FUNCTION AND STRUCTURE OF COMPLEX II OF THE RESPIRATORY CHAIN*

Gary Cecchini
Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121, and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143; email: ceccini@itsa.ucsf.edu

Key Words succinate dehydrogenase, fumarate reductase, quinone oxidoreductase, reactive oxygen species, respiratory chain

Abstract Complex II is the only membrane-bound component of the Krebs cycle and in addition functions as a member of the electron transport chain in mitochondria and in many bacteria. A recent X-ray structural solution of members of the complex II family of proteins has provided important insights into their function. One feature of the complex II structures is a linear electron transport chain that extends from the flavin and iron-sulfur redox cofactors in the membrane extrinsic domain to the quinone and $b$ heme cofactors in the membrane domain. Exciting recent developments in relation to disease in humans and the formation of reactive oxygen species by complex II point to its overall importance in cellular physiology.

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OVERVIEW OF MEMBRANE-BOUND RESPIRATORY CHAIN

Cells oxidize a variety of substrates to generate the energy used for metabolism. Membrane-associated respiratory reactions energize vectorial proton translocation to generate this energy. In mitochondria and many aerobic bacteria this process occurs through the electron transport chain with oxygen serving as the terminal electron acceptor. In anaerobic and facultative anaerobic bacteria, however, organic and inorganic compounds other than oxygen can serve as the ultimate electron acceptor. Fumarate, for example, can act as the terminal electron acceptor for growth on glycerol, lactate, formate, or molecular hydrogen during anaerobic respiration. The protein components of the respiratory chain are oligomeric complexes located in the inner mitochondrial membrane in eukaryotes and the cytoplasmic membrane of prokaryotes. In the field of bioenergetics these protein complexes are often referred to as the multisubunit electron transport complexes I, II, III, and IV, and the mitochondrial oxidative phosphorylation system is composed of these complexes along with ATP synthase (complex V) (Figure 1) (1). During the past few years great strides have been made in our understanding of the three-dimensional structures of the membrane-bound enzyme complexes that interact to form aerobic and anaerobic electron transport chains (2). This review focuses on complex II [succinate: ubiquinone oxidoreductase (SQR) and menaquinol:fumarate oxidoreductase (QFR), its homolog utilized for anaerobic respiration]; however, a brief discussion of the components of the other respiratory complexes is necessary in order to place the function of complex II in context.

The entry point for electrons into the mitochondrial electron transport chain is through NADH:ubiquinone oxidoreductase (complex I). This is the largest respiratory complex, with a molecular mass greater than 900 kDa; the bovine enzyme contains at least 45 different subunits (3). Complex I catalyzes electron transfer from NADH to quinone through a series of redox centers that include a flavin mononucleotide (FMN) moiety, seven to nine iron-sulfur centers, and up to three detectable ubisemiquinone species (4–6). Coupled to electron transfer, protons are vectorially translocated across the mitochondrial inner membrane such that complex I is one of three respiratory chain complexes where energy is conserved. Electron microscopy shows that complex I exhibits an overall L shape with the membrane domain connected by a thin collar to the stalk domain in the matrix of the mitochondrion (or cytoplasm of bacteria) (7, 8). Recent electron microscopy studies of the Escherichia coli complex I suggest, however, that the native conformation exhibits a horseshoe-shaped structure and that the complex can convert its shape depending upon ionic strength of the buffer used for isolation (9). Mammalian complex I also exhibits an active/de-active transition that can be modulated by divalent cations and other factors in intact mitochondria, and it has been suggested that conformational changes in the enzyme are responsible for this transition (10). Whether the conformational change observed by electron cryomicroscopy is responsible for the active/de-
Figure 1  Diagram of respiratory chain from mitochondria. The complex II Protein Data Bank accession code is 1FUM; complex III, 1BGY; cytochrome c, 1CXA; complex IV, 1OCC; complex V, 1QO1. The complex I structure is a representation of the architecture of NADH:ubiquinol oxidoreductase determined in Reference 9.
active transition will require further experimentation. An X-ray structure for complex I is not yet available, and further understanding of its intricate architecture and regulation will necessitate much more experimental study.

Ubiquinol produced by the action of membrane-bound dehydrogenases such as complexes I, II, and electron transfer flavoprotein-ubiquinone oxidoreductase (ETF Q-reductase) is oxidized by complex III (ubiquinol-cytochrome c oxidoreductase or cytochrome bc1 complex). Complex III in mammalian mitochondria contains 11 subunits, which include a membrane-bound diheme cytochrome b, and a membrane-anchored cytochrome c1 and [2Fe-2S]-containing Rieske iron-sulfur protein. The electrons from ubiquinol are transferred to cytochrome c and this reaction develops the protonmotive Q cycle (11). Complex III is thus another of the mitochondrial respiratory complexes where energy is conserved. The transmembrane arrangement of complex III and the topographical orientation of the redox groups and mechanistic implications of this structure have been reviewed in detail in previous volumes of this series (12, 13). The final member of the mitochondrial electron transport chain that generates a transmembrane proton gradient is the terminal cytochrome oxidase (complex IV). Complex IV is a member of a superfamily of heme-copper oxidases found in many bacteria as well as the mitochondrion. The mammalian enzyme contains 13 different subunits, 3 of which are mitochondrially encoded (12, 14, 15). Complex IV has four redox metal centers, CuA, heme a, heme a3, and CuB, that are part of a pathway from the substrate cytochrome c. Electrons are first transferred from cytochrome c to the mixed valence copper center (CuA) in subunit II. The electrons are subsequently transferred to cytochrome a in subunit I and then to the a3 CuB binuclear active site, also in subunit I, where they reduce oxygen to two water molecules (2, 12, 14, 15).

The final component of the oxidative phosphorylation system of mitochondria is the ATP synthase (complex V or F1F0 ATPase). This enzyme is functionally reversible; it can use the proton gradient generated by the electron transport system described above to synthesize ATP and it can also hydrolyze ATP and pump protons against the electrochemical gradient. The crystal structure of the F1 component of the bovine ATPase was the first reported for the members of the oxidative phosphorylation system described above (16) (Figure 1), and this structure supported the elegant binding exchange mechanism proposed for catalysis by the ATPase (17). The E. coli F1F0 ATPase contains 8 different subunits, whereas the bovine enzyme contains 16 different proteins (18). Both the bacterial and mammalian enzymes have a proton channel in the F0 portion, which is linked to the catalytic F1 portion by a stalk that is necessary for the structural rotation of the F1 portion during catalysis (19).

**COMPLEX II**

Complex II has been associated with many seminal discoveries involving the structure and function of the bioenergetic complexes over the past 50 years. An excellent description of the part that complex II played in the discovery of covalently bound flavin cofactors, labile sulfide and properties of iron-sulfur
clusters, and utilization of protein-stabilized ubiquinones by mitochondria for electron transfer can be found in a review by Helmut Beinert (20). The enzyme studied was succinate dehydrogenase (SDH), which catalyzes the oxidation of succinate to fumarate as part of the Krebs cycle. Subsequent studies showed an additional role for SDH besides its part in the Krebs cycle: Following succinate oxidation, the enzyme transfers electrons directly to the quinone pool; hence complex II is more precisely termed succinate:quinone oxidoreductase (SQR). Fumarate reductase (FRD), or menaquinol:fumarate oxidoreductase (QFR), is found in anaerobic and facultative organisms such as bacteria and parasitic helminths, where membrane-bound forms of the complex catalyze the oxidation of reduced quinones coupled to the reduction of fumarate. It was first shown in *E. coli* that separate enzymes for fumarate reduction and succinate oxidation are present depending upon growth conditions (21). In organisms that contain both a FRD and a SDH the synthesis of SDH is repressed and FRD is induced by anaerobic conditions (22–24). Nevertheless, it has been shown using genetic manipulation of *E. coli* that in vivo QFR and SQR can functionally replace each other if appropriate conditions are used to allow expression of their respective gene products (25, 26). As more information on the structure and genetic organization of SQR and QFR has become available the fact that they can functionally replace each other seems less surprising. It should be emphasized that QFR and SQR can catalyze the same reactions in vivo and in vitro, attesting to their high degree of sequence and structural similarity.

