Minireview

Progress in understanding structure–function relationships in respiratory chain complex II

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Abstract Complex II (succinate:quinone oxidoreductase) of aerobic respiratory chains oxidizes succinate to fumarate and passes the electrons directly into the quinone pool. It serves as the only direct link between activity in the citric acid cycle and electron transport in the membrane. Finer details of these reactions and interactions are but poorly understood. However, complex II has extremely similar structural and catalytic properties to quinol:fumarate oxidoreductases of anaerobic organisms, for which X-ray structures have recently become available. These offer new insights into structure–function relationships of this class of flavoenzymes, including evidence favoring protein movement during catalysis.

Key words: Complex II; Fumarate reductase; Covalent flavin; Iron–sulfur center; Cytochrome *b*; Quinone reductase

1. Introduction

Respiratory chain complex II (succinate:ubiquinone oxidoreductase; SQR; EC 1.3.5.1) and related fumarate reductases are membrane-bound, complex flavoproteins with remarkably similar physical and catalytic properties [1–3]. Each complex can both oxidize succinate and reduce fumarate (Eq. 1), but there is a clear demarcation of roles in the cell. SQR is produced in aerobic organisms, where it catalyzes the oxidation of succinate to fumarate as one step of the Krebs cycle and transfers the electrons directly to the ubiquinone pool ($E_m = +100$ mV). The fumarate reductase complex, quinol:fumarate oxidoreductase (QFR), is synthesized in anaerobes when fumarate replaces oxygen as the terminal oxidant. An accompanying synthesis of a lower potential quinone facilitates fumarate reduction. Facultative prokaryotes and eukaryotes manufacture one or other of the complexes and the appropriate quinone depending on the redox status of the cell.

$$\text{succinate} \Leftrightarrow \text{fumarate} + 2H^+ + 2e^- \quad (E_m = +10\ \text{mV},\ \text{pH}\ 7) \qquad (1)$$

Why cells should use separate enzymes for oxidizing succinate and reducing fumarate is unclear. That *Escherichia coli* QFR produces superoxide in air is relevant [4], since aberrant superoxide generation by mutated SQR in the nematode *Caenorhabditis elegans* is known to result in premature aging and death [5]. On the other hand, fumarate reduction by SQR 'shuts down' (70–90%) under conditions of high reducing potential ('tunnel diode effect') [6]. Thus, SQRs appear ill-suited as physiological fumarate reducers, in accord with the observation that *E. coli* grows much less efficiently using SQR instead of QFR for anaerobic growth [7]. QFRs characteristically maintain catalytic efficiency under highly reducing conditions [6].

Isolated complexes literally [1,2] can be resolved into two parts (Fig. 1A). The hydrophilic domain consists of a flavoprotein subunit (Fp), in which the covalent FAD co-factor of the enzyme is part of the catalytic site, and an iron–sulfur subunit (Ip) containing three different clusters $[2Fe–2S]^{2+,1+}$, $[4Fe–4S]^{2+,1+,}$ and $[3Fe–4S]^{1+,0}$ for electron transfer between the FAD and membrane quinone. The primary sequences of Fp and Ip are highly homologous among species and thus indicative of common ancestral genes. Hydrophobic domains show greater diversity, being comprised of one or two membrane subunits with differing *b*-type heme content and little sequence homology. These anchors contain the binding sites for quinones and inhibitors. Bacterial complexes are oriented with the hydrophilic domain projecting into the cytoplasm and anchor polypeptides spanning the cytoplasmic membrane. Eukaryotic complexes have this same topology with respect to the mitochondrial matrix and inner membrane.

Authoritative reviews of these enzymes [1–3] predate newly acquired X-ray structures of *E. coli* QFR [8], *Wolinella succinogenes* QFR [9], and the single-subunit flavoenzymes *E. coli* L-aspartate oxidase (LASPO) [10] and the flavocytochromes c_3 of *Shewanella frigidmarina* [11,12] and *putrefaciens* [13]. Some aspects of structure–function are therefore briefly reconsidered here in light of this new structural information.

