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Limited reversibility of transmembrane proton transfer assisting transmembrane electron transfer in a dihaem-containing succinate:quinone oxidoreductase

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ABSTRACT

Membrane protein complexes can support both the generation and utilisation of a transmembrane electrochemical proton potential (Δp), either by supporting transmembrane electron transfer coupled to protolytic reactions on opposite sides of the membrane or by supporting transmembrane proton transfer. The first mechanism has been unequivocally demonstrated to be operational for Δp-dependent catalysis of succinate oxidation by quinone in the case of the dihaem-containing succinate:menaquinone reductase (SQR) from the Gram-positive bacterium *Bacillus licheniformis*. This is physiologically relevant in that it allows the transmembrane potential Δp to drive the endergonic oxidation of succinate by menaquinone by the dihaem-containing SQR of Gram-positive bacteria. In the case of a related but different respiratory membrane protein complex, the dihaem-containing quinol:fumarate reductase (QFR) of the ε-proteobacterium *Wolinella succinogenes*, evidence has been obtained that both mechanisms are combined, so as to facilitate transmembrane electron transfer by proton transfer *via* a both novel and essential compensatory 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 dihaem-containing QFR enzymes and results in the overall reaction being electroneutral. However, here we show that the reverse reaction, the oxidation of succinate by quinone, as catalysed by *W. succinogenes* QFR, is not electroneutral. The implications for transmembrane proton transfer *via* the E-pathway are discussed.

1. Introduction

According to Peter Mitchell's chemiosmotic theory [1], the energy released by the oxidation of electron donor substrates in both aerobic and anaerobic respiration is transiently stored in the form of an electrochemical proton potential (Δp) across the energy-transducing membranes. Fundamentally, there are two mechanisms by which integral membrane proteins can act as catalysts in this coupling of electron transfer reactions to the generation of a transmembrane Δp — the redox loop mechanism and the proton pump mechanism [2]. The former essentially involves transmembrane electron transfer. Reduction reactions on one side of the energy-transducing membrane are associated with proton binding whereas oxidation reactions on the opposite side of the membrane are associated with proton release (Fig. 1A). The proton pump mechanism involves the actual translocation of protons across the membrane. As has been summarised previously [3,4], both of these mechanisms are apparently harnessed together in a specific case of a single respiratory membrane protein complex (Fig. 1B). The membrane protein in question is the dihaem-containing quinol:fumarate reductase (QFR) from the anaerobic ε-proteobacterium *Wolinella succinogenes* [5]. QFR is the terminal enzyme of fumarate respiration [6,7], a form of anaerobic respiration which allows anaerobic bacteria to use fumarate instead of dioxygen as the terminal electron acceptor (Fig. 1C). QFR couples the two-electron

