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Minireview

The bc_1 Complexes of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*

Robert B. Gennis,[1] Blanca Barquera,[1] Beth Hacker,[1] Steven R. Van Doren,[2,4] Sylvain Arnaud,[1] Antony R. Crofts,[2] Edgar Davidson,[3] Kevin A. Gray,[3] and Fevzi Daldal[3]

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Photosynthetic bacteria offer excellent experimental opportunities to explore both the structure and function of the ubiquinol-cytochrome *c* oxidoreductase (bc_1 complex). In both *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, the bc_1 complex functions in both the aerobic respiratory chain and as an essential component of the photosynthetic electron transport chain. Because the bc_1 complex in these organisms can be functionally coupled to the photosynthetic reaction center, flash photolysis can be used to study electron flow through the enzyme and to examine the effects of various amino acid substitutions. During the past several years, numerous mutations have been generated in the cytochrome *b* subunit, in the Rieske iron-sulfur subunit, and in the cytochrome c_1 subunit. Both site-directed and random mutagenesis procedures have been utilized. Studies of these mutations have identified amino acid residues that are metal ligands, as well as those residues that are at or near either the quinol oxidase (Q_o) site or the quinol reductase (Q_i) site. The postulate that these two Q-sites are located on opposite sides of the membrane is supported by these studies. Current research is directed at exploring the details of the catalytic mechanism, the nature of the subunit interactions, and the assembly of this enzyme.

KEY WORDS: Cytochrome; bc_1; complex III; Q-cycle; photosynthesis.

INTRODUCTION

The ubiquinol–cytochrome *c* oxidoreductases, or bc_1 complexes, constitute a large superfamily of enzymes found in mitochondrial and bacterial respiratory chains, in green plant electron transport chains (where it is known as the b_6f complex), and in bacterial photosynthetic electron transport chains (Crofts, 1983; Gabellini, 1988; Hauska *et al.*, 1988; Trumpower, 1990a; Knaff, 1992). These enzymes catalyze the two-electron oxidation of ubiquinol (or plastoquinol) and the one-electron reduction of cytochrome *c* (or c_2 or plastoquinol). This electron transport reaction is coupled to the generation of a proton electrochemical potential gradient across the membrane. The members of this superfamily are, thus, of central importance in both eukaryotic and prokaryotic respiratory and photosynthetic systems. The purpose of this article is to provide a brief summary of recent progress in studies of the bc_1 complexes of two photosynthetic bacteria, *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*.

[1]School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801.
[2]School of Life Sciences, University of Illinois, Urbana, Illinois 61801.
[3]Department of Biology, Plant Science Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104.
[4]Current address: Biophysics Research Division, University of Michigan, 2200 Bonisteel Blvd., Ann Arbor, Michigan 48109–2099.

1.1. Experimental Advantages of Photosynthetic Bacteria

Rb. sphaeroides and *Rb. capsulatus* provide several experimental advantages that complement those advantages offered by other organisms, such as yeast or bovine: (1) The bc_1 complexes of the photosynthetic bacteria contain fewer subunits than

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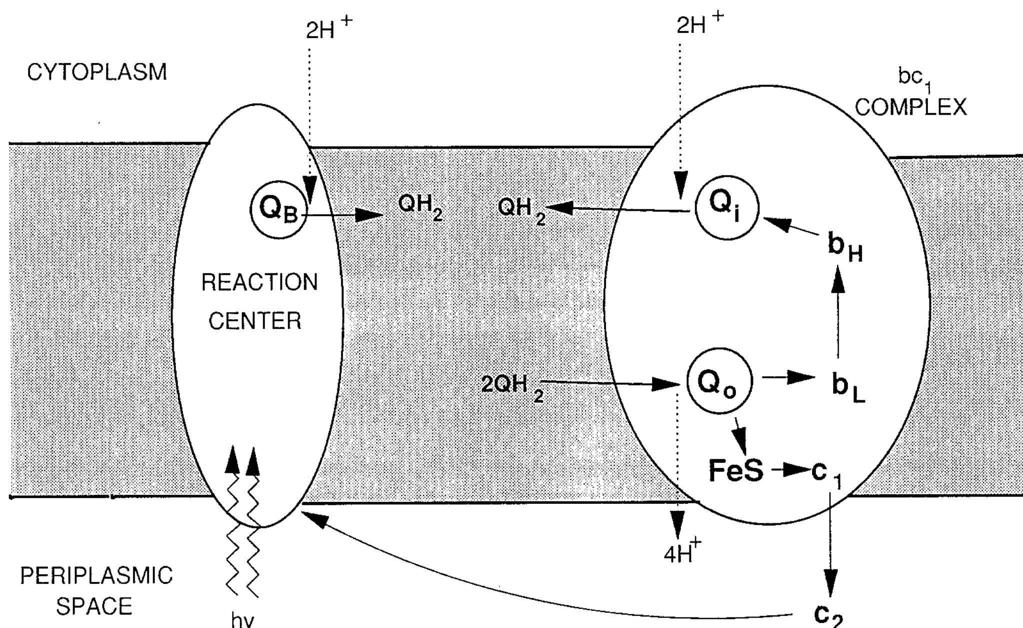

Fig. 1. Schematic diagram illustrating the role of the bc_1 complex in photosynthetic cyclic electron transfer. The chromatophore membrane, reaction center, and bc_1 complex are depicted. The modified Q-cycle mechanism is also shown. Two cycles of QH_2 oxidation at the Q_o site are required to generate the two-electron reduction of Q to QH_2 at the Q_i site. The quinone (Q) which serves as the reactant at the Q_i site (and Q_B site) and which is the product of the reaction at the Q_o site is not shown explicitly.

do their eukaryotic counterparts. The enzyme from *Rb. capsulatus* contains only three subunits, each of which contains essential metal centers required for function (Gabellini, 1988; Robertson, 1993). The *Rb. sphaeroides* complex contains only one additional subunit, whose function is not known (Andrews *et al.*, 1990; Usui and Yu, 1991; Yu and Yu, 1991). Hence, the prokaryotic systems are well suited for studies directed at the structure and functional mechanism of the catalytic core of the enzyme (2). The reactions catalyzed by the bc_1 complexes of the photosynthetic bacteria can be photoinitiated in chromatophores (Crofts, 1983), which are vesicular membranes prepared from photosynthetically grown or oxygen-limited bacteria. Figure 1 shows the elements within the chromatophores that are involved in cyclic photosynthetic electron transport: the bc_1 complex, the reaction center, ubiquinol, and cytochrome c_2. Light absorbed by the reaction center during a flash results in the rapid oxidation of cytochrome c_2 and the generation of reduced ubiquinol in the chromatophore membrane. These react with the bc_1 complex to initiate the electron transfer reactions within the enzyme, which can be monitored as a function of time by optical spectroscopy of the heme components of the complex. In this way, the step or steps which may be impeded by the addition of inhibitors or by specific mutations can be determined. (3) Defective bc_1 complexes that do not function physiologically can be examined using biochemical and biophysical techniques. In photosynthetic bacteria, the bc_1 complex is not essential for aerobic growth due to the presence of quinol oxidases that constitute a cytochrome *c*-independent branch of the respiratory systems (Daldal *et al.*, 1987; Yun *et al.*, 1990). Hence, bacterial strains which have defective bc_1 complexes will still grow aerobically. By lowering the oxygen concentration in the growth medium after the cells have grown to mid-exponential phase, the photosynthetic apparatus is induced. In this way, chromatophore preparations with defective or nonfunctional bc_1 complexes can be prepared and both thermodynamic and flash kinetics methods can be used to assess the reason for the bc_1 defect. (4) In eukaryotic systems, such as yeast, the cytochrome *b* subunit is encoded in the mitochondrion and, hence, is not yet convenient for site-directed mutagenesis techniques. The prokaryotic systems, on the other hand, are well suited for the application of reverse genetics. In both *Rb. sphaeroides* and *Rb. capsulatus* the metal-containing subunits are encoded by genes organized in an

