 was cross-linked with EDC in the presence of various concentrations of ethylene glycol to see whether its presence affected the formation of the β-ε cross-link. The cross-linking reaction was terminated at various points of time to observe the rate of cross-link formation under the various conditions. MgADP was present in the cross-linking buffer to simulate nucleotide concentrations used in ATPase activity assays. MgATP was not used as its hydrolysis by the enzyme during the

course of the experiment would lead to different ratios of MgATP and MgADP concentrations at each time point. A Coomassie Blue stained gel of such an experiment is shown in Figure 4.5. The band that migrated with an apparent molecular weight of 68,000 is the β-ε cross-linked product. To confirm its identity, a Western blot using an identical gel was probed with iodinated anti-subunit monoclonal antibodies. The 68,000-dalton band was recognized by both the anti-β and anti-ε antibodies (data not shown). Panel A shows the EDC cross-linking pattern of F_1 in the absence of ethylene glycol and each lane shows a different time point; panels B and C show the same experiment performed in the presence of 30% and 40% ethylene glycol, respectively. In panel A one observes the increase in the formation of the cross-link over time. The amount of cross-link formed in the presence of 30% ethylene glycol (panel B) was less than that formed in its absence (panel A). Its formation in the presence of 40% ethylene glycol was even less still (panel C). Maximal ethylene glycol activation occurs at approximately 30% concentration. Activation could thus result from an alteration of the interaction between β and ε. Note the presence of high molecular weight cross-linked products in the three panels which were formed efficiently regardless of the presence of ethylene glycol. This indicates that ethylene glycol or impurities in it did not block cross-linking between β and ε by reacting with the EDC.

As inhibition by ε could also be mediated through the γ subunit, it was necessary to determine whether ethylene glycol altered the interaction of ε with γ. As EDC does not cross-link these subunits efficiently, DSP was used. Figure 4.6 shows radioautographs of Western blots of DSP cross-linked F_1. Due to the number of cross-links formed

Figure 4.5. EDC cross-linking of F_1 in the presence of various concentrations of ethylene glycol. F_1 was cross-linked with EDC in the presence of 0% (panel A), 30% (panel B) and 40% (panel C) ethylene glycol as described in section 4.2.5. Samples were prepared for electrophoresis using DTT-containing SDS-sample buffer, electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel which was stained with Coomassie Brilliant Blue R-250 (as described in section 2.2.8). Lanes contained 5 µg of protein. Samples in each lane were cross-linked for the following lengths of time: lane 1, "zero time;" lane 2, 5 min; lane 3, 10 min; lane 4, 20 min; lane 5, 40 min; lane 6, 60 min.

Figure 4.6. DSP cross-linking of F_1 in the presence of various concentrations of ethylene glycol. F_1 was cross-linked with DSP for 2 min in the presence of 0%, 30% and 40% ethylene glycol as described in section 4.2.5. Samples were prepared for electrophoresis using NEM-containing SDS-sample buffer, electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel and electroblotted onto nitrocellulose as described in section 3.2.5. Each lane contained 1.5 µg of protein. Panels show blots probed as follows: A, ^{125}I-β-2; B, ^{125}I-ε-1 plus ^{125}I-ε-4; C, ^{125}I-γ-1. Lanes 1, 3 and 5 show non-cross-linked zero time controls; lanes 2, 4 and 6 show cross-linked samples. Lanes 1 and 2 show F_1 in the presence of 0% ethylene glycol; lanes 3 and 4, 30% ethylene glycol; lanes 5 and 6, 40% ethylene glycol.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1 2 3 4 5 6

by this reagent, the patterns observed on a stained gel would be difficult to interpret and thus a gel is not shown. Each pair of lanes shows an experiment performed with a different concentration of ethylene glycol in the same buffer used in the previous EDC cross-linking experiment. In each pair of lanes, the first shows a zero-time point, and the second, a 2 min time point. DSP reacted far more efficiently than EDC even at a concentration of 0.1 mM and thus only the 2 min point was informative as subunit dimers were rapidly cross-linked to form high molecular weight aggregates. The blots shown in panels A, B and C were probed with iodinated β-2, ε-4 and γ-1 respectively. It can be seen that the amount of 44,000-dalton γ-ε cross-linked product was slightly greater in the presence of 30% ethylene glycol (panels B and C, lane 4) than in its absence (panels B and C, lane 2); however the amount of product was lower in the presence of 40% ethylene glycol (panels B and C, lane 6). This implies that at maximum ethylene glycol activation, the ε subunit is still bound to F_1 and its interaction with the γ subunit has not changed appreciably. This is consistent with ethylene glycol activation resulting from a change in the interaction between β and ε. This is also consistent with ε-inhibition resulting from β-ε interaction rather than γ-ε interaction. It is also interesting to observe that there does not appear to be a substantial decrease in the 68,000-dalton β-ε product formed by DSP at 30% ethylene glycol (panels A and B, lane 4). This contrasts with the result obtained using EDC. This probably results from the fact that EDC forms a zero-length cross-link whereas DSP can cross-link residues 1.2 nm apart. Thus EDC cross-linking will be more sensitive to subtle changes in quaternary structure. This result would

then imply that ϵ is still in close proximity to a β subunit, but that ethylene glycol moves specific regions of the two molecules further apart.

The detergent LDAO also leads to activation of F_1 (Lötscher *et al.*, 1984). Bragg and Hou (1986b) published results showing that the presence of 0.5% LDAO did not affect the formation of the β-ϵ cross-linked product by EDC. The experiment was repeated using conditions analogous to the ethylene glycol experiments and only a very small progressive decrease in the production of the β-ϵ cross-link in the presence of 0.1% and 0.3% LDAO was shown to occur (data not shown). Thus LDAO activation results from a different mechanism than that observed with ethylene glycol.

4.3.4 Determination of the Region of the ϵ-Binding Site on the β Subunit

Changes in the interaction of ϵ with the region of β to which it can be cross-linked by EDC activates the enzyme. Consequently, this region of β may have an important functional role in the activity of the enzyme. Knowledge of the location of this region within the primary structure of the β subunit would allow one to orient it with respect to other catalytically important regions as determined by modification of amino acid residues by chemical or genetic means. This could lead to a better understanding of the structure/function relationships within the β subunit. In section 3.3.3, the locations of the epitopes of the anti-β antibodies were determined through the use of partial cleavages of the β subunit and the identification of

fragments recognized by the antibodies using SDS-gel electrophoresis and Western blots. One cleavage method that proved to be very useful was weak acid hydrolysis using conditions that hydrolyze only aspartyl-prolyl peptide bonds. The β subunit contains three such bonds involving aspartyl residues 305, 334 and 345. The ϵ subunit contains none and is thus not affected by weak acid. The following experiment was performed to locate the site of the EDC cross-link between the β and ϵ subunits relative to the β subunit's aspartyl-prolyl bonds. Purified F_1 was cross-linked with EDC and the products were separated by SDS-polyacrylamide gel electrophoresis. Lanes containing the β-ϵ product were subjected to weak acid hydrolysis under conditions cleaving only a portion of the aspartyl-prolyl bonds, and then the fragments were separated on another SDS-polyacrylamide gel and electroblotted onto nitrocellulose. The Western blot was probed with either β-2 or ϵ-1 monoclonal antibodies and this experiment is shown in Figure 4.7.

The major components detected by antibody β-2 were residual cross-linked β-ϵ (68,000 daltons) and fragments with apparent molecular weights of 27,000, 28,000 and 34,000. The same set of proteins was recognized by antibody ϵ-1. These fragments must therefore contain the ϵ subunit and the carboxyl-terminal region of β. The mobilities of the fragments are also consistent with this assignment (compare with the fragments produced in chapter 3 from pure β in Figure 3.3). One should also note the trace amounts of β and ϵ subunit in these lanes, implying slight reversal of the cross-link during the acidic treatment. Thus the ϵ subunit makes contact with the carboxyl-terminal portion of β. It is interesting to note that this is the same region of β that

Figure 4.7. Weak acid cleavage of F_1-ATPase after EDC cross-linking. F_1 was cross-linked with EDC, then the β-ϵ product was separated from other proteins by SDS-polyacrylamide gel electrophoresis. The β-ϵ product was subjected to weak acid hydrolysis within the gel as described in section 4.2.6. Fragments were separated by electrophoresis on a 10 to 20% SDS-polyacrylamide gradient gel, then electroblotted onto nitrocellulose. The blot was blocked with BSA and exposed to ^{125}I-β-2 (lane 1) or ^{125}I-ϵ-1 (lane 2) and then radioautographed. These procedures are described in section 3.2.5.

Figure 4.8. Cross-linking of F_1 subunits to β-6 Fab fragment using BS^3. F_1 was cross-linked with BS^3 in the presence or absence of β-6 Fab as described in section 4.2.7. Samples were either alkylated with NEM or reduced with DTT, electrophoresed on a 10 to 20% SDS-polyacrylamide gradient gel and electroblotted onto nitrocellulose (as described in section 3.2.5). Each lane contained 1.5 µg of F_1. Blots were probed as follows: panel A, ^{125}I-β-2; panel B, ^{125}I-ϵ-1; panel C, ^{125}I-δ-2. Lanes in panels A, B and C are as follows: lane 1, F_1 cross-linked with BS^3 and alkylated; lane 2, F_1 and β-6 Fab cross-linked with BS^3 and alkylated; lane 3, alkylated F_1; lane 4, alkylated F_1 and β-6 Fab; lane 5, F_1 cross-linked with BS^3 and reduced; lane 6, F_1 and β-6 Fab cross-linked with BS^3 and reduced; lane 7, reduced F_1; lane 8, reduced F_1 and β-6 Fab.

