Biochimica et Biophysica Acta, 723 (1983) 91–103
Elsevier Biomedical Press

BBA 41279

THE EFFECT OF pH, UBIQUINONE DEPLETION AND MYXOTHIAZOL ON THE REDUCTION KINETICS OF THE PROSTHETIC GROUPS OF UBIQUINOL: CYTOCHROME *c* OXIDOREDUCTASE

SIMON DE VRIES, SIMON P J ALBRACHT, JAN A BERDEN, CARLA A M MARRES and E C SLATER *

Laboratory of Biochemistry, B C P Jansen Institute, University of Amsterdam, P O Box 20151, 1000 HD Amsterdam (The Netherlands)

(Received November 19th, 1982)

Key words *Ubiquinol cytochrome c oxidoreductase, ESR, Myxothiazol, Reduction kinetics, Electron transfer, Semiquinone*

(1) The kinetics of the reduction by duroquinol of the prosthetic groups of QH_2: cytochrome *c* oxidoreductase and of the formation of ubisemiquinone have been studied using a combination of the freeze-quench technique, low-temperature diffuse-reflectance spectroscopy, EPR and stopped flow. (2) The formation of the antimycin-sensitive ubisemiquinone anion parallels the reduction of both high-potential and low-potential cytochrome *b*-562. (3) The rates of reduction of both the [2Fe-2S] clusters and cytochromes ($c + c_1$) are pH dependent. There is, however, a pH-dependent discrepancy between their rate of reduction, which can be correlated with the difference in pH dependencies of their midpoint potentials. (4) Lowering the pH or the Q content results in a slower reduction of part of the [2Fe-2S] clusters. It is suggested that one cluster is reduced by a quinol/semiquinone couple and the other by a semiquinone/quinone couple. (5) Myxothiazol inhibits the reduction of the [2Fe-2S] clusters, cytochrome c_1 and high-potential cytochrome *b*-562. (6) The results are consistent with a Q-cycle model describing the pathway of electrons through a dimeric QH_2: cytochrome *c* oxidoreductase.

Introduction

The way in which electron transfer is coupled to proton translocation in QH_2 : cytochrome *c* oxidoreductase is still unknown. Even the exact sequence of electron-transfer reactions has not yet been established, but considerable progress is being made After the successful isolation of the beef-heart enzyme [1,2] and the characterization of the prosthetic groups many kinetic studies have been undertaken to unravel its mechanism of action Wikstrom and Berden [3] have proposed a scheme in which the quinol is oxidized in two successive steps with the semiquinone as an intermediate Their model elegantly describes the mechanism of oxidant-induced reduction of cytochrome *b* in the presence of antimycin, but it is inconsistent with the observations that the reduction of cytochrome *b* is inhibited by antimycin under conditions that the [2Fe-2S] cluster is, in one way or another, incapable of electron transfer [4–9] As a general result of this type of so-called 'double kill' experiments, it may be concluded that cytochrome *b* is reducible by two independent pathways, one involving the [2Fe-2S] cluster and the other the 'antimycin-binding domain'. The Q-cycle proposed by Mitchell [10] and derivatives of this proposal [5,11,12] and also the b-cycle of Wikstrom and Krab [13] incorporate two pathways for the reduction of cytochrome *b*. The latter proposal is, however, inconsistent with the observation that

* To whom correspondence should be addressed

Abbreviations DQH_2, duroquinol; Q^-, ubisemiquinone anion, DQ^-, durosemiquinone anion, BAL, British Anti-Lewisite (2,3-dimercaptopropanol), Mops, 4-morpholinepropanesulphonic acid

two different semiquinone anions can be stabilized on QH_2 : cytochrome *c* oxidoreductase. One species is sensitive to antimycin [14–17] but not to BAL + O_2 treatment [18] and probably corresponds to $Q_{in}^{\cdot-}$ of the Q-cycle. The other species corresponds to $Q_{out}^{\cdot-}$ of the Q-cycle, since it is formed during oxidant-induced reduction of cytochrome *b* in the presence of antimycin [18,19] and is sensitive to treatment with BAL + O_2. Also the finding that a Q-analogue binds to two sites on the enzyme with different affinities is in contradiction with the *b*-cycle scheme [20]

