STUDIES ON THE CYTOCHROME $\underline{BC}_1$ COMPLEXES OF PURPLE NON-SULFUR PHOTOSYNTHETIC BACTERIA

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ABBREVIATIONS

a	Effective radius of interacting pairs
Bchl	Bacteriochlorophyll
b_H	High potential b-heme
b_L	Low potentilal b-heme
C	Degrees celsius
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CDNP	4-carboxy-2,6-dinitrophenyl
CM	Carboxymethyl
DEAE	Diethylaminoethyl
EDTA	Ethylenediaminotetraacetic acid
E_h	Ambient redox potential
E_m	Midpoint redox potential
EPR	Electron paramagnetic resonance
I	Ionic strength
k	Rate constant
k_∞	Rate constant at infinite ionic strength
kDa	Kilodalton
K_m	Michaelis constant
MMD	35 mM MOPS, pH 7.40, 1 mM $MgSO_4$, 0.1 g/L dodecyl maltoside
MOPS	4-morpholinopropane sulfonic acid
M_r	Average molecular weight
n	Number of interacting pairs

PAGE	Polyacrylamide gel electrophoresis
Q	Oxidized form of quinone
Q_2H_2	Reduced form of quinone; quinol
Q_i	Quinone reduction site
Q_o	Quinol oxidation site
r	Distance between the amino and carboxylate groups of interacting pairs
S	Substrate
SDS	Sodium dodecylsulfate
TFC	Trifluoromethylphenylcarbamoyl
Tris	Tris (hydroxymethyl)aminomethane
UHDBT	5-undecyl-6-hydroxy-4,7-dioxobenzothiazole
V	Velocity
V_{max}	Maximal velocity
μ	Micron

CHAPTER 1

INTRODUCTION AND STATEMENT OF PURPOSE

Green plants, algae, and certain bacteria are involved in the transformation of light energy to chemical energy, a process known as photosynthesis. Organisms that are able to absorb light energy and convert it into chemical energy then use chemical energy to synthesize organic compounds for their growth, reproduction, and maintenance. In addition, they provide essential foods for animals to survive. Thus all life on the earth continues to depend on the products of photosynthesis.

Considerable research has been done to establish the structure and composition of the photosynthetic systems in plants and bacteria. Investigations of how these systems function in the conversion of light energy and how energy is utilized to fix carbon dioxide are still in progress. So far, a great deal of information on the mechanisms of photosynthesis has been obtained, but many significant problems remain unsolved.

The overall photosynthetic process in all photosynthetic organisms consists of four essential phases. The first event is light absorption by pigment-protein molecules in the lipid membrane. The light energy is then transferred to a reaction center which contains a chlorophyll molecule in a specialized environment (Sauer and Austion, 1978; Youvan and Marrs, 1987; Pierson and Olson, 1987; Amesz and Knaff, 1988). The second event involves charge separation. The absorption of a photon by the reaction center results in excitation of the primary electron donor, probably a dimer of chlorophyll in all cases, followed by the loss of an electron to an acceptor molecule. The loss of an electron from the reaction center chlorophyll molecule is the first step in electron transport. In the third phase of photosynthesis, the electron moves to the final acceptor molecule through a series of electron carriers, arranged vectorially within the membrane. Charge separation results in

the formation of a membrane potential and a proton gradient across the membrane. The electrochemical proton gradient generated is utilized to drive the formation of ATP from ADP and inorganic phosphate, the transport of metabolites and other energy-requiring processes (Crofts and Wraight, 1983; Rich, 1984; Dutton, 1986). The final event in photosynthesis is biosynthesis in which the chemical energy produced from light energy by the events; light absorption, charge separation, and electron transport is utilized in the synthesis of organic compounds from carbon dioxide (Sauer and Austion, 1978; Youvan and Marrs, 1987; Pierson and Olson, 1987; Amesz and Knaff, 1988).

Photosynthetic bacteria have been extremely useful in the study of photosynthesis. The photosynthetic systems of these bacteria are simpler than those of plants and it has been easier to isolate and purify the pigment-protein complexes which are the components of the bacterial photosynthetic systems (Pierson and Olson, 1987). Although there are important differences between photosynthesis in plants and bacteria, the photochemical reactions that utilize light energy are similar.

Photosynthetic bacteria are highly diverse (Pierson and Olson, 1987). The purple and the green photosynthetic bacteria are the two main group of the photosynthetic bacteria (Pierson and Olson, 1987; Amesz and Knaff, 1988). They can reduce carbon dioxide to organic compounds. In contrast to higher plants, algae and cyanobacteria, they use hydrogen sulfide, sulfide, hydrogen, organic acids and a variety of other organic or inorganic compounds, instead of water, as electron donors.

The green photosynthetic bacteria are divided into two families (Pierson and Olson, 1987; Amesz and Knaff, 1988). The green sulfur bacteria are strictly anaerobic and they depend on both hydrogen sulfide and light for their growth and development. The gliding green bacteria carry out an anoxygenic photosynthesis under anaerobic conditions but are facultative organisms that are also capable of respiratory growth (Pierson and Olson, 1987; Amesz and Knaff, 1988).

The purple bacteria are the largest, most diverse and best studied group of the photosynthetic bacteria (Pierson and Olson, 1987; Amesz and Knaff, 1988). Recent studies on the phylogenetic relationship among the various species of this group have influenced modern ideas on the evolutionary significance of photosynthetic bacteria. Physiologically, this group is subdivided into the purple sulfur and purple non-sulfur bacteria. The purple sulfur bacteria can oxidize sulfur compounds photosynthetically. The non-sulfur purple bacteria are heterotrophs, and they can oxidize organic compounds photosynthetically or via respiratory pathways, in darkness (Pierson and Olson, 1987; Amesz and Knaff, 1988). Work during the past ten years has revealed that there is a strong similarity between the electron transfer pathways in purple non-sulfur bacteria and in mitochondria (Hauska et al., 1983; Malkin, 1988; Trumpower, 1990a). Therefore, purple non-sulfur bacteria have proven to be an extremely useful bacterial family for the study of respiration, as well as photosynthesis. One of the big experimental advantages of using bacterial cytochrome $\underline{bc}_1$ complexes instead of mitochondrial is that although bacterial complexes contain 3-4 subunits (Knaff, 1992), complexes of mitochondria are composed of about ten peptide subunits (Weiss, 1987; Weiss et al., 1990).

Remarkable similarities exist among the electron transfer chains of mitochondria, chloroplasts, photosynthetic and respiratory bacteria (Hauska et al., 1983). A comparison of the electron transfer chains is outlined in Fig. 1.1. Electron transfer in mitochondria involves three membrane-bound enzyme complexes (Complex I or NADH dehydrogenase, Complex III or the cytochrome $\underline{bc}_1$ complex, and Complex IV or cytochrome oxidase) and two mobile components, ubiquinone and cytochrome $\underline{c}$. Electron transfer through these multipeptide enzyme complexes is coupled to proton translocation across the mitochondrial membrane. A fourth membrane-bound enzyme (Complex II or succinate dehydrogenase) is also involved in mitochondrial electron transport. However, the oxidation of succinate to

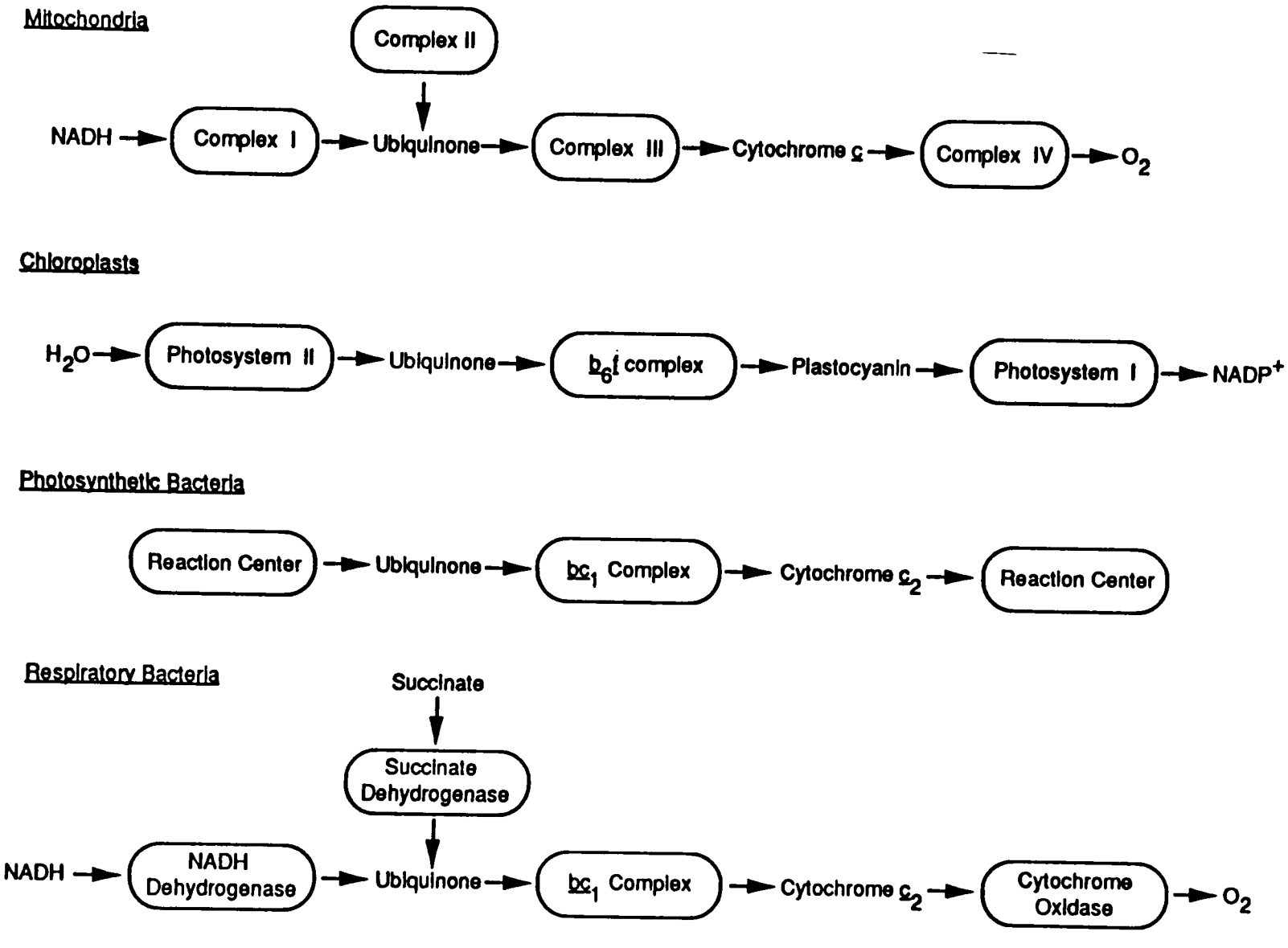

Figure 1.1. Electron Transport Patterns in Organelles and Bacteria. The boxes represent multipeptide complexes while the carriers outside of the boxes are soluble components (Tzagoloff, 1982; Hoober, 1984).

fumarate and reduction of ubiquinone catalyzed by Complex II is not coupled to translocation of protons (Tzagoloff, 1982).

The electron transport chains of chloroplasts and cyanobacteria utilize two light-dependent photosystems, photosystem I and photosystem II, located within the chloroplast membrane. Electrons are transferred from photosystem II to photosystem I by a linear chain which contains the membrane-bound cytochrome $\underline{b_6f}$ complex which is functionally similar to the cytochrome $\underline{bc_1}$ complex found in mitochondria (Hauska et al., 1983) and two mobile electron carrying components, plastoquinone and plastocyanin. Plastoquinones, which are structurally similar to the ubiquinones found within the membranes of mitochondria and bacteria in being hydrophobic substituted p-benzoquinones, function as mobile carriers between photosystem II and the cytochrome $\underline{b_6f}$ complex. Plastocyanin is a copper-containing soluble protein located on the lumen side of the membrane and accepts electrons from the cytochrome $\underline{b_6f}$ complex and donates electrons to photosystem I, as shown in Fig. 1.1. (Hoober, 1984).

In purple photosynthetic bacteria the light-dependent electron transfer pathways are cyclic in nature and involve only a single photosystem (Amesz and Knaff, 1988; Brune, 1989). First, absorption of a photon by a reaction center produces a charge separation via a series of electron transfer reactions. Two membrane bound protein complexes, the reaction center and the cytochrome $\underline{bc_1}$ complex, and two soluble components -quinones (within the membrane) and cytochrome $\underline{c_2}$ (in the periplasmic space)- are involved in these reactions. Electrons are transferred from the reaction center to the cytochrome $\underline{bc_1}$ complex by mobile quinols, and are returned to reaction center from the cytochrome $\underline{bc_1}$ complex by cytochrome $\underline{c_2}$ (Hauska et al., 1983; Crofts and Wraight, 1983; Rich, 1984; Pierson and Olson, 1987; Knaff, 1990a) (see Fig. 1.2.). When these bacteria are grown in the dark under aerobic conditions the cyclic pathway is replaced by linear electron flow from respiratory substrates to O_2. Under these conditions, ubiquinone is reduced by a series of

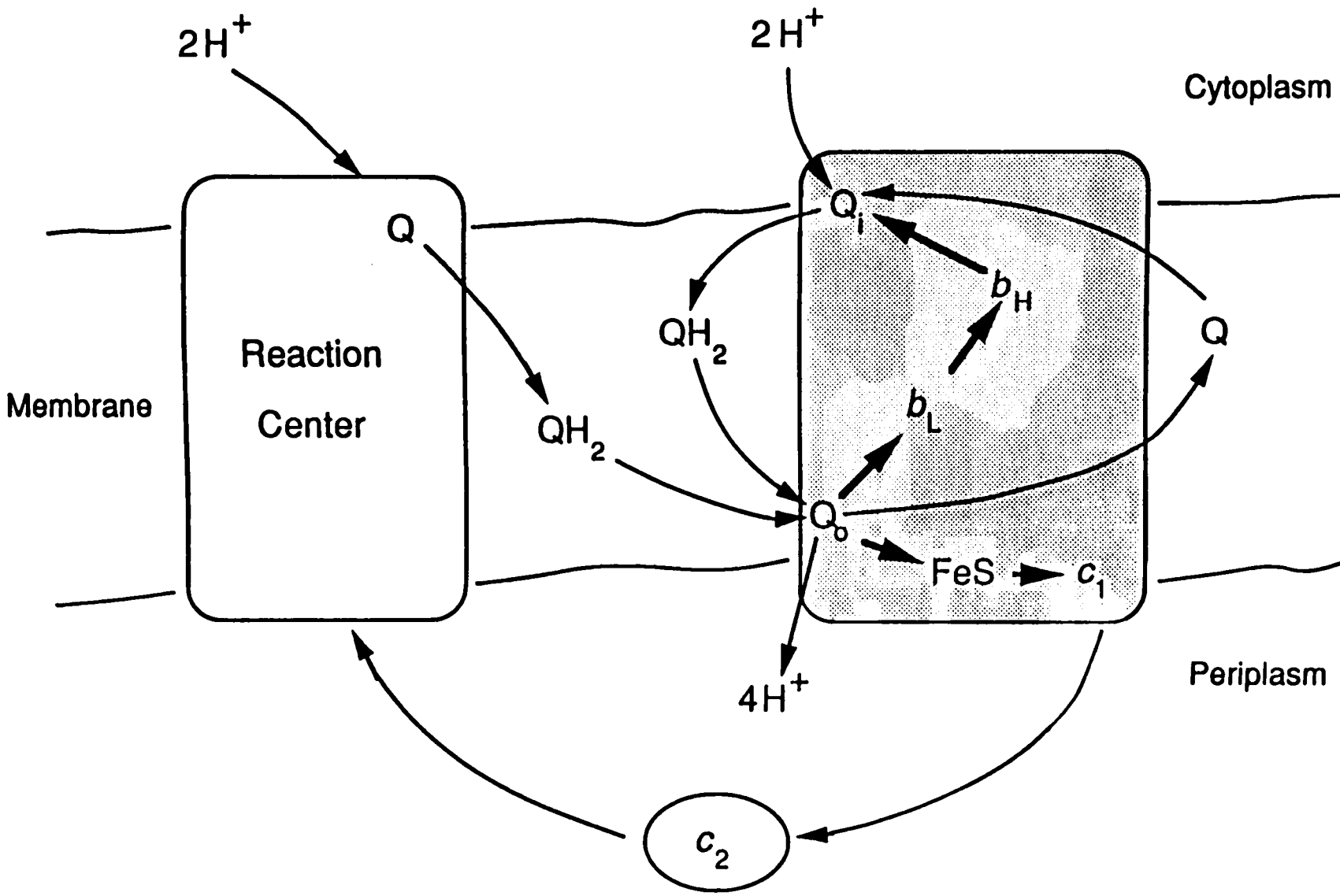

Figure 1.2. Q-cycle mechanism for the electron transport chain of photosynthetic bacteria.

dehydrogenases and then ubiquinol is oxidized by a terminal cytochrome oxidase via the cytochrome $\underline{bc}_1$ complex. Electron transfer between the cytochrome $\underline{bc}_1$ complex and cytochrome oxidase involves cytochrome $\underline{c}_2$ as an electron carrier (Saunders and Jones, 1974). Most of the purple photosynthetic bacteria have at least one additional oxidase that transfers electrons directly from ubiquinol to oxygen, a respiratory pathway that does not involve cytochromes $\underline{bc}_1$ complex and $\underline{c}_2$ (Venturoli et al., 1987).

Rhodospirillum rubrum is one of the purple nonsulfur photosynthetic bacteria which can grow either photosynthetically or using respiration for its energy needs (Pierson and Olson, 1987; Amesz and Knaff, 1988). Many aspects of its metabolism, physiology, biochemistry and genetics have been extensively studied. In the last a few years, some of the proteins involved in the photosynthetic electron transfer pathway in this bacteria have been isolated, purified and characterized. More recently, it has been documented that R. rubrum contains a three subunit, membrane bound cytochrome $\underline{bc}_1$ complex (Kriauciunas et al., 1989) which is a component of both photosynthetic and respiratory electron transport chains (Venturoli et al., 1987) of this bacterium. The genes coding for the three prosthetic group-containing subunits of this complex protein have been sequenced (Majewski and Trebst, 1990; Shanker et al., 1992).

The interaction of the cytochrome $\underline{bc}_1$ complex with cytochrome $\underline{c}_2$ from R. rubrum and Rhodobacter sphaeroides have been studied in detail by examining the effects of chemical modification of specific lysines and variations in ionic strength on the reaction kinetics (Hall et al., 1987b; Hall et al., 1987c; Hall et al., 1989). Kinetic studies using the cytochrome $\underline{c}_2$ derivatives indicated that the "front side" lysine residues surrounding the exposed heme edge play an important role in the interaction with the cytochrome $\underline{bc}_1$ complex. In the case of R. rubrum, it has also been shown that complex formation with the cytochrome $\underline{bc}_1$ complex specifically protects three "front side" lysines on cytochrome $\underline{c}_2$ against chemical modification (Bosshard et al., 1987). Reports have shown that

electrostatic interactions are involved in the reaction of the Rb. sphaeroides (Hall et al., 1987c; Hall et al., 1989) and R. rubrum (Bosshard et al., 1987) cytochrome bc_1 complex with cytochrome c_2 as well as with the structurally similar equine cytochrome c. If cytochrome c_2 contributes the positive charges responsible for electrostatic complex formation, then the cytochrome bc_1 complex and, most likely, its cytochrome c_1 subunit (Meinhardt et al., 1982; Snozzi and Crofts, 1985) must contribute the negatively charged groups. Two acidic regions implicated in binding of mitochondrial cytochrome c to cytochrome c_1 (Broger et al., 1983; Stonehuerner et al., 1985) are conserved in the four cytochromes c_1 from photosynthetic bacteria for which sequences are available, suggesting that these regions are also involved in binding cytochrome c_2 in photosynthetic bacteria (Hall et al., 1987b; Hall et al., 1987c; Hall et al., 1989).

Additional evidence for the electrostatic nature of the complex formation between cytochrome c_2 and the cytochrome bc_1 complex was obtained from analysis of the effect of ionic strength on kinetic parameters in both R. rubrum and Rb. sphaeroides (Hall et al., 1987b; Hall et al., 1987c; Hall et al., 1989). It has been observed that the reduction rate of cytochrome c_2 in the electron transfer from quinol to cytochrome c_2 catalyzed by the cytochrome bc_1 complex is ionic strength-dependent. Although there is little or no effect of ionic strength on V_{max}, large increases in K_m were observed as the ionic strength was increased. This ionic strength dependent rate of cytochrome c_2 reduction has been utilized in estimating the number of interacting charged pairs involved in cytochrome c_1/c_2 binding, using a semiempirical relationship (Stonehuerner et al., 1979).

The work reported in this dissertation involves the study of three aspects of photosynthetic purple non-sulfur bacterial cytochrome bc_1 complexes. In the first part of this work, the cytochrome bc_1 complex from R. rubrum was characterized in detail by: (1) Western blotting; (2) inhibition experiments; (3) ultraviolet-visible spectroscopy; (4) electron paramagnetic resonance spectroscopy; (5) proton pumping experiments.

The second part of this work focuses on examining the local environment of the heme groups of the cytochrome $\underline{bc}_1$ complex using resonance Raman spectroscopy. Resonance Raman spectroscopy has been applied to biological molecules containing specific chromophores for the past fifteen years. Hemes, flavins, chlorophyll and several copper or iron containing metalloprotein sites have been studied succesfully by resonance Raman spectroscopy (Spiro and Strekas, 1974; Adar and Yonetani, 1978; Spiro and Li, 1988; Spiro et al., 1990; Hobbs et al., 1990; Hobbs et al., 1991; Hildebrant et al., 1992). The technique involves Raman scattering from a light-absorbing sample. As the monochromatic laser light selectively excites the chromophores, it produces detailed vibrational spectra of chromophoric sites in the molecule. Since a vibrational spectrum is sensitive to chromophoric environment, this technique can be utilized in identification of the ligands of all the heme-containing chromophoric sites in the cytochrome $\underline{bc}_1$ complex.

The final portion of this work involves the characterization of the interaction with cytochrome $\underline{bc}_1$ complexes and cytochrome $\underline{c}_2$ in Rhodobacter capsulatus and Rhodopseudomonas viridis. The formation of an electrostatically stabilized complex between the Rb. capsulatus cytochrome $\underline{bc}_1$ and cytochrome $\underline{c}_2$ has been studied by gel filtration chromatography and membrane ultrafiltration. Kinetic studies have been performed with the cytochrome $\underline{bc}_1$ complexes and cytochrome $\underline{c}_2$ from Rb. capsulatus and Rps. viridis and the ionic strength dependencies of ubiquinol-cytochrome $\underline{c}_2$ oxidoreductase activity for the cytochrome $\underline{bc}_1$ complexes from these two different photosynthetic bacteria are compared to previous results obtained with R. rubrum (Hall et al., 1987b) and Rb. sphaeroides (Hall et al., 1987c; Hall et al., 1989) in order to correlate the results with negative charges on cytochrome $\underline{c}_1$. We have also looked at the effect of "front side" lysines altered by site-directed mutagenesis on the reduction of cytochrome $\underline{c}_2$ by the cytochrome $\underline{bc}_1$ complex of Rb. capsulatus.

The spatial arrangement of the cytochrome $\underline{bc_1}$ complex within the membrane is a subject of intensive investigation. Recently, diffraction quality crystals of a cytochrome $\underline{bc_1}$ complex from mitochondria have been obtained (Yue et al., 1991; Kubota et al., 1991; Berry et al., 1992), and crystallization studies on the R. rubrum cytochrome $\underline{bc_1}$ complexes are in progress in collaboration with Prof. Deisenhofer in Dallas.

CHAPTER 2

LITERATURE REVIEW

In the membranes of all purple photosynthetic bacteria grown under photosynthetic conditions, secondary electron transfer appears to require a cytochrome $\underline{bc}_1$ complex (Crofts and Wraight, 1983; Hauska et al., 1983; Rich, 1984; Dutton, 1986; Knaff, 1990). This membrane bound enzyme complex is composed of three peptides directly involved in redox reactions: The Rieske iron-sulfur protein, one $\underline{c}$-type and one $\underline{b}$-type cytochrome. The Rieske protein contains a single [2Fe-2S] cluster, cytochrome $\underline{c}_1$ contains a single covalently bound heme $\underline{c}$ and cytochrome $\underline{b}$ contains two unequivalent, non-covalently bound protohemes (Crofts and Wraight, 1983; Hauska et al., 1983; Rich, 1984; Dutton, 1986; Gabellini, 1988; Hauska et al., 1988). The cytochrome $\underline{bc}_1$ complex functions as a ubiquinol-cytochrome $\underline{c}_2$ oxidoreductase, catalyzing the transfer of electrons from a reduced quinone to cytochrome $\underline{c}_2$. During light-driven cyclic electron flow in the membranes of photosynthetic bacteria, electron transfer between the reaction center and the cyochrome $\underline{bc}_1$ complex is mediated by a mobile pool of quinone (Q pool) and the periplasmically located soluble cytochrome $\underline{c}_2$, with the reaction center reducing ubiquinone and oxidizing cytochrome $\underline{c}_2$ (Crofts, 1983; Hauska et al., 1983; Crofts and Wraight, 1983; Rich, 1984; Knaff, 1990).

Structurally and functionally similar cytochrome $\underline{bc}_1$ complexes are found in mitochondria and the related cytochrome $\underline{b}_6\underline{f}$ complex is found in cyanobacteria, algae and plants (Hauska et al., 1983; Rich, 1984; Snozzi and Kälin, 1987; Hauska et al., 1988; Wynn et al., 1988). In all photosynthetic systems, the cytochrome $\underline{bc}_1$ or $\underline{b}_6\underline{f}$ complex couples electron transfer to proton translocation . This proton translocation activity of the cytochrome $\underline{bc}_1$ complexes produce an electrochemical proton gradient across the

membrane. The proton gradient which is produced during the electron transfer reactions is utilized in the synthesis of ATP (McCarty and Carmeli, 1982).

2.1. The cytochrome bc_1 complex

Recent research on the cytochrome bc_1 complexes of photosynthetic bacteria has provided more information on the peptide composition (Kriauciunas et al., 1989; Purvis et al., 1990; Andrews et al., 1990) and gene sequences of the complex (Daldal et al., 1987; Gabellini and Sebald, 1988; Verbist et al., 1989; Majewski and Trebst, 1990; Yun et al., 1990; Shanker et al., 1992). To date, cytochrome bc_1 complexes have been extensively studied in four photosynthetic bacteria; Rb. capsulatus (Gabellini et al., 1982; Ljungdahl et al., 1987; Daldal et al., 1987a; Davidson and Daldal, 1987a; Davidson and Daldal; 1987b; Gabellini, 1988; Robertson et al.,1992), Rb. sphaeroides (Ljungdahl et al., 1987; Gabellini and Sebald, 1988; Yun et al., 1990; Purvis et al., 1990), R. rubrum (Wynn et al., 1986; Kriauciunas et al., 1989; Purvis et al., 1990; Majewski and Trebst, 1990; Shanker et al., 1992) and Rps. viridis (Wynn et al., 1985; Wynn et al., 1986; Verbist et al., 1989; Cully et al., 1989; Cully, 1990). All of the cytochrome bc_1 complexes isolated, purified, and characterized from photosynthetic bacteria contain a minimum of three subunits: the Rieske iron-sulfur protein, cytochrome c_1 and cytochrome b (Hauska et al., 1983; Crofts and Wraight, 1983; Rich, 1984; Wynn et al., 1986; Kriauciunas et al., 1989). In addition to these three peptide subunits, the Rb. sphaeroides cytochrome bc_1 complex contains a 14.3 kDa-fourth subunit which has been reported to be important for catalytic activity of complex and in quinone binding (Yu et al., 1984; Knaff, 1990; Usui et al.,1990; Purvis et al., 1990; Andrews et al., 1990; Yu and Yu, 1991; Usui et al., 1991; Usui and Yu, 1991). In contrast to this relatively simple composition, mitochondrial cytochrome bc_1 complexes contain approximately ten subunits (Rieske et al., 1964; Schägger et al., 1986; Ljungdahl et al., 1987; Malkin, 1988).

The cytochrome $\underline{bc_1}$ complexes from both mitochondria and photosynthetic purple bacteria have been extensively studied by utilizing biochemical, biophysical, molecular biological and spectroscopic techniques. Recently, the genes for the electron carrying subunits of the cytochrome $\underline{bc_1}$ complexes have been cloned and sequenced. In photosynthetic bacteria, the genes coding for the three peptide subunits are arranged in an fbc or pet operon with the order of the genes being: petA, encoding the Rieske protein; petB, encoding cytochrome b and petC, encoding cytochrome $\underline{c_1}$ (Davidson and Daldal, 1987a; Daldal et al., 1987a; Davidson and Daldal, 1987b; Gabellini and Sebald, 1988; Verbist et al., 1989; Yun et al., 1989; Shanker et al., 1992). The primary structure of the three electron-carrying subunits of the cytochrome $\underline{bc_1}$ complexes was deduced from the DNA base sequence of the pet operon and the data has revealed that the structure of the cytochrome $\underline{bc_1}$ complexes is highly conserved among photosynthetic purple bacteria (Hauska et al., 1988). Biochemical studies and hydropathy profiles have provided information about the likely topology of the cytochrome $\underline{bc_1}$ complex, its subunits and the relative arrangement of their prosthetic groups within the membrane (Gabellini and Sebald, 1986; Davidson and Daldal, 1987b; Verbist et al., 1989; Yun et al., 1990). The circular dichroism spectrum of the mitochondrial cytochrome $\underline{bc_1}$ complex has been resolved in the visible region into the contributions from the individual prosthetic groups (Solaini et al., 1987). Magnetic circular dichroism studies have revealed useful information on the likely axial ligands to the redox centers of b and c cytochromes (Simpkin et al., 1989). Very recently, the cytochrome $\underline{bc_1}$ complex from beef heart mitochondria has been crystallized (Kubota et al., 1991; Yue et al., 1991; Berry et al., 1992) but the crystals diffract x-rays to less than atomic resolution (Kubota et al., 1991; Berry et al., 1992).