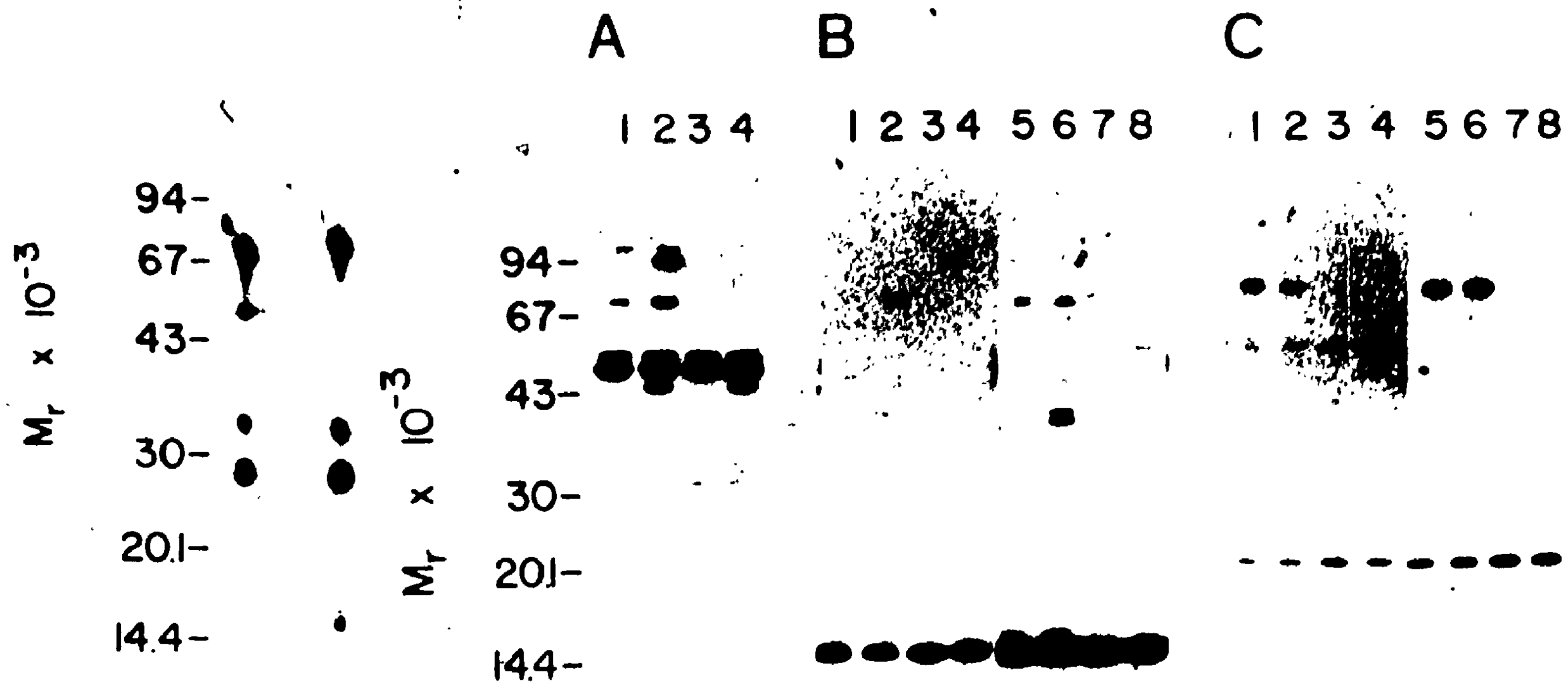


Figure 4.7

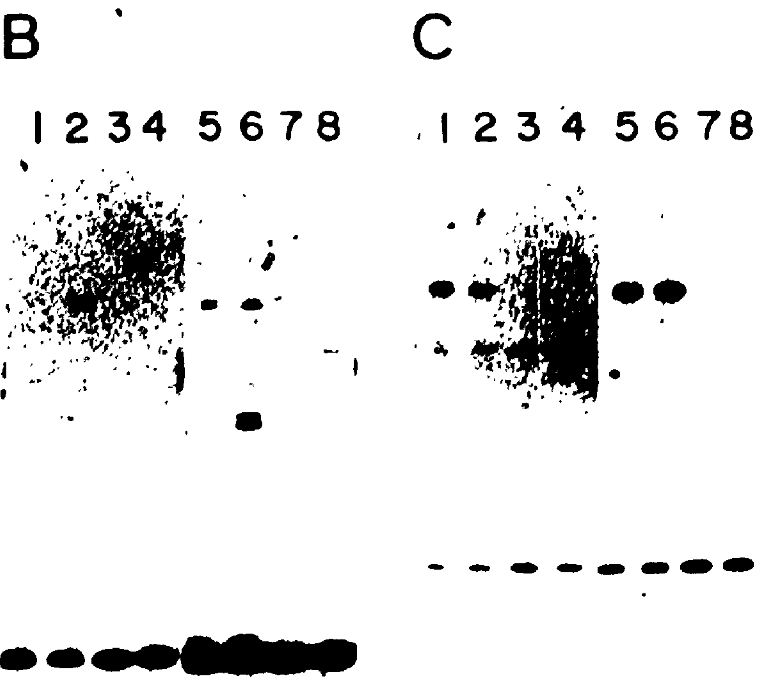


Figure 4.8

contains the epitopes of the inhibitory B-I anti-β monoclonal antibodies. This suggests that this region of the β subunit functions in a catalytic or regulatory role, as two agents which interact with it both exhibit inhibitory activity effects.

EDC also forms a slight amount of γ-ϵ cross-linked product. The γ subunit has a single aspartyl-prolyl bond located at position 211 which is cleaved by weak acid. Using the same procedure described for the β-ϵ cross-linked product, the γ-ϵ cross-linked product was subjected to weak acid hydrolysis. A fragment with an apparent molecular weight of 40,000 was generated that was recognized by both anti-γ and anti-ϵ monoclonal antibodies (data not shown). Its size is consistent for it being the weak acid fragment containing the first 211 residues of γ cross-linked to ϵ. Thus the ϵ-binding site on γ is located within the first 211 residues.

4.3.5 Proximity of Binding Sites for B-I Antibodies and ϵ on β in Native F_1

The similar effects of ϵ and the B-I antibodies on ATPase activity, and the proximity of their binding sites in the primary structure of β, suggested a relationship between their binding sites in the native enzyme. This possibility was investigated by treating mixtures of F_1-ATPase and Fab fragment prepared from the B-I antibody β-6 with various cross-linking reagents. Products were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. Some samples were reduced with DTT before electrophoresis to allow separation of the two chains of the Fab, while others were treated with

NEM. The blots were probed with iodinated monoclonal antibodies to each of the subunits of ATPase.

An experiment performed with BS^3, a non-reversible reagent that links lysyl residues, is shown in Figure 4.8. The blot in panel A was probed with ^{125}I-β-2, and contains samples which were not reduced before electrophoresis. The sample shown in lane 1 was cross-linked without Fab present, while the sample in lane 2 was cross-linked in the presence of β-6 Fab. Lanes 3 and 4 contain non-cross-linked controls. The labelling of β-6 Fab, which migrated just below β, by ^{125}I-β-2 probably reflects binding of free β which desorbed from the nitrocellulose by the Fab, and subsequent recognition of this bound β by the ^{125}I-β-2 (see section 4.3.1). In support of this hypothesis, the β-6 Fab was not labelled by iodinated monoclonal antibodies of the B-I group, as they possess the same binding site. Furthermore, ^{125}I-β strongly labelled Fab on the blot. Note the presence of a major product, with an apparent molecular weight of approximately 94,000, in lane 2, indicating formation of a cross-link between β and the Fab, as would be expected.

Panel B shows blots which were probed with ^{125}I-ϵ-1 to detect products containing ϵ. Lanes 1 to 4 are otherwise equivalent to those of panel A. A major ϵ-containing product with an apparent molecular weight of 70,000 can be seen in lane 2 but not in lane 1, suggesting the formation of an ϵ-Fab product. Potential ambiguity in this assignment arises because β-ϵ, which is produced with low efficiency by BS^3, migrates to an identical position in the gel. This ambiguity was resolved by a similar experiment in which the samples were reduced with DTT before electrophoresis (lanes 5 to 8). The major ϵ-containing

product in the reduced sample migrated with an apparent molecular weight of 39-41,000 (lane 6). The large decrease in size of this product upon reduction indicates that the ε was linked to the β-6 Fab. This result indicates that the ε subunit and the antibody β-6 bind to adjacent, non-overlapping sites when the β subunit is in its native form in F_1-ATPase.

No other subunits were cross-linked to the Fab in these experiments. An example is shown in panel C, which is similar to panel B, except that the blot was probed with ^{125}I-δ-2. The cross-linked product with an apparent molecular weight of 74,000 was produced regardless of the presence of Fab, and was unaffected by reduction. Thus it is the product of δ and another F_1 subunit. Recognition of this product by an anti-α antibody confirmed that it is an α-δ product. The specific cross-linking of β-6 Fab to the β and ε subunits was also demonstrated using sulfo-SMCC, a heterobifunctional, non-reversible cross-linking agent that cross-links lysyl and cysteinyl residues (results not shown).