operon (*fbc*FBC or *pet*ABC operon) (Gabellini and Sebald, 1986; Daldal *et al.*, 1987; Davidson and Daldal, 1987a, b; Yun *et al.*, 1990). The Rieske subunit, cytochrome *b* subunit, and cytochrome c_1 subunit are encoded respectively by the *fbc*F, *fbc*B, and *fbc*C genes in each organism. In both *Rb. sphaeroides* and *Rb. capsulatus* the *fbc* operon has been deleted from the chromosome and systems are well established to express site-directed mutations in each of these three subunits (Daldal *et al.*, 1987; Yun *et al.*, 1990; Atta-Asafo-Adjei and Daldal, 1991). In the case of *Rb. spaeroides*, there is a fourth subunit of unknown function which is encoded by a gene that is not linked to the *fbc* operon (Andrews *et al.*, 1990; Usui and Yu, 1991; Yu and Yu, 1991).

1.2. The Modified Q Cycle and Inhibitors of the bc_1 Complex

The bc_1 complex contains four metal redox centers: cytochrome b_L and cytochrome b_H, both located within the cytochrome *b* subunit, cytochrome c_1, and the Rieske 2Fe–2S cluster, which are each located on separate subunits. It is now generally accepted that the bc_1 complex generates a proton-motive force by a mechanism known as the modified Q cycle (Mitchell, 1976; Crofts and Wraight, 1983; Trumpower, 1990b), which is shown schematically in Fig. 1. The key feature of this mechanism is the postulate of two separate quinone (Q) sites, one where ubiquinol is oxidized (Q_o or Q_z site) and one where ubiquinone is reduced (Q_i, Q_R, or Q_c site). The oxidation of quinol is catalyzed at the Q_o site by the Rieske 2Fe2S cluster and cytochrome b_L. The quinol oxidation site must be located at the interface of the Rieske subunit and the cytochrome *b* subunit. Oxidation by the high potential FeS cluster ($E_{m,7}$ 300 mV) generates a transient ubisemiquinone species which is rapidly oxidized in a concerted reaction by cytochrome b_L ($E_{m,7}$ -90 mV). The reduced Riseke FeS cluster is, in turn, oxidized by the cytochrome c_1 component of the bc_1 complex. Cytochrome c_1 is oxidized by cytochrome c_2, or other soluble cytochromes in the periplasm in the photosynthetic bacteria. The Rieske cluster and cytochrome c_1 constitute the high-potential branch of the bc_1 complex. The low-potential branch consists of cytochrome b_L, located at the quinol oxidase site, and cytochrome b_H, which is located at the quinone reductase site (see Fig. 1). Electron transfer from cytochrome b_L to cytochrome b_H contributes about half of the transmembrane potential that is generated by turnover of the bc_1 complex. An additional contribution to the transmembrane potential comes from the electron transfer from cytochrome b_H to the quinone at the Q_i site (Glaser and Crofts, 1984; Robertson *et al.*, 1984; Robertson and Dutton, 1988).

Quinone is reduced at the Q_i site located near cytochrome b_H in two separate steps. The first electron from the cytochrome b_H to the fully oxidized quinone (Q) forms a stable semiquinone ($Q^{\dot{-}}$) which is EPR-detectable. A second turnover at the Q_o (quinol oxidase) site is required to generate the second electron to form fully reduced ubiquinol (QH_2).

Oxidation of two QH_2 species at the Q_o site releases $4\,H^+$ into the bacterial periplasm (equivalent to the intermembrane space in the mitochondrial systems), and the formation of QH_2 utilizes $2H^+$ from the cytoplasmic side of the membrane. The system, thus, generates a proton and electrical gradient across the bilayer without any ion channel traversing the membrane. Rather, the chemistry catalyzed at the Q_o and Q_i sites are the central elements in the net proton translocation effected by this enzyme. Much of the work on the bc_1 complex is directed at experimentally testing the expectations of the Q cycle mechanism, in particular, by defining the structural determinants of the putative two Q sites. These are expected to be physically located on opposite sides of the membrane and to have quite distinct characteristics.

The elucidation of the two Q sites and the development of the modified Q cycle mechanism sites has largely resulted from determining the effects of various inhibitors of the bc_1 complex using the spectroscopic techniques. These have been valuable not only for the biochemical and biophysical characterization of the enzyme, but also for guiding the application of genetic techniques to the study of the enzyme. One set of inhibitors, including myxothiazol, UHDBT (undecylhydroxydioxobenzothiazole), stigmatellin, and mucidin, blocks the oxidation of quinol and is thought to bind at or near the Q_o site (Meinhardt and Crofts, 1982; von Jagow and Ohnishi, 1985; von Jagow *et al.*, 1986; diRago *et al.*, 1989; Brandt and von Jagow, 1991). A second set of inhibitors, including antimycin, HQNO (2-n-heptyl-4-hydroxy-quinoline-N-oxide), and diuron, binds to a different locus on the enzyme and blocks the quinone reductase (Q_i) site (diRago *et al.*, 1986; Myatt *et al.*, 1987; diRago and Colson, 1988; Weber and Wolf, 1988). The effects of these inhibitors can be illustrated by the use of both steady-state kinetics and flash kinetics.

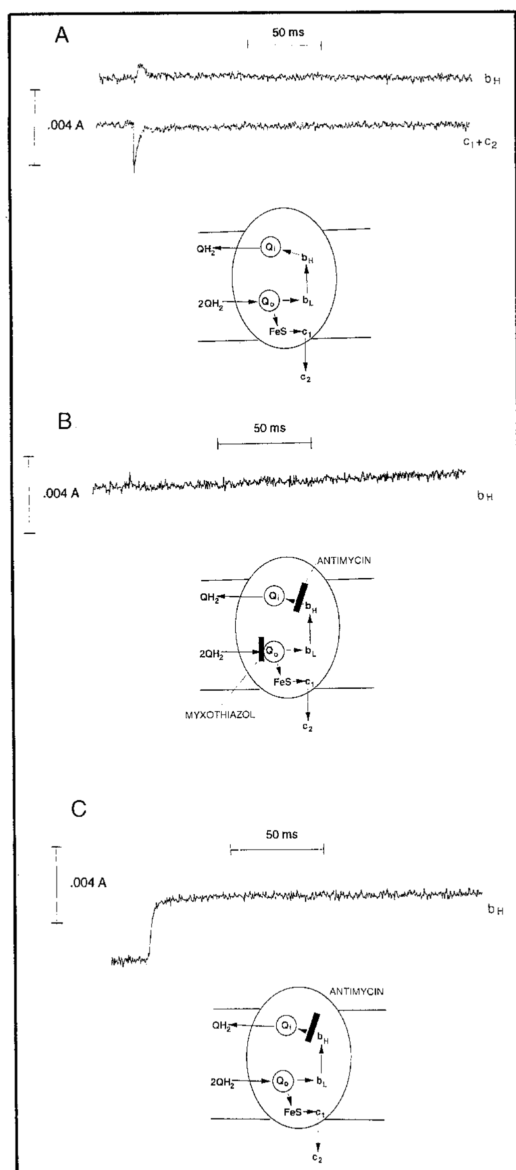

Fig. 2. Flash kinetics of a preparation of *Rb. sphaeroides* chromatophores, in the absence of inhibitors (A) and in the presence of either antimycin (B) or antimycin plus myxothiazol (C). The chromatophores were poised at 100 mV. The redox status of cytochromes ($c_1 + c_2$) is monitored by (A_{551}–A_{542}) and for cytochrome b_H (A_{561}–A_{569}).