Abbreviations: b_D, distal haem; b_H, high-potential haem; b_L, low-potential haem; b_P, proximal haem; CCCP, carbonyl cyanide *m*-chloro-phenylhydrazone; DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, 2,3-dimethyl-1,4-naphthoquinol; Δp, electrochemical proton 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; 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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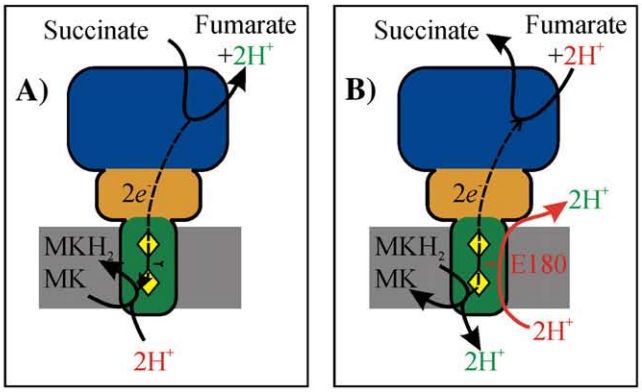
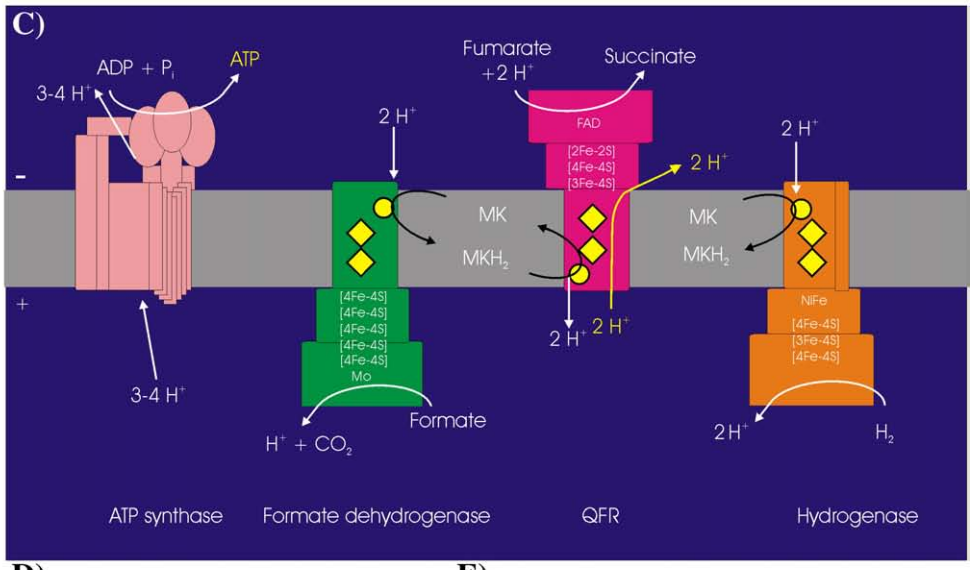
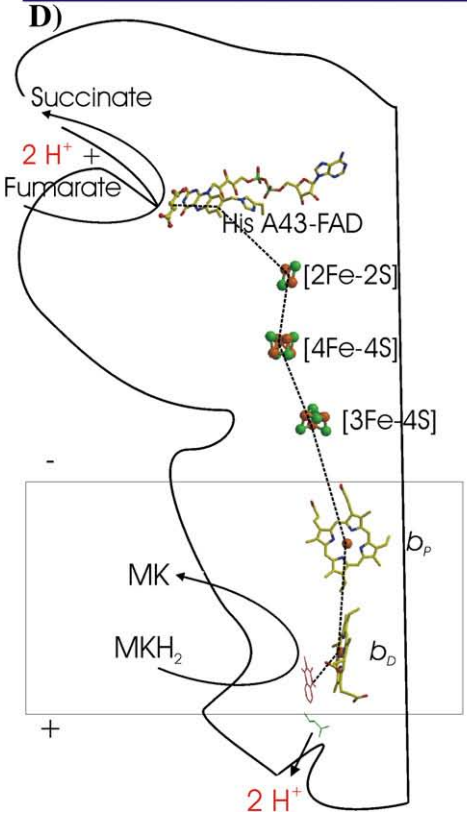
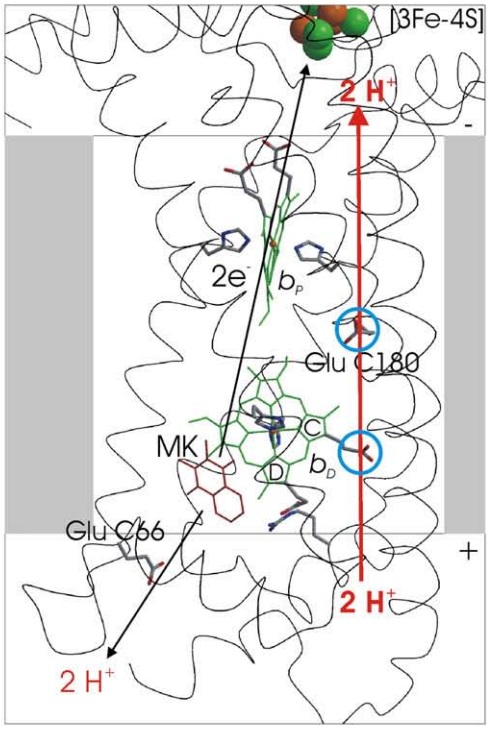

A)
Succinate
Fumarate
+2H+
2e-
MKH2
MK
2H+
B)
Succinate
Fumarate
+2H+
2e-
2H+
MKH2
MK
E180
2H+
2H+


C)
ADP + Pi
ATP
3-4 H+
2 H+
Fumarate
+2 H+
Succinate
FAD
[2Fe-2S]
[4Fe-4S]
[3Fe-4S]
2 H+
2 H+
MK
MKH2
MK
MKH2
[4Fe-4S]
[4Fe-4S]
[4Fe-4S]
[4Fe-4S]
Mo
NiFe
[4Fe-4S]
[3Fe-4S]
[4Fe-4S]
3-4 H+
2 H+ 2 H+
Formate
H+ + CO2
2 H+
H2
ATP synthase
Formate dehydrogenase
QFR
Hydrogenase


D)
Succinate
2 H+ +
Fumarate
His A43-FAD
[2Fe-2S]
[4Fe-4S]
[3Fe-4S]
bP
MK
MKH2
bD
2 H+


E)
[3Fe-4S]
2 H+
2e-
bP
Glu C180
MK
C
D
bD
Glu C66
2 H+
2 H+

reduction of fumarate to succinate (reaction *a*) to the two-electron oxidation of hydroquinone (quinol) to quinone (reaction *b*):

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

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

Genes in *W. succinogenes* encoding for a second membrane protein complex exhibiting fumarate reductase activity have been expressed and the membrane complex produced has been characterized recently [8]. Regarding the QFR from *W. succinogenes*, its crystal structure has been determined, by X-ray crystallography, in three different crystal forms, initially at a resolution of up to 2.2 Å [9]. The relevance of this structure determination for the superfamily of succinate:quinone oxidoreductases [10] has been discussed and reviewed [11–20]. In all three crystal forms, two heterotrimeric complexes of A, B, and C subunits form a dimer of 260 kDa. Based on analytical gel filtration and analytical ultracentrifugation experiments [21], 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 harbours three different iron–sulphur clusters, a [2Fe–2S], a [4Fe–4S], and a [3Fe–4S] centre, and the membrane-embedded subunit C binds two haem *b* groups (Fig. 1D). Based on their proximity to the hydrophilic subunits, these are referred to as the proximal haem b_P and the distal haem b_D. Although it has long been known [22] that the two haem groups have different oxidation–reduction midpoint potentials, it has only recently been possible to assign the "high-potential" haem to b_P and the "low-potential" haem to b_D [23].

