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Review

The di-heme family of respiratory complex II enzymes ☆

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ABSTRACT

The di-heme family of succinate:quinone oxidoreductases is of particular interest, because its members support electron transfer across the biological membranes in which they are embedded. In the case of the di-heme-containing succinate:menaquinone reductase (SQR) from Gram-positive bacteria and other menaquinone-containing bacteria, this results in an electrogenic reaction. This is physiologically relevant in that it allows the transmembrane electrochemical proton potential Δp to drive the endergonic oxidation of succinate by menaquinone. In the case of the reverse reaction, menaquinol oxidation by fumarate, catalysed by the di-heme-containing quinol:fumarate reductase (QFR), evidence has been obtained that this electrogenic electron transfer reaction is compensated by proton transfer *via* a both novel and essential transmembrane proton transfer pathway ("E-pathway"). Although the reduction of fumarate by menaquinol is exergonic, it is obviously not exergonic enough to support the generation of a Δp. This compensatory "E-pathway" appears to be required by all di-heme-containing QFR enzymes and results in the overall reaction being electroneutral. In addition to giving a brief overview of progress in the characterization of other members of this diverse family, this contribution summarizes key evidence and progress in identifying constituents of the "E-pathway" within the framework of the crystal structure of the QFR from the anaerobic epsilon-proteobacterium *Wolinella succinogenes* at 1.78 Å resolution. This article is part of a Special Issue entitled: Respiratory complex II: Role in cellular physiology and disease.

1. Introduction

As for the superfamily of succinate:quinone oxidoreductases (SQORs, EC 1.3.5.1) as a whole [1,2, this Special Issue], the di-heme family of respiratory complex II enzymes couple the two-electron oxidation of succinate to fumarate (reaction *a*) to the two-electron reduction of quinone to quinol (reaction *b*):

$$\text{succinate} \rightarrow \text{fumarate} + 2\,H^+ + 2e^- \quad \text{(a)}$$

$$\text{quinone} + 2\,H^+ + 2\,e^- \rightarrow \text{quinol} \quad \text{(b)}$$

They can also catalyse the opposite reaction, the coupling of quinol oxidation to the reduction of fumarate [3]. Depending on the direction of the reaction catalysed *in vivo*, these members can be classified as either succinate:quinone reductases (SQR) or quinol:fumarate reductases (QFR) [1]. SQR and QFR complexes are anchored in the cytoplasmic membranes of eubacteria and archaebacteria, and in the inner mitochondrial membrane of eukaryotes with the hydrophilic domain extending into the cytoplasm and the mitochondrial matrix, respectively.

SQR (respiratory complex II) is involved in aerobic metabolism as part of the citric acid cycle and of the aerobic respiratory chain. QFR participates in anaerobic respiration with fumarate as the terminal electron acceptor. This process is termed fumarate respiration (Fig. 1) [4–6].

SQORs generally contain two hydrophilic protein subunits, referred to as A and B, and one large or two small integral membrane proteins, referred to as C or C and D. While subunits A and B have high sequence homology among species, that for the hydrophobic subunits is much lower. While subunit A harbors the site of fumarate reduction and succinate oxidation, the hydophobic subunit(s) contain the site of quinol oxidation/quinone reduction. Based on their hydrophobic domain and heme content [7,8], the enzymes can be classified in five types (Fig. 2A) [9,10], of which two contain two heme groups and are therefore within the focus of this review. Type A anchors contain two small hydrophobic subunits and two heme groups, e.g. in the SQR enzyme from the thermo- and acidophilic archaea *Thermoplasma acidophilum* [11] and the extreme thermophilic bacterium *Thermus thermophilus* [12]. Type B anchors contain one large hydrophobic subunit (C), which, based on sequence [7,1] and structure comparisons [13,14], is thought to have evolved from a fusion of the genes for the two smaller subunits C and D of the other membrane anchors. These anchors are found in a wide range of bacteria, such as in the SQR enzymes the Gram-positive bacteria *Corynebacterium glutamicum* [15], *Bacillus subtilis* [16], *Paenibacillus macerans* [17], and *Bacillus licheniformis* [18], some cyanobacteria, e.g. *Synechocystis* sp. PCC6803 [19–21], the

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thermohalophilic *Rhodothermus marinus* [22], and in the QFR enzymes of the photosynthetic bacterium *Chloroflexus aurantiacus* [23], the anaerobic sulfur-reducing delta-proteobacteria *Geobacter sulfurreducens* [24,25] and *Desulfovibrio gigas* [26], and the epsilon-proteobacteria *Campylobacter jejuni* [27,28], *Helicobacter pylori* [27], and *Wolinella succinogenes* [29]. Although a broad range of these enzymes within the di-heme family have been characterized extensively (see Table 1 for an overview), and diffracting crystals of some preparations have been reported [12,13,23,30–33], the currently available three-dimensional crystal structures are exclusively from the QFR from *W. succinogenes* and will be discussed in the next section. A second set of genes encode for a further membrane protein complex exhibiting fumarate reductase activity have been expressed and the enzyme with a type-E anchor (Fig. 2A) has been characterized as an 8-methylmenaquinol:fumarate reductase (MFR) with its hydrophilic subunits oriented towards the periplasm [34]. This enzyme, whose physiological role is yet unclear, is also found in the human pathogen *C. jejuni* [35].