In the Q cycle [10–12] only one type of [2Fe-2S] cluster and two types of cytochrome *b* are considered but it has been established that there exist three or four different types of cytochrome *b* [21–24]. Based on quantitative EPR studies we have proposed that QH_2 : cytochrome *c* oxidoreductase contains four types of cytochrome *b* and two types of the [2Fe-2S] cluster and that the concentration of each prosthetic group is one-half that of the cytochrome c_1 concentration [19,25], suggesting that the basic enzymic unit is a dimer containing two molecules of cytochrome c_1. Ultracentrifuge analysis [26,27] and electron microscopy [28] show that the enzyme is indeed in a dimeric aggregation state both in detergent solution and when incorporated in a phospholipid bilayer

Recently, we have undertaken a study of the reduction kinetics of the prosthetic groups and the semiquinone anions [19]. The anomalous reduction kinetics of the two cytochromes *b*-562 (see also Ref. 29) and of the [2Fe-2S] clusters, in the presence of antimycin, have led us to propose a Q-cycle scheme for a dimeric enzyme, in which in one protomer, as in the schemes in Refs 5 and 10–12, QH_2 reduces the [2Fe-2S] cluster, and in the other protomer it reduces cytochrome *b* (see also Fig 8) One complete turnover involves the oxidation of two molecules of QH_2 and the regeneration of one molecule of QH_2. The latter is formed by the sequential oxidation of high- and low-potential cytochrome *b*-562 by Q and $Q_{in(II)}^{\cdot-}$, respectively, so that the enzyme functions as a self-contained QH_2 oxidoreductase [10]

In this paper the effect of varying the pH, Q depletion and addition of the inhibitor myxothiazol on the reduction kinetics of the prosthetic groups and the formation of the semiquinone anions has been studied It is shown that at low pH and in Q-depleted particles the two [2Fe-2S] clusters have different rates of reduction These results and others described are consistent with the scheme of the double Q-cycle proposed previously [19] but incompatible with the other Q-cycle models [5,10–12].

