Progress to determine whether p-ISP is converted to i-ISP in the yeast bc\textsubscript{1} complex, and whether the 22 amino acid presequence removed by the yeast MPP protease may be retained as a small subunit in the yeast bc\textsubscript{1} complex, analogous to the situation in bovine bc\textsubscript{1} complex.


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**Topographical distribution of redox centres and the Q\textsubscript{a} site in ubiquinol–cytochrome-c oxidoreductase (Complex III) and ligand structure of the Rieske iron–sulphur cluster**

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**Introduction**

Ubiquinol–cytochrome-c oxidoreductase, which is also known as the bc\textsubscript{1} complex, contains cytochromes b\textsubscript{11}, b\textsubscript{1}, and c\textsubscript{1}, and a 2Fe–2S cluster (generally known as the Rieske iron–sulphur cluster). Physicochemical properties of these redox components have been intensively studied by many investigators [1–3]. To date, the modified Q-cycle model [4, 5] has been most generally accepted as the mechanism of electron and proton transfer in the cytochrome bc\textsubscript{1} complex.

Two essential features of the modified Q-cycle model [4, 5] are: (i) the presence of two separate quinone reaction sites – Q\textsubscript{a} (also designated as

Q\textsubscript{a} or Q\textsubscript{b}) and Q\textsubscript{i} (also named as Q\textsubscript{a}, Q\textsubscript{i} or Q\textsubscript{c}), which are each in equilibrium with the aqueous phase, on opposite sides of the membrane; and (ii) the occurrence of an electrogenic transmembranous electron transfer between them [6–8] (see Figure 1). At the Q\textsubscript{a} site, two electrons from quinol are split [4, 5]; the first electron reduces the Rieske iron–sulphur cluster in the high-potential arm, while the second electron cycles back along the low-potential arm via cytochromes b\textsubscript{11} and b\textsubscript{1} to regenerate ubiquinol at the Q\textsubscript{i} site [4–8]. The cytochrome b and the Rieske iron–sulphur apoproteins form the Q\textsubscript{a} pocket in close association [6–9]. The Q\textsubscript{a} site does not favour the formation of a thermodynamically stable semiquinone, and it is specifically inhibited by various Q\textsubscript{a} inhibitors as exemplified by myxothiazol, 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), or stigmatellin [10]. The site of quinone reduction (Q\textsubscript{a} site) has been shown to contain a thermodynamically stable semiquinone species (Q\textsuperscript{a}\textsuperscript{−} at neutral pH) which is completely destabilized by the specific Q\textsubscript{a} inhibitor, antimycin [11, 12]. This Q\textsuperscript{a}\textsuperscript{−} species catalyses the net reduction of Q to
Figure 1
Schematic illustration of the modified Q-cycle mechanism

Cytochrome bc
Complex

QH
2
2H+

Q
QH
pool

2Q

2H+

N-side

P-side

Spatial organization of redox centres and the two Q reaction sites in the bc
complex