2.2. Subunits of the cytochrome bc_1 complex

2.2.1. Cytochrome b

It has been shown that all photosynthetic bacterial cytochrome bc_1 complexes contain a cytochrome b subunit contributing structural elements to both quinol oxidation, Q_o, and quinone reduction, Q_i, sites on the protein complex (Yun et al., 1992). The cytochrome b peptide contains two non-covalently bound protohemes in different environments, resulting in different spectra and E_m values (Wynn et al., 1986; Dutton, 1986). The mid-point potentials have been measured and values near -90 mV and +50 mV for low potential; b_L, and high potential; b_H cytochrome, respectively have been obtained in the cytochrome bc_1 complexes from four photosynthetic bacteria, values similar to those obtained for mitochondrial complexes (Malkin, 1988). In the mitochondrial cytochrome bc_1 complex and the complexes from Rb. sphaeroides and Rb. capsulatus, the α-band spectrum of reduced high potential b-cytochrome exhibits a maximum at approximately 560 nm, and the α-band spectrum of reduced low potential b-cytochrome is split, with maxima at 566 nm and 559 nm (Dutton, 1986).

Molecular genetics has been used to identify structurally and functionally important residues in the cytochrome bc_1 complex. The cytochrome b subunit of the cytochrome bc_1 complex is well conserved and is a hydrophobic protein of approximately 400 amino acid residues. The earliest models for cytochrome b , based on hydropathy analysis, predicted the presence of nine transmembrane helices (Widger et al., 1984; Saraste, 1984). The nine-helix model has been questioned on the basis of more careful analysis of hydropathy profiles and a model containing eight membrane-spanning helices and one amphipathic helix was proposed (Rao and Argos, 1986; Crofts et al., 1987). One additional advantage of the eight helix model is that it groups amino acids that, when altered confer resistance to specific inhibitors, close to one another (Daldal et al., 1989). In contrast, the nine helix model places some of this amino acids on opposite sides of the membrane (Daldal et al.,

1989). Recent gene fusion experiments (Yun et al., 1991a) have provided additional support for the eight-transmembrane helices model of the cytochrome b subunit. The eight helix is also more consistent with results obtained from site directed mutagenesis experiments on the four conserved histidine residues in the cytochrome b subunit thought to be axial ligands, two each, to the two hemes of b_L and b_H (Yun et al., 1991b). It has been shown from the results of electron paramagnetic resonance spectroscopy (Carter et al., 1981) and magnetic circular dichroism measurements (Simpkin et al., 1989) that both hemes in cytochrome b of the related mitochondrial cytochrome bc_1 complex are likely to have bis-histidyl ligation. Several highly conserved residues on the cytochrome b subunit of the cytochrome bc_1 complex were altered by site-directed mutagenesis and found to make clear changes in the spectroscopic, kinetic and electrochemical properties of the complex. However, none of these residues appear to be absolutely essential for the function of the cytochrome bc_1 complex (Tron et al., 1991; Yun et al., 1992).

2.2.3. Cytochrome c_1

One of the two c-type cytochromes which participates in cyclic electron flow in purple nonsulfur bacteria is cytochrome c_1. While cytochrome c_2, the second c-type cytochrome component, is soluble and located in periplasmic space, cytochrome c_1 is a membrane-bound constituent of the cytochrome bc_1 complex. The folding of the cytochrome c_1 peptide appears to be conserved within the photosynthetic bacteria (Gabellini, 1987; Hauska et al., 1988). It has been demonstrated by proteolytic digestion of the membrane surfaces that the two high potential prosthetic groups of the cytochrome bc_1 complex, 2Fe-2S and heme c_1, form an electron transfer pathway along the outer surface of the membrane, facing towards the soluble acceptor cytochrome c_2 (Gabellini, 1988). Although the spectral and redox properties are very similar, the amino acid sequences of cytochrome c_1 and cytochrome c_2 (c) show almost no homology (Schägger et al., 1986). Cytochrome

c_1 carries a covalently bound heme as an electron carrying prosthetic group and binds a heme through two thioether linkages to two cysteines (Yu et al., 1986). Spectroscopic (Simpkin et al., 1989) and site-directed mutagenesis (Gray et al., submitted for publication) data on cytochrome c_1 subunit of the photosynthetic bacteria have suggested that two residues, histidine and methionine, are the ligands to heme iron. These two residues are well conserved in cytochrome c_1 from various organisms (Meyer and Kamen, 1982; Gabellini and Sebald, 1986; Davidson and Daldal 1987a; Nakai et al., 1990). The α-band spectrum of reduced cytochrome c_1 has a maximum at 552-553 nm (Hauska et al., 1983; Ljungdahl et al., 1987; Kriauciunas et al., 1989; Robertson et al., 1992). Its molecular weight has been calculated from the amino acid sequences to be 28.5 kDa, 31.2 kDa, 29.4 kDa and 30.3 kDa in Rb. sphaeroides, Rps. viridis, R. rubrum, and Rb. capsulatus, respectively (Davidson and Daldal, 1987a; Gabellini and Sebald, 1988; Verbist et al., 1989; Majewski and Trebst, 1990; Shanker et al., 1992). The cytochromes c_1 of both mitochondrial and photosynthetic bacterial cytochrome bc_1 complexes have midpoint potentials close to +280 mV (Gabellini et al., 1982; Hauska et al., 1983, Malkin, 1988; Andrews et al., 1990; Robertson et al., 1992). From hydropathy analysis, cytochrome c_1 is predicted to be largely polar, with a single transmembrane helix. Studies, in which elimination of this hydrophobic helix by either proteolytic cleavage (Li et al., 1983) or site-directed mutagenesis (Konishi et al., 1991) produced a soluble cytochrome c_1, are consistent with this model. The purified soluble cytochrome c_1 retains all the physical characteristics of the intact subunit (Konishi et al., 1991).

2.2.4. The Rieske iron-sulfur protein

The Rieske iron-sulfur protein was first observed in the mitochondrial cytochrome bc_1 complex by Rieske (Rieske et al., 1964). Later it was also detected in photosynthetic bacteria and in chloroplasts (Hauska et al., 1983, Malkin, 1988). The protein has a

characteristic EPR signal, in the reduced form, with g-values of 1.76, 1.90 and 2.01 and this signal is affected by the presence of quinone or quinol at the Q_o site and can be altered by quinol analog inhibitors of the cytochrome $\underline{bc}_1$ complexes (von Jagow and Ohnishi, 1985; Andrews et al., 1990). The EPR spectra of Rieske iron-sulfur protein of photosynthetic bacteria are similar to those of the mitochondrial Rieske proteins. E_m values have been reported for the protein to lie between +280 mV and +315 mV in several photosynthetic bacteria and in mitochondria (Evans et al., 1974; Prince et al., 1975; Malkin, 1988; Güner et al., 1991; Robertson et al., 1992). Amino acid sequences for the iron-sulfur protein are available from mitochondria, cyanobacteria, chloroplasts, and for photosynthetic bacteria (Hauska et al., 1988; Davidson et al., 1992a). Their hydropathy patterns are very similar, although the overall amino acid sequences of iron sulfur proteins are only weakly conserved. The molecular weights of the protein deduced from the amino acid sequence data have been calculated to be 19.8 kDa, 18.9 kDa, 19.5 kDa and 20.4 kDa in Rb. sphaeroides, Rps. viridis, R. rubrum, and Rb. capsulatus, respectively (Gabellini and Sebald, 1988; Verbist et al., 1989; Majewski and Trebst, 1990; Shanker et al., 1992). The prosthetic group of the protein is a [2Fe-2S] cluster with the two nonheme irons and two inorganic sulfides that combine to serve as a one electron carrier. Studies on the Rieske iron-sulfur protein from R. rubrum, Rb. sphaeroides, Rb. capsulatus, spinach chloroplasts and mitochondria have shown that the protein contributes two histidine nitrogen and two cysteine sulfur ligands to the iron-sulfur cluster instead of the more common four cysteine pattern (Telser et al., 1987; Britt et al., 1991; Gurbiel et al., 1991). Recent site directed mutagenesis experiments and comparison to the sequence of Rieske-like bacterial enzymes (Davidson et al., 1992a) have allowed a tentative assignment of the two histidine and two cysteine ligands to the cluster. Optical spectroscopy and fluoroescence binding assays with specific inhibitors (Brandt et al., 1991) and site-directed mutagenesis experiments (Davidson et al., 1992b) have suggested that the domains of the

iron-sulfur protein are directly involved in the formation of the quinol oxidation site and important for the structure of the iron-sulfur protein of the cytochrome $\underline{bc}_1$ complex.

The hydropathy plot for the Rieske iron-sulfur protein indicates that the major part of the peptide is hydrophilic and is exposed to the aqueous environment. This hydrophilic domain is well conserved among different photosynthetic bacterial species (Davidson and Daldal, 1987a; Verbist et al., 1989; Majewski and Trebst, 1990; Yun et al., 1990). Recent experiments on the Rieske protein of chloroplasts have suggested that it has at least one membrane-spanning helix (Szczepaniak et al., 1991).

2.3. The proton motive Q-cycle

The cytochrome $\underline{bc}_1$ complexes have a dual function in photosynthetic bacteria. They not only transfer electrons from ubiquinol to cytochrome $\underline{c}_2$ in the electron transfer chain, but also couple these electron transfer reactions to translocation of protons across the chromatophore membrane. The Q-cycle mechanism, originally proposed by Mitchell, describes how electron transfer reactions are coupled to proton translocation by cytochrome $\underline{bc}_1$ complexes (Mitchell, 1976). A "modified Q-cycle" has been used successfully to analyze the structural, kinetic and thermodynamic properties of the cytochrome $\underline{bc}_1$ complex (Ohnishi and Trumpower, 1980; de Vries et al., 1981; de Vries et al., 1982; Crofts and Wraight, 1983; van der Wal and van Grondelle, 1983; Crofts et al., 1983; Yang and Trumpower, 1988; Trumpower, 1990a). In the first step of the cycle, a ubisemiquinone anion is formed by the transfer of one electron from ubiquinol to the Rieske iron-sulfur protein at a quinol oxidation, Q_o, site. One electron from the reduced iron-sulfur protein passes through cytochrome $\underline{c}_1$ to reduce cytochrome $\underline{c}_2$. The ubisemiquinone anion reduces the low potential cytochrome $\underline{b}$ heme, which then transfers one electron to the low potential cytochrome $\underline{b}$ (Glazer and Crofts, 1984). The oxidation of quinol at the Q_o site results in the release of two protons to the periplasmic space. The high

potential cytochrome b reduces a ubiquinone to a ubisemiquinone anion at a quinone reduction site, Q_i, near the cytoplasmic surface of the membrane (Glazer and Crofts, 1984; Robertson and Dutton, 1988; McCurley et al., 1990). The oxidation of a second ubiquinol molecule at Q_o is needed for a complete Q-cycle. One electron is again transferred to cytochrome c_2 via the Rieske protein and cytochrome c_1 and another electron is transferred to cytochrome b_L and then to cytochrome b_H. Finally, cytochrome b_H reduces the ubisemiquinone anion formed at the Q_i during the previous turnover to ubiquinol and two protons are taken up from cytoplasm. As a result of these electron transfer and proton translocation processes, while two electrons are transferred through the cytochrome bc_1 complex, two protons are taken up on the cytoplasmic site and four protons are deposited to the periplasmic site of the bacterial membrane. Two molecules of ubiquinol are utilized for reduction of two molecules of cytochrome c_2 but, as one ubiquinone is reduced at Q_i site, the net stoichiometry is one ubiquinol oxidized per two cytochrome c_2 reduced.

Strong support for the Q-cycle model came from the demonstration of the phenomenon of "oxidant-induced reduction of cytochrome b " in the cytochrome bc_1 complexes from mitochondria (Wikström and Berden, 1972; Baum et al., 1987) and photosynthetic bacteria (Dutton and Prince, 1978). Support for the Q-cycle mechanism also came from experiments demonstrating that removal of the iron-sulfur protein from cytochrome bc_1 complex blocks reduction of cytochrome c_1 and prevents reduction of cytochrome b at the quinol oxidation site, Q_o, but allows reduction of cytochrome b by reverse electron flow through the quinol reduction site, Q_i (Crofts et al., 1983; Robertson and Dutton, 1988; Trumpower, 1990a).

Detailed kinetic studies on cytochrome bc_1 complexes also strongly support the modified Q-cycle model (Crofts et al., 1983; Robertson and Dutton, 1988). Studies of the electron transfer reactions from Q_o site to Q_i site through cytochrome b have also allowed identification of all the steps in cytochrome bc_1 complexes that are electrogenic and produce

charge separation across the membrane (Crofts and Wraight, 1983; Robertson and Dutton, 1988; Daldal et al., 1989; Robertson et al., 1990). Additional evidence for a Q-cycle model comes from observations with inhibitors (von Jagow and Bohrer, 1975; Hauska et al., 1983; Crofts et al., 1983; Matsuura et al., 1983; Rich, 1984; von Jagow and Link, 1986; Yang and Trumpower, 1988; Trumpower, 1990a; Trumpower, 1990b). Several inhibitors, isolated from microorganisms, specifically inhibit electron transfer through the cytochrome $\underline{bc}_1$ complex (von Jagow and Link, 1986; Robertson et al., 1987). These inhibitors, myxothiazol, stigmatellin, UHDBT and antimycin A, appear to be quinol analogs structurally, but their site of action differs in the cytochrome $\underline{bc}_1$ complex. Myxothiazol and stigmatellin block the oxidation of ubiquinol by Rieske iron-sulfur protein (von Jagow and Engels, 1981; Meinhardt and Crofts, 1982). UHDBT eliminates electron transfer from Rieske iron-sulfur protein to cytochrome $\underline{c}_1$ by binding directly to iron-sulfur protein (von Jagow and Link, 1986). Antimycin A, however, inhibits the oxidation of the reduced high potential cytochrome $\underline{b}$ by ubiquinone (Robertson et al., 1984). Data obtained with inhibitor resistant mutations shows that the antimycin A site maps in a different part of the protein than those for stigmatellin and myxothiazol. This is also consistent with a Q-cycle model that includes two separate quinone redox sites (Daldal et al., 1989) (see Fig.1.2.). Recent studies on the internal reactions of the cytochrome $\underline{bc}_1$ complex of photosynthetic bacteria have suggested that the rate determining step is the formation of semiquinone at the quinol oxidation site (Crofts and Wang, 1989).

The three subunits of the photosynthetic bacterial cytochrome $\underline{bc}_1$ complex form two active domains involved in quinol reduction (Q_i) located on the periplasmic side of the membrane and quinol oxidation (Q_o) located on the cytoplasmic side of the membrane (Bowyer and Crofts, 1981; Meinhardt and Crofts, 1982; Meinhardt and Crofts, 1983; Glaser et al., 1984; Robertson et al., 1984a; Robertson et al., 1984b; Crofts et al., 1987; Robertson and Dutton, 1988; Ohnishi, 1989; Meinhardt and Ohnishi, 1992). It has been

suggested from functional studies that iron-sulfur cluster and cytochrome $\underline{b_L}$ are the components of the quinol oxidation site and cytochrome $\underline{b_H}$ forms the quinol reduction site in the cytochrome $\underline{bc_1}$ complex (Rich, 1984). The location of these sites were defined by analysis of inhibitor-resistant mutants (Howell and Gilbert , 1988; di Rago and Colson, 1988; Daldal et al., 1989). Recently a stable cytochrome $\underline{bc_1}$ complex having functional quinone reduction (Q_i) site but lacking the quinol oxidation (Q_o) site has been demostrated by experiments on several mutants of photosynthetic bacterial cytochrome $\underline{bc_1}$ complex (Robertson et al., 1986; Robertson et al., 1990). Recent evidence has resulted in a proposal for a "Double Occupancy Model" that features the binding of the two quinones at the quinol oxidation site (Ding et al., 1992).

2.4. Electron donor and acceptor of the cytochrome bc_1 complex

2.4.1. Quinone

Quinones play a central role in the electron transfer chains of both mitochondria and photosynthetic bacteria. Several experiments have been performed to help to understand the interaction of quinone with protein complexes and to define its role in proton translocation and electron transfer (Vermeglio, 1977; Wraight, 1977; Ohnishi and Trumpower, 1980). The majority of the quinone is dissolved in the lipid membrane constituting a functionally homogenous pool (Crofts et al., 1983; Suzuki and Ozawa, 1984; Venturoli et al., 1986; Venturoli et al., 1988). It is the only membrane soluble, mobile component of the electron transfer chain and shuttles electrons among the membrane-bound electron transfer proteins. It has been demonstrated that the cytochrome $\underline{bc_1}$ complex of both mitochondria and photosynthetic bacteria contain significant amounts of quinone (Yu et al., 1978). It has been shown that quinones do not appear to be permanent, tightly bound quinone at either the Q_o or Q_i site and that all the reactions at these sites occur with

bimolecular kinetics and free diffusion (Crofts et al., 1983; Zannoni and Melandri, 1985; Venturoli et al., 1988). Recently, It has been shown that several short chain ubiquinols, although not duroquinol can interact with the proteins of electron transfer chain of mitochondria (Fato et al., 1986).

2.4.2. Cytochrome c_2

In photosynthetic purple non-sulfur bacteria, photosynthesis or respiration involves a periplasmic c-type cytochrome, cytochrome c_2 (Bartsch, 1978). This soluble electron acceptor for the cytochrome bc_1 complex is very well characterized. C-type cytochromes share a common characteristic absorption spectrum (Ambler, 1991) and they carry a covalently bound heme as a prosthetic group (Wood, 1991). Cytochromes c_2 isolated from different photosynthetic bacteria have molecular weights ranging from 10 to 14 kDa and mid-point potentials ranging from +290 to +380 mV (Bartsch, 1978). Amino acid sequence data for cytochrome c_2 from several photosynthetic bacteria are available (Ambler et al., 1979; Daldal et al., 1986; Donohue et al., 1986). Crystals have been obtained for cytochrome c_2 from four photosynthetic bacteria (Salemme et al., 1973; Miki et al., 1986; Holden et al., 1987; Axelrod et al., 1992), and a three-dimensional structure has been reported for that of R. rubrum, Rb. capsulatus and Rb. sphaeroides (Salemme et al., 1973; Benning et al., 1991; Axelrod et al., 1992). Considerable homologies in amino acid sequence and similarities in three-dimensional structure exist between mitochondrial cytochrome c and photosynthetic bacterial cytochrome c_2 (Ambler et al., 1979; Dickerson, 1980; Meyer and Kamen, 1982; Meyer, 1991). Recently the heme side groups, ligands and other related important resonances of the cytochrome c_2 have been studied by nuclear magnetic resonance spectroscopy (Pettigrew et al., 1978; Moore et al., 1984; Yu and Smith, 1990) and detailed analysis of the pH dependence of the redox potential of cytochrome c_2 have been carried out (Pettigrew et al., 1978). It was thought that

cytochrome c_2 of photosynthetic purple bacteria was required for photosynthetic electron flow in all photosynthetic bacteria. However recent studies by deletion of the gene coding for cytochrome c_2 of Rb. capsulatus has shown that photosynthetic growth can occur in this bacterium without cytochrome c_2. It has been suggested from these results that another pathway operates in Rb. capsulatus cells in the absence of cytochrome c_2 (Daldal et al., 1986; Prince et al., 1986; Prince and Daldal, 1987; Jones et al., 1990; Zannoni et al., 1992). Additional experiments by site-directed mutagenesis have provided evidence on involvement of a c-type cytochrome in the transfer of electrons from the cytochrome bc_1 complex to the photosynthetic reaction center (Jones et al., 1990). In contrast, Rb. sphaeroides cells were unable to grow photosynthetically without cytochrome c_2 (Donohue et al., 1986; Donohue et al., 1988).

Electrostatically stabilized complex formation occurs between the R. rubrum cytochrome bc_1 complex and both R. rubrum cytochrome c_2 or equine cytochrome c (Wynn et al., 1986; Bosshard et al., 1987). Kinetic studies have established that, in photosynthetic bacteria, cytochrome c_1 in the cytochrome bc_1 complex is the direct electron donor to cytochrome c_2 (Meinhardt and Crofts, 1982; Snozzi and Crofts, 1985) and lysine residues surrounding the exposed heme edge of cytochrome c_2 play an important role in binding to the cytochrome bc_1 complex of R. rubrum and Rb. sphaeroides (Bosshard et al., 1987; Hall et al., 1987b; Hall et al., 1987d; Hall et al., 1989). These lysines are also important in cytochrome c_2/reaction center docking in R. rubrum, Rb. sphaeroides and Rb. capsulatus (Hall et al., 1987a; Hall et al. 1987c; van der Wal et al., 1987; Caffrey et al., 1992a). Although these results implicate negatively charged groups on cytochrome c_1 as the binding site for cytochrome c_2, no direct evidence for this hypothesis exists in photosynthetic bacteria. However, cross-linking and chemical protection experiments have implicated two acidic regions on the mitochondrial cytochrome c_1 in the formation of an electrostatically stabilized complex with mitochondrial cytochrome c (Capaldi et al., 1982;

Broger et al., 1983; Stonehuerner et al., 1985). These acidic regions are conserved in most photosynthetic bacterial species, indicating that glutamate and aspartate residues on the bacterial cytochrome $\underline{c}_1$ are likely to be involved in binding to cytochrome $\underline{c}_2$ (Davidson and Daldal, 1987a; Verbist et al., 1989; Majewski and Trebst, 1990; Yun et al., 1990; Shanker et al., 1992).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Antimycin A, valinomycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP), equine cytochrome c (type VI), Sephadex G-75 and Dowex 1-X were purchased from Sigma Chemical Company. n-dodecyl-β-D-maltoside was obtained from Anatrace, Inc.. Diethylaminoethyl-sepharose CL-6B, carboxymethyl-sephadex, "Phast" polyacrylamide gels and buffers for electrophoresis in the presence of sodium dodecylsulfate (SDS) were obtained from Pharmacia LKB Biotechnology. Molecular weight standards for electrophoresis and Biogel P-100 were purchased from Bio-Rad Laboratories, Richmond, California. YM-5, YM-10 and YM-30 kDa cut-off ultrafiltration membranes and microconcentrators were obtained from Amicon, Co.. Diethylaminoethyl-cellulose (DE 52) was obtained from Whatman, Inc.. Myxothiazol was purchased from Boehringer Mannheim Biochemicals. Stigmatellin was a generous gift from Dr. G. Hofle (Gesellschaft für Biotechnologische Forschung, Braunschweig) and 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was supplied by Prof. B. L. Trumpower (Department of Biochemistry, Dartmouth Medical School). 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol, the beef-heart mitochondrial cytochrome bc_1 complex and polyclonal antibodies against the cytochrome b and 12 kDa subunits of the Rhodobacter sphaeroides cytochrome bc_1 complex were provided by Dr. C. -A. Yu (Department of Biochemistry, Oklahoma State University) and 2,3-dimethoxy-5-decyl-6-methyl-1,4-hydrobenzoquinone (DBH) was kindly supplied by Dr. D. Birney (Department of Chemistry and Biochemistry, Texas Tech University). Monoclonal antibodies against Rhodobacter capsulatus cytochromes b and c_1 were generous gift from Prof. F. Daldal (Department of Biology,

University of Pennsylvania). Azolectin was obtained from Associate Concentrate. All other chemicals were obtained from commercially available sources. The cytochrome c_2 mutants were provided by Prof. M. A. Cusanovich and TFC-derivatives of equine cytochrome c provided by Prof. F. Millett.

All solutions were prepared either with double distilled water which was passed through three combination mixed bed/organic cartridges from Barnstead or, when necessary, a Milli-Q water purification system from Millipore.

3.2. Methods

3.2.1. Growth and chromatophore membrane preparation

3.2.1.1. Growth and source of cell material

Wild-type Rhodospirillum rubrum (strain S1) cells were grown photosynthetically on a malate-containing medium described by Pfennig and Trüper (1981). The cultures were incubated for three to four days at an intensity of about 250 lux in 9 liter bottles. The cells were harvested by centrifugation at 7000 x g for 10 minutes using a Beckman model J2-21 centrifuge at 0-5 °C. The cells were washed twice with 25 mM Tris-HCl buffer, pH 8.00, stored at -20 °C and used when needed.

Rhodopseudomonas viridis NHTC 133 cells were supplied by Mr. Y. L. Chen (Department of Chemistry and Biochemistry, Texas Tech University).

3.2.1.2. Chromatophore membrane preparation

Both R. rubrum and Rps. viridis chromatophore membranes were prepared by the same procedure, described below, and all steps were performed at 5 °C. The cells were suspended in 25 mM Tris-HCl, pH 8.00, to a final concentration of about 0.1 g wet cell weight/ ml of buffer. These cell suspensions were sonicated, 50 ml at a time, using a Branson Model 200 sonifier. Sonication was done at 50 % duty cycle at a power setting of

7 for a total of 3 minutes. The container containing the cell suspension was immersed in an ice-sodium chloride bath to prevent excessive heating. Any undisrupted cells were pelleted by centrifugation at 7,000 x g for 10 minutes using a JA-10 rotor and a Beckman Model J2-21 centrifuge. The pellet was suspended with 25 mM Tris-HCl buffer, pH 8.00, resonicated and centrifuged at 7,000 x g for ten minutes. The pooled supernatants from the 7,000 x g centrifugations were then recentrifuged at 12,000 x g for 20 minutes, using a JA-20 rotor to remove larger cell debris. The supernatant was then centrifuged at 200,000 x g for 2 hours to pellet chromatophore membranes using either a Beckman Ti-70 or a Ti-60 Beckman rotor and a Beckman Model L7-65 ultracentrifuge. Both the pelleted chromatophore membrane fragments and supernatant were stored at -20 °C if not used immediately.

3.2.2. Preparation of cytochrome bc_1 complexes

3.2.2.1. Isolation and purification of the R. rubrum cytochrome bc_1 complex

The R. rubrum cytochrome bc_1 samples used for most of the experiments described below were prepared as described previously (Kriauciunas et al., 1989). The chromatophore pellet obtained from 200,00 x g centrifugation was suspended in 20 mM Tris-HCl, pH 8.00, 5 mM EDTA and centrifuged at 200,000 x g for 75 min. The resulting pellet was suspended in 35 mM MOPS buffer, pH 7.40, and recentrifuged at 200,000 x g for 75 min. The resulting pellet was suspended in a small amout of 35 mM MOPS buffer, pH 7.40, 1 mM $MgSO_4$, 0.1 g/ml dodecyl maltoside then the chromatophore membrane concentration was adjusted to a protein concentration between 10 and 11 mg/ml by diluting with the same buffer. In some cases, the detergent solubilization step was carried out in the presence of 20% (v/v) glycerol. The suspended chromatophores were made 25 mM dodecyl maltoside, incubated at 4 °C for 1 hour, and then centrifuged at 200,000 x g for 90

minutes. Sodium chloride was added to the resulting supernatant to a final concentration of 100 mM and incubated at 4 °C for 30 minutes. The detergent solubilized solution was applied to a DEAE-Biogel A (1.5 x 15 cm) column equilibrated with 35 mM MOPS, pH 7.40, 1 mM $MgSO_4$, 0.1 g/ml dodecyl maltoside (MMD buffer) containing 100 mM NaCl. In some cases 20% (v/v) glycerol was included. The column was washed with 10 column volumes of MMD buffer containing 200 mM NaCl and 20 % glycerol. Finally, the sample was eluted from the column with MMD buffer containing 350 mM NaCl and 20 % glycerol. Pooled fractions containing the cytochrome $\underline{bc}_1$ complex were diluted 1:1 with MMD buffer containing no sodium chloride and then loaded on a DEAE-Sepharose (1.5 x 15 cm) column equilibrated with MMD buffer containing 100 mM NaCl. The column was washed with 10 column volumes of MMD buffer containing 250 mM NaCl and then the sample was eluted with MMD buffer containing 500 mM NaCl. Column fractions containing the purified complex were pooled, glycerol was added to a final concentration of 5% (v/v), and the samples were stored at -196 °C until needed.

The inclusion of 20% glycerol in the buffers used for the detergent solubilization and first chromatography steps in the purification protocol resulted in an increased yield of the complex but did not affect any of the properties (i.e., subunit composition, absorbance spectrum, prosthetic group content, bacteriochlorophyll content or specific activity) of the purified complex. However, in the presence of 20% glycerol, the cytochrome $\underline{bc}_1$ complex was eluted from the first anion exchange column by buffer containing 200 mM NaCl, compared to the 300 mM NaCl required to elute the complex in the absence of glycerol. The purified complex was stored unfrozen at -20 °C in buffer containing 50% (v/v) glycerol. The complex proved to be considerably more stable when stored in this fashion than when stored frozen in 20% (v/v) glycerol at -196 °C.