The Application of Flash Kinetics

Flash kinetics can be used to analyze the electron transfer reactions of the bc_1 complex in photosynthetic bacteria. The redox states of cytochromes b_L, b_H, and c_1 can be monitored by optical spectroscopy as a function of time fillowing a pulse of light. Figure 2 shows an example using chromatophores containing the wild type bc_1 complex in the presence or absence of different inhibitors. Prior to the flash, the solution potential is poised at 100 mV, so the Rieske FeS center, cytochrome c_1, and cytochrome c_2 are reduced, whereas cytochromes b_L and b_H are both oxidized. At 100 mV, about half of the quinone pool is reduced prior to the flash, and oxidation of quinol at the Q_o site is not limited by the rate of diffusion of quinol to this site. The flash-induced rapid oxidation of both cytochromes c_1 and c_2 (not resolved in the experiment) by the reaction center (see Fig. 1) is followed by re-reduction via the Rieske center and from turnover at the Q_o site. By monitoring different wavelengths, the redox status of cytochrome b_H is observed as it rapidly goes reduced and is subsequently reoxidized by the quinone at the Q_i site.

The addition of antimycin prevents electron transfer from the reduced cytochrome b_H to quinone at the Q_i site, as seen in Fig. 2. However, the quinol oxidation site (Q_o) is still functional in the presence of antimycin (not shown). In contrast, the addition of myxothiazol prevents turnover of the quinol oxidation (Q_o) site, so no reduction of cytochrome b_H is observed after the light flash.

Flash kinetics has proven to be a valuable technique for understanding the effect of mutations in the bc_1 complex of the photosynthetic bacteria. The following sections will highlight some of the recent results obtained relating to each of the three metal-containing subunits of the bc_1 complex.

THE CYTOCHROME *b* SUBUNIT

Topology and Heme Ligation

The cytochrome *b* subunit is remarkably conserved, as evidenced by the comparison of the amino acid sequences of this subunit from hundreds of different species (Hauska *et al.*, 1988; Degli Esposti *et al.*, 1993). Figure 3 is an alignment of the sequences of the subunit from *Rb. Sphaeroides*, *Rb. capsulatus*, and yeast (*Saccharomyces cerevisiae*), and Fig. 4 is a two-dimensional hypothetical model of how the subunit folds in the membrane. The eight transmembrane helices (A–H) in this model are suggested by conserved hydrophobic spans in the sequences of this subunit. Originally, the helix depicted on the periplasmic surface between helix C and D was postulated to span the membrane (Saraste, 1984; Widger *et al.*, 1984). However, the conserved amphipathic character of this helix is consistent with it being located at the membrane

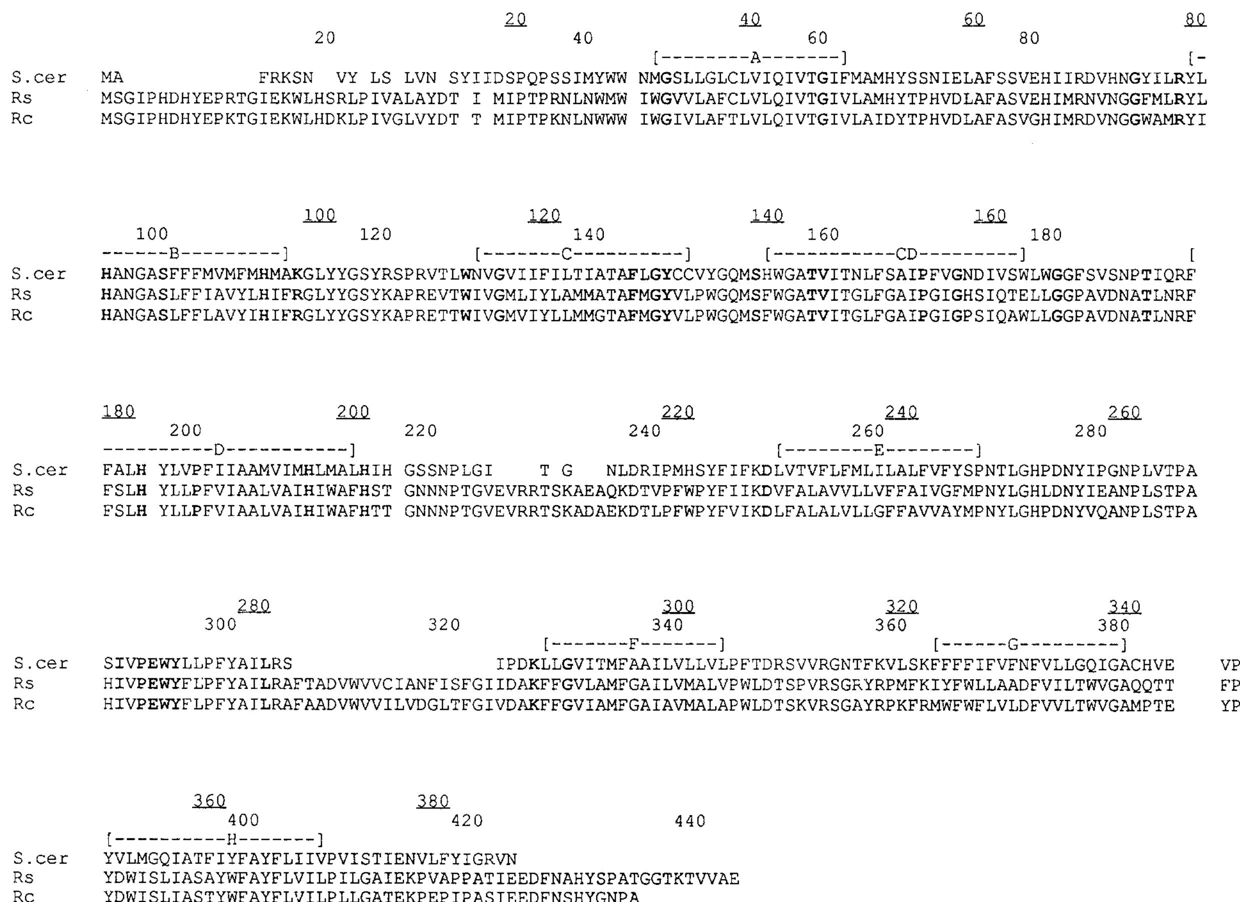

Fig. 3. A sequence alignment of the cytochrome *b* subunits of *Rb. sphaeroides*, *Rb. capsulatus*, and yeast (*Saccharomyces cerivisiae*). The putative transmembrane spans (A–H) are indicated, as are highly conserved residues (in bold). The numbers that are underlined refer to the yeast (residue under the first leftmost digit), and the second set of numbers refer to both bacterial sequences. Sequences are from Nobrega and Tzagoloff, (1980), Davidson and Daldal (1987b) and Yun *et al.*, (1990).

surface (Crofts *et al.*, 1987). Direct experimental evidence to support this model comes from the use of the gene fusion technique to determine those portions of the *Rb. sphaeroides* subunit which are on or near the periplasmic side of the membrane (Yun *et al.*, 1991b). This identified the interhelical connections *ab*, *cd*, and *ef* as periplasmic.

