Studies on the cytochrome oxidase of

Pseudomonas stutzeri

Dominic J.B.Hunter

Thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh

1989

Declaration

The work described in this thesis is my own, unless otherwise indicated. Some of the results presented here have previously been published in Hunter, D.J.B., Brown, K.R. and Pettigrew, G.W. (1989) Biochem. J. 262 232-240, bound in the Appendix.

Acknowledgements

My principal thanks must go to Dr Graham Pettigrew for his supervision over the past three years. His support, encouragement and enthusiasm for research have been an inspiration to me. I must also thank the staff and research students of the Biochemistry Department and of the Royal (Dick) School of Veterinary Studies. In particular, my thanks go to Celia Goodhew, Elaine Sutherland, Kevin Brown and John Leaver for making my stay at the 'Dick Vet' enjoyable and productive.

My thanks also go to Dr David Apps and Ian Flynn for their help and advice, to members of the Department of Veterinary Pathology for their assistance in the preparation of bacteriological media and to Colin Warwick for his help in the preparation of this thesis. I am also grateful to my friends in Edinburgh and in particular, my flatmates, Justin Rye and Iain MacLennan, for their help and support.

Finally, I must thank my parents for their help and encouragement in the course of this work. I would like to dedicate this thesis to my late father.

Abstract

The presence of an o-type cytochrome oxidase in the membranes of the denitrifying soil bacterium *Pseudomonas stutzeri* 224 was suggested by difference spectroscopy and by carbon monoxide-binding spectra. A small amount of a d-type cytochrome was also detected. The sensitivities of the oxidase activities of the membranes of *P. stutzeri* to cyanide leads to the conclusion that cytochrome d is associated with NADH oxidase activity and cytochrome o is associated with ascorbate-TMPD oxidase activity.

Cytochrome c_4 has been implicated in the o-type oxidase of *Azotobacter vinelandii* (Jurtshuk et al., 1981). The role of this cytochrome in the *P. stutzeri* o-type oxidase was investigated by selective removal of the cytochrome c_4 from *P. stutzeri* membranes. Sodium iodide, at concentrations above 1.5M, and propan-2-ol, at concentrations above 20% (v/v) were found to remove cytochrome c_4 from *P. stutzeri* membranes selectively with respect to other c-type cytochromes, as judged by haem-stained SDS-PAGE gels. Cytochrome c_4 could also be removed from octyl glucoside-solubilised membranes after frozen storage by chromatography on Sephadex G-75. Removal did not occur when freshly solubilised membranes were used.

Ascorbate-TMPD and cytochromes c oxidase activities of the membranes were retained, despite the removal of cytochrome c_4. Duroquinol oxidase activity was partially lost, whilst NADH, succinate and lactate oxidase activities were completely lost. Purified cytochrome c_4, or that removed by iodide treatment, could be reconstituted with cytochrome c_4-depleted membranes with no effect on the oxidase activities of the membrane. Return of purified cytochrome c_4 was shown to be by a specific mechanism, rather than being due to non-specific co-precipitation with the membranes.

It was therefore concluded that cytochrome c_4 is not involved in the ascorbate-TMPD or cytochromes c oxidase activities. The losses of duroquinol, NADH, succinate and lactate oxidase activities are probably due to damage to other components of the electron transport chain, since the treatments used to remove cytochrome c_4 remove other non-haem proteins in addition. This conclusion implies that cytochrome c_4 is not involved in the o-type cytochrome oxidase of *P. stutzeri* . A similar conclusion can be drawn for *A. vinelandii* (Hunter et al., 1989).

Iodide treatment of cytochrome c_4 caused its partial denaturation. The midpoint potentials of the cytochrome c_4 in sodium iodide (+5 and -80mV) were considerably lower than the native values (+310 and +195mV), indicating a greater exposure of the haems to solvent. Additionally, the near-infrared band of the ferricytochrome, indicative of methionyl-haem coordination was lost on iodide treatment. Removal of the iodide allowed 60% of the cytochrome c_4 to regain its native properties.

Purification of the o-type oxidase of *P. stutzeri* 224 was undertaken. The ascorbate-TMPD oxidase activity was solubilised intact by octyl glucoside, dodecyl maltoside and Triton X-100, but not by CHAPS, MEGA-9 or deoxycholate. When solubilised by dodecyl maltoside and Triton X-100, the oxidase proved refractory to purification. The following methods were also of no use in the purification: affinity chromatography on yeast cytochrome c-sepharose; hydrophobic chromatography on octyl Sepharose; chromatography on hydroxyapatite; ion exchange chromatography of the crude detergent extract; molecular exclusion chromatography of the crude detergent extract and molecular exclusion chromatography on columns equilibrated with octyl glucoside.

The ascorbate-TMPD oxidase was partially purified by ammonium sulphate fractionation of an octyl glucoside extract, the oxidase activity precipitating as a red oil at 70% saturation. This step removed most of the cytochromes c, along with many of the proteins of the extract. Further purification was achieved by molecular exclusion chromatography on Fractogel HW-55 in Triton X-100, which was capable of separating much of the remainder of the cytochromes c from the oxidase. This step was followed by ion exchange chromatography on DE-cellulose.

The final product contained cytochromes b and c and possessed cyanide inhibitable ascorbate-TMPD oxidase activity. The polypeptide composition of this material was too complex to permit identification of the subunits of the oxidase. The removal of most of the cytochrome c_4 from the oxidase activity during this preparation supports the conclusion that cytochrome c_4 is not involved in the o-type oxidase in *P. stutzeri* . In the light of this, the possible function of cytochrome c_4 as a link between the o-type oxidase and its reductase is discussed.

Contents

Chapter 1: Introduction

Bacterial electron transport systems give the impression of great diversity and complexity when compared with the system found in mitochondria. There are many different redox components associated with such systems, including a large number of cytochromes of different types. The electron transport chains formed by these components are frequently branched, with a multitude of substrates and terminal electron acceptors capable of being used. In addition, electron flow through a series of redox components may be reversed with respect to the analogous situation in mitochondria. However, despite this complexity, the underlying structure of such systems is relatively simple, with various respiratory complexes being linked by diffusible electron carriers.

Bacterial cytochromes c often play a central role in electron transport. A large group of the many bacterial cytochromes c probably act as diffusible links between cytochrome c reductases and terminal electron accepting reactions, whilst other cytochromes c form components of these reductases and the enzymes involved in the terminal reactions. The central role of bacterial cytochromes c is illustrated in Fig.1.1. Not all these pathways occur in a single organism, although it is common for several to be present in parallel, to allow the organism to adapt to changing environmental conditions.

I: Cytochromes c

Cytochromes c possess one or more protohaem IX prosthetic groups (Fig.1.2a) attached covalently to the polypeptide chain by thioether linkages formed by condensation of the protohaem IX vinyl groups with cysteine residues in the polypeptide. This forms mesohaem (Fig.1.2b). Normally, two such linkages are made, however, in some protozoan cytochromes c, a single thioether linkage alone is present. Examples of such cytochromes c are the cytochrome c-557 of *Crithidia oncopeltii* (Pettigrew, 1972) and cytochromes c-558 and c_1 of *Euglena gracilis* (Pettigrew et al., 1975; Mukai et al., 1988). These unusual cytochromes c resemble cytochromes b in spectral properties.

The haem iron is coordinated by five or six ligands, four of which are

Fig.1.1 The central role of cytochrome c in the terminal reactions of bacterial electron transport.

Cytochromes c also form part of complex III (cytochromes c_1 and f) or occur in association with cytochrome c oxidases, photosynthetic reaction centres, denitrifying enzymes and enzymes involved in substrate oxidation (From Pettigrew and Moore, 1987).

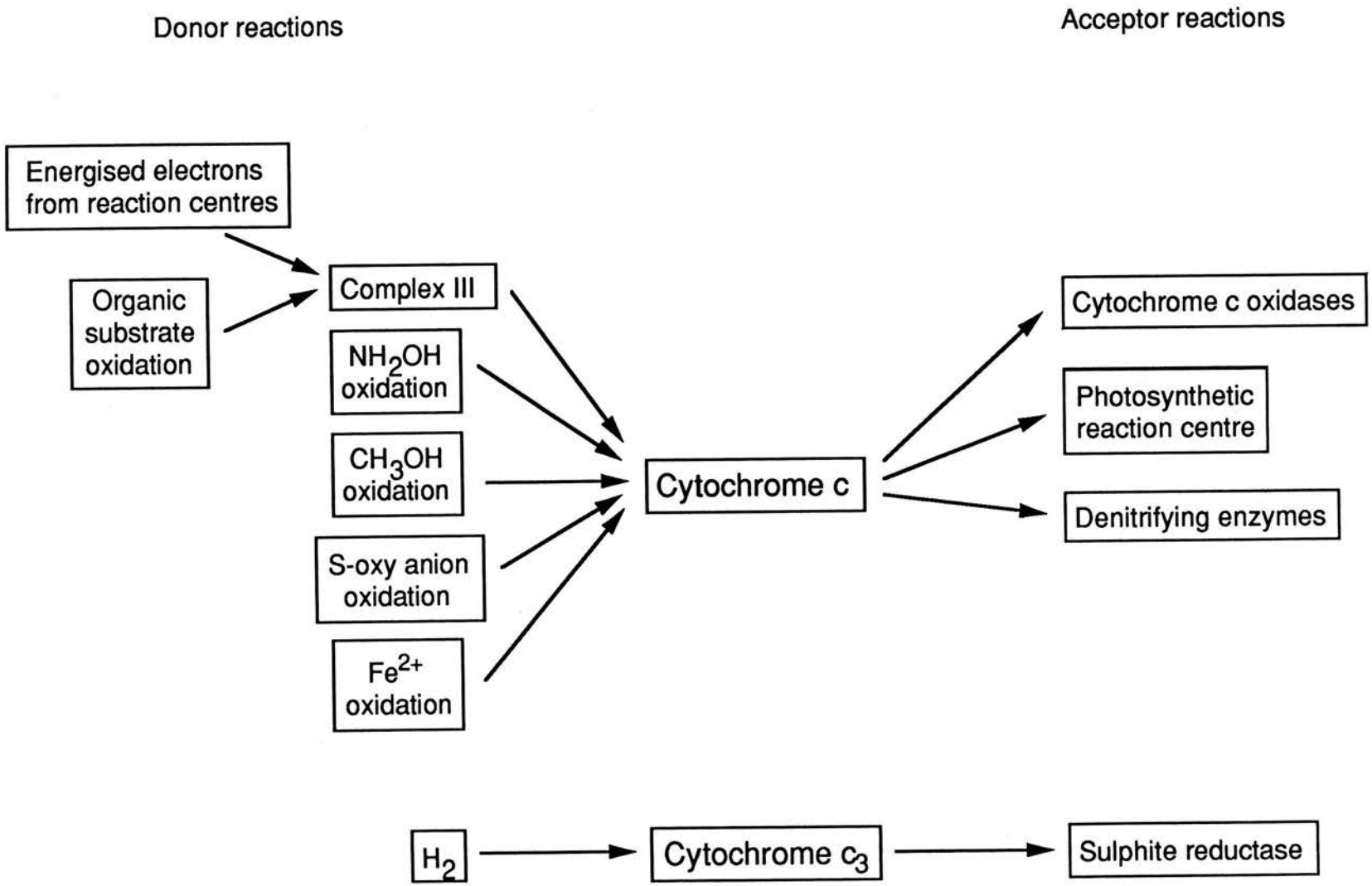
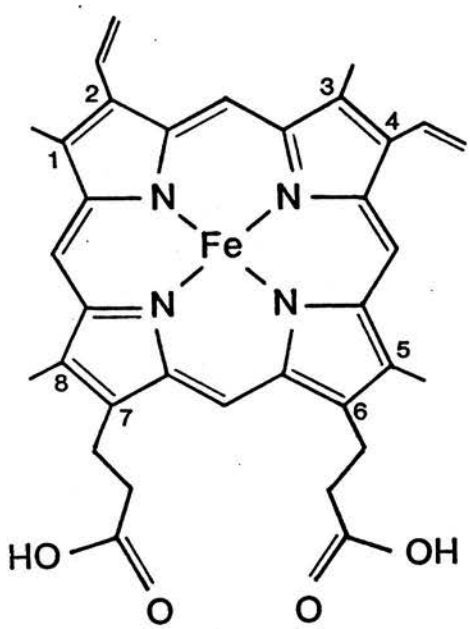

Donor reactions
Acceptor reactions
Energised electrons from reaction centres
Organic substrate oxidation
Complex III
NH_2OH oxidation
CH_3OH oxidation
S-oxy anion oxidation
Fe^{2+} oxidation
Cytochrome c
Cytochrome c oxidases
Photosynthetic reaction centre
Denitrifying enzymes
H_2
Cytochrome c_3
Sulphite reductase

Fig.1.2 Structures of the haems of cytochromes b and c

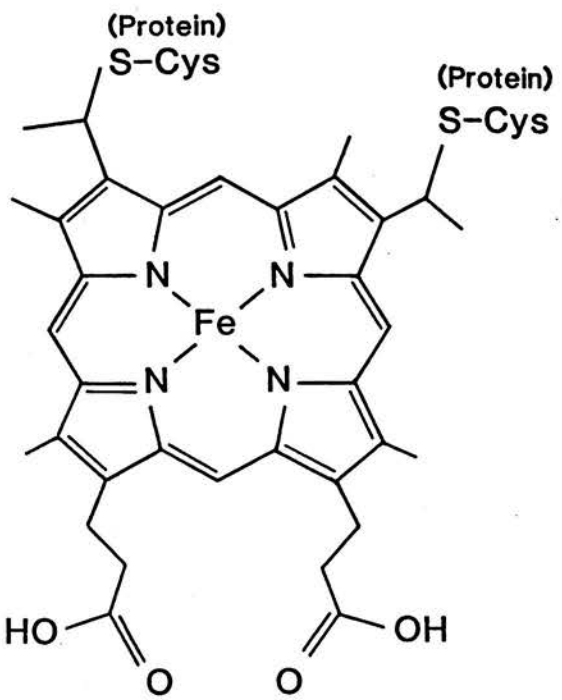
(a): Protohaem IX (haem b)

(b): Mesohaem (haem c)

(a)


1
2
3
4
5
6
7
8
N
N
Fe
N
N
HO
O
O
OH

(b)


(Protein)
S–Cys
(Protein)
S–Cys
N
N
Fe
N
N
HO
O
O
OH

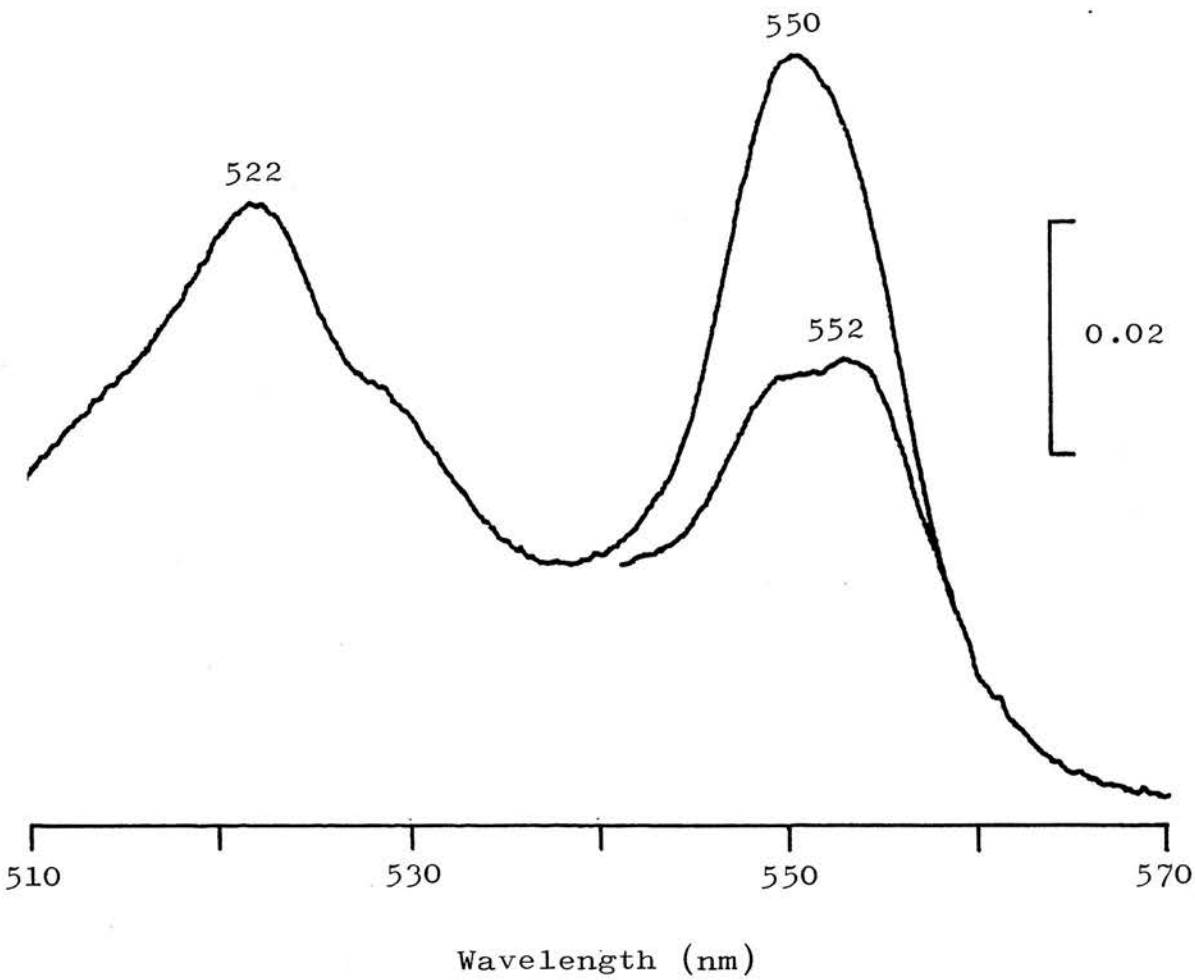
provided by the porphyrin pyrrole nitrogens, the remaining ligands being provided by the apoprotein. In all cytochromes c so far discovered, the fifth ligand is a histidine nitrogen. The sixth ligand may be absent or may be formed by a second histidine or by a methionine sulphur. The nature of the sixth haem ligand determines the spin state of the haem iron. An absent or weak field sixth ligand results in a high spin state. Examples of high spin cytochrome c are the cytochromes c', which exhibit a broad absorption band in the oxidised state at around 630nm when oxidised. This is typical of the high spin state. Low spin cytochromes c lack this band, but may possess a band at 695nm in the oxidised state, if methionine is their sixth ligand. Bishistidinyl coordination does not give rise to a 695nm band, as illustrated by cytochromes c_3.

Cytochromes c function by a reversible change in oxidation state between Fe(II) and Fe(III), allowing them to receive and donate electrons. Ferryl (Fe(IV)) iron occurs only in the peroxidatic centre of *Pseudomonas aeruginosa* cytochrome c peroxidase (Ronnberg et al., 1981) and possibly in the mammalian cytochrome oxidase haem a_3 (Kumar et al., 1988). 'Reduced' and 'oxidised' cytochrome therefore refers to the Fe(II) and Fe(III) states respectively.

The haem group of cytochromes c confers a distinctive absorption spectrum in the visible range (Fig.1.3). The α- and β-peak maxima for reduced cytochromes c are at 550 to 555nm and 520 to 525nm respectively. The visible spectrum is influenced only by the sixth haem ligand and by the environment the haem experiences, since the structure of the haem group is believed to be constant. Pure cytochromes c can therefore be identified on the basis of the characteristics of their visible spectrum (α-peak maximum, α/β peak ratio, assymetry of the α-peak in the reduced state, presence or absence of bands at 630 and 695nm in the oxidised state). However, a mixture of cytochromes c, such as that found in a respiratory membrane, yields a composite spectrum with little usable information on the constituents of the mixture. Recording the spectrum at a reduced temperature, usually 77K, may

Fig.1.3 The visible absorption spectrum of a c-type cytochrome in the region of the α- and β-peaks

This is a native spectrum of membrane-bound cytochrome c_4 (3.2μM) reduced with a few crystals of sodium dithionite. Note the α-peak of this cytochrome is at 550nm when fully reduced, but shifts to 552nm on partial oxidation and the two haem groups of the cytochrome c_4 become spectroscopically distinct.


550
522
552
0.02
510
530
550
570
Wavelength (nm)

allow resolution of the components of the spectrum, but identification may still prove difficult by this means.

A further property conferred by the haem group is a peroxidase activity. When exposed to solvent by denaturation of the cytochrome c, the haem group possesses a peroxidase activity which can be exploited in the haem-staining method outlined in Chapter 2. Here, reduction of hydrogen peroxide to water at the haem group is coupled to oxidation of colourless, soluble 3,3',5,5' tetramethylbenzidine to an insoluble, blue-coloured precipitate. Therefore the position of haem on an acrylamide gel may be detected. Since the haem of cytochromes c is covalently attached to the apoprotein, the method detects the position of the apoprotein as well in this case.

As many cytochromes c are relatively small proteins which are soluble and available in large quantities, many have been purified and characterised. A large number of cytochrome c amino acid sequences have been obtained and from this data, a classification of cytochromes c has been proposed (Ambler, 1980).

Class I cytochromes c are all low spin, with methionyl-haem coordination. The haem group is attached close to the N-terminus of the polypeptide chain, which is approximately 80 to 140 amino acids in length. The midpoint potentials of these cytochromes are generally high (+150 to +400mV). Class I cytochromes are all structural homologues of mitochondrial cytochrome c. They all have the same general folding pattern and haem environment, differing mainly by the addition or deletion of loops of chain on the surface of the molecule (Dickerson and Takano, 1978, Fig.1.4). The class is subdivided into three.

Large class I cytochromes c include the cytochromes c_2 of photosynthetic bacteria and cytochrome c-550 from *Paracoccus denitrificans* (Fig.1.4a). Medium class I cytochromes c include mitochondrial cytochromes c (Fig.1.4b). Small class I cytochromes c include *Pseudomonas* cytochromes c-551 (Fig.1.4c), *Chlorobium* cytochrome c-555, cytochromes c_5 and cytochromes c_4. This latter is included in class I cytochromes c despite its large size

Fig.1.4 Structures of class I cytochromes c

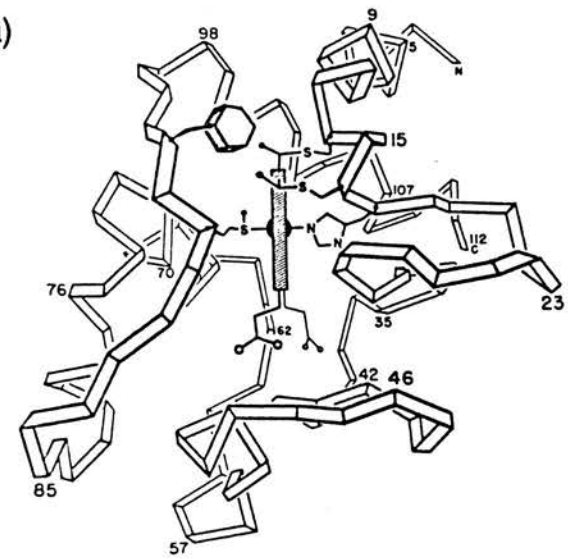
(taken from Dickerson and Takano, 1978).

(a) Large class I cytochrome c - cytochrome c2 from *Rhodospirillum rubrum.*

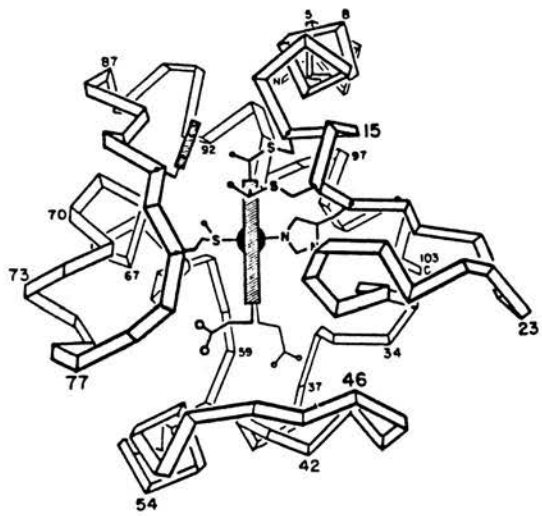
(b) Medium class I cytochrome c - mitochondrial cytochrome c from tuna heart.

(c) Small class I cytochrome c - cytochrome c-551 from *P. aeruginosa.*

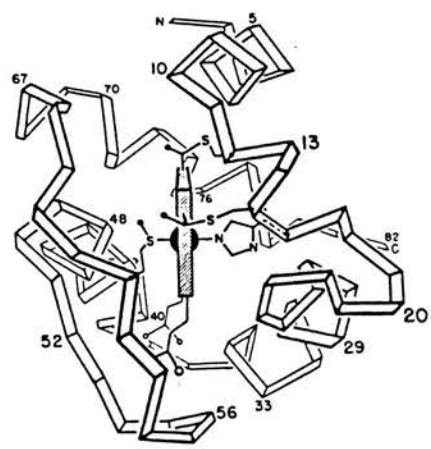
(a)


9
98
5
N
15
S
S
107
S
N
N
112
C
70
76
H62
35
23
42
46
85
57

(b)


5
8
87
NC
15
92
S
S
97
70
S
N
N
103
C
73
67
23
59
34
77
37
46
42
54

(c)


N
5
10
67
70
S
13
76
48
S
S
N
N
82
C
40
20
52
29
33
56

(approx. 180 amino acids) and possession of two haem groups, since it is believed to be the result of a gene duplication and fusion of two small class I cytochromes c. Its other properties (midpoint potential, spin state, haem coordination and folding pattern) all support this classification. Class I cytochromes c generally function as diffusible electron carriers between reductases and terminal acceptors of electrons and are therefore usually soluble. Cytochromes c_4 and c_5 are exceptions to this, being largely membrane-bound, although a soluble portion exists in both cases (Pettigrew and Brown, 1988).

Class II cytochromes c all possess a single haem group attached close to the C-terminus of the polypeptide. This class originally included only the high spin cytochromes c', but low spin homologues of these cytochromes have since been discovered. High spin state is therefore no longer a defining characteristic of this group. The midpoint potentials of class II cytochromes c are lower than those of class I, in the range -10 to +150mV. Examples of class II cytochromes c include the previously mentioned cytochromes c' from photosynthetic bacteria and the low spin cytochrome c-556 from *Agrobacterium*. Class III cytochromes c are distinguished by the possession of multiple haem groups. Cytochromes c with three to eight haem groups are known, although four haems are more usual. The midpoint potentials of these cytochromes are very low, between -100 and -300mV, and the haems are low spin, with bishistidinyl coordination. This class comprises the cytochromes c_3.

A final class, class IV has also been proposed, to contain those cytochromes with a second prosthetic group, such as flavocytochrome c, *Pseudomonas* cytochrome cd1 and cytochrome c peroxidase. However, such cytochromes c may possess domains which resemble class I cytochromes c in sequence and folding pattern and may merit inclusion in this class rather than in a new class. For example, *P. aeruginosa* cytochrome c peroxidase, possesses a high potential haem c-containing domain resembling a class I cytochrome c (Ronnberg, 1987).

Although bacterial cytochromes c are known to be important in electron transport, their role in many cases in not clearly defined. Often a great deal is

known about the structure and properties of a cytochrome c, whilst its function remains unclear. Definitive proof that a cytochrome c is involved in a particular electron transfer process is difficult to obtain, not least because several c-type cytochrome may be found in one bacterial species. For example, *P. stutzeri* possesses at least five soluble cytochromes of which only two have a defined function. These are cytochrome cd_1, the nitrite reductase and cytochrome c-551, which is believed to be a diffusible electron donor to cytochrome cd_1 and other terminal electron acceptors. In addition, there are a further five membrane-bound cytochromes c, including cytochromes c_4 and c_5, whose functions are also unknown.

A second complication is that bacteria may grow using different respiratory modes depending on the conditions of growth, which may use alternative electron transport components. One example of this phenomenon is the utilisation of multiple terminal oxidases in many species, as discussed in Chapter 7. Other examples include the switch from aerobic growth to denitrification under anaerobic conditions in certain pseudomonads and *Pa. denitrificans*.

Cytochromes c are found in large quantities in denitrifying bacteria, where their role is, in general, poorly defined. A probable function is to act as carriers of electrons between the electron transport chain and the enzymes of denitrification. Cytochrome c-551 has already been mentioned in this regard. *Pa. denitrificans* cytochrome c-550 probably also has a similar function under anaerobic conditions.

Finally, the lack of specificity in the interactions of redox components observed in vitro may reflect the state of affairs in vivo. If this is the case, then the description of unique pathways of electron transport may not be justified, and a defined role for bacterial cytochromes c may not be obtained. An example of the lack of unique pathways of electron transport is the parallel function of cytochrome c-551 and azurin in *P. aeruginosa*. These diffusible electron carriers are believed to be synthesised reciprocally, depending on the availability of iron and copper in the medium (Pettigrew and Moore, 1987) by analogy with established reciprocal relationships between algal

cytochromes c-553 and plastocyanin (Wood, 1978b).

Because of these complexities, several lines of evidence are required to demonstrate the involvement of a cytochrome c in a particular electron transport process and to define its function. Firstly, the cytochrome c and the process should share a coincident biological distribution. However, care is required when the presence of a cytochrome c and a respiratory or photosynthetic process are being correlated, since if an occurrence of the cytochrome is overlooked, a false picture of its involvement in a process may be obtained. For example, cytochrome c_4 occurs in several species of pseudomonads and an *Alcaligenes* species (Pettigrew and Brown, 1988). Since these species are all capable of denitrification, a role in this process for cytochrome c_4 could be put forward. However, cytochrome c_4 also occurs in the strict aerobe *Azotobacter vinelandii* (Tissieres, 1956), so a unique role for cytochrome c_4 in denitrification can be discounted.

Secondly, the cytochrome may be induced in the presence of a specific electron donor or terminal acceptor of electrons. In this case, the cytochrome complement of the organism under investigation under normal growth conditions would be compared with that when the donor or acceptor was present. Any changes in the cytochrome levels could then be identified and any induction by the donor or acceptor distinguished.

An example of this is the cytochrome cd_1 of *P. aeruginosa*, which is involved in denitrification. Under aerobic conditions, no cytochrome cd_1 is present. When the organism in grown under anaerobic conditions in the presence of nitrate, cytochrome cd_1 is induced, showing clearly its involvement in the process of denitrification. However, not all cases are clear-cut. *P.aeruginosa* cytochrome c-551 is also induced by anaerobic, denitrifying conditions, but is present under aerobic conditions as well. This indicates that cytochrome c-551 is not uniquely involved in denitrification and has a separate function under aerobic conditions. A further complication is that the conditions of growth must be correct for induction to occur. Denitrification only occurs in the absence of oxygen and is repressed even at

low oxygen levels (Alefounder and Ferguson, 1980). Therefore, no induction of cytochrome cd_1 would occur under aerobic conditions even if nitrate were present. This might lead to the erroneous conclusion that cytochrome cd_1 is not involved in denitrification.

Thirdly, the cytochrome should interact with its proposed redox partners such that the rates of electron transport for the whole process in vivo could be supported. This is often investigated by the measurement of the rates of electron transfer in vitro, between purified redox components. These rates may not be representative of the rates in vivo since the conditions under which the components interact in vivo may be significantly different from those used in vitro. Also the components interacting in vitro may not be capable of doing so in vivo since they may be spatially separated. Measurements in the intact organism are therefore more desirable, but are difficult if several redox partners with similar properties to that under investigation are present. The use of specific antibodies to block the reactions of these contaminants and allow only that of the component under investigation to proceed is a useful approach in this case.

Fourthly, loss of the cytochrome c by genetic manipulation should lead to predictable changes in the metabolism of the organism. Similarly, selective removal of the cytochrome c by chemical means or blocking of its interactions by specific antibodies should have a comparable effect.

Finally, the physical properties of the cytochrome c give clues to its function. Sequence homologies to other cytochromes c with known function may indicate a similar function for that under investigation. The midpoint potential of the cytochrome c suggests a position for the cytochrome in the electron transport chain, as the more positive the potential, the closer the cytochrome c is likely to be to the terminus of the chain. Furthermore, the midpoint potential may also allow potential electron donors and acceptors to the cytochrome c to be discounted on the grounds that their midpoint potentials make such a reaction unfavourable. The cellular location of the cytochrome c and its proposed redox partners also determines whether or not their interaction could take place. If the proposed partners are located on

opposite sides of a membrane then direct interactions between them would be impossible.

Wood (1983) has proposed that cytochromes c are all located in the periplasm or on the periplasmic face of the cell membrane. This is because cytochromes c are unique among cytochromes in possessing a covalently attached haem group. In all other cytochromes, there is a possibility that the haem group will dissociate from the apoprotein. This is not a problem in the cytoplasm of the cells since the concentration of haem will be relatively high and reassociation is likely to occur. However, in the periplasm, dissociation of the haem is likely to lead to its loss to the medium, so that reassociation is extremely unlikely. This problem does not occur with cytochromes c. This hypothesis is receiving increasing experimental support (for example, Hunter et al., 1989).

Lastly, co-purification of the cytochrome c and another redox component might occur, suggesting that their functions could be related. However, the co-purification could also be fortuitous.

Cytochrome c_4 has recently been extensively studied (Leitch et al., 1985; Pettigrew and Brown, 1988; Brown, 1988; Hunter et al., 1989) and many of its properties are now well known. Thus its cellular location, midpoint potentials, sequence, three-dimensional structure, biological distribution and the effects of anaerobic, denitrifying growth on cellular levels are all known. These are described in the next section.

II: Cytochrome c_4

A. Distribution Cytochrome c_4 was first purified from *A. vinelandii* (Tissieres, 1956; Tissieres and Burris, 1956) and examples have now been isolated from *P. aeruginosa, P. mendocina* (Pettigrew and Brown, 1988; Ambler and Murray, 1973), *P. stutzeri* (van Niel strain, Kodama and Shidara, 1969; strain 224, Pettigrew and Brown, 1988), *Alcaligenes* sp. (Shidara, 1981; Pettigrew and Brown, 1988) and possibly from *P. perfectomarinus*, now reclassified as *P. stutzeri* strain ZoBell. The latter is of interest since a cytochrome o has recently been purified from this organism (Heiss et al.,

1989). *P. stutzeri* strain ZoBell (f. *P. perfectomarinus*) contains a cytochrome c-552 which has some of the characteristics of a cytochrome c_4 although its spectrum is atypical and one haem group has a very low midpoint potential (Liu et al., 1981). Classification of this cytochrome as a cytochrome c_4 must await comparison of its sequence with those of cytochromes c_4, although statistical analysis of amino acid compositions does predict a low level of divergence (37%) from *A. vinelandii* cytochrome c_4 (Pettigrew and Brown, 1988). A cytochrome "c-551" was also present and may be a more typical cytochrome c_4.

Cytochrome c_4 may have a wider biological distribution than its known presence in the pseudomonads and *A. vinelandii* (Pettigrew and Brown, 1988), since few studies of bacterial cytochromes c bound to membranes have been made. For example, a M_r 22 000 membrane-bound cytochrome c was isolated along with the ubiquinol oxidase complex from *Pa. denitrificans* (Berry and Trumpower, 1985). This may be related structurally or functionally to cytochrome c_4.

B. Structure of cytochrome c_4 Cytochrome c_4 is a dihaem cytochrome of molecular weight approximately 20 000. The amino acid sequences of the cytochromes c_4 from *P. aeruginosa* and *A. vinelandii* have been determined (Ambler, 1980; Ambler et al., 1984). These show evidence of a gene duplication event, with each half of the protein resembling a typical small class I monohaem cytochrome c (Ambler et al., 1984).

Structural studies support this conclusion, showing that each half of the cytochrome c_4 folds into a separate domain containing a haem group (Sawyer et al., 1981). These domains resemble small class I cytochromes c in folding patterns. Fig.1.5 shows a tentative structure for cytochrome c4 (Sawyer, personal communication). The two domains are linked by an extended length of polypeptide chain and lie together with the 'bottom' of the haem crevices facing each other. The haem propionates are therefore in close proximity and may hydrogen bond to each other. The 'front' faces of the domains lie on opposite sides of the molecule, as shown diagrammatically in Fig.1.6a.

Fig.1.5 Structure of cytochrome c_4 from *P. aeruginosa*

Stereopair of the tentative structure of cytochrome c_4 (courtesy of Dr L. Sawyer, Dept. of Biochemistry, University of Edinburgh).

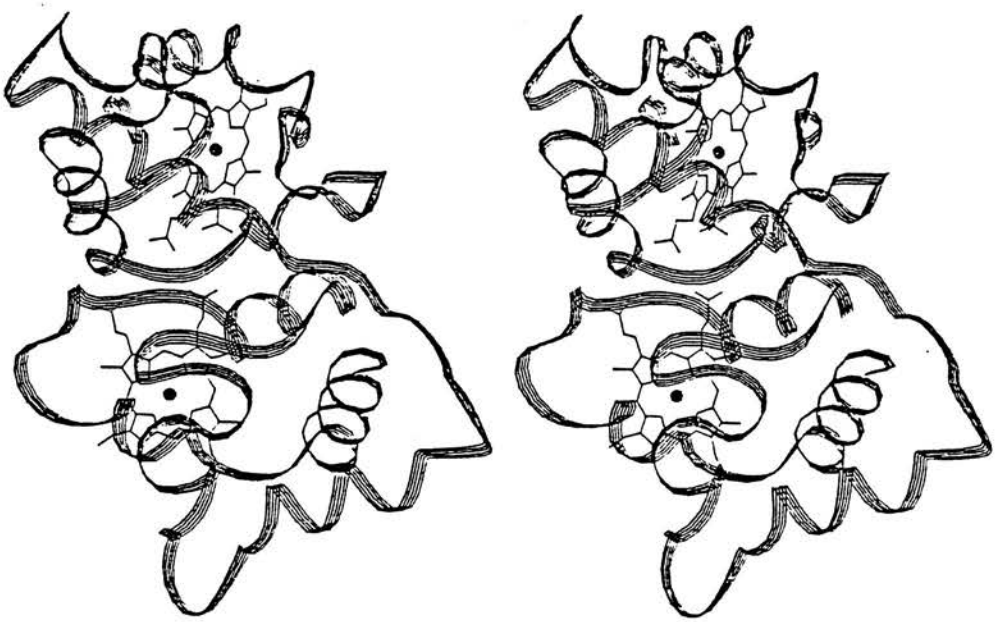
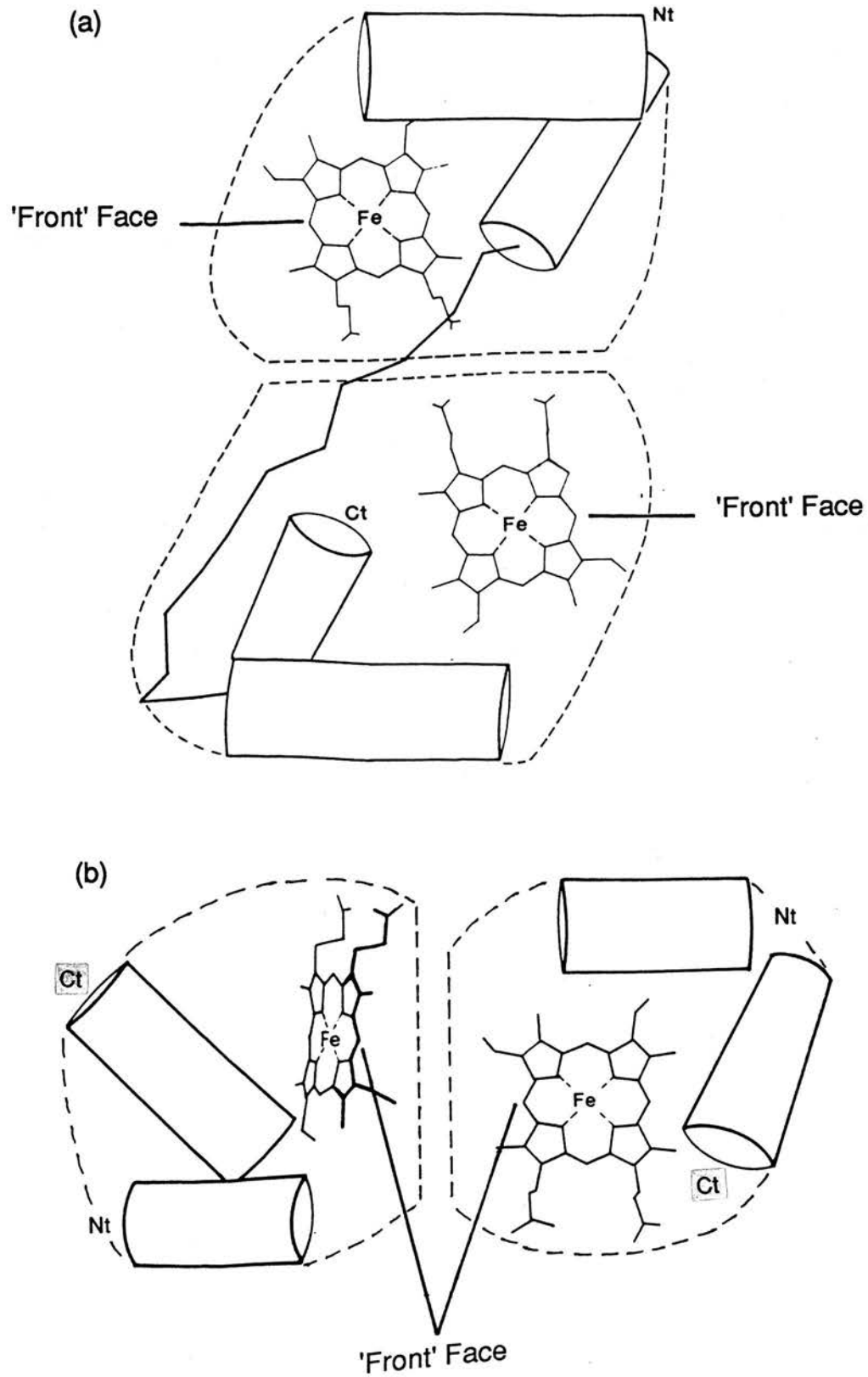


Fig.1.6 Comparison of the structures of cytochrome c_4 and the cytochrome c_5 dimer

These diagrammatical representations of cytochrome c_4 (a) and the cytochrome c_5 dimer (b) show the orientations of the haem groups and the major helices of the cytochromes. Nt indicates the N-terminus of the cytochromes.

Cytochrome c_5 is a small monohaem cytochrome c of unknown function, usually found as a non-covalent dimer of identical, M_r approx. 12 000, monomers (Pettigrew and Moore 1987). Its structure is given here to allow comparison with that of cytochrome c_4, which may be viewed as a covalent dimer.

Cytochromes c_5 are characterised by a red-shifted α-peak in the reduced state (typically around 554nm), by the possession of a second Cys-X-Y-Cys site, lacking bound haem and by N-terminal sequence heterogeneity. The latter has given rise to speculation that cytochrome c_5 derives from a larger cytochrome by the action of endogenous protease activity. However, no precursor cytochrome has yet been identified.


(a)
Nt
'Front' Face
Fe
Ct
Fe
'Front' Face
(b)
Ct
Fe
Nt
Nt
Fe
Ct
'Front' Face

The structure of cytochrome c_4 differs considerably from that of the cytochrome c_5 dimer, in which the haem propionates face in opposite directions and the front faces of the two cytochromes c_5 are involved in the interdomain surface (Fig.1.6b; Carter et al., 1985).

C. Spectral properties Ferrocytochrome c_4 has a characteristic α/β region, with an α-peak maximum in the range 550 to 552nm, a low ratio of α- to β-absorbance (typically 1.2) and a low α–absorbance (Fig.1.3; Pettigrew and Brown, 1988). Cytochrome c_4 from *P. stutzeri* 224 has an α-peak maximum at 550 nm when fully reduced, but on partial oxidation the two haem groups become spectroscopically distinguishable and the α-peak becomes asymmetric and shifts to 552nm (Fig.1.3). This complexity is not seen in the cytochromes c_4 from *P. aeruginosa* and *A. vinelandii*.

Ferricytochrome c_4 has a near infrared band at 701 to 704nm which indicates methionyl-iron coordination (Fig.5.4; Schechter and Saludjian, 1967). The absorption coefficient of this band indicates that both haem groups possess this type of coordination. In addition, a broad band at 630nm is present (Fig.5.4) suggesting that a
small amount of the cytochrome c_4 is high spin. This is discussed in Chapter 5.

D. Redox properties Redox titration of cytochromes c_4 (Leitch et al., 1985; Brown, 1988; this work) show that two redox components are present. In the cytochrome c_4 from *P. stutzeri*, the midpoint potentials of the two haem groups are +300 and +190mV, with cytochromes c_4 from other organisms showing similar, though less widely separated midpoint potentials (Leitch et al., 1985).

Two explanations have been advanced for the existence of a difference between the midpoint potentials of the two haems of cytochrome c_4 (Brown, 1988). Firstly, the two haems may exist in chemically distinct environments, which influence their midpoint potentials sufficiently to account for the observed difference. Secondly, anticooperativity could be operating between

the two haems, such that the two haems are initially identical, but reduction of the second haem group is made less easy by reduction of the first.

These models have been investigated by separation of the two domains and characterisation of each individually with respect to midpoint potential (Brown, 1988). If the difference in midpoint potentials is due to anticooperativity then the midpoint potentials of the separated haems would be expected to be very similar, otherwise they would be expected to retain their difference in midpoint potentials.

The two domains of *P. stutzeri* cytochrome c_4 can be separated by proteolysis using subtilisin or chymotrypsin followed by molecular exclusion chromatography. One domain remains intact as a fragment of M_r approximately 10 000 which retains methionyl-iron coordination and has a midpoint potential of +110mV. The second domain is extensively degraded and the fragment does not retain methionyl-iron coordination. The midpoint potential of the fragment is -190mV, suggesting that the haem group has been exposed to the solvent (Harburg and Loach, 1959). Thus no distinction between the two models proposed could be made (Brown, 1988)

E. Properties on SDS-PAGE Cytochromes c_4 behave anomalously on SDS-PAGE. Oxidised holocytochromes c_4 migrate more rapidly than would be expected from their molecular weight of approximately 20 000. For example, *P. aeruginosa* cytochrome c_4 migrates at a position corresponding to a M_r of 17 000, whereas its true molecular weight, from sequence analysis is 19 900. Reduction of the cytochromes c_4 prior to electrophoresis, or removal of the haem groups results in a slower migrating form. Pettigrew and Brown (1988) proposed that, when oxidised, the cytochromes c_4 retain some compact structure around the haem group, even when in SDS, allowing a faster than expected migration. Reduction of the cytochrome c_4 by 2-mercaptoethanol causes the iron to be lost from the haems (Wood, 1981), disrupting this structure. Similarly, removal of the haem groups entirely allows the protein to unfold completely and thus to migrate at its native molecular weight. Such treatments also prevent haem staining, since the intact haem

group is necessary for the peroxidase activity this technique relies on. All gel samples in this work were in an oxidised state prior to SDS treatment, to prevent this loss of staining.

The holocytochromes c_4 from *P. aeruginosa*, *P. stutzeri* and *A. vinelandii* also show a multiple banding pattern (Pettigrew and Brown, 1988). This multiple banding is abolished by conversion to the apocytochrome and so is not a result of contaminating species in the purified cytochromes c_4. In the case of *P. aeruginosa* cytochrome c_4, heating caused the conversion of the slower migrating to the faster migrating form. This effect was more pronounced if the cytochrome c_4 was first pre-reduced with ascorbate. The ascorbate was then removed by desalting. Note that this treatment differs from the 2-mercaptoethanol treatment in that the reducing agent was removed before denaturation in SDS. Multiple banding of cytochrome c_4 from *P. stutzeri* was not pronounced and the minor band of slightly lower molecular weight than cytochrome c_4 discussed in Chapter 4 was not due to this phenomenon.

F. Immunological properties Immunological analysis of purified cytochromes c_4 from *P. aeruginosa*, *P. stutzeri*, *A. vinelandii* and *Alcaligenes* sp. with antisera directed against *P. stutzeri* and *A. vinelandii* cytochromes c_4 showed that pseudomonad cytochrome c_4 and *A. vinelandii* cytochrome c_4 were weakly cross-reactive, but that the c_4-like cytochrome from *Alcaligenes* sp. was not recognised. This pattern of cross-reactivity agrees with the pattern of sequence identities between these cytochromes on the basis of amino acid compositions (Pettigrew and Brown, 1988). However, despite their usefulness with purified cytochromes c_4, the antisera were of no use when membranes were present since a major membrane protein with mobility very close to that of cytochrome c_4 cross-reacted with the antiserum and cytochrome c_4 could not be resolved from this protein.

G. Cellular location Pettigrew and Brown (1988) showed that two forms of cytochrome c_4 exist, a tightly membrane-bound form and a soluble form, the relative proportions of which vary from organism to organism and with the

growth conditions used. Most of the cytochrome c_4 is membrane-bound. In *P. aeruginosa*, no soluble cytochrome c_4 is detectable, whereas in *P. stutzeri* and *A. vinelandii*, a small percentage (15 to 20%) of the cytochrome c_4 is soluble. More soluble cytochrome c_4 is present under anaerobic, denitrifying conditions, with a corresponding reduction in the amount of membrane-bound cytochrome c_4. This is believed to be the result of a reduction in the number of binding sites for cytochrome c_4 on the membranes under these conditions (Pettigrew and Brown, 1988; Chapter 7).

Binding of cytochrome c_4 to membranes is believed to occur by hydrophobic interactions with the binding sites on the membranes (Pettigrew and Brown, 1988; Chapter 7). When cytochrome c_4 is removed from the membrane environment, a conformational change is believed to occur to hide the now-exposed hydrophobic surfaces. This is discussed in more detail in chapters 5 and 7 and is illustrated in Fig.1.7.

Soluble cytochrome c_4 is located in the periplasm of *P. stutzeri* and *A. vinelandii*. Formation of spheroplasts of these organisms by lysozyme/EDTA treatment released the cytochrome with no release of cytoplasmic marker proteins (Hunter et al., 1989). The membrane-bound cytochrome c_4 is attached to the periplasmic face of the cytoplasmic membrane, as it is degraded by proteases added to intact spheroplasts of *P. stutzeri*. These proteases are only able to attack the outer face of the cytoplasmic membrane, being membrane-impermeable. Hence the cytochrome c_4 must face the periplasm in this organism. *A. vinelandii* cytochrome c_4 was not degraded in this way and a periplasm-facing location can only be inferred (Hunter et al., 1989). Cytochrome c_4 therefore appears to conform to the hypothesis of Wood (1983), discussed earlier, in that it is located in the periplasm and on the periplasmic face of the cytoplasmic membrane.

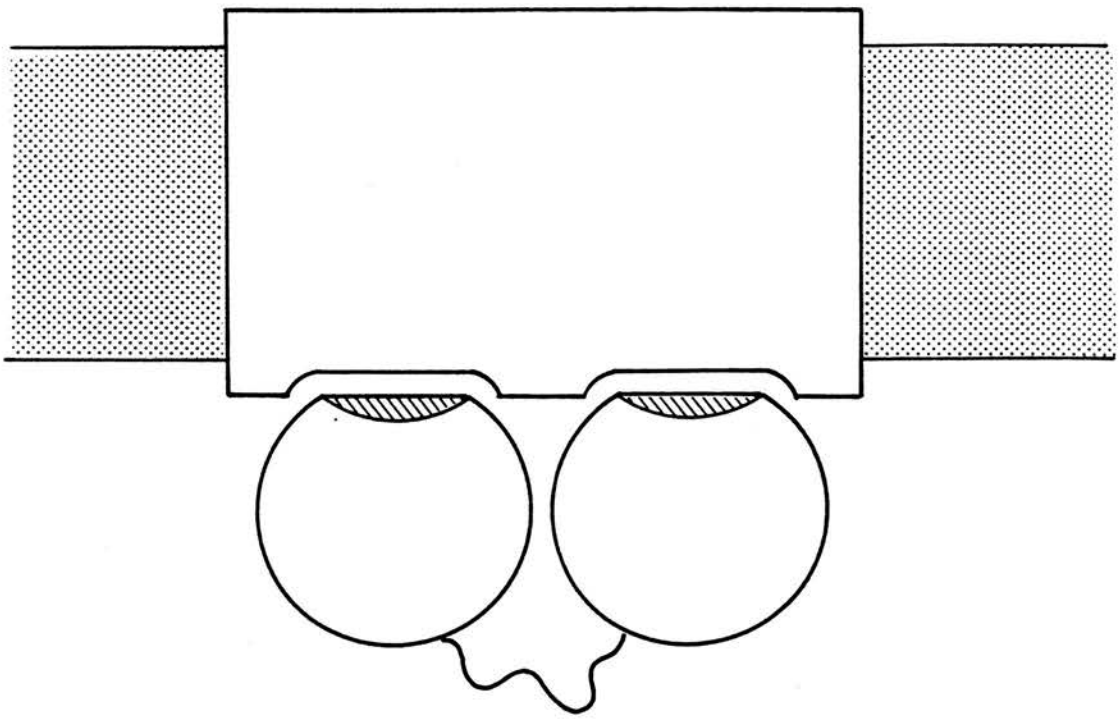
Despite all the information about cytochrome c_4 now available, the function of this cytochrome is still not clear. The aim of the work undertaken

Fig.1.7 Model of the binding of cytochrome c_4 to membranes

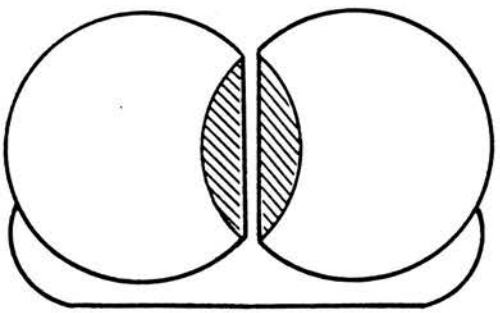
(a) Membrane-bound cytochrome c_4 attached to the membrane by interaction of hydrophobic surfaces on the molecule (shaded) with a specific binding site on the membrane.

(b) Soluble cytochrome c_4. A conformation change has occurred to hide the hydrophobic surfaces (shaded).

(a)



(b)



here was therefore to investigate the properties of cytochrome c_4 further and to try to determine the function of this unusual class I cytochrome c.

III: *Pseudomonas stutzeri* 224

The organism chosen for this investigation was *Pseudomonas stutzeri* strain 224. This choice was made since much of the data gathered about cytochrome c_4 refers to the cytochrome from this organism. The only drawback to this choice is that the amino acid sequence of the *P. stutzeri* cytochrome c_4 is not known. However, the sequence is believed to be closely related, on the basis of amino acid composition (Pettigrew and Brown (1988), to the cytochromes c_4 from *P. aeruginosa* and *A. vinelandii*, whose sequences have been elucidated (Ambler, 1980; Ambler et al., 1984).

The aerobic Pseudomonads (*Pseudomonas* and related genera) are one of the largest groups of chemoheterotrophic Gram-negative bacteria known. The cells of this group are small straight or curved rods, with one or more polar flagellae. Metabolically, they are among the most versatile organisms known, capable as a group of using over 100 different organic compounds as sole energy and carbon sources. These include many exotic compounds such as terpenes, steroids and polycyclic aromatic hydrocarbons. The nutritional requirements of most species are simple, with no requirement for growth factors. Some species are capable of denitrification under anaerobic conditions, but unless this ability is possessed, the organisms are strictly aerobic.

Pseudomonas aeruginosa is perhaps the best known of the aerobic pseudomonads as it is occasionally pathogenic for man. In common with many pseudomonads, it is a free living soil and water organism. It is capable of denitrification. Infection with *P. aeruginosa* only occurs if the individual has a reduced resistance to infection and *P. aeruginosa* is believed to be an 'accidental' pathogen. However, once infected, treatment is difficult as P. aeruginosa is resistant to antibiotic
therapy.

P. aeruginosa is a member of the fluorescent group of pseudomonads,

which produce water-soluble, yellow-green, fluorescent pigments, particularly when grown in media with a low iron content. Other species of this group include *P. putida*, *P. fluorescens* and *P. syringae*, the latter species probably containing many of the organisms responsible for many plant diseases.

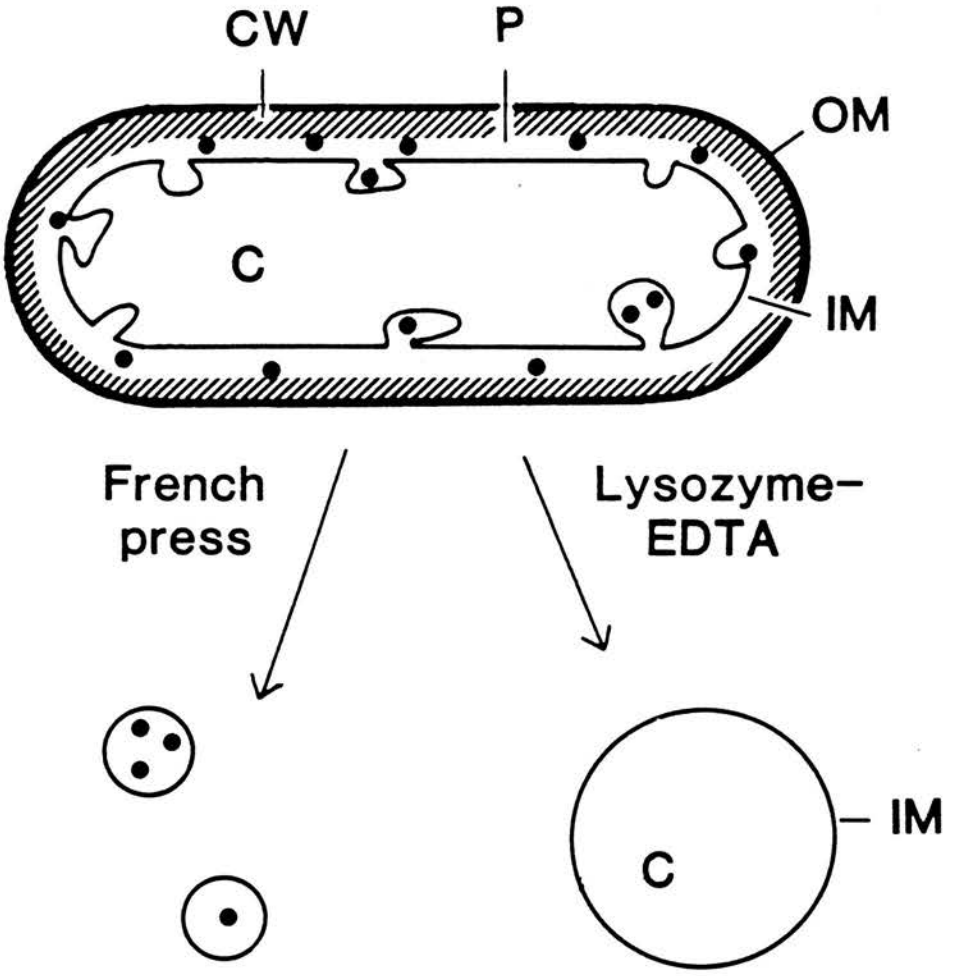
P. pseudomallei is the causative agent of meliodosis, a highly fatal disease of man and animals. As with *P. aeruginosa*, the organism is only an 'accidental' pathogen, infecting individuals with a lowered resistance. It is also a free-living soil and water organism and is a powerful denitrifier. A related species, *P. mallei*, is a true parasite and is responsible for the equine disease glanders.

Pseudomonas stutzeri is a non-fluorescent, denitrifying member of the pseudomonads which is easily isolated from soil samples (van Niel and Allen, 1952). However, the strain used in this work (strain 224, ATCC 17591) was isolated from a clinical specimen (Stanier et al., 1966). Further studies on several strains of *P. stutzeri* revealed two sub-groups, based on the GC content of their DNA (Mandel, 1966). Group B, containing the neotype of *P. stutzeri*, had a GC content of 65%, whilst group A had a GC content of 62.1% and contained strain 224. A new species, "*P. stanieri*", was proposed for this latter group, but this has not been adopted. Recently, *P. perfectomarinus*, a marine pseudomonad, has been reclassified as *P. stutzeri* (strain ZoBell, Dohler et al., 1987) and is placed in the low GC group, along with strain 224.

P. stutzeri 224 is a Gram-negative organism, with the structure shown diagrammatically in Fig.1.8. Such bacteria possess three cellular compartments in which c-type cytochromes might occur, these being the cytoplasm, the cytoplasmic membrane and the space between the cytoplasmic membrane and the outer membrane, the periplasm. Periplasmic proteins can be selectively released by treatment with lysozyme and EDTA in the presence of sucrose, which provides osmotic support for the spheroplasts formed. The method by which this is achieved is given in Chapter 2. The spheroplasts may then be lysed by osmotic shock to release the cytoplasm, so that periplasmic, cytoplasmic and membrane fractions of the cells may be separated.

Fig.1.8 Structure of a Gram-negative bacterium such as *P. stutzeri*

OM: outer membrane, IM: inner (cytoplasmic) membrane, C: cytoplasm, P: periplasm, CW: cell wall. French pressing of the bacterium yields small vesicles which may contain periplasmic proteins (●). The likelihood of this occurring is increased if the inner membrane of the bacterium is highly invaginated. Lysozyme/EDTA treatment removes the outer membrane and cell wall to leave spheroplasts (inner membranes containing an intact cytoplasmic compartment) if osmotically supported. Osmotic shock can then be used to liberate the cytoplasmic contents.


CW
P
OM
C
IM
French
press
Lysozyme–
EDTA
C
IM

Chapter 2: Materials and Methods

Materials:

All chemicals and reagents used were of the best available grade.

Methods:

I: Growth of bacteria

Pseudomonas stutzeri (Stanier strain 224, ATCC 17591) was obtained from Joan Fleming, Dept. of Molecular Biology, University of Edinburgh and was maintained on nutrient agar plates (1% peptone, 1% NaCl, 0.5% yeast extract and 1.5% agar) at 32°C. *P. stutzeri* was grown in liquid culture aerobically at 32°C in a medium containing trisodium citrate (5g/l), potassium dihydrogen phosphate (1g/l), magnesium sulphate (0.5g/l) and yeast extract (Oxoid, 4g/l), adjusted to pH7.0 with 3.0M NaOH, in 1l flasks on an orbital shaker or in a 10l microfermentor (New Brunswick), with sterile air at 10l/min. Anaerobic growth, in the presence of nitrate, was performed as described in chapter 3, section IVB. Cells were harvested at early stationary phase, at A_{600} = 1.2 to 1.5 unless otherwise stated, by centrifugation at 4000g, 30 min, 4°C. The cells were then resuspended in approximately 5 volumes of 10mM sodium phosphate, pH7.0 and were centrifuged as before, to remove traces of the medium. The cells were finally resuspended to 0.5g cells/ml in 10mM sodium phosphate, pH7.0 for storage. *Azotobacter vinelandii* (strain o, ATCC 12837) was grown in modified Burks' medium (Newton et al., 1953) in the 10l microfermentor at 32°C, with vigorous aeration (15l sterile air/min). Cells were harvested as described above.

II: Preparation of cytoplasmic membranes

A. By French press breakage. Cells were centrifuged from their storage medium and were resuspended in three volumes of 10mM sodium phosphate, pH7.0 containing a trace of DNase II (Sigma). They were then disrupted by

passage through a French pressure cell at 83MPa (French and Milner, 1955). Membranes were collected by centrifugation (100 000g, 1 hour, 4°C) and were washed once by resuspension in three volumes of 10mM sodium phosphate, pH7.0 followed by centrifugation as before. The membranes were then resuspended in three volumes of 10mM sodium phosphate, pH7.0, the membrane protein concentration was assayed as described in section VII and the concentration was adjusted to give 10 or 15 mg protein/ml.