In eubacteria the genes for complex II are usually encoded as part of a compact operon. For example, *E. coli* SQR genes are in the order *sdhCDAB*, whereas those for QFR are ordered *frdABCD*. In both cases the C and D genes encode hydrophobic membrane anchor proteins that interact with quinones and are necessary to anchor the catalytic domain to the membrane surface. The gene order for archaeal complex IIs, however, can vary; for many enzymes termed SQRs the gene order is *sdhABCD*, like the QFR sequence (27). Similarly, the *Wolinella succinogenes* QFR is ordered *frdCAB* (28), as is the SQR from the gram-positive organism *Bacillus subtilis* (*SdhCAB*) (29). These latter organisms are examples of the class of complex II in which the hydrophobic C and D polypeptides have apparently fused into a single membrane anchor subunit. In all cases the polypeptide containing the dicarboxylate binding site and the flavin cofactor is encoded by the A gene, and the iron-sulfur-containing subunit of complex II is encoded by the B gene. In the case of eukaryotic organisms, mitochondrial DNA encodes a number of the protein components of the other electron transport and oxidative phosphorylation complexes (complexes I, III, IV, and V); however, with only a few exceptions all the genes for complex II are nuclear encoded. The exceptions include mitochondrial genomes from red algae such as *Porphyra purpurea* and heterotrophic zooflagellates such as *Reclinomonas americana*, in which the *sdhB, sdhC*, and *sdhD* genes are mitochondrially
encoded (30). The high degree of sequence similarity across species for the hydrophilic subunit genes (A and B) and for the membrane anchor C subunit, as well as their nuclear and mitochondrial locations, has been used to support the idea that mitochondria and citric acid cycle evolution originated from within the α-proteobacterial branch of eubacteria (30, 31).

It had been predicted, based upon nucleotide and amino acid sequence comparison of QFR and SQR as well as biochemical analysis of their proposed structures, that they have a common evolutionary precursor (32–36). A class of soluble fumarate reductases is found in many anaerobic or microaerophilic bacteria such as from the genus Shewanella (37), yeast (38), and unicellular parasites like Trypanosoma brucei (39). Although these enzymes do not exhibit many of the properties of the classical complex II, the flavoprotein domain is homologous to the flavoprotein subunit of SDH and FRD. Two characteristics of this soluble class of FRD differ from classical complex II: (a) the flavin is noncovalently bound to the enzyme; and (b) the enzymes are essentially unable to oxidize succinate (37–40). Similar to what is found in nature, when site-directed mutants of *E. coli* QFR (41) or *Saccharomyces cerevisiae* SQR (42) were constructed that produce enzymes containing noncovalently bound flavin, the enzymes had lost the ability to oxidize succinate, although they retained the ability to reduce fumarate. This led to the suggestion that during evolution of complex II the primordial form of the enzyme contained a noncovalently linked flavin cofactor and that this protein was able only to reduce fumarate (34, 36). Upon acquisition of the covalent flavin linkage the enzyme would become able to catalyze succinate oxidation, which is the physiological reaction for SDH. It would also have been necessary for the enzymes to acquire other intermediate electron carriers of suitable redox potential, such as iron-sulfur clusters or cytochromes as in the *Shewanella* fumarate reductases (37). In order for the enzyme to become a complex II, the capacity to bind to the membrane domain and interact with electron carriers such as quinones would have to be acquired. The high degree of sequence conservation of the flavin and iron-sulfur domain of complex II is in agreement with suggestions that FRD and SDH evolved from a common evolutionary ancestor. The membrane domain of complex II has less sequence conservation across species; nevertheless there is a common structural motif of a four-helix bundle for the transmembrane domain. The sites of quinone reduction/oxidation are found in the membrane domain. It is presumed that once the soluble forms of SDH and FRD bound to the membrane domain over the course of evolution, subtle changes in the potential of the redox cofactors allowed the enzyme to interact with different quinone species in the membrane. As ubiquinone has a higher redox potential than menaquinone, the more positive redox potential of the iron-sulfur clusters of SQR (compared to QFR) allows a thermodynamically more favorable reaction for reduction of ubiquinone compared to menaquinol oxidation by QFR.
OVERALL STRUCTURE OF COMPLEX II

After more than 50 years of biochemical study on complex II, within a span of six months in 1999 a number of high-resolution X-ray structures for QFR and soluble forms of fumarate reductase became available (43–48). These structures came from several different laboratories, and the enzymes were isolated from different organisms, which has resulted in the most significant advances in our understanding of the overall architecture required for fumarate reduction and succinate oxidation. A gratifying aspect of the structural analysis is that to a large extent it has confirmed many of the speculations about the probable structure of the enzymes based on biochemical and biophysical analysis (20, 34–36, 49, 50). Of the available structures, however, only two are from membrane-bound forms of QFR, those from *E. coli* (43) and *W. succinogenes* (45). The other structures are for soluble forms of fumarate reductase from bacteria of the genus *Shewanella* (46–48) or l-aspartate oxidase from *E. coli* (44). l-Aspartate oxidase is a member of this family, as it can function as an l-aspartate/fumarate oxidoreductase generating iminoaspartate and succinate (51), in addition to its role in the biosynthesis of NAD\(^+\). Although this review focuses on the properties of the membrane-bound forms, important mechanistic information about the catalytic process of fumarate reduction has been derived from studying the soluble fumarate reductases and they are discussed in that context.

Various classification schemes have been proposed for complex II based upon in vivo function, particularly quinone substrate used by the enzyme, differences in b heme composition, and number of membrane domain polypeptides (35, 52–55). In these schemes the *E. coli* and *W. succinogenes* QFR complexes would fall into two separate classes. The *E. coli* QFR enzyme, like mammalian and *E. coli* SQR, contains two hydrophobic membrane anchor subunits; however, it lacks the b heme moiety. The *W. succinogenes* QFR is of the class that contains two \(b\) type hemes but only a single hydrophobic membrane anchor subunit. Mammalian complex II and *E. coli* SQR would be part of the same class in which the enzymes are poised to reduce ubiquinone, contain a single \(b\) heme, and are anchored to the membrane by two hydrophobic subunits. Although no X-ray structure is available for any SQR, one is anticipated soon (56).

In addition to the reports of the X-ray structures (43–48), several insightful reviews have discussed the details of the fumarate reductase structures (44, 50, 52, 57, 58). As predicted from analysis of the sequences of the subunits and biochemical data, complex II is essentially a modular protein complex. The structure of the *E. coli* QFR in Figure 2 clearly shows a demarcation between the hydrophilic and hydrophobic subunits. In all of the crystal forms analyzed to date for either the *E. coli* or *W. succinogenes* QFR, the complexes in the asymmetric unit are associated in a fashion that forms a dimer. In the *E. coli* QFR the crystal contact buries 325 Å\(^2\) of surface area and this is mediated by molecules of the detergent Thesit (C\(_{12}\)E\(_9\)) (43), whereas in the *W. succinogenes* QFR structure 3665 Å\(^2\) is buried upon formation of the dimer (45). Although this dimer has also
been reported in the detergent-solubilized *W. succinogenes* enzyme, based upon gel filtration experiments (58), no compelling evidence suggests that the dimer is a necessary prerequisite for function of QFR. In contrast to the dimers found in the QFR crystals, the *E. coli* SQR crystals available show that SQR is packed as a trimer with the monomers related by a crystallographic threefold symmetry axis (56); however, as for QFR there is no indication that this arrangement has any functional significance.

Both QFR structures have an overall similar length. They are oriented perpendicular to the membrane; *W. succinogenes* QFR is 120 Å long and *E. coli* QFR is 110 Å long (57, 58). Parallel to the membrane, the maximum width (FrdAB catalytic domain) of the monomer of both QFRs is 70 Å. The enzymes are attached to the membrane domain by interactions between the iron-sulfur subunit (FrdB) and their respective hydrophobic membrane anchor subunits. It has been suggested that an intact [3Fe-4S] cluster is necessary in order for succinate dehydrogenase and fumarate reductase hydrophilic domains (SdhAB

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**Figure 2** Ribbon diagram of the *E. coli* QFR structure (1FUM) is shown on the left. The FrdA flavoprotein subunit is shown in yellow, the FrdB iron-sulfur protein in brown, the FrdC subunit in blue, and the FrdD subunit in purple. Cofactors are shown as space-filling models. On the right of the figure the distances between the redox cofactors (edge-to-edge) in *E. coli* QFR are shown.
and FrdAB) to bind to the membrane and thus be active in quinone reduction (34, 60–62). The QFR structures show a close interaction between the [3Fe-4S] iron-sulfur cluster and the membrane anchor subunit(s). The \textit{E. coli} QFR membrane domain is composed of two subunits (FrdC and FrdD), each of which contains three membrane-spanning segments with helical secondary structures. These helices have been termed helices I–VI (I–III in FrdC, and IV–VI in FrdD). The single subunit FrdC of \textit{Wolinella} QFR contains five transmembrane helices; it lacks the one corresponding to helix III in \textit{E. coli} FrdC. This may indicate that a gene fusion between a corresponding \textit{frdD} gene and the 5′ end of an ancestral \textit{frdC} gene is responsible for the five transmembrane helices and single FrdC subunit forms of QFR and SQR. As mentioned, the structures of the hydrophobic anchor subunits from the two organisms can be aligned to a significant degree; however, this requires a rotation around the membrane normal and involves a difference in the relative orientation of the soluble subunits for the \textit{E. coli} and \textit{Wolinella} QFRs (45). As pointed out by Lancaster and coworkers, in the membrane domain of its QFR the \textit{E. coli} enzyme, which lacks heme, has the four transmembrane helices forming the helical core bundle packed closer together than in the \textit{Wolinella} QFR, which contains two \textit{b} hemes (45). Thus, in \textit{E. coli} QFR there does not seem to be room for heme insertion. Also, the \textit{E. coli} enzyme contains additional bulky amino acid residues in this core as compared to \textit{Wolinella} QFR; both factors probably contribute to the lack of a \textit{b} heme moiety (45). For example, whereas the distances between the Ca atoms of the \textit{W. succinogenes} heme axial \textit{b}_\text{P} ligands are 12.7 Å (HisC93-HisC182), the corresponding residues in \textit{E. coli} (FrdC His82 and FrdD Cys77) are only 11.2 Å apart (45). Mutagenesis to change FrdD Cys77 to His in \textit{E. coli} QFR, in order to provide a second heme axial ligand, fails to allow heme \textit{b} insertion in the \textit{E. coli} enzyme (D.A. Berthold & G. Cecchini, unpublished data) in agreement with the idea that the tight packing of the helices in the \textit{E. coli} QFR membrane domain precludes heme insertion.