Materials and Methods

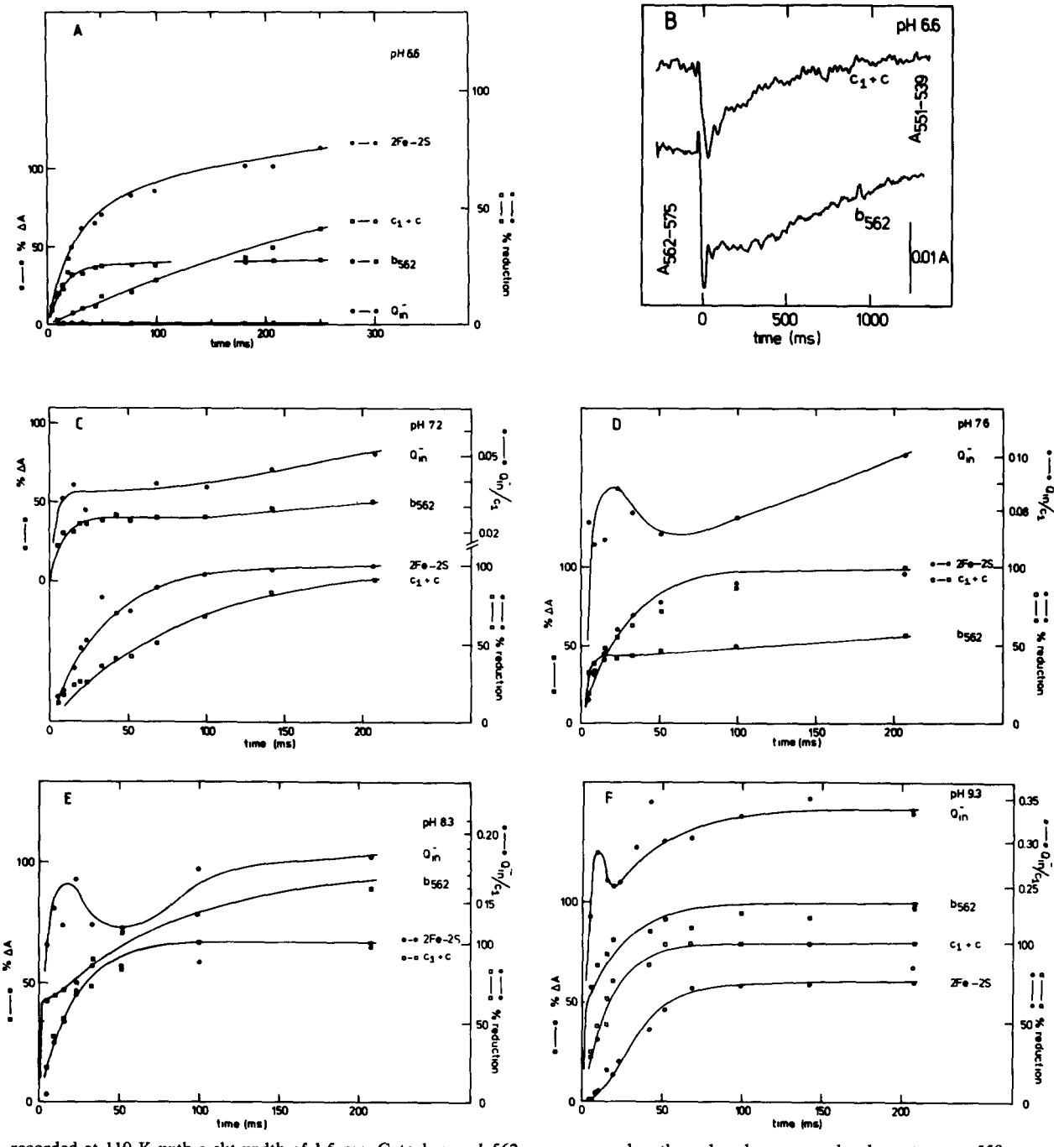
Beef heart submitochondrial particles were prepared as described in Ref. 30 Succinate: and QH_2 · cytochrome *c* oxidoreductase were prepared as described in Refs. 1 and 31, respectively. EPR, low-temperature diffuse-reflectance spectroscopy and freeze-quench experiments were carried out as described previously [19,25]. Stopped-flow experiments were performed with an Aminco stopped-flow apparatus supplied with a home-built four-jet tangential mixer. Pentane extraction was carried out as described in Ref 32 and BAL + O_2 treatment as in Ref. 33.

Results

Effect of pH on the kinetics of the reduction of the prosthetic groups and of the formation of the semiquinone anion $Q_{in(II)}^{\cdot-}$

Cytochromes b-562 and $Q_{in(II)}^{\cdot-}$. It has been shown before that, in the absence of inhibitors, cytochrome *b*-562 becomes reduced in two phases separated by a lag time [29,34,19]. We have ascribed the initial rapid phase to the reduction of

Fig 1 Effect of pH on the reduction kinetics of the prosthetic groups and on the formation of $Q_{in}^{\cdot-}$ of QH_2 cytochrome *c* oxidoreductase as present in submitochondrial particles, measured by EPR and low-temperature diffuse reflectance spectroscopy using the freeze-quench technique (A, C–F) and by stopped flow (B) For freeze-quench experiments one syringe contained particles (70 mg/ml) suspended in 0 25 M sucrose, 50 mM Mops-Tris buffer The other syringe contained an N_2-saturated solution of 0 25 M sucrose, 1 mM acetic acid and 600 μM DQH_2 For stopped-flow experiments the same solutions were used except that the particle concentration was 3 5 mg/ml and 2 mM KCN was present (B) After mixing, the DQH_2 concentration was 300 μM The reaction temperature was 22 ± 2°C A, pH 6 6, B, pH 6 6, C, pH 7 2, D, pH 7 6, E, pH 8 3, F, pH 9 3, Diffuse-reflectance spectra were



