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THE PATHWAY OF ELECTRONS THROUGH QH_2:CYTOCHROME *c* OXIDOREDUCTASE STUDIED BY PRE-STEADY-STATE KINETICS *

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(1) The kinetic behaviour of the prosthetic groups and the semiquinones in QH_2:cytochrome *c* oxidoreductase has been studied using a combination of the freeze-quench technique, low-temperature diffuse-reflectance spectroscopy, EPR and stopped flow. (2) In the absence of antimycin, cytochrome *b*-562 is reduced in two phases separated by a lag time. The initial very rapid reduction phase, that coincides with the formation of the antimycin-sensitive $Q^{\cdot}_{in}$, is ascribed to high-potential cytochrome *b*-562 and the slow phase to low-potential cytochrome *b*-562. The two cytochromes are present in a 1:1 molar ratio. The lag time between the two reduction phases decreases with increasing pH. Both the [2 Fe-2 S] clusters and cytochrome c_1 are reduced monophasically under these conditions, but at a rate lower than that of the initial rapid reduction of cytochrome *b*-562. (3) In the presence of antimycin and absence of oxidant, cytochrome *b*-562 is still reduced biphasically, but there is no lag between the two phases. No $Q^{\cdot}_{in}$ is formed and both the Fe-S clusters and cytochrome c_1 are reduced biphasically, one-half being reduced at the same rate as in the absence of antimycin and the other half 10-times slower. (4) In the presence of antimycin and oxidant, the recently described antimycin-insensitive species of semiquinone anion, $Q^{\cdot}_{out}$ (De Vries, S., Albracht, S.P.J., Berden, J.A. and Slater, E.C. (1982) J. Biol. Chem. 256, 11996–11998) is formed at the same rate as that of the reduction of all species of cytochrome *b*. In this case cytochrome *b* is reduced in a single phase. (5) The reversible change of the line shape of the EPR spectrum of the [2Fe-2S] cluster 1 is caused by ubiquinone bound in the vicinity of this cluster. (6) The experimental results are consistent with the basic principles of the Q cycle. Because of the multiplicity, stoicheiometry and heterogeneous kinetics of the prosthetic groups, a Q cycle model describing the pathway of electrons through a dimeric QH_2:cytochrome *c* oxidoreductase is proposed.

Introduction

As early as in 1962 it was recognized by Deul and Thorn [1] that the pathway of electrons in the cytochrome bc_1 region of the mitochondrial respiratory chain could not be adequately described by a simple linear sequence of electron-transfer reactions. This conclusion was based on the finding that the reduction of cytochrome *b* in BAL + (O_2)-treated preparations is inhibited by antimycin whereas in the presence of only one of these inhibitors cytochrome *b* is still reducible by substrate. A similar effect of antimycin was found by Trumpower [2], after extraction from QH_2:cytochrome *c* oxidoreductase of a protein, called Oxidation Factor. Later, both Oxidation Factor [3]

* Part of this work has been recently published [25].
Abbreviations: DQH_2, duroquinol; $Q^{\cdot}$, ubisemiquinone anion; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; BAL, British Anti-Lewisite (2,3-dimercaptopropanol); Mops; 4-morpholinepropanesulphonic acid.

and the BAL-labile factor [4] were identified as the Rieske Fe-S protein and cluster, respectively. It was also shown [5] that this cluster is involved in the oxidant-induced reduction of cytochrome *b*. Although the model of Wikström and Berden [6] provides an elegant explanation for the latter phenomenon, it does not do so for the experiment of Deul and Thorn [1]. The Q cycle proposed by Mitchell [7] and the *b* cycle of Wikström and Krab [8], which can be considered as extensions of the model of Wikström and Berden [6], are consistent with both phenomena (see, however, Ref. 9). A modification of the Q cycle that takes account of the experimental results obtained since the original proposal in 1976, in particular of the role and position of the [2Fe-2S] cluster [3,4] and of the finding that the semiquinones are in the anionic form [10,11], has been published previously [9,12,13]. An essential feature of the Q cycle, in contrast to the *b* cycle, is that two semiquinone anions are involved during the catalytic action of the enzyme. The antimycin-sensitive semiquinone anions described in Refs. 10, 11,14–17 are very likely all the same species and probably correspond to what has been called $Q_{in}^{\cdot-}$ of the Q cycle, where 'in' refers to the matrix face of the inner mitochondrial membrane. Recently, we showed [12] that the concentration of $Q_{in}^{\cdot-}$ is not affected by BAL + (O_2) treatment and also that during oxidant-induced reduction of cytochrome *b* in the presence of antimycin, a second species of semiquinone anion bound to QH_2:cytochrome *c* oxidoreductase is formed. This species differs in EPR properties from $Q_{in}^{\cdot-}$, and is in contrast to $Q_{in}^{\cdot-}$ stabilized in the presence of antimycin and destabilized or not formed after BAL ($+O_2$) treatment. This species of semiquinone anion has the properties that one expects for the $Q_{out}^{\cdot-}$ proposed in the Q cycle. Thus, it seems that the Q cycle gives a satisfactory description of electron transfer through the mitochondrial QH_2:cytochrome *c* oxidoreductase.

The analogous enzyme in chromatophores of photosynthetic bacteria is similar to the mitochondrial enzyme, with respect to the composition of the prosthetic groups and their midpoint redox potentials [18–22] and the effects of antimycin [23]. No clear picture as to the mechanism has, however, emerged from the extensive kinetic studies performed in photosynthetic bacteria [19–23]. Thus, although many results are consistent with a Q-cycle type of mechanism, some that are not have led to proposals of 'complex' linear models [21,24]. In this paper, we report the results of a kinetic study on the mitochondrial QH_2:cytochrome *c* oxidoreductase using a combination of the freeze-quench technique, low-temperature diffuse-reflectance spectroscopy, EPR and stopped flow. By means of these techniques it is possible to establish the kinetic behaviour of the cytochromes, the Fe-S clusters and the semiquinone anions, $Q_{in}^{\cdot-}$ and $Q_{out}^{\cdot-}$. Many observations are consistent with the version of the Q cycle previously proposed [9,12,13], but particularly the finding that in the presence of antimycin only one-half of the [2Fe-2S] clusters and cytochrome c_1 present becomes rapidly reduced, have led us to propose a Q cycle type of mechanism for a dimeric QH_2:cytochrome *c* oxidoreductase.