2. Structure

The structure of the QFR membrane protein complex been determined by X-ray crystallography in three different crystal forms, initially at a resolution of up to 2.2 Å [13] and subsequently at an improved resolution of 1.78 Å (PDB ID: 2BS2 [33]). The relevance of this structure determination for the superfamily of succinate:quinone oxidoreductases has been discussed and reviewed earlier [2,9,10,14,36–41]. In all three crystal forms, two heterotrimeric complexes of A, B, and C subunits form a homodimer of 260 kDa (Fig. 2B). Based on analytical gel filtration and analytical ultracentrifugation experiments [27], this homodimer represents the aggregation state as isolated and is not an artefact of crystallization. Subunit A contains a covalently bound FAD and the site of fumarate reduction, subunit B harbors three different iron-sulfur clusters, a [2Fe–2S], a [4Fe–4S], and a [3Fe–4S] cluster, and the membrane-embedded subunit C binds two heme *b* groups (Fig. 3A). Based on their proximity to the hydrophilic subunits, these are referred to as the proximal heme b_P and the distal heme b_D. It has long been known [42] that the two heme groups have different oxidation–reduction midpoint potentials and an assignment for *B. subtilis* SQR based on the effects of site-directed mutagenesis has been made [43]. Although in retrospect this assignment is probably correct, the interpretation of such mutagenesis effects can be misleading, as demonstrated recently [44] by the stronger effect of the replacement of a *W. succinogenes* QFR heme b_L ligand on the midpoint potential of heme b_H rather than on that of heme b_L. A mutagenesis-independent assignment of the "high-potential" heme to b_P and the "low-potential" heme to b_D was achieved on the basis of structure-based electrostatic calculations [45]. These calculations also provide explanations for the higher midpoint potential of heme b_P. This is due to approximately equal contributions of the more buried nature of this heme and the close presence of a larger number of positively charged basic side chains [45,61].

As noted earlier [13], the binding of the two heme *b* molecules by an integral membrane protein four-helix bundle described here is very different from that described for the four-helix bundle of the cytochrome bc_1 complex [46]. In *W. succinogenes* QFR, the axial ligands for heme binding are located on four different transmembrane segments. This we refer to as a "four-helix motif" (Fig. 3D). This motif is also found in eukaryotic cytochrome b_{561} enzymes (e.g. [47]) In the cytochrome bc_1 complex [46] and membrane-bound nitrate reductase [48,49], only two transmembrane segments provide two axial heme *b* ligands each ("two-helix motif", Fig. 3B). Examples for a "three-helix motif", where one transmembrane helix provides two heme *b* ligands, and two others provide one heme *b* ligand each (Fig. 3C), may be found in the cases of membrane-bound hydrogenases [48,50], and formate dehydrogenases [51]. One consequence of this difference is that the distance between the two heme iron centers is distinctly shorter in *W. succinogenes* QFR (15.6 Å) than it is in the mitochondrial cytochrome bc_1 complex (21 Å [46]) and in *E. coli* formate dehydrogenase-N (20.5 Å [51]).

The representative structures of *W. succinogenes* QFR, *E. coli* QFR ([52], a type D enzyme, Fig. 2A) and *E. coli* SQR ([53], a type C enzyme, Fig. 2A) could be superimposed on the basis of the Cα positions of the conserved hydrophilic subunits A and B [13,14]. In spite of differences in polypeptide and heme composition, the overall topology of the membrane anchors and their relative orientation to the conserved hydrophilic subunits is strikingly similar in the case of *W. succinogenes* QFR and *E. coli* SQR (Fig. 4A and B), but not for *E. coli* QFR (Fig. 4C). However, in an alternate orientation (Fig. 4D), the transmembrane subunits of *E. coli* QFR and of *W. succinogenes* QFR could be overlayed in spite of the respective absence and presence of the two heme groups. Consequently, the relative orientations of the hydrophilic subunits and the transmembrane subunits are similar in *W. succinogenes* QFR and *E. coli* SQR and different in *E. coli* QFR. Given that the small C and D subunits of the type C and D membrane anchors superimpose surprisingly well with the large C subunit of the type B membrane anchor, it is expected that, when a structure becomes available, this will also apply to the C and D subunits of the type A membrane anchor.

Subunit C also contains the active site of menaquinol oxidation, close to the heme b_D. Structural and functional characterization of the *W. succinogenes* QFR variant enzyme E66Q, in which the nearby residue

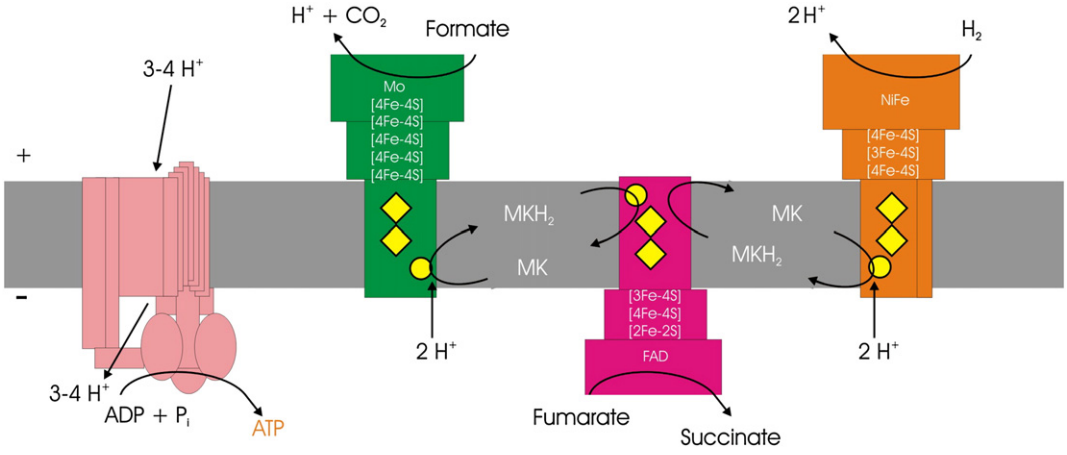

Fig. 1. Electron and proton transfer in fumarate respiration. Positive and negative sides of the membrane are the periplasm and the cytoplasm, respectively. Heme groups are indicated by yellow diamonds. The key enzymes involved in fumarate respiration are shown. From left to right, these are ATP synthase, formate dehydrogenase, quinol:fumarate reductase, and hydrogenase. Sites of quinone reduction/quinol oxidation are indicated by yellow circles. This figure was modified from reference [2].

