ELSEVIER

Biochimica et Biophysica Acta 1320 (1997) 107–141

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Review

Succinate: quinone oxidoreductases
Variations on a conserved theme

Cecilia Hägerhäll *

Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104, USA

Received 21 January 1997; accepted 28 January 1997

Keywords: Succinate:quinone oxidoreductase; Succinate dehydrogenase; Fumarate reductase; Flavoprotein; Iron-sulfur protein; Cytochrome *b*; Quinone binding site; Inhibitor; Electron transfer; 'Respiration-early'

Contents

* c/o Dr. Lars Hederstedt, Department of Microbiology, Lund University, Sölvegatan 12, S-223 62 Lund, Sweden. Fax: +46-46157839; E-mail: caroline.hagerhal@lpal.slu.se

PII S0005-2728(97)00019-4

1. General introduction

Succinate:quinone oxidoreductase (EC 1.3.5.1) refers collectively to succinate:quinone reductase (SQR, or Complex II) and quinol:fumarate reductase (QFR). The former enzyme is found in aerobic organisms, and catalyses the oxidation of succinate to fumarate in the citric acid cycle and donates the electrons to quinone in the membrane. The latter enzyme can be found in anaerobic cells respiring with fumarate as terminal electron acceptor. SQR and QFR are very similar in composition and seemingly also in structure in spite of that they catalyze the opposite enzymatic reactions in vivo. Succinate:quinone oxidoreductases consist of a peripheral domain, exposed to the cytoplasm in bacteria and to the matrix in mitochondria, and a membrane-integral domain that spans the membrane (Fig. 1). The peripheral part, which contains the dicarboxylate binding site, is composed of a flavoprotein (FP; 64–79 kDa) subunit, with one covalently bound FAD, and an iron-sulfur protein (IP; 27–31 kDa) subunit containing three iron-sulfur clusters, a [2Fe-2S] cluster denoted S1 (in SQR) or FR1 (in QFR), a [4Fe-4S] cluster denoted S2 or FR2, and a [3Fe-4S] cluster denoted S3 or FR3. The membrane-integral domain functions to anchor the FP and IP subunits to the membrane and is required for quinone reduction and oxidation. The anchor domain shows the largest variability in composition and primary sequence. It consists of one larger (SdhC/FrdC; 23–30 kDa) or two smaller hydrophobic polypeptides (SdhC/FrdC/C_{II-3}/QPs1 and SdhD/FrdD/C_{II-4}/QPs2; 13–18 kDa and 11–16 kDa) and contains either one or two protoheme IX groups, with hexa-coordinated iron, or no heme at all. Anchors with two polypeptides con-

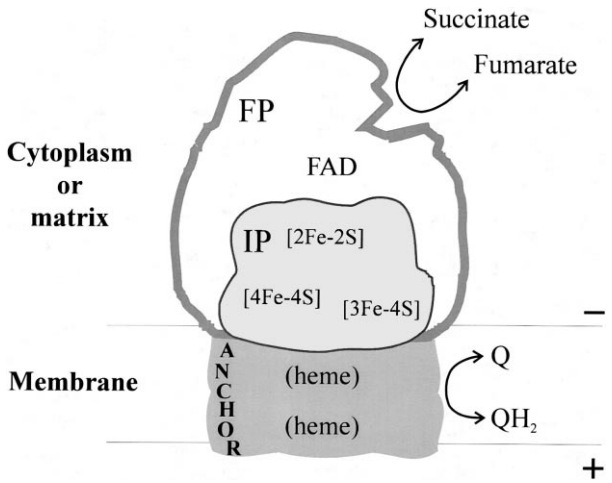


Fig. 1. A schematic outline of succinate:quinone oxidoreductase.

taining two hemes are denoted Type A, those comprising one polypeptide and two hemes — Type B, two polypeptide anchors with one heme group are denoted Type C whereas anchors with two polypeptides and no heme are referred to as Type D. In spite of the differences, these SQR and QFR membrane anchors share a common origin, based on recent evidence to be discussed later.

Succinate dehydrogenase (EC 1.3.99.1) activity was first detected in frog tissue in 1909 [1]. Since then a lot of information about the enzyme has been accumulated. Many review articles with emphasis on various aspects of the topic are currently available, cf. those by Kröger, [2], Hederstedt and Rutberg, [3], Ohnishi and Salerno, [4], Beinert and Albracht, [5], Singer and Johnson, [6], Hatefi, [7], Cole et al. [8], Vinogradov, [9], Ohnishi, [10], Jaramillo and Escamilla, [11], Kita et al. [12], Kröger et al. [13], Ackrell et al. [14], Hederstedt and Ohnishi, [15], van Hellemond and Tielens [16] and Hägerhäll and Hederstedt [17].

Vinogradov [9] expressed his love for this enzyme when he wrote ''The history of the study of succinate dehydrogenase is associated with the names of such prominent biochemists as D. Keilin, O. Warburg, L.S. Stern, J. Hopkins, D. Green, R. Morton, M. Dixon, E. Slater, T. King, T. Singer, H. Beinert and their students. It is therefore not surprising that the study of succinate dehydrogenase led to the formulation of a number of concepts which have become classical in enzymology and have given rise to the development of whole areas of present day biochemistry.'' Singer and Johnson [6], a year earlier, put the emphasis on the hard work invested when they state that their review ''traces the tortuous path, the many pitfalls and false leads, which have led us from the discovery of non-heme iron and bound flavin in the enzyme to elucidation of their structures''. More recently, the combination of molecular genetics, biochemistry and biophysical techniques have accelerated progress and improved our understanding of the enzyme. Nevertheless, Complex II is not a coupling site in the respiratory chain, and has thus received less attention than the other mitochondrial respiratory complexes.

It is notable that Complex II has the same general composition in pro- and eukaryotes, in contrast to Complex I, III and IV where, in higher organisms, various numbers of subunits are present in addition to those corresponding to the prokaryotic 'minimal functional units' of the enzymes. Saraste coined the expression 'Complex I is complex but Complex II is complex too' perhaps in response to Ohnishi's 'Complex I — the most complex Complex' [18]. Considering the relatively simple function of SQR it is indeed a remarkably complex enzyme. For example, the bacterial NADH:quinone reductase Type II, which have a similar function in the sense that it is a non-energy coupled enzyme that carries electrons from a substrate to quinone, consists of a single polypeptide with one FAD as a cofactor [19]. The unique position of Complex II as member of both the citric acid cycle and the respiratory chain implies that it may have a key regulatory role. Succinate:quinone oxidoreductases are also particularly interesting as model systems to further understand directionality of long range electron transfer, since they illustrate how the same components and structure are tuned to carry out reverse reactions. No high resolution structural data of SQR/QFR is currently available, although structural data for one SQR and one QFR may become available in the nearest future.

2. Scope of review

The material in this review is assembled with a bias towards the authors own research interests. Even so, I have attempted to focus on the most recent progress in the field, and to provide comprehensive structural and functional comparisons of SQRs and QFRs from different sources. Such a compilation of currently available data is particularly useful when X-ray crystallographic structural information becomes available for the enzyme from one or a few species. Progress in the field during the last few years makes it possible to better understand the functional directionality of these enzymes, and to propose new models related to their current function and past evolution. The 'respiration early' hypothesis, which is based on sequence analyses of terminal oxidases and other respiratory enzymes, convincingly argues an early origin of terminal oxidases and other respiratory chain components prior to oxygenic photosynthesis [20,21]. Although this theory is quite different from that converse, more widely accepted and

presented in textbooks, I am sure that many members of the scientific community just like myself find it both logic, plausible and attractive. SQR/QFR from all species, archaebacteria, eubacteria and eukaryotes also seems to have a common origin, and was presumably present in the last common ancestor. It follows from the 'respiration early' hypothesis that the primordial enzyme was not necessarily a fumarate reductase. The puzzling complexity of SQR/QFR, and the large variability particularly in the membrane anchor domain can be better explained as a development of an enzyme with a more complex function to enzymes with simpler functions. The current succinate:quinone oxidoreductases can be divided into three functional classes, with a varying degree of complexity that will be discussed in this context. Furthermore, I will place a particular emphasis on the nature of two quinone binding sites in SQR/QFR, and propose a common framework on which future experiments can be based.

3. Organization of the *sdh* and *frd* genes

In most prokaryotes the genes encoding the SQR/QFR subunits are organized as an operon. The first genes to be cloned and sequenced were those for *Escherichia coli* QFR. These genes where named *frd A, frdB, frdC* and *frdD*, and encode FP, IP and the two membrane anchor polypeptides, respectively [22,23]. When the operon encoding *E. coli* SQR was sequenced the A, B, C, and D nomenclature was kept for each polypeptide, and the operon became *sdhCDAB* [24,25]. The same gene order was found for the *sdh* operon in *Paracoccus denitrificans* [U31902], *Coxiella burnettii*, [26] and other gram-negative bacteria. In *Bacillus subtilis, Bacillus macerans* and *Wolinella succinogenes*, which have only three subunits in SQR/QFR, the gene order is *sdhC, sdhA, sdhB* [27,28] [Y08563] and *frdC, frdA, frdB* [29,30], respectively. In *Thermoplasma acidophilum sdhB* is followed by *sdhC* and *sdhD*, the location of *sdhA* is unknown [31]. Another archaebacterium, *Natronobacter pharaonis* contains a *sdhCDBA* operon [Y07709]. It appears that regardless of whether the operon encodes a SQR or a QFR the gene for FP is always located upstream of that for IP, with the exception of *N. pharaonis*. The genes encoding the membrane anchor polypeptides are however always clustered and in the order *C, D*, either before or after the genes encoding FP and IP. *Haemophilus influensae* [P44891], *Proteus vulgaris* [P20921] and *Mycobacterium tuberculosis* [Q10760] contain an operon similar to the *E. coli frd* operon. The exceptions are cyanobacteria (*Synechosystis*) where two genes homologous to *sdhB* and one to *sdhA* [sll1625, sll0823, slr1233] were found flanked by other genes, and *Methanococcus jannashi* where one *sdhA* and one *sdhB* [MJ0033, MJ0092] have been assigned so far, located apart and surrounded by unrelated genes.

In eukaryotes the FP, IP and the anchor polypeptides are generally encoded by unlinked nuclear genes, cf., [32–35] and imported into the mitochondrion. Very recently it was found that the mitochondrial genomes of the photosynthetic red algae *Porphyra purpurea*, *Chondrus crispus* and *Cyanidium caldarium*, the zooflagelate *Rectinomonas americana* and the liverworth *Marchantia polymorpha* carry *sdhC* and *sdhD* and in some cases also *sdhB* [36,37]. In *P. purpurea* and *R. americana* the *sdhC* and *sdhD* genes are arranged as in bacteria, whereas in *C. crispus* and *M. polymorpha* they are separated.

The many ongoing, large scale genome sequencing projects are providing us with SQR and QFR sequence information from various organisms. Unfortunately the usefulness of these data is limited, since in many cases no biochemical data exists for the corresponding enzymes. In other cases, when more than one set of genes are found, for example in the case of *Saccharomyces cerevisiae sdhA* and *sdhC*, it is unclear which genes to assign to the previously characterized enzyme. Nevertheless, the information is welcome since sequence comparisons in many cases allow us to make some predictions about the uncharacterized enzymes. Since user-friendly databases and search engines are readily available I will not attempt to provide a complete listing of available SQR/QFR sequences. The number of sequences, particularly from various eukaryotes, grow rapidly and a listing would very soon become outdated. Instead I have tried to provide sequence comparisons emphasizing differences rather than similarities, showing a few typical sequences from each type of SQR/QFR. These comparisons will by discussed throughout the text. Presented sequence data are generally referred to by accession number, for the readers convenience.

4. SQR and QFR enzyme activity

The two 'half cell reactions' of SQR/QFR are:

fumarate + $2H^+ + 2e^- \rightarrow$ succinate ($E_m = +30$ mV)

quinone + $2H^+ + 2e^- \rightarrow$ quinol ($E_m = -75$ mV to $+120$ mV) (see Table 1, Fig. 2)

In vitro, SQRs and QFRs can generally catalyze both succinate oxidation and fumarate reduction, but at different rates. However, in the in vitro assays of

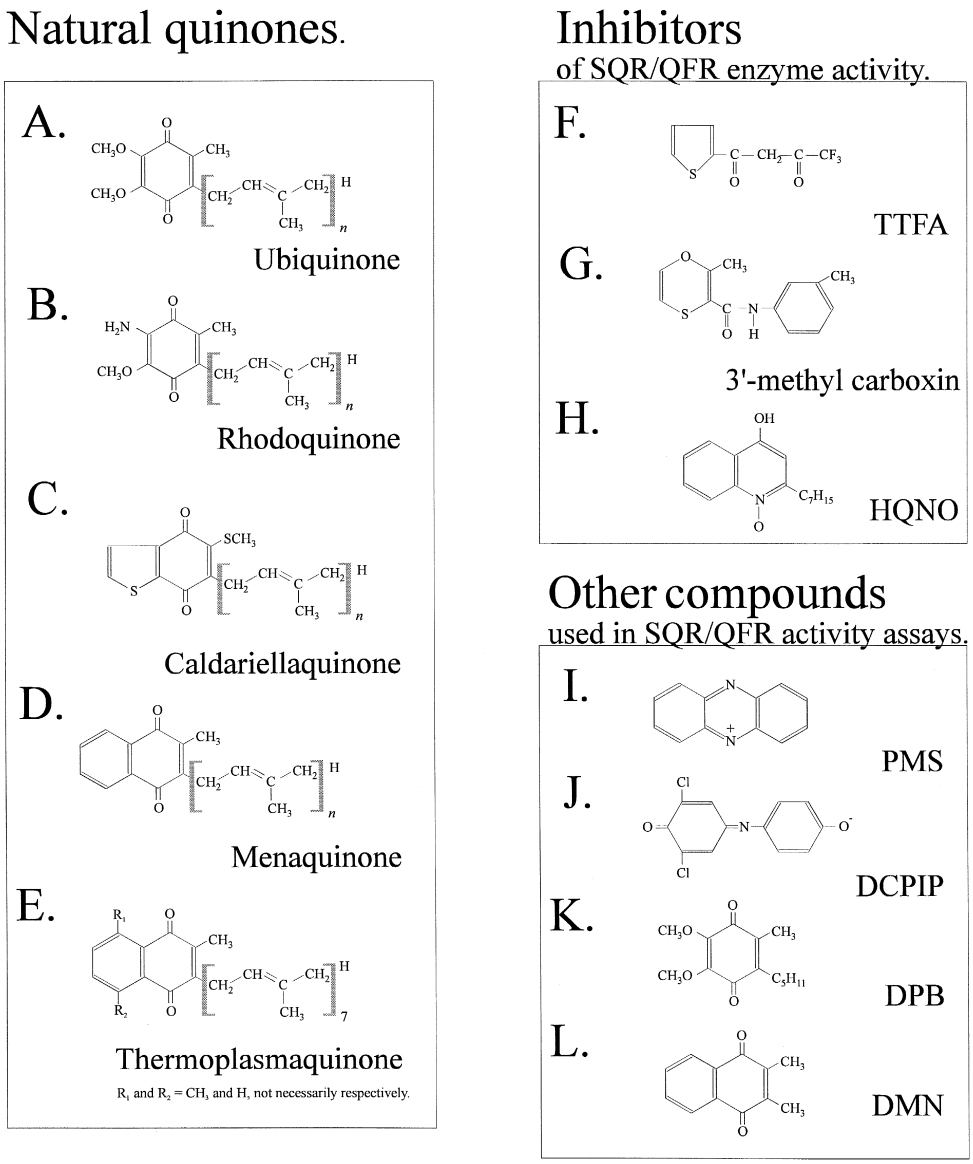


Fig. 2. Structures of naturally occurring quinone substrates, quinone analogues, inhibitors and other compounds commonly used in SQR/QFR studies. The E_m of ubiquinone (A) is $+112$ mV and of menaquinone (B) -74 mV ([77] and references therein), as determined in organic solvent. The E_m of the menaquinone pool in the membrane of *Bacillus* PS3 is -60 ± 20 mV [170]. The E_m of rhodoquinone is -63 mV according to [51]. The redox midpoint potential of caldariellaquinone was determined as $+100$ mV at pH 6.5 [132]. Thermoplasmaquinone is a methylated menaquinone [171], presumably with a similar E_m. TTFA stands for theonyltrifluoroacetone. Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) is a systemic fungicide of which the 3-methyl derivative is often used as SQR inhibitor. HQNO stands for 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. Phenazinemethosulphate (PMS) and 2,6-dichlorophenolindophenol are redox dyes. DPB (2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone) is a ubiquinone analogue whereas DMN (2,3-dimethyl-1,4-naphtoquinone) is a menaquinone analogue.

Table 1
A summary of properties of selected SQRs/QFRs

	Thermoplasma acidophilum	*Bacillus subtilis*	*Wolinella succinogenes*	*Micrococcus luteus*	*Escherichia coli*	*Bos taurus*	*Ascaris suum*	*Escherichia coli*	*Saccharomyces cerevisiae*
Type of organism	aerobic [a], thermo- and acidophilic archaebacterium	aerobic [a], G^+ soil bacterium	anaerobic, G^- in rumen of herbivores	aerobic, G^+, found on human skin	facultative G^-, gut bacterium	mammal, (mitochondria)	parasite worm(muscle mitochondria)	facultative, G^-, gut bacterium	bakers yeast (mitochondria)
Function Sequence data	SQR, Class 3 *sdhBCD* (location and sequence of *sdhA* is unknown)	SQR, Class 3 *sdhCAB*	QFR Class 2 *frdCAB*	SQR, Class 3? not available	SQR Class 1 *sdhCDAB*	SQR, Class 1 *sdhAsdhBsdhCsdhD*(not clustered)	QFR Class 2 *frdAfrdD* (*A. suum* also contains an SQR)	QFR Class 2 *frdABCD*	SQR or QFR? *sdhA*; Chr. XI, *sdhA**; Chr. X, *sdhB*; Chr. VII, *sdhC*; Chr. XI, *sdhC**; Chr. XIII, *sdhD*; Chr. IV
Polypeptides: number and mass	4, ?; 26; 15; 13 kDa	3, 65; 28; 23 kDa	3, 79; 31; 25 kDa	4, 72; 30; 17; 15 kDa	4, 64; 27; 14; 13 kDa	4, 70;27;15;13 kDa	4, 66;27;12;11 kDa	4, 69;27;15;13 kDa	4, 67;27;17;17 kDa
Membrane anchor	Type A	Type B	Type B	Type A or C?	Type C	Type C	Type C	Type D	Type D
Quinone [c]: type and E_m	thermoplasma quinone [b], low E_m	menaquinone -74 mV	menaquinone -74 mV	menaquinone -74 mV	ubiquinone $+113$ mV	ubiquinone $+113$ mV	rhodoquinone -63 mV	menaquinone -74 mV	ubiquinone $+113$ mV

FAD_{cov}	?	present	−20 mV	present	present	−79 mV	present	−55 mV	present
[2Fe-2S] (S1)	+68 mV(at pH 5.5)	+80 mV	−59 mV	+70 mV	+10 mV	±0 V	?	−20/−79 mV	Cys-motif
[4Fe-4S] (S2)	−210 mV (at pH 5.5)	−240 mV	−250 mV	−295 mV	−175 mV	−260 mV	?	−320 mV	Cys-motif
[3Fe-4S] (S3)	+60 mV (at pH 5.5)	−25 mV	−24 mV	+10 mV	+65 mV	+60 mV/+120 mV	?	−50/−70 mV	Cys-motif
Heme b_H	+75 mV	+16 mV	−20 mV	present	+36 mV	−185 mV	−34 mV	absent	absent
Heme b_L	−150 mV	−132 mV	−200 mV	present?	absent	absent	absent	absent	absent
Inhibitor sensitivity [d]	HQNO	HQNO	not sensitive to TTFA, carboxin or HQNO	?	HQNO	TTFA, carboxin	not sensitive to TTFA	HQNO	?
References:	[31,139,173]	[27,28,56,66,39]	[29,30,79,174]	[175,176]	[25,24,177–179].	[75,112,135], [180–184], [U50987]	[48,64,164,185]	[22,23,76], [177,186]	[34,187,188], [P3342]

[a] *Thermoplasma acidophilum* is a chemoheterotrophic aerobic archaebacterium with growth optimum at pH 2 and 59°C, but it can also grow anaerobically by sulfur respiration. *B. subtilis* can grow anaerobically with nitrate as electron acceptor.