B. By production of spheroplasts. Spheroplasts were produced by lysozyme/EDTA treatment (Wood, 1978a; Hunter et al., 1989). Cells were resuspended to 0.5g cells/ml in 10mM tris/HCl, pH8.0 at 20°C after harvesting and washing. 10ml were added to a sucrose/tris/EDTA solution of volume 35ml containing 5mg lysozyme (Sigma) per g cells. After incubation for 2 minutes at 30°C, 5ml 0.1M $MgCl_2$ were added to give final concentrations of 0.5M sucrose, 40mM tris/HCl, pH8.0 at 20°C, 4mM EDTA and 10mM $MgCl_2$. After a further 30 minutes incubation at 30°C, the periplasmic fraction was separated from the spheroplasts by centrifugation (11 000g, 20 min, 4°C). The spheroplasts were resuspended in 50ml of 10mM tris/HCl, pH8.0 at 20°C containing 2mM EDTA, to cause their lysis by osmotic shock. After 15 minutes at 20°C, $MgCl_2$ was added to a final concentration 4mM and the lysed spheroplasts were centrifuged (19 000g, 30 min, 4°C) to separate the cytoplasmic contents from the spheroplast membranes. The latter were resuspended in three volumes of 10mM sodium phosphate, pH7.0 and their protein content was adjusted to 15mg/ml. DNase II was incorporated in both incubations to reduce the viscosity caused by released DNA.

III: Spectroscopy

Difference spectroscopy and carbon monoxide-binding spectroscopy were carried out as described in the legends to Fig.3.2 and 3.3, using a Varian Cary 219 recording spectrophotometer or a Philips PU 8740 uv/vis spectrophotometer. Spectrophotometric quantitation of cytochromes c was performed as follows. A baseline of 10mM sodium phosphate, pH7.0 was recorded. 20µl of the cytochrome to be quantitated was added to 980µl of

10mM sodium phosphate, pH7.0 and was reduced by the addition of a few crystals of sodium dithionite. The spectrum of the reduced cytochrome in the α-region was recorded (530 to 570nm) allowing the α-peak absorbance to be measured. The concentration of the reduced cytochrome c could then be obtained by the use of the millimolar extinction coefficient for the α-peak absorbance of that cytochrome. The published value of 29.0$mM^{-1}cm^{-1}$ was used for the extinction coefficient of cytochrome c from horse heart (Pettigrew and Moore, 1987). The extinction coefficients of *P. stutzeri* cytochrome c-551 at pH7.0 and of *Saccharomyces cerevisiae* cytochrome c were calculated from the pyridine ferrohaemochrome spectra of these cytochromes, using a millimolar extinction coefficient of 31.18mM-1cm-1 for the α-peak absorbance (Bartsch, 1971).

Pyridine ferrohaemochromes were formed by the addition of a 0.1ml sample of the cytochrome under investigation to a pyridine (BDH) and sodium hydroxide solution of volume 1.1ml, to give final concentrations 2.0M pyridine and 0.15M NaOH. The solution was reduced with a few crystals of sodium dithionite. The spectrum was then recorded against an identical pyridine/NaOH solution in the reference cuvette. The native spectrum of an identical sample of the cytochrome, at the same concentration was recorded in a similar way. This enabled the native millimolar extinction coefficient of the cytochrome to be determined, since

$$E_{mM}\ (550nm, native) = 31.18 \times A_{550}\ (native)/A_{550}\ (pyridine\ haemochrome)$$

The values obtained for *P. stutzeri* cytochrome c-551 and *Saccharomyces cerevisiae* cytochrome c were 34.3$mM^{-1}cm^{-1}$ and 29.9$mM^{-1}cm^{-1}$ respectively.

IV: Oxidase assays

A. Ascorbate-TMPD oxidase activity. Ascorbate-TMPD oxidase activity was measured using a Clark oxygen electrode, thermostatted at 25°C. The chamber of the oxygen electrode contained 10mM sodium phosphate, pH7.0,

1mM EDTA, 1mM neutralised ascorbate (BDH), 0.3mM TMPD (Sigma) and water to give a total volume, after the addition of sample, of 2ml. A 50 to 200µl sample of the material to be assayed was added to the chamber to initiate the reaction. The initial rate of activity was recorded.

B. NADH, lactate and succinate oxidase activities. These oxidase activities were measured as described for ascorbate-TMPD oxidase activity , with 20mM NADH (Sigma), sodium lactate (BDH) or sodium succinate (Sigma) replacing the ascorbate and TMPD.

C. Duroquinol oxidase activity. Duroquinol oxidase activity was also measured as described for ascorbate-TMPD oxidase activity , with 0.5mM duroquinol replacing the ascorbate and TMPD. Duroquinol was obtained by the reduction of duroquinone with sodium borohydride (Nelson and Gellefors, 1978). Duroquinone (Sigma) was dissolved in methanol to give a 30mM solution. This was then made anaerobic by gentle bubbling with argon (B.O.C) for 15 minutes. Evaporation of the methanol during this period was minimal as no change in the volume of the solution was detected Sodium borohydride (Hopkin and Williams, Chadwell Heath, Essex, UK) was added to give a 1:1 molar ratio of sodium borohydride to duroquinone. The pH of the solution rose from 7.0 to 10.5, copious quantities of hydrogen were evolved and the yellow duroquinone solution became colourless, indicating its reduction to duroquinol. When all the colour was gone, the pH of the solution was lowered to 2 by the addition of 3.0M HCl. This acidification destroys the remaining sodium borohydride and stabilises the duroquinol.

Duroquinol prepared in this way was stable to air oxidation for long periods. However, the duroquinol slowly precipitated on storage, so a fresh solution was prepared each time before use. Inhibition of the duroquinol oxidase activity with 50µM antimycin A was achieved by the addition of the appropriate volume of 1mM antimycin A in 30% (v/v) ethanol. 30% (v/v) ethanol alone had no effect on the duroquinol oxidase activity. After each assay including antimycin A, the chamber of the oxygen electrode was thoroughly rinsed with ethanol to remove traces of the antimycin A, then with water.

D. Cytochromes c oxidase activities. Cytochromes c oxidase activities were measured spectrophotometrically, by following the oxidation of ferrocytochromes c by the material to be assayed in the presence of oxygen. Horse heart cytochrome c (Sigma, type VI), yeast cytochrome c (Sigma, type VIII-B from *Saccharomyces cerevisiae*) and *P. stutzeri* cytochrome c-551, purified by K.R.Brown, were used as substrates. The cytochromes c (300 to 400μM) were reduced by the addition of neutralised ascorbate to 1mM and the excess ascorbate was removed by desalting on a 1x10cm column of sephadex G-25 superfine (Pharmacia) into 10mM sodium phosphate, pH7.0 containing 1mM EDTA. The concentration of reduced cytochrome was then determined (section III) and sufficient ferrocytochrome c was added to the reaction mixture to give a final concentration of approximately 10μM. The reaction mixture, in a 3ml stirred quartz cuvette, contained ferrocytochrome c (approx. 10μM), 10mM sodium phosphate, pH7.0, 1mM EDTA and water to give a final volume, after the addition of the sample to be assayed, of 2ml. A 10μl sample of the material to be assayed was added to initiate the reaction and the oxidation of the ferrocytochrome c was monitored using a Pye-Unicam SP-1800 uv/vis spectrophotometer at 550nm (horse heart and yeast cytochromes c) or 551nm (*P. stutzeri* cytochrome c-551). The end-point of the oxidation was determined by the addition of a crystal of potassium ferricyanide to completely oxidise the cuvette contents.

The initial velocities for each reaction were calculated from a plot of $\log(A_t - A_\infty)$ against time, where A_t is the absorbance at time t and A_∞ is the absorbance after full oxidation. If C_r is the concentration of ferrocytochrome, then

$$-d[C_r]/dt = k[C_r]$$

$$d[C_r]/[C_r] = -kdt$$

$$\ln[C_r] = -kt + c$$

$$\log[C_r] = -kt/2.303 + c'$$

C_r is proportional to (At - A_∞) so that a plot of log(At - A_∞) against time will give a line of slope -k/2.303. Hence k may be calculated. Assuming the reaction is first order

$$V = -d[C_r]/dt = k[C_r]$$

The concentration of ferrocytochrome is known at the start of the reaction so the initial velocity can be obtained, as

$$V_{initial} = k[C_r]_{initial}$$

The plot of log(At - A_∞) against time was linear for most of the assays performed, showing the reaction to be first order. However, in a few cases a non-linear plot was obtained, showing a deviation from first order kinetics during the early portion of the reaction. The plots were sufficiently linear during the later portions of the reaction to allow straight lines to be drawn through the data for the purposes of comparing rates, but the deviation from first order kinetics should be noted.

V: <u>Redox titrations.</u>

Redox titrations were carried out in an anaerobic cuvette, the contents of which were bubbled with argon (B.O.C) for 15 minutes prior to the titration. The bubbling was continued throughout the titration to ensure the cuvette contents remained anaerobic. The cuvette was stirred constantly using a magnetically driven cell stirrer (Bel-Art). Typically, the cuvette contained 25 to 30nmol of cytochrome c_4, 20mM sodium phosphate, pH7.0, 20µM phenazine methosulphate (Sigma), 20µM phenazine ethosulphate (Sigma), 20µM 2,3,5,6 tetramethylbenzene-1,4-diamine (diaminodurol, Aldrich), 20µM ferric ammonium sulphate and 0.4mM EDTA. the total volume in the cuvette was 3.5ml.

Oxidation and reduction of the cuvette contents was achieved by the

addition of µl volumes of potassium ferricyanide solution (approx. 50mM) and sodium dithionite solution (approx. 40mM, in 0.1M sodium phosphate, pH7.0). After each addition, the ambient potential was measured using a combined platinum pin electrode and a Ag/AgCl reference electrode (Russell pH Ltd, Auchtermuchty, Scotland, UK). The measured potential was referred to that of the standard hydrogen electrode by the addition of 198mV. This value is the standard potential of the Ag/AgCl reference electrode (Bates, 1954). At each ambient potential measured, the state of reduction of the cytochrome c_4 was recorded spectrophotometrically over the wavelength range 530 to 570nm, using a Pye-Unicam SP-1800 uv/vis spectrophotometer. The data obtained were then analysed as described in chapter 4.

VI: <u>SDS-polyacrylamide gel electrophoresis (SDS-PAGE)</u>

SDS-polyacrylamide gels were prepared as 130x150x1mm slabs, with 15% (w/v) acrylamide (Sigma) and 0.4% (w/v) bisacrylamide (Sigma). Alternatively, an acrylamide gradient was employed, from 10% (w/v) acrylamide/0.2% (w/v) bisacrylamide to 25% (w/v) acrylamide/0.5% (w/v) bisacrylamide. A 4% (w/v) acrylamide stacking gel was employed in either case. Electrophoresis was carried out using the buffer system of Laemmli (1970) lacking 2-mercaptoethanol and with the addition of 2mM EDTA, for approximately 16 hours at 50V or for 4 to 5 hours at 180V.

Samples were prepared for electrophoresis by dissolving in 62.5mM tris/HCl, pH6.8 at 20°C, containing 2mM EDTA, 2% (w/v) SDS and 10% (v/v) glycerol. Samples with volumes over 30µl were lyophilised prior to dissolving in the above buffer. Samples were heated to 90°C for 2 minutes before loading on to the gel.

Gels were stained for haem by the method of Goodhew et al. (1986). Gels were soaked in a 1.25mM solution of 3,3',5,5' tetramethylbenzidine (Sigma) in methanol/0.25M sodium acetate, pH5.0 (30:70) for 30 minutes with constant shaking. 30% (w/v) hydrogen peroxide ('Analar' BDH) was then added to 26mM and the staining reaction was allowed to proceed for a further 15 minutes, The gels were then washed twice in propan-2-ol/0.25M sodium

acetate, pH5.0 (30:70) for 15 minutes each wash and were immediately photographed and scanned. Gels were stained for protein using Coomassie brilliant blue R; C.I.42660 (Sigma) in methanol/acetic acid/water (4.5:1:4.5) for approximately 30 minutes and were then destained in methanol/acetic acid/water (3:1:6).

Scanning of gels was performed using a Shimadzu CS-930 tlc scanner at 690nm (haem-stained gels) or 600nm (protein-stained gels). The scanner was used in transmission mode, with beam dimensions 0.05x2mm and using 2mm steps. Gels were photographed through a yellow filter (Cokin A.001).

VII: Protein estimation

Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as a standard. Estimations in the presence of detergents were carried out using the modified procedure of Dulley and Grieve (1975). This modified procedure is necessary as otherwise a yellow precipitate forms on the addition of the Folin-Ciocalteau reagent. Addition of 0.5% SDS to the alkaline copper reagent prevents this precipitate formation and allows the protein concentration to be estimated as before.

CHAPTER 3: Properties of *P. stutzeri* cytoplasmic membranes

Cytoplasmic membranes were prepared from *P. stutzeri* by two methods, as described in Chapter 2; French press treatment and lysozyme/EDTA treatment (Wood, 1978a). The latter method was used to produce membranes by lysis of the spheroplasts by osmotic shock. The properties of membranes prepared by these two methods were identical (data not shown) and the choice of method used depended on the purpose for which the membranes were required.

Membranes prepared by French pressing required a high g_{av} to pellet them on centrifugation (100 000g against 10 to 15 000g for spheroplast membranes). This is presumably due to the smaller size of vesicles generated by French pressing. Spheroplast membranes, whilst easier to pellet, tended to be more viscous than French press membranes due to undegraded DNA associated with them. DNase II was incorporated in the production procedure to minimise this problem. However, French press membranes were preferred for spectral studies, where rapid and even mixing of membrane suspensions was required. Only in exceptional cases was it possible to use spheroplast membranes. Spheroplast membranes were usually preferred for manipulations involving many centrifugation steps (see Chapter 4).

I: Properties of membranes on SDS-PAGE

Fig.3.1a shows the pattern of staining obtained after SDS-PAGE of *P. stutzeri* spheroplast membranes (M) and staining for haem by the method of Goodhew et al.(1986). Also included on the gel are the periplasmic (P) and cytoplasmic (C) fractions from the spheroplast preparation. S is pure cytochrome c_4 loaded as a standard. Fig.3.1b shows densitometer traces obtained after scanning the gel shown in Fig.3.1a at 690nm.

The cytochromes of the periplasm of *P. stutzeri* are well characterised with respect to molecular weight and may easily be identified on the gel and

Fig.3.1 SDS-PAGE of samples from a spheroplast preparation of *P. stutzeri*

(a) 15% slab SDS-PAGE gel 30µl samples from the periplasmic (P), cytoplasmic (C) and membrane (M) fractions of a spheroplast preparation of P. stutzeri were loaded. The gel was stained for haem by the method of Goodhew et al. (1986) as described in Chapter 2. 25pmol cytochrome c_4 were loaded as a standard (S). I_{Ps}, II_{Ps} and III_{Ps} refer to the three major unidentified cytochromes c of *P. stutzeri* cytoplasmic membranes.

(a)

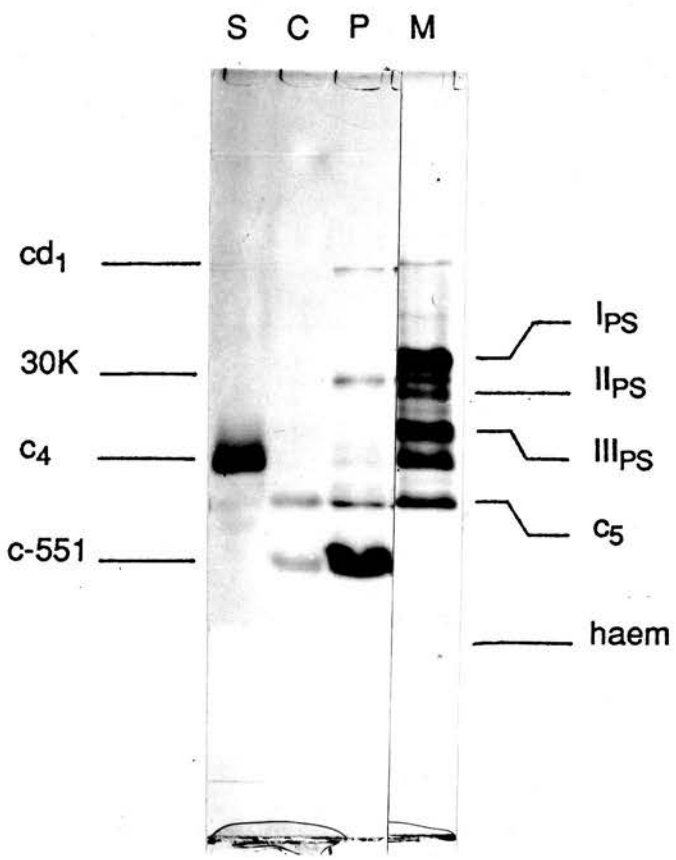
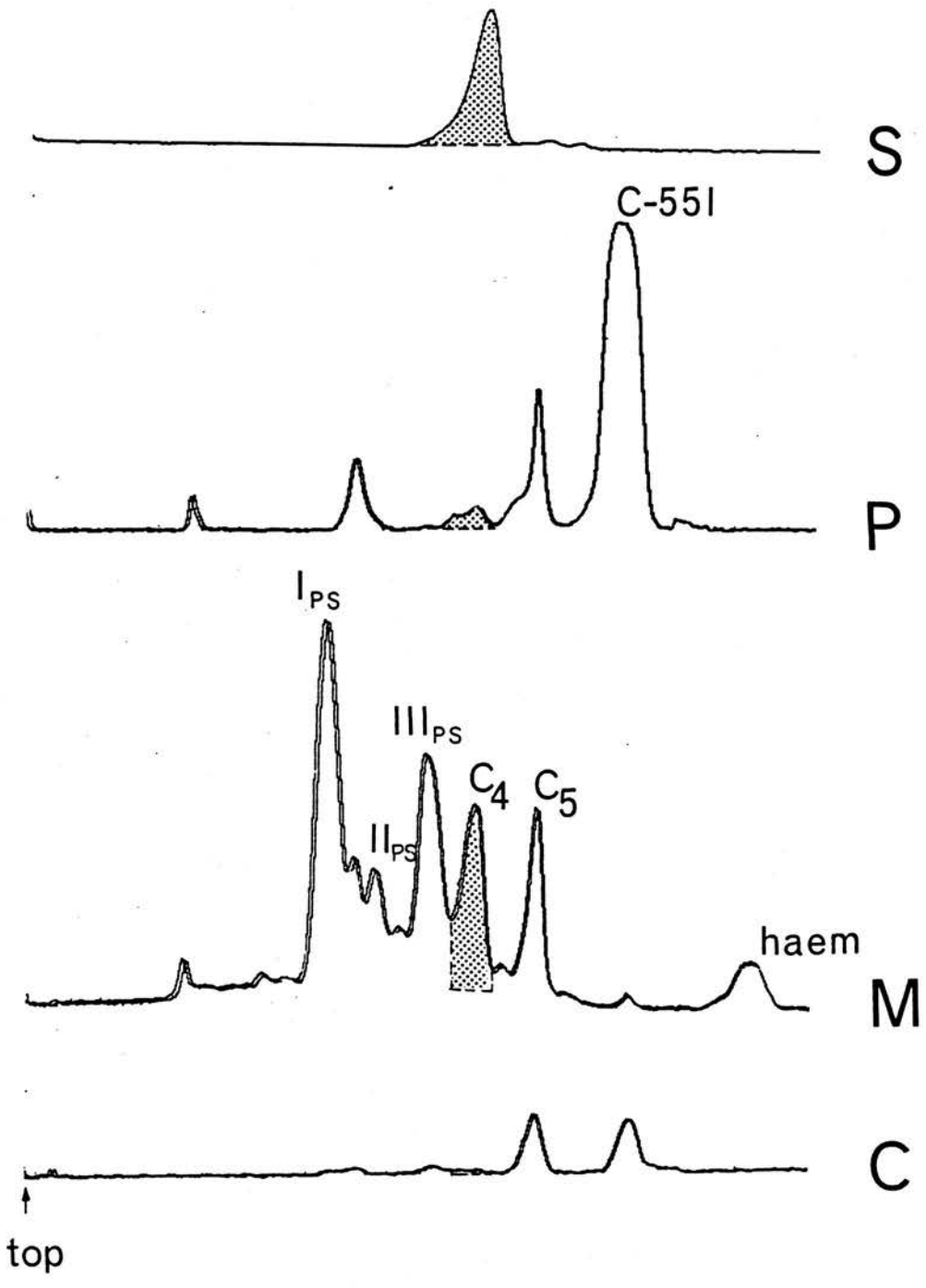


Fig.3.1 cont.

(b) Densitometric traces of the gel in (a) Five major membrane bands are labelled: I_{Ps} (M_r 38 000); II_{Ps} (M_r 32 000); III_{Ps} (M_r 25 000); cytochrome c_4 (shaded, M_r 19 000); cytochrome c_5 (M_r 12 000). Also labelled is periplasmic cytochrome c-551 (M_r 9000). Taken from Hunter et al. (1989).

(b)



densitometer traces. These are cytochrome cd_1 (nitrite reductase, two subunits each of M_r 62 000), an unidentified cytochrome of M_r approx. 30 000 (the 30K cytochrome of Brown, 1988), soluble cytochrome c_4 (M_r 19 000), soluble cytochrome c_5 (M_r 12 000) and cytochrome c-551 (M_r 9000).

Two of these c-type cytochromes may be identified in the membrane fraction (M), these being the membrane bound portions of cytochromes c_4 and c_5. In addition to these identifiable cytochromes, three other major membrane c-type cytochromes are present which I have named I_{PS} (M_r 38 000), II_{PS} (M_r 32 000) and III_{PS} (M_r 25 000), along with several minor bands. These minor bands may represent cytochromes c or may be due to the association of free haem from denatured b-type cytochromes with other proteins (Goodhew et al., 1986). The pattern of haem staining of *P. stutzeri* membranes is very similar to that observed with *A. vinelandii* membranes and the three major membrane bands may be common to both species, along with cytochromes c4 and c5 (Hunter et al., 1989). The functions of these three cytochromes is not known.

Free haem is also present in the membrane fraction after SDS-PAGE. This derives from non-covalently bound haem groups in cytochromes a, b and d, which dissociate from the apoprotein on denaturation in SDS. The free haem migrates as a poorly staining, distorted band just behind the solvent front. The presence of free haem in the membranes indicates that, as well as the various c-type cytochromes, one or more cytochromes with non-covalently bound haem are present. Unfortunately, it is not possible to identify the apoprotein from which the free haem derives, as the staining caused by residual haem cannot be distinguished from that caused by artifactual transfer of haem to non-haem proteins (Goodhew et al., 1986).

A number of cytochromes c are apparently also present in the cytoplasmic fraction (C). A little of these apparently cytoplasmic cytochromes c are actually the result of contamination of the cytoplasmic fraction by membranes. Most is due to contamination of the spheroplasts by periplasm prior to their lysis. However, the amount of cytochrome c_5 in the cytoplasm is too great to be explained by such contamination and is probably due to

membrane-bound cytochrome c_5 removed from the spheroplast membranes by the low ionic strength treatment used to lyse the spheroplasts (Hunter et al., 1989).

II: Spectroscopy of *P. stutzeri* membranes

Further characterisation of the cytochromes present in *P. stutzeri* membranes used difference spectroscopy. Fig.3.2 shows the reduced minus oxidised spectrum of *P. stutzeri* membranes between 520 and 680nm (a) together with an expansion of the 580 to 680nm region (b). The main features of the spectrum obtained are:

1. A peak at 550nm, due to c-type cytochromes previously detected by SDS-PAGE and haem-staining.
2. A shoulder on the 550nm peak, at 560nm. This is due to b-type cytochromes.
3. A peak at 630nm and a trough at 650nm, typical of cytochrome d.

No indications of the presence of a-type cytochromes (peaks at 590 to 610nm) are visible.

The presence of cytochrome d was found to be variable, depending on the particular batch of cells from which the membranes where prepared. In general, however, cytochrome d was not prominent and was often completely absent. The effect of growth conditions on cytochrome d is discussed in section IV, but the cytochrome is generally induced by low oxygen tensions. This is not the whole story and other factors known to enhance the appearance of the E. coli enzyme include growth with cyanide, growth of cells to late log or stationary phase, growth with glucose or anaerobic growth (Poole, 1983). The variable appearance of cytochrome d in *P. stutzeri* may be due to a variation in some condition of growth which was not controlled.

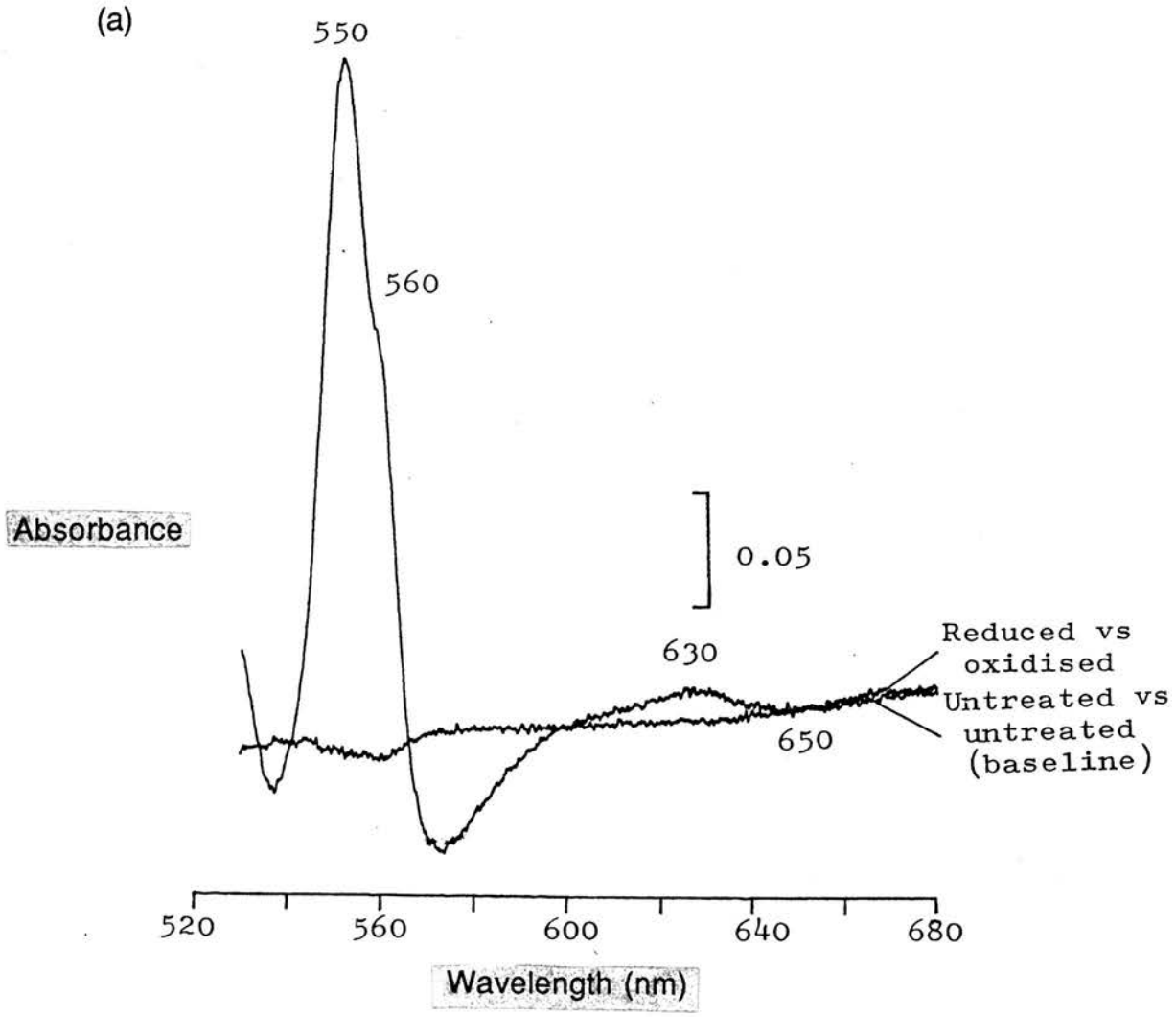
In order to confirm the presence of cytochrome d and to investigate which of the cytochromes might be involved in oxidase activity, carbon monoxide-binding spectra were recorded. Carbon monoxide binding is the most convenient method for distinguishing haem proteins where the iron has an absent or replaceable sixth ligand. It is used to tentatively identify oxidases

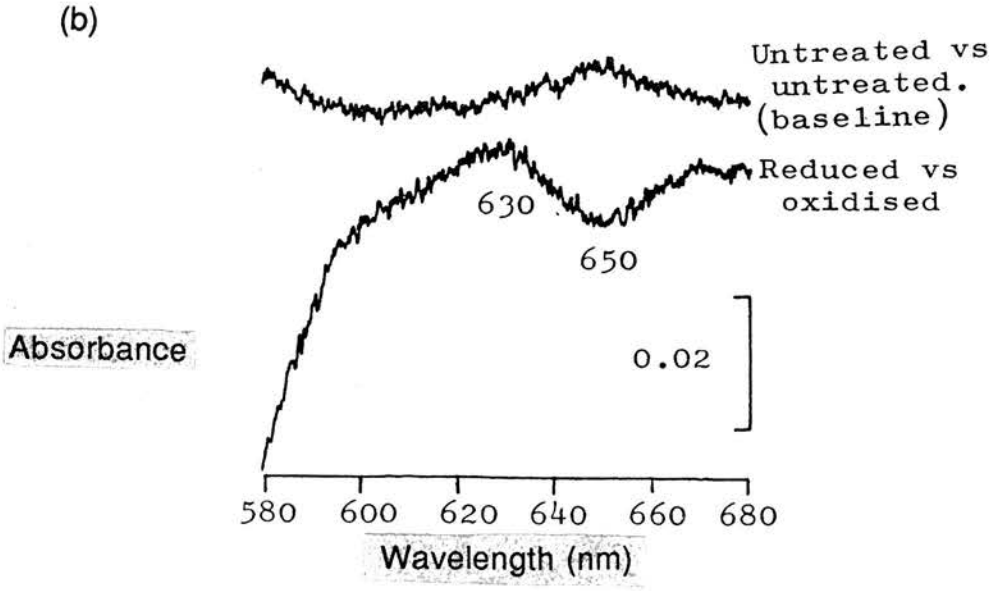
Fig.3.2 Reduced minus oxidised spectra of *P. stutzeri* cytoplasmic membranes

Spheroplast membranes (7.5 mg protein/ml) in 0.1M sodium phosphate, pH7.0 were dispensed into two 1ml cuvettes and a baseline was recorded. The reference cuvette was oxidised with a crystal of potassium ferricyanide, the sample cuvette was reduced with a few crystals of sodium dithionite and the reduced minus oxidised spectrum was recorded in a Varian Cary 219 recording spectrophotometer.

(a) α-peak region (520 to 680nm)

(b) Expansion of (a) to show the α-peak of cytochrome d.


(a)
550
560
Absorbance
0.05
630
650
Reduced vs oxidised
Untreated vs untreated (baseline)
520
560
600
640
680
Wavelength (nm)


(b)
Untreated vs untreated. (baseline)
Reduced vs oxidised
630
650
Absorbance
0.02
580
600
620
640
660
680
Wavelength (nm)

due to the similarity between the oxygen and carbon monoxide complexes of these enzymes.

Carbon monoxide spectra were recorded as described in the legend to Fig.3.3, which shows the carbon monoxide spectrum of *P. stutzeri* membranes between 520 and 680nm. Binding of carbon monoxide to cytochrome d is evident (peak at 640nm, trough at 620nm). Binding also occurs to cytochromes b (trough at 560nm) and to cytochromes c (shoulder on the 560nm trough at 550nm). This suggests that d-type and b-type (o-type) cytochrome oxidases may be present.

Cytochromes c usually possess six coordinating ligands, thus carbon monoxide binding would not be expected. However, most cytochromes will bind carbon monoxide if denatured. This may be the explanation for the binding observed here, however, there are many documented cases of carbon monoxide binding cytochromes c (Wood, 1984). For example, the cytochrome c from *M. methylotrophus* cytochrome co binds carbon monoxide (Carver and Jones, 1983). There may be a parallel situation here, with *P. stutzeri*.

Because of the problems of carbon monoxide binding to denatured or damaged cytochromes, to cytochromes c and to other non-oxidase proteins (Wood, 1984), it is not possible to use carbon monoxide binding to demonstrate an oxygen binding function for a cytochrome. In the case of cytochrome oxidases, it is necessary to link inhibition of oxidase activity by carbon monoxide to the carbon monoxide binding spectrum to distinguish them from other carbon monoxide binding cytochromes. This is done by photochemical action spectra, where the oxidase activity is inhibited be carbon monoxide. High intensity light of varying wavelength is then used to photodissociate the carbon monoxide from the haem, relieving the inhibition. This relief of inhibition occurs with a spectrum characteristic of the cytochrome under investigation and can therefore be used to identify the carbon monoxide binding centre. It was not possible to record photochemical action spectra for *P. stutzeri* membranes and the assignment of oxidase activity to cytochromes d and b (cytochrome o) on the basis of carbon monoxide binding must

Fig.3.3 Carbon monoxide-binding spectrum of *P. stutzeri* cytoplasmic membranes

Spheroplast membranes (7.5 mg protein/ml) in 0.1M sodium phosphate, pH7.0 were reduced with a few crystals of sodium dithionite, dispensed into two 1ml cuvettes and a reduced minus reduced baseline was recorded. Reagent grade carbon monoxide (British Oxygen Company) was bubbled through the contents of the sample cuvette for 1 minute and the reduced + carbon monoxide minus reduced spectrum was recorded. Further sodium dithionite was then added to the sample cuvette to check that traces of oxygen in the carbon monoxide had not partially oxidised the sample.

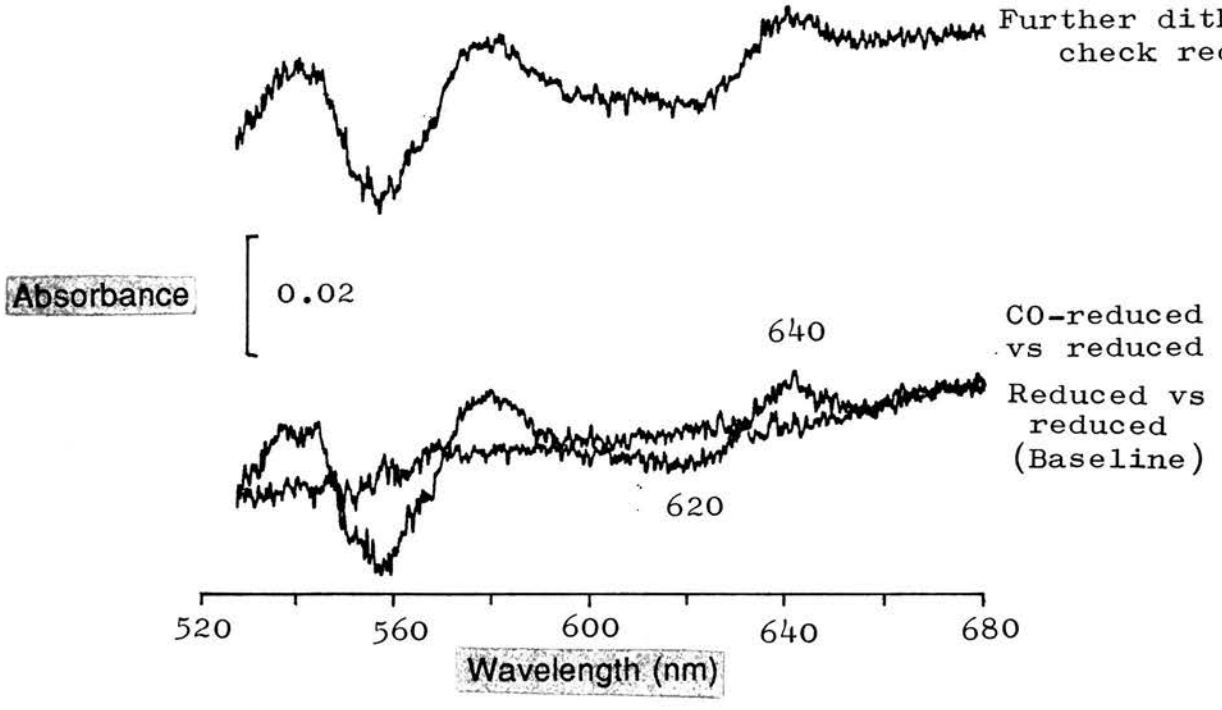


Fig.3.4 Reduction of *P. stutzeri* cytoplasmic membranes with other reductants

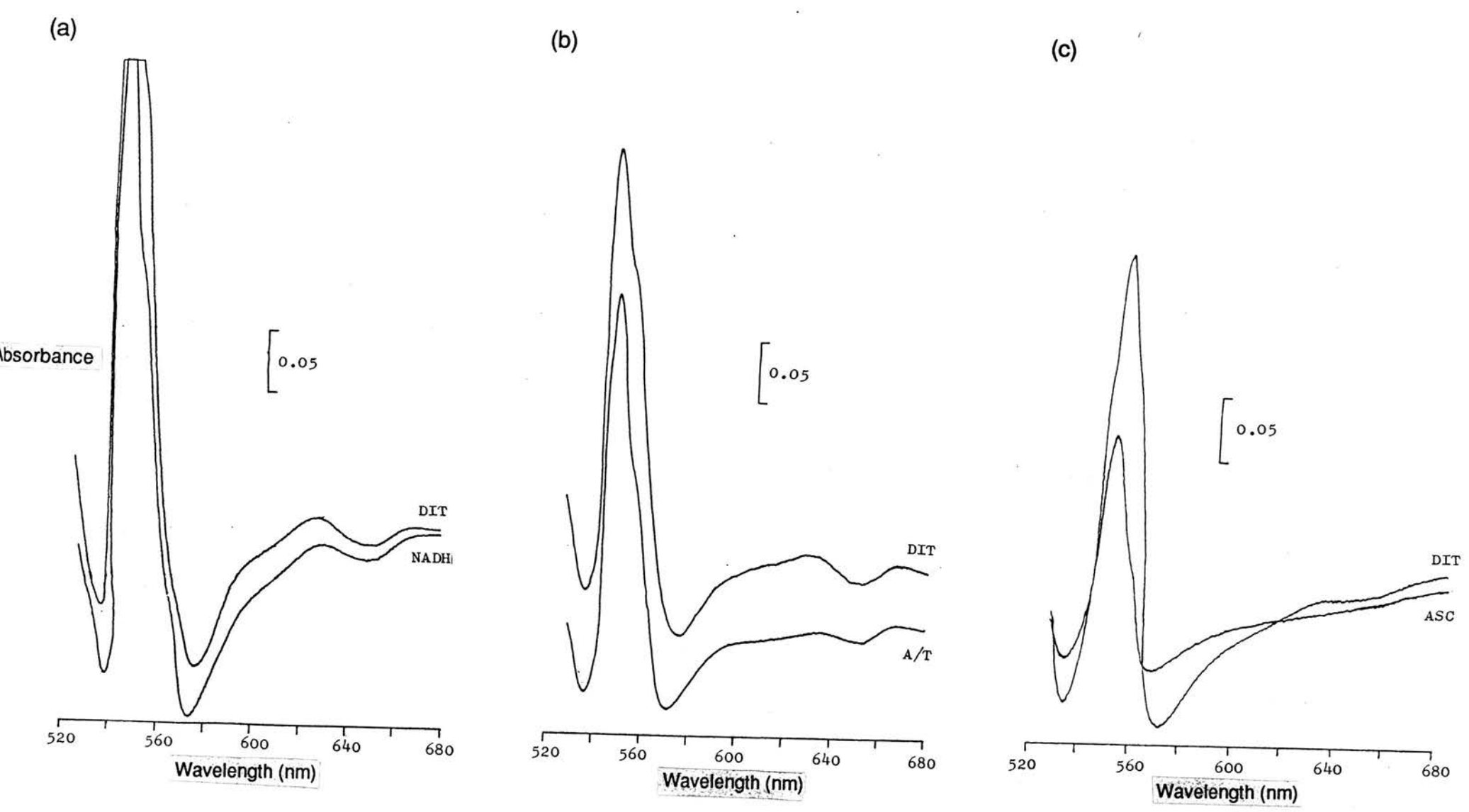
Reduced minus oxidised spectra of P. stutzeri cytoplasmic membranes were recorded as described in the legend to Fig.3.2. The following reductants were used to reduce the contents of the sample cuvette:

(a) NADH: 20mM NADH

(b) A/T: 1mM ascorbate + 0.3mM TMPD

(c) ASC: 1mM ascorbate

DIT indicates spectra recorded after the use of sodium dithionite as a reductant, as described in the legend to Fig.3.2


(a)
Absorbance
0.05
DIT
NADH
520
560
600
640
680
Wavelength (nm)
(b)
0.05
DIT
A/T
520
560
600
640
680
Wavelength (nm)
(c)
0.05
DIT
ASC
520
560
600
640
680
Wavelength (nm)

therefore only be tentative.

Sodium dithionite reduces the respiratory components of *P. stutzeri* membranes fully. A number of other reducing agents were also investigated (Fig.3.4). NADH reduces cytochrome d, but does not reduce cytochromes b or c as completely as does dithionite (Fig.3.4a). NADH oxidase activity has been associated with cytochrome d (see section III) so this result is not surprising. Ascorbate-TMPD and ascorbate alone (Fig 3.4b and c) are poor reductants for cytochrome d, although ascorbate-TMPD does partially reduce the cytochrome d. This suggests that ascorbate-TMPD oxidase activity is not associated with this cytochrome, a conclusion also suggested in section III. D-type cytochrome oxidases have often been found not to be ascorbate-TMPD oxidases (for example, Sweet and Peterson, 1981; Jones and Redfearn, 1967). A well studied example is that of *E. coli*, where the cytochrome d is known to be a quinol oxidase (Kita et al., 1984), although in this case ascorbate-TMPD oxidase activity was present.

Ascorbate-TMPD fully reduced the cytochromes c, but only partially reduced the cytochromes b (Fig.3.4b). Ascorbate alone was a poor reductant, reducing cytochromes b and c partially (Fig.3.4c). Ascorbate-TMPD reduction is generally thought to reflect reduction via cytochrome c, electrons from TMPD (Em +260mV) entering the electron transport chain at the level of cytochrome c (see Chapter 7). Reduction by ascorbate alone occurs in the same way, but at a much lower rate (Kimelberg and Nicholls, 1969). Reduction of cytochromes b and c, with only partial reduction of cytochrome d, by ascorbate-TMPD suggests that the putative o-type cytochrome oxidase is associated with ascorbate-TMPD oxidase activity (see section III) and that its reduction proceeds via cytochrome c.

III: Oxidase activities associated with *P. stutzeri* membranes

Table 3.1 lists the oxidase activities investigated in *P. stutzeri* cytoplasmic membranes. Ascorbate-TMPD, NADH, succinate and lactate oxidase activities were assayed as described in Chapter 2, by monitoring oxygen utilisation by membranes in the presence of substrate, in an oxygen

Table 3.1

(a) Oxygen electrode assays: (rates in μmol O_2/min/mg protein)

Substrate	Concentration	Rate	Notes
Ascorbate-TMPD	1mM/0.3mM	88.1	Biphasic inhibition by cyanide with K_i approx. 0.5 and 5.0μM
NADH	20mM	51.9	Not inhibited by cyanide at concentrations up to 1mM
Succinate	20mM	7.6	-
Lactate	20mM	N.D.	Rate negligable
Duroquinol	0.5mM	7.7	70-80% inhibition with 50μM antimycin A

(b) Spectrophotometric assays: (Rates in nmol cytochrome oxidised/min/mg protein)

Substrate	Concentration	Rate	Notes
Horse Heart cytochrome c	8.93μM	148.9	Assays carried out on different membranes to those in (a), hence rates are not directly comparable
Pseudomonas cytochrome c-551	9.56μM	30.2	
Saccaromyces cerevisiae cytochrome c	9.18μM	156.2	

electrode. Ascorbate-TMPD and NADH are oxidised rapidly by membranes, whilst succinate and lactate are only poorly oxidised (rates respectively approx. 9% and less than 1% of that for ascorbate-TMPD, Table 3.1). Duroquinol oxidase activity was also assayed in the oxygen electrode, in preference to monitoring duroquinol oxidation spectrophotometrically at 270nm. This activity is markedly inhibited by 50μM antimycin A, indicating that the activity is mediated by a complex III and is not due to the quinol oxidase activity of cytochrome d or to a non-specific oxidation of duroquinol by membrane components.

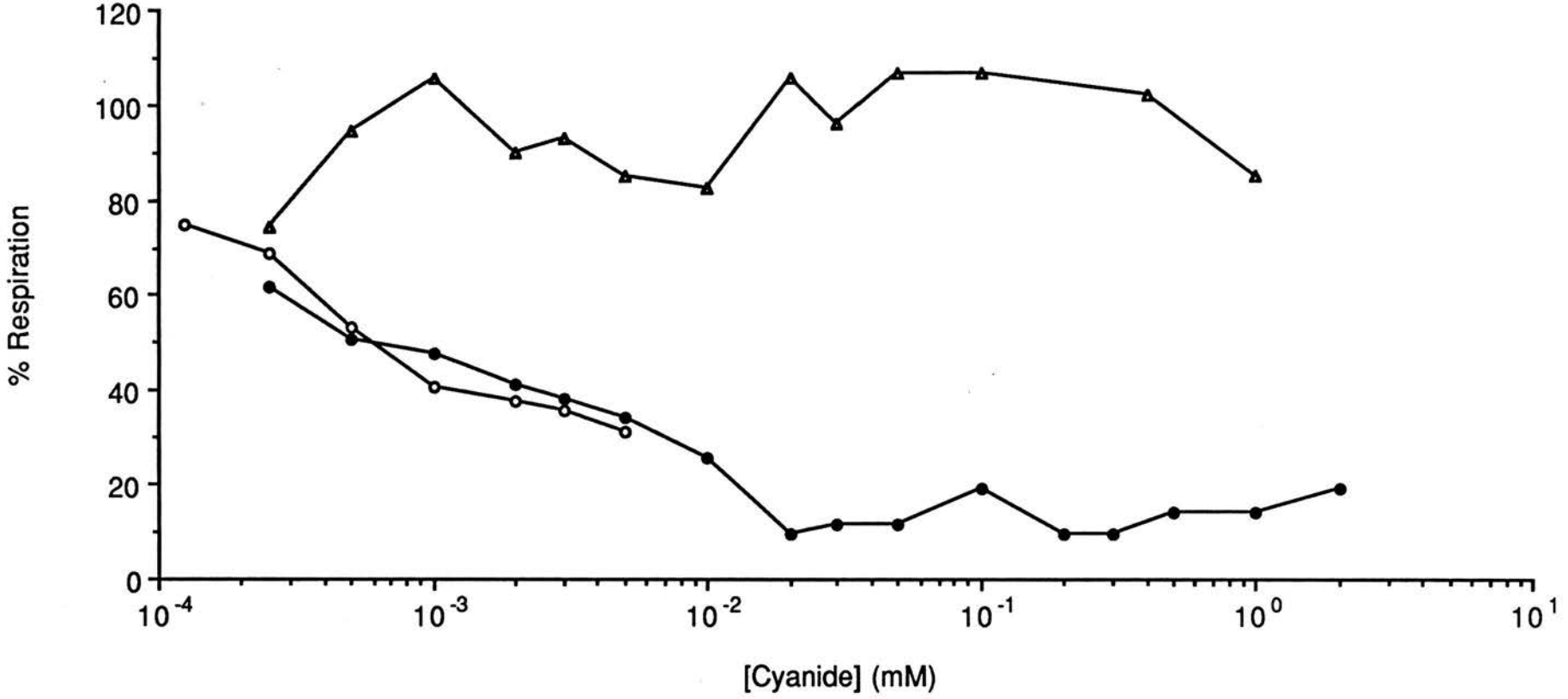
Cytochrome c oxidase activities can be assessed using ascorbate-TMPD (Goodhew, 1988). However, the activities were also assayed directly, by monitoring the oxidation of reduced cytochromes c by membranes in the presence of oxygen spectrophotometrically, at 550nm (*Saccharomyces cerevisiae* cytochrome c, horse heart cytochrome c) or 551nm (*P. stutzeri* cytochrome c-551). Initial velocities for each reaction were calculated as described in Chapter 2.

The results (Table 3.1) show that all three cytochromes are oxidised by *P. stutzeri* membranes. This is unexpected, as cytochrome c-551 is an acidic cytochrome whilst *S. cerevisiae* and horse heart cytochromes c are basic cytochromes and would not therefore be expected to interact with the same cytochrome oxidase. A second unexpected finding is that cytochrome c-551 oxidase activity is lower than the activities observed with the two basic cytochromes. One would expect the *Pseudomonas* cytochrome to interact better with a *Pseudomonas* cytochrome oxidase than exogenous cytochromes c. This difference in activity with acidic and basic cytochromes c may reflect the presence of two cytochrome oxidases, or a single cytochrome oxidase with differing rates for reactions with acidic and basic cytochromes c.

The cyanide sensitivities of ascorbate-TMPD oxidase and NADH oxidase are shown in Fig.3.5. NADH oxidase activity is unaffected by cyanide at concentrations up to millimolar, whereas ascorbate-TMPD oxidase activity is highly sensitive to the presence of cyanide, displaying a biphasic response with K_i values of 0.5 and 5.0μM. A low sensitivity to cyanide is characteristic of

Fig.3.5 Inhibition of ascorbate-TMPD and NADH oxidase activities by cyanide

Spheroplast membranes, in 5mg aliquots, were added to sodium cyanide of the appropriate final concentration (0 to 2mM) in the chamber of an oxygen electrode thermostatted to 25°C. After a 1 minute incubation to allow binding of the cyanide to the membranes, ascorbate-TMPD (○ ,●) or NADH (△) oxidase activity was assayed as described in Chapter 2. The percentage respiration was then calculated for each cyanide concentration, with 100% respiration at 0mM cyanide, and the results plotted as shown here. Two experiments are shown for the ascorbate-TMPD oxidase activity.


120
100
80
60
40
20
0
% Respiration
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
[Cyanide] (mM)

cytochrome d (Poole, 1983) and the results obtained therefore suggest that NADH oxidase activity is associated with this cytochrome. This agrees with the conclusions drawn from spectral studies of the membranes (section II).

Cytochromes o are more sensitive to cyanide. The ascorbate-DCIP oxidase reaction of *A. vinelandii*, known to proceed via cytochrome o has a K_i for cyanide of 0.15μM (Jones and Redfearn, 1967). The K_i values for cyanide observed with *P. stutzeri* membranes are close to this value and suggest that the ascorbate-TMPD oxidase activity is associated with cytochrome o. Again, this conclusion is supported by the results of membrane spectroscopy discussed in section II.

IV: Effect of growth conditions on the composition of *P. stutzeri* membranes

Previous experiments have suggested that cytochrome o is associated with ascorbate-TMPD oxidase activity and therefore its reduction proceeds via a cytochrome c. Jurtshuk et al. (1981) have proposed that the cytochrome o of *A. vinelandii* contains cytochrome c_4. As cytochrome c_4 is also present in *P. stutzeri*, the possibility of its association with cytochrome o was investigated. The effects of growth conditions - time of growth and growth under anaerobic vs aerobic conditions - on the levels of cytochrome c_4 and of ascorbate-TMPD oxidase activity were studied, in order to see if any correlation between the two could be discovered. Additionally, other changes in the membrane composition were monitored.

A. Time of Growth Cells were grown in a 10l microfermentor to set values of A_{600} (a measure of cell density), then were harvested and their membrane compositions were analysed. The microfermentor (New Brunswick), containing 10l sterile citrate medium, described in Chapter 2, was inoculated at time=0 with a 1l aerobic culture of *P. stutzeri* in stationary phase (after 18 to 20 hours growth). Sterile air was supplied at 10l/min and the fermentor was maintained at 32°C, with vigorous agitation. A_{600} was monitored at 1/2 to 1 hour intervals. Cells were harvested by centrifugation as described in Chapter

2, in 2l batches when the A_{600} reached values of approx 0.6, 0.9, 1.2 and 1.5. The remaining cells were harvested after 15 hours growth at an A_{600} of approx. 1.8.

Fig.3.6a shows the increase in A_{600} with time and the corresponding increase in cells harvested as g cells/l (wet weight). Both curves show a gradual tapering off of growth as stationary phase is approached. Little sign of a lag phase is detectable. The growth seems to follow a pattern whereby the rate of growth gradually decreases.

Spheroplast membranes were then prepared from each batch of cells by treatment with lysozyme and EDTA as described in Chapter 2, except that no osmotic support was provided during the lysozyme treatment. The spheroplasts formed therefore lysed immediately, eliminating the need for a separate lysis step. This procedure produced membranes and a soluble fraction comprising both periplasmic and cytoplasmic contents. Membrane ascorbate-TMPD oxidase activity was assayed and samples of the soluble and membrane fractions were run on SDS-PAGE and stained for haem (Fig.3.7a) or protein (Fig.3.7b), as described in Chapter 2. The amount of cytochrome c_4 in each membrane fraction was then estimated by scanning the haem-stained gel at 690nm and quantitating the area under the cytochrome c_4 peak on the densitometer trace.

Fig.3.6b shows the changes in ascorbate-TMPD oxidase activity and the amount of cytochrome c_4 on the membranes with time. The greatest ascorbate-TMPD oxidase activity occurs early in course of growth, activity peaking after 4.5 hours, then decreasing. In contrast, cytochrome c_4 and the other membrane cytochromes c are markedly induced between 2.5 and 7 hours. The levels of cytochromes c then reach a plateau and fall slightly after 15 hours. No correlation between the amount of cytochrome c4 on the membranes and the membrane ascorbate-TMPD oxidase activity is observed.

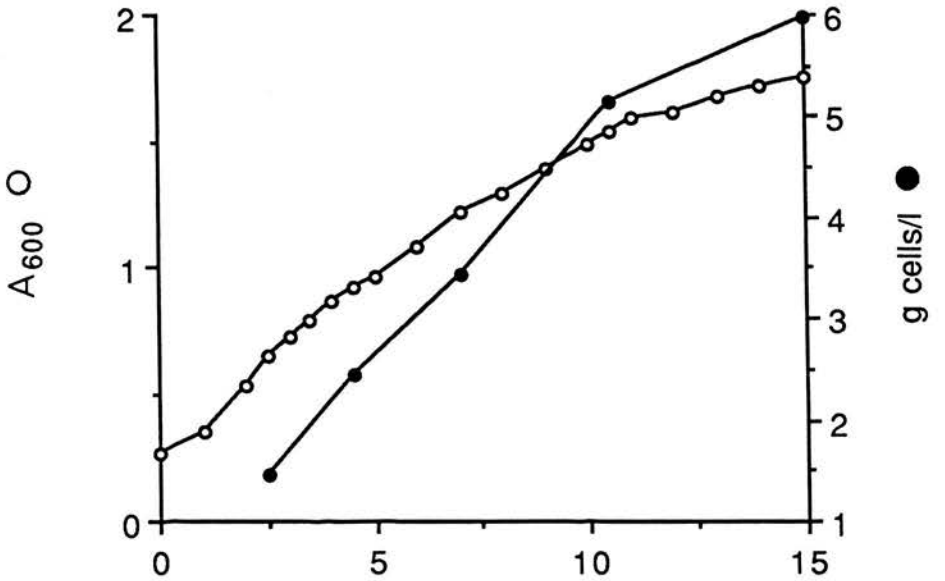
Quantitation of the amounts of cytochromes b and d by means of an SDS-PAGE gel is not possible, for the reasons discussed in section I. It was therefore necessary to record spectra of the membrane fractions to allow

Fig.3.6 Time course of growth of *P. stutzeri*

(a) Increase in A_{600} (○) and grammes of cells obtained per litre (●) (wet weight) with time of growth of an aerobic culture of *P. stutzeri*. Inoculation and growth of the culture were performed as described in the text. A_{600} was measured using a Philips PU8740 UV/Vis spectrophotometer. Grammes of cells obtained per litre of culture was measured after harvesting of the cells as described in Chapter 2.

(b) Changes in membrane ascorbate-TMPD oxidase activity (□) and cytochrome c_4 content (■) with time of growth. Ascorbate-TMPD oxidase activity was measured as described in Chapter 2. Cytochrome c_4 content of membranes was estimated from densitometric traces of the haem-stained gel of Fig.3.7a.

(a)


A_{600} O
g cells/l ●
2
1
0
6
5
4
3
2
1
0
5
10
15

(b)

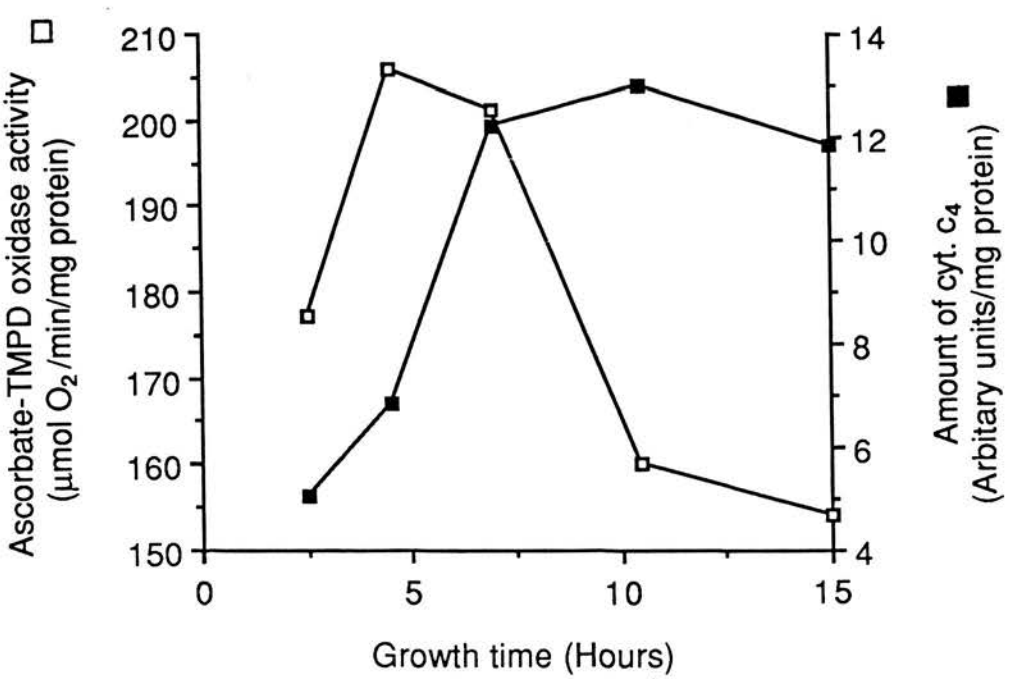

□
Ascorbate-TMPD oxidase activity
(µmol O_2/min/mg protein)
■
Amount of cyt. c_4
(Arbitary units/mg protein)
210
200
190
180
170
160
150
14
12
10
8
6
4
0
5
10
15
Growth time (Hours)

Fig.3.7 Time course of growth of P. stutzeri - SDS-PAGE

10-25% gradient SDS-PAGE gels of samples of the soluble fraction and membranes of cells harvested after different periods of growth as described in the text. 10μl samples of the undiluted fractions produced after spheroplast lysis were loaded.

(a) Haem-stained gel

Lane	Contents
1	Standards (protein-stained gel only)
2	Soluble fraction, t = 2.5 hours
3	Membranes, "
4	Soluble fraction, t = 4.5 hours
5	Membranes, "
6	Soluble fraction, t = 7.0 hours
7	Membranes, "
8	Soluble fraction, t = 10.5 hours
9	Membranes, "
10	Soluble fraction, t = 15.0 hours
11	Membranes, "

(a)

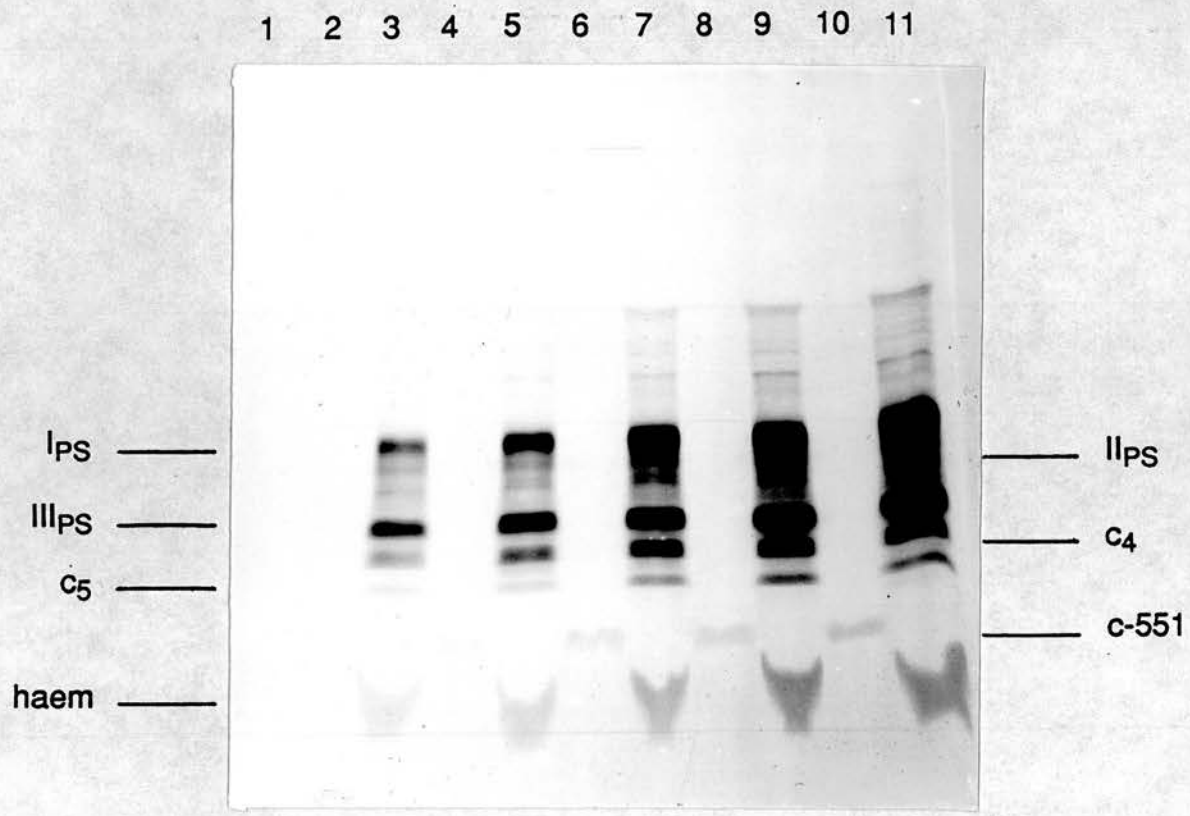
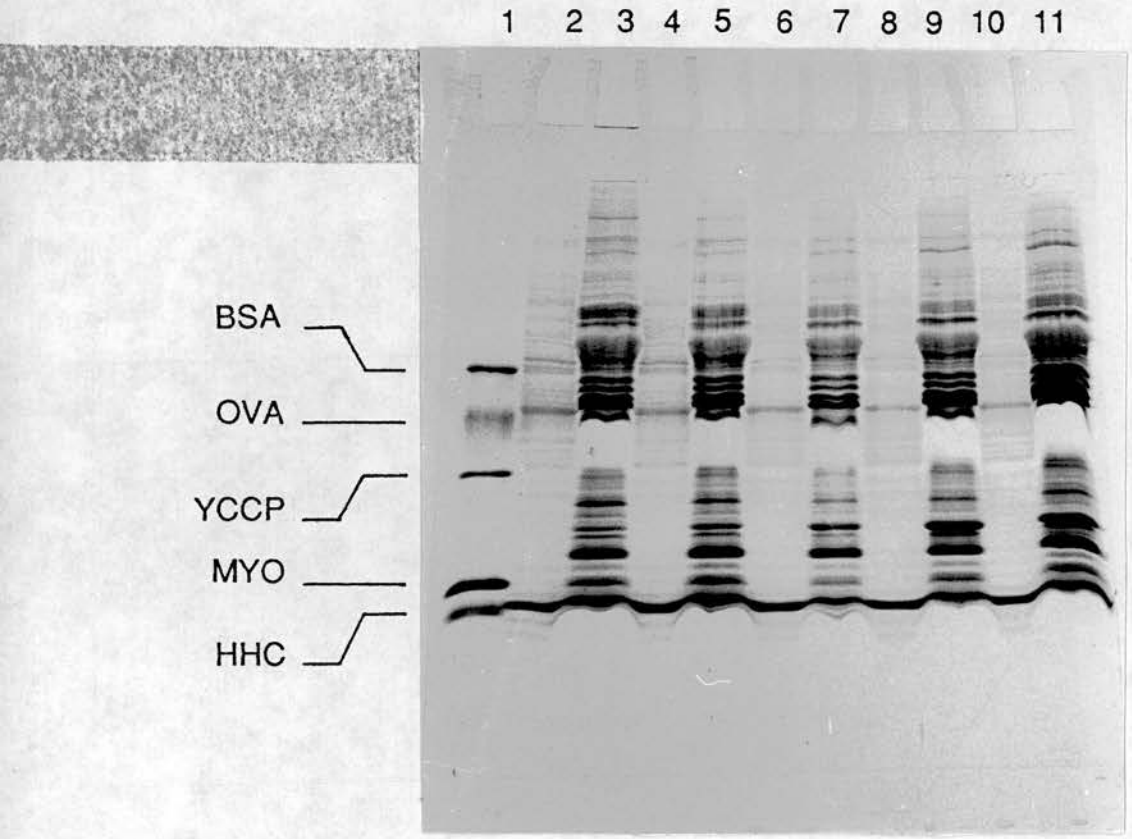


Fig.3.7 cont.