In \textit{E. coli} QFR the interaction between the membrane domain and the iron-sulfur subunit is through amino acid residues near the N terminus of the FrdC and FrdD subunits on the cytoplasmic side of the membrane. The first 21 amino acid residues of the FrdC subunit extend away from the membrane into the cytoplasm and appear to help stabilize the binding of the hydrophilic domain to the membrane. The first eight residues of FrdD may also stabilize this interaction. In the \textit{Wolinella} QFR there is also a short helix in the cytoplasm between helices II–IV (FrdC residues 105–118) that may stabilize the binding of the FrdAB subunits to FrdC. In \textit{E. coli} QFR there is an equivalent loop in FrdC between helices II and III (FrdC residues 91–104) and in FrdD between helices V and VI (FrdD residues 89–97) that also may help bind FrdB to the membrane domain. It should also be noted that the quinone binding site termed Q\textsubscript{P} (proximal to the [3Fe-4S] cluster) is part of this binding region. No one factor by itself appears to be responsible for the binding of the hydrophilic subunits to the membrane.
domain. Reconstitution of the catalytic subunits with the membrane anchor subunits is sensitive to ionic strength, certain types of anions, and pH, indicative of a strong electrostatic attraction between the membrane subunits and the iron-sulfur subunit of complex II (34). It has been suggested that amino groups on the surface of the catalytic subunits of mammalian complex II are important for the stability of the complex, based on chemical modification studies (63, 64), and some of these groups were predicted to be near the [3Fe-4S] cluster of the enzyme (34). Several conserved lysine residues are near the C terminus of the iron-sulfur subunit (in \textit{E. coli} FrdB Lys216, Lys228, Lys241), and it is probable that modification of these residues could be responsible for the dissociation of the catalytic subunits from the membrane domain (64). For example, \textit{E. coli} FrdB Lys228 seems to be in hydrogen bond contact with the quinone, indicative of close association between the membrane subunits and the C terminus of FrdB.

Clearly the presence of redox cofactors such as iron-sulfur clusters and \textit{b} heme in the case of mammalian and \textit{E. coli} SQR (61, 65, 66), \textit{Wolinella} QFR (67), and \textit{Bacillus subtilis} SQR (35, 54, 68) are necessary for proper assembly of an intact complex II. In addition to these factors, other protein components have been suggested as requirements for proper assembly of complex II in eukaryotic systems. Two chaperone-like proteins, ABC1 (69) and TCM62 (70), have been implicated in assembly of complex II in yeast. Deletion of the \textit{ABC1} gene leads to deficiencies in complex II and IV, suggesting that its role is not specific to complex II (69). The role of TCM62 seems more specific to complex II and it has been suggested to play a role in the assembly of the iron-sulfur clusters of the enzyme (70). Recent results, however, suggest that TCM62 is part of a large protein complex in the mitochondrial matrix with a mass similar to chaperonins and is required for essential mitochondrial functions at high temperature (71), not just for stability or assembly of SQR. The factors required for the assembly of iron-sulfur clusters into proteins is a complex process requiring numerous factors (72). One of these factors shown to affect SQR assembly is a cysteine desulfurase (Nfs1p), which plays a central role in iron-sulfur cluster synthesis (73). In addition to these specific factors it is known that nutritional iron deficiency affects complex I and II levels in the mitochondrial membrane, although the respiratory complexes that do assemble are fully functional (74). In the case of complex II this has been shown to be the result of a fully functional iron-responsive element (IRE) in the mRNA for the iron-sulfur protein subunit SdhB. This IRE functions to mediate translational repression by iron regulatory proteins and thus affects overall levels of complex II in the mitochondrial membrane (75). The overall picture that emerges for assembly of complex II is that although it contains only four subunits and is the smallest of the electron transport complexes, the numerous cofactors it contains (flavin, iron-sulfur clusters, heme) mean that interference with their insertion into the complex affects assembly. Thus, like other members of the mitochondrial respiratory chain, complex II may turn out to require additional assembly factors (76).
FLAVOPROTEIN SUBUNIT AND FORMATION OF THE COVALENT FAD LINKAGE

Although there were indications that SDH was a flavoprotein as far back as 1939, it was 15 years later before direct spectral evidence was obtained that flavin is present in the enzyme [reviewed in (77)]. It became apparent that SDH contained flavin adenine dinucleotide (FAD) attached to the protein through a covalent flavin linkage (78), the first covalent flavin linkage reported for any protein. A decade later, the covalent flavin was identified as an $8\alpha$-N3-histidyl-FAD linkage (79) and the primary amino acid sequence of the flavin peptide was determined (80). Since this early work on succinate dehydrogenase, a number of proteins have been identified that contain covalent flavin linkages and a variety of modes of linkage to the peptide backbone have become apparent (81). Despite this vast increase in our knowledge of the types and number of covalent flavin proteins in nature, the reason for the presence of a covalent flavin linkage in a particular enzyme remains enigmatic.

All SQR and QFR complexes from both prokaryotes and eukaryotes that have been examined in detail contain a histidyl-FAD covalent linkage. By contrast, soluble fumarate reductase homologs of the flavoprotein subunit of complex II such as those from yeast (38), bacteria from the genus Shewanella for example (37), and unicellular parasites (39) contain noncovalently bound FAD. It has been speculated that the covalent linkage prevents the loss of the flavin cofactor from proteins in a membrane or periplasmic environment where concentrations of flavin mononucleotide (FMN) and FAD would be low and unable to replace the lost flavin (81). Now that structures are available for both membrane-bound QFRs (43, 45) and soluble fumarate reductase homologs (46–48), however, there is not an obvious structural difference as to how the covalent flavin linkage is acquired. The X-ray structures of the membrane-bound QFRs and the noncovalent flavin-containing structures from Shewanella show that the overall topology of the flavin and capping domains and the site of flavin binding are conserved. The evolution and maintenance of the covalent flavin link suggest that it may serve mechanistic requirements of these enzymes (81).

Whether covalent flavin attachment is protein mediated or self-catalytic has been discussed in detail (81). A general consensus is that the process is self-catalyzed and not mediated by another enzyme, although a mitochondrial chaperone, heat shock protein 60 (hsp60), has been shown to assist in the covalent flavinylation process in yeast SDH (82). Formation of the covalent flavin linkage is nevertheless a complex process that appears to require the flavoprotein to fold into the proper scaffold in order for the flavin cofactor and the amino acid to which it will covalently link to be in the proper steric orientation for the self-catalysis to occur. Insightful work on covalent flavinylation has come from the laboratories of Edmondson (83) and McIntire (84, 85) and their colleagues. The latter group has studied this process in $p$-cresol methylhydroxylase (PCMH) from Pseudomonas putida (81, 84, 85). Although PCMH
contains an 8α-N3-tyrosyl FAD linkage rather than the 8α-N3-histidyl FAD linkage found in SQR and QFR, the proposed mechanism for covalent flavin linkage most likely has remarkable similarity to the process occurring in SQR/QFR. PCMH is an \( \alpha_2\beta_2 \) flavocytochrome composed of a flavoprotein (\( \alpha_2 \)) and cytochrome \( c \) (\( \beta_2 \)) subunits. In PCMH it was found that it was necessary for the cytochrome \( c \) subunit to bind to the flavoprotein subunit before covalent flavinylation could occur (86). This suggests that structural alterations in the flavoprotein subunit induced by cytochrome \( c \) binding are necessary before covalent flavinylation can occur (81, 84, 86). Recently these authors have also shown that the redox potential of the FAD cofactor is significantly increased when the FAD binds to the apoflavoprotein and then increases again upon formation of the covalent flavin bond (85). This increase in reduction potential of the noncovalently bound FAD would make the isoalloxazine moiety of FAD a better electrophile for attack at its 8α-carbon by the nucleophile tyrosine O\(^-\) (85).

