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Structure and function of the *bc*-complex of *Rhodobacter sphaeroides*

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The ubiquinol : cytochrome c_2 oxidoreductase (*bc*-complex) of *Rhodobacter sphaeroides* has three main subunits, which bear the prosthetic groups, and contribute to three catalytic sites and internal electron transfer pathways which define the modified Q-cycle mechanism. In this paper, we report on progress in modelling the structure of the *bc*-complex, and experiments using site directed mutagenesis and biophysical assay to probe the structural and function consequences of specific modifications to these subunits.

Model of cytochrome *b*

Fig. 1 shows a preliminary model of the cytochrome (cyt) *b* subunit. In A, the structure shows six (out of eight predicted) transmembrane helices (A–F), two amphipathic helices (ab and cd), the two heme groups (cyt b_h and b_l), and two ubiquinone molecules identifying the two quinone reactive catalytic sites. In B (Q_R-(quinone reducing) site) and C (Q_O-(quinol oxidizing) site), the quinone processing sites are shown in greater detail. The following constraints were used in building the model:

(1) The primary sequence as previously reported [1].

(2) Conserved hydrophobic spans which identify putative membrane spanning helices [2–4]. These spans are also identified on the basis of profiles using probability parameters from the distribution of residues in known or predicted membrane helices [5], or in buried helices identified in known structures in the Brookhaven Protein Data Bank [6].

(3) Spans showing conserved amphipathy at the helical repeat (hydrophobic moment) [4,7–9]. The identification of helix cd as an amphipathic helix was of importance in suggesting removal of this span from the membrane, to convert the original 9-helix model [2,3] to a structure containing 8 transmembrane helices, A–H [4,9]. Conserved amphipathic spans lie at the N-terminal end (a helix), between helices A and B (ab helix), C and D (cd helix), and possibly between E and F (ef helix).

(4) A pattern of conservation of residues suggesting a helical motif (mutability moment) [9–12]. The mutability moment shows a vector such that in membrane helices at the lipid/protein interface, the unconserved face is the more hydrophobic face, and faces the lipid. In more amphipathic helices suggested to be at the protein water interface, the conserved face is the more hydrophobic face. This complementary pattern of mutability and hydrophobic moment provides a strong indication of the orientation of these helices at the protein/lipid or protein/water interface. Helices A and ab show strong mutability moments; several of the other membrane helices show at their ends strong moments for both hydrophobicity and mutability; helices a and cd show weaker mutability moments.

(5) The topological organization with eight transmembrane spans has been strongly supported by our *pho*A fusion experiments [13].

(6) Four conserved histidines, two each in helices B and D, which are spaced 14 residues apart so as to fall on the same side of the helix, have been suggested as likely ligands for the two heme groups [2,3]. Site-directed mutagenesis of these residues has demonstrated which histidines ligate which hemes [14].

(7) Four conserved glycines, 2 each in helices A and C, spaced like the liganding histidines 14 residues apart, are suggested to accommodate the packing needs of the hemes [15]. In support of this suggestion, in helix

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A, which shows a strong mutability moment, and in helix C, the glycines are on the conserved face of the helix (Fig. 1).

(8) Inhibitor resistance mutations. An important feature leading to support of the 8-helix model was the mapping of lesions giving rise to resistance to inhibitors at the two quinone processing catalytic sites. In the 8-helix model, residue changes conferring resistance to inhibitors (diuron, antimycin, HQNO) acting at the Q_R-site all fall on the N- (protochemically Negative) side identified by the location of the heme of cyt b_h, while those giving resistance to inhibitors (stigmatellin,