Subunit C also contains the active site of menaquinol oxidation, close to the haem b_D. Structural and functional characterization of the variant enzyme E66Q, in which the nearby residue Glu C66 had been replaced by a Gln by site-directed mutagenesis, demonstrated that this residue is selectively essential for quinol oxidation [24]. The orientation of the catalytic sites of fumarate reduction [25], associated with proton binding, and menaquinol oxidation [24], associated with proton release, towards opposite sides of the membrane indicated that quinol oxidation by fumarate should be an electrogenic process in *W. succinogenes* (Fig. 1D), i.e. associated directly with the establishment of an electrochemical proton potential across the membrane.

This electrogenic catalysis indeed appeared to be the case for some dihaem-containing representatives of the superfamily of QFRs and succinate:menaquinone reductases (SQRs). 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 *Bacillus subtilis* and in other prokaryotes containing succinate:menaquinone reductases [26–28]. This is the analogous reaction to that suggested in Fig. 1D, but in the opposite direction (Fig. 1A). However, the experiments on the SQR had previously been performed only with whole cells and isolated membranes and it has been questioned [29] whether the observed effects are associated specifically with the SQR. Recently, it has been shown that the dihaem-containing succinate:menaquinone reductase, isolated from the Gram-positive bacterium *Bacillus licheniformis*

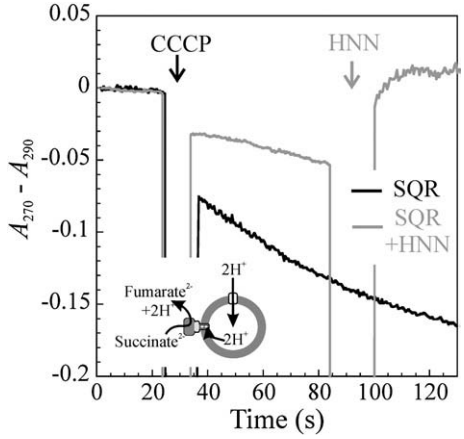


Fig. 2. Addition of the uncoupler CCCP stimulates the reduction of EMN by succinate as catalysed by the proteoliposomal SQR. Proteoliposomal SQR was unable to support the reduction of EMN by succinate without the presence of the uncoupler (black trace). This activity could be inhibited by the addition of 60 μM of the inhibitor HNN (grey trace with 30 μM HNN, addition of further HNN to 60 μM indicated by grey arrow). Figure adapted from reference [30].

and reconstituted into proteoliposomes, is unable to support the reduction of the soluble menaquinone analog 2-ethyl-3-methyl-1,4-naphthoquinone (EMN) unless the protonophore carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP) is added [30] (Fig. 2). 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; Fig. 3). 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 ($MMANH_2$; Fig. 3) [31].

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 [30] (Fig. 4A). Conversely, reduction of the high-potential quinone EQ-0 was found to be associated with the increase of the luminal pH [30] (Fig. 4B). The membrane potential $\Delta\Psi$ was measured with the help of electrodes respectively selective for the lipophilic ions tetraphenylphosphonium (TPP^+) and tetraphenylborate (TPB^-). Both in the case of quinol oxidation (Fig. 5A), as well as in the case of quinone reduction (Fig. 5B), a membrane potential could be measured, thus unequivocally demonstrating electrogenic catalysis in the case of this enzyme [30].

However, as discussed earlier [24], analogous experiments for isolated *W. succinogenes* QFR reconstituted into liposomes had shown

Fig. 1. Electron and proton transfer in *B. licheniformis* SQR (A), in *W. succinogenes* QFR (B, D, E), and in fumarate respiration (C). Positive and negative sides of the membrane are the periplasm and the cytoplasm, respectively. Haem groups are indicated by yellow diamonds. This figure was modified from references [31] and [38]. See text for details. (C) The key enzymes involved in fumarate respiration are shown. Sites of quinone reduction/quinol oxidation are indicated by yellow circles. (D) Hypothetical Δp generation as suggested by the essential role of Glu C66 for menaquinol oxidation by *W. succinogenes* QFR [24]. The prosthetic groups of the *W. succinogenes* QFR dimer are displayed (coordinate set 1QLA; [9]). 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 entry 2BS4 [35]). The position of bound fumarate (Fum) is taken from PDB entry 1QLB [9]. Figure adapted from reference [4]. (E) "E-pathway hypothesis": The two protons that are liberated upon oxidation of menaquinol (MKH_2) are released to the periplasm (bottom) *via* the residue Glu C66. In compensation, coupled to electron transfer *via* the two haem groups, protons are transferred from the periplasm *via* the ring C propionate of the distal haem 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. In the oxidised state of the enzyme, the "E-pathway" is blocked.

