Journal of Bioenergetics and Biomembranes, Vol. 18, No. 3, 1986

The Pathway of Electron Transfer in the Dimeric QH_2: Cytochrome *c* Oxidoreductase

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Received November 18, 1985

Abstract

The experimental data currently available suggest that QH_2: cytochrome *c* oxidoreductase functions according to a Q-cycle type of mechanism. The molecular weight of the enzyme in a natural or artificial phospholipid bilayer or in solution corresponds to that of a dimer. The pre-steady state kinetics of reduction of the prosthetic groups indicate that the enzyme is functionally dimeric. A double Q cycle is proposed, describing the pathway of electron transfer in the dimeric QH_2: cytochrome *c* oxidoreductase. According to this scheme, the two monomeric halves of the enzyme act in a cooperative fashion to complete the catalytic cycle. It is proposed that high-potential cytochrome *b*-562 and low-potential cytochrome *b*-562 act cooperatively, viz. as a functional pair, in the antimycin-sensitive reduction of ubiquinone to ubiquinol.

Key Words: QH_2: cytochrome *c* oxidoreductase; EPR; pre-steady state kinetics; electron transfer.

Introduction

The QH_2: cytochrome *c* oxidoreductases[2] play a central role in the energy metabolism of mitochondria, aerobic bacteria, photosynthetic bacteria,

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[2] Abbreviations: QH_2: cytochrome *c* oxidoreductase: refers in general to the enzyme from (phototrophic) eukaryotes and (phototrophic) prokaryotes; Q, Q^-, and QH_2: (ubi)quinone, (ubi)semiquinone anion, and (ubi)quinol, respectively; BAL: British Anti-Lewisite (2,3-dimercaptopropanol); DBMIB: 2,5-dibromo-6-methyl-3-isopropyl-1,4-benzoquinone; DQ, DQ^-, and DQH_2: duroquinone, durosemiquinone anion, and duroquinol, respectively; DTNB: 5,5′-dithiobis(2-nitrobenzoate); HMHQQ: 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone; HQNO: 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; NQNO: 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; UHDBT: 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazol; UHNQ: 3-undecyl-2-hydroxyl-1,4-naphthoquinone; *b*-562: cytochrome *b*-562 from mitochondria or its counterpart in chromatophores, cytochrome *b*-560; *b*-562(hp): high-potential cytochrome *b*-562 (b_{155} in chromatophores); *b*-562(lp): low-potential cytochrome *b*-562 (b_{50} in chromatophores); E_h: the ambient redox potential; E_m: redox midpoint potential.

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chloroplasts, and cyanobacteria. The QH_2: cytochrome *c* oxidoreductase from mitochondria and aerobic bacteria constitutes the middle segment of the respiratory chain; during its catalytic action protons are translocated across the membrane and the protonmotive force that is consequently generated drives the synthesis of ATP from ADP + P_i, catalyzed by the ATP-synthase. Likewise, the QH_2: cytochrome c_2 oxidoreductase from photosynthetic bacteria is involved in cyclic photophosphorylation, and the PQH_2: plastocyanin (*c*-553) oxidoreductase from chloroplasts and cyanobacteria in both cyclic and linear photophosphorylation.

The basic content of prosthetic groups of these three types of enzyme is the same, i.e., one 2Fe-2S cluster, one cytochrome c_1 (or *f*), and one cytochrome *b*, carrying two heme groups. In addition, the values of the midpoint potentials of the electron-transferring groups are similar in the various enzymes, and the polypeptides containing prosthetic groups are also structurally closely related, as indicated, for instance, by the extensive homology of the primary structure of cytochrome *b* from different organisms (Saraste, 1984; Widger *et al.*, 1984). This, then, suggests that the molecular mechanism of electron transfer coupled to proton translocation is similar, if not identical, in the QH_2: cytochrome *c* oxidoreductases from the different organisms.

In the following, diagrams describing electron transfer in QH_2: cytochrome *c* oxidoreductase are discussed. After the notion that linear and branched-linear schemes do not adequately describe the pathway of electron transfer in the enzyme, the Q-cycle and the *b*-cycle schemes are treated shortly. The reader is referred to the accompanying papers and the references cited there and in this paper for a more complete discussion on the two "cycles." In this paper the distinction between the Q cycle and the *b* cycle is emphasized and the experimental data favoring the Q cycle are discussed. After this, structural and kinetic data are presented revealing the dimeric state and mechanism of action, respectively, of the QH_2: cytochrome *c* oxidoreductase. The observations are interpreted in terms of a double Q-cycle scheme describing the pathway of electron transfer in the dimeric enzyme.

Electron Transfer Schemes

Linear and Branched-Linear Pathways of Electron Transfer

It was found by Deul and Thorn (1962) in 1962 that after addition of antimycin to particles treated with BAL ($+O_2$) cytochrome *b* is no longer reducible by substrate, whereas in the presence of either antimycin or BAL cytochrome *b* is still reducible (Slater, 1949; Chance, 1958). From this observation Deul and Thorn concluded that antimycin and BAL ($+O_2$) act

on different sites, but more importantly that this experimental finding is "difficult to explain in terms of conventional (viz. linear) representations of the respiratory chain."

About 10 years later, Wikström and Berden (1972) proposed a scheme that may be called a branched-linear scheme to describe the well-known phenomenon of oxidant-induced reduction of cytochrome *b* in the presence of antimycin. Although their kinetic interpretation gives a full account of this phenomenon, also under conditions that extra reduction of cytochrome *b* occurs in the absence of antimycin (Erecińska and Wilson, 1972), this scheme does not explain the observation made by Deul and Thorn. Likewise the scheme proposed by Papa *et al.* (1982) is not consistent with this observation.

Somewhat ironically the outcome of the experiment of Deul and Thorn was unknown to many and forgotten by others (cf. Slater, 1981), and it was the Wikström and Berden model that served as an important source of inspiration for both the Q cycle and the *b* cycle.

Cyclic Electron-Transfer Pathways

The Q-cycle diagram proposed by Mitchell (Mitchell, 1975a, b, 1976; Kröger, 1976) and the *b* cycle of Wikström and Krab (1980) both elegantly describe the phenomenon of oxidant-induced reduction of cytochrome *b* and explain the observations of Deul and Thorn.

Shortly after the proposal of the Q cycle, experimental evidence supporting a cyclic pathway of electron transfer was reported by Trumpower (1976) who showed that extraction of a protein, called *oxidation factor*, from the reductase prevents the reduction of cytochrome *b* by substrate if antimycin is also present, i.e., similar to Deul and Thorn's observation. When it was later found that both the oxidation factor and the BAL-labile factor are the Rieske Fe-S protein (Trumpower and Edwards, 1979) and cluster (Slater and de Vries, 1980), respectively, the role and function of this prosthetic group became clear (Slater, 1981; Bowyer *et al.*, 1981a; Bowyer and Trumpower, 1981).

Several variants of the experiment of Deul and Thorn have since been published. Thus instead of antimycin, any of the inhibitors HQNO, NQNO (van Ark and Berden, 1977; van Ark, 1980), diuron (Convent and Briquet, 1978), and funiculosin (Briquet *et al.*, 1981) can be employed in combination with any one of the "BAL type" of inhibitors mucidin (Briquet and Goffau, 1981; Subik *et al.*, 1974), myxothiazol, oudemansin, and strobilurines (Thierbach and Reichenbach, 1981, von Jagow and Engel, 1981; von Jagow *et al.*, 1984), the –SH reagent DTNB (Marres *et al.*, 1982). UHDBT (Bowyer and Trumpower, 1981; Bowyer *et al.*, 1981a, 1982), HMHQQ (Zhu *et al.*, 1982a), UHNQ (Matsuura *et al.*, 1983), and stigmatellin (Thierbach *et al.*,

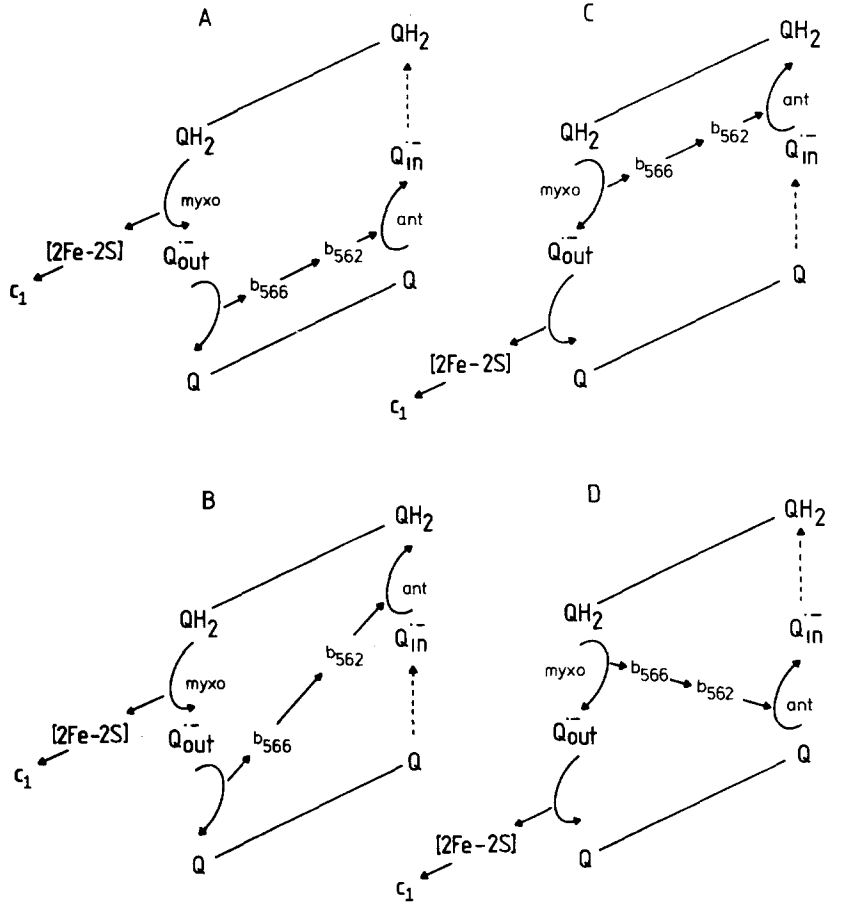


Fig. 1. Four variants of the Q cycle (A, B, C, D) and the *b*-cycle scheme (E see p. 199) showing the pathway of electron transfer in QH_2: cytochrome *c* oxidoreductase. Straight full arrows represent electron transfer; curved arrows, chemical equations. The pathway of the protons is omitted. Dotted arrows represent a possible (direct) electron transfer from the dehydrogenases (Mitchell, 1976; van Ark, 1980; Bowyer and Trumpower, 1981; Bowyer *et al.*, 1982) and/or a reversed dismutation reaction (van Ark, 1980; Slater, 1981; Zhu *et al.*, 1982a, b). Q and QH_2 are freely diffusable; the semiquinone anions are fixed. Antimycin-binding sites are represented by ant, those of myxothiazol by myxo. See also Wikström and Krab (1980), Wikström and Saraste (1984), Crofts *et al.* (1983), Rich (1984), Bendall (1982), and Hauska *et al.* (1983) for detailed discussions on Q cycles and *b* cycles.

1984; von Jagow and Ohnishi, 1985). In all cases, the combination of an antimycin-type inhibitor with a BAL-type inhibitor prevents the reduction of cytochrome *b* by substrate, and in fact prevents reduction of any prosthetic group including the formation of semiquinone anions (cf. de Vries *et al.*, 1981a). If the quinone analogues UHDBT or HMHQQ are added at

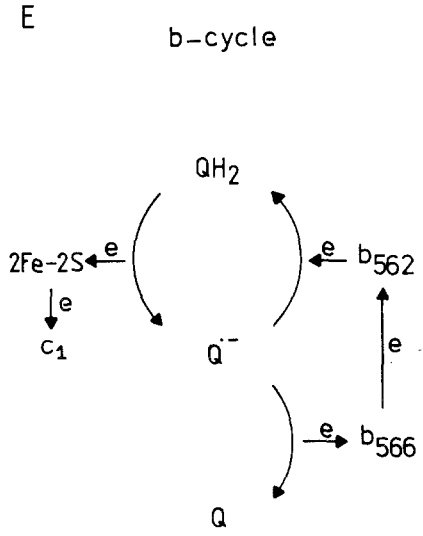


Fig. 1. Continued

sufficiently high concentrations, they alone will inhibit all electron-transfer reactions in the enzyme (Zhu *et al.*, 1982a; Bowyer and Crofts, 1978; de Vries and Dutton, 1985).