4.4 Discussion

It appears that the quaternary structure of the soluble and membrane-bound forms of F_1 are very similar as the cross-linked products observed using soluble F_1 (see section 4.1) can be produced in membrane-bound F_1. It is striking that a cross-link has yet to be observed either between the δ and ε subunits or between these subunits and the subunits of F_0. Though some of the protein bands seen produced by the cross-linking reagents on Western blots are suggestive, they are

either not recognised by anti-sera raised against subunits b and c or their molecular weights are too small to involve subunit a. As stated in the introduction, several models of F_1 structure place the δ and ε subunits across from one another on the face of the holoenzyme that interacts with F_0 (Bragg and Hou, 1980; Aris and Simoni, 1983; Futai and Kanazawa, 1983 and see Figure 1.1). The inability to cross-link δ to ε implies that either the subunits are not actually located near one another or that the the amino acid side chains that would react with the cross-linking reagents are not favourably oriented. The same argument applies to the location of δ and ε with respect to F_0 subunits. It is interesting to note that although δ and ε are required for F_1 binding to F_0, neither the individual subunit nor both subunits together have been shown to bind to membranes (Dunn and Heppel, 1981). Attempts are in the process of being made by Dunn and coworkers to locate the δ and ε subunits within F_1 using anti-δ monoclonal antibodies, colloidal gold-labelled ε subunit, ε-depleted F_1 and electronmicroscopy.

The activation of F_1 by two different agents, ε-4 Fab and ethylene glycol, was studied by observing the effects these agents had upon cross-link formation. Changes in the nature or amount of products formed would reflect conformational changes brought about by these agents. The anti-ε antibody, ε-4 led to no detectable displacement of the ε subunit from the β subunit, suggesting two possibilities. Either any conformational change induced between the β and ε subunits is too subtle to be detected by this method or it is an alteration in the association of γ with ε that is crucial. Unfortunately change in cross-link formation between the γ and ε subunits was not easily

measured in this experiment. Nevertheless this demonstrates that simple association of ε with β is not always inhibitory. A similar effect was observed with the detergent LDAO. In contrast, the activation of the F_1 in the presence of 30% ethylene glycol was concurrent with a decrease in the amount of ε that was cross-linked to β by EDC. The association of ε with γ actually increased slightly at maximum ethylene glycol activation. This result implies that inhibition is probably mediated through a β subunit rather than the γ subunit. It was interesting that the changes in association of β with ε brought about by this agent, were not detectable when DSP cross-linking was studied, implying that this effect involves a fairly localized displacement of ε from β. Another agent which activates F_1 is the antibody ε-1 and its effect results from competition with F_1 for the binding of ε (Dunn and Tozer, 1987).

The binding of the B-I antibodies or the ε subunit to the carboxyl-terminal domain of β inhibits ATP hydrolysis by 85-90%. The anti-β antibodies and the ε subunit are able to bind simultaneously to sites in this region of β and are able to be cross-linked to one another. This shows that this area contains regions of primary structure that are folded into a compact structure. This lends credence to the idea put forth in chapter 3 that this region is organized into a distinct domain. Studies of the effects of the ε subunit during unisite catalysis indicate that ε reduces the rate of product release but does not alter the equilibrium between ATP and ADP·P_i at the catalytic site (Dunn *et al.*, 1987). The ε subunit also increases the probability of oxygen exchange during ATP hydrolysis (Wood *et al.*, 1987). As stated in chapter 3, it is possible that

movement of this carboxyl-terminal domain is necessary for product binding and release. Perhaps the presence of ϵ subunit bound to this carboxyl-terminal domain hinders its motion, slowing the release of product. Alternatively, if one accepts the rotational theory (Cox *et al.*, 1984; Mitchell, 1985), in which the single-copy subunits rotate relative to the α/β hexamer during catalysis, product release could require ϵ to move onto the next β subunit.

It has been demonstrated that ϵ binds tightly to γ (Dunn, 1982). Based on the effects of sulfhydryl reagents, McCarty (1979) suggested that the γ subunit of chloroplast F_1-ATPase plays a role in channelling the flow of protons through the enzyme. Though ϵ is a partial inhibitor of the activity of soluble F_1 (see section 4.1), it is present in F_1F_0 and the enzyme is active. It has even been demonstrated that the binding of ϵ-inhibited F_1 to membranes leads to its activation (Sternweis and Smith, 1980). This may indicate that ϵ interacts with β or γ differently in the two complexes. This does not appear to be the result of any gross change in quaternary structure as ϵ can be cross-linked to both β and γ in the two complexes (especially notable in the efficiency of EDC cross-linking of ϵ to β in membranes).

The above suggest a possible role for the ϵ subunit in F_1F_0. The ϵ subunit could serve as a means for coupling the flow of protons through F_0 and γ to the conformational changes that occur in the β subunits during catalysis. Chloroplast F_1 lacking ϵ will bind to F_0 but will not prevent proton leakage through F_1 (Richter *et al.*, 1984) also suggesting a role for ϵ in coupling. ϵ may also serve as a means to ensure that F_1 is only active in the cell when coupled to F_0 thus preventing potentially deleterious hydrolysis of ATP.

Areas currently under investigation by other workers in the laboratory concern the effects of the anti-β antibodies upon unisite catalysis particularly in comparison to those seen with ε. As these proteins bind to the same region of β one might expect to observe some similarities in effects. However it is notable that the anti-β monoclonal antibodies and ε subunit are able to partially counteract one another's effects (Dunn and Zadorozny, unpublished results).

5.0 FORMATION OF A DISULFIDE BRIDGE IN F_1-ATPASE

5.1 Introduction

During the preparation of F_1 for several of the cross-linking studies (chapter 4), it was necessary to change the buffer of the sample using column centrifugation. This method, developed by Penefsky (1979) is used primarily for small volumes. It is faster than dialysis and has reasonably good sample recovery. After experiments using centrifuge columns equilibrated with certain buffers a high molecular weight product was observed using SDS-polyacrylamide gel electrophoresis. It was discovered that a cross-link had formed between two subunits and that the reaction was quantitative.

In the study of *E. coli* F_1-ATPase, the formation of a such a cross-link could serve as a means to test a recent model of the mechanism of the enzyme. This model was originally proposed by Gresser and coworkers (1982) and was developed more fully by Cox and coworkers (1984). A slightly different version was also proposed by Mitchell (1985). According to the binding change mechanism (Cross, 1981), there are two or three sites that alternate between catalytic and promotional roles during the catalytic cycle. If this mechanism is correct, during the catalytic cycle each α/β pair of subunits should be in different conformations reflecting differences in their respective promotional or catalytic roles. A certain amount of asymmetry has already been shown for the β subunits in terms of chemical reactivity and also for the α subunits in terms of nucleotide-binding site occupancy (see section 1.3). It is important to note that the asymmetry observed in these

experiments may reflect either transient or permanent differences between subunits; these alternatives could not be distinguished.

The γ subunit is required for proper assembly of the α and β subunits into a catalytically competent $\alpha_3\beta_3\gamma$ complex. Its function has therefore been defined as organizational (Dunn and Futai, 1980). This may be a simplification as antibodies raised against the *E. coli* γ subunit and modification of chloroplast γ subunit sulfhydryl groups both inhibit enzyme activity (Smith and Sternweis, 1982; Nalin *et al.*, 1983). Thus important interactions must occur between the γ and the α and β subunits that are not purely organizational in nature and may have some influence upon the enzyme's catalytic activity (McCarty, 1979). It is notable that although a single γ subunit interacts with three α/β pairs of subunits that may undergo identical cycles of conformational change, the γ subunit shows no evidence of threefold symmetry in its amino acid sequence (Walker *et al.*, 1984). Thus each α/β pair of subunits interacts with a distinct γ surface. The rotational model proposes that cyclical conformational and functional changes are concurrent with rotation of the α/β pairs around the γ subunit. A different surface of the γ subunit would be associated with each α/β pair depending upon the stage in the catalytic cycle. The remaining two minor subunits, δ and ε, are proposed to remain associated with the γ subunit and thus the α/β hexamer should rotate with respect to them also. One means of testing this hypothesis would be to cross-link a major subunit to a minor subunit.

In this chapter, the conditions that lead to the formation of the cross-link in F_1 following column centrifugation are examined. In addition, its effects upon the activity of the enzyme and the ability

of F_1 to interact with F_0 are detailed. Finally the implications that the formation of this cross-link has upon the rotational model are discussed.

5.2 Materials and Methods

5.2.1 Materials

Sephadex G-50 was purchased from Pharmacia P-L Biochemicals (Uppsala, Sweden). Sodium periodate and o-1,10-phenanthroline were purchased from Sigma (St. Louis, Mo.). Whatman 3MM paper was obtained from Canlab (Mississauga, Ont.). The sources of other materials were listed in previous materials sections.

5.2.2 Preparation of F_1, Subunits and Membranes

E. coli strains ML308-225 (*unc*$^+$) (Simoni and Shallenberger, 1972), AN120 (*uncA401*) (Butlin *et al.*, 1971) and AN1460(*unc*$^+$) (Downie *et al.*, 1980) were grown as described previously (see section 2.2.2). F_1 was prepared from strain ML308-225 and δ and ϵ subunits were prepared from strain AN1460 using published procedures (see section 2.2.2). AN120 F_1-depleted membranes were prepared using the methods for F_1 purification except that the final-low-ionic strength wash was repeated twice and the membranes were saved.

5.2.3 Column Centrifugation

Centrifuge columns were prepared essentially as described by Penefsky (1979) using 1-ml or 3-ml disposable syringes, a glass wool plug and Bio-Gel P-10 or Sephadex G-50 resin. The column was equilibrated with 5 column volumes of the appropriate buffer and centrifuged for 2 min at 2000 rpm in a Sorvall General Laboratory Centrifuge. The relative centrifugal force generated at the tip of the syringe was 540 x g. The protein was then applied to the top of the partially dehydrated resin, the column was centrifuged as before, and the eluate was collected. Unless otherwise specified, the eluate was incubated for 10 min at room temperature before EDTA was added to a final concentration of 2 mM.