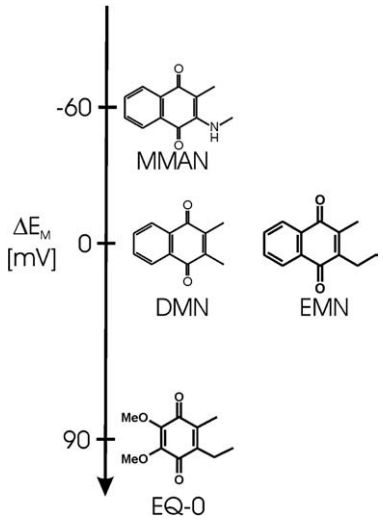


Fig. 3. Chemical structures of the quinones used for the summarised conclusive characterization experiments [30,31]. 2-methyl-3-methylamino-1,4-naphthoquinone (MMAN), 2,3-dimethyl-1,4-naphthoquinone (DMN), 2-ethyl-3-methyl-1,4-naphthoquinone (EMN), 2,3-dimethoxy-5-ethyl-6-methyl-1,4-benzoquinone (EQ-0), and the oxidation–reduction midpoint potentials of the associated quinone/quinol pairs (as determined earlier [30,31]) relative to the DMN/$DMNH_2$ couple (which corresponds approximately to that of the MK/MKH_2 couple). The *in situ* midpoint potential of the DMN/$DMNH_2$ couple in *W. succinogenes* QFR has been determined to be −35 mV [31], i.e. 60 mV lower than that of the fumarate/succinate couple [11]. Figure adapted from reference [4].

that the oxidation of quinol by fumarate as catalysed by this enzyme is electroneutral [31–34].

To reconcile these apparently conflicting experimental observations, the so-called "E-pathway hypothesis" (Fig. 1E) was proposed [3]. 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 haem groups and is closed in the oxidised enzyme. The two most prominent constituents of the proposed novel pathway were suggested to be the ring C propionate of the distal haem b_D and, in particular, the amino acid residue Glu C180, after which the "E-pathway" was named (Fig. 1E). Since the first proposal of this hypothesis, a number of theoretical [23] and experimental results [35–37] have been obtained that support it (reviewed in [38]).

A possible haem propionate involvement as a participant in the E-pathway has been investigated by combining ^{13}C labelling of the haem propionates with redox-induced FTIR spectroscopy [37]. The redox-transition of the distal haem led to protonation and/or environmental changes of (at least) one of the two distal haem propionates. Since it was established that the ring D propionate of the low-potential haem is involved in an extensive salt-bridge interaction with a nearby Arg residue [9,23], 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 [23], which predicted that this residue undergoes the combination of a change in protonation and conformation upon reduction of the haem groups, a result that was also obtained experimentally by the combination of FT-IR difference spectroscopy [36] and site-directed mutagenesis, involving the replacement of Glu C180 with a Gln residue [35]. 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 [35].

The variant enzyme, after reconstitution into proteoliposomes, is unable to support the oxidation of $DMNH_2$ unless CCCP is added [31] (Fig. 6A). Oxidation of the low-potential quinol is supported even in the absence of CCCP (Fig. 6B). 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) [31] (Fig. 6C) as well as TPB^- entry into the proteoliposomes (indicative of $\Delta\psi$ generation) [31] (Fig. 6D). Taken together with the results obtained

