THE UNIVERSITY OF CALGARY

PURIFICATION AND CHARACTERIZATION OF *BACILLUS SUBTILIS* CYTOCHROME OXIDASE

by

JOE ALBANESE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, " Purification and Characterization of *Bacillus subtilis* Cytochrome Oxidase " submitted by Joe Albanese in partial fulfillment of the requirements for the degree of Master of Science.

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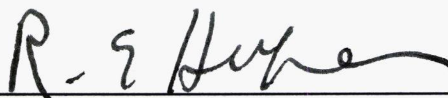

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ABSTRACT

Purification and Characterization of *Bacillus subtilis* Cytochrome Oxidase.

Cytochrome oxidase from *Bacillus subtilis* was purified to a heme A to protein ratio of 7.8 nmoles heme A/mg protein. The protein was extracted from membrane vesicles with a 3:1 Triton X-100:sodium cholate detergent mixture and purified by anion exchange chromatography on a DEAE-cellulose or a Biorad Econo-Pac® Q Cartridge followed by chromatography on a Pharmacia Mono® Q column. The enzyme has absorption maxima at 444 nm and 601 nm in the reduced *minus* oxidized spectrum, which are typical of a cytochrome-aa_3-type oxidase. In contrast to bovine heart cytochrome oxidase, *Bacillus subtilis* cytochrome oxidase lacks an absorption band in the near infrared at 830 nm. *Bacillus subtilis* oxidase reacts with carbon monoxide in its reduced state to yield a difference spectrum with absorption peaks at 429 nm and 590 nm and a trough at 444 nm. The carbon monoxide complex is photosensitive and exposure of the carbon monoxide complex to light gives a difference spectrum with a peak at 430 nm and a trough at 445 nm. The rate of carbon monoxide binding is determined by following carbon monoxide recombination after photolytic dissociation. The observed rate of 50 sec^{-1} is close to the value seen with the carbon monoxide complex of beef heart cytochrome oxidase. Addition of cyanide to the oxidized enzyme shifts the Soret maximum from 416 nm to 424 nm. *Bacillus subtilis* cytochrome oxidase reveals three principle bands (55, 37 and 29 kDaltons) when subjected to sodium dodecyl sulphate polyacrylamide gel electro-phoresis. The purified enzyme appears to bind 1 equivalent of horse heart

cytochrome *c* per cytochrome oxidase, but no respiratory activity is observed when the protein is assayed with sodium ascorbate and tetramethyl-*p*-phenylenediamine in the presence or absence of horse heart cytochrome *c*. The enzyme is also inactive when assayed with tetrachlorohydroquinone, the coenzyme Q analogue. These results will be discussed in the context of structural and functional Models of the bacterial and mitochondrial terminal oxidases.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
cyt	Cytochrome
Na_2EDTA	Disodium ethylenediaminetetraacetate
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
glycerol-3-P	Glycerol-3-phosphate
FeS	Iron sulphur cluster
MQ-7	Menaquinone-7
Mol. wt.	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced form)
Reduct.	Reductase
SDS	Sodium dodecyl sulphate
succ.	Succinate
TCHQ	Tetrachlorohydroquinone
TMPD	Tetramethyl-*p*-phenylenediamine
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UQ-8	Ubiquinone-8

INTRODUCTION

In eukaryotic cells oxidative phosphorylation occurs in the inner mitochondrial membrane (Hatefi, 1985). Reduced substrates, such as succinate donate electrons to the electron transport chain and in the process become oxidized. The composition of the four integral membrane protein complexes involved in electron transport, which supplies the free energy for the synthesis of ATP, are given in Table 1.

TABLE 1
Enzymes responsible for the catalysis of oxidative phosphorylation

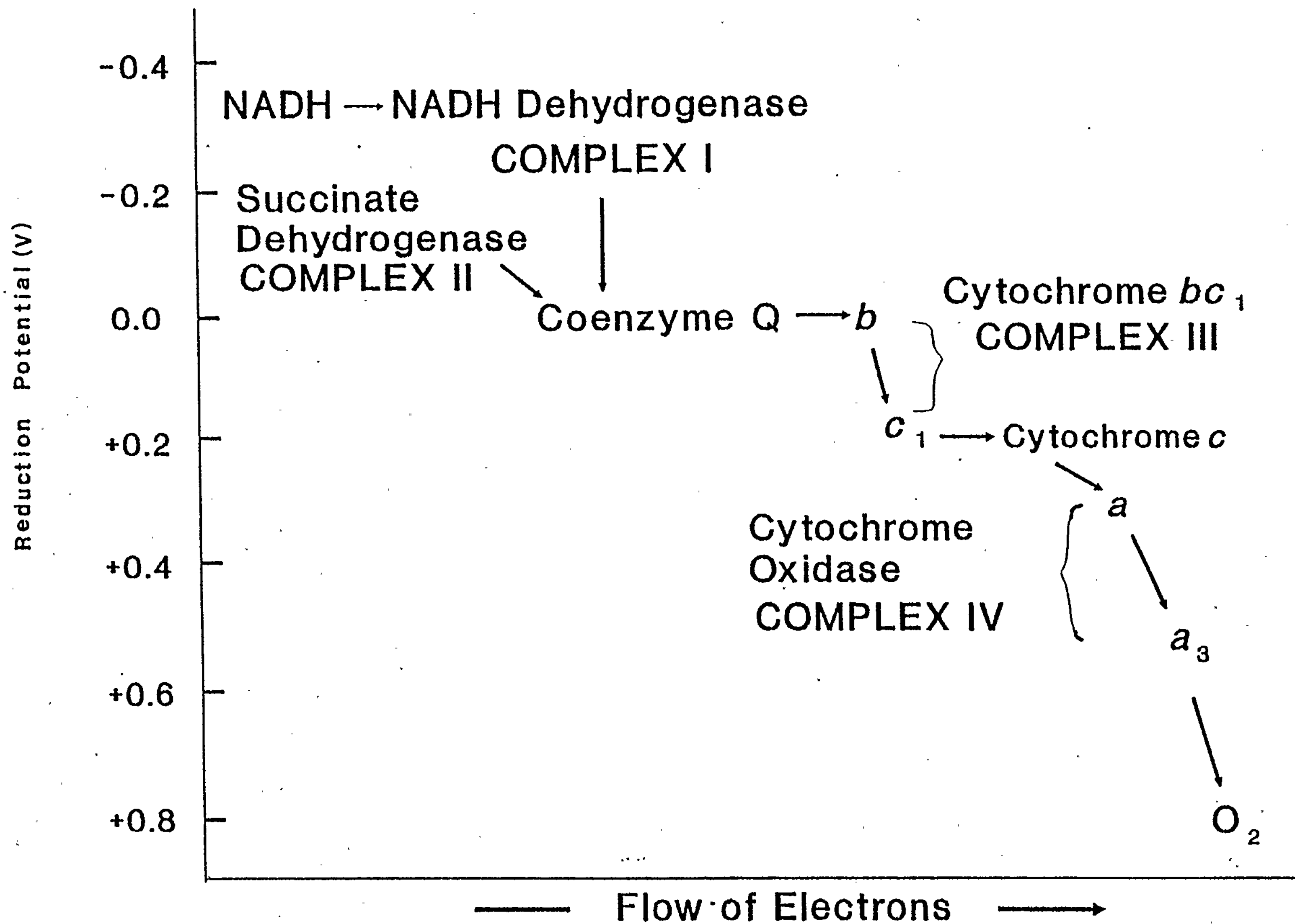
Complex	Mol. wt.	Prosthetic Groups
I NADH Dehydrogenase	1×10^6	FMN, FeS (23 Fe/FMN)
II Succinate Dehydrogenase	1×10^5	FAD, FeS (9 Fe/FAD)
III Cytochrome bc_1	2×10^5	*b*- and *c*-type hemes FeS (2 Fe/cyt c_1)
IV Cytochrome oxidase	2×10^5	*a*-type hemes (a, a_3) Cu (2Cu/ cyt *a*)

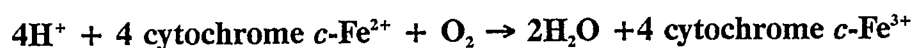
(Modified from Zubay, 1988)

These complexes are organized such that electrons donated from substrates (eg., NADH, succinate, etc.) flow down a free energy gradient from a centre with the most negative redox potential to a redox centre with a more positive potential (see figure 1). In this scheme, cytochrome oxidase is the terminal oxidase (Hatefi *et al.*, 1974a and 1974b).

Cytochrome oxidase (ferrocytochrome *c*: O_2 oxidoreductase; E.C.1.9.3.1) catalyzes electron transfer from reduced cytochrome *c* to molecular oxygen, thereby reducing it to water. This is a four electron process and proceeds according to:

Figure 1. Schematic representation of the functional relationship of the four enzyme complexes of the mitochondrial electron transport-oxidative phosphorylation system. Redox potentials are given in volts, with NADH having the most negative redox potential and oxygen the most positive redox potential.


-0.4
-0.2
0.0
+0.2
+0.4
+0.6
+0.8
Reduction Potential (V)
NADH → NADH Dehydrogenase
COMPLEX I
Succinate
Dehydrogenase
COMPLEX II
Coenzyme Q → b
Cytochrome bc 1
COMPLEX III
c 1 → Cytochrome c
a
Cytochrome
Oxidase
COMPLEX IV
a 3
O 2
Flow of Electrons



$$4H^{+} + 4 \text{ cytochrome } c\text{-}Fe^{2+} + O_2 \rightarrow 2H_2O + 4 \text{ cytochrome } c\text{-}Fe^{3+}$$

Equation (1)

where cytochrome *c*-Fe^{2+} is reduced cytochrome *c* and cytochrome *c*-Fe^{3+} is oxidized cytochrome *c*.

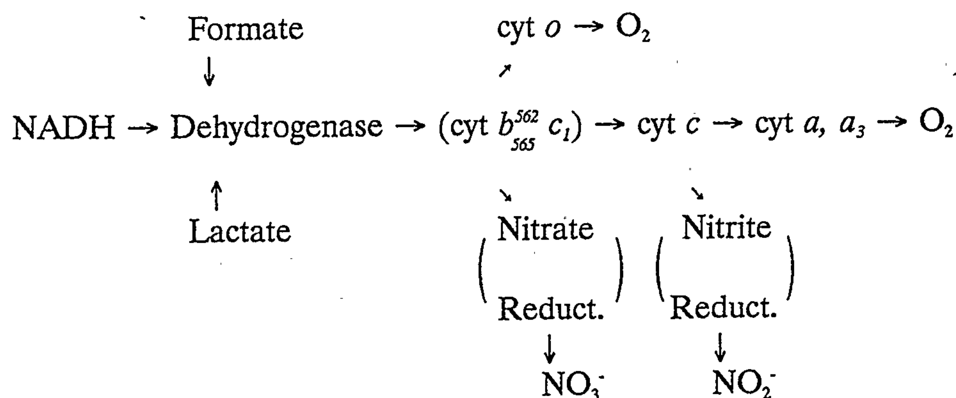
The Gibbs free energy derived from the redox reaction shown in equation 1 is conserved via two different mechanisms. The electron transfer reaction takes place such that electrons from cytochrome *c* are delivered on the cytosolic surface and are passed to oxygen which binds at the matrix side. This vectorial electron transfer generates an electrochemical potential across the inner mitochondrial membrane; positive outside, negative inside. The electron transfer is accompanied by movement of positive charge (H^{+}) from the matrix to the cytoplasmic side of the membrane and results in formation of an additional potential gradient (Mitchell and Moyle, 1965). The combination of these two gradients form the driving force (i.e. proton motive force) responsible for ATP synthesis (Mitchell, 1979).

Bacterial Respiratory Chains.

Some bacteria (e.g., *Paracoccus denitrificans*, and *Bacillus subtilis*) possess respiratory chains which display similarities to the mitochondrial electron transport chain with respect to their electron carriers (Dawes, 1986). In these bacteria the major role of the respiratory chain is the electrogenic translocation of protons out of the cytoplasm. The ensuing electrochemical proton gradient across the cytoplasmic membrane can be utilized to drive adenosine triphosphate synthesis (Anraku and Gennis, 1987). John and Whatley, (1975) have demonstrated that when grown

aerobically, *Paracoccus denitrificans* synthesizes a respiratory chain which, in its constituents and functional design, more closely resembles the electron transport chain of the inner mitochondrial membrane than that of other bacteria. Some features which are common to the mitochondrial respiratory chain and the aerobic electron transport chain of *Paracoccus denitrificans* include (1) ubiquinone-10 as the only functional ubiquinone of the respiratory chain, (2) two spectrally distinguishable b-type, as well as, two c-type cytochromes and (3) cytochrome $a + a_3$ acting as terminal oxidase.

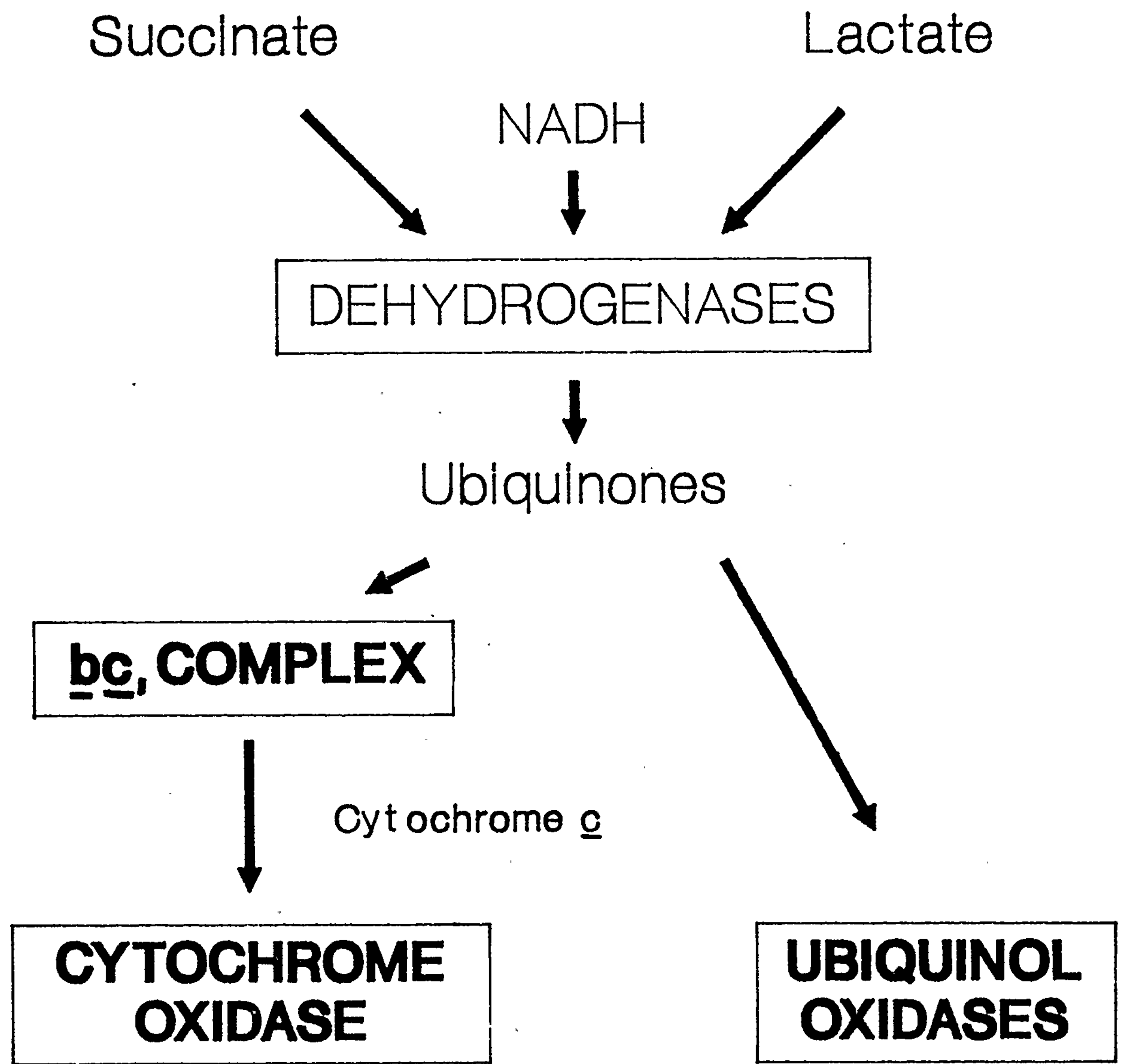
Unlike the mitochondrial system, the respiratory chain of most bacteria is branched (see figure 2 and Anraku and Gennis, 1987); this is also true of *Paracoccus denitrificans* (see scheme 1).



Scheme 1: Respiratory chain of *Paracoccus denitrificans*.

In this bacterium, electrons donated by reduced substrates (eg., NADH) flow to a bc_1-type complex where, under aerobic conditions, these electrons are transported to cytochrome aa_3 via a c-type cytochrome. Under low oxygen concentration, electrons are donated from the bc_1 complex directly to cytochrome o. In the absence of

Figure 2. Branching of a bacterial respiratory system.

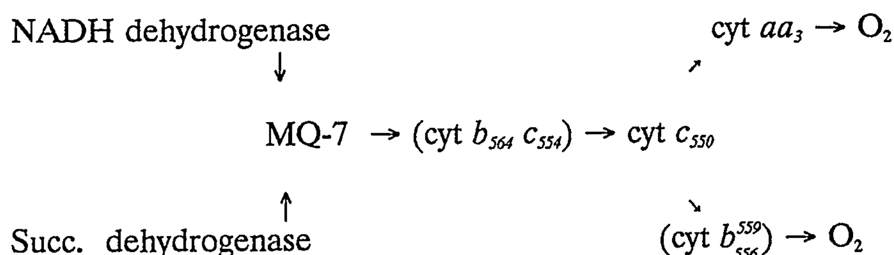


Succinate
NADH
Lactate
DEHYDROGENASES
Ubiquinones
bc1 COMPLEX
Cytochrome c
CYTOCHROME OXIDASE
UBIQUINOL OXIDASES

oxygen, electrons can be shuttled from cytochrome *bc*$_1$ directly to nitrate reductase, where NO_3^- serves as a terminal electron acceptor, or via cytochrome *c* to nitrite reductase (Gottschalk, 1988). The latter enzyme is composed of both a *c*-type cytochrome and a *d*-type cytochrome and, in addition to its NO_2^- reductase activity, it also can use oxygen as a terminal electron acceptor (Lam and Nicholas, 1969 and Newton, 1969).

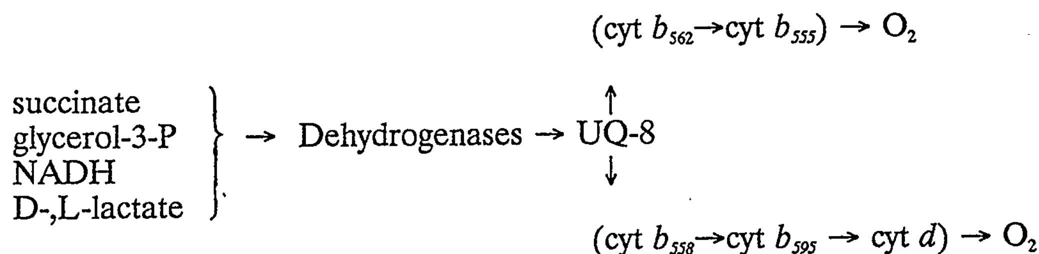
As is the case for *Paracoccus denitrificans*, the respiratory chain of *Bacillus subtilis* consists of cytochromes which are spectrophotometrically similar to mammalian mitochondrial cytochromes (Lemberg and Barrett, 1973). Two terminal oxidases have been identified in the electron transport chain: *aa*$_3$-type cytochrome oxidase (Miki *et al.*, 1967) and cytochrome *o* (James *et al.*, 1989 and de Vrij *et al.*, 1987). The latter oxidase contains two cytochrome *b* components, cytochrome b_{556} and cytochrome b_{559}. Both terminal oxidases have CO binding properties. In addition to the oxidases there is evidence to suggest the existence of a *bc*$_1$ complex in the respiratory chain of *Bacillus subtilis* (de Vrij *et al.*, 1987). This complex consists of cytochrome b_{564} associated with cytochrome c_{554}. The respiratory chain of *Bacillus subtilis* includes at least two dehydrogenases: succinate dehydrogenase (Hederstedt, 1980) and a NADH dehydrogenase (Bergsma *et al.*, 1982). As in *Escherichia coli*, the succinate dehydrogenase complex contains a *b*-type cytochrome; in *Bacillus subtilis*, this cytochrome *b* species has an absorption maximum in its difference spectrum at 558 nm as opposed to 556 nm for *Escherichia coli* (Anraku, 1988). A cytochrome c_{550} has also been isolated from *Bacillus subtilis* (Miki and Okunuki, 1969), and recently the gene coding for the protein was isolated and sequenced (von Wachenfeldt and

Hederstedt, 1990). In contrast to the water soluble cytochrome *c* of *Paracoccus denitrificans* and mitochondria, cytochrome c_{550} of *Bacillus subtilis* is membrane bound. Although tentative, the following scheme has been proposed for the operational sequence of cytochromes in the respiratory chain from *Bacillus subtilis* (de Vrij *et al.*, 1987);



<u>Scheme 2</u>: Tentative representation of the respiratory chain in *Bacillus subtilis.*

Escherichia coli can express two terminal oxidases which use oxygen as a final electron acceptor. These are the cytochrome *o* complex at high oxygen concentrations and the cytochrome *d* complex at low oxygen concentrations. Each functions as a ubiquinol oxidase (Chepuri *et al.*, 1990). Thus, in contrast to *Paracoccus denitrificans*, *Bacillus subtilis* and mitochondrial respiratory chains, the respiratory chain of *Escherichia coli* contains no cytochrome *c*-dependent branch under aerobic conditions (see scheme 3). Like the cytochrome oxidases of *Para-*



<u>Scheme 3:</u> Aerobic respiratory chain of *Escherichia coli.*

coccus denitrificans, *Bacillus subtilis* and mitochondria, both *Escherichia coli* oxidases react with carbon monoxide (Revsin and Brodie, 1969 and Lemberg and Barrett, 1973). The cytochrome *o* complex has been shown to contain one cytochrome b_{555}, one cytochrome b_{562} and two copper atoms per enzyme molecule (Anraku, 1988). It is suggested that cytochrome b_{555} is the component responsible for binding CO and O_2. The component responsible for the CO reactivity with cytochrome *d* complex is cytochrome *d* (Lorence *et al.*, 1986). Cytochromes b_{558} and b_{595} comprise the remaining two components of the complex.