2. Flavoprotein subunit

Fp polypeptides (73–60 kDa) are folded into four domains, including a large FAD binding domain containing the Rossmann-type fold seen in FAD binding proteins and a capping domain. Flavocytochrome c_3 uniquely contains one domain for a linear array of four hemes carrying electrons to FAD [11–13]. A feature of the LASPO [11] and flavocytochrome [12,13] structures lacking bound substrate is that the capping domain shows mobility (Fig. 1B) by rotating away from the FAD domain. Active site residues supplied by each of these

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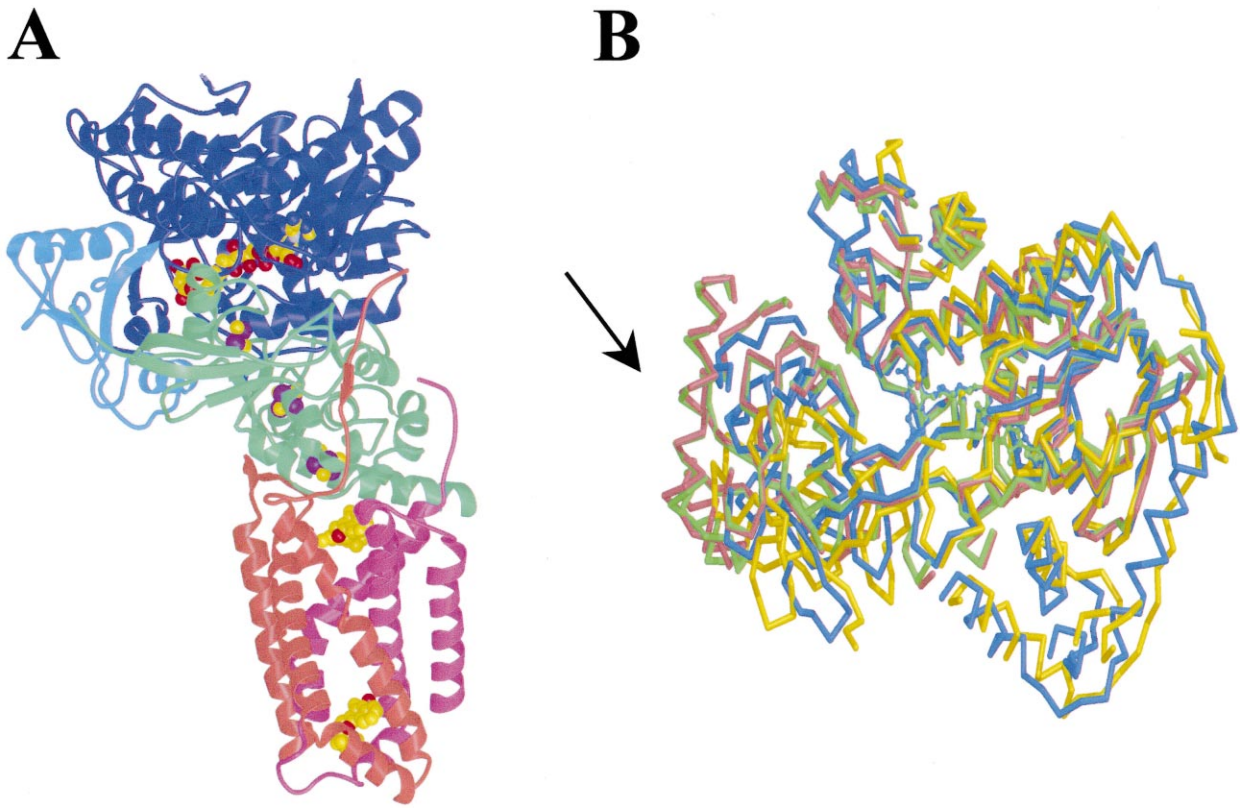

Fig. 1. A: Structure of *E. coli* QFR. In the Fp subunit (blue) the bound oxaloacetate (yellow) is between the covalent FAD (yellow) and the capping domain (cyan); Ip (green) contains three Fe–S clusters (purple); the C (red) and D (violet) anchor polypeptides contain two bound menaquinones (yellow), one close to Ip and one which would be close to the outer (+) surface of the membrane. B: Movement of capping domain. Overlay of the 'closed' forms of *E. coli* Fp (blue) and the fumarate reductases of *S. frigidmarina* (red) and *S. putrefaciens* (green), and the 'open' form of *E. coli* L-aspartate oxidase (yellow). Capping domain is indicated by arrow.