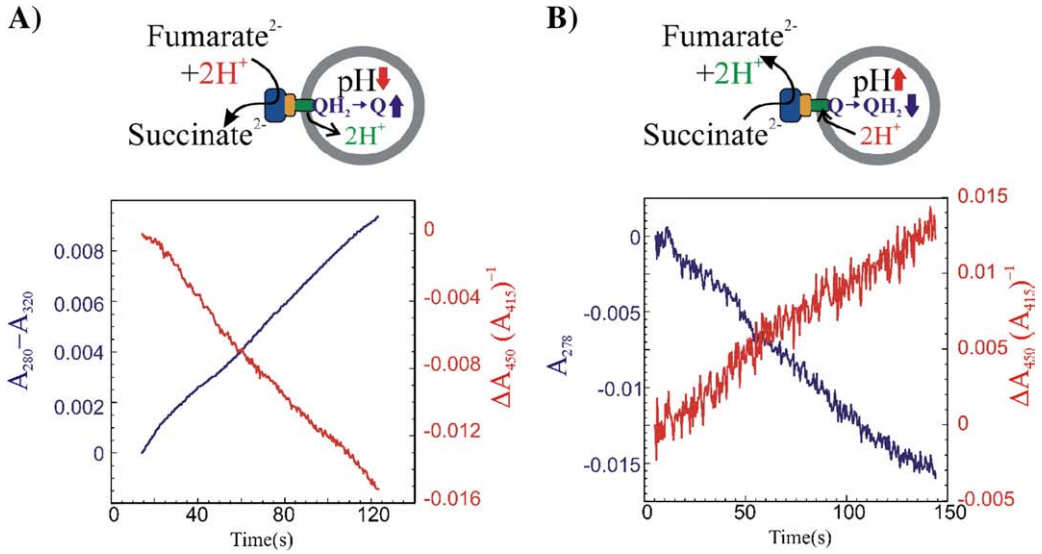


Fig. 4. Catalytic activity of proteoliposomal SQR from *B. licheniformis*, $MMANH_2$ oxidation (A) and EQ-0 reduction (B), shown in blue, and the monitoring of the respective generation of ΔpH, shown in red. Proteoliposomes (200 μg phospholipid) containing the SQR and the respective quinone substrate (10 mM/mg phospholipid) were suspended in N_2-flushed buffer. In case of MMAN, $NaBH_4$ was added to reduce the quinone and the reaction was started by addition of fumarate (20 μM). In case of EQ-0, the reaction was started with succinate (200 μM) without previous reduction of the quinone. Figure adapted from reference [30].

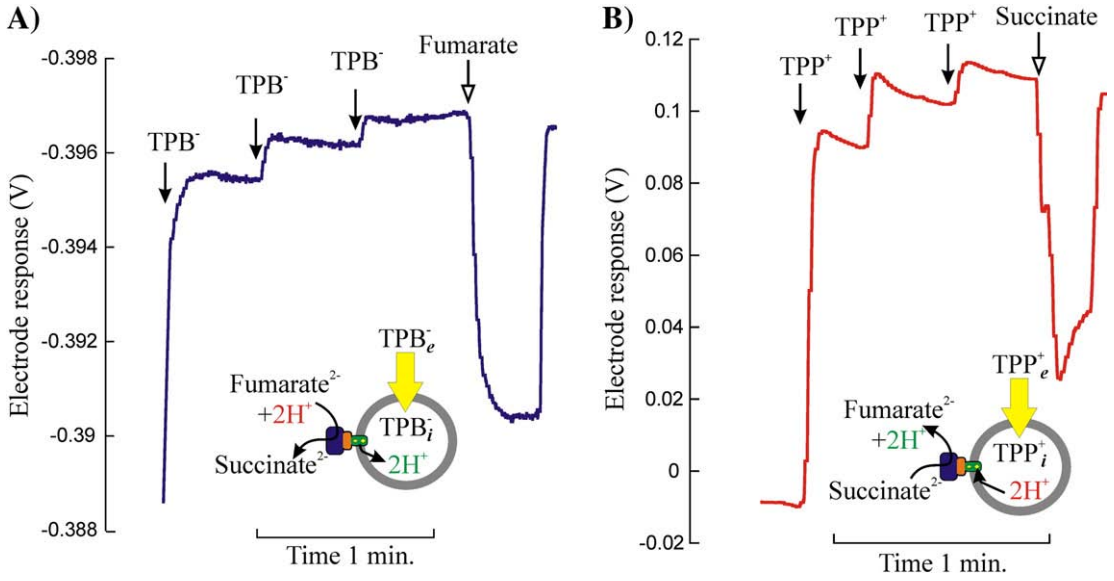


Fig. 5. TPB^- and TPP^+ accumulation upon (A) $MMANH_2$-oxidation and (B) EQ-0-reduction. The $\Delta\Psi$ was monitored as uptake of TPB^- (A) or uptake of TPP^+ (B) with an electrode sensitive to TPB^- or TPP^+ respectively. Proteoliposomes (400 μg phospholipid) containing the SQR and the respective quinone substrate (10 mM/mg phospholipid) were suspended in N_2-flushed buffer. Prior to the reaction, the electrode was calibrated by addition of TPP^+ or TPB^- (solid arrows) as annotated in the plot. In case of MMAN, $NaBH_4$ was added to reduce the quinone and the reaction was started by addition of fumarate (20 μM; open arrow). In case of EQ-0, the reaction was started with succinate (200 μM; open arrow) without previous reduction of the quinone. Figure adapted from reference [30].

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 [31].

While the E-pathway hypothesis as depicted in Fig. 1E is the simplest model compatible with the experimental data obtained so far, it is by no means unique. The simplifying assumption that both protons released upon quinol oxidation are released *via* the same pathway may turn out not to be true, as may 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 (Fig. 6E) also explains the available data [4]. However, there is no such proton transfer connectivity apparent from the structure of the oxidised enzyme, so this scenario would require an appropriate conformational change during catalysis [4].