In addition to the NADH and succinate dehydrogenases present in mitochondria, *Bacillus subtilis* and *Paracoccus denitrificans*, *Escherichia coli* also contains D-(-)-lactate, L-lactate-, and *sn*-glycerol-3-phosphate dehydrogenases which can also transfer electrons to the terminal oxidases via ubiquinone-8 (Anraku and Gennis, 1987 and Anraku, 1988).

Although ubiquinone-8 accounts for 85% of the total ubiquinone found in *Escherichia coli*, also present in variable quantities are ubiquinones-1 through -7 (Daves *et al.*, 1967 and Bragg, 1980). Under anaerobic conditions, Menaquinone-8 (vitamin K12) functions to transfer electrons to the cytochrome *d* complex.

Structure of Beef Heart Cytochrome Oxidase.

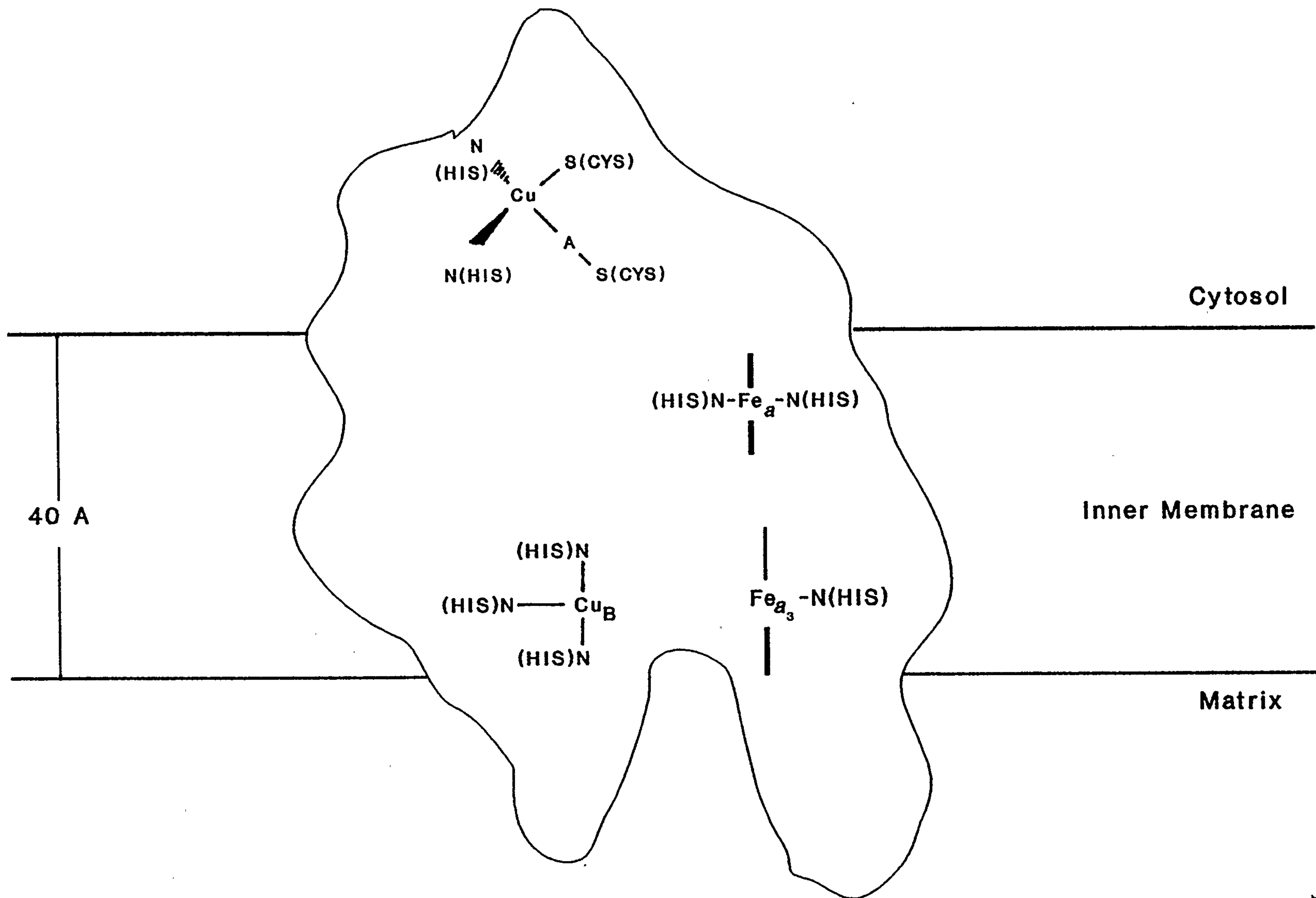
The isolation of mammalian cytochrome oxidase can be achieved by a variety of methods (Yonetani, 1961; Fowler *et al.*, 1962 and Kuboyama *et al.*, 1972). These methods are based on detergent extraction followed by fractional ammonium sulphate precipitation to separate other membrane proteins (eg., cytochrome bc_1) from

cytochrome *aa*$_3$. A pure preparation of beef heart cytochrome *c* oxidase contains 10 nmoles of heme A/mg protein (Yonetani, 1961). The enzyme is composed of at least 13 different subunits which can be resolved by polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (Azzi, 1980 and Chan and Li, 1990). Subunits I, II and III are encoded by mitochondrial DNA and are synthesized in the mitochondrial matrix, the remaining subunits are encoded by nuclear DNA and are synthesized in the cytosol. Beef heart cytochrome oxidase contains four redox metal centres; two hemes A, designated as cytochromes *a* and a_3 and two copper ions, Cu_A and Cu_B (see figure 3) (Wikström *et al.*, 1981; Van Gelder, 1966 and Malmström, 1979). One zinc and one magnesium atom per molecule of oxidase are also present in purified beef heart cytochrome oxidase (Einarsdottir and Caughey, 1984, 1985).

Heme A in cytochrome *a* is liganded by two histidine residues (Babcock *et al.*, 1981), Cu_A by two cysteine residues and two histidine residues (Stevens *et al.*, 1982), heme A of cytochrome a_3 by one histidine (Stevens and Chan, 1981) and Cu_B by three cysteine residues (Cline *et al.*, 1983). The distance between heme A of cytochrome *a* and Cu_A in the enzyme has been measured as 8 - 10 Å (Goodman and Leigh, 1985), that between cytochrome *a* and cytochrome a_3 as 12 - 16 Å (Ohnishi *et al.*, 1982) and between cytochrome a_3 and Cu_B as 3 - 4 Å (Powers *et al.*, 1981). The Zn atom is liganded by three sulphur containing residues, either cysteine or methionine residues and one histidine (Naqui *et al.*, 1988).

Cu_A and cytochrome *a* form a binuclear redox centre to which electrons from reduced cytochrome *c* are transferred. Electrons from this centre are relayed to a

Figure 3. Schematic representation of beef heart cytochrome oxidase in the inner mitochondrial membrane. The redox centres and the distances between these centres are not drawn to scale. S(CYS), cysteine residue. N(HIS), histidine residue.



N
(HIS)
Cu
S(CYS)
A
N(HIS)
S(CYS)
Cytosol
(HIS)N-Fe$_a$-N(HIS)
40 A
Inner Membrane
(HIS)N
(HIS)N— Cu$_B$
(HIS)N
Fe$_{a_3}$-N(HIS)
Matrix

second binuclear redox centre formed by cytochrome a_3 and Cu_B, which binds dioxygen and in turn, reduces it to water (Chan and Li, 1990). It has been suggested that cytochrome *a*, cytochrome a_3, and Cu_B reside in subunit I, whereas Cu_A is contained in subunit II and that this latter subunit also forms the binding site for cytochrome *c* (Holm *et al.*, 1987). The proximity of Cu_A to the cytochrome *c* binding site in subunit II raises the possibility that Cu_A is the primary electron acceptor from cytochrome *c* (Bisson *et al.*, 1980; Bisson *et al.*, 1978; and Bisson *et al.*, 1982). This view is not unanimous, see Brunori *et al.*, (1981). The roles of Zn and Mg remain undefined (Capaldi, 1990). Subunit III has been linked to proton pumping (Wikström *et al.*, 1981; and Capaldi, 1990). Removal of this subunit from the enzyme, however, does not abolish proton translocation by the enzyme, but the rate of translocation is decreased significantly. This suggests that subunit III is not fully responsible for proton pumping, although, it is an important component for this function. It has been suggested that those subunits encoded by nuclear DNA contain sites responsible for controlling the assembly of cytochrome oxidase in the inner mitochondrial membrane, the quantity of enzyme synthesized, as well as, the regulation of cytochrome oxidase activity (Capaldi, 1990).

Structure of Bacterial Cytochrome Oxidases.

In contrast to the structural complexity exhibited by beef heart cytochrome oxidase, aa_3-type cytochrome oxidases from bacterial sources (*Bacillus subtilis, Paracoccus denitrificans, Thiobacillus novellus, Bacillus stearothermophilus, Thermus thermophilus*) reveal no more than 3 subunits when the enzymes are subjected to

sodium dodecyl sulphate polyacrylamide gel electrophoresis (de Vrij *et al.*, 1983; Ludwig and Schatz, 1980; Berry and Trumpower, 1985; Yamanaka and Fujii, 1980; de Vrij *et al.*, 1989; Sone and Yanagita, 1982 and Fee *et al.*, 1980). The three subunits of bacterial aa_3-type cytochrome oxidases are homologous to the three subunits of mammalian cytochrome oxidase encoded by mitochondrial DNA (Raitio *et al.*, 1987). In *Paracoccus denitrificans*, subunits I and II form the catalytic core which binds and subsequently reduces molecular oxygen. These two subunits contain the four metal centres: two hemes A and Cu_B present in subunit I and Cu_A contained in subunit II (Raitio *et al.*, 1987 and Holm *et al.*, 1987). Subunit III of *Paracoccus denitrificans* cytochrome oxidase may be involved in a proton translocation role similar to that of subunit III of beef heart cytochrome oxidase.

Despite the fact that mammalian cytochrome oxidase has been under scrutiny for the past 50 years, the present understanding of the functional activity of the enzyme regarding the electron transfer pathway and the proton pumping mechanism is rudimentary. The binding ligands of the redox centres, in particular, those of zinc, heme A in cytochrome a_3, and those of Cu_B are only partially elucidated. The positions of the four metal centres, as described by Holm *et al.*, (1987), only represents a Model intended to show that it is possible to arrange the redox centres of cytochrome oxidase in subunits I and II. The identification of the subunits which contain the metal centres remains uncertain.

A more complete understanding of the enzyme structure is necessary for the elucidation of structure-function relationships which exist in cytochrome oxidase. Ac-

cess to well-ordered crystals would allow the use of x-ray diffraction methods for structure determination of the enzyme (Ollis and White, 1990). Crystallization of beef heart cytochrome oxidase can also be used for enzyme purification and the subsequent identification of the *true* components of the native enzyme (Scopes, 1987). Unfortunately, membrane proteins are difficult to crystallize (Michel, 1983): to date, well-ordered crystals of eukaryotic, membrane proteins have not been reported and despite much effort, crystals of beef heart cytochrome oxidase suitable for x-ray crystallography have not been obtained. In contrast, crystallization of bacterial membrane proteins has been reported; these include the *lamB* and the *ompA* genes products from *Escherichia coli* (Garavito *et al.*, 1984), the photosynthetic reaction centre of *Rhodopseudomonas viridis* (Deisenhofer *et al.*, 1985), and bacteriorhodopsin from *Halobacterium halobium* (Michel and Oesterhelt, 1980). A structurally simpler, bacterial cytochrome oxidase may crystallize easier than the more complex, mammalian enzyme.

Perhaps, as important as the structural simplicity of the bacterial enzyme is the technology of molecular genetics can be applied to bacteria. This approach may supply another powerful tool in the understanding of the catalytic and proton pumping mechanisms of cytochrome oxidase.

It has been observed that bacteria are capable of synthesizing individual redox components in response to environmental changes (e g., oxygen tension and growth substrates) (Tochikubo, 1971; Haddock and Jones, 1977; Bragg, 1980 and Downey, 1964). The approach taken in this thesis to enhance the expression of cytochrome

oxidase in *Bacillus subtilis*, hopefully resulting in large yields of purified enzyme, involves growing the bacteria aerobically in the presence of succinate.

Various detergents are tested for their ability to solubilize the enzyme from *Bacillus subtilis* membrane vesicles. Difference spectroscopy is employed to follow the protein through its purification. This extremely sensitive and rapid technique is also used to calculate the protein's concentration at various stages of the isolation procedure.

Two anion exchange chromatography steps are used to purify cytochrome oxidase. Anion exchange performed on a DEAE cellulose/Sepharose column or a Biorad Econo-Pac Q cartridge is used primarily, for bulk separation of negatively charged proteins from cytochrome oxidase. Chromatography on a Pharmacia Mono Q column is employed to separate the aa_3-type cytochrome from *b*- and *c*-type cytochromes.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis will be performed on the purified protein to determine its subunit composition. Carbon monoxide and cyanide ligand binding, as well as horse heart cytochrome *c* binding experiments will be performed in order to characterize the isolated protein as a potential ferrocytochrome c:O_2 oxidoreductase. Oxygen uptake activity of the enzyme, and its ability to oxidize reduced cytochrome *c* will also be investigated in the work presented in this thesis.

MATERIALS AND METHODS

Chemical suppliers: NaCl, KCl, $Co(NO_3)_2 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, K_2HPO_4, $MgCl_2$, ethylenediaminetetra acetic acid (EDTA), sodium dithionite (AnalR), L-ascorbic acid (AnalR), concentrated sulphuric acid, concentrated hydrochloric acid, sodium hydroxide pellets (AnalR), ammonium sulphate, D-glucose, glycerol and H_3BO_3, were obtained from BDH Chemical Co. $MnCl_2$, $ZnSO_4 \cdot 7H_20$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$, 2-amino-2-hydroxymethyl-1, 3-propanediol (Tris), lysozyme (grade I from chicken egg white), N, N, N', N'-tetramethyl-*p*-phenylenediamine, tetrachlorohydroquinone, deoxyribonuclease I (from bovine pancreas), ribonuclease (I-AS from bovine pancreas), Triton X-100, Triton X-114, sodium dodecyl sulphate (SDS), succinic acid, bicinchoninic acid solution, sucrose and horse heart cytochrome *c* (type III) were obtained from Sigma Chemical Co. Acrylamide, bisacrylamide and urea were supplied by Biorad. Cholic acid, deoxycholic acid and Tween 80 were obtained from Fluka. Laurylmaltoside was supplied by Boehringer Mannheim. Unless specified, water used in all procedures was purified with a Milli Q® water purification system.

Spectra: All spectra were obtained on a Shimadzu UV-160 UV-VIS recording spectrophotometer.

***Bacillus subtilis* grow-up in Liquid Medium:** A 2 litre culture, made with deionized water of *Bacillus subtilis* cells was grown with vigorous shaking (300 rpm) in two 5 litre flasks with an air to medium volume of 4:1 using a New Brunswick incubator (Model G25). The flasks were incubated at 37 °C for approximately 14 h until a cell density corresponding to an absorbance of 1.5 - 1.8 absorbance (λ = 660 nm) was

attained.

Composition of the Medium: The culture medium contained 0.8% (w/v) tryptone (Difco), 0.5% (w/v) NaCl, 25 mM KCl, 2% succinic acid and 150 μl/l micronutrient solution. The micronutrient solution contained; 2.2% $MnCl_2$, 0.05% $ZnSO_4 \cdot 7H_20$, 0.5% H_3BO_3, 0.016% $CuSO_4 \cdot 5H_2O$, 0.025% $Na_2MoO_4 \cdot 2H_2O$, 0.46% $Co(NO_3)_2 \cdot 6H_2O$ and 0.5 ml/100 ml concentrated H_2SO_4. The medium was supplemented with 50 mg/l $CaCl_2 \cdot 2H_2O$ and 50 mg/l $FeSO_4 \cdot 7H_2O$. The medium, containing $CaCl_2 \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ was adjusted to pH 7.4 with 50% NaOH (w/v) and auto-claved for 20 min. The micronutrient solution was sterilized by filtration using a millipore 0.22 μm (type GS) filter, and added to the medium once it had cooled to room temperature.

Harvesting *Bacillus subtilis* cells: The 2 litre culture was centrifuged at 3000 rpm at 4 °C for 5 min. The harvested cells were washed by resuspending them in 50 ml of a 50 mM K_2HPO_4 solution. The cells were centrifuged again at 3000 rpm at 4 °C for 5 min.

Preparation of Membranes Vesicles: To the harvested cells was added 50 ml of lysing solution containing 0.375 mg/ml lysozyme, 0.025 mg/ml DNAase, and 0.025 mg/ml RNAase in the presence of 50 mM Tris-HCl pH 7.4. This mixture was transferred to a 300 ml Erlenmeyer flask containing glass beads. The flask was vigorously shaken for 15 to 20 min. The mixture was decanted and transferred to a 1 litre Erlenmeyer, and to it was added 350 ml of the above lysing solution. To the mixture was added 0.55 g magnesium chloride (anhydrous) was added and the flask was incubated for 45 min at 37 °C with gentle shaking (100 rpm). Then 25.8 ml of 250 mM Na_2-EDTA, pH 7.0, was added to the lysate and 3 min later, 3.45 g

magnesium sulphate was added. The lysate was incubated for an additional 15 min. The lysate was centrifuged at 10 000 rpm for 1.5 h. The pellet was homogenized with 35 ml of 50 mM Tris-HCl, 1 mM Na_2-EDTA, pH 7.4. The suspension was centrifuged at 18 000 rpm for 1.5 h to collect the membrane vesicles.

Solubilization of Membrane Vesicles: The pellet of the membrane vesicles was suspended in 25 ml of a 1.5% :0.5% mixture of Triton X-100:Na-cholate in the presence of 20 mM Tris-HCl, pH 7.8, 1 mM Na_2-EDTA and homogenized once more. This mixture was stirred slowly overnight at 4 °C. The greyish-white suspension was centrifuged at 12 000 rpm for 10 min and the pellet was discarded.

DEAE Ion Exchange Chromatography: The supernatant was applied to 5 ml of DEAE cellulose using a flow rate of 0.5 ml/min. The column had been previously equilibrated with 0.1% Triton X-100, 20 mM Tris-HCl pH 7.8. The loading buffer used was the same as the equilibration buffer. The bound protein was eluted using a linear gradient from 0 to 700 mM NaCl in the presence of 0.1% Triton X-100, 20mM Tris-HCl pH 7.8. The fractions with the highest heme *a*:hemes *bc* ratio (this ratio was approximately 0.5:1) were pooled and dialysed in 4 litres of 10 mM Tris-HCl pH 7.8, 0.05 mM Na_2-EDTA and 0.1% Triton X-100 for 24 h. The pooled fractions were then concentrated to approximately 3 ml, and centrifuged in a minifuge at 13 000 rpm for 10 min.

Anion Exchange Chromatography on Mono Q: Aliquots of 500 μl of the 3 ml sample were injected onto a Mono Q (5/5 HR) column until all the sample was loaded.