Materials and Methods

Beef-heart submitochondrial particles and succinate: and QH_2:cytochrome *c* oxidoreductase were prepared as described in Refs. 26 and 27, respectively. DQH_2 (0.1 M) and antimycin (20 mM) were dissolved in Me_2SO. BAL ($+O_2$) treatment was carried out as described in Ref. 28, pentane extraction and reincorporation according to Ref. 29 and EPR and low-temperature diffuse-reflectance spectroscopy as detailed in Ref. 30. Kinetic studies were performed with an Aminco stopped-flow apparatus connected to an LS-II microcomputer and with a self-built freeze-quench apparatus consisting of a hydraulic ram [31] with a driving force of 3000 N, a four-jet tangential mixer [32] and a quenching bath of isopentane at 131 K. The dead time of this set up including mixing and quenching is about 5 ms. For freeze-quench experiments one syringe contained particles (70 mg/ml) suspended in 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 8.5) or succinate:cytochrome *c* oxidoreductase (40 mg/ml) suspended in the same buffer containing 1% sodium cholate. The other syringe contained an N_2-saturated solution of 0.25 M sucrose, 1 mM acetic acid and DQH_2. In this solution the rate of auto-oxidation of DQH_2 is negligible. For stopped-flow experiments an aerobic DQH_2 solution was used. The other syringe

contained the particles (4 mg/ml) suspended in 0.25 M sucrose, 50 mM Mops-Tris buffer (pH 7), or succinate : cytochrome *c* oxidoreductase (2 mg/ml) suspended in a similar buffer (pH 7.0, 7.5 or 8.0) containing 1% sodium cholate. In both freeze-quench and stopped-flow experiments the pH after mixing was the same, within 0.05 pH unit, as that of the syringe containing the enzyme. Stopped-flow traces were analysed with a computer programme similar to that in Ref. 33. The values of $t_{1/2}$ and the spectral contribution of a component calculated from the stopped-flow traces had standard deviations of 16 and 12%, respectively. Each value of a parameter in Table I is the mean of 4–12 experiments.

Results

Effect of Q on EPR spectrum of Fe-S cluster

It has been shown by several groups [30,34–36] that the line shape of the EPR spectrum of the Fe-S cluster of QH_2 : cytochrome *c* oxidoreductase depends on the redox state of the system. We have proposed [30] that the EPR signal receives contributions from two different $S = 1/2$ systems, cluster 1 and cluster 2, and that the concentration of each cluster is equal to one-half of that of cytochrome c_1. The line shape of cluster 1 broadens on lowering the potential, whereas that of cluster 2 is unaffected. This is also shown in traces A and B of Fig. 1. In the earlier paper [30] it was suggested that this broadening might be related to the redox state of Q. In agreement with this proposal, it has now been found that, after extraction of Q, cluster 1 has the 'broad' line shape, when ascorbate is the reducing agent (see trace C of Fig. 1). This spectrum is not significantly different from that of succinate-reduced particles (trace B). Reincorporation of Q (trace D) restores the 'sharp' line shape of cluster 1. It seems therefore that cluster 1 has the 'sharp' line shape only in the presence of oxidized Q (cf. Ref. 30). Antimycin does not affect the EPR spectrum of ascorbate-reduced particles (Fig. 1, trace E), indicating that it neither removes the Q bound near cluster 1 nor significantly alters the three-dimensional structure of the Fe-S clusters.

Kinetic studies

In the kinetic studies the QH_2 analogue DQH_2

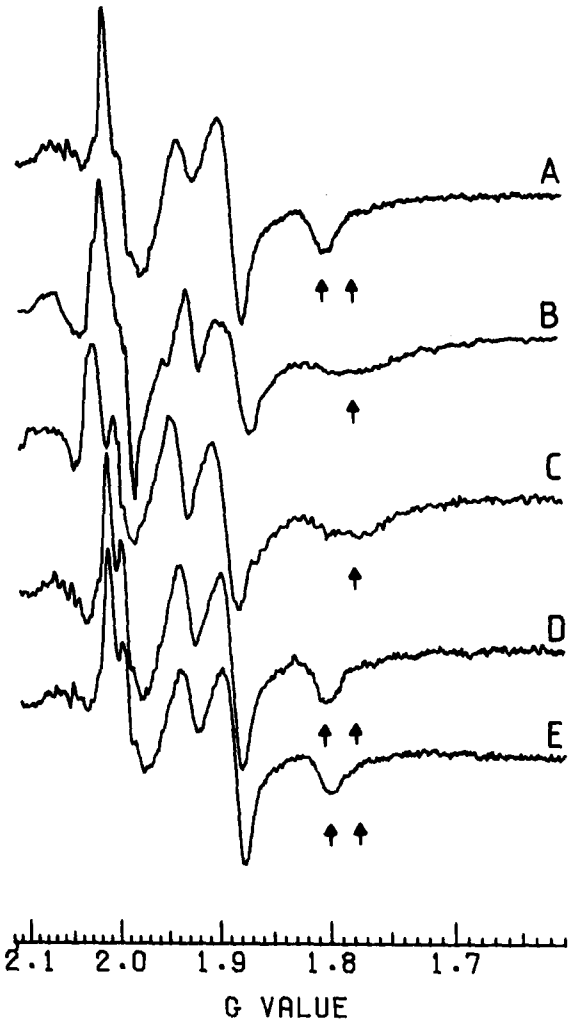


Fig. 1. Effect of the amount of Q on the EPR spectrum of the [2Fe-2S] clusters of QH_2 : cytochrome *c* oxidoreductase. Lyophilized submitochondrial particles were suspended in 0.25 M sucrose, 50 mM potassium phosphate buffer (pH 7.3). (A) Particles to which 4 mM KCN, 15 μM cytochrome *c*, 10 mM ascorbate and 3 μM TMPD were added were incubated for 3 min at room temperature and frozen in liquid N_2. The spectrum is identical to that of non-lyophilized particles (cf. Ref. 30). (B) 4 mM KCN, 100 mM sodium fumarate and 10 mM sodium succinate were added (the lines at *g* 2.02 and 1.94 originate from the [2Fe-2S] cluster of succinate : Q oxidoreductase). (C) Pentane-extracted (five times) particles. Additions as in A. (D) Q incorporated (about 7 nmol/mg protein). Additions as in A. (E) Same as A, but 3 mol antimycin/mol cytochrome c_1 was also added. Arrows indicate the g_x values of clusters 1 and 2. EPR conditions: frequency , 9.25 GHz; modulation amplitude, 0.63 mT; power, 5 mW; temperature, 36 K; scanning rate, 12.5 mT/min.

was used as a reductant. It can be concluded from previous work [37–40] that DQH_2 can reduce the enzyme both directly and indirectly via Q in the enzyme preparation. It has been suggested that DQH_2 directly reduces cytochrome *b* [37–39], but this is in contradiction with the finding that cytochrome *b* is reducible by DQH_2 in BAL ($+O_2$)-treated particles in the absence of antimycin (not shown) but not in its presence (trace D of Fig. 2, cf. Ref. 40). Since we have never been able to detect durosemiquinone (anion) radical signals in pentane-extracted particles, it is likely that the necessity of Q for DQH_2-cytochrome *c* activity [41] or DQH_2-O_2 activity [40] is due to the fact that no oxidant, i.e., $Q^{\cdot}_{in}$, is formed in the absence of Q.