(b) Protein stained gel The standards used on this gel were 5 μg each of bovine serum albumin (BSA, M_r 66 000), ovalbumin (OVA, M_r 45 000), yeast cytochrome c peroxidase (YCCP, M_r 34 000), myoglobin (MYO, M_r 17 000) and horse heart cytochrome c (HHC, M_r 12 000)

(b)



these cytochromes to be quantitated. Reduced minus oxidised and carbon monoxide binding spectra of each membrane fraction were recorded (data not shown). These spectra indicated firstly that cytochrome d was not detectable in this particular cell growth, suggesting that the ascorbate-TMPD oxidase activity observed must be due to cytochrome o.* Secondly, the levels of cytochromes b (and indeed cytochromes c) follow a pattern similar to that observed for cytochrome c_4; an induction between 2.5 and 7 hours, then a plateau and slight fall in levels by 15 hours of growth.

Fig.3.7b shows the protein-stained gel of soluble and membrane fractions from the growth experiment. Due to the complex pattern of staining seen, it is difficult to see any induction of specific protein bands. However, certain bands are increased in intensity during the growth and are presumably being induced as the growth progresses. However, there is little evidence of a major induction of specific proteins.

In this experiment, the membrane ascorbate-TMPD oxidase activity peaked after 4.5 hours, yet in later experiments, cells were grown to stationary phase (18 to 20 hours of growth). This was because the cell yield after 4.5 hours of growth was lower than that obtained after 18 hours of growth and the higher ascorbate-TMPD oxidase activity was sacrificed in favour of a greater cell yield.

B. Anaerobic vs aerobic growth Cells were grown in parallel in 1l flasks on an orbital shaker, aerobically, as described in Chapter 2, and anaerobically in the presence of nitrate. In the latter case, the 1l cultures were inoculated and allowed to grow aerobically until cell density reached approximately 30% of that at stationary phase. Sodium nitrate (5g/l) was then added aseptically and the flasks were sealed. This procedure was necessary as *P. stutzeri* does not survive low level inoculation into nitrate-containing medium. Because of the high initial level of aerobic cells, some of the proteins associated with aerobic growth may persist into the stationary, denitrifying stage. This problem has, however, been shown to be minor (Goodhew et al., 1986).

The aerobically and anaerobically grown cells were then French pressed

and centrifuged (100 000g, 1 hour, 4°C) to produce soluble and membrane fractions. Membrane ascorbate-TMPD, horse heart cytochrome c and *P. stutzeri* cytochrome c-551 oxidase activities were assayed and samples of the soluble and membrane fractions were run on SDS-PAGE and stained for haem to allow quantitation of the amounts of the various membrane cytochromes.

Table 3.2 summarises the results of these oxidase assays, showing that the membranes from aerobically grown cells possess significantly greater amounts of the oxidase activities than do membranes from anaerobically grown, denitrifying cells. It is interesting to note that whilst the ascorbate-TMPD and cytochrome c-551 oxidase activities of the anaerobic membranes are around 15% of those of the aerobic membranes, the corresponding figure for horse heart cytochrome c oxidase activity is 45%. This suggests that horse heart cytochrome c oxidase activity is less affected by the anaerobic, denitrifying conditions than the other oxidase activities measured.

Reduced minus oxidised and carbon monoxide-binding spectra were again recorded to investigate the composition of the membranes with respect to non-c-type cytochromes. Figs.3.8 and 3.9 show the spectra obtained. Anaerobic membranes contain a lower level of cytochrome d than do aerobic membranes, although the levels in aerobic membranes varied, with some membranes having little or no cytochrome d (data not shown). Anaerobic membranes also possessed a higher carbon monoxide-binding cytochrome b content, which suggests a higher cytochrome o content. This may not, however, be the case, since binding of carbon monoxide by cytochromes b is not solely due to cytochrome o, as discussed in section II.

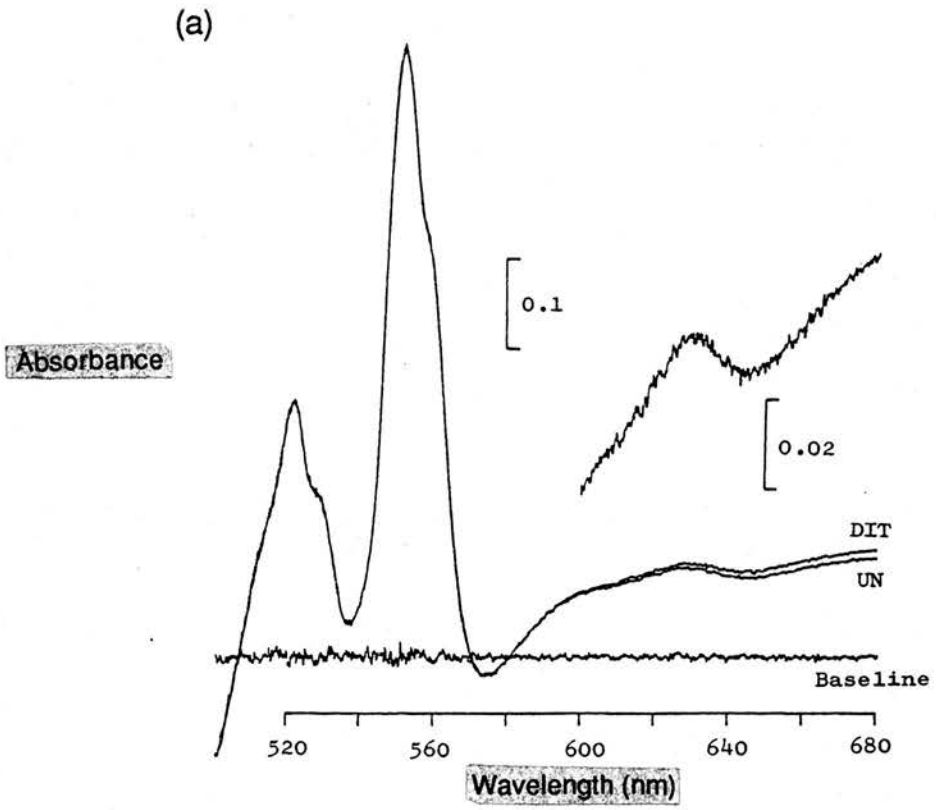
The total cytochrome c content of the two membrane types appears to be very similar, as judged by reduced minus oxidised spectra (Fig.3.8). Cytochrome c content was also investigated by SDS-PAGE and haem staining, the results of which are shown in Fig.3.10. The gel clearly shows induction of periplasmic cytochrome cd_1 and 30K protein, but does not show the previously reported induction of cytochrome c-551 (Pettigrew and Brown,

Table 3.2

Oxidase activity	Aerobic membranes	Anaerobic membranes	Aerobic/ Anaerobic
Ascorbate-TMPD (μmol O_2/min/mg protein)	241.0	37.0	6.5
Horse Heart Cytochrome c (nmol cytochrome oxidised /min/mg protein)	137.7	62.1	2.2
Pseudomonas cytochrome c-551 (nmol cytochrome oxidised/min/mg protein)	79.4	11.0	7.2

Fig.3.8 Comparison of aerobic and anaerobic, denitrifying growth - reduced minus oxidised spectra

French press membranes (7.5 mg protein/ml) from aerobically (a) or anaerobically (b) grown cells, suspended in 0.1M sodium phosphate, pH 7.0 were dispensed into 1ml cuvettes and their reduced minus oxidised spectra were recorded as described in the legend to Fig.3.2.


(a)
Absorbance
0.1
0.02
DIT
UN
Baseline
520
560
600
640
680
Wavelength (nm)

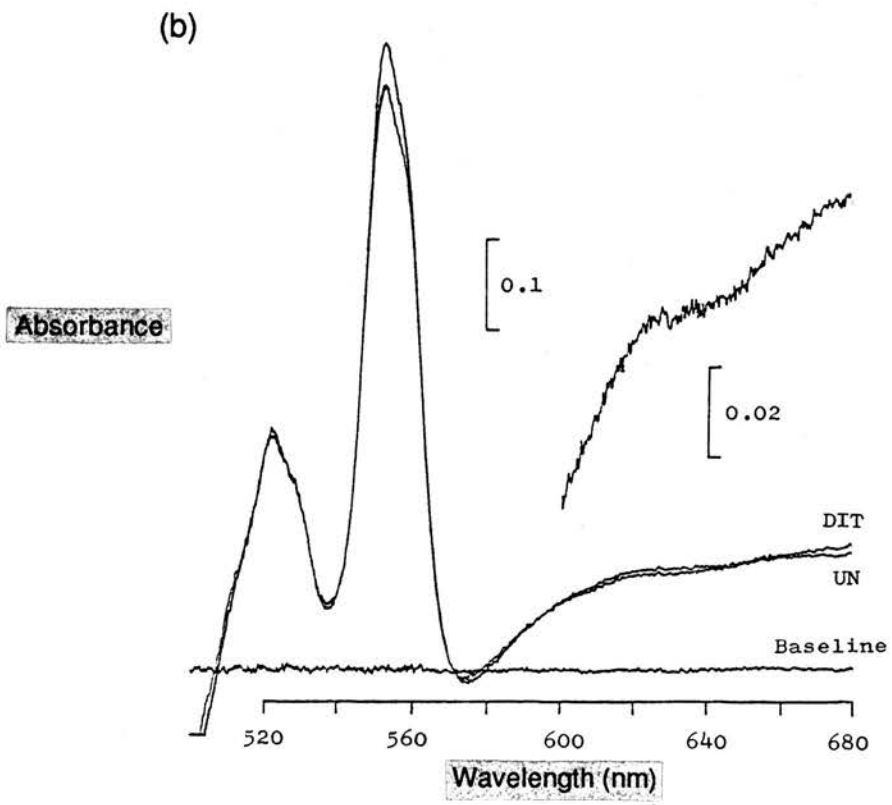
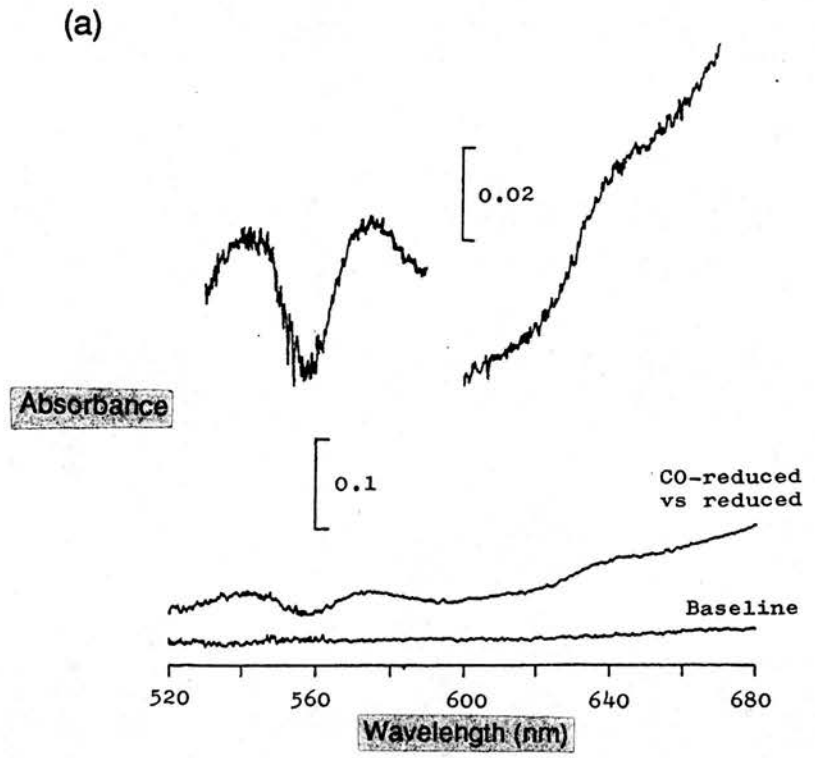

(b)
Absorbance
0.1
0.02
DIT
UN
Baseline
520
560
600
640
680
Wavelength (nm)

Fig.3.9 Comparison of aerobic and anaerobic, denitrifying growth - carbon monoxide -binding spectra

French press membranes (7.5 mg protein/ml) from aerobically (a) or anaerobically (b) grown cells, suspended in 0.1M sodium phosphate, pH 7.0 were dispensed into 1ml cuvettes and their carbon monoxide-binding spectra were recorded as described in the legend to Fig.3.3.


(a)
0.02
Absorbance
0.1
CO-reduced
vs reduced
Baseline
520
560
600
640
680
Wavelength (nm)

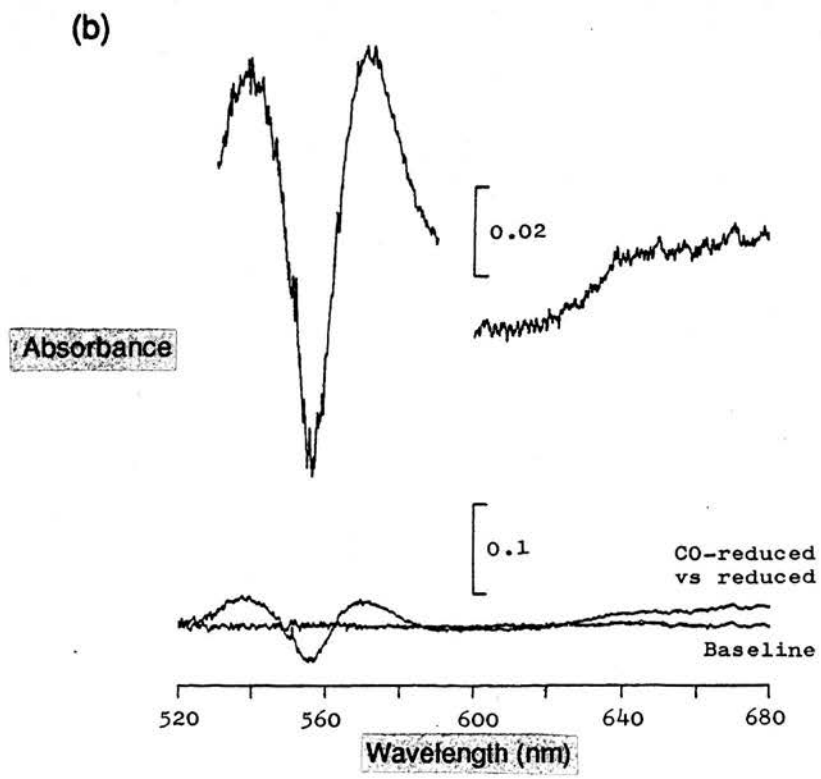
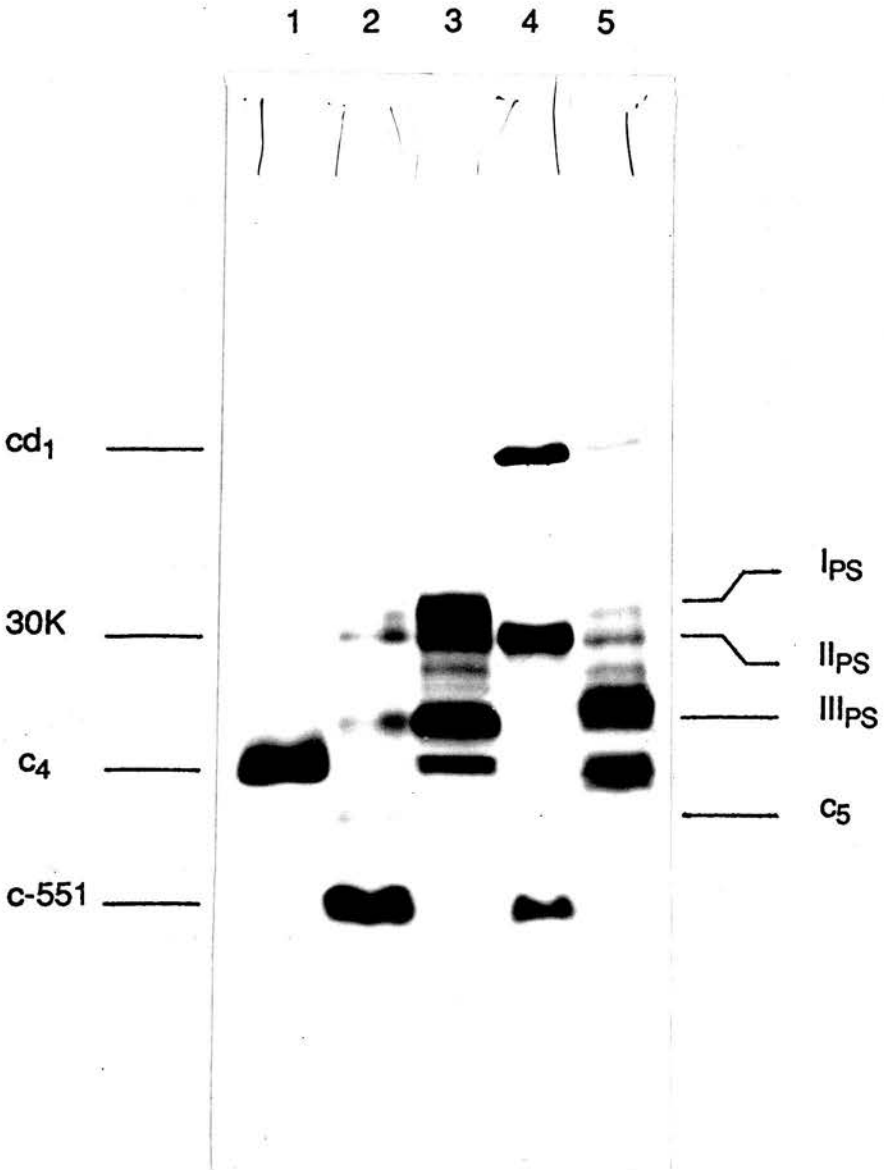

(b)
0.02
Absorbance
0.1
CO-reduced
vs reduced
Baseline
520
560
600
640
680
Wavelength (nm)

Fig.3.10 Comparison of aerobic and anaerobic, denitrifying growth - SDS-PAGE

10-25% gradient SDS-PAGE gel of soluble and membrane fractions from aerobically and anaerobically grown *P. stutzeri*, stained for haem. Samples containing 15μg protein were loaded.

Lane	Contents
1	50pmol cytochrome c_4
2	Aerobic soluble fraction
3	Aerobic membrane fraction
4	Anaerobic soluble fraction
5	Anaerobic membrane fraction


1
2
3
4
5
cd1
30K
c4
c-551
IPS
IIPS
IIIPS
c5

1988). This may be due to the poor staining of cytochrome c-551, which, due to its low molecular weight, is easily leached from gels.

The membrane cytochrome c composition of *P. stutzeri* changes markedly on anaerobic, denitrifying growth. Band I_{Ps} is markedly reduced in amount, as is band III_{Ps}, which is replaced by a form of slightly higher molecular weight. Cytochrome c_4 levels are also slightly reduced, but estimation of the exact amount of reduction is made impossible by the appearance of a strongly-staining band of slightly lower molecular weight which is not resolved from the cytochrome c_4 on scanning. Pettigrew and Brown (1988) reported that the levels of membrane-bound cytochrome c_4 fall by approximately 25% on anaerobic growth, although the overall amount of cytochrome c_4 remains constant.

V: Discussion

The evidence presented here suggests that in *P. stutzeri* two terminal cytochrome oxidases are present and a branched electron transport pathway exists. Spectroscopy and analysis of the oxidase activities of *P. stutzeri* cytoplasmic membranes, including their sensitivity to cyanide, suggests that cytochrome d and cytochrome o are both present and active. However, the presence of cytochrome d is variable and the conditions of growth affecting its expression were not investigated. The exact nature of the d-type cytochrome oxidase is also unknown, but since cytochrome c_4 has been linked with cytochrome o (Yang and Jurtshuk, 1978a; Jurtshuk et al., 1981), this oxidase was further investigated, rather than cytochrome d.

Changes in the pattern of cytochromes in the membranes of *P. stutzeri* with changing growth conditions depend on what these changes are. Little change in the relative amounts of the various cytochromes with time of growth is seen, rather a general induction occurs early in the growth. Cytochrome d was absent from the cells used in this experiment, so that no information as to whether this cytochrome follows the same pattern could be obtained.

The situation observed with anaerobic, denitrifying growth is more

complex. Here, several changes in the pattern of cytochromes were noted. Although the overall level of cytochromes c remains constant, there is a change in the cytochrome c composition of the membranes. This change involves the repression of cytochromes presumably involved in aerobic respiration and induction of those associated with denitrification. An example of this is the repression of band I_{Ps}, which is greatly reduced in intensity on anaerobic membranes. This cytochrome c may correspond to a cytochrome c_1-like protein of P. aeruginosa (Wood, 1980) However, its repression is somewhat surprising if this is the case as complex III, of which cytochrome c_1 is a part, is thought to mediate electron flow to cytochrome cd_1, the nitrite reductase and to nitrous oxide reductase in *Pa. denitrificans* (Ferguson, 1982).

Anaerobic, denitrifying cells possess a lower level of cytochrome d than do aerobic cells. This would suggest, at first sight, that the lower levels of ascorbate-TMPD and cytochromes c oxidase activities in anaerobic cells are due to the reduced amount of this oxidase. However, cytochrome d has been associated with NADH oxidase activity by spectral means and by its low sensitivity to cyanide, rather than ascorbate-TMPD oxidase activity. Also, the difference in oxidase activities between anaerobically and aerobically grown cells were seen when the membranes contained little or no cytochrome d. Cytochrome d is therefore probably not involved in this difference in oxidase activities.

The higher levels of carbon monoxide-binding cytochromes b in anaerobic membranes are not associated with cytochrome o. Cytochrome o is linked to ascorbate-TMPD oxidase activity, higher in aerobic membranes, by spectra and its sensitivity to cyanide (sections II and III). A higher level of cytochrome o in aerobic membranes would therefore be expected. The carbon monoxide-binding cytochromes b of anaerobic membranes are therefore more likely to be associated with the mechanism of denitrification than with cytochrome o. For example, cytochrome b is associated with the membrane-bound nitrate reductase of *E. coli* and *Pa. denitrificans* (Pettigrew and Moore, 1987). *P. stutzeri* would be expected to possess a similar enzyme

under denitrifying conditions. *P. stutzeri* (strain ZoBell) possesses a membrane nitric oxide reductase which also contains a b-type cytochrome subunit (Heiss et al., 1989).

The results of these growth experiments make an association between cytochrome c_4 and the cytochrome o unlikely. No correlation between membrane cytochrome c_4 levels and ascorbate-TMPD oxidase activity during growth was observed. Difficulties in quantitating the amount of cytochrome c_4 on anaerobic membranes makes the results of comparing aerobic and anaerobic membranes less clear cut. However, cytochrome c_4 levels are not greatly affected by anaerobic growth (Fig.3.10). Pettigrew and Brown (1988) report that the levels of cytochrome c_4 on membranes fall by approximately 25% on anaerobic growth, far less than the 85% fall in ascorbate-TMPD and cytochrome c-551 oxidase activities, again making an association between cytochromes c_4 and o unlikely. This conclusion is tentative and the relationship between cytochrome c_4 and ascorbate-TMPD oxidase activity (and hence cytochrome o) is explored in more detail in Chapter 4.

Chapter 4: Removal of cytochrome c_4 from the membranes of *P. stutzeri*

The results of the growth experiments described in Chapter 3 suggest that an association between cytochrome c_4 and cytochrome o is unlikely. However, the results obtained in these experiments are only suggestive and do not provide conclusive evidence for this lack of association. It was therefore necessary to investigate the proposed association more directly, by examining the effect of selective removal of cytochrome c_4 from the cytoplasmic membranes of *P. stutzeri* on the ascorbate-TMPD oxidase activity of the membranes. This required the development of methods for removing cytochrome c_4 from the membranes without the removal of other membrane proteins or significant disruption of the membranes. A number of methods were investigated, none of which met these criteria fully, but which were nonetheless useful in establishing the role of cytochrome c_4 in the oxidase activities of *P. stutzeri* cytoplasmic membranes.

I: Removal of cytochrome c_4 from native membranes

A. Manipulation of ionic strength and pH *P. stutzeri* spheroplast membranes (10mg protein/ml) were centrifuged from their storage medium (11 000g, 20 min, 4°C) in 1ml aliquots and were taken up in 1ml of the appropriate treatment solution. After incubation for 30 min at 0°C, with thorough mixing, the samples were centrifuged as before to separate solubilised from insoluble material. The pellets were then resuspended in their original incubation volume (1ml 10mM sodium phosphate, pH 7.0) and samples of equal volume from each supernatant and pellet produced were run on a 10-25% gradient SDS-PAGE gel which was then stained for haem.

The treatments used in this case were those normally employed in the removal of extrinsic membrane proteins. High ionic strength (10mM sodium phosphate, pH7.0 containing 2.5M sodium chloride) and low ionic strength (10mM tris/HCl, pH8.0 at 4°C) have commonly been employed for the removal

of membrane-associated proteins. In addition, a high pH treatment (10mM sodium carbonate, pH 9.5) was also investigated. This treatment was used by Berry and Trumpower (1985) in the purification of ubiquinol oxidase from *Pa. denitrificans*, to remove membrane proteins prior to detergent extraction of the oxidase. Treatment with 10mM sodium phosphate, pH7.0 alone, which does not normally remove any membrane proteins, was used as a control.

Fig.4.1 shows the results of the treatments used. None of the treatments removed cytochrome c_4 from the membranes. Also, no other membrane cytochromes were removed. These treatments were therefore not investigated further. Although not investigated, the treatments presumably removed some non-haem proteins from the membranes. This did not, however, interfere with the ascorbate-TMPD oxidase activity of the membranes, the rates of ascorbate-TMPD oxidation by the treated membranes being the same as that of untreated membranes (data not shown).

B. Use of chaotropes Chaotropic agents have been extensively used in the solubilisation of membrane proteins and complexes (Hatefi and Hanstein, 1974). For example, the chaotropic ion perchlorate has been used in the solubilisation of the succinate dehydrogenases of beef heart mitochondria (Davies and Hatefi, 1971) and Rhodospirillum rubrum (Hatefi and Davies, 1972). Chaotropes act by weakening hydrophobic interactions which contribute greatly to the stability of the native conformations of most biological macromolecules, multimeric proteins and membranes.

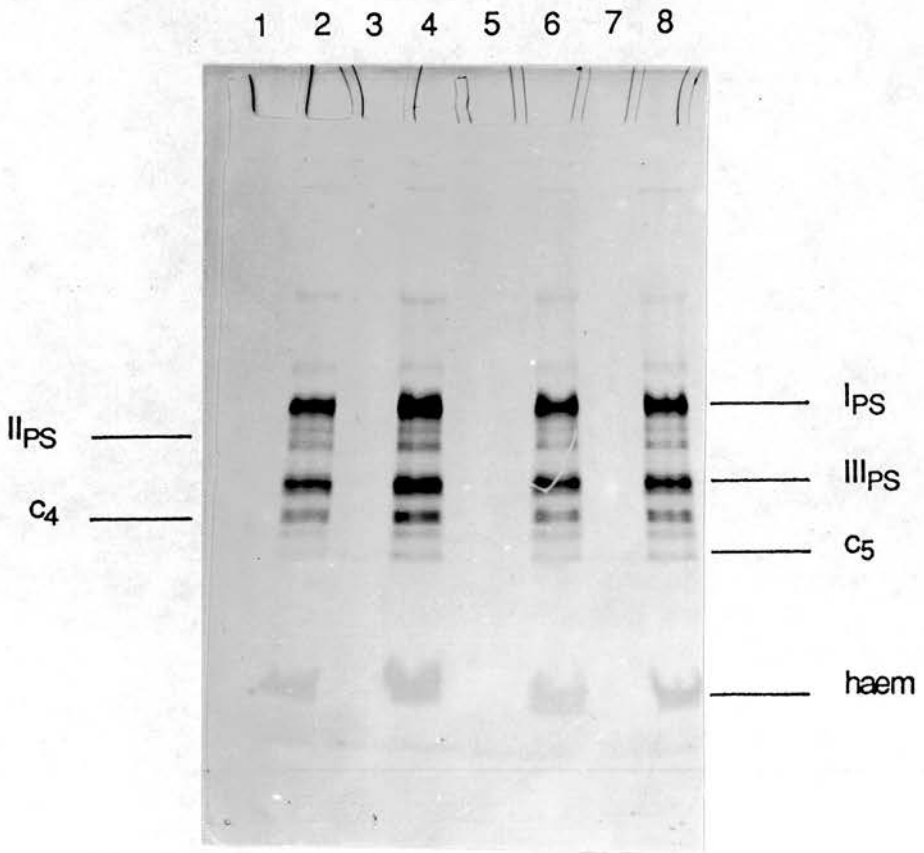
These hydrophobic interactions are the result of the thermodynamically unfavourable interaction of apolar groups with water, rather than because of an attraction of apolar groups for each other. The repulsion of apolar groups from water is mainly due to the large negative entropy involved in the transfer of apolar groups from an apolar to an aqueous environment. This occurs as a consequence of the ordering of water molecules around the apolar group. Chaotropes disorder water, disrupting the hydrogen bonding lattice of the water and therefore weaken the hydrophobic interactions by lowering the entropic barrier to transfer of apolar groups into the aqueous phase.

Chaotropes are, in general, ions with a low charge density; they have

Fig.4.1 Washing of *P. stutzeri* membranes: high ionic strength, low ionic strength and alkali

10-25% gradient SDS-PAGE gel of the supernatants and pellets from the washing of P. stutzeri spheroplast membranes with high ionic strength, low ionic strength and alkaline solutions, as described in the text. The gel was stained for haem as described in Chapter 2. 10µl samples were loaded.

Lane	Contents	
1	supernatant	control (10mM sodium phosphate,
2	pellet	pH7.0)
3	supernatant	High ionic strength (2.0M NaCl in
4	pellet	10mM sodium phosphate, pH7.0)
5	supernatant	Low ionic strength (10mM tris/HCl,
6	pellet	pH8.0 at 4°C)
7	supernatant	Alkali (10mM sodium carbonate,
8	pellet	pH9.5)


1 2 3 4 5 6 7 8
II$_{PS}$
c$_4$
I$_{PS}$
III$_{PS}$
c$_5$
haem

large radii and usually only a single charge. Among a related series of ions, such as the halides, the relative chaotropic potency of the ions is inversely proportional to the charge density. Thus iodide is a more potent chaotrope than bromide. A sequence of chaotropic potencies of ions can be constructed which correlates well with the ability of the ions to disrupt hydrophobic interactions and so solubilise membrane proteins and complexes.

$$CBr_3COO^- > CCl_3COO^- > SCN^- > \text{Guanidinium} > I^-, ClO_4^-$$
$$> CHCl_2COO^- > NO_3^-, Br^- > CF_3COO^- > CH_2ClCOO^-$$

(Hatefi and Hanstein, 1974)

Hydrophobic interactions have been postulated to be involved in the attachment of cytochrome c_4 to *P. stutzeri* membranes (Pettigrew and Brown, 1988, see Chapters 1 and 7). However, the cytochrome is not thought to be an integral membrane protein. Chaotropes would therefore be expected to solubilise membrane-bound cytochrome c_4 at concentrations not capable of solubilising the other integral membrane cytochromes. *P. stutzeri* spheroplast membranes were therefore treated with chaotropes of different potencies in order to try to obtain the selective removal of cytochrome c_4.

P. stutzeri spheroplast membranes (10mg protein/ml) were centrifuged from their storage medium in 1ml aliquots and were taken up in 1ml of the appropriate treatment solution, as described previously. Following a 30 minute incubation at 0°C, with thorough mixing, the samples were centrifuged again. Prior to this centrifugation, it proved necessary with certain chaotropes, notably iodide, to dilute the sample to 1M chaotrope . Otherwise, following centrifugation (11 000g, 20 min, 4°C) the washed membranes formed a floating pellet which was very difficult to separate completely from the supernatant. The supernatants were then dialysed against two volumes of 1.5l 10mM sodium phosphate, pH7.0 for 2 hours per change to remove residual chaotrope, lyophilised, and the freeze-dried material, and the membrane pellets, were taken up in their original incubation volume (1ml 10mM sodium

phosphate, pH7.0). Samples of equal volume could then be run on 10-25% gradient SDS-PAGE gels and stained for haem.

Fig.4.2 shows the results obtained with three of the chaotropes investigated. In addition to these chaotropes, urea and guanidine thiocyanate were also used. However, these two chaotropes proved to be of no use in the selective removal of cytochrome c_4 as they totally disrupted the membranes, solubilising large quantities of protein and cytochromes and destroying the oxidase activity (data not shown). Use of these chaotropes was not pursued.

Bromide and nitrate treatment of membranes removes no cytochrome c_4, even at 4.0M concentration. However, at 4.0M, sodium iodide removes cytochromes c_4 and c_5 from the membranes selectively with respect to other cytochromes c. Also, no removal of cytochromes b or of protohaem IX from such cytochrome occurs, as indicated by the lack of free haem in the supernatant from the 4.0M sodium iodide treatment (Fig.4.2, lane 4). However, treatment with sodium iodide does remove a large number of other non-cytochrome proteins in addition to cytochromes c_4 and c_5 (data not shown). Nevertheless, treatment with sodium iodide was selective in its removal of cytochrome c_4 with respect to other cytochromes c and merited further investigation.

Fig.4.3 shows the effect of increasing concentrations of sodium iodide on the removal of cytochrome c_4 (and cytochrome c_5) and on the ascorbate-TMPD oxidase activities of the treated membranes. 1ml aliquots of *P. stutzeri* spheroplast membranes (15 mg protein/ml) were treated with 0 to 3.5M sodium iodide as described previously. Supernatants and pellets were prepared and taken up in their original incubation volume to allow comparisons of ascorbate-TMPD oxidase activity and of cytochrome c_4 levels to be made. Ascorbate-TMPD oxidase activities were assayed as described in Chapter 2, immediately after the treatment or after dialysis as described previously. This was necessary as residual iodide in the membrane pellets otherwise caused a progressive inactivation. In all cases, the supernatants possessed negligible activity.

Fig.4.2 Washing of *P. stutzeri* membranes: chaotropes

10-25% haem-stained SDS-PAGE gels of the supernatants and pellets from the washing of *P. stutzeri* spheroplast membranes with the chaotropic ions iodide, bromide and nitrate as described in the text. 10µl samples were loaded.

Lane	Contents	
1	pellet	0.5M sodium iodide
2	supernatant	"
3	pellet	4.0M sodium iodide
4	supernatant	"
5	pellet	0.5M sodium bromide
6	supernatant	"
7	pellet	4.0M sodium bromide
8	supernatant	"
9	pellet	0.5M sodium nitrate
10	supernatant	"
11	pellet	4.0M sodium nitrate
12	supernatant	"
13	pellet	control (10mM sodium phosphate,
14	supernatant	pH7.0)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
I_{PS}
II_{PS}
III_{PS}
c_4
c_5
haem

The amounts of cytochrome c_4 remaining on the membranes after each treatment were obtained from densitometric traces of haem-stained 10-25% gradient SDS-PAGE gels of the samples produced (Fig.4.3a). The cytochrome c_4 peak from each trace was excised and weighed to give an estimate of cytochrome c_4 amount. Variations in the staining intensity over the gels were allowed for by normalising each cytochrome c_4 peak relative to band III_{Ps}, which was unaffected by iodide treatment.

Fig.4.3b shows that cytochrome c_4 is removed from *P. stutzeri* membranes at concentrations of sodium iodide greater than 1.0M, with at least 85% being removed with 2.5M sodium iodide. A similar profile of cytochrome c_4 removal is shown by *A. vinelandii* membranes (Fig.4.3c). These were treated with sodium iodide as described for *P. stutzeri* membranes (Hunter et al., 1989). In both organisms, removal of cytochrome c_4 is apparently incomplete, with approximately 15% of the total amount remaining on the membrane. However, the actual amount of cytochrome c_4 remaining is probably less than this, for two reasons.

Firstly, several minor bands which stain for haem migrate close to the position of cytochrome c_4 on SDS-PAGE. One of these has already been mentioned in connection with the estimation of cytochrome c_4 on anaerobic membranes of *P. stutzeri* (Chapter 3) where it is much more prominent and makes the estimation impossible. It is difficult to distinguish staining due to residual cytochrome c_4 from the staining due to these bands and the amount of staining due to residual cytochrome c_4 is overestimated for this reason. Secondly, cytochrome c_4 and band III, also present in *A. vinelandii* (Chapter 3; Hunter et al., 1989), are not completely resolved and the latter makes some contribution to the staining taken as being due to cytochrome c_4. It is not possible to allow for these contributions accurately and consequently the value of approximately 15% overestimates the amount of cytochrome c_4 remaining on the membrane.

This problem of over-estimation of the amount of cytochrome c_4

Fig.4.3 Removal of cytochrome c_4 from membranes by sodium iodide and effect on ascorbate-TMPD oxidase activity

(a) SDS-PAGE 10-25% gradient haem-stained SDS-PAGE gel of supernatants and pellets from washing of *P. stutzeri* spheroplast membranes with increasing concentrations of sodium iodide, as described in the text. 10µl samples were loaded.

Lane	Contents	
1	supernatant	0.0M sodium iodide (control)
2	pellet	"
3	supernatant	1.0M sodium iodide
4	pellet	"
5	supernatant	1.5M sodium iodide
6	pellet	"
7	supernatant	2.0M sodium iodide
8	pellet	"
9	50pmol pure cytochrome c_4	
10	supernatant	2.5M sodium iodide
11	pellet	"
12	supernatant	3.0M sodium iodide
13	pellet	"
14	supernatant	3.5M sodium iodide
15	pellet	"
16	50pmol pure cytochrome c_4	

(a)

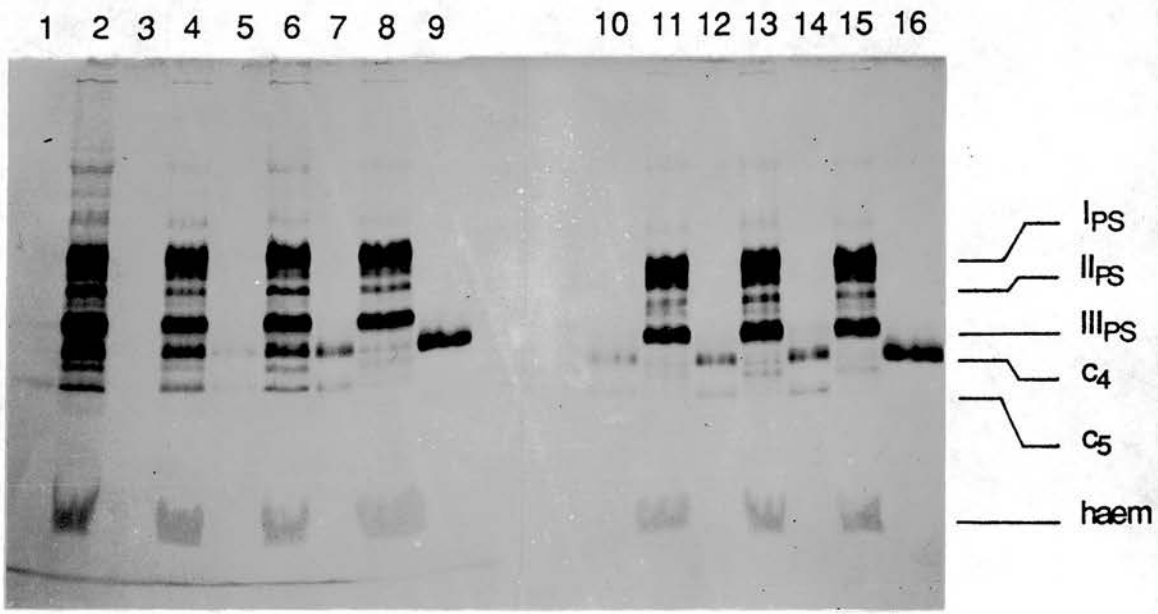


Fig.4.3 cont.

(b) Ascorbate-TMPD oxidase activity Graph to show the effect of removal of cytochrome c_4 on the ascorbate-TMPD oxidase activity of *P. stutzeri* spheroplast membranes. (○) Ascorbate-TMPD oxidase activity of membrane samples after sodium iodide treatment, relative to control membranes (0.0M sodium iodide). (●) Percentage of cytochrome c_4 remaining associated with the membranes after each treatment. Cytochrome c_4 amounts were obtained from densitometric traces of the gels in (a) as described in the text. (Redrawn from Hunter et al., 1989).

(b)

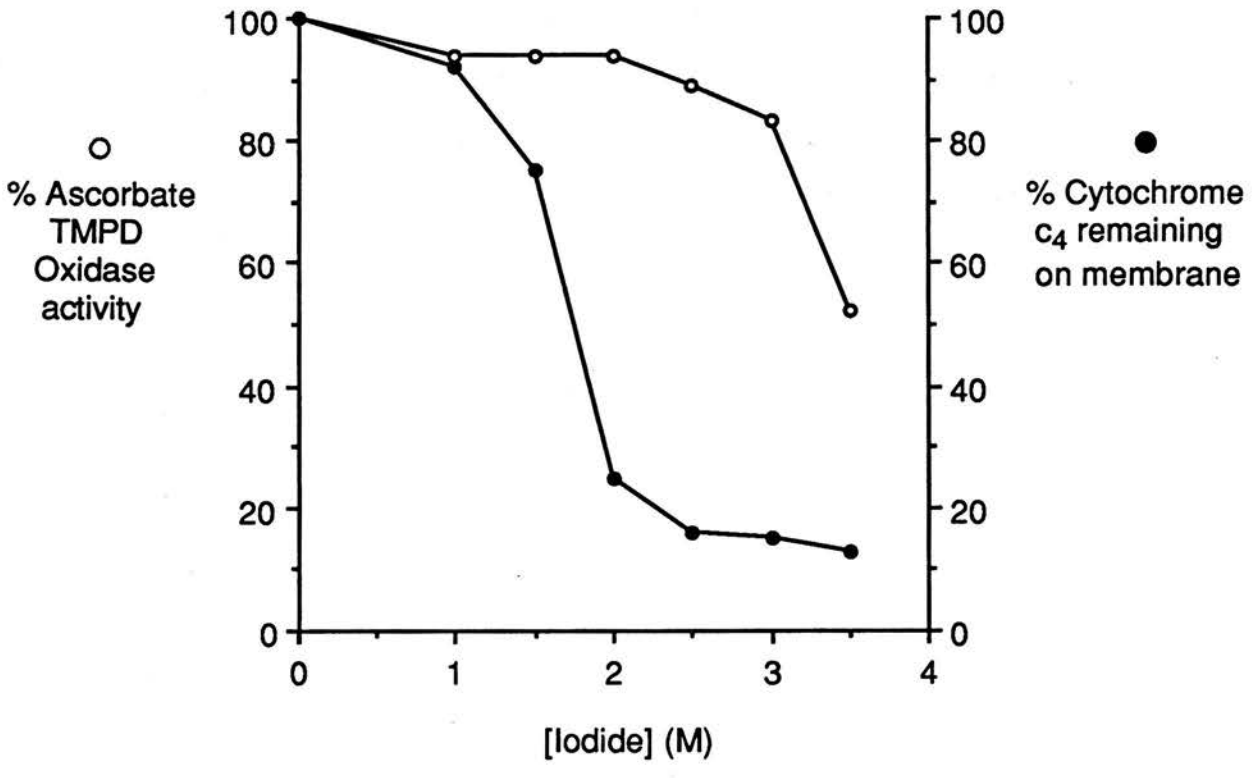
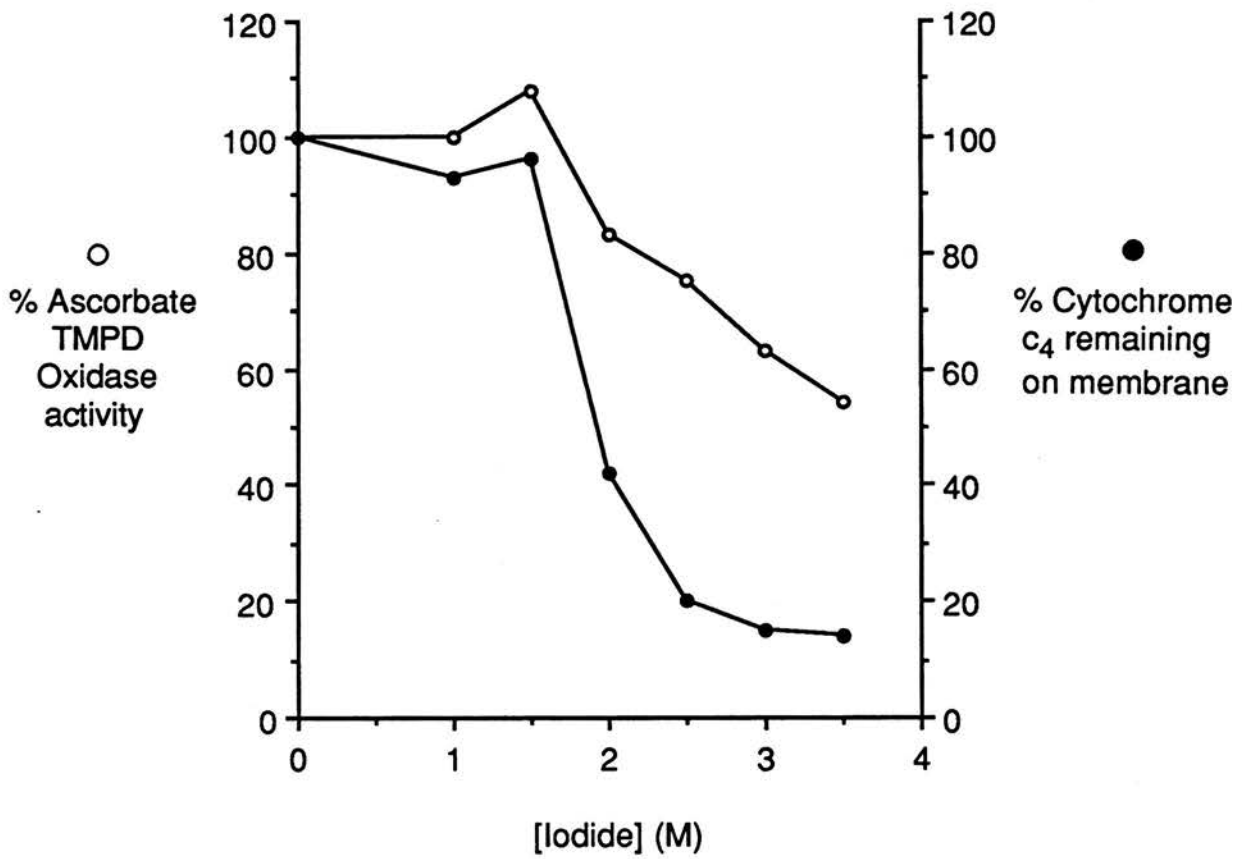

100
80
60
40
20
0
0
1
2
3
4
[Iodide] (M)
% Ascorbate TMPD Oxidase activity
% Cytochrome c_4 remaining on membrane

Fig.4.3 cont.

(c) A. vinelandii Removal of cytochrome c_4 from spheroplast membranes of *A. vinelandii* by sodium iodide and the effect on ascorbate-TMPD oxidase activity. (○) Percentage of ascorbate-TMPD oxidase activity remaining. (●) Percentage of cytochrome c_4 remaining. (Redrawn from Hunter et al., 1989).

(c)


120
100
80
60
40
20
0
0
1
2
3
4
[Iodide] (M)
% Ascorbate TMPD Oxidase activity
% Cytochrome c_4 remaining on membrane

remaining after a treatment to remove it occurs with all the methods of removal used as it is inherent in the method used to estimate the amounts of cytochrome c_4. Consequently, the residual amount of cytochrome c_4 should be regarded as an upper estimate.

Ascorbate-TMPD oxidase activity is essentially unaffected by the removal of cytochrome c_4 from the membranes of *P. stutzeri* at concentrations of sodium iodide of up to 3.0M. At higher concentrations, the activity declines. This is probably due to damage to the oxidase at these concentrations, rather than to the removal of cytochrome c4 as at 3.0M sodium iodide, 80% of the activity is retained. In *A. vinelandii*, removal of cytochrome c_4 by sodium iodide is associated with the loss of ascorbate-TMPD oxidase activity. However, the loss of activity does not parallel the removal of cytochrome c_4, and with 3.0M sodium iodide, approximately 63% of the activity remains, with only 15% of the cytochrome c_4 still present. This loss of activity is also probably due to damage to the oxidase caused by the sodium iodide treatment. These results suggest that cytochrome c_4 is not required for ascorbate-TMPD oxidase activity . However, as the complete removal of cytochrome c_4 from the membranes of both organisms could not be demonstrated, it is not possible to state this with complete certainty.

Cytochrome c_5 is also removed by sodium iodide in parallel to cytochrome c_4 in both organisms. This suggests that cytochrome c_5 has a similar method of attachment to the membrane to cytochrome c_4 and also that it too is not involved in ascorbate-TMPD oxidase activity.

Table 4.1 shows the effect of 2.0M sodium iodide treatment on the other oxidase activities possessed by *P. stutzeri* membranes. In this particular instance, removal of 82% of the membrane-bound cytochrome c_4 results in the loss of only 10% of the ascorbate-TMPD oxidase activity, comparable to the values presented in Fig.4.3b. However, succinate and NADH oxidase activities are essentially eliminated by this treatment and duroquinol oxidase activity is reduced by 41%. These figures imply that either cytochrome c_4 is

Table 4.1

Activity (%)	Control membranes	I^- washed membranes	Membranes + removed c_4	Membranes + pure c_4
Asc-TMPD oxidase	100	90	78	82
Succinate oxidase	100	5	7	2
NADH oxidase	100	2	2	2
Duroquinol oxidase	100	59	41	57
Cytochrome c_4 on membrane	100	18	63	43

involved in these activities and that its removal causes the observed losses of activities, or that iodide treatment damages the membrane in such a way that these activities are lost or reduced. To distinguish these two possibilities it was necessary to return cytochrome c_4 to the washed membranes to see if the activities were thereby restored. These experiments are discussed in section III.

C. Use of propan-2-ol A second method discovered for the selective removal of cytochrome c_4 from *P. stutzeri* membranes was the use of the water-miscible organic solvent propan-2-ol. Membrane-bound cytochrome c_4 is initially extracted from the membranes during its purification by 50% (v/v) butan-1-ol (Tissieres, 1956; Brown, 1988; Pettigrew and Brown, 1988). This treatment disrupts the membranes completely and results in the irreversible denaturation of much of the membrane protein. Propan-2-ol, at concentrations up to 50% (v/v) is much gentler in its action and does not cause the gross denaturation seen with butan-1-ol.

Fig.4.4 shows the results of washing *P. stutzeri* spheroplast membranes with propan-2-ol. Membranes (15mg protein/ml) were centrifuged from their storage medium (11 000g, 20 min, 4°C) in 1ml aliquots and were resuspended in 10mM sodium phosphate, pH7.0 containing 0 to 50% (v/v) propan-2-ol. After incubation for 30 minutes at 0°C, with thorough mixing, the samples were centrifuged to separate solubilised from insoluble material (11 000g, 30 min, 4°C). The pellets were then resuspended in their original incubation volume (1ml) in 10mM sodium phosphate, pH7.0 and were immediately assayed for ascorbate-TMPD oxidase activity, as residual propan-2-ol in the pellets caused a gradual inactivation of the oxidase activity, as does sodium iodide (section IB). Alternatively, the pellets could be dialysed against 1.5l 10mM sodium phosphate, pH7.0 overnight.

Samples of equal volume of each supernatant and pellet were run on a 15% slab SDS-PAGE gel and stained for haem, to allow quantitation of the amount of cytochrome c_4 removed (Fig.4.4a). This was carried out as described for sodium iodide. However, as band III_{Ps} was removed at high

Fig.4.4 Removal of cytochrome c_4 from membranes by propan-2-ol and effect on ascorbate-TMPD oxidase activity

(a) SDS-PAGE 15% slab haem-stained SDS-PAGE gel of supernatants and pellets from washing of *P. stutzeri* spheroplast membranes with increasing concentrations of propan-2-ol, as described in the text. 20µl samples were loaded.

Lane	Contents	
1	pellet	0% (v/v) propan-2-ol (control)
2	supernatant	"
3	pellet	20% (v/v) propan-2-ol
4	supernatant	"
5	pellet	30% (v/v) propan-2-ol
6	supernatant	"
7	pellet	40% (v/v) propan-2-ol
8	supernatant	"
9	pellet	50% (v/v) propan-2-ol
10	supernatant	"

(a)

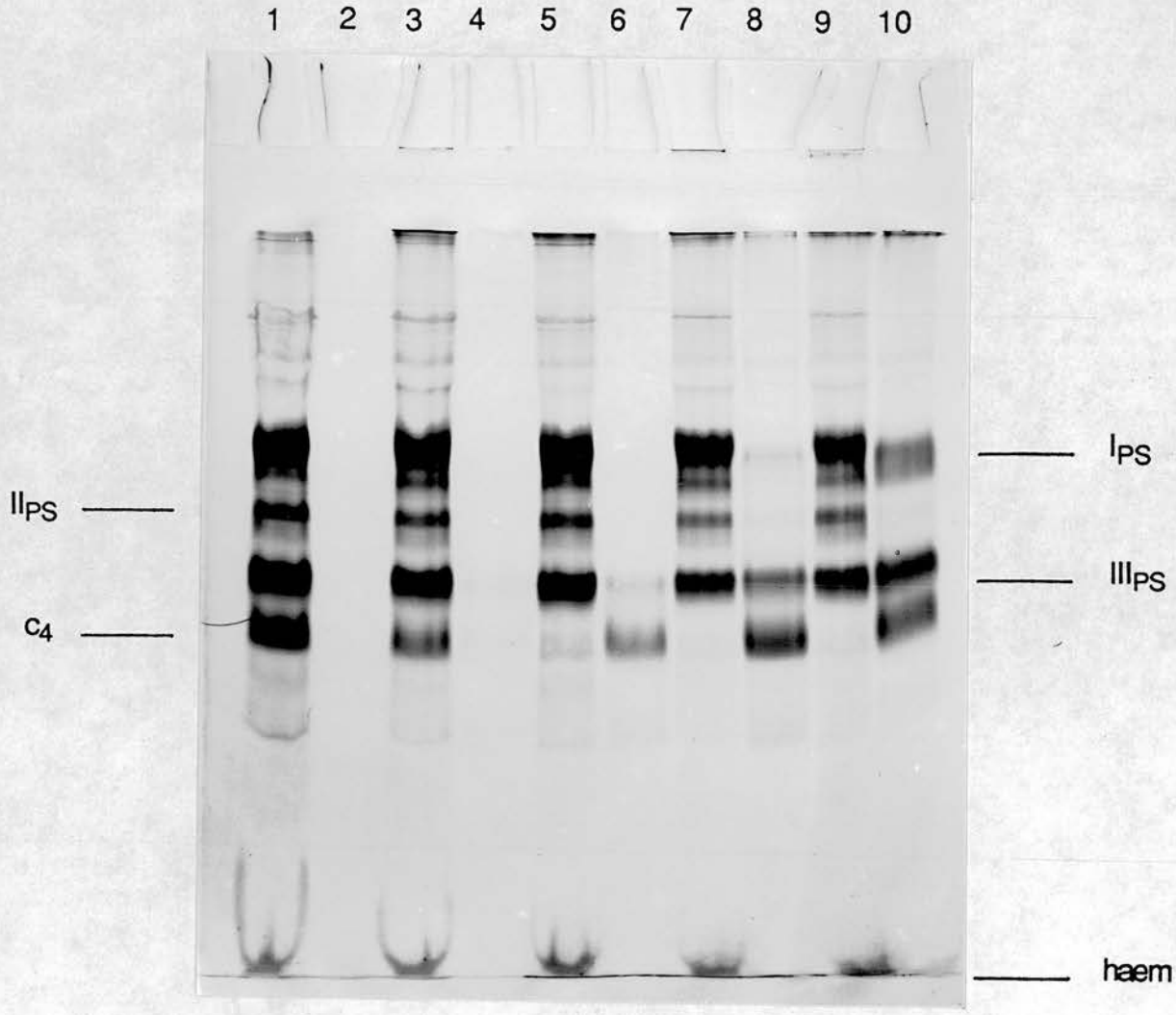
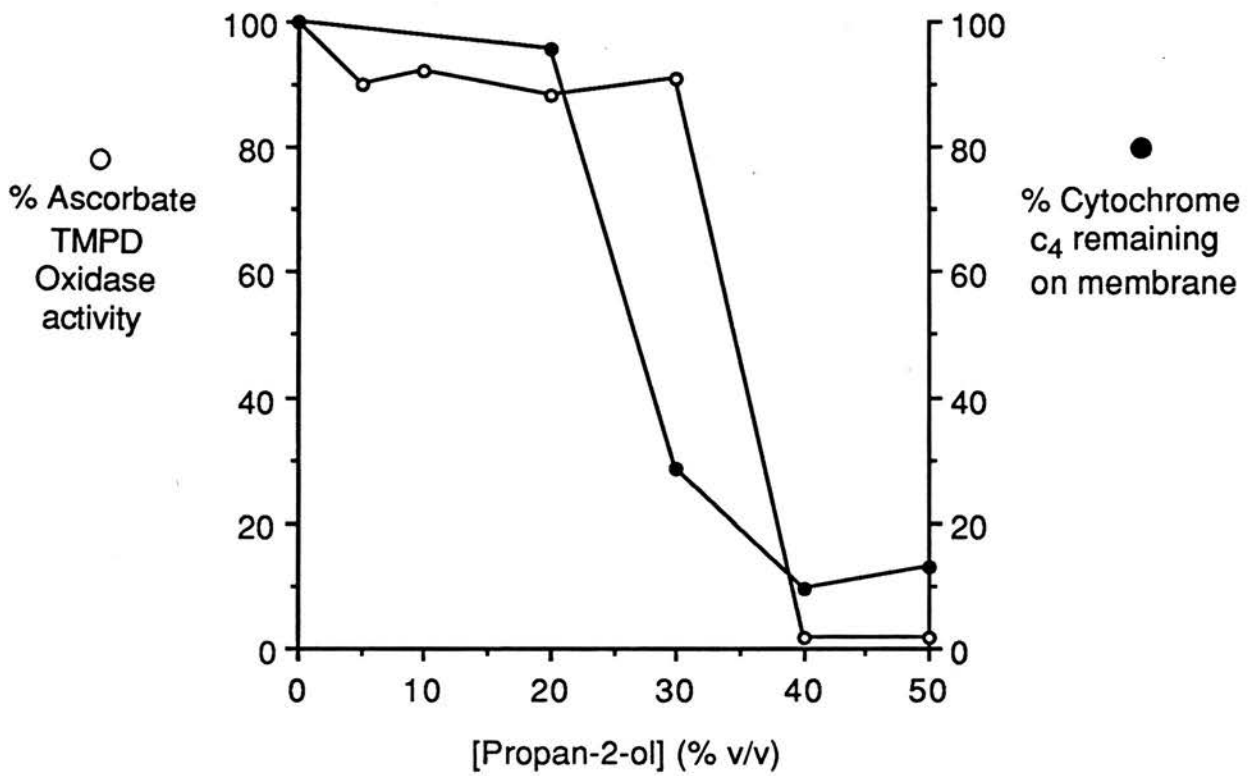


Fig.4.4 cont.

(b) Ascorbate-TMPD oxidase activity Graph to show the effect of removal of cytochrome c_4 by propan-2-ol on the ascorbate-TMPD oxidase activity of *P. stutzeri* spheroplast membranes. (○) Ascorbate-TMPD oxidase activity of membrane samples after propan-2-ol treatment, relative to control membranes (0% propan-2-ol). (●) Percentage of cytochrome c_4 remaining associated with the membranes after each treatment. Cytochrome c_4 amounts were obtained from densitometric traces of the gels in (a) as described in the text.

(b)


○
% Ascorbate TMPD Oxidase activity
100
80
60
40
20
0
0
10
20
30
40
50
[Propan-2-ol] (% v/v)
●
% Cytochrome c_4 remaining on membrane

propan-2-ol concentrations, the normalisation procedure adopted was different. The total amount of cytochrome c_4 (that solubilised and that remaining on the membrane) was calculated for each treatment and these totals were normalised with respect to the value obtained for the control membranes.

Washing of P. stutzeri membranes with propan-2-ol removes cytochrome c_4 from the membranes at concentrations greater than 20% (v/v). However, propan-2-ol is less selective in its removal of cytochrome c_4 than sodium iodide, in that other membrane cytochromes c are also removed, particularly at higher concentrations (40 to 50% v/v). Propan-2-ol also removes more non-haem protein (data not shown).

Fig.4.4b shows the effect of propan-2-ol treatment on the ascorbate-TMPD oxidase activity and the amount of cytochrome c_4 remaining on the membranes. Oxidase activity is essentially unaffected by the treatment except at 40 and 50% (v/v) propan-2-ol, where the activity is reduced to zero. This loss of activity is accompanied by removal of cytochromes c in addition to cytochrome c_4 and presumably is the result of damage to the oxidase at these concentrations.

Cytochrome c_4 is removed from membranes at propan-2-ol concentrations above 20% (v/v). This removal of cytochrome c_4 does not parallel the loss of ascorbate-TMPD oxidase activity and at 30% (v/v) propan-2-ol, 90% of the ascorbate-TMPD oxidase activity remains, whilst 72% of the cytochrome c_4 is removed. Once again, complete removal of cytochrome c_4 from membranes, with the retention of full ascorbate-TMPD oxidase activity cannot be demonstrated. However, the results do support those obtained with sodium iodide and suggest that cytochrome c_4 is not involved in ascorbate-TMPD oxidase activity.