5.2.4 Assays

Binding of F_1 to membranes was measured by preparing F_1 with columns of Bio-Gel P-10 equilibrated with 50 mM Mops-NaOH, pH 7.5, 10 µM $CuCl_2$, with or without the addition of 2 mM EDTA. Once the enzyme had been eluted and incubated at room temperature for 10 min, glycerol was added to 10%, ATP to 0.5 mM, and EDTA to 2 mM final concentrations. Various amounts of cross-linked or non-cross-linked F_1 were added to AN120 F_1-depleted membranes and binding was allowed to occur by incubating the mixture at 37°C for 10 min in a reconstitution buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10% glycerol and 100 µg/ml defatted BSA. Controls in which no membrane was present were also prepared. One hundred-µl samples were centrifuged at 111,000 x g

for 3 min in a Beckman Airfuge ultracentrifuge using an 18° A-100 rotor. The supernatant was recovered and assayed for ATPase activity using the method of Futai and coworkers (1974).

Protein concentrations and ATPase activity were assayed as described in section 2.2.8. When the activity of membrane-bound F_1 and soluble F_1 were measured in the same experiment, the incubation buffer contained 50 mM Tris-HCl, pH 8.0, 13 mM $MgCl_2$, 4 mM ATP and 10 μg/ml defatted BSA. ATP-dependant transhydrogenase activity was measured by the method of Smith and Sternweis (1977).

5.2.5 Other Methods

Gel electrophoresis and Western blotting were described previously (sections 2.2.8 and 3.2.5). Immunoprecipitations were performed as described previously (section 2.2.5) except that the Sepharose was not washed after incubation with purified F_1. The supernatant was removed by aspiration, excess liquid was removed using a small strip of Whatman 3MM paper and the proteins were solubilized as in section 2.2.5. Rabbit anti-rat IgG was coupled to Sepharose as described previously (section 2.2.4). Densitometry was performed using a Beckman DU-8 Vis-UV spectrophotometer equipped with a slab gel scanner system. Gels were scanned at 550 nm.

Cupric phenanthrolinate cross-linking of the α and δ subunits was performed using methods described by Bragg and Hou (1980). Cross-linking of the subunits with sodium periodate was performed by incubation of 1 mg/ml F_1 in 50 mM glycylglycine-NaOH, pH 7.5, 5 mM $MgCl_2$, 100 μM $NaIO_4$ for 30 min in the dark. The reaction was

terminated by the addition of ethylene glycol to 20% final concentration. Cross-linking of the subunits by aeration of the dialysis buffer was performed as follows. Purified F_1 at a concentration of 2 mg/ml was dialyzed for 5 h against 50 mM sodium phosphate, pH 7.5, 10 µM $CuCl_2$, 20% glycerol. The buffer was aerated throughout the experiment by bubbling air into it and stirring vigorously.

Distilled, deionized water was used in all experiments.

5.3 Results

5.3.1 Formation and Identification of Cross-linked Product

When *E. coli* F_1-ATPase was passed through a centrifuge column consisting of Bio-Gel P-10 or Sephadex G-50 equilibrated with 50 mM sodium phosphate, pH 7.5, as described in section 5.2.3, and the eluate was electrophoresed on an SDS-polyacrylamide gel under non-reducing conditions, a new protein band was observed migrating with an apparent molecular weight of 72,000 (Figure 5.1, lane 2). The product was not observed if 50 mM DTT was incorporated in the SDS-sample buffer indicating that the product resulted from the formation of a disulfide bridge (Figure 5.1, lane 6).

Experiments were performed to determine the nature of the reaction leading to the formation of this product and these are described below and shown in Figure 5.2. The band was not observed if F_1 was passed through a column equilibrated with sodium phosphate, with the addition of 2 mM EDTA (lane 3). The formation of the product was not observed

Figure 5.1. Chemical nature of the cross-link. F_1-ATPase was run through a centrifuge column equilibrated with 50 mM sodium phosphate pH 7.5, as described in section 5.2.3. Portions of the sample were prepared for electrophoresis as described below and electrophoresed on a 12% SDS-polyacrylamide slab gel as described in section 2.2.8. The lanes are as follows: lane 1, F_1 standard; lane 2, column-centrifuged F_1 alkylated in SDS-sample buffer containing 10 mM NEM; lane 3, column-centrifuged F_1 reduced in SDS sample buffer containing 50 mM DTT. Numbers on the right indicate molecular mass in kilodaltons.

Figure 5.2. Formation of the 72,000-dalton product upon column centrifugation of F_1. Purified F_1-ATPase was run through centrifuge columns and electrophoresed on a 12% SDS-polyacrylamide slab gel as described in section 5.2. Lanes 1 and 7 contain alkylated F_1 standards. The centrifuge column buffers of lanes 2-6 were as follows: lane 2, 50 mM sodium phosphate, pH 7.5; lane 3, 50 mM sodium phosphate, pH 7.5, 2 mM EDTA; lane 4, 50 mM triethanolamine-HCl, pH 7.5; lane 5, 50 mM triethanolamine-HCl, pH 7.5, 10 µM $CuCl_2$; lane 6, 50 mM triethanolamine-HCl, pH 7.5, 10 µM $CuCl_2$, 2 mM EDTA; for lanes 8-12, F_1 was centrifuged in 50 mM sodium phosphate pH 7.5, and the concentrations of EDTA and NEM were brought up to 2 mM and 10 mM, respectively at the indicated times: lane 8, zero time (F_1 spun directly into EDTA and NEM); lane 9, 5 min; lane 10, 10 min; lane 11, 15 min; lane 12, 30 min.

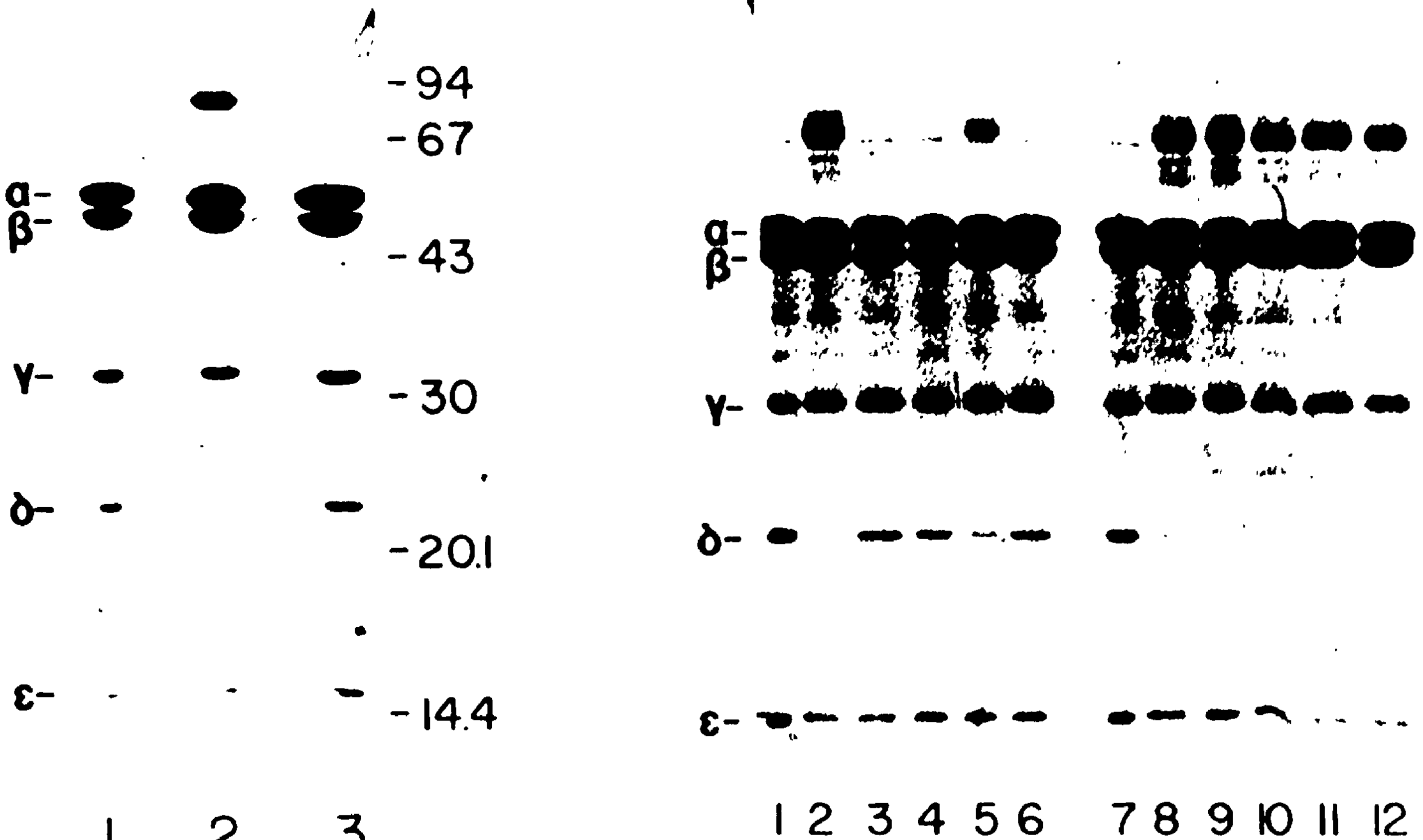


Figure 5.1

Figure 5.2

if the column was equilibrated with a triethanolamine buffer (lane 4). If 10 µM $CuCl_2$ was added to the triethanolamine buffer, the product did appear (lane 5). If 2 mM EDTA was added to the $CuCl_2$-supplemented triethanolamine buffer, the product was not observed (lane 6). Further experiments (not shown) revealed that a set of buffers including carbonate, Mops, Hepes, Pipes, Tes and imidazole all led to the formation of the 72,000-dalton product only if $CuCl_2$ was added. Glycylglycine, ethylenediamine, and tricine buffers did not lead to the appearance of the product even with the addition of $CuCl_2$. These buffer effects all indicated that there was an involvement of heavy metal ions such as copper (II) in the formation of the product. The appearance of the product in a buffer not supplemented with $CuCl_2$ implied that the buffer had sufficient heavy-metal-ion contamination to catalyze the reaction leading to product formation. Most of the buffers that did not lead to the formation of the product even after supplementation with $CuCl_2$ were found to be fairly strong metal ion chelators (Hughes, 1972).