Knowledge of the spatial organization of the redox active centres, of the multi-subunit complexes in the mitochondrial membrane, provides a basis for understanding electron and proton transfer processes. To date, high resolution structural information on respiratory chain complexes of mitochondria or bacteria is still not available, although three-dimensional crystals of cytochrome bc
complex have been reported [13-15]. In our study, the positions of paramagnetic centres were measured through the spin relaxation enhancement effect induced by external paramagnetic probes having extremely fast spin relaxation rates [16, 17]. The spin relaxation rate is obtained as the half-saturation parameter, \( P_{1/2} \), which is measured by progressive microwave power saturation using a cryogenic electron paramagnetic resonance (e.p.r.) technique. Under our e.p.r. conditions, \( P_{1/2} \) is proportional to the concentration of the spin probe as reported previously [16]. The \( \Delta P_{1/2} \) (mW/mM probe) value is converted to the effective distance using a high potential iron–sulphur protein (HiPIP)-type [4Fe–4S] globular protein with a known X-ray structure as a model [18]. The effective distance obtained by this method reflects the \( r^{-6} \)-weighted average distance of all randomly distributed probe molecules. Thus, the ratio of \( \Delta P_{1/2} \) between a paramagnetic intrinsic component and its counterpart of the standard varies with the \(-3\) to \(-4\)th power dependence on the ratio of their effective distances, depending on the shape of the system being analysed. In general, the effective distance of an intrinsic component is somewhat longer than the closest distance from the barrier surface of the probes [19-21]. This paramagnetic probe technique is a direct, but low-resolution method because of the use of simplified model systems. However, it is a useful method to obtain topography of multiple redox centres relative to the membrane, especially when the effective depth can be compared from the P- and N-side surfaces of the membrane, as in the case of haem \( b_H \) and Rieske iron–sulphur cluster [21]. This technique was applied to the reconstituted bovine heart bc
proteoliposome, with probes added to the outside medium, or trapped inside the vesicles, at varying concentrations. Using dysprosium complexes (very strong magnets with total angular momentum, \( J=15/2 \)) as the probe, the location of cytochrome \( b_L \) haem has been estimated to be approximately equidistant from both surfaces of the membrane phospholipid bilayer (see Figure 1). The Rieske iron–sulphur cluster has been assigned to be near the P-side surface of the membrane [21]. The \( g=3.8 \) signal of \( b_L \) haem exhibits an extremely fast intrinsic spin relaxation closest to that of the probe complexes. Thus, the assignment of cytochrome \( b_L \) membrane topography using this paramagnetic probe technique is the least reliable among redox components of the bc
complex. In addition, its spin relaxation enhancement could be detected only from the P-side surface of the membrane. However, we revealed that the spin relaxation of the Rieske iron–sulphur cluster is enhanced by both \( c_1 \) and \( b_H \) spins but not by the \( b_L \). We
Therefore assigned the locations of the Rieske cluster, \( b_1 \) and \( c_1 \) haems, as well as the \( Q_o \) site, near the P-side surface of the membrane. By selectively measuring the antimycin-sensitive portion of the semiquinones \( g = 2.00 \) e.p.r. signal in *Rhodobacter capsulatus* chromatophores, the effective distance of the \( Q_o \) site was estimated. To ensure the necessary accuracy of these measurements, we devised a standard which closely approximated the spin relaxation profile of the organic free radical of \( Q_{10}^- \) [19]. We used the nitroxide free radical of the doxyl spin label which has been incorporated at known positions along the fatty acid hydrocarbon tail as a model system. For the analysis of free radicals, a Holmium complex (\( J = 7/2 \)) was chosen as a suitable extrinsic paramagnetic probe. The location of the \( Q_{10}^- \) species was found to be 6–10 Å from the N-side surface of the lipid bilayer [19]. Consistent with this result is the electron nuclear double resonance (e.n.d.o.r.) analysis which reported \( Q_{10}^- \) to be approximately 6 Å from the exchangeable protons within the mitochondrial \( bc_1 \) complex [22]. These findings are in agreement with the data obtained using carotenoid bandshifts [6, 7], where the individual distances of \( b_1 - b_4 \) and of \( b_1 - Q \) represent about half of the membrane dielectric (see also [23]). Combining these independent lines of spectroscopic data for the spatial organization of the redox centres, supports the notion that the transmembrane charge separation in the \( bc_1 \) complex is driven by electron transfer from the \( Q_o \) to the \( Q_e \) site via two \( b \) haems. It should be pointed out that the aforementioned information predicts that the two \( b \) haems are not symmetrically placed (relative to the centre of the phospholipid bilayer), in contrast to the initial and revised models derived from the predicted secondary structure based on the primary sequence of the cytochrome \( b \) polypeptide [24–27].

**Histidine ligands to the Rieske cluster and their possible role in the \( Q_e \) site**

Recent spectroscopic studies of the \( Q_e \) site inhibitor-resistant mutants [28–30] have revealed considerable structural analogy between the cytochrome \( b \) domains of the \( Q_e \) pocket [31] and that of the \( Q_A \) and \( Q_B \) pockets of the bacterial reaction centre whose high resolution X-ray crystallographic structure is known [32–34]. In contrast to the wealth of information obtained concerning the cytochrome \( b \) domain, no structural information on the Rieske domain of the \( Q_e \) site became available utilizing inhibitor-resistant mutants, because no \( Q_e \) site inhibitor-resistant mutations were mapped in the Rieske protein. New information on the molecular architecture of the Rieske domain of the \( Q_e \) site has arisen from biophysical analyses of the Rieske-type and Rieske iron–sulphur proteins as well as from induced and site-directed mutagenesis studies of Rieske iron–sulphur proteins, as summarized below.