D. Use of proteolysis Proteolysis of *P. stutzeri* spheroplasts with subtilisin has been used to to demonstrate that membrane-bound cytochrome c_4 is located on the periplasmic face of the cytoplasmic membrane in this organism

(Brown, 1988; Hunter et al., 1989). Pure cytochrome c_4 is cleaved by subtilisin into two fragments of approximately equal size, which are thought to correspond to the two domains of the cytochrome (Brown, 1988). Membrane-bound cytochrome c_4 is also digested by subtilisin to produce the two fragments. In intact spheroplasts, since the added subtilisin has access only to the exterior, periplasmic face of the membrane, digestion of the membrane-bound cytochrome c_4 implies a periplasm-facing location for the cytochrome (Brown, 1988; Hunter et al., 1989). In addition to cytochrome c_4, only cytochrome c_5 and a portion of band II_{Ps} are removed by subtilisin digestion (Hunter et al., 1989). Subtilisin digestion of spheroplasts therefore offers a third method for the removal of cytochrome c_4 from membranes.

2ml *P. stutzeri* cells (0.5g cells/ml, in 10mM sodium phosphate, pH7.0) were incubated with with 2.5mg lysozyme in a sucrose/tris/EDTA solution of volume 7ml for 2 minutes at 30°C. 1ml 0.1M $MgCl_2$ was then added to give final concentrations 0.5M sucrose, 40mM tris/HCl, pH8.0 at 4°C, 3mM EDTA, and 10mM $MgCl_2$, in a total volume 10ml. 0.5mg DNase II was also present to reduce the viscosity caused by released DNA. Following a further incubation of 15 minutes at 30°C, 1ml aliquots of the spheroplast mixture were treated with subtilisin at two digestion intensities, in the presence or absence of the protease inhibitor, phenylmethylsulphonylfluoride (PMSF).

The digestion intensities used were 20:1 and 100:1 accessible protein:subtilisin (w/w). Accessible protein refers to the total amount of protein assumed to be accessible to the subtilisin. This was assumed to comprise periplasmic protein, including added lysozyme and DNase II, and membrane protein. This overestimates the actual amount of accessible protein as an unknown portion of the membrane protein will be cytoplasm-facing, deeply buried in the membrane or otherwise inaccessible to the subtilisin. Accessible protein was estimated after the preparation of periplasmic and membrane fractions from the spheroplast mixture used, by Lowry protein assays.

The periplasmic fractions were obtained by centrifuging the aliquots of spheroplast mixture (11 000g, 20 min 4°C), which separated the spheroplasts

from the released periplasm. The isolated spheroplasts were then lysed by resuspending the pellets in 900 µl each of 10mM tris/HCl, pH8.0 at 20°C containing 2mM EDTA and a trace of DNase II. After 15 minutes at 20°C, $MgCl_2$ was added to give a final concentration 4mM, in a total volume of 1ml. Centrifugation (19 000g, 30 min, 4°C) separated the cytoplasm from the spheroplast membranes, which were then resuspended in 1ml each 10mM sodium phosphate, pH7.0.

Treatment of the spheroplast aliquots with subtilisin proceeded as follows. The 1ml aliquots of spheroplast mixture were exposed to the appropriate treatment for 30 minutes at 30°C. PMSF, in propan-2-ol was added to give a 25-fold molar excess over the subtilisin and a final concentration of 1% propan-2-ol. PMSF addition occurred prior to subtilisin addition where digestion was undertaken in its presence, or after incubation with the subtilisin, to stop the reaction. At a molar ratio of 25:1, PMSF is known to completely inhibit the digestion of pure cytochrome c_4 (Brown, 1988). Periplasmic, cytoplasmic and membrane fractions were then prepared and samples of the membrane fractions were assayed for ascorbate-TMPD oxidase activity. Samples of the membranes were also run on a 15% slab SDS-PAGE gel and stained for haem, to detect the digestion of cytochrome c_4.

Fig.4.5 shows the haem-staining pattern obtained from the samples after treatment. In addition, the ascorbate-TMPD oxidase activities of each sample are given, relative to the activity of membranes prepared directly from the spheroplast mixture, with no incubation for 30 minutes at 30°C. Several points are of note. Firstly, the membrane samples have run poorly on the gel. This is probably because they contain whole cells, which release DNA on lysis with SDS. All the membrane samples are contaminated with periplasmic cytochrome c-551 indicating that cells have remained intact throughout the spheroplast production procedure.

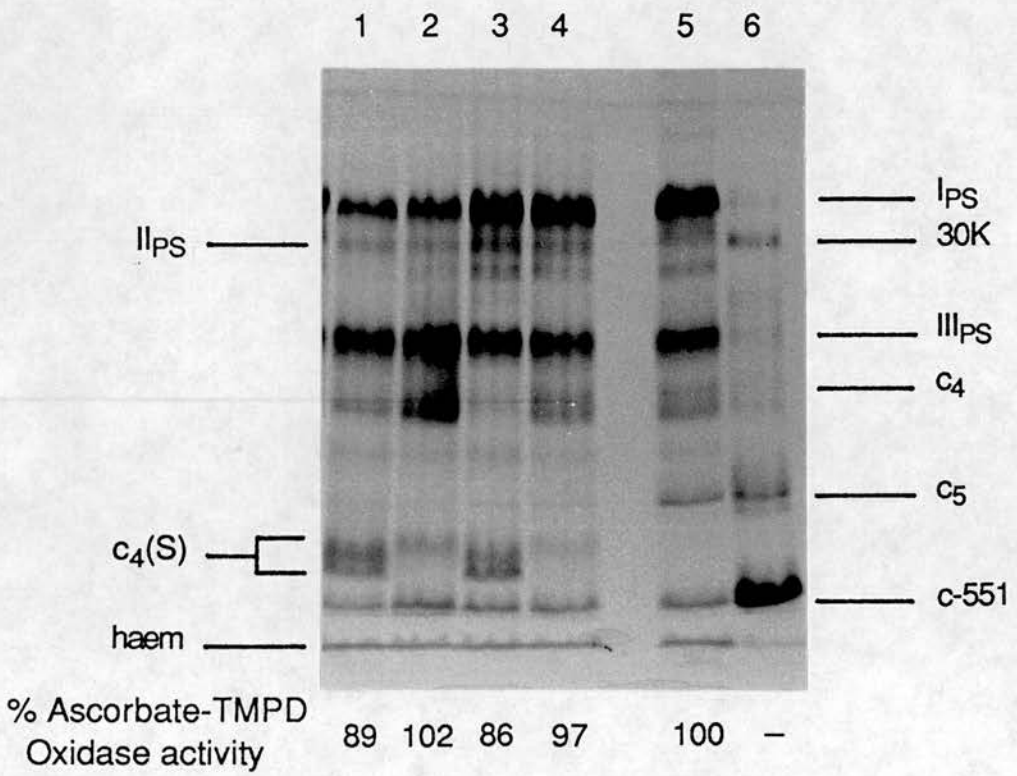
Secondly, a minor band with molecular weight close to that of cytochrome c_4 has stained unusually strongly. This makes the loss of

Fig.4.5 Proteolysis of cytochrome c_4 on *P. stutzeri* spheroplast membranes

15% slab haem-stained SDS-PAGE gel of membrane fractions prepared after digestion of spheroplasts with subtilisin, as described in the text. 30µl samples were loaded.

Lane	Contents
1	20:1 protein:subtilisin (w/w)
2	20:1 protein:subtilisin (w/w) pretreated with PMSF
3	100:1 protein:subtilisin (w/w)
4	100:1 protein:subtilisin (w/w) pretreated with PMSF
5	Untreated membranes
6	Untreated periplasm

The figures below each lane are the ascorbate-TMPD oxidase activities of each sample, relative to untreated membranes.


1 2 3 4 5 6
II_{PS}
$c_4(S)$
haem
I_{PS}
30K
III_{PS}
c_4
c_5
c-551
% Ascorbate-TMPD Oxidase activity
89 102 86 97 100 –

cytochrome c_4 difficult to detect. Nevertheless, at both digestion intensities, cytochrome c_4 is digested and produces fragments of lower molecular weight (Fig.4.5, c_4(S)). PMSF reduces, but does not totally eliminate the appearance of these fragments. This contrasts with the results of Brown (1988) and Hunter et al. (1989) who found no digestion of pure or membrane-bound cytochrome c_4 when PMSF was present.

Thirdly, the fragments produced remain attached to the membrane, rather than being released as soluble fragments by the subtilisin treatment. A similar observation was made by Hunter et al. (1989). Additionally, band II_{Ps} is partially degraded at a protein:subtilisin ratio of 20:1, but is apparently undigested at a ratio of 100:1.

The ascorbate-TMPD oxidase activities of the membrane samples are not greatly affected by the digestion of cytochrome c_4. When the digestion is carried out in the presence of PMSF, no activity is lost. In the absence of PMSF, 11 to 14% of the activity is lost, depending on the protein:subtilisin ratio used. Horse heart cytochrome c and *P. stutzeri* cytochrome c-551 oxidase activities are also not affected by the subtilisin treatments (data not shown). This indicates that the ascorbate-TMPD oxidase is not damaged by the proteolysis and suggests that removal of cytochrome c_4 by this means does not affect ascorbate-TMPD oxidase activity. However, it is difficult to assess the completeness of the removal of cytochrome c_4 by subtilisin because of the difficulties in resolving cytochrome c_4 from the close-running minor band. Furthermore, it is possible that although cytochrome c_4 is digested by the subtilisin, it may remain functional.

On digestion with subtilisin, cytochrome c_4 is cleaved into two fragments of similar molecular weight which remain associated with the membrane. The two fragments correspond to the two domains of cytochrome c_4 (Brown, 1988) which may remain associated with each other as well as the membrane. Only on denaturation in SDS would the two fragments dissociate and lose their function. The ascorbate-TMPD oxidase activity would therefore not be

affected, even if cytochrome c_4 was essential for this activity. As this possibility exists, no information on the role of cytochrome c_4 in ascorbate-TMPD oxidase activity could be obtained and these experiments were not pursued further.

II: Removal of cytochrome c_4 from detergent-solubilised membrane complexes

In addition to methods for the removal of cytochrome c_4 from native membranes of *P. stutzeri*, the removal of cytochrome c_4 from membrane complexes after their detergent solubilisation was undertaken. This investigation stemmed from the observation that *A. vinelandii* cytochrome c_4 is partially removed from Triton X-100-solubilised membrane complexes on molecular exclusion chromatography (E.R.T.Lightly, personal communication). Molecular exclusion chromatography of detergent extracts of *P. stutzeri* spheroplast membranes was therefore investigated, both as a method for the removal of cytochrome c_4 and as a prelude to the purification of the ascorbate-TMPD oxidase from *P. stutzeri* (Chapter 6).

Triton X-100, octyl glucoside and dodecyl maltoside were selected for the solubilisation of the membranes (see Chapter 6). Detergent extractions were carried out as described in Chapter 6, with 1.5mg detergent per mg membrane protein for 30 minutes at 0°C. Insoluble material was removed by centrifugation after this incubation. The efficiencies of each detergent in extraction are discussed in Chapter 6.

1ml of each detergent extract was loaded onto a 1x20cm column of Sephadex G-75-50 (Sigma), equilibrated with 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl. The column was previously calibrated with blue dextran, horse heart cytochrome c and cytochrome c_4 to determine the void volume and that the column was sufficiently resolving to be able to separate cytochrome c_4 from material eluting at the void volume. 1.3ml fractions were collected and A_{410} was monitored. Samples of each fraction were also run on 15% slab SDS-PAGE gels and haem stained. This allowed the cytochrome

composition of the fractions to be investigated.

Fig.4.6 shows the results of chromatography of an octyl glucoside extract on Sephadex G-75 as described above. When a freshly prepared extract is used (Fig.4.6a), a single peak of material at the void volume is eluted. After SDS-PAGE and haem staining, followed by scanning of the gel, cytochrome c_4 (shaded) is seen to remain associated with the solubilised membrane particles. However, when the extract was stored (frozen, 48 hours) prior to chromatography, two peaks are present in the profile of A_{410} (Fig.4.6b).

Investigation of these peaks by haem-stained SDS-PAGE gel and gel scanning reveals that cytochrome c_4 (shaded) has become dissociated from the solubilised membrane particles and chromatographs as a peak eluting after the void volume peak. Comparison with the previously performed calibration indicates that the cytochrome c_4 is eluting at approximately its native molecular weight.

The ascorbate-TMPD oxidase activities of the applied samples and of pooled fractions from the columns indicate that no activity is lost on chromatography and that removal of cytochrome c_4 from the solubilised membrane complexes causes no loss of activity. Note, however, that removal of cytochrome c_4 was apparently incomplete and that the previously discussed problems (section IB) of assessing the completeness of cytochrome c_4 removal by means of haem-stained SDS-PAGE gels apply here also.

Chromatography of a Triton X-100 extract on Sephadex G-75 also resulted in the removal of cytochrome c_4 from the Triton X-100 solubilised membranes. Removal varied from approximately 50 to 90% and was not influenced by storage. Again, no activity was lost on the removal of the cytochrome c_4. Chromatography of a stored dodecyl maltoside extract also resulted in the partial removal of cytochrome c_4. However, the effect of removal of cytochrome c_4 on the ascorbate-TMPD oxidase activity and the effect of storage on this removal were not investigated.

The release of cytochrome c_4 from octyl glucoside solubilised membrane

Fig.4.6 Removal of cytochrome c_4 from detergent solubilised membranes of *P. stutzeri* by chromatography on Sephadex G-75

P. stutzeri spheroplast membranes were extracted with octyl glucoside and chromatographed on Sephadex G-75 as described in the text. Profiles of A_{410} and scans of 15% slab haem-stained SDS-PAGE gels of selected fractions are shown. Shading indicates cytochrome c_4.

(a) Fresh extract

(a)

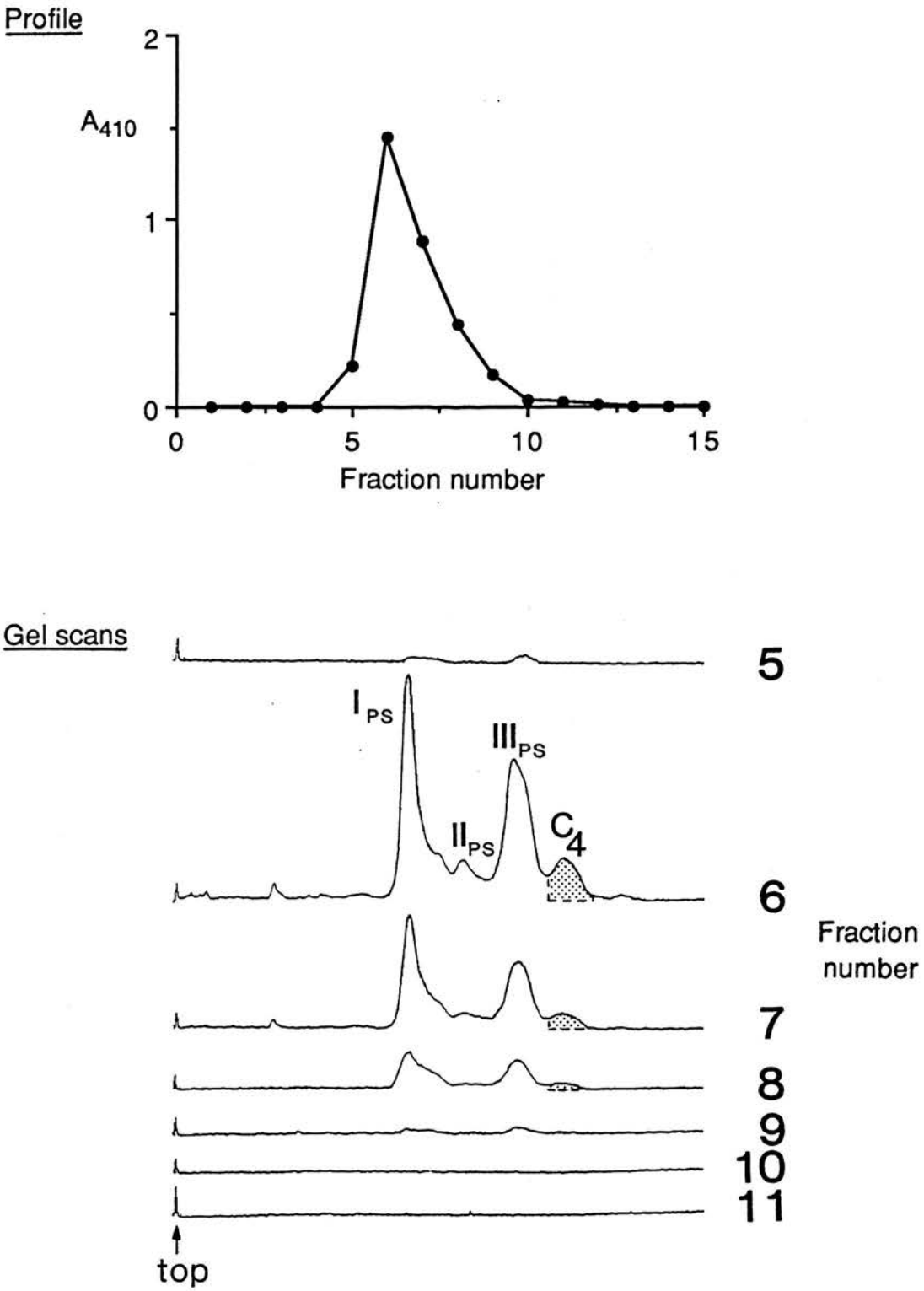
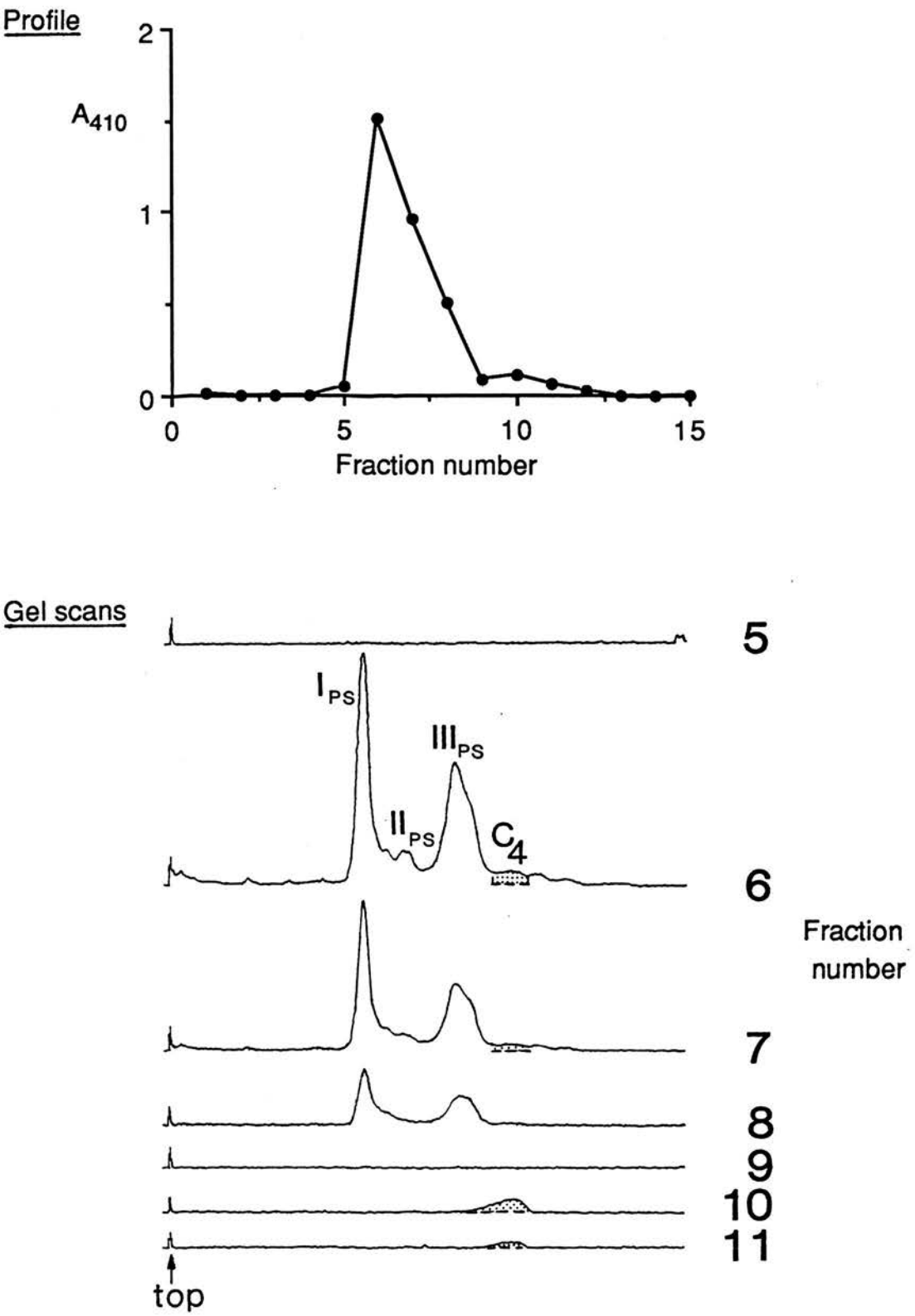

Profile
2
A_{410}
1
0
0
5
10
15
Fraction number
Gel scans
I_{PS}
III_{PS}
II_{PS}
C_4
5
6
7
8
9
10
11
Fraction number
top

Fig.4.6 cont.

(b) Frozen extract

(b)


Profile
2
A_{410}
1
0
0
5
10
15
Fraction number
Gel scans
5
I_{PS}
III_{PS}
II_{PS}
C_4
6
Fraction number
7
8
9
10
11
top

complexes on frozen storage suggests that binding of the cytochrome c_4 to the solubilised membrane complexes in cold labile. Cold lability is also displayed by the proton-translocating ATPase of beef heart mitochondria, which becomes inactive on storage at 0°C. This inactivation is due to the dissociation of the 11.9S enzyme into 9.1S and 3.5S moieties, a process which is reversed by rewarming. Reassociation of the cytochrome c_4 with the membrane complexes was not investigated, but the parallels with cold-labile ATPase are clear. The implications of this cold lability for the method of attachment of cytochrome c_4 to membranes are discussed in Chapter 7.

III: Reconstitution of cytochrome c_4 with cytochrome c_4-depleted membranes

In order to decide whether the observed losses of NADH, succinate and duroquinol oxidase activities on treatment of the membranes with 2.0M sodium iodide (section IB, table 4.1) were due to a requirement for cytochrome c_4 or due to damage to the membranes by the sodium iodide, a method was developed for the reconstitution of cytochrome c_4 with membranes previously washed free of cytochrome c_4. In this way any restoration of the lost activities by reconstituted cytochrome c_4 could be investigated.

Two types of cytochrome c_4 were available for reconstitution with the membranes. Firstly, cytochrome c_4 contained in the supernatant from the sodium iodide wash could be used. In addition to cytochrome c_4, this material also contained all the non-haem proteins removed by iodide treatment. Secondly, purified cytochrome c_4 from *P. stutzeri* could be used. The amount of purified cytochrome c_4 used in the reconstitution experiments was decided on by comparing the intensity of haem-staining on SDS-PAGE produced by various loadings of cytochrome c_4 with that produced by cytochrome c_4 bound to the membranes used in the reconstitution. An amount of purified cytochrome c_4 giving a staining intensity equivalent to that produced by the membrane-bound cytochrome c_4 was selected for use in the reconstitution

experiments.

The procedure adopted to reconstitute cytochrome c_4 with the membranes was to resuspend cytochrome c_4-depleted membranes and the cytochrome c_4 to be reconstituted in sodium iodide. The sodium iodide was then gradually removed by dialysis. Recombination of cytochrome c_4 and membranes in the absence of sodium iodide failed to reconstitute the cytochrome c_4 with the membranes. The presence of sodium iodide may be necessary to allow the cytochrome c_4 to adopt a conformation capable of binding to the membranes. Sodium iodide may be acting as a chaotrope, weakening hydrophobic interactions and allowing hydrophobic surfaces normally hidden in soluble cytochrome c_4 to become exposed and available for interaction with the membranes (see Chapter 1). Alternatively, sodium iodide may denature the cytochrome c_4 allowing it to renature on the membranes when the sodium iodide is removed by dialysis. These possibilities are further addressed in Chapter 5.

P. stutzeri spheroplast membranes (15mg protein/ml) were centrifuged from their storage medium (11 000g, 30 min, 4°C) in three 1ml aliquots and were resuspended in 1ml each of 10mM sodium phosphate, pH7.0 containing 2.0M sodium iodide. A further 1ml aliquot of membranes was centrifuged from the storage medium and was resuspended in 1ml 10mM sodium phosphate, pH7.0 alone, to act as a control (a). After a 30 minute incubation, with thorough mixing, the samples were treated in one of the following ways:

(a) Control - Centrifuged (11 000g, 30 min, 4°C) to separate the supernatant and pellet. the latter was resuspended in its original incubation volume (1ml 10mM sodium phosphate, pH7.0)

(b) Iodide washed membranes - The sample was diluted to 1M sodium iodide then was centrifuged as in (a). After dialysis against 2 volumes of 500ml 10mM sodium phosphate, pH7.0 for 2 hours per change, the supernatant was lyophilised and the freeze-dried material and the membrane pellet were resuspended in their original incubation volume (1ml 10mM sodium phosphate, pH7.0)

(c) Reconstitution with removed cytochrome c_4 - After incubation and prior to centrifugation, the sodium iodide treated membranes were dialysed as in (b). In this way the removed material was directly recombined with the membranes. The dialysed sample was then centrifuged (11 000g, 30 min, 4°C) and the resulting supernatant and pellet were treated as in (a).

(d) Reconstitution with purified cytochrome c_4 - The sample was diluted to 1M sodium iodide and was centrifuged (11 000g, 30 min, 4°C). The supernatant, containing endogenous cytochrome c_4 and other removed proteins, was discarded. The pellet was resuspended in 1ml 10mM sodium phosphate, pH7.0 containing 2.0M sodium iodide and 1nmol purified cytochrome c_4 was added. The sample was incubated for 30 minutes at 0°C, with thorough mixing. The sample was then dialysed and a supernatant and pellet prepared as described in (c).

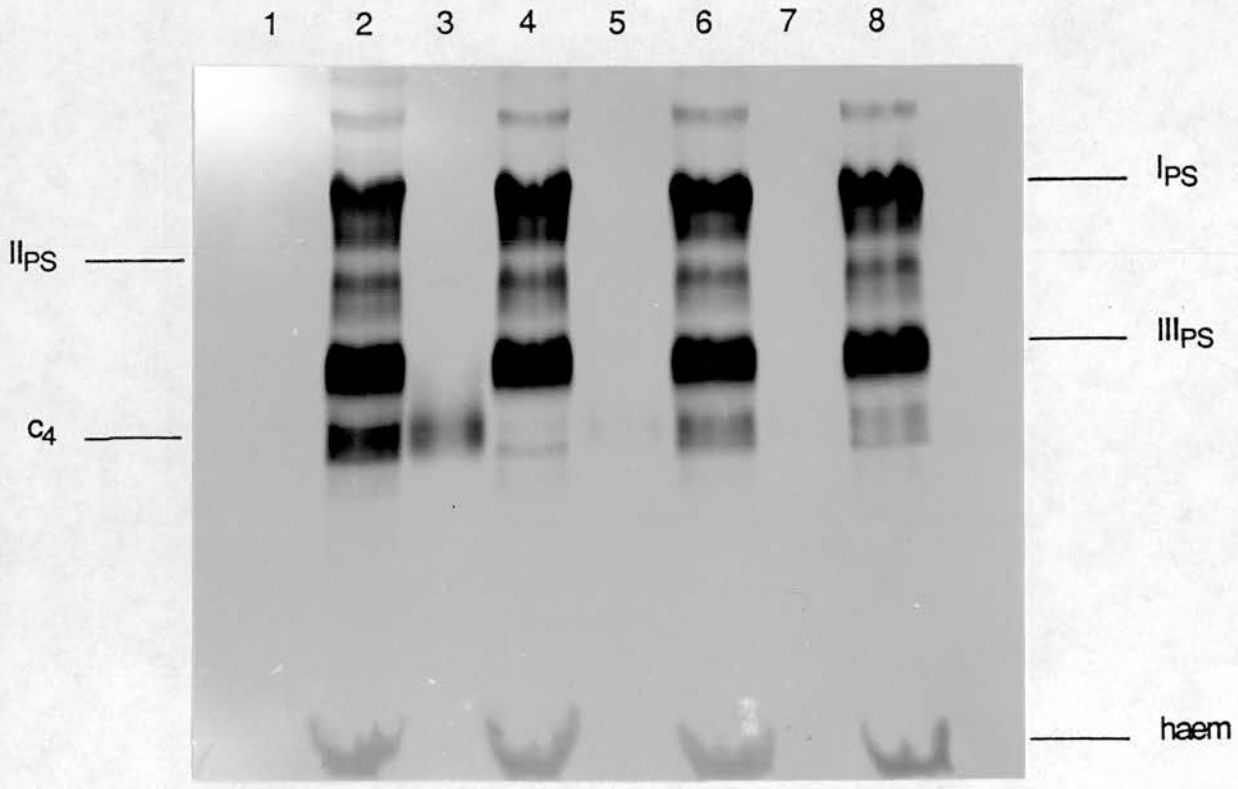
The membrane samples prepared in this way were assayed for ascorbate-TMPD. NADH, succinate and duroquinol oxidase activities. Samples of equal volume of each supernatant and pellet were run on a 15% slab SDS-PAGE gel and stained for haem (Fig.4.7). The amount of cytochrome c_4 on each membrane sample was then estimated by scanning the gel and comparing the cytochrome c_4 peaks by weight, after normalising with respect to band III_{Ps}, as described in section IB, for Fig.4.3a.

Fig.4.7 shows the haem-stained SDS-PAGE gel after such an experiment. Table 4.1 summarises the oxidase activities obtained and records the amount of cytochrome c_4 bound to the membrane in each case. As noted previously (section I), washing with 2.0M sodium iodide removes 82% of the membrane-bound cytochrome c_4 whilst removing only 10% of the ascorbate-TMPD oxidase activity. Reconstitution of the endogenous cytochrome c_4 and associated proteins removed by 2.0M sodium iodide restores cytochrome c_4 levels to 63% of the control (ie. 45% above that remaining after sodium iodide treatment. A portion of the associated non-haem proteins are also reconstituted with the membrane, but the actual amount cannot be estimated (data not shown). Reconstitution of purified

Fig.4.7 Reconstitution of cytochrome c_4 with cytochrome c_4-depleted membranes

15% slab haem-stained SDS-PAGE gel of samples from the reconstitution of cytochrome c_4 with cytochrome c_4-depleted membranes. Removal of the cytochrome c_4 and its return were accomplished as described in the text.

Lane	Contents	
1	supernatant	control (10mM sodium phosphate,
2	pellet	pH7.0)
3	supernatant	2.0M sodium iodide wash
4	pellet	"
5	supernatant	Reconstitution with removed
6	pellet	cytochrome c_4
7	supernatant	Reconstitution with pure cytochrome c_4
8	pellet	"


1
2
3
4
5
6
7
8
IIPS
c4
IPS
IIIPS
haem

cytochrome c_4 restores cytochrome c_4 levels to 43% of the control (ie. 25% above that remaining after sodium iodide treatment).

Despite the reconstitution of cytochrome c_4 with the membranes, no restoration of the oxidase activities assayed is observed. A further 10% of the ascorbate-TMPD oxidase activity is lost during the treatments to reconstitute cytochrome c_4. This is probably due to prolonged exposure to sodium iodide, which causes progressive inactivation of this activity (see section I). Some duroquinol oxidase activity is also lost and no restoration of this activity or of the NADH or succinate oxidase activities is achieved by the reconstitution of cytochrome c_4. This suggests that cytochrome c_4 is not required for these activities and that the losses of activities seen on sodium iodide treatment are due to damage or loss of other components.

This is not unexpected in the case of succinate oxidase at least, as one essential component, succinate dehydrogenase, is removed from the membranes of *Rhodospirillum rubrum* by 0.85M perchlorate (Hatefi and Davies, 1972), a chaotrope with a similar potency to sodium iodide. It is therefore likely that succinate dehydrogenase is one of the non-haem proteins removed along with cytochrome c_4 on treatment of membranes with 2.0M sodium iodide. Reconstitution of the non-haem proteins with the membranes probably does not return the succinate dehydrogenase in an active form, so that succinate oxidase activity would not be restored even with the presence of cytochrome c_4.

The lack of a positive control in this experiment, whereby reconstitution of cytochrome c_4 restores an activity it is known to be essential for, means that changes to the cytochrome c_4 during its removal from the membranes, rendering it incapable of restoring activity, cannot be ruled out. Similarly, there may be changes to cytochrome c_4 during its purification or during the return procedure which also cannot be ruled out.

Two further experiments were carried out, to address the problem of whether the return of cytochrome c_4 is to a specific site or not. One explanation for the return of cytochrome c_4 to the membranes and the lack of

restoration of oxidase activities is that the sodium iodide treatment denatures the cytochrome c_4 (see Chapter 5) and following its removal by dialysis, the denatured cytochrome c_4 non-specifically precipitates onto the membrane, either alone, or as a co-precipitate with the other removed proteins. Centrifugation then pellets the precipitated cytochrome c_4 along with the membranes.

To investigate this possibility, 1ml of the removed cytochrome c_4 (and associated proteins) or 1nmol pure cytochrome c_4, suspended in 4.0M sodium iodide, were dialysed against 10mM sodium phosphate, pH7.0 as described previously. The samples were then centrifuged (11 000g, 20 min, 4°C) and any pelleted material was resuspended in the original incubation volume (1ml 10mM sodium phosphate, pH7.0). Analysis by haem-stained SDS-PAGE gel showed that no detectable precipitation occurred with pure cytochrome c_4, but that there was precipitation when removed cytochrome c_4 was used (data not shown). Therefore, in the absence of the other proteins removed by sodium iodide treatment, cytochrome c_4 remains soluble when the sodium iodide is dialysed away.

This experiment suggests that at least part of the reconstitution of cytochrome c_4 observed with removed cytochrome c_4 is due to non-specific precipitation. However, as pure cytochrome c_4 remains soluble, this cannot be happening during the reconstitution of pure cytochrome c_4. This experiment does not rule out the possibility that pure cytochrome c_4 co-precipitates non-specifically with membrane components when dialysed with the membranes during the reconstitution procedure. A second control experiment was carried out to investigate this.

An attempt was made to 'return' to membranes a cytochrome which is not normally membrane-bound. Cytochrome c-551 has a periplasmic location and shows no association with membranes (Hunter et al., 1989). It would not therefore be expected to bind to the membranes by a specific mechanism. 'Return' of cytochrome c-551 to *P. stutzeri* membranes would suggest that

co-precipitation with membrane components is occurring and would imply that return of cytochrome c_4 occurred by a similar mechanism. Lack of 'return' would suggest that this is not the case, although it could be argued that cytochrome c_4, being membrane-bound in the first place, would be more susceptible to co-precipitation than cytochrome c-551. A control for this eventuality is difficult to devise.

Cytochrome c_4-depleted membranes were incubated with 6.4nmol purified cytochrome c-551 from *P. stutzeri*, as described previously for cytochrome c_4 (method d). Samples of the supernatant and pellet so produced were run on an SDS-PAGE gel and haem-stained. No binding of cytochrome c-551 to membranes was observed (data not shown) suggesting that co-precipitation with membrane components is not involved in this and in the reconstitution of cytochrome c_4 with membranes. A specific binding of cytochrome c_4 to the membranes is therefore occurring, at least in the case of pure cytochrome c_4.

Chapter 5: Properties of cytochrome c_4 in sodium iodide

In order to investigate the mechanism by which cytochrome c_4 is returned to the membranes of *P. stutzeri* (Chapter 4, section III) and the structure of cytochrome c_4 when bound to the membrane, the properties of the cytochrome in sodium iodide were studied. It was hoped that the presence of sodium iodide would allow cytochrome c_4 to adopt a conformation similar to that found when bound to the membrane by allowing the exposure of hydrophobic surfaces, hidden when the membrane-bound cytochrome c_4 was removed from the membrane during the purification procedure (Chapter 1, Fig.1.7). To monitor the conformation of cytochrome c_4, two methods were employed.

Redox potentiometry enables the midpoint potential(s) of a cytochrome to be measured. Midpoint potential is highly sensitive to the conformation adopted by the cytochrome under investigation, allowing redox potentiometry to be used to monitor changes in the conformation of cytochrome c_4. However, information about the conformation change is difficult to obtain by this method. The spectrum of a cytochrome is also sensitive to its conformation. In particular, the near infrared spectrum is sensitive to changes in the axial ligand coordination of the haem and therefore may also be used to monitor conformation changes. Spectroscopy was therefore also employed in these experiments.

I: Redox potentiometry

The redox potentials of cytochrome c_4 were measured in its native form , in 4.0M sodium iodide and after treatment with 4.0m sodium iodide followed by dialysis to remove the iodide. Redox titrations were carried out as described in Chapter 2. During each titration the ambient potential relative to the standard hydrogen half cell (E_h) and the ratio of oxidised cytochrome c_4 to reduced cytochrome c_4 were monitored, to allow the midpoint potential (E_m) of

the cytochrome to be calculated. This was performed using the Nernst equation

$$E_h = E_m + \frac{RT}{nF} \ln \frac{[\text{oxidised}]}{[\text{reduced}]}$$

where n is the number of electrons transferred during the reaction. Biological reactions are usually measured at 30°C, hence the equation simplifies to

$$E_h = E_m + \frac{0.06}{n} \log \frac{[\text{oxidised}]}{[\text{reduced}]}$$

A plot of E_h against log [oxidised]/[reduced] allows the midpoint potential, E_m to be obtained. In the case of a monohaem cytochrome, such as horse heart cytochrome c, such a plot will yield a straight line with slope 0.06V (n = 1) and an intercept of E_m (volts). However, in the case of cytochrome c_4, which possesses two haem groups with different midpoint potentials, a plot of E_h against log [oxidised]/[reduced] will give a sigmoidally-shaped line (Fig.5.1b, 5.2b and 5.3). This is typical of a two component system (Wilson and Dutton, 1970). The midpoint potentials of the two components can be obtained by arithmetic manipulation of such a plot. The data contained in the spectra of Fig.5.1a and plotted in Fig.5.1b will be used to illustrate the method by which this is achieved.

Firstly, the relative contributions of the two components (component I, high potential; component II, low potential) to the absorption spectra were estimated from the antilogarithm of the value of log [ox]/[red] on the abscissa of Fig.5.1b corresponding to the inflexion point of the curve (Fig.5.1b, arrow). A problem here is that the position of the point of inflexion is often hard to estimate. Therefore lines are extrapolated from the parallel linear portions of the sigmoidal curve and the point of inflexion lies on the sigmoidal curve equidistant from the two lines.

$$\text{Log [ox]/[red]} = 0.176$$

$$\text{[ox]/[red]} = 1.5 = \text{component II/component I}$$

Total divisions of reduction = 40.5 (Fig.5.1a) so that the total divisions of reduction due to component I are

$$\frac{1}{(1.5 + 1)} \times 40.5 = 16.2 \quad (40\%)$$

and total divisions of reduction due to component II are

$$\frac{1.5}{(1.5 + 1)} \times 40.5 = 24.3 \quad (60\%)$$

The raw data for Fig.5.1 are given below. Using the new values for total divisions of reduction due to each component, new values for divisions of reduction and oxidation could then be calculated, assuming that for all the points falling within the titration due to one of the components, the contribution of the other component to this change in reduction level is negligible.

E_h (mV)	Divisions of oxidation	Divisions of reduction	New divisions of oxidation	New divisions of reduction
371	38.5	2.0	14.2	2.0
344	36.7	3.8	12.4	3.8
325	35.0	5.5	10.7	5.5
294	30.7	9.8	6.4	9.8
274	28.0	12.5	3.7	12.5
247	25.0	15.5	0.7	15.5
244	17.2	23.3	17.2	7.1
193	12.0	28.5	12.0	12.3
173	7.5	33.0	7.5	16.8
142	3.0	37.5	3.0	21.3
135	2.5	38.0	2.5	21.8

Values of log [ox]/[red] could then be calculated for each component individually, and plotted against E_h.

From such a plot, the midpoint potential of each component could be obtained in the same manner as for a monohaem cytochrome, by fitting a straight line to the data. Further mathematical manipulation of the data then allows a theoretical sigmoidal curve to be constructed through the original

Fig.5.1 Redox titration of native cytochrome c_4

Reductive redox titration of cytochrome c_4, performed as described in Chapter 2.

(a) Changes in the α-peak spectrum during the titration Each spectrum corresponds to a measured ambient potential (E_h). Note the asymmetry of the α-peak when the cytochrome is partially reduced and the two isosbestic points (arrows).

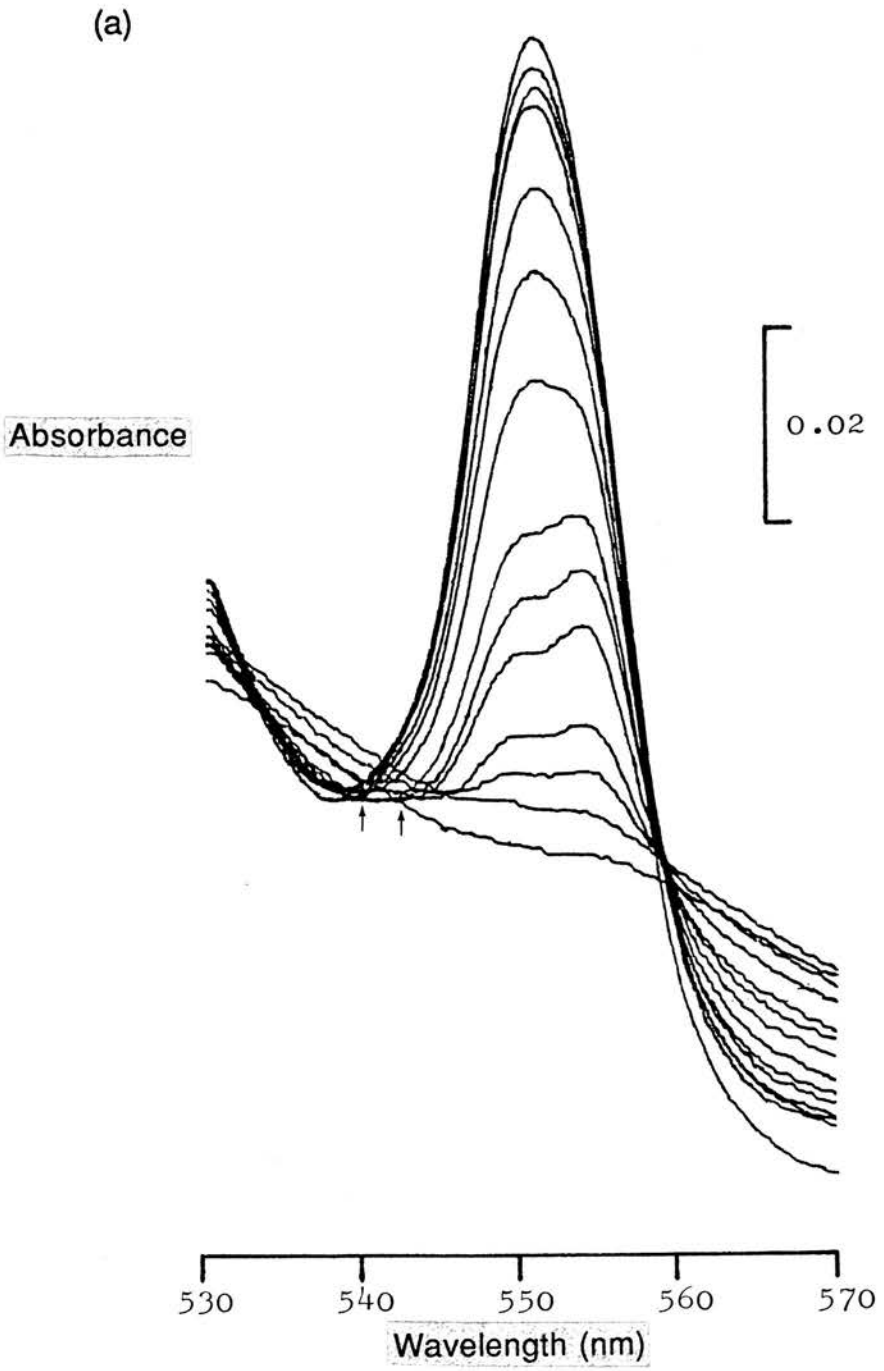
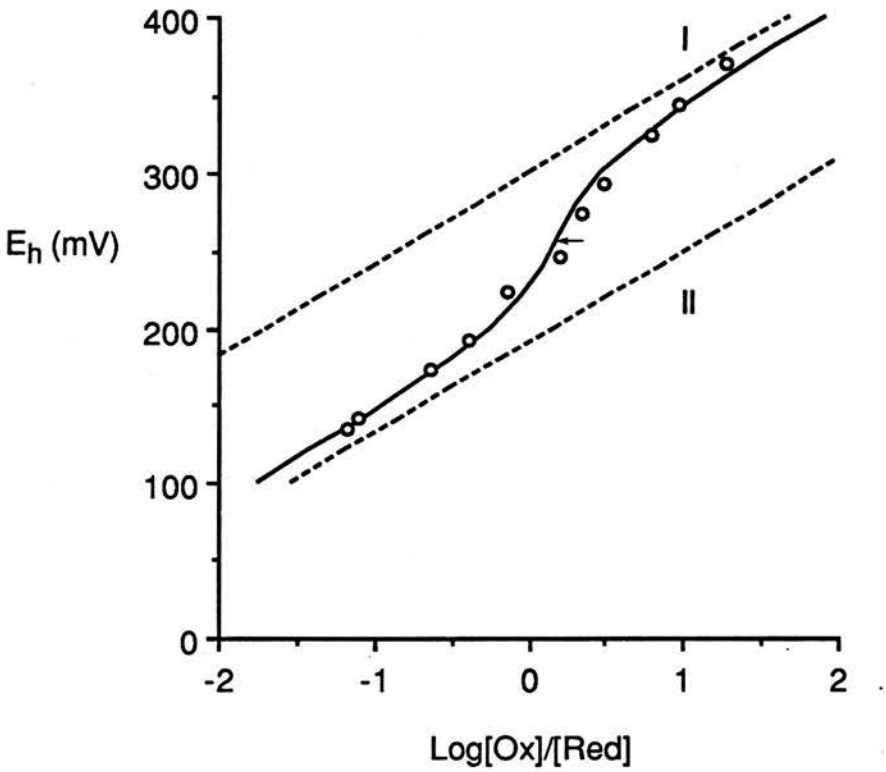

(a)
Absorbance
0.02
530
540
550
560
570
Wavelength (nm)

Fig.5.1 cont.

(b) Plot of E_h against log [ox]/[red] for the titration in (a) A curve (solid line) was fitted to the data and the midpoint potentials and percentage contributions for each component were obtained by the use of a computer program. Values in parentheses are from Leitch et al. (1985). The broken lines represent the fitting of lines of 0.06V slope to the data for each component individually. The arrow indicates the point of inflexion for the curve.

(b)



	Midpoint Potential (mV)	Percentage Contribution
Component I	+310 (+300)	40 (41)
Component II	+190 (+190)	60 (59)

data for each component. Unfortunately, the data fit very poorly to a straight line in this case and the broken lines shown are actually derived from the theoretical sigmoidal curve (solid line). Due to the laborious nature of the calculations outlined, a simple BASIC program for the BBC microcomputer was used, which allows the contribution and the midpoint potential of each component to be varied until a good fit of the theoretical curve produced to the data is obtained. The theoretical curves of Fig.5.1, 5.2 and 5.3 were all produced by this means.

Fig.5.1 shows the reductive titration of native cytochrome c_4. Fig.5.1a shows the spectra recorded during the titration, each spectrum corresponding to a measured ambient potential (E_h). The spectra show the presence of two spectrally distinct components in the cytochrome. When fully reduced, the α-peak maximum is at 550nm, whereas on partial reduction the α-peak is shifted to approx. 552nm. Additionally, two isosbestic points are present, corresponding to the two components. This spectral complexity means that the divisions of reduction can be measured at 550nm, at 552nm or at the α-peak maximum (variable wavelength). For convenience, 550nm was chosen, but there is little difference in the analysis if 552nm or the α-peak maximum are used (Leitch et al., 1985).

Fig.5.1b shows the corresponding plot of E_h against log [ox]/[red], together with the theoretical curve obtained by the use of the computer program and the midpoint potentials and contributions of the two components. These are in good agreement with the published values for *P. stutzeri* cytochrome c_4 (Leitch et al., 1985), although the quality of the data was not very good, as indicated by the poor fit of the data for each component to the 0.06V lines (broken lines).

Fig.5.2 shows an identical titration of cytochrome c_4 with the exception that 4.0M sodium iodide was present. The midpoint potential of the iodide/iodine couple

Fig.5.2 Redox titration of cytochrome c_4 in 4.0M sodium iodide

(a) Changes in the α-peak spectrum of cytochrome c_4 during the titration

Note the loss of the α-peak asymmetry seen in Fig.5.1.

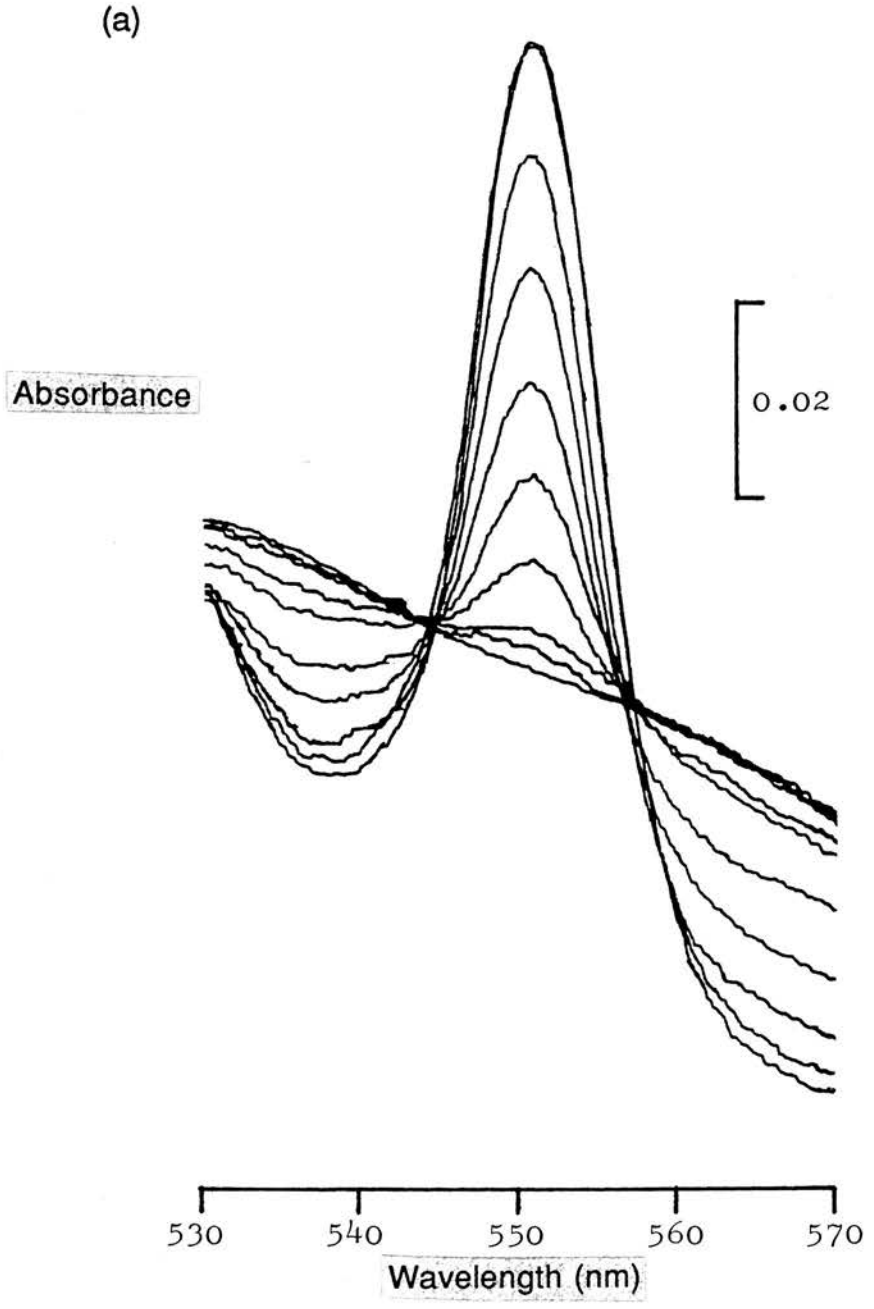
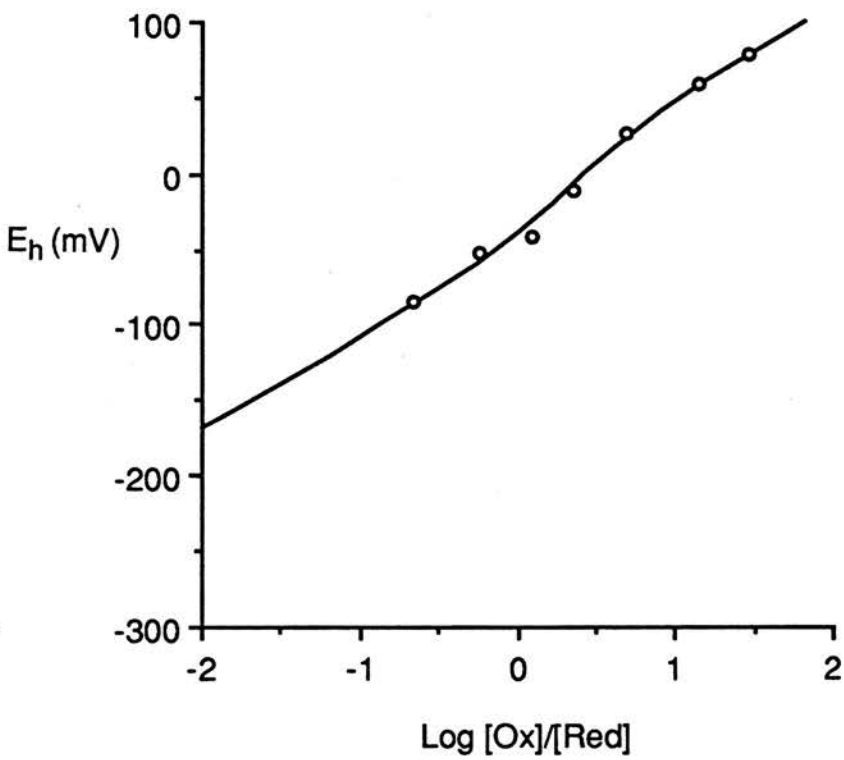

(a)
Absorbance
0.02
530
540
550
560
570
Wavelength (nm)

Fig.5.2 cont.

(b) Plot of E_h against log [ox]/[red] for the titration in (a) Midpoint potentials and the percentage contribution of each component were obtained as described in the text.

(b)



	Midpoint Potential (mV)	Percentage Contribution
Component I	+20	30
Component II	-60	70

$$I_2 + 2e^- = 2I^-$$

is +535mV. Therefore the presence of sodium iodide should not interfere with the titration as long as iodine is not present. Sodium iodide was prepared fresh prior to each experiment, to avoid this.

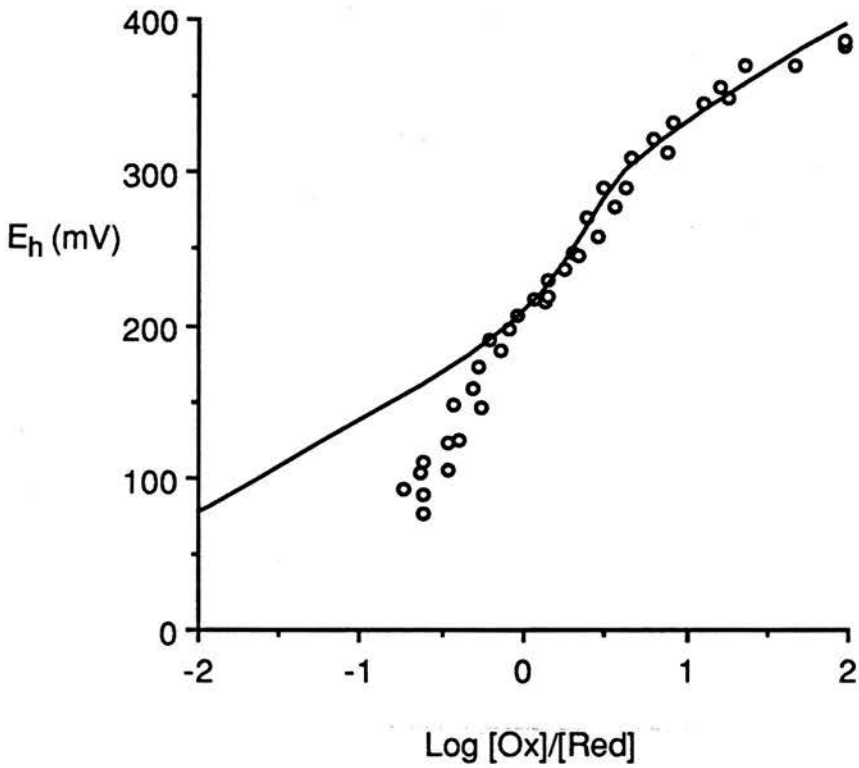
The results obtained from the redox titration of cytochrome c_4 in the presence of 4.0M sodium iodide show that the two spectral components have become identical. Fig.5.2a shows that the α-peak asymmetry seen in native cytochrome c_4 (Fig.5.1a) is no longer detectable . In parallel with this loss, the midpoint potentials of the two components are closer together (80mV separation compared with 120mV separation in the native state). Also, the higher potential component makes less of a contribution and the midpoint potentials of both components are over 250mV lower than in the native state. This latter observation suggests that the two haems are more exposed to solvent when cytochrome c_4 is in sodium iodide (Harburg and Loach, 1959).

Fig.5.3 shows a redox titration of cytochrome c_4 after treatment with 4.0M sodium iodide followed by dialysis to remove the iodide. 70nmol cytochrome c_4 in 10mM sodium phosphate, pH7.0 was added to sodium iodide in 10mM sodium phosphate, pH7.0 to give 4.0M sodium iodide and a final volume 3.3ml. After thorough mixing, the cytochrome c_4 was dialysed against two changes of 500ml 10mM sodium phosphate, pH7.0 for two hours per change. The change in volume on dialysis was negligible. 3.1ml (approx. 66nmol) of the iodide treated, dialysed cytochrome c_4 was taken for the redox titration, performed as described in Chapter 2.

Although the data in Fig.5.3 are scattered, it still proved possible to fit a theoretical curve to them. The data clearly do not follow a simple sigmoidal curve. At low ambient potentials, there is significant deviation from the curve fitted. However, approximately 60% of the cytochrome c_4 behaves in a manner very similar to the native, untreated cytochrome c_4. Furthermore, the α-peak asymmetry on partial reduction, characteristic of *P. stutzeri*

Fig.5.3 Redox titration of cytochrome c_4 after 4.0M sodium iodide treatment and removal of iodide by dialysis

Plot of E_h against log [ox]/[red] for cytochrome c_4 following treatment with 4.0M sodium iodide and removal of the iodide by dialysis as described in the text. The midpoint potentials and percentage contributions of each component were obtained as described in the text. The presence of a third component in this titration, which could affect the fit shown here, should be noted.



	Midpoint Potential (mV)	Percentage Contribution
Component I	+310	30
Component II	+185	30

cytochrome c_4, is restored indicating that this portion of the cytochrome c_4 is probably in its native state. The remaining 40% titrates with a lower midpoint potential (approx. 80%) suggesting that this portion of the cytochrome c_4 has been permanently affected by the iodide treatment so that removal of the iodide does not restore native behaviour.

Leitch et al. (1985) noted that cytochrome c_4 treated with butan-1-ol during extraction from membranes showed a similar, though much smaller, deviation from the theoretical curve and proposed that this was due to a small portion of the cytochrome c_4 damaged as a consequence of the butan-1-ol extraction. Iodide treated cytochrome c_4 may be affected in a similar way, although on a larger scale. Note that the +80mV midpoint potential is probably a composite of the midpoint potentials of several partially denatured states. The titration does not extend to sufficiently low values of ambient potential to allow these to be resolved.

II: Spectroscopy

Spectra of ferricytochrome c_4 were recorded under the same conditions as the redox potentiometry was performed ie. in the native state, in 4.0M sodium iodide and after treatment with 4.0m sodium iodide, followed by dialysis to remove the iodide. Spectra of the ferricyanide-oxidised cytochrome were recorded between 500 and 750nm in order to detect characteristic spectral features at 630 and 695nm. Cytochromes with methionyl haem coordination possess a band at around 695nm in their oxidised spectra (Chapter 1). The presence or absence of such a band in cytochrome c_4, known to possess methionyl coordination to both its haem groups, is a sensitive indicator of whether this coordination is intact. A band at 630nm is indicative of high spin state and may also give information on the conformation of the cytochrome.

21nmol cytochrome c_4, in 1ml 0.1M sodium phosphate, pH7.0 alone, or containing 4.0M sodium iodide, were oxidised with a crystal of potassium ferricyanide. The oxidised spectra of the cytochrome c_4 were then recorded

against a baseline of 0.1M sodium phosphate, pH7.0. using a Varian Cary 219 recording spectrophotometer. The cuvette contents from the spectrum of cytochrome c_4 in sodium iodide were then dialysed against two changes of 500ml 0.1M sodium phosphate, pH7.0 for two hours per change. The volume change on dialysis was negligible. The spectrum of the iodide-treated, dialysed cytochrome c_4 was then recorded after reoxidation with a crystal of potassium ferricyanide. Fig.5.4 shows the spectra obtained in the three cases.

The native spectrum (Fig.5.4a) shows peaks at 523 and around 705 to 710nm, with shoulders at 560 and 620nm. This spectrum is typical of a class I cytochrome c except that the 523nm peak is more usually at 530nm (529nm for horse heart cytochrome c). The exact absorbance maximum of the near infrared band is difficult to determine due to distortions in the baseline around this wavelength, but it appears to be 707nm, slightly more than the published value of 701nm (Pettigrew and Brown, 1988). The small feature at 620nm is indicative of the presence of a small portion of cytochrome c_4 in a high spin state. Both this band and the near infrared 707nm band are abolished in the presence of iodide, indicating that in 4.0M sodium iodide, all the cytochrome c_4 is low spin and that the methionyl haem coordination has been lost (Fig.5.4b). In 4.0M sodium iodide, horse heart cytochrome c and *P. stutzeri* cytochrome c-551 show a dramatic loss of the trough at 500nm (data not shown). Cytochrome c_4 does not show this loss, although the 500nm trough to 523nm peak ratio is somewhat reduced. This may indicate a difference in behaviour between cytochrome c_4 and its smaller relatives.