The kinetics of the formation of the 72,000-dalton product were studied by centrifuging F_1 through a column and then stopping the reaction at various time intervals by the addition of 10 mM NEM and 2 mM EDTA (Figure 5.2). A zero-time point was obtained by centrifuging the F_1 into a solution containing NEM and EDTA. The reaction was virtually complete at this point (lane 8) and no additional product was formed even with a 30-min incubation period before the addition of EDTA and NEM (lane 12). The majority of the reaction must therefore take place on the column and since Penefsky (1979) found that most of the applied protein passes through centrifuge columns in the space of 30 s,

the reaction must be extremely rapid.

The 72,000-dalton product could be generated by three other methods. It could be generated by copper phenanthrolinate (data not shown), a reagent commonly used to generate disulfide bridges. Sodium periodate also catalyzed the formation of the cross-link (data not shown). The cross-link could also be generated by aerating the buffer against which the enzyme was being dialyzed. None of these methods resulted in as high a yield of the 72,000-dalton product as column centrifugation. Sodium periodate was the least efficient method overall.

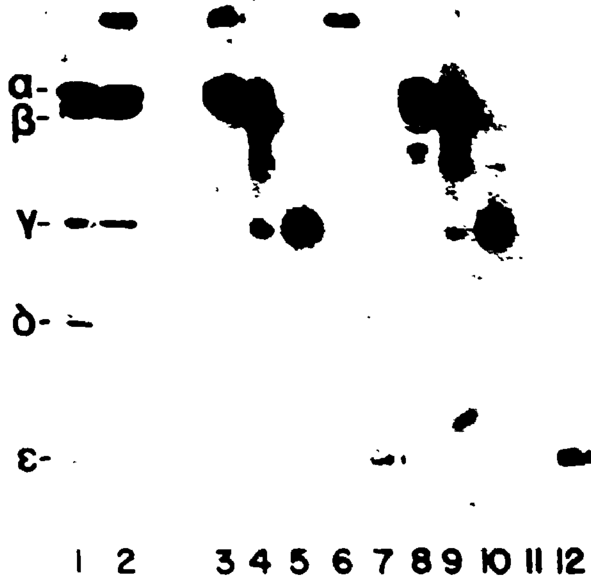
The product was identified by running column-centrifuged F_1 on a 12% SDS-polyacrylamide gel, electroblotting the protein onto nitrocellulose and then probing the nitrocellulose with subunit-specific monoclonal antibodies. As can be seen in Figure 5.3, the 72,000-dalton product was recognized only by antibodies specific to the α and δ subunits (lanes 3 and 6), indicating that the product is an α subunit cross-linked to a δ subunit. The apparent molecular weight of the product was also reasonable for a complex of the 55,000-dalton α subunit and the 19,000-dalton δ subunit.

5.3.2 Integrity of F_1 After Formation of the Cross-link

It seemed possible that the cross-linked product was formed from dissociated subunits rather than on the intact enzyme. It was also possible that if the cross-link formed on the intact enzyme, it would lead to dissociation of the subunits. To determine whether either situation was occurring, immunoprecipitations were performed with

Figure 5.3. Identification of the 72,000-dalton product using subunit-specific monoclonal antibodies. Purified F_1-ATPase was run through centrifuge columns as described in section 5.2.3. Samples were electrophoresed on a 12% SDS-polyacrylamide slab gel. Portions of the gel were stained with Coomassie brilliant blue R-250 and other portions were electroblotted onto nitrocellulose. Nitrocellulose strips were blocked with BSA, exposed to 1/100 dilutions of hybridoma culture media, followed by ^{125}I-anti-rat IgG, and then radioautographed. Details are described in section 3.2.5. Centrifuge column buffer contained 50 mM sodium phosphate, pH 7.5 with (lanes 1 and 8-12) or without (lanes 2 and 3-7) 2 mM EDTA. Lanes 1 and 2 are from the stained gel. The remaining lanes are from the radioautograph: lanes 3 and 8, α-3; lanes 4 and 9, β-1; lanes 5 and 10, γ-1; lanes 6 and 11, δ-2; lanes 7 and 12, ϵ-1.

Figure 5.4. Immunoprecipitations of column-centrifuged F_1-ATPase. F_1 was run through a centrifuge column equilibrated with 50 mM sodium phosphate. Immunoprecipitations were performed and the samples electrophoresed on a 10-20% SDS-polyacrylamide gradient gel. These methods are described in section 5.2. The lanes are as follows: lane 1, column-centrifuged F_1 immunoprecipitated by δ-2 monoclonal antibody; lane 2, column-centrifuged F_1, control with no antibody; lane 3, column-centrifuged F_1 with one equivalent of added δ subunit, immunoprecipitated by β-1 monoclonal antibody; lane 4, column-centrifuged F_1 with one equivalent of added δ subunit, control with no antibody; lane 5, column-centrifuged F_1; lane 6, column-centrifuged F_1 with one equivalent of added δ subunit; lane 7, F_1; lane 8, F_1 with one equivalent of added δ subunit.


α-
β-
γ-
δ-
ε-
1 2 3 4 5 6 7 8 9 10 11 12

α-
β-
γ-
δ-
ε-
1 2 3 4 5 6 7 8

column centrifuged F_1 to see if the cross-linked subunits would be immunoprecipitated with the rest of the complex. Immunoprecipitations were performed as described in section 2.2.5 and are illustrated in Figure 5.4. Note that in lanes 1 to 4, the band migrating just above the band corresponding to the α subunit is non-reduced BSA and that the band migrating near the top of the gel in lanes 1 and 3 is non-reduced monoclonal antibody. As isolated F_1 is generally partially deficient in δ subunit (Sternweis, 1977), one molar equivalent of δ subunit was added to F_1 prior to centrifugation on the column for use in the immunoprecipitation using the anti-β monoclonal antibody. Both the anti-δ (lane 1) and the anti-β (lane 3) monoclonal antibodies were capable of immunoprecipitating the α-δ cross-linked product as well as the rest of the enzyme. This indicated that the α-δ cross-linked subunits were still associated with the rest of the complex.

Densitometry was used to quantify this result. The gel was scanned as described in section 5.2.5 and the areas under the peaks were calculated. In each lane the area of each subunit was normalized with respect to the area corresponding to the γ subunit. The normalized area of the α-δ cross-linked product was 2.7 in the anti-δ immunoprecipitation and it was 2.2 in the anti-β immunoprecipitation. The similarity of these figures indicates that the α-δ cross-linked product is being immunoprecipitated in a constant ratio to the γ subunit and therefore must be an integral part of the enzyme complex. Comparison of the areas of the subunit peaks between cross-linked and non-cross-linked F_1 revealed that approximately 25% of the α subunit took part in the formation of the α-δ cross-linked product which is very close to the 33% of the α subunits one would expect if the

reaction was quantitative with respect to the α subunit. All of the δ subunit was cross-linked to the α subunit, as shown by the complete disappearance of the δ band. Thus with respect to both the α and δ subunits, the formation of this cross-link is quantitative.

5.3.3 Effects of α-δ Cross-link on Activity of the Enzyme

Since a cross-link could be specifically and quantitatively generated between two subunits in F_1 without leading to subunit dissociation, it was of interest to determine whether the cross-link had any effects on the activity of the soluble and membrane-bound forms of the enzyme. Soluble F_1 with one added equivalent of purified δ subunit was passed through a centrifuge column equilibrated with 50 mM Tris-HCl, pH 7.5 and 10 μM $CuCl_2$. Non-cross-linked control F_1 was produced by the addition of 2 mM EDTA to the column buffer. As shown in Table III, the specific activities of the cross-linked and non-cross-linked enzymes were the same. Table III also shows the extent of inhibition of cross-linked and non-cross-linked F_1 by the ε subunit. The amount of ε-inhibition at various amounts of added ε and the maximal extent of ε-inhibition were the same for both the cross-linked and non-cross-linked forms of the enzyme. Thus neither the affinity of ε for F_1, nor the amount of residual activity at maximum ε-inhibition was changed by the formation of the cross-link.

Experiments were performed to determine whether the cross-linked enzyme was able to function when bound to F_0. Cross-linked F_1 was unable to stimulate ATP-dependent transhydrogenase activity in F_1-depleted membranes (data not shown) and this implied that the enzyme

TABLE III

ATPase activity of cross-linked F_1 and inhibition by ε subunit

ε added	Specific activity of F_1	
	control	cross-linked
ng	units/mg	
0	56.1	54.0
6.5	32.8	33.8
13	26.4	25.9
52	16.4	16.1
210	10.1	10.5

Cross-linked F_1 was produced by centrifugation through columns equilibrated with 50 mM Tris-HCl, pH 7.5, 10 μM $CuCl_2$. Non-cross-linked (control) F_1 was produced in the same manner except that 2 mM EDTA was added to the column buffer. Note that one equivalent of purified δ subunit was added to each sample before column centrifugation. The F_1 solution was brought up to 10% glycerol, 0.5 mM ATP and 2 mM EDTA following a 10 min incubation at room temperature. Assays were performed as in section 5.2.4 and each contained 0.16 μg of F_1.

was unable to couple the pumping of protons across the membrane to the hydrolysis of ATP. One possible explanation for this result was that F_1 was unable to bind to F_0 and proton pumping was not coupled to ATP hydrolysis.