3.2.2.2. Isolation and purification of the Rhodopseudomonas viridis cytochrome bc_1 complex

Rps. viridis cytochrome bc_1 samples used for the experiments described below were prepared by a purification procedure very similar to that described above for R. rubrum except that the final concentration of detergent used in the detergent solubilization step was 15 mM dodecyl maltoside. The first and second columns was equilibrated with MMD buffer containing 70 mM NaCl and sample containing the cytochrome bc_1 complex was eluted from the first column with MMD buffer containing 150 mM NaCl and from the second column with MMD buffer containing 200 mM NaCl. This purification method is similar to one described previously (Cully et al., 1989; Cully, 1990) except that the sucrose-gradient step was eliminated and 15 mM detergent was used for solubilization of the cytochrome bc_1 complex of Rps. viridis.

3.2.3. Preparation of soluble components

All the native cytochome c_2 purification experiments were carried out essentially as described previously (Bartsch, 1978).

3.2.3.1. Purification of cytochrome c_2 of Rhodopseudomonas viridis

The supernatant obtained from the first high speed (200,000 x g) ultracentrifugation step of the Rps. viridis chromatophore membrane preparation (see section 3.2.2.2.) was dialyzed against 2 mM Tris-HCl, pH 7.10, with 4 or 5 changes of buffer, and applied to a CM-sephadex column (2.5 x 30 cm) equilibrated with the same buffer. The conductivity of dialyzing buffer was measured with a conductivity meter to ensure equilibration before and after each buffer change. The column was washed with Tris-HCl buffer, pH 7.10,

gradually increasing its concentration (5 mM, 10 mM, 15 mM) and the cytochrome $\underline{c}_2$ containing fraction was eluted from the column by washing the column with 20 mM Tris-HCl buffer, pH 7.10. The fractions containing cytochrome $\underline{c}_2$ were combined and concentrated using an Amicon concentrator with a YM-5 membrane. Cytochrome $\underline{c}_2$ was further purified on a Sephadex G-75 column (2.5 x 75) by using 20 mM Tris-HCl buffer, pH 7.10, as elution buffer. The purity of cytochrome $\underline{c}_2$ was determined by using the A_{277}/A_{416} ratio which has a value of 0.16 for the pure protein. The sample was kept at -20 °C until it was needed.

3.2.3.2. Purification of cytochrome $\underline{c}_2$ from crude extracts of Rhodobacter capsulatus cells

Crude extracts containing soluble components of Rb. capsulatus were kindly provided by Dr. D. E. Robertson (University of Pennsylvania). The crude extracts of Rb. capsulatus were centrifuged at 200,000 x g using a Ti-70 Beckman rotor and a Beckman Model L7-65 ultracentrifuge for 5-6 hours to remove cell debris. The supernatant was dialyzed against 2 mM Tris-HCl, pH 8.00 with 4 or 5 changes of buffer and applied to a DEAE-cellulose column (2.5 x 30 cm) equilibrated with the same buffer. The conductivity of dialyzing buffer was measured with a conductivity meter to determine equilibration before and after each buffer change. The column was washed with Tris-HCl buffer, pH 8.00, gradually increasing its concentration (first 5 mM, and then 10 mM), and then with Tris-HCl buffer, pH 8.00, containing 20 mM NaCl. Cytochrome $\underline{c}_2$ was eluted from the column by washing the column with 10 mM Tris-HCl buffer, pH 8.00, containing 40 mM NaCl. The cytochrome $\underline{c}_2$-containing fractions were combined and concentrated using an Amicon concentrator with a YM-5 membrane. Concentrated cytochrome $\underline{c}_2$ was further purified on a Sephadex G-75 column (2.5 x 75), using 20 mM Tris-HCl buffer, pH 8.00, as the elution buffer. The purity of cytochrome $\underline{c}_2$ was determined by using the A_{273}/A_{415}

ratio which has a value of 0.17 the pure protein. The sample was kept at -20 °C until it was needed.

3.2.4. Analytical determinations

3.2.4.1. Ultraviolet-visible spectroscopy

Shimadzu Model UV-2100 and UV-160A spectrophotometers were used to measure ultraviolet-visible spectra.

3.2.4.2. Bacteriochlorophyll and protein determinations

Bacteriochlorophyll (Bchl a) was determined for R. rubrum membrane fragments from the absorbance at 772 nm following extraction in 7:2 (v/v) acetone:methanol (Clayton, 1963). The protein contents of the cytochromes of R. rubrum, Rb capsulatus and Rps. viridis were determined according to Lowry et al. (1951) as modified by Peterson (1983). Bovine serum albumin was used as a protein standard.

3.2.4.3. Enzymatic activity assays of the cytochrome bc_1 complexes from the purple photosynthetic bacteria

The quinol:cytochrome c oxidoreductase activity of the complexes was assayed as described previously (Kriauciunas et al.,1989). Cytochrome c reduction was monitored by following absorbance increases at 550 nm using a Shimadzu Model UV-2100 spectrophotometer. 50 μM of 2,3-dimethoxy-5-decyl-6-methyl-1,4-hydrobenzoquinone (DBH) was used as the electron donor and 1 ml of 50 μM oxidized equine cytochrome c was used as the electron acceptor in 50 mM potassium phosphate buffer, pH 7.40.

3.2.4.4. Inhibition assays

The effect of quinol-analog inhibitors on the quinol-cytochrome c oxidoreductase activities of the cytochrome bc_1 complexes from R. rubrum, Rb. capsulatus, and Rps. viridis was monitored at 550 nm as described in (section 3.2.4.3.). The assay mixture contained 10 nM cytochrome bc_1 complex. The rates were corrected, by subtraction, for the small amount of direct electron transfer from the quinol to equine cytochrome c that was observed in the absence of the complex. Inhibitors, antimycin A, myxothiazol, stigmatellin and 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) were used as electron transport inhibitors at concentrations ranging from 0 to 0.8 μM of concentrated ethanol stock solutions which were determined by the individual extinction coefficients of the inhibitors (von Jagow and Link, 1986). Control experiments were done to show that ethanol itself does not inhibit the activity of the cytochrome bc_1 complex.

3.2.4.5. Kinetic assays

These assays were performed with purified cytochrome c_2 and the cytochrome bc_1 complexes from Rb. capsulatus and Rps. viridis using a Shimadzu Model UV-2100 spectrophotometer. The quinol-cytochrome c/c_2 oxidoreductase activity of the Rb. capsulatus cytochrome bc_1 complex was assayed by the following the rate of reduction of native or mutant of Rb. capsulatus cytochrome c_2 and native or modified equine cytochrome c at 550 nm after addition of DBH, as reported by Berry and Trumpower (1985). Prior to the kinetic measurements Rb. capsulatus cytochrome c_2 and equine cytochrome c were oxidized with potassium ferricyanide and chromatographed on a Dowex 1-X column (1.5 x 5 cm) equilibrated with potassium phosphate buffer, pH 7.40 containing 290 μM EDTA. Each assay mixture contained 2.90 nM Rb. capsulatus cytochrome bc_1 complex, 75 μM DBH, 290 μM EDTA and cytochrome c_2 or equine cytochrome c at concentrations ranging from 0.5-50 μM in 50 mM potassium phosphate

buffer, pH 7.40. The ionic strength of the buffer was varied by addition of NaCl. Eadie-Hofstee plot of the velocities, V, versus V/[S] values were obtained by fitting the kinetic data using a CricketGraph program, and V_{max} and K_m values were extracted from the plots on a Macintosh SE computer. The data obtained from the ionic strength dependency of the reduction rate of cytochrome $\underline{c}_2$ by the cytochrome $\underline{bc}_1$ complex of Rb. capsulatus were fitted to the electrostatic pair model (Stonehuerner et al., 1979) using a Quattro spreadsheet program kindly provided by Prof. F. Millett (University of Arkansas). Similar kinetic experiments were carried out with Rps. viridis for comparative purposes.

3.2.4.6. Antimycin A-induced shift of cytochrome $\underline{b}_H$ of the Rhodospirillum rubrum cytochrome $\underline{bc}_1$ complex

The antimycin A-induced red shift of high potential b-cytochrome was measured in 25 mM Tris-HCl, pH 8.00, with the cytochrome $\underline{bc}_1$ complex (1 μM cytochrome $\underline{c}_1$) in the presence of sodium dithionite to fully reduce all the cytochromes as described in Gabellini et al. (1982), using a Shimadzu Model UV-2100 spectrophotometer. Antimycin A (in ethanol) was added to 5 μM in sample cuvette and an equal volume of solvent to reference cuvette. A baseline was recorded before the addition of antimycin A to the sample cuvette.

3.2.4.7. Proton translocation experiments

The beef-heart mitochondrial cytochrome $\underline{bc}_1$ complex was prepared according to the method of Yu and Yu (1980) and incorporated into azolectin phospholipid vesicles (liposomes) using the cholate dialysis method of Racker and Kagawa (1971). The R. rubrum cytochrome $\underline{bc}_1$ complex was incorporated into phospholipid vesicles in the same manner. In some cases, the R. rubrum cytochrome $\underline{bc}_1$ complex was precipitated with 60% saturated ammonium sulfate to remove the glycerol in which the complex was stored and the precipitate, after collection by centrifugation, was dissolved directly in azolectin

cholate solution prior to the dialysis step. Proton translocation coupled to electron flow through the complex was measured at 25 °C using a Beckman Model pH meter and Model 39532 combination pH electrode. The reaction mixture contained, in a volume of 1.4 ml, 0.15 M KCl, 2.9 μM equine ferrocytochrome c, 18 μM Q_2H_2, 0.26 μM valinomycin and 25 μl of liposomes. Electron flow was initiated by the addition of 5 nmol of potassium ferricyanide, which oxidizes the cytochrome c and thus provides an electron acceptor for the complex. Electron flow under conditions where no transmembrane ΔpH is formed was measured in an identical manner except that the protonophore CCCP, at a concentration of 0.5 μM, was added to make the membrane permeable to protons.

3.2.4.8. Co-migration and ultrafiltration experiments

Gel-filtration co-chromatography experiments were carried out in 20 mM Tris-HCl buffer, pH 8.00, containing 0.05 % (w/v) dodecyl maltoside using a Biogel P-100 column (1 x 30 cm) at a flow rate of 7.5 ml/hr. On this column, the Rb. capsulatus cytochrome bc_1 complex was eluted with the void volume, well separated from either cytochrome c_2 or equine cytochrome c. Complex formation was also studied using an Amicon stirred ultrafiltration cell with a YM-30 membrane that completely retained the Rb. capsulatus cytochrome bc_1 complex but through which both equine cytochrome c and Rb. capsulatus cytochrome c_2 passed completely. The filtrates from each assay were collected, and the amount of cytochrome c_2 or equine cytochrome c was quantitated from ascorbate-reduced minus ferricyanide-oxidized difference spectra.

3.2.5. Western blots

Western blots, using monoclonal antibodies against Rb. capsulatus cytochromes b and c_1, were performed according to the method of Davidson et al. (1987). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to

Laemmli (1970). After SDS-PAGE of the detergent-solubilized cytochrome bc_1 complex of R. rubrum (50 μg protein per lane) proteins were immunoblotted to nitrocellulose membranes and incubated with antisera raised against cytochromes c_1 and b, eluted from SDS-PAGE of purified cytochrome bc_1 complex isolated from Rb. capsulatus. Nonspecific binding of antibody to the nitrocellulose was minimized by preincubating filters with 3 % fish gelatin. Horseradish peroxidase IgG was used to detect antigen-antibody interactions.

3.2.6. Electron paramagnetic resonance spectroscopy

Oxidation-reduction titrations under anaerobic conditions were performed at University of Pennsylvania in collaboration with Dr. D. E. Robertson as described by Dutton (1978). Absorbance spectra, taken at defined E_h values during oxidation-reduction titrations, were obtained using a Biomedical Instrumentation Group (University of Pennsylvania) spectrophotometer interfaced with an IBM personal computer. Low-temperature EPR spectra of the Rieske iron-sulfur center were obtained using a Varian Model E-109 X-band spectrometer equipped with an Air Products Model LTD 3-110 variable temperature, flowing helium cryostat.

3.2.7. Resonance Raman spectroscopy

Raman samples were prepared by concentrating the purified complex using Amicon Centricon 30 microconcentrator to a final concentration of about 100 μM in cytochrome c_1. Samples were then placed in an anaerobic optical cell to which one cytochrome c_1 equivalent of potassium ferricyanide was added. Samples were rigorously degassed, placed under a slightly positive nitrogen atmosphere and allowed to incubate for 1 h to obtain the fully oxidized complex. A gas-tight syringe containing a sodium dithionite solution, which had been standardized using an equal volume of 100 μM equine

cytochrome c (Type VI), was then fitted to the optical cell. Single reduction equivalents were added to the sample and the level of reduction monitored by ultraviolet/visible absorption using an HP8452 diode array spectrophotometer interfaced to an Amstrad 512PC computer. Raman spectra corresponding to the fully oxidized and two, three- and four-electron reduced equilibrium species were then obtained using a Molectron UV-24 nitrogen-pumped dye-laser (tunable range, 380 to 830 nm) using a 160 degree backscattering collection geometry. Laser energy density was varied from 10-100 mJ/cm^{-1} at the sample using either cylindirical or spherical focusing optics. The scattered light was collected in a Spex 1403 double monochromator with a water-cooled photomultiplier tube (Hamamatsu R928). The spectral band pass was 6-10 cm^{-1} for all spectra. Data were stored and processed on a Spex Industries DM3000 XT system. All spectra are the unsmoothed average of three to five scans.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Properties and characterization of the cytochrome bc_1 complex of Rhodospirillum rubrum

4.1.1. Western blots

Recent evidence on cytochrome bc_1 complexes isolated and characterized from four purple non-sulfur photosynthetic bacteria has shown that the complexes from Rhodobacter capsulatus (Robertson et al., 1992), Rhodopseudomonas viridis (Cully et al., 1989) and R. rubrum (Kriauciunas et al., 1989) contain three peptide subunits. However, the Rhodobacter sphaeroides cytochrome bc_1 complex has been reported to contain four rather than three subunits (Yu et al., 1984; Purvis et al., 1990; Andrews et al., 1990). Despite the fact that work performed in our laboratory (Wynn et al., 1986; Kriauciunas et al., 1989) and by two other groups (Purvis et al., 1990; Majewski and Trebst, 1990) had failed to detect a 12-14 kDa subunit in the R. rubrum complex, it seemed possible that such a subunit could be present in situ and subsequently lost during purification of the complex. The observation that an antibody prepared against the 12-14 kDa subunit of the Rb. sphaeroides cytochrome bc_1 complex inhibited the quinol:cytochrome c oxidoreductase activity of the Rb. sphaeroides complex (Yu and Yu, 1991) suggested that this antibody might be able to detect a related peptide in unfractionated R. rubrum membranes. Of course, in order for such an approach to be useful it is necessary that the subunits of the two complexes be sufficiently similar that an antibody raised against a subunit isolated from one bacterial species would be capable of recognizing the corresponding subunit from another bacterial species. In fact, it has been shown that antibodies raised against the cytochrome c_1 (Kriauciunas et al., 1989) and cytochrome b peptides (Güner et al., 1991)

of the Rb. sphaeroides complex recognize the corresponding subunits of the R. rubrum complex. These antigenic similarities between two subunits of the two bacterial complexes suggested that a 12-14 kDa subunit of the R. rubrum complex, if one existed, might be detected by an antibody against the Rb. sphaeroides peptide. Western blots, done by Dr. Linda Yu at Oklahoma State University, of solubilized R. rubrum membranes (chromatophores), prepared in our laboratory, failed to detect any component that cross reacted with this antibody. These results indicate that either the R. rubrum cytochrome bc_1 complex does not contain a fourth subunit or that, if a fourth subunit is present, it is antigenically unrelated to the 12-14 kDa subunit of the Rb. sphaeroides complex.

It had previously been demonstrated that polyclonal antibodies raised against the Rb. capsulatus Rieske iron-sulfur protein recognized the corresponding R. rubrum protein (Kriauciunas et al., 1989). To further explore possible antigenic similarities between the two other subunits of the cytochrome bc_1 complexes of these two bacteria, four separate Western blots were run with the R. rubrum complex using three monoclonal antibodies raised against Rb. capsulatus cytochrome c_1 (Davidson et al., 1987) and one monoclonal antibody raised against Rb. capsulatus cytochrome b (Davidson et al., 1987). Fig. 4.1 (lanes 1-3) shows that three monoclonal antibodies raised against Rb. capsulatus cytochrome c_1 recognize R. rubrum cytochrome bc_1. It is interesting to note that one of these monoclonal antibodies against Rb. capsulatus cytochrome c_1 (antibody D42, used in lane 1) does not recognize Rb. sphaeroides cytochrome c_1 (Davidson et al., 1989), raising the possibility that greater structural similarity may exist between the R. rubrum and Rb. capsulatus cytochrome c_1 than between the Rb. capsulatus and Rb. sphaeroides cytochromes. Monoclonal antibody D42 (lane 1) also recognizes a band of a higher molecular weight than that of monomeric cytochrome c_1 (Wynn et al., 1986; Kriauciunas et al., 1989), indicating the presence of some aggregated material. Such aggregates have been detected in previous studies of the R. rubrum complex (Wynn et al., 1986;

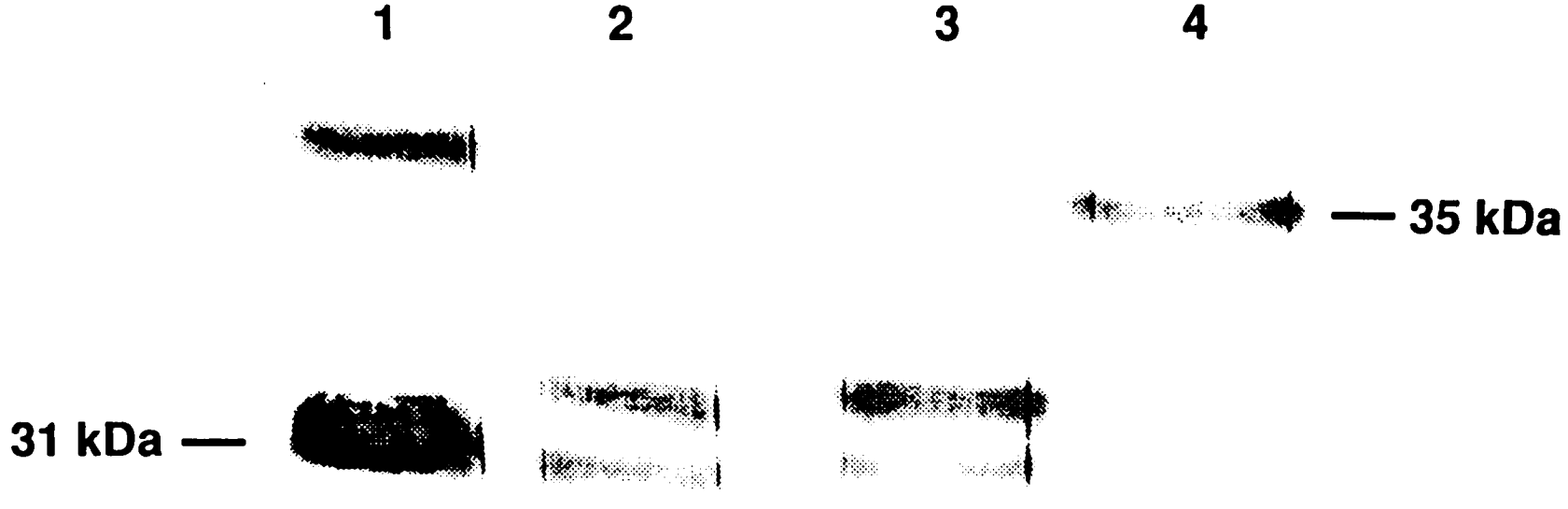

Figure 4.1. Recognition of Rhodospirillum rubrum cytochrome c_1 and cytochrome b peptides by monoclonal antibodies against the Rhodobacter capsulatus peptides. Western blots were performed as described in Materials and Methods. Lanes 1-4 show the results of separate experiments in which approximately equal amounts (10-15 μg of protein per electrophoresis run) of the R. rubrum cytochrome bc_1 complex were separated into constituent subunits by electrophoresis in the presence of sodium dodecyl sulfate, blotted, and treated with monoclonal antibodies raised against Rhodobacter capsulatus cytochrome c_1 (1, antibody D42; 2, antibody D3; and 3, antibody D1) or Rb. capsulatus cytochrome b (4, antibody D50). The antibody designations correspond to those of Ref. Davidson et al., 1987 (Güner et al., 1991).

Kriauciunas et al., 1989; Purvis et al., 1990). Examination of lanes 2 and 3 in Fig. 4.1 indicates the presence of two closely spaced bands, rather than a single band, in the resolved R. rubrum preparation that are recognized by two different monoclonal antibodies raised against Rb. capsulatus cytochrome c_1. This is probably also the case for the third monoclonal antibody against Rb. capsulatus cytochrome c_1 (lane 1), but the intense staining makes it difficult to resolve the two bands. As the upper of the two bands has an apparent molecular mass of 31 kDa, equal to that for the single cytochrome c_1 band previously observed in our laboratory (Wynn et al., 1986; Kriauciunas et al., 1989), we conclude that some proteolysis of the cytochrome occured under the conditions used for the current experiments. Fig. 4.1 (lane 4) also shows that R. rubrum cytochrome b is recognized by a monoclonal antibody raised against Rb. capsulatus cytochrome b.

4.1.2. Inhibition experiments

Recent characterization of Rb. capsulatus mutants that are resistant to myxothiazol and stigmatellin, two specific inhibitors of the cytochrome bc_1 complexes, indicate that the binding sites for these inhibitors are probably located on the cytochrome b subunit of the complex (Daldal et al., 1989). Mutations that confer resistance to antimycin A in aerobic eukaryotes indicate that the binding site for this specific inhibitor of the complex is also likely to be on the cytochrome b peptide (Howell et al., 1987; di Rago et al.,1988; Brasseur, 1988; Howell and Gilbert, 1988, Weber and Wolf, 1988; di Rago et al., 1989). These three inhibitors of the cytochrome bc_1 complexes have structures that suggest that they can function as quinone or quinol analogs and may inhibit the complex by competing with the normal quinone substrates for their binding sites (Rich, 1984; von Jagow and Link, 1986). As the 12-14 kDa subunit of the Rb. sphaeroides complex has been implicated in quinone binding (Yu and Yu, 1988), it seemed important to determine the sensitivity of the three subunit R. rubrum complex to these inhibitors. A fourth inhibitor of

these complexes, UHDBT, has been shown to affect the properties of the Rieske iron-sulfur protein in Rb. sphaeroides (Bowyer et al., 1980; Andrews et al., 1990). As UHDBT is likely to act as a quinone analog (Bowyer et al., 1980), it also seemed advisable to determine the sensitivity of the three subunit R. rubrum complex to this inhibitor. Fig. 4.2 shows inhibition curves for all four of these inhibitors of the quinol:cytochrome c oxidoreductase activity of the R. rubrum complex. It is clear from these results that this solubilized, three subunit complex retains high sensitivity to these four specific inhibitors of cytochrome bc_1 complexes.

Inhibition of the ubiquinol:cytochrome c oxidoreductase activity of the R. rubrum cytochrome bc_1 complex by myxothiazol and antimycin A had previously been demonstrated (Wynn et al., 1986; Kriaciunas et al., 1989), but the concentration dependence of the inhibition had not been reported. Another effect of antimycin A observed with cytochrome bc_1 complexes from other organisms, including detergent-solubilized complexes from the photosynthetic bacteria Rb. capsulatus and Rb. sphaeroides, is the shift to longer wavelength the inhibitor produces in the α-band maximum of reduced cytochrome b_H, the higher potential protoheme (van den Berg et al., 1979; Gabellini et al., 1982; Andrews et al., 1990). This band-shift had not been previously investigated with the solubilized R. rubrum complex. We have now observed such a band-shift with the detergent solubilized R. rubrum complex (Fig. 4.3). Difference spectra (+ antimycin A minus control) of the dithionite-reduced R. rubrum complex show a maximum at 564 nm and a minimum at 554 nm, similar to those reported previously for the detergent-solubilized Rb. capsulatus (Gabellini et al., 1982) and Rb. sphaeroides (van den Berg et al., 1979; Andrews et al., 1990) complexes. Thus, in so far as the antimycin A-induced red-shift in the α-band absorbance maximum of cytochrome b_H provides a measure of the interaction of antimycin A with the complex, the presence of a fourth 12-14 kDa subunit does not appear to be required for normal binding of the inhibitor.

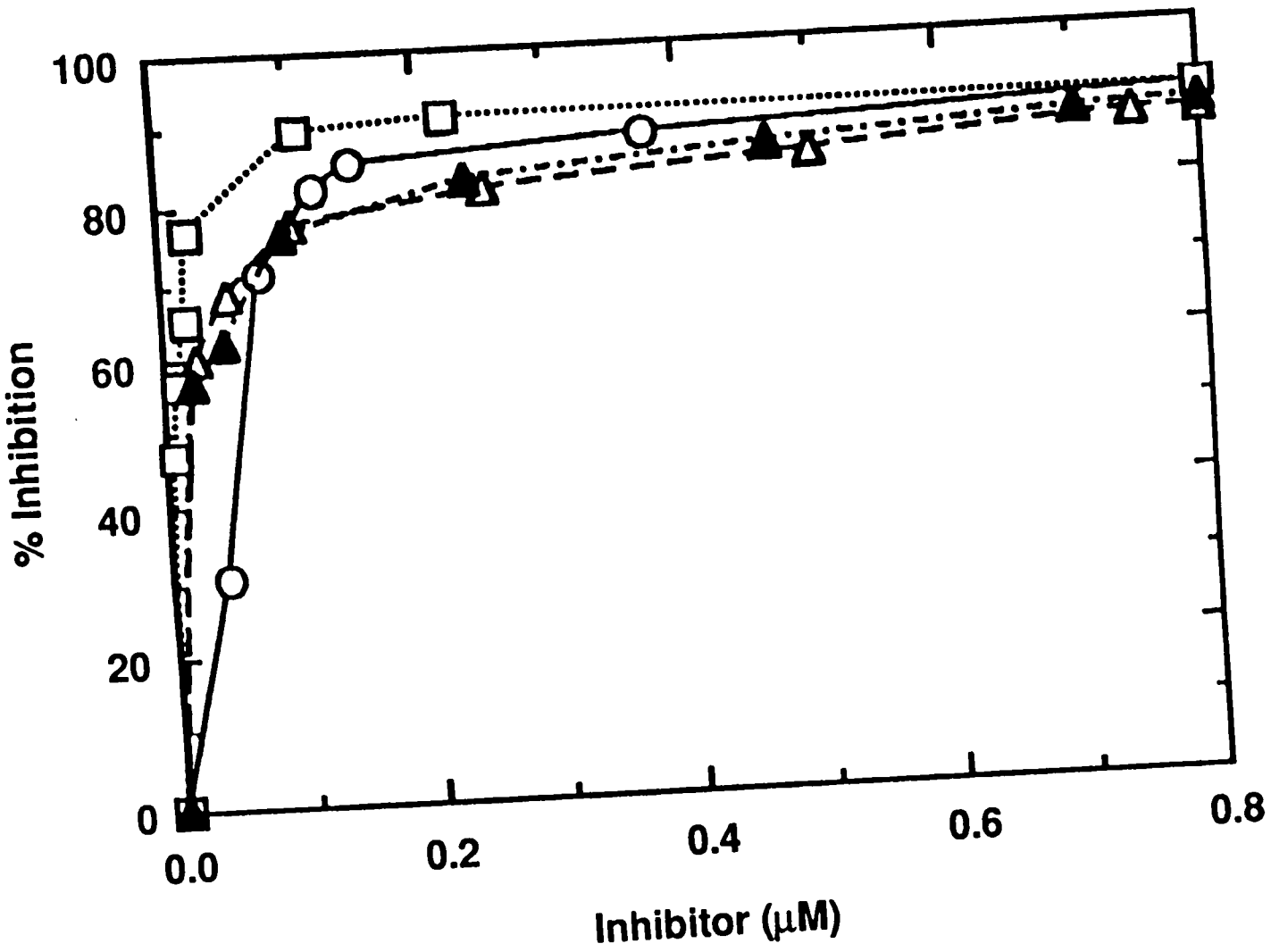

Figure 4.2. The effect of inhibitors on the quinol:cytochrome c oxidoreductase activity of the Rhodospirillum rubrum cytochrome bc_1 complex. The electron transfer activity of the complex was assayed as described in Materials and Methods, using an assay mixture contained 10 nM cytochrome bc_1 complex. The rates were corrected, by substraction, for the small amount of direct electron transfer from the quinol to equine cytochrome c that was observed in the absence of the complex. Inhibitors, present at concentrations indicated, were added as small aliquots of concentrated ethanol stock solutions. Antimycin A (△), myxothiazol (○), stigmatellin (□), UHDBT (▲) (Güner et al., 1991).