Dialysis of iodide treated cytochrome c_4 reverses the spectral changes noted on treatment with 4.0m sodium iodide (Fig.5.4c). In particular, there is recovery of the spectral features at 620 and 707nm, indicating that a small portion of the cytochrome c_4 becomes high spin once more and that the methionyl coordination of the haems is restored. However, a significant decrease in the 523nm peak is observed which is probably not due to dilution of the cytochrome c_4 during dialysis as the near infrared band is restored to its former intensity. The reason for this decrease in absorbance over the range

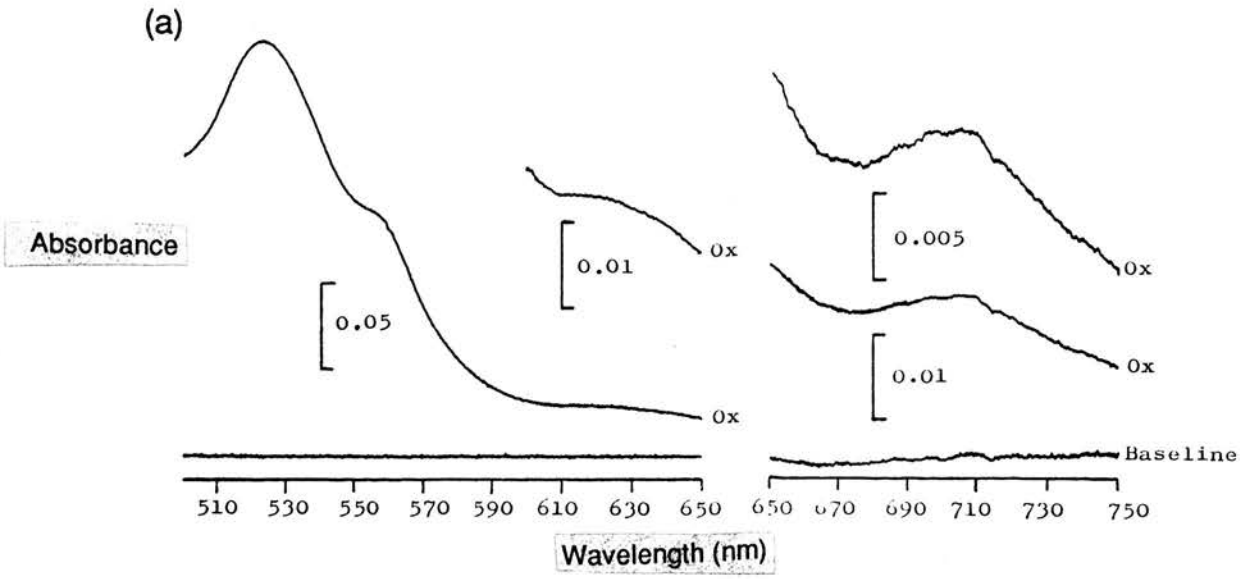
Fig.5.4 Spectra of oxidised cytochrome c_4

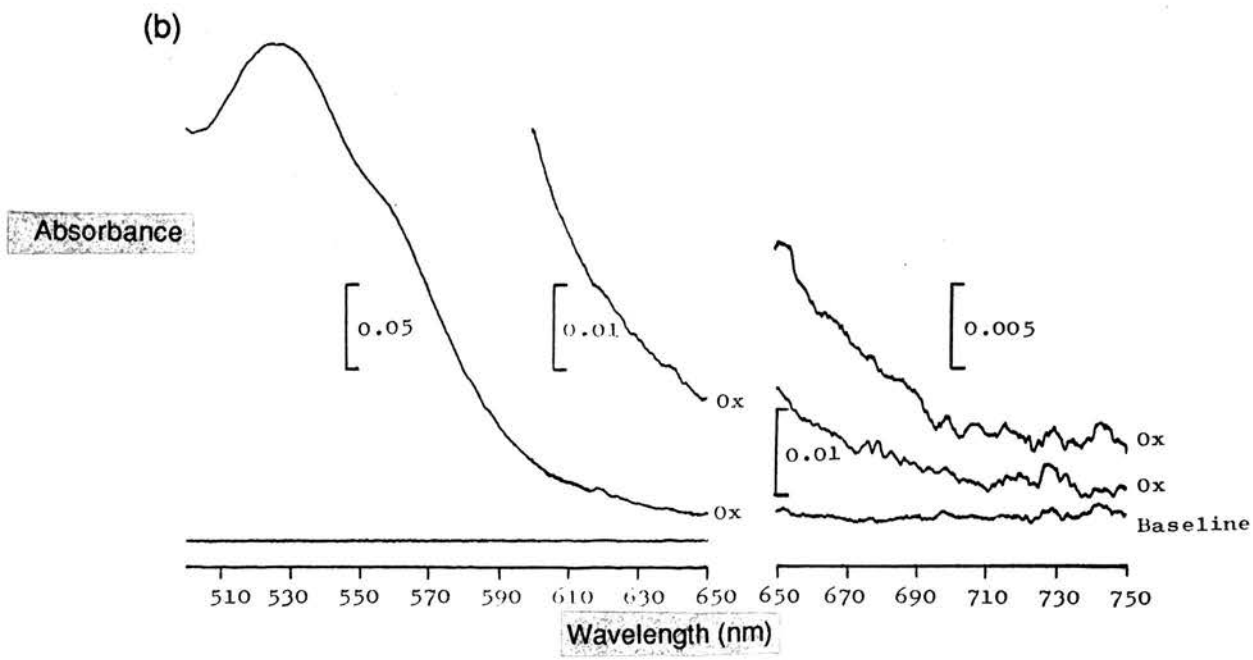
Spectra of oxidised cytochrome c_4 were recorded between 500 and 750nm as described in the text.

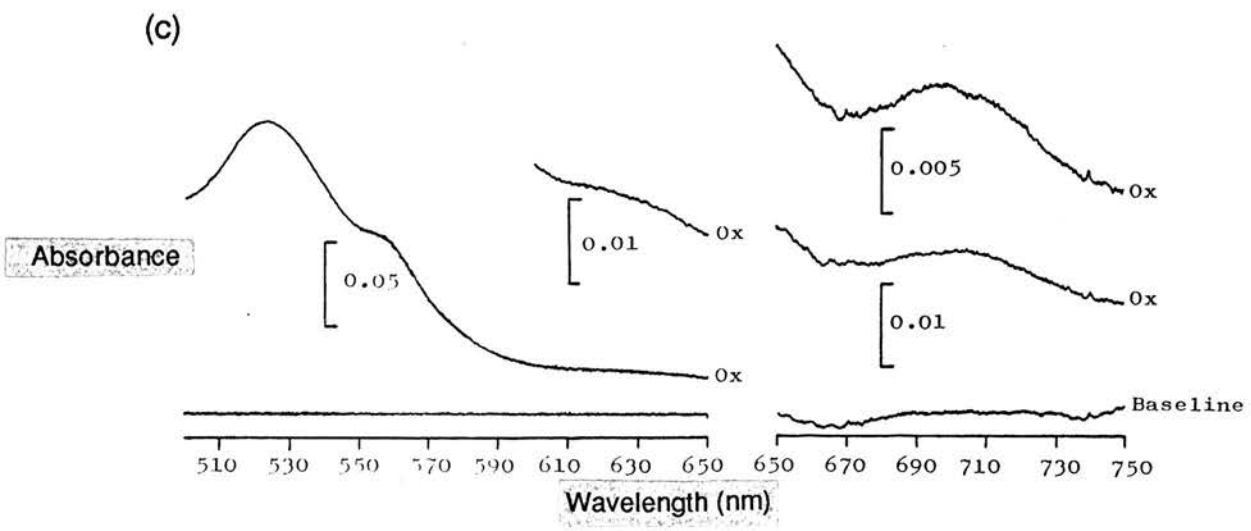
(a) Native cytochrome c_4

(b) Cytochrome c_4 in 4.0M sodium iodide

(c) Cytochrome c_4 following sodium iodide treatment and dialysis


(a)
Absorbance
0.05
0.01
0x
0.005
0x
0.01
0x
Baseline
510 530 550 570 590 610 630 650 650 670 690 710 730 750
Wavelength (nm)


(b)
Absorbance
0.05
0.01
0x
0.005
0x
0.01
0x
Baseline
510 530 550 570 590 610 630 650 650 670 690 710 730 750
Wavelength (nm)


(c)
Absorbance
0.05
0.01
0x
0.005
0x
0.01
0x
Baseline
510 530 550 570 590 610 630 650 650 670 690 710 730 750
Wavelength (nm)

500 to 600 nm is not known.

III: Discussion

The properties of cytochrome c_4 under native conditions were found to be very similar to those published (Leitch et al., 1985; Pettigrew and Brown, 1989). It was noted that a portion of the cytochrome c_4 was high spin, giving rise to the observed band at 620nm. This finding is discussed later.

The properties observed for cytochrome c_4 in 4.0M sodium iodide indicate that it is at least partially denatured. The large decrease in midpoint potential observed indicates that the haem groups may be more exposed to solvent than in the native state and the reduction in the difference between the two observed potentials suggests that the two haem environments have become more similar, or that anticooperativity between the haems is less strong. However, despite this, and despite the loss of the methionyl-haem coordination, indicated by the disappearance of the near infrared band in the oxidised spectrum, the cytochrome is not completely denatured. This can be stated for two reasons.

Firstly, although the two midpoint potentials become more similar, they do not become identical, suggesting that sufficient structure is retained around the haems to provide differing environments and hence midpoint potentials in the two cases. Secondly, the cytochrome remains low spin, indicating that methionine is replaced as sixth ligand rather than a sixth ligand being absent (which would give rise to high spin).

Replacement of methionine as the sixth haem ligand by another group is reminiscent of the alkaline transition of horse heart ferricytochrome c (Dickerson and Timkovich, 1975). This transition occurs between pH9.35 and 12.8 and results in the loss of ascorbate reducibility and a lowered midpoint potential. Spectroscopic studies are all consistent with a conformation change in the cytochrome leading to replacement of methionine 80 as the sixth haem ligand. Equilibrium studies have shown a single proton ionisation to occur, with apparent pK9 to 9.4. However, stopped flow and temperature jump experiments have shown that this apparent pK is composed of an ionisation of

pK11 and a conformational change which shifts the $pK_{overall}$ to 9 (Davies et al., 1974). The group ionising with pK11 may act as the replacement ligand for methionine 80 or as the trigger for the conformational change resulting in the replacement of methionine 80 by another group. Spectroscopic studies have suggested that a lysine acts as the replacement sixth ligand, but many chemical modification studies are incompatible with this. The identity of the ionising group is also the subject of much debate, with lysine, histidine 18, haem propionate 6 and water associated with the protein all being put forward.

Although cytochrome c_4 is treated in a different way to bring about the observed changes in properties in this instance, similarities to the alkaline transition are clear. The midpoint potentials of the cytochrome are lowered, although ascorbate irreducibility was not investigated. Cytochrome c_4 loses methionyl coordination of the haems, but remains low spin, suggesting replacement of the methionines by other groups, the identity of which is even less certain than for the alkaline transition.

Under native conditions, a small portion of cytochrome c_4 is high spin. An explanation for this observation lies in the fact that in solution, the compact, globular cytochrome c_4 is in equilibrium with various unfolded states ranging from small local fluctuations in side chain positions to gross unfolding. At any one time, therefore, it is likely that a portion of the cytochrome c_4 will have temporarily lost methionyl coordination and, in the absence of a replacement ligand, will be high spin. The amount of high spin form is governed by the position of the equilibrium. In native cytochrome c_4, this is such that a small portion of the cytochrome is high spin. When in iodide, however, a new equilibrium is set up which is shifted such that no high spin cytochrome c_4 is present.

A similar example is the case of cytochrome c_2 from *Rhodomicrobium vannielii* (Pettigrew et al., 1978). Treatment of this cytochrome with increasing pH causes the production, then the loss of a high spin form. This observation can be explained by considering that the increasing pH shifts the equilibrium

between low and high spin forms towards high spin. Methionyl coordination of the haem is lost and no replacement ligand is present. At still more alkaline pH, a side chain ionises and becomes available as a replacement sixth ligand, changing the equilibrium. Further increases in pH shift the new equilibrium such that the high spin form is lost due to increasing coordination by the replacement sixth ligand.

Cytochrome c_4 in sodium iodide does not adopt a 'membrane-bound' conformation. Rather, it is partially denatured and shows extensive changes to its properties. The mechanism by which cytochrome c_4 is returned to *P. stutzeri* membranes, by the method described in Chapter 4, section III, cannot therefore involve the adoption of a conformation where hydrophobic surfaces, hidden in the native, soluble protein (see Fig.1.7), are exposed and able to interact with the membranes. A more likely mechanism is one where the partially denatured cytochrome c_4 renatures on dialysis whilst in contact with the membrane, thus allowing hydrophobic interactions with the membranes to be restored. This mechanism, involving renaturation of cytochrome c_4 whilst interacting with membrane components, also explains why cytochrome c-551 does not 'return' to the membrane; no interaction with membrane components occurs and the cytochrome simply renatures in solution.

Redox titration of pure cytochrome c_4 after iodide treatment and dialysis indicates that only 60% returns to its native conformation. This may explain the partial nature of the return of cytochrome c_4 to membranes. If renaturation is required for reassociation of the cytochrome c_4 with membranes, a maximum of 60% of the cytochrome c_4 present will be able to be returned to the membranes, a figure close to that actually observed (table 4.1).

Elucidation of the structure of cytochrome c_4 whilst bound to the membrane and comparison with the structure in solution is not possible by means of the methods discussed here. A probe of conformation specific to cytochrome c_4 is required for this to be possible. Specific labelling of cytochrome c_4 followed by return to unlabelled membranes might allow the conformation to be investigated. However, it would be necessary to show that

returned cytochrome c_4 is identical to membrane-bound cytochrome c_4 as the experiments described here do not allow this conclusion to be drawn.

Chapter 6: Partial purification and characterisation of the cytochrome o of *P. stutzeri* 224

In order to resolve some of the questions remaining about the involvement of cytochrome c_4 in the terminal oxidase of *P. stutzeri*, an attempt was made to purify the ascorbate-TMPD oxidase activity associated with cytochrome o from this organism, with a view to characterising the interactions of this oxidase with other respiratory components and investigating the involvement of cytochrome c_4 in these interactions. Additionally, the preparation could be compared with cytochromes o from other organisms. It did not prove possible to purify the cytochrome o from *P. stutzeri* to homogeneity, despite a very extensive investigation in to the conditions for purification. These investigations are outlined in sections I to VII and proved so time-consuming that only one preparation of the ascorbate-TMPD oxidase using the final procedure chosen for its purification could be attempted. In addition, the activity of this final preparation was unstable and was lost before a thorough characterisation could be carried out. However, the level of purification achieved did allow some conclusions to be drawn about this enzyme.

Ascorbate-TMPD oxidase activity, in conjunction with difference and carbon monoxide-binding spectra was used to monitor the purification. Spectroscopy was used to ensure that the purification of the ascorbate-TMPD oxidase activity was linked to carbon monoxide-binding cytochrome b at each stage and was not due to non-specific ascorbate-TMPD oxidase activity. Additionally, the sensitivity of the final preparation to cyanide was investigated as part of its characterisation. This investigation also served to show that the activity purified was specific and cyanide-sensitive. Ascorbate-TMPD oxidase activity assays and spectroscopy were carried out as described in Chapter 2 and the legends to Fig.3.2 and 3.3.

I: Solubilisation of the ascorbate-TMPD oxidase activity

The first step in the purification of the ascorbate-TMPD oxidase activity (and hence the cytochrome o) was to solubilise the activity from the membranes of *P. stutzeri*. French press membranes were used in all preparations of the oxidase undertaken and were prepared as described in Chapter 2. The choice of detergent for the solubilisation of the ascorbate-TMPD oxidase activity was very important. For a detergent to be of use in the solubilisation, it must solubilise the membrane whilst retaining the native protein structure and the enzyme activity of the protein. Since each protein has its own individual characteristics, it is not possible to predict the most useful detergent nor the conditions of extraction that will allow solubilisation with the retention of enzyme activity. An illustration of this is given by the example of Triton X-100, which does not solubilise all membranes alike and has different powers of solubilisation for different components of the same membrane (McDonald, 1980). This unpredictability means that a process of trial and error is required to find the best detergent for the solubilisation being attempted. Despite this, however, a number of generalisations may be made about the properties of of detergents commonly used.

Firstly, ionic detergents, such as SDS, cholate and deoxycholate are able to solubilise proteins well and disaggregate artifactual protein aggregations. They are however, charged and therefore interfere with ion exchange chromatography. SDS is strongly denaturing and is therefore of no use in the solubilisation of native proteins. Cholate and deoxycholate, whilst non-denaturing, are less mild than non-ionic detergents and are therefore less likely to fully retain enzyme activity.

Secondly, non-ionic detergents are, in general, less able to solubilise membranes and disaggregate protein aggregates. They are, however, considerably more gentle towards the enzyme activity being solubilised and for this reason are generally preferred for solubilisations. Note that in certain cases it is desirable to add a small quantity of ionic detergent to facilitate solubilisation. An example of this occurs in the redissolving of ammonium

sulphate precipitates, where 0.1% cholate was added to 0.5% Triton X-100 to allow the precipitates to redissolve (section V).

A number of other factors influence the choice of detergent which are not related to the initial solubilisation of the enzyme but which may become important in the later stages of purification. These are discussed below, with reference to the detergents investigated in this instance. The following detergents were investigated. Their chemical structures are given in Fig.6.1.

Triton X-100 (Sigma, Fig.6.1a) is a polyethylene oxide derivative of iso-octyl phenol, with nine to ten ethylene oxide units. The variable number of ethylene oxide units is due to the method of synthesis, whereby ethylene oxide is added to iso-octyl phenol. A mixture of adducts in obtained, with the mean number of ethylene oxide units corresponding to the moles of ethylene oxide used. Triton X-100 is therefore not chemically homogeneous unless specially purified. It is generally considered to be the standard non-ionic detergent and is known to be non-denaturing and good at retaining enzyme activity on solubilisation in most cases. However, it has the disadvantages that its absorbance at 280nm is high, interfering with the detection of protein by means of A_{280}. It is subject to autooxidation, yielding peroxide forms which can interfere with peroxidase-linked reactions and can damage proteins. In addition, it is very hard to separate from isolated proteins as its low critical micellar concentration (the concentration at which dissolved detergent monomer aggregates to form micelles) means that micelles are present at all the concentrations usually employed. Removal of Triton X-100 by dialysis is therefore difficult, as the micelles must dissociate for dialysis of the Triton X-100 to occur. Triton X-100 also interferes with the Lowry protein assay, necessitating a modification to the procedure, described in Chapter 2 (Dulley and Grieve, 1975)

n-Octyl β-D glucoside (Sigma, Fig.6.1b), n-dodecyl β-D maltoside (Calbiochem, Sigma, Fig.6.1d) and MEGA-9 (octanoyl n-methyl glucamide, Cambridge Research Biochemicals Ltd, Fig.6.1c) are all alkyl glycoside type detergents. Such detergents have the advantage of being chemically homogeneous and of possessing a high critical micellar concentration,

Fig.6.1 Chemical structures of the detergents used in the solubilisation experiments

(a) Triton X-100 (n = 9 to 10)

(b) n-Octyl β-D glucoside

(c) MEGA-9 (octanoyl N-methyl glucamide)

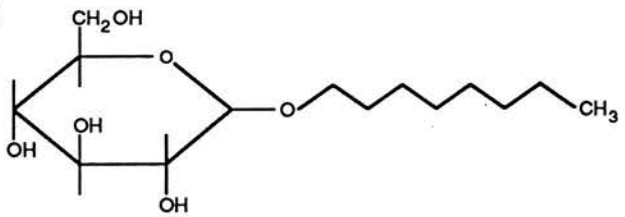
(d) n-Dodecyl β-D maltoside

(e) Sodium deoxycholate

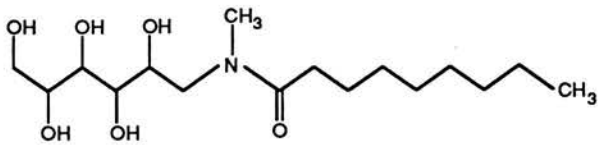
(f) CHAPS (3[(3 cholamidopropyl) dimethylammonio]-1-propane sulphonate)

(a)

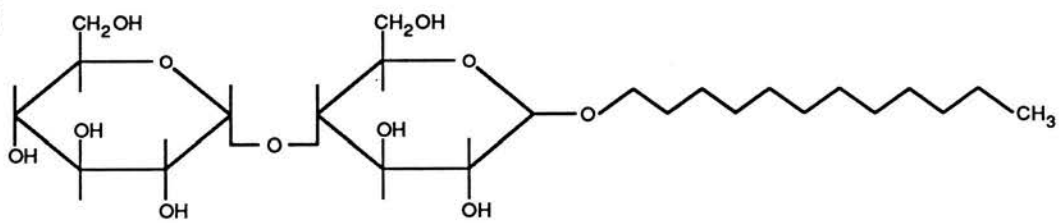
(b)



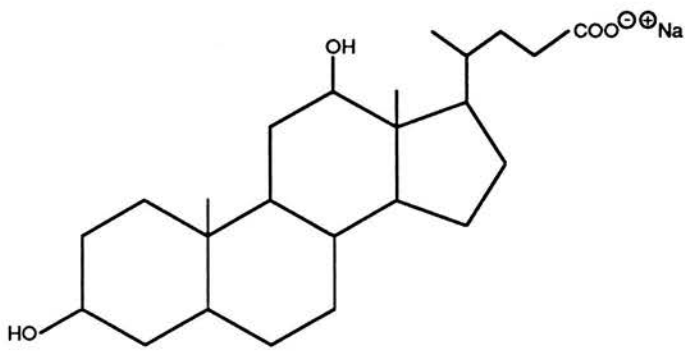
(c)



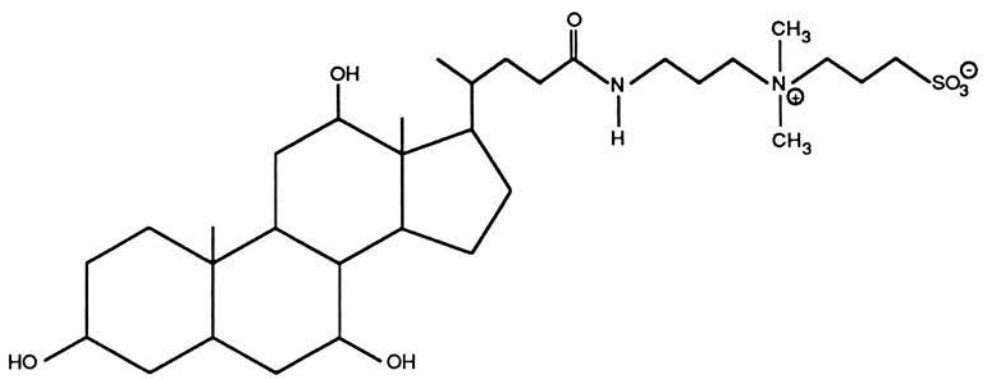
(d)



(e)



(f)



allowing easy removal by dialysis. In addition, they have been shown to be very good at retaining enzyme activity. Dodecyl maltoside has been shown to be an excellent detergent for the solubilisation of beef heart and *Neurospora* cytochrome oxidases, giving up to tenfold higher activities than other detergents (Rosevear et al., 1980). However, the stability of alkyl glycoside solubilised proteins may be less than that of Triton X-100 solubilised proteins (Dencher and Heyn, 1978). MEGA-9 is a less expensive alternative to octyl glucoside, with reportedly similar properties (Hildreth, 1982). Note that MEGA-9 supplied by Sigma is described as nonanoyl n-methyl glucamide. Two batches of dodecyl maltoside from different suppliers (Calbiochem and Sigma) were investigated. The properties of the two were identical.

CHAPS (3 [(3 cholamidopropyl) dimethylammonio]-1-propane sulphonate, Sigma, Fig.6.1f) is a non-denaturing, electrically neutral derivative of cholate. It possesses the advantages of ionic detergents, in that it is a powerful solubilising agent, but being zwitterionic, does not interfere with ion exchange chromatography. It also does not interfere with protein estimation at 280nm or with the Lowry protein assay (Hjelmeland, 1980).

Sodium deoxycholate (Sigma, Fig.6.1e) is an anionic detergent which has been used extensively in membrane solubilisations. In particular, it has been used to isolate respiratory complexes from mitochondria, since it is capable of disrupting lipid-lipid interactions, whilst leaving protein-protein interactions intact. This detergent must be used at pH7.0 or above, since it precipitates at lower pH due to protonation of its carboxyl group.

Each detergent investigated was tried at two concentrations, 0.5 and 1.5mg detergent per mg membrane protein. It is important to note that the detergent concentration was related to the membrane protein concentration rather than to a fixed amount of membranes. This was to ensure that the detergent:protein ratio remained constant in the event of variations in the membrane protein content.

0.5ml aliquots of French press membranes (15mg protein/ml) in 10mM sodium phosphate, pH7.0, were treated with detergents at 0.5 and 1.5 mg detergent per mg membrane protein. Detergents were added as 5% (w/v or

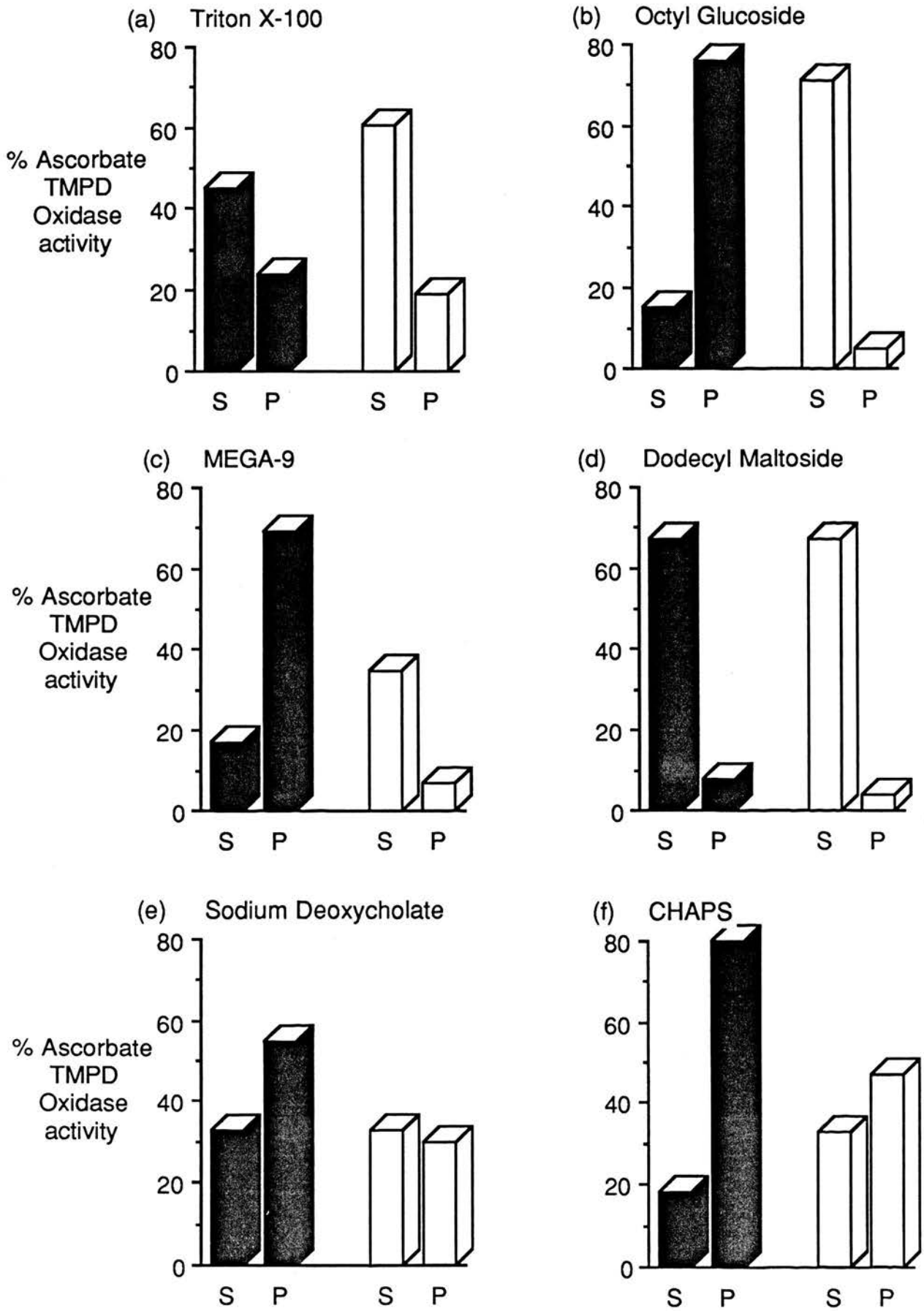
v/v) aqueous solutions, along with 10mM sodium phosphate, pH7.0 to give the appropriate final detergent concentrations and final volumes of 1.5ml. After incubation for 30 minutes at 0°C, the samples were centrifuged at 11 000g, 30 min, 4°C to separate the solubilised from the insoluble material. The latter was resuspended in 1.5ml aliquots of 10mM sodium phosphate, pH7.0 for assay of the ascorbate-TMPD oxidase activity. The ascorbate-TMPD oxidase activities of both supernatants and pellets were assayed and compared to the control activity obtained when an identical membrane sample was treated in the same way but with no detergent added. Samples of the supernatants and pellets were also run on haem-stained 15% slab SDS-PAGE gels to determine the degree of solubilisation of the membranes. It was found that solubilisation of the membrane cytochromes c, as judged by this method, matched the pattern of solubilisation of the ascorbate-TMPD oxidase activity, hence only the latter data is presented.

Fig.6.2 shows the solubilisation of ascorbate-TMPD oxidase activity from *P. stutzeri* French press membranes by the six detergents investigated. Three of these, Triton X-100, octyl glucoside and dodecyl maltoside, solubilise over 60% of the ascorbate-TMPD oxidase activity at 1.5mg detergent per mg membrane protein (Fig.6.2a,b and d). Triton X-100 and dodecyl maltoside also solubilise the activity at 0.5mg detergent per mg membrane protein, although the former only solubilised 45% of the activity at this concentration. Octyl glucoside solubilises poorly at this lower concentration (Fig.6.2b), suggesting that membranes could be washed free of some membrane proteins by 0.5mg octyl glucoside per mg membrane protein, then the oxidase activity could be solubilised by the higher concentration of octyl glucoside.

The remaining three detergents, deoxycholate, CHAPS and MEGA-9 solubilised the ascorbate-TMPD oxidase activity poorly. Deoxycholate and CHAPS solubilised less than 40% of the ascorbate-TMPD oxidase activity at both the detergent concentrations used (Fig.6.2e and f). The membrane cytochromes c were also not fully solubilised by these detergents. In the case of sodium deoxycholate, it is clear that the detergent inactivates the ascorbate-TMPD oxidase activity , since no more activity is solubilised at

Fig.6.2 Choice of detergent for solubilisation of the ascorbate-TMPD oxidase activity

0.5ml aliquots of *P. stutzeri* French press membranes were treated with detergent at 0.5 (solid bars) and 1.5 (open bars) mg detergent per mg membrane protein, then were centrifuged to separate the solubilised material (S) from detergent insoluble material (P) as described in the text. The ascorbate-TMPD oxidase activities of each pair of samples was assayed. The histograms show these activities, expressed relative to that of untreated membranes.


(a) Triton X-100
(b) Octyl Glucoside
(c) MEGA-9
(d) Dodecyl Maltoside
(e) Sodium Deoxycholate
(f) CHAPS
% Ascorbate TMPD Oxidase activity
80
60
40
20
0
S
P

1.5mg deoxycholate per mg membrane protein than at 0.5mg per mg membrane protein, yet the activity associated with the particulate material falls markedly. MEGA-9 showed a similar pattern of solubilisation to octyl glucoside (Fig.6.2c, compare with Fig.6.2b). However, at the higher MEGA-9 concentration, despite solubilising the membrane cytochromes c to the same extent as octyl glucoside at this concentration, less than 40% of the ascorbate-TMPD oxidase activity is recovered, indicating that this detergent is inhibitory. In addition, MEGA-9 was prone to precipitation at low temperatures (0 to 4°C) which made it of little use for further purification.

Triton X-100, octyl glucoside and dodecyl maltoside were therefore selected for further study. However, a problem was encountered with octyl glucoside which necessitated the use of a modified procedure for solubilisation of membranes when this detergent was used. Extraction with octyl glucoside at 1.5mg per mg membrane protein gave a variable recovery of ascorbate-TMPD oxidase activity (30 to 80%) although the solubilisation of the membranes, as judged by haem-stained SDS-PAGE gels, was reproducible. Several experiments were therefore performed to investigate this variability and to attempt to find a means of maximising the recovery of activity.

The variable recovery of activity was not due to the slight differences in the procedures used for extraction, as variations observed did not correlate with the differences in procedures. The variability in the recovery of activity was also not due to aging of the 5% (w/v) octyl glucoside solution, as freshly prepared octyl glucoside solution showed the same variability as a solution stored for several weeks. The age of the cells used also had no bearing on the recovery of activity after octyl glucoside solubilisation. Log phase cells showed the same variable recovery of ascorbate-TMPD activity as did stationary phase cells and the method of preparation of membranes used also had no effect on the recovery of activity. Spheroplast membranes gave similar results to French press membranes in this respect. Octyl glucoside at a concentration of 1.0mg per mg membrane protein also displayed this variability in the recovery of ascorbate-TMPD oxidase activity, although the

extraction in this case was poorer than with 1.5mg octyl glucoside per mg membrane protein. The extraction procedure of Bogonez and Koshland (1985) was therefore investigated.

These workers found that octyl glucoside was capable of solubilising the *E. coli* aspartate receptor involved in chemotaxis in an inactive form. However, by incubating the solubilised receptor with 40% (v/v) glycerol and *E. coli* phospholipids, the activity of the receptor could be restored. They proposed that the glycerol and phospholipids created an environment which allowed the receptor to regain its native conformation and hence its activity. Treatment of the octyl glucoside extract of *P. stutzeri* membranes in this way, to restore the ascorbate-TMPD oxidase activity, was investigated.

Incubation of a 1.5mg octyl glucoside per mg membrane protein extract of *P. stutzeri* membranes with 40% glycerol and *P. stutzeri* phospholipids (0.7mg/ml) restored the activity to approximately 100% of that of untreated membranes. *P. stutzeri* phospholipids were prepared by chloroform/methanol (2:1 v/v) extraction of *P. stutzeri* French press membranes. The extracted phospholipids were then taken up in 10mM sodium phosphate, pH7.0 by sonication to give 7mg phospholipids/ml.

However, in the course of this investigation, it was discovered that a control incubation, using 10mM sodium phosphate, pH7.0 instead of glycerol and phospholipids, produced the same restoration of activity. This procedure, being simpler than that of Bogonez and Koshland (1985), was therefore adopted. Freshly prepared octyl glucoside extracts were diluted with an equal volume of 10mM sodium phosphate, pH7.0 and were incubated for 1 hour at 25°C before any further manipulations were performed. This allowed the retention of 60 to 100% of the activity on extraction.

The reason for this reactivation, by dilution and incubation of the extracts, is not known. It may be that at the concentration necessary to solubilise the ascorbate-TMPD oxidase activity , octyl glucoside, or an impurity in the octyl glucoside used, is inhibitory. If this inhibitor is allowed to remain in contact with the ascorbate-TMPD oxidase at this concentration, it permanently inactivates the oxidase. Dilution of the extract reduces the concentration of the

inactivating species such that no inactivation occurs. This does not, however, explain why, in some extractions, little loss of ascorbate-TMPD oxidase activity was observed, despite the use of the same octyl glucoside, cells and conditions of extraction.

Having successfully solubilised the ascorbate-TMPD oxidase activity a large number of methods for its further purification were then investigated. These methods are presented in sections II to VII.

II: <u>Yeast cytochrome c affinity chromatography</u>

Chromatography on a yeast cytochrome c affinity column has been used to purify beef heart cytochrome c oxidase in a single step from a Triton X-100 extract of beef heart mitochondrial membranes (Bill et al., 1980). This method was therefore investigated as a way of rapidly purifying the ascorbate-TMPD oxidase activity of *P. stutzeri* membranes. Interaction of the ascorbate-TMPD oxidase with a yeast cytochrome c affinity column seemed possible, since *P. stutzeri* membranes possess a high yeast cytochrome c oxidase activity (Chapter 3).

Yeast cytochrome c is preferred for use in affinity chromatography as it possesses a single free cysteine (cys 107) which lies at the "back" of the molecule. Yeast cytochrome c can therefore be coupled to a matrix via this cysteine, leaving the "front face" of the molecule free for interaction with redox partners. Coupling is carried out to thiol Sepharose, a disulphide bond being formed between the "back face" cysteine 107 and the thiol moiety on the Sepharose. Horse heart cytochrome c does not possess a free cysteine and must therefore be coupled to a matrix by a lysine residue. The surface lysines of horse heart cytochrome c cluster around the "front face" of the molecule and are involved in the interaction of cytochrome c with its redox partners. Coupling of cytochrome c via a lysine, to cyanogen bromide-activated Sepharose, is more likely to produce severe steric hindrance to the interaction of cytochrome c with its redox partners, as well as preventing the involvement of the lysine in such interactions. Such columns have been used in the purification of cytochrome oxidase (Ozawa et al., 1975), but have a much

lower binding capacity than yeast cytochrome c columns. For this reason, the latter are preferred.

Yeast cytochrome c-Sepharose 4B was synthesised by the method of Madden and Cullis (1985). Yeast cytochrome c was firstly reduced, to dissociate any dimers formed by disulphide formation between the "back face" cysteines of two monomers and to ensure that the "back face" cysteines were otherwise available for interaction. 50mg yeast cytochrome c (Sigma type VIII-B, from *Saccharomyces cerevisiae*) were dissolved in 2ml 50mM tris/HCl, pH8.0 at 4°C, containing 1mM EDTA, 100mM NaCl and 20mM dithiothreitol and were incubated for 12 hours at 4°C. After incubation, the reduced cytochrome c was desalted on a 1x10cm column of Sephadex G-25 superfine into 50mM tris/HCl, pH8.0 at 4°C, containing 1mM EDTA and 100mM NaCl, to remove the dithiothreitol.

The Sepharose was then prepared for the coupling reaction. 2g activated thiol Sepharose 4B (Sigma) were swollen in 15ml distilled water for 4 hours at 20°C and were then washed (400 ml distilled water) and equilibrated in 50mM tris/HCl, pH8.0 at 4°C containing 1mM EDTA and 100mM NaCl by repeated filtering on a sintered glass filter. The swollen gel, volume approximately 8ml, was finally resuspended in an equal volume of the above equilibration buffer.

The coupling reaction was then performed. Half of the reduced, desalted yeast cytochrome c was added to the gel and the mixture was dialysed for 24 hours against 2 changes of 1l of equilibration buffer, for 12 hours per change. The remainder of the yeast cytochrome c was then added and the dialysis was repeated. Vigorous stirring was used during the dialysis to ensure that the Sepharose remained suspended. After dialysis, the Sepharose was allowed to settle and a sample of the gel-free supernatant was removed to allow quantitation of the amount of cytochrome c remaining unbound to the thiol Sepharose. From this figure, the amount of cytochrome c bound to the Sepharose could be obtained and hence the efficiency of coupling of the cytochrome c to the thiol Sepharose. Quantitation of the amount of cytochrome c was performed as described in Chapter 2, using the millimolar extinction

coefficient 29.9mM^{-1}cm^{-1}. 3627 of the 3970nmoles of yeast cytochrome c used were bound to the Sepharose, a coupling efficiency of 91%. This compares with the greater than 95% coupling efficiency obtained by Madden and Cullis (1985).

A 1x5cm column of the yeast cytochrome c-Sepharose was then packed and thoroughly washed with equilibration buffer. This contained 5 to 20mM tris/HCl, pH8.0 at 4°C containing 1mM EDTA and 1mM ascorbate. The latter was incorporated in the equilibration buffer to maintain the column in a reduced form as bound ferrocytochrome c is known to have a higher affinity for cytochrome oxidase than the ferricytochrome (Bill and Azzi, 1984).

Octyl glucoside extracts were then prepared for chromatography on the column. 1ml aliquots of French press membranes (15mg protein/ml) were centrifuged from their storage medium (10mM sodium phosphate, pH7.0) and were resuspended in 2ml each of equilibration buffer containing octyl glucoside to give 1.5 mg octyl glucoside per mg membrane protein. The samples were incubated for 30 minutes at 0°C, with vigorous mixing before centrifugation (100 000g, 1 hour, 4°C). The extracts were then diluted with an equal volume of column incubation buffer, then were incubated for 1 hour at 25°C. 2ml samples of the diluted extracts were applied to the column, which was operated at a flow rate of 2ml per hour during the loading of samples, to allow binding of the oxidase to occur. The flow rate was then increased to 10ml per hour. Initially, the column was run in 20mM tris/HCl, so that the ionic strength of the equilibration buffer was sufficient to prevent non-specific interactions of the loaded extract with the matrix. However, later column runs used a reduced ionic strength in an attempt to achieve binding.

Fig.6.3 shows a typical elution profile from the yeast cytochrome c-Sepharose. In this case, the equilibration buffer contained 5mM tris/HCl. At this low ionic strength, non-specific binding to the column would be expected. However, the sample, applied to the column at (a), fails to bind to the column and is eluted in the void volume. Assays of ascorbate-TMPD oxidase activity in the peak fractions indicate that all the applied activity is associated with this unbound peak. The column was then washed with increasing concentrations

Fig.6.3 Affinity chromatography on YCC-Sepharose

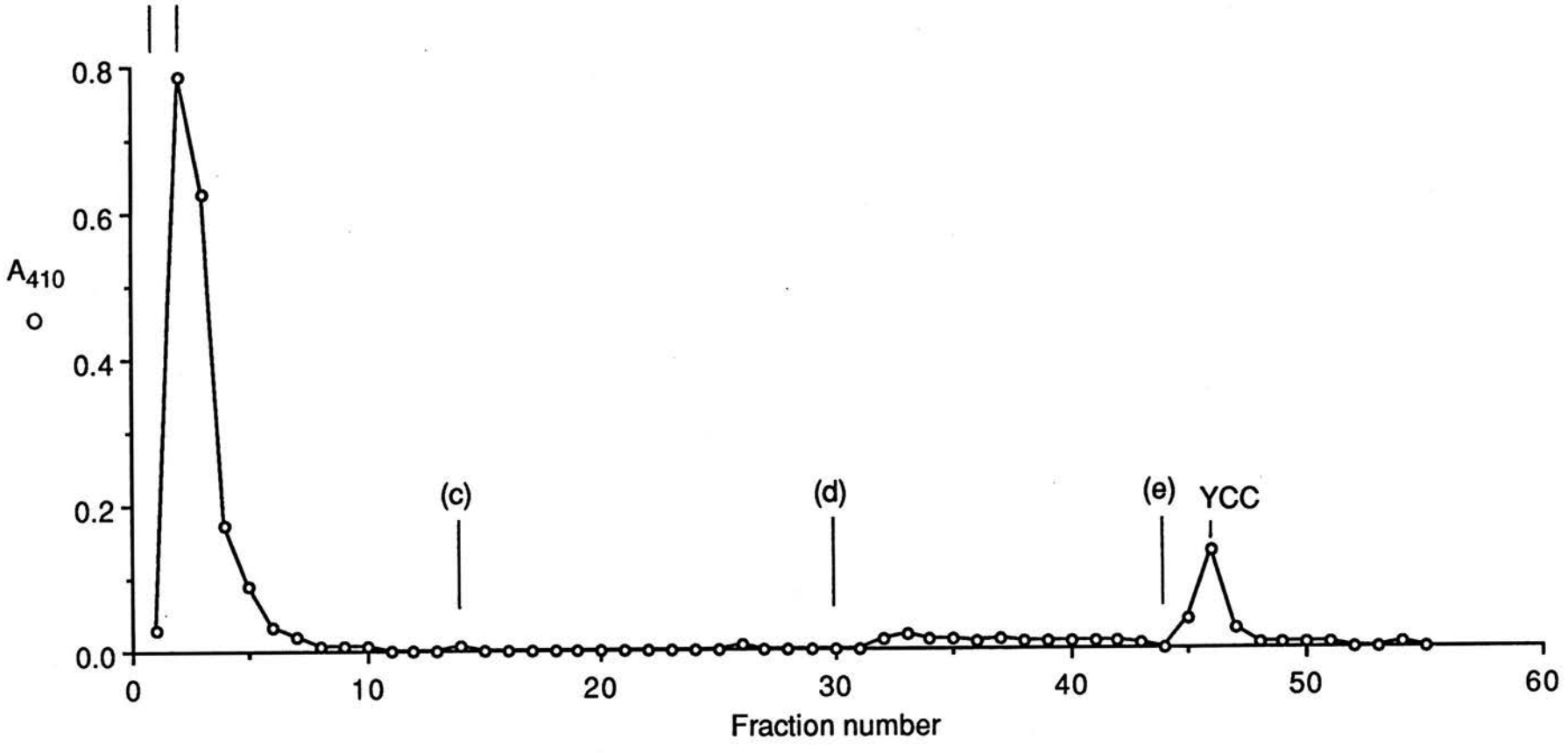
1ml of a 1.5mg octyl glucoside per mg membrane protein extract of *P. stutzeri* French press membranes, diluted with an equal volume of equilibration buffer, was loaded onto a 1x5cm column of yeast cytochrome c-Sepharose equilibrated with 5mM tris/HCl, pH8.0 at 4°C containing 1mM EDTA and 1mM ascorbate at (a), as described in the text. 1.4ml fractions were collected. The column was washed with approx. 20ml equilibration buffer at (b), then was eluted with:

(c) 50mM NaCl in equilibration buffer

(d) 100mM NaCl in equilibration buffer

(e) 0.5M NaCl in equilibration buffer

YCC indicates removal of a protein, identified as yeast cytochrome c by SDS-PAGE and haem-staining, from the column.


0.8
0.6
0.4
0.2
0.0
A_{410}
o
(c)
(d)
(e)
YCC
0
10
20
30
40
50
60
Fraction number

of NaCl, in equilibration buffer (50mM (c); 100mM, (d); 0.5M, (e)). No bound material was removed by these salt washes, except that 0.5M NaCl removed some yeast cytochrome c from the Sepharose. This probably represents yeast cytochrome c bound tightly to the column by interactions other than covalent linkage via cys 107. Although the column is run in the presence of ascorbate, the reduction potential is not low enough to to cause the breakage of the disulphide links binding the yeast cytochrome c to the Sepharose.

To check that binding of material to the column was possible, a mammalian cytochrome oxidase preparation was used. Rat liver mitochondria were prepared and were washed free of endogenous cytochrome c by the method of Jacobs and Sanadi (1960). They were then extracted at 1.5mg Triton X-100 per mg protein and the extract was chromatographed on the yeast cytochrome c-Sepharose column as described in the legend to Fig.6.3. No binding of the extract to the column was observed, so the method was abandoned.

III: Hydrophobic chromatography

Chromatography of octyl glucoside extracts of *P. stutzeri* French press membranes on octyl Sepharose, a hydrophobic affinity matrix, was investigated. The mechanism proposed for the action of hydrophobic affinity matrices (Shaltiel, 1974) is that proteins bind to the hydrophobic side chains of the affinity matrix via hydrophobic interactions with apolar regions on the protein molecules. Elution is then achieved by disrupting the hydrophobic interactions by changing the ionic strength or by the use of chaotropes in the elution buffer.

Two main classes of hydrophobic affinity matrix exist. Those which are charged, due to the method of coupling the hydrophobic side chains to the matrix, bind proteins by a combination of hydrophobic and electrostatic interactions. Examples of this type of matrix are the ω-aminoalkyl-Sepharoses, produced by the coupling of alkyl amines to cyanogen bromide-activated Sepharose. This coupling procedure introduces a positive charge into the matrix, which is then important in electrostatic interactions with the bound

protein. The importance of such interactions can be seen by the observation that elution of bound material is accomplished by an increase in ionic strength. Hydrophobic interactions are strengthened by increased ionic strength, demonstrating the involvement of electrostatic interactions in binding to such matrices (Hjerten et al., 1974).

Coupling of aliphatic or aromatic alcohols to agarose as glycidyl ethers produces electrically neutral hydrophobic affinity matrices, of which octyl Sepharose is an example. As no charge is introduced into the matrix by the coupling process, the matrix operates almost entirely due to hydrophobic interactions. This is demonstrated by the effects of ionic strength and temperature on the binding of material to such a matrix. Decreased ionic strength or lowered temperature results in a decrease in binding of protein to the hydrophobic affinity matrix. This reflects the decrease in strength of hydrophobic interactions on such changes in conditions. The usual procedure for running this type of column is to load the material to be chromatographed in high ionic strength buffer. This increases the strength of hydrophobic interactions and promotes binding. The column is then eluted using low ionic strength buffer, which decreases the strength of the hydrophobic interactions and allows elution of the bound material.

Octyl glucoside extracts of *P. stutzeri* French press membranes were prepared as described in section II and 4ml samples were applied to a 1x5cm column of octyl Sepharose CL-4B (Sigma) equilibrated with 10mM tris/HCl, pH8.0 at 4°C containing 0.5% (w/v) octyl glucoside and 0.5M NaCl (High ionic strength buffer). After loading, the column was washed with approximately 2 volumes of the equilibration buffer and then was eluted with 10mM tris/HCl, pH8.0 at 4°C containing 0.5% (w/v) octyl glucoside (low ionic strength buffer).

Approximately 20% of the loaded activity was recovered, having passed through the column without binding (data not shown). Washing of the column with low ionic strength buffer did not elute any more ascorbate-TMPD oxidase activity and a good deal of red colour remained irreversibly bound to the column. A small quantity of material with absorbance at 410nm was eluted by the low ionic strength wash, but no spectrum was obtained from this material

and no staining for haem was observed after SDS-PAGE of samples. This column was therefore of no use in the purification of the ascorbate-TMPD oxidase, as a majority of the oxidase activity bound irreversibly to it. No conditions were discovered which allowed the elution of the remainder of the bound ascorbate-TMPD oxidase activity, so its use was abandoned.

IV: Chromatography on Hydroxyapatite

Hydroxyapatite, a form of calcium phosphate, has often been used to resolve complex mixtures of substances, so its suitability for use in the purification of the ascorbate-TMPD oxidase activity was investigated. Hydroxyapatite ($Ca_5(PO_4)_3OH$) is produced by the treatment with alkali of the precipitate formed on mixing equimolar quantities of calcium chloride and disodium hydrogen phosphate (Tiselius et al., 1956). In general, high molecular weight material is bound by hydroxyapatite, whilst low molecular weight material, such as dyes and amino acids, is not bound. Phosphate buffers are commonly used for chromatography on hydroxyapatite, with proteins being bound to the column at low phosphate concentrations and eluted at by increasing the phosphate concentration. Stepwise elution is preferable, as the use of a phosphate gradient gives a poorer resolution of the separated proteins. Stepwise elution may, however, give rise to extensive tailing of the protein bands, with concomitant loss of separation (Tiselius et al., 1956).

Binding of proteins to hydroxyapatite is believed to occur both by non-specific attraction between protein positive charges and the hydroxyapatite matrix and by specific complexing of protein carboxyl groups with calcium loci on the matrix. In addition, the presence of free calcium or magnesium ions allows the formation of additional bonds between hydroxyapatite phosphate groups and protein carboxyls. Elution occurs as a result of non-specific ion screening of charges or by specific displacement of the proteins from the hydroxyapatite by ions (Gorbunoff, 1984).

4g Bio-Gel HTP (Bio-Rad) were suspended in 6 volumes of 10mM tris/HCl, pH8.0 at 4°C, containing 0.5% octyl glucoside. After gentle swirling,

the hydroxyapatite was allowed to settle and the cloudy supernatant and fines were decanted, This procedure was repeated and the hydroxyapatite was suspended in an equal volume of the above buffer. A 2x2cm column of the resuspended material was packed using a low (<5cm water) pressure, to maximise flow rate. Octyl glucoside extracts of French press membranes were prepared as described in section II and 4ml samples of the diluted extract were loaded onto the column, equilibrated with 10mM tris/HCl, pH8.0 at 4°C, containing 0.5% octyl glucoside. The column was then washed with two column volumes of equilibration buffer and was eluted with 0.2M sodium phosphate, pH7.0 containing 0.5% octyl glucoside.

As with octyl Sepharose, around 20% of the applied ascorbate-TMPD oxidase activity passed through the column without binding, the remainder of the activity remaining irreversibly bound to the column (data not shown). 0.2M sodium phosphate, pH7.0 did not elute any further activity. However, cytochrome c_4 was removed from the column by this treatment and was identified by difference spectroscopy and its mobility on haem-stained SDS-PAGE gels (data not shown). Again, as the majority of the ascorbate-TMPD oxidase activity bound irreversibly to this column, despite the use of a variety of conditions of equilibration and elution, chromatography on hydroxyapatite was of no use in the purification of the ascorbate-TMPD oxidase activity.

V: <u>Ion exchange chromatography</u>

Chromatography on DEAE-cellulose is a commonly used technique in protein purification. The purifications of many bacterial cytochromes use this method. Octyl glucoside extracts of French press membranes were prepared as described in section II and 5ml samples of the diluted extracts were applied to a 1x5cm column of DEAE-cellulose (Whatman DE52) equilibrated with 10mM tris/HCl, pH8.0 at 4°C, containing 0.5% octyl glucoside. The ascorbate-TMPD oxidase activity could then be eluted from the column by several means.

200mM NaCl in the equilibration buffer stripped the ascorbate-TMPD

oxidase activity from the DE52 column if applied immediately after loading was completed. However, if the column was allowed to sit for a time before elution, the recovery of activity was markedly reduced. Stripping the column with 200mM NaCl did not give rise to any purification, rather it served to concentrate the applied extract. This was necessary to allow a minimum volume of material to be applied to molecular exclusion columns (see section VII).

Elution of the column with a 0 to 0.5M gradient of NaCl in equilibration buffer resulted in the irreversible binding of most of the ascorbate-TMPD oxidase activity. This is probably due to the length of time taken for the NaCl concentration to reach a sufficiently high value to elute the ascorbate-TMPD oxidase activity, as irreversible binding with time occurs. Elution of the column with NaCl concentrations below 200mM (100mM, 150mM, 100 to 200mM gradient) removed a portion of the bound activity (approx. 30% of that loaded), but the remainder of the activity remained bound irreversibly unless 0.5M NaCl was used immediately to elute it. No separation of the ascorbate-TMPD oxidase activity from other proteins was observed. The two peaks of ascorbate-TMPD oxidase activity produced (after elution with <200mM NaCl and after 0.5M NaCl) were of identical polypeptide composition, except that cytochrome c_4 eluted almost exclusively at the lower concentration of NaCl (data not shown).

DEAE-Sepharose (Sigma) was also investigated, using the same chromatographic conditions as described for DEAE-cellulose. Approximately half of the loaded material failed to bind. The remainder of the material bound and was stripped by 0.5M NaCl. No difference in the composition of the two peaks of activity thus obtained was observed with respect to protein and cytochrome composition, except that cytochrome c_4 was present in the eluted peak only. As no useful separation was achieved by this column, its use was not further investigated.

Ion exchange chromatography of crude extracts of *P. stutzeri* membranes was of little use in the purification of the ascorbate-TMPD oxidase activity, since the material applied either bound irreversibly or eluted with no

separation of its components. However, such extracts could be concentrated by loading onto a DE52 column and stripping with a high ionic strength buffer into a minimum volume, prior to other methods of purification. At later stages of purification, ion exchange chromatography using DE52 was more successful.This is described in section VIII.

VI: Ammonium sulphate fractionation

Ammonium sulphate fractionation of detergent extracts of *P. stutzeri* French press membranes was investigated, both as a method for purification of the ascorbate-TMPD oxidase activity and as a means of concentrating dilute detergent extracts prior to molecular exclusion chromatography. Fractional precipitation of proteins by salt is a commonly used technique in purification. Ammonium sulphate is most commonly used as it is highly soluble in water and is also very effective. Additionally, it has few harmful effects on proteins and stabilises most enzymes (Dixon and Webb, 1979). Precipitation of proteins by salt (salting out) is believed to occur as the result of competition between the salt ions and the protein for water molecules for the purpose of solvation. At high concentrations of salt, hydration of the salt ions means that insufficient water remains to maintain the hydration shell of the protein and protein-protein interactions become more important than protein-water interactions. The protein therefore precipitates (Green and Hughes, 1955). Ammonium sulphate is effective in the salting out of proteins as it produces highly hydrated ions and is soluble at high concentrations.

Detergent extracts, using Triton X-100, octyl glucoside and dodecyl maltoside were prepared from *P. stutzeri* French press membranes as described in section I, except that 10, 20 or 50ml aliquots of membranes (15mg protein/ml) were used, depending on the volume of extract required. Detergents were added as 5% (w/v or v/v) aqueous solutions to give 1.5mg detergent per mg membrane protein, except in the case of dodecyl maltoside. Here a concentration of 0.5mg dodecyl maltoside per mg membrane protein was used, for reasons discussed later. The final volume, after detergent addition, was 1.5 times that of the aliquot of membranes used. After incubation

for 30 minutes at 0°C, the mixture was centrifuged and the supernatant obtained was diluted as described in section I. Extracts were then fractionated with ammonium sulphate.

Solid ammonium sulphate (Fisons Plc, 'AR' grade) was added slowly to the diluted extract, stirring on ice, to give 20 to 70% saturation. The amount of ammonium sulphate required for each addition was obtained from Dawson et al. (1969). The ammonium sulphate was ground prior to use to ensure large lumps were not added, creating locally high concentrations of ammonium sulphate. After incubation for 15 minutes, the extract was centrifuged (11 000g, 30 min, 4°C) to remove the precipitate formed at that percentage saturation and more ammonium sulphate was added to the resulting supernatant, as described previously, to give the next percentage saturation.

The precipitates obtained were redissolved in a minimum volume of 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl, 0.5% Triton X-100 or octyl glucoside and 0.1% sodium cholate. The latter was purified by recrystallisation and was a gift from Dr D.K.Apps, Dept. of Biochemistry, University of Edinburgh. It was included to aid the redissolving of the ammonium sulphate precipitates. When octyl glucoside and dodecyl maltoside were fractionated, the precipitates produced at 70% saturation with ammonium sulphate did not pellet on centrifugation at 11 000g, so a 100 000g centrifugation for 1 hour at 4°C was employed.

Fig.6.4 shows the fractionation of an octyl glucoside extract of *P. stutzeri* membranes. 20ml membranes were extracted and fractionated as described above. Fig.6.4a shows the recovery of ascorbate-TMPD oxidase activity in the precipitates produced by the fractionation, after redissolving each in 1ml 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl, 0.5% Triton X-100 and 0.1% cholate. The majority of the ascorbate-TMPD oxidase activity recovered is found in the 70% saturation precipitate This appeared as a floating red oil after centrifugation. Difference and carbon monoxide-binding spectra show that this material contains mainly b-type cytochrome, with some contaminating cytochrome c. The membrane cytochromes c mainly precipitate at 50 to 60% saturation.

Fig.6.4 Ammonium sulphate fractionation of an octyl glucoside extract of *P. stutzeri* French press membranes

(a) Recovery of ascorbate-TMPD oxidase activity Recovery of activity in the ammonium sulphate precipitates, expressed relative to the original activity of the supernatant.

(a)

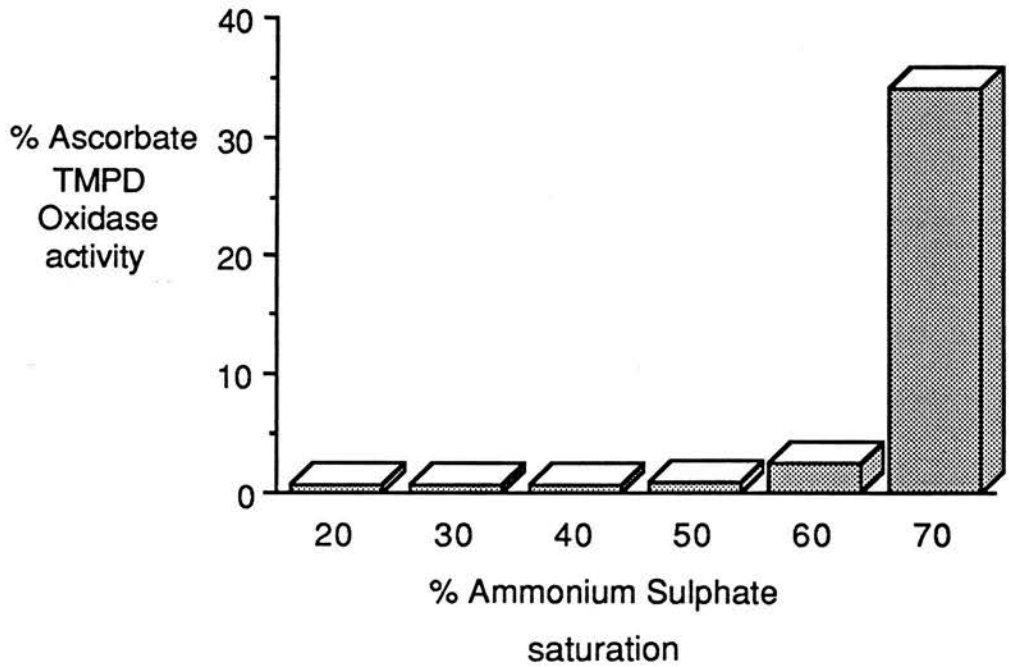

40
30
20
10
0
% Ascorbate TMPD Oxidase activity
20
30
40
50
60
70
% Ammonium Sulphate saturation

Fig.6.4 cont.

(b) Haem-stained SDS-PAGE gel 10-25% gradient SDS-PAGE gel of the ammonium sulphate precipitates, stained for haem. 10µl samples were loaded.