When submitochondrial particles are mixed with DQH_2, cytochrome *b*-562 becomes reduced as illustrated in Fig. 2. A fraction of the total amount of cytochrome *b*-562 is reduced very rapidly and the rest more slowly. These two reduction phases are more clearly defined when purified succinate:cytochrome *c* oxidoreductase is used. As shown by Jin et al. [42], the initial very rapid reduction phase is followed by a stationary phase (see Fig. 3A, trace C), in which sometimes cytochrome *b*-562 even becomes slightly more oxidized, and then by a second relatively slow phase. The lag time between the two phases decreases and the rate of the two phases increases at increasing pH

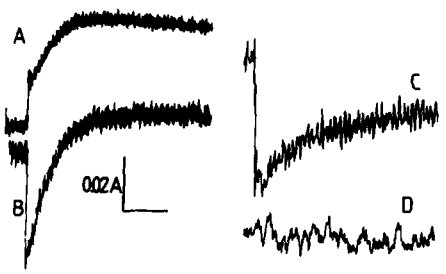


Fig. 2. Stopped-flow traces showing the reduction kinetics of cytochrome *b* and cytochrome c_1 as present in submitochondrial particles. Experimental conditions: reaction temperature, 2°C; pH 7.0; DQH_2, 20 μM; cytochrome *b* was measured at 562–575 nm or at 563–575 nm when antimycin was present and cytochrome c_1 at 552–539 nm. (A) Cytochrome *b*, (B) cytochrome *b* in the presence of antimycin, (C) cytochrome c_1, (D) cytochrome *b* in BAL ($+O_2$)-treated particles in the presence of antimycin. The horizontal bar indicates 1 s (A and B) or 2 s (C and D).

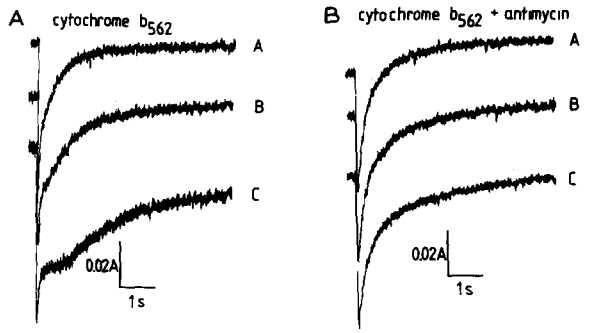


Fig. 3.(A) Stopped-flow traces of the reduction kinetics of cytochrome *b*-562 in succinate:cytochrome *c* oxidoreductase. Experimental conditions as in Fig. 2 except that 100 μM DQH_2 was used. Trace A, pH 8.0, trace B, pH 7.5, trace C, pH 7.0. (B) Same as A but 3 mol antimycin/mol cytochrome c_1 was added.

values (Fig. 3A) and increasing DQH_2 concentrations (see also Table I). The extent of the first phase comprises approx. 38% of the total, independent of pH, both in succinate:cytochrome *c* oxidoreductase and submitochondrial particles. In the presence of antimycin, separate phases of reduction are not clearly visible (Fig. 3B), but a computer simulation of the traces using a single exponential gave a very bad fit. The best fit [33] was obtained when the traces were simulated as a sum of two exponentials. The spectral contribution of the most rapid phase comprises 62% of the total absorbance change (Table I). The rates of both reduction phases of cytochrome *b*-562 in the presence of antimycin are lower than that of the initial rapid reduction phase in the absence of antimycin. Both rates increase with increasing pH, but that of the slower phase increases more.

Fig. 4A shows the kinetics of reduction of cytochrome c_1 in the absence of antimycin. The values of the kinetic parameters are summarized in Table I. Computer analysis of the traces showed that the absorbance increment at 552–539 nm is best simulated as a sum of two exponentials with a ratio of about 10 for the two rate constants. The rapid phase contributes about 70–80% of the total absorbance change at this wavelength pair. The other 20–30% is mainly due to spectral overlap of the slowly reduced cytochrome *b*-562. The spectral contribution of the rapid phase increases up to 80–90% when 550–539 nm is used as the measuring wavelength and decreases to 50–60% at 555–

TABLE I

KINETIC PARAMETERS AT 2°C AND 100 μM DQH_2 FOR REDUCTION OF CYTOCHROME *b*-562 AND CYTOCHROME c_1 IN SUCCINATE: CYTOCHROME *c* OXIDOREDUCTASE AS DETERMINED BY SIMULATION OF THE STOPPED-FLOW TRACES

A_1 is the relative spectral contribution of the more rapidly reduced component. $(t_{1/2})_1$ and $(t_{1/2})_2$ are the times for 50% reduction of the rapid and slow component, respectively. N.B., with 20 μM DQH_2 all values for the half-times of reduction and also the lag time were 4–5-times greater than those in the table.

Cytochrome	Antimycin	pH	A_1 (% of total ΔA)	$(t_{1/2})_1$ (ms)	$(t_{1/2})_2$ (s)	Lag time [a] (ms)
c_1	−	7.0	70	340	3.3	
	−	7.5	76	191	1.9	
	−	8.0	82	126	1.4	
	+	7.0	49	358	3.4	
	+	7.5	50	229	1.7	
	+	8.0	50	166	1.4	
b-562	−	7.0	40	63	1.5	≈900
	−	7.5	39	44	0.7	≈300
	−	8.0	37	<35	0.4	≈160
	+	7.0	61	232	2.0	
	+	7.5	61	190	1.2	
	+	8.0	63	164	0.8	

[a] Lag time between two phases (see Fig. 3).

539 nm, but the ratio of the rate constants remains the same, irrespective of the wavelength pair. In the presence of antimycin, the total absorbance change at 552–539 nm is 10–15% lower, owing to the red shift of cytochrome *b*-562. A similar observation was made by Bowyer and Trumpower [43]. We conclude that about 90% of all cytochrome c_1 is reduced in one rapid phase. The rate increases with increasing pH (Table I). In the presence of antimycin, cytochrome c_1 becomes reduced biphasically (Fig. 4B and Table I). The spectral contribution of each phase is about 50% of the total, independently of whether 550–539, 552–539 or 554–539 nm was used as the measuring wavelength pair. This shows that the spectral overlap of cytochrome *b*-562 at these wavelength pairs is negligible when antimycin is present. Also, the ratio of the rate constants remains constant at the different wavelength pairs and at different pH values (between pH 7.0 and 8.0). At pH 7.0, the rate constant of the first reduction phase of cytochrome c_1 is not affected by antimycin whereas at pH 7.5 and 8.0 antimycin seems slightly to increase the half-time of reduction of the first phase.

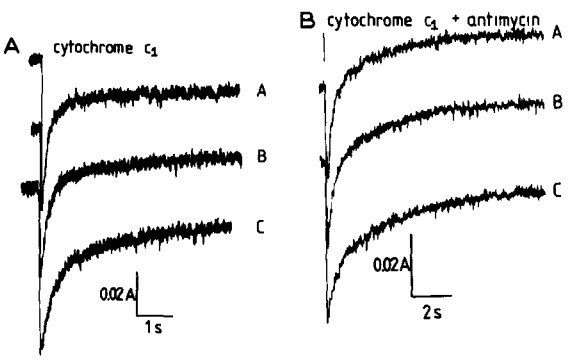


Fig. 4. (A) Stopped-flow traces of the reduction kinetics of cytochrome c_1 in succinate: cytochrome *c* oxidoreductase. Experimental conditions as in Fig. 3A. Trace A, pH 8.0; trace B, pH 7.5; trace C, pH 7.0. (B) Same as A but 3 mol antimycin/mol cytochrome c_1 was added.