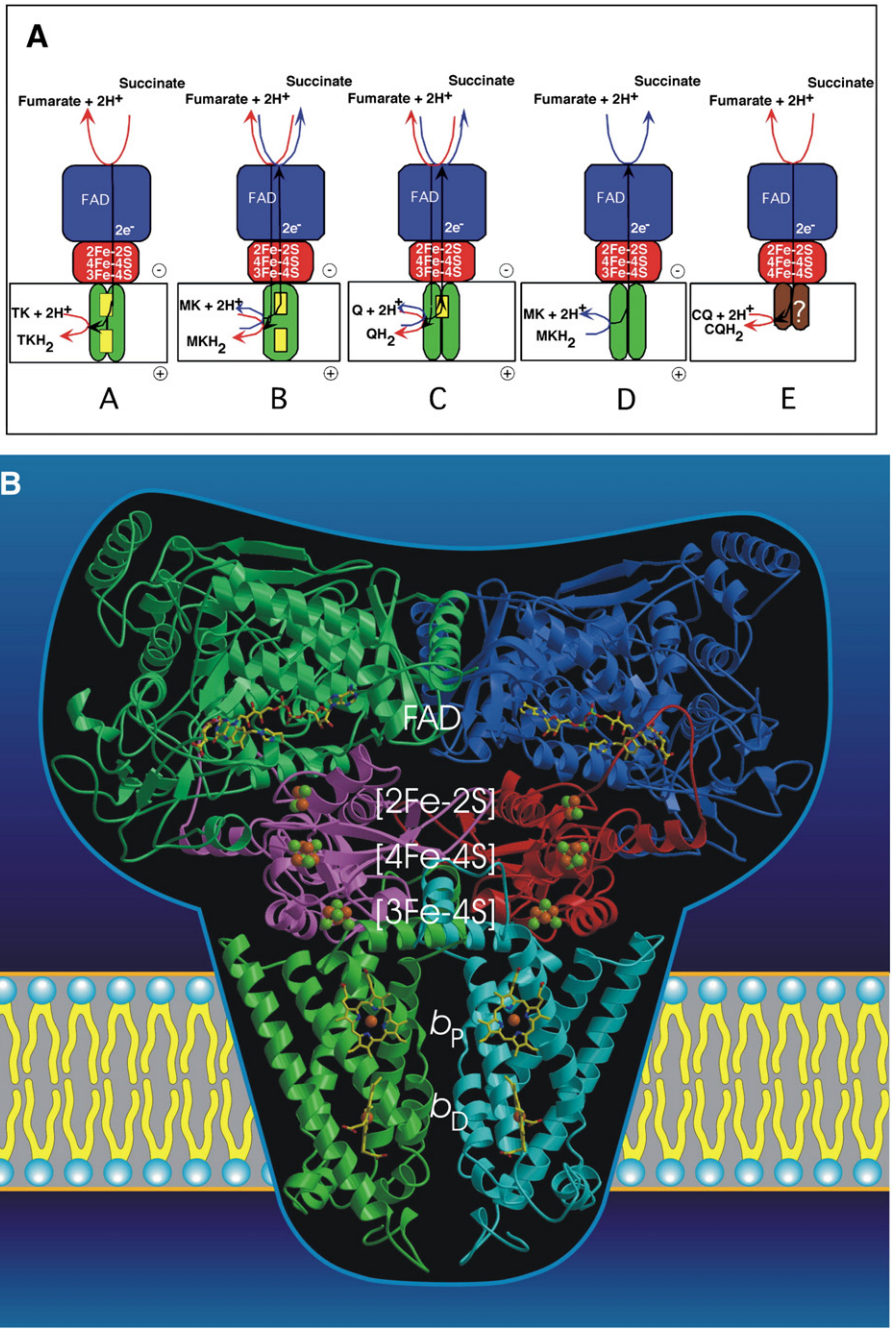

Fig. 2. Modified from Reference [60]. A) (from [2]) Classification (A to E) of succinate:quinone oxidoreductases [9,10] based on their hydrophobic domain and heme content [7,8]. The hydrophilic subunits A and B are drawn schematically in blue and red, respectively, the hydrophobic subunits C and D in green or brown. Heme groups are symbolized by yellow rectangles. The directions of the reactions catalysed by SQR and QFR are indictaed by red and blue arrows, respectively. White rectangles symbolize the respective cytoplasmic or inner mitochondrial membrane bilayer. The positive (+) and negative (−) sides of the membrane are indicated. In bacteria, the negative side is the cytoplasm ("inside"), the positive side the periplasm ("outside"). For mitochondrial systems, these are the mitochondrial matrix and the intermembrane space, respectively. The type of quinone transformed *in vivo* is not necessarily unique for each type of enzyme. The examples given are thermoplasma-quinone (TK), menaquinone (MK), ubiquinone (Q), and caldariella quinone (CQ) [1]. More recently, representatives of type E with hydrophilic subunits oriented towards the periplasm were found to display fumarate reductase activity [34,35]. B) *W. succinogenes* QFR homodimer of heterotrimers. Three-dimensional structure of the QFR dimer of heterotrimeric complexes of A, B, and C subunits. The Cα traces of the two A subunits are shown in blue and blue-green, those of the two B subunits in red and purple and those of the two C subunits in green and light blue. The atomic structures of the six prosthetic groups per heterotrimer are superimposed for better visibility. From top to bottom, these are the covalently bound FAD, the [2Fe–2S], the [4Fe–4S], and the [3Fe–4S] iron-sulfur centers, the proximal and the distal heme *b* groups. Atomic colour coding is as follows: C, N, O, P, S, and Fe are displayed in yellow, blue, red, light green, green, and orange, respectively. The figure is drawn from the coordinate set with PDB ID: 1QLA [13]. The position of bound fumarate close to the isoalloxazine ring of FAD is taken from the coordinate set with PDB ID: 1QLB [13].

Glu C66 had been replaced by a Gln by site-directed mutagenesis, demonstrated that this residue is selectively essential for quinol oxidation [30]. The orientation of the catalytic sites of fumarate reduction [31], associated with proton binding, and menaquinol oxidation [30], associated with proton release, towards opposite sides of the membrane and linked by "edge-to-edge" distances between the redox centers shorter than the 14 Å upper limit compatible with physiological electron transfer [54] indicated that quinol oxidation by fumarate should be an electrogenic process in *W. succinogenes* (Fig. 5A), i.e. associated directly with the establishment of an electrochemical proton potential across the membrane. This will be discussed further in the next two sections.