recorded at 110 K with a slit width of 1 5 nm Cytochrome *b*-562 was measured in the reduced minus oxidized spectrum as 559 nm minus 570 nm and cytochrome ($c_1 + c$) as 552 nm minus 542 nm A $\%\Delta A$ of 40–43% corresponds to the complete reduction of high-potential cytochrome *b*-562 (cf. Ref 19) which has a smaller extinction coefficient at this wavelength pair than low-potential cytochrome *b*-562 (○———○) Reduction of the [2Fe-2S] clusters (□———□) Reduction of cytochromes ($c_1 + c$) (■———■) Reduction of cytochrome *b*-562. (●———●) Formation of Q^-_{in}, which corresponds to $Q^-_{in(II)}$ of Fig 8 The lines through the points of cytochromes $c_1 + c$ are first-order exponentials with $t_{1/2}$ equal to 320 ms (pH 6 6, cf. A and B), 60 ms (pH 7 2), 20 ms (pH 7 6) and 15 ms (pH 8 3 and 9 3). The lines through the points of the [2Fe-2S] clusters are also first-order exponentials in C, D and E with $t_{1/2}$ equal to 25 ms (pH 7 2), 20 ms (pH 7.6) and 15 ms (pH 8 3) At pH 6 6 the points of the [2Fe-2S] clusters are simulated as a sum of two exponentials, with equal contributions and $t_{1/2}$ equal to 18 and 250 ms

high-potential cytochrome *b*-562 and the second relatively slow phase to that of low-potential cytochrome *b*-562 Furthermore, it was shown that in purified succinate. cytochrome *c* oxidoreductase the lag time between the two reduction phases decreases and the rate of the second phase increases when the pH is raised. The same behaviour of the two cytochromes *b*-562 is found in submitochondrial particles (Fig 1) The lag time decreases from about 400 ms at pH 6.6 (Fig. 1A and B) to less than 5 ms at pH 9.3 (Fig 1F), while the rate of reduction of low-potential cytochrome *b*-562 increases with increasing pH Also, the half-time of reduction of high-potential cytochrome *b*-562 decreases with increasing pH, being about 15 ms at pH 6.6, about 5 ms at pH 7 2 and less than 5 ms at higher pH values.

As we have shown previously [19,34], the formation of the antimycin-sensitive semiquinone anion, $Q^{-}_{in(II)}$, coincides with the reduction of high-potential cytochrome *b*-562 (see also Fig 1). The amount of $Q^{-}_{in(II)}$ formed increases with increasing pH. At pH 6 6 (Fig. 1A) no $Q^{-}_{in(II)}$ is detectable and even at pH 7.2 only a small amount of the radical is formed It seems that at higher pH values the formation of $Q^{-}_{in(II)}$ not only parallels the reduction of high-potential cytochrome *b*-562 but also accompanies the relatively slow reduction of low-potential cytochrome *b*-562. Moreover, there is an apparent overshoot in the formation of $Q^{-}_{in(II)}$ The overshoot is real and is not due to the scatter in results (see, e g , Fig. 2). The standard deviation in this kind of experiment is about 6% of the signal amplitude This overshoot and the increase in the radical concentration at long reaction times were also observed in our previous communication (see Figs 5 and 8 of Ref. 19) but not interpreted as such

The close correspondence between the redox state of low-potential cytochrome *b*-562 and $Q^{-}_{in(II)}$ is also evident in the pH-jump experiment of Fig 2 Particles poised with the fumarate/succinate oxidation-reduction couple at pH 7 3 were mixed with a buffer of pH 9.1. While the redox states of the [2Fe-2S] clusters, cytochromes *c*, c_1, *a* and a_3, and high-potential cytochrome *b*-562 remain unaltered, i.e., fully reduced, low-potential cytochrome *b*-562 becomes more reduced and the concentration of $Q^{-}_{in(II)}$ increases (cf. Refs 15 and