The results of freeze-quench experiments with purified succinate: cytochrome *c* oxidoreductase are shown in Figs. 5 and 6. The behaviour of cytochrome *b*-562 is qualitatively similar to that obtained in the stopped-flow experiments, i.e., an initial very rapid reduction ($t_{1/2}$ about 5 ms), followed by a stationary phase of about 40 ms and finally a second relatively slow reduction phase

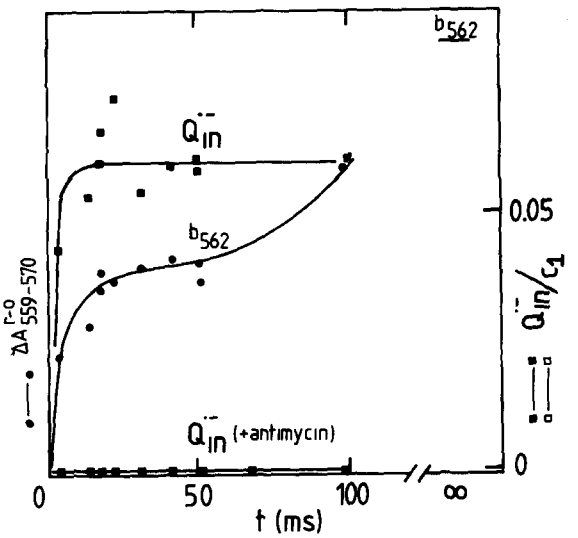


Fig. 5. Kinetics of cytochrome *b*-562 reduction and formation of $Q_{in}^{\cdot-}$ in succinate:cytochrome *c* oxidoreductase measured by EPR and low-temperature diffuse-reflectance spectroscopy using the freeze-quench technique. Experimental conditions: reaction temperature, 22±2°C; pH 8.5; DQH_2, 300 μM; diffuse-reflectance spectra were recorded at 110 K. Slit width, 1.5 nm. (■———■) Formation of $Q_{in}^{\cdot-}$ (the amount of $Q_{in}^{\cdot-}$ at 100 ms was 0.06 mol/mol cytochrome c_1); (●———●) reduction of cytochrome *b*-562, measured as 559 nm minus 570 nm in the reduced minus fully oxidized spectrum ($\Delta A^{r-o}_{559-570}$). The amount of DQH_2-reducible cytochrome *b*-562, which is more than 95% of all cytochrome *b*-562, is indicated by the bar at the top right of the figure ($t=\infty$); (□———□) amount of $Q_{in}^{\cdot-}$ in the presence of 3 mol antimycin/mol cytochrome c_1. The reduction of cytochrome *b*-562 in the presence of antimycin is not shown. See Table II for the values of the kinetic parameters.

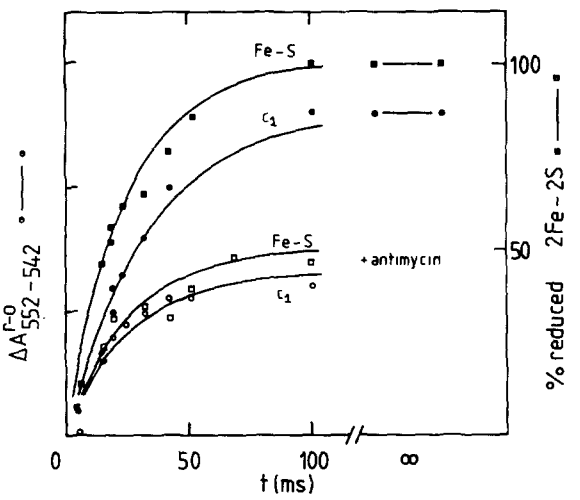


Fig. 6. Kinetics of reduction of the [2Fe-2S] clusters and cytochrome c_1 as present in succinate:cytochrome *c* oxidoreductase and the effect of antimycin. Experimental conditions as in the legend of Fig. 5 (■———■). Reduction of the Fe-S clusters; (●———●) reduction of cytochrome c_1 measured as $\Delta A^{r-o}_{552-542}$; (□———□) reduction of the Fe-S cluster in the presence of antimycin; (○———○) reduction of cytochrome c_1 in the presence of antimycin. The curves through the points are first-order exponentials. The values of $t_{1/2}$ are listed in Table II. In the presence of antimycin 50% of the amount of the [2Fe-2S] clusters and cytochrome c_1 is reduced at 100 ms with the same $t_{1/2}$ as in the absence of antimycin. However, in both cases, these two prosthetic groups are fully reducible ($t=\infty$; ◨———◨, ◑———◑).

(Fig. 5). Concomitantly with the initial rapid reduction of cytochrome *b*-562 the semiquinone anion, $Q_{in}^{\cdot-}$, is formed ($t_{1/2}$ about 5 ms). Both cytochrome c_1 and the Fe-S clusters are reduced monophasically (Fig. 6), with nearly the same values for $t_{1/2}$ (Table II). They become reduced after the formation of $Q_{in}^{\cdot-}$ and the completion of the rapid reduction phase of cytochrome *b*-562. In the presence of antimycin, only one-half of the Fe-S clusters and cytochrome c_1 is reduced with the same $t_{1/2}$ as in the absence of antimycin (Fig. 6). The other half becomes very slowly reduced (Table II). The semiquinone anion $Q_{in}^{\cdot-}$ is not formed in the presence of antimycin (Fig. 5) and also the rapid reduction of cytochrome *b*-562 is absent (not shown).

Fig. 7 shows that the extinction coefficients of high- and low-potential cytochrome *b*-562 are different and that the two cytochromes are present in a 1:1 molar ratio. Purified QH_2:cytochrome *c* oxidoreductase was reduced at various potentials with ascorbate (which selectively reduces cytochrome c_1), with ascorbate and TMPD (which reduces cytochrome c_1 and high-potential cytochrome *b*-562) and by titration with the fumarate/succinate oxidation-reduction couple until all the cytochrome *b*-562 was reduced but none of the cytochromes *b*-566 and *b*-558. The optical difference spectra show some differences in the β-band region for the two cytochromes, and the EPR difference spectra reveal that they have different *g* values (cf. Ref. 30). The computed ratio cytochrome *b*-562 (high-potential)/*b*-562 (low-potential) equals 1.02:1.0 when corrected for T^{AV} [44]. The total amount of cytochrome *b*-562 is

TABLE II

KINETIC PARAMETERS FOR THE FORMATION OF SEMIQUINONE ANIONS AND THE REDUCTION OF THE PROSTHETIC GROUPS OF QH_2:CYTOCHROME *c* OXIDOREDUCTASE AS DETERMINED BY A COMBINATION OF THE FREEZE-QUENCH TECHNIQUE, EPR AND LOW-TEMPERATURE DIFFUSE-REFLECTANCE SPECTROSCOPY

Experimental conditions as in the legends of Figs. 5–10. n.f., not formed; r.o., remains oxidized.