Additional supporting evidence for the model comes from the identification of the ligands to the two heme prosthetic groups bound to this subunit. Spectroscopic studies indicate that both cytochromes b_L and b_H are bound to histidines (Simpkin *et al.*, 1989) and that they are oriented with the heme planes perpendicular to the plane of the membrane (Erecinska *et al.*, 1978). Figure 4A shows the most highly conserved residues in the cytochrome b subunit, and these include four totally conserved histidines (*His*97 and 111 in helix B and *His* 198 and 212 in helix D). The presumption that these are the heme ligands (Saraste, 1984; Widger *et al.*, 1984) is supported by site-directed mutagenesis experiments on the *Rb. sphaeroides* subunit (Yun *et al.*, 1991a) as well as on *Rb. capsulatus* (Daldal *et al.*, unpublished). Substitutions for any of these four residues results in inactive bc_1 complex. Furthermore, substitutions for either *His*111 or *His*212 result in the specific elimination of cytochrome b_H, with a functional cytochrome b_L and Q_o site. Hence, the two hemes are ligated to histidines within helix B and helix D, so these helices must be adjacent and aligned parallel to each other. These data also support the location of cytochrome b_H near the cytoplasmic surface and of cytochrome b_L near the periplasmic surface, as one would expect from the modified Q cycle mechanism shown in Fig. 1.

Figure 5 shows a helical wheel representation of how six of the membrane-spanning helices might be arranged in relation to the two hemes, seen edge-on (Crofts *et al.*, 1992). Four totally conserved glycines

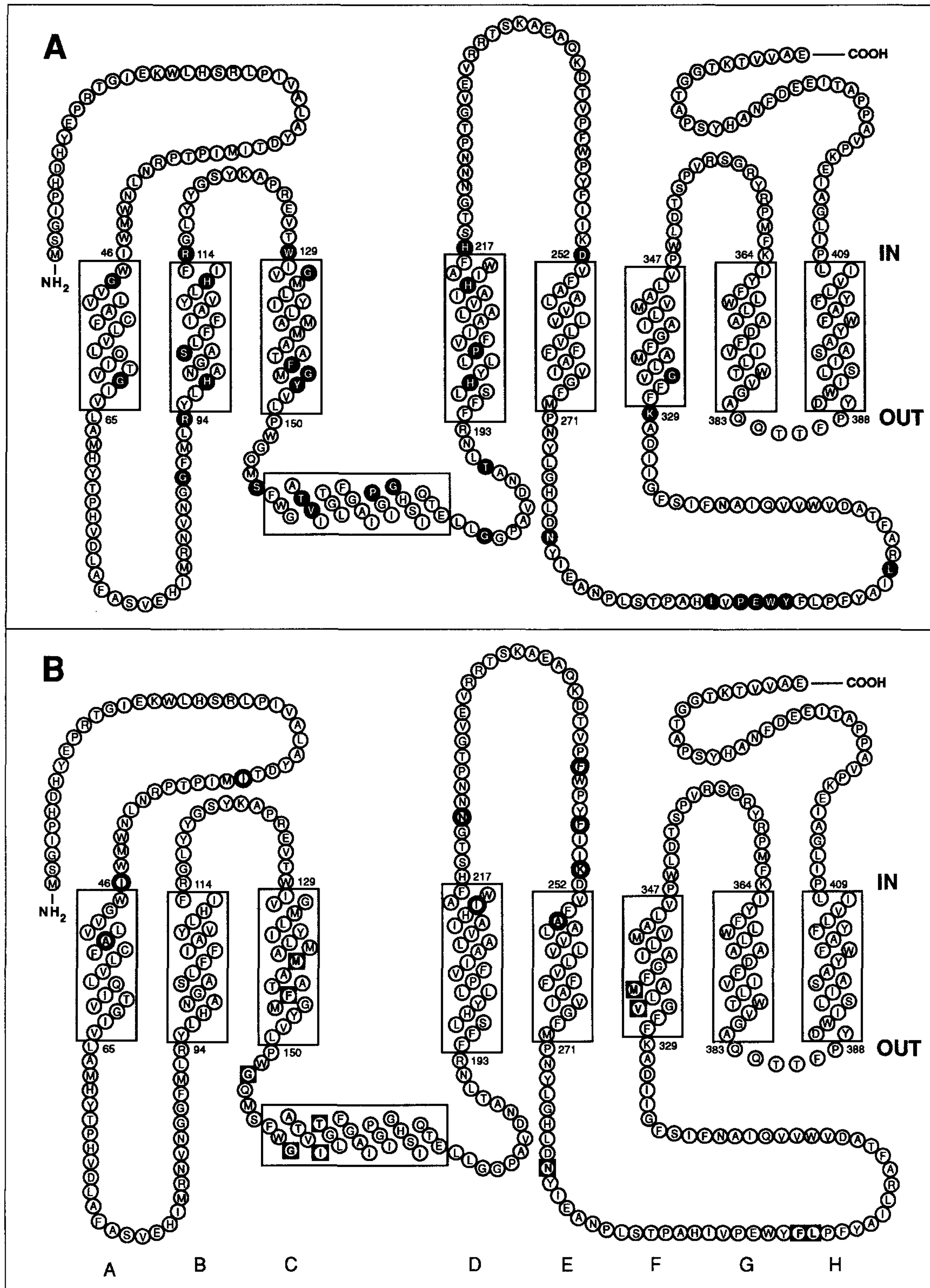

Fig. 4. Proposed topology of the cytochrome *b* subunit of *Rb. sphaeroides*. In A, residues that are very highly conserved in all known sequences of the cytochrome *b* subunit are highlighted. In B, the loci of mutants exhibiting inhibitor resistance for the Q_o site (dark squares) or the Q_i site (dark rings) are shown. These include mutations from yeast (diRago *et al.*, 1986; diRago and Colson, 1988; Weber and Wolf, 1988; diRago, *et al.*, 1989, 1990) and mouse (Howell *et al.*, 1987; Howell and Gilbert, 1988). Mutations in *Rb. capsulatus* that specifically confer inhibitor resistance are located at *Met*140, *Phe*144, *Gly*152, *Gly*158, *Thr*163, and *Val*333 (Daldal *et al.*, 1989).

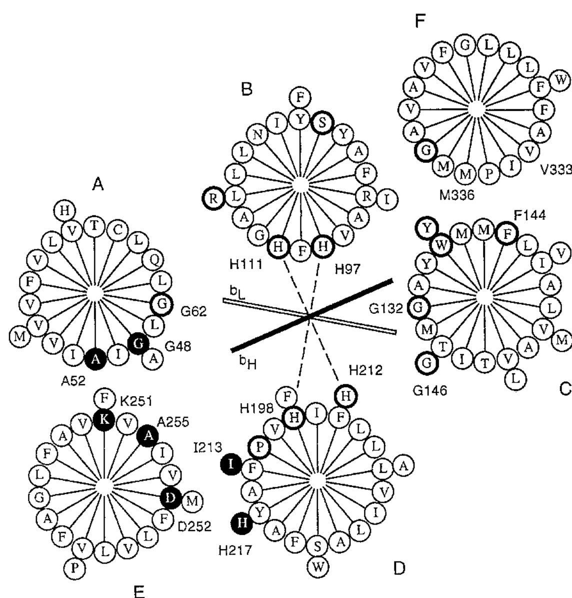

Fig. 5. A helical wheel diagram showing the arrangement of six of the helices (A–F) of the cytochrome *b* subunit shown from the cytoplasmic side (Crofts *et al.*, 1992). The two hemes (cytochromes b_L and b_H) are shown edge-on ligated to the indicated histidine ligands in helices B and D. Highly conserved residues are highlighted with dark rings. Filled circles denote residues implicated as being at or near the Q_i site, based on the analysis of mutants. In a three-dimensional version of this model, all these residues define a common volume.