In summary, it has previously been shown that both fumarate reduction by quinol as well as succinate oxidation by quinone, as catalysed by the dihaem-containing SQR from *B. licheniformis*, i.e. both in the non-physiological and physiological directions, are electrogenic reactions, whereas fumarate reduction by quinol, i.e. catalysis by the dihaem-containing QFR from *W. succinogenes* in the physiological direction, is electroneutral. Here we present the characterization of catalysis by the QFR from *W. succinogenes* in the non-physiological direction, i.e. succinate oxidation by quinone. Surprisingly, we show that this reaction is clearly electrogenic. The implications for transmembrane proton transfer *via* the E-pathway are discussed.

2. Materials and methods

Wild-type QFR was produced and purified as described earlier [9,39–41]. The quinone substrates EMN and EQ-0 had been synthesized previously [30]. Proteoliposomes were prepared as described earlier [34,30,31].

2.1. Enzymatic assays

The reduction of EMN was monitored by recording the difference at 270 nm minus 285 nm and that of EQ-0 by the absorption change at 278 nm ($\varepsilon = 14.7\ mM^{-1}\cdot cm^{-1}$) [30].

2.2. Acidification measurements, H^+/e^- ratio

The acidification measurements upon succinate oxidation by EQ-0 were performed as described [30,31].

2.3. Determination of $\Delta\Psi$

The generation of $\Delta\Psi$ during the reduction of EQ-0 by succinate was monitored using a TPP^+-selective electrode as described for measurements on *B. licheniformis* SQR [30]. Proteoliposomes were suspended in 15 mM HEPES buffer (adjusted to pH 7.5 with NaOH) containing 100 mM KCl. Reaction was started by addition of succinate. The TPP^+ uptake was calculated from the amplitudes between the first succinate addition and the level where the TPP^+ concentration reached a minimum. $\Delta\Psi$ was calculated as described [31].

3. Results and discussion

After reconstitution into proteoliposomes, we attempted to force the QFR from *W. succinogenes*, now bound by sealed membrane vesicles, to catalyse the reduction of quinone by succinate. This is the reverse direction of the reaction to that catalysed physiologically by *W. succinogenes* QFR. In spite of supporting succinate oxidation by EMN with a specific activity of 2.5 U/mg in its detergent solubilised state, when reconstituted into proteoliposomes *W. succinogenes* QFR exhibited no detectable enzymatic activity of succinate oxidation by EMN (left part of Fig. 7). We attributed this non-detectable activity to the unfavourable 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 favourable enough, even with a large excess of starting material over product to support sustained Δp generation. These conclusions are supported by our finding that the addition of CCCP to the ostensibly inactive proteoliposomes enabled the catalysis of EMN reduction (right part of Fig. 7).

In order to measure any generation of a membrane potential Δp, we replaced EMN with the high-potential quinone EQ-0. By