To determine whether the modified enzyme was capable of binding to F_0, the following experiment was performed. Various amounts of cross-linked or non-cross-linked F_1 were added to F_1-depleted membranes and the mixtures were incubated under conditions that would promote F_1 binding to F_0. The sample was centrifuged and the supernatant, containing soluble F_1, was assayed for ATPase activity. If F_1 was capable of binding to F_0, it should have sedimented with the membranes during centrifugation, resulting in a depletion of ATPase activity in the supernatant. Once the F_1-binding sites became saturated with F_1, the amount of F_1 in the supernatant would begin to increase. As can be seen in Figure 5.5, panel A, a control using non-cross-linked F_1, approximately 2 μg of F_1 specifically bound to the membranes. The same experiment performed with cross-linked F_1 (Figure 5.5, panel B) demonstrated no specific binding to membranes. The slightly greater amount of cross-linked F_1 that was sedimented in the presence of membranes in contrast to the amount sedimented in their absence may be due to a small population of F_1 that was not cross-linked and thus still capable of binding to F_0. Non-specific binding to membranes and trapping by membranes may also have contributed to the small amount of binding.

A final experiment determined whether the α-δ cross-link could be formed while the enzyme was still associated with F_0. These experiments are illustrated in Figure 5.6. *E. coli* membranes were

Figure 5.5. Binding of cross-linked (B) and non-cross-linked (A) F_1-ATPase to membranes. F_1 was prepared by column centrifugation and incubated with AN120 F_1 depleted membranes. Free and membrane-bound F_1 were separated by centrifugation. Depletion of F_1 in the supernatant was measured by a colorimetric assay. Controls in which no membrane was present were also prepared. Details are described in section 5.2. The panels are as follows: panel A, experiment performed using non-cross-linked F_1 ; panel B, experiment performed using cross-linked F_1. (●) In presence of membranes; (▲) in absence of membranes.

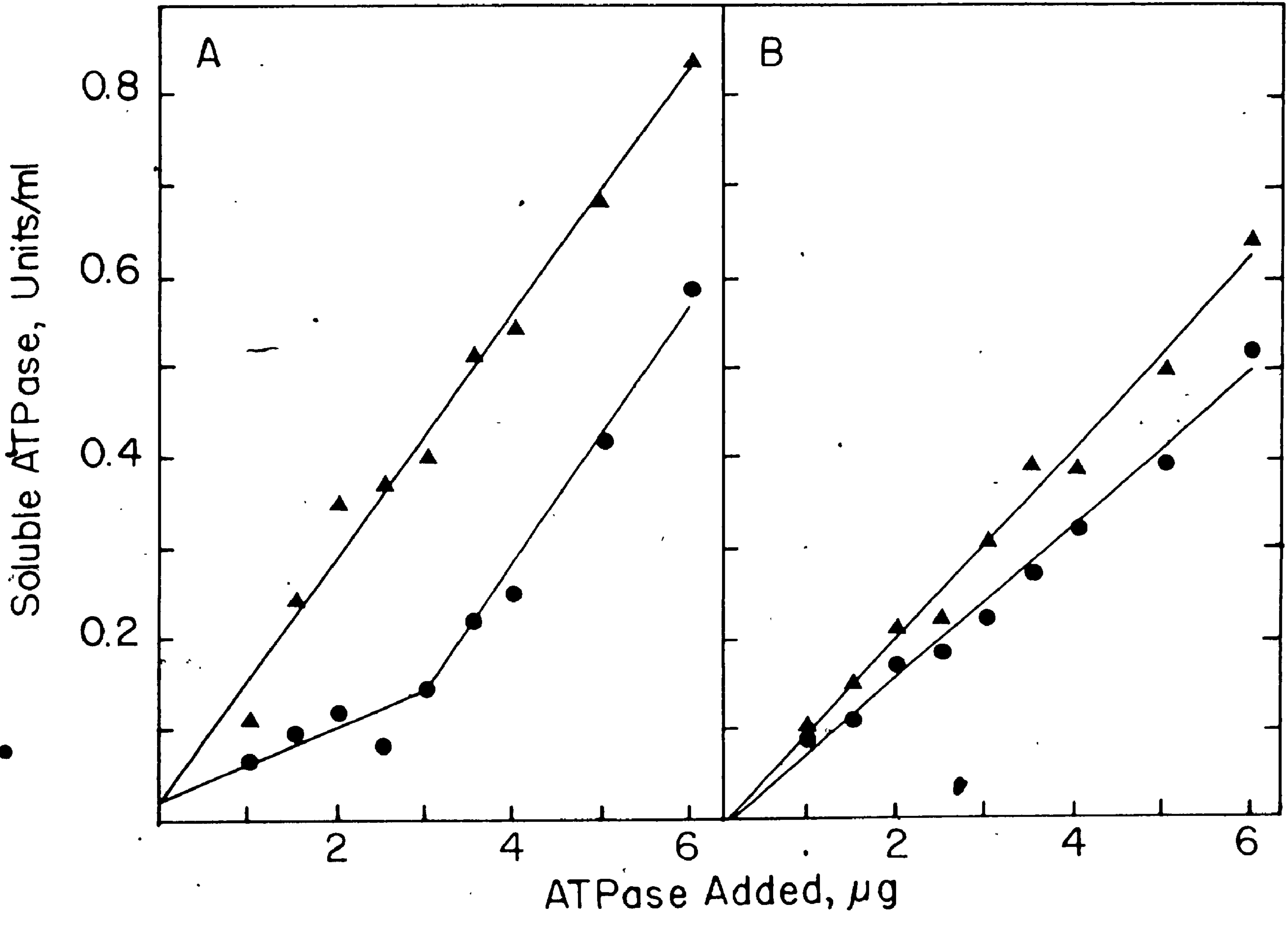
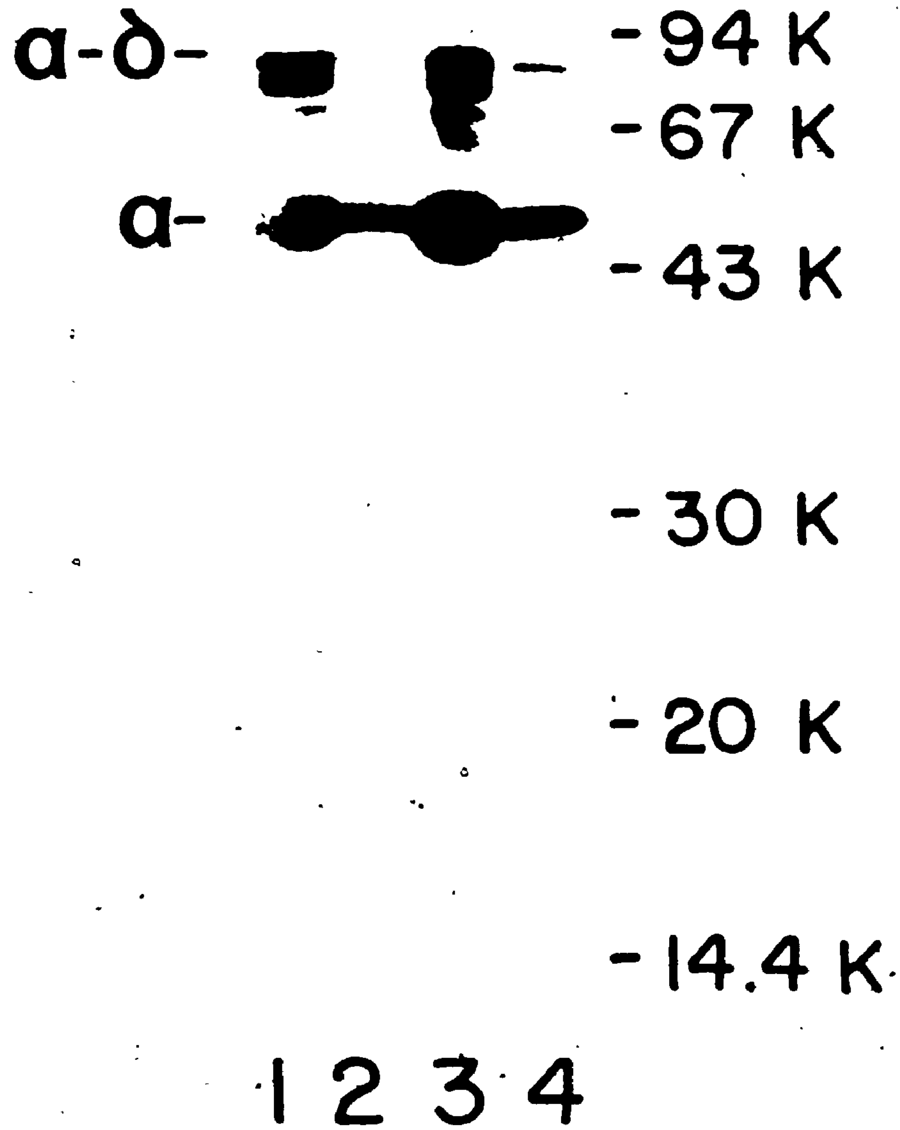

A
B
0.8
0.6
0.4
0.2
Soluble ATPase, Units/ml
2
4
6
ATPase Added, μg

Figure 5.6. Formation of α-δ cross-link on membranes containing F_1-ATPase in the presence or absence of $MgCl_2$. Fifty µg of purified F_1 or membranes obtained from *E. coli* strain AN1460 were run through centrifuge columns equilibrated with 1 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 µM $CuCl_2$. Samples in lanes 3 and 4 were prepared with the same buffer but without the addition of 5 mM $MgCl_2$. Samples were electrophoresed on a 12% SDS-polyacrylamide slab gel and electroblotted onto nitrocellulose. The nitrocellulose was blocked with BSA, exposed to a 1/100 dilution of α-3 hybridoma culture supernatant, followed by ^{125}I-anti-rat IgG and then radioautographed. Details are described in section 5.2. The lanes are as follows: lane 1, F_1 with $MgCl_2$; lane 2, membranes with $MgCl_2$; lane 3, F_1 without $MgCl_2$; lane 4, membranes without $MgCl_2$. Numbers on the right indicate molecular mass in kilodaltons.