The authors suggest that this would increase the equilibrium concentration of the iminoquinone methide intermediate required for nucleophilic attack at the 8α-carbon. These results are consistent with the quinone-methide mechanism of covalent flavin linkage that has been proposed from several different laboratories (83, 87, 88). In this mechanism a quinone-methide tautomer is formed by loss of a proton from the 8α-position of the isoalloxazine ring, followed by an attack by a nucleophilic amino acid residue at the 8α-methide. This forms a reduced 8α-flavin adduct, which it is important to note must then be oxidized to produce the active covalently bound flavin (83).

Although the formation of the covalent flavin linkage described above has not been experimentally demonstrated for SQR or QFR, evidence suggests that the same mechanism applies to complex II. An interesting series of studies has used the yeast SQR to investigate when in the process of assembly of complex II the flavin is inserted covalently. In \( S. \text{cerevisiae} \), His90 is the attachment site in the SdhA subunit for the covalent FAD. When this residue is mutated, it was found that the SdhA subunit could still be translocated to the mitochondrion and assembled into the enzyme complex; however, the flavin was attached noncovalently (42). Further studies showed that cofactor attachment occurs within the mitochondrial matrix following cleavage of the mitochondrial targeting pre-sequence (82). In a situation reminiscent of PCMH discussed above, FAD attachment was stimulated by the presence of the iron-sulfur subunit (SdhB). Flavinylation was also stimulated by the dicarboxylic acid intermediates such as succinate, malate, or fumarate, all of which would bind at the active site of the enzyme. An additional observation was that C-terminal-truncated SdhA subunit imported by the mitochondrion was not able to incorporate covalent flavin, suggesting that the imported protein must fold properly for covalent FAD insertion to take place (82). Although the mitochondrial chaperonin hsp60 apparently does interact with the SdhA subunit, it was not absolutely required for FAD attachment (89). Complex II in the mutated protein assembled in \( S. \text{cerevisiae} \) contained tightly bound noncovalent FAD, and was also shown to be
catalytically competent for fumarate reduction but inactive in its normal physiological reaction, succinate oxidation (42).

Similar results were found for *E. coli* QFR. When His44, which is the site of the covalent FAD linkage, was mutated to a Ser, Cys, or Tyr residue, QFR retained tightly bound FAD but in noncovalent form (41), and the enzyme complexes assembled normally within the membrane. These mutant enzyme forms also essentially lost the ability to oxidize succinate, although they retained significant fumarate reductase activity. The tightly bound noncovalent flavin cofactor could be removed by dialysis against potassium bromide, which resulted in inactive enzyme. Fumarate reductase activity, however, could be restored by reconstitution of the enzyme with FAD (41). In both the yeast and *E. coli* studies it was suggested that the reason the noncovalent-FAD-containing QFR or SQR was unable to oxidize succinate was that the redox potential of the FAD cofactor had lowered sufficiently to preclude this reaction (41, 42). Unfortunately, no direct measurement of the redox potential of the flavin in the mutant enzymes has been done in order to address this hypothesis.

Recently additional site-directed mutations of *E. coli* QFR have been constructed that allow normal enzyme assembly; however, the enzymes contain noncovalent FAD (I. Schröder, E. Maklashina, Y. Sher, G. Cecchini, unpublished data). This was accomplished by mutating conserved residues thought to be part of the catalytic site in the FrdA subunit. In Figure 3 the spatial location of the residues that cause noncovalent flavin incorporation is shown. It is noteworthy that the residues which cause noncovalent flavin assembly are also those implicated as being involved in substrate binding and catalysis of the enzyme. As shown in Figure 3, FrdA Glu245 and Arg287 are two residues from the capping domain that when mutated result in noncovalent flavin assembly in QFR. In addition, FrdA His355 and Arg390 from the flavin domain also produce noncovalent flavins. Although not shown in Figure 3, when alanine insertions are placed in the hinge region connecting the capping and flavin domains, the enzyme contains noncovalent FAD. These results are consistent with previous observations that suggest that proper folding and alignment of residues around the site of flavin attachment are required for self-catalysis to be initiated in flavoproteins containing covalent flavin linkages. FrdA His355 and Arg390 are hydrogen bonded to dicarboxylate oxygens at the C4 position of fumarate, and in the closed conformation Arg287 also would bind this region of the substrate/inhibitor. FrdA His232, by contrast, is hydrogen bonded to the other end of the dicarboxylate at the C1 position, and mutation of His232 still allows the covalent FAD linkage to form (90). FrdA Glu245 is part of the capping domain and may be part of a proton pathway to Arg287, so it may have a similar effect by affecting the protonation state of the arginine. The alanine insertions would be expected to affect the movement of the hinge region connecting the capping and flavin domains. They might be expected to affect the molecular architecture of the active site such that there would be a misalignment of amino acid residues around the flavin and substrate binding site that could affect the covalent flavin
binding. An additional point is that when a covalent FAD linkage forms based on the quinone-methide mechanism, a positively charged amino acid residue in the vicinity of the N1 and C2 positions of the flavin ring would facilitate this reaction. The positively charged amino acid would help stabilize the negative charge in the N1/C2 region of the flavin ring system to make the 8α-position more electrophilic and thus more reactive with the nucleophilic His44. The most likely candidate for this amino acid is FrdA Arg390, since it is the only positively charged amino acid near the N1/C2 position (3.4 Å away in E. coli QFR). This amino acid residue thus is critical to covalent bond formation, and in humans the equivalent residue (an Arg408 to Cys mutation in human SdhA) has been shown to be responsible for late-onset neurodegenerative disease (91). In total these data suggest that the precise orientation of the flavin ring system, amino acids at the active site, and overall topology of the flavin and capping domains must be exquisitely maintained in order for covalent bond formation to occur similar to

**Figure 3** View of the substrate and FAD binding site region in *E. coli* QFR. The flavoprotein domain of the FrdA subunit is shown in blue and the capping domain is shown in yellow-gold. Inhibitory oxaloacetate (OA), which binds tightly at the substrate binding site, is shown in green. FrdA His232, which binds to the C1 carboxylate of fumarate, is shown in purple. FrdA Arg390 and His44 are shown in cyan. FrdA His355 is in purple, and Arg287 and Glu245 in beige. Mutation of His232 allows covalent FAD incorporation into the enzyme, whereas for all other residues shown, mutations cause the flavin to bind noncovalently.
the situation in vanillyl-alcohol oxidase, another covalent flavoprotein (92). The presence of a substrate or dicarboxylic acid inhibitor at the active site region also appears to be necessary in order to maintain the proper conformation by causing closure of the capping domain. The stimulation of covalent flavinylation shown by the presence of the SdhB subunit (82) may also be explained in two ways. One, it may contribute to the conformational changes necessary for proper alignment of the amino acid residues around the flavin; second, it may act as an electron acceptor for the reduced histidyl-FAD product that would be formed upon nucleophilic attack on the quinone-methide (83).

The noncovalent mutant proteins described above maintain fumarate reductase activity but lack succinate oxidase activity. Recently Heffron, Armstrong, and colleagues have been able to determine by protein film voltammetry the redox potential of the noncovalent flavin in the FrdA Arg287 mutation. The direct measurement of the FAD redox potential in this noncovalent mutation showed the redox potential of the flavin to be some 100 mV lower than wild-type covalent FAD in fumarate reductase (93) [wild-type QFR, FAD \( E_{m,7} = -50 \) mV (94)]. This lowered redox potential in \( E. \ coli \) fumarate reductase noncovalent FAD mutants is consistent with the redox potential reported for those fumarate reductase homologs that naturally contain a noncovalent flavin. For example, the redox potential of the noncovalent FAD from \( L \)-aspartate oxidase of \( E. \ coli \) is \( -216 \) mV (95) and that for \( Shewanella \ frigidimarina \) flavocytochrome \( c_3 \) is \( -152 \) mV (96). Both of these enzymes, although they reduce fumarate proficiently, are essentially unable to oxidize succinate. The significant rise in the redox potential upon formation of a covalent flavin bond is a consequence of both the covalent linkage and the protein environment around the flavin and is consistent with that for other enzymes containing this linkage (81, 83, 85). Thus, the higher FAD redox potential is also a prerequisite for succinate oxidation.