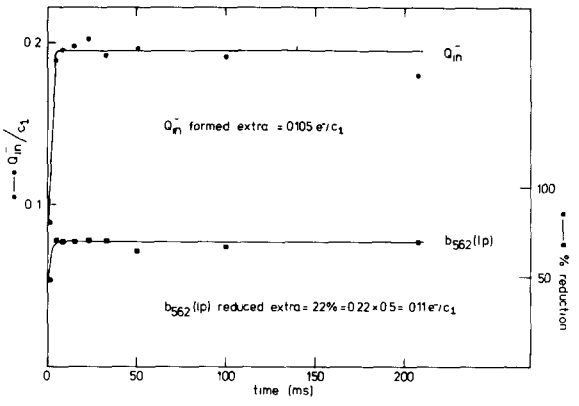


Fig 2 Kinetics of the effect of a change of pH on the redox state of cytochrome *b*-562 and the concentration of $Q^{-}_{in(II)}$ in partly reduced submitochondrial particles Submitochondrial particles dissolved in 0 25 M sucrose, 20 mM Mops buffer (pH 7 3) were incubated with 4 mM KCN, 150 mM sodium fumarate and 25 mM sodium succinate and mixed with a solution of 0 25 M sucrose, 200 mM Tris-HCl buffer (pH 9 1) The pH after mixing was 8 8 The initial redox state of cytochrome *b*-562 and the concentration of $Q^{-}_{in(II)}$ were determined by mixing the partly reduced particle suspension with a solution of 0 25 M sucrose, 200 mM Tris-HCl buffer (pH 7 3) Other experimental conditions and measurements as in Fig 1

16). Both events take place within 5 ms, after which time their redox state remains constant

The [2Fe-2S] clusters and cytochromes ($c + c_1$) It can be seen from Fig 1 that the rate of reduction of the [2Fe-2S] clusters and cytochromes ($c + c_1$) increases with increasing pH At pH 7.6 and 8 3, these prosthetic groups show identical half-reduction times (both 20 ms (pH 7.6) and 15 ms (pH 8.3)), but at pH 6 6 and 7.2 the reduction of cytochromes ($c + c_1$) clearly lags behind that of the [2Fe-2S] clusters. In addition, at pH 6.6 (Fig. 1A), the reduction kinetics of the [2Fe-2S] clusters do not follow a single exponential, whereas they apparently do so at pH 7 2, 7.6 and 8.3 At pH 6.6 about half of the [2Fe-2S] clusters is reduced with a $t_{1/2}$ of 18 ms and the other half with a $t_{1/2}$ of about 250 ms (Fig 1A) There are no indications that at pH 6.6 also cytochromes ($c + c_1$) are reduced biphasically (cf. Fig 1A and B)

At pH 9 3 (Fig. 1F) cytochromes ($c + c_1$) are reduced in a single rapid phase ($t_{1/2} = 12$ ms) but the [2Fe-2S] clusters behave abnormally. After a short lag period they become reduced relatively slowly ($t_{1/2} = 30$ ms) but even after 200 ms the