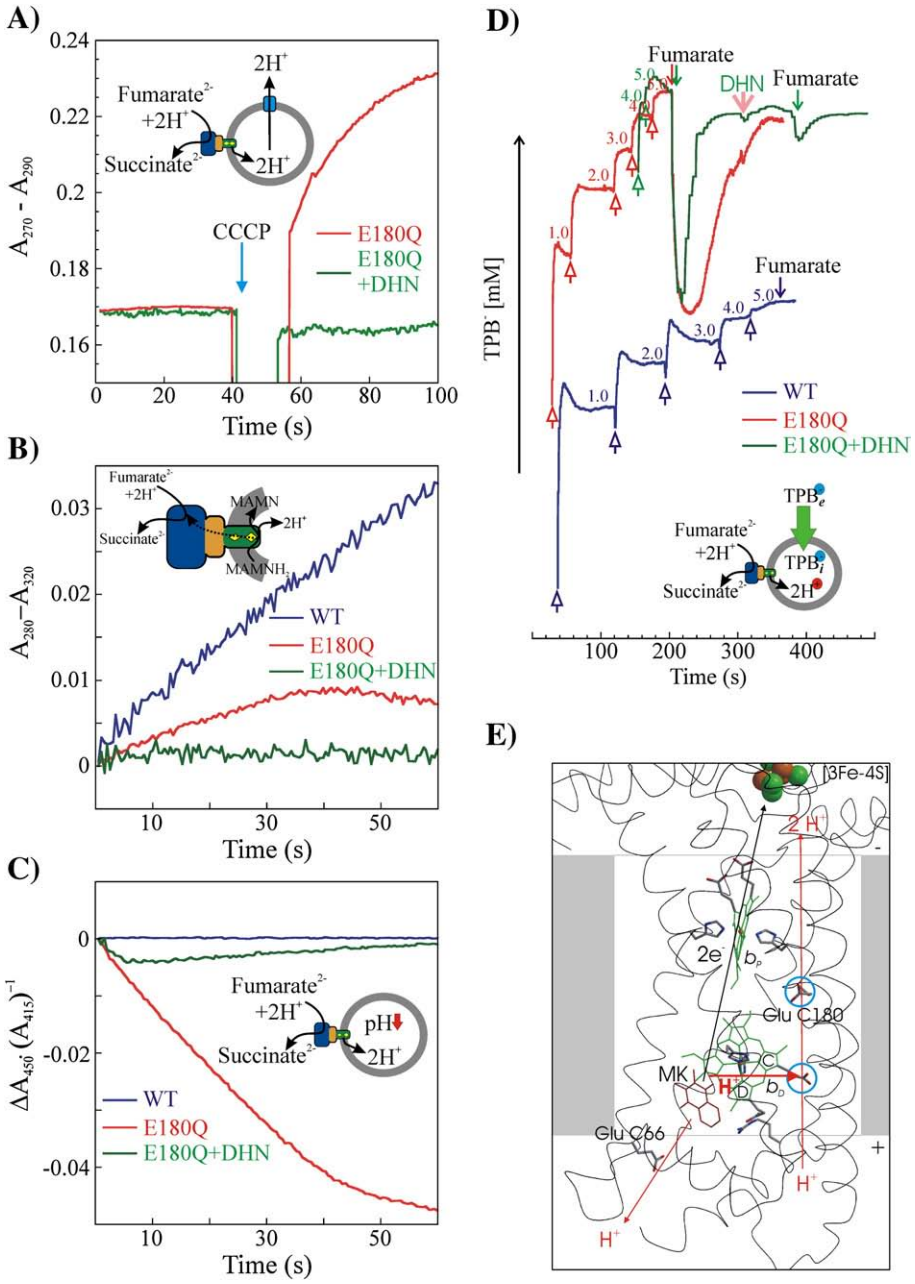


Fig. 6. Catalytic activity (A, B), and monitoring of any generation of ΔpH (B) and $\Delta\Psi$ (C) by wild-type and E180Q-QFR and a modified version of the E-pathway hypothesis (E). Panels A–D are adapted from reference [31], panel E is from reference [4]. (A) $DMNH_2$ oxidation by E180Q-QFR reconstituted in proteoliposomes (500 μg). The traces were recorded under the same conditions as for panel B, except that $DMNH_2$ (20 μM) was used as the electron donor and the fumarate concentration was 40 μM. Catalytic activity was significantly detectable only after the addition of 25 μM protonophore CCCP. (B) Normalized, apparent progress curves of $MMANH_2$ oxidisation by fumarate as catalysed by wild-type QFR (blue trace) and by E180Q-QFR (red) and not catalysed by E180Q-QFR in the presence of the inhibitor DHN (20 μM, green). Proteoliposomes (200 μg) containing the respective QFR enzyme and $MMANH_2$ (10 mM/mg phospholipid) were suspended in N_2-flushed buffer. The reaction was started by the addition of fumarate (20 μM). The absorbance difference A_{280}–A_{320} was monitored as function of time. The traces were normalized by setting the absorption difference of A_{280}–A_{320} to zero and the time of fumarate addition to zero. The progress curves were recorded in the same experiment as in panel C using a diode array spectrophotometer. (C) Normalized progress curves of the pyranine absorbance ratio offset as an indicator of the acidification of the proteoliposomal interior upon $MMANH_2$ oxidation by fumarate. The colour-coding is as defined for panel A. The absorbance ratio offset $\Delta(A_{450}/A_{415})$ depended only on the H^+ concentration in the liposomes and was monitored as function of time. The reaction was started by the addition of fumarate with the time set to 0. The traces were normalized by setting the initial absorbance ratio of A_{450}/A_{415} to zero. (D) Recording of the external TPB^- concentration (logarithmic scale) in a suspension of proteoliposomes during fumarate reduction by $MMANH_2$. To a solution containing proteoliposomes (200 μg in 50 mM HEPES, pH7.5) with reconstituted *wt* QFR (blue trace) or E180Q-QFR (red trace), $MMANH_2$ (10 mM/mg phospholipid), TPB^- was added in 1 mM aliquots for calibration (open arrows pointing upwards). Reaction was started by the addition of 20 μM fumarate (arrows pointing downwards). The E180Q-QFR in presence of 20 μM DHN (green trace, addition of DHN indicated by green arrow) did not generate any appreciable $\Delta\Psi$. The TPB^- uptake was calculated from the amplitude between the first fumarate addition and the level where the TPB^- concentration reached a minimum. The concentration inside the proteoliposomes was corrected according to Eq. (1) in reference [31]. Because the electrodes had different Nernst factors the respective TPB^- concentrations are indicated in the plot in respective colour. (E) Alternative implementation of the E-pathway hypothesis [4]. See text for details.

measuring the uptake of TPP^+ into proteoliposomes after starting the reaction with the addition of succinate (Fig. 8), we 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, analogous to those shown in Figs. 4A, B and 6C (Fig. 9), by monitoring the absorbance properties of pyranine, we could measure in the course of the catalysis a drop in the proton concentration within the proteoliposomes, corresponding to between

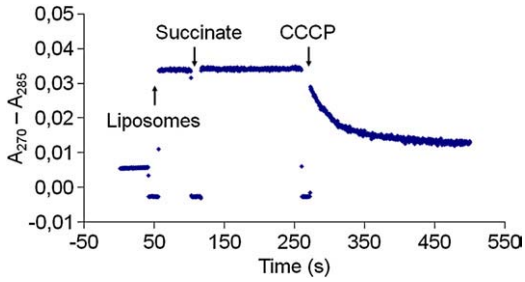


Fig. 7. Addition of the uncoupler CCCP (25 μM) stimulates the reduction of EMN (40 μM) by succinate (20 mM) as catalysed by the proteoliposomes (500 μg) containing QFR (right half). Proteoliposomal QFR was unable to support the reduction of EMN by succinate without the presence of the uncoupler (left half).