are located in helix A (*Gly*48 and 62) and in helix C (*Gly*132 and 146). These have the same spacing as do the conserved histidines that are the heme ligands, and it has been proposed that these glycines are important for the packing of the two hemes in the protein (Tron *et al.*, 1991). In this model, residues that appear to be at the Q_i site on the basis of the analyses of mutants are highlighted (see below). These include residues on the cytoplasmic side of helix A (*Gly*48, *Ala*52), Helix E (*Lys*251, *Asp*252), and Helix D (*His*217). In addition, *Ile*213 and *Ala*255 correspond to positions where eukaryotic mutants resistant to Q_i-site inhibitors are located (Fig. 4B) (Howell *et al.*, 1987; diRago *et al.*, 1990). All of these residues occupy a common volume in this model. This arrangement of the transmembrane helices (Fig. 4) also brings together, on the opposite side of the membrane, those residues within helix C and helix F that are implicated as being at the Q_o site (Fig. 4B) (Crofts *et al.*, 1992)

Spontaneous Inhibitor-Resistant Mutations

Photosynthetic growth of *Rb. spaeroides* and *Rb. capsulatus* is dependent on a functional bc_1 complex. Those natural inhibitors of the bc_1 complex that are directed at the Q_o site (stigmatellin, myxothiazol, and mucidin) are also potent growth inhibitors of these bacteria under photosynthetic conditions. In contrast, those inhibitors affecting the Q_i site (antimycin, funiculosin, and HQNO) have no effect on bacterial growth, even though they are potent inhibitors of the bc_1-catalyzed reactions *in vitro* (Daldal *et al.*, 1989). Various classes of spontaneous mutants of *Rb. capsulatus* resistant to myxothiazol, stigmatellin, and/or mucidin have been isolated and characterized (Daldal, *et al.*, 1989). Those mutations that are due to changes in the *fbc* operon are all single base-pair changes in codons corresponding to phylogenetically conserved amino acid residues. Most of these are located between positions 140 and 163 in the cytochrome *b* subunit (helix C and the *cd* helix, see Fig. 4B). One exception is a mutation in *Val*333, within helix F. These data indicate that at least two distinct regions of this subunit participate in forming the cytochrome *b* portion of the Q_o site. These two regions, which are both located on the periplasmic side of the subunit, are also delineated by Q_o site inhibitor-resistant mutants in mitochondrial bc_1 complexes. Biochemical analyses of the Q_o inhibitor-resistant mutants of *Rb. capsulatus* indicate a weakening of inhibitor binding and impaired catalytic rates of Q_o site reactions which parallel a loss of affinity for the quinol substrate at this site (Robertson *et al.*, 1990).

The reason why *Rb. capsulatus* and *Rb. sphaeroides* are able to grow photosynthetically in the presence of Q_i site inhibitors such as antimycin is not known. However, it has recently been shown that another photosynthetic bacterium, *Rhodospirillum rubrum*, is sensitive to antimycin, and mutants resistant to this inhibitor have been obtained (Güner *et al.*, 1991; Knaff, 1992). Preliminary analysis indicates that these mutants carry an Asp252Glu mutation in the cytochrome *b* subunit (Park, S.-Y. and Daldal, F., unpublished).These ongoing analyses should further contribute to our understanding of the structure of the Q_i site. Several mutations resulting in Q_i site inhibitor resistance have already been identified in the eukaryotic bc_1 complexes (diRago *et al.*, 1986; Howell *et al.*, 1987; diRago and Colson, 1988; Weber and Wolf, 1988; diRago *et al.*, 1990), and these are also shown in Fig. 4B. Consistent with the expectations of the modified Q cycle mechanism, these mutants are located on the opposite side of the membrane from the Q_o site lesions. Two cytoplasmic regions are implicated as comprising the Q_i site on the basis of these mutants: a

span near the amino terminus including a portion of helix A, and the span running from helix D to helix E (Fig. 4B).

The distribution of both Q_o and Q_i site inhibitor-resistance loci supports the helical arrangement shown in Fig. 5 for the cytochrome *b* subunit.

Site-Directed Mutations

The inhibitor-resistant mutations alter residues thought to be at or in the immediate vicinity of the substrate binding sites (Q_o or Q_i). This interpretation follows the analogous situation of the mutations conferring herbicide resistance to the photosynthetic reaction centers of bacteria and of photosystem II of green plants and algae. The structure of the reaction center, determined by X-ray crystallography, has confirmed that the herbicide-resistant lesions map to the Q_B site, where quinone is reduced (Baciou *et al.*, 1991; Paddock *et al.*, 1991; Sinning, 1992). It is evident, however, that the residues identified by inhibitor resistance of the bc_1 complex cannot be essential to the function of the enzyme, since these mutant bc_1 complexes are still functional. Presumably, residues critical for the function of the Q_i or Q_o sites are spatially near those positions identified by the inhibitor-resistance phenotype. It is also reasonable to expect that residues essential for the function of the Q sites will be highly conserved. The most highly conserved residues are shown in Fig. 4A. These, along with the mutations conferring inhibitor resistance, provide a rational guide for selecting targets for site-directed mutagenesis. Some of the data are summarized in Table I.

Q_o-*Site Mutations*

Most of the work on *Rb. sphaeroides* has focused on the loop connecting helix E and helix F. This region contains the highly conserved PEWY sequence, which is adjacent to Q_o-site inhibitor-resistant loci (see Figs. 4A and B). Most of the mutations in the PEWY residues impair the function of the Q_o site and cause the midpoint potential of cytochrome b_L to be somewhat higher than in the wild type (Crofts *et al.*, 1992). The most dramatic result obtained so far is the 50-fold inhibition of the rate of quinone oxidation, as measured by flash kinetics, when *Glu*295 is replaced by an asparagine. Figure 6 shows the flash kinetics of the mutant, where the rate of reduction of cytochrome b_H in the presence of antimycin is too small to observe over the time range displayed. This mutation blocks Q_o turnover and behaves as if myxothiazol were present. Remarkably, even the slow turnover of the bc_1 complex permitted by this mutation is sufficient to support photosynthetic growth of *Rb. sphaeroides*. *Glu*295 cannot be considered to be essential; replacement by aspartic acid has only a modest inhibitory effect on Q_o function.

By performing the flash kinetics experiments at different solution potentials, the rate of cytochrome b_H reduction as a function of the relative concentration of QH_2 can be determined (Crofts *et al.*, 1992). These data can be simply analyzed to yield K_m and V_{max} values for QH_2 at the Q_o site for each mutant. When *Trp*296 is replaced by phenylalanine, for example, the kinetic effect is due to a reduction in V_{max}, but for other mutation both K_m and V_{max} are influenced by the amino acid substitution (Barquera, Hacker, Crofts, and Gennis, unpublished). These data support the contention that the PEWY region of the cytochrome *b* subunit is intimately involved in the architecture of the Q_o site (Doyle *et al.*, 1989).