**CATALYSIS IN COMPLEX II**

The availability of high-resolution X-ray structures of QFR has contributed significantly to our understanding of the mechanism of fumarate reduction by the succinate dehydrogenase/fumarate reductase family of flavoproteins. In particular the structures of the soluble fumarate reductases, which have been solved to \( 1.8 \) Å (47), have provided important insights into the mechanism of fumarate reduction. The residues shown to be part of the substrate binding/active site and involved in catalysis are absolutely conserved throughout the family of fumarate reductases and succinate dehydrogenases whether they are membrane bound or soluble forms. The mechanism originally proposed for the \( S. \ frigidimarina \) fumarate reductase (47) has been supported by elegant studies using site-directed mutagenesis and X-ray crystallography of mutant enzyme forms (96–99). The basic mechanism proposed by these workers is shown in Figure 4 with the amino acids from the \( E. \ coli \) FrdA subunit used for comparison. The structures of all
Fumarate reductases in the closed conformation are similar, suggesting that this is the catalytically competent state of the enzyme (100). In this conformation, as shown in Figure 4, the C4 carboxylate of fumarate is in a highly polar environment and bound by hydrogen bonds and electrostatic attraction to FrdA Arg287 and Arg390 and by a hydrogen bond to FrdA His355 (101). The C1 carboxylate of fumarate is in a less polar environment and hydrogen bonded to FrdA His232 (47, 48, 57, 58, 96–101). The bound substrate is also distorted during closure of the capping domain, which induces polarization of the substrate (45, 48, 101). The polar nature of the hydrogen bonding environment around the C4 carboxylate group has been suggested to polarize the fumarate (101). The combined effect of twisting of the substrate and electronic effects generates a positive charge at the C2 position, making it a candidate for nucleophilic attack.

Figure 4  Catalytic mechanism proposed for fumarate reduction. Fumarate is polarized by interactions with His232, Arg390, and His355, which facilitates hydride transfer from the N5 position of the isoalloxazine ring of the reduced FAD cofactor. Arg287 is then positioned to donate a proton to fumarate, which results in succinate formation. Reprotoxation of Arg287 is accomplished via a proton pathway that includes Arg248 and Glu245. (Adapted from References 97, 98.)
from the N5 position of the flavin ring. Hydride transfer from the N5 of the flavin to C2 is followed by protonation at the C3 position by FrdA Arg287 (96, 101). Reprotonation of FrdA Arg287 is accomplished by a proton pathway involving FrdA Glu245 and FrdA Arg248 (96). The product of this reaction succinate is then released by movement of the capping domain, which also would allow entrance of another molecule of the substrate. In principle, the reverse reaction (succinate oxidation), which is catalyzed efficiently by membrane-bound QFRs containing covalently bound flavin, would proceed by the reverse of the mechanism described above. Mutation of the amino acid residues indicated in Figure 4 has been done in E. coli QFR, and results are consistent with the mechanism proposed using the S. frigidimarina enzyme [(47, 90, 96–99); I. Schröder, E. Maklashina, & G. Cecchini, unpublished data].

Another interesting aspect of catalysis that is restricted to succinate dehydrogenase and not shown by fumarate reductase is the “diode effect” (102). This effect shows that catalytic fumarate reduction by succinate dehydrogenase abruptly slows to a diminished catalytic rate below a redox potential of approximately −60 to −80 mV despite an increase in the driving force of the reaction (103, 104). At redox potentials above −60 mV, however, succinate dehydrogenase will reduce fumarate very rapidly, and in that sense the enzyme is fully reversible, like fumarate reductase containing covalently bound flavin. The diode effect has been used to classify fumarate reductase and succinate dehydrogenase as to their normal physiological function (105). It is suggested that at pH values below 7.64, SDH is energetically poised to catalyze fumarate reduction under conditions of low driving force (106). It was concluded that the reduction of FAD is the factor responsible for the diode effect and that a conformational change may occur upon formation of FADH₂ (106). This hypothesis seems reasonable in light of the domain movements that obviously occur in QFR, and presumably SQR, around the FAD binding site. Nevertheless there must be differences between succinate dehydrogenase and fumarate reductase to account for their different catalytic behavior even though their flavoprotein subunits are likely to have similar structures. It is pertinent therefore that for E. coli FrdAB the two-electron oxidation of the anionic FAD hydroquinone is associated with the loss of one proton, whereas the reoxidation of the hydroquinone in SdhAB appears to involve two protons (94, 103, 104). Additionally, in fumarate reductase the flavin semiquinone appears to be neutral and most likely protonated at the N5 position (94), whereas succinate dehydrogenase is thought to contain an anionic flavin semiquinone (34).

The physiological significance of the diode effect is unclear. It has been suggested, however, that it could provide a means for control of the Krebs cycle during periods of hypoxia, in which the quinone pool would become reduced (106), by shutting down succinate dehydrogenase so that it would not reduce fumarate and thus interfere with the Krebs cycle. This effect might be more relevant in bacteria that vary their ratio of low- and high-potential quinones in response to environmental stimuli (107) but also could be useful in mammalian...
cells where a hypoxic response is initiated by mutations in SQR genes, for example.

**IRON-SULFUR CLUSTERS OF COMPLEX II AND THE ELECTRON TRANSFER PATHWAY**

After years of controversy the fact that three distinct types of iron-sulfur clusters are present in prototypical SQR/QFRs finally became apparent during the 1980s. The history of these controversies and discovery of the numbers and types of iron-sulfur clusters have been extensively reviewed (20, 34, 49). It was determined that the cluster composition of complex II is a [2Fe-2S]^{2+,1+} cluster, often termed Center 1; a [4Fe-4S]^{2+,1+} cluster, Center 2; and a [3Fe-4S]^{1+,0} cluster, Center 3. The application of biophysical technologies such as electron paramagnetic resonance (EPR), Mössbauer, low-temperature magnetic circular dichroism (MCD), and other spectroscopies (20) allowed these answers about the numbers and types of iron-sulfur clusters. It is thus quite appropriate that the new knowledge obtained from the X-ray crystal structures of complex II (43, 45) has helped to answer a question about the role of the [4Fe-4S] cluster of the enzyme. The midpoint potential (pH 7) of the [4Fe-4S] cluster has been reported to range from $-320$ mV for *E. coli* QFR to $-175$ to $-260$ mV for *E. coli* and mammalian SQR, respectively (108). The low midpoint potential of the [4Fe-4S] cluster had led to speculation that this cluster was not part of the electron transfer pathway but rather might play a structural or regulatory role in the enzyme (34, 49, 109). The X-ray structures show, however, that the [4Fe-4S] cluster is part of a linear electron transport chain between the FAD cofactor and the quinone or b heme(s) located in the membrane domain, as indicated in Figure 2. The relatively close physical association of the iron-sulfur clusters ($\sim 11$ Å, edge-to-edge) suggests that they all participate in electron transfer and that the low potential of the [4Fe-4S] cluster does not present a thermodynamic barrier (52). Studies of *E. coli* FrdAB subunits using protein film voltammetry show a boost in catalytic current becoming apparent at the redox potential of the [4Fe-4S] cluster. This is also consistent with the [4Fe-4S] cluster relaying electrons to FAD as part of the electron transport chain within the enzyme (110).

The reported reduction potentials of all three iron-sulfur clusters in QFR are lower than the respective counterparts in SQR in accord with their physiological donors/acceptors. In *E. coli* QFR the reduction potentials are $-74$, $-67$, $-310$, $-35$, $-50$ mV, respectively, for menaquinol, [3Fe-4S], [4Fe-4S], [2Fe-2S], FAD (94), whereas for *E. coli* SQR the reduction potentials are $-60$ to $-79$ for FAD, and $+10$, $-175$, $+65$, $+36$, $+90$ for [2Fe-2S], [4Fe-4S], [3Fe-4S], heme b, and ubiquinone, respectively (34, 66, 111–113). The reduction potential for FAD has not actually been measured for *E. coli* SQR; however, a potential of $-79$ mV has been determined for beef SQR (111). The value of $-60$ mV listed above is derived from the potential at which the diode effect makes itself apparent in *E.
coli SQR (104) versus a potential of $-80$ mV for the beef enzyme (103). The different value for the reduction potentials of the iron-sulfur clusters in QFR versus SQR most likely reflects differences in the protein environment surrounding the clusters. The arrangement of the iron-sulfur clusters in SQR is without a doubt the same as in QFR, considering the high degree of sequence similarity of the FrdB and SdhB subunits from different species, including the absolute arrangement of conserved cysteinyl ligands for the Fe-S clusters (34, 35, 108). *E. coli* SQR is unusual in one respect in that the third cysteine of the [2Fe-2S] signature sequence (CxxxxCxxC.... C) is replaced by an aspartate residue (114); however, site-directed mutagenesis of the equivalent residue in *E. coli* QFR has shown that the [2Fe-2S] cluster is retained albeit with a slightly higher redox potential (115). Based on sequence and EPR studies it had been predicted that the iron-sulfur protein of complex II would fold into two domains (34, 35), which is as found in the QFR structures (43, 45). The N-terminal domain containing the [2Fe-2S] cluster folds similarly to plant-type ferredoxins, whereas the C-terminal domain containing the [4Fe-4S] and [3Fe-4S] clusters is similar in topology to bacterial ferredoxins (43, 45, 57, 58). The C-terminal domain of FrdB also contains several helices that associate with the membrane anchor subunits and thus are required to hold the hydrophilic subunits to the membrane domain.