After each injection, 2 ml of buffer A (20 mM Tris-HCl pH 7.8, 0.1% laurylmaltoside and 0.1 mM EDTA) was run through the column at a flow rate of 0.5 ml/min, collecting 1 ml fractions. After the final injection, 10 ml of buffer A was run through the column. The bound cytochrome *c* oxidase was then eluted with buffer B (same as buffer A, + 1 M NaCl) using a step gradient as follows; flow rate was set at 0.35 ml/min. The change in salt concentration for the first 7 ml was 5%/min. This change was decreased to 1.8%/min for the next 3 ml, and the change was further decreased to 1.2%/ml for the subsequent 4 ml. For the final 26 ml, the change in salt concentration was set at 1.1%/ml, at this point in the gradient, the final concentration of salt in the elution buffer was 1 M.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis: A 200 μl sample of cytochrome oxidase was dialysed overnight in 250 ml of a solution containing 20 mM Tris-HCl pH 6.8, 1% β-mercaptoethanol and 10 g of recrystallized SDS. Electrophoresis was performed on a 12% polyacrylamide gel in the presence of 4% SDS and 12% urea. A sample of 15 μl was loaded per well. Coomassie-Blue was used to stain the protein.

Preparation of Submitochondrial Particles: The procedure for the preparation of submitochondrial particles was adapted from Yonetani, (1966). All steps of the preparation were carried out at 4 °C. The fat and connective tissue was trimmed off of approximately 2 kilograms of beef heart. The muscle was ground in a precooled meat grinder (Moulinex). The mince was washed by suspending it in 10 volumes of 0.02 M Na_2HPO_4 buffer pH 7.4, for 15 min with constant stirring using an overhead

stirrer. The mince was collected by squeezing the suspension through four layers of cheesecloth. This procedure was repeated 7 times. To 300 g portions of mince 150 ml of 0.2 M Na_2HPO_4 buffer, pH 7.4, and 150 grams of ice were added. The mixture was homogenized in a Waring Blender (1 litre capacity) for 30 seconds at low speed and 5.5 min at high speed. The homogenate was pooled, diluted 1.5 times with ice cold distilled water and centrifuged at 3500 rpm for 20 min using an IEC (Model DPR 6000) centrifuge. The supernatant was decanted and saved. The pellet was rehomogenized with 1 litre of 0.02 M sodium phosphate buffer, pH 7.4, for 3 min at high speed and centrifuged as described above. The second supernatant was combined to the first and the pellet discarded. Using a magnetic stirrer to slowly stir the supernatant, 1 M acetic acid was added dropwise to acidify the mixture to a pH of 5.6. The mixture was centrifuged at 3500 rpm for 10 min using the IEC (Model DP 6000) centrifuge. The pellet was washed with 10 volumes of ice cold distilled water and centrifuged at 3500 rpm for 10 more min. The pellet was layered with a minimal amount of 0.2 M sodium phosphate, pH 7.4, and stored at 4 °C overnight.

Preparation of Beef Heart Cytochrome Oxidase: A modified procedure presented by Kuboyama *et al.*, (1972), was used to purify cytochrome oxidase from the prepared submitochondrial particles. All manipulations were carried out at 4 °C. The precipitate was suspended in 300 ml of 0.2 M phosphate buffer, pH 7.4. To the mixture was added 550 ml 3 M NH_4OH to adjust the pH to 7.4, the final volume of the suspension was 550 ml. The protein concentration was 19.4 mg/ml. Sixty one ml of a 10% (w/v) sodium cholate solution, pH 7.4, was added to bring the mixture to

a final detergent concentration of 1% (v/v). Solid ammonium sulphate was added to give 25% saturation and the pH of the mixture was adjusted to 8.0 with 1 M NaOH. This latter addition was made slowly with mechanical stirring over a period of 20 - 30 min. Solubilization of the submitochondrial particles was achieved by letting the mixture stand at 4 °C for 1 h with occasional stirring. At this point, solid ammonium sulphate was added to obtain 35% saturation. After allowing the mixture to stand for 10 min, the suspension was centrifuged in an IEC centrifuge, Model B-20, at 8500 rpm with a rotor No. 872. The supernatant was discarded and the precipitate homogenized in 0.1 M sodium phosphate buffer, pH 7.4, to a final volume of 300 ml using a Potter-Elvehjem homogenizer. 10% sodium cholate solution was added to the suspension to give a final detergent concentration of 2% (v/v). Solid ammonium sulphate was added slowly to the mixture to 25% saturation, the pH was adjusted to 7.6 and the suspension was stirred slowly overnight using a magnetic stirrer. After centrifugation at 8500 rpm for 30 min, solid ammonium sulphate was added to the clear, greenish brown supernatant to 40% saturation. The mixture was stirred for 10 min and centrifuged for 15 min. The supernatant was discarded and the pellet dissolved in 100 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 1.5% (v/v) cholate. Saturated, neutralized ammonium sulphate solution was added to give 25%. The mixture was allowed to stand for 20 min and then centrifuged for 20 min at 8500 rpm. The pellet was discarded and the supernatant was brought to 37% saturation with saturated ammonium sulphate solution. After centrifugation, the pellet was dissolved in 80 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 1.5%

cholate. Saturated ammonium sulphate was added to the greenish brown solution until a slight turbidity was observed (20 - 25%), then, the solution was centrifuged and the precipitate discarded. Enough saturated ammonium sulphate solution was added to the supernatant to give 36% saturation. After centrifugation, the pellet was redissolved in 60 ml of 0.1 M sodium phosphate, pH 7.4, containing 1% cholate. At this stage the absorbance ratio, $A_{280} : A_{420}$, of the oxidized enzyme in 0.1 M sodium phosphate/cholate buffer was determined and found to be 3.7. The preparation was treated with saturated ammonium sulphate solution, until a slight turbidity was visible, let stand for 10 - 15 min, centrifuged, more saturated ammonium sulphate solution was added until a definite, heavy precipitate was observed, centrifuged again and the pellet redissolved in 50 ml of sodium phosphate/detergent buffer. This cycle was repeated until the $A_{280} : A_{420}$ ratio was 2.5. At this point, enough (31 - 33%) saturated ammonium sulphate solution was added to precipitate the enzyme and the solution was centrifuged as previously described. The surface of the pellet was washed with ice cold distilled water and then the pellet dissolved in 25 mM potassium phosphate buffer, pH 7.4, containing 25 mM KCl and 1 mg/ml laurylmaltoside. The heme A to protein ratio (as determined by equation 2) of this preparation was 9.5.

$$\text{Heme A:Protein in nmoles heme A/mg protein} = \frac{\text{Concentration of heme A } (\mu M)}{\text{Protein concentration (mg/ml)}}$$

Equation (2)

The concentration of heme A was determined from the difference spectrum (dithionite reduced *minus* air oxidized) of cytochrome oxidase by dividing the

difference in absorbance between 605 nm and 630 nm by 27 $mM^{-1}cm^{-1}$, the extinction coefficient for this wavelength pair (reduced *minus* oxidized) and then multiplying this value by 2. The protein concentration in equation 2 was determined by performing a bicinchoninic acid assay as adapted from Smith *et al.*, (1985), and described below.

Bicinchoninic Acid Assay: Six protein standards were used to prepare a calibration curve for the determination of unknown protein concentrations. The protein standards contained 0, 20, 40, 60, 80 and 100 μg of bovine serum albumin. The concentration of stock bovine serum albumin was 1 mg/ml. Sufficient Milli Q® water was added to each standard tube so that the final volume of each tube was 100 μl. Similarly, the unknowns were prepared by diluting a fixed volume of unknown protein solution to a final volume of 100 μl with Milli Q water. Two ml of protein determination reagent (1 part $CuSO_4 \cdot 5H_2O$, 50 bicinchoninic acid solution) was added to all tubes, the tubes were vortexed and incubated at 37 °C for 30 min. Tubes were allowed to cool to room temperature (ca. 22 °C) and the absorbance at 562 nm was determined. The absorbance of the tube containing no bovine serum albumin was subtracted from the absorbance of the remaining assay tubes to obtain the net absorbance due to protein.

Preparation of Ferrocytochrome *c*: To a 1 ml solution of 5 mM horse heart cytochrome *c* was added 50 mM sodium dithionite. This solution was applied to a column (1 cm x 50 cm) containing 30 ml of Sephadex® G25 preequilibrated with 20 mM Tris-HCl, pH 7.4 and 100 mM KCl. The concentration of cytochrome *c* (ferrocytochrome *c*) was determined spectrophotometrically from the absorbance at

550 nm of reduced cytochrome *c* using an extinction coefficient of 27.6 $mM^{-1}cm^{-1}$.

Photodissociation of Carbon Monoxide Bound Complexes: The light source used to measure the optical density change of the CO bound complexes was provided by a 150 watt xenon arc lamp (Oriel Corporation, Model 66003). A clear, flat sided culture bottle was used as a heat and ultraviolet filter. Photolysis of the CO complexes was carried out by flashes from a dye (rhodamine) laser (Candela Laser Corporation, Model SLL 250) positioned at 90° to the observation beam. The laser beam was focused on the reaction mixture contained in a cuvette (No. 117.004F-OS) supplied by Hellma. A shutter placed between the sample and the xenon arc lamp was opened 2 ms prior to the triggering to the laser flash. Delay time between opening the shutter and the laser flash was controlled by a Uniblitz Model SD 10 shutter drive timer supplied by Vincent Associates. The observation beam was focused through the sample cuvette and then onto the entrance slit of a f3.4 monochromator supplied from Applied Physics. The beam was then focused onto the head of an optical waveform analyzer (Model 1500XP) supplied by Photodyne Inc. The signal from the analyzer was converted to voltage readings by a Phillips digital oscilloscope (Model PM 3323/41). The conversion of the voltage signals to absorbances was performed with the help of the Assystant data processing package. Absorbance changes with time were fitted to a double exponential equation as shown below:

$$A_t = A\, e^{-k_1 t} + B\, e^{-k_2 t}$$

Equation (3)

The CO recombination rate constants (k_1 and k_2) and the preexponential factors (A and B) used to fit the overall absorbance change as a function of time (A_t) were obtained from equation 3. When purified *Bacillus subtilis* cytochrome oxidase bound to carbon monoxide was photolysed, the absorbance changes with time fit a the single exponential equation shown below:

$$A_t = A\, e^{-k_1 t}$$

Equation (4)

The CO recombination rate constant, k_1, and the preexponential factor, A used to plot the difference spectrum between the CO-aa_3 complex and the reduced form of cytochrome oxidase, were obtained from equation 4.

RESULTS

Effects of Growth Substrates on Heme A Expression in *Bacillus subtilis*.

In an attempt to establish conditions yielding the highest possible quantities of aa_3-type cytochrome oxidase, various substances were supplied as oxidizable food sources in the medium used to grow *Bacillus subtilis*. The results are shown in Table 2.

TABLE 2
Heme A content of *Bacillus subtilis* grown on simple medium supplemented with the indicated growth substrates

Additional energy	[Heme A] (μmolar)	[Protein] (mg/ml)	Heme:Protein
Tryptone alone	0.87	1.13	0.77
Succinate 2%	1.76	1.20	1.50
Glucose 2%	1.00	1.85	0.54
Glycerol 2%	1.02	1.71	0.60
Succinate 3%	1.61	1.43	1.13
Sucrose 2%	0.95	1.87	0.51

- All cultures grown in 50 ml of medium.
- Cell density at time of harvesting was 1.8 O.D at 660 nm.
- Preparation of membrane vesicles was as described in the legend for figure 4.
- Membrane vesicles were dissolved with 0.5 ml of 2% Triton X-100 in 20 mM Tris-HCl pH 7.8, and containing 1 mM disodium EDTA.
- Heme A and protein concentrations determined as described in *Materials and Methods*.
- The values given in this table were obtained from one experiment.

Table 2 shows the heme A content and protein concentration of Triton X-100 extracts from plasma membranes of *Bacillus subtilis* grown under different conditions. The culture grown in the presence of 2% succinate provides the highest quantity of

heme A of the treatments tested. To further maximize the expression of the heme A containing enzyme (Gel'man *et al.*, 1967) and to prevent anaerobic respiration, *Bacillus subtilis* is grown with vigorous agitation to assure proper oxygenation of the culture (Downey, 1964).

Detergent Solubilization of Cytochrome Oxidase.

The isolation of membrane vesicles from *Bacillus subtilis*, a gram positive bacterium, can be achieved by enzymatic digestion of the cell wall with lysozyme (Dawes, 1986) in the presence of disodium-EDTA. The cytochrome oxidase contained within these membrane vesicles can be extracted by employing detergents. Table 3 shows the efficiency of cytochrome oxidase solubilization, as monitored by the heme A concentration in the extract, using different detergents for the extraction. Laurylmaltoside, Triton X-100 and the detergent mixture of Triton X-100 and sodium cholate provide the largest yield of oxidase, but Triton X-100 appears to be more selective in the extraction of cytochrome oxidase with respect to other proteins in the membrane vesicles. Triton X-100 extractions give the highest heme to protein ratio of the detergents tested. Figure 4 shows the relative ability of Tween-80, Triton X-100 and laurylmaltoside to extract cytochromes from membranes of *Bacillus subtilis*.

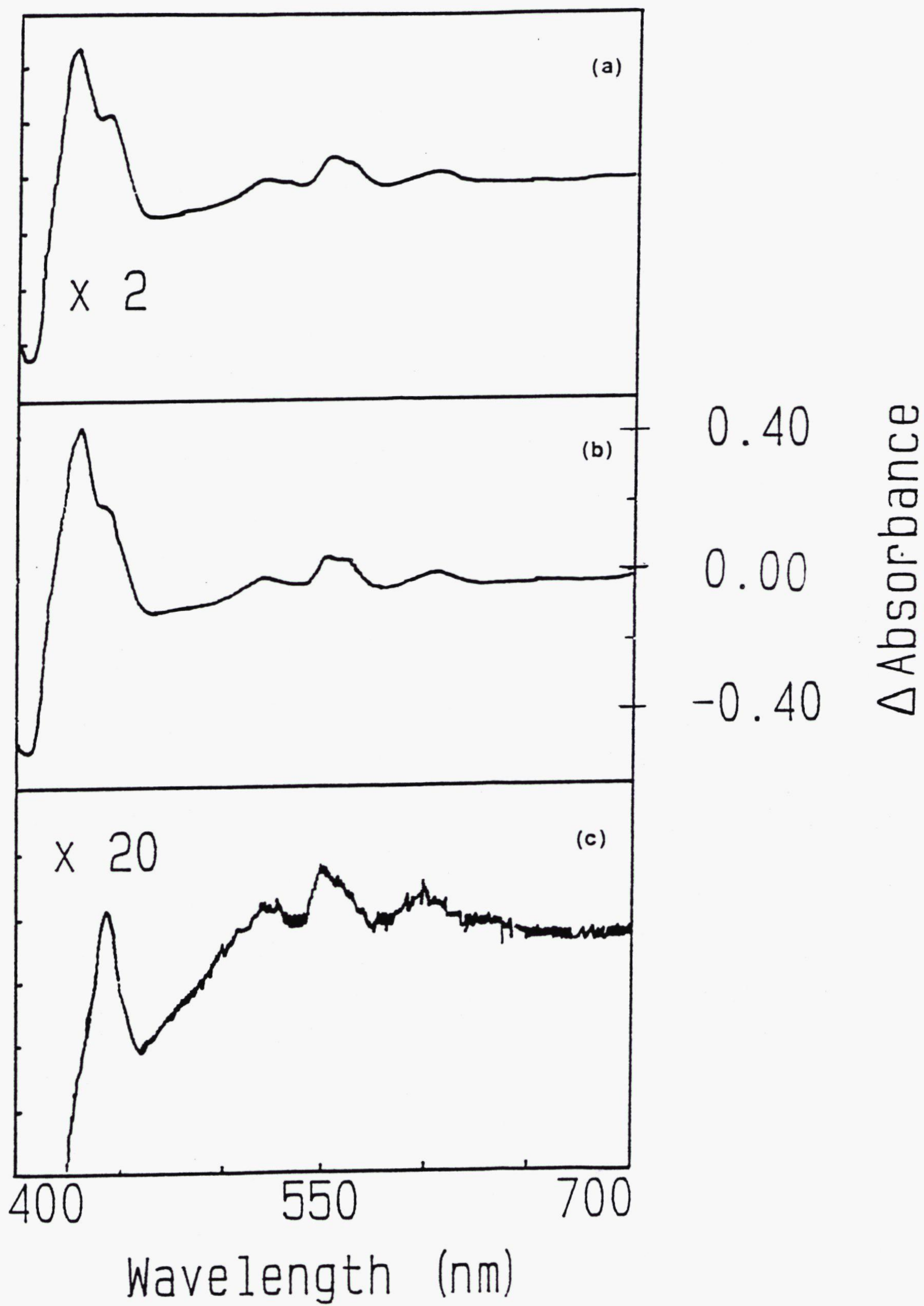
TABLE 3
Efficacy of various detergents to solubilize cytochrome oxidase from membrane vesicles of *Bacillus subtilis*

DETERGENT	HEME A CONC. (μmolar)	PROTEIN CONC. (mg/ml)	HEME A: PROTEIN RATIO
2% Na-CHOLATE	0.59	1.71	0.35
2% Na-DEOXY-CHOLATE	0.66	2.77	0.24
2% TRITON X-114	1.40	2.32	0.60
2% TRITON X-100	1.72	1.17	1.47
1.5% TRITON X-100 0.5% Na-CHOLATE	1.60	1.20	1.33
2% TWEEN 80	0.23	0.78	0.29
1% LAURYLMALTO-SIDE	2.80	3.10	0.58

- A 2 litre culture of *Bacillus subtilis* was grown, harvested and lysed as described in *Materials and Methods*. The lysate was divided into seven - 50 ml aliquots, and the aliquots centrifuged at 10 000 rpm for 1.5 h. Each pellet was homogenized with 3 ml of 50 mM Tris-HCl, 1 mM Na_2-EDTA, pH 7.4, and centrifuged at 18 000 rpm for 1.5 h. The pellets were incubated in 2.5 ml of the stated detergents for 14 h. at 4 °C. The soluble material was separated by centrifugation at 13 000 rpm for 20 min.
- Protein and heme A concentrations were determined as described in the *Materials and Methods*.
- Concentrations of Na-cholate and deoxycholate, as well as, lauryl-maltoside are w/v percentages; Triton X-100 and Tween are v/v percentages.
- Values in this table were obtained from one experiment.

Figure 4 shows the dithionite reduced *minus* air oxidized difference spectra of vesicle extracts obtained by solubilization of *Bacillus subtilis* plasma membranes with (a) laurylmaltoside (b) Triton X-100 and (c) Tween-80. In these spectra the reference cuvette contains oxidized detergent extract and an excess (i.e., 2 - 3 mM)

Figure 4. Difference spectra of *Bacillus subtilis* membrane vesicles solubilized with (a) 1% (w/v) laurylmaltoside (b) 2% (v/v) Triton X-100 and (c) 2% (v/v) Tween 80. *Bacillus subtilis* was grown as described in the section titled *Materials and Methods.* From the 2 litre culture, three 50 ml aliquots were harvested as described in *Materials and Methods* with the following exceptions; 5 ml of K_2HPO_4 solution was used to wash the cells of each aliquot. To each sample was added 10 ml of lysing solution followed by 14 milligrams magnesium chloride. The samples were incubated at 37 °C, afterwhich 645 μl Na_2-EDTA was added to the lysate. Three min later 86 milligrams magnesium sulphate was added and the lysate incubated at 37 °C, centrifuged and homogenized with 1 ml Tris-HCl buffer. Following centrifugation, the membrane vesicle were solubilized overnight (at 4 °C) using 0.5 ml of the above detergents contained in a 20 mM Tris-HCl buffer, adjusted to pH 7.8 and made 1 mM in disodium EDTA. The membrane vesicles were centrifuged in a minifuge (13 000 rpm for 15 min) and an air oxidized spectrum of the detergent extract was recorded from 400 nm to 700 nm. The spectrum was stored in memory. In order to reduce the sample, 2 mM sodium dithionite was added to the sample and the sample allowed to stand undisturbed for 15 min. At this point, a second spectrum was scanned and stored in a second memory channel. At increments of 0.5 nm the air oxidized spectrum was subtracted from the reduced spectrum to produce the difference spectrum.