[b] Thermoplasmaquinone is a methylated menaquinone (see Fig. 2 in [171]).

[c] The E_m values stated for ubiquinone and menaquinone are determined in solvent ([77] and references therein). The E_m of the menaquinone pool in the membrane of *Bacillus* PS3 is -60 ± 20 mV [170]. The E_m of rhodoquinone is from [51] (see also Fig. 2).

[d] Unfortunately, there are few systematic studies of SQR/QFR inhibitor sensitivity. For HQNO many are listed in [159]. For other references, see text.

succinate:quinone oxidoreductase activity directionality is usually achieved by providing the enzyme with excess substrates, and a quinone with a favorable redox potential.

Q_2 and Q_2H_2 bind preferentially to the reduced enzyme with dissociation constant (k_D) values of 0.3 and 0.9 μM, respectively in the bovine enzyme [14]. As mentioned, SQR/QFR activity assays involve the use of quinones or other redox compounds that provide an energetically favorable reaction. The succinate/ fumarate couple has a E_m of +30 mV. The commonly used artificial electron acceptor phenazinemethosulfate (PMS, Fig. 2) has an E_m of +80 mV. In assays of SQR activity using PMS, dichlorophenolindophenol (DCPIP, $E_m = +220$ mV, Fig. 2) serves as final electron acceptor. DCPIP can also be used in combination with water soluble quinone analogues. In the presence of excess DCPIP the concentration of quinol will be very low throughout the assay. When quinone reduction is measured directly, using the different extinction coefficients for oxidized and reduced form of the quinone in the UV-region, quinol will accumulate in the cuvette during the assay. Thus, assays without DCPIP often gives lower apparent turnover numbers, most likely reflecting product inhibition of enzyme activity due to the similar k_D's for quinone and quinol. Product inhibition may affect the assay more or less severely depending on assay conditions and instrumentation, i.e., ability to monitor the initial rate. It should also be noted that there are cases were electron transfer from quinol to DCPIP was found to be rate-limiting. The rate of quinone reduction by bovine SQR measured with DCPIP was only 75% compared to that using N,N,N',N'-tetramethyl-*p*-phenylenediamine (Wurster's Blue) as terminal electron acceptor, or the initial rate observed when quinone reduction was monitored directly [38]. The efficiency of DCPIP or other terminal electron acceptors may also vary depending on the quinone being used as primary acceptor [39]. QFR activity measurements encounter a similar problem, i.e., accumulation of oxidized quinone, and in addition the technical problems with anaerobic handling of the assay. A new QFR activity assay method that avoids these problems was designed by Kotlyar et al. [40] and Grivennikova et al. [41], using NAD(P)H-quinone reductase (DT-diaphorase) to continuously regenerate quinol.

The in vitro conditions poorly reflect the different substrate availabilities in the cell. SQR is a member of the citric acid cycle, that influences the production of succinate and the removal of fumarate whereas for QFR, in a cell respiring on fumarate, one has to expect more accumulation of succinate inside the cell. In the former case quinol is consumed by the respiratory chain at some rate, and in the latter case quinol is being produced at some rate, by various hydrogenases and dehydrogenases. In addition, $\Delta\mu_H^+$ can influence turnover rates in a positive sense, as will be discussed later, a condition more difficult to reproduce in vitro. Furthermore, one has to bear in mind the difficulties of estimating the actual working concentrations of quinones or inhibitors in the assays since these compounds often are hydrophobic and will accumulate to various degrees in lipid or detergent phase. Thus, turnover numbers from in vitro activity assays of SQRs/QFRs from different laboratories will not be presented since the assays are performed using different protocols and conditions, which hampers a meaningful comparison. In general, results from in vitro activity assays must be interpreted with caution, since they in many respects poorly reflect the in vivo conditions.

A difference in behavior of SQR and QFR was seen in the direct (unmediated) catalytic electrochemistry of the FP/IP subunits from bovine mitochondria [42] and *E. coli* QFR [43] when adsorbed on an electrode surface, i.e., cyclic voltammetry experiments. For the SQR it was found that catalysis in the direction of fumarate reduction was controlled by a potential gating effect, with activity decreasing as the electrochemical driving force is increased. No such effect was seen with the QFR. The fumarate reductase activity of SQR was maximal at −85 mV. Recently, a more detailed study of the *B. taurus* FP/IP showed that the two electron/two proton reduction of FAD most likely is responsible for the tunnel diode effect. Furthermore, binding and release of the competitive inhibitor oxaloacetate is observed when cycling between FAD_{ox} (tight binding) and FAD_{red} (weaker binding) [44]. This will be further discussed in Section 7.2.

A similar contrasting behavior of SQR and QFR can be seen in assays where fumarate reduction is measured using benzyl viologen radical ($E_m = -359$ mV) as electron donor. The fumarate reductase activ-

ity of a number of QFRs decreases as expected when the substrate (electron donor) concentration becomes lower whereas for a number of SQRs the activity increases. Notably, the SQR of *B. subtilis*, that do not support fumarate respiration in vivo, behaved in this assay as a QFR enzyme [45]. This method was later used to compare a number of eukaryotic succinate:quinone oxidoreductases. The 'diode' behavior was observed in rat and bovine heart SQR, in the facultative anaerobic organisms *Mytilus edulis* (mussels), *Crassostrea angulata* (oysters) and *Arenicola marina* (lugworms), and in aerobic, free living life stages of *Fasciola hepatica* (metacercariae) and *Haemonchus contortus* (L3) but was also seen in the anaerobic life stages of *Ascaris suum* and *F. hepatica*, although both these organisms completely depend on QFR activity. Adult *H. contortus* and *Dictyocaulus viviparus* on the other hand, which are not solely dependent on fumarate reductase activity do not show the 'diode' behavior [46]. Thus the absence or presence of 'diode' behavior seems to give no indication to the importance of fumarate reduction in vivo.

5. Succinate oxidation and fumarate reduction are generally carried out by separate enzymes in vivo

Some succinate:quinone oxidoreductases, i.e., those of *Desulfobulbus elongatus* [47] and *A. suum* [48], have been suggested to function as both SQR and QFR in vivo. In the case of *D. elongatus* this conclusion was based on the fact that the same apparent K_m values for succinate oxidation and fumarate reduction were observed in crude cell extract and in a purified enzyme. Furthermore, resting cells of *Db. elongatus* grown on a lactate-sulfate medium can oxidize propionate and produce propionate from lactate without any lag. In the case of *A. suum* it was more recently shown that both the parasitic nematodes *A. suum* and *H. contortus* contain different, growth stage specific forms of succinate:quinone oxidoreductase [49,50]. In addition, these and other eukaryotes were shown to switch from the use of ubiquinone (E_m +112 mV) to rhodoquinone (E_m −63 mV, [51], Fig. 2) as electron carrier under anaerobic conditions [46]. Other organisms, such as *E. coli*, have long been known to have a SQR and a QFR expressed during aerobic and anaerobic growth, respectively [52], the former using ubiquinone and the latter using menaquinone as electron acceptor/donor [53]. Some controversy remains as to whether these enzymes can substitute for each other in vivo [54].

Bacillus macerans (belonging to the *B. polymyxa* group of the *Bacilli*) contains only menaquinone regardless of growth condition, but was shown to express different succinate:quinone oxidoreductase enzymes during aerobic and anaerobic growth [55]. *Bacillus subtilis* (and other members of the *B. subtilis* subgroup) also contains only menaquinone [56] and can grow anaerobically only by nitrate respiration, and not by fumarate respiration. *B. subtilis* contains one SQR and in vitro it exhibits higher fumarate reductase rates than succinate oxidation rates, when provided with quinones with a favorable redox potential [39,57]. Schirawski and Unden [55] concluded that the potential fumarate reductase activity of the *B. subtilis* SQR must somehow be inhibited in vivo.

As mentioned, the DNA sequencing efforts in *S. cerevisiae* has so far identified two genes homologous to those encoding FP, one for IP, two for SdhC and one for SdhD [Z28148x1, Z49320x1, J05487x1, Z28141x1, Z49702x19, Z46727x11]. Some SQR activity remained in a yeast *sdhD* knockout mutant [58]. Thus it is possible that yeast also contains a SQR and a QFR expressed under different growth conditions. Other organisms also contain multiple sets of postulated SQR/QFR encoding genes, but no information is available as to whether they are functional genes. A search for a gene causing a respiratory chain deficiency in man turned up two *sdhA* genes, one on the distal long arm of chromosome 3 and the other on the distal short arm of chromosome 5. Only the latter gene was expressed in the somatic cell hybrids tested [59], but the authors conclude that both genes may still be functional and could be tissue-specific and/or developmentally regulated. If so, they may be responsible for the clinical heterogeneity of SQR deficiency in humans.

It must be concluded that SQR and QFR are functionally distinctly different enzymes in spite of similarities in structure and composition. To vary the quinone type in the membrane from ubiquinone to menaquinone or other low potential quinones facilitates, but is apparently not enough to turn a SQR into

a QFR. It is probably the rule more than an exception that organisms carry a separate enzyme for each of the two functions. Thus, intrinsic properties must determine whether a succinate:quinone oxidoreductase functions as SQR or QFR.

6. Three functional classes of succinate:quinone oxidoreductases

A compilation of available information for selected, relatively well characterized succinate:quinone oxidoreductases is provided in Table 1. To be able to rationalize these data it is helpful to have a functional classification of the enzymes. SQRs and QFRs in different organisms can be divided into three functional classes (Fig. 3), based on the quinone substrate and the in vivo function of the enzyme.

Class 1. SQRs that in vivo catalyze oxidation of succinate and reduction of a high potential quinone such as ubiquinone. Enzymes of this class are common, and are often perceived as typical. They are found in mammalian mitochondria and also in many other pro- and eukaryotes.

Class 2. All QFRs so far studied are included in

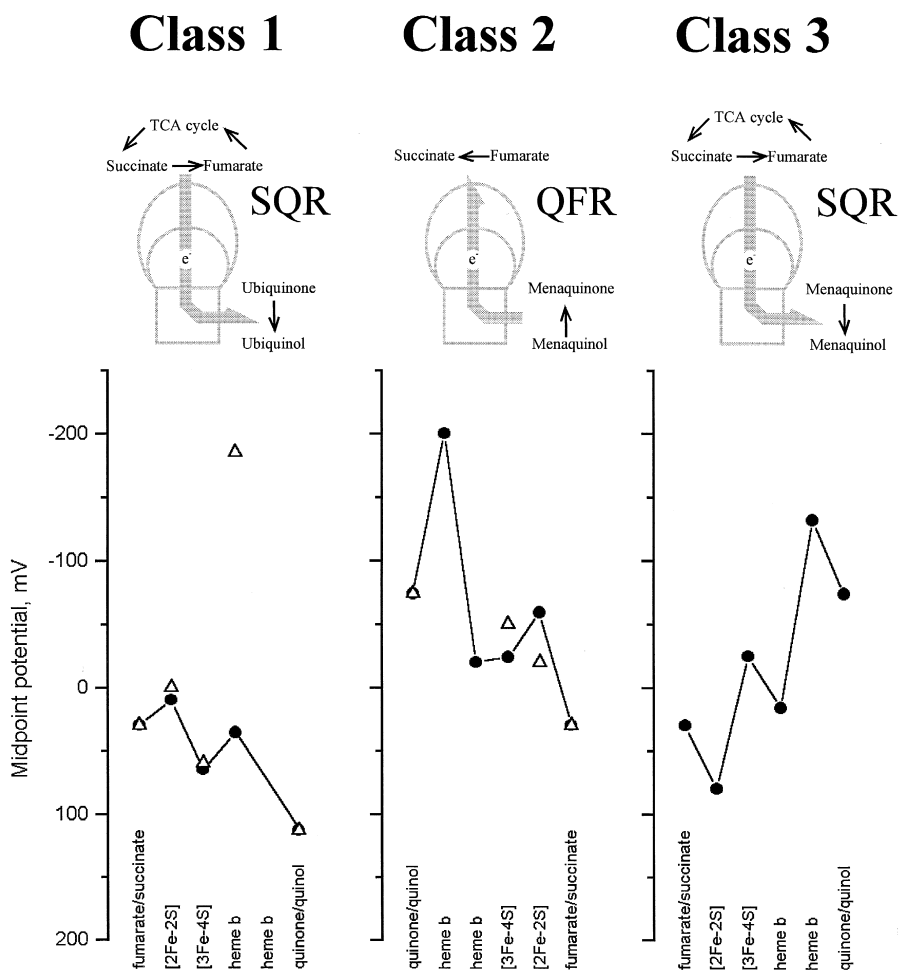


Fig. 3. The cartoons and graphs illustrate electron transfer in three principally different enzyme classes of SQR/QFR. Examples of Class 1 enzymes are *E. coli* SQR (filled circles) and *B. taurus* SQR (open triangles) transferring electrons from succinate to ubiquinone. Examples of Class 2 enzymes are *W. succinogenes* QFR (filled circles) and *E. coli* QFR (open triangles) transferring electrons from menaquinol to fumarate. These enzymes are chosen as representatives of somewhat different members of Class 1 and 2 respectively (see text and Table 1). The Class 3 enzyme is *B. subtilis* SQR, catalyzing oxidation of succinate and reduction of menaquinone. Reference to the various E_m values can be found in Table 1. The low potential iron-sulfur cluster S2/FR2 have been omitted from the plots for clarity.

this class. They always catalyze oxidation of a low potential quinol such as menaquinol or rhodoquinol and reduction of fumarate.

Class 3. SQRs that in vivo catalyze oxidation of succinate and reduction of a low potential quinone such as menaquinone or thermoplasmaquinone. This class of enzyme is found in *B. subtilis*, *B. macerans*, *T. acidophilum* and seemingly in other gram-positive bacteria and archaebacteria using exclusively low potential quinones in the respiratory chain.

As seen in Table 1 and Fig. 3, and as will be further discussed, the midpoint redox potentials of the iron-sulfur clusters are differently tuned in the three enzyme classes. In addition, the Class 3 SQRs seem to predominantly contain membrane anchors of Type A and B, i.e., membrane anchors that contain two transmembraneously arranged hemes, whereas Class 1 and 2 enzymes contain membrane anchors of various types. The Class 1 and Class 2 enzymes both carry out reactions that are energetically favorable, i.e., involve an overall decrease in ΔG. Class 3 enzymes are catalyzing an 'uphill' reaction (Fig. 3), i.e., the final electron acceptor has a lower reduction potential than the succinate/fumarate couple. Thus the functional mechanism of these SQRs deserve special attention. So far we have failed come up with any convincing explanation to what prevents extensive electron 'back flow' in these SQRs, when the in vitro assays predict a large bias towards fumarate reduction in these enzymes.

B. subtilis SQR, a Class 3 enzyme, can be readily overexpressed without any apparent growth impairment of the cells. An effect on cell growth would be expected if a delicate balancing of succinate and

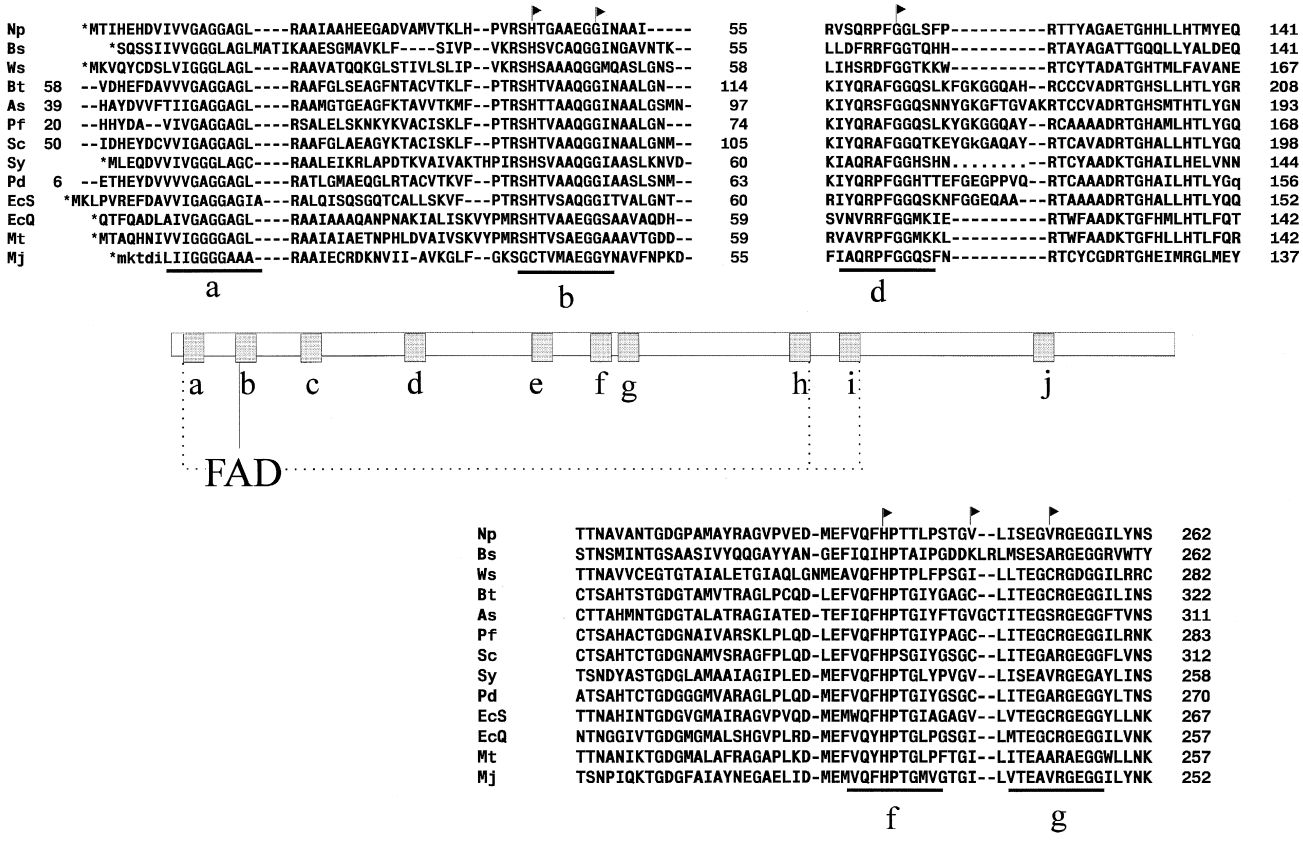


Fig. 4. Schematic representation of different conserved segments in the FP polypeptide. The indicated segments a–j are of importance for FAD binding or part of the active site. The positions of mutations discussed in the text are indicated by flags. Sequences are from *N. pharaonis* (Np, Y07709), *B. subtilis* (Bs, P08065), *W. succinogenes* (Ws, P17412), *B. taurus* (Bt, P31039), *A. suum* (As, D30650), *Plasmodium falsiparium* (Pf, D86573), *S. cerevisiae* (Sc, Q00711), *Synechosystis* sp. (Sy, slr1233), *P. denitrificans* (Pd, U31902), *E. coli* SQR (EcS, P10444), *E. coli* QFR (EcQ, P00363), *M. tuberculosis* (Mt, Q10760) and *M. jannashi*.(Mj, MJ0033).

oxidized menaquinone levels facilitate the appropriate reaction.