These experiments indicate that the QH_2: cytochrome *c* oxidoreductase contains two *independent* routes to reduce cytochrome *b*. One, the Q_{out} route, involves the Rieske Fe-S cluster and is inhibited by the "BAL type" of inhibitors; the other one, the Q_{in} route involving cytochrome *b*-562, is inhibited by the antimycin-like inhibitors (see Fig. 1).

According to the Q cycle (Mitchell, 1975a, b, 1976) two different specific semiquinones are involved in each electron-transfer pathway during steady-state turnover. One, namely $Q_{out}^{\cdot-}$, reduces cytochrome *b* (i.e., cytochrome *b*-566) and the other one, $Q_{in}^{\cdot-}$, oxidizes cytochrome *b* (i.e., cytochrome *b*-562). In fact, two different semiquinone anions have been detected by EPR, and, more importantly, their thermodynamic and kinetic characteristics as well as their responses to various inhibitors are exactly those expected for the semiquinones of the Q cycle (Mitchell, 1976). Thus, the $Q_{in}^{\cdot-}$ is both detectable under conditions of redox equilibrium (Konstantinov and Ruuge, 1977; Ohnishi and Trumpower, 1980; de Vries *et al.*, 1980, 1982b; Wei *et al.*, 1981; Robertson *et al.*, 1984b) and in the pre-steady state (de Vries *et al.*, 1982a, 1983), its maximal concentration amounting to $0.5\,Q_{in}^{\cdot-}/c_1$. It is absent in the presence of antimycin, but not affected by BAL ($+O_2$) treatment or addition of myxothiazol. The maximal concentration of $Q_{in}^{\cdot-}$ in the bovine heart enzyme is obtained at $E_h = 80$–85 mV (pH 7) and since $E_{m7}(Q_{pool}) = 90$ mV (Takamiya and Dutton, 1979), this indicates that Q and QH_2 bind to this site

with about equal affinity. However, in the chromatophore system QH_2 binds 100 times more tightly to this site than Q (Robertson *et al.*, 1984b).

The other semiquinone anion, $Q^{\cdot-}_{out}$, is only detectable under nonequilibrium conditions, e.g., during oxidant-induced reduction of cytochrome *b* in the presence of antimycin, and its maximal concentration amounts to $0.1-0.15\, Q^{\cdot-}_{out}/c_1$ (de Vries *et al.*, 1981a). In particles treated with BAL ($+O_2$), $Q^{\cdot-}_{out}$ could not be detected.

All these observations are in perfect agreement with the Q cycle. Moreover, the mere detection of $Q^{\cdot-}_{out}$ under conditions of oxidant-induced reduction of cytochrome *b* in the presence of antimycin, combined with the finding that a functional Fe-S cluster is required for the reduction of cytochrome *b* in the presence of antimycin, proves (cf. Mitchell, 1976; Slater, 1981) that (a) oxidation of QH_2 via the Q_{out} route is not a concerted reaction (contrast Crofts *et al.*, 1983) but rather a consecutive one (de Vries *et al.*, 1981a; Rich, 1984), and (b) that QH_2 is the electron donor to the Fe-S cluster and that the product of this reaction $Q^{\cdot-}_{out}$ is the reductant for cytochrome *b* (*b*-566). Furthermore, the experimental detection of $Q^{\cdot-}_{out}$ itself seems to invalidate the proposal of the *b* cycle since according to this scheme the enzyme contains only one Q ($Q^{\cdot-}$, QH_2) binding site. However, in a recent paper Wikström and Saraste (1984) outline that the existence of two different semiquinone anions can be accommodated in the *b*-cycle scheme. They suggest that $Q^{\cdot-}$ is bound in a pocket and that the quinone ring is able to flip-flop between two domains, the Fe-S/*b*-566 domain (in which case the semiquinone anion has the properties of $Q^{\cdot-}_{out}$) and the *b*-562 domain (in which case $Q^{\cdot-}$ shows up as $Q^{\cdot-}_{in}$).

According to the *b* cycle a full catalytic cycle of the enzyme comprises two turnovers by the Fe-S cluster. The electron-transfer reactions that occur can be written as (protons omitted):

First turnover:
$$QH_2 + c^{3+} \xrightarrow[c_1]{\text{Fe-S}} Q^{\cdot-}_{out} + c^{2+} \quad (1)$$

$$Q^{\cdot-}_{out} + b\text{-}562^{3+} \xrightarrow{b\text{-}566} Q + b\text{-}562^{2+} \quad (2)$$

Second turnover:
$$QH_2 + c^{3+} \xrightarrow[c_1]{\text{Fe-S}} Q^{\cdot-}_{out} + c^{2+} \quad (1')$$

$$Q^{\cdot-}_{out} \longrightarrow Q^{\cdot-}_{in} \text{ (flip-flop)} \quad (3)$$

$$Q^{\cdot-}_{in} + b\text{-}562^{2+} \rightleftharpoons QH_2 + b\text{-}562^{3+} \quad (4)$$

Sum
$$2QH_2 + 2c^{3+} \longrightarrow QH_2 + Q + 2c^{2+} \quad (5)$$

The recognition that two species of semiquinone anion are also active according to the *b* cycle makes the distinction between the Q cycle and the *b* cycle rather small inasmuch as electron transfer is concerned. In fact, studies of

cyclic electron transfer in bacterial chromatophores or in the so-called hybrid system are consistent with both Q-cycle (Crofts *et al.*, 1983; Crofts and Wraight, 1983; Meinhardt and Crofts, 1983; Dutton and Prince, 1978; Prince *et al.*, 1982) and *b*-cycle (cf. Matsuura and Dutton, 1981) types of electron-transfer schemes. Recently, Crofts *et al.* (1983) proposed a double-turnover Q cycle, but, in my opinion, their experiments do not discriminate between Q or *b* cycle and may even favor the latter.

The Q cycle and the *b* cycle are distinct in two ways. The first distinction concerns the number of Q binding sites which is equal to *two* for the Q cycle and *one* for the *b* cycle. Recently, Robertson *et al.* (1985) showed that the QH_2: cytochrome c_2 oxidoreductase from a mutant strain of *Rhodopseudomonas capsulata* still contains the antimycin-sensitive $Q_{in}^{\cdot-}$, but lacks all the characteristics associated with the Q_{out} binding domain as evidenced by the absence of quinol-mediated reduction of cytochromes ($c_1 + c_2$) and by the absence of the effect on the EPR spectrum of the Fe-S cluster induced by ubiquinone, i.e., Q_{out}, which is bound closely to this cluster (cf. de Vries *et al.*, 1982a). Furthermore, it is shown by de Vries and Dutton (1985) that in the hybrid system the extent of reduction of cytochrome *b* titrates out upon addition of *one* UHDBT per cytochrome c_1, either in the presence of antimycin (in which case UHDBT inhibits electron transfer via the Q_{out} route), or in the presence of myxothiazol [when UHDBT inhibits (the reversal of) reaction (4), viz. the reduction of cytochrome *b*-562 via the Q_{in} route]. However, *two* UHDBT per cytochrome c_1 are required to completely inhibit reduction of cytochrome *b* in the absence of additional inhibitors, in which case UHDBT inhibits both the Q_{in} and the Q_{out} route. These experimental findings are difficult to reconcile with the *b* cycle.

The second distinction between the two cycles is related to the rate of reaction (3). According to the *b* cycle this reaction is simply a flip-flop that must occur as fast as or faster than turnover. According to the Q cycle, such a reaction designates a net electron transfer than can formally be written as

$$Q_{out}^{\cdot-} + Q_{in} \longrightarrow Q_{out} + Q_{in}^{\cdot-} \qquad (6)$$

This reaction is not permitted at the time scale of turnover unless, of course, the electron transfer goes via *b*-566 and *b*-562. This, then, allows the following experimental distinction between the two models (Bowyer and Trumpower, 1981; Wikström and Saraste, 1984): A pulse of O_2 given to fully reduced submitochondrial particles should lead to the instantaneous oxidation of cytochrome *b* according to the *b* cycle, but according to the Q cycle the oxidation is expected to start after a lag period, the length of which may be given by the rate of reaction (6) and/or by the rate of dismutation of $Q^{\cdot-}$. Such a lag period has, indeed, been observed (de Vries, 1983). In addition, in a variant of this type of experiment carried out in the hybrid system (de Vries

and Dutton, 1985), the rate of oxidation of cytochrome *b* (*b*-562 as well as *b*-566) is found to decrease with increasing redox state of the Q pool, whereas the rate of turnover, viz. the rate of reduction of cytochrome $c + c_1$, remains constant, exactly as expected from the Q-cycle scheme but in conflict with the *b* cycle.

The Dimeric Structure of QH_2: Cytochrome *c* Oxidoreductase

Determination of the Molecular Weight

Tzagoloff *et al.* (1965) have determined the molecular weight of the purified QH_2: cytochrome *c* oxidoreductase from bovine heart dispersed in cholate. They determined the *s* value but neither the diffusion constant nor the partial specific volume. Their calculated molecular weight, corrected for bound lipid and detergent, corresponds to that of the monomeric enzyme.

We have repeated these experiments (de Vries, 1983) and found the same *s* value as Tzagoloff *et al.* (1965). In addition, the diffusion constant and the partial specific volume ($\bar{v}$ = 0.83 ml/g!) were determined. The molecular weight, corrected for bound lipid and detergent, equals 430,000, corresponding to that of the dimer.

The enzyme from *Neurospora crassa* or bovine heart dispersed in Triton X-100 is also dimeric (von Jagow *et al.*, 1977; Weiss and Kolb, 1979).

Recently, Nalecz *et al.* (1985) and Nalecz and Azzi (1985) proposed that the enzyme dispersed in lauryl maltoside is predominantly monomeric at low salt concentrations and dimeric in 50 mM KCl. The monomeric and dimeric enzymes can be separated on a gel filtration column.

Ultracentrifuge experiments with the enzyme preparation treated exactly as by Nalecz *et al.* (1985) were performed at 0 and 50 mM KCl (Fig. 2). In both media a single homogenous species was observed sedimenting with the same *s* value. Essentially the same *s* value (16.2) is found for the dimeric enzyme dispersed in cholate (de Vries, 1983). Under all conditions tested the enzyme is dimeric.

EPR Properties of the Two Rieske [2Fe-2S] Clusters

QH_2: cytochrome *c* oxidoreductase shows a rhombic EPR spectrum when reduced with ascorbate with $g_{z,y,x}$ = 2.02, 1.90, 1.80 as first described by Rieske *et al.* (1964a, b, c). The signal strongly resembles those of [2Fe-2S] clusters (Gibson *et al.*, 1966).

It was noticed that the g_x resonance shifts from g_x = 1.80 to 1.78 and broadens when, instead of ascorbate, succinate or dithionite is used as the reducing agent (Rieske *et al.*, 1964a, b, c). Orme-Johnson *et al.* (1971)

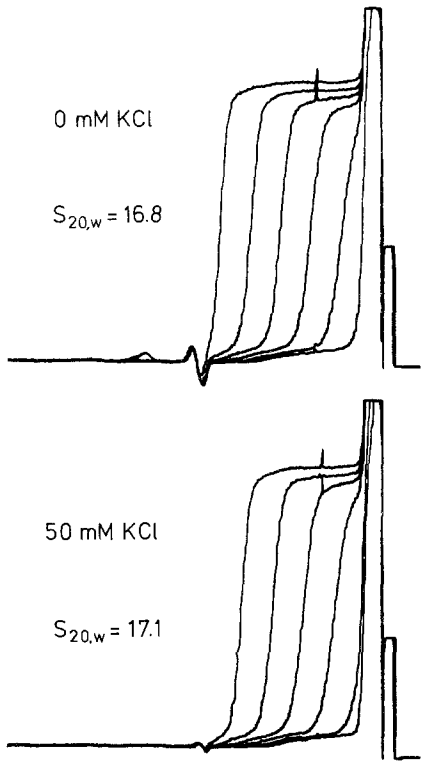


Fig. 2. Traces of a sedimentation velocity experiment with the QH_2: cytochrome *c* oxidoreductase purified according to Rieske (1967). The enzyme was treated with lauryl maltoside and subsequently dialyzed exactly as by Nalecz *et al.* (1985). Prior to ultracentrifugation, the enzyme was diluted 20-fold to a protein concentration of 0.5 mg/ml with a 10 mM Tris-HCl buffer (pH 7.4), 0.1% lauryl maltoside with or without 50 mM KCl. The traces were recorded at 6 min interval, 45.000 rpm, 20°C and 429 nm.

suggested that this effect accompanies the reduction of cytochrome *b*-562. We found that it is the redox state of Q that is responsible for this effect (de Vries *et al.*, 1978, 1979) and that in the presence of *oxidized* Q g_x is at $g = 1.80$, whereas in the presence of QH_2 or in the total absence of ubiquinone the g_x line is broadened and shifted to $g = 1.78$. Similar findings have been made for the enzyme from yeast mitochondria (Siedow *et al.*, 1978).