(a)
x 2
(b)
0.40
0.00
-0.40
Δ Absorbance
(c)
x 20
400
550
700
Wavelength (nm)

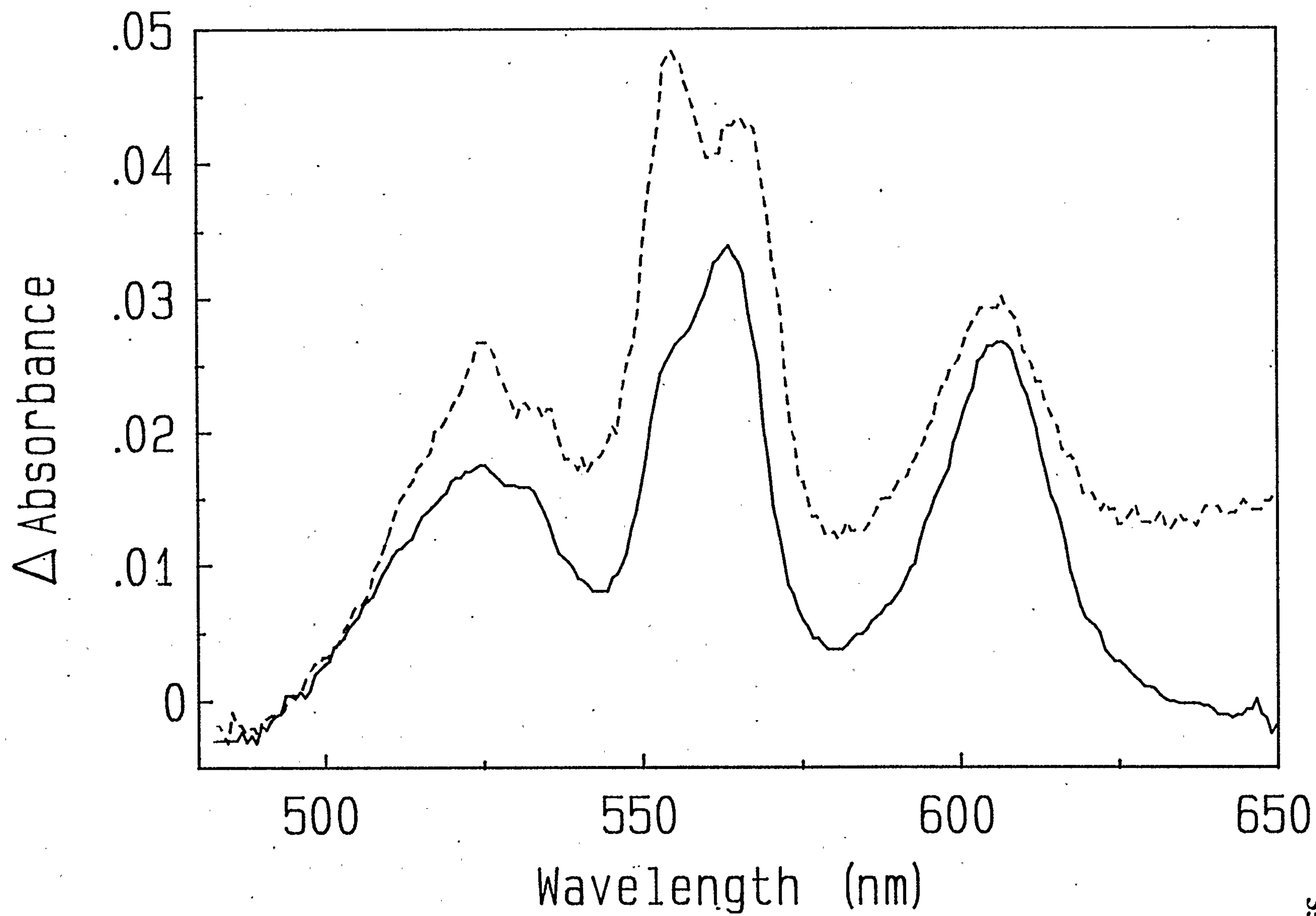
of the reducing agent sodium dithionite has been added to the sample cuvette. Evident in these spectra are the peaks at 429 nm and 562 nm indicating the presence of a *b* type cytochrome. The absorbance maxima at 551 nm and 521 nm, as well as the shoulder appearing to the left of the 429 nm peak (approx. 417 nm) are associated with the α, β, and γ, bands of *c*-type cytochromes. Cytochrome aa_3 gives rise to peaks at 443 nm and 602 nm in the visible region.

The UV-visible spectral properties of detergent solubilized *Bacillus subtilis* membrane vesicles are similar to those of submitochondrial particles from bovine heart muscle (see figure 5). A noticeable difference is the ratio of the different visible absorption bands found in the *Bacillus subtilis* membrane extract and in the submitochondrial particles. In the bacterial extract, the ratio of the 602 nm, 562 nm, and 550 nm absorption bands are 0.5:1:1.3, whereas, this ratio in beef heart submitochondrial particles is 1:1:0.7. Presumably, this reflects the different content of cytochromes *a*, *b*, and *c* in the two membrane extract.

Purification of Cytochrome Oxidase by Anion Exchange Chromatography.

Ammonium sulphate fractionation of cytochromes *b* and *c* from cytochrome oxidase does not appear to work for the Triton X-100 solubilized bacterial membranes. An alternative approach in attempting to segregate these cytochromes is to employ anion exchange chromatography. DEAE cellulose, was the resin of choice since this has been used successfully with beef heart cytochrome oxidase. The reductive substrate of cytochrome oxidase, cytochrome *c*, contains a ring of six or seven positively charged lysine residues around its heme edge and this is proposed

Figure 5. Comparison of solubilized submitochondrial particles (——) and solubilized *Bacillus subtilis* membrane vesicles (----). Solubilized submitochondrial particles and *Bacillus subtilis* membrane vesicle extract were prepared as describe in *Materials and Methods*. The difference spectrum was obtained by scanning an air oxidized sample from 450 to 650 nm. The recorded spectrum was stored in memory. Sodium dithionite (2 - 3 mM) was added to reduce components present in the sample. After 15 min the sample was scanned again from 450 to 650 nm and the spectrum was stored in a second memory channel. At increments of 0.5 nm, the absorbance of the oxidized sample was subtracted from the absorbance of the reduced sample to produce a difference spectrum. The *Bacillus subtilis* extract was contained in 20 mM Tris-HCl buffer, pH 7.8, containing 1 mM Na_2EDTA, 1.5% Triton X-100 and 0.5% sodium cholate.


.05
.04
.03
.02
.01
0
Δ Absorbance
500
550
600
650
Wavelength (nm)

to be the area on the surface of cytochrome *c* which interacts ionically with beef heart cytochrome oxidase (Staudenmayer *et al.*, 1977). The lysine residues of cytochrome *c* pair with complementary carboxyl containing residues on cytochrome oxidase (Reider and Bossard, 1980). If the structures of the bacterial cytochrome *c* and cytochrome oxidase are similar to their beef heart counterparts, one would expect the bacterial oxidase to bind to the anionic resin.

When a sample of Triton X-100 solubilized *Bacillus subtilis* plasma membranes is applied to a DEAE column at low ionic strength a protein characteristic of cytochrome *c* elutes. In these fractions, there is no spectrophotometric evidence suggesting the presence of *a* or *b*-type cytochromes. The elution of DEAE cellulose-bound cytochromes is performed using a linear, NaCl concentration gradient (0 to 700 mM) contained in a Tris-HCl/Triton X-100 buffer. The first cytochrome to come off during the salt gradient elutes at 400 mM NaCl and shows the same cytochrome *c* type spectrum as found for the protein eluting at low ionic strength. Again, difference spectroscopy shows no evidence of cytochromes *a* or *b*. The appearance of a heme A containing protein occurs in fractions eluting between 470 mM and 530 mM NaCl. These fraction also contained *b* and *c*-type cytochromes.

In an attempt at obtaining better resolution of the components present in the detergent extract, DEAE resin was replaced by a Biorad Econo-Pac Q cartridge. The resin in this cartridge is a strongly basic anion exchanger, and provides a better separation of the cytochrome components in the detergent extract, relative to the weaker, DEAE exchange resin. As with the DEAE resin, when a sample of Triton

X-100 solubilized, *Bacillus subtilis* membrane vesicles is applied to the Biorad Econo-Pac Q cartridge at low ionic strength, a characteristic *c*-type cytochrome elutes from the cartridge. The difference spectra of these fraction show no evidence that *a* or *b*-type cytochromes are present in these fractions. Cytochromes bound to the Q Sepharose are eluted using a linear, NaCl concentration gradient (0 to 1 M) contained in a Tris-HCl/Triton X-100 buffer.

The first cytochrome to come off the cartridge at a salt concentration of 200 mM NaCl shows the same *c*-type spectrum as found for the protein eluting at low ionic strength. These fractions contain no *a* or *b*-type cytochromes. A second fraction with spectral characteristics of a *c*-type cytochrome elutes between 350 mM and 450 mM NaCl. At salt concentrations between 500 mM and 600 mM, fractions containing a mixture of *b* and *c*-type cytochromes begin to elute. Fractions containing cytochrome *a* elute at a salt concentration of 750 mM NaCl. These fractions are not spectrally clean and in addition to cytochrome *a*, also present are cytochromes *b* and *c*. A typical elution profile for detergent extract chromatographed on the Biorad Econo Q cartridge is shown in figure 6.

Fractions with the greatest heme A to protein ratio from the DEAE or Biorad Econo-Pac Q cartridge were pooled, dialyzed, and rechromatographed on a Pharmacia Mono Q column. A typical chromatogram is shown in figure 7. The Mono Q column is developed as with the DEAE column, except that the range of NaCl is from 0 to 1 M, and the detergent used is laurylmaltoside. The pattern of

Figure 6. Elution profile of detergent solubilized membrane vesicles chromatographed on a Biorad Econo-Pac Q cartridge. A quantity of 75 milligrams of protein in a total volume of 30 ml were loaded with a low ionic strength buffer using a flow rate of 1 ml/min. Bound cytochromes were eluted using a linear, sodium chloride gradient from 0 to 1 M. Peaks I and II correspond to the elution of a *c*-type cytochrome, peak III represents those fractions containing cytochromes *b* and *c*, cytochrome oxidase is contained in peak IV. The chart speed was set at 12 cm/hr. The wavelength used to monitor protein elution was 280 nm. The start of the gradient is marked with an arrow. The composition of buffers A and B are described in *Materials and Methods*.

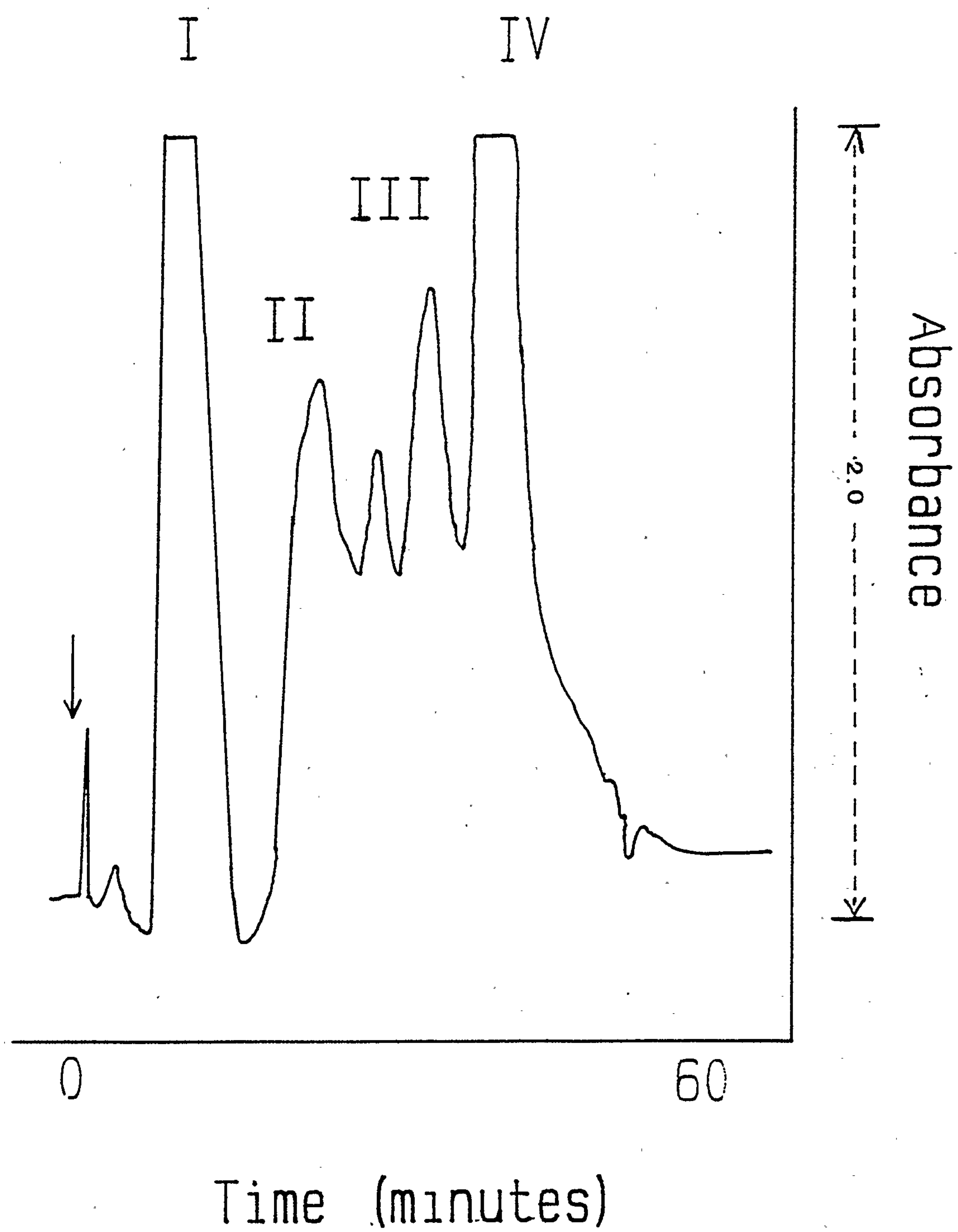
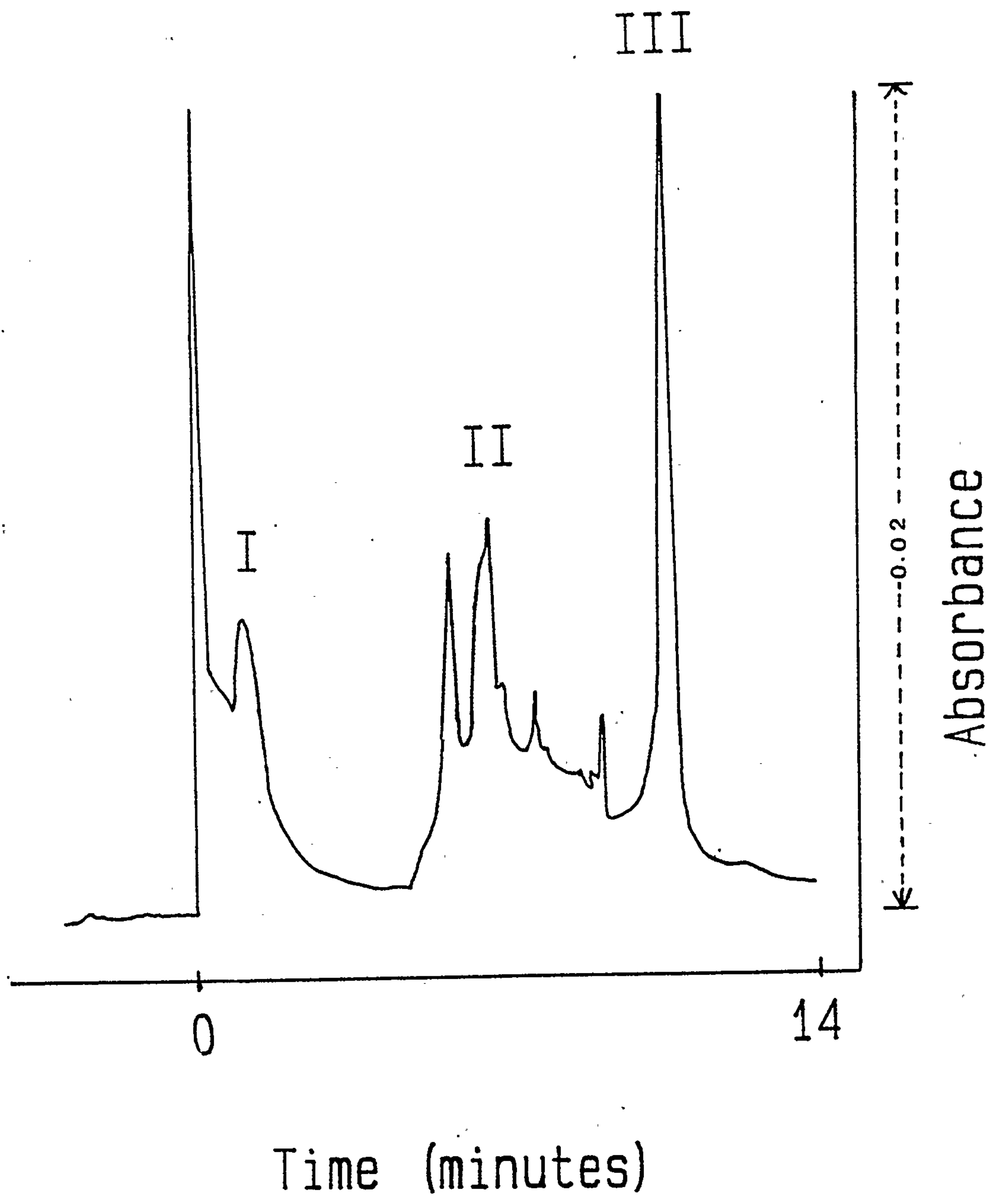

I
II
III
IV
Absorbance
2.0
0
60
Time (minutes)

Figure 7. Elution profile of partially purified *Bacillus subtilis* cytochrome oxidase chromatographed on a Pharmacia Mono Q column. The compositions of buffers A and B, as well as, the gradient used to elute the protein are described in the *Materials and Methods*. Peaks I, II, and III represent those fractions containing a *c*-type cytochrome, *b* and *c*-type cytochromes, and an aa_3-type cytochrome, respectively. The flow rate was 0.35 ml/min and the wavelength used to monitor protein elution was 280 nm. The amount of protein loaded on to the column was 10 milligrams.


III
II
I
0.02
Absorbance
0
14
Time (minutes)

cytochromes eluting from the Mono Q column is similar to that of DEAE cellulose column and the Biorad Econo-Pac Q cartridge. At this stage, however, no cytochrome *c* type protein elutes at 0 mM NaCl. Instead the first appearance of a cytochrome *c* protein occurs at 100 mM NaCl. This fraction contains no cytochrome *a* or *b*. At sodium chloride concentrations between 450 to 550 mM, fractions containing both cytochromes *c* and *b* are eluted from the Mono Q column. The cytochrome *a* protein elutes from the column at a salt concentration of 750 mM. This protein is the last to elute from the column. The difference spectrum in figure 8 shows that this fraction is enriched in cytochrome *a* and virtually free of other contaminating cytochromes; the difference spectrum of mammalian cytochrome oxidase is shown for comparative purposes. The reduced *minus* oxidized difference spectrum of purified cytochrome oxidases is scanned from 400 to 700 nm. The UV-vis spectrum of the bacterial enzyme, relative to the oxidase from bovine heart, is blue shifted. Maxima of the bacterial enzyme are at 601 nm (α-band) and 443 nm (γ-band), compared to 605 nm and 445 nm for bovine heart oxidase.

Thus, the critical difference between the DEAE column and the Mono Q column is that the latter provides a higher degree of resolution, as well as, much tighter binding of cytochromes and in particular, of cytochrome aa_3. Despite the fact that Biorad Econo-Pac Q cartridge binds cytochrome oxidase as tightly as Mono Q column resin, the latter seems to give better separation of *b* and *c* type cytochromes from cytochrome oxidase. A summary of *Bacillus subtilis* cytochrome oxidase purification is given in table 4.

TABLE 4
Summary of purification of *Bacillus subtilis* cytochrome oxidase

Stage of purification	Quantity of heme A (nmoles)	Quantity of protein (mg)	Heme: Protein ratio	% Recovery	
				Protein	Heme A
Solubilized Membrane Vesicles prior to Centrifugation	1776	2528	0.70	100	100
Solubilized Membrane Vesicles after Centrifugation	1432	748	1.71	29.6	81
Biorad Q Cartridge	664	176	3.77	7.0	37
Pharmacia Mono Q	141	18	7.82	0.7	7.9

- Anion exchange using DEAE cellulose (as described in Materials and Methods) was replaced by anion exchange using Q resin. Sample was loaded onto the cartridge using the same buffer as the DEAE chromatography. Subsequently, 10 ml of loading buffer was run through the cartridge. The cytochrome oxidase was eluted with loading buffer containing 1 M NaCl.
- Protein and Heme A concentrations were determined as described in *Materials and Methods*.
- Values given in table 2 represent those obtained from a single experiment.