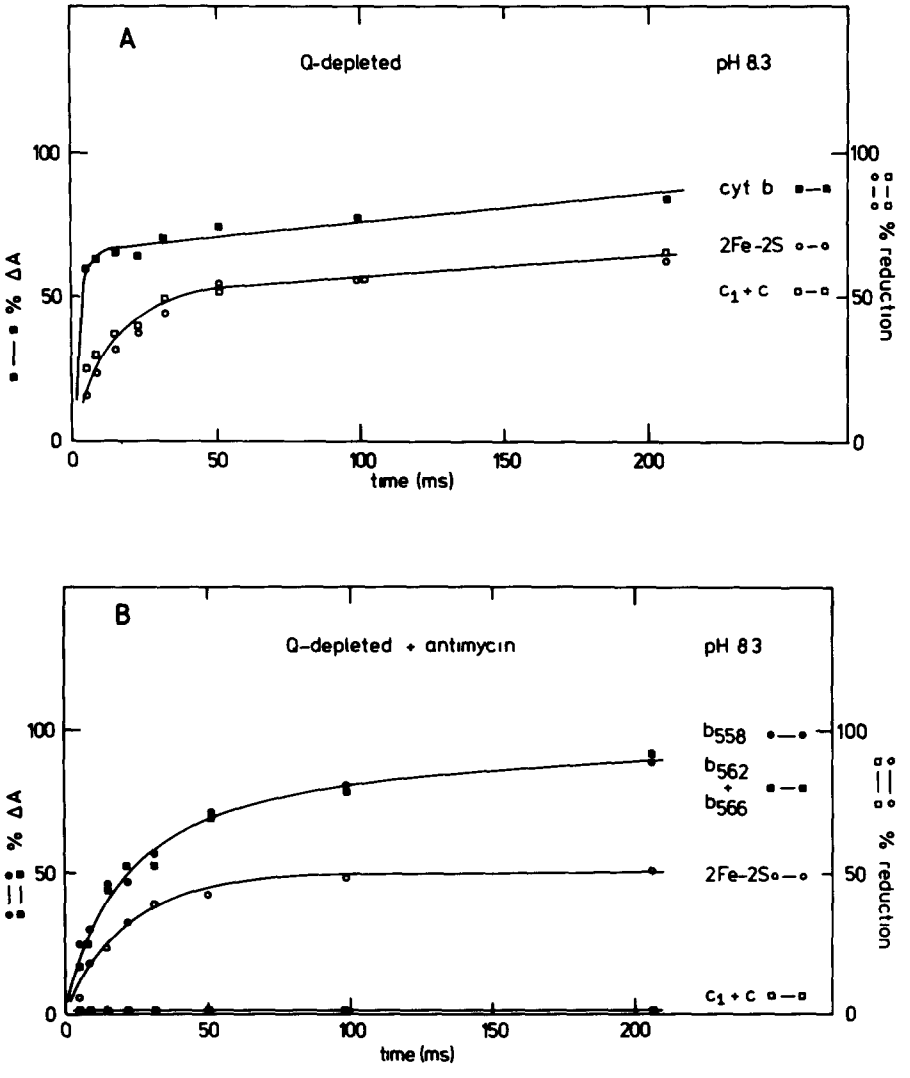


Fig 3 Kinetics of the reduction of the prosthetic groups of QH_2 cytochrome *c* oxidoreductase as present in Q-depleted submitochondrial particles Experimental conditions as in Fig 1 The pH after mixing was 8 3 A, no further additions, (■———■) reduction of cytochrome *b*, the reduction level of 60% after 5 ms corresponds to the reduction of all cytochrome *b*-562 (see also text) B, 2 mol antimycin/mol c_1 was added, (●———●) reduction of cytochrome *b*-558, (■———■) reduction of cytochromes *b*-562 and *b*-566 In A, 25% reduction of cytochromes ($c_1 + c$) and the [2Fe-2S] clusters is obtained after 11 ms In B, 25% of the [2Fe-2S] clusters is reduced after 15 ms, and 50% of cytochromes *b*-558, *b*-562 and *b*-566 after 25–30 ms In this case cytochromes ($c_1 + c$) remain oxidized

reduction level is only about 75–80%. Thus, at pH 9.3 cytochromes ($c + c_1$) become reduced before the [2Fe-2S] clusters.

Effect of Q depletion on the reduction kinetics of the prosthetic groups

After extraction with pentane (four times) and pentane + 10% acetone [32] no chemically detectable Q was present in the particles. The total amount of Q extracted was 12.1 mol Q/mol cytochrome c_1 After reduction (cf. Fig. 3A) a very small amount of radical could be detected and its intensity decreased to zero in the presence of antimycin (cf. Fig. 3B). Due to its low concentration, estimated to be about 1–2% of that of cytochrome c_1, and the poor signal-to-noise ratio, the

signal could not be identified as either $Q^{-}_{in(II)}$ or $Q^{-}_{out(II)}$, but the effect of antimycin on the signal intensity suggests that the radical may be $Q^{-}_{in(II)}$.

The experiment of Fig. 3A shows that in Q-depleted particles cytochrome *b* becomes reduced by DQH_2 in two phases. Within 5 ms all cytochrome *b*-562 is reduced and not only high-potential cytochrome *b*-562 as in normal particles By measuring the optical difference spectra with the sample of 5 ms as the reference, it was shown that the slower reduction phase is due to the reduction of cytochromes *b*-566 and *b*-558. Even after 200 ms the latter are only partly reduced

In the Q-depleted particles only 50% of the total amount of [2Fe-2S] clusters becomes reduced with a similar $t_{1/2}$ (12 ms) as the total amount of [2Fe-2S] clusters in normal particles (see Fig. 1E) The second half is reduced much more slowly, about 15% after 200 ms Cytochromes $(c + c_1)$ behave in exactly the same way