It should be noted that the succinate to oxygen respiratory activity of *B. subtilis* is 13 times lower in membranes isolated in uncoupled state compared to in intact bacteria and furthermore, 90% of the respiratory activity in intact bacteria was lost upon addition of small amounts of protonophore (tetrachloro-2-trifluoro-methyl-benzimidazol) [56]. This indicates that a mechanism to control directionality is present in the Class 3 enzymes, and it seems to be associated with the membrane part of the enzyme.

There is also an important evolutionary aspect to this issue. The reason for the current, seemingly unnecessary complexity of Complex II may be that the primordial enzyme had a more complex function. The Class 3 SQRs may in this respect closely resemble the last universal ancestor enzyme, whereas the enzymes of the two other classes represent degenerate, simplified forms. This aspect will be further discussed toward the end of this review. Let us now take a closer look at the components of succinate:quinone oxidoreductase.

7. The flavoprotein subunit

The flavoprotein (FP) subunit is a soluble 79–65 kDa polypeptide, exposed on the matrix side of the mitochondrial inner membrane or the prokaryotic cytoplasmic membrane. The FP subunit contains the dicarboxylate binding site. Amino acid sequence comparisons have revealed several particularly conserved regions in the FP polypeptide (Fig. 4). Oxidation of succinate to fumarate is a trans-dehydrogenation. L-Chlorosuccinate but not D-chlorosuccinate is a substrate for SQR [60]. Malonate and oxaloacetate are not substrates, but potent competitive inhibitors of SQR, although the sensitivity to oxaloacetate varies greatly between different SQR/QFR [14]. D- and L-malate are poor substrates for SQR since they are oxidized at the active site to enol-oxaloacetate that inhibits the enzyme [61]. More extensive data concerning substrate binding and catalysis as well as various aspects on the covalently bound FAD can be found in [14] and in [15]. A model for the dicarboxylate binding site is shown by van Hellemond and Tielens in [16].

7.1. The dicarboxylate binding site

Chemical modifications of substrate protectable residues in combination with mutant studies have been used to identify residues in the FP polypeptide that are located in or close to the active site. *B. taurus* SQR, *E. coli* SQR and QFR and *W. succinogenes* QFR are very sensitive to thiol-modifying reagents and substrate or substrate analogues protect these enzymes from inactivation ([15] and refs. therein). *B. subtilis* SQR is not sensitive to thiol-modifying reagents. In the former species there is a cysteine residue in segment g (Fig. 4), that is not conserved in *B. subtilis*. When the alanine in this position in *B. subtilis* FP was changed to cysteine the SQR remained fully functional, but became sensitive to thiol-modifying reagents, and could be protected by substrate [62]. Analogously, when the indicated cysteine in the FP of *E. coli* QFR was changed to alanine the enzyme became less sensitive to modification [63]. *A. suum* and *Caenorhabditis elegans* FP have serine in the place of this cysteine. SQR activity in these organisms is nevertheless sensitive to sulfhydryl reagents. A cysteine residue six residues from the serine (see Fig. 4) was suggested to be responsible for the effect. Furthermore, QFR activity in *A. suum* was not at all affected by the reagents [64], in contrast to in *E. coli* QFR and *B. taurus* SQR [41]. Next to the cysteine in segment g there is a conserved arginine residue that is suggested to form a bidentate ionic pair with one of the carboxylate groups of succinate, thereby orienting the substrate at the active site [65]. Diacetyl modification of *E. coli* QFR suggests that two arginines are functionally important [63]. A *B. subtilis* SQR mutant has one of the conserved glycines in segment d replaced by a glutamate. This mutant enzyme is inactive although all prosthetic groups are present and have normal properties [66]. Segment f in FP (Fig. 4) contains a conserved triad, H-P-T. An *E. coli* QFR mutant with the histidine in this triad replaced by a serine can not oxidize succinate [63]. However, fumarate reductase activity of this mutant QFR is close to wild type levels. It has been suggested that an unprotonated histidine may assist in the deprotonation of succinate [67]. The active site of the flavoenzymes glutathione reductase and lipoamide reductase contain an identical triad motif [15]. The combined data indicate that

residues in segments f, g and d are part of the dicarboxylate binding site. Mammalian SQR and *E. coli* QFR have different affinities for substrate and the inhibitor oxaloacetate depending on whether the enzyme is in the reduced or oxidized state as summarized in [14]. This indicates that the structure of the dicarboxylate binding site must be different depending on the redox state of enzyme.

A mutation causing SQR deficiency in two human siblings was shown to be a R555 → W substitution in FP. About 60% of wild type SQR activity was observed in patient cells. The defect Complex II showed increased sensitivity to oxaloacetate [59].

7.2. FAD

Segment b contains the histidine residue, to which FAD is covalently bound via N(3)-8 α-riboflavin linkage [68]. The sequence of segment b is not conserved in other types of enzymes containing His-N(3)-8 α-FAD [14], which suggests that this sequence is more related to enzyme function than FAD binding. Segments a, h and i are probably in contact with the AMP part of FAD ([14] and references therein). It is not known by which mechanism the FAD is incorporated, but FAD is covalently bound to FP before enzyme assembly [69]. An autocatalytic mechanism have been suggested. However, *B. subtilis* SQR expressed from cloned genes in *E. coli* is not flavinylated [70]. In yeast flavinylation of FP occurs in the mitochondrial matrix, after import of the polypeptide and removal of the presequence. FAD attachment was stimulated by, but not dependent on presence of IP [71]. A matrix processing peptidase, but not any mitochondrial chaperonins seem required for covalent attachment of FAD [72].

Succinate oxidation is only possible when the FAD is covalently bound. *Desulfovibrio multispirans* QFR contains non-covalently bound flavin and can not oxidize succinate [73]. Replacement of the FAD-binding histidine by various amino acids in *E. coli* QFR results in mutant enzymes that contain stoichiometric amounts of non-covalently bound FAD that can not oxidize succinate. Some fumarate reductase activity was retained in the mutants in vivo and in vitro [74]. Sequence data predict that FP from the anaerobic archaebacterium *M. jannashi* contains non-covalently bound FAD and therefore most likely is a component of a QFR (Fig. 4).

The flavin is probably located close to the substrate binding site and the most likely first electron acceptor during succinate oxidation. In *B. subtilis* mutants lacking flavin (i.e., a G → D mutation in segment b) the iron-sulfur centers are not reduced by succinate, but can be reduced by reversed electron transfer from menaquinol in the membrane [66,70]. FAD is a two electron carrier, in contrast to the other prosthetic groups in SQR/QFR. Redox titrations can be performed on the flavin free radical EPR signal at $g = 2.00$. The midpoint potentials of the two consecutive electron transfer steps of FAD in *B. taurus* SQR were determined to be -127 mV and -31 mV at pH 7, corresponding to a midpoint potential of -79 mV for the overall reaction [75]. Similar redox potentials for FAD in SQR/QFR have been determined in other species (Table 1). Free FAD has a midpoint potential of -219 mV [76]. Thus, both electron transfer steps to enzyme-bound FAD are endergonic with respect to the succinate/fumarate couple ($+30$ mV; [77]). The FAD free radical state in bovine SQR has a stability constant of $2.5 \cdot 10^{-2}$ [75], i.e., it is much more stable than typical $n = 2$ components such as free $NAD^+/NADH_2$ or Q/QH_2. These observations indicate that FAD performs the $n = 2$ to $n = 1$ conversion required to transfer electrons from succinate to the iron-sulfur clusters and vice versa within SQR/QFR.

In *B. taurus* SQR in the presence of succinate a maximum of 10% flavin free radical was observed at pH 7 and up to 20% at pH 9 [75]. In contrast, in reconstitutivly inactive preparations of the same enzyme, i.e., FP/IP lacking iron-sulfur cluster S3, the flavin radical was 80% of maximal at pH 7.8. [78]. Interestingly, in *B. subtilis* SQR, and only in the presence of succinate, we observe a similar, large flavin free radical in both the membrane bound and intact, isolated enzyme (Hägerhäll and Sled', unpublished). A flavin free radical showing anomalous behavior has also been observed in *W. succinogenes* QFR [79].

In the previously mentioned cyclic voltammetry experiments using *B. taurus* FP/IP it was shown that the two electron/two proton reduction of FAD most likely is responsible for the tunnel diode effect. Furthermore, binding and release of the competitive

inhibitor oxaloacetate is observed when cycling between FAD_{ox} (tight binding) and FAD_{red} (weaker binding) [44]. The 'diode' behavior is absent in the *E. coli* QFR, but both enzymes are sensitive to oxaloacetate and exhibit different affinities for the inhibitior in the oxidized and reduced states. Redox titrations of deactivated SQR [80] and QFR [76] demonstrated that reductive activation results from reduction of FAD. However, the processes differed for the two enzymes; activation of *B. taurus* SQR involved $2e^-$ and $2H^+$, whereas the process requires $2e^-$ and $1H^+$ in *E. coli* QFR, consistent with reduction of the flavin to the anionic hydroquinone form. The reason for the difference is not understood. The redox potential of the $FAD/FADH_2$ couple in QFR (E_m −55 mV, Table 1) is slightly more positive than that in *B. taurus* SQR.

8. The iron-sulfur protein (IP) subunit

This subunit is 31–24 kDa, and harbors three iron-sulfur clusters. The IP polypeptide contains three groups of cysteine residues, which are ligands to three iron-sulfur clusters. *B. subtilis* and *E. coli* mutant IPs, truncated before and after the second group of cysteines respectively, retain the [2Fe-2S] cluster but have lost the other two clusters [81,82]. Thus, IP can be described as consisting of two domains. The N-terminal domain harbors the [2Fe-2S] cluster (S1/FR1), ligated by a CxxxxCxxC.....C motif, resembling a plant-type ferredoxin (Fig. 5A). The C-terminal domain ligates the [4Fe-4S] (S2/FR2) and [3Fe-4S] (S3/FR3) clusters and contains cysteine motifs characteristic for bacterial ferredoxins (Fig. 5B). Information about the spatial organization of the iron-sulfur centers is provided from EPR:detectable magnetic spin–spin interactions. The distance between FAD and S1 is 12–18 Å, cluster S1 and S2 are 9–12 Å apart whereas cluster S1 and S3 are estimated to be 10–20 Å apart. Strong spin–spin coupling also between cluster S2 and S3 has been detected, indicating close mutual distance of these clusters [10,15].

Two peculiar exceptions among IPs are the postulated IP from *M. jannashi* which contains a very long additional tail in the C-terminal end (Fig. 6) and one of the postulated IPs in *Synechosystis* which also contains a C-terminal extension, though much shorter than the one in *M. jannashi*. The archaebacterial polypeptide comprises 489 amino acids and the cyanobacterial 331 amino acids, whereas typical IPs contain 240–250 amino acids. The deduced amino acid sequence in the C-terminal part of the *M. jannashi* polypeptide shows sequence similarity to a subunit of heterodisulfide reductase, HdrB, from *Methanobacterium thermoautotrophicum* (Fig. 6 [83]). In addition both postulated IP polypeptides contain cysteine motifs indicative of two [4Fe-4S] clusters, in addition to S1. These polypeptides will be further discussed in following sections. Apart from in the former species, the structure of IP in various organisms seems very well conserved, but the midpoint potential of the respective iron-sulfur clusters varies depending on how the enzyme operates (Table 1, Fig. 3).

Ackrell et al. [14] give an excellent overview of the EPR properties of the respective iron-sulfur clusters in SQR/QFR. For a recent review on FeS cluster containing proteins in general see that by Johnson, [84].

8.1. The [2Fe-2S] cluster

In general, the midpoint potential of S1/FR1 in SQR/QFR is high compared to that of [2Fe-2S] centers in plant type ferredoxins [85]. The [2Fe-2S] center of the Rieske protein in the bc_1 complex

Fig. 5. A: sequence comparison of the N-terminal domain of IP. The sequence and structure of a plant type ferredoxin [172] is shown as a reference to this domain. The sequence following the [2Fe-2S] cluster ligating domain is typical for IP, and is not found in other iron-sulfur proteins. B: sequence comparison of the C-terminal domain of IP. The sequence and structure of a bacterial type ferredoxin [94] is shown as a reference. Mutations discussed in the text are indicated by flags. Sequences are from: *T. acidophilum*, (Ta, X70908), *N. pharaonis* (Np, Y07709), *B. subtilis* (Bs, P08066), *W. succinogenes* (Ws, P17596), *E. coli* SQR (EcS, P07014), *Homo sapiens* (Hs, P21912), *P. falsiparium* (Pf, D86574), *S. cerevisiae* (Sc, P21801), *U. maydis* (Um, P32420), *C. crispus*, (Ccr, P48932), *Synechosystis* sp. (Sy1, sll0823, Sy2, sll1625), *M. jannashi*.(Mj, MJ0092), *E. coli* QFR (EcQ, P00364) and *M. tuberculosis* (Mt, Q10761).

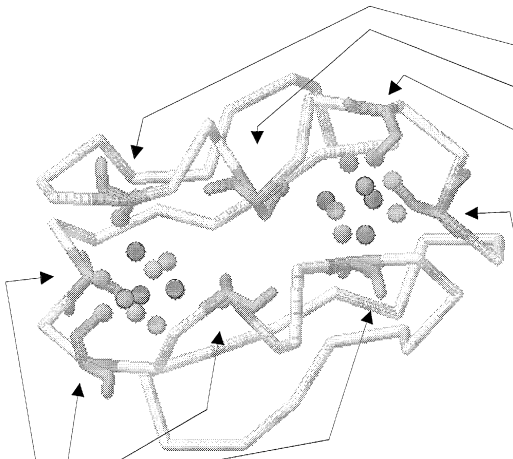
A

Sequence and structure of plant-type [2Fe-2S] ferredoxin from *Anabaena* sp. strain PCC7120. (Brookhaven PDB file 1fxa)

Stretch implicated in membrane binding using yeast/human chimeric IP.

```
                --ATFKVTLINEAEGTKHEIEVPDDEYILDAAGGGGYDLPFSCRAGACSTCAGKLVSGTVLTCVAYPTSAVVIQTHK---
     *MADEYEFEIKRKDQSGKVYYSTYKVPKDKISTV.LEGLLYIKENLDQSLSFRYPCRMEICGSCGMEIDGKPRMACSTIVEDLKKDKIRIE.........PLRHYKVIRDLVVDIDPFFEKYREVKPYIIRDDDGKYDKELP---  Ta
44-ADETVQIKVFRYDPEVEGKKDPRFDTFEIPFTKGMTV.LDALIYARDHFDSTLTFRHSCRQAICGSDALFVNGAQRLGCKTQMVDLEWPVRIEP.........LPHAEVVKDLVVDMEHFYDQMESVEPYFQTNELPDGELEEQR--  Np
 8-IITRQDTADSTPYDEEFEIPYRPNLNVISALMEIRRNP...VNVKGEKTTP.VTWDMNCLEEVCGACSMVINGKPRQSCTALIDQLEQPIRLK.........PMKTFPVVRDLQVDRSRMFDSLKKVKAWIPIDGTYDLGPGP----  Bs
 9-FKYDPQSAVSKPHFQEYKIEEAPSMTIFIVLNMIRETYDPD.........LNFDFVCRAGICGSCGMMINGRPSLACRTLTKDFEDGVITLL.........PLPAFKLIKDLSVDTGNWFNGMSQRVESWIHAQKEHDISKLEE--  Ws
     *MRLEFSIYRYNPDVDDPRMQDYTLEADEGRDMMLLDALIQLKEKDPS.LSFRRSCREGVCGSDGLNMNGKNGLACITPISALNQPGKKIVIR.......PLPGLPVIRDLVVDMGQFYAQYEKIKPYLLNNGQNPPAREHL---  EcS
34-TAPRIKKFAIYRWDPDKAGDKPHMQTYKVDLNKCGPMVLDALIKIKNEVDSTLTFRRSCREGICGSCAMNINGGNTLACTRRIDTNLNKVSKIY.........PLPHMYVIKDLVPDLSNFYAQYKSIEPYLKKKDESQEGKQQYL--  Hs
61-EVVKRKKKFSIFRYNPTNKKRPQMETFEVDIDNCGPMVLDVLIKIKDEIDSTLSFRRSCREGICGSCAMNINGKNGLACLTEVNRDKKEITEIQ.........PLPNLYVMKDLVPDLTNFYNQYKSIDPWLKRKTKKEKGQKEFY--  Pf
28-HTPRLKTFKVYRWNPDEPSAKPHLQSYQVDLNDCGPMVLDALLKIKDEQDSTLTFRRSCREGICGSCAMNIGGRNTLACICKIDQNESKQLKIY.........PLPHMFIVKDLVPDLTNFYQQYKSIQPYLQRSSFPKDGTEVL---  Sc
47-KPQHLKQFKIYRWNPDKPSEKPRLQSYTLDLNQTGPMVLDALIKIKNEIDPTLTFRRSCREGICGSCAMNIDGVNTLACLCRID..KQNDTKIY.........PLPHMYIVKDLVPDLTQFYKQYRSIEPFLKSNNTPSEGEHL----  Um
14-KTLSDLKLIKIFRWDSSEKKDPWYSTYVVSLKNCGPIVLDALIKIKNECDSTVSFRRSCREGICGSCAININGTNSLACLQKLNI.KNNIIYVY.........PLPHIFVLKDLVVDLTNFYAQYRLIQPWLQSSLNIKSKKEIY---  Ccr
        *MEIVCKIQRQLTDGDRHWQNYYLDVDPQTSILNCLNQIKWQQDGSLAFRKNCRNAICGSCAVRVNDRPVLACKESIKSLGQWLAESNQENTGVFTISPLGNLPVIKDLVVDMEKFFAGLDAVYPYLINKHQGEKGAEFS---  Sy1
        *MQVQFQILRQKPQQSPYLEKFDLEVEPGATILECLNQIKWEQDGSLNFRKNCRNTICGSCSMRVNGRSALACKENVGSETRLFTQVNEAGIPVVTVAPLGNLPVIKDLIVDMQPFWDDLERVEPYVSTQGRKVPEREFL---  Sy2
           *MIKITVKRFNGEKEYLESYEVPENITVLEALEYINKHYEANILFRASCRNAQCGSCAVTINGEPRLACETKVEDGMIIE.............PLRGFKVIRDLIVDREPYYKKLLGIKNYLIRKNYPEELEI-----  Mj
   *AEMKNLKIEVVRYNPEVDTAPHSAFYEVPYDATTSLLDALGYIKDNLAPDLSYRWSCRMAICGSCGMMVNNVPKLACKTFLRDYTDGMKVE.........ALANFPIERDLVVDMTHFIESLEAIKPYIIGNSRTADQGTNI---  EcQ
    *MMDRIVMEVSRYRPEIESAPTFQAYEVPLTREWAVLDGLTYIKDHLDGTLSFRWSCRMGICGSSGMTINGDPKLACATFLADYLPGPVRVE.........PMRNFPVIRDLVVDISDFMAKLPSVKPWLVRHDEPPVEDGEYR--  Mt
```