The Rieske iron–sulphur cluster differs greatly from typical ferredoxin-type [\( 2Fe-2S \)] clusters in regard to its unique e.p.r. spectral line shape and its unusually high redox mid-point potential [1–4, 35]. The first experimental evidence suggesting the involvement of at least one non-sulphur ligand in the Rieske-type protein, isolated from the *Thermus thermophilus* HB-8 membranes, was reported in 1984, based on the chemical, e.p.r., Mössbauer, and resonance Raman analyses [36]. Subsequently, X-band e.n.d.o.r. and electron spin-echo envelope modulation (e.s.e.e.m.) analysis detected signals arising from nitrogen ligands in the Rieske-type protein of *Pseudomonas cepacia* phthalate dioxygenase (PDO) as well as in the Rieske [\( 2Fe-2S \)] cluster of yeast \( bc_1 \) complex [37, 38], but the number of nitrogen ligands was not established. More rigorous e.n.d.o.r. analysis (X-band as well as Q-band) of the Rieske-type [\( 2Fe-2S \)] cluster of *P. cepacia* PDO, using uniformly or selectively \( 14N \)- or \( 15N \)-labelled enzymes, demonstrated definitively that two distinct nitrogen ligands co-ordinate the [\( 2Fe-2S \)] cluster [39]. This contrasts with classical ferredoxin-type [\( 2Fe-2S \)] clusters in which all ligation is provided by the sulphur of cysteine residues [40]. By combining Mössbauer and resonance Raman data [36], it was also concluded that both histidines are directly co-ordinated to the \( Fe^{2+} \) site of the cluster and that the geometry of ligation at \( Fe^{2+} \) is approximately tetrahedral [39]. The iron-ligating nitrogen was found to be \( \delta N \), as in the case of superoxide dismutase but contrary to the fifth haem liganding histidine [41]. This line of work was extended to the ligand structure of the Rieske iron–sulphur cluster in the \( bc_1 \) complex utilizing the \( 14N \)- as well as the \( 15N \)-enriched *Rb. capsulatus* system combined with the Q-band e.n.d.o.r. technique [42]. The results demonstrated that two nitrogen ligands also co-ordinate with the respiratory chain Rieske iron–sulphur cluster [42]. E.s.e.e.m. data obtained with the Rieske iron–sulphur cluster in various systems reached the same conclusions [43].

Recent studies of amino acid sequence homology of respiratory chain Rieske iron–sulphur proteins from more than 19 different sources revealed two universally conserved regions, which contained four cysteine and two histidine residues as potential ligands and were in close proximity to
the C-terminus (see [44] and refs. therein). These two regions, namely (133/129CTHLGC)--(153/148CPCHG), were designated Box I and II, respectively. The residue numbers were from the Rieske protein sequence of *Rb. capsulatus*/*Saccharomyces cerevisiae*. As a first genetic approach to determine the ligand residues of the iron-sulphur cluster, Gatti et al. [45] isolated 11 respiratory-deficient yeast mutants with a single amino acid replacement in the Rieske apoprotein. An important piece of information, which came from analysis of this limited number of mutants, was that within the flexible loop domain of the C-terminal region containing Boxes I and II, mutations of non-ligand amino acid residues often resulted in diminished electron transfer activity, diminished intensity of e.p.r. active Rieske cluster and lowered its mid-point potentials. In contrast, a mutation that occurred on the ligand candidate residue resulted in no detectable iron-sulphur signal and no electron transfer activity even in the presence of 10% apoprotein. In this study, the first Cys residue in Box II was proposed as one of the ligands [45].

In a subsequent site-directed mutagenesis approach, we used *Rb. capsulatus* chromatophores which harbour genetically overexpressed (by 5–10-fold) *bc*$_1$ complex [46]. Each of the six ligand candidate residues was placed with one of several alternate residues by site-directed mutagenesis. All of the strains containing altered Rieske protein were found to be photosynthetically non-competent (Ps$^−$). Therefore, they were grown aerobically in the dark. By probing with a highly sensitive polyclonal antibody procedure, we detected low levels of Rieske apoproteins in at least one of each of the site-directed mutants of Cys-138, Cys-153, Cys-155, and His-156 (numbers in *Rb. capsulatus* sequence) residues. We detected about a 5% level of both apoprotein and of e.p.r. signals of the Rieske cluster in the C155S mutant. Using appropriate e.p.r. conditions, we could detect, with reasonable accuracy, a Rieske signal even at 1% that of the wild-type overproducer. This mutant protein exhibits a somewhat modified e.p.r. feature and shows a mid-point potential lowered by about 120 mV. This mutant also does not sense the redox states of the Q pool as in the case of the yeast G133D mutant [45]. Thus, the second Cys in Box II is disqualified as a ligand of the iron–sulphur cluster. C155S mutation, however, affects the stability of the Rieske protein in the *bc*$_1$ complex and also the integrity of the Q$_O$ site.