When Mono Q purified cytochrome oxidase is run on a SDS polyacrylamide gel (see figure 9), three prominent bands corresponding to molecular weights of 55 000, 38 000 and 29 000 can be seen. In addition to these bands, two faint bands of 126 and 67 kDal are also present. Molecular weights of the three major bands are in close agreement with those described by de Vrij *et al.*, (1983) for *Bacillus subtilis*

Figure 8. Comparison of beef heart cytochrome oxidase (----) and *Bacillus subtilis* cytochrome oxidase (———). The concentration of beef heart cytochrome oxidase is 4.5 μM in a 25 mM Tris-HCl, pH 7.4 buffer containing 1 mg/ml laurylmaltoside. The concentration of *Bacillus subtilis* cytochrome oxidase is 450 ηM in a 25 mM Tris-HCl, pH 7.4 buffer containing 0.1% Triton X-100 and 1 mM Na_2EDTA. The spectra were obtained using the same conditions as described in figure 4. For purposes of comparison, the *Bacillus subtilis* difference spectrum is amplified by a factor of ten in order to place it on the same scale as the beef heart cytochrome oxidase difference spectrum.

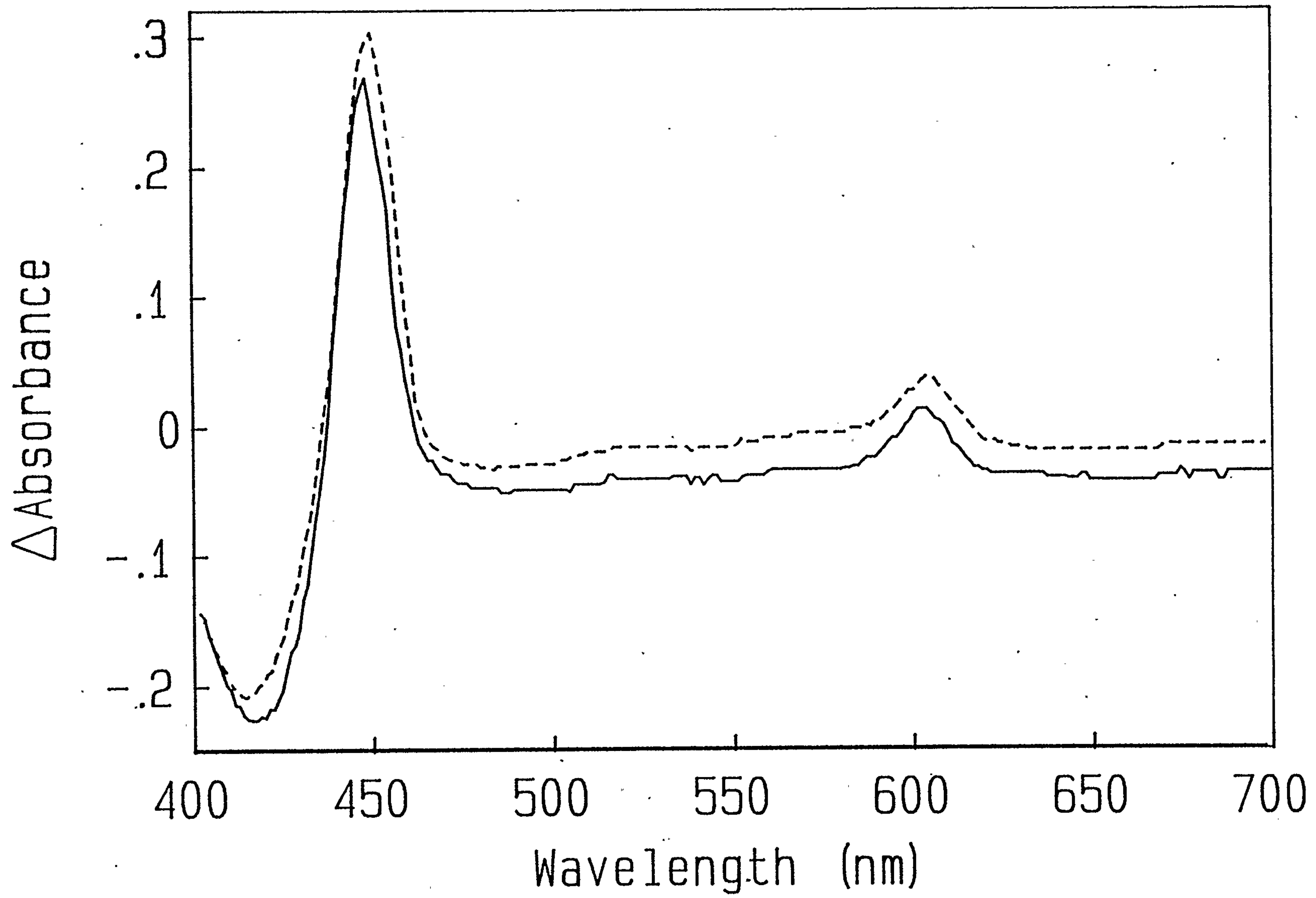
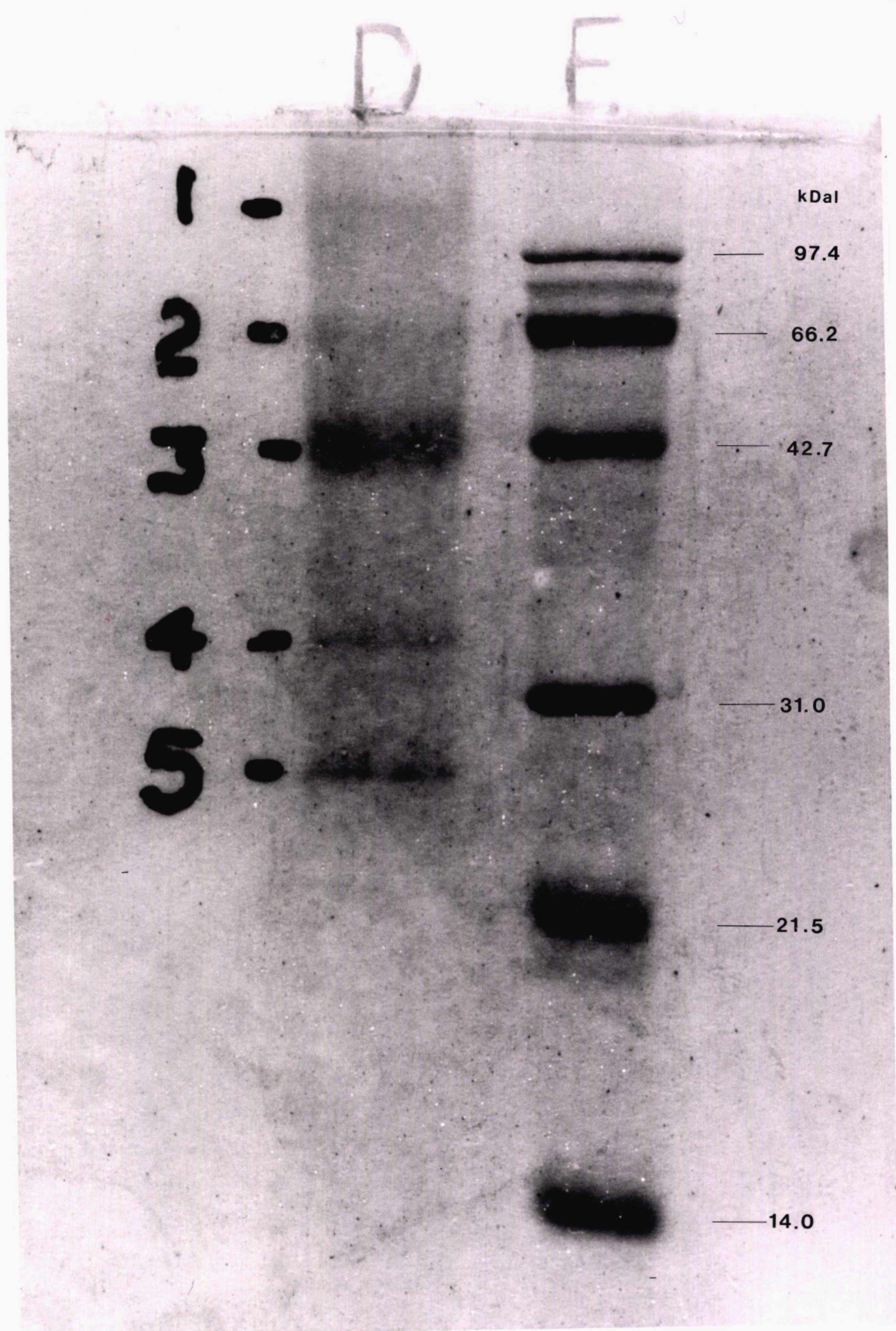

.3
.2
.1
0
-.1
-.2
ΔAbsorbance
400
450
500
550
600
650
700
Wavelength (nm)

Figure 9. *Bacillus subtilis* cytochrome oxidase run on a SDS denaturing gel. The lane on the left shows the band pattern produce by the bacterial oxidase. Five bands with molecular mass of 126 000, 67 000, 55 000, 37 000 and 29 000 Daltons are observed. The lane on the right shows the pattern produced by low molecular weight markers. Six principle bands have molecular masses of 97 400, 66 200, 42 700, 31 000, 21 500, and 14 000 Daltons. The molecular weights of bands in lane 'D' were determined from a standard curve constructed by plotting log molecular weight of the standards against their relative mobility. Molecular weights of bands 1 and 2 in lane 'D' were obtained from a linear regression line performed on standards with weights of 97.4, 66.2 and 42.7 kDal. A second regression line, constructed by using all standard point of the semi-log curve, was used to determine weights of bands 3, 4 and 5 in lane 'D'. Gel conditions are described in *Materials and Methods*.


D
E
1
2
3
4
5
kDal
97.4
66.2
42.7
31.0
21.5
14.0

cytochrome *c* oxidase (Mol. Wts. = 55 000, 37 000 and 21 000) and for that reported for cytochrome *c* oxidase from *B. stearothermophilus* (Mol. Wts. = 55 000, 37 000 and 22 000) (de Vrij *et al.*, 1989).

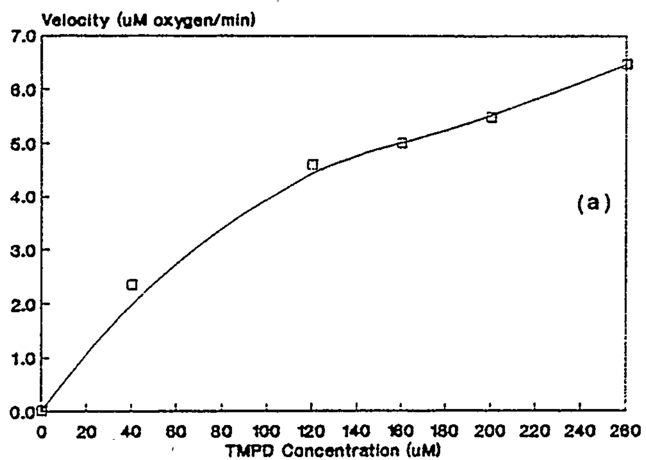
Respiratory Activity of *Bacillus subtilis* Membrane Vesicle Extract.

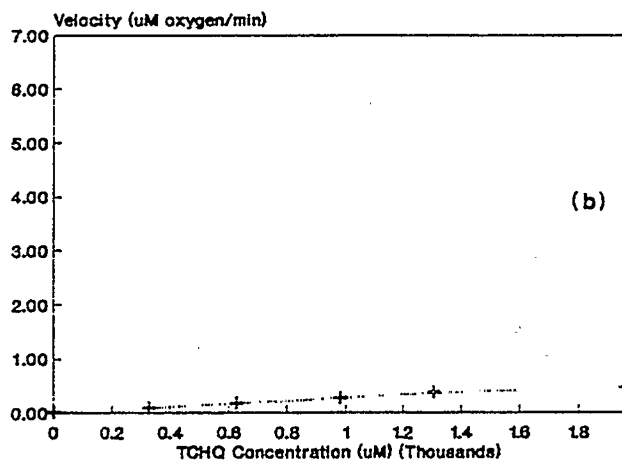
Membrane vesicles and purified cytochrome oxidase from *Bacillus subtilis* have also been characterized for their abilities to interact with various substrates. Solubilized plasma membrane vesicles of *Bacillus subtilis* show respiratory activity using the artificial electron donors ascorbate and TMPD which act to feed electrons to cytochrome *c*. However, when tetrachlorohydroquinone is used as a substrate, a significant decrease in respiratory activity is observed. Horse heart cytochrome *c* is not oxidized by the membrane vesicles, see figure 10. The purified enzyme shows only low turnover when assayed with ascorbate and TMPD. In addition, neither pure horse heart cytochrome *c* nor partially purified bacterial cytochrome *c* promote oxygen uptake with *Bacillus* oxidase.

Carbon Monoxide and Cyanide Binding to Membrane Vesicles and Cytochrome Oxidase.

Figure 11 illustrates the CO binding properties of solubilized membrane vesicles from *Bacillus subtilis*. A difference spectrum indicative of two CO binding hemes is produced when CO is added to dithionite reduced membranes. In this difference spectrum, the 430 nm peak and the 445 nm trough of cytochrome a_3-CO are clearly discernable. The 590 nm band also characteristic of cytochrome a_3-CO appears as a shoulder to the right of a band at 573 nm. In addition to the 573 nm

Figure 10. Oxidation rates of (a) tetramethyl-*p*-phenylenediamine, (b) tetrachlorohydroquinone and (c) horse heart cytochrome *c* by *Bacillus subtilis* membrane vesicles. Oxygen consumption was monitored polarographically in a 5 ml reaction mixture, using a Clark-type electrode (Yellow Spring Instrument Co.). The assay buffer contained 25 mM sodium phosphate, pH 7.0, 25 mM KCl, 0.1% laurylmaltoside and 1 mM disodium EDTA. When TMPD and tetrachlorohydroquinone were used as substrates the assay was performed as follows; 5 ml of assay buffer was placed in the reaction vessel (Model 5301) and equilibrated for 15 min at 29 C. To the mixture was added 5 mM ascorbate. After 5 min, the substrate was added. This rate of oxygen consumption constituted the background rate. Five min later, membrane vesicles were added to the assay mixture so that the final concentration of heme A was 30 nM. The background rate was subtracted from the enzymatic rate. When cytochrome *c* was used as the substrate, the assay was performed as described above except that the background rate was determined by monitoring the rate of oxygen consumption after additions of 5 mM ascorbate, 400 μM TMPD and membrane vesicles. Horse heart cytochrome *c* was added to the mixture and from the observed rate was subtracted the background rate. For each substrate concentration, the assay was repeated four times.


Velocity (uM oxygen/min)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0 20 40 60 80 100 120 140 160 180 200 220 240 260
TMPD Concentration (uM)
(a)


Velocity (uM oxygen/min)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8
TCHQ Concentration (uM) (Thousands)
(b)

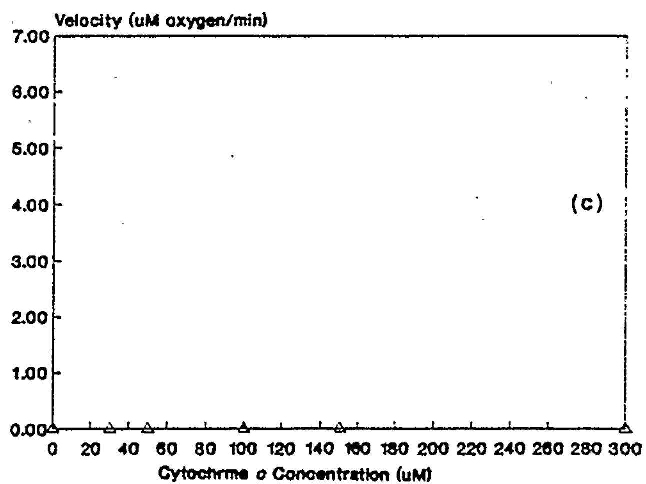

Velocity (uM oxygen/min)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Cytochrme c Concentration (uM)
(c)

Figure 11. Difference spectrum of carbon monoxide binding to *Bacillus subtilis* membrane vesicle extract. The difference spectrum was obtained by subtracting the absolute spectrum of the reference cuvette from the absolute spectrum of the sample cuvette. The reference cuvette contained dithionite reduced cytochromes present in the solubilized membranes. The sample cuvette contained dithionite reduced cytochromes present in solubilized membranes treated with CO gas. The membrane vesicles were suspended in 20 mM Tris-HCl buffer, pH 7.8, containing 1 mM Na_2EDTA, 1.5% Triton X-100 and 0.5% sodium cholate. CO gas was bubbled into the sample cuvette for 2 min.

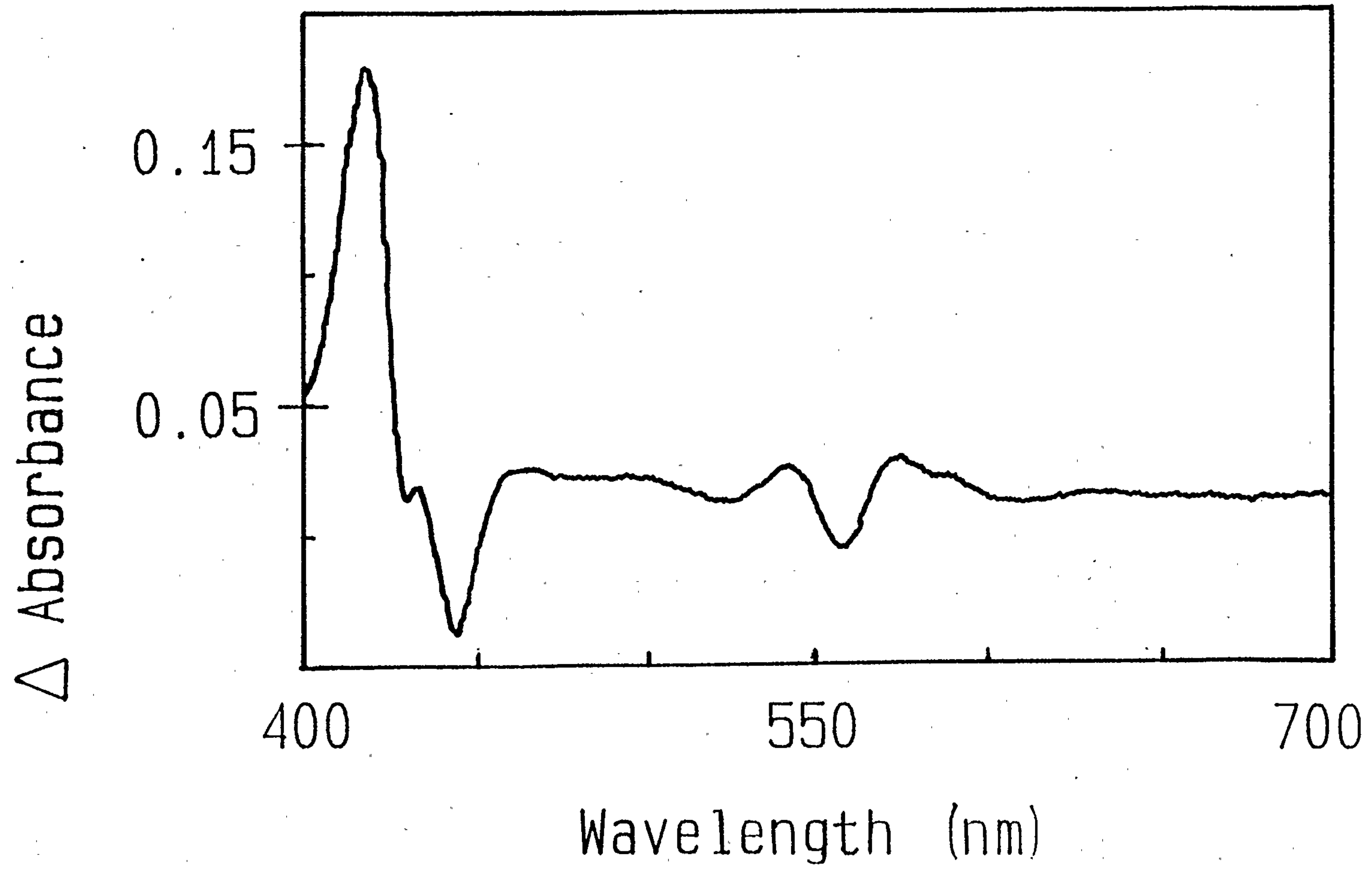
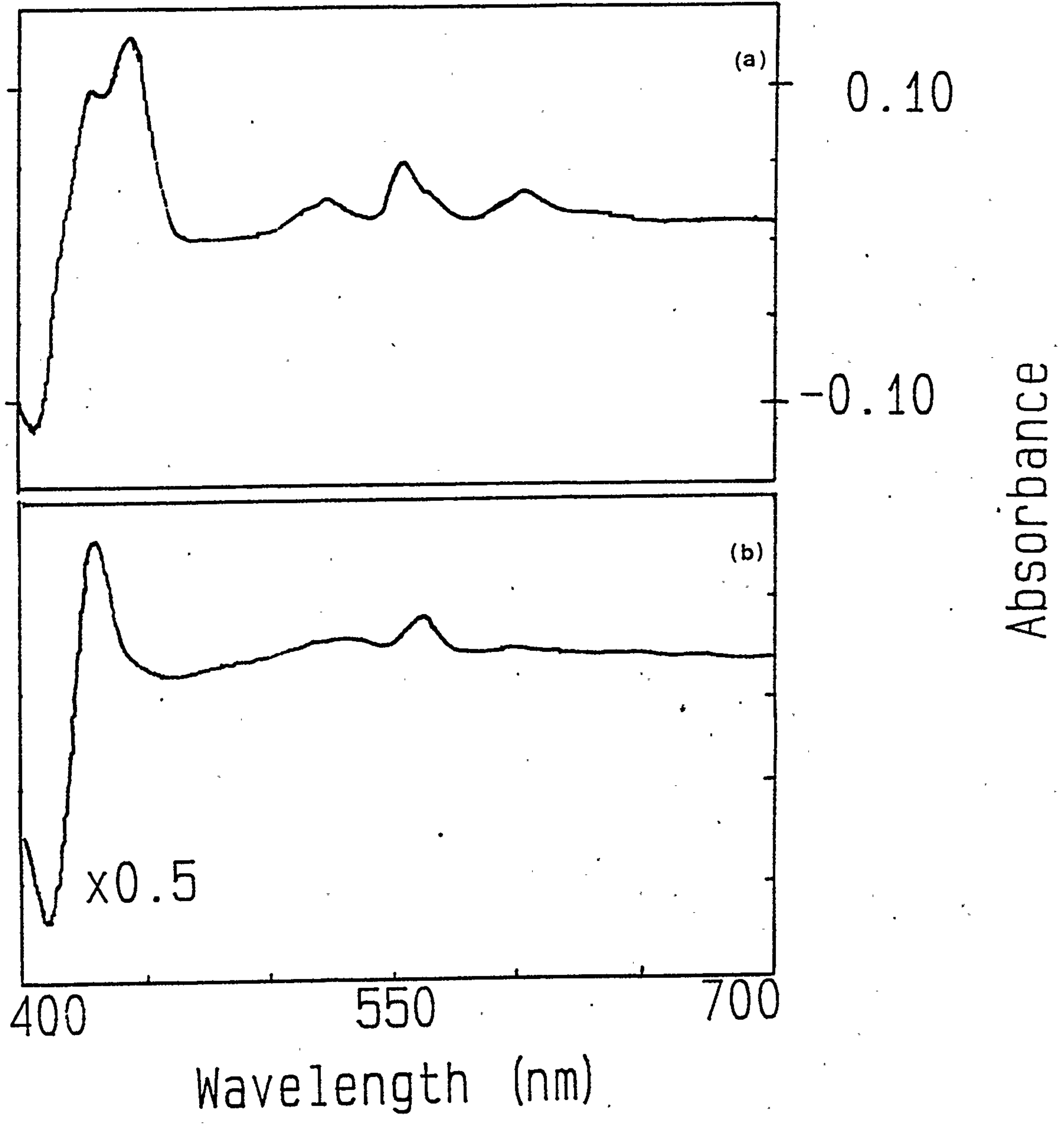