domains thus become farther apart. The residues are in place in the *Wolinella* and *Shewanella* enzymes containing bound fumarate [11,13] or succinate [13], and in *E. coli* QFR bound with oxaloacetate, a competitive inhibitor. One inference, clearly, is that binding of substrate induces movement of the capping domain to close the active site to solvent. It might also explain why the presence of substrate should greatly enhance covalent FAD attachment to apo-Fp [14,15].

LASPO and flavocytochrome c_3 are among unusual fumarate reductases that are soluble, unable to oxidize succinate, and have non-covalent rather than covalent 8α-(*N*3)-histidyl–FAD. Interactions with the protein environment and covalent linkage combine to raise the redox potential of free FAD (−219 mV) to allow reduction by succinate. Thus, the E_m of the FAD/$FADH_2$ couple in flavocytochrome c_3 (−152 mV) [16] is appreciably lower than in, for example, *W. succinogenes* QFR (−20 mV) and beef SQR (−79 mV), which have covalent flavin [1,2].

Interpretations of the *Wolinella* and *Shewanella* enzyme structures provide slightly different detail to the mechanism of *trans* hydrogenation of fumarate first suggested by Vik and Hatefi [17]. In the former case, fumarate docking is seen as involving hydrogen bonding of one carboxylate to Arg A301 and the other carboxylate to both Arg A404 and His A369 (Fig. 2). Hydride exchange between N5 of reduced flavin and the substrate β-methylene carbon is then accompanied by protonation of the α-methylene position by a water molecule hydrogen bonded between Arg A301 and Arg A404. Based on the *S. frigidmarina* and *putrefaciens* enzyme structures, however, residue Arg A402/401 (equivalent to *Wolinella* Arg A301) is the proton donor, not H_2O, and the carboxylate group is instead bound by His A365/364 (not shown). Steric constraints at the active site force the fumarate out of its planar conformation. This and electron redistribution due to the strong polar hydrogen bonding at one end of the molecule

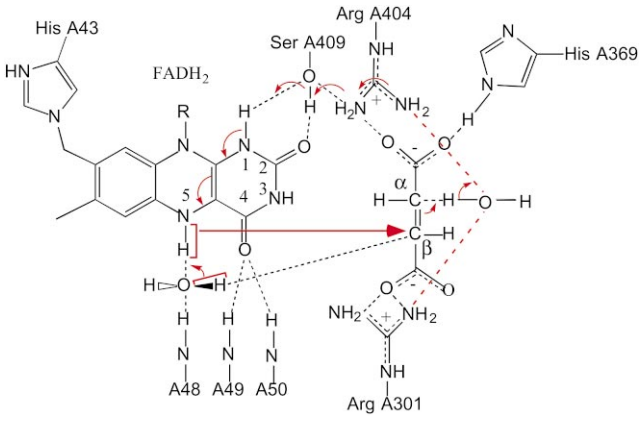

Fig. 2. Proposed mechanism of fumarate reduction by *W. succinogenes* QFR. Also indicated are backbone amino groups from residues A48–50 and hydrogen bonded water molecules. Fumarate reduction occurs by hydride transfer from the isoalloxazine N(5) position to the β-carbon and a proton from water to the α-position. Taken from [9].