Redox group	$t_{1/2}$ (ms)	
	−antimycin	+antimycin
Succinate:cytochrome *c* oxidoreductase		
Cytochrome c_1	23	18 [a]
Cytochrome *b*-562 (hp)	≈5	≈25
Cytochrome *b*-562 (lp)	≈60 [b]	≈25
Fe-S clusters	17	17 [a]
$Q_{in}^{\cdot-}$	≈5	n.f.
Submitochondrial particles		
Cytochromes $c + c_1$	22	r.o.
Cytochrome *b*-562 (hp)	<5	33
Cytochrome *b*-562 (lp)	≈40 [b]	33
Cytochromes *b*-566 + *b*-558	r.o.	33
Fe-S clusters	17	[c]
$Q_{in}^{\cdot-}$	<5	n.f.
$Q_{out}^{\cdot-}$	[d]	33

[a] Values of $t_{1/2}$ for the first half. The $t_{1/2}$ for the other half is greater than 150 ms.

[b] The lag times were about 40 and 15 ms in succinate:cytochrome *c* oxidoreductase and submitochondrial particles, respectively.

[c] Transiently reduced.

[d] The concentration is probably to low to detect.

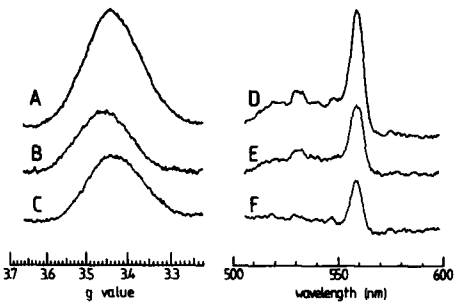


Fig. 7. EPR and low-temperature optical difference spectra of QH_2:cytochrome *c* oxidoreductase poised at various potentials. EPR difference spectra showing: (A) total cytochrome *b*-562 at *g* 3.440 (ascorbate minus succinate); (B) low-potential cytochrome *b*-562 at *g* 3.455 (ascorbate/TMPD minus succinate) and (C) high-potential cytochrome *b*-562 at *g* 3.429 (ascorbate minus ascorbate/TMPD). (D–F) Optical difference spectra of the samples of A–C, respectively. The relative spectral contributions of the high- and low-potential cytochromes *b*-562 are 43 and 57%, respectively, in the low-temperature spectra (cf. Figs. 5 and 8). Experimental conditions: the ascorbate sample was prepared by incubating QH_2:cytochrome *c* oxidoreductase (pH 7.2) with 2 mM ascorbate for 30 s at 0°C before freezing. The ascorbate/TMPD sample (pH 7.2) was incubated with 2 mM ascorbate, 250 μM TMPD for 10 min at room temperature. The succinate sample (pH 8.1) was incubated with 100 mM sodium fumarate, 1.5 mM sodium succinate for 10 min at room temperature. EPR conditions: frequency, 9.23 GHz; modulation amplitude, 3.2 mT; power, 0.16 mW; temperature, 10 K; scanning rate, 20 mT/min. The traces are an average of 10 spectra. Conditions for optical spectra: temperature, 110 K; scanning rate, 0.2 nm/s; slit width, 1.0 nm.

equal to the amount of cytochrome c_1 [30].

The pre-steady-state kinetics of the components of QH_2:cytochrome *c* oxidoreductase in submitochondrial particles are reproduced in Fig. 8. Again a very rapid formation of $Q_{in}^{\cdot-}$ and reduction of a part of cytochrome *b*-562, followed by a somewhat slower reduction of the rest of this cytochrome, are observed. The lag time between the two phases is considerably shorter than in purified succinate:cytochrome *c* oxidoreductase (cf. Fig. 8. with Fig. 5, and Fig. 2 with Fig. 3A). The Fe-S clusters and cytochromes $c + c_1$ are reduced monophasically (cf. Fig. 2). The half-times of reduction are nearly the same as in the purified enzyme (Table II). In the presence of antimycin and oxygen, the well known oxidant-induced reduction of cytochrome *b* takes place (see also Fig. 2), while cytochromes $c + c_1$ and aa_3 remain oxidized. Fig. 9 shows the optical diffuse-reflectance spectra of submitochondrial particles at different reaction times, under conditions of the oxidant-induced reduction of cytochrome *b*. The peak at 560 nm is a composite of the absorbances of cytochromes *b*-562 and *b*-566, that at 554.5 nm originates from cytochrome *b*-558. Though the latter is rather close to the peak of cytochromes $c + c_1$, the spectrum is sufficiently different from the control spectrum in which only cytochromes $c + c_1$ and cytochrome *b*-562 are reduced (lower trace), with respect to peak position and shape, to conclude that practically no cytochromes $c + c_1$ and aa_3 become reduced in the first 50 ms. Also,

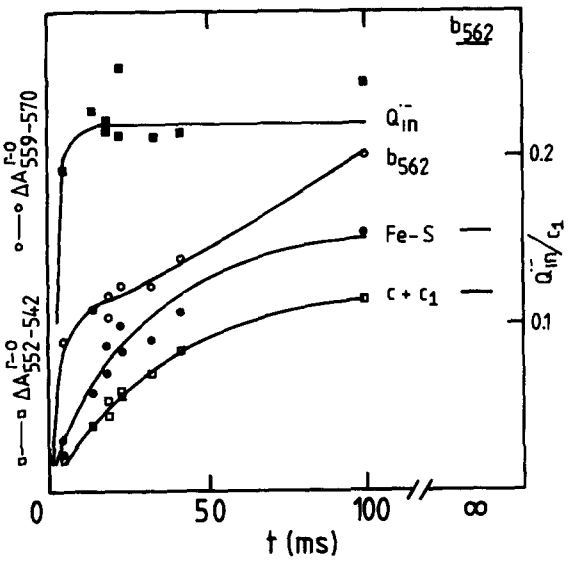


Fig. 8. Kinetics of the formation of $Q_{in}^{\cdot-}$ and reduction of the Fe-S clusters, cytochrome c_1 and cytochrome *b*-562 as present in submitochondrial particles. Experimental conditions as in the legends of Figs. 5 and 6. (■———■) Formation of $Q_{in}^{\cdot-}$ (the amount of $Q_{in}^{\cdot-}$ of the plateau corresponds to 0.22 mol/mol c_1); (○———○) reduction of cytochrome *b*-562; (●———●) reduction of the Fe-S clusters; (□———□) reduction of cytochromes $c + c_1$. The total amounts of DQH_2-reducible cytochrome *b*-562, Fe-S clusters and cytochrome c_1 are shown on the right-hand side of the figure. The curves for the reduction of the Fe-S cluster and cytochrome c_1 are first-order exponentials. Although no KCN is present, the prosthetic groups of QH_2:cytochrome *c* oxidoreductase become fully reduced, presumably owing to the low cytochrome *c* content (about 0.1 mol/mol c_1).