In addition to ubiquinone the (oxidized) inhibitory quinone analogues HMHQQ, UHDBT, and UHNQ all specifically affect the EPR lineshape (Bowyer *et al.*, 1982; Zhu *et al.*, 1982a; Matsuura *et al.*, 1983), as does myxothiazol but not antimycin (de Vries *et al.*, 1983). The effect of Q (and HMHQQ) is not observed in preparations that are not able to oxidize QH_2 via the Q_{out} route (as, e.g., in the enzyme modified by DTNB (Marres *et al.*,

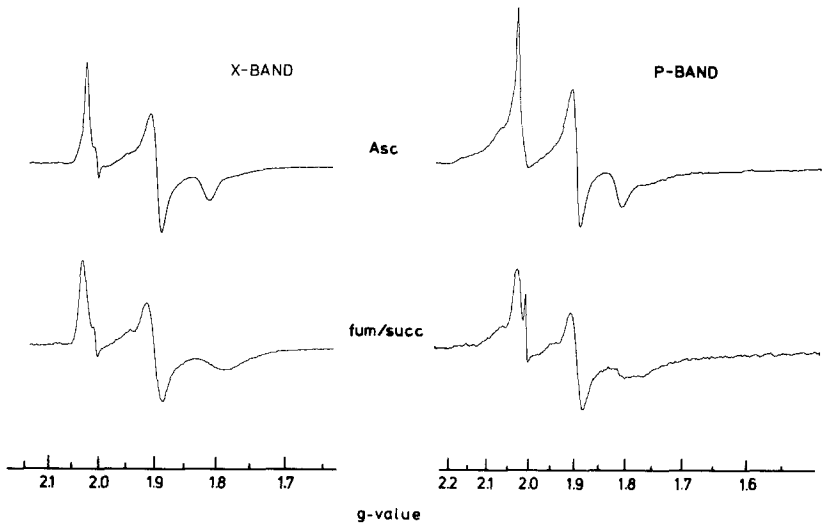


Fig. 3. EPR spectra recorded at X-band (9.3 GHz) and P-band (14.7 GHz) frequencies of the Rieske Fe-S clusters as present in purified QH_2:cytochrome *c* oxidoreductase reduced with ascorbate (Asc) or with the fumarate/succinate (fum/succ) redox couple. See the legends to Figs. 1 and 8 in de Vries *et al.* (1979) for the method of sample preparation. The same sample (in the same tube) was run on the X-band and P-band spectrometers. The resolution in two Fe-S clusters [called cluster 1, with the relatively sharp resonances, and cluster 2 (cf. de Vries *et al.* 1978, 1979)] is much better visible in the P-band spectra. The molar ratio of cluster 1 and cluster 2 (1 : 1.17) is the same for the two P-band spectra and similar (1 : 1.22) to that of the X-band spectrum of the sample reduced with ascorbate. The values of g_y and g_x of clusters 1 and 2 as determined by the simulation of the X-band spectrum (top left) are the same as in the P-band spectra. The values of g_z of clusters 1 and 2 in the P-band are not the same as found in the X-band. Upon reduction of Q (compare upper and lower traces) the position of the maxima of the resonance lines shifts in the X-band spectra but not in the P-band spectra. EPR conditions X(P)band: frequency 9.318 (14.784) GHz; temperature 36 (25) K; microwave power 2 (2) mW; modulation amplitude 0.63 (0.4) mT.

1982) or in a specific mutant of *Rhodopseudomonas capsulata* (Robertson *et al.*, 1985) but otherwise contain an intact Rieske Fe-S cluster.

The effect of changing the redox state of Q on the EPR lineshape of the Rieske Fe-S cluster is not confined to the g_x resonance, but also manifest in the g_y and g_z lines (cf. Fig. 3 and de Vries *et al.*, 1979)).

Also, the latter lines broaden and shift, although the shift in g_y is very small.

The X-band EPR spectra of the Fe-S cluster reduced with ascorbate or the fumarate/succinate couple (Fig. 3) cannot be satisfactorily simulated as a single component. Computer simulation of the former signal, recorded at the X-band, indicated that it is an overlap of two signals in a 1 : 1 weighted molar ratio originating from two slightly different Rieske Fe-S clusters. The relatively sharp signal is, arbitrarily, called cluster 1, and the broader one

cluster 2. It was further concluded from the X-band spectra that only the resonance lines of cluster 1 broaden and shift upon reduction of Q and, in fact, in such a way that cluster 1 and 2 are no longer clearly distinguishable (de Vries *et al.*, 1978, 1979, 1982a).

The interpretation that the signal in Fig. 3 (top left) is an overlap of two signals was recently criticized by Hagen (1981, 1982). However, later Hagen *et al.* (1985a, b) showed that this signal indeed cannot be simulated as a single component, not even with a sophisticated simulation program that includes the concept of *g*-strain. Even more convincing evidence that the signal is an overlap of two signals comes from the spectra of the same samples recorded at P-band frequencies (Fig. 3). The resonance lines of the two clusters are now much better separated and it is clear that also the spectrum of the sample reduced with the fumarate/succinate couple is an overlap of two signals. However, and this is at variance with our previous interpretation from the X-band spectra, one has to conclude from the P-band spectra that the lines of *both* cluster 1 and cluster 2 broaden upon reduction of Q and that the *g*-values remain the same (cf. upper and lower traces in Fig. 3).

The X-band EPR spectrum of the Rieske Fe-S clusters of *Paracoccus denitrificans* can be simulated with exactly the same set of parameters used for the spectrum of bovine heart particles reduced with ascorbate (Albracht *et al.*, 1980). In addition, the spectra of the Rieske clusters in horse heart (de Vries, unpublished), in yeast (Siedow *et al.*, 1978), and in bacterial chromatophores (Matsuura *et al.*, 1983; Prince, 1983) are all very similar if not identical to that of bovine heart. Nevertheless, the EPR spectrum of the Rieske Fe-S cluster in chloroplasts or the purified b_6/f complex does not show any of the above-mentioned features (Malkin, 1981; Hurt *et al.*, 1981). In the presence of DBMIB, however, the spectrum seems to consist of two signals in an approximate 1 : 1 ratio (Hurt *et al.*., 1981).

It is concluded that the QH_2: cytochrome *c* oxidoreductase contains two EPR-distinguishable [2Fe-2S] clusters, each in a concentration *half* of that of cytochrome c_1, and both have similar if not identical redox midpoint potentials (Prince and Dutton, 1976).

Multiplicity of Cytochrome b as Determined by EPR

Previously we have analyzed the EPR spectrum of the cytochromes in purified QH_2: cytochrome *c* oxidoreductase (de Vries *et al.*, 1979, 1982a) and concluded that the spectrum of the enzyme reduced with ascorbate is an overlap of four g_z resonances originating from *b*-566 (g_z = 3.78 or 3.71), *b*-558 (g_z = 3.71 or 3.78), *b*-562(lp) (g_z = 3.45), and *b*-562(hp) (g_z = 3.42). The area under each curve was determined and, using a newly devised method (de Vries and Albracht, 1979), the concentration of each of the

resonances was calculated to correspond to half the concentration of cytochrome c_1. Both the four-component analysis and the method of quantitation have been criticized by Salerno (1984).

The method of quantitation was tested with several low-spin heme compounds. It was also used by others (Aasa *et al.*, 1981; Bergström and Vänngård, 1982) to determine the concentration of the cytochromes in several different species or of the a_3–CN complex with $g_z = 3.6$ (Hagen, unpublished). In all these cases the optically determined concentrations and those determined by EPR are in good agreement. When applied to the purified reductase, the concentration of cytochrome c_1 determined by the two methods agrees to within 4%. In addition, the b/c_1 ratio determined by EPR gives 1.95 or 2.11, dependent on whether the resonances at $g = 3.78$ and 3.71 are treated as a single species or as two (de Vries *et al.*, 1979). Both these ratios are not significantly different from that determined optically or chemically, justifying the assumptions made in the quantitation procedure, namely that the orbital reduction factor is close to 1 and that the sum of the squares of the g values is close to 16. In fact, Salerno (1984) arrives at the same conclusion concerning the values of these quantities. Also Salerno must *implicitly* make use of an algorithm similar to the one derived by de Vries and Albracht (1979) to estimate the concentration of the cytochromes, because his simulation is limited to the absorption-like features of the EPR-powder spectrum (i.e., the g_z line, in which case only a single orientation is calculated), instead of simulating and integrating the whole spectrum (in which case an "infinite" number of orientations are calculated; cf. Hagen, 1981, 1982; Hagen *et al.*, 1985a, b). Of course, I agree with Salerno's explanation as to why the resonance line at $g_z = 3.78$ is so asymmetric. However, and in contrast to the statement by Salerno, this asymmetric line was *not* simulated by de Vries *et al.* (1979) but, instead, the lineshape, obtained at 200 mW microwave power, was used in the simulation of the spectrum, assuming that such an asymmetric line originates from a *single* cytochrome *b* (i.e., *b*-566 or *b*-558).

The resolution of the EPR spectrum of the enzyme reduced with ascorbate into four components, three symmetrical and one asymmetrical, has also been criticized by Salerno (1984). As explicitly mentioned by him, his spectra were recorded under power-saturating conditions, which, in fact, is clearly seen from the small amplitudes of the lines at $g = 3.44$ and 3.71 relative to that at $g = 3.78$ (i.e., about two times smaller than in the spectra recorded under nonsaturating conditions shown by de Vries *et al.*, 1979). Power saturation often leads to deformation of the lineshape (Hagen, 1982), in particular to that of the lines at $g = 3.44$ and 3.71 (cf. Fig. 12 of de Vries *et al.*, 1979). In addition, the 20-G modulation amplitude used by Salerno is much too large to obtain the true lineshape of the resonance at $g = 3.78$.

Power saturation and overmodulation are very likely the reasons why Salerno concluded that the EPR spectrum of the enzyme reduced with ascorbate contains the resonances of only two cytochromes *b*.

On the Nature of High-Potential Cytochrome b-562

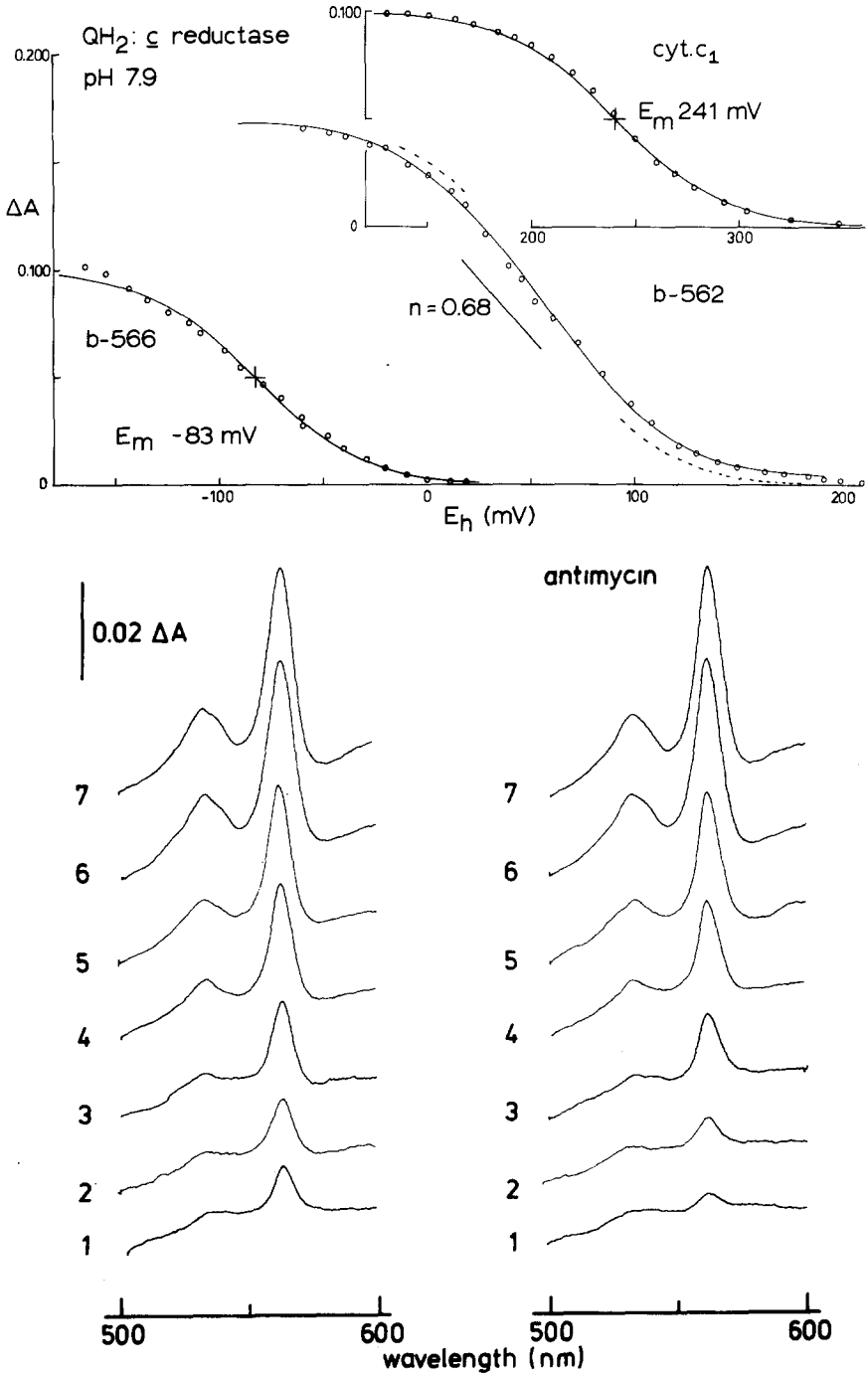
Potentiometric titrations in mitochondria (Dutton *et al.*, 1970, 1972; Berden and Opperdoes, 1972; Berden *et al.*, 1972) and bacterial chromatophores (Dutton and Jackson, 1972; Bowyer *et al.*, 1981b) reveal, in addition to cytochrome *b*-566/558, the presence of two other cytochromes *b*. In mitochondria they are called high-potential cytochrome *b*-562 (E_{m7} = 130–150 mV) and low-potential cytochrome *b*-562 (E_{m7} = 40–60 mV), in the chromatophores b_{155} and b_{50}, respectively. In both types of membranes, the spectral contribution of the cytochrome with the lower value of E_m is about twice that of the one with the higher value of E_m.