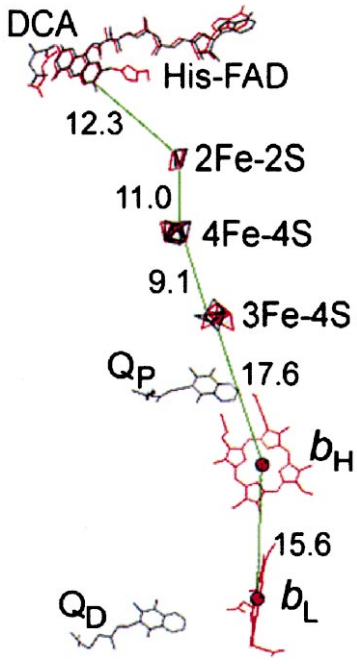

Fig. 3. Electron pathways in *E. coli* (black) and *W. succinogenes* (red) QFRs. Distances are in Å. DCA is oxaloacetate (*E. c.*) or fumarate (*W. s.*). Distance between Q_P, proximal menaquinone, and Q_D, distal menaquinone, is 27 Å [8]. E_m values for FAD, [2Fe–2S], [4Fe–4S], [3Fe–4S], b_H, and b_L in *W. s.* are −20, −59, −250, −24, −20, and −200 mV, respectively, and for FAD and respective Fe–S clusters in *E. c.*, −55, −20 to −79, −320, and −70 mV, respectively [2,30]. Structures were superimposed based on the Cα atoms of the Fp and Ip subunits.

opens up the fumarate to hydride attack at the β-carbon. Importantly, the malate-like intermediate detected snugly bound within the 'closed' active site of the oxidized *Shewanella* enzyme [9] offers convincing proof that, in the absence of hydride, the β-carbon is attacked by H_2O and that this step precedes the proton transfer step. The trapping and nature of the hydrated intermediate may well also explain the extraordinarily tight binding of oxaloacetate ($K_D = 10^{-8}$ M) to these complexes [2] and the higher potency of its enol tautomer [18]. Unfortunately, the form of oxaloacetate bound in the *E. coli* QFR structure (3.3 Å) cannot be discriminated. A previously undetected Na^+ ion with possible structural or regulatory roles is located close to the active site [11]. Oxidation of succinate would be the reverse of a scheme such as presented in Fig. 2.

A highly conserved cysteine is responsible for the sensitivity of many complexes to *N*-ethylmaleimide (NEM). Protected by substrate and competitive inhibitors such as oxaloacetate and malonate, the thiol has been assumed to be part of, or close to, the active site (see [2]). The crystal structures now show the cysteine in question, Cys A248 in *E. coli* QFR and Cys A272 in *W. succinogenes* QFR, is located on the capping domain and some 8–9 Å further away than bound substrate from the isoalloxazine ring. While the NEM-modified cysteine conceptually might block passageway to the active site, it is difficult to see how bound substrate/competitive inhibitor makes a distal cysteine inaccessible other than by inducing a conformational change in the protein. Spectral changes [19] and loss of flavin fluorescence [20] on binding competitive inhibitors provide tangible evidence of induced conformation changes in the oxidized enzyme. Importantly, the dissociation constants ($K_D = k_{off}/k_{on}$) for such complexes increase up to an order of magnitude when the FAD moiety of the enzyme becomes reduced, the effect being solely on k_{off} [2,21]. Without bound substrate, the oxidized and reduced forms of the enzyme are equally 'open' to attack by NEM [21]. These data argue that ligand binding to oxidized enzyme closes the active site and that the site is open in the catalytic cycle whenever the FAD moiety is in the fully reduced state.

The conserved Arg A404 residue (Fig. 2) not only binds substrate, but also is essential for formation of the histidyl–FAD bond. This role was revealed in our studies (unpublished) of a complex II deficiency caused by an Arg A450 Cys mutation in the human Fp subunit. The mutated complex neither oxidizes succinate nor reduces fumarate, in keeping with the loss of hydrogen bonding to substrate (Fig. 2), and contains non-covalent FAD. The patients survive because they have a second allele producing normal Fp. The mechanism proposed for flavinylation requires a positive charge in the region of the N1/C2 positions of the isoalloxazine ring in order to stabilize a negative charge developing during production of the iminoquinone methide intermediate [22]. Of seven covalent flavoproteins with known structures, now including those of the *E. coli* and *Wolinella* QFR, six have an arginine in that position and one has a positive charge at the end of a α-helix. LASPO and flavocytochrome c_3 have non-covalent FAD because they lack the His that is flavinylated. The positive charge would also stabilize the negative charge localized