In Box I, neither Cys-133 nor His-135 gave rise to any detectable Rieske apoprotein assembled into the *bc*$_1$ complex, and only the C138S mutant showed an extremely low and variable level of the iron–sulphur cluster and apoprotein. To circumvent gross modification of the *bc*$_1$ complex in the

**Figure 2**

**E.p.r. spectra of toluene-permeabilized wild-type and C138S mutant Rb. capsulatus cells**

Both cells were suspended in 50 mM 3-[N-Morpholino]propanesulphonic acid (Mops) containing 0.1 M KCl and 1.2 mM EDTA (pH 7.0). (a) Cytochrome *bc*$_1$ overproducer wild-type (MTO-40/M+MT-RBC1) cells (35.3 mg protein/ml) were reduced with 10 µM phenazine methosulphate and 5 mM ascorbate. (b) As for (a) plus stigmatellin (50 µM). (c) The cytochrome *bc*$_1$ overproducing mutant (C138S/M+MT-RBC1) cells (76 mg protein/ml) were reduced with 10 µM phenazine methosulphate (PMS) and 5 mM dithionite. E.p.r. conditions: microwave power, 20 mW; modulation amplitude, 1.25 x 10$^{-2}$ T; time constant, 0.128 s; sample temperature, 17 K.
Mitochondria

Electron Transport Inhibitors

C138S mutant to its apparent extreme instability, we have recently analysed the Rieske signal directly in toluene-permeabilized cells of this mutant, and compared it with its wild-type counterpart. In both cases, the bc1 complex was genetically over-expressed, as before. As shown in Figure 2 (spectrum a), the ascorbate-reduced wild-type cells show an intense Rieske iron-sulphur signal with a g

line shape which senses the redox state of the Q pool. Upon treatment with stigmatellin, the resonance feature is sharpened with a small shift at both g

and g

transitions (spectrum b). In contrast, a very small Rieske e.p.r. signal is seen in C138S mutant cells only after reduction with dithionite, indicating its greatly lowered E

value (spectrum c). Rieske line shape of this mutant resembles that of the cluster in C155S mutant [44]. Likewise, stigmatellin did not affect the e.p.r. line shape. Thus Cys-138 was also suggested as a non-ligand residue. These mutant data favour Cys-133 and His-135 of Box I, and Cys-153 and His-156 of Box II as ligand residues of the Rieske iron–sulphur cluster [44]. The proposed ligand assignment of the Rieske cluster is supported by the full conservation of these four residues with counterpart residues of the bacterial mono- and dioxygenase proteins, which have an iron–sulphur cluster with e.p.r. characteristics similar to those of the Rieske iron–sulphur proteins [44, 47]. In contrast, neither Cys-138 nor Cys-155 are conserved.

An important outcome which has emerged from the aforementioned Rieske mutation studies [44, 45] is that some non-ligand mutants of Box I and II region exhibit g

e.p.r. signature with no response to the different redox state of the Q pool. To obtain independent empirical evidence for the

Figure 3

(A) Effects of ethoxyformic anhydride (EFA) on the e.p.r. signature of the Rieske cluster and reversal of its effect by hydroxyamine and (B) Competition between EFA and stigmatellin effect on the Rieske e.p.r. line shape