0.15
0.05
Δ Absorbance
400
550
700
Wavelength (nm)

Figure 12. Spectroscopic identification of a *b-o* type cytochrome in *Bacillus subtilis* membrane vesicles. (a) Ascorbate/TMPD reduced *minus* oxidized spectrum of *Bacillus subtilis* membrane vesicles. Membrane vesicles were reduced using 200 μM TMPD in the presence of excess ascorbate (5 mM). (b) Dithionite reduced difference spectrum *minus* ascorbate/TMPD reduced difference spectrum. The membrane vesicle extract was dissolved in 20 mM Tris-HCl buffer, pH 7.8, containing 1 mM Na_2EDTA, 1.5% Triton X-100 and 0.5% sodium cholate. The concentration of dithionite was 2 - 3 mM.


(a)
0.10
-0.10
(b)
x0.5
400
550
700
Wavelength (nm)
Absorbance

absorption band, there are peaks at 418 nm and 541 nm, as well as a trough at 558 nm. These latter spectral properties do not correspond to an aa_3-type cytochrome oxidase. The presence of these peaks are due to a second CO binding pigment also present in the membrane solubilized extract.

The second CO-binding component is spectrally resolved in the following way: When the membrane extract is treated with ascorbate and TMPD (see figure 12a), the reduced *minus* oxidized spectrum shows reduction of cytochrome *c* and cytochrome oxidase predominantly. This is in contrast to the dithionite reduced difference spectra shown in figure 4, cytochrome *b* is only partially reduced by ascorbate/TMPD as is evident by the decreased absorption peaks at 429 and 562 nm. The difference in the absorption intensities is directly observed by subtracting the ascorbate/TMPD reduced difference spectrum from the dithionite reduced difference spectrum, as is shown in panel (b) of figure 12. The spectrum shows maxima at 429 and 558 nm. The positions of the α and γ bands observed in this spectrum are analogous to the positions of the α and γ bands observed for the difference spectrum of cytochrome b_{562}-*o* complex, a terminal oxidase in the respiratory chain of aerobically grown *Escherichia coli* (Kita *et al.*, 1984). When the CO-ascorbate/TMPD reduced *minus* ascorbate/TMPD reduced spectrum is subtracted from the CO-dithionite reduced *minus* dithionite reduced spectrum, the resulting difference spectrum shows a maximum at 415 nm and a trough at 428 nm in the Soret region (figure 13). These features are similar to those reported for the CO difference spectrum of the cytochrome b_{562}-*o* complex (Anraku, 1988). Additionally, photolysis

Figure 13. Carbon monoxide reaction with a *b-o*-type cytochrome complex in *Bacillus subtilis* membrane vesicles. The difference spectrum illustrated in this figure was obtained by subtracting the CO-ascorbate/TMPD reduced *minus* ascorbate/TMPD reduced difference spectrum from the CO-dithionite reduced *minus* dithionite reduced difference spectrum of *Bacillus subtilis* membrane vesicles. The contents in the reference cuvette was reduced by adding 200 μM TMPD in the presence of 5 mM ascorbate, that of the sample cuvette was reduced by addition of 2 mM sodium dithionite. After addition of the reducing agents the samples were allowed to incubate undisturbed for 30 min. CO gas was bubbled into the sample cuvettes for 2 min. In both cases, ascorbate/TMPD reduction and sodium dithionite reduction, the spectrum of the reference cuvette was subtracted from that of the sample cuvette and the difference stored into one of two memory channels. The spectrum shown in this figure is achieved by subtracting the CO-ascorbate/TMPD difference spectrum from the CO-dithionite difference spectrum. The membrane vesicles were suspended in 20 mM Tris-HCl buffer, pH 7.8, containing 1 mM Na_2EDTA, 1.5% Triton X-100 and 0.5% sodium cholate.

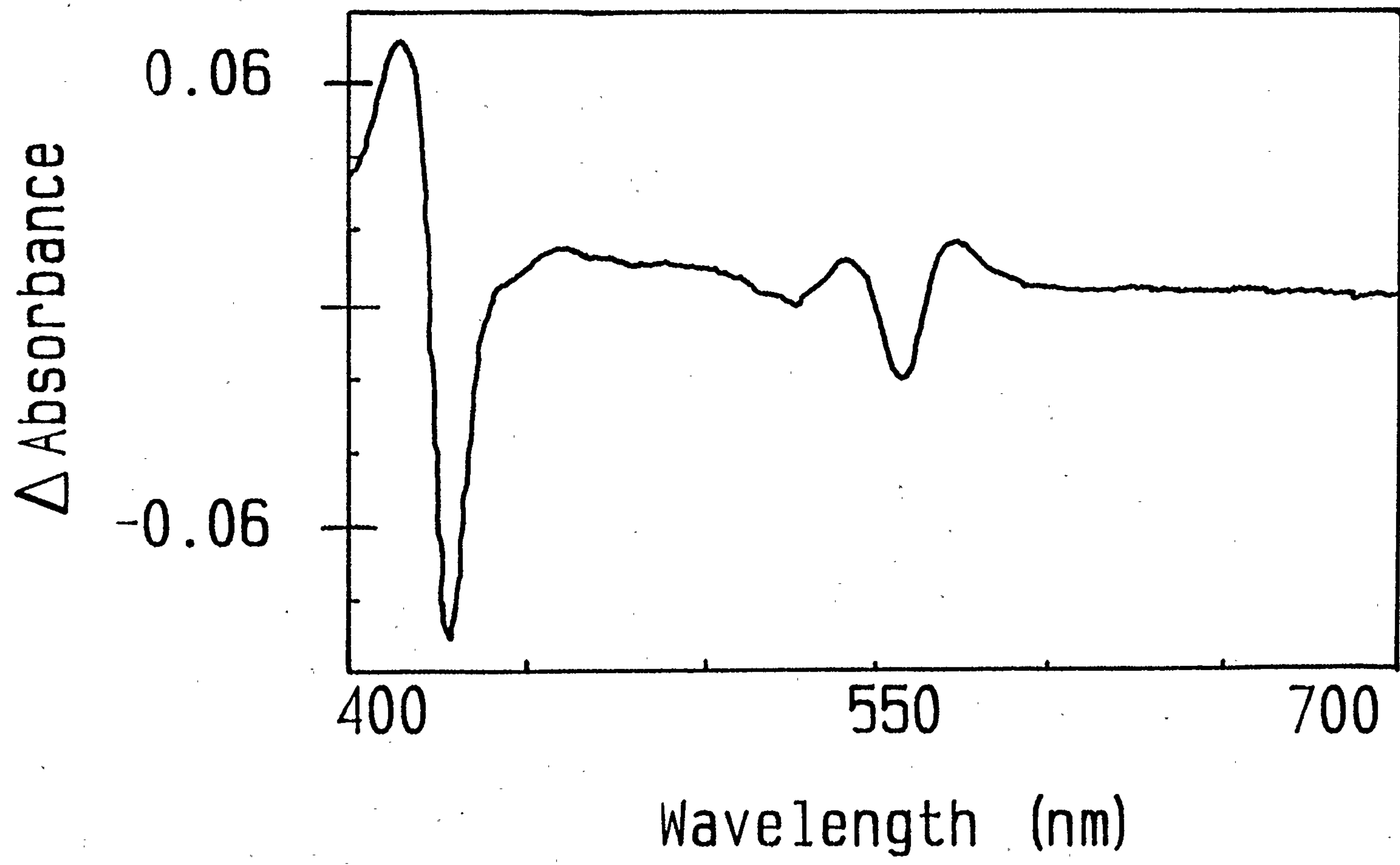
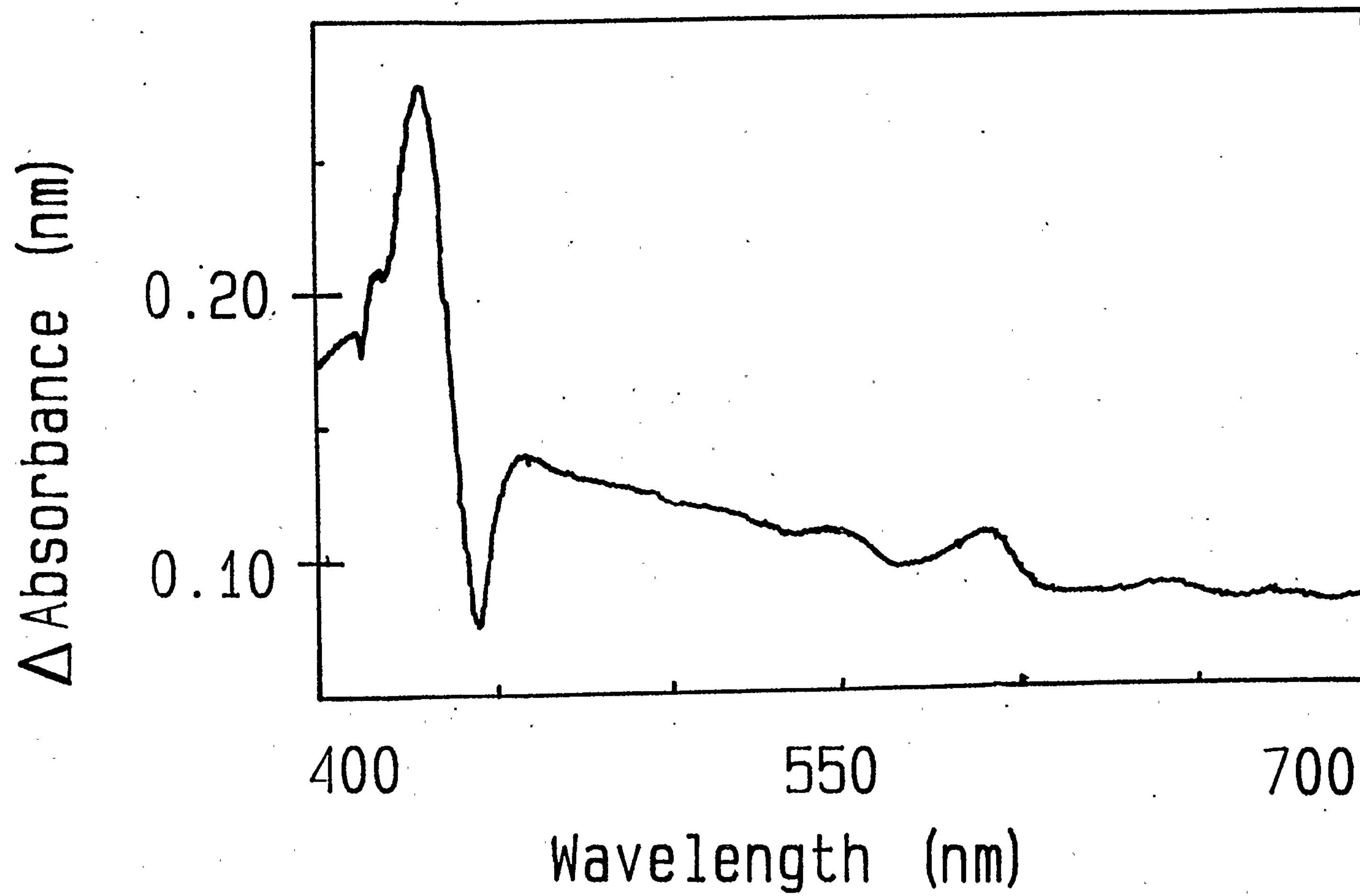

0.06
-0.06
Δ Absorbance
400
550
700
Wavelength (nm)

Figure 14. CO-bound spectrum of *Bacillus subtilis* cytochrome oxidase. To the sample and reference cuvettes was added 2 mM sodium dithionite and the cuvettes allowed to stand undisturbed for 15 min. CO gas was bubbled into the sample cuvette for 2 min. In order to obtain a difference spectrum, the spectrum of the reference cuvette is subtracted from the spectrum of the sample cuvette. The enzyme concentration is 2 μM and is dissolved in a 20 mM Tris-HCl buffer, pH 7.8, containing 1% Triton X-100 and 1 mM Na_2EDTA.


0.20
0.10
Δ Absorbance (nm)
400
550
700
Wavelength (nm)

of ascorbate/TMPD reduced, CO-liganded cytochromes of solubilized *Bacillus subtilis* membranes vesicles generates two CO recombination rate constants, indicating the presence of two CO binding pigments. At room temperature, the slow component has an observed rate constant of 50 s^{-1} while the fast component has an observed rate constant of 3 x 10^3 s^{-1} at a CO concentration of 1 mM. These observations suggest that there are two terminal oxidases in *Bacillus subtilis*, an aa_3-type oxidase and an *o*-type oxidase.

The CO-treated, dithionite-reduced *minus* dithionite-reduced spectrum of purified *Bacillus subtilis* cytochrome oxidase shows features characteristic of cytochrome a_3-CO complex. Peaks at 430 nm and 590 nm, as well as the trough at 445 nm indicate that the CO binding protein is an aa_3 oxidase (see figure 14).

The binding of carbon monoxide to *Bacillus subtilis* cytochrome oxidase is photodissociable. The photodissociation is measured as a change in absorbance in the Soret region after exposing CO bound cytochrome oxidase to a short flash of intense light. By varying the wavelength of the measuring light beam, a spectrum representing the difference between carbon monoxide bound enzyme and reduced cytochrome oxidase is obtained. The dissociation spectrum has characteristics resembling the difference spectrum of cytochrome oxidase, "CO-dithionite reduced *minus* dithionite reduced" (see figure 15). The trough at 445 nm and the peak at 430 nm is indicative of a cytochrome aa_3-type oxidase.

Figure 16 shows the spectra recorded for fully oxidized *Bacillus subtilis* cytochrome oxidase and oxidized, cyanide bound cytochrome oxidase after an

Figure 15. The difference spectrum obtained by illuminating CO bound *Bacillus subtilis* cytochrome oxidase with light. Carbon monoxide gas was bubbled for 2 min into a cuvette holding 600 μl of a 20 mM Tris-HCl buffer, pH 7.8, containing 1% Triton X-100, 1 mM disodium EDTA and 2 μM *Bacillus subtilis* cytochrome oxidase. The concentration of enzyme is determined was described in *Materials and Methods*. The experiment is carried out at ambient temperature.