Table 1
A summary of properties of selected di-heme SQOR family members (modified from references [1,37]. [a]The assignment of b_P and b_D follows the assignment of [45] for *W. succinogenes* QFR.

Organism	Enzyme function	Polypeptides (mass [kDa])	Membrane anchor type	Oligomeric state	E_M [mV] of …						Inhibitor sensitivity	References
					FAD_{cov}	[2Fe-2S]	[4Fe-4S]	[3Fe-4S]	[a]Heme b_P	[a]Heme b_D		
Thermoplasma acidophilum	SQR	A (63) B (27) C (15) D (14)	A			+68	-210	+60	+75	-150	HQNO	[11]
Thermus thermophilus	SQR	A (64) B (27) C (14) D (15)	A	Homo-trimer		-10		>+50	-20	-160		[12]
Rhodothermus marinus	SQR	A (70) B (32) C (18)	B					+130	+75	-65		[22]
Chloroflexus aurantiacus	QFR	A (73) B (28) C (27)	B	Homo-dimer					-40	-190		[23]
Desulfovibrio gigas	QFR	A (71) B (31) C (22)	B			-140		+20	-45	-175	HQNO	[26]
Geobacter sulfurreducens	QFR / SQR	A (71) B (28) C (24)	B								HQNO	[24,25]
Corynebacterium glutamicum	SQR	A (75) B (27) C (28)	B	Homo-trimer							HQNO	[15]
Bacillus subtilis	SQR	A (65) B (28) C (23)	B			+80	-240	-25	+16	-132	HQNO	[1,3] [16,55]
Bacillus licheniformis	SQR	A (65) B (28) C (23)	B	Homo-trimer							HNN	[18]
Campylobacter jejuni	QFR / SQR	A (74) B (28) C (30)	B	Homo-dimer	-101	-5	-235	+42	+1	-129		[27]
Helicobacter pylori	QFR / SQR	A (80) B (28) C (29)	B	Homo-dimer	-70	+26	-260	+33	+8	-106		[27]
Wolinella succinogenes	QFR	A (73) B (27) C (30)	B	Homo-dimer	-125	-112	-340	-61	-9	-152	DHN, not sensitive to HQNO	[1,9,27] [30,33]

3. Electrogenicity of succinate oxidation by menaquinone

This electrogenic catalysis indeed appeared to be the case for some representatives of the di-heme SQOR family. Succinate oxidation by menaquinone, an endergonic reaction under standard conditions, had been proposed to be driven by the electrochemical proton potential in the Gram-positive bacterium *B. subtilis* [56,17], where the quinone reduction site was deduced to be close to the low-potential heme [55]), and in other prokaryotes containing succinate:menaquinone reductase [25]. This is the analogous reaction to that suggested in Fig. 5A, but in the opposite direction (Fig. 6A). However, the experiments on the SQR had previously been performed only with whole cells and isolated membranes and it has been questioned [57] whether the observed effects are associated specifically with the SQR. It has since been shown that the di-heme-containing succinate:menaquinone reductase, isolated from the Gram-positive bacterium *B. licheniformis* and reconstituted into proteoliposomes, is unable to support the reduction of the soluble menaquinone analog 2-ethyl-3-methyl-1,4-naphthoquinone (EMN)

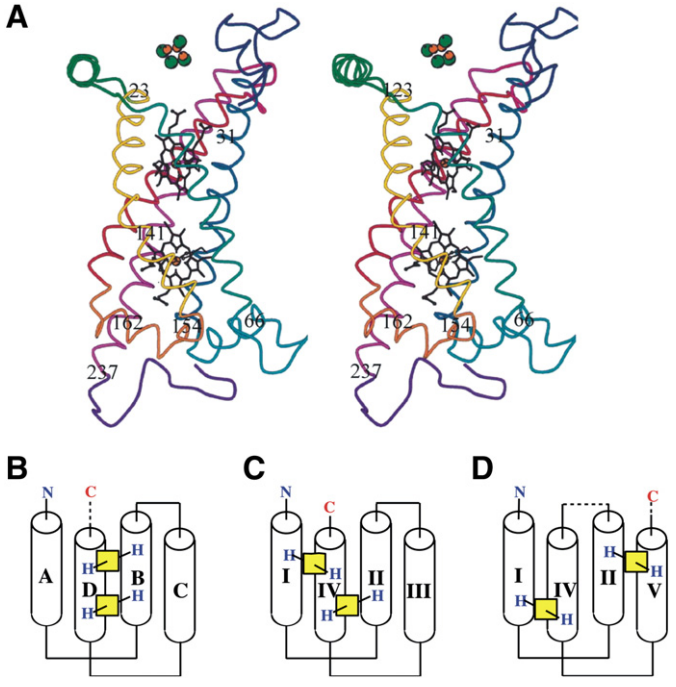

Fig. 3. *W. succinogenes* QFR subunit C. A) (from [38]) Subunit C, the di-heme cytochrome *b* of *W. succinogenes* QFR (stereo view). Also shown are the proximal (upper) and distal (lower) heme groups and the position of the [3Fe–4S] cluster (top), which is bound by the B subunit (Fig. 2B). Selected C subunit residues are labeled by their residue number. The Cα trace is drawn in dark blue (amino terminus), blue (transmembrane helix I), light blue (periplasmic I-II connection), blue-green (transmembrane helix II), green (cytoplasmic II–IV connection), yellow (transmembrane helix IV), orange (periplasmic IV-V connection), red (transmembrane helix V), pink (transmembrane helix VI), and purple (carboxy terminus). B)-D) (from [60]) Diheme binding by integral membrane protein four helix bundles. B) "Two-helix motif": His ligands from two transmembrane helices (mitochondrial cytochrome bc_1 complex). C) "Three-helix motif": His ligands from three transmembrane helices (e.g. hydrogenase, formate dehydrogenase). D) "Four-helix motif": His ligands from four transmembrane helices (di-heme succinate:quinone oxidoreductases).

unless the protonophore carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP) is added [18]. Apparently the driving force of the reaction, even with a large excess of starting material over product, was insufficient to support the establishment of a Δp across the proteoliposomal membrane. In order to increase this driving force, the quinone substrate was modified to increase its oxidation/reduction midpoint potential, as is the case for the soluble ubiquinone analog 2,3-dimethoxy-5-ethyl-6-methyl-1,4-benzoquinone (EQ-0). For an increased driving force of the reverse reaction, a lower redox midpoint potential than that of EMN and 2,3-dimethyl-1,4-naphthoquinone (DMN) was achieved by designing the substrate 2-methyl-3-methylamino-1,4-naphthoquinol ($MMANH_2$) [33].