Lane	Contents
1	Octyl glucoside extract
2	20% saturation ammonium sulphate precipitate
3	30% saturation " " "
4	40% saturation " " "
5	50% saturation " " "
6	60% saturation " " "
7	70% saturation " " "
8	Standards - 5µg each of bovine serum albumin (BSA, M_r 66 000), ovalbumin (OVA, M_r 43 000), yeast cytochrome c peroxidase (YCCP, M_r 34 000), myoglobin (MYO, M_r 17 000) and horse heart cytochrome c (HHC, M_r 12 000)

(b)

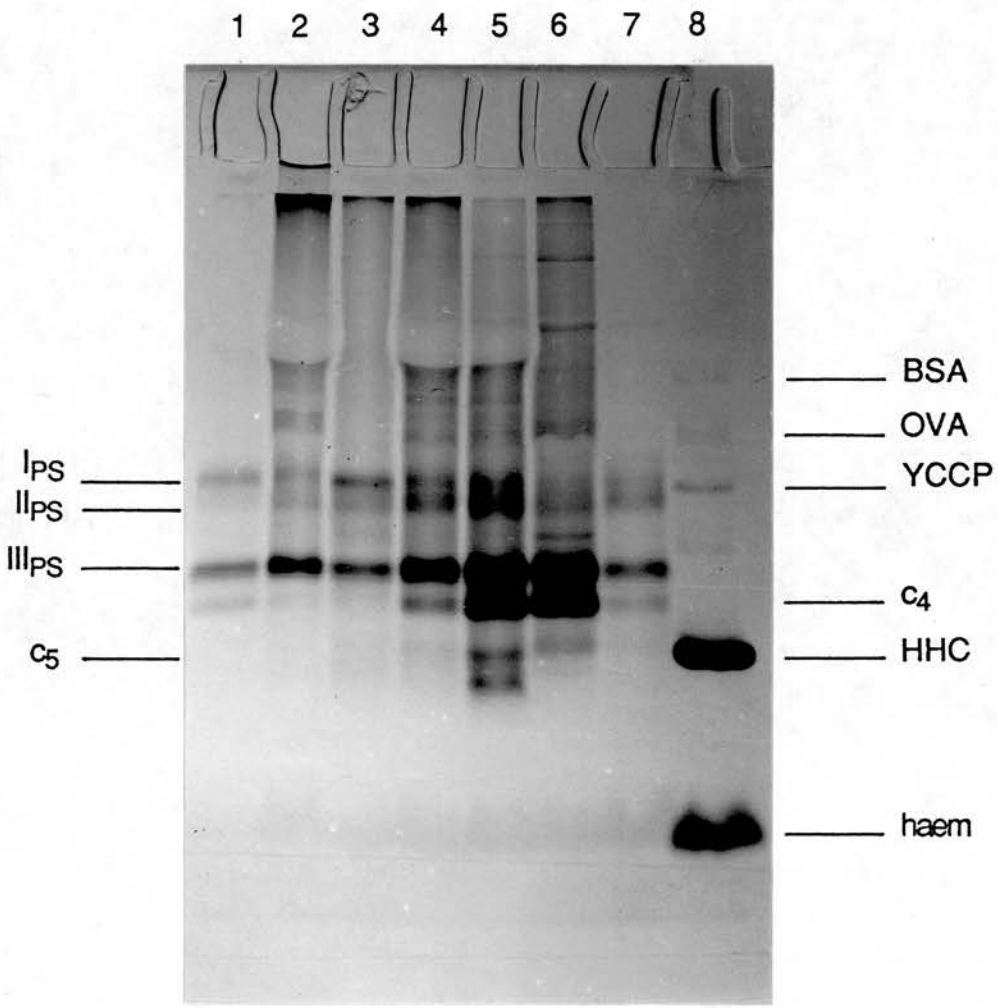
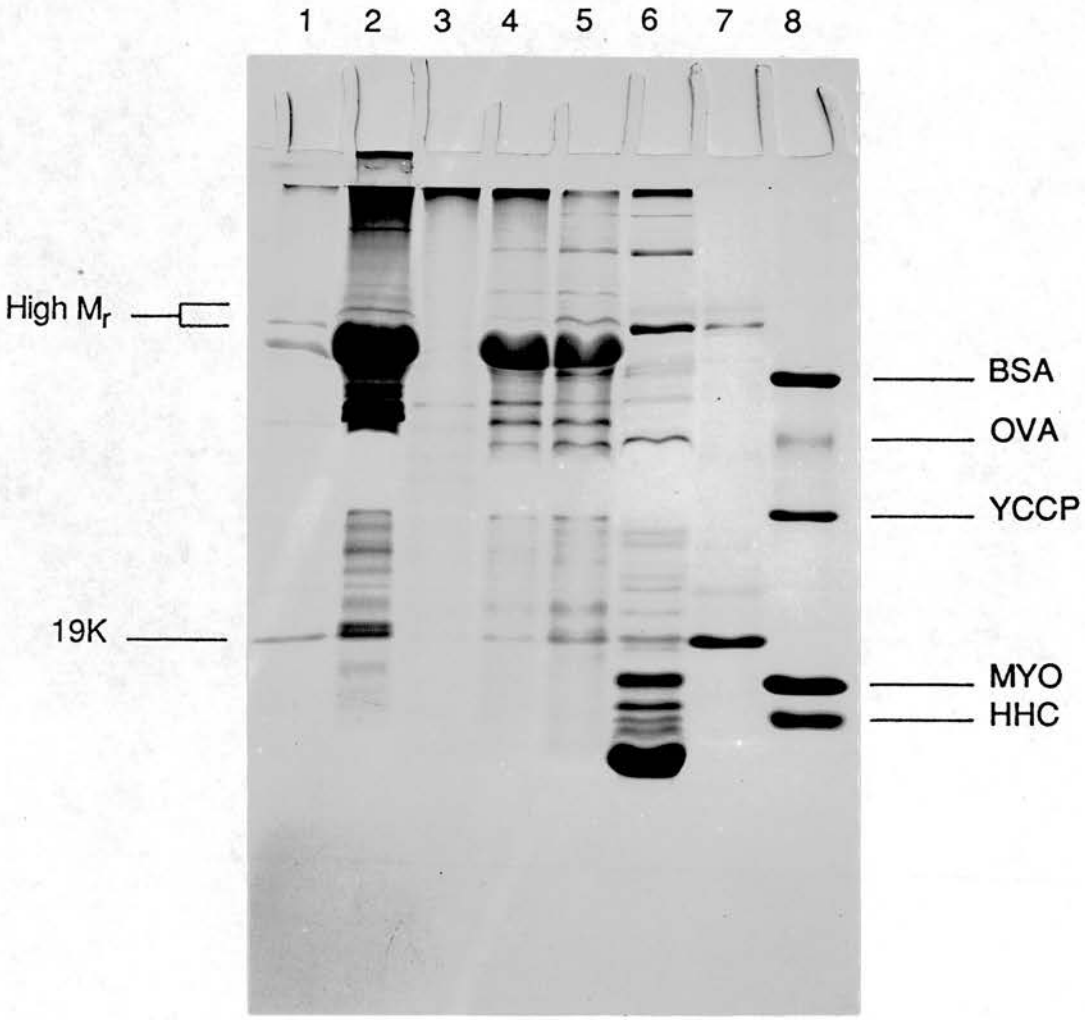


Fig.6.4 cont.

(c) Protein-stained SDS-PAGE gel Layout as in (b)

(c)



This distribution of cytochromes can clearly be seen in Fig.6.4b, which shows a haem-stained SDS-PAGE gel of the ammonium sulphate precipitates. Note the strong cytochrome c bands in the 50 and 60% precipitates (lanes 5 and 6) and the removal of much of the c-type cytochrome from the 70% ammonium sulphate precipitate, containing the ascorbate-TMPD oxidase activity (lane 7). At lower percentage saturations (20 to 40%), the precipitated material contains much denatured and lipid-containing material.

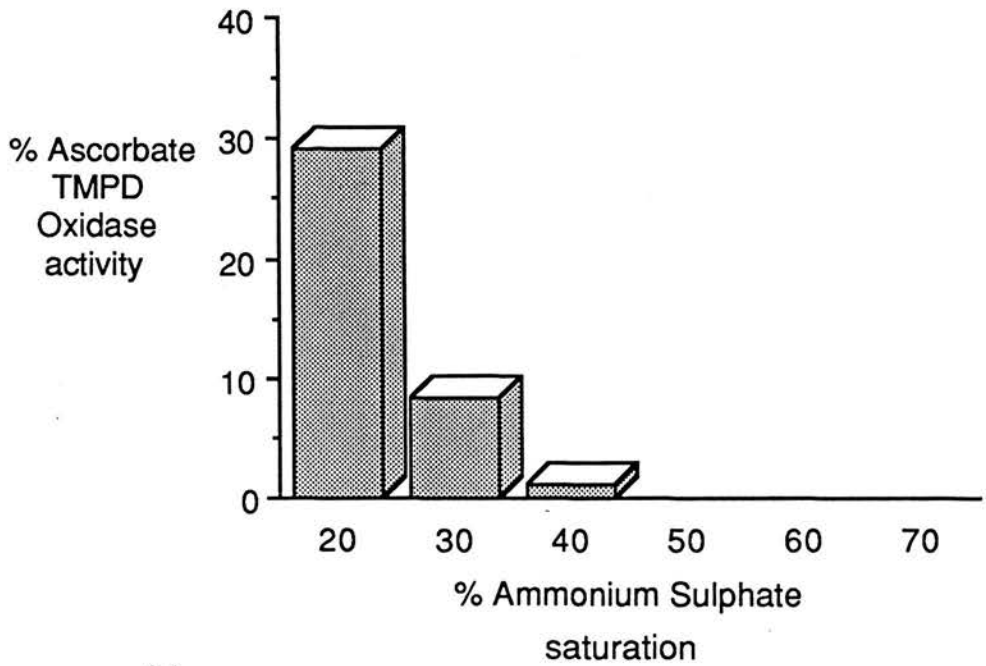
The recovery of ascorbate-TMPD oxidase activity in the 70% ammonium sulphate precipitate means that a great deal of contaminating material is removed by precipitation at lower percentage saturations. The removal of the majority of the membrane cytochromes c from the ascorbate-TMPD oxidase activity has already been discussed. The removal of contaminating non-haem proteins can best be seen in Fig.6.4c, which shows a protein-stained SDS-PAGE gel of the ammonium sulphate precipitates, with a layout identical to the haem-stained gel of Fig.6.4b. A large quantity of contaminating protein is removed by precipitation at lower percentage ammonium sulphate saturations. Very few major protein bands remain in the 70% saturation precipitate (lane 7). Most notable are a band of M_r 19 000 and a complex of bands of higher molecular weight (80 to 100 000). In some preparations, for example that of Fig.6.6, a strongly staining band of M_r 48 000 was also present.

Fig.6.5 shows ammonium sulphate fractionations of a Triton X-100 extract (a) and a dodecyl maltoside extract (b) of *P. stutzeri* French press membranes. Fractionation of the Triton X-100 extract of membranes causes the precipitation of the ascorbate-TMPD oxidase activity at 20 to 30% ammonium sulphate saturation. The activity is accompanied by a great deal of contaminating protein and lipid-containing material, and no purification of the activity, as seen with the octyl glucoside extract, is observed, as essentially all the extracted material is precipitated at these saturations (data not shown). Ammonium sulphate treatment of Triton X-100 extracts is therefore of no use in the purification of the ascorbate-TMPD oxidase activity, other than as a

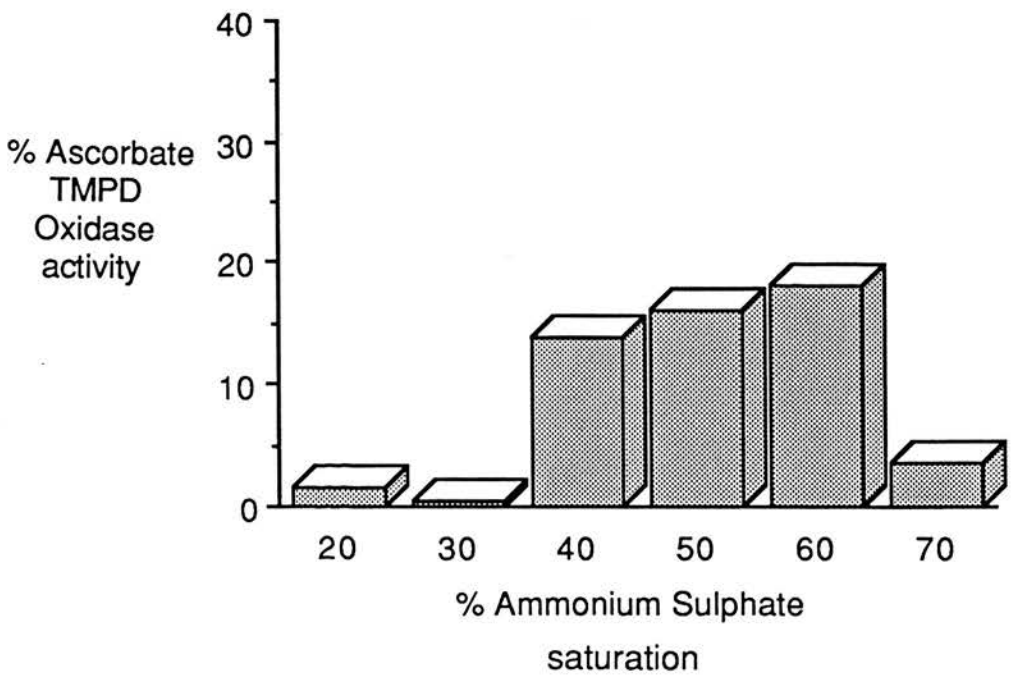
Fig.6.5 Ammonium sulphate fractionation of other detergent extracts

The histograms show the recovery of ascorbate-TMPD oxidase activity in the ammonium sulphate precipitates from fractionations of (a) 1.5mg Triton X-100 per mg membrane protein and (b) 0.5mg dodecyl maltoside per mg membrane protein, expressed relative to the original activity of the extract.

(a)


% Ascorbate TMPD Oxidase activity
40
30
20
10
0
20
30
40
50
60
70
% Ammonium Sulphate saturation

(b)


% Ascorbate TMPD Oxidase activity
40
30
20
10
0
20
30
40
50
60
70
% Ammonium Sulphate saturation

means for concentrating the Triton X-100 extract.

As described previously, dodecyl maltoside was employed at a concentration of 0.5mg per mg membrane protein, rather than at 1.5mg per mg membrane protein. This was because dodecyl maltoside extracts membranes equally well at either concentration (Fig.6.2d) and at the higher concentration of dodecyl maltoside, 80 to 90% saturation with ammonium sulphate was required to precipitate all the ascorbate-TMPD oxidase activity. The combination of the high dodecyl maltoside concentration and high percentage saturation with ammonium sulphate caused the extract to become very viscous and difficult to manipulate. Use of the lower dodecyl maltoside concentration prevented these problems occurring.

Fractionation of the 0.5mg dodecyl maltoside per mg membrane protein extract with ammonium sulphate results in the precipitation of the majority of the ascorbate-TMPD oxidase activity at between 40 and 60% saturation (Fig.6.5b). Haem- and protein-stained SDS-PAGE gels (not shown) reveal that there was little separation of the cytochrome and other protein components of the extract by the fractionation. the compositions of each precipitate at 40, 50 and 60% saturation were essentially the same as that of the original extract, indicating that no purification was achieved by the fractionation. Therefore, as with Triton X-100, ammonium sulphate fractionation of a dodecyl maltoside extract was useful only as a method for the concentrating the extract for use in other purification procedures.

VII: Molecular exclusion chromatography

Molecular exclusion chromatography was used to further separate detergent extracts after fractionation with ammonium sulphate or concentration by means of a DE52 column or ammonium sulphate treatment. A variety of media and conditions were investigated, the majority of which did not allow further purification of of the ascorbate-TMPD oxidase activity. However, conditions were found under which some separation of the ascorbate-TMPD oxidase activity from contaminating proteins could be achieved. The following molecular exclusion media were used.

Sephadex G-150-50 (Sigma) is a commonly used molecular exclusion medium, produced by cross-linking a dextran polymer with epichlorohydrin. Its fractionation range is M_r 5000 to 150 000. Fractogel HW-55 (S) (Merck) is based on a hydrophilic vinyl polymer and is mechanically very stable, enabling a high pressure to be used during chromatography. Its fractionation range is M_r 1000 to 1 million. Sephacryl S-300 (SF) (Pharmacia) is an acrylamide cross-linked dextran polymer with a fractionation range M_r 10 000 to 1.5 million. As with Fractogel, it is mechanically stable, allowing high pressures to be used. Sepharose CL-4B-200 (Sigma) is an agarose gel, cross-linked to give increased physical and chemical stability. Even so, only moderate pressures can be used with this matrix. Its fractionation range is M_r 60 000 to 20 million.

A. Dodecyl maltoside extracts 20ml French press membranes (15mg protein/ml) were extracted using dodecyl maltoside at 0.5mg dodecyl maltoside per mg membrane protein as described in section VI. The extract was then concentrated using ammonium sulphate. Ammonium sulphate was slowly added to the extract, stirring on ice, to give 70% saturation. After incubation for 8 hours at 0°C, the precipitate produced was collected by centrifugation (11 000g, 30 min, 4°C) and was redissolved in 5ml 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl, 0.5% Triton X-100 and 0.1% cholate, by homogenising and incubation for a further 12 hours at 0°C. Undissolved material, which comprised mainly lipid-containing material, was removed by centrifugation (11 000g, 30 min, 4 C) leaving a clear red concentrate. This was then loaded onto either Sephadex G-150-50 (2.2x74cm column) or Sepharose CL-4B (2.2x80cm column) equilibrated with 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.5% Triton X-100. 3 to 4 ml fractions were collected.

Chromatography on both columns results in the exclusion of the ascorbate-TMPD oxidase activity from the column and no separation of the activity from cytochromes or proteins other than cytochrome c_4 (data not shown). Cytochrome c_4 is separated from the ascorbate-TMPD oxidase

activity and elutes at volumes corresponding approximately to its native molecular weight. Separation of cytochrome c_4 from detergent solubilised membrane particles has been discussed previously (Chapter 4). In this case, removal of cytochrome c_4 from the solubilised membrane particles was essentially complete. This contrasts with the previous observations (Chapter 4, section II) and is probably the result of the long incubations used in the ammonium sulphate treatment and the relatively long time taken to run the Sepharose CL-4B column.

It seems that in dodecyl maltoside, the ascorbate-TMPD oxidase forms part of a very large detergent-containing particle which also contains most, if not all, of the proteins in the concentrated dodecyl maltoside extract (not shown). This particle is excluded from both columns, indicating a molecular weight greater than 20 million. Chromatography of pooled extracts from these columns on DE52 also gave no separation of the constituents of the dodecyl maltoside particle (data not shown). The loaded material eluted as a single peak of constant composition when a 0 to 0.5M NaCl gradient was used.

As no separation of the components of this particle is possible by the methods used, dodecyl maltoside solubilisation, in conjunction with molecular exclusion chromatography, appears to be of no use in the purification of the ascorbate-TMPD oxidase and was not investigated further.

B. Octyl glucoside extracts

(i) Columns equilibrated with octyl glucoside Samples were prepared for chromatography on molecular exclusion columns equilibrated with octyl glucoside in one of two ways, either by concentration on a DE52 column or by ammonium sulphate fractionation. 10ml portions of *P. stutzeri* French press membranes (15mg protein/ml) were extracted with octyl glucoside at 1.5mg octyl glucoside per mg membrane protein as described in section IV. The extract was then concentrated. For concentration by means of ion exchange chromatography (see section V) the extract was loaded onto a 1x5cm column of DE52 equilibrated with 10mM tris/HCl, pH8.0 at 4°C containing 0.5% octyl glucoside. The bound extract was then eluted in to a minimum volume (approx. 3ml) using 0.5M NaCl in the above buffer. Alternatively, the extract

was fractionated using ammonium sulphate, as described in section VI, except that the 70% ammonium sulphate saturation precipitate was redissolved in 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.1% cholate and 0.5% octyl glucoside, rather than 0.5% Triton X-100.

Samples prepared in either way were loaded on to either Sephadex G-150-50 (1.2x78cm column) or Sephacryl S-300 (SF, 1.2x90cm column) equilibrated with 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.5% octyl glucoside. Sepharose CL-4B was not used, as calibration with molecular weight marker proteins showed that its resolution was very poor. A similar observation was made for Sephacryl S-300, which was not used again.

As with dodecyl maltoside, the ascorbate-TMPD oxidase activity is excluded from both columns and no separation of proteins or cytochromes is observed, other than the removal of cytochrome c_4, as described previously, in section VIIA (data not shown). Once again, it seems that under these conditions, the ascorbate-TMPD oxidase activity forms part of a large detergent particle containing many components, whose molecular weight is such that it is excluded from the columns used.

The ammonium sulphate fractionated sample has many fewer proteins than that simply concentrated by ion exchange chromatography (data not shown) and the octyl glucoside-protein particle it comprises is probably smaller than that of the unfractionated sample. Nevertheless, it is still excluded by the Sephacryl S-300, indicating that its molecular weight is in excess of 1.5 million. The use of molecular exclusion columns equilibrated with octyl glucoside therefore suffers from the same problems as molecular exclusion chromatography of dodecyl maltoside extracts and so was not investigated further.

(ii) Columns equilibrated with Triton X-100 Samples were prepared for chromatography on columns equilibrated with Triton X-100 by ammonium

sulphate fractionation, as described in section V. After redissolving the 70% ammonium sulphate saturation precipitate in 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl, 0.5% Triton X-100 and 0.1% cholate, the material was dialysed against 1.5l of column equilibration buffer (20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.5% Triton X-100) for 12 hours prior to loading onto the columns used. This was necessary to remove residual traces of ammonium sulphate which otherwise interfered with the smooth running of the columns. Sephadex G-150-50 and Fractogel HW-55 (S) were used in these experiments, the latter being preferred to Sephacryl S-300 (SF) as it was better at resolving molecular weight marker proteins.

Fig.6.6 shows the results of chromatography of a 70% ammonium sulphate saturation precipitate on Sephadex G-150-50 (2.2x74cm column). Fig.6.6a shows the profile of elution of material absorbing at 410nm and of the ascorbate-TMPD oxidase activity. Fig.6.6b shows a protein-stained SDS-PAGE gel of selected fractions from the column, to illustrate the separation of proteins achieved.

Despite the elution of the ascorbate-TMPD oxidase activity close to the exclusion limit of the column, there is clearly some separation of the major protein bands of the applied material (Fig.6.6b). The beginnings of separation of the oxidase activity (Fig.6.6a, filled circles) from the majority of the material absorbing at 410nm (Fig.6.6a, open circles) is also noticeable, with the oxidase activity eluting slightly ahead of the A_{410} peak. Comparison of Fig.6.6a and 6.6b shows that the ascorbate-TMPD oxidase activity co-elutes with the major protein of M_r 19 000, and with associated minor proteins. This co-elution might imply a role for the M_r 19 000 protein in the ascorbate-TMPD oxidase activity. However, further experiments show that this association is fortuitous and that the M_r 19 000 protein has no role in ascorbate-TMPD oxidase activity. It is also clear, from a comparison of Fig.6.6a and 6.6b that the M_r 48 000 and high M_r protein bands are also not associated with the ascorbate-TMPD oxidase activity, as they elute just before the peak of the activity.

Fig.6.6 Chromatography on Sephadex G-150

(a) Profile of A_{410} and ascorbate-TMPD oxidase activity 3.5ml 70% ammonium sulphate precipitate, prepared from an octyl glucoside extract of *P. stutzeri* membranes, were chromatographed on Sephadex G-150-50 equilibrated with 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.5% Triton X-100. 3.5ml fractions were collected.

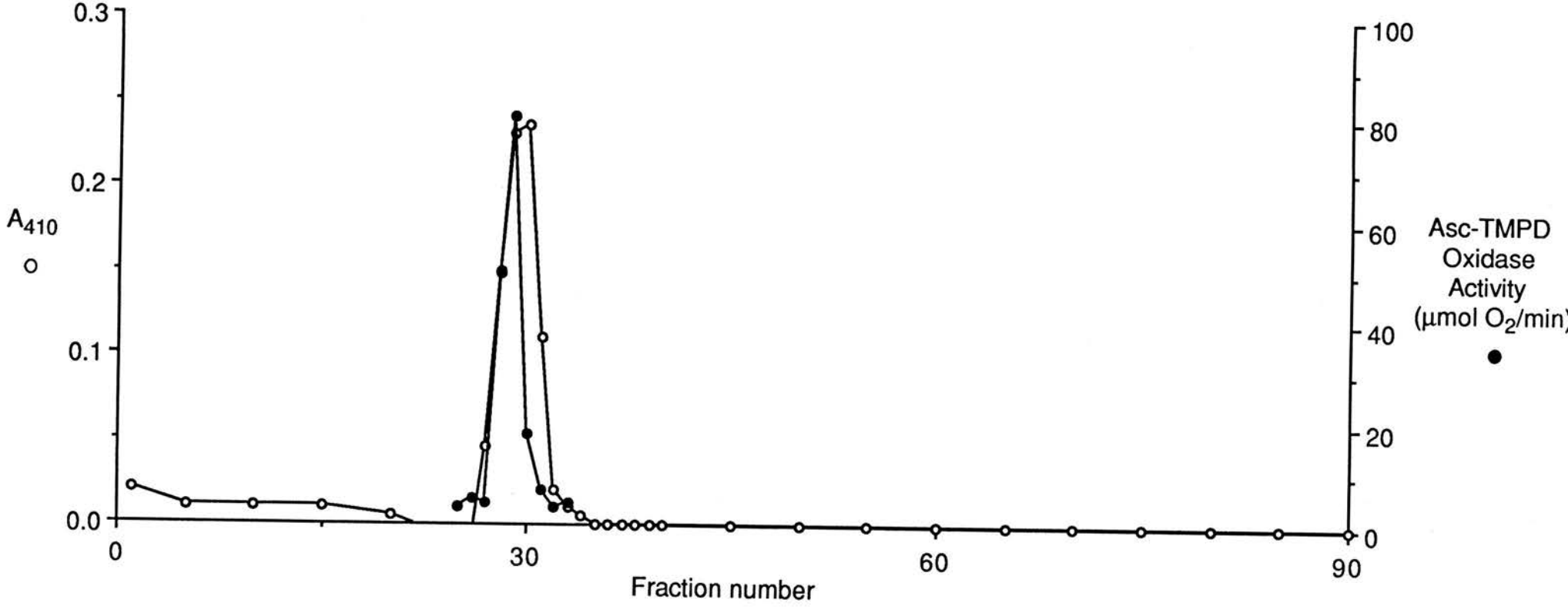
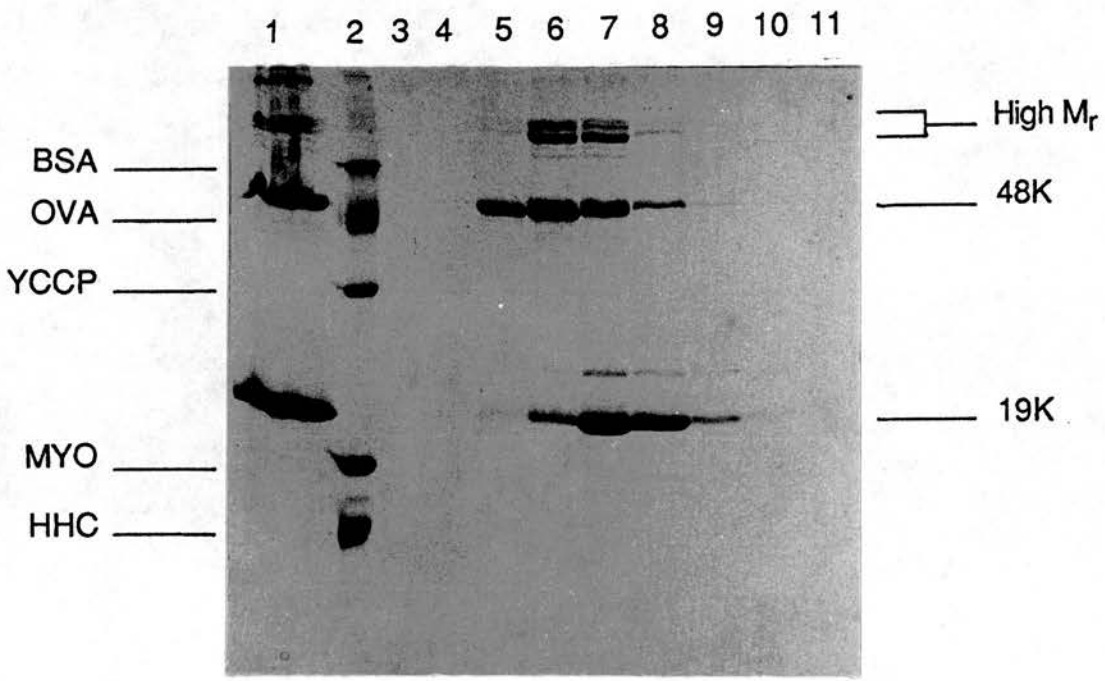

0.3
0.2
0.1
0.0
A_{410}
○
0
30
60
90
Fraction number
100
80
60
40
20
0
Asc-TMPD
Oxidase
Activity
(μmol O_2/min)
●

Fig.6.6 cont.

(b) SDS-PAGE of selected fractions Fractions were run on a 10-25% gradient gel and stained for protein.

Lane	Contents
1	70% ammonium sulphate precipitate
2	Standards
3	Fraction 25
4	" 26
5	" 27
6	" 28
7	" 29
8	" 30
9	" 31
10	" 32
11	" 33

(b)



A haem-stained SDS-PAGE gel of identical layout to the protein-stained gel presented in Fig.6.6b (not shown) shows that the membrane cytochromes c do not co-chromatograph with the ascorbate-TMPD oxidase activity, eluting slightly after the oxidase activity peak (Fig.6.6a, filled circles), at the peak of A_{410} (Fig.6.6a, open circles). Because of the difficulties in quantitating b-type cytochromes on a haem-stained gel (Chapter 3, section I), the profile of elution of cytochromes b could not be determined. Note that cytochrome c_4 remained associated with the other c-type cytochromes in this instance, rather than chromatographing at its native molecular weight.

Fig.6.7 shows an identical experiment to that in Fig.6.6 except that Fractogel HW-55 (S) was used in this case. As Fractogel has a higher exclusion limit than Sephadex G-150 (1 million as opposed to 150 000), a clearer separation of the ascorbate-TMPD oxidase activity from contaminating proteins was expected.

Fig.6.7a shows the profile of elution of ascorbate-TMPD oxidase activity (filled circles) and material absorbing at 410nm (open circles) from a 2.2x80cm column of Fractogel HW-55 (S) equilibrated with 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.5% Triton X-100. As with Sephadex G-150, the ascorbate-TMPD oxidase activity elutes before the main peak of A_{410}, which contains the membrane cytochromes c. In this case, cytochrome c_4 is removed from the membrane and chromatographs at approximately its native molecular weight.

The peak of ascorbate-TMPD oxidase activity is asymmetrical, with a shoulder of activity in the later fractions. This may be due to heterogeneity in the size of the ascorbate-TMPD oxidase activity-containing particle, giving rise to two elution volumes. Alternatively, inactivation of a portion of the ascorbate-TMPD oxidase activity eluting later in the profile may be occurring, the residual activity appearing as the shoulder on the peak of ascorbate-TMPD oxidase activity. This latter possibility is discussed later.

The Fractogel HW-55 column was calibrated, so that the approximate molecular weight of the ascorbate-TMPD oxidase peak could be obtained.

Fig.6.7 Chromatography on Fractogel HW-55

(a) Profile of A_{410} and ascorbate-TMPD oxidase activity 6.5ml dialysed 70% ammonium sulphate precipitate, prepared from an octyl glucoside extract of *P. stutzeri* membranes, were chromatographed on Fractogel HW-55 (S) equilibrated with 20mM tris/HCl, pH8.0 at 4C containing 100mM NaCl and 0.5% Triton X-100. 2.75ml fractions were collected.

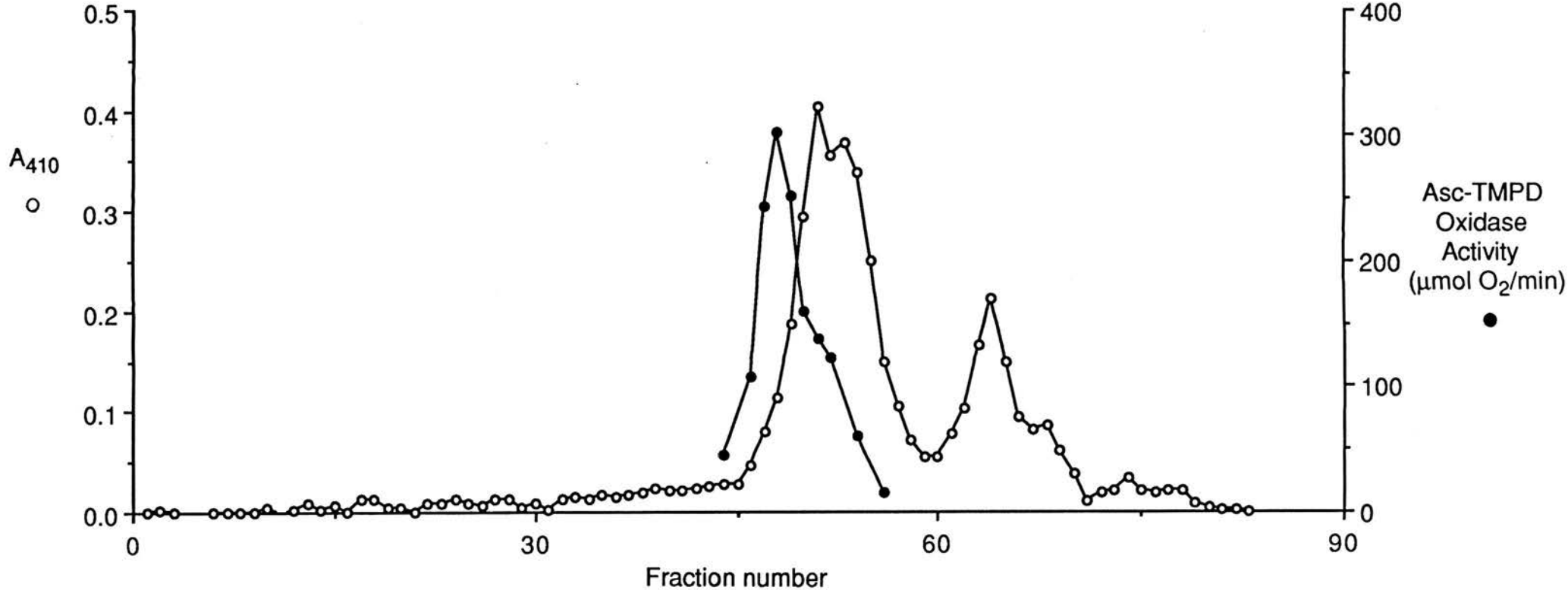

0.5
0.4
0.3
0.2
0.1
0.0
A410
0
400
300
200
100
0
Asc-TMPD
Oxidase
Activity
(µmol O2/min)
0
30
60
90
Fraction number

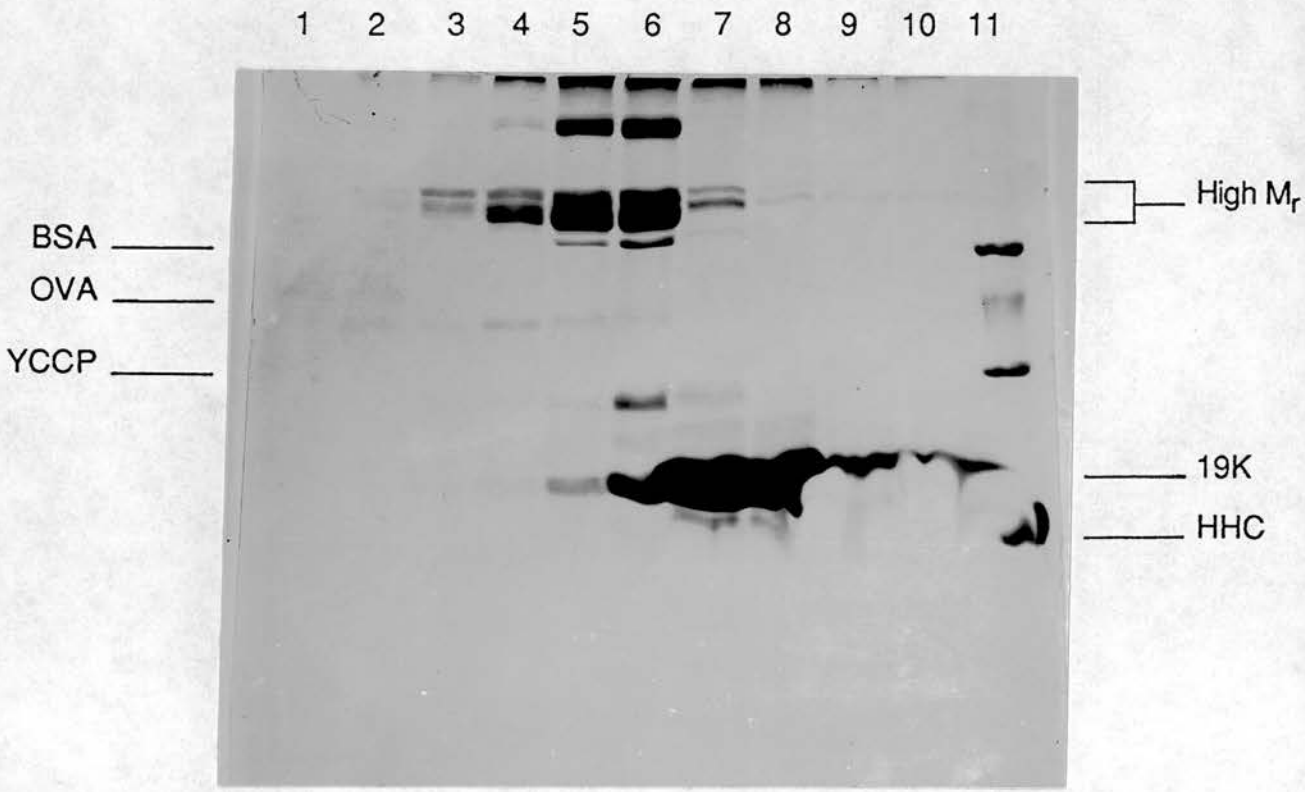
Fig.6.7 cont.

b) SDS-PAGE of selected fractions Fractions were run on a 10-25% gradient gel and stained for protein.

Lane	Contents
1	Fraction 44
2	" 46
3	" 47
4	" 48
5	" 49
6	" 50
7	" 51
8	" 52
9	" 53
10	" 54
11	Standards

Samples were concentrated fourfold by freeze-drying prior to loading, as described in Chapter 2.

(b)



The calibration used thyroglobulin (M_r 669 000), apoferritin (M_r 443 000), catalase (M_r 100 000), bovine serum albumin (M_r 66 000) and horse heart cytochrome c (M_r 12 000), all obtained from Sigma. The ascorbate-TMPD oxidase activity eluted at a volume corresponding to a molecular weight of approximately 220 000, with the peak of A_{410} eluting at molecular weights between 130 000 and 90 000. Note, however, that this calibration underestimates the molecular weight of the separated cytochrome c_4 by approximately 6000.

Fig.6.7b shows a protein-stained SDS-PAGE gel of selected fractions from the Fractogel column. Again, separation of the major protein bands of the applied material occurs, with the high M_r bands eluting from the column prior to the M_r 19 000 protein and associated minor bands. This is a similar pattern to that seen with Sephadex G-150. However, in contrast to the profile of elution obtained with this latter column, the ascorbate-TMPD oxidase activity elutes before, rather than after, the high M_r bands. The activity is not associated with the M_r 19 000 protein, or with any of the other major protein bands in the applied material. It is therefore likely that the ascorbate-TMPD oxidase is associated with a minor protein band. A possible candidate is visible on the gel of Fig.6.7b, with a molecular weight slightly less than that of ovalbumin (M_r 43 000). This band appears to co-chromatograph with the ascorbate-TMPD oxidase activity, but as it is not visible on other gels, its association with the activity must be extremely tentative.

A haem-stained SDS-PAGE gel with a layout identical to the protein-stained gel of Fig.6.7b was also run (not shown) and revealed that the membrane cytochromes c, with the exception of cytochrome c_4, co-chromatographed with the main A_{410} peak and not with the ascorbate-TMPD oxidase activity. again, it was not possible to determine the profile of elution of the membrane cytochromes due to distortion of the free haem bands. This distortion was very marked in lanes 7 to 10 of the gel, corresponding to fractions 51 to 54 from the column.

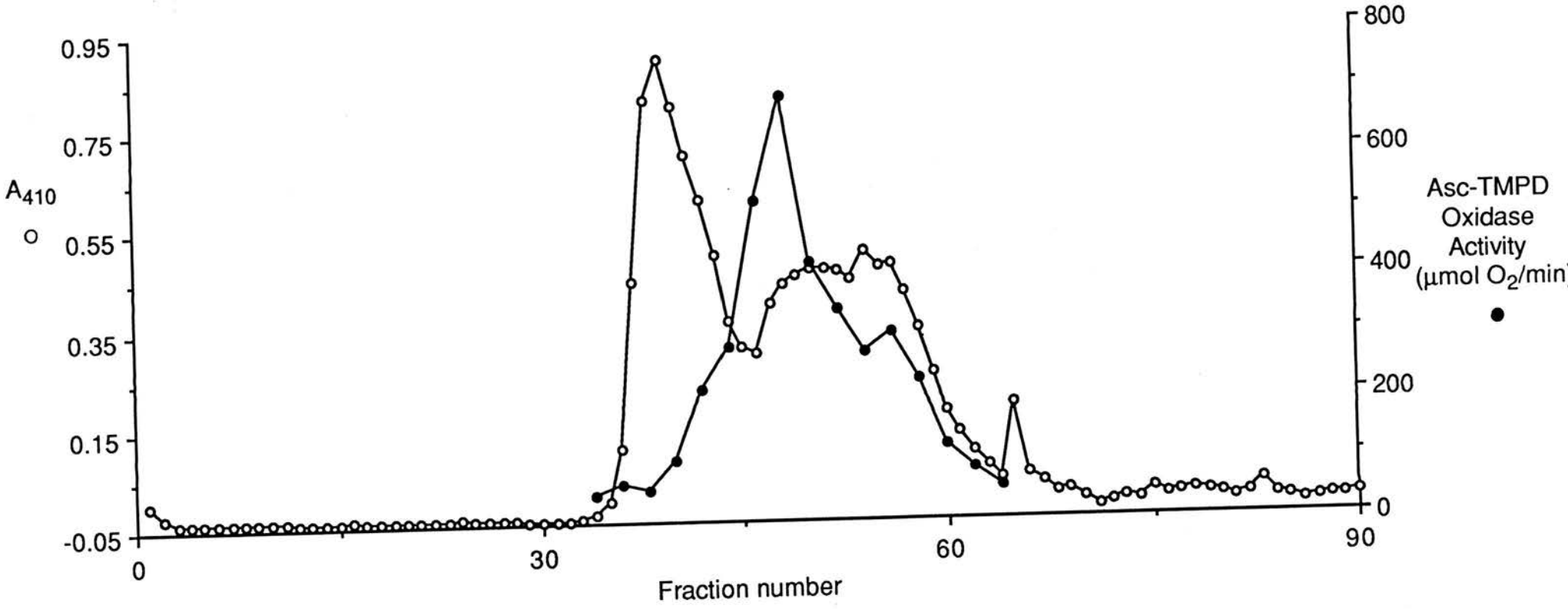
Fig.6.7b also shows distortion of the electrophoretic patterns from samples of these fractions, the peak fractions of the A410 profile. This suggests that the distortion observed is not simply due to the poor running of free haem on SDS-PAGE. The fractions giving rise to distortion were noted to be slightly viscous following the addition of SDS, prior to loading the gel. This may account for the poor running and distortion observed. The cause of the viscosity in these fractions is not known. Possibly these fractions contain a high concentration of bound detergent which interferes with the dissolving in SDS. This may also account for the shoulder in the ascorbate-TMPD oxidase activity profile (Fig.6.7a), as these viscous fractions may contain partially inactivated ascorbate-TMPD oxidase.

Fig.6.8 shows the elution profile obtained when membranes treated with sodium iodide, to remove cytochrome c_4, are used. The membrane sample was prepared for chromatography exactly as described previously (section VIIB(ii)) except that the 20ml *P. stutzeri* French press membranes were treated with 2M sodium iodide prior to extraction with octyl glucoside. The membranes were centrifuged from their storage medium (100 000g, 1 hour, 4°C) and were resuspended in 20ml 2M sodium iodide in 10mM sodium phosphate, pH7.0. After incubation for 30 minutes at 0°C, with vigorous mixing, a further 20 ml of 10mM sodium phosphate, pH7.0 were added, to allow the membranes to pellet and the mixture was centrifuged (100 000g, 1 hour, 4°C). The treated membranes were resuspended in 20ml 10mM sodium phosphate, pH7.0 and were dialysed against 2 changes of 1.5l 10mM sodium phosphate, pH7.0 for 2 hours per change, to remove residual iodide. The protein content of the treated membranes was then assayed, to allow extraction at 1.5mg octyl glucoside per mg membrane protein to be performed.

The profile obtained after chromatography on Fractogel HW-55 (Fig.6.8) is very similar to that shown in in Fig.6.7a, with two exceptions. Firstly, as expected, the amount of cytochrome c_4 present is greatly reduced, with only a minor peak of cytochrome c_4 at fraction 65 remaining. The presence of this minor peak of cytochrome c_4 suggests that the removal of cytochrome c_4 from membranes by 2M sodium iodide treatment is not complete in this case.

Fig.6.8 Chromatography on Fractogel HW-55: Iodide washed membranes

Profile of A_{410} and ascorbate-TMPD oxidase activity obtained on chromatography on Fractogel HW-55 of 8ml of 70% ammonium sulphate precipitate prepared from 2.0M sodium iodide washed membranes as described in the text. Chromatographic conditions were as described in the legend to Fig.6.7.


0.95
0.75
0.55
0.35
0.15
-0.05
A_{410}
o
0
30
60
90
Fraction number
800
600
400
200
0
Asc-TMPD
Oxidase
Activity
(µmol O_2/min)
●

Secondly, a large peak of material apparently absorbing at 410nm is present, eluting before the peak of ascorbate-TMPD oxidase activity (filled circles). This peak is separate from the previously seen peak of A_{410}, which contains the membrane cytochromes c. The peak is composed of turbid material with no detectable difference spectrum and which gives no protein- or haem-staining after SDS-PAGE. The absorbance at 410nm observed is therefore probably spurious, due to detergent or protein which has come out of solution and which then gives rise to the observed turbidity. The reason for the presence of this material is not known, but a similar phenomenon is seen in Fig.6.9, where fraction 49 has an abnormally high A_{410} due to its turbidity.

A function proposed for cytochrome c_4 is that of a direct link between the reductase and terminal oxidase (Chapter 7). In this model, cytochrome c_4 is bound to the reductase and the oxidase simultaneously. Removal of cytochrome c_4 prior to detergent extraction, ammonium sulphate fractionation and molecular exclusion chromatography was therefore employed to investigate whether the absence of cytochrome c4 would allow the further dissociation and separation of the oxidase from other respiratory chain components. If the model is correct and cytochrome c_4 does bind to both oxidase and reductase, it may hold these two components together during the purification, preventing their resolution. However, as the removal of cytochrome c_4 does not significantly affect the pattern of separation of the membrane components on ammonium sulphate fractionation or chromatography on Fractogel HW-55, this does not seem to be occurring.

VIII: Partial purification of the ascorbate-TMPD oxidase activity

Following the preliminary investigations into purification methods for the ascorbate-TMPD oxidase activity of *P. stutzeri* , discussed in the preceding sections, a procedure for the partial purification of the ascorbate-TMPD oxidase activity and hence the cytochrome o, was adopted. Time allowed only one preparation to be performed. 50ml *P. stutzeri* French press membranes were used as a starting material, so that sufficient material was available in

the later stages of the purification to allow the ascorbate-TMPD oxidase activity to be monitored. Briefly, the procedure involved the extraction of membranes with octyl glucoside, fractionation of the octyl glucoside extract with ammonium sulphate, chromatography on Fractogel HW-55 and ion exchange chromatography on Whatman DE52. The oxidase preparation produced was then characterised by means of spectra, SDS-PAGE and the sensitivity of the ascorbate-TMPD oxidase activity to cyanide.

A. Purification procedure 50ml aerobic *P. stutzeri* French press membranes (15mg protein/ml) were extracted with octyl glucoside at 1.5mg octyl glucoside per mg membrane protein. Octyl glucoside was added to the membranes, suspended in 10mM sodium phosphate, pH7.0, as a 5% (w/v) aqueous solution. 10mM sodium phosphate, pH7.0 was added to give a final volume 75ml and the mixture was incubated for 30 minutes at 4°C, with vigorous mixing. After incubation, the mixture was centrifuged (100 000g, 1 hour, 4°C) and the supernatant was removed and diluted with an equal volume of 10mM sodium phosphate, pH7.0. The diluted extract was incubated for 1 hour at 25°C and was then fractionated with ammonium sulphate.

Solid ammonium sulphate (Fisons Plc, 'AR' grade) was slowly added to the diluted octyl glucoside extract, stirred on ice, to give 20 to 70% saturation, in 10% steps. After each addition of ammonium sulphate, the precipitate formed after 15 minutes incubation was collected by centrifugation (11 000g, 30 min, 4°C) and the supernatant was brought to the next percentage saturation with ammonium sulphate. After the extract was brought to 70% saturation, the precipitate obtained was collected as a floating red oil by centrifugation (100 000g, 1 hour, 4°C). The slightly turbid supernatant was carefully removed and recentrifuged (100 000g, 1 hour, 4°C) to recover residual precipitate and thus maximise the recovery of activity. The combined 70% precipitates from the two centrifugations were redissolved in 3ml 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl, 0.5% Triton X-100 and 0.1% cholate. The redissolved material was then dialysed against 1.5l 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.5% Triton X-100.

15ml of the dialysed 70% precipitates were then loaded onto a 2.2x80cm

column of Fractogel HW-55 (S) equilibrated with 20mM tris/HCl, pH8.0 at 4°C containing 100mM NaCl and 0.5% Triton X-100. 3.0ml fractions were collected and the A_{410} and ascorbate-TMPD oxidase activity of each was measured.

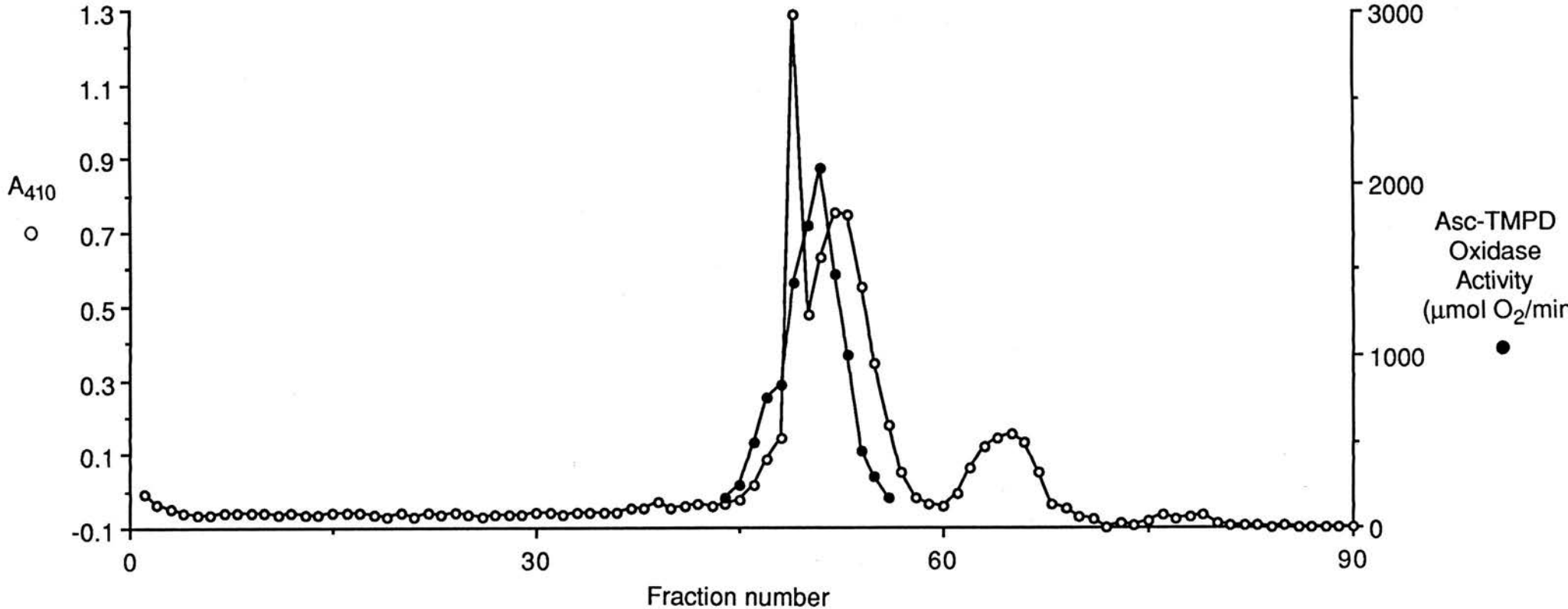
Fig.6.9 shows the elution profile obtained from this column. Note that the separation of the ascorbate-TMPD oxidase activity (filled circles) from the main peak of A_{410} (open circles) is poorer than seen previously. This is due to the amount of material loaded onto the column, which resulted in the poor resolution of the ascorbate-TMPD oxidase activity from the contaminating material of the A_{410} peak. The lack of resolution was also apparent when a protein-stained SDS-PAGE gel of selected fractions from the column was run (not shown). This clearly showed the poor resolution of the major protein bands of the applied sample. The unusually high absorbance of fraction 49 is due to the presence of turbid material in this fraction (see section VIIB(ii)). The ascorbate-TMPD oxidase activity eluted at a volume corresponding to a molecular weight of 210 000, similar to that obtained in previous experiments (section VIIB(ii)). Cytochrome c_4 was removed from the other membrane cytochromes c in the main peak of A_{410} and chromatographed at approximately its native molecular weight (peak at fraction 65).

Because of the poor resolution displayed by the Fractogel column in this instance, it was decided to pool all fractions containing ascorbate-TMPD oxidase activity, rather than discarding those heavily contaminated by material from the peak of A_{410}. fractions 46 to 54 from the Fractogel column were pooled and the pooled fractions were dialysed against 1.5l of 10mM tris/HCl, pH8.0 at 4°C containing 0.5% Triton X-100 before chromatography on DEAE-cellulose.

The pooled, dialysed Fractogel fractions were then loaded onto a 1x6cm column of DE52 (Whatman) equilibrated with 10mM tris/HCl, pH8.0 at 4°C containing 0.5% Triton X-100. The column was fully saturated with bound material and a small quantity of that applied was unable to bind for this reason and passed through the column. The column was then eluted using a 0 to

Fig.6.9 Chromatography on Fractogel HW-55: Increased loading

Chromatography on Fractogel HW-55 was performed as described in the legend to Fig.6.7, except that a greater amount of material was employed in order to allow further purification steps to be undertaken (see text). 16ml dialysed 70% ammonium sulphate precipitate, prepared from an octyl glucoside extract of 50ml *P. stutzeri* membranes, were chromatographed. 3.0ml fractions were collected.


1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
A410
3000
2000
1000
0
Asc-TMPD
Oxidase
Activity
(µmol O2/min
0
30
60
90
Fraction number

250mM linear NaCl gradient in the above equilibration buffer.

Fig.6.10a shows the profile of elution of A_{410} and ascorbate-TMPD oxidase activity of the pooled Fractogel fractions on DE52. The unbound portion of the applied material elutes at fractions 18 and 19. The gradient of NaCl elutes two peaks of A_{410}, both of which have associated ascorbate-TMPD oxidase activity, although only the material in the second peak has a specific activity greater than 1.4 μmol O_2/min/mg protein. The first peak is low and broad, eluting early in the gradient, between approximately 40 and 95mM NaCl. The later peak is also broad, due to tailing of the material in it, and elutes between approximately 125 and 180mM.

Fig.6.10b shows a haem-stained gel of samples from the elution profile. Both peaks of A_{410} eluted by the NaCl gradient contain the four major membrane cytochromes c, although the later peak is enriched with band I_{Ps}, cytochrome c_4 and cytochrome c_5. Note that these latter two cytochromes have not been completely removed from the detergent particle by chromatography on Fractogel HW-55. Lane 10 of this gel contains a sample of the pooled Fractogel fractions. Unfortunately, due to overloading of this sample, and due to it containing viscous fractions (discussed in section VIIB(ii)), severe distortion of the staining pattern in this lane was observed. Traces of a similar distortion can also be seen in lane 1, which contains material which was unable to bind to the column and passed through it.

Fig.6.10c shows a protein-stained SDS-PAGE gel of identical layout to that in Fig.6.10b. The gel shows that the first peak eluted from the column by the NaCl gradient contains mainly M_r 19 000 protein, with little high M_r material. In contrast, the later peak, which contains the majority of the ascorbate-TMPD oxidase activity , also contains mainly high M_r material, with little M_r 19 000 protein. This separation of the M_r 19 000 protein from the majority of the ascorbate-TMPD oxidase activity confirms that the M_r 19 000 protein is not involved in the ascorbate-TMPD oxidase. As the high M_r material has also been shown not to be involved in the ascorbate-TMPD oxidase activity, since it does not co-chromatograph with the activity on Fractogel

Fig.6.10 Chromatography on DEAE-cellulose

(a) Profile of A_{410} and ascorbate-TMPD oxidase activity Pooled fractions 46 to 54 from the Fractogel column of Fig.6.9 were chromatographed on DE52. The pool was loaded at (a) and the column was eluted with a 0 to 250mM gradient of NaCl in equilibration buffer (10mM tris/HCl, pH8.0 at 4°C containing 0.5% Triton X-100) at (b). 1.3ml fractions were collected.

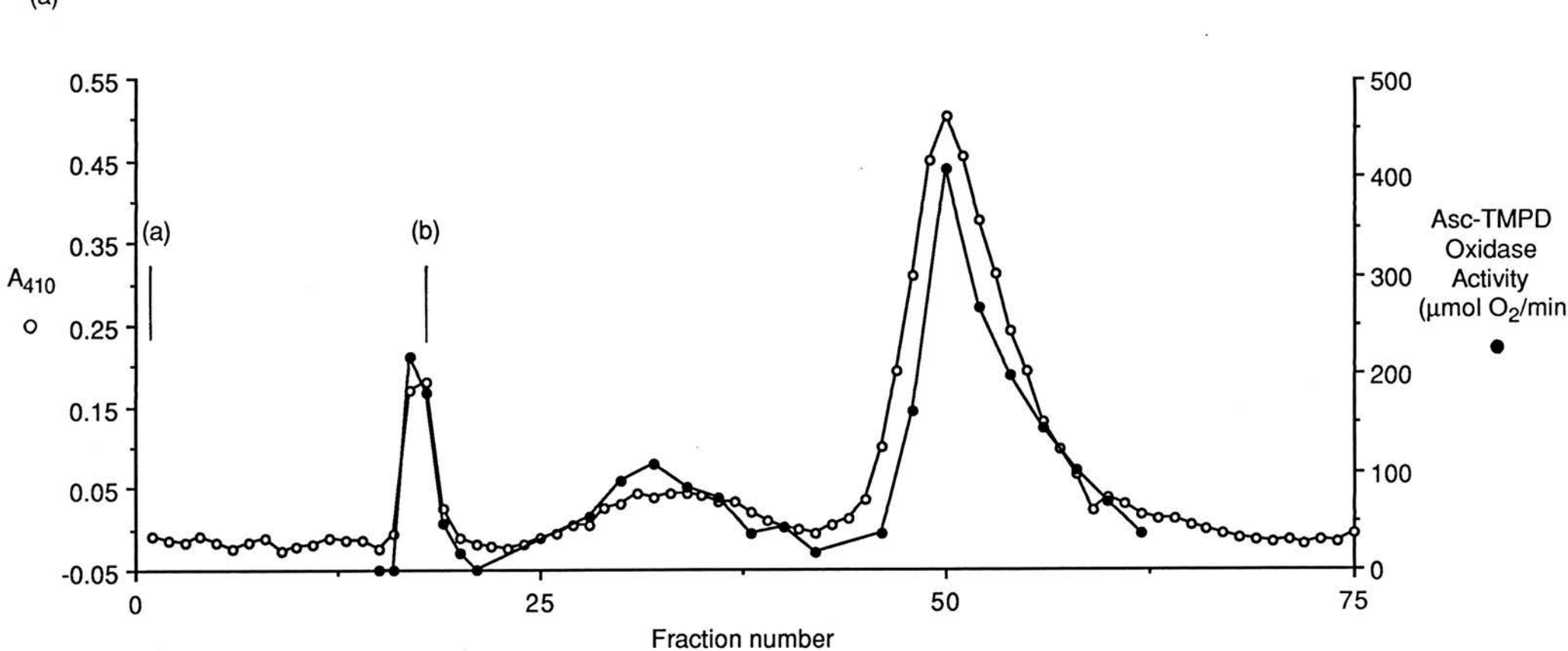

(a)
0.55
0.45
0.35
0.25
0.15
0.05
-0.05
(a)
(b)
A_{410}
o
500
400
300
200
100
0
Asc-TMPD
Oxidase
Activity
(µmol O_2/min
●
0
25
50
75
Fraction number

Fig.6.10 cont.

(b) SDS-PAGE of selected fractions Fractions were run on a 10-25% gradient gel and stained for protein.

Lane	Contents
1	Fraction 18
2	" 33
3	" 35
4	" 48
5	" 50
6	" 52
7	" 54
8	" 56
9	" 58
10	Pooled Fractogel fractions
11	Standards

Samples were concentrated fourfold by freeze-drying prior to loading, as described in Chapter 2.

(b)

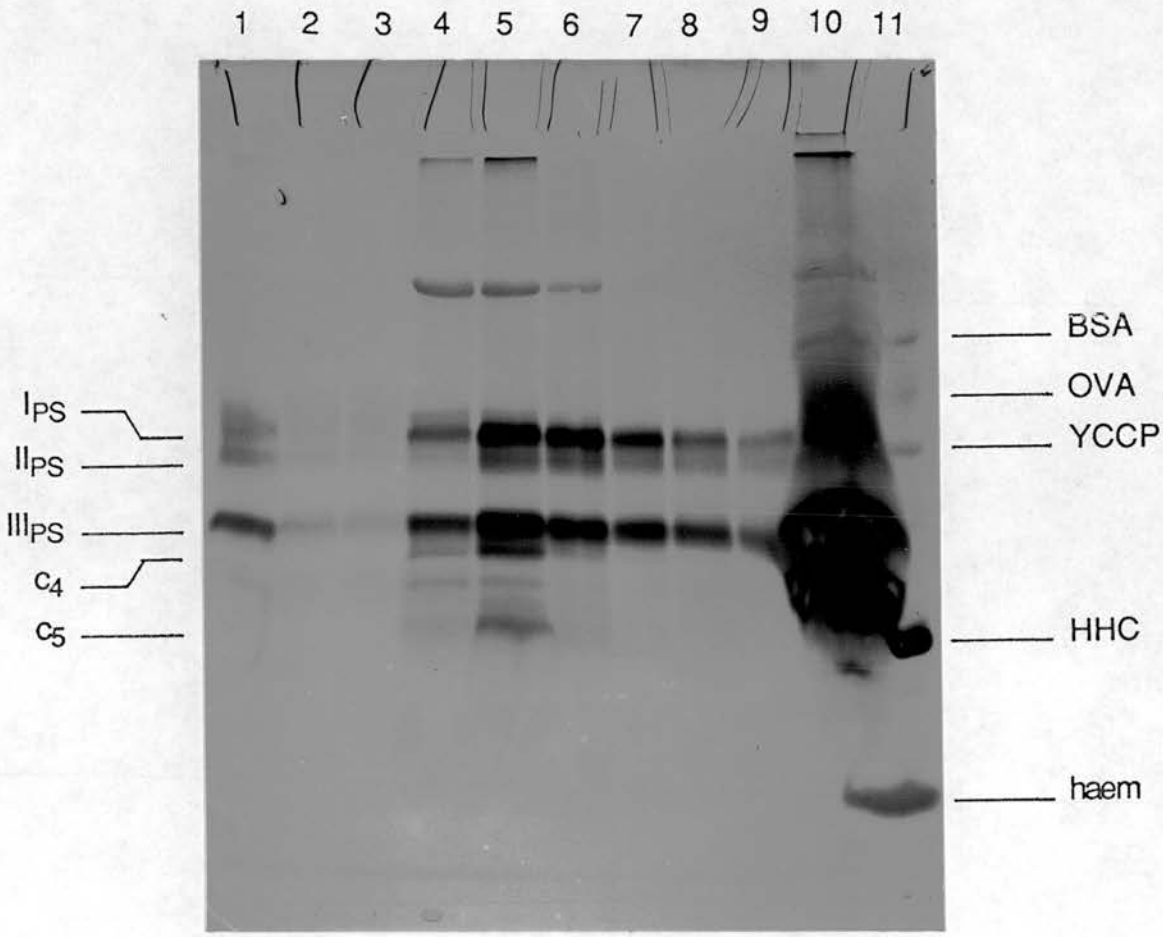
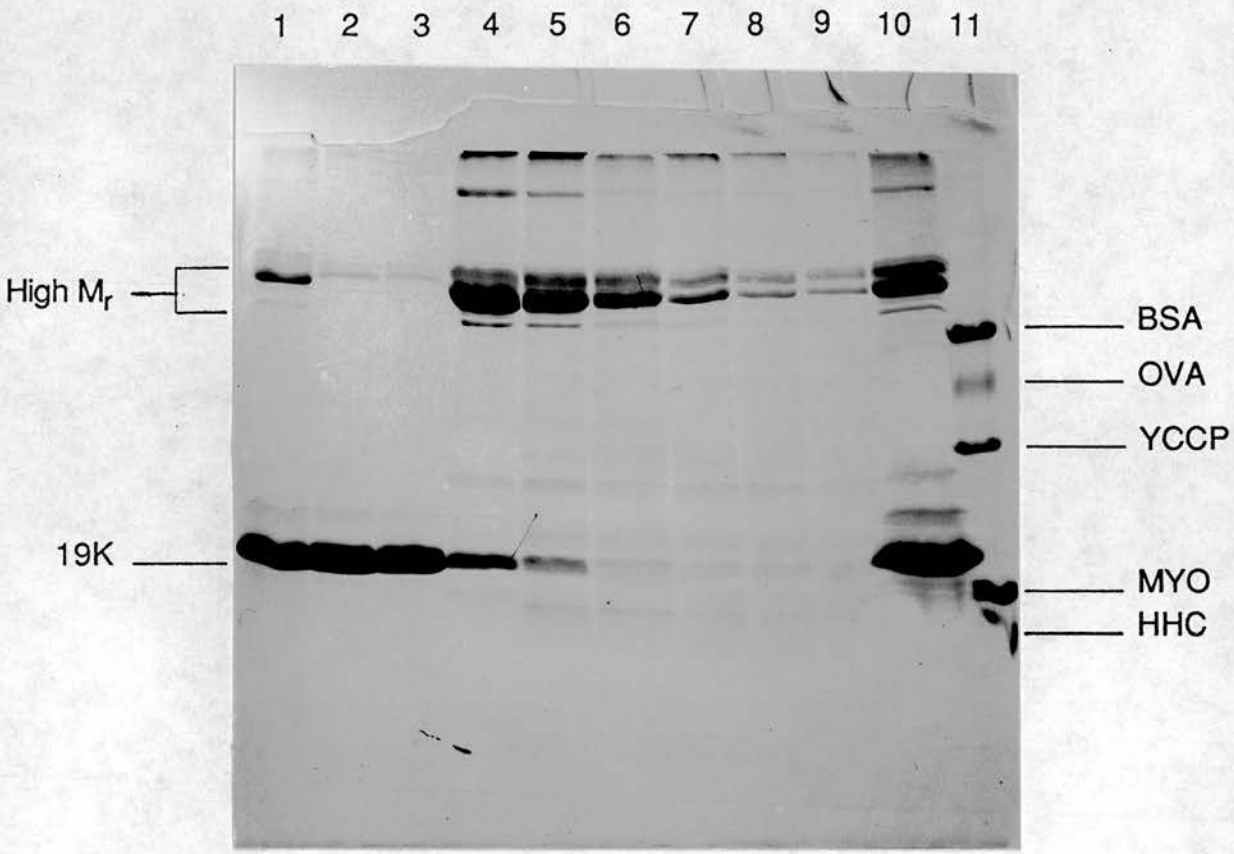


Fig.6.10 cont.