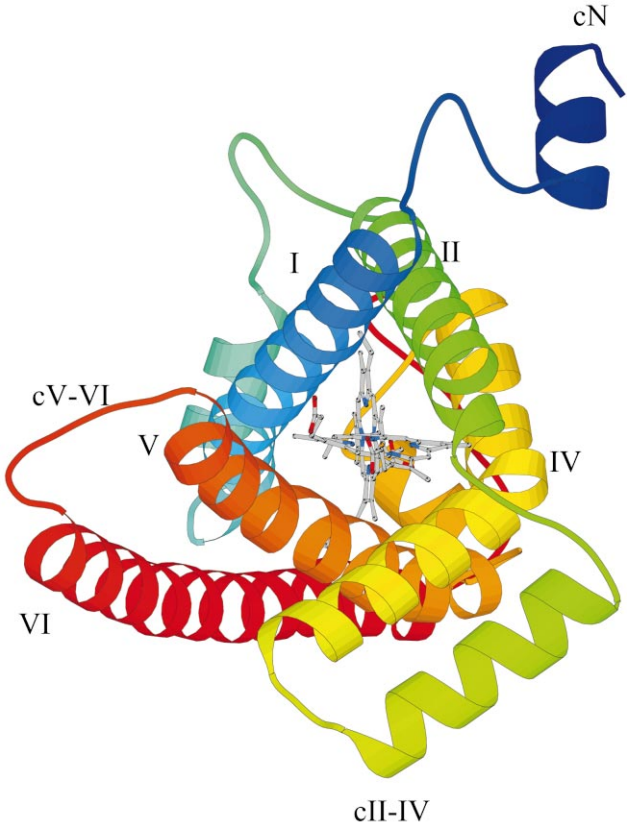

Fig. 4. View of the helices of a subunit C monomer of *W. succinogenes* from the cytoplasmic (−) surface of the membrane. Transmembrane helices are labelled I, II, IV, and VI after [30]. Hemes viewed edge-on are at the center of the pore.

at N1/C2 in the anionic (red) radical generated during normal catalysis [23].

3. Iron–sulfur subunit

Ip subunits (24–31 kDa) have an N-terminal 'plant ferredoxin' domain containing the $[2Fe–2S]^{2+,1+}$ cluster and a C-terminal 'bacterial ferredoxin' domain with the $[4Fe–4S]^{2+,1+}$ and $[3Fe–4S]^{1+,0}$ clusters. Three highly conserved groups of cysteines in the sequence are the ligands (discussed in [1,2]). A novel group of SQRs and QFRs from archaea (e.g. *Acidianus ambivalens*) and bacteria (e.g. *Methanococcus jannaschii*) lack the EPR signal for the canonical [3Fe–4S], but have an extra cysteine in the sequence suggestive of a second [4Fe–4S] [24]. Whereas replacement of the native 3Fe by a 4Fe cluster could be achieved by introduction of a cysteine into *E. coli* QFR [25], this was not so for *Bacillus subtilis* SQR [26]. Modification of clusters is deleterious to stability and/or activity. The crystal structures of *E. coli* and *W. succinogenes* QFRs emphasize the conservative nature of Ip by showing that the clusters (Fig. 3) and a majority of Cα atoms superimpose. It explains why chimeras of human and yeast Ips will confer activity on Ip-deficient yeast [27].

X-ray crystallography now confirms the proximity of the [2Fe–2S] cluster to FAD and the interposition of the [4Fe–4S] cluster between [2Fe–2S] and [3Fe–4S] in a linear array (Fig. 3). It dispels past misgivings about a role for [4Fe–4S] in electron transport because of its characteristic low potential (−200 to −300 mV; for discussions, see [1,2]), one explanation for which is anti-cooperative electrostatic interactions [28]. The integrity of [3Fe–4S] is essential for the binding of Ip to the anchor domain and for electron exchange with bound quinone. Distances of 9–12 Å are appropriate for the paramagnetic spin–spin interactions reported between neighboring centers [1,2] and for efficient and directional electron transfer between FAD and quinone [29]. The relatively high stability constants of the free radical forms of FAD (2.5×10^{-2}) and bound quinones ($\sim$10) allow ready interfacing with single-electron [2Fe–2S] and [3Fe–4S], respectively (see [1,2]).