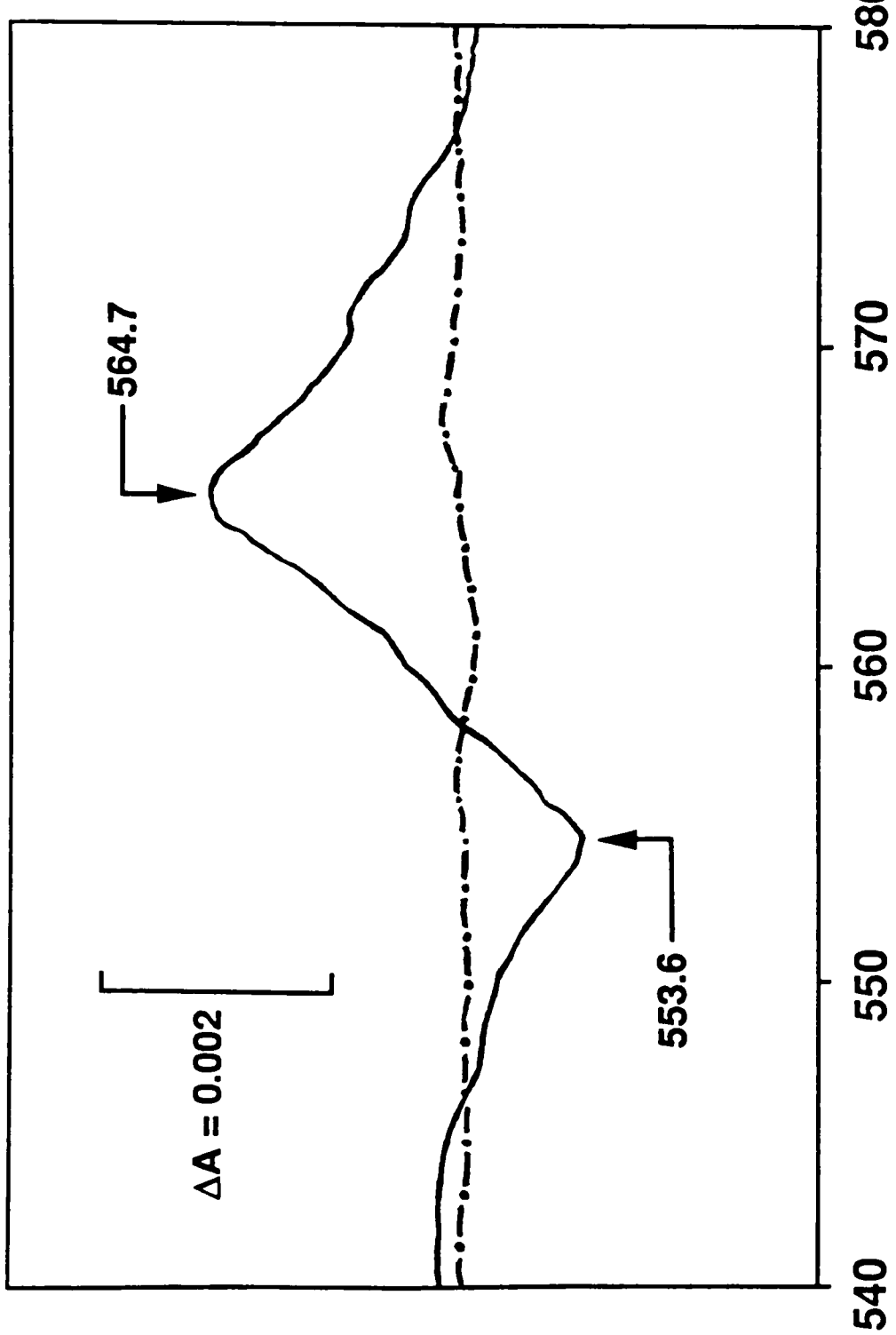

Figure 4.3. Antimycin A-induced shift spectrum (——) of cytochrome b_H of the Rhodospirillum rubrum cytochrome bc_1 complex. Baseline (– · –)was taken in a reaction mixture contained the cytochrome bc_1 complex, 1 μM in cytochrome c_1, in the presence of dithionite. Antimycin A was added to 5 μM in sample quvette and an equal volume of solvent to reference quvette.

4.1.3. Oxidation-reduction titrations

4.1.3.1. Electron paramagnetic resonance spectroscopy

Oxidation-reduction titrations, using absorbance changes in the cytochrome α-band region to monitor oxidation state, had previously been performed on cytochrome c_1 and the two hemes of cytochrome b in the solubilized R. rubrum complex (Kriauciunas et al., 1989). The EPR spectrum of the reduced Rieske protein in the R. rubrum had also been reported (Wynn et al., 1986). However, no measurements of the EPR spectrum at defined redox potentials, which would have allowed a determination of the E_m value of this component, were made. Fig. 4.4 shows an oxidation-reduction titration of the [2Fe-2S] cluster of the Rieske protein in the solubilized R. rubrum cytochrome bc_1 complex, using the amplitude of the g = 1.90 feature in the EPR spectrum to monitor the oxidation state of the cluster. The average of three titrations gave E_m = +305±10 mV, a value similar to that reported for the Rieske protein in other cytochrome bc_1 complexes (Prince et al., 1975; Bowyer et al., 1980; Hauska et al., 1983; Crofts and Wraight, 1983; Rich, 1984; Dutton, 1986; Andrews et al., 1990). Unlike the case for the cytochrome components of the R. rubrum complex (see below), the E_m value of the Rieske protein was not affected by the conditions used for storage of the complex.

Although studies with inhibitor-resistant mutants, cited above, suggested that the binding site for stigmatellin is likely to involve, at least in part, the cytochrome b subunit of these complexes, in species other than R. rubrum stigmatellin has been shown to also case changes in the midpoint potential and EPR spectrum of the Rieske iron-sulfur protein (von Jagow and Ohnishi, 1985; Andrews et al., 1990). Fig. 4.5 demonstrates that stigmatellin produces a large positive shift in the E_m value of the R. rubrum Rieske protein, similar to that previously observed with other cytochrome bc_1 complexes (von Jagow and Ohnishi, 1985; Andrews et al., 1990). Fig. 4.5 shows that stigmatellin also shifts the g values

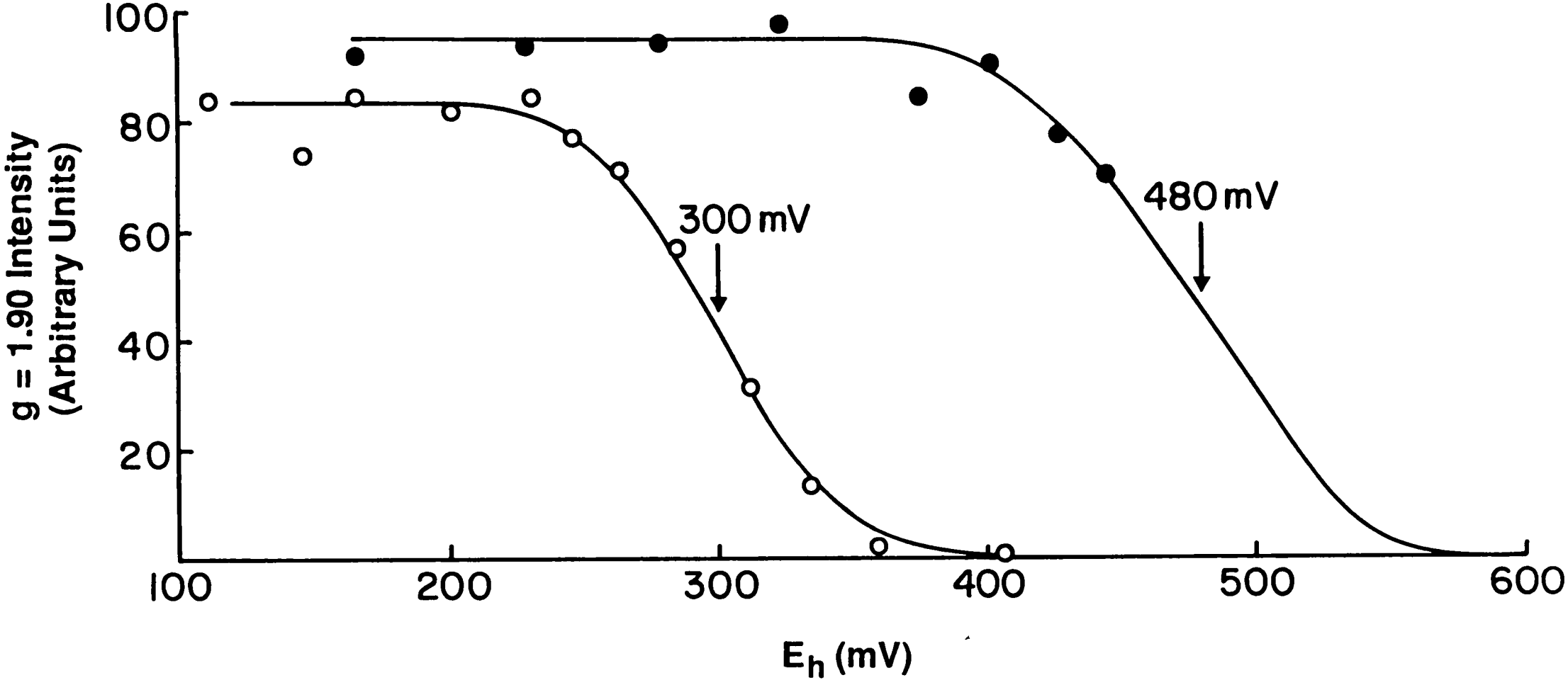

Figure 4.4. Oxidation-reduction of the Rhodospirillum rubrum Rieske iron-sulfur protein and the effect of stigmatellin on its E_m value. Open circles and closed circles represent the titrations in the absence and in the presence of 25 μM stigmatellin, respectively. The reaction mixture contained the R. rubrum cytochrome bc_1 complex at a concentration equivalent to 24 μM cytochrome c_1 in 35 mM MOPS buffer, pH 7.40, containing 1 mM $MgSO_4$ and 0.1 mg/ml dodecyl maltoside and the following oxidation-reduction mediators: 40 μM 2,3,5,6-tetramethyl-p-phenylenediamine; 40 μM quinhydrone; 20 μM phenazine methosulfate and 20 μM N,N,N',N'-tetramethyl-p-phenylenediamine. Samples equilibrated at the indicated E_h values were transferred anaerobically to EPR tubes and frozen. EPR conditions: Frequency, 9.33 GHz; power, 1 mW; modulation amplitude, 10 G; temperature, 20 K (Güner et al., 1991).

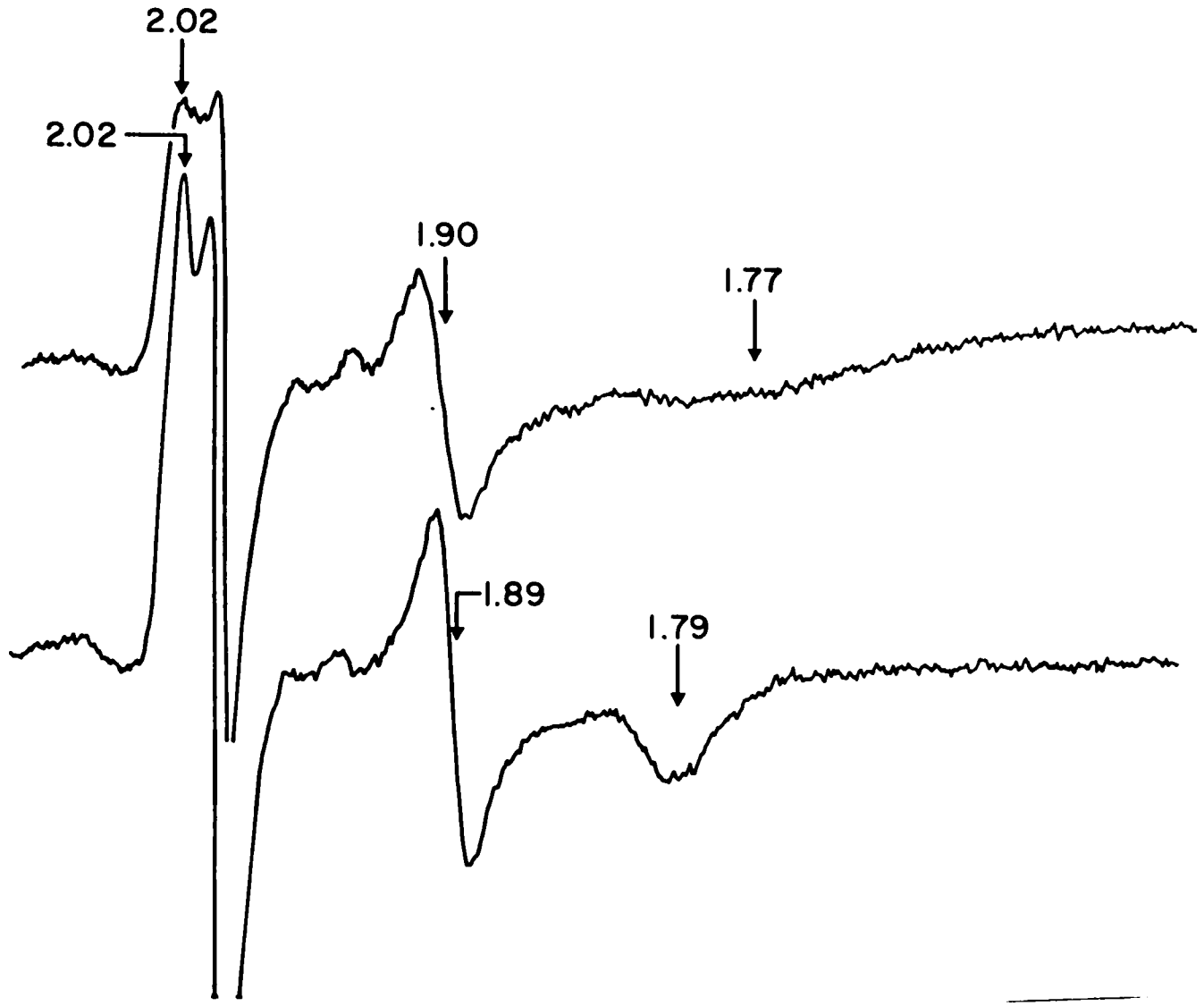

Figure 4.5. The effect of stigmatellin on the EPR spectrum of the Rhodospirillum rubrum Rieske iron-sulfur protein. The upper trace shows the EPR spectrum of the iron-sulfur protein in the absence of stigmatellin and the lower trace shows the EPR spectrum in the presence of 25 μM stigmatellin. The spectra were obtained with the samples that had been poised at E_h values of +168 mV and +174 mV, respectively, prior to freezing. Other conditions were as in Fig. 4.4 (Güner et al., 1991).

observed in the EPR spectrum of the reduced R. rubrum Rieske protein in a manner similar to that observed with cytochrome bc_1 complexes from other species (von Jagow and Ohnishi, 1985; Andrews et al., 1990). It has been demonstrated that shifts in the EPR spectrum of the reduced Rieske protein can also be observed, in the absence of inhibitors of the complex, when the quinol oxidizing site of the complex (Q_o) is occupied by quinol rather than by quinone (de Vries et al., 1979; Matsuura et al., 1983; Robertson et al., 1986; Andrews et al., 1990). No change in the EPR spectrum of the reduced Rieske protein in the solibilzed R. rubrum complex was observed when the E_h value of the sample was lowered from +200 mV to less than -300 mV (data not shown). The absence of any observable shift in Rieske protein g values over a range of E_h values where the ubiquinone known to be present in the solubilized R. rubrum complex (Wynn et al., 1986) should go from fully oxidized to completely reduced suggests either that this ubiquinone is not at the Q_o site or the g value shift does not occur in the solubilized R. rubrum complex.

4.1.3.2. Reduced-minus-oxidized difference spectra of cytochromes

Previous oxidation-reduction titrations of the solubilized R. rubrum complex resolved the two different E_m values of the two inequivalent protohemes of cytochrome b (Kriauciunas et al., 1989). The values obtained in this earlier study, -30 and -90 mV for the high potential (cytochrome b_H) and low potential (cytochrome b_L) components, respectively, differed somewhat from values obtained with solubilized cytochrome bc_1 complexes isolated from other photosynthetic bacteria (Gabellini et al., 1982; Cully et al., 1989; Andrews et al., 1990) and from values obtained with intact R. rubrum membranes (Venturoli et al., 1987). Some, but not all, of these differences could perhaps be attributed to the different pH values at which the measurements were conducted, as cytochrome b has been reported to have pH-dependent E_m values (Hauska et al., 1983; Gabellini and Hauska, 1983; Crofts and Wraight, 1983; Rich, 1984; Dutton, 1986; Cully et al., 1989).

As our earlier titrations were performed electrochemically, using an optically transparent gold mesh electrode, while the other studies used chemical oxidants and reductants to adjust E_h values, it seemed appropriate to repeat the redox titrations using conventional rather than electrochemical methods. In the course of these measurements, it became clear that considerable variability could be observed in the E_m values measured for cytochromes $\underline{b}_H$ and $\underline{b}_L$. This variability correlated with the manner in which the complex was stored rather than the manner in which the titrations were performed. Fig. 4.6 shows the result of a titration on a sample that had been stored unfrozen in 50 % glycerol. Average E_m values, from redox titrations of three separate samples of the R. rubrum complex that had been stored unfrozen in 50 % glycerol at -20 °C, of +20 and -85±35 mV were obtained for cytochromes $\underline{b}_H$ and $\underline{b}_L$, respectively. The significant variation observed from one titration to another (indicated by the ±35 mV average deviation) appears to arise, in part, from slow equilibration of the sample because of presence of residual glycerol in some of the samples. Considerably more negative E_m values (e.g., as low as -50 mV for cytochrome $\underline{b}_H$ and -175 mV for cytochrome $\underline{b}_L$) were obtained with samples that had been stored frozen at -196 °C in 20 % glycerol (data not shown). As samples stored unfrozen in 50 % glycerol exhibited considerably higher ubiquinol:cytochrome $\underline{c}$ oxidoreductase activity than those that had been stored frozen at -196 °C, it seems reasonable to take the more positive values as most representative of the native E_m values. These E_m values of +20 and -85 mV for cytochromes $\underline{b}_H$ and $\underline{b}_L$, respectively, are also in closer agreement with the E_m values of +35 and -40 mV determined for the two hemes of cytochrome b in intact R. rubrum membranes (Venturoli et al., 1987), than the more negative values obtained with samples that had been stored frozen.

The effect of sample storage on the E_m value of cytochrome $\underline{c}_1$ was also investigated. Fig. 4.7 shows a representative oxidation-reduction titration of cytochrome $\underline{c}_1$ in a sample of the R. rubrum cytochrome $\underline{bc}_1$ complex that was stored unfrozen at -20 °C in 50 %

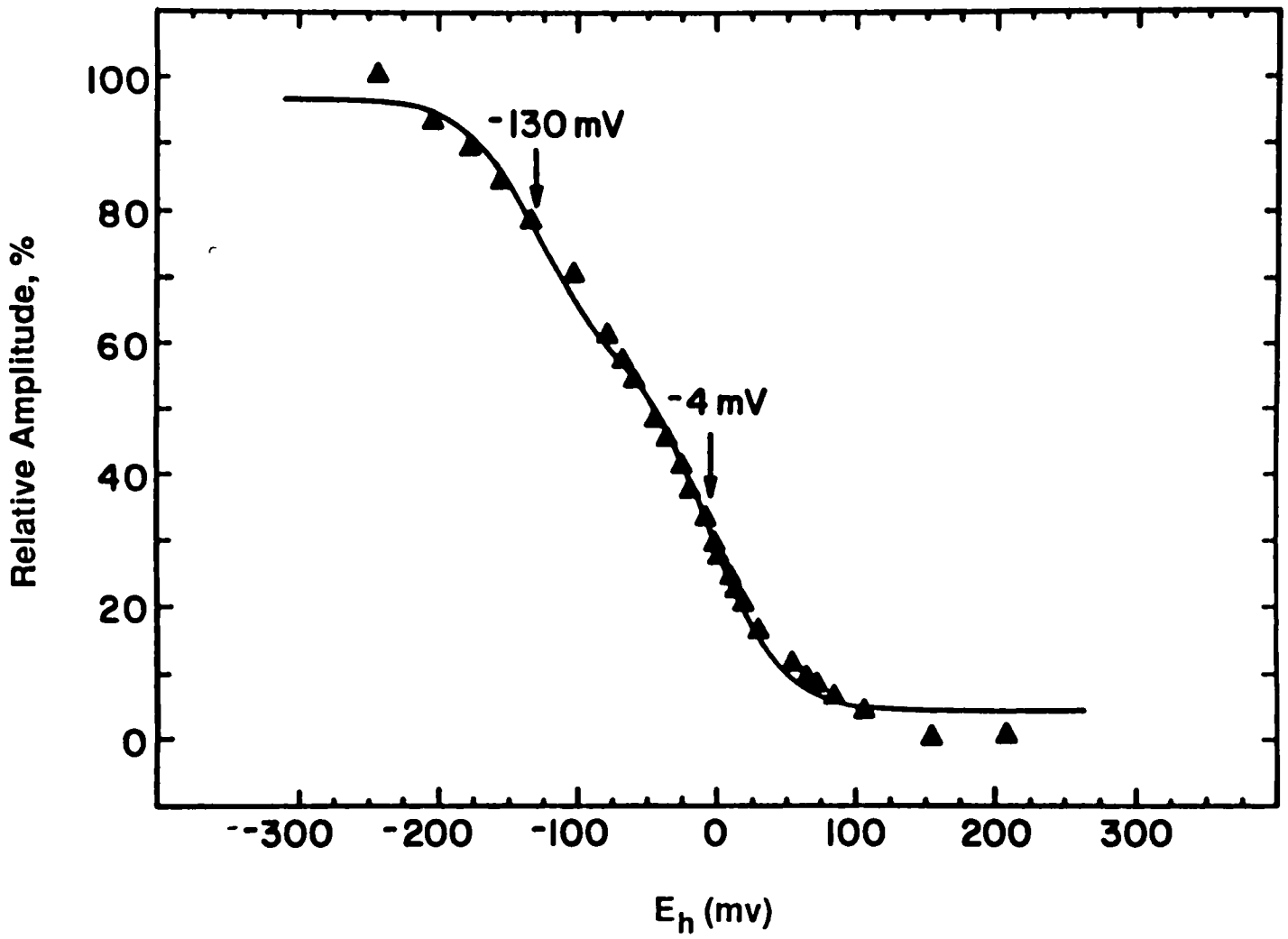

Figure 4.6. Oxidation-reduction titrations of Rhodospirillum rubrum cytochrome b_H and b_L. The reaction mixture contained the R. rubrum cytochrome bc_1 complex at a concentration equivalent to 2 μM cytochrome c_1 in 35 mM MOPS buffer, pH 7.40, containing 100 mM NaCl and 1 mM $MgSO_4$ and the following redox mediators; 20 μM phenazine methosulfate; 20 μM phenazine ethosulfate; 20 μM phenazine; 20 μM pyocyanine; 25 μM benzoquinone; 25 μM 1,2-naphtoquinone; 25 μM 1,4-naphtoquinone; 50 μM duroquinone; 70 μM 2,3,5,6-tetramethyl-p-phenylenediamine and 15 μM 2-hydroxy-1,4-naphtoquinone. Absorbance changes were monitored at 562 minus 577 nm and the data plotted to give the best fit to the Nernst equation for two n = 1 components (Güner et al., 1991).

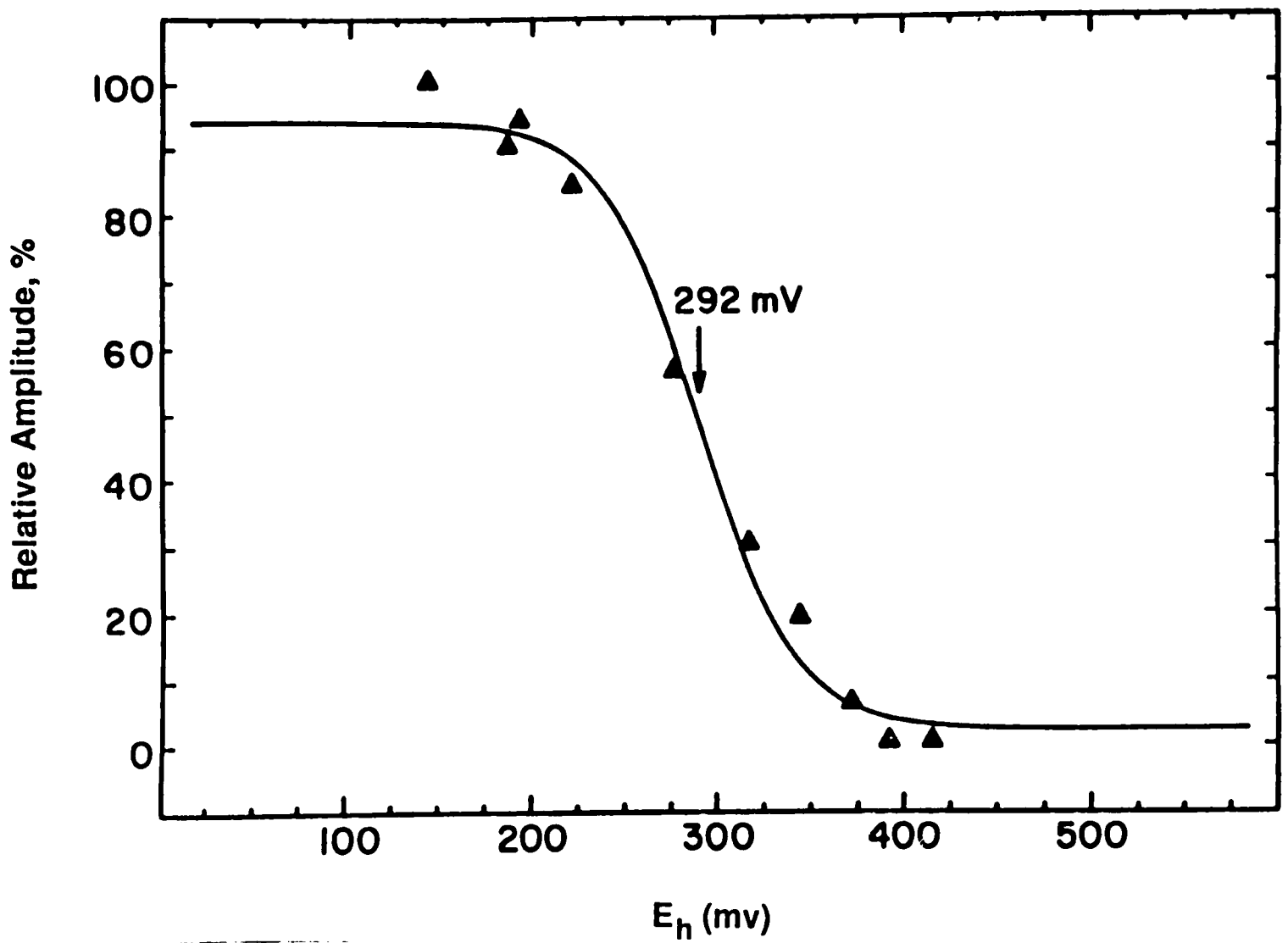

Figure 4.7. Oxidation-reduction titration of Rhodospirillum rubrum cytochrome c_1. Conditions were as in Fig. 4.6 except that observation and reference wavelengths of 552 and 540 nm, respectively, were used and the data fitted to a single n = 1 component (Güner et al., 1991).

glycerol. An average value of +280±15 mV was obtained for the E_m of the cytochrome in these samples, compared to an average value of +265±20 mV (three titrations) for the E_m of cytochrome $\underline{c}_1$ in samples of the R. rubrum cytochrome $\underline{bc}_1$ complex that had been stored frozen in 20 % glycerol at -196 °C. Thus, storage conditions for the complex had little or no effect on the E_m value of cytochrome $\underline{c}_1$. The E_m value obtained for cytochrome $\underline{c}_1$ in samples of the R. rubrum complex that had been stored unfrozen is similar to values obtained for cytochrome $\underline{c}_1$ in detergent-solubilized complexes isolated from other photosynthetic bacteria (Gabellini et al., 1982; Andrews et al., 1990; Cully et al., 1989) and the value measured for cytochrome $\underline{c}_1$ in intact R. rubrum membranes (Venturoli et al., 1987). Both the E_m values obtained in this study with samples that had been stored unfrozen and those that had been stored frozen were less positive than the +320 mV value previously obtained in our laboratory using electrochemical methods. The reasons for this discrepancy are not known.