Most of the work in *Rb. capsulatus* has been directed at the region connecting helix C and helix D (see Fig. 4) and, in particular, *Gly*158 (Atta-Asafo-Adjei and Daldal, 1991). In this organism, genetic systems have been developed to allow mutations to be expressed either in the normal amount, using a single gene copy, or overproduced by 3–5-fold, using a multicopy vector. These systems allow one to optimize for the differing requirements of physiological, genetic, biochemical, and biophysical characterization of mutant complexes. *Gly*158 was selected for an intensive study to try to correlate the perturbations to the function or the assembly of the bc_1 complex, to the physicochemical properties of the substituting side-chains. A mutation in this position in *Rb. capsulatus* to aspartic acid was originally found as a spontaneous nonphotosynthetic mutant (strain R126) (Daldal *et al.*, 1989). Flash kinetics and EPR spectroscopy data indicate a defect in its Q_o function while its Q_i site is normal (Robertson *et al.*, 1986). A spontaneous mutation at the equivalent position in the mouse bc_1 complex has also been found (Howell and Gilbert, 1988). In this case, the bc_1 mutant is functional but is resistant to myxothiazol.

Fifteen site-directed mutants have been made at this locus in *Rb. capsulatus* and all had fully assembled bc_1 complex with the prosthetic groups incorporated (Atta-Asafo-Adjei and Daldal, 1991). With the exception of alanine and serine, all of the substitutions for *Gly*158 resulted in inactive bc_1 complexes and failed to

Table I. Mutations Constructed at Predicted Quinol Oxidizing (Q_o) and Quinone Reducing (Q_i) Sites

Residue	cytb_L	cytb_H	Photosynthetic growth	Characteristics
			Mutations at the Q_i site *Rb. sphaeroides*	
G48A	−90	40	+++	Turnover indistinguishable from wild type
G48V	−90	23	−	Cyt.b_H oxidation blocked, Q_o site normal
G48D	none	none	−	Complex not assembled, but trace of FeS center
A52V	−90	32	++	Q_i site slowed by 5-fold, antimycin resistant
H217A	−40	50	−	Complex assembled, cyt.b_H oxidation blocked
F244L	none	none	−	Complex not assembled
K251M	−59	47	++	Q_i site slowed by 3-fold, antimycin resistant, normal Q_o site
K251I	−71	42	++	Q_i site slowed by 2-fold, normal at Q_o site
D252N	−59	68	++	Cyt.b_H oxidation inhibited, normal at Q_o site
D252A	−50	48	−	Cyt.b_H oxidation blocked, normal Q_o site
			Rb. capsulatus	
I213L	n.d.	n.d.	++	—
H217L	n.d.	n.d.	−	—
H217D	n.d.	n.d.	++	—
			Mutations at the Q_o site *Rb. sphaeroides*	
F144S	−90	50	+++	Q_o site inhibited by 3-fold, myxothiazol resistant
N279Y	n.d.	n.d.	++	Q_o site inhibited by 10-fold
E295D	−89	75	+++	Q_o site inhibited by 2-fold
E295G	−62	44	+++	Q_o site inhibited by 9-fold
E295Q	−30	64	++	Q_o site inhibited by 50-fold
W296F	−20	60	+++	Q_o site weakly inhibited
W296L	−19	58	++	Q_o site inhibited by 2-fold
Y297F	−92	60	+++	Q_o site weakly inhibited
Y297S	−66	46	++	Q_o site inhibited by 25-fold
			Rb. capsulatus	
G158A	n.d.	n.d.	+++	Myxothiazol resistant
G158S	n.d.	n.d.	+++	Myxothiazol resistant
G158P	n.d.	n.d.	++	Myxothiazol resistant
G158C,D, T,N,E,V,H, L,I,R,Y,W,	n.d.	n.d.	−	—

(Plus many mutations at positions M140, F144, G152 and T163).

support photosynthetic growth. The photosynthetically competent mutants (G158A and G158S) had lower quinol:cytochrome *c* reductase activities, and were resistant to myxothiazol but not to stigmatellin. The overall data indicate, not surprisingly, that the chemical nature of the amino acid sidechain at position 158 is not important, but that size is of crucial importance. The sidechains of either alanine (methyl) or serine (hydroxymethyl) result in hindered access to both the quinol substrate and myxothiazol, leading

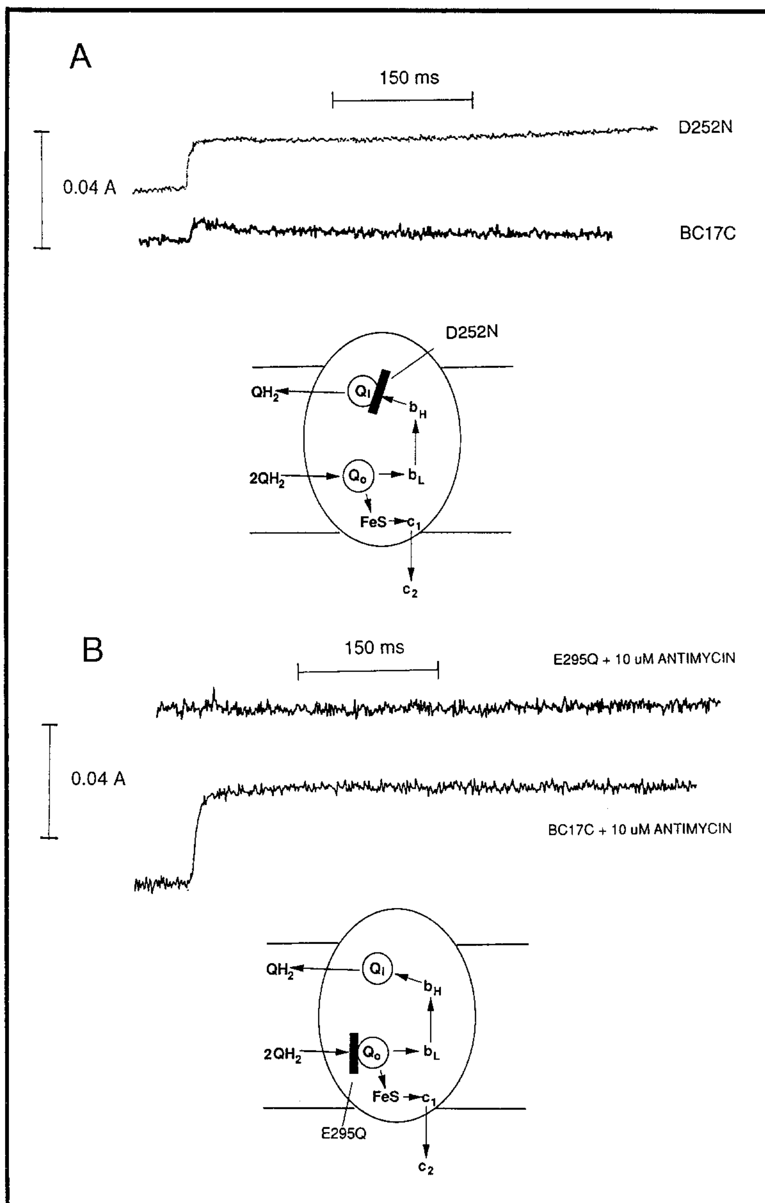

Fig. 6. Flash kinetics of chromatophores showing the redox status of cytochrome b_H for the wildtype control and two mutants. *Asp*252*Asn* effectively blocks the reoxidation of cytochrome *b* but has no effect on the rate of its reduction (A). *Glu*295*Gln* dramatically slows the turnover at the Q_o site, so no reduction of cytochrome b_H is observed on this time scale (B). Taken from (Crofts *et al.*, 1992) and manuscripts in preparation.

to a partially active, myxothiazol-resistant species. Larger sidechains lead to assembled but inactive complexes. Photosynthetically competent revertants obtained from either a leucine or histidine substitution at position 158 yielded a proline substitution (unpublished). The *Pro*158 mutant is functional but myxothiazol-resistant (Atta-Asafo-Adjei and Daldal, 1991).