4. Anchor polypeptides

The most prevalent anchor domain consists of two polypeptides, C and D, of 13–18 kDa and 11–16 kDa, respectively, each contributing three transmembrane α-helices (I, II, III, and IV, V, VI). The N-terminus of each polypeptide is at the (−) side of the membrane and the C-terminus at the (+) surface. Such anchors contain one or two low spin ($g = 3.6$) protohemes IX, or, as in *E. coli* QFR, no heme. The other type of anchor is a single polypeptide (C; 23–30 kDa) providing all but α-helix III and liganding two hemes (discussed in [1,30]). The unifying model for anchor domains [30], now supported by two crystal structures [8,9], has a core of four transmembrane anti-parallel helices (α-helices I, II, IV, and V) with the hemes coordinated inside the bundle. Helices II and V each provide a histidine ligand to the heme (b_H) closest to the [3Fe–4S] cluster, and helices I and IV provide the histidines for any second, lower potential heme (b_L) located towards the (+) membrane surface (Fig. 3). That side chains from all four helices help bond b_H emphasizes the importance of heme to assembly of the complex. The planes of the two hemes are normal to the membrane surface and 95° to each other (Fig. 4).

The generation of two protein-stabilized and interacting ubisemiquinones ($Q^{\bullet-}Q^{\bullet-}$) during reduction of ubiquinone by beef SQR is reviewed in detail in [1,2]. By analogy to the bacterial reaction center [31], a linear series of events is assumed in which electrons are passed singly from [3Fe–4S] to a primary Q_A acceptor functioning between the Q and $Q^{\bullet-}$ state, and thence to the secondary acceptor Q_B, which becomes fully reduced before exchanging into the Q pool [1,32]. The *Wolinella* QFR crystal contains no quinone [9]. That of *E. coli* QFR [8] contains two bound menaquinones in pockets also formed by helices I, II, IV, and V, one (Q_P) being proximal to the [3Fe–4S] cluster and the other (Q_D) near the outside (+) surface of the membrane (Figs. 1 and 3). Mutation of residues at these sites affects menaquinone oxidation or reduction [32]. Similarly spaced sites have been indicated in yeast [33,34] and beef SQRs [35,36]. The dilemma is that in *E. coli* QFR, which contains no heme, the extended distance of 27 Å between Q_P and Q_D [8] precludes efficient electron transfer without movement of the quinones or discovery of an intervening electron carrier. Nor do distant sites reconcile with the 8 Å estimated to separate the $Q^{\bullet-}Q^{\bullet-}$ pair in beef SQR [37] and detected also in green plants [38] and *Neurospora crassa* [39]. A previous argument for double occupancy at the Q_P site is not yet substantiated [1].

That at least one of the semiquinones is close to the [3Fe–4S] and b_H cluster is indicated by the interdependent EPR properties of the cluster and $Q^{\bullet-}Q^{\bullet-}$ signal [1,2], and by the fact that resistance to inhibitory thenoyltrifluoroacetone and carboxanilides is conferred by a mutation within the cysteine motif liganding the [3Fe–4S] cluster [40,41] and by one in the D anchor of *Paracoccus denitrificans* predicted also to be part of this pocket [42]. Further, studies utilizing *n*-heptyl-4-hydroxyquinoline-*N*-oxide, which is considered to inhibit the $Q^{\bullet-} \leftrightarrow QH_2$ partial reaction, localized the menaquinol site to the cytoplasmic (−) side of the membrane in *E. coli* QFR [43] but at b_L on the opposite side in the diheme *B. subtilis* SQR [44]. The data would support the classification of SQRs into two types: those that interact with ubiquinone, have one heme, and conduct quinone chemistry on the negative side of the membrane, as opposed to those that are diheme and reduce menaquinone at the (+) side of the membrane [1]. For the latter category, the fact that reduction of menaquinone ($E_m = -74$ mV) by succinate is a thermodynamically uphill reaction ($\Delta G^{0'} = +20$ kJ/mol) and inhibited in *B. subtilis* by uncouplers or disruption of the membrane would suggest the membrane potential is the driving force [45].

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