Fig. 4.8A and B shows reduced minus oxidized difference spectra for cytochromes $\underline{b}_L$ and $\underline{b}_H$, respectively, obtained during an oxidation-reduction titration of a sample of the R. rubrum complex that had been stored unfrozen. A spectrum recorded at an ambient potential of -79 mV was subtracted from one recorded at -244 mV to produce the difference spectrum of cytochrome $\underline{b}_L$ in Fig. 4.8A, which shows an asymmetric α-band with a maximum at 563 to 564 nm and a shoulder at 559 nm. The reduced minus oxidized difference spectrum obtained for the cytochrome $\underline{b}_L$ component of the solubilized R. rubrum cytochrome $\underline{bc}_1$ complex differs from those reported for cytochrome $\underline{b}_L$ in the complexes isolated from Rb. capsulatus and Rb. sphaeroides, which exhibit a split α-band with two maxima located at 565 to 566 nm and 558 nm, respectively (Gabellini et al., 1982; Gabellini and Hauska, 1983; Andrews et al., 1990; Robertson et al., 1992). In intact R. rubrum membranes the component with E_m = -40 mV that is likely to be cytochrome $\underline{b}_L$ has been demonstrated to have a reduced-minus-oxidized difference

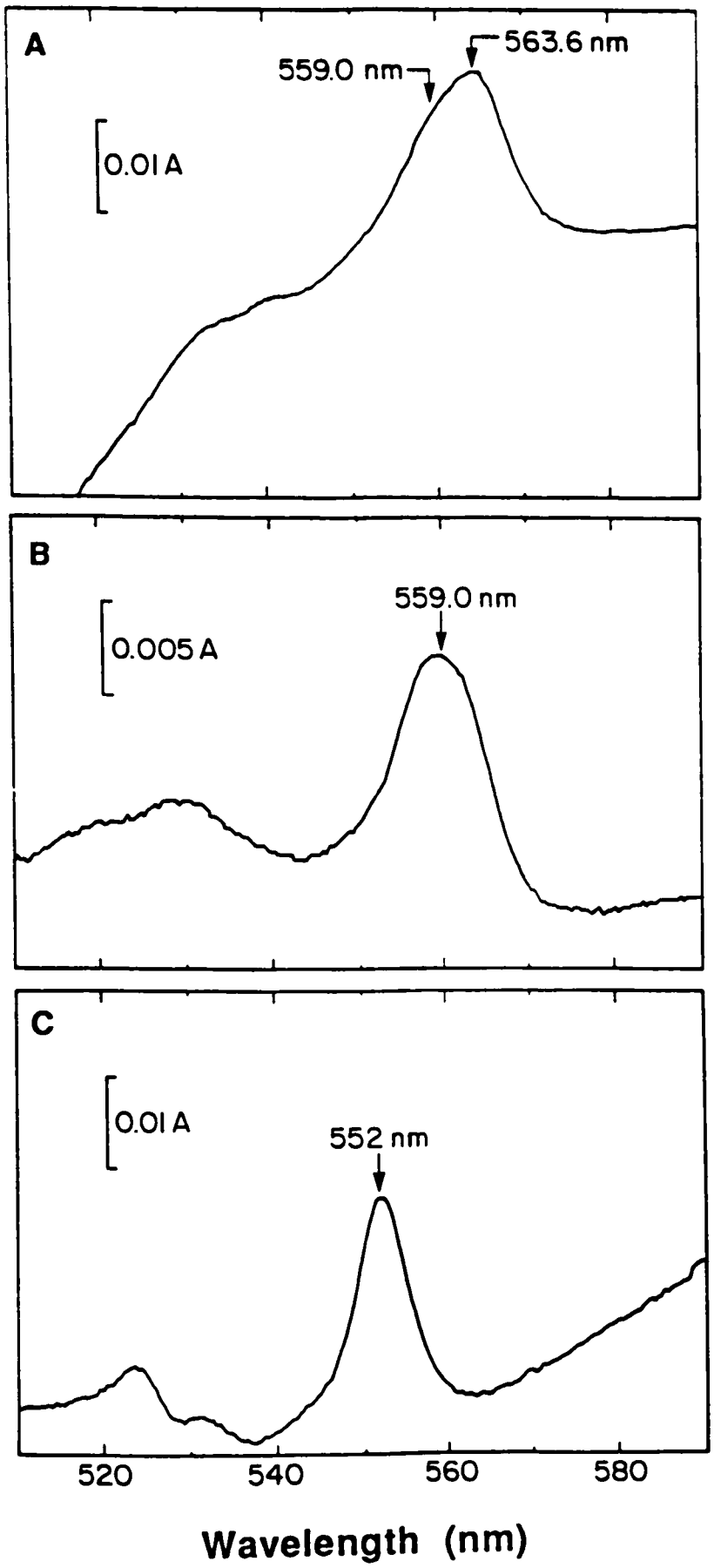

Figure 4.8. Reduced minus oxidized difference spectra for the cytochrome components of the Rhodospirillum rubrum cytochrome bc_1 complex. Reaction conditions were as Fig. 4.6. (A) Cytochrome b_L; (B) cytochrome b_H; (C) cytochrome c_1 (Güner et al., 1991).

spectrum similar to that of Fig. 4.8A, with an α-band maximum at 564 nm and a shoulder at 558 nm (Venturoli et al., 1987). It is thus possible that R. rubrum cytochrome b_L has an α-band reduced-minus-oxidized difference spectrum that differs somewhat from those of this component in other photosynthetic bacteria and does not exhibit two well-separated maxima in the α-band region. The β-band region in the difference spectrum of R. rubrum cytochrome b_L exhibits a broad maximum centered at 552 nm. Reduced-minus-oxidized difference spectra for cytochrome b_L obtained using R. rubrum cytochrome bc_1 complex samples that had been stored frozen were similar to that of Fig. 4.8A except that α-band was slightly broader.

The reduced-minus-oxidized difference spectrum of cytochrome b_H (Fig. 4.8B), obtained by subtracting a spectrum recorded at an ambient potential of +134 mV from one recorded at E_h = -4 mV, shows a single, symmetric α-band centered at 559±1.0 nm, a value similar to that observed for the α-band absorbance maxima of cytochrome b_H in other cytochrome bc_1 complexes (Gabellini et al., 1982; Gabellini and Hauska, 1983; Andrews et al.,1990) and to that observed for the component detected in intact R. rubrum membranes that is likely to be cytochrome b_H (Venturoli et al., 1983). The β-band maximum in the cytochrome b_H difference spectrum is centered at 520 nm. Reduced-minus-oxidized difference spectra for cytochrome b_H, obtained using samples that had been stored frozen, exhibited an α-band centered at 560 nm and the band was somewhat broader than that shown in Fig. 4.8B.

Fig. 4.8C shows the reduced-minus-oxidized difference spectrum in the α- and β-band regions for cytochrome c_1 in the solubilized R. rubrum complex. This difference spectrum was obtained by subtracting a spectrum, measured during the course of one of the oxidation-reduction titrations described above, recorded at an ambient potential of + 392 mV for one recorded at E_h = +187 mV. The α-band maximum, located at 552±1.0 nm, is identical to that previously observed in ascorbate-reduced-minus-ferricyanide-oxidized

difference spectra of the complex (Wynn et al., 1986; Kriauciunas et al., 1989). The c-type cytochrome with E_m = +260 mV, likely to be cytochrome c_1, that has been detected in intact R. rubrum membranes exhibits an α-band maximum at 553 nm (Venturoli et al., 1987), a value that is identical (within the experimental uncertainities) to that observed for cytochrome c_1 in the solubilized complex. The reduced-minus-oxidized difference spectrum contains maxima at 514 and 521 in the β-band region. Storage conditions for the complex had no detectable effect on the reduced-minus-oxidized difference spectrum of cytochrome c_1. The shape of the difference spectrum shown in Fig. 4.8C is also essentially identical to that of the previously reported ascorbate-reduced-minus-ferricyanide-oxidized complex (Wynn et al., 1986; Kriauciunas et al., 1989), indicating that cytochrome c_1 is the only ascorbate-reducible cytochrome in the solubilized R. rubrum complex.

4.1.4. Proton translocation by cytochrome bc_1 complex of Rhodospirillum rubrum

One important property of cytochrome bc_1 complexes in situ is the ability to couple electron flow from quinol to cytochrome c to proton translocation across the membrane in which the complex is located (Hauska et al., 1983; Crofts and Wraight, 1983; Rich, 1984; Dutton, 1986). It has proven possible to demonstrate this property with several detergent-solubilized, purified cytochrome bc_1 complexes, including the beef-heart mitochondrial complex (Leung and Hinkle, 1975) and the three subunit complex isolated from the non-photosynthetic bacterium Paracoccus dentrificans (Yang and Trumpower, 1986), after incorporation of the complexes into liposomes. As mentioned above, it is at least possible in principle that the R. rubrum cytochrome bc_1 complex contains four subunits in situ and the fourth subunit is lost during purification. If the putative fourth subunit were to play some role in vectorial proton translocation by the complex, then the purified complex

would not be expected to couple electron flow to proton translocation. It was thus of interest to determine whether the R. rubrum complex, after incorporation into liposomes, was capable of proton translocation. Before attempting such measurements with the R. rubrum complex, a series of control experiments were conducted using liposomes containing the beef-heart cytochrome bc_1 complex in the Dr. Yu's Laboratory at Oklahoma State University. This system had previously been shown to translocate protons, with the ratio of protons translocated to electrons transferred (H^+/e^-) approaching 2.0 (Leung and Hinkle, 1975).

Fig. 4.9A shows the results of a typical proton ejection that occurs when liposomes containing beef-heart cytochrome bc_1 complex catalyze electron flow from Q_2H_2 to cytochrome c (cytochrome c, originally present in the reduced form, is oxidized by the addition of potassium ferricyanide to initiate the electron flow from Q_2H_2 through the complex). After pH equilibration was reached, the uncoupler CCCP was added to render the liposome membranes freely permeable to protons and a second aliquot of ferricyanide added. The protons released under these conditions are the scalar protons released by the oxidation of Q_2H_2, as no contribution from the accumulation of vectorially translocated protons is possible in the presence of CCCP (Jagendorf, 1975). The ratio of the pH changes produced by the addition of equal amounts of ferricyanide, in the absence and presence of CCCP, was taken as a measure of the H^+/e^- ratio for electron flow through the beef-heart complex. Ratios between 1.6 and 2.0 were routinely observed with liposomes containing the beef-heart complex. The average value of 1.8 is equal, within the experimental uncertainties of the measurements, to the value of 2.0 predicted by the Q-cycle model for electron flow (Crofts and Wraight, 1983; Rich, 1984; Dutton, 1986).

Fig. 4.9B shows the results of a similar proton translocation experiment, conducted with liposomes containing the R. rubrum cytochrome bc_1 instead of the beef-heart complex. The results are qualitatively similar to those shown in Fig. 4.9A, indicating that

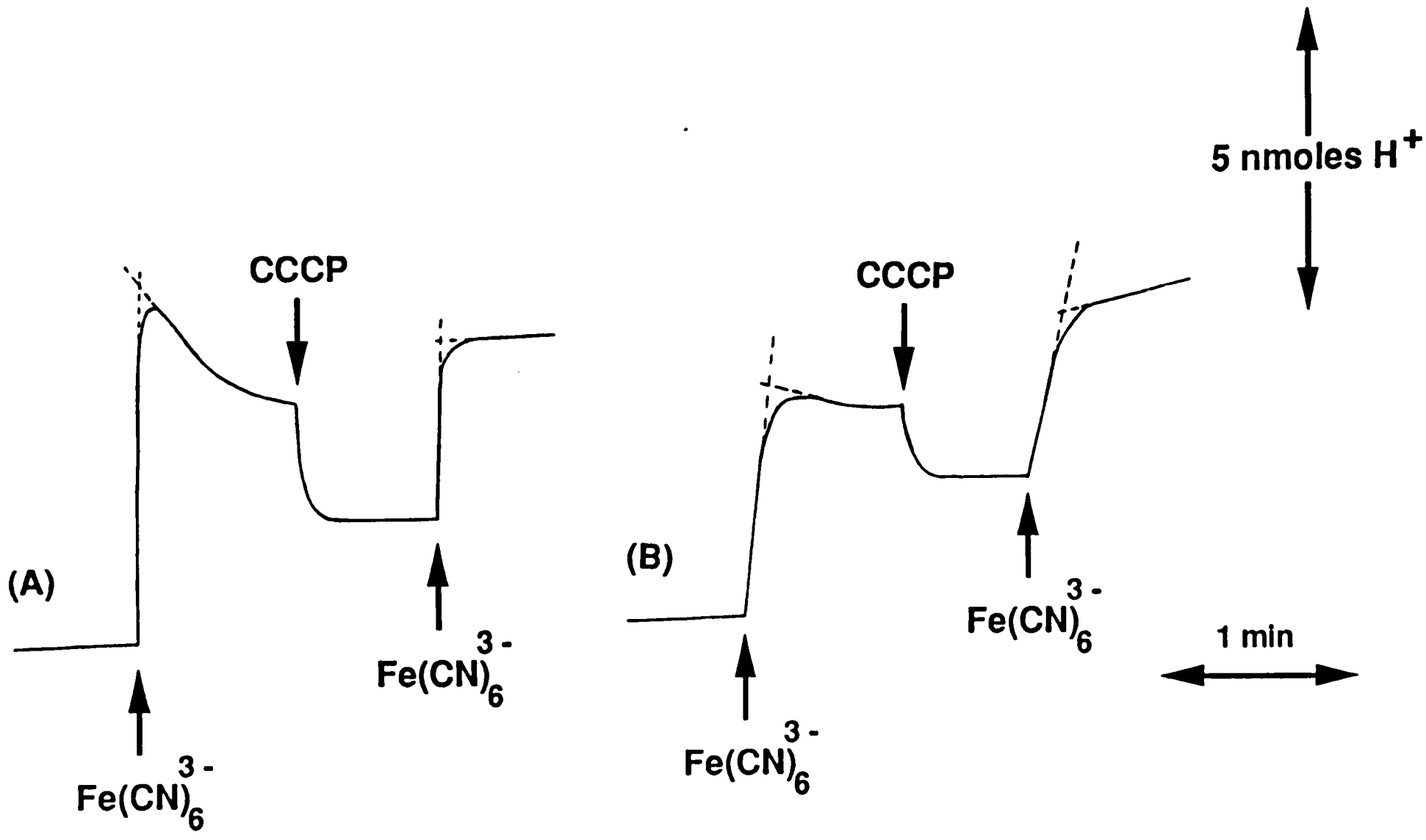

Figure 4.9. Proton translocation by liposomes containing cytochrome bc_1 complexes. Reaction conditions were as described in Materials and Methods. The final liposome concentrations were equivalent to 0.4 mg protein/ ml. (A) Liposomes containing the beef-heart complex; (B) liposomes containing the Rhodospirillum rubrum complex (Güner et al., 1991).

the solubilized, purified R. rubrum complex is capable of proton translocation, but differ quantitatively from those obtained with liposomes containing the beef-heart cytochrome bc_1 complex. In particular, the H^+/e^- ratios obtained with liposomes containing the R. rubrum complex ranged from 1.3 to 1.6, indicating a lower effiency of coupling between proton translocation and electron flow than was observed with liposomes containing the beef-heart complex. Removal of the glycerol in which the R. rubrum complex was stored by precipitating the complex with ammonium sulfate prior to incorporation of the complex into the liposomes did not increase the H^+/e^- ratio observed for the R. rubrum complex. Likewise, initiating the reaction by adding Q_2H_2 to a reaction mixture containing ferricyanide and cytochrome c rather than using the reverse sequence did not increase the H^+/e^- ratio observed for the R. rubrum complex. In fact, the H^+/e^- ratios observed were consistently lower for both liposomes containing the R. rubrum complex and those containing the beef-heart complex when electron flow was initiated by the addition of Q_2H_2 compared to those observed when electron flow was initiated by the addition of ferricyanide. The liposomes containing the R. rubrum cytochrome bc_1 complex also exhibited respiratory control. The rate of electron flow from quinol to cytochrome c observed in continuous electron flow assays (Wynn et al., 1986) was approximately 3-fold higher in the presence of the uncoupler CCCP than that in the absence of an uncoupler (data not shown).

One other difference in the properties of the two types of liposomes, in addition to the difference in H^+/e^- ratios, should be mentioned. As can be seen in Fig. 4.9A, the initial proton ejection observed with liposomes containing the beef-heart complex is followed by a slow proton uptake, presumably due to some leakage of protons back across the liposome membrane. This proton uptake was typically slower and less extensive with liposomes containing the R. rubrum complex (Fig. 4.9B) and in some cases no proton uptake was

observed (data not shown). The reasons for this difference in apparent proton leakage between the two types of liposome are not known.

4.1.5. Discussion

Evidence presented above indicates that the prosthetic groups of the R. rubrum cytochrome bc_1 complex have oxidation-reduction characteristics very similar to those observed with the cytochrome bc_1 complexes isolated from other photosynthetic bacteria. Storage conditions for the complex have effects on both the E_m values and spectral properties of the cytochromes in the complex and on its activity. The cytochrome b and c_1 subunits of the R. rubrum are recognized by antibodies raised against the corresponding subunits of the Rb. capsulatus and Rb. sphaeroides complexes. These results, combined with the earlier observation of antigenic similarities between the Rieske iron-sulfur proteins of R. rubrum and Rb. capsulatus (Kriauciunas et al.,1989), no doubt reflect the structural similarities between the complexes of these three photosynthetic bacteria that could be expected from the amino acid sequence homologies that have been found in their three prosthetic group-containing subunits (Majewski and Trebst, 1990; Shanker et al., 1992).

Results presented above clearly indicate that the solubilized, purified three subunit R. rubrum cytochrome bc_1 complex is sensitive to four specific inhibitors of those complexes at inhibitor concentrations similar to those necessary for inhibition of complexes in other prokaryotic species and eukaryotes. Thus, if the R. rubrum complex were to contain a fourth, 12-14 kDa subunit in situ, this subunit would appear to play no role in binding the inhibitors antimycin A, myxothiazol, stigmatellin and UHDBT. The fact that one can observe the antimycin A-induced red shift in the α-band of cytochrome b_H and stigmatellin-induced changes in the E_m value and EPR g-values of the Rieske iron-sulfur cluster with the isolated R. rubrum complex further suggest that a fourth subunit is not required for normal binding of these quinone analogs.

It has also been clearly established that the solubilized, three subunit R. rubrum cytochrome bc_1 complex, after incorporation into liposomes, is capable of coupling electron flow to the translocation of protons across a membrane and exhibits respiratory control. Thus, the presence of a fourth, 12-14 kDa subunit is not an absolute requirement for proton translocation by the complex. However, the maximum H^+/e^- ratio observed was 1.6, significantly lower than the 2.0 value predicted by the Q-cycle (Crofts and Wraight, 1983; Rich, 1984; Dutton, 1986) and the average H^+/e^- value of 1.5 was lower than the average value of 1.8 observed for liposomes containing the beef-heart complex. It should be mentioned that kinetic studies conducted with R. rubrum chromatophores support the likely operation of a Q-cycle pathway during light-driven cyclic electron flow (van der Wal and van Grondelle, 1983). The reason for suboptimal proton translocation by liposomes reconstituted with the R. rubrum complex is not clear, but it cannot be attributed to the presence of different liposome populations with opposite orientations of the cytochrome bc_1 complex with respect to the lipid membrane. As the electron acceptor used in the assay, cytochrome c, cannot cross the lipid membrane of the liposome, only those liposomes containing R. rubrum complex with cytochrome c/c_2-binding site facing out towards the external medium will be active in electron transfer and proton translocation. The ferricyanide anion, due to its substantial negative charge, would also be expected to be unable to cross the liposome membrane. Liposomes, if any present, containing cytochrome bc_1 complexes with orientations other than that resulting in proton ejection, cannot produce proton translocation in the opposite direction that would diminish the H^+/e^- ratio, as such liposomes will not catalyze electron flow due to the absence of an accesible electron acceptor.

In summary, it seems clear that the three peptide subunit R. rubrum cytochrome bc_1 complex is capable of catalyzing electron flow from quinol to cytochrome c/c_2 at high rates and that the complex exhibits high affinity, normal binding of four specific, quinone analog

inhibitors of cytochrome bc_1 complexes. Previous observations of the interaction of a ubiquinone photoaffinity analog with the R. rubrum complex also indicated that a fourth subunit is not required for binding of the analog to cytochrome b (Kriauciunas et al., 1989). Thus neither quinol:cytochrome c/c_2 activity of the cytochrome bc_1 nor the interaction of quinone with the complex appears to require any components other than the three prosthetic group-containing subunits of the complex in R. rubrum. A similar situation appears to be true for the Rb. capsulatus cytochrome bc_1 complex (Robertson et al., 1992).

The three subunit R. rubrum complex is also capable of coupling electron transfer to proton translocation, albeit with a H^+/e^- ratio less than the value of 2.0 predicted by Q-cycle models (Crofts and Wraight, 1983; Rich, 1984; Dutton, 1986) and observed for the beef-heart (see Fig. 4.9A). Thus it is possible that, if the R. rubrum complex does contain a fourth subunit in situ, then this subunit could perhaps play some role in optimal proton translocation. It is also possible that the fourth subunit, if one exists, is involved in some still unrecognized function. However, such speculations must await the appearance of evidence for the existence of such a subunit in R. rubrum. At the present, there is no evidence for the presence of a fourth subunit in the R. rubrum cytochrome bc_1 complex, nor does evidence for the presence of more than three subunits in the corresponding complex isolated from Rps. viridis (Wynn et al., 1986; Gabellini, 1988; Cully et al., 1989). The situation for the complex isolated from Rb. capsulatus is less clear. Earlier reports (Hauska et al.,1983; Ljungdahl et al., 1987), based on the appearance of four protein-staining bands after electrophoresis under denaturing conditions, concluded that four discrete subunits were present in the Rb. capsulatus complex. Recent evidence, based on recognition by monoclonal antibodies and on peptide sequencing, has revealed that the isolated Rb. capsulatus complex, in fact, contains only three subunits (Robertson et al., 1992). Should the R. rubrum, Rps. viridis and Rb. capsulatus cytochrome bc_1 complexes

turn out to contain only three peptide subunits in situ and the fourth subunit found associated with preparations of the detergent-solubilized Rb. sphaeroides complex (Yu et al., 1984; Ljungdahl et al.,1987; Andrews et al., 1990; Purvis et al., 1990) prove to be a bona fide constituent of the complex and not a fortuitous contaminant, an interesting case of species differences in this energy-transducing electron transfer complex will exist in closely related bacteria.

4.2. Interaction between cytochrome c_2 and the cytochrome bc_1 complexes of Rhodobacter capsulatus and Rhodopseudomonas viridis

4.2.1. Co-migration and ultrafiltration assays

Binding between the cytochrome bc_1 complex of Rb. capsulatus and cytochrome c_2 was measured by gel-filtration chromatography on Biogel P-100 at either 10 or 310 mM ionic strength as shown in Fig. 4.10. Equimolar amounts of the cytochrome bc_1 complex and cytochrome c_2 were used in each run. At high ionic strength, cytochrome c_2 and the cytochrome bc_1 complex eluted separately at positions expected from the differences in their molecular masses. However at low ionic strength, cytochrome c_2 co-migrated with the cytochrome bc_1 complex and no free cytochrome c_2 was detected. The cytochrome containing fractions obtained at low ionic strength were completely retained by a YM-30 ultrafiltration membrane but when the ionic strength was increased to 0.31 M, most of the cytochrome c_2 but none of the cytochrome bc_1 complex was found in the filtrate. Experiments in which equine cytochrome c replaced Rb. capsulatus cytochrome c_2 showed the same pattern at both high and low ionic strengths, indicating the cytochrome c, like cytochrome c_2, forms an electrostatically stabilized complex with the Rb. capsulatus cytochrome bc_1 complex. Additional evidence for complex formation was obtained from ultrafiltration experiments. Although control experiments showed that M_r = 30 kDa Rb.

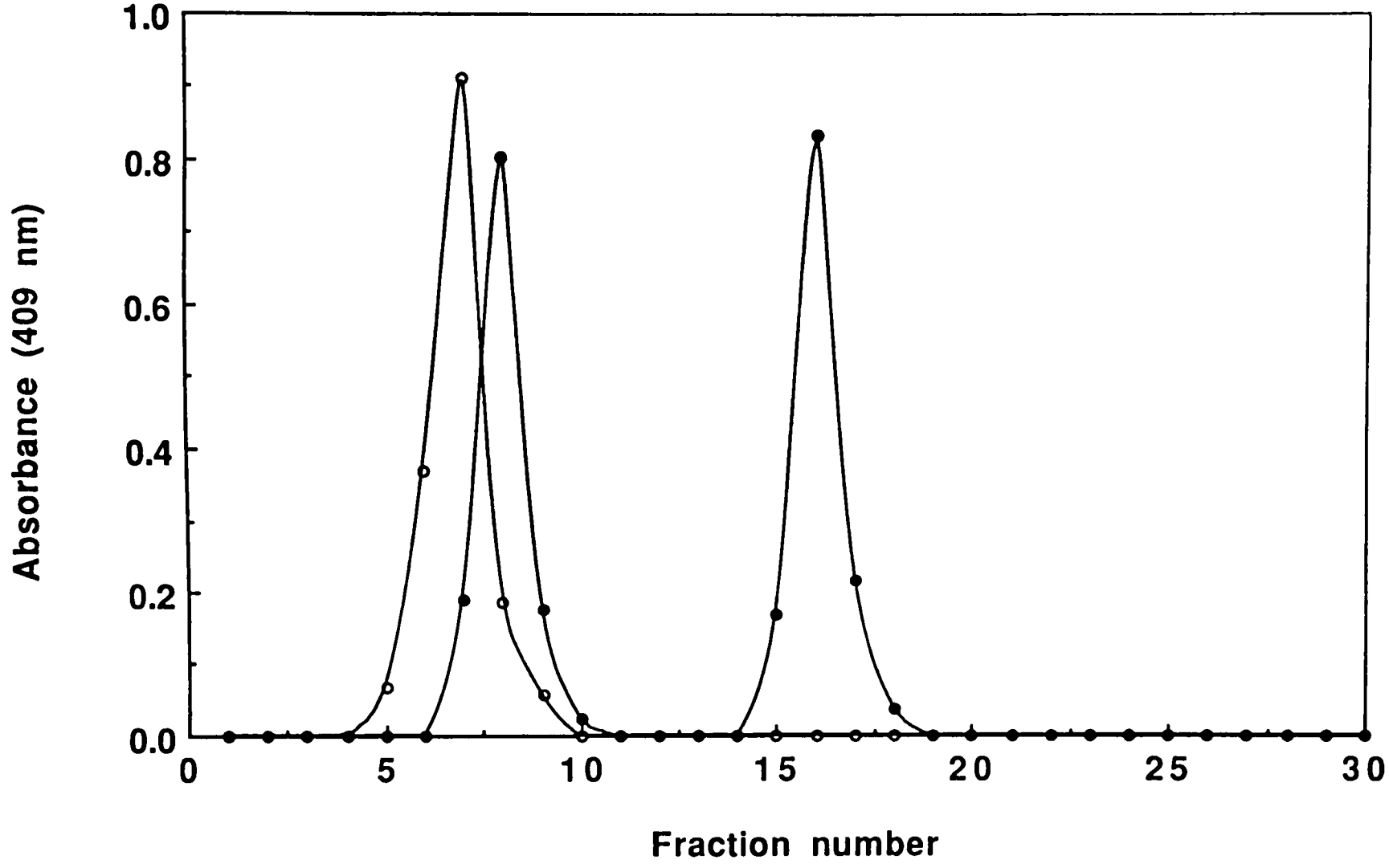

Figure 4.10. Complex formation between Rhodobacter capsulatus cytochrome c_2 and the Rb. capsulatus cytochrome bc_1 complex. Equimolar amounts (10 nmoles) of each protein in a total volume of 0.5 ml were chromatographed at low ionic strength (open circles) and high ionic strength (closed circles) on a Biogel P-100 column, as described in Materials and Methods.

capsulatus cytochrome c_2 passes freely through the M_r = 30 kDa cut-off filter, an equimolar mixture of the cytochrome bc_1 complex and cytochrome c_2 at low ionic strength is completely retained by the ultrafiltration membrane, with no detectable cytochrome c_2 appearing in the filtrate. This is as expected if all of the Rb. capsulatus cytochrome c_2 is tightly bound to the Rb. capsulatus cytochrome bc_1 complex ($M_r \approx 100$ kDa). However, if the ionic strength was raised to 0.31 M, > 85 % of the cytochrome c_2, but none of the cytochrome bc_1 complex, was recovered in the filtrate. This is exactly the predicted result if the complex between cytochrome c_2 and the cytochrome bc_1 complex is stabilized by electrostatic forces that weaken at high ionic strength.

4.2.2. Kinetics of reduction of cytochrome c_2 by the cytochrome bc_1 complexes of Rhodobacter capsulatus and Rhodopseudomonas viridis

The kinetics of electron transfer from DBH to Rb. capsulatus cytochrome c_2 catalyzed by the Rb. capsulatus and Rps. viridis cytochrome bc_1 complexes obeyed Michaelis-Menten kinetics at all ionic strengths examined. Varying the ionic strength from 0.25 to 1 M had little or no effect on V_{max} but K_m increased significantly with increasing ionic strength. Fig. 4.11 and Fig. 4.12 summarize the variation of V/K with ionic strength observed in these experiments. Similar dependencies have been observed previously for R. rubrum (Hall et al., 1987b) and Rb. sphaeroides (Hall et al., 1987d; Hall et al., 1989). The effects appear to be entirely due to changes in ionic strength rather than to specific ion effects, as similar results were obtained using KNO_3, $MgCl_2$ and Na_2SO_4 (data not shown).