Two components contribute to Δp, the membrane potential $\Delta\Psi$ and the proton gradient ΔpH. The latter was measured by monitoring pH changes in the lumen of the proteoliposomes *via* the absorption properties of the proton-sensitive dye pyranine. Oxidation of the low-potential quinol $MMANH_2$ by fumarate as catalysed by the proteoliposomal SQR from *B. licheniformis* was found to be associated with the lowering of the luminal pH [18]. Conversely, reduction of the high-potential quinone EQ-0 was found to be associated with the increase of the luminal pH [18]. The membrane potential $\Delta\Psi$ was measured with the help of electrodes respectively selective for the lipophilic ions tetraphenylphosphonium (TPP^+) and tetraphenylborate (TPB^-). In the case of quinol oxidation as well as in the case of quinone reduction, a membrane potential could be measured, thus unequivocally demonstrating electrogenic catalysis in the case of this enzyme [18].

4. Electroneutrality of fumarate reduction by menaquinol

As discussed earlier [30], analogous experiments to those mentioned above in the case of the SQR from Gram-positive bacteria, for isolated *W. succinogenes* QFR reconstituted into liposomes had shown that the oxidation of quinol by fumarate as catalysed by this enzyme is electroneutral [5,33,58,59].

To reconcile these apparently conflicting experimental observations, the so-called "E-pathway hypothesis" (Fig. 5B) was proposed [60]. According to this working hypothesis, the transmembrane transfer of two electrons in *W. succinogenes* QFR is coupled to the compensatory, parallel translocation of one proton per electron from the periplasm to the cytoplasm. The proton transfer pathway used is transiently established during reduction of the heme groups and is closed in the oxidized enzyme. The two most prominent constituents of the proposed novel pathway were suggested to be the ring C propionate of the distal heme b_D and, in particular, the amino acid residue Glu C180, after which the "E-pathway" was named (Fig. 5B). Since the first proposal of this

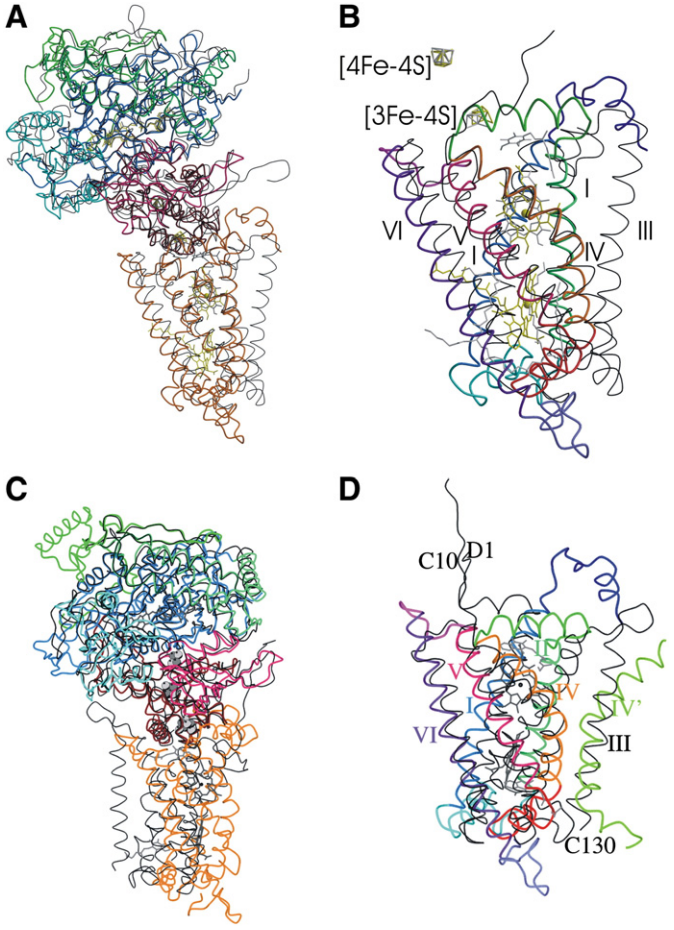

Fig. 4. (From [13,14]) Structure of the subunits of *W. succinogenes* QFR ([13], in colour) and comparison to the structure of (A,B) *E. coli* SQR ([53], in black, with cofactors in gray) and (C,D) *E. coli* QFR ([52], in black, with cofactors in gray). Single letters in labels identify the respective subunit. Structures in (A) and (C) are superimposed based on the Cα atoms of the A and B subunits. Structures in (B) and (D) are superimposed based on the Cα atoms of the transmembrane subunits. A) Subunit A of *W. succinogenes* QFR is drawn in blue (FAD-binding domain), light blue (capping domain), green (helical domain), and light green (C-terminal domain), subunit B is shown in pink (N-terminal domain) and brown (C-terminal domain), and subunit C in orange. The positions of 782 Cα atoms could be superimposed with an r.m.s. deviation of 1.7 Å. B) *W. succinogenes* QFR subunit C is colour-coded as for Fig. 3A. The [4Fe–4S] and [3Fe–4S] clusters are shown to aid orientation. See reference 14 for details of the alignment. C) *W. succinogenes* QFR subunit A and B domains and subunit C are drawn as described for panel A. D) *W. succinogenes* QFR subunit C is colour-coded as for Fig. 3A. See reference 13 for details of the alignment.