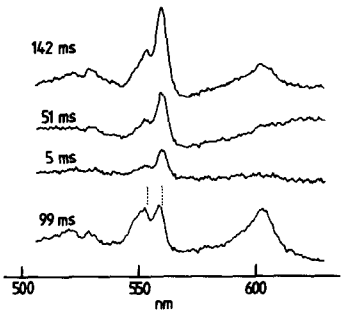


Fig. 9. Low-temperature diffuse-reflectance spectra of submitochondrial particles measured at different reaction times after addition of DQH_2, showing the effect of antimycin on the reducibility of the cytochromes. In the presence of antimycin (upper three traces), cytochrome *b*-558 (maximum at 554.5 nm) and cytochromes *b*-566 + *b*-562 (at 560 nm) become reduced whereas cytochromes $c + c_1$ remain oxidized. In the absence of antimycin (lower trace), cytochromes $c + c_1$ (at 552 nm) and cytochrome *b*-562 (at 559 nm) are reduced. The dotted lines indicate 554.5 and 560 nm. It can be seen from the lower trace that the absorption maxima of both peaks are closer to the blue end of the spectrum in the absence of antimycin. The reaction time with DQH_2 is indicated in the figure. The upper three traces are taken from the corresponding samples of Fig. 10 and the lower trace from the 99 ms sample of Fig. 8.

the difference spectrum dithionite-reduced particles minus the spectrum after 142 ms (cf. Fig. 9) showed that more than 90% of cytochrome *b* was reduced while cytochromes $c + c_1$ remained oxidized. Fig. 10 shows that all the cytochromes *b* are reduced at the same rate. The semiquinone anion ascribed to $Q_{out}^{\cdot-}$ [12] is formed at the same rate as the reduction of cytochromes *b* (see also Table II). The maximal amount of $Q_{out}^{\cdot-}$ was 0.06 mol/mol cytochrome c_1, i.e., 3–4-times lower than that of $Q_{in}^{\cdot-}$ under similar conditions, but in the absence of antimycin. The Fe-S cluster behaves rather strangely. After an apparent lag time of about 10 ms, it becomes maximally reduced at 50 ms (about 30% of the total amount) and then slowly oxidizes (to 90%).

Discussion

Both in submitochondrial particles and purified succinate : cytochrome *c* oxidoreductase, cytochrome *b*-562 is reduced in two phases separated by a lag time [25,42]. The relative spectral contributions of the rapid and the slow phase, 38 and 62%, respectively, agree closely with the contribution estimated for high- and low-potential cytochrome *b*-562, respectively, as determined by potentiometric titrations [45]. A similar ratio for the spectral contributions of high-potential (155 mV) and low-potential (50 mV) cytochrome *b* as present in photosynthetic bacteria may be calculated from Fig. 7 in Ref. 46. Thus, it seems as if there are two species of cytochrome *b*-562 with different midpoint potentials, with slightly different *g* values (cf. Ref. 30) and with a different kinetic behaviour. From the EPR spectra in Fig. 7, a 1:1 molar ratio for the two cytochromes was calculated.

High-potential cytochrome *b*-562 becomes reduced and $Q_{in}^{\cdot-}$ is formed before the Fe-S clusters and cytochrome c_1 are reduced. Thus, the electrons

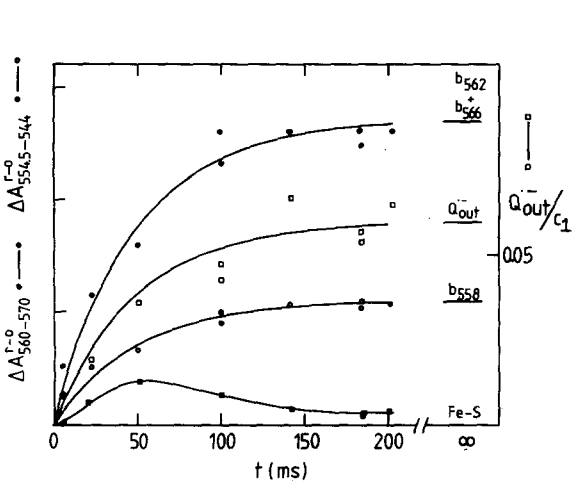
reaching high-potential cytochrome *b*-562 and $Q_{in}^{\cdot-}$ do not originate from QH_2 oxidized by the Fe-S clusters. This is in agreement with the findings that in particles devoid of the Fe-S clusters, $Q_{in}^{\cdot-}$ is still formed [12] and (all) cytochrome *b*-562 is reducible [28,47]. When antimycin is present, no $Q_{in}^{\cdot-}$ is formed and the rapid reduction of high-potential cytochrome *b*-562 is abolished (cf. Refs. 25 and 42). A computer simulation of the stopped-flow traces of cytochrome *b*-562 in the presence of antimycin reveals that the absorbance increase is biphasic, the more rapidly reduced component contributing 62% to the spectral change. This suggests that in the presence of antimycin, low-potential cytochrome *b*-562 becomes reduced first. This finding is somewhat surprising, since even in the presence of antimycin the mid-point redox potential of high-potential cytochrome *b*-562 is still 50 mV more positive than that of low-potential cytochrome *b*-562. This could mean that there exists a barrier for high-potential cytochrome *b*-562 to accept electrons. This barrier is apparently lower at high pH, since the ratio of the reduction rates of the two cytochromes then decreases (see Table I). This phenomenon is possibly related to the so-called accessibility barrier [48]. This barrier is apparently absent in submitochondrial particles under conditions of oxidant-induced reduction of cytochrome *b*. Trace B of Fig. 2 was fitted best with a single exponential and also the points of Fig. 10 fit reasonably well with a single exponential. The version of the Q cycle previously proposed [9,12,13] predicts that in the presence of antimycin, cytochrome *b*-562 would be reduced at a rate comparable to that of the Fe-S cluster and cytochrome c_1. As shown in Table I, this is approximately true for low-potential cytochrome *b*-562 although at pH 7 the discrepancy is rather large (232 vs. 358 ms).

One may wonder why, in the absence of antimycin, this cytochrome is so slowly reduced and only after an appreciable lag time. A possible explanation is that in the absence of antimycin, low-potential (lp) cytochrome *b*-562 is more rapidly oxidized than reduced. In this case, the antimycin-sensitive $Q_{in}^{\cdot-}$ could serve as the oxidant according to the equation:

$$b^{2+}\text{-562 (lp)} + Q_{in}^{\cdot-} + 2H_{in}^{+} \rightarrow b^{3+}\text{-562 (lp)} + QH_2 \qquad (1)$$

It is clear from Tables I and II that low-potential cytochrome *b*-562 becomes reduced after the Fe-S clusters and cytochrome c_1 are nearly fully reduced, so that this cytochrome can no longer accept electrons via this route. It can, however, accept electrons by the reverse of Eqn. 1, which is apparently slow but is very likely to become more rapid at higher pH. The onset of this reaction, i.e., the lag time between the two reduction phases of cytochrome *b*-562, is thus determined by the turnover rate of the Fe-S clusters and cytochrome c_1 and since this increases with increasing pH and DQH_2 concentration, the lag time decreases. That the lag time in submitochondrial particles is usually shorter than in isolated succinate:cytochrome *c* oxidoreductase, although the Fe-S clusters and cytochromes $c + c_1$ are reduced at the same rate, is partly due to the presence of detergent (results not shown) and maybe also to the lower Q content of the isolated enzyme.