The ionic strength dependence of the reaction rates could be used to analyze the electrostatic interactions in terms of the number, n, of charged pairs involved in the interaction between cytochrome c_2 and the cytochrome bc_1 complex. The data were fitted

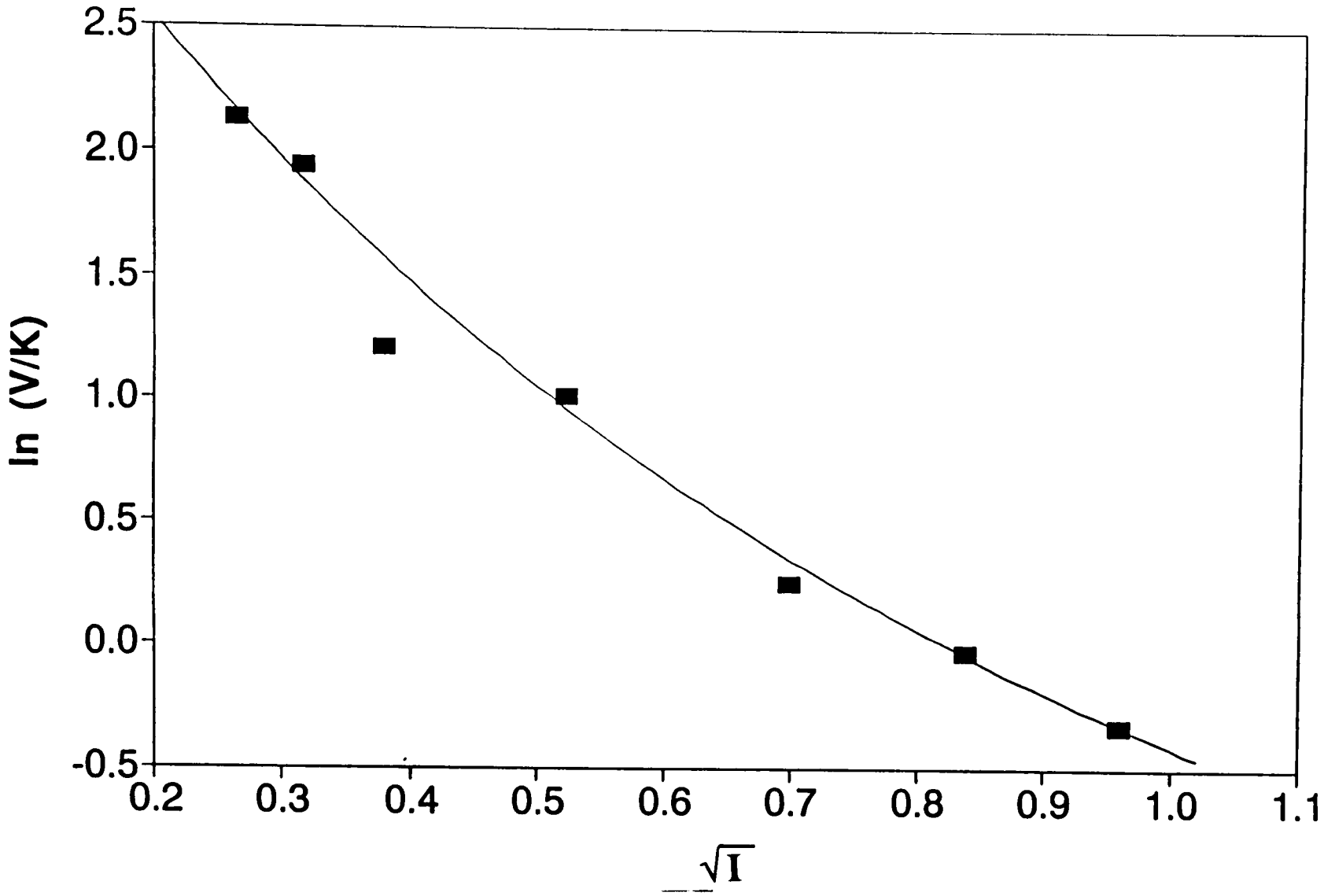

Figure 4.11. Ionic strength dependence of the kinetic parameters for the reduction of Rhodobacter capsulatus cytochrome c_2 catalyzed by the Rb. capsulatus cytochrome bc_1 complex. V_{max}/K_m is reported in units of min^{-1}. The assays were carried out in 50 mM phosphate buffer, pH 7.40, with 0-1 M NaCl. The solid line was obtained by fitting the data to an electrostatic pair model (Stonehuerner et al., 1979) using a Quattro spreadsheet program with r = 4, n = 3.5 and $\ln (V_{max}/K_m)_{inf} = -2.3$ for cytochrome c_2.

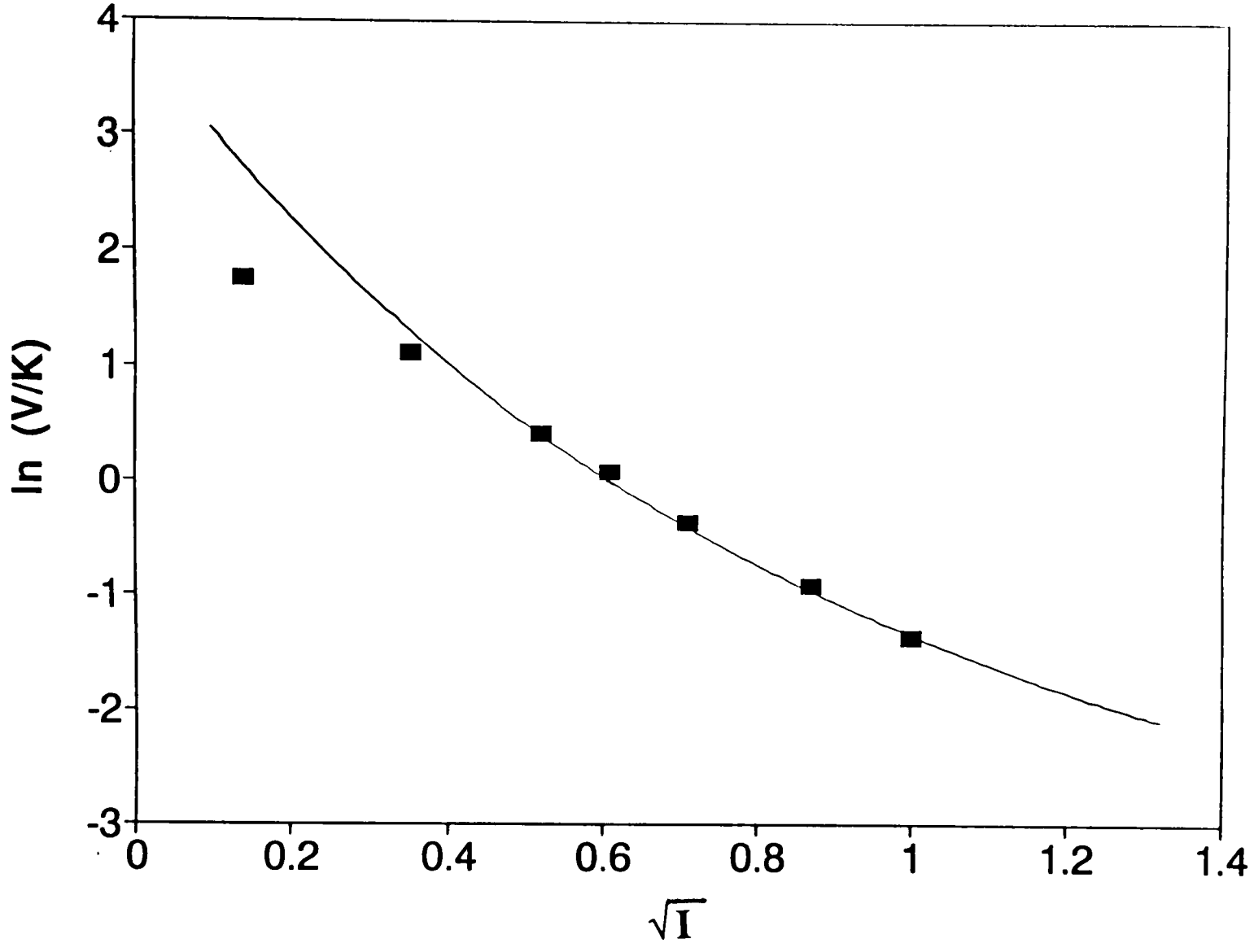

Figure 4.12. Ionic strength dependence of the kinetic parameters for the reduction of Rhodopseudomonas viridis cytochrome c_2 catalyzed by the Rps. viridis cytochrome bc_1 complex. The assay conditions were as in Fig. 4.11. The best fit was obtained with r = 3.5, n = 4 and ln $(V_{max}/K_m)_{inf}$ = -4.25 for cytochrome c_2.

to a semiempirical equation (4.1) designed to analyze such interactions (Stonehuerner et al., 1979). The best fit was obtained with n = 3.5, r = 4 Å and n = 4, r = 3.5 Å for the reactions between cytochrome $\underline{c}_2$ and cytochrome $\underline{bc}_1$ complexes from Rb. capsulatus and Rps. viridis, respectively. Similar analysis of kinetic data for the reactions catalyzed by the cytochrome $\underline{bc}_1$ complexes of R. rubrum (Hall et al., 1987b) and Rb. sphaeroides (Hall et al., 1987d; Hall et al., 1989) gave n values of 5 and 8, respectively.

$$\ln (k/k_{\infty}) = \frac{7.152\, n\, e^{K (a - r)}}{(1 + K a)\, r} \qquad (4.1)$$

where

a = effective radius of interacting pairs,

r = distance between the amino and carboxylate groups of interacting pairs,

n = number of interacting pairs,

k = rate constant,

k_{∞} = rate constant at infinite ionic strength,

$K = 0.329 \sqrt{I}$,

I = ionic strength.

Having established the importance of electrostatic forces in the interaction of Rb. capsulatus cytochrome $\underline{c}_2$ with the Rb. capsulatus cytochrome $\underline{bc}_1$ complex, experiments were designed to test the hypothesis (Ambler et al., 1979), that conserved lysine residues located near the exposed heme edge of cytochrome $\underline{c}_2$ (Benning et al., 1991) contribute the positive charges involved in complex formation. Three lysine residues, K12, 14 and 32 (Fig. 4.13), all of which are highly conserved among c-type cytochromes (Ambler et al., 1979) were changed to either aspartate or glutamate by site-directed mutagenesis (Caffrey et

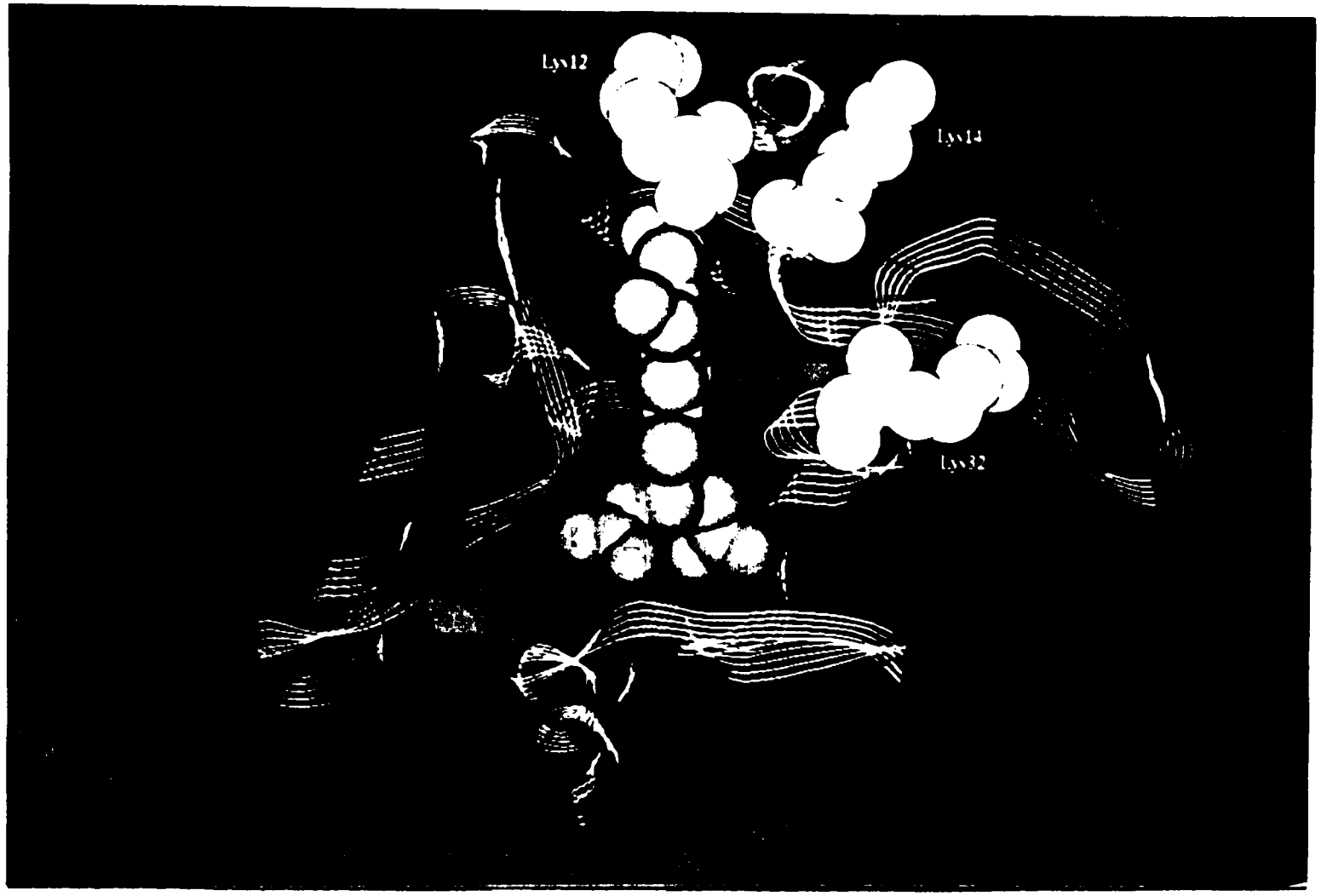

Figure 4.13. Ribbon structure of Rhodobacter capsulatus cytochrome c_2. The heme group is shown edge-on. Lysine residues that have been altered by mutagenesis are indicated in white by amino acid sequence number (Cytochrome c_2 co-ordinates from the Brookhaven Protein Data Bank).

al., 1992b). Fig. 4.14 shows the effect of these mutations; K12D, K14E, K32E or K14E/K32E, of cytochrome $\underline{c}_2$ of Rb. capsulatus on the kinetic parameters associated with the activity of the Rb. capsulatus cytochrome $\underline{bc}_1$ complex. The reduction of these cytochrome $\underline{c}_2$ mutants, like that of the wild-type cytochrome, obey Michaelis-Menten kinetics. Replacing the positive charge normally contributed by these lysines with negative charges had virtiually no effect on V_{max} but significantly increased K_m (Table 4.1).

Equine cytochrome $\underline{c}$ shows significant sequence homology to Rb. capsulatus cytochrome $\underline{c}_2$ (Ambler et al., 1979) and serves an effective acceptor of electrons from the cytochrome $\underline{bc}_1$ complexes of R. rubrum (Hall et al., 1987b) and Rb. sphaeroides (Hall et al., 1987d; Hall et al., 1989). Derivatives of cytochrome $\underline{c}$ are available in which chemical modification of specific lysine residues eliminates the positive charge normally present and replaces it with an uncharged side chain derivative. Trifluoromethylphenylcarbamoylation of cytochrome $\underline{c}$ at lysines 13 and 72 have previously been found to have significant effects on the reduction of cytochrome $\underline{c}$ by the cytochrome $\underline{bc}_1$ complexes of photosynthetic bacteria (Hall et al., 1987b; Hall et al., 1987d; Hall et al., 1989) and mitochondria (Ahmed et al., 1978). Fig. 4.15 shows that eliminating the positive charge on either of these lysine residues, both of which are located near the exposed heme edge of equine cytochrome $\underline{c}$ (Fig. 4.16), has little effect on V_{max} but resulted in significant increases in K_m (Table 4.2).

4.2.3. Discussion

Affinity chromatography of cytochrome $\underline{bc}_1$ complexes from the photosynthetic bacteria R. rubrum and Rps. viridis on cytochrome $\underline{c}$-coupled Sepharose provided early evidence for electrostatically stabilized complex formation between the cytochrome $\underline{bc}_1$ complex and cytochrome $\underline{c}_2$ of these bacteria. In the case of R. rubrum, co-chromatography and differential chemical modification experiments have provided additional evidence for complex formation (Bosshard et al., 1987). Analysis of kinetic data

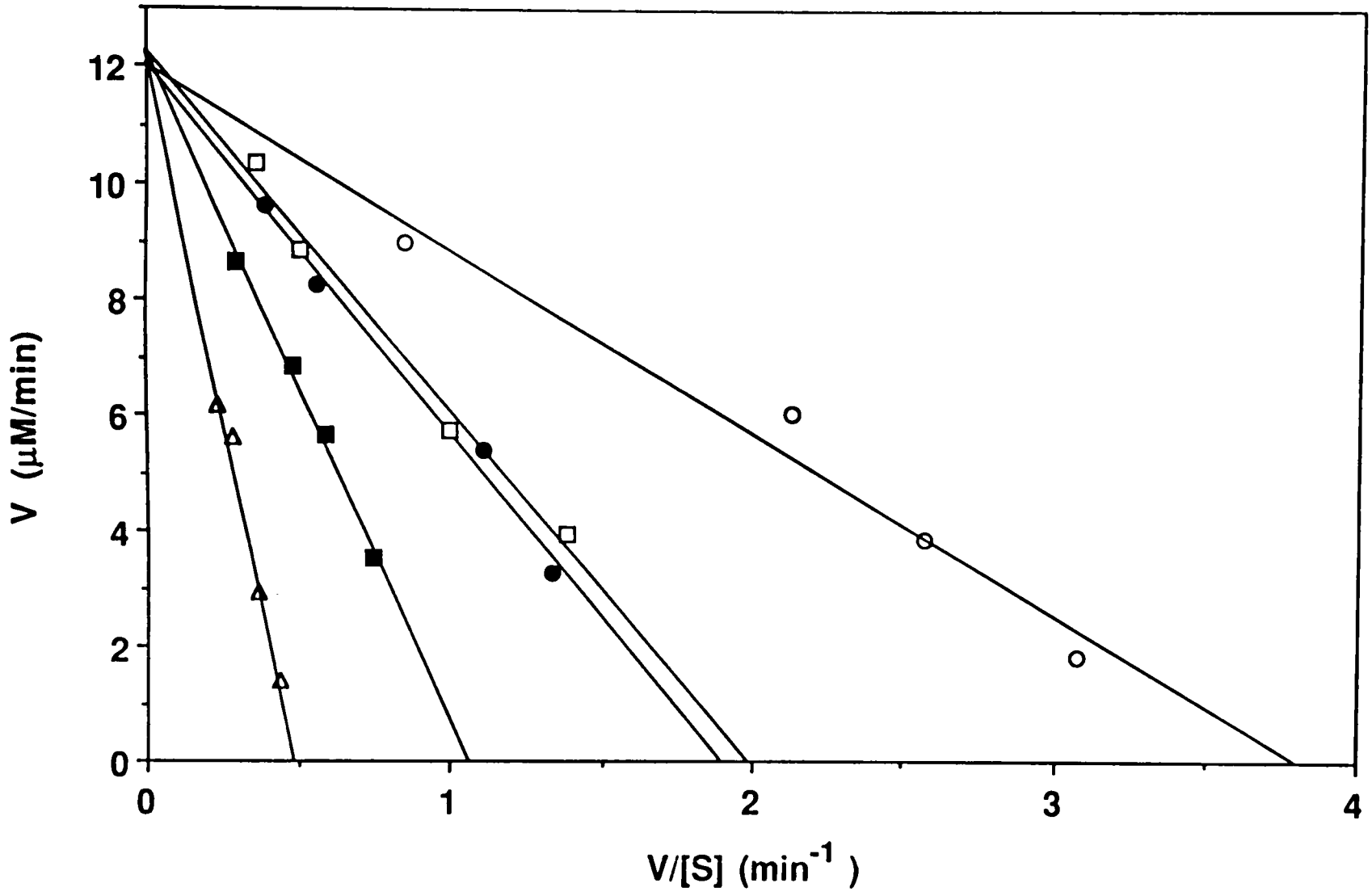

Figure 4.14. Steady state kinetics for the reduction of wild type and mutated Rhodobacter capsulatus cytochrome c_2 catalyzed by the Rb. capsulatus cytochrome bc_1 complex. Native cytochrome c_2 (o), K12D cytochrome c_2 (●), K14E cytochrome c_2 (□), K32E cytochrome c_2 (■), K14E/K32E cytochrome c_2 (Δ). See Materials and Methods for details.

Table 4.1. Effect of specific lysine mutations on the kinetic parameters for the reduction of cytochrome $\underline{c}_2$ catalyzed by the cytochrome $\underline{bc}_1$ complex of Rhodobacter capsulatus. V_{max} is reported in μM/min, K_m is in μM, and experimental error is expressed as standard deviation from the mean of two data sets.

Condition	V_{max}	K_m
Native cytochrome $\underline{c}_2$	12.0 ± 0.3	3.17 ± 0.36
K12D cytochrome $\underline{c}_2$	12.1 ± 0.4	6.37 ± 0.77
K14E cytochrome $\underline{c}_2$	12.3 ± 0.4	6.17 ± 0.81
K32E cytochrome $\underline{c}_2$	12.3 ± 0.8	11.6 ± 1.6
K14E/K32E cytochrome $\underline{c}_2$	12.2 ± 1.1	25.2 ± 2.7

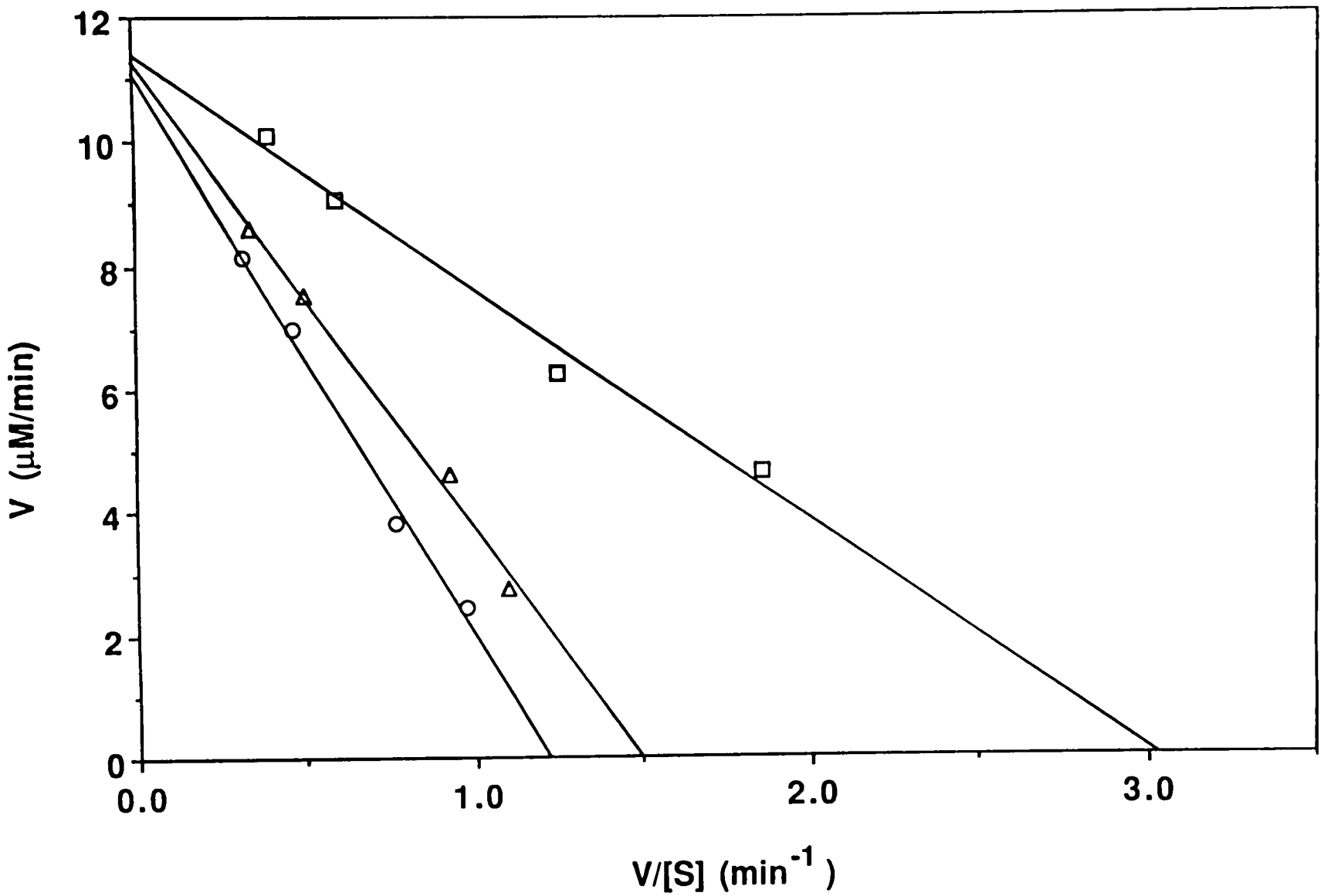

Figure 4.15. Steady state kinetics for the reduction of native and lysine-modified derivatives of equine cytochrome c catalyzed by the Rhodobacter capsulatus cytochrome bc_1 complex. Native cytochrome c (□), TFC-lys-13 cytochrome c (Δ), TFC-lys-72 cytochrome c (○). See Materials and Methods for details.

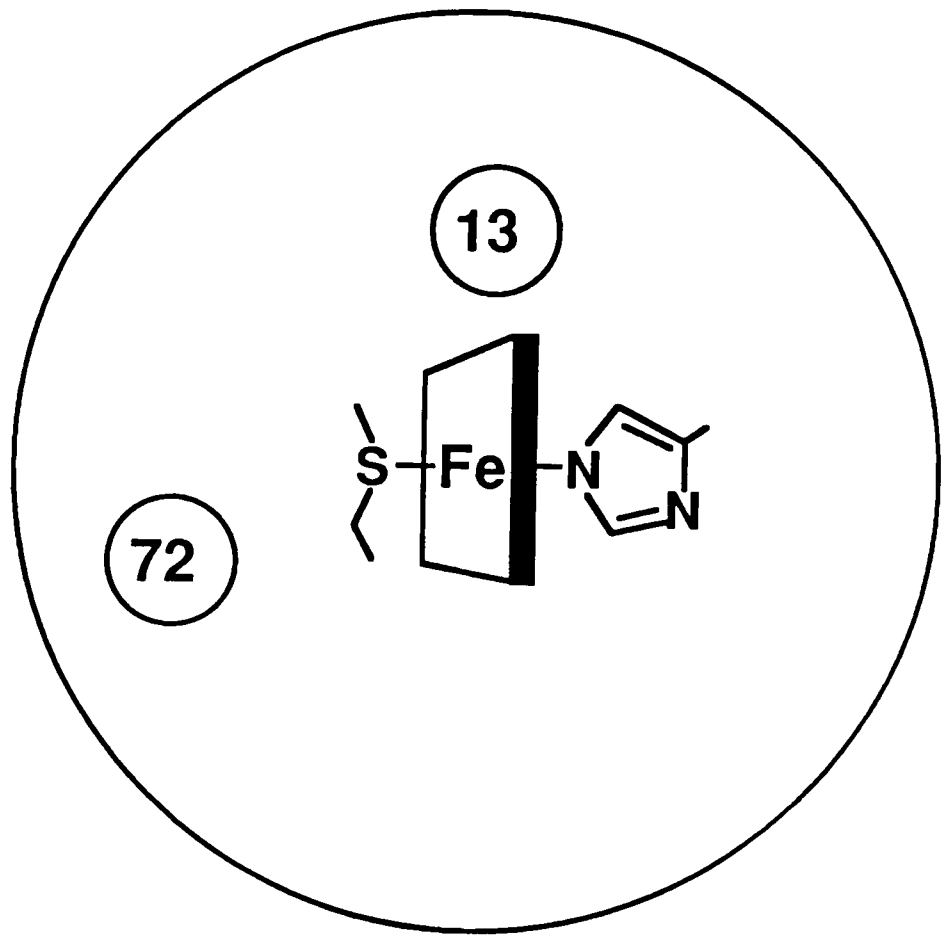

Figure 4.16. A schematic diagram of equine cytochrome c. Lysine residues that have been chemically modified are indicated by amino acid sequence number in small circles.

Table 4.2. Effect of specific lysine modifications on the kinetic parameters for the reduction of equine cytochrome c catalyzed by the Rhodobacter capsulatus cytochrome bc_1 complex. V_{max} is reported in μM/min, K_m is in μM and experimental error is expressed as standard deviation from the mean of two data sets.

Condition	V_{max}	K_m
Native cytochrome c	11.4 ± 0.7	3.75 ± 0.23
TFC-lys-13 cytochrome c	11.3 ± 0.5	7.50 ± 0.74
TFC-lys-72 cytochrome c	11.1 ± 1.3	9.09 ± 0.62

obtained from investigations of the reduction of cytochrome $\underline{c}_2$ catalyzed by the cytochrome $\underline{bc}_1$ complexes of R. rubrum and Rb. sphaeroides have provided substantial evidence for the existence of an electrostatically stabilized complex between these two reaction partners. These studies have also provided considerable evidence for the involvement of conserved lysine residues located at the exposed heme edge on the cytochrome $\underline{c}_2$ of photosynthetic bacteria in complex formation with the cytochrome $\underline{bc}_1$ complexes of these two bacteria (Hall et al., 1987b; Hall et al., 1987d; Hall et al., 1989). It has been found that these conserved lysine residues also play similar role in docking the photosynthetic reaction center in photosynthetic bacteria (van der Wal et al., 1987; Hall et al., 1987a).

It has been demonstrated that cytochrome $\underline{c}_1$ subunit of the cytochrome $\underline{bc}_1$ complex is the direct reductant for cytochrome $\underline{c}_2$ in photosynthetic bacteria (Meinhardt and Crofts, 1982; Snozzi and Crofts, 1985). A considerable amount of evidence suggests that cytochrome $\underline{c}_1$ provides the negative charges necessary for electrostatic binding to positively charged lysine residues on mitochondrial cytochrome $\underline{c}$ (Margoliash and Bosshard, 1983) which is structurally related to cytochrome $\underline{c}_2$ (Ambler et al., 1979; Dickerson, 1980; Meyer and Kamen , 1982). Observations on the interaction between the cytochrome $\underline{bc}_1$ complex and cytochrome $\underline{c}$ of mitochondria implicate two acidic regions on mitochondrial cytochrome $\underline{c}_1$ in binding cytochrome $\underline{c}$. One of these two regions is well conserved in all four photosynthetic bacterial species for which cytochrome $\underline{c}_1$ sequences are available, while the other region is conserved in some but not all of these photosynthetic bacteria (Gabellini and Sebald, 1986; Davidson and Daldal, 1987b; Verbist et al., 1989; Majewski and Trebst, 1990; Yun et al., 1990).