A (a) 41 μM bc1 complex was reduced with 1 mM Q2H2. (b) 94.3 μM bc1 complex was incubated with 7.6 mM EFA at 1°C for 10 min, then diluted to 41 μM and reduced as in (a). (c) EFA-treated bc1 complex (94.3 μM) was treated with 30 mM hydroxyamine for 30 min at 0°C before dilution and reduction as in (b). B (a) Same as A (b). (b) Sample B (a) was treated with 125 μM stigmatellin. (c) EFA-untreated bc1 complex was reduced with 2.5 mM ascorbate, and treated with 125 μM stigmatellin. E.p.r. conditions: microwave power, 1 mW; modulation amplitude, 1 × 10⁻⁵ T; time constant, 0.128 s; sample temperature, 15 K.
possible involvement of the Rieske protein histidine residues in Q binding reactions occurring in the Q\(_b\) pocket, we examined the effects of a well-established specific modifier of histidine or tyrosine residues, namely ethoxyformic anhydride (EFA) [48], using bovine heart bc\(_1\) complex. The EFA derivative of histidine with one imidazole nitrogen (but not two) is known to be readily reversed by hydroxylamine treatment [48]. As shown in Figure 3A (a) the Rieske cluster, reduced with \(\text{Q}_{b}\), shows an e.p.r. feature of \(g_{xx}=1.76, 1.90, 2.02\). After incubation with EFA, the e.p.r. signal is converted to \(g_{xx}=1.80, 1.90, 2.017\) [Figure 3A (b)]. The total spin concentration of the Rieske iron-sulphur was not significantly diminished, which suggests that EFA does not destroy the direct ligation site, namely \(4\)N of the histidine. This altered line-shape is fully reversed upon subsequent treatment of the EFA-modified bc\(_1\) complex with hydroxylamine [Figure 3A (c)]. EFA inhibits electron transfer within the bc\(_1\) complex via the Q\(_b\) site: for example, oxidant-induced reduction of cytochromes b\(_1\) and b\(_{1b}\), but not antimycin-sensitive b reduction via the Q\(_b\) site [49]. After Rieske protein is derivatized by EFA, stigmatellin does not cause any line-shape change [Figure 3B (a) and (b)], contrary to the stigmatellin effect in the absence of EFA treatment [Figure 3B (c)]. Conversely, pretreatment of Rieske protein with stigmatellin greatly hinders subsequent EFA reaction with the protein, judging from the e.p.r. line-shape change of the iron-sulphur cluster. Brandt et al. [9] showed that once Rieske protein is completely deleted from bc\(_1\) complex, stigmatellin loses its tight binding capacity to the bc\(_1\) subcomplex. Stigmatellin, on the other hand, has no effect on the isolated Rieske iron-sulphur protein. In contrast, EFA induces the same e.p.r. line shape change to the isolated Rieske protein as one would expect for a direct amino acid modifier. These observations strongly indicate that the stigmatellin-binding site covers both the Rieske and cytochrome b domains of the Q\(_b\) pocket, and it is very close to the 'N position of the histidine ligand. These combined results suggest that the two His residues which directly co-ordinate to the iron of the Rieske cluster may also be involved in the binding of ubiquinone to the protein in the Q\(_b\) pocket; this is consistent with the 'functional double-occupancy Q\(_b\) site model' proposed recently by Dutton and coworkers [31, 50].

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Introduction
The bc₁ complexes are ubiquitous components of electron transfer chains of bacteria and mitochondria; the homologous bc complexes are found in chloroplasts. The bc₁ complexes catalyse the transfer of electrons from hydroquinone to the electron acceptor cytochrome c. The redox energy is converted into a chemi-osmotic membrane potential through the translocation of 4H⁺/2e⁻ across the membrane. The Q-cycle mechanism is now widely accepted as the scheme of electron and proton transfer [1−3].

Abbreviations used: e.p.r., electron paramagnetic resonance; UHDBT; 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole; MOA, E-β-methoxyacrylate; Q₁ centre, hydroquinone oxidation centre (Q₁ centre); QN centre, quinone reduction centre (Q centre).

Modelling the three-dimensional structure of cytochrome b

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This mechanism requires a bifurcation of the path of electrons. The first electron goes from hydroquinone to the high-potential electron pathway at the P-side of the membrane comprised of the Rieske iron-sulphur cluster and cytochrome c₁, and from there to cytochrome c. This exergonic redox reaction drives the second electron across the membrane; it is shuttled along the low-potential electron pathway formed by the two haem groups of cytochrome b and transferred back to ubiquinone.

The protonmotive Q-cycle mechanism requires the existence of two quinone reaction sites on opposite sides of the membrane. These reaction sites must catalyse a concerted sequence of electron and proton transfers to prevent dissociation of intermediate semiquinone species and thus dissipation of redox energy.

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