MEMBRANE DOMAIN OF COMPLEX II

Although the hydrophilic flavoprotein and iron-sulfur protein subunits seem highly conserved among eukaryotic and prokaryotic organisms, there is a much greater variation in the primary amino acid sequences of the membrane-spanning subunits. The single-subunit membrane anchor complexes such as QFR from *W. succinogenes* (45) or *B. subtilis* SQR (29) have five membrane-spanning helices rather than the six found in the two-subunit membrane anchor forms. The suggestion that the single membrane anchor subunits evolved by a fusion event where the third helix of the C-subunit has been deleted (35, 36) is supported by the two available QFR structures. The complexes that contain a single membrane-spanning polypeptide also contain two heme groups while the mammalian, *S. cerevisiae*, and *E. coli* SQR contain a single heme group, and *E. coli* QFR lacks heme.

There is no evidence in the single b heme–containing complex II or *E. coli* QFR that the enzymes can act as proton pumps. It has been suggested that this is because the reactions catalyzed are not sufficiently exergonic to promote proton translocation (116). Nevertheless, in the single-subunit diheme containing *B. subtilis* SQR the uphill electron transfer reaction from menaquinol to succinate has shown to be sensitive to uncouplers (53, 117), and this process is thought to be driven by the $\Delta \mu_{H^+}$ produced by the aerobic respiratory chain as discussed by Ohnishi and coworkers (52). It is also possible that the *W. succinogenes* QFR
may produce $\Delta \mu_{H^+}$, which may occur by a Mitchellian type Q loop [reviewed in (52, 58, 67)]. This contention awaits experimental verification, as the available evidence is not entirely consistent with this hypothesis (118). Therefore, it is quite pertinent that a recent report shows that in *W. succinogenes* QFR mutation of FrdC Glu180 almost completely abolishes the ability of the enzyme to interact with quinones (119). This glutamate residue is conserved in all diheme QFRs but not in diheme SQRs and lies in the middle of the membrane domain in FrdC in a helical region parallel to the $b$ heme electron transfer pathway. Fourier transform infrared (FTIR) spectroscopy data support the idea that this glutamate is involved in proton exchange, and it is suggested that FrdC Glu180 is part of a proton translocation pathway that would take protons from the periplasm back across the membrane to the cytoplasm, a process termed the E-pathway hypothesis (119). The net effect would be a coupled proton/electron transfer system so that *W. succinogenes* QFR is not electrogenic, which would agree with previous data showing that *W. succinogenes* QFR is not a classical proton pump (58, 118, 119). This result seems to suggest that diheme QFRs and diheme SQRs have a different mechanism with regard to the process of proton translocation, in agreement with the different thermodynamics of fumarate reduction and succinate oxidation in the two enzymes.

The interactions of quinones with complex II is an area of active interest, and the structural data from *E. coli* QFR in particular has shed light on the amino acid residues that line the binding site(s). In the *E. coli* 3.3-Å structure, two menaquinone binding sites were observed (43). That two binding sites were found for quinones in the structure for complex II was not unexpected based on previous site-directed mutagenesis studies in *E. coli* QFR (120) and *S. cerevisiae* SQR (121) and by photoaffinity labeling studies of mitochondrial and *E. coli* SQR (122, 123). It had also been known that a stabilized semiquinone pair exists in complex II from beef heart (124), and EPR studies had suggested that this interacting pair were approximately 8–9 Å apart. The two quinones seen in the *E. coli* QFR structure were, however, some 25 Å apart (Figure 2) and localized on opposite sides of the membrane (43), suggesting that the quinone at Q$_D$ was not part of the semiquinone pair. In addition the large spatial separation between the two quinones would suggest that they were not part of an electron transfer chain unless some other redox active species was placed between them to shorten the electron transfer distances (52). A single stabilized semiquinone species has been found in an *E. coli* QFR mutant (FrdC Glu29Leu) with a signal of $g = 2.005$, a midpoint potential of $-57$ mV, and a stability constant of $\sim 1.2 \times 10^{-2}$ at pH 7.2 (125). This stability constant is some eight orders of magnitude greater than that found for free quinone within the membrane. These same studies also showed the presence of a stabilized semiquinone species in wild-type QFR, however, with a stability constant four orders of magnitude lower than in the mutant enzyme (125). The results indicate that the FrdC Glu29 residue destabilizes the semiquinone produced during electron transfer at the proximal Q$_D$ site in *E. coli* QFR (125). The EPR studies also indicated that the [3Fe-4S] cluster was the dominant
spin relaxation enhancer (125), which is consistent with the close spatial separation (8 Å) between the two entities. The quinone site inhibitor 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) was shown to affect the EPR spectrum of the [3Fe-4S] cluster, suggesting that its binding site was also at or near the Q_p site. This suggestion was confirmed with the recent 2.7-Å resolution of the X-ray structure of E. coli QFR containing HQNO that binds at the Q_p site (126). HQNO is nearly isosteric with menaquinol and it was found that two hydrogen bond donors, FrdB Lys228 and FrdD Trp14, were positioned within hydrogen bonding distance of the negatively charged N-oxide (126). The hydroxyl group on the other side of the HQNO ring, like that for menaquinone, is within hydrogen bonding distance of FrdC Glu29 and FrdD Arg81. Thus, as noted above, replacement of FrdC Glu29 with a neutral amino acid such as leucine apparently disrupts this hydrogen bonding interaction and stabilizes the semiquinone species present at Q_p. Another inhibitor of quinone reactions in E. coli QFR, DNP-19 (2-[1-(p-chlorophenyl)ethyl]4,6-dinitrophenol) (127), was also found to bind at the Q_p site (126). The finding was interpreted to mean that this inhibitor bound at the Q_p site such that it would sterically prevent quinol binding in agreement with inhibitor studies (126, 127). Importantly, in both the HQNO and DNP-19 structures, quinone was absent at the Q_D site even though electron density had been very strong in the original 3.3-Å-resolution structure determined in the absence of inhibitors. This is reminiscent of the binding of inhibitors at the Q_o site of the cytochrome bc_1 complex where their binding reduces the affinity for quinol at the second Q_o site in the dimer (128).

The structure for the W. succinogenes QFR did not contain bound quinones (45), although they are necessary for function with this enzyme. Further study using site-directed mutagenesis approaches has identified a glutamate residue (Glu-C66) that is essential for menaquinol oxidation (100). This residue was chosen for mutagenesis, as it was positioned between two cavities on the periplasmic side of the FrdC subunit and modeling suggested that a hydrogen bond could form from one of the hydroxyl groups of menaquinol to the carboxylate oxygen of Glu-C66. Substitution of Glu-C66 with a Gln residue did not significantly alter the structure or change the redox potential of the two b hemes of the Wolinella QFR, but it did dramatically alter the ability of the enzyme complex to oxidize menaquinol (100). Thus, it was concluded that this residue probably accepts a proton from the menaquinol during electron transfer to the distal heme b_D (100), a role analogous to that previously proposed for FrdC Glu29 in E. coli QFR (120, 129). Mechanistically, however, the different spatial location of these two residues has important implications. In E. coli QFR the glutamate residue is localized on the cytoplasmic side, and thus protons liberated during menaquinol oxidation would stay in the cytoplasm. By contrast, in Wolinella QFR the glutamate residue is located near the periplasm and the protons released by menaquinol oxidation would likely be delivered there. Thus, it was noted by Lancaster and coworkers (100) that since protons resulting from the reduction of fumarate are taken up from the cytoplasmic side of the
membrane, the oxidation of quinol by fumarate should be coupled to the
generation of an electrochemical proton gradient across the membrane (100). However, as noted above it is possible that protons are transported back across the membrane so that the net process is not electrogenic (119). In *B. subtilis* SQR a similar residue (SdhC Asp52) has been suggested to be part of the menaquinone reduction site (130). This site would likely be located on the periplasmic side of the membrane and sensitive to the inhibitor HQNO based on EPR studies and their effect on the potential of heme \(b_L\) (131). Overall all these studies suggest that the sites for menaquinol oxidation are on different sides of the membrane in *E. coli* and *W. succinogenes* QFR and that HQNO also inhibits *B. subtilis* SQR on the periplasmic side of the membrane (125). As noted above, the *B. subtilis* SQR oxidation of succinate by menaquinone is sensitive to uncouplers and may be driven by the electrochemical proton gradient (53, 117). The topology of the sites for quinone oxidation/reduction in *B. subtilis* SQR and *W. succinogenes* QFR and the presence of two \(b\) hemes spanning the membrane in these complexes suggest that they both carry out analogous reactions but in different directions (52, 100). Thus, for membrane-spanning, diheme-containing complex IIs, a major difference between those with a single subunit and those containing two subunits may be their coupling to a proton potential across the membrane.