Observations in several laboratories have demonstrated that as the ionic strength increased, the affinity of the cytochrome $\underline{bc}_1$ complex for cytochrome $\underline{c}_2$ and the rate of electron flow from quinol to cytochrome $\underline{c}_2$ catalyzed by the cytochrome $\underline{bc}_1$ complex decreased substantially (Bosshard et al., 1987; Hall et al., 1987b; Hall et al., 1997d; Hall et

al., 1989). The ionic strength dependency of these reactions has been related to the number of charged pairs in electrostatic interaction of these two proteins utilizing a semiempirical relationship developed by Stonehuerner et al. (1979) and Smith et al. (1981). The data obtained for complexes from R. rubrum and Rb. sphaeroides have been found to be consistent with this theoretical relationship (Hall et al., 1987b; Hall et al., 1987d) and with the negative net charge present in the acidic regions which thought to be involved in electrostatic interaction with cytochrome c_2.

In this part of our work, the earlier studies on the interaction between cytochrome bc_1 complexes and cytochrome c_2/c in Rb. sphaeroides and R. rubrum have been extended to Rb. capsulatus and Rps. viridis. Ultrafiltration and co-migration chromatography experiments for Rb. capsulatus (see above) strongly support the idea that electrostatically-stabilized complex formation also occurs between cytochrome c_2 and the cytochrome bc_1 complex in this bacterium. The effect of ionic strength on the kinetics of the reactions catalyzed by the cytochrome bc_1 complexes of Rb. capsulatus and Rps. viridis supports the idea that electrostatic interactions between cytochrome c_2 and the cytochrome bc_1 complex are important in these two bacteria.

Fitting the data to the electrostatic theory used previously to analyze the Rb. sphaeroides and R. rubrum data (Hall et al., 1987b; Hall et al., 1987d), gave a best fit with $n = 3.5$ and $n = 4$ for the number of the charged pairs involved in the interaction between cytochrome bc_1 complexes and cytochrome c_2 of Rb. capsulatus and Rps. viridis, respectively (Table 4.3). The fact that two values are so similar despite the considerable differences in negative charge in one of the regions proposed to be involved in cytochrome c_2 binding, raises the possibility that this site may in fact not play a role.

To examine the interaction site on cytochrome c_2 and equine cytochrome c for the cytochrome bc_1 complex, we have used four mutants of cytochrome c_2 of Rb. capsulatus in which specific lysine residues were altered by site-directed mutagenesis and

Table 4.3. Comparison of the number of interacting pairs (n) in the interaction of cytochrome bc_1 complexes and cytochrome c_2 of purple non-sulfur photosynthetic bacteria and of the net negative charge on the region I and II of cytochrome c_1.

Bacterium	n	Net charge on c_1		Reference
		Region I	Region II	
Rhodospirillum rubrum	5	-3	-4	Hall et al., 1987b
Rhodobacter sphaeroides	8	-5	-4	Hall et al., 1987d
Rhodobacter capsulatus	3.5	-4	-4	This work
Rhodopseudomonas viridis	4	0	-2	This work

threefluoromethylphenylcarbamoylated equine cytochrome c in which specific lysine residues were chemically modified. Mutation of cytochrome c_2 lysines at positions 12,14, or 32 (Fig. 4.13) and modification of cytochrome c lysines at positions 13 or 72 (Fig. 4.16), all of which are expected to be involved in the electrostatic interaction of cytochromes c_2 and c_1, markedly increased the K_m values for these two cytochromes as shown in Figs. 4.14 and 4.15, indicating the importance of these specific lysine residues located at the exposed heme edge of the cytochrome c_2 of Rb. capsulatus and equine cytochrome c in interacting with the cytochrome bc_1 complex.

4.3. Resonance Raman spectroscopy

In this part of the study, characterization of the heme electron transfer sites of the complex in various stages of reduction by resonance Raman spectroscopy is demonstrated. This is possible due to the differences in midpoint potentials among the three hemes present. The measured E_m values (pH 7.40) are: cytochrome c_1, +280 mV, cytochrome b_H, +20 mV, and cytochrome b_L, -85 mV (see section 4.1.3.2.). The distinctive redox potentials of the hemes allow for the specific reduction of the higher potential sites by either the addition of stochiometric amounts of a strong reductant or the use of redox mediators having appropriate reduction potentials. Further, because the Raman intensity of normal modes of vibration is greatly enhanced when the excitation frequency of the laser light source is tuned to a strongly allowed electronic transition (Spiro, 1983), such as the Soret or Q bands observed in hemes, the differences in the absorption bands of the b- and c-type hemes present may be exploited in differentiating their relative contributions to the Raman spectrum.

4.3.1. Fully oxidized and fully reduced complexes

Soret excitation (406-430 nm) preferentially enhances scattering from polarized and depolarized heme bands corresponding primarily to in-plane porphyrin ring vibrations (Spiro and Li, 1988). These skeletal modes are numbered based on calculated and assigned normal-mode frequencies for model compounds (Kitagawa et al., 1978; Abe et al.,1978). These bands are most prominent in the high-frequency (1100-1700 cm^{-1}) region of the spectrum and are highly reliable indicators of the heme environment, especially the oxidation and spin-state of the iron ion (Spiro and Strekas, 1974; Spiro et al.,1979).

Fig. 4.17 shows the high-frequency spectra obtained with 406 nm excitation of the complex as isolated and the sodium ascorbate reduced cytochrome $\underline{bc}_1$ complex. Sodium ascorbate is a relatively mild reductant and should only reduce heme components having E_m values greater than approximately +50 mV. Raman spectra in Fig. 4.17 are consistent with reduction of only heme $\underline{c}_1$, as evidenced by ν_4 at 1360 cm^{-1}. The presence of oxidized heme $\underline{b}$ is observed by ν_4 intensity at 1371 cm^{-1}. Absorption spectra (Fig. 4.18) corroborate that ascorbate reduces only the $\underline{c}$-type heme as reported earlier by Kriauciunas et al. (1989). The close correspondence between the two spectra indicates that, in the freshly prepared complex, only the heme $\underline{c}_1$ is reduced.

The effects of two other redox reagents, dithionite and ferricyanide, upon high-frequency Raman spectrum of the cytochrome $\underline{bc}_1$ complex are shown in Fig. 4.19. Addition of ferricyanide (E_m = +430 mV) to the solution completely oxidizes all heme sites in the cytochrome $\underline{bc}_1$ complex. Raman bands ν_4 (1372 cm^{-1}), ν_3 (1505 cm^{-1}) and ν_2 (1581 cm^{-1}) appear (Fig. 4.19a) at frequencies that are characteristic of low-spin ferric hemes. Small additions of sodium dithionite produce changes in the Raman spectrum indicative of formation of low-spin ferrous hemes (Fig. 4.19b).

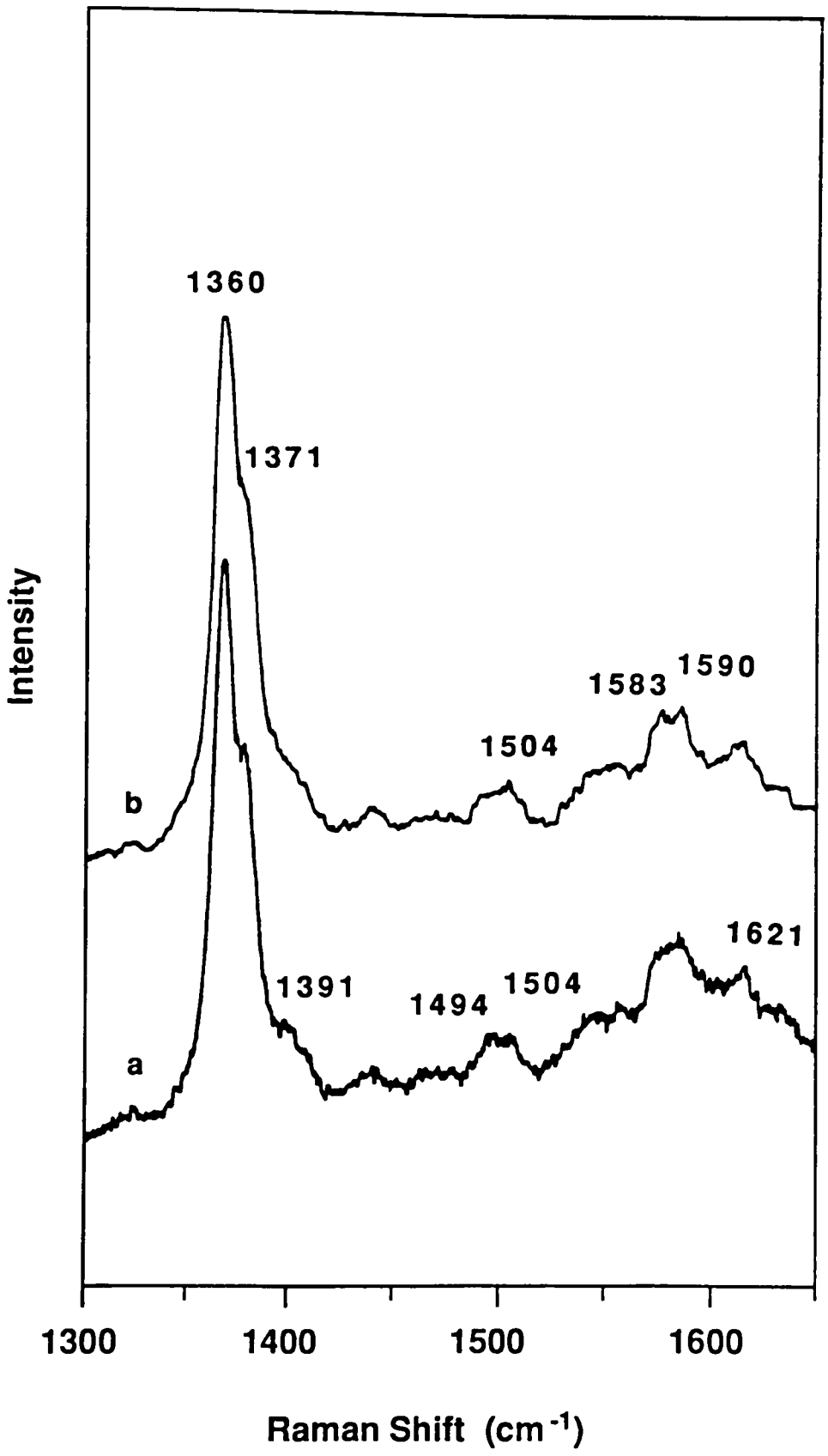

Figure 4.17. High-frequency resonance Raman spectra of the cytochrome $\underline{bc}_1$ complex from Rhodospirillum rubrum (a) as prepared and (b) sodium ascorbate reduced. All samples were approximately 50 μM cytochrome $\underline{bc}_1$ complex in 35 mM MOPS buffer, pH 7.40, 1 mM $MgSO_4$, 0.1 mg dodecylmaltoside per ml and 5 % (v/v) glycerol. Spectra are the unsmoothed sum of three scans with an average laser power of approximately 5 mW at 15 Hz using 406 nm excitations. The spectral bandpass was 5-8 cm^{-1} for all spectra (Hobbs et al., 1990).

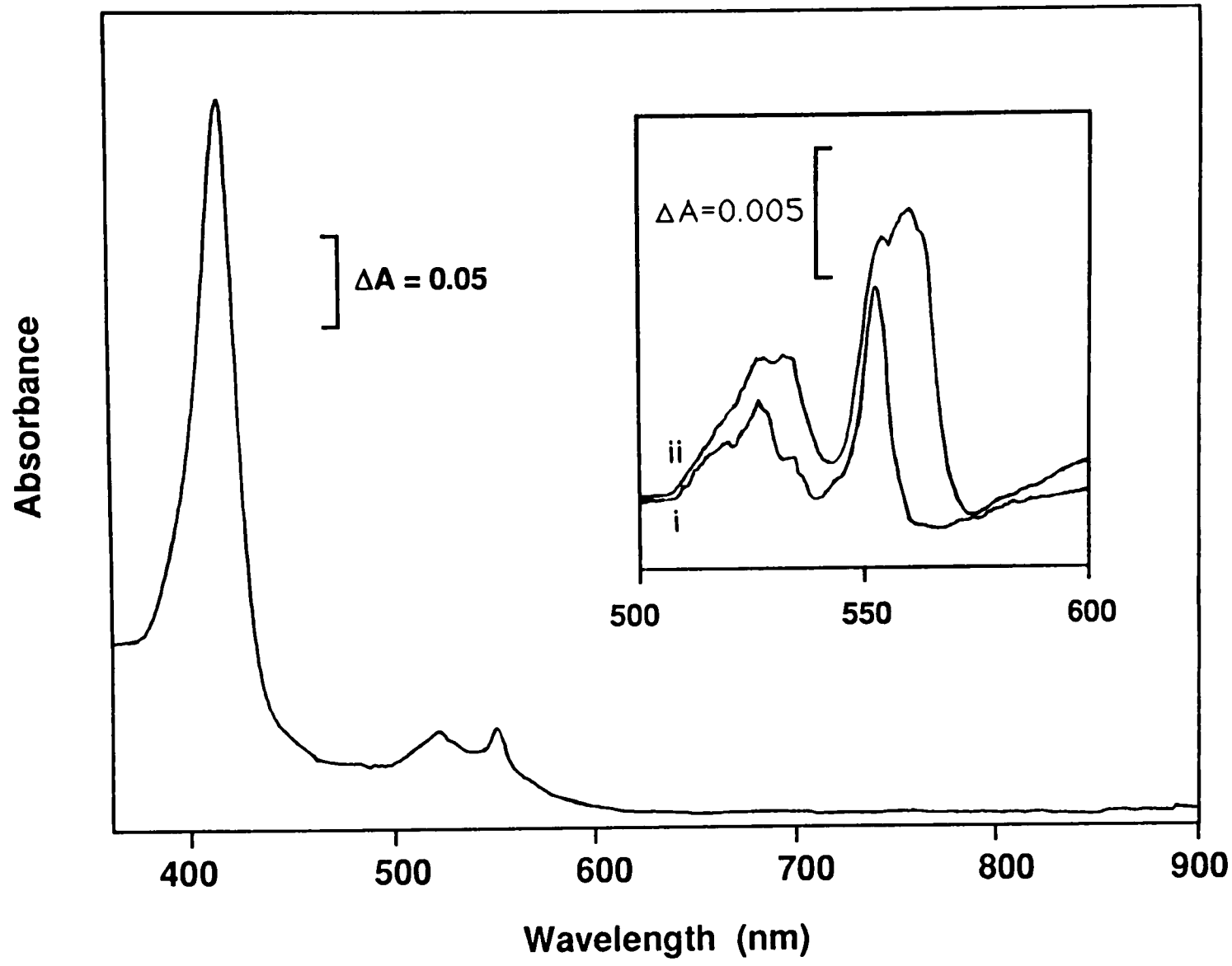

Figure 4.18. Absorption spectra of the Rhodospirillum rubrum cytochrome bc_1 complex. The spectra were obtained in 35 mM MOPS, pH 7.40. Sodium ascorbate-reduced minus potassium ferricyanide-oxidized (ii) and sodium-dithionite reduced minus potassium ferricyanide-oxidized (i) spectra were shown within the inset. Spectral resolution was 1 nm and optical path length was 1 cm.

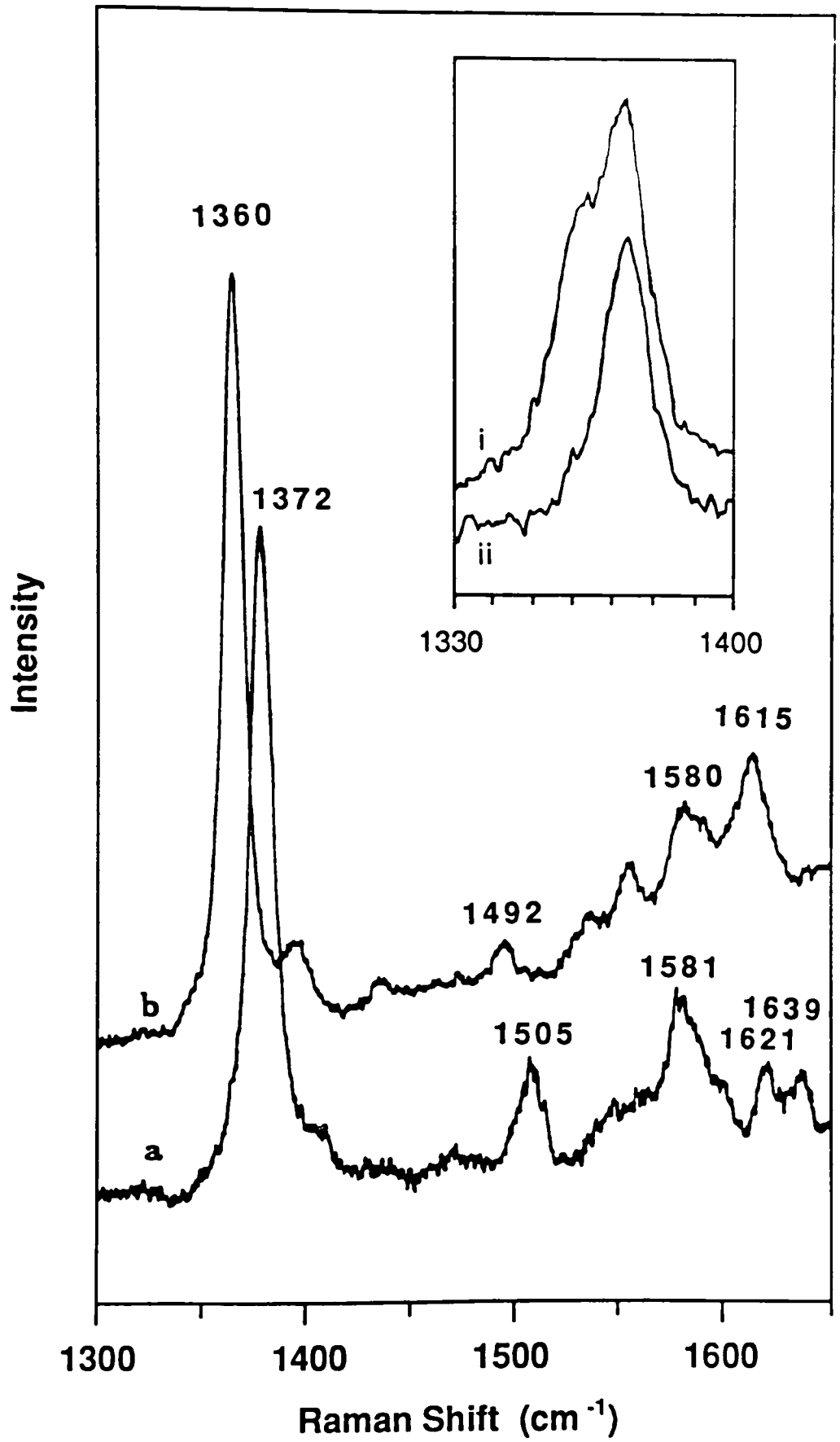

Figure 4.19. High-frequency resonance Raman spectra of the Rhodospirillum rubrum cytochrome bc_1 complex (a) potassium ferricyanide oxidized, and (b) sodium dithionite reduced, using 406 nm excitation. All other conditions were the same as in Fig. 4.17. The inset depicts the behaviour of υ_4 for the ferricyanide oxidized sample under high-power (i) and low-power (ii) laser excitation (Hobbs et al., 1990).

The fully oxidized complex displayed a propensity for photoreduction even in the presence of ferricyanide. The reductant for this photoreduction is unknown. Photoreduction even in the presence of ferricyanide of a fraction of the heme sites was evident in the dependence of ν_4 upon laser intensity (see the inset of Fig. 4.19). At high laser powers, a lower frequency shoulder (about 1360 cm^{-1}) is clearly evident, suggesting that one or more of the hemes in the complex are photoreducible. In the absence of ferricyanide, high power 440 nm excitation yields a spectrum very similar to that of the ascorbate reduced complex. In Fig. 4.20, the effects of changes in the excitation frequency within the Soret absorption band of the fully reduced cytochrome $\underline{bc}_1$ complex are demonstrated. Excitations at 406 nm (Fig. 4.20b) preferentially enhance reduced heme $\underline{c}_1$, while excitations at 430 nm (Fig. 4.20a) yield increased scattering from the reduced heme $\underline{b}$ chromatophores. Evidence of this may be observed by changes in relative intensity of Raman bands appearing at 1581 cm^{-1} and 1615 cm^{-1}.

Similar effects are observed using Q-band (520-560 nm) excitation (Fig. 4.21). Excitation at 550 nm enhances the reduced heme $\underline{c}$ chromatophore, while 560 nm excitation leads to enhancement of the protoheme chromophores of cytochrome $\underline{b}$. Moreover, the Q band resonance Raman spectra are generally dominated by non-totally symmetric vibrational modes, which are responsible for vibronic mixing of the Q_0 and B absorption bands. Thus Q band excitation allows for further separation of the individual heme contributions to the Raman spectrum. For example, using 550 nm excitation, ν_{19} appears at 1589 cm^{-1} (Fig. 4.21b), but shifts to 1585 cm^{-1} when enhanced at 560 nm (Fig. 4.21a). Also ν_{10}, which can be readily identified with 550 nm excitation, is obscured in the Raman spectrum obtained with 560 nm excitation. The band appearing at 1504 cm^{-1} is prominent using either excitation. However, its relative intensity is clearly sensitive to the excitation wavelength. Finally, a band observed at 1542 cm^{-1} (ν_{11}) in Fig. 4.21b is consistent with a

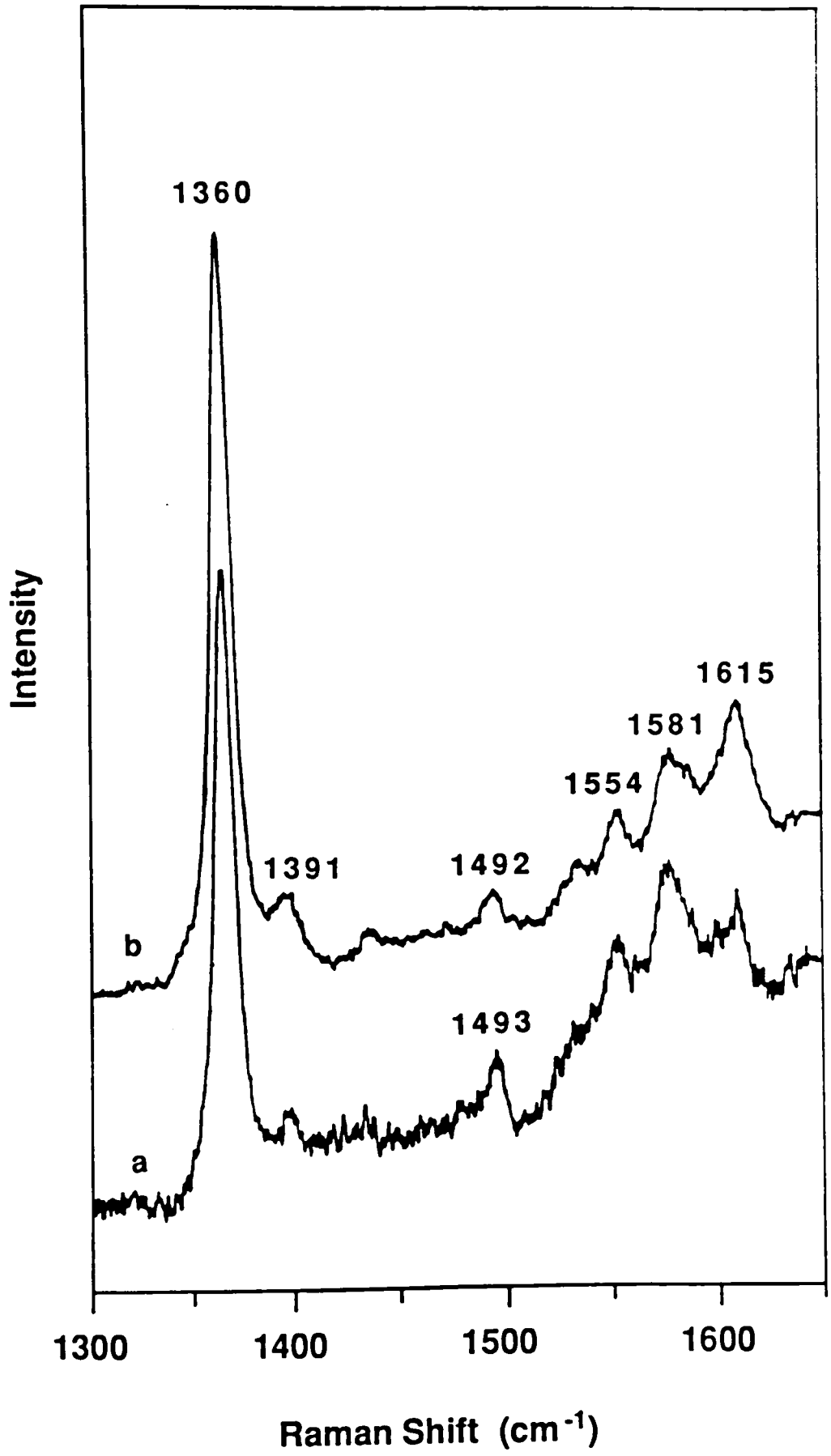

Figure 4.20. High-frequency resonance Raman spectra of sodium dithionite fully reduced Rhodospirillum rubrum cytochrome bc_1 complex using (a) 430 nm excitation and (b) 406 nm excitation. All other conditions were the same as in Fig. 4.17 (Hobbs et al., 1990).

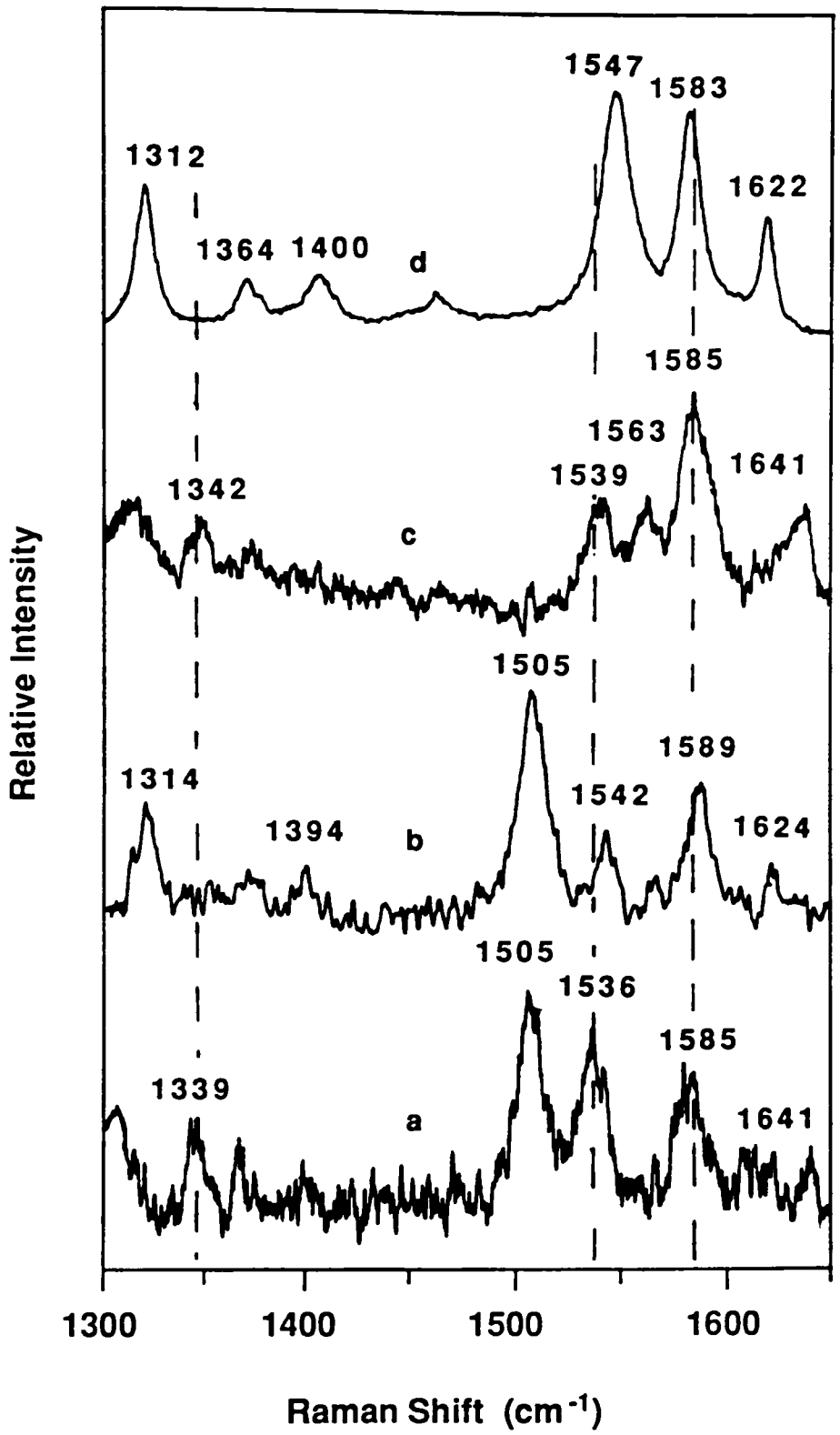

Figure 4.21. High-frequency resonance Raman spectra of sodium dithionite fully reduced Rhodospirillum rubrum cytochrome bc_1 complex using (a) 560 nm excitation and (b) 550 nm excitation. All other conditions were the same as in Fig. 4.17, except the samples were about 200 μM in cytochrome bc_1 complex. (c) Raman spectrum of reduced mitochondrial cytochrome b_5. (d) Raman spectrum of reduced equine cytochrome c. Raman spectra of reduced cytochrome c and b_5 were obtained under conditions similar to those used for the cytochrome bc_1 spectra in (a) and (b). In spectra (a) and (b) the baselines have been adjusted to facilitate presentation (Hobbs et al., 1990).

c-type cytochrome, while in Fig. 4.21a this band shifts to 1536 cm^{-1}. Attempts to obtain resonance Raman spectra of the fully oxidized cytochrome bc_1 complex using Q band excitation wavelengths were unsuccessful due to the relatively low optical absorbance of the heme chromophores in this region of the absorption spectrum.