Since, however, *b*-562(hp) or b_{155} are apparently not present in the purified enzyme preparations from mitochondria or chromatophores (Leigh and Erecínska, 1975; Riccio *et al.*, 1977; T'sai and Palmer, 1983; Gabellini and Hauska, 1983), and since they are sometimes not observed in pigeon-heart mitochondria or in intact chromatophores (Dutton *et al.*, 1972; Crofts *et al.*, 1983; Glaser *et al.*, 1984), it is often assumed that *b*-562(hp) and b_{155} do not form an integral part of the QH_2: cytochrome *c* oxidoreductase. In the following this view is challenged.

Employing potentiometric titrations, van Wielink *et al.* (1982) detected *b*-562(hp) in the enzyme purified according to Hatefi *et al.* (1961).

Its presence is also inferred by the potentiometric titrations shown in Fig. 4 using the preparation of Rieske (1967). In addition, the kinetics of reduction of *b*-562 by DQH_2 in both submitochondrial particles and the succinate: cytochrome *c* reductase (Jin *et al.*, 1981; Tsou *et al.*, 1982; de Vries *et al.*, 1981b, 1982a, 1983) shows two phases of reduction, separated by a lag period, that can be ascribed to reduction of *b*-562(hp) and *b*-562(lp), respectively (de Vries *et al.*, 1982a, 1983). Also, two different EPR signals and two different low-temperature optical spectra are observed in the course of reduction of cytochrome *b*-562 (de Vries *et al.*, 1979, 1982a; Salerno, 1984).

Furthermore, it was shown by Berden and Opperdoes (1972) that in mitochondria from bovine heart the E_m of *b*-562(hp) is decreased by 50 mV upon addition of antimycin, without any effect on the E_m of *b*-562(lp). The effect of antimycin in itself implies that *b*-562(hp) forms part of the enzyme. As shown in Fig. 4, antimycin in the purified enzyme has the same effect as in the mitochondria. It is concluded that *b*-562(hp) is a functional prosthetic group of the enzyme and that its apparent absence in the more purified preparations, where the E_m values of the two cytochromes are closer than


QH2: c reductase
pH 7.9
0.200
0.100
0
ΔA
cyt.c1
Em 241 mV
0.100
0
200
300
b-562
n = 0.68
b-566
Em -83 mV
-100
0
100
200
Eh (mV)
0.02 ΔA
antimycin
7
6
5
4
3
2
1
500
600
wavelength (nm)

in the particulate preparations, cf. Fig. 4, is due to the limits of resolution of the technique of potentiometric titrations (Dutton, 1978). However, kinetic studies easily resolve the two species of *b*-562.

In the previous sections evidence has been reviewed indicating that the minimal enzymic unit of the QH_2: cytochrome *c* oxidoreductase is twice that based on its cytochrome c_1 content. The dimeric enzyme consists of two monomers with identical primary structure as inferred by genetic studies (van Loon *et al.*, 1984). However, when brought together in the dimeric enzyme the one monomer may be distinguished from the other by the presence of either Fe-S cluster 1 or 2 or by the presence of *b*-562(hp) or *b*-562(lp). As to cytochrome *b*-566/558, the possibility that the absorption maxima reflect a split α-band, despite the observations by Wikström (1973) and Higuti *et al.* (1975), must be seriously considered as well as the possibility that the sum spectra of the asymmetric $g_z = 3.78$ line and the symmetric $g_z = 3.71$ line originates from two identical cytochromes *b*-566/558 (cf. Salerno, 1984) present in the two monomeric halves, as in the case of cytochrome c_1. The small differences between the two Fe-S clusters and the cytochromes *b*-562 may be caused by specific interactions, e.g., hydrophobic and/or electrostatic, along the boundary joining the two monomeric halves.

Mechanism of Action of the Dimeric QH_2: *c* Oxidoreductase

We have previously proposed a scheme describing the pathway of electron transfer in the dimeric enzyme (de Vries *et al.*, 1982a, 1983; Slater, 1983). This proposal is based on observations as discussed in the previous section and on an analysis of the pre-steady state kinetics of reduction of the

Fig. 4. Potentiometric titrations (top) and the effect of antimycin on the redox state of cytochrome *b*-562 (bottom) in QH_2: cytochrome *c* oxidoreductase purified according to Rieske (1967). Potentiometric titrations were carried out as by Dutton (1978). Top: the points of cytochrome *b*-566/558 and cytochrome c_1 are fitted with an $n = 1$ Nernst curve. The dotted (truncated) lines through the data points of cytochrome *b*-562 is an $n = 1$ curve with $E_{50\%} = 55$ mV. The continous line through these points is a simulation assuming the presence of two cytochromes *b*-562 (both $n = 1$) with $E_{m7.9} = 40$ and 95 mV and in a 2 : 1 spectral ratio, respectively. Bottom: optical difference spectra (the sample reduced with ascorbate served as the reference) in which the redox state of cytochrome *b*-562 is varied with the addition of (1) 5 μM Q_2H_2; (2) 100 μM ascorbate + 1 μM TMPD; (3) 1 mM ascorbate + 10 μM TMPD; (4) 50 μM DQH_2; (5) 100 μM DQH_2; (6) 100 μM DQH_2 + 100 μM dithionite (from a 100 mM solution); (7) a few grains of solid sodium dithionite. In traces 6 and 7, cytochrome *b*-566/558 is partly (60%) and completely reduced, respectively. The reduction level of cytochrome *b*-562 in 1 corresponds roughly to the level of reduction of the first phase of reduction seen in experiments in which DQH_2 is used as the donor (cf. Fig. 7). The effect of antimycin is consistent with a change in the E_m of high-potential cytochrome *b*-562 of 40–60 mV, while the E_m of low-potential cytochrome *b*-562 is not altered (cf. Berden and Opperdoes, 1972). In the presence of antimycin, the values of E_m of the two species of cytochrome *b*-562 are similar.

prosthetic groups by DQH_2 in both submitochondrial particles and succinate: cytochrome *c* reductase. The experimental conditions were varied by varying the pH or the ubiquinone content of the particles. In addition, the effect of various inhibitors on the oxidation–reduction kinetics of the prosthetic groups was examined.

It was observed that under specific conditions the Rieske Fe-S cluster (and cytochrome c_1) are reduced in two distinct phases (de Vries *et al.*, 1982a, 1983), each phase corresponding to *half* of the total amount of these prosthetic groups. Therefore we proposed that in each reduction phase a single Fe-S cluster, either cluster 1 or 2, is completely reduced.

Similarly, we concluded from the triphasic reduction of cytochrome *b*-562 by DQH_2 that the initial rapid phase ($t_{1/2} \leqslant 5$ ms, 38% of the total *b*-562 extent) corresponds to reduction of *b*-562(hp) and the second, slower phase to reduction of *b*-562(lp). In the following, some of the experimental observations that have led to the proposal of the double Q cycle are discussed in more detail and, if necessary, reevaluated.

Kinetics of Reduction of the Fe-S Clusters and Cytochrome c

In succinate: cytochrome *c* reductase with antimycin present but without oxidant, only one Fe-S cluster is rapidly reduced and only 50% of cytochrome c_1 (de Vries *et al.*, 1982a). We suggested that antimycin, in addition to inhibiting the oxidation of *b*-562, specifically inhibits the reduction of cluster $[2Fe\text{-}2S]_1$ (cf. de Vries *et al.*, 1983 and Fig. 5) and consequently of the cytochrome c_1 connected to it in the same monomeric half. I realize that the thermodynamic interpretation for the partial reduction of these prosthetic groups given by Rich (1983, 1984) and Crofts *et al.* (1983) is much simpler and have therefore revised the original double Q cycle (de Vries *et al.*, 1982a, 1983) to the one in Fig. 5 with respect to the site of inhibition by antimycin. However, as discussed by Rich (1983, 1984), the observed rates and extents of reduction of the prosthetic groups in the presence of antimycin and absence of oxidant are quantitatively not in agreement with those expected on the basis of their E_m values, so that other factors may also be of importance.

In Fig. 6 the pre-steady state kinetics of reduction of the prosthetic groups in ubiquinone-depleted particles is reproduced. Both in the absence and presence of antimycin only one of the two Fe-S clusters is rapidly and completely reduced. Also, no $DQ^{\cdot -}$ or $Q^{\cdot -}$ is formed in the course of oxidation of DQH_2, in contrast to the $Q_{in}^{\cdot -}$ and $Q_{out}^{\cdot -}$ formed after a pulse of DQH_2 to ubiquinone-containing particles, probably because $DQ^{\cdot -}$ does not contain a hydrophobic tail that may help in the binding and thus in the stabilization of the radical. These two findings strongly suggest that $(D)QH_2$

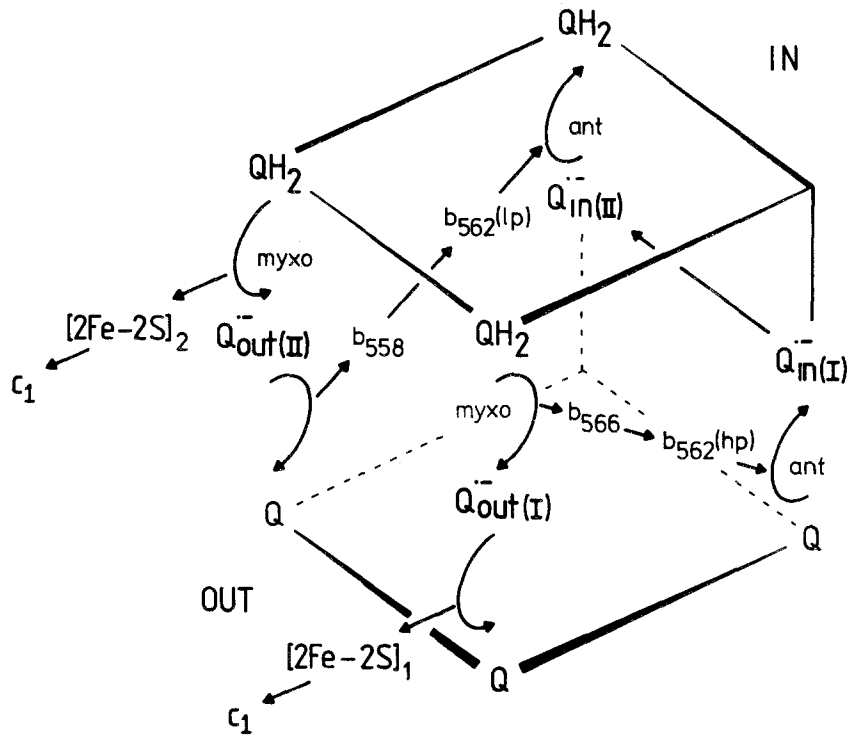


Fig. 5. Diagram showing a three-dimensional representation of a double Q cycle that describes electron transfer in the dimeric QH_2: cytochrome *c* oxidoreductase. The meaning of the various symbols is the same as in Fig. 1. The front and posterior face, carrying the cytochrome *b* polypeptides, represent the membrane fractions of protomers I and II, respectively. Indices I or II indicate that $Q^{\cdot -}$ is located in protomers I and II, respectively. Cytochrome *b*-566 is placed arbitrarily in protomer I, and cytochrome *b*-558 in protomer II. It is equally possible that cytochrome *b*-558 is in protomer I and cytochrome *b*-566 in protomer II, or that a single type of cytochrome *b*-566/558 is present in both protomer I and protomer II (see also text). Also, the Fe-S cluster called cluster 1 on the basis of the EPR spectrum is not necessarily the Fe-S cluster located in protomer I. The same uncertainty holds for Fe-S cluster 2. In contrast to the previous proposals (de Vries *et al.*, 1982a, 1983; Slater, 1983), the oxidation of QH_2 in protomer I is not inhibited by antimycin.