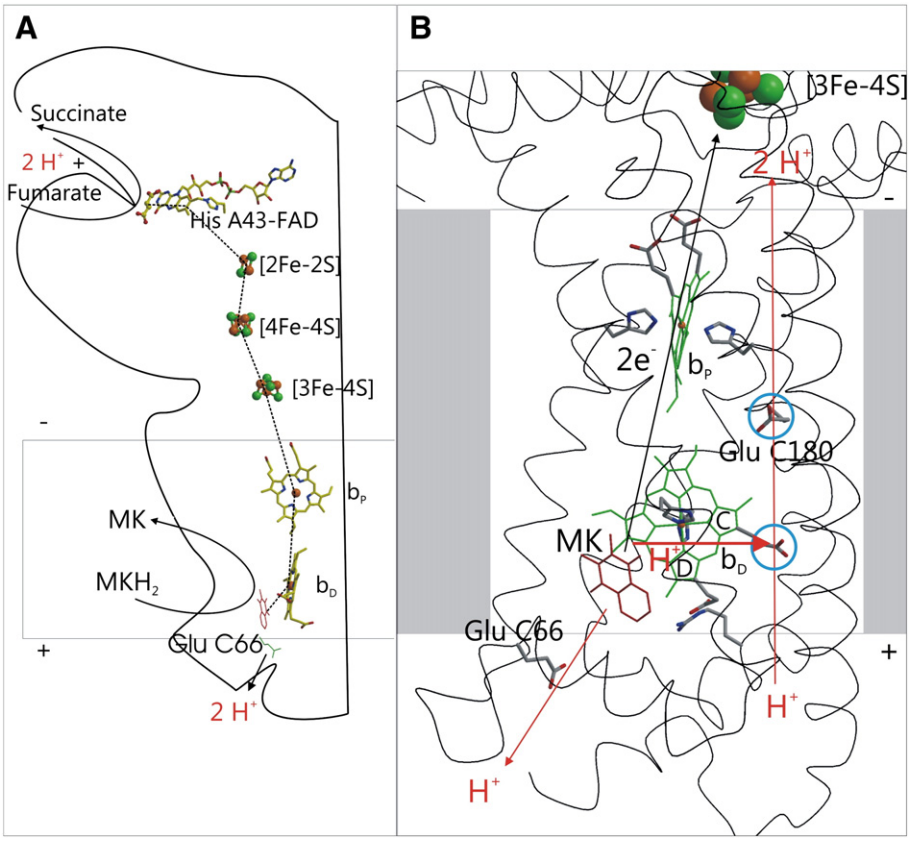

Fig. 5. A) Hypothetical Δp generation as suggested by the essential role of Glu C66 for menaquinol oxidation by *W. succinogenes* QFR [30]. The prosthetic groups of the *W. succinogenes* QFR dimer are displayed (coordinate set PDB ID: 1QLA; [13]). Also indicated are the side chain of Glu C66 and a tentative model of menaquinol (MKH_2) binding, based on the coordinates of QFR-bound DMN (PDB ID: 2BS4 [32]). The position of bound fumarate (Fum) is taken from PDB ID: 1QLB [13]. Figure adapted from references [64,65]. B) "E-pathway hypothesis" [60], revised [64,65]: One of the two protons that are liberated upon oxidation of menaquinol (MKH_2) is released to the periplasm (bottom) *via* the residue Glu C66. In compensation, coupled to electron transfer *via* the two heme groups, protons are transferred from the periplasm *via* the ring C propionate of the distal heme b_D and the residue Glu C180 (indicated by the blue circles) to the cytoplasm (top), where they replace those protons which are bound during fumarate reduction. One of these protons is taken up from the periplasm, the other transferred directly from the menaquinol oxidation site [44]. In the oxidized state of the enzyme, the "E-pathway" is blocked.

hypothesis, a number of theoretical [45] and experimental results [32,61,62] have been obtained that support it (reviewed in [63]).

A possible heme propionate involvement as a participant in the E-pathway has been investigated by combining ^{13}C labeling of the heme propionates with redox-induced FTIR spectroscopy [62]. The redox-transition of the distal heme led to protonation and/or environmental changes of (at least) one of the two distal heme propionates. Since it was established that the ring D propionate of the low-potential heme is involved in an extensive salt-bridge interaction with a nearby Arg residue [13,45], the obvious candidate for the observed effects is the ring C propionate, which is fully consistent with the proposed role of this residue in the E-pathway hypothesis.

The role of Glu C180 in this context was first supported by multi-conformation continuum electrostatics calculations [45], which predicted that this residue undergoes the combination of a change in protonation and conformation upon reduction of the heme groups, a result that was also obtained experimentally by the combination of FT-IR difference spectroscopy [61] and site-directed mutagenesis,

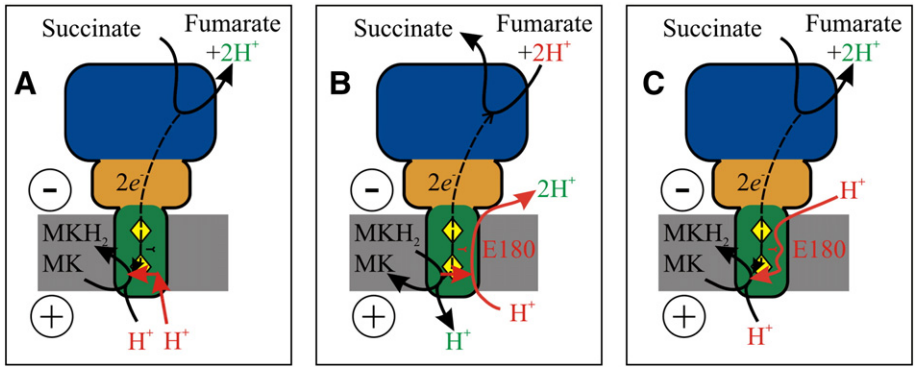

Fig. 6. Electron and proton transfer in *B. licheniformis* SQR (A), as a representative of type A and B SQR enzymes, and in *W. succinogenes* QFR (B), as a representative of type B QFR enzymes. Positive and negative sides of the membrane are the periplasm and the cytoplasm, respectively. Heme groups are indicated by yellow diamonds. See text for details. C) Possible mechanism underlying catalysis by proteoliposomal *W. succinogenes* QFR of quinone reduction by succinate with an H^+/e^- ratio of 0.5. Panels from this figure were modified from references [33,63,65].

involving the replacement of Glu C180 with a Gln residue [32]. The mutant E180Q was unable to grow with fumarate as the terminal electron acceptor, an observation that we can now understand as a demonstration of the essential nature of this pathway for life under the conditions of fumarate respiration. The mutant did grow when fumarate was replaced by nitrate and the variant QFR was produced. After refining the structure of the variant QFR at 2.2 Å resolution, any major structural changes compared to the structure of the wild-type enzyme could be ruled out [32].