B



Sequence and structure of bacterial type ferredoxin from *Peptococcus aerogenes*. (Brookhaven PDB file 1fdx)

```
                ----AYVINDSCIACGACKPECPVNIIQGSIYAIPA.............................DSCIDCGSCASVCPVGAPNPED----
Ta   ---QTPEQFHEYA.NFAMCIKCGLCMAACPIEGSDPQYL.GPAPLAAAWRYIADNRDKGA..KYRVEIVDGEEGTSRCHFAGECTEVCPKGVDPSFAIQKLRRTALKIELASIFGR*
Np   ---QTRENREKVK.MSTRCIWCSACMSSCNIAAGDNEYL.GPAAINKAYRFAMDKREGEEMKQHRLNIIEQEHGVWRCQTQFSCTEVCPKDIPLTEHIQELKREAVKNNLKFW*
Bs   ---RMPEKRRQWAYELSKCMTCGVCLEACPNVNSKSKFM.GPAPMSQVRLFNAHPTGAMN.KSERLEALMDEGGLADCGNSQNCVQSCPKGIPLTTSIAALNRDTNLQAFRNFFGSDRV*
Ws   ---RIEPEVAQEVFELDRCIECGCCIAACGTKIMREDFV.GAAGLNRVVRFMIDPHDERT.DEDYYELIGDDDGVFGCMTLLACHDVCPKNLPLQSKIAYLRRKMVSVN*
EcS  ---QMPEQREKLD.GLYECILCACCSTSCPSFWWNPDKFIGPAGLLAAYRFLIDSRDTET..DSRLDGLSDAFSVFRCHSIMNCVSVCPKGLNPTRAIGHIKSMLLQRNA*
Hs   ---QSIEEREKLD.GLYECILCACCSTSCPSYWWNGDKYLGPAVLMQAYRWMIDSRDDFT..EERLAKLQDPFSLYRCHTIMNCTRTCPKGLNPGKAIAEIKKMMATYKEKKASV*
Pf   ---QSIEDRKKLD.GLYECIMCASCSTSCPSYWWNPEYYLGPATLMQAYRWIVDSRDEYT..KERLMEVNDTMKLYRCHGIMNCTMCCPKGLDPAKAIKDMKNLVQENFSEDTIKEHSQYIKSKMEKTK*
Sc   ---QSIEDRKKLD.GLYECILCACCSTSCPSYWWNQEQYLGPAVLMQAYRWLIDSRDQAT..KTRKAMLNNSMSLYRCHTIMNCTRTCPKGLNPGLAIAEIKKSLAFA*
Um   ---QSPEERRRLD.GLYECILCACCSTSCPSYWWNQDEYLGPAVLMQAYRWMADSRDDFG..EERRQKLENTFSLYRCHTIMNCSRTCPKNLNPGKAIAQIKKDMAVGAPKASERPIMASS*
Ccr  ---QSKQDRLYLD.GLYECILCACCSASCPSYWWNHDKYLGPAVLLQAYRWIVDSRDDNT..LSRLLSLKDSYKLYRCHTIMNCTKTCPKHLNPGKVIASIKKRLLNLEVLGEKRDLNS*
Sy1  ---QSPEQRAALN.DVSNCVLCGVCYSDCDAKKKNENFV.GPHALAKANRLILDNRDMAT.KARLEALGKDKQGVWGCNNCRMCSDSCPTGVAPLSQIEALKIRLFDLMDPL*
Sy2  ---QTPAEREKLN.QMGNCILCGACYSECNAKSVNPDFV.GPHALAKAQRLLTDSRDGAT.ADRLESYNNATAGAWGCTRCYLCNEVCPMEVAPMDQIGKIKSALLAQKTAQDSRPVRHRKVMVDLVKAGGWVD-----
Mj   ---LIPKYVEENK.ELRGCIDCLSCLSVCPAREVSDYP..GPTFMRQLARFAFDKRDEDG.....REITAYFENIYNCTTCAKCVEVCPKEIDIVHRAIEKLRALAFSKGYYIENHLKVRENVLKYNRSVVEEE-----
EcQ  ---QTPAQMAKYH.QFSGCINCGLCYAACPQFGLNPEFI.GPAAITLAHRYNEDSRDHGK..KERMAQLNSQNGVWSCTFVGYCSEVCPKHVDPAAAIQQGKVESSKDFLIATLKPR*
Mt   ---QTPAELDAFK.QFSMCINCMLCYSACPVYALDPDFL.GPAAIALGQRYNLDSRDQGA..ADRRDVLAAADGAWACTLVGECSTACPKGVDPAGAIQRYKLTAATHALKKLLFPWGGG*
```

(Complex III) also has a much higher midpoint potential than the clusters in ferredoxins. In the bc_1 complex two cysteines and two histidines are ligating the [2Fe-2S] cluster [86,87]. The higher midpoint potential is in this case explained by that nitrogen is less electron-donating than sulfur.

The ligation of the [2Fe-2S] cluster (S1/FR1) has been studied in detail in *E. coli* QFR, where the four cysteines were individually changed to serine [88]. The mutants retained all iron-sulfur clusters, as well as at least some enzymatic activity. The EPR properties of cluster FR2 and FR3 were normal in these mutants. It was shown that the [2Fe-2S] cluster is assembled in each of the four mutants, but the mutations cause changes in g tensor anisotropy and/or redox midpoint potential. The potential of the cluster was lower than in the wild type (−79 mV) in the C57 → S (−182 mV), the C62 → S (−322 mV) and the C77 → S (−110 mV) mutants, but higher than in wild type in the C65 → S (−49 mV) mutant [88]. It should be noted that the third cysteine in the CxxxxCxxC.....C motif is not fully conserved; the IP of *E. coli* and *N. pharaonis* SQR has an aspartate whereas *M. tuberculosis* QFR has a serine in this position (see Fig. 5A). Werth et al. [89] concluded that C65 in *E. coli* QFR is not essential for function although it may be a cluster ligand in the wild type enzyme. They proposed that S1/FR1 is coordinated by three cysteines and a water molecule, the latter being hydrogen bonded to an aspartate or a cysteine. This seems to be an attractive scheme, since it could explain the higher E_m of S1/FR1 compared to ferredoxin [2Fe-2S] clusters.

The iron-sulfur cluster S1 is most likely the first electron acceptor after flavin in SQRs and FR1 is the electron donor to flavin in QFRs. It should be noted that S1 has a high E_m (higher than S3) in Class 3 SQRs, an intermediate E_m (but lower than S3) in Class 1 SQRs and a low E_m in QFRs (Table 1, Fig. 3). Growth properties and enzyme activities of the above mentioned *E. coli* QFR mutants demonstrated near wild type properties of the C65 → S mutant but a decreased enzyme activity in the remaining mutants [88] perhaps indicative of some redox imbalance.

8.2. The [4Fe-4S] and [3Fe-4S] clusters

The C-terminal part of the IP ligates the [4Fe-4S] and [3Fe-4S] clusters and contains cysteine-motifs characteristic for bacterial ferredoxins (Fig. 5B). Assuming a ferredoxin-like structure of the C-terminal part of IP these two clusters are located close to each other. Attempts have been made to identify the ligands to FR2 and FR3 in *E. coli* QFR by site-specific mutagenesis. Replacement of C154, C158, C204, C210 and C214 by serine resulted in the loss of both FR2 and FR3, but did not affect FR1 [90,91]. The C148 → S and C151 → S mutants retained all three clusters, with a modest decrease in E_m of FR1 and FR3 (about −10 to −15 mV) and a somewhat larger decrease, −60 to −70 mV, of FR2. The mutant enzymes showed pronounced instabilities and signs of increased oxygen sensitivity. However, EPR analyses of the C148 → S mutation revealed much more complex resonances for FR2 than in the wild type enzyme, interpreted as perturbation of the FR2–FR3 intercluster spin–spin interaction. The FR1-FR2 spin–spin interaction was not affected by this mutation. The C151 → S mutant lacked resonances other than those originating from FR1 in the spin 1/2

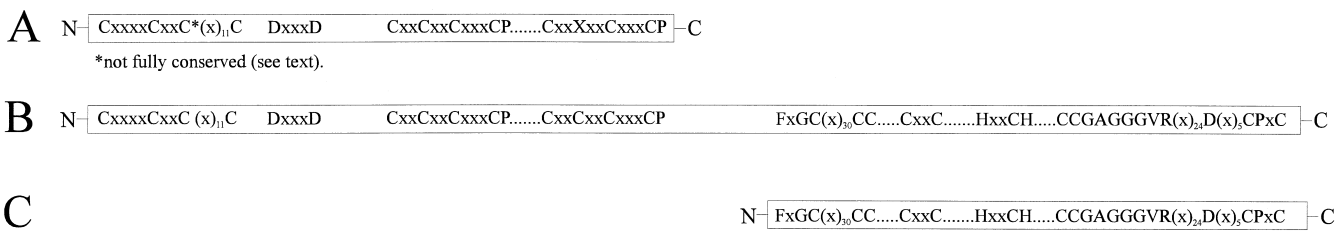


Fig. 6. A schematic outline of a classical IP polypeptide (A, Figure 5) compared to the postulated IP from *M. jannashi* (B, MJ0092) and the HrdB polypeptide, a subunit of heterodisulfide reductase from *M. thermoautotrophicum* (C, X81133). Detailed sequences of IP are in Figure 5A and B (see also FP from *M. jannashi*, Fig. 4). The C-terminal end of *M. jannashi* IP and HrdB are similar to the SdhC polypeptide in *S. acidocaldarium* ([Y09041], see text).

region and exhibited new resonances at $g = 5.7$ and 5.0 indicative of a $S = 3/2$ [4Fe-4S] cluster. Furthermore, the presence of FR2 was apparent from the always observed increase in FR1 spin relaxation after dithionite reduction. It was concluded that FR2 in this mutant is assembled as a [4Fe-4S] cluster, but that the replacement of a cysteinyl ligand with a serinate ligand causes a change in the spin state of the reduced cluster from 1/2 to 3/2 [91].

When a fourth cysteine residue was introduced into the putative FR3 binding motif of *E. coli* QFR (V207 → C substitution; Fig. 5B), a [3Fe-4S] to [4Fe-4S] cluster conversion occurred, i.e., a mutant enzyme with two low potential [4Fe-4S] clusters was obtained [90]. Opposite cluster conversion, i.e., from [4Fe-4S] to [3Fe-4S], has been reported for several enzymes after replacement of a ligating cysteine by another amino acid (cf., [92,93]). These observations suggest that the prototypical cysteine sequence motif predominantly determines the cluster type. Taken together it can be concluded that the [4Fe-4S] (S2/FR2) and [3Fe-4S] (S3/FR3) clusters both probably have a cubane-like structure and are ligated by the cysteine residues arranged in the sequence as CxxCxxCxxxCP followed by CxxxxxCxxxCP. The first three cysteines in the first group and the last cysteine in the sequence are ligands to S2/FR2 and the remaining cysteines are ligands to S3/FR3, as is also deduced from sequence comparison to bacterial ferredoxins that contain cubane iron-sulfur clusters (Fig. 5B, cf., [94]).

However, when a cysteine was introduced in the S3 consensus binding motif in IP of *B. subtilis* (S214Q215 → CG, Fig. 5B), a tri- to tetranuclear cluster conversion did not occur, and a [3Fe-4S] cluster, with somewhat altered EPR properties remained in the enzyme [95]. Thus we concluded that a cysteine residue in this position in *B. subtilis* IP is somehow sterically hindered from acting as a ligand for a tetranuclear cluster, whereas in *E. coli* QFR this is not the case.

As mentioned, two different genes homologous to *sdhB* are present in the genome of *Synechosystis*, and one in *M. jannashi*. The deduced amino acid sequence reveals cysteine motifs typical for two [4Fe-4S] clusters in the IP homologous polypeptides (Fig. 5B). Unfortunately there is no biochemical information about SQR/QFR in cyanobacteria or in *M. jannashi*, or experimental evidence indicating whether either of these polypeptides are components of SQR/QFR. There are examples of polypeptides that contain sequence motifs indicative of one [2Fe-2S] cluster followed by two [4Fe-4S] clusters that are subunits in unrelated enzyme complexes, for example RnfB encoded by the *Rb. capsulatus rnf*-operon [96,97]. However, this subunit notably lacks the peptide stretch between the S1/FR1 domain and the S2/FR2-S3/FR3 domain in IP which is present and conserved in IPs as shown in Fig. 5A and Fig. 6. Results from gene fusion experiments in *S. cerevisiae* have indicated that this particular segment in IP, located between the first and second cysteine motif (Fig. 5A), is important for assembly of SQR [98]. Recent sequence information from an other archaebacteria, *Sulfolobus acidocaldarius*, provides explanations to the nature of the *M. jannashi* IP which will be discussed towards the end of Section 9.

The [4Fe-4S] cluster generally has a very low potential and may not participate in electron transfer (Table 1). It can however not be excluded that the determined low potential is an artifact, due to anticooperative interaction between the redox centers [99]. It has also been suggested that the [4Fe-4S] cluster functions as a 'voyeur' cluster with the purpose of modifying the wavefunctions of its neighbor clusters [100]. The mutagenesis studies in *E. coli* QFR demonstrate the important structural role of this cluster. In the C148 → S and C151 → S mutants there was only a modest effect on the E_m of FR2 but a 50–70% decrease in fumarate reductase activity with $DMNH_2$, the substrate most resembling the physiological electron donor (Fig. 2) [91].

The [3Fe-4S] cluster clearly has an important structural role, as demonstrated early by reconstitution studies using *B. taurus* SQR. Purified, soluble FP + IP can be rebound to the anchor domain only when cluster S3 is kept intact [101–103].

In *B. subtilis* it was shown that the S3 signal, but not the S1 or S2 signals in wild type SQR, is extremely sensitive to methanol. Within less than two minutes in the presence of 1% methanol the lineshape and power saturation behavior of the S3 signal dramatically changed, whereas the midpoint potential of the cluster remained the same [95]. The effect of methanol-modification on S3 is seen in membrane bound as well as in isolated SQR. Interestingly,

ethanol does not have these effects. The modification of S3 was reversible, i.e., methanol could be washed off from the membranes and a near normal S3 signal was restored. The S3 center of the previously mentioned SQ → CG mutant was in contrast completely insensitive to methanol. The presence of 1% methanol does not inhibit succinate reductase activity in vitro, using PMS or Q_1 as electron acceptors [95]. The drastic effect of methanol, but not ethanol, on the properties of S3 can possibly be explained by that methanol fits into a small pocket, where it affects the S3 micro-environment. This pocket is then absent or perturbed in the mutant enzyme, which makes it completely insensitive to methanol.

There are also several indications that cluster S3/FR3 is close to a quinone binding site, and functions as electron donor/acceptor to/from quinone. This quinone-binding site will be further discussed in the section on quinone interaction. Generally, in Class 1 SQRs, interacting with ubiquinone, the E_m of S3 is higher than that in Class 2 QFRs or in Class 3 SQRs, interacting with low potential quinones (Table 1, Fig. 3).

It was observed early that extraction of ubiquinone from submitochondrial particles affected the redox midpoint potential of the so called HiPIP center, later demonstrated to be cluster S3 [104]. Addition of TTFA, (a SQR specific inhibitor, Fig. 2) to pigeon heart submitochondrial particles resulted in not only quenching of the hyperfine splitting originating from the semiquinone pair in SQR (discussed in detail later), increased the EPR signal amplitude of S3, and shifted its midpoint redox potential by about −50 mV [105]. A somewhat larger shift in E_m was observed using beef heart submitochondrial particles [106]. A mutation in *Ustilago maydis* SQR, conferring resistance to a similar specific inhibitor, carboxin was found to be a histidine to leucine substitution within the S3 ligation motif (Fig. 5B) [107].

The previously mentioned *E. coli* QFR mutant with two low potential [4Fe-4S] centers in the enzyme, retained 16–17% of wild type SQR and QFR activities and 30% of the wild type succinate: PMS reductase activity [90]. From these data one can not conclude whether the change in the FR3 structure or redox potential or both these changes together impair the enzyme function. In the *B. subtilis* IP the SQ → CG mutation was sufficiently close to the trinuclear cluster to alter the EPR properties of the S3 signal, but did not significantly change the midpoint potential of the cluster (−25 mV, [66,95]). The enzyme activity of the mutant SQR is about 40% as compared to wild type enzyme. Thus this mutant demonstrates that merely a wild type redox potential is not sufficient to retain function, and amino acid residues in the putative S3 ligation motif seem to be important for binding quinone (Fig. 5B).

Another mutation in the FR3 ligation motif, P159 → Q in *E. coli* QFR resulted in an oxygen sensitive enzyme that interestingly could be protected from succinate induced inactivation not only by anaerobiosis but also by separating FP/IP from the membrane anchor or by the presence of an inhibitor blocking electron transfer to/from quinone. It was suggested that the inactivation of the mutant enzyme occurred by production of hydroxyl radicals generated by a Fenton-type reaction. As the substrate-induced inactivation was demonstrated to require the presence of quinone it was concluded that FR3 must be intimately associated with a quinone binding site [108].

Interestingly, somewhat similar types of quinone binding sites, in close contact with a cubane iron-sulfur cluster, seem to be present in several membrane bound enzymes. Two *E. coli* DMSO reductase mutants, one with a mutation in the DmsC membrane anchor, and one with a mutation in the DmsB high potential (−50 mV) iron-sulfur cluster ligation motif, were analyzed independently and as a double-mutant. The DmsC mutant was defective in menaquinone oxidation whereas the mutation in DmsB caused a tetra- to tri- nuclear conversion of the [4Fe-4S] cluster. The EPR properties of the resulting [3Fe-4S] cluster were affected by the menaquinone-like inhibitor HQNO, an effect that was abolished in the double mutant, indicating a close functional link between the quinone binding site and the iron-sulfur cluster [109]. Furthermore, DMSO reductase is representative of a whole group of iron-sulfur-molybdoenzymes such as *E. coli* formate dehydrogenase, *E. coli* nitrate reductases and *W. succinogenes* polysulfide reductase. Another example is the [4Fe-4S] cluster N2 in NADH:ubiquinone oxidoreductase which was shown to be in close contact with a quinone binding site. The spin–spin interaction between N2, located close to the membrane surface, and a stabilized semiquinone, presumably located in a

membrane-spanning subunit, is EPR detectable as split peaks of the $g_{\|} = 2.05$ signal originating from cluster N2, with a coupling constant of 1.65 mT, indicating a distance of 8–11 Å between the interacting species [110].

9. The membrane anchor subunits

The membrane anchor consists of one larger or two smaller polypeptides. The understanding of the membrane anchor was confused for a long time due to the varying number of polypeptides and hemes in different species and preparations. The anchor polypeptides from different species show little apparent sequence similarity, and can for instance not be picked out from databases as easily as FP and IP sequences, using conventional comparison programs (cf. [31]). Recently, an increasing amount of experimental data as well as sequence information made it possible to clarify some of the confusion. As mentioned in the introduction, at our present state of knowledge there are four types of SQR/QFR membrane anchors (Table 1, Fig. 7A): Type A anchors have two polypeptides, with three transmembrane helices each, and contain two protoheme IX molecules (denoted heme b_{H} and heme b_{L}, indicating a high and a low redox midpoint potential respectively). Type B anchors have one polypeptide with five transmembrane helices and contain heme b_{H} and heme b_{L}. Type C anchors have the same polypeptide arrangement as Type A, but contains only one protoheme IX molecule, corresponding to heme b_{H} although its midpoint potential varies in different organisms. Type D membrane anchors have two polypeptides, with three transmembrane helices each, and are without heme. We have recently shown that the four different types of anchors have a common origin, and presented a three-dimensional structural model for the membrane-spanning part of the enzyme [17]. The structural model is schematically shown in Fig. 7A, and is based on sequence comparisons in combination with experimental data.

9.1. Transmembrane topology

Surface labeling experiments using mammalian and *B. subtilis* SQR in situ demonstrated that the anchor domain spans the membrane [111–113]. Nevertheless, even recent textbook illustrations of the respiratory chain often represent Complex II as membrane bound, but not membrane-spanning. Hydropathy analyses of available sequences reveal three hydrophobic stretches per polypeptide in the Type A, C and D membrane anchors whereas five such stretches are present in the Type B anchors. Their respective orientation in the membrane can be predicted using von Heijnes 'positive inside rule' [114]. By analyzing various *B. subtilis* truncated SdhC with alkaline phosphatase fused at the C-terminal ends [115] it was demonstrated that this anchor polypeptide contains five transmembrane segments as predicted, with the N-terminal exposed to the cytoplasm [116]. This transmembrane arrangement is further supported by effects of a *B. subtilis* SdhC mutation, G168 → D, (Fig. 7C). Residue 168 is located in a hydrophilic stretch facing the cytoplasm, and the mutation blocks binding of FP and IP to the membrane anchor, although the cytochrome is assembled, and shows normal spectroscopic properties [117].