is the electron donor to the one Fe-S cluster, viz. $[2Fe\text{-}2S]_2$, and $(D)Q^{\cdot -}_{out}$ to the other one, $[2Fe\text{-}2S]_1$. As to the latter, its reduction involves the reactions (cf. Fig. 5)

$$(D)QH_2 + b\text{-}566^{3+} \longrightarrow (D)Q^{\cdot -}_{out(I)} + b\text{-}566^{2+} \quad (7)$$

$$(D)Q^{\cdot -}_{out(I)} + [2Fe\text{-}2S]^{2+}_{1} \longrightarrow (D)Q + [2Fe\text{-}2S]^{1+}_{1} \quad (8)$$

Since $DQ^{\cdot -}_{out(I)}$ is not formed, reaction (8) does not occur, but neither will reaction (7) to any measurable extent because it is thermodynamically too unfavorable (Kröger, 1976; Slater, 1981).

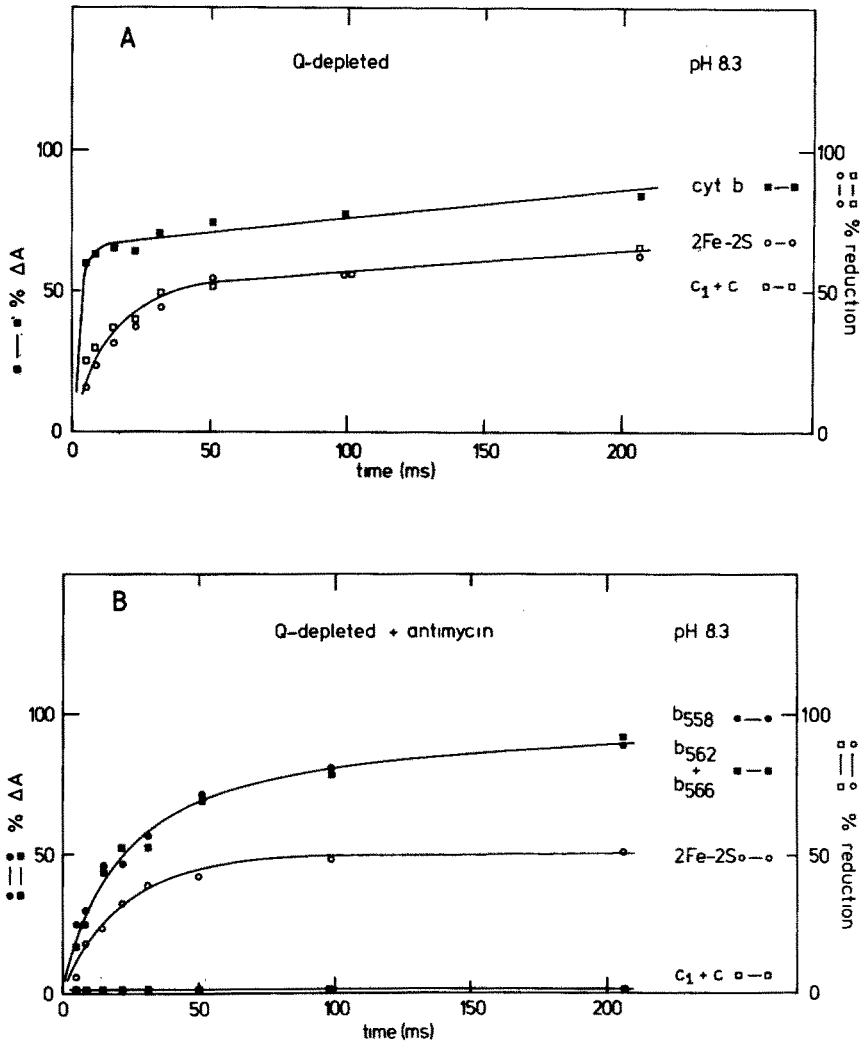


Fig. 6. Kinetics of reduction of the prosthetic groups of QH_2 : cytochrome *c* oxidoreductase by DQH_2 in ubiquinone-depleted submitochondrial particles (pH 8.3). See de Vries *et al.* (1983) for the complete details. (A) All cytochrome *b*-562 is reduced within 5 ms. The increase in the reduction of cytochrome *b* after 5 ms is due to reduction of cytochrome *b*-566/558. (B) With antimycin present, half reduction of the cytochrome *b* is obtained after 25–30 ms, but the reduction of the cytochromes does not follow a first-order exponential as in ubiquinone-containing particles (cf. de Vries *et al.*, 1981a, 1982a). The reduction of the Fe-S clusters is biphasic, both in the presence and absence of antimycin. The rapid phase comprises 50% of the total amount of EPR-detectable Fe-S and has $t_{1/2}$ = 11 and 15 ms in (A) and (B), respectively. Note that in (B) no net reduction of cytochromes c_1 (+ *c*) is observed. (Reprinted with permission of *Biochim. Biophys. Acta.*)

It was further observed by de Vries *et al.* (1983) that the rate of reduction of the Fe-S clusters is dependent on pH in a manner indicating that the rate of reduction of the one is pH dependent and of the other is not, suggesting that the rate is determined by the redox properties of, for example, the $QH^-/Q^{\cdot -}$ couple (cf. Rich and Bendall, 1980; Rich, 1981) for $[2Fe\text{-}2S]_2$ and by that of the $Q^{\cdot -}/Q$ couple for $[2Fe\text{-}2S]_1$.

The finding that in Q-depleted particles with antimycin present (Fig. 6) cytochromes *b*-566/558 and *b*-562 are not reduced in a single phase, whereas they are in Q-containing particles, is difficult to explain in any of the schemes of Fig. 1, especially, since the thermodynamic argument mentioned above (cf. Rich, 1984) no longer applies. This observation suggests that reduction of all the species of cytochrome *b* requires the operation of two, independent, Q_{out} routes as depicted in Fig. 5.

The Triphasic Reduction of Cytochrome b-562

When submitochondrial particles or succinate : cytochrome *c* reductase are mixed with DQH_2, cytochrome *b*-562 is reduced in two phases separated by a lag time (Jin *et al.*, 1981; Tsou *et al.*, 1982; de Vries *et al.*, 1981b, 1982a, 1983; Fig. 7a). The initial rapid phase (completed within 5 ms) is ascribed to reduction of *b*-562(hp) and the subsequent relatively slow phase to reduction of *b*-562(lp). The lag time between the two phases decreases when the pH or concentration of DQH_2 is raised, whereas the rate of the second phase increases. The slow phase indicating the reduction of *b*-562(lp) starts when the Rieske Fe-S clusters and cytochrome c_1 are (nearly) fully reduced and therefore the reduction of *b*-562(lp) occurs via the reversal of reaction (4). It was also observed that the amount of the antimycin-sensitive $Q_{in}^{\cdot -}$ increases in parallel with the reduction of *b*-562(hp) *and b*-562(lp) (cf. Fig. 7a) and that during the lag period (which is more clearly visible in the traces recorded at pH values below 8.3; cf. Fig. 1 of de Vries *et al.*, 1983) the amount of $Q_{in}^{\cdot -}$ decreases.

In preparations treated with BAL ($+O_2$) or inhibited by myxothiazol the kinetics of reduction of cytochrome *b*-562 were found to differ in three respects compared to the kinetics observed in uninhibited preparations (cf. Figs. 7A and 7B): (1) The lag time between the two reduction phases is apparently absent; (2) the amount of $Q_{in}^{\cdot -}$ steadily increases with time and does not show a transient behavior; (3) the amount of *b*-562 reduced within 5 ms comprises 60% of the total absorbance change at 562–575 nm and was therefore ascribed to reduction of *b*-562(lp).

On the basis of the above-mentioned findings and others, de Vries *et al.* (1982a, 1983) concluded that the reduction of *b*-562(hp) is inhibited by

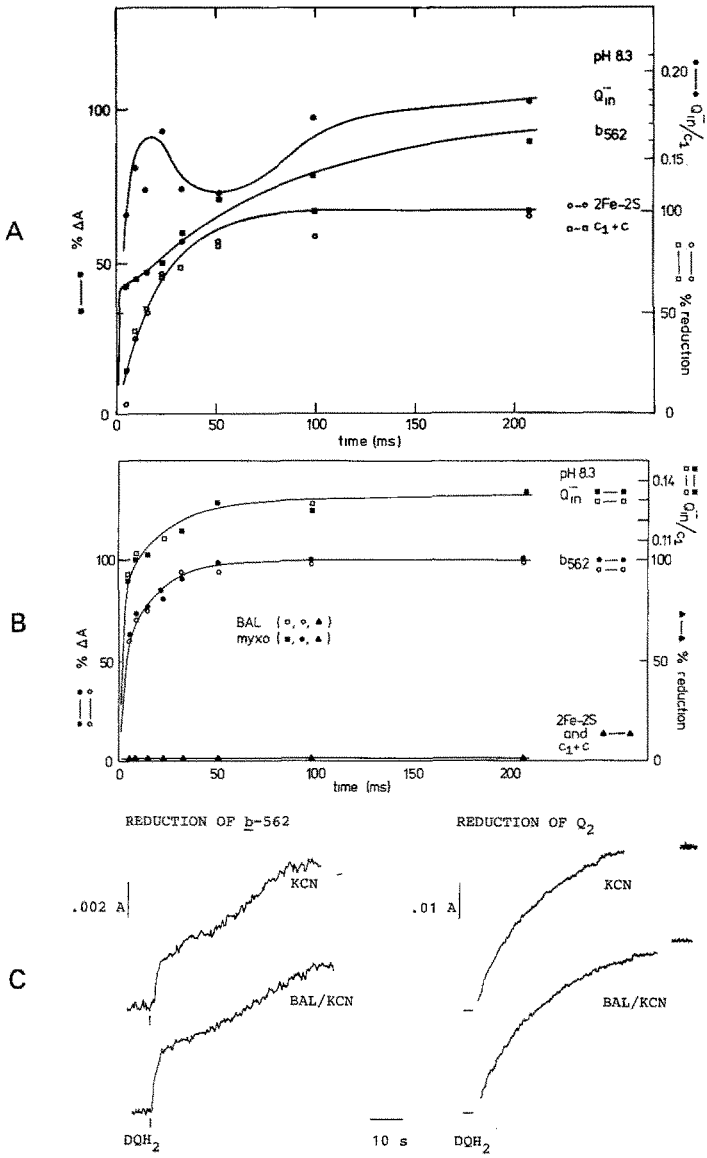


Fig. 7. Examples of the triphasic reduction of cytochrome *b*-562 (A, B, C), a simulation of the triphasic reduction according to the $n = 1$ and $n = 2$ models discussed in the text (D), and the results of the potentiometric titrations of cytochrome *b*-562(lp) with the fumarate/succinate couple (E). A and B: Kinetics of reduction of the prosthetic groups and the formation of the antimycin-sensitive Q_{in}^{-} in untreated submitochondrial particles (A) and in particles treated with BAL ($+O_2$) (B). See Figs. 1 and 6 in de Vries *et al.* (1983) for the experimental details. Note that the transient in the formation of Q_{in}^{-} is not seen in (B). C: traces of the reduction of cytochrome *b*-562 (562–575 nm) and Q_2 (300–284 nm) by DQH_2 in mitoplasts [0.5 mg/ml in 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 8.4)], freed of cytochrome *c*. $[Q_2] = 10\,\mu M$;