Similar systematic studies have been carried out on more than 50 different mutations covering positions 140, 144, 152, 163, and 333, which were previously defined as inhibitor-resistant loci (Tokito and Daldal, unpublished). In most cases, unlike the experience with position 158, the mutant bc_1 complexes are photosynthetically competent. However, these mutations have perturbed steady-state quinol oxidation kinetics and altered inhibitor recognition, indicating that they are important for the function of the Q_o site. Detailed analysis of a subset of these mutants by EPR spectroscopy have indicated that the kinetically defective mutants do not have a fully occupied Q_o-site (Ding *et al.*, unpublished).

Q_i-site Mutants

Figure 4B shows the location of several positions

where mutations in the eukaryotic bc_1 complexes confer resistance to Q_i site inhibitors such as antimycin. Conserved residues in the vicinity of these positions are reasonable targets for site-directed mutagenesis to search for important determinants of the Q_i site. Substitutions for either *His*217 or *Asp*252 (Figs. 4 and 5) can have dramatic effects on the function of the Q_i site (Table I). Figure 6 shows the status of cytochrome b_H in a flash kinetics experiment. In a mutant where *Asp*252 has been replaced by asparagine, for example, cytochrome b_H becomes rapidly and fully reduced but no reoxidation is observed (Hacker, Barquera, Crofts, and Gennis, unpublished). This mutant exhibits behavior similar to that observed when antimycin is used to block the Q_i site (Fig. 2). Although antimycin has no additional effect on the flash kinetics observed with this mutant, the inhibitor can be demonstrated to bind the mutant bc_1 complex by observing the spectroscopic perturbation of cytochrome b_H that accompanies inhibitor binding. Substitutions for *His*217 in *Rb. capsulatus* also point to this residue as being extremely important for proper function of the Q_i site.

THE RIESKE FeS PROTEIN

In contrast to the wealth of information concerning the cytochrome *b* subunit, relatively little is known about the Rieske protein, whose function is to oxidize QH_2 at the Q_o site and subsequently pass an electron to cytochrome c_1. The bulk of the protein, with its 2Fe-2S cluster, is exposed to the periplasm, and the subunit is probably anchored to the membrane by a single amino-terminal membrane-spanning helix (Theiler and Niederman, 1991; Van Doren *et al.*, 1992b). The Rieske subunit of *Rb. sphaeroides* can be expressed both in *Rb. sphaeroides* and in *Escherichia coli* in the absence of the other subunits of the bc_1 complex (Van Doren *et al.*, 1992b). The independently expressed subunit contains the FeS cluster and is membrane-bound, although the levels are low and the subunit is very labile under these circumstances. Protease treatment of the *Rb. sphaeroides* bc_1 complex yields a soluble form of the Rieske subunit, which results from cleavage after *Leu*34 (Van Doren *et al.*, 1992b). Within the first 34 residues of the *Rb. sphaeroides* sequence is a single hydrophobic span that could serve both as a signal sequence and as a membrane anchor.

The Ligands to the 2Fe-2S Cluster

The reduced form of the 2Fe-2S Rieske cluster has a distinct electron spin resonance (EPR) spectrum ($g_{x,y,z}$ = 1.80, 1.90, and 2.03, respectively with an average *g* value, g_{av} = 1.91). The redox midpoint potential of the cluster is around +300 mV, which is considerably higher than that of typical ferredoxins. Spectroscopic studies indicate that the cluster is bound to the protein by two nitrogen ligands and two sulfur ligands (Britt *et al.*, 1991; Gurbiel, *et al.*, 1991). Comparison of the sequences of Rieske subunits (Davidson *et al.*, 1992a; Van Doren *et al.*, 1992a) shows two totally conserved histidines and four totally conserved cysteines (Fig. 7). These are located at the carboxyl terminus of the protein within two totally conserved stretches of six residues each: CTHLGC (starting at *Cys*133 in *Rb. capsulatus*) and CPCHGS (starting at *Cys*153 in *Rb. capsulatus*). Further information is obtained by comparing the sequences of the Rieske subunit with the sequences of "Rieske-like" 2Fe-2S proteins associated with benzene and naphthalene dioxygenases (Irie *et al.*, 1987; Kurkela *et al.*, 1988), which have similar spectroscopic properties. As seen in Fig. 7, the sequences have in common the two histidines and two of the four cysteines found in all the Rieske subunits. Based on these considerations, one would expect the four ligands to the FeS cluster to be *Cys*133, *His*135, *Cys*153, and *His*156 (*Rb. capsulatus* numbers).

Mutagenesis results with both *Rb. capsulatus* (Davidson *et al.*, 1992a) and *Rb. sphaeroides* (Van Doren *et al.*, 1992a) are consistent with this assignment. In these species, amino acid substitutions for either of the two conserved histidines results in elimination of the cluster. In *Rb. capsulatus*, this is accompanied by degradation of the Rieske subunit of the bc_1 complex, but in *Rb. sphaeroides*, substitution for *His*131 (equivalent to *Rb. capsulatus His*135) by cysteine results in wild type levels of immunodetectable Rieske subunit and only slight perturbation of the cytochrome components of the bc_1 complex. As a control, *His*159 in *Rb. capsulatus*, which is not highly conserved, was altered by mutagenesis without any detectable effects.

With only one exception, substitutions for any of the four conserved cysteine residues in either *Rb. capsulatus* or *Rb. sphaeroides* eliminates the EPR-detectable cluster. Similar results have also been obtained in a study of the subunit in yeast (Graham and Trumpower, 1991). The one exception is the *Cys*155*Ser*

```
                               129   134                   149 151
                                 G  C DS                     Y S
Rb. sphaeroides bc1        WLVMWGVCTHLGCVPIGGVSGDFGGWFCPCHGSHYDSAGR

Yeast bc1                  WLIMIGICTHLGCVPIGE-AGDFGGWFCPCHGSHYDISGR

Bovine bc1                 WVILIGVCTHLGCVPIAN-AGDFGGYYCPCHGSHYDASGR

Spinach b6f                TFGINAVCTHLGCVVPFN-AAE-NKFICPCHGSQYNNQGR

R. capsulatus bc1          WLVMLGVCTHLGCVPMGDKSGDFGGWFCPCHGSHYDSAGR

                                 R L   R                 R  SL  A
                                 S P   S                 S  GF  S
                               133     F               153  DP  159
                                     138                     T
                                                             Y
                                                            156

                                 *  *                    *   *
nap. dioxygenase ndoB        FLNVCRHRGKTLVSVEAGNAKGFVCSYHGWGFGSNGD

ben. dioxygenase   P3        FLNQCRHRGMRICRADAGNAKAFTCSYHGWAYDTAGN
```

Fig. 7. Sequence comparison of a portion of the Rieske subunit from several sources, along with the sequences of benzene and naphthalene dioxygenases, which have Rieske-like 2Fe-2S clusters. Conserved residues are shown in bold residues, and the asterisks indicate the probable cysteine and histidine ligands to the FeS cluster. Mutants which have been examined in either *Rb. sphaeroides* or *Rb. capsulatus* are indicated by letters adjacent to the appropriate sequences. Sequences are taken from Beckmann *et al.*, (1987), Davidson and Daldal (1987b) Irie *et al.* (1987), Schägger *et al.* (1987), Steppuhn *et al.* (1987), Kurkela *et al.* (1988) and Yun *et al.* (1990).

mutant in *Rb. capsulatus*, in which very low levels (5%) of the cluster are observed relative to the wild type control (Davidson, *et al.*, 1992a). This suggests that *Cys*155 is not a ligand, consistent with the sequence comparison with the dioxygenases (Fig. 7).