The biphasic kinetics of the Fe-S clusters and cytochrome c_1 (see below) do not easily fit the models of the Q cycles previously proposed [9,12,13], but are compatible with the scheme of Fig. 11. This scheme retains all the important characteristics of the Q cycle [7], but also takes account of the dimeric aggregation state of the enzyme [49–51], and of the multiplicity and stoicheiometry of the prosthetic groups, i.e., c_1: *b*-562 (hp): *b*-562 (lp): *b*-566: *b*-558: cluster 1: cluster 2 = 2:1:1:1:1:1:1 [11,30,52,53]. Four sites per dimer where, in principle, semiquinone anions can be formed and Q and QH_2 can be bound are proposed. We have detected two different species of semiquinone anion and therefore assume that the other two cannot be stabilized, under the experimental conditions, to an extent that they are observable by EPR. We propose that the latter are the $Q_{in}^{\cdot-}$ and $Q_{out}^{\cdot-}$ species of protomer I. The Q bound near cluster 1 could be reduced to $Q_{out(II)}^{\cdot-}$ (see below) or $Q_{out(I)}^{\cdot-}$ which is not observable by EPR. If the latter proposal is true we can distinguish three different Q-binding domains. The finding that the maximal concentration of $Q_{in}^{\cdot-}$ amounts to 50% of the cytochrome c_1 concentration [10,16], i.e., to one per protomer II, is very readily explained by the scheme of Fig. 11. The assignment of cytochromes *b*-566 and *b*-558 to protomers I and II, respectively, is arbitrary, but



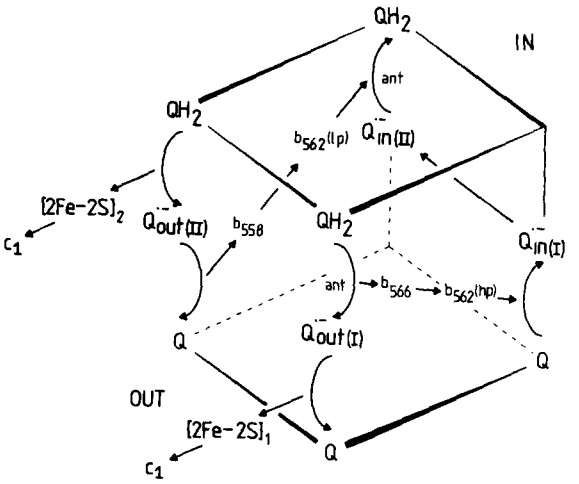


Fig. 10. Kinetics of formation of $Q^{\bar{}}_{out}$, reduction of cytochrome *b* and the Fe-S cluster in submitochondrial particles under conditions of oxidant-induced reduction of cytochrome *b* in the presence of antimycin. Experimental conditions as in the legends of Figs. 5 and 6, but now 3 mol antimycin/mol c_1 was also added. (●———●) Reduction of cytochromes *b*-562 and *b*-566, that are indistinguishable under these conditions; (□———□) formation of $Q^{\bar{}}_{out}$ (the maximum amount of $Q^{\bar{}}_{out}$ corresponds to 0.06 mol/mol cytochrome c_1); (○———○) reduction of cytochrome *b*-558 (see also Fig. 9 and text); (■———■) reduction of the Fe-S cluster. At 51 ms about 30% of the total amount of Fe-S clusters is reduced. The curves through the top three traces are all first-order exponentials with $t_{1/2} = 33$ ms. Cytochromes $c + c_1$ and aa_3 remain nearly fully oxidized throughout the reaction (see also text and Fig. 9).

Fig. 11. Diagram showing a three-dimensional representation of a double Q-cycle that describes electron transfer in a dimeric QH_2:cytochrome *c* oxidoreductase. Curves arrows represent chemical equations, straight full arrows the pathway of electron transfer. Q and QH_2 are freely diffusible, the semiquinone anions are fixed. The pathway of protons is omitted. The front and posterior faces, carrying the cytochrome *b* polypeptides, represent the membrane fractions of protomers I and II, respectively. Indices I or II indicate that $Q^{\bar{}}$ is located in protomers I and II, respectively. Cytochrome *b*-566 is placed arbitrarily in protomer II and cytochrome *b*-558 in protomer I. It is equally possible that cytochrome *b*-558 is in protomer I and cytochrome *b*-566 in protomer II. The same uncertainty applies to $[2Fe-2S]_1$ and $[2Fe-2S]_2$. The cytochrome c_1 in protomer I is reduced via an antimycin-sensitive pathway. Direct electron transfer between the two cytochrome *b* polypeptides is not shown in the diagram. Antimycin-binding sites are represented by ant. See text for further details.

not that of the high- and low-potential cytochromes *b*-562. The reduction kinetics of the latter cytochromes are given by Eqns. 2–6.

$$QH_2 + b^{3+}\text{-}566 \rightarrow Q^{\bar{}}_{out(I)} + b^{2+}\text{-}566 + 2H^+_{out} \quad (2)$$

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

$$b^{2+}\text{-}562\ (hp) + Q \rightarrow b^{3+}\text{-}562\ (hp) + Q^{\bar{}}_{in(I)} \quad (4)$$

$$Q^{\bar{}}_{in(I)} + Q \rightarrow Q + Q^{\bar{}}_{in(II)} \quad (5)$$

$$b^{2+}\text{-}562\ (lp) + Q^{\bar{}}_{in(II)} + 2H^+_{in} \rightarrow b^{3+}\text{-}562\ (lp) + QH_2 \quad (6)$$

Eqn. 5 could, in principle, be omitted if $Q^{\bar{}}_{in(I)}$ and $Q^{\bar{}}_{in(II)}$ are identical. The reactions of Eqns. 2–5 occur within 5 ms (see Figs. 5 and 8) and cytochrome *b*-562 (lp) becomes reduced, via Fe-S cluster 2, with a $t_{1/2}$ greater than 17 ms. Provided that the forward rate of Eqn. 6 is fast, cytochrome *b*-562 (lp) can be rapidly oxidized, the more so as the oxidant, $Q^{\bar{}}_{in(II)}$, is formed much faster than cytochrome *b*-562 (lp) becomes reduced, which explains the low reduction level of this cytochrome. When, however, all Fe-S and cytochrome c_1 are reduced, $Q^{\bar{}}_{in(II)}$ can no longer be formed and cytochrome *b*-562 (lp) equilibrates with the Q/QH_2 couple. According to our proposal, antimycin, which inhibits the reaction of Eqn. 6 and thus blocks the oxidation of cytochrome *b*-562 (lp), also inhibits the rapid reduction of cytochrome *b*-562 (hp) and formation of $Q^{\bar{}}_{in(II)}$ (see Figs. 2, 3B, 5 and 10) by inhibiting the reaction of Eqn. 2.

At first sight, the antimycin-binding sites in protomers I and II look very dissimilar. However,

in both protomers antimycin inhibits a reaction in which the $QH_2/Q^{\cdot}$ couple and one of the haems in cytochrome *b* is involved. The spectroscopic data suggest that the three-dimensional structure in the region of the haems is different for all four haems. This might be due to a structural asymmetry in the dimeric enzyme and is represented in Fig. 11 by the fact that the two cytochrome *b* polypeptides run in opposite directions. As a consequence, the two antimycin-binding sites are located diametrically opposed to each other.