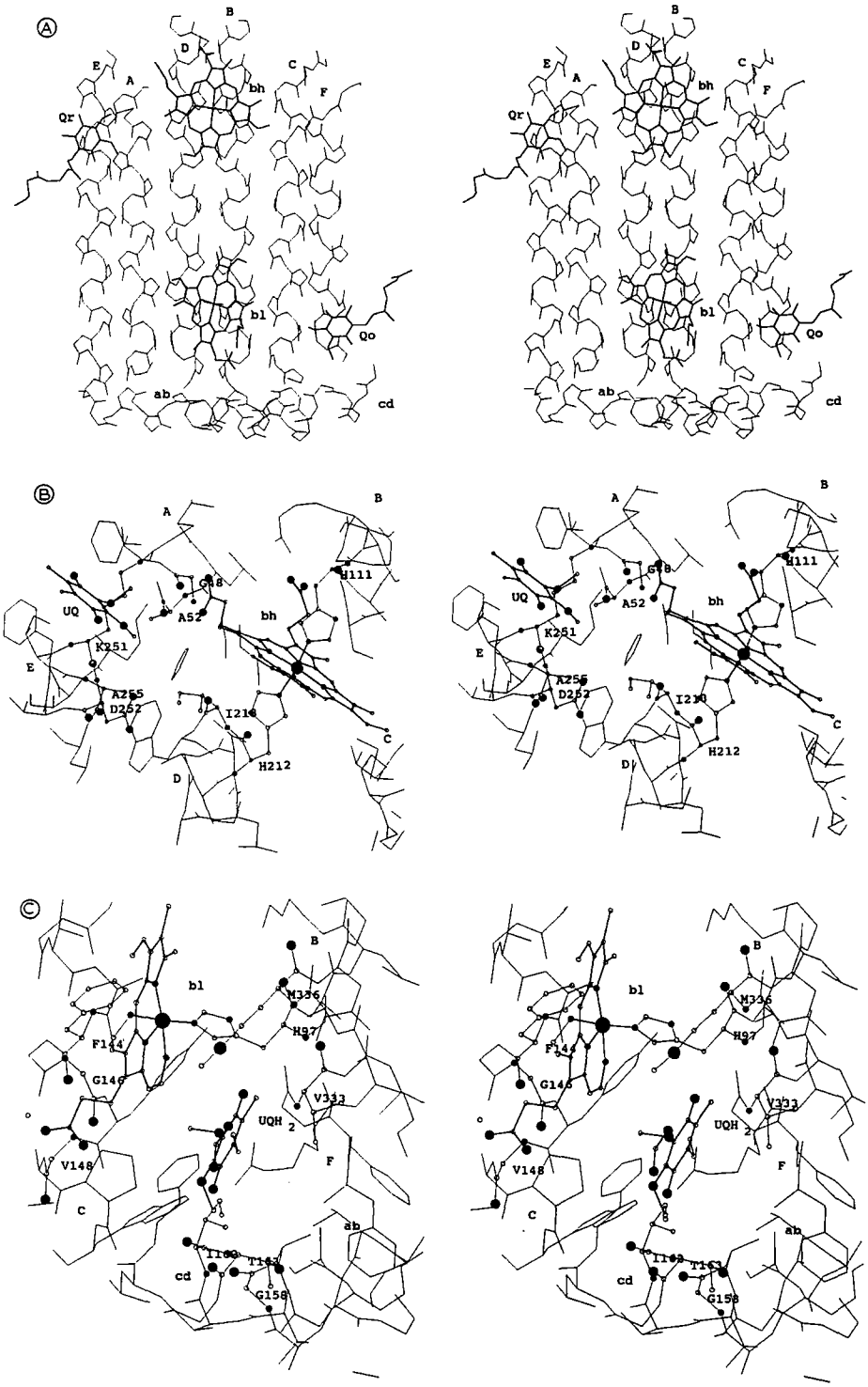

Fig. 1. Stereo views of a preliminary model of the cytochrome *b* subunit. (A) Six transmembrane helices (A–F) (from eight predicted), two amphipathic helices (ab and cd), the two heme groups cytochrome b_h and b_l and two ubiquinone molecules identifying the two quinone reactive catalytic sites. (B) Q_R in greater detail. (C) Q_O in greater detail.

myxothiazol, mucidin) acting at the Q_O-site fall on the P-side [16–20], close to cyt b_l. In helices A and C, the lesions conferring resistance to inhibitors are rotated around the helical axis with respect to the conserved glycines, so as to come at the predicted protein/lipid interface.

(9) Specific mutagenesis.

(a) In reaction centers from several bacteria, and photosystem II, mutations giving rise to resistance to herbicides have all mapped to the Q_B-site (where quinone is reduced) blocked by these inhibitors. This pattern could be used to infer that similar mutations in cyt *b* would identify residues which contribute to the catalytic sites at which the affected inhibitors bind [4]. Work on mutagenesis of residues thought to contribute to the Q_O-site in *Rb. capsulatus* by Daldal's group [20], and characterization of these mutant strains by Robertson, Dutton and colleagues [21] has been of importance in defining this site. Our own work exploring both Q_O- and Q_R-sites in *Rb. sphaeroides* is summarized in Table I, and discussed below, and we have reported similar work on mutagenesis of conserved residues elsewhere [14,22].

Site-directed mutagenesis of residues at predicted quinone processing catalytic sites

Although much work has been described on inhibitor resistance of spontaneous mutants, more detailed characterization of the effects on kinetic parameters has only been reported for mutants in photosynthetic bacteria [1,14,20–22]. We have extended our own work in this area to cover mutations in the loop connecting E and F helices which contains the highly conserved -PEWY- span (Table I). Preliminary characterization of these strains shows that non-conservative changes in the PEWY region lead to modifications of quinol oxidation and the binding of inhibitors specific to the Q_O-site, suggesting a role in catalysis or structure. Surprisingly, none of the mutants was completely devoid of activity, and none failed to assemble.