4.3.2. Reductive titrations

Figures 4.22 and 4.23 summarize the results of studies in which reductant was added stochiometrically to fully oxidized samples. Electrochemical redox titrations of the cytochrome bc_1 complex indicate that the cytochrome c_1 component has a midpoint redox potential of +280 mV (Kriauciunas et al., 1989) slightly greater than that observed in redox titrations of the complex in situ using R. rubrum chromatophore membranes at pH 7.00 (Venturoli et al., 1987). Potentiometric titrations (see section 4.1.3.2.) indicate that the Rieske iron-sulfur protein (E_m = +305 mV) is approximately isopotential to or perhaps slightly more electropositive than, cytochrome c_1 in the isolated R. rubrum complex. Thus one would predict that addition of one reducing equivalent to the fully oxidized complex would result in approximately 50 % reduction of cytochrome c_1 and 50 % reduction of the Rieske protein. However, the behavior of the Q-band absoption of cytochrome bc_1 complexes during stochiometric titrations with sodium dithionite indicates that the initial electron added to the complex resides on the c_1 heme. Furthermore, addition of the second electron initially reduces the b-type heme sites. It may be possible that the Rieske protein is not oxidized by the ferricyanide pretreatment and so is already reduced before the addition of any exogenous reductant or that it does not equilibrate well with dithionite. This is followed by a kinetically slow ($\tau_{1/2} \approx 2$ h) reoxidation of the cytochrome b. The oxidant for this reaction is not known.

Fig. 4.23 consists of resonance Raman spectra from the partially reduced cytochrome bc_1 complexes. The absorption spectra of these partially reduced samples (shown in

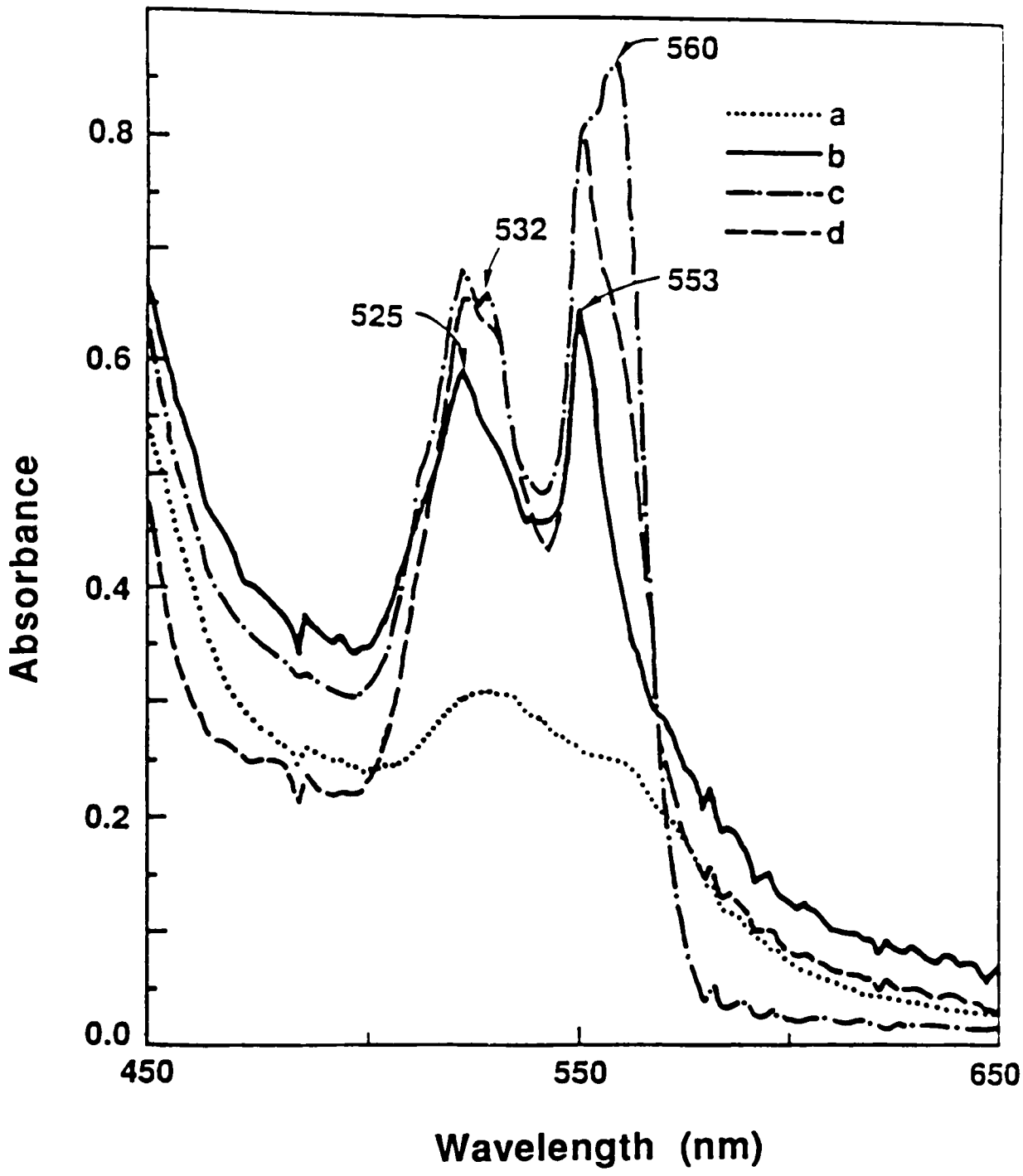

Figure 4.22. Ultraviolet-visible absorption spectra of the Rhodospirillum rubrum cytochrome bc_1 complex which has been stochiometrically reduced with sodium dithionite. (a) Zero electron equivalent (fully oxidized). (b) One electron equivalent (only heme c_1 reduced). (c) Three electron equivalents (heme c_1 and heme b reduced). (d) Four electron equivalents (fully reduced) (Hobbs et al., 1990).

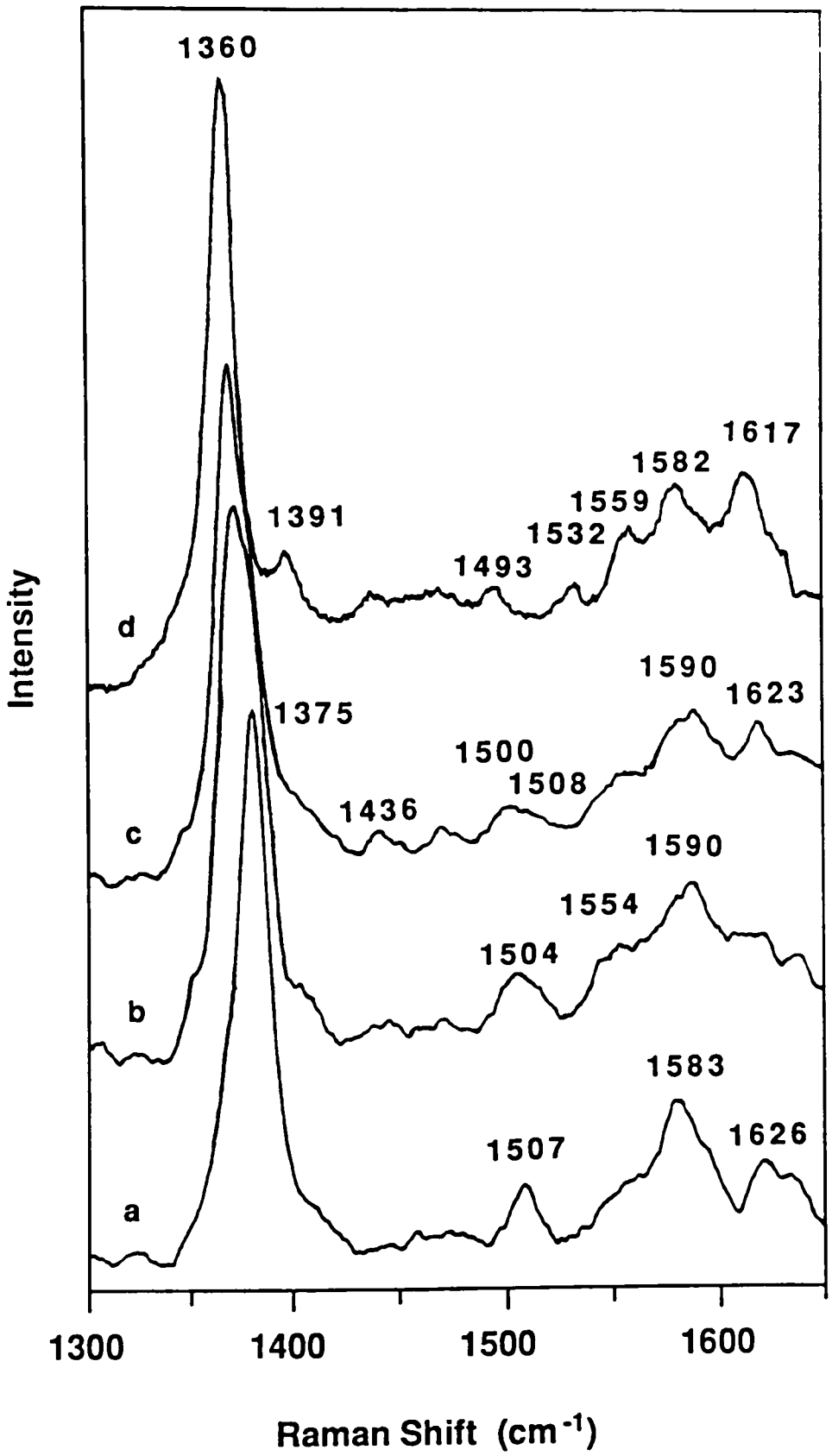

Figure 4.23. Sodium dithionite redox titration of the Rhodospirillum rubrum cytochrome bc_1 complex which had been completely oxidized with a stochiometric amount of potassium ferricyanide. (a) Zero-electron equivalents. (b) One-electron equivalent. (c) Three-electron equivalents. (d) Four-electron equivalents. All spectra taken using 410 nm laser excitation. Sample and spectral parameters were the same as in Fig. 4.17 (Hobbs et al., 1990).

Fig. 4.22) are consistent with a fully oxidized, cytochrome $\underline{c}_1$, partial heme $\underline{b}$ reduction, and the fully reduced complex, respectively. For these initial Raman experiments, a single laser excitation wavelength at 410 nm was employed. The behavior of ν_4 (Fig. 4.23) is indicative of the progressive reduction of the hemes. The addition of one electronequivalent of dithionite to the complex (Fig. 4.23b) produce a mode at 1590 cm^{-1} consistent with ν_2 observed for most ferrous $\underline{c}$-type cytochromes. Further additions of dithionite shift ν_2 to 1582 cm^{-1}, with a shoulder at 1590 cm^{-1} (Fig. 4.23d). A similar shift occurs in the position of ν_{10} for the complex. In Fig. 4.19B, ν_{10} at 1623 cm^{-1} is consistent with that found for other $\underline{c}$-type cytochromes; while in Fig. 4.19D, ν_{10} is observed at 1617 cm^{-1} (with a shoulder at 1623 cm^{-1}), a position similar to that observed for cytochrome $\underline{b}_5$.

4.3.4. Discussion

Recent studies have provided substantial molecular details for some of the protein complexes involved in the initial phases of light transduction in photosynthetic bacterial membranes. For instance, high-resolution crystallographic data are now available for the reaction centers from Rps. viridis (Deisenhofer et al., 1985) and Rb. sphaeroides (Allen et al., 1987). These important structural studies have revealed the molecular framework responsible for the initial reaction in bacterial photosynthesis. Our attempts in collaboration with Prof. J. Deisenhofer to crystallize the cytochrome $\underline{bc}_1$ complex of R. rubrum are in progress however no crystal structures exist for the cytochrome $\underline{bc}_1$ complex of photosynthetic bacteria. These complexes are responsible for secondary electron and proton transfers that lead to the reduction of the photoreduced reaction centers via cytochrome $\underline{c}_2$ (Crofts et al., 1983; Hauska et al., 1983; Dutton, 1986, Hauska et al., 1986). Thus, the resonance Raman data for the cytochrome $\underline{bc}_1$ complexes are very helpful

for the determination of the roles played by the structure and dynamics of their heme active sites in photosynthetic electron transfer.

Resonance Raman spectra for the cytochrome $\underline{bc}_1$ complex isolated from the photosynthetic bacterium, R. rubrum, have been obtained in successive states of reduction for the heme $\underline{b}$ and heme $\underline{c}$ components, and resonance Raman enhancement techniques have been employed to further separate the relative contributions of the different heme chromophores to the spectra. The analysis of the resonance Raman data presented in this work was done by Prof. M. Ondrias at University of New Mexico.

Spectra of the fully oxidized or reduced heme complex were obtained by addition of excess ferricyanide or dithionite, respectively. Excess ferricyanide was required to maintain complete oxidation of the hemes within the complex during the course of the scattering experiments. Heme photoreduction is quite evident in spectra of the fully oxidized cytochrome $\underline{bc}_1$ complexes obtained at moderate to high laser powers. Similar phenomena have been observed in other membrane bound protein complexes, such as mitochondrial cytochrome oxidase (Adar and Yonetani, 1978; Ogura et al., 1985) and chloroplast $\underline{b_6f}$ complexes (Hobbs et al., 1991). Photoreduction of cytochrome $\underline{bc}_1$ complexes is evidently a very efficient process, competing effectively with the rapid reoxidation of the complex by ferricyanide. This implies that the heme photoactivity in cytochrome $\underline{bc}_1$ complexes is a direct consequence of active site environment(s) within the complex.

Preliminary studies indicated that both B and Q-band excitation are effective in producing reduced heme $\underline{c}_1$ within the approximately 10 ns laser pulsewidth. This strongly suggest that the heme $\underline{c}_1$ itself is locus of the photoactivity and that heme excited states accessible from both B and Q-band optical transitions act as electron acceptors. The fact that photoreduction is apparent within 10 ns of excitation further suggests that the unidentified immediate eletron donor must be close to the heme $\underline{c}_1$ site.

Spectra of the fully oxidized complex (Fig. 4.19a) obtained using low power Soret excitations (406-430 nm) exhibit the polarized and activated depolarized modes typically enhanced via scattering from hemes. The positions and relative intensities of these modes are consistent with all of the heme sites possesing a low-spin six-coordinate configuration. The oxidation state marker, ν_4, was observed at 1372 cm^{-1} and has a linewidth at half maximum approximately equal to 11 cm^{-1}, a value similar to the homogeneous linewidths obtained from proteins containing a single heme. Thus the ν_4 bands for the ferric $\underline{c}_1$ and ferric $\underline{b}$ hemes in the complex apparently have coincident positions and linewidths despite the large differences in their redox potentials and porphyrin peripheral substituents. This is somewhat surprising in view of the dependence of ν_4 upon metal $\rightarrow$ porphyrin ($d_\pi \rightarrow e_g$ (π^*)) backbonding (Spiro, 1983). Another Raman band, ν_3 which has been identified as being sensitive to the iron spin-state and number of the axial ligands (Spiro and Burke, 1976), is also relatively narrow and thus is consistent with similar ligand field strengths among the (six-coordinate, low-spin) oxidized hemes present. Raman bands appearing in the 1500-1700 cm^{-1} region of the spectrum are diagnostic of the iron spin state as well as the porphyrin core size (Spiro and Strekas, 1974). In our results, these bands were broad as compared to ν_4 and thus represent more than one distinct heme configuration. These were not directly assignable to the $\underline{b}$ or $\underline{c}_1$ hemes using Soret excitation wavelengths.

Raman spectra of the fully reduced complex obtained with Soret excitation (Fig. 4.19b) also give evidence for similar electronic ground states of the heme $\underline{b}$ and $\underline{c}_1$ of the complex. Here again, the linewidths of ν_4 (1360 cm^{-1}) and ν_3 (1494 cm^{-1}) composite bands are relatively narrow, indicating that the ferrous hemes in the complex have similar metal-porphyrin and ligand field interactions. Varying excitation wavelengths within the Soret band produced some changes in the relative intensity of several core-size-sensitive bands (Fig. 4.20), but no obvious shifts were observed in vibrational modes that were specifically useful in assigning heme $\underline{c}_1$ and heme $\underline{b}$ bands.

Spectral isolation of the different hemes within the fully reduced complex proved more effective in the Q band spectral region of the chemically reduced species for several reasons. The reduced hemes have larger scattering cross-sections due to their greater extinction coefficients for both Q_{00} and Q_{01} transitions. Moreover, relative absorption differences between the two heme types are greater in the α-band. Excitation at 550 nm preferentially enhances scattering from ferrous heme $\underline{c}_1$ sites, while slightly redder excitation (560 nm) was used to maximally enhance the contributions of the ferrous $\underline{b}$ hemes to the composite spectrum. As points of comparison, spectra of equine cytochrome $\underline{c}$ and mitochondrial $\underline{b}_5$ were obtained using similar excitations and sample conditions (Figs. 4.21d and 4.21c, respectively).

The sensitivity of the resonance Raman spectra (Fig. 4.21) to excitation wavelength can be used to assign bands at 1589 cm^{-1} and 1542 cm^{-1} to ν_{19} and ν_{11} of heme $\underline{c}_1$, respectively. With 560 nm excitation, these bands shift to 1585 cm^{-1} and 1536 cm^{-1}, respectively, and are assigned to be predominantly ν_{19} and ν_{11} of the ferrous heme b sites. Two porphyrin ring modes at 1304 cm^{-1} are also preferentially enhanced with excitation into the heme $\underline{b}$ α-band (560 nm). These modes are diagnostic for the presence of vinyl substituents.

Both ν_{19} and ν_{11} are quite sensitive to axial ligation and/or geometric distortions of the heme macrocycle (Kitagawa et al., 1975; Spiro and Li, 1988). Their positions in the $\underline{bc}_1$ spectra are generally consistent with low-spin six-coordinate heme $\underline{c}_1$ and heme $\underline{b}$ in the complex. The near coincidence of positions of these modes for cytochrome $\underline{b}_5$ and the heme $\underline{b}$ sites of the cytochrome $\underline{bc}_1$ complex strongly suggests that they possess identical axial ligation (His-His) and heme geometries (Hauska et al., 1988). On the other hand, comparison of ν_{11} and ν_{19} for heme $\underline{c}_1$ and equine cytochrome $\underline{c}$ reveals significantly lower frequencies for the heme $\underline{c}_1$ site, even though the axial ligands of these species are presumed to be equivalent (Gabellini and Sebald, 1982; Davidson and Daldal, 1987;

Simpkin et al., 1989; Gray et al., 1992). This indicates that the putative His-Met heme $\underline{c}_1$ configuration may be perturbed from the rhombic symmetry observed for most cytochrome $\underline{c}$ (Cartling, 1988).

The intense mode appearing at 1504 cm^{-1} (see Fig. 4.17) in the Q-band spectra is anomalous, but is probably not due to contaminants such as bacteriochlorophylls or carotenoids since the spectra of the detergent-solubilized cytochrome $\underline{bc}_1$ complexes from R. rubrum suggest that the complex is almost entirely free of bacteriochlorophyll and carotenoids. These adventitious contaminants are not observed in the ultraviolet absorption or as background fluorescence in the Raman spectra. This band appears more intensely in the spectra obtained at 550 nm and thus may be associatedwith the $\underline{c}_1$ heme component of the complex. No such band, however, is observed in the Raman spectra of either equine cytochrome $\underline{c}$ or mammalian cytochrome $\underline{b}_5$. Further studies will address the assignment of this band in an isolated heme $\underline{c}_1$ subunit.

Sequential reduction of the cytochrome $\underline{bc}_1$ complexes proved to be an effective means of separating and identifying contributions from the $\underline{b}$ and $\underline{c}_1$ heme sites to the Raman spectra using Soret band excitation. Since the redox potentials of the three heme sites are well separated, the stochiometric addition of strong reductants ($E_m < -300$ mV) allows for the preparation of equilibrium $\underline{c}_1^{3+}\underline{b}_H^{3+}\underline{b}_L^{3+}$, $\underline{c}_1^{2+}\underline{b}_H^{3+}\underline{b}_L^{3+}$, $\underline{c}_1^{2+}\underline{b}_H^{2+}\underline{b}_L^{3+}$, and $\underline{c}_1^{2+}\underline{b}_H^{2+}\underline{b}_L^{2+}$ complexes.

This, in turn, permits the direct assignment of several important Raman shift frequencies for reduced $\underline{c}_1$ and the still oxidized $\underline{b}$ type hemes. In general, these assignment are consistent with those observed for reduced equine cytochrome $\underline{c}$ and oxidized cytochrome $\underline{b}_5$. Further mode assignments were made following this general method while stochiometrically reducing the complex.

As in the ascorbate reduced samples, addition of one cytochrome $\underline{c}_1$ electron equivalent to the fully oxidized complex reduced only the higher potential cytochrome $\underline{c}_1$

site. Several vibrational mode assignments for the heme c (FeII) and the heme b (FeIII) can be made from the spectra of this species. In the spectra of the one-electron reduced complex, the contributions of the reduced heme c_1 dominate that of the oxidized b type hemes. This is clearly observed in the greater intensity of the 1360 cm^{-1} oxidation state band for the single heme c_1 versus that at 1373 cm^{-1}, which represents the two oxidized b-type hemes. By and large, the Raman modes assignable to cytochrome c_1 (ν_4, 1362; ν_2 1590; ν_3, 1491 and ν_{10} , 1622 cm^{-1}) resemble those determined for equine cytochrome c (ν_4 , 1362; ν_2, 1590; ν_3, 1493; and ν_{10} , 1622 cm^{-1}) .

Comparison of the spectra of the equilibrium two-electron reduced complex (heme c_1 reduced) with that of the four-electron, fully reduced complex (hemes c_1, b_H and b_L reduced) allows assignment of vibrational frequencies associated with ν_2, ν_3, and ν_{10} of the ferrous b-type hemes . The positions of these porphyrin modes compare well with those reported for ferrocytochrome b_5, corroborating the behavior of ν_{19} and ν_{11} observed with Q-band excitation. In particular ν_3 (1493 cm^{-1}), ν_{10} (1617 cm^{-1}) and ν_{19} (1585 cm^{-1}) determined in this part of study for the b-type hemes of the cytochrome bc_1 complex are virtually identical with those found for mitochondrial cytochrome b_5 and thus reflect strong similarities in porphyrin core-size and ligand field strength for these species.

It is possible to exploit the spectral differences among the hemes in bc_1 complexes to obtain insight into their structures and environments. Selective resonance enhancement and sequential stochiometric reduction of the complex are particularly useful for isolating the spectra of the c- and b-type hemes. Using these methods, it is possible to assign most of the high-frequency Raman bands for these redox sites in the R. rubrum cytochrome bc_1 complex. In general, Raman modes observed for the hemes b and c_1 of the complex are similar to those reported for isolated mammalian cytochrome b_5 and cytochrome c. In particular, ν_4, the Raman band most associated with electron density of the porphyrin π^* molecular orbital, is narrow for both the fully oxidized and fully reduced complex. This

finding is consistent with similar interactions between the d_π orbital of the heme iron and the porphyrin $e_g(\pi^*)$ orbitals in the ferric b- and c-type as well as the ferrous b- and c-type hemes.

The core-size sensitive Raman bands (1500-1700 cm^{-1}), however, are broad (> 20 cm^{-1}) and suggest that different heme environments exist among the heme sites. This result is plausible in view of the differences in the axial ligation state and peripheral substituents of the two heme types. These variances in core size and porphyrin bond strengths are consistent with those observed between equine cytochrome c (which has a His/Met axial ligand configuration and covalent linkages to the apoprotein at two adjacent pyrrole rings) and mammalian cytochrome b_5 (which has a bis-histidine axial ligand configuration and two free pyrrole vinyls). Thus, Raman spectra using B band excitation alone would imply that there are few significant differences in the low-spin b- and c-type hemes of the cytochrome bc_1 complex as compared to other low-spin b- and c-type hemes. It is clear, however, from Raman excitations within the Q_{00} absorption bands for the heme b (560 nm) and heme c (550 nm) chromophores that the heme sites of the complex differ from those of structurally related b- and c-type heme proteins. Moreover, since Q band Raman excitations enhance primarily antisymmetric A_{2g}, B_{1g} and B_{2g} vibrational modes in D_4 point group symmetry, it is possible that perturbations to the heme c_1 environment are of similar symmetry. For instance, ν_{11} (B_{1g}) and (A_{2g}) observed, using 550 nm laser excitations, at 1542 and 1589 cm^{-1}, respectively, for cytochrome c_1 and differ from the values obtained for equine cytochrome c (ν_{11}, 1547 cm^{-1} and ν_{19}, 1585 cm^{-1}). Although these modes have been shown to be influenced by heme ligation state and core size, it is clear from the absence of shifts in the core-size sensitive A_{1g} vibrational modes, ν_2 and ν_3, that core-size is not the determinative factor in these differences. More likely, ν_{11} and ν_{19} are affected by peripheral heme-protein interactions. In particular, ν_{11} has been shown to be sensitive to the conformation of vinyl pyrrole substituents, and their planarity (Cartling,

1988), relative to the heme plane, could easily be directed through van der Waal's contacts with individual peptides of the protein. In the cytochrome $\underline{bc}_1$ complex of R. rubrum, we observed shifts in ν_{11} to lower frequency for both the b- and c-type of the complex. These shifts to lower vibrational energy may be due to out-of-plane distortion of the heme substituents and thus loss of π-bonding with the tetrapyrrole ring.

CHAPTER 5

SUMMARY AND CONCLUSIONS

An antibody against the M_r = 12-14 kDa subunit of Rhodobacter sphaeroides did not recognize the corresponding protein in solubilized Rhodospirillum rubrum membranes, indicating that R. rubrum cytochrome bc_1 complex either does not contain a fourth subunit or, if present, it is antigenically different than that of Rb. sphaeroides cytochrome bc_1 complex.

Antibodies raised against the Rhodobacter capsulatus cytochrome bc_1 peptides recognized corresponding R. rubrum proteins indicating that similarities exist between the peptides of these two photosynthetic bacteria.

The ubiquinol-cytochrome c_2 oxidoreductase activity of solubilized and purified R. rubrum cytochrome bc_1 complex is highly sensitive to specific inhibitors of cytochrome bc_1 complexes. Antimycin A shifts the α-band maximum of reduced cytochrome b_H to longer wavelength, providing a measure of the interaction of this inhibitor with the purified R. rubrum cytochrome bc_1 complex. Stigmatellin produces a shift in the E_m value and in the EPR g-value of the reduced Rieske iron-sulfur protein of R. rubrum. These results suggest that a fourth subunit, if present, is not required for normal binding of these inhibitors to the R. rubrum cytochrome bc_1 complex.

The solubilized and purified three subunit R. rubrum cytochrome bc_1 complex, after incorporation into liposomes, is capable of proton translocation coupled to electron transfer. Thus, the presence of a fourth subunit is not a requirement for proton translocation by the complex. However, the H^+/e^- ratio is somewhat lower than that obtained with the beef heart cytochrome bc_1 complex.

The effects of ionic strengths on the kinetic parameters of cytochrome $\underline{c}_2$ or cytochrome $\underline{c}$ reduction catalyzed by the Rb. capsulatus cytochrome $\underline{bc}_1$ complex are consistent with the involvement of an electrostatic complex formation between the cytochrome $\underline{bc}_1$ complex and its electron accepting substrate. The number of charged pairs in the interaction of these two proteins have been estimated to be n = 3.5 and n = 4.0 for Rb. capsulatus and Rps. viridis, respectively, using a semiempirical relationship. This is similar to the number estimated previously for other photosynthetic bacteria. The interaction between Rb. capsulatus cytochrome $\underline{c}_2$ (and its analog equine cytochrome $\underline{c}$) and the cytochrome $\underline{bc}_1$ complex involves lysine residues surrounding the exposed heme edge on cytochrome $\underline{c}/\underline{c}_2$.

The resonance Raman spectra of cytochrome $\underline{bc}_1$ complexes from R. rubrum have been obtained at various resonance conditions and by stochiometric redox titrations of the complex. The results obtained were used to isolate and identify the contributions of the heme sites to the observed spectra. The complex was found to partially photoreduced when exposed to laser excitation.

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