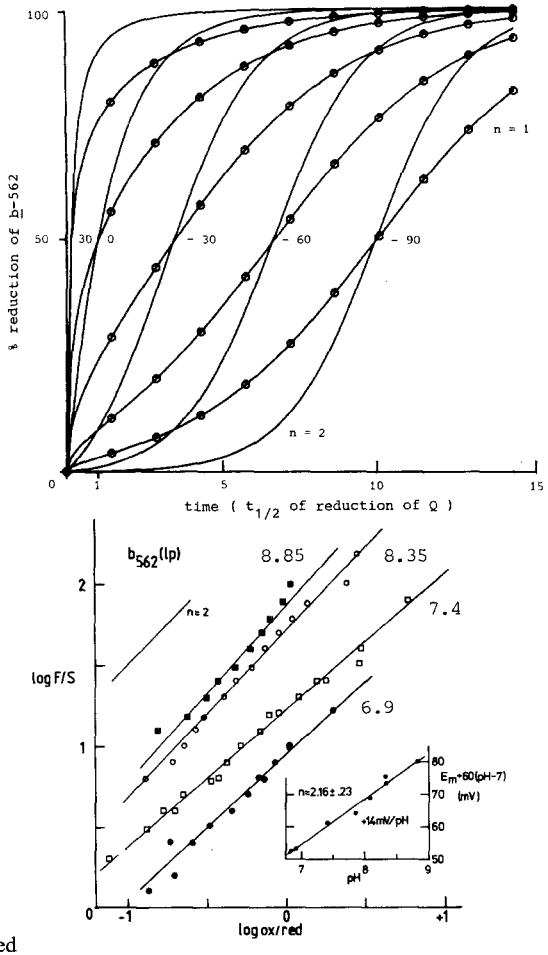


Fig. 7. Continued

$[DQH_2]$ = 80 μM; [KCN] = 1 mM; temperature 2°C; DQH_2 was added to a cuvet mounted in a magnetic stirrer assembly (mixing time 1–2 s); cytochrome c_1, (*c*) and *a* are reduced within the time of mixing (viz. in the untreated mitoplasts); the wavelength pair 300–284 nm is isosbestic for changes in the redox state of DQH_2. The nonenzymic rate of reduction of Q_2 is 10% at pH 8.4 D: Simulation of the triphasic reduction of cytochrome *b*-562. (○) Simulation on the basis of the model given by Eqs. (12) and (13), i.e., n = 1, assuming a single type of *b*-562; (——) simulation on the basis of Eq. (14), i.e., n = 2, assuming the same values of E_m for *b*-562(hp) and *b*-562(lp). The numbers refer to the difference $E_m(Q_2) - E_m(b\text{-}562)$ expressed in mV; the unit of time used on the *x*-axis is the time required to obtain 50% reduction of the Q pool (i.e., at 5 the Q pool is reduced for 96.9%, at 10 for 99.9%). Half reduction of *b*-562(lp), experimentally, occurs at 2–3 on such a time scale. E: Potentiometric titrations of cytochrome *b*-562 in submitochondrial particles with the fumarate/succinate redox couple at different pH values. The anaerobic titrations were performed in the oxidative (starting with 5 mM succinate) and in the reductive (starting with 150 mM fumarate) direction in the presence of 4 mM KCN. Under all conditions *b*-562(hp) was (remained) reduced. The numbers refer to the pH of the experiment. The average value of n, obtained at the various pH's, equals 2.16. Note that the E_m of *b*-562(lp) varies by −46 (60–14) mV/pH. E_{m7} = 55 mV. (Figures 7A and 7B are reprinted with permission of *Biochim. Biophys. Acta.*)

myxothiazol or treatment with BAL ($+O_2$) and proposed that the pathway of reduction of *b*-562(hp) in the uninhibited enzyme is *not* via the reversal of reaction (4) but rather via reaction (7), occurring in protomer I of the dimer (Fig. 5) followed by

$$b\text{-}566^{2+} + b\text{-}562(\text{hp})^{3+} \longrightarrow b\text{-}566^{3+} + b\text{-}562(\text{hp})^{2+} \quad (9)$$

and in order to account for the formation of $Q_{in}^{\cdot-}$ within 5 ms, the equilibrium

$$b\text{-}562(\text{hp})^{2+} + Q \rightleftharpoons b\text{-}562(\text{hp})^{3+} + Q_{in(I)}^{\cdot-} \quad (10)$$

When, however, the reduction of *b*-562 was studied in mitoplasts with a large Q pool present (Fig. 7C) the pattern of reduction is essentially the same in untreated mitoplasts and mitoplasts treated with BAL ($+O_2$), at least at pH 8.4, although there is a clear difference at pH 6.2 (de Vries and Marres, 1985). Thus the proposal that the reduction of *b*-562(hp) occurs via the Q_{out} route in protomer I within 5 ms is not true in general and the reduction may equally well proceed via the reversal of reaction (4). This is not meant to imply that reactions (7)–(10) would not occur at all and evidence for such a pathway of electron transfer is shown in the previous section. Rather it means that the rates of oxidation of quinol in protomer I and protomer II are about the same ($t_{1/2}$ = 10–15 ms) and that the oxidation of quinol in protomer I via reactions (7)–(10) is not completed within 5 ms as originally proposed (de Vries *et al.*, 1982a, 1983).

Kinetic and Thermodynamic Properties of the b-562/Q_{in} domain

The traces showing the triphasic reduction of *b*-562 (Fig. 7C) are obtained in mitoplasts in which the size of the Q pool has been increased by the addition of Q_2 to about 200 Q_2/c_1. Due to this large Q pool the rate of net reduction of *b*-562 by DQH_2 decreases, whereas the length of the lag period increases (de Vries and Marres, unpublished observations). These two phenomena are understandable in terms of a (rapid) equilibrium between *b*-562 and the Q pool (Kröger and Klingenberg, 1970, 1973). The reduction of Q_2 by DQH_2 can be spectroscopically monitored and follows quite well a (pseudo) first-order exponential as expected from the concentrations used in the experiment and the equation

$$DQH_2 + Q_2(\text{pool}) \xrightarrow{b\text{-}562} DQ + Q_2H_2(\text{pool}) \quad (11)$$

Assuming that a single *b*-562 is in equilibrium with the Q_2 pool, the following equations hold:

$$Q_2H_2 + 2b\text{-}562^{3+} \rightleftharpoons Q + 2b\text{-}562^{2+} + 2H_{in}^{+} \quad (12)$$

and

$$\log K = \log Q/QH_2 \cdot (b^{2+}/b^{3+})^2 = [E_m(Q_2) - E_m(b\text{-}562)]/30 \quad (13)$$

The difference $E_m(Q_2) - E_m(b\text{-}562)$ can be directly estimated from traces as in Fig. 7C and equals about -10 mV at pH 8.4.

A simulation on the basis of this simple model is shown in Fig. 7D. Although this model predicts a lag in the reduction of *b*-562, such a lag would only be clearly visible when the $E_m(b\text{-}562)$ is at least 60 mV lower than $E_m(Q_2)$, in contrast to the experimental difference in E_m. In addition, the model predicts an apparent multiphasic pattern of reduction of the single *b*-562, but the shape of the simulation curves is totally different from the experimental traces (Figs. 7A–7C).

The introduction of two species of *b*-562, viz. *b*-562(hp) and *b*-562(lp), with appropriate values of E_m does not yield a satisfactory fit to the data either (not shown). The main reason for this is that the degree of reduction of, particularly, *b*-562(lp) increases much more steeply (i.e., $n \gg 1$) than predicted by Eq. (13). In fact, a direct plot of the redox state of Q_2 vs. that of *b*-562 indicates that $n = 3$–4. There is no simple rationale for such a high value of n other than that the implicit assumption of true equilibrium [Eqs. (12) and (13)] is not valid.

Indeed, considering the kinetics of formation of $Q_{in}^{\cdot-}$, true equilibrium may not be obtained. One expects to see an increase in the amount of $Q_{in}^{\cdot-}$ until the Q pool is half reduced followed by a decrease on further reduction of the Q pool. Assuming that the relative degrees of reduction of Q_2 and *b*-562 in the experiments in Fig. 7C are the same as in the experiments in Figs. 7A and 7B, one may conclude that the Q_{10} pool is completely reduced after 200 ms. Thus, no significant amount of $Q_{in}^{\cdot-}$ is expected to be present after 200 ms, in contrast to the amount found experimentally. The transient in the formation of $Q_{in}^{\cdot-}$ (between 10–50 ms, Fig. 7A) corresponds roughly to the period in which the redox state of the Q_{10} pool changes from less than half reduced to more than half reduced. Suprisingly, such a transient is not observed in the particles inhibited by BAL ($+O_2$). It seems therefore that only part of the amount of $Q_{in}^{\cdot-}$ present is in thermodynamic equilibrium with the Q pool. The other part, which kinetically follows the reduction of *b*-562(hp) and *b*-562(lp), is apparently not in rapid equilibrium with the Q_{10} pool for reasons that may be related to the mechanism by which it is formed (cf. Glaser *et al.*, 1984; Robertson *et al.*, 1984a).

In similar types of experiments performed in chromatophores the reduction of *b*-562 via the Q_{in} route has been studied at high pH or in preparations (partially) depleted of ubiquinone (Glaser *et al.*, 1984; Roberston *et al.*, 1984a). The extent of reduction of *b*-562 after a flash was found to follow an $n = 2$ Nernst curve (see also Matsuura and Dutton, 1981). In potentiometric titrations performed with the fumarate/succinate redox couple, *b*-562(lp) also titrates according to $n = 2$ [Fig. 7E; Wikström and Berden (1972); de Vries (1983)], in contrast to the titrations performed in the presence of mediators

(Fig. 4). Although $n = 2$ may indicate that no true equilibrium is obtained, the system is in equilibrium in the sense that the data points from oxidative and reductive titrations fall on the same line. In these experiments (Fig. 7E) *b*-562(hp) was already reduced at the highest values of E_h that can be obtained with fumarate/succinate couple.

The value of $n = 2$ for the reduction of *b*-562 can be rationalized in the following model. It is proposed that the two cytochromes *b*-562(hp) and *b*-562(lp) act as a pair, for instance, according to

$$QH_2 + b\text{-}562(hp)^{3+} - b\text{-}562(lp)^{3+} \rightleftharpoons Q + b\text{-}562(hp)^{2+} - b\text{-}562(lp)^{2+} + 2H^+_{in} \quad (14)$$

i.e., reduction (oxidation) of the one species of *b*-562 is coupled *mechanistically* to reduction (oxidation) of the other species. Such a mechanistic constraint can be any constraint, preventing one or several of the possible routes of electron transfer by which the system equilibrates according to the respective E_m values of *b*-562(hp), *b*-562(lp), Q pool, Q_{in}, $Q_{in}/Q_{in}^{\cdot-}$, and $Q_{in}^{\cdot-}/QH_2$.

This model yields values of n close to 2 (but not $n = 3$–4) for the reduction of *b*-562(hp) and *b*-562(lp) by quinol. A simulation of the kinetic traces (Fig. 7D) with E_m(*b*-562(hp)) = E_m(*b*-562(lp)) shows that a lag in the reduction of *b*-562 is obtained as soon as its midpoint potential is lower than that of the Q pool, in agreement with the experiment. However, the pattern of reduction of *b*-562 does not show the apparent multiphasic behavior as before. Therefore, in order to improve the simulation one has to assume further that the values of the midpoint potentials of *b*-562(hp) and *b*-562(lp) are different.

Although the $Q_{in}^{\cdot-}$ is left out of Eq. (14), this does not imply that the reaction is a concerted one. Thus reaction (14) can equally well be split in two reactions with the $Q_{in}^{\cdot-}$ as an intermediate. As long as one of the possible routes of electron transfer is prevented by some constraint, values of n close to 2 will be observed. The double Q cycle (Fig. 5) contains such a constraint, namely that QH_2 can *directly* reduce *b*-562(lp) but not *b*-562(hp), but one can imagine many more possible constraints. Furthermore, Eq. (14) is not meant to imply that only a single molecule of quinone can be accommodated in the Q-binding domain close to the cytochrome *b*-562 pair. In fact, the studies by de Vries *et al.* (1980, 1982b), de Vries and Dutton (1985), and Robertson *et al.* (1984b) imply that the number of Q-binding sites in the Q_{in}-binding domain is one per monomeric enzyme and thus two per dimeric enzyme.