(c) Protein-stained SDS-PAGE gel Layout as in (b)

(c)



(section VII), the ascorbate-TMPD oxidase activity must be associated with one of the minor membrane proteins in the second peak and is therefore not readily identified.

Little trace is detectable of the minor band of M_r approximately 40 000 previously suggested as a possible candidate for involvement in the ascorbate-TMPD oxidase. The obvious explanation is that this band is removed by chromatography on DE52. However, a significant amount of the material loaded onto the gel in lanes 4 to 8 has failed to migrate into the gel, which may offer an alternative explanation for its absence. This phenomenon is also noticeable in several of the other gels presented in this chapter and is probably the result of aggregation of denatured membrane proteins on heating in the presence of SDS prior to loading. This heating was necessary to prevent proteolysis of the samples by endogenous proteases, by ensuring that these were rapidly denatured. This aggregation problem means that SDS-PAGE does not always separate the protein components of a sample and makes identification of the components of the ascorbate-TMPD oxidase difficult. A similar problem was found in the purification of the cytochrome oxidase from *Pa. denitrificans* (Ludwig and Schatz, 1980; Schatz, personal communication).

Fractions 50 to 60, with specific activities greater than 1.4 μmol O_2/min/mg protein, were pooled as the final product of this purification. The small peak of activity which eluted before this peak was not included in the pool since it contained high levels of the contaminating M_r 19 000 protein and had a specific activity less than 1.4 μmol O_2/min/mg protein. Further purification of the ascorbate-TMPD oxidase was not possible in this case due to lack of time. The activity was lost on storage, whether at 4°C or frozen, which also prevented an exhaustive characterisation of the partially purified ascorbate-TMPD oxidase from being carried out.

B. Summary of the purification The results of the purification are summarised by Fig.6.11 and Table 6.1. Fig.6.11 shows haem-stained (a) and protein-stained (b) gels of samples from each stage of the purification. In order to facilitate comparison of the samples, the volume of each sample loaded

Fig.6.11 Summary of the purification of the ascorbate-TMPD oxidase of *P. stutzeri*

10-25% gradient SDS-PAGE gels of samples from the stages of purification of the ascorbate-TMPD oxidase of *P. stutzeri*. The volume of each sample was adjusted so that 60μg of protein was loaded in each case.

(a) Haem-stained gel

Lane	Contents
1	French press membranes
2	Octyl glucoside extract
3	70% ammonium sulphate precipitate
4	Fractogel HW-55 pool
5	DE52 pool (final product)
6	50pmol cytochrome c_4
7	Standards

(a)

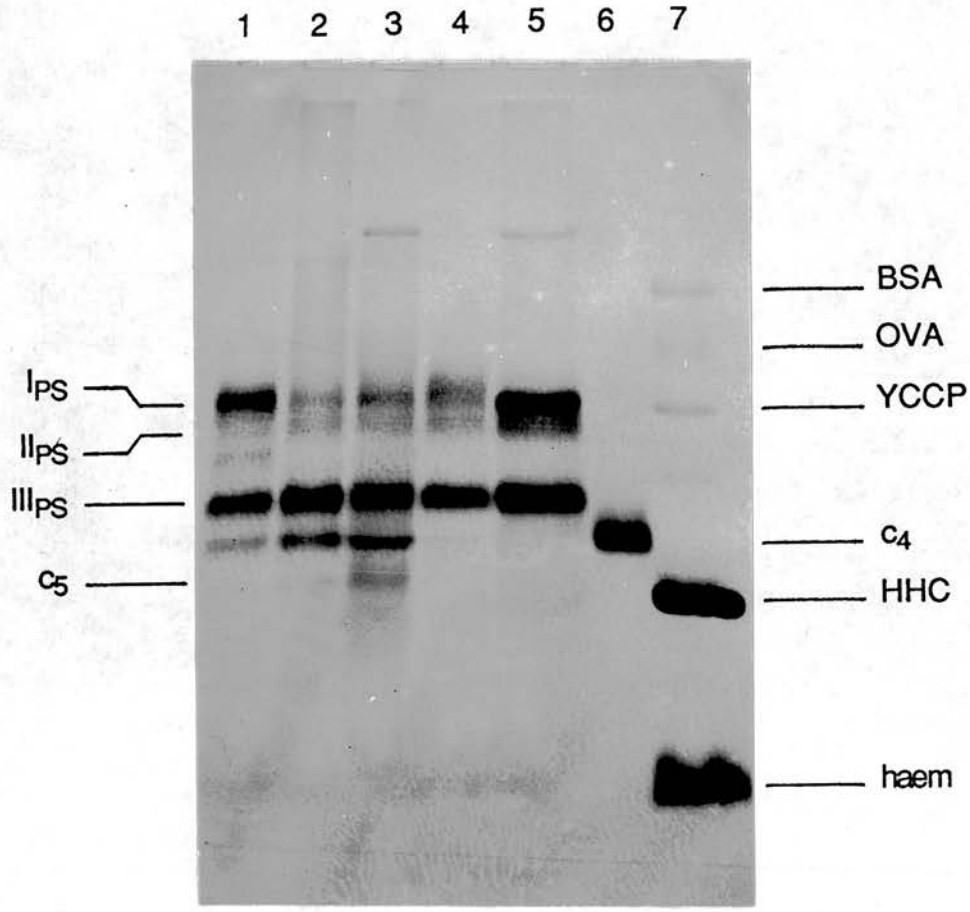
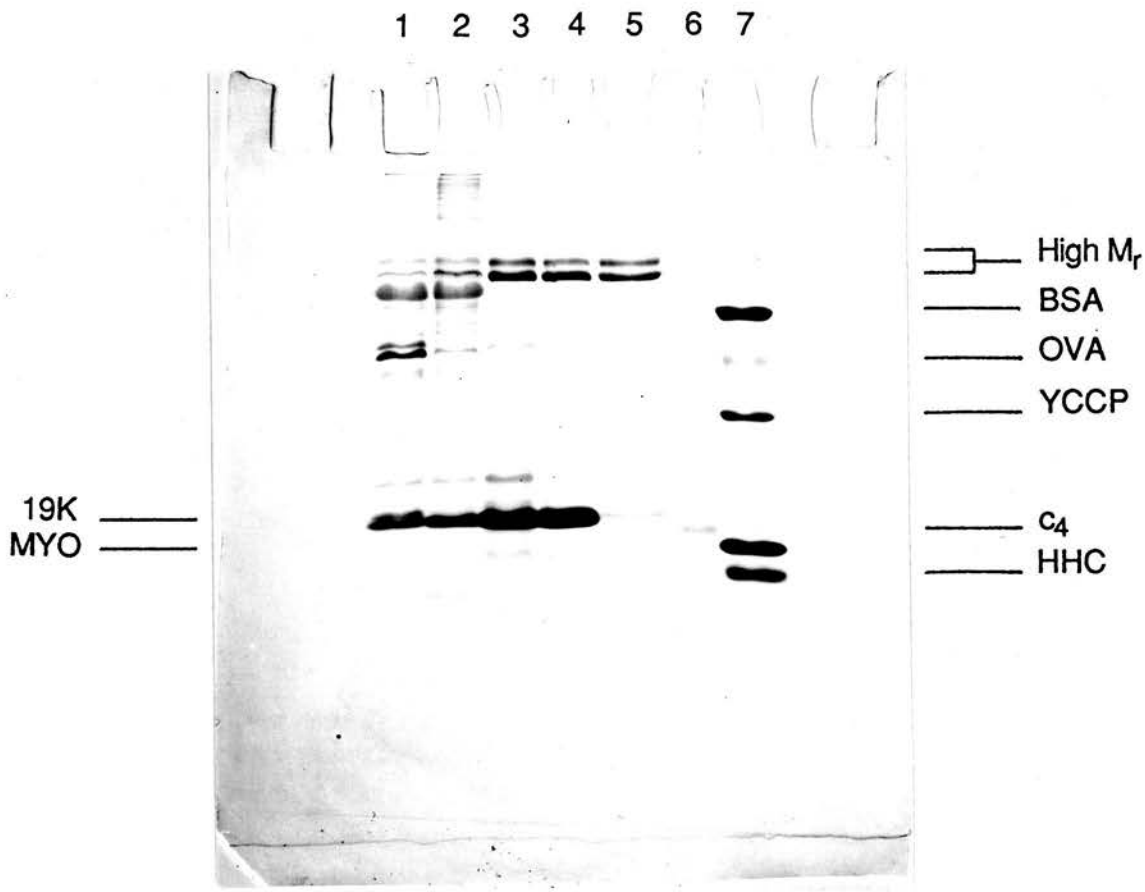


Fig.6.11 cont.

(b) Protein-stained gel Layout as in (a) except that lane 6 contains 0.5nmol cytochrome c_4

(b)



onto the gels was adjusted such that the amount of protein loaded in each case was 60µg.

The haem-stained gel, Fig.6.11a, shows that the majority of cytochromes c_4 and c_5 are removed from the preparation by chromatography on Fractogel HW-55. The remaining major membrane cytochromes c, I_{Ps}, II_{Ps} and III_{Ps} are still present in the final product, despite the removal of the majority of the membrane cytochromes c by the ammonium sulphate fractionation. Samples of the material at each stage of the purification, with equal protein contents, give approximately the same intensity of haem-staining. This indicates that the level of cytochromes c, as a proportion of protein content, remains relatively constant throughout the purification.

The protein-stained gel (Fig.6.11b) clearly shows the removal of many contaminating proteins from the ascorbate-TMPD oxidase activity. The ammonium sulphate fractionation step is particularly effective, leaving only the M_r 19 000 protein and the high M_r bands in addition to minor bands. Chromatography on Fractogel HW-55 was unsuccessful in removing these major bands as the column was overloaded, resulting in poor resolution of the ascorbate-TMPD oxidase activity from contaminating proteins (compare Fig.6.7a and 6.9). This chromatographic step should have separated the high M_r and the M_r 19 000 bands from the ascorbate-TMPD oxidase activity (section VIIB(ii)). However, the M_r 19 000 protein was removed by chromatography on DE52, with the high M_r bands remaining as contaminants. The identity of the proteins comprising the ascorbate-TMPD oxidase is not clear.

Table 6.1 summarises the purification. A 12.84-fold purification was achieved, with a recovery of 11% of the initial ascorbate-TMPD oxidase activity. This compares fairly well with values obtained for other purifications of cytochrome o. For example: Jurtshuk et al. (1981) (*A. vinelandii*) 13-fold purification, 18% recovery; Matsushita et al. (1982) (*P. aeruginosa*) 15.6-fold purification, 10.7% recovery; Yang and Jurtshuk (1978a) (*A. vinelandii*) 12-fold purification, 22% recovery; Yang (1982) (*P. aeruginosa*) 12.8-fold

Table 6.1

Sample	Volume (ml)	Total Activity (μmol O_2/min)	Recovery (%)	[Protein] (mg/ml)	Specific Activity (μmol O_2/min/mg protein)	Fold Purification
French press membranes	50.0	31250	100	17.0	36.8	-
Octyl Glucoside extract	73.0	19872	64	4.6	59.2	1.61
Diluted extract	138.0	32583	104	3.0	78.6	2.14
70% Amm. Sulphate pellet	9.0	4575	15	3.4	149.4	4.06
Fractogel pool	27.0	10275	33	1.7	223.9	6.08
DE52 pool	14.3	3378	11	0.5	472.2	12.84

purification, 43% recovery. The specific activity of the final product was 472.2 μmol O_2/min/mg protein, but this activity fell markedly on storage of the material, as discussed previously, limiting the characterisation of the ascorbate-TMPD oxidase.

French press membranes of nominal protein concentration 15 mg/ml were used in the purification. However, Lowry protein assay of the membranes gave a protein concentration of 17.0 mg/ml. This discrepancy is due to errors in the dilution of the membranes to 15 mg protein/ml during their preparation and to errors in the Lowry protein assay. Octyl glucoside extraction of these membranes removes approximately 40% of the membrane protein, with 64% of the original activity being recovered. In this case, little inhibition of the ascorbate-TMPD oxidase activity by the high concentration of octyl glucoside used in the extraction was observed. Nevertheless, the extract was diluted, enabling 100% of the activity to be recovered.

Fractionation of the diluted extract with ammonium sulphate removed approximately 93% of the protein content of the extract and resulted in a significant purification of the ascorbate-TMPD oxidase as judged by protein-stained SDS-PAGE gel (Fig.6.11b). However, only 15% of the starting activity was recovered. This is poor, since previous fractionations had yielded up to 40% recovery of activity (for example, Fig.6.4a).

Chromatography of the fractionated extract on Fractogel HW-55 was more successful. A restoration of some of the activity lost on ammonium sulphate fractionation was observed, probably due to the removal of residual ammonium sulphate by the pre-chromatography dialysis. A worrying increase in the protein content of the material was noted at this point (30.6 mg protein applied to the column, 45.9 mg recovered). The reason for this increase is not known, but the increase was reproducible.

Finally, chromatography on DE52 removed approximately 80% of the protein in the applied material, with 11% of the starting ascorbate-TMPD oxidase activity being recovered. A part of the loss of activity observed at this stage of the purification is due to the discarding of the small initial peak of ascorbate-TMPD oxidase activity eluted from the DE52 column, to avoid

pooling material containing the contaminating M_r 19 000 protein as a portion of the final product.

C. Characterisation of the final product The loss of ascorbate-TMPD oxidase activity on storage of the final product prevented its rigorous characterisation. However, two aspects of the final product were investigated, these being its spectral properties and the sensitivity of the ascorbate-TMPD oxidase activity to cyanide, although the latter was affected by the loss of activity on storage.

Fig.6.12 shows the reduced minus oxidised spectrum (a) and the carbon monoxide-binding spectrum (b) of the final product. These spectra are noisy as the dilute nature of the final product required a sensitive absorbance scale to be used. Nevertheless, this does not prevent the key spectral features from being seen. Both spectra show the presence of cytochromes c (551nm) as well as the expected cytochromes b (560nm). No evidence of the presence of cytochrome d is visible. This is not unexpected, as the membranes used in the purification contained little, if any, cytochrome d. The reduced minus oxidised spectrum indicates that the b-type cytochromes of the final product make a smaller contribution to the spectrum, relative to that of the cytochromes c.* However, the carbon monoxide-binding spectrum shows that there is approximately equal binding of carbon monoxide to the b-type cytochromes and the c-type cytochromes. This may indicate enrichment of the final product in carbon monoxide-binding cytochromes b, which is expected since the ascorbate-TMPD oxidase activity is believed to be associated with carbon monoxide-binding cytochrome o.

Fig.6.13 shows the inhibition of the ascorbate-TMPD oxidase activity of the final product by cyanide (open circles). In addition, the inhibition of the ascorbate-TMPD oxidase activity of the starting French press membranes is also shown (closed circles). Unfortunately, the activity of the final product was lost before cyanide concentrations above 1.5 μM could be investigated. Note, however, that this loss of activity is not the cause of the reduction in rates of ascorbate-TMPD oxidation observed in the presence of cyanide.

The French press membranes show a pattern of inhibition of

Fig.6.12 Spectra of the final product

(a) Reduced minus oxidised spectrum 0.5mg of the final product, in 2ml 0.1M sodium phosphate, pH7.0 were dispensed into two 1ml cuvettes and a reduced minus oxidised spectrum was recorded as described in the legend to Fig.3.2, using a Philips PU 8740 uv/vis spectrophotometer.

(b) Carbon monoxide-binding spectrum 0.8mg of the final product, in 2ml 0.1M sodium phosphate, pH7.0 were dispensed into two 1ml cuvettes and a carbon monoxide-binding spectrum was recorded as described in the legend to Fig.3.3.

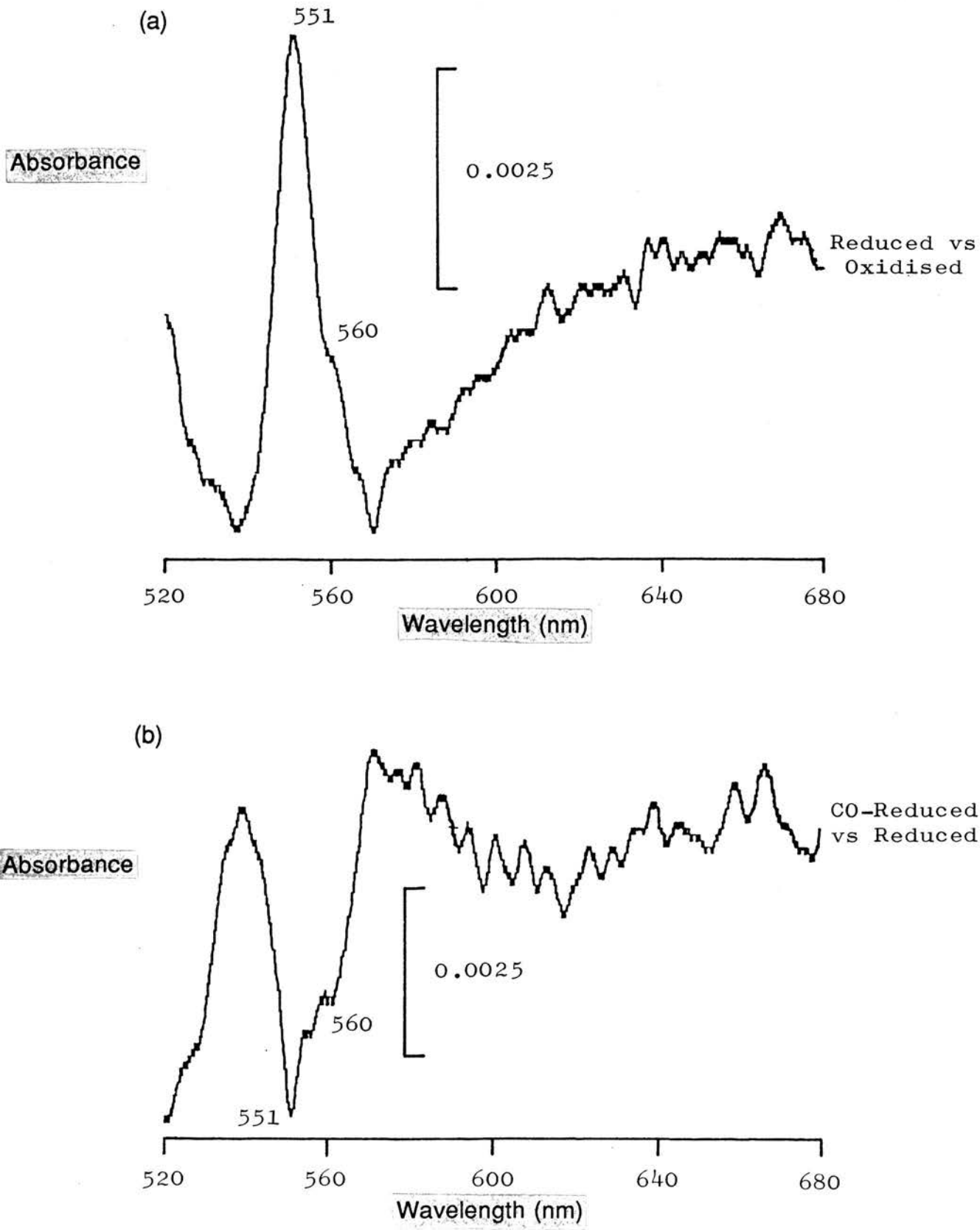
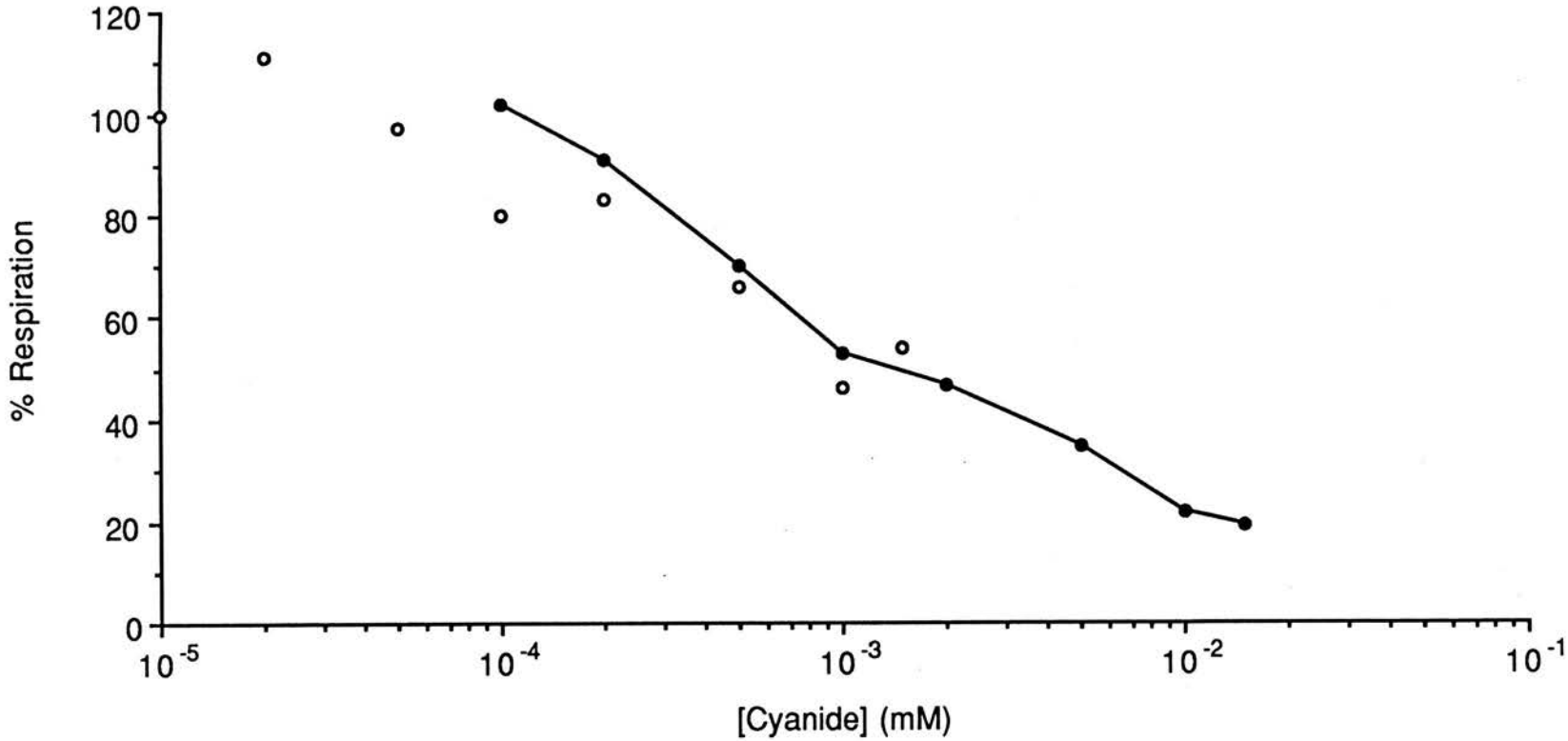

(a)
551
Absorbance
0.0025
Reduced vs
Oxidised
560
520
560
600
640
680
Wavelength (nm)
(b)
CO-Reduced
vs Reduced
Absorbance
0.0025
560
551
520
560
600
640
680
Wavelength (nm)

Fig.6.13 Inhibition of the final product by cyanide

Cyanide inhibition of the ascorbate-TMPD oxidase activity of the final product (○) and of the starting French press membranes (●) was investigated, as described in the legend to Fig.3.5. Samples, containing 0.05mg (final product) or 1.5mg (French press membranes) protein, were added to sodium cyanide of the appropriate concentration (0 to 1mM) and the ascorbate-TMPD oxidase activity was then assayed. The percentage respiration could then be calculated and the results plotted as shown.


120
100
80
60
40
20
0
% Respiration
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
[Cyanide] (mM)

cyanide at concentrations above 0.05 μM and is 50% inhibited at approximately 1.0 μM cyanide. The exact profile of inhibition of the final product is not clear, as cyanide concentrations above 1.5 μM could not be investigated due to the loss of ascorbate-TMPD oxidase activity. Also, the data obtained at lower concentrations of cyanide do not follow a smooth curve, as the rates of ascorbate-TMPD oxidation were low and there were therefore significant errors in their measurement. Nevertheless, it seems clear that the inhibition of the ascorbate-TMPD oxidase activity of the final product by cyanide parallels that of the starting French press membranes over the range of cyanide concentrations used. It is not possible, however, to see if a biphasic pattern of inhibition similar to that of the starting membranes was followed by the final product.

The loss of the ascorbate-TMPD oxidase activity of the final product precluded the investigation of other oxidase activities possessed. Thus NADH, succinate, duroquinol and cytochromes c oxidase activities were not investigated. NADH and succinate oxidase activities would be expected to be absent if the oxidase complex was no longer associated with the rest of the electron transport chain. In any case, NADH oxidase activity should be absent since cytochrome d, with which this activity is believed to be associated, is not present in the final product. Duroquinol oxidase activity would be expected if the cytochrome o co-purified with a cytochrome c reductase, in a similar way to the co-purified cytochromes bc_1 and aa_3 of *Pa. denitrificans* , which form a quinol oxidase complex (Berry and Trumpower, 1985). Cytochrome c oxidase activities are monitored by ascorbate-TMPD oxidase activity and therefore their presence would be expected. However, it would be interesting to see if the relative rates of the two types of cytochromes c used (basic and acidic) were altered by the partial purification of the ascorbate-TMPD oxidase achieved here.

Chapter 7: Discussion

I: Function of cytochrome c_4

In this study, the effect of the selective removal of cytochrome c_4 from the membranes of *P. stutzeri* on the various oxidase activities of these membranes has been used to try to define the function of cytochrome c_4. A number of methods by which the cytochrome c_4 can be selectively removed from *P. stutzeri* membranes were developed. These included washing with sodium iodide, washing with propan-2-ol and molecular exclusion chromatography of detergent extracts of membranes. Cytochrome c_4 was not removed from membranes by treatments commonly used to wash off extrinsic proteins.

Treatment of *P.stutzeri* membranes with 2 to 3M sodium iodide or 30% propan-2-ol resulted in the removal of at least 75 to 85% of the membrane-bound cytochrome c_4 with almost no loss of ascorbate-TMPD oxidase activity. Purified cytochrome c_4, or that contained in the sodium iodide extract, could be reconstituted with the washed membranes with no recovery of activity.

Parallel experiments with *A. vinelandii* (Hunter et al., 1989) show that removal of cytochrome c_4 by iodide treatment does cause a partial loss of ascorbate-TMPD oxidase activity. However, with 3M iodide, approximately 63% of the ascorbate-TMPD oxidase activity remains, with only 15% of the cytochrome c_4 still present. Reconstitution of purified cytochrome c_4, or that contained in the iodide extract, with cytochrome c_4-depleted membranes resulted in no recovery of activity.

Cytochrome c_4 was also removed from detergent solubilised respiratory particles by molecular exclusion chromatography after cold storage of the detergent extracts. This removal of cytochrome c_4 also did not affect the ascorbate-TMPD oxidase activity of the particles.

These results show that cytochrome c_4 is not essential for

ascorbate-TMPD oxidase activity. However, this cannot be stated with complete certainty for two reasons. Firstly, a small residual amount of cytochrome c_4 remains on the membranes after each removal procedure. Secondly, no positive control is present in the reconstitution of cytochrome c_4 with the membranes, so that changes to the cytochrome c4 during removal from the membrane environment cannot be ruled out.

Treatment of *P. stutzeri* membranes to remove cytochrome c_4 also causes the loss of NADH, succinate and lactate oxidase activities. Rather than necessarily implying a role for cytochrome c_4 in these activities, the losses of activities seen are probably the result of damage to the respiratory chain components involved in the activities. The lack of restoration of these activities on reconstitution of cytochrome c_4 supports this statement.

Duroquinol oxidase activity is partially lost on removal of cytochrome c_4. This loss of activity is not restored on reconstitution of cytochrome c_4. However, the variability in the duroquinol oxidase activity, and the autooxidation of duroquinol, make interpretation of these results difficult. The observed losses of activity may be due to damage to the respiratory components involved in the duroquinol oxidase activity or may reflect a requirement for cytochrome c_4, the residual duroquinol oxidation being due to electron transport via an alternative, lower capacity route.

In the light of these results, the function of cytochrome c_4 remains uncertain. A role in denitrification for this cytochrome has been discounted (Brown, 1988) since it is present in the obligate aerobe *A. vinelandii* and little induction of cytochrome c_4 in *P. stutzeri* and *P. aeruginosa* on denitrifying growth is seen (Pettigrew and Brown, 1988). The relatively high midpoint potentials of cytochrome c_4, +300 and +190mV for the *P. stutzeri* cytochrome, +317 and +263mV for the *A. vinelandii* cytochrome (Leitch et al., 1985) suggest that it is positioned close to the terminal oxidase in these organisms.

Jurtshuk et al. (1981) have proposed that cytochrome c_4 forms the c-type cytochrome component of the co-type oxidase of *A. vinelandii*. In this case,

the function of cytochrome c_4 might be to act as the electron accepting pole of the *A. vinelandii* co-type oxidase, in a manner analogous to haem a/ Cu_A in the mammalian cytochrome c oxidase. The cytochrome c_4 would accept electrons from a small, soluble cytochrome c, possibly cytochrome c-551, acting as a soluble carrier of electrons from the reductase. This model is shown diagrammatically in Fig.7.1. However, the lack of a requirement for cytochrome c_4 for both ascorbate-TMPD oxidase activity and for cytochromes c oxidase activities suggests that such a function is unlikely.

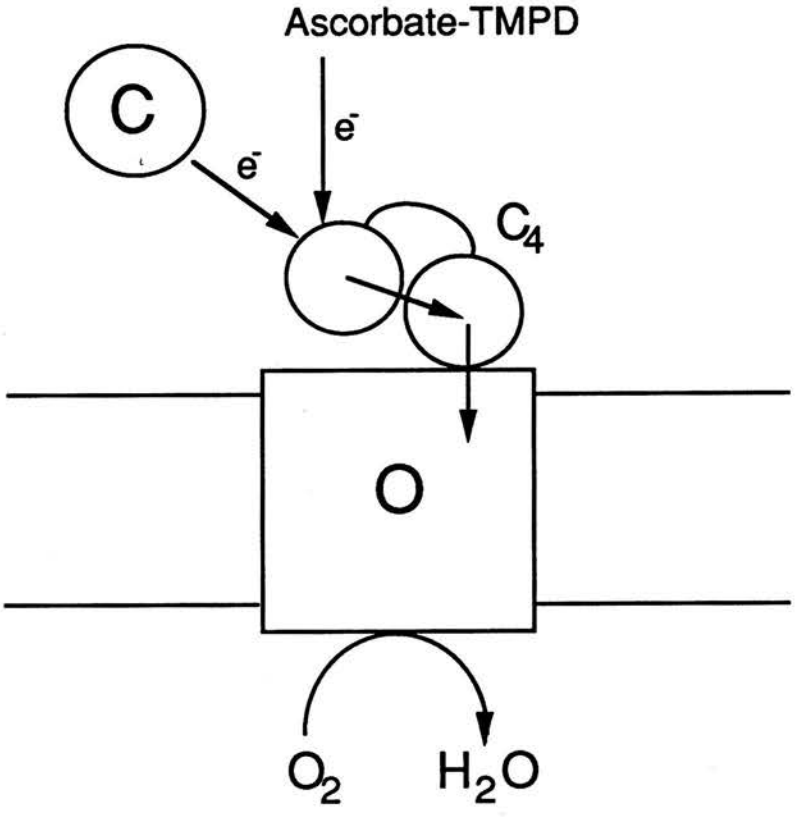
As ascorbate-TMPD and cytochrome c oxidase activities in *P. stutzeri* do not require the presence of cytochrome c_4, cytochrome c_4 must be positioned in the electron transport chain before the point of entry for electrons from ascorbate-TMPD and cytochromes c. Electrons from ascorbate-TMPD are believed to enter the electron transport chain at the level of cytochrome c. Claims have also been made that electrons from TMPD may bypass cytochrome c and donate directly to the oxidase (Sagi-Eisenberg and Gutman, 1979a, 1979b). However, several workers have found that removal of the c-type cytochrome component from cytochromes co causes loss of ascorbate-TMPD oxidase activity, suggesting that direct donation of electrons from TMPD to the cytochrome o does not occur.

Thus Froud and Anthony (1984) found that separation of the b and c-type components of the *M. methylotrophus* enzyme caused the loss of ascorbate-TMPD oxidase activity. Similarly, Carver and Jones (1983), working on the same enzyme found that cytochrome o alone could not accept electrons from TMPD. Jurtshuk et al. (1981) showed that the TMPD-dependent oxidase activity of *A. vinelandii* was proportional to the cytochrome c content of the oxidase preparation. Previous preparations of this enzyme, lacking the c-type component, were unable to oxidise TMPD (Yang and Jurtshuk, 1978a, 1978b).

The c-type component of the *A. vinelandii* cytochrome o has been identified by these workers as cytochrome c_4, implying cytochrome c_4 is essential for ascorbate-TMPD oxidase activity. However, the results presented

Fig.7.1 Cytochrome c_4 as the electron accepting pole of a co-type oxidase

C is a small soluble cytochrome c such as Pseudomonas cytochrome c-551, O is the co-type oxidase.


Ascorbate-TMPD
C
e⁻
e⁻
C4
O
O2
H2O

in this work (Chapter 4) and in Hunter et al. (1989) show that cytochrome c_4 is not required for ascorbate-TMPD oxidase activity in either *A. vinelandii* or *P. stutzeri*. An explanation for this apparent contradiction lies in the fact that the identification by Yang and Jurtshuk (1978b) of the c-type component of the *A. vinelandii* cytochrome co is based on the very undiscriminating criterion of membrane spectra only; no positive identification of the c-type component with pure cytochrome c_4 was made. The current results, taken together with those of Yang, Jurtshuk and co-workers, suggest that the c-type component of the *A. vinelandii* cytochrome co is not cytochrome c_4, as the removal of cytochrome c_4 does not affect ascorbate-TMPD oxidase activity, whereas removal of the c-type component of the cytochrome co does abolish ascorbate-TMPD oxidase activity.

The suggested position of cytochrome c_4 in the electron transport chain, before the point of entry for electrons from ascorbate-TMPD and cytochromes c suggests that its function could be as a link between the co-type oxidase and its reductase. Brown (1988) has suggested that cytochrome c_4 is associated with the cytochrome o of *P. stutzeri* such that it forms an electron transporting 'bridge' linking the oxidase and its reductase (Fig.7.2). In this model, one domain of the cytochrome c_4 binds to the reductase, one to the oxidase, with electrons being transported by an intramolecular transfer between the two haem groups of the cytochrome c_4. The resulting complex of reductase, cytochrome c_4 and o-type oxidase would act as a quinol oxidase.

A quinol oxidase supercomplex, with a structure similar to that suggested in Fig.7.2, has been isolated from *Pa. denitrificans* by Berry and Trumpower (1985). The ubiquinol oxidase isolated contained cytochromes corresponding to those of the bc_1 complex and cytochrome aa_3, together with a M_r 22 000 membrane-bound cytochrome c. This cytochrome c may bear some structural or functional relation to cytochrome c_4. A possible model of the *Pa. denitrificans* quinol oxidase supercomplex is given in Fig.7.3.

If cytochrome c_4 does function as an electron 'bridge' in *P. stutzeri*, then

Fig.7.2 Cytochrome c_4 as an electron translocating 'bridge'

In this model, cytochrome c_4 acts as a pathway for electron flow from the reductase (R) to the co- type oxidase (O).

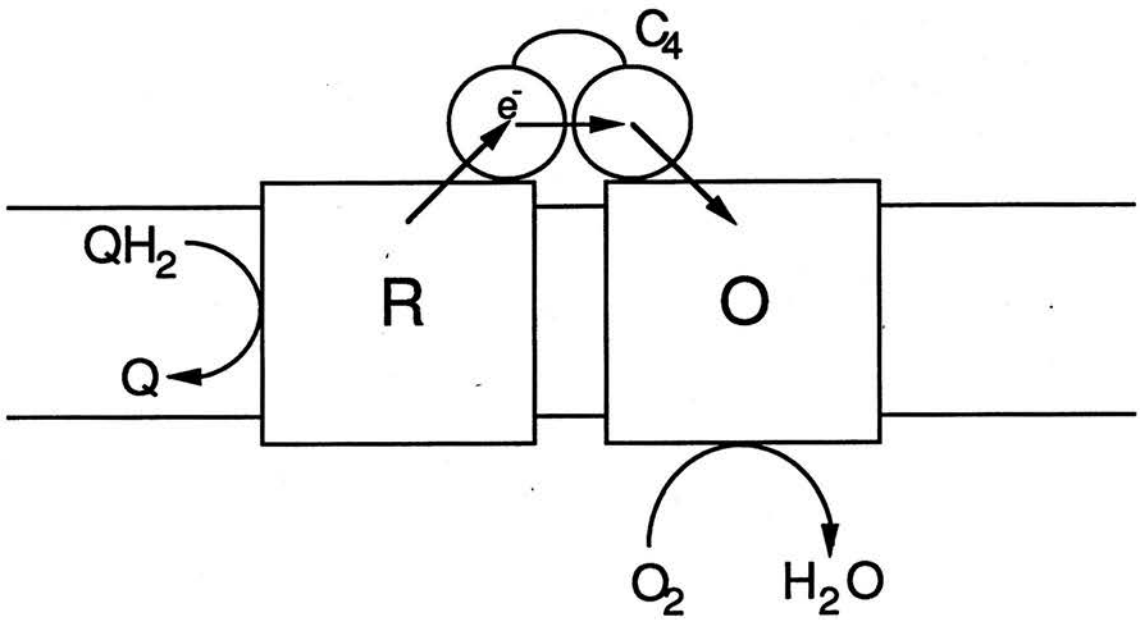
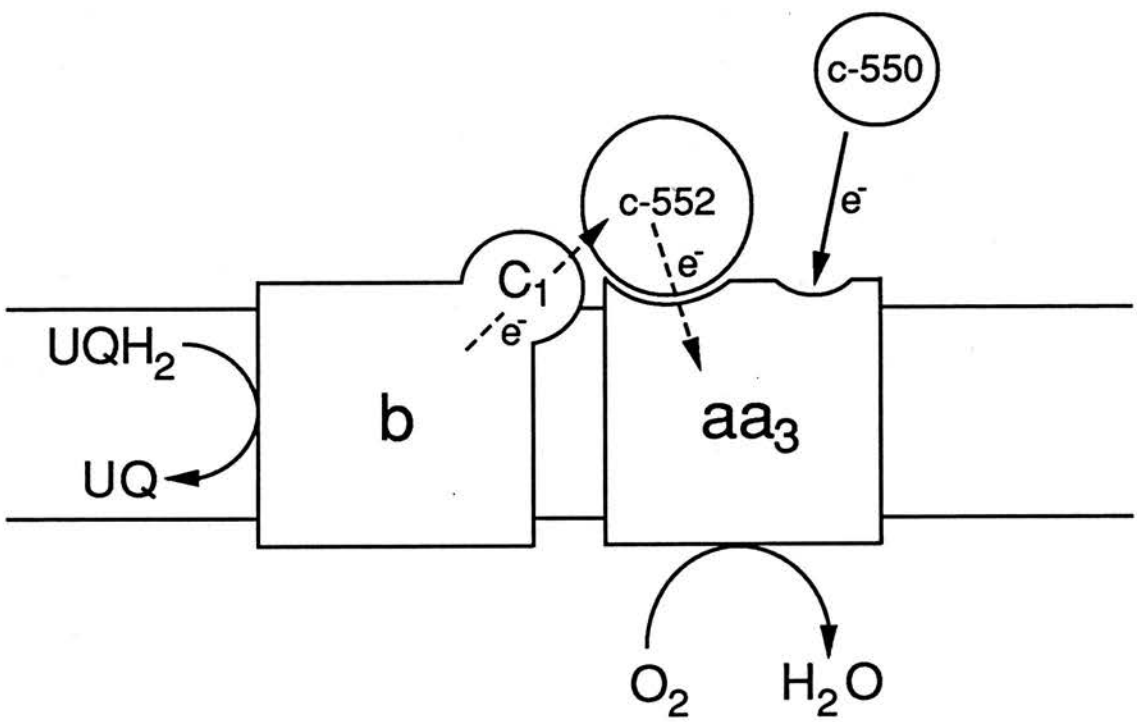

C4
e-
QH2
Q
R
O
O2
H2O

Fig.7.3 A model for the ubiquinol oxidase supercomplex of *Pa. denitrificans*

The function of the membrane-associated cytochrome c-552 is not known, but it may be involved in the transfer of electrons from the reductase (cytochrome bc_1) to the aa_3-type oxidase as shown here. This cytochrome may be structurally or functionally related to cytochrome c_4.


c-550
c-552
e⁻
e⁻
C1
e⁻
UQH2
UQ
b
aa3
O2
H2O

one would expect removal of the cytochrome c_4 to cause the loss of quinol oxidase activity. The results presented here, although of doubtful quality, suggest that such a loss of activity does not occur when cytochrome c_4 is removed from the membrane. A partial loss of activity is seen, which is not restored by the reconstitution of cytochrome c_4 with the membranes. This suggests that the loss of activity observed is not due to loss of the cytochrome c_4. However, an alternative possibility is that a parallel pathway of electron transport occurs, with electrons being transferred directly from the reductase to the o-type oxidase.

Evidence for a direct electron transfer comes from work with mutants of *Rhodopseudomonas capsulata*. Here, a mutant lacking cytochrome c_2, homologous to the mitochondrial cytochrome c, was able to sustain electron transfer from the ubiquinol cytochrome c reductase to the reaction centre, implying an alternative electron transfer protein or direct electron transfer mechanism. Removal of any alternative electron transfer proteins, by the formation of spheroplasts of the mutant cells, had no effect on the electron transfer, implying a direct electron transfer mechanism (Prince et al., 1986).

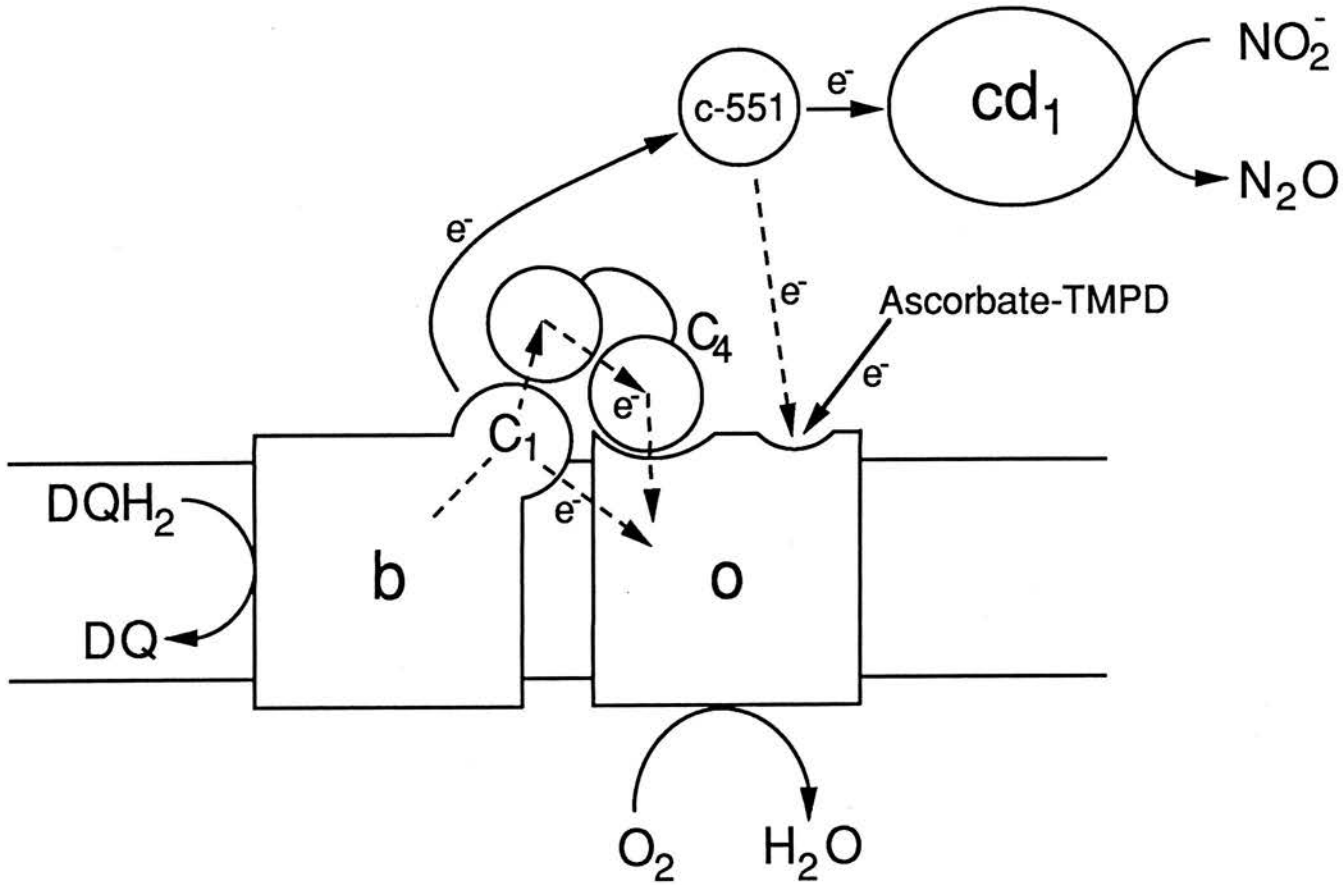
Cytochrome c_4 may therefore function as an additional, possibly higher capacity, electron transfer route from the reductase to the cytochrome co. A model of the *P. stutzeri* co-type oxidase is given in Fig.7.4, with the cytochrome c_4 functioning in this manner. It should, however, be emphasised that this model is tentative and the exact function of cytochrome c_4 remains in doubt. It may be that the only way to determine the function of cytochrome c_4 will be to construct mutants deficient in this cytochrome and then to look at the effect of the total lack of cytochrome c_4 on all the activities the cytochrome is proposed to be involved in.

II: Attachment of cytochrome c_4 to the membranes of P. stutzeri

The cytochrome c_4 of *P. stutzeri*, in common with those of *P. aeruginosa*, *A. vinelandii* and *Alcaligenes* sp. is firmly attached to the cytoplasmic

Fig.7.4 Model of the terminal electron transport chain in *P. stutzeri* 224

In this model, duroquinol is oxidised by a quinol cytochrome c oxidoreductase (cytochrome bc_1) and electrons are transferred to the terminal o-type via cytochrome c_4 or via a direct route. Electrons are also passed to cytochrome c-551, which forms the substrate of cytochrome cd1, the nitrite reductase. Cytochrome c-551 may also donate electrons to the o-type oxidase (O). Electrons from this source, and from ascorbate-TMPD, bypass cytochrome c_4 and donate directly to the o-type oxidase, which may or may not possess a cytochrome c component as well as oxygen-binding cytochrome b.


c-551
e-
cd1
NO2-
N2O
e-
e-
Ascorbate-TMPD
e-
C4
e-
C1
e-
DQH2
DQ
b
o
O2
H2O

membrane and is not removed by methods commonly used to release extrinsic membrane proteins (Chapter 4, section I; Pettigrew and Brown, 1988; Hunter et al., 1989). The nature of the attachment of the cytochrome c_4 to the membranes is not known, but hydrophobic interactions with specific protein sites are believed to be involved.

The involvement of hydrophobic interactions in the attachment of cytochrome c_4 to the membranes is suggested by the nature if the methods required for its solubilisation. The purification of membrane-bound cytochrome c_4 requires the disruption of the membranes with butan-1-ol in order to liberate the cytochrome c_4 (Pettigrew and Brown, 1988). The use of an organic solvent in the removal of cytochrome c_4 strongly suggests a hydrophobic attachment of the cytochrome c_4 to the membranes. Similarly, propan-2-ol has been used to solubilise cytochrome c_4 from *P. stutzeri* membranes (Chapter 4, section II; Hunter et al., 1989). Finally, the chaotrope, sodium iodide, which also disrupts hydrophobic interactions, removes cytochrome c4 from the membranes whilst sodium chloride, which is not a chaotrope, does not remove cytochrome c4 when used at the same concentration.

The involvement of hydrophobic interactions in the attachment of cytochrome c_4 to the membranes does not necessarily imply that it is an integral membrane protein. Firstly, it is not a particularly hydrophobic protein; cytochromes c_4 from *A. vinelandii* and *P. aeruginosa* have polarity indices (Capaldi and Vanderkooi, 1972) significantly higher than those of most integral membrane proteins (Pettigrew and Brown, 1988). Secondly, cytochrome c_4 exists as a stable monomer in aqueous solution, both in vivo (Pettigrew and Brown, 1988) and after removal from the membranes by a variety of techniques (iodide washing, propan-2-ol washing, butan-1-ol extraction). An integral membrane protein would be expected to aggregate under such conditions. No such aggregation has been detected. Finally, sodium iodide, although capable of disrupting hydrophobic interactions sufficiently to release cytochrome c_4, does not cause the solubilisation of the

other membrane cytochromes c, suggesting that these are more tightly bound to the membrane and are probably integral membrane proteins.

Binding of the cytochrome c_4 to the membranes is believed to occur to specific protein sites (Pettigrew and Brown, 1988). The number of such attachment sites is thought to be limited. During anaerobic, denitrifying growth, the amount of cytochrome c_4 present is approximately the same as that during aerobic growth. However, the distribution of cytochrome c_4 between membrane-bound and soluble forms shifts so that less cytochrome c_4 is bound to the membrane. This is interpreted as being the result of a reduction in the number of binding sites for the cytochrome c_4 during anaerobic growth (Pettigrew and Brown, 1988). The identity of these binding sites is of interest since they will be related to the function of cytochrome c_4.

Candidates for the cytochrome c_4 binding sites include the co-type oxidase and its reductase (see section I). Band I_{PS}, which may be the cytochrome c_1 in this organism, and hence a part of the reductase complex, is markedly reduced in amount in membranes from cells grown under anaerobic, denitrifying conditions (Chapter 3, Fig.3.10). This may indicate the repression of the reductase and hence the reduction in numbers in at least one possible binding site for cytochrome c_4. Furthermore, the ascorbate-TMPD and cytochrome c oxidase activities of membranes from cells grown under such conditions are significantly lower than those of membranes from aerobically grown cells (Chapter 3, section IV). This is believed to indicate a decreased cytochrome co content for these membranes which would also reduce the number of possible binding sites for cytochrome c_4.

The binding of cytochrome c_4 to these specific sites is cold-labile, following solubilisation of the membranes with detergent. This is a reflection of the hydrophobic nature of the binding, since the strength of hydrophobic interactions is reduced at lower temperatures. The reduction is because such interactions are driven by entropy and

$$\Delta G = \Delta H - T\Delta S$$

so that if T falls, the contribution of ΔS to the free energy of the interaction will fall, making the reaction less favourable.

Binding of cytochrome c_4 to native membranes was not detectably influenced by temperature. This difference in binding of cytochrome c_4 to native membranes and detergent solubilised membranes probably reflects the destabilising effect of the detergents used on the hydrophobic interactions binding cytochrome c_4 to the membrane, such that, in the absence of detergent, the decrease in the strength of the hydrophobic interactions with low temperature is insufficient to cause removal of the cytochrome.

Removal of cytochrome c_4 from the membranes probably results in a conformation change to hide the now-exposed hydrophobic surfaces, usually involved in interaction with the membrane (Fig.1.7). An alternative mechanism by which this hiding could occur is by the formation of dimers of cytochrome c_4. However, as discussed previously, cytochrome c_4 is monomeric. Although the three-dimensional structure of cytochrome c_4 is known (Chapter 1), the resolution available at present is insufficient to be able to detect regions on the molecule which might be involved in interactions with the membrane. Such studies must await a higher resolution structure for cytochrome c_4.

An attempt was made to crudely reproduce the conditions found on the membrane, in order to try to characterise the cytochrome c_4 in its membrane-bound conformation. Since 4M sodium iodide removed cytochrome c_4 from the membrane by weakening hydrophobic interactions, it should also prevent the hiding of hydrophobic surfaces when cytochrome is not bound to the membrane. Hence a 'membrane-bound' conformation of cytochrome c_4, with exposed hydrophobic surfaces, might be expected. However, extensive characterisation of cytochrome c_4 in 4M sodium iodide (Chapter 5) revealed that the conformational changes caused by this treatment were consistent with a partial denaturation of the cytochrome c_4,

rather than with a change in conformation to expose hydrophobic surfaces. In particular, methionyl-haem coordination was lost, indicating a denaturation had occurred, although the retention of a difference in midpoint potentials between the two haems suggests that the denaturation was only partial. The properties of cytochrome c_4 in a membrane-bound conformation therefore could not be determined.

Reproducing the conditions experienced by cytochrome c_4 on the membrane so that the cytochrome adopts its membrane-bound conformation is very difficult, not least because this membrane-bound conformation is unknown. It may therefore prove easier to investigate the conformation of cytochrome c_4 in situ on the membrane. One possible way of doing this is to perform a redox titration of whole membranes. If the midpoint potentials of cytochrome c_4 are sufficiently distant from those of the other membrane cytochromes c, it should prove possible to resolve them. Comparison of the midpoint potentials of membrane-bound and soluble cytochrome c_4 should give valuable information on the nature of the membrane-bound cytochrome c_4.

A second possible method by which the conformation of cytochrome c_4 on membranes could be probed requires a method for the removal of cytochrome c_4 from membranes and its return. This then allows a probe of conformation to be inserted into cytochrome c_4 alone, which is then re-bound to the membranes. Examples of probes which could be used include fluorescent groups, NMR and ESR probes and ^{57}Fe for Mossbauer spectroscopy. However, for this method to be of value, one must be sure that the conformation change observed is due to binding to the membrane and not to an artifact of the reconstitution process.

Cytochrome c_4 can be removed from and returned to membranes of *P. stutzeri* by iodide treatment (Chapter 4, section III). Most of the cytochrome c_4 is believed to return to the membranes in its native state, but work would be required to ensure that the preparations were sufficiently free of artifactual binding to give meaningful results.

III: The cytochrome oxidase of P. stutzeri 224

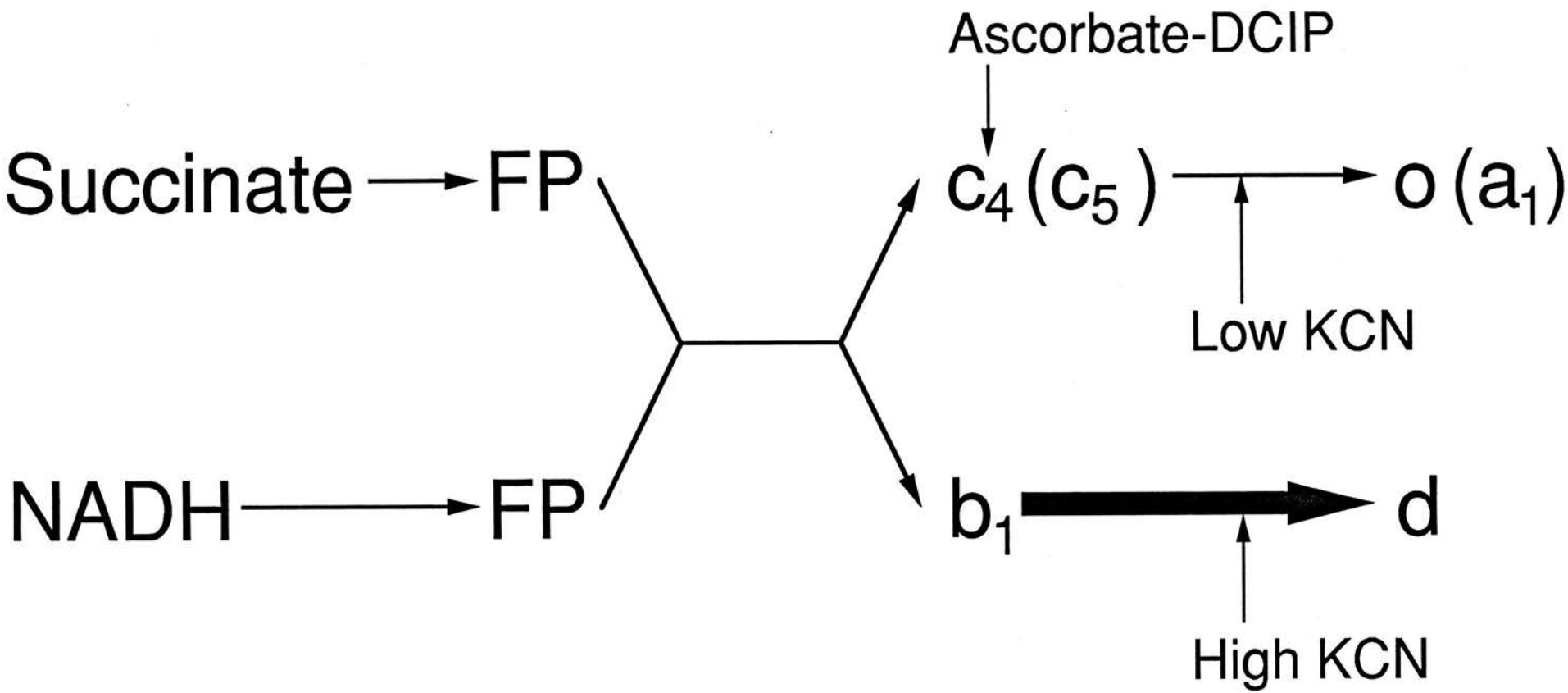
At least four types of membrane-bound bacterial cytochrome oxidases which reduce molecular oxygen to water have been discovered. These are cytochromes of the aa_3, a_1, d (formerly a_2) and o-types. Cytochrome cd_1 is also able to reduce oxygen to water and was believed to be a cytochrome oxidase when first purified (Horio et al., 1958). It is now thought to function primarily as a nitrite reductase and will not be discussed further. In addition, a ba_3-type cytochrome oxidase, where haem b replaces haem a, has recently been discovered (Zimmerman et al., 1988) as a second terminal oxidase in the extreme thermophile *Thermus thermophilus*.

Comparison of these enzymes with the 'classical' mitochondrial cytochrome oxidase reveals that, in addition to possessing different redox centres to the mitochondrial cytochrome aa_3 in most cases, the bacterial cytochrome oxidases are generally simpler with respect to polypeptide composition. Also, a direct role for many of them as proton pumps has not been demonstrated and the components of the oxidases are the product of a single genetic system, rather than two, as in the mitochondrial instance. A further complication, of great importance, in the study of bacterial cytochrome oxidases is the existence of two or more oxidase types in the same organism.

Linear electron transport chains, terminating in a single cytochrome oxidase, as in the mitochondrion, appear to be the exception rather than the rule in bacteria. Even under constant growth conditions, more than one type of cytochrome oxidase may be present as the termini of limbs of a branched electron transport chain. An example of this is seen in *A. vinelandii* where cytochromes a_1, d and o form the terminal oxidases of a branched respiratory chain (Fig.7.5; Jones and Redfearn, 1967).

In addition to this, growth conditions influence the relative contributions of the two or three cytochrome oxidases to electron transport. For example, oxygen limitation may induce cytochrome o (Sapshead and Wimpenny, 1972; but see footnote *), although synthesis of this terminal oxidase is regarded by some to be constitutive (Poole, 1983). Cytochrome d is believed

Fig.7.5 Branched electron transport in *A. vinelandii* (taken from Jones and Redfearn, 1967). The large arrow from cytochrome b_1 to cytochrome d indicates that the majority of electron flow is believed to take this route under aerobic conditions.


Ascorbate-DCIP
Succinate
FP
NADH
FP
c_4 (c_5)
o (a_1)
Low KCN
b_1
d
High KCN

to be induced by the presence of cyanide in the growth medium, since its low sensitivity to this inhibitor allows the organism to continue aerobic respiration in the presence of cyanide (Henry, 1981; Jones, 1977), although the correlation between cyanide resistant respiration and cytochrome d is disputed (Akimenko and Trutko, 1984). This complexity in bacterial respiration, the presence of branched electron chains, multiple terminal oxidases, cytochrome c peroxidase, anaerobic terminal electron acceptors such as cytochrome cd1 and multiple electron donor systems (Fig.1.1) allows adaptation of the organism to changing conditions of growth.