The variant enzyme, after reconstitution into proteoliposomes, is unable to support the oxidation of $DMNH_2$ unless CCCP is added [33]. Oxidation of the low-potential quinol $MMANH_2$ is supported even in the absence of CCCP. In contrast to the results obtained with the wild-type enzyme, quinol oxidation by the E180Q variant was clearly associated with an acidification of the interior of the proteoliposomes (indicative of ΔpH generation) [33] as well as TPB^- entry into the proteoliposomes (indicative of $\Delta\psi$ generation) [33]. Taken together with the results obtained for the proteoliposomal wild-type enzyme, these results clearly demonstrate the presence and absence of the "E-pathway" in the WT and E180Q-variant enzymes, respectively [33].

While the E-pathway hypothesis as proposed originally [60] was the simplest model compatible with the experimental data obtained initially, it was by no means unique. The simplifying assumption that both protons released upon quinol oxidation were released *via* the same pathway is probably not true, as is the assumption that both E-pathway protons have the same entry point. In particular, the scenario that one proton is transferred directly from the quinol oxidation site to the E-pathway (Figs. 5B, 6B) also explains the available data [64]. However, there is no such proton transfer connectivity apparent from the structure of the oxidized enzyme, so this scenario would require an appropriate conformational change during catalysis [64].

Subsequently [65], during characterization of catalysis by the QFR from *W. succinogenes* in the non-physiological direction, i.e. succinate oxidation by quinone, it turned out that this reaction is clearly electrogenic. In spite of supporting succinate oxidation by EMN with a specific activity of 2.5 U/mg in its detergent solubilized state, when reconstituted into proteoliposomes *W. succinogenes* QFR exhibited no detectable enzymatic activity of succinate oxidation by EMN [65]. This non-detectable activity was attributed to the unfavorable difference in oxidation–reduction midpoint potential between the EMN/$EMNH_2$ couple and the fumarate/succinate couple, rendering the overall reaction mildly endergonic under standard conditions (at pH 7) and apparently not favorable enough, even with a large excess of starting material over product, to support sustained Δ*p* generation. These conclusions were supported by the finding that the addition of CCCP to the ostensibly inactive proteoliposomes enabled the catalysis of EMN reduction [65].

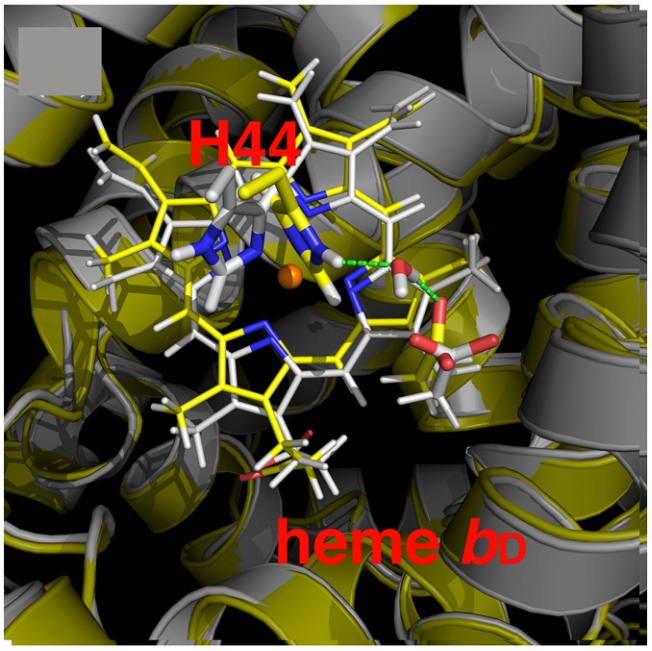

Fig. 8. (From [44]) Superposition of an MD snapshot without hydrogen bonding paths (helix and carbon atoms coloured in white) and one with formed hydrogen bonding paths (helix and carbon atoms coloured in yellow). Side chains of His C44 (labeled "H44"), a bridging water molecule as well as the carboxyl group of the heme b_D propionate are highlighted by using thicker sticks.

In order to measure any generation of a membrane potential Δ*p*, EMN was replaced with the high-potential quinone EQ-0. By measuring the uptake of TPP^+ into proteoliposomes after starting the reaction with the addition of succinate [65], it was found that the reduction of EQ-0 by succinate, as catalysed by proteoliposomal QFR, generated a significant $\Delta\Psi$ of 92 mV (negative inside). In a separate experiment, by monitoring the absorbance properties of pyranine in the course of the catalysis, a drop in the proton concentration could be measured within the proteoliposomes, corresponding to between 0.86 and 1.1 mol H^+ per 1 mol of EQ-0 reduced. This yielded H^+/e^- ratios of between 0.43 and 0.55 [65].