During steady-state electron transfer the Q_{in} route acts as a quinone-reducing route. It is the natural function of the bacterial reaction center and the succinate : Q oxidoreductase to reduce Q to QH_2. The structure of their Q-binding domains is remarkably similar. In both enzymes a Q–Q pair or a

$Q^{\cdot -}$–$Q^{\cdot -}$ pair (and other possible pairs dependent on pH and redox potential) is present either in close contact with an Fe atom as in the reaction center (cf. Crofts and Wraight (1983) and references therein) or with an Fe-S cluster, in the succinate: Q oxidoreductase (Ingledew *et al.*, 1976; Salerno *et al.*, 1977; Salerno and Ohnishi, 1980). In both enzymes the semiquinones are very stable. Also $Q_{in}^{\cdot -}$ is stable. However, $Q_{in}^{\cdot -}$ is (magnetically) isolated and not in close contact with another $Q^{\cdot -}$ or *b*-562, as has to be concluded from its slow rate of relaxation in EPR experiments performed from 4.2 K to 37°C (de Vries *et al.*, 1980, 1981a, 1982b). Possibly, the role played by the $Q^{\cdot -}$–$Q^{\cdot -}$ pairs in the reduction of Q to QH_2 is played by the *b*-562(hp)–*b*-562(lp) pair at the quinone-reducing site in the QH_2: cytochrome *c* oxidoreductase.

Steady-State Oxidation of QH_2 Requires a Dimeric Enzyme

Recently, Linke *et al.* (1985) have presented reconstitution experiments with the QH_2: cytochrome *c* oxidoreductase from *Neurospora crassa*. Starting with a dimeric bc_1 subcomplex (Hovmöller *et al.*, 1981; Li *et al.*, 1981; Karlsson *et al.*, 1983) lacking the core proteins and the Fe-S subunit, they reconstituted an active dimeric QH_2: cytochrome *c* oxidoreductase (Wingfield *et al.*, 1979; Weiss and Kolb, 1979) by adding back a dimeric or a monomeric core-complex in the presence of excess Fe-S protein. It was found that one dimeric or two monomeric core-complex(es) per dimeric reductase is (are) required to reconstitute maximal activity. Likewise, in the presence of excess core-complex, two Fe-S proteins per dimeric reductase are necessary to regain full reconstitutive activity, and since the relation between the amount of added subunit and reconstituted activity was found to be sigmoidal, this indicates that only the dimeric QH_2: cytochrome *c* oxidoreductase is catalytically active (Linke *et al.*, 1985).

Similar conclusions were arrived at by Graan and Ort (1985) for the PQH_2: plastocyanin oxidoreductase from spinach chloroplasts. They found that one molecule of DBMIB per dimeric b_6/f complex is sufficient to completely inhibit the steady-state oxidation of DQH_2, again indicating that the enzyme functions as a dimer.

Concluding Remarks

Several independent lines of evidence suggest that the QH_2: cytochrome *c* oxidoreductase is both structurally and functionally a dimer. The molecular weight of the mitochondrial enzyme in solution and incorporated in an artificial phospholipid bilayer corresponds to that of a dimer. There is also evidence that the b_6/f complex is in a dimeric state (Hurt and Hauska, 1981; Mörschel and Staehelin, 1983). The results of the reconstitution experiments

mentioned above and the finding that a single DBMIB per dimeric b_6/f complex completely inhibits turnover imply that only the dimeric enzyme is catalytically active. The latter finding is, indeed, very surprising considering that the enzyme from mitochondria (cf. Nalecz and Azzi, 1985) or photosynthetic bacteria (van den Berg *et al.*, 1979) requires the binding of *two* molecules of inhibitor per dimer for complete inhibition. In fact, this finding argues against a dimer as the functional unit, unless one assumes that the binding of the inhibitors is strongly positive cooperative.

Spectroscopic studies reveal the existence of two types of Fe-S clusters present in equal amounts and two types of cytochrome *b*-562, i.e., *b*-562(hp) and *b*-562(lp) (also present in a 1 : 1 molar ratio), with different extinction coefficients and different midpoint potentials, indicating that the enzyme is dimeric in a natural membrane. The analysis of the pre-steady state kinetics of reduction of the prosthetic groups by DQH_2 shows separate phases of reduction of the two Fe-S clusters and of the two species of cytochrome *b*-562.

All of the above-mentioned results are incorporated in the scheme of the double Q cycle shown in Fig. 5, illustrating the pathways of electron transfer in the dimeric QH_2 : cytochrome *c* oxidoreductase. This scheme differs in two respects from the original proposal (de Vries *et al.*, 1982a, 1983; Slater, 1983) as discussed above, but all the (chemical) reactions that take place are the same as in the scheme previously proposed. The double Q cycle is a Q cycle. The arguments that the Q-cycle scheme describes the pathway of electron transfer in QH_2 : cytochrome *c* oxidoreductase more adequately than the *b*-cycle scheme does, apply equally well to the comparison between a double Q-cycle scheme and a double *b*-cycle scheme.

The double Q cycle of Fig. 5 is a construct of the two Q cycles shown in Figs. 1B and 1D. It is asymmetrical in the sense that the Fe-S cluster in protomer II is reduced by quinol and the Fe-S cluster is protomer I by semiquinone. Likewise, *b*-562(hp) is oxidized by quinone and *b*-562(lp) is oxidized by the product of the foregoing reaction, semiquinone. It is exactly the coupling of these two reactions that makes the enzyme functionally dimeric.

One can imagine more symmetrical variants of a double Q cycle, for instance, the construct of the two Q cycles shown in Figs. 1A and 1B. This symmetrical variant can be considered as the dimeric extension of the double-turnover Q cycle proposed by Crofts *et al.* (1983). In such a variant both Fe-S clusters are reduced by quinol, but on the basis of the experimental observations, I prefer the version of Fig. 5. However, in the more symmetrical variant the asymmetry with respect to the reactions catalyzed by the two species of cytochrome *b*-562 still remains and also this variant requires the cooperative action of the two monomeric halves. In this respect, a symmetrical double Q cycle is essentially different from a double-turnover Q cycle.

The molecular basis that the QH_2: cytochrome *c* oxidoreductase functions as a cooperative dimer is perhaps related to the fact that, at the quinone-reducing (Q_{in}) site, the electron transfer changes from a one-electron transfer process to a two-electron transfer process. Although at the quinol-oxidizing (Q_{out}) site a similar change occurs, no cooperative dimeric enzyme is required for the oxidation of (QH_2), possibly because the Q_{out}-binding domain itself contains a cooperative electron-accepting pair, i.e., Fe-S/*b*-566.

Analogous to the quinone-reducing bacterial reaction center and the succinate: Q oxidoreductase, which both contain a Q–Q pair involved in the reduction of Q to QH_2, and in view of the different properties of $Q_{in}^{\cdot-}$ with respect to those of the semiquinone pairs, it is proposed that a functional *b*-562(hp)–*b*-562(lp) pair is required to catalyze the reduction of Q to QH_2 at the Q_{in}-binding domain. Such a pair is only active in a functionally dimeric QH_2: cytochrome *c* oxidoreductase.

Acknowledgment

I like to thank Drs. S. P. J. Albracht, J. A. Berden, C. A. M. Marres, E. C. Slater, and Q. S. Zhu for their numerous suggestions and contributions in the period of my stay at the B. C. P. Jansen Institute. I am grateful to Drs. W. R. Hagen and W. R. Dunham for providing me with the P-band spectra. The help and support of H. Plat with the ultracentrifuge experiments and the hospitality of the Landbouw Hogeschool in Wageningen in the performance of some of these experiments are gratefully acknowledged as well as the assistance of B. van Swol in writing the computer simulation program. I also thank Drs. J. Whitmarsh, D. R. Ort, T. Graan, and H. Weiss for making available to me their papers and Ms. G. E. E. van Noppen for reading and correcting the manuscript.

References

Aasa, R., Ellsfolk, N., Rönnberg, M., and Vänngård, T. (1981). *Biochim. Biophys. Acta* **670**, 170–175.

Albracht, S. P. J., van Verseveld, H. W., Hagen, W. R., and Kalkman, M. L. (1980). *Biochim. Biophys. Acta* **593**, 173–186.

Bendall, D. S. (1982). *Biochim. Biophys. Acta* **683**, 119–152.

Berden, J. A., and Opperdoes, F. R. (1972). *Biochim. Biophys. Acta* **267**, 7–14.

Berden, J. A., Opperdoes, F. R., and Slater, E. C. (1972). *Biochim. Biophys. Acta* **256**, 594–599.

Bergström, J., and Vänngård, T. (1982). *Biochim. Biophys. Acta* **682**, 452–456.

Bowyer, J. R., and Crofts, A. R. (1978). In *Frontiers of Biological Energetics* (Dutton, P. L., Leigh, J. S., and Scarpa, A., eds.), Vol. 1, Academic Press, New York, pp. 326–333.

Bowyer, J. R., and Trumpower, B. L. (1981). In *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P., and Hinkle, P., eds.), Addison-Wesley, Reading, Massachusetts, pp. 105–122.

Bowyer, J. R., Edwards, C. A., and Trumpower, B. L. (1981a). *FEBS Lett.* **126**, 93–97.

Bowyer, J. R., Meinhardt, S. W., Tierney, G. V., and Crofts, A. R. (1981b). *Biochim. Biophys. Acta* **635**, 167–186.
Bowyer, J. R., Edwards, C. A., Ohnishi, T., and Trumpower, B. L. (1982). *J. Biol. Chem.* **257**, 8321–8330.
Briquet, M., and Goffau, A. (1981). *Eur. J. Biochem.* **117**, 333–339.
Briquet, M., Purnelle, B., Faber, A. M., and Goffau, A. (1981). *Biochim. Biophys. Acta* **638**, 116–119.
Chance, B. (1958). *J. Biol. Chem.* **233**, 1223–1229.
Convent, B., and Briquet, M. (1978). *Eur. J. Biochem.* **82**, 473–481.
Crofts, A. R., and Wraight, C. A. (1983). *Biochim. Biophys. Acta* **726**, 149–186.
Crofts, A. R., Meinhardt, S. W., Jones, K. R., and Snozzi, M. (1983). *Biochim. Biophys. Acta* **723**, 202–218.
Deul, D. H., and Thorn, M. B. (1982). *Biochim. Biophys. Acta* **59**, 426–436.
De Vries, S. (1983). "Pathway of Electrons in QH_2:cytochrome *c* oxidoreductase", Ph.D. Thesis, University of Amsterdam, Rodopi, Amsterdam.
De Vries, S., and Albracht, S. P. J. (1979). *Biochim. Biophys. Acta* **546**, 334–340.
De Vries, S., and Dutton, P. L. (1985). In Achievements and Perspectives of Mitochondrial Research (Quagliariello, E., Slater, E. C., Saccone, C. and Kroon, A. M., eds.), Elsevier, Amsterdam, Vol. I, pp. 103–110.
De Vries, S., Albracht, S. P. J., and Leeuwerik, F. J. (1978). In *Mechanisms of Oxidizing Enzymes* (Singer, T. P., and Ondarza, R. N., eds.), Elsevier/North-Holland, New York, pp. 181–188.
De Vries, S., Albracht, S. P. J., and Leeuwerik, F. J. (1979). *Biochim. Biophys. Acta* **546**, 316–333.
De Vries, S., Berden, J. A., and Slater, E. C. (1980). *FEBS Lett.* **122**, 143–148.
De Vries, S., Albracht, S. P. J., Berden, J. A., and Slater, E. C. (1981a). *J. Biol. Chem.* **256**, 11996–11998.
De Vries, S., Albracht, S. P. J., den Bakker, C. W., Berden, J. A., and Slater, E. C. (1981b). In *Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria* (Palmieri, F., Quagliariello, E., Siliprandi, N., and Slater, E. C., eds.), Elsevier/North-Holland, Amsterdam, pp. 173–177.
De Vries, S., Albracht, S. P. J., Berden, J. A., and Slater, E. C. (1982a). *Biochim. Biophys. Acta* **681**, 41–53.
De Vries, S., Berden, J. A., and Slater, E. C. (1982b). In *Function of Quinones in Energy-Conserving Systems* (Trumpower, B. L., ed.), Academic Press, New York, pp. 235–246.
De Vries, S., Albracht, S. P. J., Berden, J. A., Marres, C. A. M., and Slater, E. C. (1983). *Biochim. Biophys. Acta* **723**, 91–103.
De Vries, S. and Marres, C. A. M. (1985). In *Proton Pumping in Respiration and Photosynthesis*, Table Ronde Roussel–Uclaf, no. 52, Paris, pp. 46–48.
Dutton, P. L. (1978). *Methods Enzymol.* **54**, 411–435.
Dutton, P. L., and Jackson, J. B. (1972). *Eur. J. Biochem.* **30**, 609–613.
Dutton, P. L., and Prince, R. C. (1978). In *The Photosynthetic Bacteria* (Clayton, R. K., and Sistrom, W. R., eds.), Academic Press, New York, pp. 523–584.
Dutton, P. L., Lindsay, J. G., and Wilson, D. F. (1972). In *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E., and Siliprandi, N., eds.), Academic Press, New York, pp. 165–176.
Dutton, P. L., Wilson, D. F., and Lee, C. P. (1970). *Biochemistry* **9**, 5077–5082.
Erecínska, M., and Wilson, D. F. (1972). *FEBS Lett.* **24**, 269–272.
Gabellini, N., and Hauska, G. (1983). *FEBS Lett.* **153**, 146–150.
Gibson, J. F., Hall, D. O., Thornley, J. H. M., and Whatley, F. R. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **56**, 987–990.
Glaser, E. G., Meinhardt, S. W., and Crofts, A. R. (1984). *FEBS Lett.* **178**, 336–342.
Graan, T., and Ort, D. R. (1985), in press.
Hagen, W. R. (1981). *J. Magn. Reson.* **44**, 447–469.
Hagen, W. R. (1982). In "Electron Paramagnetic Resonance of Metalloproteins", Ph.D. Thesis, University of Amsterdam, Rodopi, Amsterdam.
Hagen, W. R., Hearshen, D. O., Harding, L. J., and Dunham, W. R. (1985a). *J. Magn. Reson.* **61**, 233–244.