The presence of an acidic amino acid residue (like the Glu and Asp residues discussed above) as part of a quinone binding site is prevalent in respiratory proteins. There is a conserved aspartate in the cytochrome \(bc_1\) complex that is hydrogen bonded to ubiquinone, and quinol oxidase also contains a conserved aspartate (132). Particularly relevant to complex II is that in *Paracoccus denitrificans* SQR a mutation of SdhD Asp88 confers resistance to carboxins, which are specific inhibitors of the reaction of SQR with quinones (133). In eukaryotic complex II there are also conserved aspartate residues that have been localized as part of the quinone binding sites either by site-directed mutagenesis or by chemical labeling with azido-quinones (123, 134).

The role of the quinone found at the Q\(_D\) site in *E. coli* QFR remains enigmatic. It does not appear to play an essential structural role, since it is absent in the enzyme containing HQNO (126), which otherwise shows a normal structure. Nevertheless, mutagenesis data on both *E. coli* QFR (120) and *S. cerevisiae* SQR (121) suggest that residues in the region of the hydrophobic Q\(_D\) binding site have effects on enzymatic quinone activity. Yeast SQR has recently been reported to contain a \(b\) heme (135), and it is not clear if the reported mutations (121) affect the properties of the heme. In the case of *E. coli* QFR, the large spatial separation between the Q\(_P\) and Q\(_D\) quinones (25 Å) makes electron transfer seem unlikely over such a long distance (52). The \(b\) heme in yeast SQR by contrast might play a role as an intervening redox cofactor to allow electron transfer across the membrane, similar to the case in *B. subtilis* and *W. succinogenes* complex II. It should be stated, however, that no data suggest that yeast SQR is involved in producing a transmembrane proton gradient, nor does it respond to the electrical potential of the membrane. In the case of *E. coli* QFR, available data suggest that
electron transfer occurs near the cytoplasmic side of the membrane (108), making it seem even less likely that Q₃ plays a role in catalysis with quinones. This question requires further study and it is relevant that an unassigned density in the membrane-spanning domain of *E. coli* QFR has been reported (126). This density, which became more apparent at the higher resolution (2.7 Å) structure, coincides with the only major cavity found in the *E. coli* QFR structure. This factor, termed M, appears about equidistant from the Q₉ and Q₃ binding sites, and the amino acids lining the cavity where it apparently resides are polar, giving the cavity characteristics of a quinone binding site (126). It remains to be proven what the M factor is and whether it participates in electron transfer in *E. coli* QFR.

**ROLE OF THE b HEME IN COMPLEX II**

It is apparent that the b hemes in *W. succinogenes* QFR and *B. subtilis* SQR participate in electron transfer to/from quinone during menaquinol oxidation/reduction, respectively. In *B. subtilis* SQR there is direct evidence that the high-potential heme b is reduced by succinate at the same rate as enzyme turnover (130). In *W. succinogenes* QFR the high-potential heme b is partially reduced by succinate, whereas it is fully reduced by low-potential menaquinol analogues and reoxidized by fumarate at the same rate as enzyme turnover (136). The axial ligands of the heme seem to be the same in all SQRs so far examined. Measurements using near infrared MCD spectroscopy and EPR have suggested that all of the hemes have a bis-histidine axial ligation (137–139). This is also consistent with what is observed in the *W. succinogenes* QFR structure (45).

The role of the heme in the complex IIs containing only a single b heme is, however, not understood. As this is the type found in mammalian mitochondria, it is important to our understanding of how complex II participates in the disease process to understand the function of the single heme in SQR. One well-established fact is that there is a structural role for the b heme in those complex IIs containing heme (29, 35). The structural role for the b hemes in *B. subtilis* SQR is well studied (29, 35), and using heme biosynthesis mutants in both *B. subtilis* (68) and *E. coli* SQR (65), it was found that the hydrophilic subunits failed to assemble with the membrane subunits unless heme was present. The crystal structure of *W. succinogenes* QFR also shows significant interaction between four of the transmembrane helices and the heme(s), indicating an important role in assembly of that complex (45). The histidyl ligands for the b hemes are conserved throughout complex II whether they contain one or two hemes (35, 54). In the *E. coli* SQR containing a single b heme, SdhC His84 and SdhD His71 have been identified as the axial heme ligands (66, 140). Mutation of the SdhC His84 residue results in formation of a hexacoordinated low spin heme, but since this mutant is able to bind carbon monoxide, the data suggest that carbon monoxide is able to displace an alternative ligand that presumably...
replaces the imidazole nitrogen of SdhC His84 (66). Mutation of SdhD His71 has, however, a more severe effect on the enzyme in that the heme becomes high spin and pentacoordinate and its redox potential is lowered by \( \sim 100 \) mV (66). The mutant enzymes are also less stable and the catalytic subunits more easily dissociate from the membrane domain, in agreement with results that heme is important for assembly (35, 54, 65, 68).

Therefore, one reason the role of the \( b \) heme in single-heme-containing SQR remains enigmatic is that in the bovine SQR the measured redox potential is too low for the heme to be fully reduced by succinate. The \( b \) heme redox potential for bovine SQR was found to be \(-185\) mV, although this potential was raised somewhat (\(-144\) mV) in the isolated SdhCD peptide fraction (141). The isolated SdhCD-containing fraction, however, was reactive with carbon monoxide, whereas SQR itself is not reactive toward carbon monoxide. This suggests that the isolated heme \( b \)-containing peptides may have altered their axial ligands such that the heme becomes pentacoordinate and thus reactive with carbon monoxide (141). The heme \( b \) in bovine SQR when fully reduced is, however, rapidly oxidized by fumarate, suggesting a possible role for the heme in fumarate reduction (139). This implies that the heme may play a role in fumarate reduction but not succinate oxidation in the mammalian enzyme. In contrast to the bovine enzyme, the \( E.\ coli \) SQR \( b_{556} \) heme, which has a much higher potential (\(+36\) mV), is fully reducible by succinate (142). The rate of the \( b \) heme reduction is, however, significantly affected by the presence of UQ/UQH\(_2\); reduction is slower than turnover at low concentration of quinone and accelerated by anaerobic conditions and saturating levels of ubiquinone (143). Like the bovine enzyme, the heme in \( E.\ coli \) SQR, once it is reduced, can be oxidized by fumarate at the same rate as the turnover of the enzyme. One interpretation of these data is that the [3Fe-4S] cluster directly reduces quinone without participation of the \( b \) heme, but in reverse electron transfer the \( b \) heme, which must be in close spatial proximity to the quinone and [3Fe-4S] cluster, becomes part of the electron transfer pathway (108). These data could also be interpreted to suggest that in SQR containing a single \( b \) heme, the heme is in redox equilibrium with the quinone pool in the membrane. The understanding of the role of the \( b \) heme in SQR requires further study, as there are intriguing suggestions that it plays more than a structural role in the single \( b \) heme class of SQR.

**RELATION OF COMPLEX II TO DISEASE AND REACTIVE OXYGEN SPECIES**

The genes for complex II in mammals are all encoded in the nucleus. The \( SDHA \) gene has been localized in the human genome to chromosome 5p15; the gene contains 15 exons over 38 kb (144). The \( SDHB \) gene has been mapped to chromosome 1p35–36.1 and contains eight exons over 40 kb (145). The genes for the small hydrophobic subunits that contain the cytochrome \( b \) component were
mapped to chromosome 1q21 (SDHC) and 11q23 (SDHD) (146). The SDHC gene is composed of six exons covering 35 kb (147), and the SDHD gene contains four exons over 18 kb (148).

The availability of human genomic sequence data has generated considerable interest in human complex II gene defects. Although complex II–related diseases are relatively rare, they have been associated with a wide spectrum of clinical phenotypes (149, 150). In fact the first reported nuclear mutation that causes a respiratory chain defect was a mutation in the SDHA gene associated with Leigh syndrome (151). This mutation changed human Arg544 to a tryptophan residue and significantly lowered SDH activity. Based on the E. coli QFR structure (43), the equivalent arginine in the bacterial enzyme is near the mouth of the substrate entrance channel. This suggests, as one possibility, that the channel is altered or that closure of the capping domain is restricted, affecting substrate entry/exit. Another mutation recently described is alteration of human Arg408 to a cysteine residue, which causes late-onset neurodegenerative disease (91). This mutation results in the loss of the covalent FAD linkage in the flavoprotein subunit of SQR. The loss of covalent FAD linkage results in enzyme that is no longer able to oxidize succinate. This is what is seen in E. coli QFR when the equivalent arginine (FrdA Arg390, Figure 3) is mutated.