9.2. Heme b_H and heme b_L axial ligands

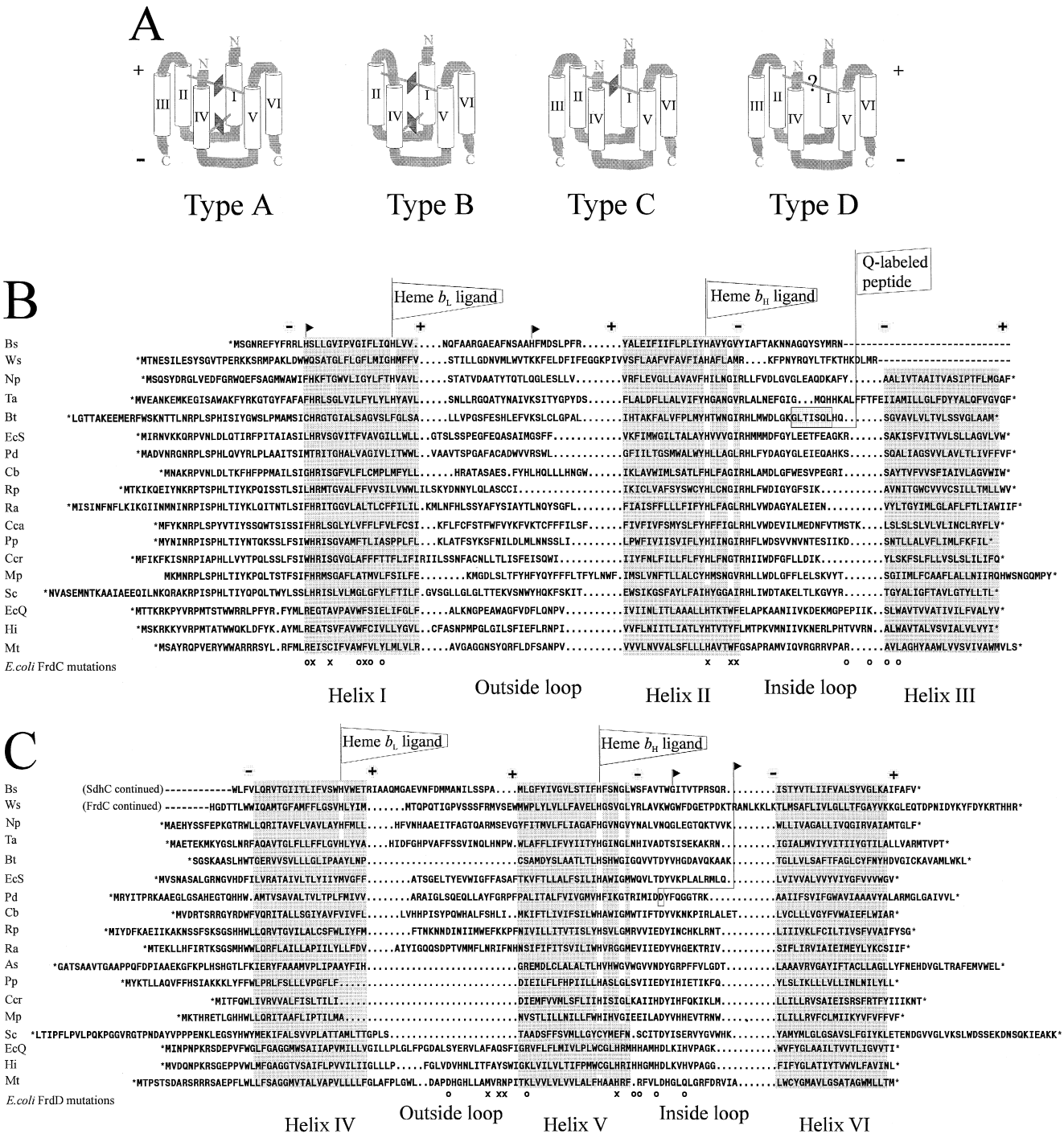
EPR and near infrared MCD spectroscopic information suggested bis-histidine axial ligation of both hemes in *B. subtilis* SQR, with a near perpendicular orientation of the imidazoles at each heme [118]. The same type of axial ligation was demonstrated for the single heme in *B. taurus* SQR [119]. The authors of the latter work further concluded that observed changes in EPR properties of the isolated cytochrome was due to perturbation of the orientation of the imidazole rings, rather than a change in axial ligation during isolation.

The six histidines present in the *B. subtilis* SdhC subunit were individually changed to tyrosine by site-directed mutagenesis. H47 could be excluded from consideration as a heme ligand, since this mutant assembles SQR with normal properties [120]. The H13 → Y mutant was shown to assemble a SQR containing two hemes, albeit with somewhat different properties [116]. The remaining four mutants did not assemble SQR. H70 and H155 mutant cytochromes were most severely affected and showed great instability whereas the H28 and H113 mutant cytochromes could be expressed in *E. coli* and isolated. They were found to contain one heme. Taken to-

gether with the topological data it could be concluded that H70 and H155, which are conserved in all heme containing anchors are the axial ligands to heme b_H and H28 and H113, which are conserved in Type A and B anchors, are axial ligands to heme b_L (Fig. 7, [116]).

9.3. Heme is important for assembly and stability

Heme has an important role for proper assembly of the membrane anchor, a prerequisite for binding of the FP and IP subunits. It was demonstrated already in 1980 that heme is essential for assembly of *B.*



subtilis SQR. In the absence of heme the apocytochrome is made and inserted in the membrane whereas FP and IP accumulate in the cytoplasm. The prosthetic groups seem to be incorporated before assembly. When heme becomes available, there is assembly of SQR from the preformed subunits [121]. *B. subtilis* mutants lacking one or both hemes in the membrane anchor can not assemble SQR [116,120]. Recently, the importance of heme for proper assembly was indicated also for a Type C anchor, that of *E. coli* SQR. Overexpression of the *sdhCDAB* operon in an *E. coli* mutant deficient in heme synthesis resulted in accumulation of FP/IP in the cytoplasm [122]. Furthermore, this study clearly demonstrated that although overexpression of SdhD alone results in an increase in the cytochrome *b* content of the *E. coli* membrane, this cytochrome showed other properties than that of the wild type cytochrome, and it was unable to bind FP and IP. Both SdhC and SdhD were required for heme insertion and assembly of a functional SQR, i.e., indicated that one ligand to heme is provided by each polypeptide. These data add further support to the current assignment of heme axial ligands in Type C anchors (Fig. 7).

9.4. A structural model for the membrane anchor

From the accumulated data it became clear that the Type A and B anchors are cytochromes with a novel type of heme arrangement, distinctly different from that of the diheme cytochrome *b* of the bc_1 and b_6f complexes. The heme axial ligands in SQRs/QFRs are distributed on four transmembrane helixes instead of on two. Based on the transmembrane topology and the position of the four heme axial ligands in *B. subtilis* SdhC only three different 3-dimensional folds remained possible for the core four helix bundle of that anchor. One is clearly favored and can be applied to membrane anchors of Type A, B, C and D (Fig. 7). The model was described in a very recent mini-review [17] and will thus be discussed more briefly here. Please bear in mind though, that Fig. 7A comprises a schematic illustration of the membrane anchors to clearly illustrate the topology of the transmembrane helices and the ligation of heme. In the actual protein the helices in the bundle are probably somewhat tilted, both relative to the membrane plane and to each other.

Primary sequence comparisons (Fig. 7B,C) in which the predicted heme axial ligands and/or hydrophobic stretches were first aligned and fixed turned out to be very informative compared to conventional alignment methods. First, it is clear that the Type B anchors arose from a fusion of SdhC and SdhD into one polypeptide, with concomitant loss of helix III. The shared sequence motifs of the two membrane anchor polypeptides and the order of their corresponding genes, *C* and *D*, in the prokaryotic *sdh* and *frd* operons, indicate that they have resulted from a gene duplication. An evolutionary early membrane anchor having the proposed three-dimensional structure, could have been composed of two identical subunits encoded by one gene. The heme ligation in such a homodimer would be the same as in the existing heterodimers. Additional functions may be better accomplished when some asymmetry of the domain is allowed, i.e., in a heterodimer. The polypeptide fusion in the Type B anchors may have provided an advantage, for example if the C and D polypeptides were very similar, and could combine in the form of CC or DD by mistake. Notably, and as mentioned before, overexpression of SdhD alone in

Fig. 7. A: proposed structure of the membrane anchor domain of SQR/QFR. This illustration is adapted from [17]. B: sequence comparison of SdhC anchor polypeptides. Positions of mutations in *E. coli* QFR [130] are indicated at the bottom row, and roughly rated as x for mutations resulting in QFR enzyme with 60% or less enzymatic activity compared to wild type, and o for mutations causing a smaller or no effect. Other mutations discussed in the text are indicated with flags. Larger flags denote heme axial ligands, and a peptide photo-labeled with a quinone analogue (see text). C: sequence comparison of SdhD polypeptides. Mutations and heme axial ligands are indicated as in (B). Note that for the Type B anchors the SdhC polypeptides continue from Figure 7B. Sequences are from: *B. subtilis* (Bs, P08064), *W. succinogenes* (Ws, P17413) *N. pharaonis* (Np, Y07709), *T. acidophilum*, (Ta, X70908), *B. taurus* (Bt, P35720, U50987), *E. coli* SQR (EcS, P10466, P10445), *P. denitrificans* (Pd, U31902), *C. burnettii*, (Cb, L33409), *Rickettsia prowazekii* (Rp, P41085), *R. americana*, (Ra, [36]), *C. caldarium*, (Cca, P48935, only SdhC), *A. suum*, (As, [164], only SdhD), *P. purpurea*, (Pp, [36]) *C. crispus*, (Ccr, P48934), *M. polymorpha*, (Mp, P35721, M68929) *S. cerevisiae* (Sc, P33421, P37298), *E. coli* QFR (EcQ, P03805, P03806), *H. influenzae*, (Hi, P44892, P44891) and *M. tuberculosis* (Mt, Q10762, Q10763).

E. coli resulted in production of a cytochrome *b* [122].

Helices I, II, IV and V, on which the histidine axial ligands to heme are distributed, form a four helix bundle in the model (Fig. 7A). Statistical analyses of the primary sequences for heme-containing anchors showed that hydrophobic residues are predominantly found on the surface of the bundle, facing the lipid, whereas several conserved residues are oriented towards the center of the bundle, particularly in the area surrounding heme b_H. Type C anchors show less sequence conservation in the lower part and contain more bulky amino acids facing the center, consistent with the absence of heme b_L [17].

The proposed structure is consistent with an important role of heme in assembly and stability of the membrane anchors. The position of helices III and VI can not be predicted with certainty, but we have chosen to position these helices in contact with the N-terminal ends, where they can act as stabilizing, 'hydrophobic zippers'. Two heme groups are always present in the Type B anchors lacking one of these helices. Type C and D anchors lacking one or both hemes may for reasons of stability retain helix III. Type A anchors, predicted to have the most stable configuration, have so far only been found in archaebacteria living under extreme conditions.

The hydrophilic stretches between helix II and III, and between helix V and VI show a high degree of sequence conservation compared to the other loops. The reason for this may be that they interact with the highly conserved FP/IP [17]. As mentioned, some SQR/QFR can be split into a soluble FP/IP domain and a membrane anchor domain. The soluble domain can be rebound to the anchor domain, provided that the [3Fe-4S] cluster is kept reduced and protected from oxidants. The strength of FP/IP binding to the membrane anchor varies among different species [14]. The polypeptide between helix II and III was labeled in a quinone photolabel study using bovine heart SQR ([123] Fig. 7B) as will be further discussed in Section 10. Interestingly, a ubiquinone analogue can protect mitochondrial SQR against splitting into FP/IP and anchor peptides [124,125]. Reconstitution of FP/IP to mammalian membrane anchor polypeptides could also be inhibited by DCCD, indicating that carboxyl groups are involved in the interaction [126]. Notably, aspartate and glutamate residues are often conserved in the hydrophilic loops between helices II and III and between helices V and VI (Fig. 7B,C).

9.5. The anchors without heme

The most well studied Type D membrane anchor is that of *E. coli* QFR, and it is also found in QFRs of related bacteria, i.e., *H. influenzae* and *P. vulgaris*. This type of membrane anchor, lacking heme, may also be present in yeast SQR but as discussed previously it is also possible that *S. cerevisiae* contains one SQR and one QFR. In spite the lack of heme it seems like the Type D anchors have a similar structure as the heme containing anchors. They bind FP and IP subunits which are well conserved in all SQRs/QFRs. Furthermore the fully conserved b_H histidine axial ligand in SdhC, is also conserved in the type D anchors. The corresponding histidine in SdhD seems to be replaced with a cysteine in Type D anchors. However, very recently the sequence of a *sdh* operon from *Mycobacterium tuberculosum*, a strictly aerobic bacterium, became available [Q10760, Q10761, Q10762, Q10763]. These SdhC and D are remarkably similar to those of *E. coli* FrdC and D but contains the histidine conserved in SdhD (Fig. 7B,C). It is not known whether this SQR contain heme, but if it does, it must be regarded as a very interesting 'missing link' between the heme-containing and the heme-less membrane anchors. FP/IP was isolated from *Mycobacterium phlei* as a complex with a cytochrome *b* component [127]. A group of functionally important amino acids pinpointed in a *E. coli* QFR mutagenesis study are located almost exclusively in the center of the four helix bundle, or in the peptide stretch between helix IV and V. These mutations are indicated in Fig. 7B,C and will be discussed in Section 10.

In *E. coli* FrdC the conserved histidine corresponding to the b_H axial ligand, H82, was subjected to extensive site-directed mutagenesis and changed to R [128,129], Q, E, L and Y [130]. The mutants assemble QFR but exhibit a latent instability observed as a detergent-induced dissociation of FP/IP from the anchor subunits. All mutants show some decrease in SQR and QFR activity but exhibit near wild type activities with the artificial electron acceptor/donors PMS and benzylviologen. The H82 → R

and H82 → Y mutations had the most drastic effect. The results can be interpreted such that H82 is part of a quinone binding site. However, in the light of the predicted common structure of all membrane anchors one may speculate whether the Type D anchors contain, for example a metal atom at this site. This would stabilize the structure and contribute to the architecture of a quinone binding site, just like heme apparently does in the other anchors.

A fifth type of 'membrane anchor', which is not similar to those in the previously known SQR/QFR may recently have been discovered. SQR purified from the obligate aerobic archaebacterium *S. acidocaldarius* was shown to lack heme, and contain FP (66 kDa), IP (31 kDa) and two smaller polypeptides (28 kDa and 12.8 kDa) [131]. This SQR was predicted to contain a Type D membrane anchor, although a 28 kDa SdhC is atypically large for a two polypeptide anchor. SQR in *S. acidocaldarius* is operating with caldariellaquinone which has a redox midpoint potential similar to that of ubiquinone (+100 mV at pH 6.5 [132]), i.e., this SQR is of Class 1. SQR was also isolated from *Sulfolobus* sp. Strain 7, a hyperthermophilic and acidophilic archaebacterium sometimes presumed to be *S. acidocaldarius.* This enzyme was comprised of 66, 37, 33 and 12 kDa subunits and lacked heme [133]. However, the *S. acidocaldarius sdhABCD* operon has recently been cloned, and the sequence determination is completed [Y09041]. SdhA and SdhB are similar to known FP/IP but the SdhC and D subunits are different, and exhibit very little hydrophobicity. The SQR is said to be loosely membrane bound. SdhC in addition contains an unusual accumulation of cysteines which are conserved in a subunit from heterodisulfide reductase from a methanogen. The SdhD may possibly contain some hydrophobic stretch. (S. Jansen, G. Schäfer and R. Moll, personal communication).

No genes for SQR/QFR membrane anchor subunits have been detected in *M. jannashi*, although the entire genome has been analysed [134]. In addition, the postulated IP subunit in *M. jannashi* contains a long C-terminal extension, also homologous to a subunit of heterodisulfide reductase (HdrB) in *M. thermoautotrophicum.* Taken together, these data indicate that the *M. jannashi* polypeptide is IP, fused to the *S. acidocaldarium* 'SdhC' equivalent polypeptide (Fig. 6). Thus, some archaebacteria contain a novel type of subunit composition for SQR/QFR in which FP and IP are conserved but the two additional polypeptides are unrelated to SdhC/FrdC and SdhD/FrdD. The 'SdhC' polypeptide is cysteine rich, but without transmembrane segments, and homologous to HdrB. Unfortunately, the function of HdrB in heterodisulfide reductase is not understood [83]. The fourth polypeptide, 'SdhD' may provide some kind of looser membrane anchoring.

9.6. Properties and functional role of heme

The role of heme in succinate:quinone oxidoreductases remains somewhat enigmatic.

It seems clear that heme has a role in assembly and structure of the membrane anchor, as demonstrated for *B. subtilis* and *E. coli* SQR. Heme b_H in most enzymes has a sufficiently high E_m to be fully succinate reducible (Table 1), but there are examples of heme b_H with extremely low midpoint redox potential, for example that in *B. taurus* [135]. Even so, *B. taurus* SQR preparations with a low heme content also show low enzyme activity, and semiquinone species are not detectable [136]. Furthermore, there may be examples of heme-less anchors among SQRs (i.e., that of yeast) as well as QFRs, although the previously mentioned *S. acidocaldarius* SQR seems to contain SdhC and D polypeptides unrelated to other anchors, and thus may operate by a different principle. It can be concluded that presence of heme is not obligatory for succinate oxidation per se.

Two transmembraneously arranged hemes in the membrane anchor is typical for Class 3 SQRs (Table 1), one with higher (heme b_H) and one with lower redox midpoint potential (heme b_L). One exception seems to be SQR from the facultative alkaliphilic *Bacillus* sp. strain YN-2000, which is reported to contain one heme with E_m −64 mV, and uses menaquinone as electron acceptor [137]. Intriguingly, this cytochrome was fully succinate reducible in spite of its rather low E_m. Another alkaliphile, *Bacillus firmus* OF4 seems to contain an SQR very similar to that of *B. subtilis*, exhibiting 50% reduction of the cytochrome *b* with succinate compared with dithionite [138]. When two hemes are present they can be discriminated based on their different optical and EPR properties [39,139], in addition to their different E_m values (Table 1). In *B. subtilis* the midpoint

potential of the two hemes are affected by the environment, i.e., they are +65 mV and −95 mV in purified SQR and +16 mV and −132 mV in the membrane bound enzyme [39]. The lower potentials are restored when the purified SQR is reconstituted into liposomes. (Hägerhäll and Konstantinov, unpublished results). At alkaline pH also heme b_L can be reduced by succinate (≈70% reduction at pH 9.5). The redox midpoint potential of heme b_H is pH independent in the interval pH6 to pH8. Above pH8 a slight decrease in E_m is seen. The midpoint potential of heme b_L seemingly shows a linear pH dependence of −30 mV/pH unit in the interval pH 6 to 9.5 (Smirnova, Hägerhäll and Hederstedt, unpublished). This corroborates a report by Cammack et al. [140] demonstrating only a weak pH dependence of mitochondrial heme b_H between pH 5.9 and 7.4.

The kinetics of heme reduction in isolated *W. succinogenes* QFR have been measured using the freeze-quench technique [79]. It was found that 40% of heme b_H is reduced by succinate within 100 ms ($k = 23\ s^{-1}$). Heme b_H was fully reduced by the water soluble, low potential quinone analogue $DMNH_2$ (Fig. 2) within 30 ms ($k = 155\ s^{-1}$) and reoxidized by fumarate at essentially the same rate. These rate constants show that electron transfer is at least as fast as the enzyme turnover, i.e., heme could be involved in electron transfer from/to quinone.