0.86 and 1.1 mol H^+ per 1 mol of EQ-0 reduced. This yields H^+/e^- ratios of between 0.43 and 0.55.

Clearly, the results from the uncoupler-stimulated activity test (Fig. 7), the measurement of $\Delta\Psi$ (Fig. 8), and the pyranine absorbance measurements (Fig. 9) are very different from what one would expect from a simple reversal of the reaction monitored in Fig. 6. Taken together, the results indicate 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 (Figs. 2 and 5), 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 are significantly lower, only about half the values of those generated under the same conditions with *B. licheniformis* SQR. Thus, our 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^- ratios of 0.5; Fig. 10). The most straightforward explanation for this phenomenon is that the E-pathway protons do not share completely identical pathways, making the scenario shown in Fig. 6E more likely than the original one shown in Fig. 1E. 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.

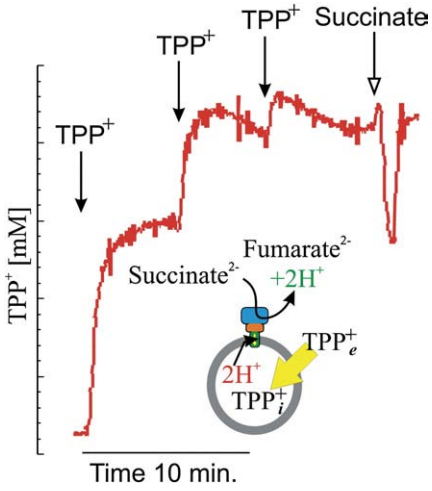


Fig. 8. Recording of the external TPP^+ concentration (logarithmic scale) in a suspension of proteoliposomes during succinate oxidation by EQ-0. To a solution containing proteoliposomes (200 μg in 50 mM HEPES, pH7.5) with reconstituted *wt* QFR, EQ-0 (10 mM/mg phospholipid), TPP^+ was added in 1 mM aliquots for calibration (black arrows). Reaction was started by the addition of 20 μM succinate (open arrow).

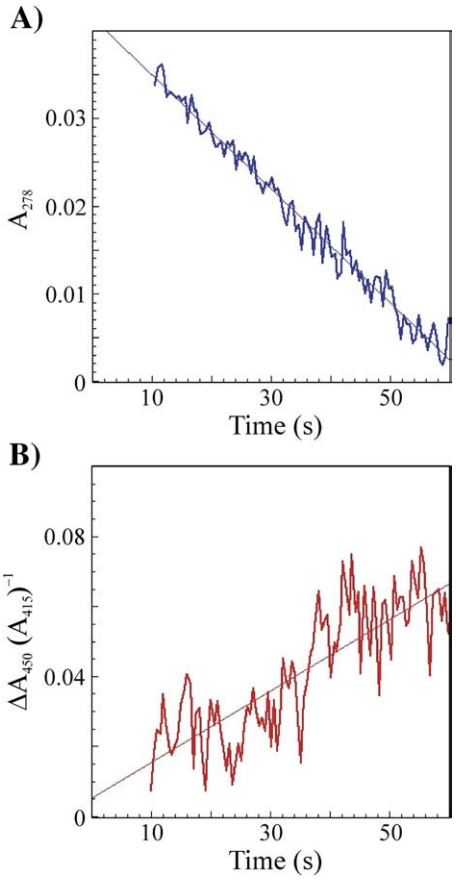


Fig. 9. Catalysis by proteoliposomal wild-type QFR (30 g/l proteoliposomes; 0.1 cm pathlength). Normalized progress curves of (A) EQ-0 reduction by succinate and (B) pyranine absorbance ratio offset as an indicator of the alkalisation of the proteoliposomal interior upon EQ-0 reduction by succinate.

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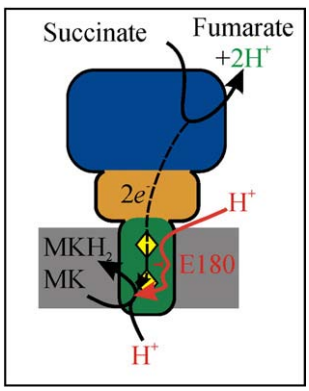

Fig. 10. Possible mechanism underlying catalysis by proteoliposomal *W. succinogenes* QFR of quinone reduction by succinate with an H^+/e^- ratio of 0.5.

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