The results of Chapter 3 show that *P. stutzeri* 224 possesses cytochrome o and d as terminal oxidases. These oxidases were identified on the basis of difference and carbon monoxide-binding spectra of membranes and on the cyanide sensitivity of the membrane oxidase activities. Identification of an oxidase is often made on the basis of the visible absorption spectrum of membranes and on the effect of carbon monoxide on such a spectrum, as in this case. However, these approaches are not rigorous, as discussed in Chapter 3 and leave open the possibility that the cytochrome identified as an oxidase merely binds carbon monoxide fortuitously. However, the cyanide sensitivities of the membrane oxidase activities of *P. stutzeri* 224 (Chapter 3, Fig.3.5) support the presence of two terminal oxidases, one with a low sensitivity to cyanide. Also, cytochrome o has been identified as a terminal oxidase in another strain of *P. stutzeri* (strain ZoBell, Heiss et al., 1989) and in related organisms (*P. aeruginosa*, Matsushita et al., 1982; Yang, 1982 and *A. vinelandii*, Yang et al., 1979; Jurtshuk et al., 1981) so that the presence of cytochrome o in *P. stutzeri* 224 is well supported. Matsushita et al. (1983) described a cyanide insensitive alternate oxidase in *P. aeruginosa*, but this oxidase was not identified as a cytochrome d. However, the results presented in Chapter 3 do suggest that cytochrome d is a terminal oxidase in *P. stutzeri* 224.

Cytochromes o were originally defined on the basis of photochemical action spectra as b-type cytochromes possessing an oxidase activity sensitive to inhibition by carbon monoxide (Chance et al., 1953). Cytochrome o

appears to be the most widely distributed of the bacterial oxidase types (Jones, 1980). A number of cytochromes o have been purified and two distinct groups of this enzyme have been identified, cytochromes bo and cytochromes co (Froud and Anthony, 1984). Cytochrome bo contains b-type haem alone and has been purified from E. coli (Kita et al., 1984). This enzyme possesses subunits of M_r 33 000 and 55 000, with two haems b and two coppers. One of the haems is carbon monoxide reactive and is believed to be the site of oxygen reduction. The other redox centres are believed to act as an electron buffer in a manner analogous to the haem a and Cu_A of the mitochondrial cytochrome oxidase. A cytochrome bo has also been purified from *Vitreoscilla* (strain C1, Georgiou and Webster, 1987). Note that this is not the 'soluble cytochrome o' previously purified from *Vitreoscilla* (Liu and Webster, 1974) which is now believed to be a bacterial haemoglobin (Orii and Webster, 1986).

This pattern of function of redox centres is also believed to occur in the second group of cytochromes o, those containing haem c in addition to carbon monoxide reactive haem b - cytochromes co. Cytochromes co have been purified from *A. vinelandii* (Yang and Jurtshuk, 1978a, 1978b; Jurtshuk et al., 1981; Wong and Jurtshuk, 1984; Yang, 1986), *P. aeruginosa* (Matsushita et al., 1982; Yang, 1982), *P. stutzeri* (strain ZoBell, Heiss et al., 1989), *M. methylotrophus* (Carver and Jones, 1983; Froud and Anthony, 1984), *Rhodopseudomonas capsulata* (Hudig and Drews, 1982; 1983), *Rhodopseudomonas palustris* (King and Drews, 1976) and organism 4025, an obligate methylotroph (Auton and Anthony, 1989). In general, two subunits are present, of M_r approximately 22 000 and 30 000. Other bands of lower M_r are also present in many cases. After SDS-PAGE, both these subunits have TMBZ peroxidase activity associated with them, suggesting the presence of haem c. However, Froud and Anthony (1984) concluded that the M_r 23 800 subunit of the M. methylotrophus enzyme is a c-type cytochrome and the M_r 31 500 subunit is a b-type cytochrome. Also, Yang (1986) showed that the M_r 29 000 subunit of the A. vinelandii enzyme is a b-type cytochrome, the M_r 20 000

subunit being referred to as the c-type cytochrome c_4, although no positive identification with cytochrome c_4 was made.

The exception to this general structure is the cytochrome o of *Rps. capsulata* (Hudig and Drews, 1982). This is a dimer of M_r 130 000, with a single haem b per dimer, although this could be due to losses during the purification. No binding of carbon monoxide to this haem b was observed and no c-type cytochrome was present in the final purified enzyme. However, a c-type cytochrome of Mr 13 000 which binds carbon monoxide co-purified with the cytochrome oxidase during the early stages of purification (Hudig and Drews, 1983). This cytochrome is not the well characterised cytochrome c_2 from Rps capsulata.

Comparison of the partially purified cytochrome o from *P. stutzeri* 224 with these purified cytochromes co reveals several things. Firstly, as discussed in Chapter 6, the fold-purification and recovery obtained in this purification compare reasonably well with those obtained in many of the other preparations. Secondly, the polypeptide composition of the partially purified material is still too complex to allow conclusions to be drawn about the subunit composition of the cytochrome o. It is interesting to note that protein-stained bands of M_r approximately 25 000 and 30 000 do exist in the partially purified material (Fig.6.10c and 6.11b). However, these bands do not co-chromatograph with the ascorbate-TMPD oxidase activity on molecular exclusion chromatography (Fig.6.7b), suggesting that they are not involved in the cytochrome o.

Spectra and haem-stained SDS-PAGE gels of the partially purified cytochrome o show that both b and c-type cytochromes are present. C-type cytochrome bands I_{PS} and III_{PS} are prominent in this preparation, with smaller amounts of band II_{PS} and cytochrome c_4. If the cytochrome o in *P. stutzeri* 224 is similar to the cytochromes co purified from other organisms, then a cytochrome c of M_r approximately 22 000 would be expected in addition to the oxygen-binding cytochrome b. This cytochrome c is not cytochrome c_4, since cytochrome c_4 is not involved in ascorbate-TMPD or cytochromes c oxidase

activity (see section I).

Band III_{Ps} is a possible candidate for this c-type component as it is of Mr 25 000. Bands I_{Ps} and II_{Ps} are of too high a molecular weight to be considered. However band III_{Ps} is largely removed from the ascorbate-TMPD oxidase activity on ammonium sulphate fractionation (Fig.6.4b) and what remains of it after this procedure does not co-chromatograph with the ascorbate-TMPD oxidase activity. This is true for all the membrane cytochromes c and leaves open the possibility that the *P. stutzeri* 224 cytochrome o has no associated cytochrome c. The exact nature of the cytochrome o of *P. stutzeri* 224 therefore remains unknown and further speculation on the basis of the current purification is not justified. Clearly, in order to resolve the unanswered questions about the possible involvement of cytochrome c_4 in its interactions with other respiratory components, a full purification of the cytochrome o of *P. stutzeri* 244 must be achieved.

References

Akimenko, V.K. and Trutko, S.M. (1984) Arch. Microbiol 138 58-63

Alefounder, P.R. and Ferguson, S.J. (1980) Biochem. J. 192 231-240

Ambler, R.P. (1980) in From Cyclotrons to Cytochromes (Robinson, A.B. and Kaplan, N.O., eds.) pp263-280, Academic Press, New York

Ambler, R.P. and Murray, S. (1973) Biochem. Soc. Trans. 1 162-164

Ambler, R.P., Daniel, M., Melis, K. and Stout, C.D. (1984) Biochem. J. 222 217-227

Auton, K.A. and Anthony, C. (1989) Biochem. J. 260 75-79

Bartsch, R.G. (1971) Meth. Enzymol. 23 344-363

Bates, R.G (1954) Electrometric pH Determination, p208, Wiley, New York

Berry, E.A. and Trumpower, B.L. (1985) J. Biol. Chem. 260 2458-2467

Bill, K., Casey, R.P., Broger, C. and Azzi, A. (1980) FEBS Lett. 120 248-250

Bill, K. and Azzi, A. (1984) Biochem. Biophys. Res. Commun. 120 124-130

Bogonez, E. and Koshland, D.E. (1985) Proc. Natl. Acad. Sci. USA 82 4891-4895

Brown, K.R. (1988) Ph.D Thesis, University of Edinburgh.

Capaldi, R.A. and Vanderkooi, G. (1972) Proc. Natl. Acad. Sci. USA 69 930-932

Carter, D.C., Melis, K.A., O'Donnell, S.E., Burgess, B.K, Furey, W.F., Wang, B. -C. and Stout, C.D. (1985) J. Mol. Biol. 184 279-295

Carver, M.A. and Jones, C.W. (1983) FEBS Lett. 155 187-191

Chance, B., Smith, L. and Castor, L.N. (1953) Biochim. Biophys. Acta 12 289-298

Davies, K.A. and Hatefi, Y. (1971) Biochemistry 10 2509-2516

Davies, L.A., Schejter, A. and Hess, G.A. (1974) J. Biol. Chem. 249 2624-2632

Dawson, R.M.C., Elliot, D.C., Elliot, W.H. and Jones, K.M. (1969) (eds.) Data for Biochemical Research, 2nd edn., Oxford University Press

Dencher, N.A. and Heyn, M.P. (1978) FEBS Lett. 96 322-326

Dickerson, R.E. and Timkovich, R. (1975) in The Enzymes (Boyer, P.D., ed.), Vol. 11, pp397-547, Academic Press, New York

Dickerson, R.E. and Takano, T. (1978) Frontiers of Biological Energetics 1 101-108

Dixon, M. and Webb, E.C. (1979) Enzymes, 3rd edn., pp31-33, Longmans, London

Dohler, K., Huss, V.A.R. and Zumft, W.G. (1987) Int. J. Syst. Bacteriol. 37 1-3

Dulley, J.R. and Grieve P.A. (1975) Anal. Biochem. 64 136-141

Ferguson, S.J. (1982) Biochem. Soc. Trans. 10 198-200

French, C.S. and Milner, H.W. (1955) Meth. Enzymol. 1 64-67

Froud, S.J. and Anthony, C. (1984) J. Gen. Microbiol. 130 2201-2212

Georgiou, C.D. and Webster, D.A. (1987) Biochemistry 26 6521-6526

Goodhew, C.F. (1988) Ph.D Thesis, University of Edinburgh.

Goodhew, C.F., Brown, K.R. and Pettigrew, G.W. (1986) Biochim. Biophys. Acta 852 288-294

Gorbunoff, M.J. (1984) Anal. Biochem. 136 440-445

Green, A.A. and Hughes, W.L. (1955) Meth. Enzymol. 1 67-90

Harburg, H.A. and Loach, P.A. (1959) Proc. Natl. Acad. Sci. USA 45 1344-1359

Hatefi, Y. and Davies, K.A. (1972) Arch. Biochem. Biophys. 152 613-618

Hatefi, Y. and Hanstein, W.G. (1974) Meth. Enzymol. 31 770-790

Heiss, B., Frunzke, K. and Zumft, W.G. (1989) J. Bacteriol. 171 3288-3297

Henry, M. -F. (1981) in Cyanide in Biology (Vennesland, B., Conn, E.E., Knowles, C.J., Westley, J. and Wissing, F., eds.), pp415-436, Academic Press, London

Hildreth, J.E.K. (1982) Biochem. J. 207 363-366

Hjelmeland, L.H. (1980) Proc. Natl. Acad. Sci. USA 77 6368-6370

Hjerten, S., Rosengren, J. and Pahlman, S. (1974) J.Chromatogr. 101 281-288

Horio, T., Higashi, T., Matsubara, H., Kusai, K., Nakai, M. and Okunuki, K. (1958) Biochim. Biophys. Acta 29 297-302

Hudig, H. and Drews, G. (1982) FEBS Lett. 146 389-392

Hudig, H. and Drews, G. (1983) FEBS Lett. 152 251-255

Kumar, C., Naqui, A., Powers, L., Ching, Y.-C. and Chance, B. (1988)
Biol. Chem. 263 7159-7163

Ronnberg, M. (1987) Biochim. Biophys. Acta 916 112-118

Ronnberg, M., Araiso, T., Ellfolk, N. and Dunford, H.B. (1981) Arch. Biochem
Biophys 207 197-204

Hunter, D.J.B., Brown, K.R. and Pettigrew, G.W. (1989) Biochem. J. 262 233-240

Jacobs, E.E. and Sanadi, D.R. (1960) J. Biol. Chem. 235 531-534

Jones, C.W. (1977) in 27th Symposium of the Society for General Microbiology (Haddock, B.A. and Hamilton, W.A., eds.), pp23-59, Cambridge University Press, Cambridge

Jones, C.W. (1980) in Microbiological Classification and Identification (Goodfellow, M. and Board, R.G., eds.), pp127-138, Academic Press, London

Jones, C.W. and Redfearn, E.R. (1967) Biochim. Biophys. Acta 143 340-353

Jurtshuk, P., Mueller, T.J. and Wong, T.-Y. (1981) Biochim. Biophys. Acta 637 374-382

Kimelberg, H.K. and Nicholls, P. (1969) Arch. Biochem. Biophys. 133 327-335

King, M. -T. and Drews, G. (1976) Eur. J. Biochem. 68 5-12

Kita, K., Konishi, K. and Anraku, Y. (1984) J. Biol. Chem. 259 3368-3374

Kodama, T. and Shidara, S. (1969) J. Biochem. 65 351-360

Laemmli, U.K. (1970) Nature 227 680-685

Leitch, F.A., Brown, K.R. and Pettigrew, G.W. (1985) Biochim. Biophys. Acta 808 213-218

Liu, C.Y. and Webster, D.A. (1974) J. Biol. Chem. 249 4261-4266

Liu, M.C., Peck, H.D., Payne, W.J., Anderson, J.L., DerVartanian, D.V. and LeGall, J. (1981) FEBS Lett. 129 155-160

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193 265-275

Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77 196-200

MacDonald, R.I. (1980) Biochemistry 19 1916-1922

Madden, T.D. and Cullis, P.R. (1985) Biochim. Biophys. Acta 808 219-224

Mandel, M (1966) J. Gen. Microbiol. 43 273-292

Matsushita, K., Shinegawa, E., Adachi, O. and Ameyama, M. (1982) FEBS Lett. 139 255-258

Matsushita, K., Yamada, M., Shinegawa, E., Adachi O. and Ameyama, M. (1983) J. Biochem. 93 1137-1144

Mukai, K., Yoshida, M., Yao, Y., Wakabayashi, S. and Matsubara, H. (1988) Proc. Japan. Acad. 64B 41-44

Nelson, D.B. and Gellefors, P. (1978) Meth. Enzymol. 53 80-91

Newton, J.W., Wilson, P.w. and Burris, R.H. (1953) J. Biol. Chem. 204 445-451

Orii, Y. and Webster, D.A. (1986) J. Biol. Chem. 261 3544-3547

Ozawa, T., Okumura, M. and Yagi, K. (1975) Biochem. Biophys. Res. Commun. 65 1102-1107

Pettigrew, G.W. (1972) FEBS Lett. 22 64-66

Pettigrew, G.W. and Brown, K.R. (1988) Biochem. J. 252 427-435

Pettigrew G.W. and Moore, G.R. (1987) Cytochromes c - biological aspects, Springer-Verlag, Heidelburg

Pettigrew, G.W., Leaver, J.L., Meyer, T.E. and Ryle, A.P. (1975) Biochem. J. 147 291-302

Pettigrew, G.W., Bartsch, R.G., Meyer, T.E. and Kamen, M.D. (1978) Biochim. Biophys. Acta 503 509-523

Poole, R.K. (1983) Biochim. Biophys. Acta 726 205-243

Prince, R.C., Davidson, E., Haith, C.E. and Daldal, F. (1986) Biochemistry 25 5208-5214

Rosevear, P., VanAken, T., Baxter, J. and Ferguson-Miller, S. (1980) Biochemistry 19 4108-4115

Sagi-Eisenberg, R. and Gutman, M (1979a) Arch. Biochem. Biophys. 197 470-476

Sagi-Eisenberg, R. and Gutman, M (1979b) Eur. J. Biochem. 97 127-132

Sapshead, L.M. and Wimpenny, J.W.T. (1972) Biochim. Biophys. Acta 267 388-397

Sawyer, L., Jones, C.L., Damas, A.M., Harding, M.M., Gould, R.O. and Ambler, R.P. (1981) J. Mol. Biol. 153 831-835

Schechter, E. and Saludjian, P. (1967) Biopolymers 5 788-790

Shaltiel, S. (1974) Meth. Enzymol. 34 126-140

Shidara, S. (1980) J. Biochem. 87 1177-1184

Stanier, R.Y., Palleroni, N.J. and Doudoroff, M. (1966) J. Gen. Microbiol. 43 159-272

Sweet, W.J. and Peterson, J.A. (1981) Arch. Biochem. Biophys. 209 256-265

Tiselius, A., Hjerten, S. and Levin, O. (1956) Arch. Biochem. Biophys. 65 132-155

Tissieres, A. (1956) Biochem. J. 64 582-589

Tissieres, A. and Burris, R.H. (1956) Biochim. Biophys. Acta 20 436-437

Van Niel, C.B. and Allen, M.B. (1952) J. Bacteriol. 64 413-422

Wilson, D.F. and Dutton, P.L. (1970) Biochem. Biophys. Res. Commun 39 59-64

Wong, T.-Y. and Jurtshuk, P. (1984) J. Bioenerg. Biomemb. 16 447-489

Wood, P.M. (1978a) FEBS Lett. 92 214-218

Wood, P.M. (1978b) Eur. J. Biochem. 87 9-19

Wood, P.M. (1981) Anal. Biochem. 111 235-239

Wood, P.M. (1983) FEBS Lett. 164 223-226

Wood, P.M. (1984) Biochim. Biophys. Acta 768 293-317

Yang, T. (1982) Eur. J. Biochem. 121 335-341

Yang, T. (1986) Biochim. Biophys. Acta 848 342-351

Yang, T. and Jurtshuk, P. (1978a) Biochim. Biophys. Acta 502 543-548

Yang, T. and Jurtshuk, P. (1978b) Biochem. Biophys. Res. Commun. 81 1032-1039

Zimmermann, B.H., Nitsche, C.I., Fee, J.A. Rusnak, F. and Munk, E. (1988) Proc. Natl. Acad. Sci. USA 85 5779-5783

Appendix

Biochem. J. (1989) **262**, 233–240 (Printed in Great Britain)

The role of cytochrome c_4 in bacterial respiration

Cellular location and selective removal from membranes

Dominic J. B. HUNTER, Kevin R. BROWN and Graham W. PETTIGREW
Department of Preclinical Veterinary Sciences, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, U.K.

The cellular location of cytochrome c_4 in *Pseudomonas stutzeri* and *Azotobacter vinelandii* was investigated by the production of spheroplasts. Soluble cytochrome c_4 was found to be located in the periplasm in both organisms. The remaining cytochrome c_4 was membrane-bound. The orientation of this membrane-bound cytochrome c_4 fraction was investigated by proteolysis of the cytochrome on intact spheroplasts. In *P. stutzeri*, 78 % of the membrane-bound cytochrome c_4 could be proteolysed, whilst 82 % of the spheroplasts remained intact, suggesting that the membrane-bound cytochrome c_4 is on the periplasmic face of the membrane in this organism. Cytochrome c_4 was not susceptible to proteolysis on *A. vinelandii* spheroplasts, in spite of being digestible in the purified state. Cytochrome c_5 was shown to have a similar cellular distribution to cytochrome c_4. Selective removal of cytochrome c_4 from membranes of *P. stutzeri* was accomplished by the use of sodium iodide and propan-2-ol, with the retention of most of the ascorbate–TMPD (*NNN'N'*-tetramethylbenzene-1,4-diamine) oxidase activity associated with the membrane. Sodium iodide removed most of the cytochrome c_4 from *A. vinelandii* membranes with retention of 62 % of the ascorbate–TMPD oxidase activity. Cytochrome c_4 could be returned to the washed membranes, but with no recovery of this enzyme activity. We conclude that cytochrome c_4 is not involved in the ascorbate–TMPD oxidase activity associated with the membranes of these two organisms.

INTRODUCTION

Cytochromes *c* play a central role in bacterial electron transport systems (Pettigrew & Moore, 1987). Many soluble cytochromes *c* have been characterized, and Wood (1983) has proposed that these cytochromes are located in the periplasm of Gram-negative bacteria. This proposal has some experimental support; thus *Paracoccus denitrificans* cytochrome *c*-550 (Scholes *et al.*, 1971), *Thermus thermophilus* HB8 cytochrome *c*-552 (Lorence *et al.*, 1981), *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* cytochromes c_2 (Prince *et al.*, 1975), *Paracoccus denitrificans* cytochrome cd_1 (Alefounder & Ferguson, 1980), *Pseudomonas aeruginosa* cytochrome cd_1 (Wood, 1978), *Desulphovibrio vulgaris* (Marburg) cytochrome c_3 (Badziong & Thauer, 1980) and cytochrome *c* peroxidase from pseudomonads (C. F. Goodhew, D. J. B. Hunter, I. B. H. Wilson and G. W. Pettigrew, unpublished work) are all periplasmic cytochromes.

Membrane-bound cytochromes *c* have received less attention and their location and orientation are uncertain in most cases. According to Wood (1983), these cytochromes *c* should also be located in the periplasm, either attached to the periplasmic face of the cell membrane or, if part of an integral membrane protein, as a periplasmic-facing domain. An example of a protein expected to have this orientation is cytochrome c_1. This protrudes into the intermembrane space in mitochondria (Leonard *et al.*, 1981) and its bacterial counterparts are expected to be periplasmic-facing (Pettigrew & Moore, 1987).

Cytochrome c_4 is a dihaem cytochrome of M_r 20000, with a low α/β radio and a low α absorbance in the ferrohaem form (Pettigrew & Brown, 1988). It has been purified from *Azotobacter vinelandii* (Tissieres, 1956; Swank & Burris, 1969), and from *Pseudomonas aeruginosa* and *Pseudomonas stutzei* (Pettigrew & Brown, 1988), and has been reported in other pseudomonads and an *Alcaligenes* species. Pettigrew & Brown (1988) showed that the majority of cytochrome c_4 in *P. aeruginosa*, *P. stutzeri* and *A. vinelandii* was membrane-bound, with a small portion soluble. According to the proposal of Wood (1983), we would expect the soluble portion of cytochrome c_4 to be located in the periplasm and the membrane-bound portion to be on the periplasmic face of the cell membrane. The present paper uses spheroplast formation and proteolysis of intact spheroplasts to address this question.

The function of cytochrome c_4 is unknown. A role in denitrification is unlikely as it is found in the strict aerobe *A. vinelandii*. Jurtshuk *et al.* (1981) have proposed that cytochrome c_4 is the *c*-type component of the *co*-type cytochrome oxidase of *A. vinelandii*. Yang (1986) found that pure cytochrome *o* showed no reactivity with molecular oxygen, whereas a partially pure preparation containing a *c*-type cytochrome, presumed to be cytochrome c_4, showed a reaction with oxygen and possessed TMPD (*NNN'N'*-tetramethylbenzene-1,4-diamine) oxidase activity. However, there was no characterization of this *c*-type component, and the proposal of Jurtshuk *et al.* (1981) remains unsupported by firm evidence. This paper investigates the effect of selective removal of cytochrome c_4 from spheroplast membranes prepared from *A. vinelandii* and *P. stutzeri* on the ascorbate–TMPD oxidase activity of these membranes.

Abbreviations used: TMPD, *NNN'N'*-tetramethylbenzene-1,4-diamine; PAGE, polyacrylamide-gel electrophoresis; ICDH, isocitrate dehydrogenase.

MATERIALS AND METHODS

Growth of bacteria

The organisms used in this study were *Pseudomonas stutzeri* (Stainer 224, A.T.C.C. 17591) and *Azotobacter vinelandii* (strain o, A.T.C.C. 12837). *P. stutzeri* was grown aerobically at 32 °C in a medium containing trisodium citrate (5 g/l), potassium dihydrogen phosphate (1 g/l), magnesium sulphate (0.5 g/l) and yeast extract (Oxoid; 4 g/l), adjusted to pH 7.0, in 11 flasks on an orbital shaker or, for the washing experiments, in a 10 l microfermentor (New Brunswick) with sterile air at 10 l/min. *A. vinelandii* was grown in a modified Burks medium (Newton *et al.*, 1953) in the 10 l microfermentor, with vigorous aeration (15 l of air/min). Cells of each type were harvested at early stationary phase, at A_{600} = 1.2–1.5, by centrifugation at 4000 ***g*** for 30 min at 4 °C. They were washed once, by resuspension in 5 vol. of 10 mM-Tris/HCl, pH 8, at 20 °C followed by recentrifugation, and were resuspended to 0.5 g of cells/ml in 10 mM-Tris/HCl, pH 8, at 20 °C.

SDS/polyacrylamide-gel electrophoresis and haem staining

SDS/PAGE and detection of haem *c* by peroxidase action on 3,3′,5,5′-tetramethylbenzidine used the method of Goodhew *et al.* (1986). Quantification of the amount of staining of each band was by means of absorbance scans at 690 nm using a Shimadzu CS-930 t.l.c. scanner.

Isocitrate dehydrogenase assay

Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) was assayed by the method of Bernt & Bergmeyer (1974). Production of NADPH was monitored at 340 nm using a Philips PU-8740 spectrophotometer.

Ascorbate–TMPD oxidase assay

Ascorbate–TMPD oxidase activity was measured using a Clark oxygen electrode, thermostatted at 25 °C. The reaction was initiated by the addition to the chamber of a 50 μl sample, containing 10 mM-sodium phosphate, pH 7.0, 1 mM-EDTA, 1 mM-ascorbate, 0.3 mM-TMPD and water to a total volume, after the addition of sample, of 2 ml. The initial rate of activity was recorded.

Production of spheroplasts

Spheroplasts were produced from both organisms by treatment of freshly harvested cells with lysozyme and EDTA as described in the legend to Fig. 1. Spheroplast membranes could then be obtained by lysis of the spheroplasts using a low ionic strength plus EDTA treatment, liberating the cytoplasmic contents of the cells.

Proteolysis of spheroplasts

Spheroplasts were produced from each organism by lysozyme/EDTA treatment and were then proteolysed as described in the legends to Figs. 2 and 3. Proteases were chosen which cleaved cytochrome c_4 into well-defined fragments; thus subtilisin was used with *P. stutzeri* spheroplasts and thermolysin was used with *A. vinelandii* spheroplasts.

Removal of cytochrome c_4 from spheroplast membranes using sodium iodide

Cytochrome c_4 was removed from spheroplast membranes using sodium iodide, as described in the legend to Fig. 4. Return of cytochrome c_4 to the membrane was accomplished by dialysing purified cytochrome c_4, or cytochrome c_4 removed from membranes by iodide treatment, together with cytochrome c_4-depleted membranes, suspended in 10 mM-sodium phosphate, pH 7.0, containing 2 M-sodium iodide (*P. stutzeri*), or in 10 mM-sodium phosphate alone (*A. vinelandii*). Dialysis was performed against two changes each of 1 litre of 10 mM-sodium phosphate, pH 7.0, for 2 h per change. Soluble cytochrome c_4 was then separated from that returned to the membrane by centrifugation (11000 ***g***, 20 min, 4 °C) and the membrane pellets were resuspended in 10 mM-sodium phosphate, pH 7.0. Ascorbate–TMPD oxidase activities could then be measured, and the amount of cytochrome c_4 reconstituted could be assessed by means of SDS/PAGE followed by haem staining and absorbance scans at 690 nm.

Removal of cytochrome c_4 from spheroplast membranes using propan-2-ol

P. stutzeri spheroplast membranes (15 mg of protein/ml) were centrifuged from their storage medium (11000 ***g***, 30 min, 4 °C) in 2 ml aliquots and were resuspended in 2 ml of the appropriate percentage (v/v) of propan-2-ol in 10 mM-sodium phosphate, pH 7.0. After incubation at 0 °C for 30 min, the samples were centrifuged (11000 ***g***, 30 min, 4 °C) to separate the solubilized proteins from the membranes. The latter were each resuspended in 2 ml of 10 mM-sodium phosphate, pH 7.0. Ascorbate–TMPD oxidase activity and the percentage of cytochrome c_4 remaining on the membrane were then measured.

RESULTS

Location of cytochromes c_4 and c_5 in *Azotobacter vinelandii* and *Pseudomonas stutzeri*

Formation of spheroplasts. Successful spheroplast formation in both species is indicated by two features (Table 1). Firstly, little ICDH, an enzyme accepted to be a cytoplasmic marker (Wood, 1978), is released into the periplasmic fraction by lysozyme/EDTA treatment. This indicates that few spheroplasts were lysed by the treatment. Secondly, very little cytochrome *c*-551 is present in the 'membrane' fraction. This cytochrome is known to be soluble (Pettigrew & Brown, 1988) and its absence from the membrane fraction indicates that very few whole cells remain after lysozyme/EDTA treatment. These results show that the lysozyme/EDTA treatment is effective in releasing the periplasmic contents whilst allowing retention of cytoplasmic integrity. A low ionic strength step was then used to lyse the spheroplasts.

Location of the soluble forms of cytochromes c_4 and c_5. In both organisms, cytochrome c_4 is predominantly membrane-bound. However, 15–25 % is soluble, in agreement with the results of Pettigrew & Brown (1988) (Table 1). The soluble cytochrome c_4 appears almost entirely in the periplasmic fraction of each organism (Fig. 1). These periplasmic fractions show no sign of other membrane cytochromes *c*, indicating that the presence of cytochrome c_4 is not the result of contamination of the

Table 1. Location of *c*-type cytochromes in *P. stuzeri* and *A. vinelandii*

The figures in parentheses are the amounts of cytochromes *c* after purification as described in Pettigrew & Brown (1988). P, periplasm; C, cytoplasm; M, membranes. Relative areas were obtained by weighing excised peaks from the 690 nm absorbance scans of Fig. 1. The amount is given in nmol of protein/100 g of cells. This was calculated assuming linearity of haem staining (see Goodhew *et al.*, 1986) and using a known amount of cytochrome standard. Note that cytochrome c_4 is dihaem and therefore gives twice the staining intensity per mol as a monohaem cytochrome such as cytochrome *c*-551. The percentage is calculated from the proportion found in a particular fraction relative to the total. M/P is the ratio of membrane-bound to periplasmic cytochrome. For the associated figures in parentheses, M/P is the ratio of membrane-bound to soluble cytochrome *c* after purification according to Pettigrew & Brown (1988).

	P			C			M			
	Relative area	Amount	%	Relative area	Amount	%	Relative area	Amount	%	M/P
P. stutzeri:										
Cytochrome c_4	1.4	97 (35)	14	0.3	21	3	8.0	556 (205)	83	5.7 (5.9)
Cytochrome c_5	4.2	583	35	1.8	250	15	6.0	833	50	1.4
Cytochrome *c*-551	20.7	2874 (1610)	92	1.6	222	7	0.2	28 (35)	1	0.01 (0.02)
ICDH			5			77			18	
A. vinelandii:										
Cytochrome c_4	5.1	123 (79)	21	0.8	19	3	18.1	433 (315)	75	3.5 (4.0)
Cytochrome *c*-551	18.7	900 (246)	91	0.8	38	4	1.1	53 (14)	5	0.06 (0.06)
ICDH			0.1			97			3	

periplasm by membranes. Very small amounts of cytochrome c_4 (3 %) are apparently present in the cytoplasmic fractions (Table 1), but these are accompanied by traces of other membrane cytochromes (see e.g. Fig. 1*a*). Thus they are probably the result of low level contamination by membranes.

Cytochrome c_5 is predominantly membrane-bound in *P. stutzeri*. However, a sizeable fraction appears in the periplasm (Table 1). In *A. vinelandii*, quantification of the amount of cytochrome c_5 in the periplasm was not possible, as there is complexity in the haem-staining pattern in the cytochrome c_5 region of this fraction (Fig. 1*b*). Purification studies have indicated that both peaks in this region contain cytochromes which are spectrally identical to cytochrome c_5. However, as the exact identity of these peaks was not certain, the amount of cytochrome c_5 could not be obtained. In addition to the periplasmic and membrane-bound cytochrome c_5, some appears in the cytoplasmic fractions of both organisms in amounts which cannot be explained either by periplasmic cytochrome c_5 trapped adventitiously in the spheroplast pellet or by membrane contamination. This is discussed below.

A further observation in *A. vinelandii* is that protohaem is detected in the periplasmic fraction in addition to that in the membrane fraction (Fig. 1*b*). This cannot be due to contamination of the periplasmic fraction by membrane *b*-type cytochromes since, as noted previously, there is no sign of contamination of the periplasm by membrane *c*-type cytochromes.

Orientation of membrane-bound forms of cytochrome c_4 and c_5. Proteolysis of intact spheroplasts was used to investigate the membrane orientation of cytochromes c_4 and c_5. Digestion intensities are expressed as the ratio of protein/enzyme (w/w). It should be noted that 'protein' refers to the total amount of protein assumed to be accessible to the protease. In the case of a purified cytochrome this is simply the amount of cytochrome present. However, in the case of spheroplasts, accessible protein was assumed to comprise that in the periplasm plus the amount of added lysozyme plus that in the membranes. This is an overestimation of the actual amount of accessible protein as an unknown proportion of the membrane protein will be cytoplasmic-facing, deeply buried in the membrane, or otherwise inaccessible to the protease.

P. stutzeri. Subtilisin was chosen for proteolysis of *P. stutzeri* cytochrome c_4 as it produces fragments of approximately equal size after digestion at an intensity of 20:1 protein/enzyme (w/w) (Fig. 2). In addition, it possesses the advantage of being easily inhibitable by phenylmethanesulphonyl fluoride. Of total membrane-bound cytochrome c_4, 78 % is proteolysed by subtilisin under these conditions, a similar yield to proteolysis of the pure cytochrome under similar conditions. This degree of removal of cytochrome c_4 was confirmed by Western blotting of subtilisin-treated membranes, using an anti-(*P. stutzeri* cytochrome c_4) antiserum, which showed that only 19 % of the cytochrome c_4 remained on the membranes after subtilisin treatment (results not shown). Approximately two-thirds of the 78 % loss in haem-staining activity can be accounted for by the appearance of the band labelled c_4(s), which corresponds to the subtilisin fragments of pure cytochrome c_4 (Fig. 2). These fragments remain attached to the membrane after proteolysis.

After incubation, the spheroplasts treated with protease were 82 % intact (Table 2). This indicates that the

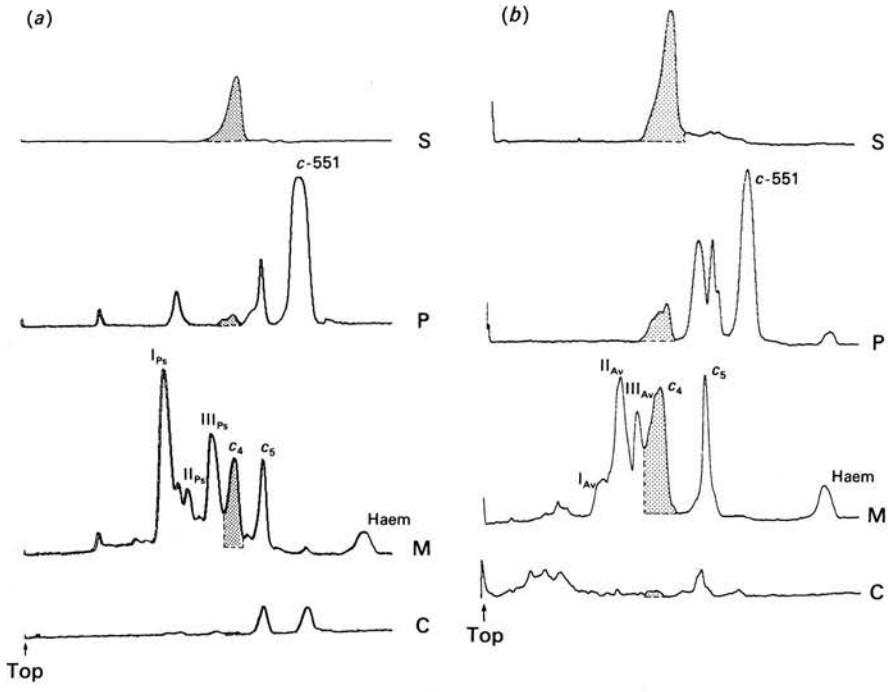


Fig. 1. Location of *c*-type cytochromes in *Azotobacter vinelandii* and *Pseudomonas stutzeri*

The periplasmic (P), cytoplasmic (C) and membrane (M) fractions from (*a*) *P. stutzeri* and (*b*) *A. vinelandii* were obtained as follows and subjected to SDS/PAGE. Washed cells (5 g; 10 ml), resuspended to 0.5 g cells/ml in 10 mM-Tris/HCl, pH 8, at 20 °C, were added to a sucrose/Tris/lysozyme/EDTA solution of volume 35 ml. The reaction mixture was incubated for 2 min at 30 °C, then $MgCl_2$ (5 ml; 100 mM) was added to give final concentrations of 0.5 M-sucrose, 40 mM-Tris/HCl, pH 8, at 20 °C, 5 mg of lysozyme (Sigma) per g of cells and 10 mM-$MgCl_2$, in a total volume of 50 ml. The final concentrations of EDTA during the production of spheroplasts were (*a*) 4 mM and (*b*) 5 mM. The mixture was then incubated for a further 30 min at 30 °C. Centrifugation (11000 ***g***, 20 min, 4 °C) separated the periplasmic fraction (P) from the spheroplasts. Lysis of the spheroplasts was then accomplished by resuspending the spheroplast pellet in 50 ml of 10 mM-Tris/HCl, pH 8/2 mM-EDTA at 20 °C. After 15 min at 20 °C, $MgCl_2$ was added to a final concentration of 4 mM. The lysed spheroplasts were then centrifuged (19000 ***g***, 30 min, 4 °C) to separate the cytoplasmic fraction (C) from the membranes (M). The latter were then resuspended in 50 ml of 10 mM-Tris/HCl, pH 7.3, at 20 °C. In the case of *A. vinelandii*, an additional low-speed centrifugation (1500 ***g***, 5 min, 4 °C) was required after spheroplast lysis, to remove a bulky white jelly which otherwise contaminated the membranes. A second centrifugation (19000 ***g***, 30 min, 4 °C) separated the glassy red membranes (M) from the clear cytoplasmic fraction (C). Samples were then subjected to SDS/PAGE on a gradient gel (10–25% polyacrylamide). The traces are absorbance scans at 690 nm following haem detection by the method of Goodhew *et al.* (1986). Three major membrane *c*-type cytochromes may be common to both species and are labelled: (*a*) I_{Ps} (38000), II_{Ps} (32000), and III_{Ps} (25000) and (*b*) I_{Av} (37000), II_{Av} (31000), and III_{Av} (25000). M_r values were obtained by comparison with marker proteins: ovalbumin, 45000; yeast cytochrome *c* peroxidase, 34000; myoglobin, 17000; horse heart cytochrome *c*, 12000 and *Pseudomonas aeruginosa* cytochrome *c*-551, 9000. These gave a linear plot of log M_r against mobility in spite of the gradient nature of the gel. Cytochromes c_4, c_5 and *c*-551 in both organisms were identified by comparison with purified samples of these proteins. Cytochrome c_5 is monomeric in SDS in agreement with the results of Carter *et al.*, (1985). Scans labelled S are lanes loaded with purified cytochrome c_4 from each organism. Only small amounts of cytochrome cd_1 were detected in the periplasm of *P. stutzeri* under these growth conditions (small peak of highest M_r).

subtilisin had access to the cytoplasmic face of the membranes in only 18% of the spheroplasts.

Both membrane-bound cytochrome c_5 and its soluble form disappear completely on proteolysis (Fig. 2). The disappearance of cytochrome c_5 from the periplasmic and membrane fractions is accompanied by the appearance of a band in the periplasm with a size just greater than that of the cytochrome c_4 fragments [c_4(s)] (Fig. 2, lane c). It is likely that this fragment derives from both soluble and membrane-bound forms of cytochrome c_5. In addition to its effect on cytochromes c_4 and c_5, proteolysis of spheroplasts with subtilisin partially removes the weakly staining band II_{Ps} from the membranes (Fig. 2, lane f). Bands I_{Ps} and III_{Ps} are not affected by the proteolysis (Table 2).

A. vinelandii. Subtilisin does not digest purified native cytochrome c_4 from *A. vinelandii*. Of other proteases tested (trypsin, chymotrypsin, thermolysin and staphylococcal V8 protease), thermolysin alone efficiently digested purified cytochrome c_4 (results not shown) to produce two fragments with an M_r of approx. 11000 and a smaller fragment whose yield increases as the intensity of digestion increases (Fig. 3, lanes e and f). At 50:1 protein/enzyme (w/w), proteolysis was essentially complete, as judged by the disappearance of the cytochrome c_4 band.

Despite this, however, thermolysin, at an intensity of 16:1 protein/enzyme (w/w), produces no loss of haem staining by the membrane-bound cytochrome c_4, indicating that no proteolysis of the cytochrome is taking place

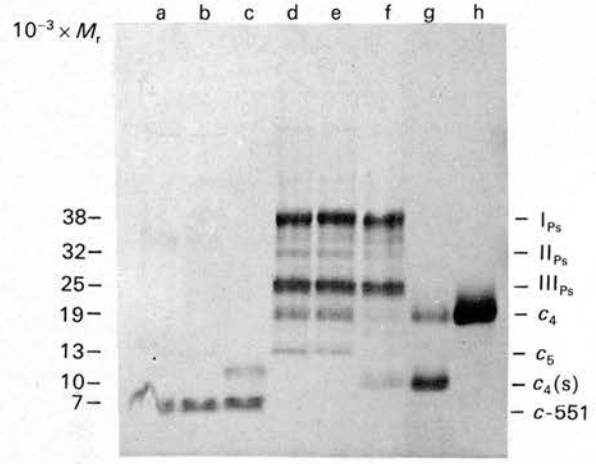


Fig. 2. Proteolysis of cytochrome c_4 on spheroplasts of *P. stutzeri*

P. stutzeri spheroplast suspension (5 ml), prepared as described in the legend to Fig. 1, was exposed to subtilisin at a digestion intensity of 20:1 (w/w) protein/enzyme for 30 min at 30 °C. Phenylmethanesulphonyl fluoride (PMSF) in propan-2-ol was then added to give a 25-fold molar excess over enzyme and a final concentration of 1% propan-2-ol. A control experiment contained no subtilisin. Periplasmic, cytoplasmic and membrane fractions were prepared from the spheroplast suspension as described in the legend to Fig. 1, and samples were subjected to SDS/PAGE. EDTA (3 mM) was used in the production of the spheroplasts. Lanes a and d contain the periplasmic and membrane fractions of untreated spheroplasts. Lanes c and f contain periplasmic and membrane fractions after subtilisin treatment. Lanes b and e contain periplasmic and membrane fractions from the control experiment. Lanes g and h each contain 1 nmol samples of purified cytochrome c_4, untreated (lane h) and treated with subtilisin (lane g) by incubation for 15 min at 30 °C at a digestion intensity of 20:1 (w/w) protein/enzyme. PMSF in propan-2-ol was then added to give a 30-fold molar excess over enzyme. The M_r values of the major membrane bands were calculated as described in the legend to Fig. 1, using the following standards: bovine serum albumin, 66000; yeast cytochrome *c* peroxidase, 34000; myoglobin, 17000; horse heart cytochrome *c*, 12000 and *P. aeruginosa* cytochrome *c*-551, 9000. c_4(s) indicates the subtilisin fragments of cytochrome c_4.

Table 2. The proteolysis of cytochrome c_4 in spheroplasts of *P. stutzeri*

Relative peak areas were obtained by weighing excised peaks from the absorbance scans of Fig. 2. Peak areas of channel f (+subtilisin) were expressed as a percentage of corresponding peaks in channel e (−subtilisin) The percentage value for c_4(s) was calculated relative to the parent c_4 peak of channel e (−subtilisin). Spheroplast intactness was calculated by expressing the combined cytoplasmic and membrane-bound ICDH activities as a percentage of the total activity. The values obtained were: −subtilisin, 72%, +subtilisin, 82%.

	Peak area		
	−Subtilisin	+Subtilisin	%
c_4	4.5	1.0	22
c_4(s)	—	2.2	49
I_{Ps}	13.8	13.1	95
III_{Ps}	10.8	11.1	103

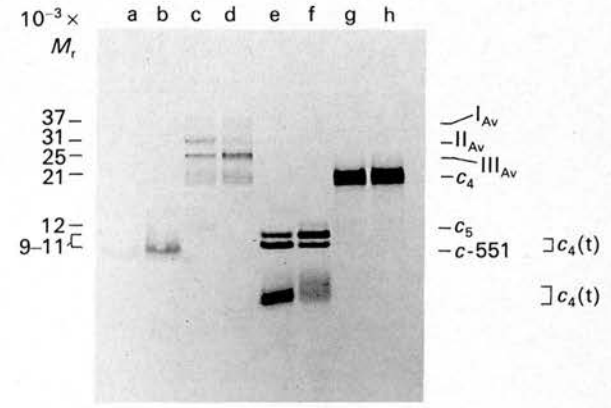


Fig. 3. Proteolysis of cytochrome c_4 on spheroplasts of *A. vinelandii*

A. vinelandii spheroplast suspension (5 ml), prepared using 5 mM-EDTA, was exposed to thermolysin at a digestion intensity of 16:1 (w/w) protein/enzyme for 30 min at 30 °C. Periplasmic, cytoplasmic and membrane fractions were prepared from the spheroplast suspension as described in the legend to Fig. 1, with the difference that no $MgCl_2$ was added after the EDTA lysis stage. This ensured inhibition of the thermolysin after the experiment by EDTA chelation of its active site metal ion. EDTA could not be added immediately after the thermolysin treatment, as premature lysis of the spheroplasts would have occurred. Lanes b and d contain periplasmic and membrane fractions treated with thermolysin as described above. Lanes a and c are corresponding fractions treated as for the proteolysis experiment, but with no addition of thermolysin. Lanes e–h contain 0.5 nmol samples of purified cytochrome c_4 untreated (lane h), treated with a 60-fold molar excess of EDTA over enzyme prior to thermolysin at an intensity of 10:1 (w/w) protein/enzyme (lane g), or treated with thermolysin at digestion intensities of 50:1 (lane f) or 10:1 (lane e) (w/w) protein/enzyme. After 30 min incubation at 30 °C, EDTA was added to those samples not already treated to stop the reaction. The M_r values of the major bands were calculated as described in the legend to Fig. 2. c_4(t) indicates the position of the thermolysin fragments of cytochrome c_4.

on the spheroplasts (Fig. 3, lanes c and d). In contrast, cytochrome c_5 is completely degraded (lane d), and probably contributes to a soluble band (M_r approx. 10000) which appears in the periplasm (lane b). Band II_{Av} is also extensively degraded and may also contribute to this soluble fragment.

The thermolysin-treated spheroplasts released only 6% of their cytoplasmic ICDH, indicating that 94% remained intact during the digestion.

Selective removal of cytochrome c_4 from membranes and effect on ascorbate–TMPD oxidase activity

Selective removal of cytochrome c_4 and cytochrome c_5 from the membranes of the two organisms was accomplished by the use of the chaotropic ion I^-. Other treatments commonly used to wash off extrinsic membrane proteins, such as high ionic strength (10 mM-sodium phosphate, pH 7.0/2.5 M-NaCl), low ionic strength (10 mM-Tris/HCl, pH 7.3) and high pH (10 mM-sodium carbonate, pH 9.5) (Berry & Trumpower, 1985) failed to remove cytochrome c_4 (results not shown). In addition, 30% (v/v) propan-2-ol removed cytochromes

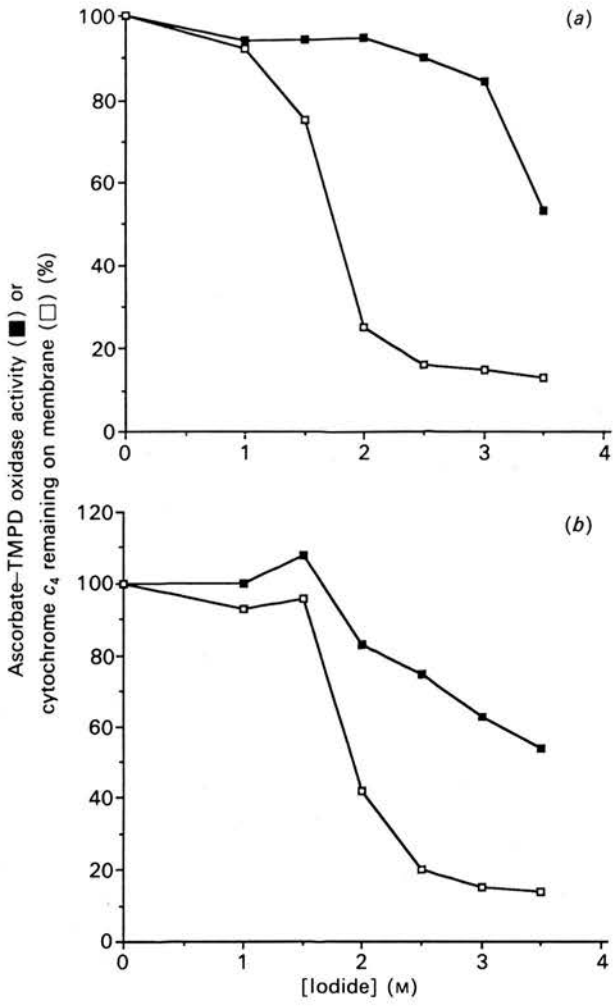


Fig. 4. Removal of cytochrome c_4 from spheroplast membranes of (*a*) *P. stutzeri* and (*b*) *A. vinelandii* by sodium iodide and the effect on ascorbate–TMPD oxidase activity

Spheroplast membranes were produced as described in the legend to Fig. 1 and were adjusted to 15 mg of protein/ml (*P. stutzeri*) or 3 mg of protein/ml (*A. vinelandii*). The iodide treatment method used was common to both organisms. Aliquots (2 ml) of membranes were centrifuged from their storage medium (11000 *g*, 20 min, 4 °C) and were then resuspended in 2 ml of the appropriate concentration of sodium iodide in 10 mM-sodium phosphate, pH 7.0. After incubation for 30 min at 0 °C, 10 mM-sodium phosphate, pH 7.0, was added to give an iodide concentration of 1 M. This allowed the membranes to pellet on centrifugation (11000 *g*, 20 min, 4 °C). The supernatants produced were dialysed against two changes of 1.5 l of 10 mM-sodium phosphate, pH 7.0, for 2 h per change. Supernatants were lyophilized and the freeze-dried material and the membrane pellets were taken up in their original incubation volume (10 mM-sodium phosphate, pH 7.0). ■, Ascorbate–TMPD oxidase activity of the membranes relative to control membranes (10 mM-phosphate, pH 7.0; 100% activity). □, Percentage of cytochrome c_4 remaining on the membrane after each treatment. The amounts of cytochrome c_4 remaining on the membrane were obtained by weighing excised peaks from 690 nm absorbance scans performed after SDS/PAGE and haem detection by the method of Goodhew *et al.* (1986). Variations in staining intensity over the gels were allowed for by normalizing each cytochrome c_4 peak relative to band III_{Ps} or III_{Av}.

c_4 and c_5 from the membranes of *P. stutzeri* but not from those of *A. vinelandii* (results not shown).

Fig. 4 shows the effect of increasing concentrations of iodide ion on the removal of cytochrome c_4 from spheroplast membranes of *P. stutzeri* and *A. vinelandii* and on the ascorbate–TMPD oxidase activities of the membranes. In both organisms, removal of cytochrome c_4 is apparently incomplete, with around 15% remaining on the membrane even when 3.5 M-iodide is used. However, the true amount of cytochrome c_4 remaining is probably less than this, for two reasons. Firstly, there are several minor bands which stain for haem and which migrate close to the position of cytochrome c_4. It is difficult to distinguish the staining by residual cytochrome c_4 from staining of these. Secondly, cytochrome c_4 and band III are not completely resolved. Band III therefore makes some contribution to the cytochrome c_4 staining which is difficult to allow for accurately. For these reasons, the amount of cytochrome c_4 shown as remaining on the membrane after 3.5 M iodide treatment should be regarded as an upper estimate.

Experiments similar to the iodide experiments were performed using propan-2-ol as the washing agent, with *P. stutzeri* spheroplast membranes. Again, removal of cytochrome c_4 was apparently incomplete, probably for the reasons stated previously. However, with 30% propan-2-ol, only 28% of the cytochrome c_4 remained on the membrane, whilst 90% of the ascorbate–TMPD oxidase activity was retained (results not shown).

Both removed and pure cytochrome c_4 could be returned to depleted membranes. In *P. stutzeri*, 65–78% of the original amount could be returned to membranes washed with 2 M-iodide with no effect on the oxidase activity. In *A. vinelandii*, 92% could be returned to membranes washed with 3 M-iodide. No recovery of activity was observed (results not shown).

DISCUSSION

Location and orientation of cytochromes c_4 and c_5

Wood (1983) has proposed that all *c*-type cytochromes are located either in the periplasm or on the periplasmic side of the cell membrane in Gram-negative organisms. This paper provides further evidence for the proposal and also confirms and extends the conclusions of Pettigrew & Brown (1988) who showed that cytochrome c_4 exists in two forms, one soluble and one membrane-bound, in *P. stutzeri* and *A. vinelandii*.

Spheroplasts were successfully produced from both organisms under investigation, allowing the cellular location of the soluble fractions of cytochromes c_4 and c_5 to be determined. Almost all of the soluble cytochromes c_4 and c_5 were found to be located in the periplasm, with a small proportion in the cytoplasm. These apparently cytoplasmic cytochromes are actually the result of contamination of the cytoplasmic fraction by membranes in the case of cytochrome c_4 from both organisms. However, the amount of cytochrome c_5 in the 'cytoplasm' of both organisms is too great to be explained by such contamination, or by periplasmic cytochrome c_5 being trapped in adventitious liquid in the spheroplast pellet, prior to lysis. We propose that this 'cytoplasmic' cytochrome c_5 is actually membrane cytochrome c_5 which has been washed off the spheroplast membranes by the treatment used to lyse the spheroplasts (low ionic strength plus EDTA).

A further proposal of Wood (1983) is that *b*-type cytochromes should not appear in the periplasm of Gram-negative organisms, as their non-covalently-bound haem would be easily lost to the medium. However, protohaem was detected in the periplasmic fraction of *A. vinelandii*. This may suggest that a soluble *b*-type cytochrome exists in the periplasm, but a number of other explanations for the presence of protohaem should be considered. Firstly, the protohaem is not due to the presence in the periplasmic fraction of membrane-bound *b*-type cytochromes attached to contaminating membranes, as no corresponding membrane-bound *c*-type cytochromes are detected. It is possible, however, that certain membrane cytochromes *b* are removed from the spheroplast membranes by the lysozyme/EDTA treatment used to produce the spheroplasts. Another possibility is that protohaem alone is removed by lysozyme/EDTA. Secondly, the protohaem may be the prosthetic group of a soluble, periplasmic peroxidase or of a haemoglobin-like protein.

Investigation of the membrane orientation of a protein requires the use of a modifying agent which is membrane-impermeant. In this respect, proteases are very useful since their size precludes their passing across the membrane. The data presented here demonstrate their use in such an investigation.

Purified cytochromes c_4 from *P. stutzeri* and *A. vinelandii* are cleavable using subtilisin and thermolysin respectively. Both proteases cleave the cytochromes c_4 into two approximately equal halves. In addition, a lower-M_r minor fragment is produced in the case of *A. vinelandii* cytochrome c_4, which increases in amount as the digestion intensity increases. This is presumably the result of further degradation of the fragments. The major fragments produced correspond to the two domains of the dihaem cytochrome, each containing a single haem. The proteases therefore probably act in the interdomain region of the cytochrome (Brown, 1988).

Using subtilisin, we have been able to show that 78% of the membrane-bound cytochrome c_4 in *P. stutzeri* is digested, whilst only 18% of the spheroplasts released their cytoplasmic ICDH. The simplest interpretation of these results is that membrane-bound cytochrome c_4 faces the periplasm. Nevertheless, we cannot exclude the possibility that a portion of the cytochrome c_4 faces the cytoplasm. An alternative possibility is that cytochrome c_4 faces the cytoplasm but possesses a transmembrane portion which is attacked by the subtilisin. However, there is no indication of a hydrophobic, membrane-spanning sequence in the amino acid sequence of *A. vinelandii* cytochrome c_4 (Ambler *et al.*, 1984) to which the cytochrome c_4 from *P. stutzeri* is thought to be closely related. Also, a significant proportion of cytochrome c_4 is soluble, which would not be expected for an integral membrane protein. Therefore, the 78% digestion of cytochrome c_4 in 82% intact spheroplasts leads us to conclude that a large proportion, and probably all, membrane-bound cytochrome c_4 is periplasm-facing in *P. stutzeri*.

Using thermolysin, it was not possible to proteolyse cytochrome c_4 on *A. vinelandii* spheroplasts. Thus the cytochrome c_4 must either face the cytoplasm in *A. vinelandii* or be in a position, or of a conformation, not susceptible to cleavage by thermolysin. By comparison with *P. stutzeri*, the latter is more likely.

In both organisms, cytochrome c_5 is digested on the spheroplast membranes, producing a smaller soluble fragment. Membrane-bound cytochrome c_5 is therefore periplasm-facing. In addition, the membrane cytochrome band II is also digested, suggesting that the haem-containing moiety faces the periplasm in this case also. The other membrane cytochromes *c* (bands I and III) are not digested, and must either face the cytoplasm or be otherwise inaccessible to the proteases used.

Involvement of cytochrome c_4 in ascorbate–TMPD oxidase activity

Jurtshuk *et al.* (1981) have proposed that cytochrome c_4 forms the *c*-type component of the *co*-type cytochrome oxidase of *A. vinelandii*. However, this was based on the very undiscriminating criterion of membrane spectra; no positive identification of the *c*-type cytochrome with purified cytochrome c_4 was made. In the present study, the effect on ascorbate–TMPD oxidase activity of selective removal of cytochrome c_4 from membranes of *P. stutzeri* and *A. vinelandii* has been investigated.

In the case of *P. stutzeri*, treatment of membranes with 2–3 M-iodide or 30% (v/v) propan-2-ol results in the removal of at least 75–85% of the cytochrome c_4 with almost no loss of ascorbate–TMPD oxidase activity. Purified cytochrome c_4, or the iodide extract, could be reconstituted with the washed membranes with no recovery of activity.

In *A. vinelandii*, removal of cytochrome c_4 by iodide is associated with loss of ascorbate–TMPD oxidase activity. However, with 3 M-iodide, approx. 63% of the activity is still present, with only 15% of the cytochrome c_4 remaining. Return of purified cytochrome c_4, or of the iodide extract, to the washed membranes results in no recovery of activity.

These results indicate that cytochrome c_4 is probably not essential for ascorbate–TMPD oxidase activity. However, this cannot be stated with complete certainty for two reasons. Firstly, there remains a small residual amount of cytochrome c_4 on the washed membranes. Secondly, the lack of a positive control in the reconstitution experiments means that changes to the cytochrome c_4 during extraction or purification, or changes to the oxidase during washing with iodide, cannot be ruled out.

Cytochrome c_5 is removed by iodide in parallel to the removal of cytochrome c_4 in both organisms. This may indicate some similarity in the mechanism of attachment of the two cytochromes to the membrane and suggests that cytochrome c_5 is also not involved in ascorbate–TMPD oxidase activity.

Chaotropic agents, of which iodide is an example, have been frequently used in the selective removal of membrane proteins (Hatefi & Hanstein, 1974). For example, succinate dehydrogenase from *Rhodospirillum rubrum* has been isolated by the use of 0.85 M-perchlorate (Hatefi & Davies, 1972). Iodide and perchlorate have similar chaotropic potencies (Hatefi & Hanstein, 1974) and the requirement here for higher iodide concentrations indicates that cytochrome c_4 is more tightly bound to the membrane than succinate dehydrogenase.

Conclusions

We conclude that cytochromes c_4 and c_5 are predominantly membrane-bound proteins which face the

periplasmic space. In the case of *P. stutzeri*, almost all the cytochrome c_4 can be removed with no effect on ascorbate–TMPD oxidase activity. In the case of *A. vinelandii*, removal of most of the cytochrome c_4 is accompanied by a loss of 50% of ascorbate–TMPD oxidase activity, but return of the pure cytochrome to the membrane does not allow recovery of activity. Thus we propose that cytochrome c_4 has no involvement in ascorbate–TMPD oxidase activity, in contrast to the proposal of Jurtshuk *et al.* (1981). If the cytochrome *o* in these organisms is a *co*-type oxidase, the *c*-type component is not cytochrome c_4. This component may, however, be one of the other *c*-type membrane bands. The relatively high redox potentials of cytochrome c_4 (in *A. vinelandii*, 317 and 263 mV; in *P. stutzeri*, 300 and 190 mV; Leitch *et al.*, 1985) do, however, suggest that it is positioned close to the terminal oxidase.

The work was supported by Science and Engineering Research Council Grant GRD 45161 and research studentships to D.J.B.H. (University of Edinburgh, Faculty of Medicine Gordon Lennie Bursary) and K.R.B. (S.E.R.C., instant award).

REFERENCES

Alefounder, P. R. & Ferguson, S. J. (1980) Biochem. J. **192**, 231–240
Ambler, R. P., Daniel, M., Melis, K. & Stout, C. D. (1984) Biochem. J. **222**, 217–227
Badziong, W. & Thauer, R. K. (1980) Arch. Microbiol. **125**, 167–174
Bernt, E. & Bergmeyer, H. U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 624–627, Academic Press, New York
Berry, E. A. & Trumpower, B. L. (1985) J. Biol. Chem. **260**, 2458–2467
Brown, K. R. (1988) Ph.D Thesis, University of Edinburgh
Carter, D. C., Melis, K. A., O'Donnell, S. E., Burgess, B. K., Furey, W. F., Wang, B.-C. & Stout, C. D. (1985) J. Mol. Biol. **184**, 279–295
Goodhew, C. F., Brown, K. R. & Pettigrew, G. W. (1986) Biochim. Biophys. Acta **852**, 288–294
Hatefi, Y. & Davies, K. A. (1972) Arch. Biochem. Biophys. **152**, 613–618
Hatefi, Y. & Hanstein, W. G. (1974) Methods Enzymol. **31**, 770–790
Jurtshuk, P., Mueller, T. J. & Wong, T. Y. (1981) Biochim. Biophys. Acta **637**, 374–382
Leitch, F. A., Brown, K. R. & Pettigrew, G. W. (1985) Biochim. Biophys. Acta **808**, 213–218
Leonard, K., Wingfield, P., Arad, T. & Weiss, H. (1981) J. Mol. Biol. **149**, 259–274
Lorence, R. M., Yoshida, T., Findling, K. L. & Fee, J. A. (1981) Biochem. Biophys. Res. Commun. **99**, 591–599
Newton, J. W., Wilson, P. W. & Burris, R. H. (1953) J. Biol. Chem. **204**, 445–451
Pettigrew, G. W. & Brown, K. R. (1988) Biochem. J. **252**, 427–435
Pettigrew, G. W. & Moore, G. R. (1987) Cytochromes *c* – Biological Aspects, Springer-Verlag, Berlin
Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandri, B. A. & Crofts, A. R. (1975) Biochim. Biophys. Acta **387**, 212–227
Scholes, P. B., Martin, G. & Smith, L. (1971) Biochemistry **10**, 2072–2075
Swank, R. T. & Burris, R. H. (1969) Biochim. Biophys. Acta **180**, 473–489
Tissieres, A. (1956) Biochem. J. **64**, 582–589
Wood, P. M. (1978) FEBS Lett. **92**, 214–218
Wood, P. M. (1983) FEBS Lett. **164**, 223–226
Yang, T. (1986) Biochim. Biophys. Acta **848**, 342–351

Received 12 January 1989/28 April 1989; accepted 5 May 1989