10. Quinone interaction

As already discussed, different organisms use different quinones as electron acceptors/donors to SQR/QFR (Table 1), Class 1 SQRs are using a quinone with a higher redox midpoint potential such as ubiquinone, whereas Class 2 QFRs and Class 3 SQRs are using a quinone with lower redox potential such as menaquinone. The structures of common quinones and quinone analogues are shown in Fig. 2. To be able to explore quinone–enzyme interactions a combination of inhibitors and mutants is useful, as demonstrated by the progress made on the bc_1-complex (Complex III, cf. [141,142]).

The quinone binding site(s) of SQR/QFR are localized to the membrane part of succinate:quinone oxidoreductase. The *B. taurus* SQR membrane anchor polypeptides were both photolabeled with [^{3}H]aryloazidoquinone derivatives [143]. Later on one peptide stretch, indicated in Fig. 7B, was singled out as quinone binding in a labeling study using the same enzyme [123].

10.1. Quinone specificity and inhibitor sensitivity

Purified *B. subtilis* SQR and *W. succinogenes* QFR were assayed in parallel and showed SQR and QFR activities with both naphto- and benzoquinones [57]. The enzymes exhibited little quinone specificity. Rather the redox potential of the quinone/quinol used determined the turnover rate. This phenomenon was seen both with water soluble and non-water-soluble quinones. Similar conclusions can be drawn from experiments with other SQRs/QFRs [138,144,145].

There are several inhibitors that interfere with quinone binding in SQR/QFR. The most well known are 2-thenoyltrifluoroacetone (TTFA), 3-methylcarboxin and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), (Fig. 2). Sensitivity to these inhibitors vary greatly and seemingly somewhat randomly among species and enzyme types (Table 1). This is in agreement with the poor sequence similarities in the areas of IP and the membrane anchor that have been indicated to be involved in quinone binding (Fig. 7B,C). Assuming that extensive variability can be tolerated at the quinone binding sites, but that the location of these sites on the protein is conserved, we can draw some conclusions about quinone binding sites by combining evidence obtained from various SQRs/QFRs.

10.2. The EPR detectable semiquinone radical pair (Q_s)

Since quinones are two electron carriers the SQR/QFR protein must be able to stabilize semiquinone species. The existence of a stable semiquinone pair in *B. taurus* SQR, denoted SQ_s, has been demonstrated with EPR spectroscopy based on a rapidly relaxing $g = 2.00$ semiquinone signal as well as low temperature detectable complex split signals arising from spin–spin interactions between two semiquinones [136,146,147]. From modeling of the dipolar coupling using a point charge approximation, the distance between the two semiquinones was estimated to be 7.7 Å [146]. Potentiometric analysis

of the signal showed two E_m's of +140 and +80 mV (at pH 7.4) for the ubiquinone to ubisemiquinone and ubisemiquinone to ubiquinol state, respectively, in submitochondrial particles [106]. These values correspond to a stability constant of approximately 10, and was an early strong indication for the existence of binding sites for the quinone species. (The stability constant of free ubisemiquinone in a hydrophobic milieu has been estimated to be 10^{-10} [148]. The two E_ms are +40 mV and +128 mV respectively, in isolated beef heart SQR [147] corresponding to a stability constant of 10^{-1}, whereas in Complex II plus III preparations the stability constant is intermediate to those observed in the two former cases. The SQ_s signal is not detected in enzyme preparations with a low heme content and not in isolated SQR unless quinone has been added to the preparation. Maximal radical concentration was observed when quinone was added in a 5:1 molar ratio to flavin [147]. Oriented multilayers of bovine heart mitochondrial membranes were used to show that the semiquinone pair most likely is ordered with the quinone–quinone vector perpendicular to the membrane plane [149]. Significantly, the Q_s semiquinone pair has only been observed in mammalian mitochondria SQR, in some green plant mitochondria [150] and in mitochondria from *Neurospora crassa* [151], but not in a wild type bacterial SQR/QFR. This has been envisaged such that an interacting semiquinone pair is being much more sensitive to perturbations than a single semiquinone. A modest shift of ~40 mV in the midpoint potential of one quinone relative to the other would cause almost complete lack of EPR signal. Also a modest decrease in the binding constants of the semiquinones would affect detection, since the observed signal intensity depends on the square of the semiquinone concentration.

10.3. A proximal quinone binding site

The mammalian SQR is sensitive to both TTFA and carboxins, two inhibitors that share some common structural features (Fig. 2). Both inhibitors were shown to interact with the same site, but carboxin with one order of magnitude lower K_i than TTFA [152]. These compounds inhibit PMS reductase activity to 50% and quinone reductase activity up to 95% [153]. These inhibitors block reoxidation of reduced cluster S3 with quinone but do not prevent S3 from being reduced by succinate [154]. Addition of TTFA to heart submitochondrial particles resulted not only in quenching of the hyperfine splitting originating from the semiquinone pair in SQR, but also increased the EPR signal amplitude of S3 and shifted its midpoint redox potential [105,106]. Furthermore, a mutation in *U. maydis*, conferring resistance to carboxin was located within the S3 ligation motif (Fig. 5B, [107]). The PMS reductase activity of soluble FP/IP preparations is not affected by these inhibitors, indicating that the membrane anchor, directly or indirectly, contributes to the inhibitor binding site [155]. A later study confirmed these observations and demonstrated carboxin binding to the membrane anchor subunits, but only in the presence of FP/IP. Irradiation of azidocarboxanilide-inhibited SQR generated labeled membrane anchor polypeptides [156]. The EPR and optical spectra of heme is sensitive to inhibitors which block electron transfer from S3 to quinone [135]. The presence of a ubiquinone analogue protected mitochondrial SQR against splitting into FP/IP and anchor peptides [125]. See [14] for a comparison of activities of soluble FP/IP and intact SQR. All these data together indicate that one quinone binding site is located close to the (negative) inside of the membrane, bordering both IP and the membrane anchor, in close contact with both iron-sulfur cluster S3 and heme b_H.

Notably, *A. suum* QFR shows low sensitivity to TTFA which was discussed in the light of its reverse function [48]. However, in *B. taurus* SQR the K_i for carboxin is very similar during succinate oxidation and fumarate reduction [41]. *E. coli* SQR, that contains the histidine implicated in carboxin interaction in *U. maydis* is not sensitive to carboxins [14], whereas the structurally and functionally similar *P. denitrificans* SQR is sensitive to this inhibitor [157]. A carboxin resistant *P. denitrificans* mutant has a mutation in SdhD in the loop connecting helix V and VI (D89 → G, Fig. 7C) further establishing the contribution of the membrane anchor part to this binding site. [158].

10.4. A distal quinone binding site

B. subtilis SQR and *E. coli* QFR are not sensitive to TTFA and carboxins but are sensitive to HQNO

(Fig. 2 [159,160]). In *B. subtilis* SQR HQNO blocks quinone reductase and quinol oxidase activity but not PMS reductase activity [39]. The *W. succinogenes* QFR, structurally very similar to *B. subtilis* SQR, is not HQNO sensitive [57]. In *B. subtilis* we have not detected a significant effect of HQNO on the EPR properties or the thermodynamic behavior of S3. Furthermore the previously mentioned *B. subtilis* SQ → CG IP mutant enzyme shows the same apparent K_i for HQNO as the wild type enzyme [95]. Binding of HQNO to *B. subtilis* SQR causes a shift in the cytochrome light absorption spectrum and in the E_m of heme b_L (about −60 mV), but has no effect on the E_m of heme b_H [160]. This indicates that the HQNO binding site in *B. subtilis* SQR is located in the vicinity of heme b_L, i.e., close to the (positive) outside of the membrane. In the isolated *B. subtilis* membrane anchor (without FP and IP bound) the heme b_H can be reduced by succinate in the presence of catalytic amounts of purified SQR and a low potential menaquinone analogue (DMN, Fig. 2). Cytochrome in the previously mentioned *B. subtilis* H28 → Y membrane anchor mutant, that lacks heme b_L but contains heme b_H, is not reducible under the same condition, in spite of a favorable redox potential of the remaining heme (+30–+50 mV, Hägerhäll and Hederstedt, unpublished data). Another *B subtilis* mutant, H13 → Y in SdhC is impaired in PMS and quinone reductase activity as well as in quinol oxidation. Residue H13 is located close to heme b_H in our structural model [116] and is conserved in all the SQR membrane anchors (Fig. 7B, [17]). The artificial electron acceptor PMS seems to accept electrons from the proximal, (analogous to the TTFA and carboxin-binding) site, and thus PMS activity is not affected by HQNO in *B. subtilis*. These observations suggest that in *B. subtilis* SQR two separate quinone binding sites are present. One site is close to the negative side of the membrane, (near heme b_H and S3), the other site, where HQNO mainly acts [160], is close to the positive side of the membrane (near heme b_L).

It should be noted that HQNO is not a specific SQR/QFR inhibitor. HQNO binds to the Q_i site of the bc_1 complex, i.e., the site where the stabilized semiquinone resides. Formate dehydrogenase and a number of quinol oxidizing enzymes, including QH_2-nitrate reductase, the *o*- and *d*-type ubiquinol oxidases [159] and DMSO reductase are HQNO sensitive. As mentioned in Section 8, a recent paper describes interaction of an engineered [3Fe-4S] cluster in DMSO reductase with HQNO [109], indicating the presence of a proximal HQNO binding site. The structure of HQNO resembles a semi(naphtho)quinone (Fig. 2). The apparent K_i for HQNO of *B. subtilis* SQR increases with increasing pH, indicating that the deprotonated inhibitor is less efficient [161]. Taken together, HQNO may preferentially bind to sites designed to have a high affinity for semiquinone, and thus even if HQNO binds to the distal quinone-binding site in *B. subtilis* SQR it may not necessarily bind to a geographically similar site in other enzymes, but rather to a functionally similar site.

In this context it should be noted that although a Q_s type semiquinone pair has not been detected in bacterial SQR/QFR we have detected an EPR signal indicative of a stabilized semiquinone in *E. coli* membranes containing the well characterized E29L QFR mutant. The signal is sensitive to HQNO and absent from membranes containing wild type QFR and membranes from the parent strain, which is deleted for QFR and SQR (Hägerhäll, Cecchini and Ohnishi, unpublished). Residue E29 (Fig. 7B) has been proposed to facilitate protonation/deprotonation of quinone [130] in analogy with a glutamate residue in the reaction center Q_B. Notably E29 is located at the position corresponding to residue H13 in *B. subtilis* SQR. The previously mentioned *B. subtilis* H13 → Y mutant does however not exhibit a similar EPR signal. Thus, it seems like HQNO binds to the opposite site in *E. coli* QFR versus in *B. subtilis* SQR, but to the analogous site in another quinol oxidizing enzyme, DMSO reductase. At this point we can however not exclude that inhibitor binding to one site causes destabilization of a semiquinone at the other site.

10.5. Where is Q_s located?

A comprehensive mutagenesis search for quinone binding sites was carried out in *E. coli* QFR. As a result Westenberg et al. [130] pinpointed a number of amino acid residues in the *E. coli* QFR membrane anchor, including the previously mentioned residue E29, that are important for enzyme activity. These residues are indicated in Fig. 7B,C, with '×' for

mutations affecting enzyme activity and 'o' for mutations with little or no such effects. The essential residues map to two different regions on the anchor (see also Fig. 7A), indicating the presence of two transmembraneously arranged quinone-binding sites. One site maps close to the (negative) inside of the membrane and another site close to the (positive) outside of the membrane, which assuming a conserved topological distribution of the quinone binding sites in SQR/QFR, correspond to the TTFA/carboxin binding site in mammalian and *P. denitrificans* SQR and the HQNO binding site in *B. subtilis* SQR respectively.

As mentioned in Section 9, the mitochondrial genome of some photosynthetic red algae, a zooflagellate, and the liverwort *M. polymorpha* carry genes similar to SQR anchor polypeptides [36,37,162]. These mitochondrial *sdhC* genes are similar to the nuclear and bacterial genes, but the corresponding *sdhD* genes from the red algae and the liverwort (but not from the zooflagellate) deviate from other SdhD polypeptides. The hydrophilic peptide stretch between helix IV and V, in *E. coli* QFR indicated to contain the distal quinone binding site, is absent from these postulated SdhDs. However, in plants it is not unusual to observe an ongoing gene-migration from mitochondria to the nucleus. Some genes present on the mitochondrial chromosomes are also simultaneously present in the nucleus, one or the other being the functional gene [163]. It was thus not clear whether these mitochondrial *sdhC* and *sdhD* encode functional proteins or if they were pseudo-genes. Very recent sequence information however reveals that the same peptide stretch is also absent from the functional *A. suum* SdhD (cybS) [164] and *B. taurus* SdhD (Shenoy and Yu, personal communication [U 50987]). Thus one member of the EPR detectable transmembrane semiquinone pair in *B. taurus* SQR can not originate from this site. In retrospect, the calculated distance between the two semiquinones is a bit short, even if the estimation can be stretched from 7.7Å to up to 15 Å, considering that S3 is located close to the membrane surface, and heme b_H is accommodated between S3 and heme b_L.

The Q_s semiquinone pair seems more likely to originate from a 'double occupancy' quinone site, similar to that recently demonstrated for the Q_o site in the bc_1-complex [165]. As discussed, the diheme Type A and B membrane anchors of SQR/QFR have a different structure and heme organization than the bc_1 complex, even if both cytochromes contain transmembraneously arranged heme b_H and heme b_L. Indeed, in the bc_1 complex the Q_o site is located toward the (positive) outside of the membrane, and catalyzes exclusively oxidation of quinol. It is however similar in the sense that it resides between an iron-sulfur cluster and a heme. The quinone pair at the Q_o site (denoted Q_{os} and Q_{ow}) are suggested to provide a conduit for the rapid movement of semiquinone away from the oxidizing side (in this case the Rieske cluster) to reduce heme b_L. This process supposedly provides directionality, minimizes the lifetime of the semiquinone and wasteful side reactions [165]. In SQR we can imagine an opposite purpose, i.e., movement of semiquinone away from the reducing (S3) side to prevent electron backflow.

In summary, accumulating evidence indicate that there are two transmembraneously arranged quinone binding sites in SQR/QFR. However, the distal site, localized towards the (positive) outside of the membrane, seems to be absent in several mitochondrial SQRs, notably also in beef heart SQR where the Q_s semiquinone pair is readily observed. It can thus be proposed that there is a proximal, Q_o-type quinone binding site localized towards the negative, inside of the membrane, in close contact with S3 and heme b_H (Fig. 8). Amino acid residues in the S3 ligation motif in IP and residues between helix II and III in SdhC

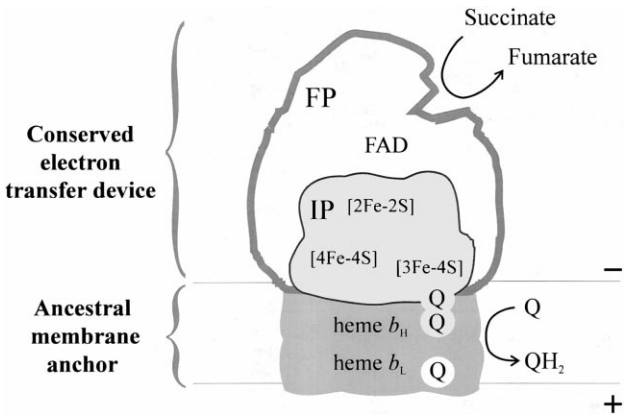


Fig. 8. Drawing to illustrate the components of a proposed ancestral succinate:quinone oxidoreductase enzyme. The indicated redox components are found to various extents in present day SQRs/QFRs.

contribute to this quinone binding site, and possibly also residues in the equivalent peptide stretch in SdhD, between helix V and VI. Two extensively conserved aspartate residues in these regions of SdhC and SdhD (Fig. 7B,C) may be of particular interest. This quinone binding site interacts with TTFA and carboxin in mammalian mitochondria and *P. denitrificans*, and accommodates two quinone molecules, giving rise to the Q_s semiquinone pair observed in mammalian SQR. PMS is proposed to accept electrons from this site or S3.

A second, distal quinone-binding site is localized towards the positive, outside of the membrane, close to heme b_L when present, and involve amino acid residues in the peptide stretch between helix IV and V. The absence of this stretch, and thus this quinone binding site in *B. taurus* SQR may in fact account for the possibility of observing the Q_s radical pair in this SQR, and not in bacterial enzymes such as *E. coli* QFR or *B. subtilis* SQR. It is at present rather ambiguous to predict which other organisms do or do not contain the distal site due to the low sequence similarity in quinone binding regions. One can also imagine such a site to be abolished by replacing amino acids rather than deleting the whole stretch. Future research will hopefully shed light on this issue.

11. Evolution of SQR/QFR

The similar primary sequence of FP and IP and the conserved structure of the membrane anchors of Type A, B, C and D in archaebacteria, eubacteria and eukaryotes demonstrate that succinate:quinone oxidoreductase was present in the last universal ancestor. The 'respiration early' hypothesis [20,21] postulates that the primordial enzyme does not have to be a fumarate reductase. It is tempting to assume that the reason for the current, seemingly unnecessary complexity of SQR/QFR may be that the original enzyme had a more complex function. The peripheral domain of the enzyme, FP/IP, comprising the dicarboxylate binding site and a set of redox groups providing the 'electric cord' in the electron transfer device has remained relatively constant over time. The membrane anchor, which probably originally was composed of two polypeptides and contained two transmembraneously arranged hemes (Type A), had key roles in the postulated, more complex function(s) of the enzyme. The Class 3 SQRs may in this respect more closely resemble the ancestral enzyme, whereas the enzymes of the two other classes represent degenerate, simplified forms. Among the respiratory chain complexes it seems like progenitors of bc_1 complexes and terminal oxidases, but not of NADH:quinone oxidoreductases (Complex I), were present in the last universal ancestor. Thus one can postulate two scenarios, or possibly a combination of the two.

(1) The original SQR enzyme was designed to carry out a thermodynamically unfavorable reaction, i.e., oxidation of succinate and reduction of a low potential quinone such as menaquinone or thermoplasmaquinone. Some organisms shifted to ubiquinone or other higher potential quinones, or to using the enzyme in the reverse direction. As a result intrinsic functions were simplified or abolished, but the original framework was maintained.

(2) The original enzyme was an energy coupling site, i.e., functioned as proton translocator. The enzyme may have retained this form in organisms lacking coupling site 1, but has in those organisms that acquired Complex I and thus sufficient proton pumping capacity, or in those organisms utilizing it as QFR, been allowed to degenerate to various degrees, loosing one or both of two transmembraneously arranged hemes, and/or loosing the distal quinone binding site. These possibilities will be further discussed in Section 12.

It is evident that larger enzyme assemblies can evolve from a combination of preexisting functional modules. For example, the *nuoE*, *nuoF* and *nuoG* genes, encoding the NADH-dehydrogenase subcomplex of Complex I, are always clustered in bacteria. In addition their deduced amino acid sequences show significant similarity to that of NAD^+ and $NADP^+$ reducing hydrogenases. Chloroplast Complex I, that prefers NADPH as a substrate, lacks the NuoG, E and F subunit analogues, and uses instead a ferredoxin-$NADP^+$ reductase module as a primary electron input device. The other components of the chloroplast enzyme complex are similar to mitochondrial and prokaryotic Complex I subunits. The NuoB, NuoC, NuoD, NuoI, NuoH and NuoL subunits of Complex I are homologous to *E. coli* formate hydro-

gen lyase, a non-energy coupled enzyme, thus representing another functional module of Complex I. The membrane-spanning NuoL, M and N subunits also show large similarity to each other, apparently a result from gene duplications of a primordial gene. More surprising is that NuoL, M and N also show remarkable sequence similarity to proposed Na^+/H^+ and K^+/H^+ antiporters. The SQR/QFR of the archaebacteria *S. acidocaldarium*, and *M. jannashi* have apparently not lost heme groups during the course of evolution, but acquired or maintained another type of anchor/accessory subunits combined with FP/IP. If this combination was present in the last universal ancestor it has seemingly been lost in all branches except some of the archae. Heterodisulfide reductase is an integral membrane protein that in some methanogens forms a functional complex with cytochrome *b* and hydrogenase or F_{420} dehydrogenase — an example of a proton pump working in a highly specialized environment [166]. Perhaps the atypical SQR is an alternative partner in such a supercomplex, providing reducing equivalents from another source.