Clearly, the results from the uncoupler-stimulated activity test, the measurement of $\Delta\Psi$, and the pyranine absorbance measurements were very different from what one would expect from a simple reversal of the QFR reaction monitored in the physiological direction. Taken

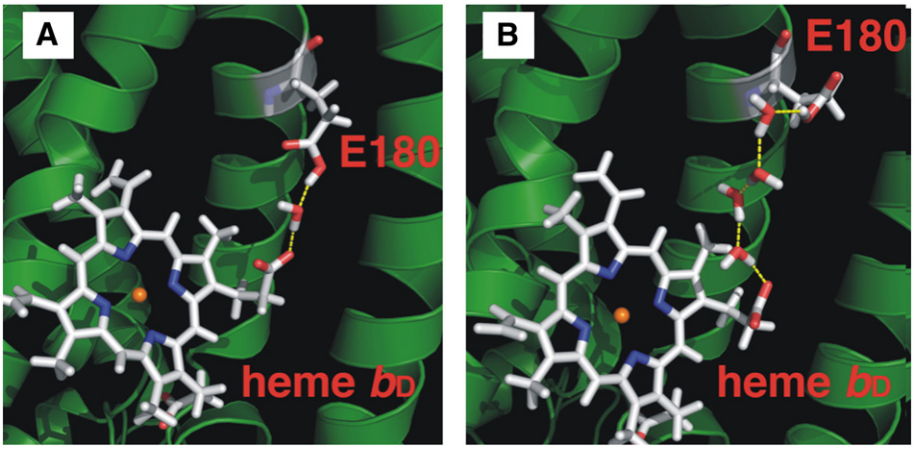

Fig. 7. (From [44]) MD snapshots (A) Shorter hydrogen bonded-path between Glu C180 (labeled "E180") and heme b_D, (B) Longer hydrogen bonded-path. The structural reason for the shorter distance, i.e. a shorter path resulting in large proton conductance, is a flipping of the side chain of Glu C180.

together, the results indicated that fully compensatory transmembrane proton transfer *via* the E-pathway is only associated with catalysis by *W. succinogenes* QFR in the physiological direction (quinol oxidation by fumarate) and only then is the catalysed reaction electroneutral. In the opposite direction, *W. succinogenes* QFR appears to function in a manner more similar to *B. licheniformis* SQR, which lacks the compensatory E-pathway, with the notable difference that both the $\Delta\Psi$ and the H^+/e^- ratio generated by succinate oxidation with *W. succinogenes* QFR were significantly lower, only about half the values of those generated under the same conditions with *B. licheniformis* SQR. Thus, the results indicate partially impaired compensatory transmembrane proton transfer through the E-pathway to be associated with quinone reduction by *W. succinogenes* QFR. This could involve only one transmembrane proton transfer being associated with the transmembrane transfer of two electrons and the second proton ultimately being bound from the inside of the proteoliposomal lumen (corresponding to an H^+/e^- ratio of 0.5; Fig. 6C). The most straightforward explanation for this phenomenon is that the E-pathway protons do not share completely identical pathways, making the revised scenario shown in Fig. 5B more likely than the original one proposed [60]. However, it has not been clear on the basis of the available crystal structures of the oxidized enzyme how proton transfer could occur between the quinol oxidation site and the E-pathway. In addition, although the heme b_D ring-C-propionate and Glu C180 were identified as the most prominent components of the proton transfer pathway, it has also not been clear, on the basis of the structure, how proton transfer could occur between them.

All available crystal structures of the QFR are those of the oxidized enzyme. However, functionality of the E-pathway requires the enzyme to be reduced [60]. Lacking an experimentally determined structure of the reduced enzyme, Herzog et al. [44] performed molecular dynamics simulations of various redox states of the enzyme. The results obtained indicated that transiently established hydrogen bonding paths involving internal water molecules are responsible for proton transfer from the heme b_D ring-C-propionate to Glu C180 (Fig. 7A,B) and that high proton conductance associated with short hydrogen bonding paths could be identified only for the reduced but not the oxidized state of the enzyme [44]. These differences were also associated with a conformational change in the orientation of the Glu C180 side chain in agreement with the predictions from multiconformation continuum electrostatics calculations [45], results from FTIR difference spectroscopy [61] and with alternate conformers in the 1.78 Å resolution crystal structure (PDB ID: 2BS2 [33]).

The functional importance of the heme b_D ligand His C44 derived from identification (Fig. 8) and analysis of transient hydrogen bonding paths between this residue and the heme b_D ring-C-propionate provides a structural basis for the previously deduced bifurcation of the E-pathway, allowing a substrate proton to be transferred from the site of menaquinol oxidation to the E-pathway. This theoretical finding regarding the functional importance of residue His C44 is also consistent with the results from site-directed mutagenesis involving replacement of His C44 with a Glu, which, apart from abolishing quinol oxidation activity, has the unusual effect of lowering the midpoint potential of the more distant high-potential heme b_P to a larger extent than that of the closer low-potential heme b_D [44]. This result clearly supports a role of His C44 in providing a proton for the compensatory E-pathway and thereby making reduction of the high-potential heme b_P more favorable. In spite of major advances in this area, further characterization is clearly required in order to fully understand the mechanism of this intriguing catalytic process.

Compensatory proton transfer *via* the E-pathway is expected to be essential for all other di-heme QFR enzymes. Since functional QFR is required, e.g. for host colonization by the human pathogens *C. jejuni* [28] and *H. pylori* [66], the E-pathway is expected to be relevant to health and disease.

Abbreviations

b_D	distal heme
b_H	high-potential heme
b_L	low-potential heme
b_P	proximal heme
CCCP	carbonyl cyanide m-chloro-phenylhydrazone
DHN	2-decyl-3-hydroxy-1,4-naphthoquinone
DMN	2,3-dimethyl-1,4-naphthoquinone
$DMNH_2$	2,3-dimethyl-1,4-naphthoquinol
Δp	electrochemical proton potential
E_M	oxidation–reduction midpoint potential
EMN	2-ethyl-3-methyl-1,4-naphthoquinone
$EMNH_2$	2-ethyl-3-methyl-1,4-naphthoquinol
EQ-0	2,3-dimethoxy-5-ethyl-6-methyl-1,4-benzoquinone
EQH_2-0	2,3-dimethoxy-5-ethyl-6-methyl-1,4-benzoquinol
FTIR	Fourier transform infrared
HNN	2-hydroxy-3-neopentyl-1,4-naphthoquinone
HQNO	2-*n*-heptyl-4-hydroxyquinoline N-oxide
MD	molecular dynamics
MMAN	2-methyl-3-methylamino-1,4-naphthoquinone
$MMANH_2$	2-methyl-3-methylamino-1,4-naphthoquinol
QFR	quinol:fumarate reductase
SQR	succinate:quinone reductase
SQOR	succinate:quinone oxidoreductases

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