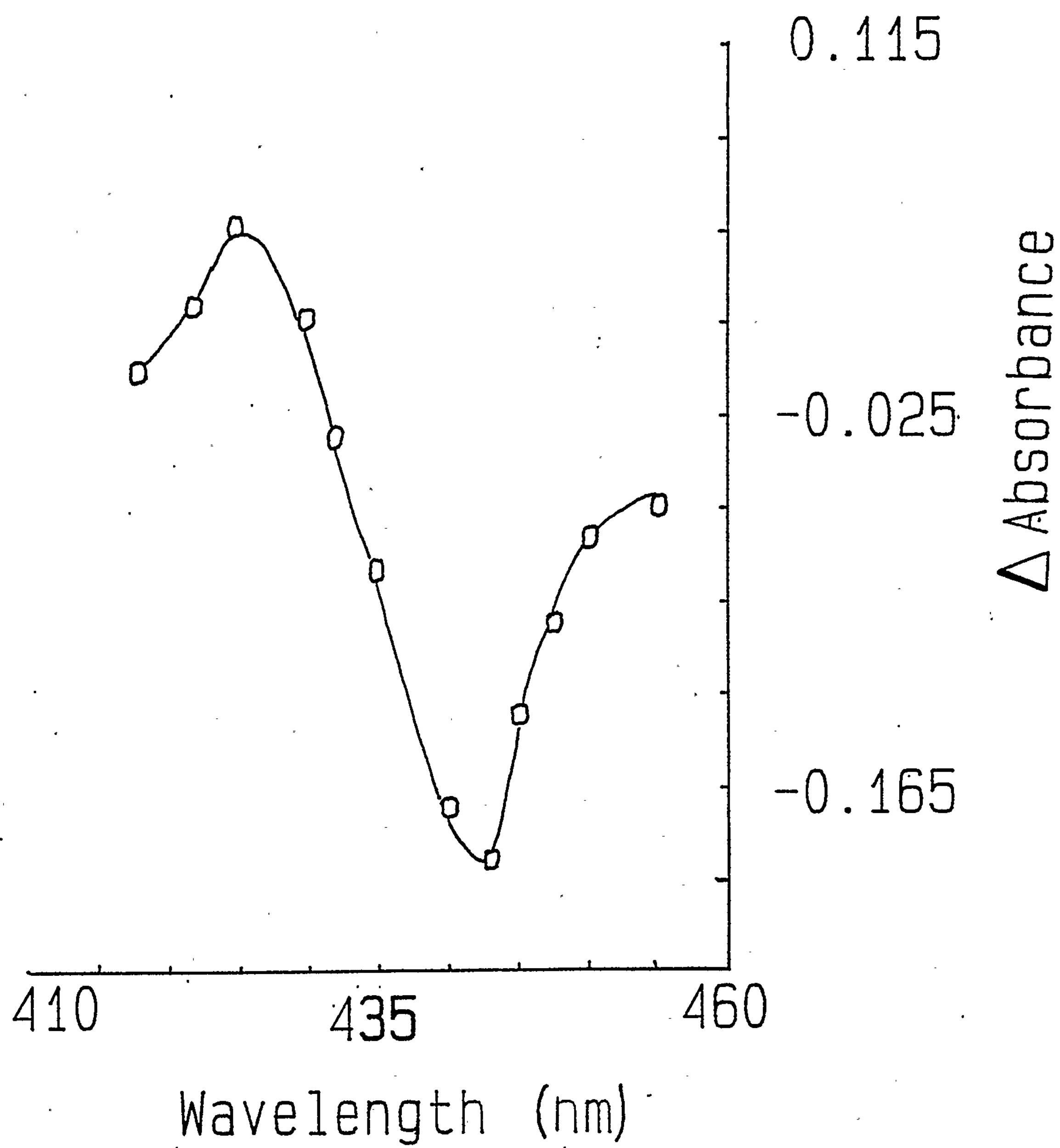
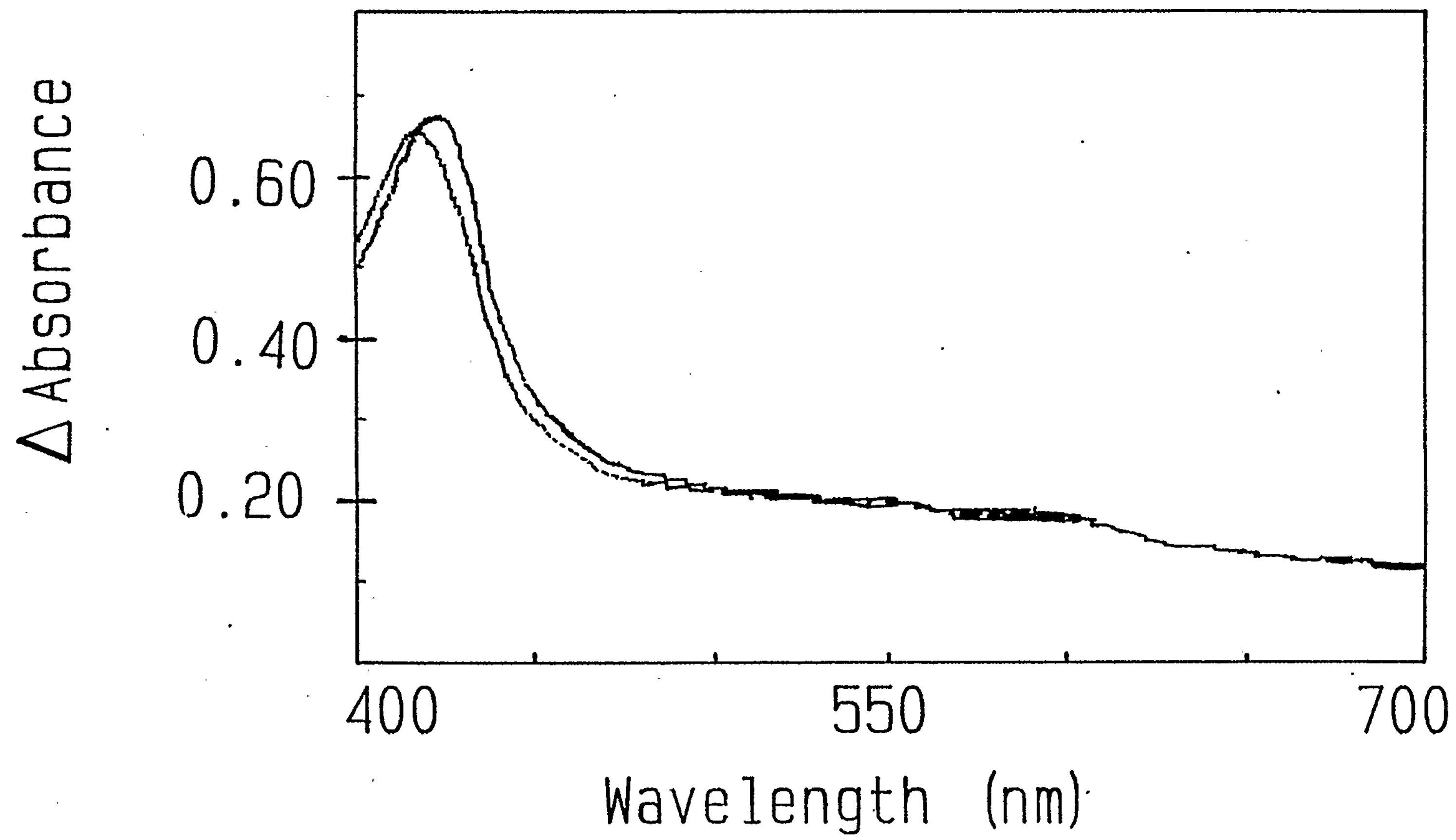

0.115
-0.025
-0.165
Δ Absorbance
410
435
460
Wavelength (nm)

Figure 16. The effects of cyanide on the spectrum of oxidized *Bacillus subtilis* cytochrome oxidase. Spectrum of cytochrome oxidase was obtained after the enzyme was incubated in the presence of 1 mM potassium cyanide overnight in a 20 mM Tris-HCl buffer containing 1% Triton X-100, 1mM disodium EDTA. The concentration of enzyme was 2 μM and was determined as described in *Materials and Methods*. The experiment was carried out at ambient temperature.



Δ Absorbance
0.60
0.40
0.20
400
550
700
Wavelength (nm)

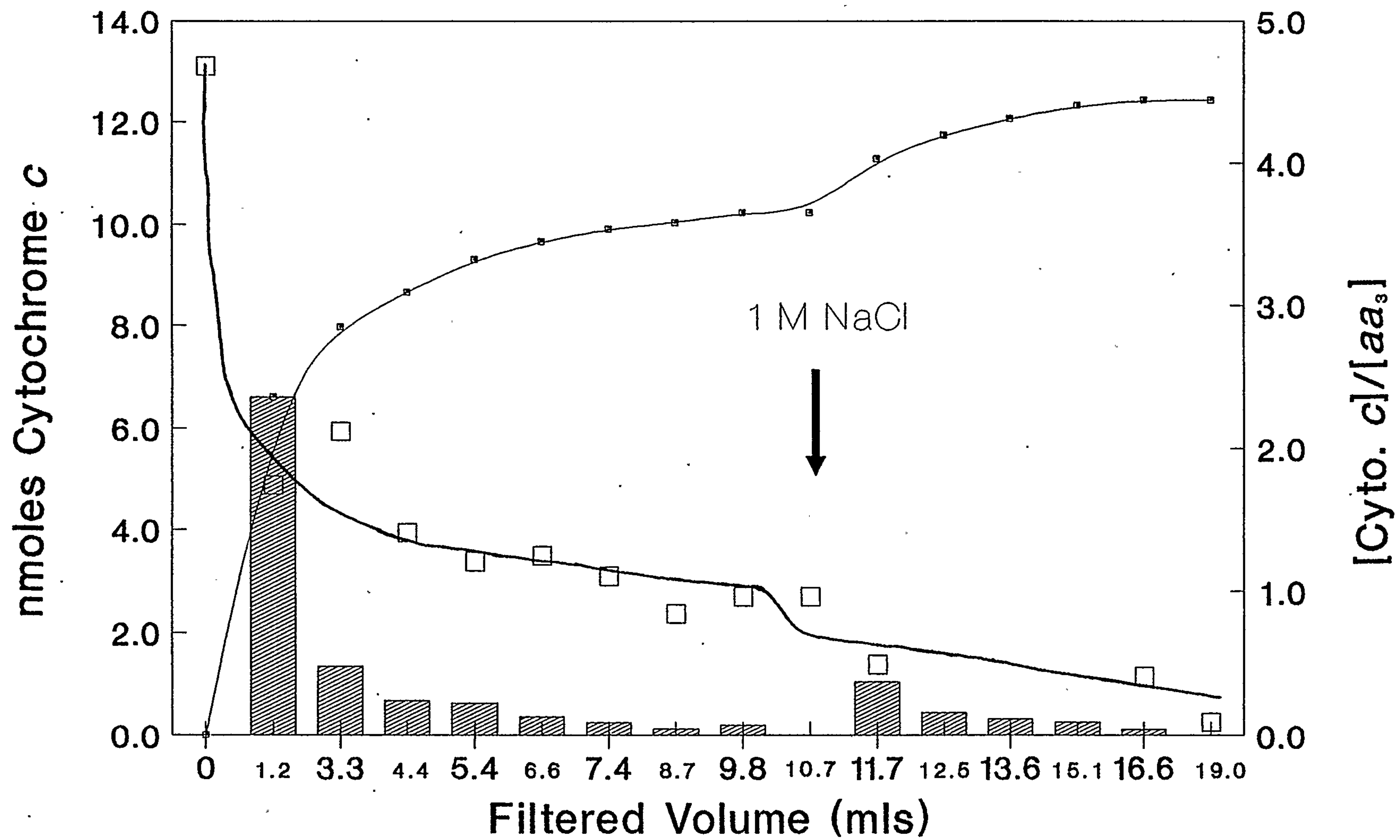
overnight incubation period. The Soret band shifts from 417 nm to 425 nm and intensifies slightly. These results are consistent with the changes observed when oxidized beef heart is incubated with cyanide (Van Buuren *et al.*, 1971). Thus this constitutes one more point of similarity between the beef heart cytochrome oxidase and the isolated enzyme of *Bacillus subtilis*.

Ferric beef heart cytochrome oxidase shows a weak absorption band at 830 nm (Griffiths and Wharton, 1961) which is assigned to a charge-transfer transition between Cu_A and one of its sulphur ligands (Beinert *et al.*, 1961). Ferric cytochrome oxidase isolated from *Bacillus subtilis* in this work fails to show this absorption band in the near infrared.

Cytochrome *c* Binding to Cytochrome Oxidase.

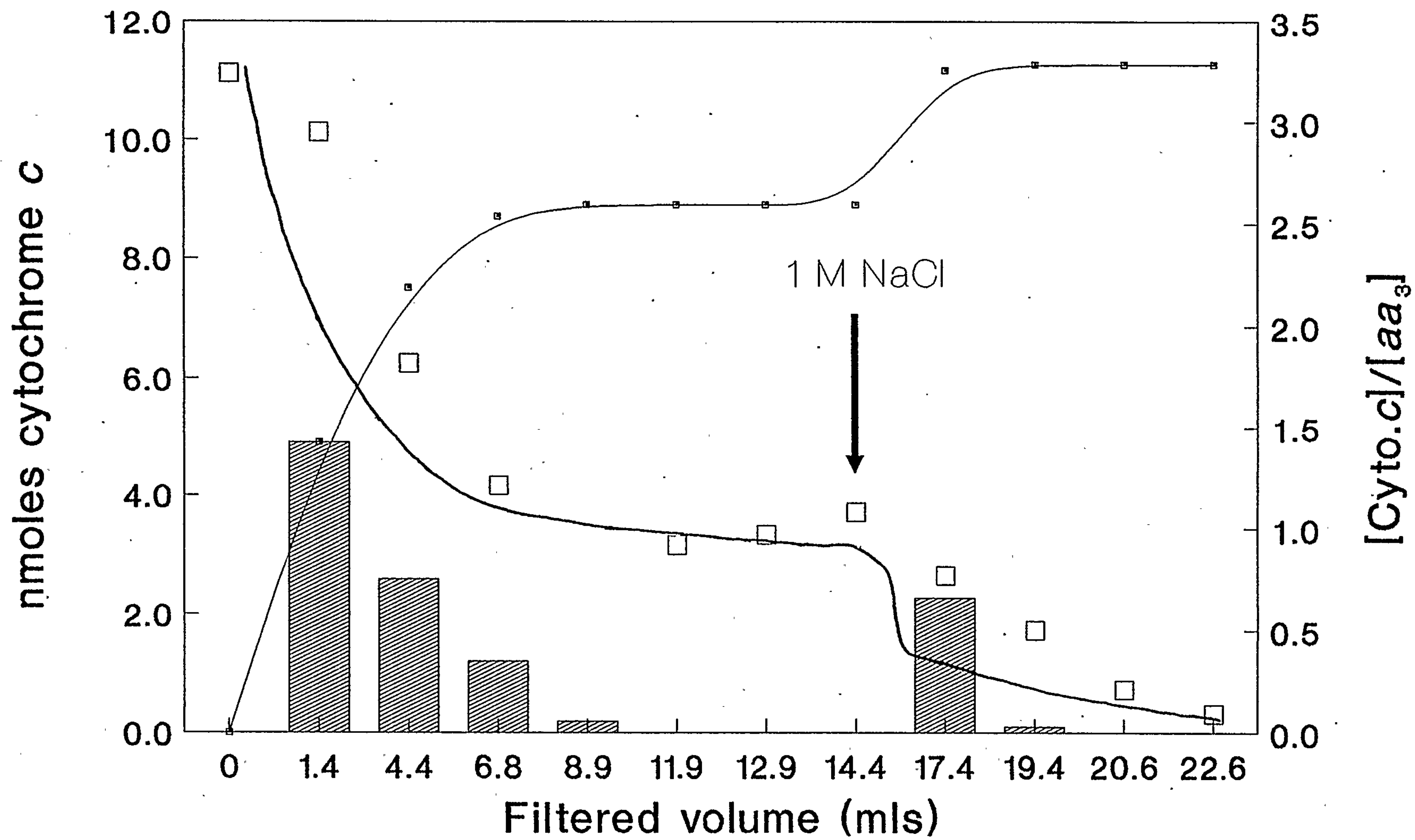
Mammalian cytochrome oxidase forms a thermodynamically stable complex with cytochrome *c*, in low ionic strength media, that is stabilized by electrostatic interactions and is dissociated in a high ionic strength environment. The binding of horse heart cytochrome *c* to beef heart cytochrome oxidase is compared to the binding of horse heart cytochrome *c* to *Bacillus subtilis* cytochrome oxidase (compare figures 17 and 18). Filtering the solution through a YM 100 membrane filter (Amicon), 54 % of the total cytochrome *c* content is collected in the first 15 min in a total volume of 1.2 ml and the cytochrome *c*/beef heart cytochrome oxidase ratio drops to 1.9. As cytochrome *c* continues to be washed through the filter, the cytochrome *c* to oxidase level reaches a limiting value of 1. At this point, no more

Figure 17. Horse heart cytochrome *c* binding to beef heart cytochrome oxidase. 2 ml of 20 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 was made 10 μM in cytochrome *c* and 2 μM cytochrome oxidase. This solution was placed in an ultrafiltration cell and the cell pressurized with nitrogen gas. The content of the cell was filtered through a YM 100 membrane filter under 15 pounds per square inch of pressure. At 15 minute intervals the pressure of the system was released and 100 μl of solution was removed from the cell, diluted six fold and a difference spectrum was recorded to determine the cytochrome *c* and cytochrome oxidase concentration. The filtrate (ca. 1.2 - 1.5 ml) was assayed for its cytochrome *c* content. The volume of the solution in the ultrafiltration cell was brought up to 2 ml with 20 mM Tris-HCl buffer, pH 7.4. After 135 min (indicated by the arrow), the volume of the cell was restored to 2 ml with 20 mM Tris-HCl buffer, pH 7.4, containing 1 M sodium chloride. Filtrate was collected for another 90 min. The ratio of cytochrome *c* to cytochrome oxidase of the retentate (-⊟-) and the cumulative amount of cytochrome *c* filtered (—) are plotted as '[Cyto. *c*]/[aa_3]'and 'nmoles Cytochrome *c*', respectively, against filtered volume. Quantity of cytochrome *c* present in each filtered fraction is shown as a histogram and plotted as 'nmoles of cytochrome *c*' against filtered volume. The experiment was carried out at 4 °C.



nmoles Cytochrome c
[Cyto. c]/[aa3]
Filtered Volume (mls)
1 M NaCl
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
5.0
4.0
3.0
2.0
1.0
0.0
0
1.2
3.3
4.4
5.4
6.6
7.4
8.7
9.8
10.7
11.7
12.5
13.6
15.1
16.6
19.0

Figure 18. Horse heart cytochrome *c* binding to *Bacillus subtilis* cytochrome oxidase. A solution of 2 μM cytochrome oxidase, suspended in 3.2 ml of 20 mM Tris-HCl, pH 7.4, containing 1% Triton X-100 was made 8 μM in cytochrome *c*. This solution was placed in an ultrafiltration cell and the cell pressurized with nitrogen gas. The content of the cell was filtered through a YM 100 membrane filter under a pressure of 15 pounds per square inch. At ca. 10 minute intervals the pressure of the system was released and 100 μl of solution was removed from the cell, diluted six fold and a difference spectrum was recorded to determine the cytochrome *c* and cytochrome oxidase concentration. The filtrate (ca. 1.0 - 3.0 ml) was assayed for its cytochrome *c* content. The volume of the solution in the ultrafiltration cell was brought up to 3.2 ml with 20 mM Tris-HCl buffer, pH 7.4. After ca. 70 min (indicated by the arrow), the volume of the cell was restored to 2 ml with 20 mM Tris-HCl buffer, pH 7.4, containing 1 M sodium chloride. Filtrate was collected for another 90 min. The ratio of cytochrome *c* to cytochrome oxidase of the retentate (-⊟-) and the cumulative amount of cytochrome *c* filtered (—) are plotted as '[Cyto. *c*]/[aa_3]'and 'nmoles Cytochrome *c*', respectively, against filtered volume. Quantity of cytochrome *c* present in each filtered fraction is shown as a histogram and plotted as 'nmoles of cytochrome *c*' against filtered volume. The experiment was carried out at 4 °C.


nmoles cytochrome *c*
12.0
10.0
8.0
6.0
4.0
2.0
0.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
[Cyto.*c*]/[*aa*3]
1 M NaCl
0
1.4
4.4
6.8
8.9
11.9
12.9
14.4
17.4
19.4
20.6
22.6
Filtered volume (mls)

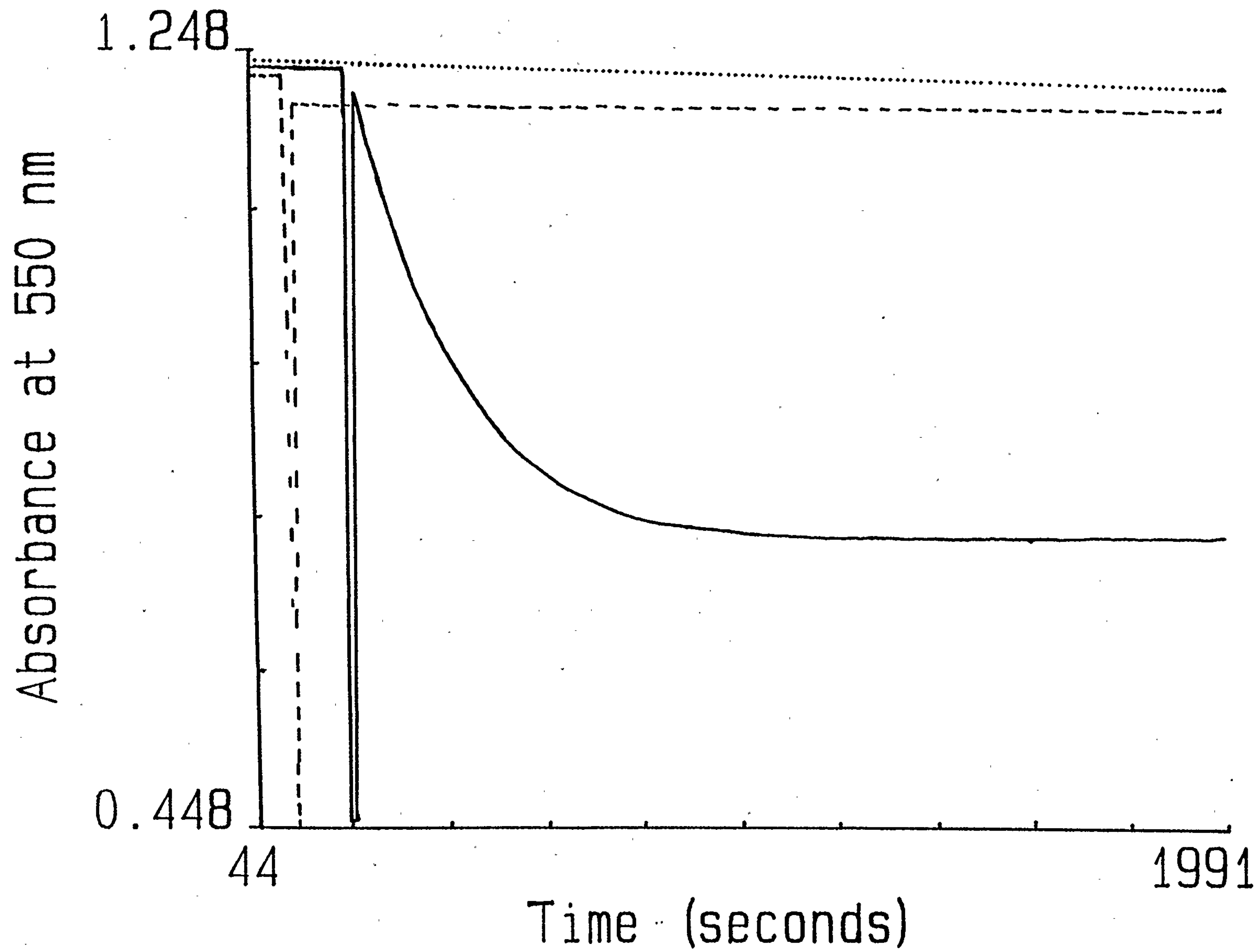
cytochrome *c* is detected in the filtrate. The time required for the excess cytochrome *c* to filter through the membrane is 135 min. At this stage 82 % of the total cytochrome *c* content has been filtered. The remaining 18% is filtered in a time span of 90 min in a total volume of 7.3 ml after the ionic strength was increased to 1 M (indicated by the arrow).