It is striking that the mutagenesis of the two cysteines that are not assigned as metal ligands (*Cys*138 and *Cys*155 in *Rb. capsulatus*) results in such a drastic decrease of the steady-state levels of the Rieske subunit in chromatophore membranes (Davidson *et al.*, 1992a). Possibly, these two cysteines form a disulfide bond that is important for the conformation of the Rieske subunit and/or the quinol oxidation site. In *Rb. sphaeroides*, *Gly*133 (equivalent to *Gly*137 in *Rb. capsulatus*), which is adjacent to one of the non ligand cysteines, has been converted to an aspartic acid (Van Doren *et al.*, 1992a), mimicking a mutant previously characterized in yeast (Gatti *et al.*, 1989). The resulting bc_1 complex is assembled and supports photosynthetic growth. However, flash kinetics shows that the turnover of the Q_o site is 20-fold lower than that of the wild type strain.

The compilation of these data support the model placing this portion of the Rieske subunit at the Q_o site where quinol is oxidized. Furthermore, the identification of the four metal ligands, plus constraints from spectroscopic studies (Salerno, *et al.*, 1979; Prince, 1983; Gurbiel *et al.*, 1989; Britt *et al.*, 1991; Gurbiel *et al.*, 1991), directs molecular modelling of this unusual 2Fe-2S cluster (Davidson *et al.*, 1992a).

THE CYTOCHROME c_1 SUBUNIT

Whereas the Rieske subunit appears to be attached to the membrane by a hydrophobic helical span near the amino terminus of the polypeptide, the cytochrome c_1 subunit is attached by a single transmembrane span near the carboxyl terminus (Konishi *et al.*, 1991). The hydrophilic domain that precedes this anchor contains a highly conserved consensus sequence (CxyCH) for binding heme C in *c*-type cytochromes. This mode of attachment to the membrane was first indicated for the *Neurospora* subunit by limited proteolysis experiments (Li, *et al.*, 1981). More recently, site-directed mutagenesis converted *Gln*228 in the *Rb. sphaeroides* subunit to a stop codon (Konishi, *et al.*, 1991). This truncates the subunit just prior to the putative membrane-spanning helix and results in a soluble form of cytochrome c_1 exported to

the periplasm. This was expected, because cytochrome c_1 has a cleaved signal sequence located at the amino terminus which would be expected to direct the subunit across the cytoplasmic membrane up to the stop-transfer signal provided by the hydrophobic span near the carboxyl terminus (Yun *et al.*, 1990). The truncated form, lacking this attachment, is left as a soluble cytochrome in the periplasm. This experiment confirms the topology of the cytochrome c_1 subunit as well as its mode of membrane attachment.

The redox and spectroscopic properties of the soluble cytochrome c_1 are very similar to those of the subunit when it is part of the bc_1 complex. The identification of the residues which ligate the heme iron has been addressed by recent mutagenesis studies in *Rb. capsulatus* (Gray *et al.*, 1992). By analogy with other *c*-type cytochromes, the two cysteines in the consensus sequence (CxyCH) are expected to covalently link the heme macrocycle to the protein through thioether bonds to the porphyrin vinyl groups, and the histidine contributes one of the ligands to the iron via the N3 imidazole nitrogen. The high midpoint potential ($E_{m,7}$ 300 mV) and magnetic circular dichroism spectroscopy suggest histidine-methionine ligation for the heme iron (Simpkin *et al.*, 1989; Gadsby and Thomson, 1990). Only one methionine is conserved in all the available sequences of cytochrome c_1, *Met*183 (*Rb. capsulatus* mature sequence numbering). Changing this residue to a leucine results in an inactive bc_1 complex which contains all the prosthetic groups. The midpoint potential of cytochrome c_1 is, however, drastically lowered to about -70 mV. All of the effects observed in the M183L mutant can be accounted for by the removal of a methionyl sulfur from the ligation sphere of the heme in this mutant. By contrast, *Met*205 is not conserved to nearly the same degree, and changing this to a valine has no measurable effect on the bc_1 complex (Gray, *et al.*, 1992). It is concluded that if the sixth axial ligand in the cytochrome c_1 subunit is a methionine, then it would be *Met*183.

SUMMARY AND FUTURE DIRECTIONS

Recent advances in studying the bc_1 complexes of photosynthetic bacteria have taken advantage of the ability to utilize the techniques of modern molecular genetics, but have built on the rich history of biophysical characterization of the photosynthetic apparatus of these organisms. New information concerning each of the three metal-containing subunits has been obtained. A variety of approaches has defined the topologies of the three subunits, experimentally indicating the number and approximate locations of the membrane-spanning helices. Site-directed mutagenesis has been useful to define the amino acids ligating to each of the metals in the bc_1 complex, confirming, in several instances, conclusions reached by amino acid sequence comparisons.

The success of using site-directed mutagenesis has been greatly assisted by the patterns of amino acid conservation which are evident from alignments of the sequences available from many species. In addition, the spontaneous mutations conferring resistance to inhibitors of the bc_1 complex has been an indispensable guide. The enormous amount of information obtained from the study of all of these mutants is compatible with the model implied by the modified Q cycle, with two distinct, physically separable Q sites. Mutations in the cytochrome *b* subunit which specifically effect the Q_i site are all located on the cytoplasmic side of the membrane. Mutations which specifically effect the Q_o site are all on the periplasmic side of the complex, either within the cytochrome *b* subunit or within the Rieske subunit. Mutations have been obtained in which the Q_o site is nonfunctional, but the Q_i site is intact and vice versa (Robertson, *et al.*, 1986; Yun *et al.*, 1991a), pointing out the remarkable degree of independence of these two sites. In the near future, the role of specific amino acid residues at both the Q_i and Q_o sites will be explored by the systematic mutational analysis. It should be possible to identify residues influencing substrate binding or the interaction with catalytic intermediates of the movement of protons into or out of the Q sites.

Both site-directed and random mutagenesis have contributed to developing three-dimensional models of the cytochrome *b* subunit (Brasseur, 1988; Crofts *et al.*, 1992). Aspects of such models are testable by additional mutagenesis experiments, leading to an iterative refinement protocol. Hopefully, at some point in the future these models can be compared to a true high-resolution structure obtained by crystallographic methods.

Another important direction in the near future is the exploration of how the subunits of the bc_1 complex interact with each other. The interface between the cytochrome *b* and Rieske subunits (Robertson *et al.*, 1990; Ding *et al.*, 1992), comprising the Q_o site, is of particular interest. Surprisingly, some mutants lacking the rieske subunit retain both the cytochrome *b* and c_1

subunits, suggesting that these two subunits form a stable unit (Davidson *et al.*, 1992b). This is consistent with biochemical studies of the bovine complex from which the Rieske subunit has been dissociated (Brandt *et al.*, 1991; Gonzalez-Halphen *et al.*, 1991). In contrast, mutants lacking the intact cytochrome c_1 subunit have greatly reduced levels of both the Rieske subunit and the cytochrome *b* subunit (Konishi *et al.*, 1991; Davidson *et al.*, 1992b). The requirement of an intact cytochrome c_1 subunit to stabilize the complex is indicated by other observations with bacterial bc_1 complexes (Gerhus *et al.*, 1990).

There are many unanswered questions that remain concerning the assembly, structure, and catalytic mechanism of the bc_1 complex. The range of experimental techniques available for examining the complexes of the photosynthetic bacteria ensure that studies utilizing these systems will continue to make a considerable contribution in the future.

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