Mutations in human SDHB, SDHC, and SDHD genes appear to cause a different clinical phenotype than that found with the SDHA mutations (149). Mutations of the iron-sulfur protein or the cytochrome b subunits cause a clinical syndrome termed familial paraganglioma and pheochromocytoma (149, 150). This condition is characterized by neural crest–derived tumors of the paraganglia, mostly in the head or neck. Frequently they are localized in highly vascular organs such as the carotid body. A more complete description of the clinical effects in patients carrying these mutations has been described (149, 150, 152). A mutation in human SDHB that causes a pheochromocytoma is substitution of human Pro197 by arginine (153). This proline residue is conserved in all QFRs and SQRs and is next to one of the cysteinyl residues involved in ligation of the [3Fe-4S] cluster. In E. coli QFR, substitution of this proline by either a histidine or glutamine causes the enzyme to lose succinate oxidase activity in an oxygen-sensitive manner (154). Although the [3Fe-4S] cluster apparently remains intact, the enzyme in the presence of quinones appears to generate reactive oxygen species, which in turn rapidly inactivate the enzyme. It is quite likely that a similar phenomenon would take place in subjects carrying this mutation in SQR.

Although fewer mutations have been located in the human SDHC gene, mutations that disrupt its start codon cause autosomal dominant hereditary paraganglioma (155). Analysis suggested that SDHC functions as a tumor-suppressor gene, since tumors have lost the wild-type gene (155). Although not reported for humans, an interesting mutation termed mev-1 has been found in the SdhC gene of the nematode Caenorhabditis elegans (156). The mev-1 mutation is a missense mutation of SdhC Gly69 (C. elegans numbering) to a glutamic acid residue (156). Nematodes harboring this mutation show a dramatic decrease in
life span and also accumulate markers of aging such as protein carbonyls. Further study in the Ishii laboratory has shown that this defect causes increased super-oxide production and that complex II containing this mutation is potentially a potent producer of reactive oxygen species (157). It was suggested that the mutated glycine residue was near a quinone binding site (156), and the *E. coli* QFR structure (43) indicates that the Qₜ site would be very near this glycine residue, based on amino acid sequence alignment. As an acidic residue at this site seems to be involved in protonation reactions in *E. coli* QFR (125, 126) and may help destabilize the quinone, it seems reasonable that alteration of this site with such a residue could cause alteration of the ability of complex II to produce reactive oxygen species. It is thus quite pertinent that studies from the Imlay laboratory have shown that *E. coli* QFR is a very potent producer of the superoxide anion (158) and recently that the more fully reduced enzyme also produces H₂O₂ (159). These studies also suggested that SQR produces only superoxide (159) and at a much lower level than QFR, although in the case of mutations like that found in *C. elegans* (156) SQR might also produce H₂O₂ like QFR by redistributing electrons more to the FAD cofactor than found in wild-type enzyme. These studies indicated that the primary site of production of superoxide and H₂O₂ was at the FAD cofactor of wild-type QFR. They also showed that wild-type SQR produced much less superoxide and virtually no H₂O₂ (159), in agreement with the known propensity of flavins to react with molecular oxygen (160). Nevertheless, the studies are also consistent with the interpretation that mutant forms of both enzymes could produce superoxide and/or H₂O₂ if the iron-sulfur or quinone reaction sites should become exposed and available to oxygen. It is reasonable to assume that both the flavin and site of mutation would contribute to reactive oxygen species formation in mutant forms of complex II, although it is likely that the primary site is at the FAD cofactor as proposed (159).

The largest number of human complex II mutations linked to disease have been found in *SDHD*; these all contribute to hereditary paraganglioma or pheochromocytoma (149). Some are in the leader sequence of SdhD or are frameshift mutations, so they likely cause assembly defects in complex II. Several of the mutations are interesting for their similarity to mutations made in the bacterial complex II, where their biochemistry has been studied in greater detail (108). For example, the human His102Leu mutation, which contributes to hereditary paraganglioma (161), is equivalent by sequence alignment to SdhD His71 in *E. coli* SQR. This residue is one of the axial heme ligands of SQR, and mutation of the residue lowers the redox potential of the b heme by 100 mV and makes the enzyme less stable (66). The enzyme did retain significant activity; however, the lower potential of the heme might contribute to increasing the electron distribution toward the FAD cofactor, which as noted above could be deleterious. Examination of the structure of *E. coli* QFR, although it lacks the b heme, suggests that other missense mutations may also affect the environment of the b heme. For example, human SdhD Arg70, Pro81, Asp92, Leu95, all reported
human mutations (149), may also reside within the second transmembrane helix of SdhD (equivalent to helix V, in QFR; see Figure 5). Thus mutation might be expected to disrupt the heme environment, which may affect the redox potential of the $b$ heme and stability of the enzyme complex (66).

Overall it has been suggested that $SDHB$, $SDHC$, and $SDHD$ act as tumor suppressor genes (150), and it is noted that their mutation mimics chronic hypoxia (161). This led to the suggestion that the gene products could be important components of the oxygen-sensing system and their malfunction would lead to hypoxic stimulation, cellular proliferation, and vascularization (161). There is no direct evidence, however, suggesting that wild-type SQR can

Figure 5  Cartoon of the human SDHD subunit representing hypothetical locations of mutations that contribute to hereditary paraganglioma. The numbered residues are the equivalent human SDHD amino acids known to contribute to hereditary paraganglioma (149, 150). Exceptions are residue C-Ile69, which is the human residue equivalent to the mev-1 mutation from $C. $ elegans that causes premature aging (156), and $E. coli$ B-Pro160, which is equivalent to human B-Pro197 associated with hereditary paraganglioma (149). Also shown is the location of the [3Fe-4S] cluster. The diagram was drawn using the $E. coli$ FrdD subunit as a template with FrdD Cys77 represented as equivalent to human His102, one of the $b$ heme ligands.
respond to oxygen, although it is possible that mutations in the \( b \) heme could provide an oxygen binding site. Alternatively, the membrane domain could respond to the state of reduction of the quinone pool, which might affect succinate oxidation and quinone reduction by SQR. This is an important question that needs further study.

**CONCLUDING REMARKS**

Significant advances in our understanding of complex II have come from the available crystal structures of QFR (43, 45). Clearly it would be advantageous to obtain the three-dimensional structure of an SQR member of the complex II family of respiratory proteins, particularly one containing a single \( b \) heme. Such a structure may help shed light on the fascinating observations of a tunnel diode effect in the enzyme (102) and help us understand the biochemical regulation of complex II. A structure may also provide information on the possible role of the \( b \) heme cofactor in the function of complex II and on whether it plays a role in regulating the hypoxic response noted in mammals carrying complex II mutations. Like complexes I and III of the respiratory chain, complex II contains quinone binding sites in the membrane domain. Elucidation of the ubiquinone binding site in SQR should aid in defining amino acid residues that may participate in the quinone binding sites in the other respiratory complexes.

As the only membrane-bound enzyme in the Krebs cycle and as part of the mitochondrial electron transport chain, complex II is uniquely positioned to act as a regulator of both metabolic pathways. Although it is the simplest respiratory complex in terms of number of subunits, it does contain multiple redox cofactors, such as complexes I, III, and IV, and it is unique because it is not a proton pump. Nevertheless, although simpler in overall structure, the way electron flow is regulated through the complex is incompletely understood. As with other respiratory complexes, understanding how the redox cofactors and individual subunits are assembled into the holoenzyme may require the discovery of additional protein assembly factors. The mitochondrial respiratory chain, particularly complexes I and III, has long been suggested as a primary source of reactive oxygen species. However, the role of complex II in this activity has been less appreciated. Recent findings in eukaryotic systems have shown that complex II can be a significant source of reactive oxygen species (157, 159). How specific complex II mutations generate reactive oxygen species and how this effects mitochondrial-related diseases are likely to receive increased attention as a result of its documented importance in such processes (149, 159). Bacterial systems will probably continue to be seminal in addressing important questions on the assembly and function of complex II because of the relative ease of biochemical, genetic, and structural studies using them. Complex II continues to fascinate researchers, and its unique physiological role in both the respiratory chain and Krebs cycle makes it an important area to study.
ACKNOWLEDGMENTS

The author gratefully acknowledges funding from the Department of Veterans Affairs and National Institutes of Health (NIH) grant GM61606. The author is also appreciative of past support from the National Science Foundation and the NIH Heart, Lung, and Blood Institute. The author would also like to thank his collaborators for many fruitful discussions and help over the years and Roy Lancaster for communicating results prior to publication. He is indebted to Tina Iverson, Elena Maklashina, and Victoria Yankovskaya for preparing the figures and helpful discussions.

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LITERATURE CITED

28. Lauterbach F, Körtner C, Albracht SP,
RESPIRATORY COMPLEX II

101. Reid GA, Miles CS, Moysey RK, Pan-