12. Electron transport in SQR / QFR

A dual pathway model for the electron flow in cardiac SQR has previously been proposed, which includes a two step reduction of quinone at different sites [140,167]. In this model cluster S2 and the single heme (corresponding to heme b_H) constitute a 'low potential' pathway linking FAD/FAD^* and Q/Q^* whereas clusters S1 and S3 comprise a 'high potential' pathway linking $FAD^*/FADH_2$ and Q^*/QH_2. In this model there is no place for the second heme (corresponding to heme b_L). Also it does not explain how succinate oxidation with a low potential final electron acceptor can occur. Theoretically there seems to be no requirement for the division of the electron pathway into high and low potential arms [99].

As mentioned in the section on FP, FAD is the most likely first electron acceptor in SQR/QFR at succinate oxidation. FAD is a two electron carrier, in contrast to the other prosthetic groups in SQR/QFR which are one electron carriers. The FAD free radical state has a high stability constant, indicating that FAD performs the $n = 2$ to $n = 1$ conversion required to transfer electrons from succinate to the iron-sulfur clusters and vice versa within SQR/QFR. The E_m of cluster S2/FR2 is generally very low, indicating that it has a structural function, or that it merely facilitates electron transfer. The E_m of cluster S1/FR1 is high in Class 3 SQRs that operate with low potential quinones, of intermediate redox potential in Class 1 SQRs using high potential quinones as electron acceptors and low in Class 2 QFRs (Table 1). This correlation offers a clue to an understanding of the differences in these enzymes. *W. succinogenes* QFR and *B. subtilis* SQR have the same type of diheme membrane anchor, and both operate with menaquinone. Yet they catalyze the reverse enzymatic reactions in vivo, and are representative of a Class 2 and Class 3 enzyme respectively. As is apparent from the scheme in Fig. 3, the main difference between the three classes is the midpoint potential of S1/FR1, which is high in the Class 3 enzyme. The high potential center S1 seems to provide an essential, energetically favorable step, needed to pull the electrons from FAD into the SQR when the final electron acceptor has a low E_m. Cluster S3 generally has a lower potential in enzymes operating with low potential quinones, regardless if they are SQRs or QFRs (Table 1). The indications that S3/FR3 is close to a quinone-binding site make it possible to conclude that the electron transfer sequence within SQR/QFR most likely proceeds as:

succinate/fumarate ↔ FAD ↔ S1/FR1 ↔ (S2/FR2) ↔ S3/FR3 ↔ quinone/quinol (see also Fig. 3).

The involvement of heme in electron transfer in SQR/QFR is unclear, as discussed in Section 9. The presence of two transmembraneously arranged hemes however intuitively implies that they should have a role in transmembrane electron transfer, and at least primarily not be present merely to stabilize the anchor. Considering the topological arrangement of heme b_H and heme b_L, located on top of each other but ligated to different transmembrane helices (Fig. 7A), one may speculate that this organization has a functional importance. The heme–heme negative cooperativity (for hemes in the oxidized state) in marquettes was reported to be 6.82–7.87 kJ/mol [168]. The heme groups may thus electrostatically attract each other when one heme is reduced and the other is

oxidized. The heme–heme attraction could possibly be accompanied by a relative movement of the transmembrane helices in the four helix bundle (see Fig. 7A). The helix pairs (I: IV and II: V) in the four helix bundle would move along with the hemes they are ligating. Such movement would provide an explanation to why succinate reduced *B. subtilis* SQR is more unstable than oxidized or fully reduced enzyme.

It seems like a majority of the Class 1 SQRs retain only one heme, heme b_H. If the topological arrangement of hemes discussed above has a functional implication this feature is concomitantly lost. Furthermore, at least in some cases the distal quinone binding site seems to be absent, and the thermodynamic behavior of quinones at the proximal site significantly altered. It seems plausible that Class 1 SQR can catalyze succinate oxidation and ubiquinone reduction using the previously drawn electron transfer scheme, without any additional accessories (Fig. 3). The single heme can be required to maintain the architecture of the intricate proximal quinone binding site, which in spite of altered thermodynamic properties of the quinone pair may still aid in preventing electron back flow, or due to these altered properties actually regulate such flow.

The QFRs (Class 2) contain zero *(E. coli)*, one *(A. suum)* or two *(W. succinogenes)* hemes, and one *(A. suum)* or two *(E. coli)* quinone binding sites. This suggest that QFRs were invented more than once during the course of evolution, and that they may not all necessarily work with identical mechanisms. *W. succinogenes* QFR contains a Type B anchor, and may thus be trapped with hemes for stability purpose, as previously discussed. The parasite mitochondrial QFRs most likely evolved from a mitochondrial type SQR, thus lacking the distal quinone binding site. Sequence comparisons of FP also favor such a development [64]. The *E. coli* type heme-less QFR evolved independently. Also menaquinol or rhodoquinol oxidation and fumarate reduction is plausible using only the electron transfer scheme above. However, some QFRs maintain two quinone binding sites. Perhaps by rather oxidizing quinol at the distal quinone binding site, protons can be deposited in a favorable location, whereas the quinone(s) at the proximal site can function merely to facilitate electron transfer from the distal site to FR3.

At our present stage of knowledge the Class 3 SQRs seem in most cases to have a 'full set' of components, i.e., two transmembraneously arranged hemes and the proximal and the distal quinone binding site as illustrated in Fig. 8.

A previously proposed functional model for the two quinone binding sites was based on the photosynthetic reaction center. The two quinone binding sites pinpointed by the *E. coli* QFR mutagenesis study were envisaged such that the proximal quinone binding site corresponded to Q_A, which is the primary acceptor of electrons from the iron-sulfur cluster and passes electrons one by one to the secondary quinone at the distal quinone binding site, corresponding to Q_B. Only this latter quinone exchanges with the quinone pool [130]. The reaction centers do however not contain transmembraneously arranged quinone binding sites. A quinone permanently occupies Q_A and mediates the two electron transfer, via an iron atom, to another quinone that is reduced, and exchanged at Q_B. This arrangement is suggested to protect the reaction center from backward electron flow, that would result if electrons reach an empty quinone binding site [169]. In this respect, a catabolic enzyme have more possibilities to control electron influx than a photosynthetic enzyme.

Quinone/quinol in the electron transfer scheme correspond to the proximal, Q_o-type quinone binding site, which presumably is present in all SQRs/QFRs. If we imagine that in analogy with in the bc_1-complex, the distal quinone binding site is of Q_i-type, i.e., can stabilize a semiquinone, we seemingly have an inverted bc_1 complex with respect to quinone-binding sites, but not with respect to the orientation of heme b_H and heme b_L. The postulated Q_i-type site in fact agrees with the observations that the Q_i site of the bc_1 complex interacts with HQNO, just as the distal quinone binding site in a Class 3 SQR (i.e., that of *B. subtilis*). We can then proceed and envisage an inverted 'Q cycle' with a proximal Q_o type site for the reduction of two quinones and a distal Q_i type site for the oxidation of one quinol during one catalytic turnover. This concept is not new, and was recently proposed by Dutton and colleagues, (manuscript in preparation) as bases for a model aiming to explain the high proton/electron stoichiometry of Complex I. In case of SQR we do not have to be concerned about increasing the proton/electron stoichiometry, in fact it would be an

unexpected surprise to translocate one proton per electron using this enzyme.

In the bc_1 complex the first step of catalytic oxidation of quinol at the Q_o site results in a highly reactive, thermodynamically unstable ubisemiquinone, that provides the driving force for electrogenic electron transfer across the membrane. This semiquinone becomes oxidized by another quinone molecule, bound at the Q_i site located towards the positive side of the membrane barrier, via two transmembraneously arranged hemes *b* (b_L and b_H). Quinone at the Q_i site serves as a collector of two single electrons from heme b_H before returning a quinol to the membrane pool. The quinone pair at the Q_o site (denoted Q_{os} and Q_{ow}) are, as mentioned, suggested to facilitate rapid movement of semiquinone away from the oxidizing side (the Rieske cluster) to reduce heme b_L. This process provides directionality, minimize the lifetime of the semiquinone and wasteful side reactions [165]. In SQR we can imagine the opposite purpose of the quinone pair, i.e., movement of semiquinone away from the reducing (S3) side to prevent electron backflow. The oxidation of quinol at the distal site would in turn be driven by the highly reactive, thermodynamically unstable semiquinone created at the proximal site, and the electron transfer facilitated by the transmembraneously arranged hemes. We do not know if any of the present day SQR functions as a coupling site, but it is quite feasible that an ancestral enzyme could have had such a function. It is possible that proton pumping abilities of present day SQRs have simply been overlooked in bacterial systems, due to difficulties of making coupled membrane particles, and the convincing negative results with the mitochondrial enzymes.

As to present day Class 3 SQRs the proposed 'inverted Q-cycle' mechanism as such could facilitate reduction of low potential quinones by succinate, since a second reductant participates in the catalytic turnover. However this mechanism does not quite comply with experimental observations. First, there is no apparent reason why this mechanism should not be able to operate in vitro as well. Furthermore, the enzyme activity would be expected to be lower in the presence of $\Delta\mu_{H^+}$, not higher. As mentioned in Section 6, the succinate to oxygen respiratory activity of *B. subtilis* is 13 times lower in membranes compared to in intact bacteria and 90% of the respiratory activity of intact bacteria is lost upon addition of a protonophore [56]. These findings rather support an arrangement of quinone binding sites as in the previously mentioned Q_A/Q_B type model in the sense that transmembrane electron transfer would be facilitated by $\Delta\mu_{H^+}$. With the 'Q_A' site exchanged for a Q_o-type site, electron backflow could be more efficiently prevented. However, this scheme is less attractive since SQR would parasite on the proton gradient built up by other enzymes. During certain growth conditions *B. subtilis* membrane contains large amounts of SQR but no detectable bc_1 complex or Complex I, that could contribute to the buildup of a proton gradient. Overexpression of SQR does also not impair growth.

One possibility would be to propose a dual function for Class 3 SQRs — a slow but energy coupled reaction in the absence of $\Delta\mu_{H^+}$ and a fast reaction utilizing $\Delta\mu_{H^+}$ when present. The suggested structural flexibility of the membrane anchor could have a role in switching between these two functions, i.e., making both or only the distal quinone binding site able to exchange with pool quinone. This hypothesis is highly speculative but can and should be experimentally tested. I hope that both this and other conjectures as well as facts presented in this review will stimulate thinking and be incentives for the design of novel experiments. It is clear that we are far from having any clear documentation on the functional mechanism of Class 3 SQRs and much future work is needed to understand the functional mechanism of all succinate:quinone oxidoreductases.

Acknowledgements

I dedicate this review to Dr. Vladimir D. Sled' who was killed on his way home from the laboratory less than a month after the manuscript was submitted for publication. His short life was devoted to science, and a large portion thereof to bioenergetics. I am obliged to Ms. Sudha Shenoy and Dr. Linda Yu for providing unpublished sequence information, Dr. Kiyoshi Kita for a manuscript and Prof. Günter Schäfer for communicating results prior to publication. Some of the unpublished experimental work cited in this review done in the laboratory of Tomoko Ohnishi was supported by NSF grant MCB-9418694.

I am also grateful to Dr. Sergei Vinogradov for invaluable computer assistance, to Dr. Lars Hederstedt for reading the manuscript, and to Dr. Vladimir Sled' for reading and assisting in the preparation of the manuscript, and for engaging in many helpful discussions throughout this work.

References

[1] T. Thunberg, Scand. Arch. Physiol. 22 (1909) 430.
[2] Kröger, A. (1980) in Diversity of bacterial respiratory systems (Knowles, C.J., Ed.), Vol. 2, pp. 1–17, CRC press, Boca Raton.
[3] L. Hederstedt, L. Rutberg, Microbiol. Rev. 45 (1981) 542–555.
[4] Ohnishi, T. and Salerno, J.C. (1982) in Iron-sulfur proteins, Vol. IV (Spiro, T.G., Ed.), pp. 285–327, Wiley, New York.
[5] H. Beinert, S.P. Albracht, Biochim. Biophys. Acta 683 (1982) 245–277.
[6] T.P. Singer, M.K. Johnson, FEBS Lett. 190 (1985) 189–198.
[7] Y. Hatefi, Ann. Rev. Biochem. 54 (1985) 1015–1069.
[8] S.T. Cole, C. Condon, B.D. Lemire, J.H. Weiner, Biochim. Biophys. Acta 811 (1985) 381–403.
[9] A.D. Vinogradov, Biokhimiia 51 (1986) 1944–1973.
[10] Ohnishi, T. (1987) in Current Topics in Bioenergetics (Lee, C.P., Ed.), pp. 37–65, Academic Press, New York.
[11] R.D. Jaramillo, J.E. Escamilla, Rev. Latinoam. Microbiol. 32 (1990) 229–248.
[12] Kita, K., Takamiya, S., Furushima, R., Villagra, E., Wang, H., Akoi, T. and Oya, H. (1991) in Mitochondrial Encephalomyopathies (Sato, T. and Dimauro, S., Eds.), pp. 21, Raven, New York.
[13] A. Kröger, V. Geisler, E. Lemma, F. Theis, R. Lenger, Arch. Microbiol. 158 (1992) 311–314.
[14] Ackrell, B.A.C., Johnson, M.K., Gunsalus, R.P. and Ceccini, G. (1992) in Chemistry and Biochemistry of Flavoenzymes (Müller, F., Ed.), Vol. III, pp. 229–297, CRC Press, Boca Raton, Florida.
[15] Hederstedt, L. and Ohnishi, T. (1992) in Molecular Mechanisms in Bioenergetics (Ernster, L., Ed.), Elsevier, Amsterdam.
[16] J.J. Van Hellemond, A.G.M. Tielens, Biochem. J. 304 (1994) 321–331.
[17] C. Hägerhäll, L. Hederstedt, FEBS Lett. 389 (1996) 25–31.
[18] T. Ohnishi, J. Bioenerg. Biomembr. 25 (1993) 325–329.
[19] T. Yagi, J. Bioenerg. Biomembr. 23 (1991) 211–225.
[20] J. Castresana, M. Saraste, TIBS 20 (1995) 443–448.
[21] G. Schäfer, W. Purschke, C.L. Schmidt, FEMS Microbiol. Rev. 18 (1996) 173–188.
[22] S.T. Cole, Eur. J. Biochem. 122 (1982) 479–484.
[23] S.T. Cole, T. Grundström, B. Jaurin, J.J. Robinson, J.H. Weiner, Eur. J. Biochem. 126 (1982) 211–216.
[24] M.G. Darlison, J.R. Guest, Biochem. J. 223 (1984) 507–517.
[25] D. Wood, M.G. Darlison, R.J. Wilde, J.R. Guest, Biochem. J. 222 (1984) 519–534.
[26] R.A. Heinzen, Y.-Y. Mo, S.J. Robertson, L.P. Mallavia, Gene 155 (1995) 27–34.
[27] K. Magnusson, M.K. Philips, J.R. Guest, L. Rutberg, J. Bacteriol. 166 (1986) 1067–1071.
[28] M.K. Phillips, L. Hederstedt, S. Hasnain, L. Rutberg, J.R. Guest, J. Bacteriol. 169 (1987) 864–873.
[29] C. Körtner, F. Lauterbach, D. Tripier, G. Unden, A. Kröger, Mol. Microbiol. 4 (1990) 855–860.
[30] F. Lauterbach, C. Körtner, S.P. Albracht, G. Unden, A. Kröger, Arch. Microbiol. 154 (1990) 386–393.
[31] M. Bach, H. Reilander, P. Gärtner, F. Lottspeich, H. Michel, Biochim. Biophys. Acta 1174 (1993) 103–107.
[32] F.G. Oostveen, H.C. Au, P.J. Meijer, I.E. Scheffler, J. Biol. Chem. 270 (1995) 26104–26108.
[33] B. Daignan-Fornier, M. Valens, B.D. Lemire, M. Bolotin-Fukuhara, J. Biol. Chem. 269 (1994) 15469–15472.
[34] A. Lombardo, K. Carine, I.E. Scheffler, J. Biol. Chem. 265 (1990) 10419–10423.
[35] K.M. Robinson, B.D. Lemire, J. Biol. Chem. 267 (1992) 10101–10107.
[36] G. Burger, B.F. Lang, M. Reith, M.W. Gray, Proc. Natl. Acad. Sci. USA 93 (1996) 2328–2332.
[37] S. Viehmann, O. Richard, C. Boyen, K. Zetsche, Curr. Genet. 29 (1996) 199–201.
[38] V.G. Grivennikova, A.D. Vinogradov, Biochim. Biophys. Acta 682 (1982) 491–495.
[39] C. Hägerhäll, R. Aasa, C. von Wachenfeldt, L. Hederstedt, Biochemistry 31 (1992) 7411–7421.
[40] A.B. Kotlyar, M. Gutman, B.A. Ackrell, Biochem. Biophys. Res. Commun. 186 (1992) 1656–1662.
[41] V.G. Grivennikova, E.V. Gavrikova, A.A. Timoshin, A.D. Vinogradov, Biochim. Biophys. Acta 1140 (1993) 282–292.
[42] A. Sucheta, B.A. Ackrell, B. Cochran, F.A. Armstrong, Nature 356 (1992) 361–362.
[43] A. Sucheta, R. Cammack, J. Weiner, F.A. Armstrong, Biochemistry 32 (1993) 5455–5465.
[44] J. Hirst, A. Sucheta, B.A.C. Ackrell, F.A. Armstrong, J. Am. Chem. Soc. 118 (1996) 5031–5038.
[45] B.A.C. Ackrell, F.A. Armstrong, B. Cochran, A. Sucheta, T. Yu, FEBS Lett. 326 (1993) 92–94.
[46] J.J. Van Hellemond, M. Klockiewicz, C.P.H. Gaasenbeek, M.H. Roos, A.G.M. Tielens, J. Biol. Chem. 270 (1995) 31065–31070.
[47] E. Samain, D.S. Patil, D.V. DerVartanian, G. Albagnac, J. LeGall, FEBS Lett. 216 (1987) 140–144.
[48] K. Kita, S. Takamiya, R. Furushima, Y.C. Ma, H. Suzuki, T. Ozawa, H. Oya, Biochim. Biophys. Acta 935 (1988) 130–140.
[49] F. Saruta, T. Kuramochi, K. Nakamura, S. Takamiya, Y. Yu, T. Aoki, K. Sekimizu, S. Kojima, K. Kita, J. Biol. Chem. 270 (1995) 928–932.