Although we have shown that there are two different [2Fe-2S] clusters, we cannot decide at the moment which cluster is located in which protomer, the more so since in all freeze-quench experiments cluster 1 always showed the broad line shape, possibly because QH_2 is bound during turnover. The different EPR properties of clusters 1 and 2, reflecting a different conformation due to bound Q, have a rational basis in the scheme of Fig. 11, since the cluster in protomer I acts as a semiquinone/quinone oxidoreductase and the other cluster as a quinol/semiquinone oxidoreductase. This demands some flexibility of the Fe-S protein, e.g., two stable conformations, since the primary structures of the two proteins are very likely identical. The same holds for cytochrome *b*.

The reduction kinetics of the two Fe-S clusters and cytochrome c_1 are explained as follows. The total amount of Fe-S clusters is apparently reduced in a single phase (Figs. 6 and 8) with the same $t_{1/2}$ in submitochondrial particles and isolated succinate : cytochrome *c* oxidoreductase. Consequently, clusters 1 and 2 have the same 'intrinsic' turnover number, irrespective of whether $(D)QH_2$ or $Q^{\cdot}_{out(I)}$ is the reductant. Taken in conjunction with the finding that electrons from DQH_2 enter the enzyme within 5 ms, one can conclude that the reactions in which clusters 1 and 2 become reduced are rate limiting (cf. Ref. 54). Electron transfer between the Fe-S cluster and cytochrome c_1 must be very rapid as judged from the slight difference in half-reduction times, particularly since the midpoint redox potential of cytochrome c_1 is 60 mV more negative [55,56]. The reduction of cytochrome c_1 followed by the freeze-quench technique is readily simulated with a single exponential (the spectral contribution of cytochrome *b*-562 in the low-temperature spectrum is negligible).

In the presence of antimycin and absence of oxidant, only 50% of the total amount of Fe-S clusters and cytochrome c_1 becomes reduced at the same rate as in the absence of antimycin (see Tables I and II and Fig. 6). This observation is very hard to reconcile with a single Q cycle, but is readily explained by the scheme in which it is proposed that the formation of the reductant for the [2Fe-2S] cluster and cytochrome c_1 of protomer I, i.e., $Q^{\cdot}_{out(I)}$, is inhibited by antimycin. Thus one Fe-S cluster and one cytochrome c_1 per dimer are reduced via an antimycin-sensitive pathway.

In the presence of antimycin and oxidant, all the cytochrome *b* becomes reduced with a $t_{1/2}$ larger than that of the reduction of the Fe-S cluster. It is proposed that the cytochromes *b* of protomer I can become reduced by direct electron transfer from the cytochromes *b* of protomer II, without mediation of Q (cf. Refs. 29 and 40). Under the same conditions, the Fe-S cluster is transiently reduced (see Fig. 10) and then becomes slowly oxidized, while cytochromes $c + c_1$ remain more than 95% oxidized. It seems, therefore, that there is no equilibration between the Fe-S cluster and cytochrome c_1. This phenomenon could be related to the effect of the regulatory component 'Y' proposed by Eisenbach and Gutman [57], which is possibly Q bound to the reduced Fe-S cluster 1 [13,58].

Concomitantly with the oxidant-induced reduction of cytochrome *b*, an antimycin-insensitive, BAL-sensitive semiquinone anion, $Q^{\cdot}_{out(II)}$, is formed (see Fig. 10). It was concluded recently [12] that this species is the reductant for cytochrome *b*, specifically, according to the scheme in Fig. 11, cytochrome *b*-558. This semiquinone anion was also detected in isolated succinate:cytochrome *c* oxidoreductase, in the presence of antimycin, when $Fe(CN)_6^{3-}$ or cytochrome *c* + cytochrome *c* oxidase were added (results not shown), but no semiquinone anion whatsoever was detected in the absence of oxidants (see Fig. 5). It appears that the concentration of $Q^{\cdot}_{out(II)}$ is usually very low. This may be because the rate of its formation, via cluster 2, is much lower than the rate at which it is oxidized by cytochrome *b*-558. Under conditions of oxidant-induced reduction of cytochrome *b*, the latter cannot be oxidized, while the (oxidized) [2Fe-2S] cluster can continuously

create the reductant, $Q^{\cdot-}_{out(II)}$, for all the cytochrome *b*. Since the half-times of formation of $Q^{\cdot-}_{out(II)}$ and reduction of cytochrome *b* are the same, they equilibrate rapidly. Thus, the concentration of $Q^{\cdot-}_{out(II)}$ will increase as long as cytochrome *b* can accept electrons (Fig. 10). This kinetic explanation for the accumulation of the reductant of cytochrome *b*, under conditions of oxidant-induced reduction of cytochrome *b*, is essentially the same as that given by Mitchell [7]. In the previously proposed variants of the Q-cycle, an electron, originating from the dehydrogenases [2,7] or from a second molecule of QH_2 via a reversed dismutation reaction [9,12,13], is required to complete the cycle. In the double Q cycle of Fig. 11, the 'extra' electron for the Q-cycle in protomer II is supplied by protomer I so that the enzyme functions as a 'self-contained' QH_2:cytochrome *c* oxidoreductase [7]. According to our proposal, a single protomer is unable to catalyze the overall reaction, but together in a dimer they can.

The experimental results described in this paper are consistent with the basic concepts of the pathway of electrons as depicted in the Q cycle. Findings that are not consistent with the 'single' Q cycles proposed earlier all derive from the fact that the enzymic unit is a dimer, consisting of two identical monomers that become two distinguishable protomers upon dimer formation. For this reason, we have proposed the 'double' Q cycle of Fig. 11. Of course, the degrees of freedom for a model that describes electron flow through a dimeric enzyme are much greater than those for a monomer. The experiment of Deul and Thorn [1,11] and the analogous experiment of Trumpower [2] are very discriminating and greatly reduce the number of possibilities. We have chosen the variant that requires the least number of assumptions by combining the data in the literature and those brought forward in this paper. In the Q cycle depicted in Fig. 11, we have omitted the protons but in Eqns. 2 and 6 we have retained the proposal of Mitchell [7] with respect to the H^+/e stoicheiometry [2] and the mechanism of H^+ translocation. However, other mechanisms [8,59,60] are also attractive possibilities and it is not certain that the H^+/e stoicheiometry equals 2.

The biological advantage of a dimeric QH_2:cytochrome *c* oxidoreductase over a monomeric enzyme for the functioning of a Q cycle is that the dimer acts as a self-contained QH_2:cytochrome *c* oxidoreductase. In rat liver mitochondria, for example, at least six enzymes can reduce Q. According to the single Q cycle envisaged by Mitchell [7], all these enzymes would require a specific binding domain for the interaction with a monomeric QH_2:cytochrome *c* oxidoreductase in order to transfer the electron that completes the cycle. Obviously, from the standpoint of evolution, a dimeric QH_2:cytochrome *c* oxidoreductase, composed of two identical monomers, reacting with the common product QH_2, is a much simpler alternative. Our previous proposal [9,12,13] was also apparently a self-contained enzyme, since it was proposed that the extra electron was provided by the reversed dismutation of one molecule of Q with a second molecule of QH_2. Since, however, the product of this reaction is two molecules of semiquinone, this mechanism also requires a dimer.

Acknowledgements

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