α-δ-
-94 K
-67 K
α-
-43 K
-30 K
-20 K
-14.4 K
1 2 3 4

spun through 1-ml centrifuge columns in the presence of 10 µM $CuCl_2$. One set of buffers contained 1 mM Tris-HCl, pH 7.5 and 5 mM $MgCl_2$, with the high $MgCl_2$ concentration ensuring that the enzyme remained on the membrane, bound to F_o. These membranes did not exhibit α-δ cross-linked F_1 when examined using Western blots and anti-α monoclonal antibodies (lane 2). When the membranes were incubated and run in the presence of the same buffer lacking $MgCl_2$, a condition promoting the release of F_1 from F_o, cross-link formation was observed (lane 4). The $MgCl_2$ did not inhibit cross-link formation in isolated F_1 (lane 1) and thus it was the association of F_1 with F_o in the presence of membranes rather than either the buffer or the mere presence of membranes that prevented formation of the disulfide bond. This result is consistent with the results of the previous experiment in which the cross-linked enzyme did not bind to F_1-depleted membranes.

5.4 Discussion

It is possible to generate a disulfide bridge between the α and δ subunits of the *E. coli* F_1-ATPase by a heavy-metal-ion-catalyzed reaction that occurs while the enzyme passes through a centrifuge column. This implies that there are two closely placed cysteinyl residues on the α and δ subunits. As the genes for all the F_1 subunits have been sequenced, the number and location of the cysteinyl residues in the α and δ subunits are known (Walker *et al.* 1984). The three cysteinyl residues of the α subunit are located towards the amino terminus of the primary structure (residues 47, 90 and 203), while the two cysteinyl residues of the δ subunit are evenly distributed

(residues 65 and 141). Stan-Lotter and coworkers (1986) have evidence that there might be an additional cysteinyl residue in the α subunit at position 243. It has been shown that the last 15 amino acid residues at the amino terminus of the α subunit interact with the δ subunit (Dunn *et al.*, 1980). The formation of an α-δ disulfide bridge indicates that a more extensive region of the amino-terminal end of the α subunit is in proximity to the δ subunit.

The α-δ cross-linked F_1 cannot bind to membranes, nor can the α-δ cross-link be generated in membrane-bound F_1. This implies that there must be some change in the arrangement or conformation of the α and δ subunits between the membrane-bound and soluble forms. Perhaps the α and δ subunits are held in place more rigidly in the membrane-bound form, preventing transient contact of the cysteinyl residues. Since the δ subunit has a role in mediating the binding of F_1 to F_0 (Sternweis and Smith, 1977), the formation of the α-δ cross-link may hold the δ subunit in a conformation that is unable to interact with F_0. It is also possible that δ mediates binding to F_0 through an indirect mechanism. There is still no evidence to place δ at the F_1/F_0 interface (see chapter 4). Bragg (1984) has even proposed the α subunits interact with the c subunits of F_0 and it is possible that it is this interaction that is affected by the α-δ cross-link.

Bragg and Hou (1986b) independently observed the formation of the α-δ disulfide. In their hands, they found that cross-linked F_1 was able to bind to membranes but that the membranes were proton-leaky as measured by ATP hydrolysis driven quenching of the florescent dye quinacrine. Other than that, they found that cross-linked and non-cross-linked F_1 were identical in terms of activity, stimulation by

LDAO, aurovertin binding and pattern of cross-linked products formed by DSP.

F_1 undergoes conformational changes during its catalytic cycle (Cross, 1981). Monoclonal antibodies have relatively little effect on ATPase activity when they have bound to the α subunit (Dunn *et al.*, 1985), so the addition of extra mass to α should not necessarily hinder conformational changes the subunit might undergo. Therefore it is not surprising that the covalent attachment of the δ subunit to an α subunit has no effect on ATPase activity. However, these results do cast some doubt on the validity of the rotational model of ATP synthesis as put forth by Cox and coworkers (1984). This model deals specifically with membrane-bound form of the enzyme in which subunit b of F_0, and the γ, δ and ε subunits of F_1 rotate relative to the α/β subunit hexamer. The changing juxtaposition of each α/β pair of subunits with respect to the minor subunits are proposed to contribute to the conformational changes that are associated with the alternating catalytic sites of F_1. In the soluble form of the enzyme one would still expect rotation of the minor subunits with respect to the major subunits. One would predict from this model that by covalently linking a major subunit to a minor subunit, the rotational movement would be blocked and thus enzymatic activity would be inhibited.

The finding that the α-δ cross-link has no inhibitory effect on ATPase activity is therefore evidence against the rotational theory. One could argue, however, that in soluble F_1, the δ subunit may not be an essential part of the rotor. An interaction has yet to be demonstrated between the δ subunit and the γ and ε subunits. It could still be a part of the rotor in intact F_1F_0 but as the cross-linked

enzyme did not bind to F_0 this cannot be tested. In some models of the structure of F_1 (Aris and Simoni, 1983, Futai and Kanazawa, 1983, Bragg and Hou 1980), the δ and ϵ subunits are shown underneath the central γ subunit, directly across from one another (see Figure 1.1). If the δ subunit was locked in position by cross-linking it to the α subunit, the rotational movement of the ϵ subunit, still a part of the rotor, would be blocked. Therefore one would predict that if the rotational model is valid, cross-linked F_1 containing both δ and ϵ subunits would be inhibited. However, equivalent residual activity is obtained in cross-linked and non-cross-linked F_1 under conditions in which the enzyme is saturated with the ϵ subunit. This argues that either the rotational model is incorrect, or the δ and ϵ subunits trace out very different paths during rotation.

Kandpal and Boyer (1987) have tested the rotational hypothesis by cross-linking *E. coli* F_1 with the reversible cross-linking reagent, DSP. They found that approximately 2/3 of the activity of the enzyme was lost upon cross-linking with the formation of intersubunit and intrasubunit cross-links. The inhibition was reversed upon cleavage of the cross-links. Though these results are consistent with large conformational changes being required for catalysis, it is not the perfect test of the rotational model due to lack of specificity of the cross-links formed. One cannot say which particular cross-links actually lead to the inhibition. It is easy to imagine the formation of α-β cross-links that prevent motion of domains without evoking a rotational model. Workers in that lab are currently attempting to specifically form a cross-link between a β subunit and the γ subunit which if formed and proven inhibitory, would be more conclusive a test

of the rotational model. Workers in this lab are attempting to create a similar cross-link by forming F_1-dimers by cross-linking F_1 with anti-α or anti-β and anti-γ monoclonal antibodies and observing effects upon enzyme activity.

It is still surprising that the formation of the cross-link has no effect upon the activity of F_1. Mutations in the α subunit can lead to inactivation of the enzyme and this has been used as evidence of the importance of cooperative interactions between the α and β subunits. Perhaps the region of the α subunit that is cross-linked to δ is not involved in these interactions. Bragg and Hou (1986b) explain the lack of effect of the cross-link upon enzyme activity by evoking the recent observations of subunit asymmetry. They propose that only the α subunits not in proximity to the δ subunit are involved in catalysis.

The results illustrate that disulfide bridges can be generated between suitably placed cysteinyl residues in proteins by a heavy-metal-ion-catalyzed oxidation reaction which occurs under the highly aerobic conditions that proteins experience while passing through centrifuge columns. This reaction presents a potential hazard in the use of centrifuge columns for procedures such as the rapid desalting of proteins. In order to prevent potential protein modification following column centrifugation, contamination by heavy metal ions of buffers used to equilibrate the centrifuge column should be controlled by the use of a suitable chelating agent. Reducing agents can also be used in the column buffer as a further precaution. As a method of generating cross-links, however, column centrifugation has advantages over the more common chemical methods, in that is gentle, rapid, specific, and most importantly, quantitative. Thus, it is ideal for studies

involving the effects of limiting conformational changes both within and between subunits.

6.0 CONCLUSION

Monoclonal antibodies can serve as useful tools for various enzymological investigations. As monoclonal antibodies consist of homogeneous populations of antibodies that recognize a single epitope, they are ideal probes for studies of enzyme structure and function. Their high specificity and often high affinity also make them useful for the analysis of complex mixtures of proteins using Western blots. This thesis described the characterization of a set of 21 monoclonal antibodies that bind to subunits of the *E. coli* F_1-ATPase and their application to the study of subunit structure/function relationships in this enzyme.

Extensive characterization of the antibodies was necessary in order to assess their utility in future studies. Workers in the laboratory had previously determined that some antibodies recognizing the α and β subunits inhibit or stimulate the activity of the enzyme. Other studies had demonstrated that some antibodies recognizing the α, β and γ subunits cross-react with ATPases from other sources (Dunn *et al.*, 1985). Studies described in chapter 2 used immunoprecipitations and membrane-binding studies to determine which antibodies recognize buried or exposed epitopes on the soluble and membrane-bound forms of the holoenzyme. It was notable that of the anti-α and anti-β antibodies, only those recognizing buried epitopes cross-react with ATPases from other organisms. This demonstrates that subunit interfaces have been highly conserved during evolution and emphasizes the importance of subunit/subunit interactions in F_1. Another interesting finding was that some anti-δ and anti-ε antibodies are able

to bind to membrane-bound F_1. This shows that despite the proposed location of δ and ϵ at the F_1/F_0 interface (Figure 1.1), a substantial region of each subunit is exposed on the external surface of F_1F_0.