[50] M.H. Roos, A.G.M. Tielens, Mol. Biochem. Parasitol. 66 (1994) 273–281.
[51] T. Erabi, T. Higuti, T. Kakuno, J. Yamashita, M. Tanaka, T. Horio, J. Biochem. 78 (1975) 795–801.
[52] C.A. Hirsch, M. Rasminsky, B.D. Davies, E.C.C. Lin, J. Biol. Chem. 238 (1963) 3770–3774.
[53] B.J. Wallace, I.G. Young, Biochim. Biophys. Acta 461 (1977) 84–100.
[54] C. Condon, J.H. Weiner, Mol. Microbiol. 2 (1988) 43–52.
[55] J. Schirawski, G. Unden, Arch. Microbiol. 163 (1995) 148–154.
[56] E. Lemma, G. Unden, A. Kröger, Arch. Microbiol. 155 (1990) 62–67.
[57] E. Lemma, C. Hägerhäll, V. Geisler, U. Brandt, G. von Jagow, A. Kröger, Biochim. Biophys. Acta 1059 (1991) 281–285.
[58] B.L. Bullis, B.D. Lemire, J. Biol. Chem. 269 (1994) 6543–6549.
[59] T. Bourgeron, P. Rustin, D. Chretien, M. Birch-Machin, M. Bourgeois, E. Viegas-Pequignot, A. Munnich, A. Roetig, Nature Genet. 11 (1995) 144–149.
[60] T.P. Singer, E.B. Kearney, W.C. Kenney, Enzymol. Rel. Areas Mol. Biol. 37 (1973) 189–27260.
[61] M.V. Panchenko, A.D. Vinogradov, FEBS Lett. 286 (1991) 76–78.
[62] L. Hederstedt, L.-O. Hedén, Biochem. J. 260 (1989) 491–497.
[63] I. Schröder, R.P. Gunsalus, B.A. Ackrell, B. Cochran, G. Cecchini, J. Biol. Chem. 266 (1991) 13572–13579.
[64] T. Kuramochi, H. Hirawake, S. Kojima, S. Takamiya, R. Furushima, T. Aoki, R. Komuniecki, K. Kita, Mol. Biochem. Parasitol. 68 (1994) 177–187.
[65] A.B. Kotlyar, A.D. Vinogradov, Biochemistry International 8 (1984) 545–552.
[66] J.J. Maguire, K. Magnusson, L. Hederstedt, Biochemistry 25 (1986) 5202–5208.
[67] S.B. Vik, Y. Hatefi, Proc. Natl. Acad. Sci. USA 78 (1981) 6749–6753.
[68] W.H. Walker, T.P. Singer, J. Biol. Chem. 245 (1970) 4224–4225.
[69] L. Hederstedt, Eur. J. Biochem. 132 (1983) 589–593.
[70] L. Hederstedt, T. Bergman, H. Jörnvall, FEBS Lett. 213 (1987) 385–390.
[71] K.M. Robinson, B.D. Lemire, J. Biol. Chem. 271 (1996) 4055–4060.
[72] K.M. Robinson, B.D. Lemire, J. Biol. Chem. 271 (1996) 4061–4067.
[73] S.H. He, D.V. DerVartanian, J. LeGall, Biochem. Biophys. Res. Commun. 135 (1986) 1000–1007.
[74] M. Blaut, K. Whittaker, A. Valdovinos, B.A. Ackrell, R.P. Gunsalus, G. Cecchini, J. Biol. Chem. 264 (1989) 13599–13604.
[75] T. Ohnishi, T.E. King, J.C. Salerno, H. Blum, J.R. Bowyer, T. Maida, J. Biol. Chem. 256 (1981) 5577–5582.
[76] B.A. Ackrell, B. Cochran, G. Cecchini, Arch. Biochem. Biophys. 268 (1989) 26–34.
[77] R.K. Thauer, K. Jungermann, K. Decker, Bacteriol. Rev. 41 (1977) 100–180.
[78] H. Beinert, B.A. Ackrell, E.B. Kearney, T.P. Singer, Eur. J. Biochem. 54 (1975) 185–194.
[79] G. Unden, S.P.J. Albracht, A. Kröger, Biochim. Biophys. Acta 767 (1984) 460–469.
[80] B.A. Ackrell, E.B. Kearney, D. Edmondson, J. Biol. Chem. 250 (1975) 7114–7119.
[81] A. Aevarsson, L. Hederstedt, FEBS Lett. 232 (1988) 298–302.
[82] M.K. Johnson, A.T. Kowal, J.E. Morningstar, M.E. Oliver, K. Whittaker, R.P. Gunsalus, B.A. Ackrell, G. Cecchini, J. Biol. Chem. 263 (1988) 14732–14738.
[83] R. Hedderich, J. Koch, D. Linder, R.K. Thauer, Eur. J. Biochem. 225 (1994) 253–261.
[84] Johnson, M.K. (1994) in Encyclopedia of Inorganic Chemistry, Vol. 4 (King, R.B., Ed.), Wiley, U.K., pp. 1896–1915.
[85] H.M. Holden, B.L. Jacobson, J.K. Hurley, G. Tollin, B.-H. Oh, L. Skjeldal, Y.K. Chae, H. Cheng, B. Xia, J.L. Markley, J. Bioenerg. Biomembr. 26 (1994) 67–88.
[86] R.J. Gurbiel, D.E. Robertson, F. Daldal, T. Ohnishi, B.M. Hoffman, Biochemistry 30 (1991) 11579–11584.
[87] T.A. Link, S. Iwata, Biochim. Biophys. Acta 1275 (1996) 54–60.
[88] M.T. Werth, G. Cecchini, A. Manodori, B.A. Ackrell, I. Schröder, R.P. Gunsalus, M.K. Johnson, Proc. Natl. Acad. Sci. USA 87 (1990) 8965–8969.
[89] M.T. Werth, H. Sices, G. Cecchini, I. Schröder, S. Lasage, R.P. Gunsalus, M.K. Johnson, FEBS Lett. 299 (1992) 1–4.
[90] A. Manodori, G. Cecchini, I. Schröder, R.P. Gunsalus, M.T. Werth, M.K. Johnson, Biochemistry 31 (1992) 2703–2712.
[91] A.T. Kowal, M.T. Werth, A. Manodori, G. Cecchini, I. Schröder, R.P. Gunsalus, M.K. Johnson, Biochemistry 34 (1995) 12284–12293.
[92] R.A. Rothery, J.H. Weiner, Biochemistry 30 (1991) 8296–8305.
[93] R.A. Rothery, J.H. Weiner, Biochemistry 32 (1993) 5855–5861.
[94] E.T. Adman, L.C. Siefker, L.H. Jensen, J. Biol. Chem. 251 (1976) 3801–3806.
[95] C. Hägerhäll, V. Sled, L. Hederstedt, T. Ohnishi, Biochim. Biophys. Acta 1229 (1995) 356–362.
[96] K. Saeki, K. Tokuda, T. Fujiwara, H. Matsubara, Plant Cell Physiol. 34 (1993) 185–199.
[97] M. Schmehl, A. Jahn, A. Meyer zu Vilsendorf, S. Hennecke, B. Masepohl, M. Schuppler, M. Marxer, J. Oelze, W. Klipp, Mol. Gen. Genet. 241 (1993) 602–615.
[98] M. Saghbini, P.L.E. Broomfield, I.E. Scheffler, Biochemistry 33 (1994) 159–165.
[99] J.C. Salerno, Biochem. Soc. Trans. 19 (1991) 599–605.
[100] Salerno, J.C. and Yan, X. (1987) in Cytochrome Systems

Molecular Biology and Bioenergetics (Papa, S., Chance, B. and Ernster, L., Eds.), pp. 467–471, Plenum Press, New York.
[101] M.L. Baginsky, Y. Hatefi, J. Biol. Chem. 244 (1969) 5313–5319.
[102] T. Ohnishi, J. Lim, D.B. Winter, T.E. King, J. Biol. Chem. 251 (1976) 2105–2109.
[103] C.A. Yu, L. Yu, T.E. King, Biochem. Biophys. Res.Commun. 79 (1977) 939–946.
[104] R.L. Lester, S. Fleischer, Arch. Biochem. Biophys. 80 (1959) 470–473.
[105] W.J. Ingledew, T. Ohnishi, Biochem. J. 164 (1977) 617–620.
[106] J.C. Salerno, T. Ohnishi, Biochem. J. 192 (1980) 769–781.
[107] P.L.E. Broomfield, J.A. Hargreaves, Curr. Genet. 22 (1992) 117–121.
[108] G. Cecchini, H. Sices, I. Schröder, R.P. Gunsalus, J. Bacteriol. 177 (1995) 4587–4592.
[109] R.A. Rothery, J.H. Weiner, Biochemistry 35 (1996) 3247–3257.
[110] A.D. Vinogradov, V.D. Sled, D.S. Burbaev, V.G. Grivennikova, I.A. Moroz, T. Ohnishi, FEBS Lett. 370 (1995) 83–87.
[111] A. Merli, R.A. Capaldi, B.A. Ackrell, E.B. Kearney, Biochemistry 18 (1979) 1393–1400.
[112] L. Yu, Y.Y. Wei, S. Usui, C.A. Yu, J. Biol. Chem. 267 (1992) 24508–24515.
[113] L. Hederstedt, L. Rutberg, J. Bacteriol. 153 (1983) 57–65.
[114] G. von Heijne, EMBO J. 5 (1986) 3021–3027.
[115] C. Manoil, J.J. Mekalanos, J. Beckwith, J. Bacteriol. 172 (1990) 515–518.
[116] C. Hägerhäll, H. Fridén, R. Aasa, L. Hederstedt, Biochemistry 34 (1995) 11080–11089.
[117] H. Friden, L. Rutberg, K. Magnusson, L. Hederstedt, Eur. J. Biochem. 168 (1987) 695–701.
[118] H. Friden, M.R. Cheesman, L. Hederstedt, K.K. Andersson, A.J. Thomson, Biochim. Biophys. Acta 1041 (1990) 207–215.
[119] B.R. Crouse, C.-A. Yu, L. Yu, M.K. Johnson, FEBS Lett. 367 (1995) 1–4.
[120] H. Friden, L. Hederstedt, Mol. Microbiol. 4 (1990) 1045–1056.
[121] L. Hederstedt, L. Rutberg, J. Bacteriol. 144 (1980) 941–951.
[122] K. Nakamura, M. Yamaki, M. Sarada, S. Nakayama, C.R.T. Vibat, R.B. Gennis, T. Nakayashiki, H. Inokuchi, S. Kojima, K. Kita, J. Biol. Chem. 271 (1996) 521–527.
[123] G.Y. Lee, D.Y. He, L. Yu, C.A. Yu, J. Biol. Chem. 270 (1995) 6193–6198.
[124] Z.M. Choudhry, E.V. Gavrikova, A.B. Kotlyar, P.R. Tushurashvili, A.D. Vinogradov, FEBS Lett. 182 (1985) 171–175.
[125] Z.M. Choudhry, A.B. Kotlyar, A.D. Vinogradov, Biochim. Biophys. Acta 850 (1986) 131–138.
[126] Yu, C.-A. and Yu, L. (1987) in Cytochrome systems (Papa, S., Chance, B. and Ernster, L., Eds.), pp. 649–656, Plenum Press, New York.
[127] T.L. Prasad Reddy, M.M. Weber, J. Bacteriol. 167 (1986) 1–6.
[128] J.H. Weiner, R. Cammack, S.T. Cole, C. Condon, N. Honore, B.D. Lemire, G. Shaw, Proc. Natl. Acad. Sci.USA 83 (1986) 2056–2060.
[129] D.J. Westenberg, R.P. Gunsalus, B.A. Ackrell, G. Cecchini, J. Biol. Chem. 265 (1990) 19560–19567.
[130] D.J. Westenberg, R.P. Gunsalus, B.A. Ackrell, H. Sices, G. Cecchini, J. Biol. Chem. 268 (1993) 815–822.
[131] R. Moll, G. Schäfer, Eur. J. Biochem. 201 (1991) 593–600.
[132] S. Anemuller, G. Schäfer, Eur. J. Biochem. 191 (1990) 297–305.
[133] T. Iwasaki, T. Wakagi, T. Oshima, J. Biol. Chem. 270 (1995) 30902–30908.
[134] C.J. Bult, O. White, G.J. Olsen et al., Science 273 (1996) 1058–1073.
[135] L. Yu, J.X. Xu, P.E. Haley, C.A. Yu, J. Biol. Chem. 262 (1987) 1137–1143.
[136] Y. Xu, J.C. Salerno, Y.H. Wei, T.E. King, Biochem. Biophys. Res. Commun. 144 (1987) 315–322.
[137] M.H. Qureshi, T. Fujiwara, Y. Fukumori, J. Bacteriol. 178 (1996) 3031–3036.
[138] R. Gilmour, T.A. Krulwich, Biochim. Biophys. Acta 1276 (1996) 57–63.
[139] P. Gärtner, Eur. J. Biochem. 200 (1991) 215–222.
[140] Cammack, R., Maguire, J.J. and Ackrell, B.A.C. (1987) in Cytochrome systems (Papa, S., Chance, B. and Ernster, L., Eds.), pp. 485–491, Plenum Press, New York.
[141] B.L. Trumpower, J. Biol. Chem. 265 (1990) 11409–11412.
[142] G. Brasseur, A.S. Saribas, F. Daldal, Biochim. Biophys. Acta 1275 (1996) 61–69.
[143] C.A. Yu, L. Yu, J. Biol. Chem. 257 (1982) 6127–6131.
[144] A.K. Tan, R.R. Ramsay, T.P. Singer, H. Miyoshi, J. Biol. Chem. 268 (1993) 19328–19333.
[145] M. Degli Esposti, A. Ngo, A. Ghelli, B. Benelli, V. Carelli, H. McLennan, A.W. Linnane, Arch. Biochem. Biophys. 330 (1996) 395–400.
[146] F.J. Ruzicka, H. Beinert, K.L. Schepler, W.R. Dunham, R.H. Sands, Proc. Natl. Acad. Sci. USA 72 (1975) 2886–2890.
[147] T. Miki, L. Yu, C.A. Yu, Arch. Biochem. Biophys. 293 (1992) 61–66.
[148] P. Mitchell, Biochem. Soc. Trans. 4 (1976) 399–430.
[149] J.C. Salerno, H.J. Harmon, H. Blum, J.S. Leigh, T. Ohnishi, FEBS Lett. 82 (1977) 179–182.
[150] P.R. Rich, A.L. Moore, W.J. Ingledew, W.D. Bonner Jr., Biochim. Biophys. Acta 462 (1977) 501–514.
[151] P.R. Rich, W.D. Bonner Jr., Biochim. Biophys. Acta 504 (1978) 345–363.
[152] P.C. Mowery, D.J. Steenkamp, A.C. Ackrell, T.P. Singer, G.A. White, Arch. Biochem. Biophys. 178 (1977) 495–506.
[153] V.G. Grivennikova, A.D. Vinogradov, Biokhimiia 50 (1985) 375–383.

[154] B.A. Ackrell, E.B. Kearney, C.J. Coles, T.P. Singer, H. Beinert, Y.P. Wan, K. Folkers, Arch. Biochem. Biophys. 182 (1977) 107–117.
[155] A. Bruni, E. Racker, J. Biol. Chem. 243 (1968) 962–971.
[156] R.R. Ramsay, B.A. Ackrell, C.J. Coles, T.P. Singer, G.A. White, G.D. Thorn, Proc. Natl. Acad. Sci. USA 78 (1981) 825–828.
[157] J.D. Pennoyer, T. Ohnishi, B.L. Trumpower, Biochim. Biophys. Acta 935 (1988) 195–207.
[158] Hederstedt, L., Matsson, M., Cochran, B. and Ackrell, B.A.C. (1996) Biochim. Biophys. Acta EBEC short reports 9, 247 (S-6).
[159] M. Degli Esposti, Biochim. Biophys. Acta 977 (1989) 249–265.
[160] I. Smirnova, C. Hägerhäll, A. Konstantinov, L. Hederstedt, FEBS Lett. 359 (1995) 23–26.
[161] Hägerhäll, C. (1994) Ph.D. Thesis, Department of Microbiology, University of Lund, Sweden, ISBN 91-628-1273-4.
[162] K. Oda, K. Yamato, E. Ohta, Y. Nakamura, M. Takemura, N. Nozato, K. Akashi, T. Kanegae, Y. Ogura, T. Kohchi et al., J. Mol. Biol. 223 (1992) 1–7.
[163] J.L. Blanchard, G.W. Schmidt, J. Mol. Evol. 41 (1995) 397–406.
[164] F. Saruta, H. Hirawake, S. Takamiya, Y.C. Ma, T. Aoki, K. Sekimizu, S. Kojima, K. Kita, Biochim. Biophys. Acta 1276 (1996) 1–5.
[165] H. Ding, C.C. Moser, D.E. Robertson, M.K. Tokito, F. Daldal, P.L. Dutton, Biochemistry 34 (1995) 15979–15996.
[166] M. Lubben, Biochim. Biophys. Acta 1229 (1995) 1–22.
[167] P.R. Rich, Biochim. Biophys. Acta 768 (1984) 53–79.
[168] D.E. Robertson, R.S. Farid, C.C. Moser, J.L. Urbauer, S.E. Mulholland, R. Pidikiti, J.D. Lear, A.J. Wand, W.F. DeGrado, P.L. Dutton, Nature 368 (1994) 425–432.
[169] H. Michel, I. Sinning, J. Koepke, G. Evald, G. Fritsch, Biochim. Biophys. Acta 1018 (1990) 115–118.
[170] U. Liebl, S. Pezennec, A. Riedel, E. Kellner, W. Nitschke, J. Biol. Chem. 267 (1992) 14068–14072.
[171] M.D. Collins, FEMS Microbiol. Lett. 28 (1985) 21–23.
[172] W.R. Rypniewski, D.R. Breiter, M.M. Benning, G. Wesenberg, B.H. Oh, J.L. Markley, I. Rayment, H.M. Holden, Biochemistry 30 (1991) 4126–4131.
[173] S. Anemüller, T. Hettmann, R. Moll, M. Teixeira, G. Schaefer, Eur. J. Biochem. 232 (1995) 563–568.
[174] A. Kröger, A. Innerhofer, Eur. J. Biochem. 69 (1976) 497–506.
[175] B.A. Crowe, P. Owen, R. Cammack, Eur. J. Biochem. 137 (1983) 185–190.
[176] B.A. Crowe, P. Owen, D.S. Patil, R. Cammack, Eur. J. Biochem. 137 (1983) 191–196.
[177] Cammack, R., Patil, D.S., Condon, C., Owen, P., Cole, S.T. and Weiner, J.M. (1985) in Flavines and Flavoproteins (Bray, R.C., Engel, P.C. and Mayhew, S.G., Eds.), pp. 551–552, Walter de Gruyter, Berlin.
[178] C. Condon, R. Cammack, D.S. Patil, P. Owen, J. Biol. Chem. 260 (1985) 9427–9434.
[179] K. Kita, C.R. Vibat, S. Meinhardt, J.R. Guest, R.B. Gennis, J. Biol. Chem. 264 (1989) 2672–2677.
[180] M.A. Birch-Machin, L. Farnsworth, B.A.C. Ackrell, B. Cochran, S. Jackson, L.A. Bindoff, A. Aitken, A.G. Diamond, D.M. Turnbull, J. Biol. Chem. 267 (1992) 11553–11558.
[181] K. Kita, H. Oya, R.B. Gennis, B.A. Ackrell, M. Kasahara, Biochem. Biophys. Res. Commun. 166 (1990) 101–108.
[182] T. Ohnishi, D.B. Winter, J. Lim, T.E. King, Biochem. Biophys. Res. Commun. 61 (1974) 1017–1025.
[183] T. Ohnishi, J.C. Salerno, J. Biol. Chem. 251 (1976) 2094–2104.
[184] B. Cochran, R.A. Capaldi, B.A. Ackrell, Biochim. Biophys. Acta 1188 (1994) 162–166.
[185] R. Furushima, K. Kita, S. Takamiya, K. Konishi, T. Aoki, H. Oya, FEBS Lett. 263 (1990) 325–328.
[186] D. Simpkin, W.J. Ingledew, Biochem. Soc. Trans. 13 (1985) 603–607.
[187] K.B. Chapman, S.D. Solomon, J.D. Boeke, Gene 118 (1992) 131–136.
[188] N. Schulke, G. Blobel, D. Pain, Proc. Natl. Acad. Sci. USA 89 (1992) 8011–8015.