Hagen, W. R., Hearshen, D. O., Sands, R. H., and Dunham, W. R. (1985b). *J. Magn. Reson.* **61**, 220–232.
Hatefi, Y., Haavik, A. G., and Jurtshuk, P. (1981). *Biochim. Biophys. Acta* **52**, 106–118.
Hauska, G., Hurt, E., Gabellini, N., and Lockau, W. (1983). *Biochim. Biophys. Acta* **726**, 93–133.
Higuti, T., Mizuno, S., and Muraoka, S. (1975). *Biochim. Biophys. Acta* **396**, 36–47.
Hovmöller, P., Gothe, A., and Weiss, H. (1981). *FEBS Lett.* **123**, 118–122.
Hurt, E., and Hauska, G. (1981). *Eur. J. Biochem.* **117**, 591–599.
Hurt, E., Hauska, G.,, and Malkin, R. (1981). *FEBS Lett.* **134**, 1–5.
Ingledew, W. J., Salerno, J. C., and Ohnishi, T. (1976). *Arch. Biochem. Biophys.* **177**, 176–184.
Jin, Y. Z., Tang, H. L., Li, S. L., and Tsou, C. L. (1981). *Biochim. Biophys. Acta* **637**, 551–554.
Karlsson, B., Hovmöller, S., Weiss, H., and Leonard, K. (1983). *J. Mol. Biol.* **165**, 287–302.
Konstantinov, A. A., and Ruuge, E. K. (1977). *FEBS Lett.* **81**, 137–141.
Kröger, A. (1976). *FEBS Lett.* **65**, 278–280.
Kröger, A., and Klingenberg, M. (1970). *Vitam. Horm. (N.Y.)* **28**, 533–574.
Kroger, A., and Klingenberg, M. (1973). *Eur. J. Biochem.* **39**, 313–323.
Leigh, J. S., Jr., and Erecínska, M. (1975). *Biochim. Biophys. Acta* **387**, 95–106.
Li, Y., De Vries, S., Leonard, K., and Weiss, H. (1981). *FEBS Lett.* **135**, 277–280.
Linke, P., Gothe, A. and Weiss, H. (1985), In Achievements and Perspectives of Mitochondrial Research, Vol. I, (Quagliariello, E., Slater, E. C., Saccone, C. and Kroon, A. M., eds.), Elsevier, Amsterdam, pp. 73–82.
Malkin, R. (1981). *FEBS Lett.* **131**, 169–172.
Marres, C. A. M., de Vries, S., and Slater, E. C. (1982). *Biochim. Biophys. Acta* **681**, 323–326.
Matsuura, K., and Dutton, P. L. (1981). In *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P., and Hinkle, P., eds.), Addison-Wesley, Reading, Massachusetts, pp. 259–270.
Matsuura, K., Bowyer, J. R., Ohnishi, T., and Dutton, P. L. (1983). *J. Biol. Chem.* **258**, 1571–1579.
Meinhardt, S. W., and Crofts, A. R. (1983). *Biochim. Biophys. Acta* **723**, 219–230.
Mitchell, P. (1975a). *FEBS Lett.* **56**, 1–6.
Mitchell, P. (1975b). *FEBS Lett.* **59**, 137–139.
Mitchell, P. (1976). *J. Theor. Biol.* **62**, 327–367.
Mörschel, E., and Staehelin, L. A. (1983). *J. Cell. Biol.* **97**, 301–310.
Nalecz, M. J., and Azzi, A. (1985). *Arch. Biochem. Biophys.* **240**, 921–931.
Nalecz, M. J., Bolli, R., and Azzi, A. (1985). *Arch. Biochem. Biophys.* **236**, 619–628.
Ohnishi, T., and Trumpower, B. L. (1980). *J. Biol. Chem.* **255**, 3278–3284.
Orme-Johnson, N. R., Hansen, R. E., and Beinert, H. (1971). *Biochem. Biophys. Res. Commun.* **45**, 871–878.
Papa, S., Guerrieri, F., Lorusso, M., Izzo, G., and Capuano, F. (1982). In *Function of Quinones in Energy-Conserving Systems* (Trumpower, B. L., ed.), Academic Press, New York, pp. 527–539.
Prince, R. C. (1983). *Biochim. Biophys. Acta* **723**, 133–138.
Prince, R. C., and Dutton, P. L. (1976). *FEBS Lett.* **65**, 117–119.
Prince, R. C., O'Keefe, D. P., and Dutton, P. L. (1982). In *Electron Transport and Photophosphorylation* (Barber, J., ed.), Elsevier, Amsterdam, pp. 197–248.
Riccio, P., Schägger, H., Engel, W. D., and von Jagow, G. (1977). *Biochim. Biophys. Acta* **459**, 250–262.
Rich, P. R. (1981). *FEBS Lett.* **130**, 173–178.
Rich, P. R. (1983). *Biochim. Biophys. Acta* **722**, 271–280.
Rich, P. R. (1984). *Biochim. Biophys. Acta* **768**, 53–79.
Rich, P. R., and Bendall, D. S. (1980). *Biochim. Biophys. Acta* **592**, 506–518.
Rieske, J. S. (1967). *Methods Enzymol.* **10**, 239–245.
Rieske, J. S., Hansen, R. E., and Zaugg, W. S. (1964a). *J. Biol. Chem.* **239**, 3017–3022.
Rieske, J. S., MacLennan, D. H., and Coleman, R. (1964b). *Biochem. Biophys. Res. Commun.* **15**, 338–344.
Rieske, J. S., Zaugg, W. S., and Hansen, R. E. (1964c). *J. Biol. Chem.* **239**, 3023–3030.
Robertson, D. E., Giangiacomo, K. M., de Vries, S., Moser, C. C., and Dutton, P. L. (1984a). *FEBS Lett.* **178**, 343–350.

Robertson, D. E., Prince, R. C., Bowyer, J. R., Matsuura, K., Dutton, P. L., and Ohnishi, T. (1984b). *J. Biol. Chem.* **259**, 1758–1763.
Robertson, D. E., Davidson, E., Prince, R. C., van den Berg, W. H., Marrs, B. L., and Dutton, P. L. (1986). *J. Biol. Chem.*, **261**, 584–591.
Salerno, J. C. (1984). *J. Biol. Chem.* **259**, 2331–2336.
Salerno, J. C., Harmon, H. J., Blum, H., Leigh, J. S., and Ohnishi, T. (1977). *FEBS Lett.* **82**, 179–182.
Salerno, J. C., and Ohnishi, T. (1980). *Biochem. J.* **192**, 769–781.
Saraste, M. (1984). *FEBS Lett.* **166**, 367–372.
Siedow, J. N., Power, S., de la Rosa, F. F., and Palmer, G. (1978). *J. Biol. Chem.* **253**, 2392–2399.
Slater, E. C. (1949). *Biochem. J.* **45**, 14–30.
Slater, E. C. (1981). In *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P., and Hinke, P., eds.), Addison-Wesley, Reading, Massachusetts, pp. 69–104.
Slater, E. C. (1983). *Trends Biochem. Sci.* **8**, 239–242.
Slater, E. C., and de Vries, S. (1980). *Nature (London)* **288**, 717–718.
Subik, J., Behun, M., and Musilek, V. (1974). *Biochem. Biophys. Res. Commun.* **57**, 17–20.
Takamiya, K. I., and Dutton, P. L. (1979). *Biochim. Biophys. Acta* **546**, 1–16.
Thierbach, G., and Reichenbach, H. (1981). *Biochim. Biophys. Acta* **638**, 282–289.
Thierbach, G., Kunze, B,., Reichenbach, H., and Höfle, G. (1984). *Biochim. Biophys. Acta* **765**, 227–235.
Trumpower, B. L. (1976). *Biochem. Biophys. Res. Commun.* **70**, 73–80.
Trumpower, B. L., and Edwards, C. A. (1979). *J. Biol. Chem.* **254**, 8697–8706.
T'sai, A. L., and Palmer, G. (1983). *Biochim. Biophys. Acta* **722**, 349–363.
Tsou, C. L., Tang, H. L., Wang, D. C., and Jin, Y. Z. (1982). *Biochim. Biophys. Acta* **682**, 315–321.
Tzagoloff, A., Yang, P. C., Wharton, D. C., and Rieske, J. S. (1965). *Biochim. Biophys. Acta* **96**, 1–8.
Van Ark, G. (1980). "Electron Transfer through the Ubiquinol: Ferricytochrome *c* Oxidoreductase of the Mitochondrial Respiratory Chain", Ph.D. Thesis, University of Amsterdam.
Van Ark, G., and Berden, J. A. (1977). *Biochim. Biophys. Acta* **459**, 119–137.
Van den Berg, W. H., Prince, R. C., Bashford, C. L., Takamiya, K., Bonner, W. D., Jr., and Dutton, P. L. (1979). *J. Biol. Chem.* **254**, 8594–8604.
Van Loon, A. P. G. M., Vijn, R. J., de Groot, R. J., Polman, J. E. M., and Grivell, L. A. (1984). *Mol. Gen. Genet.* **197**, 219–224.
Van Wielink, J. E., Oltmann, L. F., Leeuwerik, F. J., de Hollander, J. A., and Stouthamer, A. H. (1982). *Biochim. Biophys. Acta* **681**, 177–190.
Von Jagow, G., and Engel, W. D. (1981). *FEBS Lett.* **136**, 19–24.
Von Jagow, G., and Ohnishi, T. (1985). *FEBS Lett.* **185**, 311–315.
Von Jagow, G., Schägger, H., Riccio, P., Klingenberg, M., and Kolb, H. J. (1977). *Biochim. Biophys. Acta* **462**, 549–558.
Von Jagow, G., Ljungdahl, P. O., Graf, P., Ohnishi, T., and Trumpower, B. L. (1984). *J. Biol. Chem.* **259**, 6318–6326.
Wei, Y. H., Scholes, C. P., and King, T. E. (1981). *Biochem. Biophys. Res. Commun.* **99**, 1411–1419.
Weiss, H., and Kolb, H. J. (1979). *Eur. J. Biochem.* **99**, 139–149.
Widger, W. R., Cramer, W. A., Hermann R., and Trebst, A. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81**, 674–678.
Wikström, M. K. F. (1973). *Biochim. Biophys. Acta* **301**, 155–193.
Wikström, M. K. F., and Berden, J. A. (1972). *Biochim. Biophys. Acta* **283**, 403–420.
Wikström, M., and Krab, K. (1980). *Curr. Top. Bioenerg.* **10**, 51–101.
Wikström, M., and Saraste, M. (1984). In *Bioenergetics* (Ernster, L., ed.), Elsevier, Amsterdam, pp. 49–94.
Wingfield, P., Arad, T., Leonard, K., and Weiss, H. (1979). *Nature (London)* **280**, 696–697.
Zhu, Q. S., Berden, J. A., de Vries, S., Folkers, K., Porter, T., and Slater, E. C. (1982a). *Biochim. Biophys. Acta* **682**, 160–167.
Zhu, Q. S., Berden, J. A., de Vries, S., and Slater, E. C. (1982b). *Biochim. Biophys. Acta* **680**, 69–79.