Solid phase binding inhibition studies (also described in chapter 2) determined the spatial relationships between antibody epitopes on isolated F_1 subunits. Those antibodies which recognize the same or overlapping epitopes were placed into the same competition subgroup. It became apparent that members of a particular competition subgroup share common characteristics. For example, the anti-β antibodies were placed into two competition subgroups. One competition subgroup, B-I, contains antibodies that recognize external epitopes and are strong inhibitors of the enzyme. The other competition subgroup, B-II, contains antibodies that recognize buried epitopes and cross-react with ATPases from several eukaryotic and prokaryotic organisms. Nevertheless, differences in affinity for epitopes, ease of elution from affinity columns and shape of inhibition curves among members of the same competition subgroup showed that member antibodies are not identical. These results are summarized in Table II.

Studies described in chapter 3 attempted to map the location of antibody epitopes within the amino acid sequence of the α and β subunits. This was expected to provide useful information regarding subunit structure and antibody activity effects. Exhaustive cleavage of these subunits with a variety of chemical and enzymatic agents failed to produce small fragments capable of competing with whole subunit for binding of antibody. This result suggested that the epitopes are predominantly conformational in nature and possibly formed from non-contiguous sequences of each subunit's amino acid sequence.

Useful information was derived from partial enzymatic and chemical cleavages of the subunits and analysis of the resulting fragments through SDS-polyacrylamide gel electrophoresis and Western blotting. Tryptic digestion of the α subunit in the presence of ATP and partial CNBr cleavage of trypsin-treated F_1 revealed that the epitopes of the anti-α antibodies of both competition subgroups lie towards the carboxyl terminus of the subunit. Lack of useful cleavage sites within this region of the molecule prevented further progress.

A two-dimensional cleavage mapped the locations of the epitopes of the anti-β antibodies. The products of partial CNBr cleavage of the β subunit were resolved using SDS-polyacrylamide gel electrophoresis. Methanol was used to temporarily fix the fragments within the gel, and weak acid hydrolysis was performed to cleave the fragments at aspartyl-prolyl peptide bonds. The weak acid fragments were then resolved by electrophoresis in a second dimension and analyzed by Western blotting. This technique mapped the epitopes of the B-I antibodies to a region between Asp-381 and the carboxyl terminus at Leu-459 and those of the B-II antibodies to a region between Asp-345 and Met-380.

Cross-linking experiments described in chapter 4 obtained results with interesting implications regarding the ε subunit. This subunit, like the B-I antibodies, is a partial inhibitor of enzyme activity (Laget and Smith, 1979; Sternweis and Smith, 1980). ε-inhibition may result from either its interaction with a β subunit (Lötscher *et al.*, 1984a) or from its interaction with the γ subunit (Dunn, 1982). To distinguish between these possibilities, the following experiments were performed using agents that activate F_1 hydrolytic activity. Activation may result from changes in subunit interactions involving ε

and may lead to sizeable alterations in subunit arrangement. Such alterations may be observed by comparison of the patterns of cross-link formation in the presence and absence of these agents through the use of Western blotting and monoclonal antibodies.

Two cross-linking reagents were chosen. The water soluble carbodiimide EDC forms a zero-length cross-link in high yield between the β and ε subunits (Lötscher *et al.*, 1984). Formation of this cross-link should be very sensitive to changes in subunit arrangement. The homobifunctional cross-linker DSP bridges lysyl residues which are situated at most 1.2 nm apart. Although less sensitive to conformational changes because of the greater length of the cross-link formed, DSP cross-links ε to both β and γ. The activating agents studied included the viscosogen ethylene glycol, the monoclonal antibody ε-4 and the detergent LDAO. It was observed that ethylene glycol decreases the efficiency of EDC cross-linking of ε to β without affecting DSP cross-linking of ε to either β or γ. This implies that ethylene glycol activation results from a subtle change in the interaction between ε and a specific region of a β subunit. This result further implies that ε-inhibition is mediated through a β subunit rather than the γ subunit. LDAO and ε-4 activation do not decrease EDC or DSP formation of the β-ε cross-link suggesting that it is not the mere association of ε with a β subunit that is inhibitory.

The method used to map the location of epitopes of the anti-β antibodies was applied to EDC cross-linked β-ε to determine the location of the ε-binding site on β. The results showed that the ε subunit binds to the same carboxyl-terminal region of β as the inhibitory B-I antibodies. A variety of chemical cross-linking

reagents could cross-link the Fab fragment of B-I antibody β-6 to ε showing that these binding sites are adjacent in the tertiary structure of the β subunit. This suggests that this region is folded into a compact structure such as a domain. That interactions by both the ε subunit and the B-I antibodies with this putative domain inhibit ATPase activity suggests that this region of β may have an important functional role.

Results using chemical modification reagents show that this carboxyl-terminal region of β contains residues that may be located near the catalytic site (Esch and Allison, 1978; Bullough and Allison, 1986a, 1986b). Another group has proposed that the central-third of the molecule forms a domain containing the nucleotide-binding site (Duncan *et al.*, 1986). Residues at the catalytic site may be contributed by both of these putative domains. It is also possible that the carboxyl-terminal domain may function as a gate. The unique environment at the catalytic site may result from movement of the carboxyl-terminal domain relative to the central domain thereby enclosing bound substrate. A similar conformational change would be required for product release.

The ε subunit appears to inhibit a conformational change required for product release (Dunn *et al.*, 1987; Wood *et al.*, 1987). Perhaps this conformational change is movement of the carboxyl-terminal region which could be hindered by the presence of ε. As ε does not appear to inhibit membrane-bound F_1 and EDC efficiently cross-links ε to β in membrane-bound F_1, the difference between the β/ε interaction in F_1F_0 and that in soluble F_1 must be very subtle. This alteration may reflect the role of ε in F_1F_0. Perhaps ε couples proton flow

through F_0 to ATP synthesis/hydrolysis at catalytic sites through its interactions with both the γ subunit or the F_0 subunits and the carboxyl-terminal region of the β subunits. A direct interaction with F_0 subunits is possible as a role for ε in linking F_1 to F_0 has been proposed (Dunn and Heppel, 1982). Thus ε-inhibition may reflect alteration of interactions that normally result in coupling of F_1 to proton flow and the inhibition may have some sort of regulatory role (Dunn and Heppel, 1982). Furthermore, this role may be reflected in the observation that the amount of ε-4 antibody that binds to membranes as measured by a direct approach (chapter 2) is much less than that measured using a kinetic approach (Dunn and Tozer, 1987). Two possible explanations for this result are that ε-4 is lost in the membrane binding assay during washes or that the affinity of the antibody for ε is different in the two assays. The proton motive force generated during measurements of antibody activity effects may result in a change in the conformation of ε making it more accessible to antibody in one complex than another. Thus this may be suggestive of an involvement of ε in the coupling of catalysis to proton flow.

The nature of the proposed interactions between ε and F_0, as well as those proposed between δ and F_0, is not understood. The δ and ε subunits have sometimes been depicted as forming a stalk between F_1 and F_0 but the only demonstrable interactions between F_1 and F_0 involve β and subunits a and b (Aris and Simoni, 1983). Use of a wide variety of cross-linking reagents, including a non-specific, photoactivatable reagent and a sensitive means of analysis using Western blots and monoclonal antibodies failed to demonstrate proximity of ε or δ to any of the F_0 subunits. Either the cross-linkers chosen

were not suitable for the side chains exposed on the subunits, or ε and δ do not lie at the F_1/F_0 interface.

The preparative procedure of column centrifugation results in the heavy-metal-ion-catalyzed formation of a disulfide bridge between the α and δ subunits (Chapter 5). This reaction is quantitative and does not lead to subunit dissociation. The cross-link has no effect on either the enzyme's hydrolytic activity or its sensitivity to ε-inhibition. This result casts some doubt upon the rotational model of the catalytic mechanism of F_1 proposed by Cox and coworkers (1986) in which rotation of the major subunits in relation to the minor subunits is an essential feature of catalytic activity. If this model is valid, cross-linking a major subunit to a minor subunit should completely inhibit the activity of the enzyme. The lack of effect of the α-δ cross-link implies that if the model is correct, the δ subunit must not be an essential part of the rotor in soluble F_1. Furthermore, the δ and ε subunits must be located far enough apart to allow a moving ε subunit to trace a path free of obstruction. Nevertheless, as δ is required for the binding of F_1 to F_0, it would be expected to be a component of the rotor in F_1F_0. Thus the cross-link should inhibit F_1F_0. As cross-linked enzyme is unable to reconstitute with F_0 this could not be tested. This inability for α-δ cross-linked enzyme to bind to F_0 does reaffirm the importance of δ in interactions of F_1 with F_0. However one cannot determine whether this effect is mediated by δ directly, or indirectly through α. This is possible as Bragg has proposed that the α and β subunits form contacts with subunit c of F_0 (Bragg, 1984) and thus binding to F_0 may be mediated through the α subunits instead of δ.

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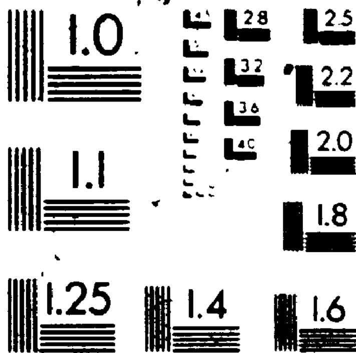

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