When the experiment is repeated with *Bacillus subtilis* cytochrome oxidase and horse cytochrome *c*, a similar pattern is observed (see figure 18). Forty three percent of the total cytochrome *c* collected is filtered in the first 10 min in a total volume of 1.2 ml. After 90 min, 78% of the total cytochrome *c* is collected. At this time the cytochrome *c*/cytochrome oxidase ratio is 1. The ionic strength is increased to 1 M (indicated by the arrow) and the remaining 22 % of the total cytochrome *c* collected is filtered.

Cytochrome *c* Oxidation by Cytochrome Oxidase.

In order to determine if *Bacillus subtilis* cytochrome oxidase is capable reoxidizing dithionite reduced, horse heart cytochrome *c*, a catalytic amount of *Bacillus subtilis* oxidase (15 nM) was combined with 30 μM reduced substrate. The rate of ferrocytochrome *c* oxidation was measured by following the decrease in the absorbency of its α-band at 550 nm. The results indicate that horse heart cytochrome *c* is not oxidized by *Bacillus subtilis* cytochrome oxidase (see figure 19). In contrast, beef heart cytochrome oxidase oxidizes reduced horse heart cytochrome *c* with a first order rate constant of 0.25 min^{-1}.

Figure 19. Time course of horse heart cytochrome *c* oxidation in the presence of beef heart cytochrome oxidase (——) or *Bacillus subtilis* cytochrome oxidase (---). Preparation of reduced cytochrome *c* and beef heart cytochrome oxidase is as described in *Materials and Methods*. Cytochrome *c* oxidation is followed by monitoring the magnitude of the α band (550 nm) of the reduced protein in the presence or absence (......) of cytochrome oxidase over a period of 1 h. This figure shows the expanded portion of the 1 hour time course for cytochrome *c* oxidation. The reaction medium contains 20 mM Tris-HCl, pH 7.4, 10 mM KCl, 0.1% Triton X-100 and 30 μM ferrocytochrome *c*. The reaction is started by making the reaction medium 15 nM in cytochrome oxidase. Reaction is allowed to proceed at ambient temperature.


1.248
0.448
44
1991
Time (seconds)
Absorbance at 550 nm

DISCUSSION

In aerobically grown *Bacillus subtilis*, at least two CO combining proteins are consistently found in membrane vesicles when solubilized with Triton X-100/sodium cholate detergent mixture. These two proteins are cytochrome (*aa_3*-type) oxidase and a cytochrome with spectral characteristics resembling the cytochrome *o* complex in *Escherichia coli*. Also present in the extract are *c*-type cytochromes. In this thesis I provide a protocol for producing a spectrally homogeneous cytochrome (*aa_3*-type) oxidase with a heme to protein ratio of 8 nmol heme A/mg of protein. The isolated protein binds horse heart cytochrome *c* and possesses similar characteristics to the bovine heart cytochrome oxidase in its reduced *minus* oxidized, carbon monoxide bound *minus* reduced, oxidized cyanide bound and photodissociation spectra. This protocol is meant for the large (100 litres) scale isolation of the bacterial cytochrome oxidase and is a modification of the procedure originally presented by de Vrij *et al.*, (1983) for the isolation of the enzyme on a smaller scale (10 litres).

A. Growth Conditions and Cell Lysis.

The composition of bacterial respiratory chains varies with growth conditions. Two major factors influence the composition of the *Bacillus subtilis* respiratory chain; (a) composition of the growth media and (b) oxygen (Downey, 1964). James *et al.*, (1989) isolated a mutant of *Bacillus subtilis* unable to grow when succinate is used as a sole source of carbon. However, they observed that the bacteria will grow aerobically by using cytochrome *o* as a terminal oxidase if the growth medium is supplemented with glucose. Spectral data of the detergent solubilized membrane

vesicles reveals that this mutant lacks aa_3-type cytochrome oxidase. One possibility to explain the failure of the mutant bacteria to grow on succinate is that, in contrast to wild-type *Bacillus subtilis*, capable of growing on succinate as a sole source of carbon, the mutant *Bacillus subtilis* lacks cytochrome aa_3, and electrons from succinate oxidation flow preferentially to cytochrome aa_3, and not to cytochrome *o*. The oxidation of glucose, on the other hand, leads to the reduction of dioxygen via type-*o* cytochrome oxidase enabling aerobic growth. In the present work, the possibility of preferential electron transfer to cytochrome aa_3 when succinate is oxidized justifies the supplementation of the original growth medium (de Vrij *et al.*, 1983) with succinate in order to try and maximize the quantity of cytochrome oxidase (aa_3) expressed by *Bacillus subtilis*. It was hoped that succinate would induce the expression of type *a* cytochrome oxidase. This might occur if succinate is converted, by the bacteria, to succinyl-CoA. Succinyl-CoA can be reacted with glycine, in a condensation reaction catalyzed by δ-aminolevulinate synthase, to form δ-amino levulinate, the immediate precursor of porphyrin needed for cytochromes.

Although it is difficult to speculate as to why membrane extracts of *Bacillus subtilis* grown on a succinate supplemented medium have the highest heme A to protein ratio, it is possible that cytochrome oxidase (aa_3) in this bacteria is inducible by growing the bacteria on a medium containing succinate.

In some bacteria it has been demonstrated that the ratio of terminal oxidases present in the cytoplasmic membrane can be controlled by varying the oxygen concentration in the growth medium (Ludwig, 1987 and Sone *et al.*, 1983). In the

thermophilic microorganism,PS-3 and in *Bacillus stearothermophilus* oxygen limitation lead to an increase in the cytochrome *o* content, whereas, under strong aeration cytochrome-aa_3-type oxidase becomes dominant (Sone *et al.*, 1983; de Vrij *et al.*, 1989). Downey, (1964) has shown that *Bacillus subtilis*, when grown at high oxygen tension expresses higher quantities of cytochrome *a* than those cells grown under the same conditions but at lower oxygen concentrations. In this work, the cells were grown in cultures exposed to air.

The membrane vesicle preparation as described by de Vrij *et al.*, (1983), is essentially a two step procedure, involving: (a) the conversion of the bacterial cells into osmotically sensitive structures, i.e., protoplasts and (b) lysis of the protoplasts due to osmotic shock. The first step is accomplished by treating the bacteria with lysozyme and EDTA. The protoplasts, suspended in a hyposmotic medium, undergo lysis and the cellular content is released (Gottschalk, 1988). Preliminary experiments (not shown) show that for a large scale isolation procedure a proportional amount of lysozyme in the lysing solution as originally described by de Vrij *et al.*, (1983), results in the solubilization of only 40-50% of the cytochrome oxidase present in the membrane vesicles during detergent extraction. However, if the lysozyme concentration is increased to 2.5 fold that proposed by de Vrij *et al.*, (1983), or if the bacterial cells are incubated at 50 °C during the lysing stage of the isolation procedure, up 75% of the total cytochrome oxidase present in the membrane vesicles can be extracted. It can be argued that if the cell wall is not digested, the cytoplasmic membrane is not exposed to the detergent molecules and protein extraction would not occur at the detergent solubilization stage.

B. Detergent Extraction of Cytochrome Oxidase.

Bacillus subtilis cytochrome oxidase is a transmembrane protein (de Vrij *et al.*, 1987). As with most membrane proteins, cytochrome oxidase can be more efficiently extracted by the more hydrophobic detergents (Helenius and Simons, 1975; and Egan *et al.*, 1976). Nonionic detergents such as Triton X-100 have been used extensively for the isolation and purification of terminal oxidases from a variety of microorganisms (Schiavo and Bisson, 1989; Kita *et al.*, 1984 and Lemberg and Barrett, 1973). A measure of relative hydrophobicity of nonionic detergents is the hydrophilic/lipophilic number. The lower this number, the greater the hydrophobicity of the detergent. Umbreit and Strominger, (1973), have shown that detergents with a hydrophilic/lipophilic balance value between 12 and 14 are optimal for solubilizing membrane proteins of *Bacillus subtilis*. The results obtained in this work agree with this observation: Tween 80, Triton X-100, Triton X-114 and laurylmaltoside have hydrophilic/lipophilic balance values of 15.0, 13.5, 12.4 and 8.9, respectively. Despite the fact that laurylmaltoside appears to solubilize cytochrome oxidase 1.6 times more efficiently than Triton X-100, the benefit is negated by the fact that this detergent solubilizes other proteins from the cell membrane which Triton X-100 does not. As a result, laurylmaltoside membrane vesicle extracts of *Bacillus subtilis* have a significantly lower heme A to protein ratios than Triton X-100 extracts. An explanation for the decreased degree in selectivity for protein solubilization of laurylmaltoside, relative to Triton X-100 involves the well established mechanism of membrane protein solubilization by detergents. This mechanism requires that the

detergent molecules partition themselves into the membrane. When the detergent concentration becomes greater than the lipid concentration in the bilayer, the equilibrium of all amphipathic molecules in the membrane mixture shifts from bilayer to lipid-detergent-protein mixed micelles (Helenius and Simons, 1975). Since the ability of a detergent to partition into a bilayer is influenced by its hydrophobic nature, one can argue that the greater hydrophobic nature of laurylmaltoside allows it to partition into the membrane's hydrophobic environment to a greater extent than Triton X-100, leading to a greater degree of protein solubilization.

When a Triton X-100/sodium cholate detergent mixture or Triton X-100 alone is used to solubilize *Bacillus subtilis* membrane vesicles, the extract contains similar spectral features as solubilized, mammalian submitochondrial particles. The major differences appear to be in the relative quantities of cytochromes *b* and *c* present in extracts. It has been postulated that all *c*-type cytochromes in Gram-positive bacteria are membrane-bound because they lack an outer membrane, and as such, soluble cytochrome *c* would be lost by diffusing through the cell wall (Pettigrew and Moore, 1987; and Wood, 1983). Furthermore, von Wachenfeldt and Hederstedt, (1990), have demonstrated that, in *Bacillus subtilis*, cytochrome c_{550} is anchored to the cytoplasmic membrane. They propose a two domain structure, one membrane-spanning anchor and the other containing the heme. In contrast, cytochrome c_{550} of mammalian mitochondria is water soluble. During the preparation of submitochondrial particles, soluble cytochrome *c* can be easily separated from the inner mitochondrial membrane. Thus, only cytochrome c_1, which is membrane-bound (Lemberg and

Barrett, 1973) and associated with complex III remains attached to the inner mitochondrial membrane (Kuboyama *et al.*, 1972). The absorbance observed in the 550 nm region for the submitochondrial extract is most likely due to cytochrome c_1. However, if in *Bacillus subtilis*, all cytochromes *c* are membrane-bound, then no cytochrome *c* is expected to be released from the periplasmic space during cell wall digestion with lysozyme and hence, the absorbance observed in the 550 nm region is due to both, cytochrome c_1 and cytochrome c_{550}; this would explain the higher absorbency observed in this region for the bacterial extract. The greater quantity of cytochrome *b*, relative to cytochrome aa_3 in the bacterial extract, as opposed to the relative quantities in beef heart submitochondrial particles of these cytochromes can be explained by the presence of cytochrome *o*, a heme B-containing protein, in *Bacillus subtilis*.

C. Purification of Cytochrome Oxidase.

Bacillus subtilis cytochrome oxidase can be isolated using a two step ion-exchange procedure. The first ion exchange is used as a bulk separation stage where negatively charged protein may be removed from the preparation. This bulk separation phase can be performed using DEAE cellulose or a Biorad Econo-Pac Q cartridge. In both cases, the eluent is less turbid suggesting that particles such as, membrane fragments, lipid micelles, protein micelles, etc., which are not pelleted during centrifugation of the solubilized membrane mixture, are being bound irreversibly to the resin and are not being eluted with high ionic strength buffer. This irreversible binding of the protein to the column is likely to occur via hydrophobic

interaction if the protein is being denatured in such a fashion as to expose the hydrophobic portion of the protein to the surface of the molecule. The low recovery (ca. 45% with Biorad Econo-Pac Q cartridge and ca. 60% with DEAE cellulose/Sepharose) of cytochrome oxidase observed at this stage of the purification may indicate that adequate solubilization of the enzyme is not achieved. In this case, the enzyme is trapped in membrane fragments which are too large to pass through the resin. A second possibility to explain the low recovery of the enzyme is that the solubilized cytochrome oxidase is not homogeneous. As such, some cytochrome oxidase molecules bind to the resin electrostatically, whereas, other molecules associate with the resin or matrix via hydrophobic interaction (Rossmondo, 1990). These latter cytochrome oxidase molecules are, therefore, not eluted using the salt gradient. Yet, a third possibility may be that the ionic interaction between the resin and the enzyme is so tight that some molecules can not be displaced by salt anions (chloride). This latter possibility may be the reason why bulk separation using DEAE cellulose/Sepharose gives a greater recovery of the enzyme than does bulk separation with Pharmacia Mono Q or Biorad Econo-Pac Q cartridge. The functional group (diethylaminoethyl) of DEAE resins is a tertiary amino group (pK_a 9) and binds cytochrome oxidase less tightly than does the quaternary amino group (trimethyl) of Q resin. Therefore, the chloride counter ion is more likely to displace cytochrome oxidase bound to DEAE than it is cytochrome oxidase bound to Q Sepharose.

D. Properties of *Bacillus subtilis* Cytochrome Oxidase.

Terminal oxidases can be identified by their ability to interact with carbon monoxide (Lemberg and Barrett 1973). Carbon monoxide cause parts of the 445 nm and 605 nm bands of reduced beef heart cytochrome oxidase to disappear and be replaced by a peak at 430 nm and a shoulder at 590 nm (Yonetani, 1960). With aa_3-type cytochrome oxidases, the high spin, pentacoordinated, ferrous cytochrome a_3 binds to carbon monoxide. Since 80% of the total absorbance of the α-bands is due to cytochrome a, the position of the α-band does not change drastically, nonetheless, a characteristic shoulder at 590 nm is observed. In the Soret region, however, fifty percent of the absorbance of the γ-band is due to cytochrome a_3 and CO causes a significant shift of the Soret band from 444 nm to 430 nm (Nicholls, 1978). The purified bacterial oxidase also shows these characteristics and the photodissociation spectrum is that of cytochrome aa_3 (Chance *et al.*, 1953). In view of these data, the identity of the isolated protein, *Bacillus subtilis* cytochrome oxidase, is established with confidence on a spectroscopic basis. Further evidence to support this conclusion is provided by the pattern observed on the sodium dodecyl sulphate polyacrylamide gel which clearly indicates the presence of three subunits corresponding to the molecular weights of the subunits of the *Bacillus subtilis* cytochrome oxidase isolated by de Vrij *et al.*, (1983).

In contrast to the results reported by de Vrij *et al.*, (1983), purified *Bacillus subtilis* cytochrome oxidase isolated in this work does not promote oxygen uptake when horse heart cytochrome *c*, in the presence of tetramethyl-*p*-phenylenediamine

and ascorbate, is used as substrate. Two possibilities can explain this observation; (a) oxygen is unable to bind to the enzyme and/or (b) the cytochrome *c* binding site is either damaged or not present. Binding studies performed using carbon monoxide and cyanide suggest that the ligand binding site is not obstructed and oxygen is free to interact with the oxygen binding site. The role of Cu_B in the dioxygen reduction reaction is well documented (Hill *et al.*, 1986; and Chan *et al.*, 1988). Unlike Cu_A which has an absorption band at 830 nm, Cu_B in cytochrome oxidase shows no detectable spectral features with absorption spectroscopy. Thus, from the spectra presented in this thesis, it cannot readily be assumed that Cu_B is present at the oxygen reduction site of *Bacillus subtilis* cytochrome oxidase. On this basis, it is possible to argue that the enzyme shows no oxygen uptake because Cu_B is absent. However, indirect evidence for the presence of Cu_B in *Bacillus subtilis* is provided by the carbon monoxide difference spectrum. Lindsay *et al.*, (1975), and Wilson and Miyata, (1977) have shown that carbon monoxide binding in mitochondrial cytochrome oxidase requires the reduction of both, cytochrome a_3 and Cu_B. On the assumption that *Bacillus subtilis* cytochrome oxidase interacts with carbon monoxide in the same fashion as beef heart cytochrome oxidase, the binding of CO to the bacterial oxidase implicates the presence of a Cu_B centre. Furthermore, it is observed that if an ascorbate/TMPD reduced sample of *Bacillus subtilis* cytochrome oxidase is exposed to oxygen, the 601 nm band disappears and the Soret peak shifts from 442 nm to 416 nm in the absolute spectrum. This further suggests that oxygen is able to interact with the enzyme.

It has been suggested that subunit III of *Paracoccus denitrificans* cytochrome oxidase is lost when the protein is isolated using the original protocol (Saraste *et al.*, 1986). Although subunit III depleted *Paracoccus denitrificans* cytochrome oxidase is capable of oxidizing horse heart cytochrome *c*, the enzyme is a poor proton translocator (Saraste *et al.*, 1986). This precedence for the loss of a subunit from a bacterial oxidase raises the possibility that purification of *Bacillus subtilis* cytochrome oxidase according to the procedure presented in this thesis results in the loss of subunit II. The loss of this subunit, which contains the cytochrome *c* binding site (Holm *et al.*, 1987), would explain the lack of respiratory activity exhibited by the enzyme. However, sodium dodecyl sulphate polyacrylamide gel electrophoresis of the purified enzyme reveals that this is not the case. The molecular weight of the subunits present in *Bacillus subtilis* cytochrome oxidase isolated by de Vrij *et al.*, (1983) correspond to those bands for the enzyme isolated in this work.

Cytochrome *c* binding studies confirm the presence of a cytochrome *c* binding site on *Bacillus subtilis* cytochrome oxidase. The dissociation of the 1:1 cytochrome *c*:cytochrome oxidase complex at high salt concentration indicates that substrate binding to the oxidase is electrostatic. Although not conclusive, the fact that the cytochrome *c* : *Bacillus subtilis* cytochrome oxidase value reaches a limiting value of 1 in a low ionic strength environment and then drops at a high salt concentration infers that the binding of horse heart cytochrome *c* to *Bacillus subtilis* cytochrome oxidase is selective. These results are also observed for the interaction of beef heart cytochrome oxidase with horse heart cytochrome *c* (Ferguson-Miller *et al.*, 1976).

One aspect of the cytochrome *c* binding to beef heart cytochrome oxidase experiment merits consideration. It involves the percentage recovery of total cytochrome *c* added to the system. Out of 20 nmoles cytochrome *c* originally combined with cytochrome oxidase, only 63 % was recovered. This low recovery may indicate that cytochrome *c* binds nonspecifically to the sides of glassware and to the filtering apparatus.

The failure of *Bacillus subtilis* cytochrome oxidase to oxidize horse heart cytochrome *c* or partially purified *Bacillus subtilis* cytochrome *c*, coupled with the evidence obtained from the cytochrome *c* binding experiment suggests the presence of a kinetically inactive cytochrome *c* binding site. The absence of a 830 nm absorption band in the air oxidized *Bacillus subtilis* cytochrome oxidase spectrum, suggests that Cu_A is not present in the protein. There is structural data to support the argument that the position of cytochrome *c*, in a cytochrome *c*/cytochrome oxidase (beef heart) complex, is close to the Cu_A site of the enzyme and that this redox centre is directly reduced by substrate (Capaldi, 1990). Thus, the absence of Cu_A in *Bacillus subtilis* cytochrome oxidase may be responsible for the lack of respiratory activity exhibited by the enzyme. This conclusion is supported by the work done on *Halobacterium halobium* by Fujiwara *et al.*, (1989). They isolated cytochrome oxidase from *Halobacterium halobium* grown under copper deficient conditions. The enzyme contains no copper and is devoid of electron transfer and dioxygen reduction activity.

Future studies on the *Bacillus subtilis*, aa_3-type cytochrome oxidase, as isolated by the protocol presented in this thesis, should include the determination of the

copper content associated with the protein. This determination can be achieved with the use of atomic absorption spectroscopy (Fujiwara *et al.*, 1989). Alternatively, colorimetric assays can be performed to quantify the copper present in the protein (Brumby and Massey, 1967). If the protein is found to be copper deficient (less than 2 mol Cu/mol aa_3), it would be interesting to incubate the purified aa_3-type protein in a solution containing copper, and then test for cytochrome *c* oxidase activity.

Another point of interest is the oxygen uptake activity observed when membrane vesicle extract is assayed using TMPD as an artificial electron donor. It is still not clear what component(s) is responsible for this activity. Testing each fraction as it elutes from the Pharmacia Mono Q column may reveal which protein is responsible for TMPD oxidase activity.

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