In contrast to Q_O-site mutations, those constructed around the putative Q_R-site showed a wider range of effects. No stable spontaneous mutations giving rise to resistance at this site have previously been reported in the photosynthetic bacteria. The K251M change mimics a mutation found to confer antimycin resistance in yeast. Our strain showed a slowed oxidation of cyt b_h, as seen in the kinetics of cyt b_h turn-over or the slow phase of the carotenoid change in the absence of antimycin, but no dramatic change in antimycin titre. However, the shape of the titration curve suggested a weaker affinity. The K251I mutant showed similar kinetic effects but no antimycin resistance. Mutants at D252 showed a much more marked block, and behaved as if constitutively inhibited by antimycin. In D252N, a slow antimycin sensitive turn-over could be detected from electrochromic measurements, but D252A was more strongly affected. Both showed weak antimycin resistance. The first three strains could grow photo-

TABLE I

Mutations constructed at predicted quinol oxidizing (Q_O) and quinone reducing (Q_R) sites

Mutations at the Q_R-site				
Residue	cyt b_l	cyt b_h	Photosynth.	Characteristics
G48A	−90	40	+ + +	turn-over indistinguishable from WT
G48V	−90	23	−	complex assembled, electron transfer through Q_l-site inhibited, normal at Q_O-site
G48D	none	none	−	complex not assembled, but trace of FeS center
A52V	−90	32	+ +	complex assembled, electron transfer through Q_l-site slowed by 5, weak antimycin resistance
A52D	none	none	−	complex not assembled
H217A	−40	50	−	complexed assembled, electron transfer through Q_l-site partly inhibited
F244L	none	none	−	complex not assembled
K251M	−59	47	+ +	electron transfer through Q_l-site 2-fold slower, weak antimycin resistance, normal Q_O-site
K251I	−71	42	+ +	electron transfer through Q_l-site 2-fold slower, no antimycin resistance, normal Q_O-site
D252N	−59	68	+ +	electron transfer through Q_l-site 100-fold slower, weak antimycin resistance, normal at Q_O-site
D252A	−50	48	−	electron transfer through Q_l-site inhibited, weak antimycin resistance, normal Q_O-site
Mutations at the Q_O-site				
Residue	cyt b_l	cyt b_h	Photosynth.	Characteristics
F144S	−90	50	+ + +	electron transfer at Q_O-site inhibited by 3, myxothiazol resistant
N279Y	−140	30	+ +	electron transfer at Q_O-site inhibited by 10, stigmatellin and myxothiazol resistant
E295D	−89	75	+ + +	electron transfer at Q_O-site inhibited by 2, stigmatellin resistant
E295G	−62	44	+ + +	electron transfer at Q_O-site inhibited by 9
E295Q	−30	64	+ +	electron transfer at Q_O-site inhibited by 50, stigmatellin resistant
W296F	−20	60	+ + +	electron transfer at Q_O-site uninhibited, no inhibitor resistance
W296L	−19	58	+ +	electron transfer at Q_O-site inhibited by 2, stigmatellin and myxothiazol resistant
Y297F	−92	60	+ + +	electron transfer at Q_O-site uninhibited
Y297S	−66	46	+ +	electron transfer at Q_O-site inhibited by 25

synthetically, although at reduced rate in some cases. In all strains assembling a complex containing cyt b_h, redox titrations showed the high potential form, cyt b_{150} ($E_{m,7} = 150$ mV in the absence of antimycin). Addition of antimycin induced oxidation of cyt b_{150} in all strains, even those with strongly inhibited turn-over at the Q_R-site. Antimycin induced oxidation showed the largest amplitude in strains H217A, K251M and K251I, and was also larger in D252A and D252N than in wild-type. This could indicate over-stabilization of Q^- at the site (see Ref. 23 for suggested mechanism).

Discussion

The constraints on the model discussed above provide limitations on the general topology of the cyt *b* subunit. For six of the transmembrane helices, A–F, choices among alternative arrangements for packing the structure around the liganding helices B and D depend on the weight attached to the suggested role of the conserved glycines (Ref. 15 and item 7 above). Tertiary models of the helices showing distribution of the inhibitor resistance lesions define the catalytic sites, but with reference to the packing of helices A and C against the hemes. Support for the orientation shown comes from our characterization of strains with specific mutations of G48 (G33 in yeast) (see Table I), which show that only residues of small volume are tolerated. The location of the Q_R-site is suggested by mutations at A52 (G37 in yeast, conferring antimycin resistance) and from the L198F mutation in yeast which confers funiculosin resistance. This residue is next to H197, which is one of the ligands to cyt b_h, and its position is therefore defined with respect to the heme and membrane planes. Both lesions suggest a location for the Q_R-site as shown in Fig. 1. We are presently exploring the roles of the other conserved glycines, I213 (equivalent to L198 in the *Rb. sphaeroides* sequence), and other residues shown as projecting into the volume of the Q_O- and Q_R-sites in the model. Examination of the pattern of conserved residues on the surface of the model suggests possible sites for interaction with other subunits of the protein, or in dimeric association.

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