In the presence of antimycin (Fig. 3B) cytochromes $(c + c_1)$ remain oxidized In this case only one-half of the [2Fe-2S] clusters is reduced ($t_{1/2}$ = 15 ms) and the second slow reduction phase is absent. All species of cytochrome *b* become reduced but in contrast to normal particles [19], the reduction is not monophasic. After 25 ms half reduction is obtained

The effect of myxothiazol on the EPR properties and reduction behaviour of the prosthetic groups

The recently introduced inhibitor myxothiazol was shown to inhibit electron transfer in the QH_2 : cytochrome *c* oxidoreductase between cytochromes *b* and c_1 [7,35]. Furthermore, it shifts the α-band maximum of cytochrome *b*-566 to the red and this shift is additive to that of antimycin [7,35], showing that these two inhibitors can bind simultaneously. Addition of the two inhibitors together inhibits the reduction of cytochrome *b* (Ref. 7 and cf. Refs. 4, 5 and 9) In order to define the site of action of myxothiazol more precisely we have studied its effect on the EPR spectra of the prosthetic groups

The EPR spectrum of ascorbate-reduced QH_2 : cytochrome *c* oxidoreductase is composed of the resonances of the four cytochromes *b* [25] (Fig. 4, trace A). In agreement with previous work [36], antimycin shifts the maximum at $g = 3\,44$,

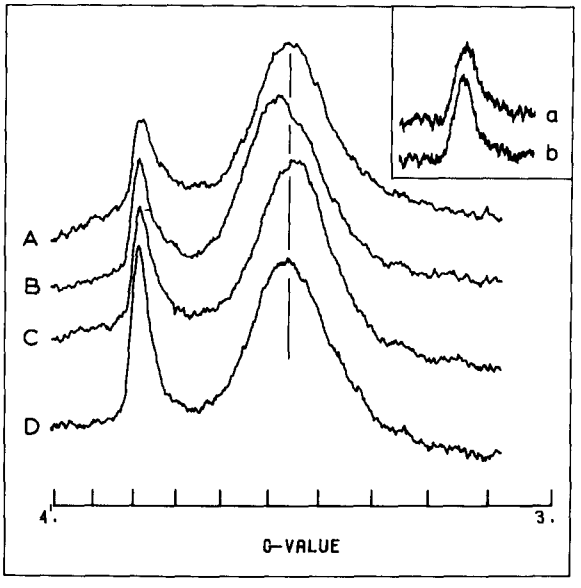


Fig 4 The effect of antimycin and myxothiazol on the EPR spectra of the *b* cytochromes in QH_2 cytochrome *c* oxidoreductase QH_2 cytochrome *c* oxidoreductase prepared by the method of Von Jagow et al [31] was diluted 2-fold with glycerol All samples contained a final concentration of 1 5% (v/v) dimethyl sulphoxide and 2 3% (v/v) ethanol, the solvents for antimycin and myxothiazol, respectively Each inhibitor was allowed to react with the enzyme for 3 min at 0°C before the mixture was frozen or before the second inhibitor was added EPR spectra were then recorded (two examples shown in the inset) after which the samples were thawed and mixed with 13 mM sodium ascorbate for 1 min at 0°C The samples were then frozen again in liquid nitrogen A, only solvents added, B, in the presence of 5 5 mol antimycin/cytochrome c_1, C, in the presence of 5 0 mol myxothiazol/cytochrome c_1, D, both antimycin and myxothiazol were present in the same amounts as in B and C The inset shows an enlargement of the $g = 3\,8$ region as present in the enzyme before the addition of ascorbate a, only solvents added, b, antimycin and myxothiazol were both present as in D Note the lack of effect of the inhibitors in contrast to the effect in the presence of ascorbate EPR conditions microwave frequency, 9258 4 MHz, microwave power, 11 7 mW, temperature, 10 K, modulation amplitude, 1 25 mT (traces A–D) and 2 5 mT (inset) All spectra were corrected for differences in the experimental microwave frequency

belonging to high- and low-potential cytochromes *b*-562 [19,25], to lower field values (trace B). Moreover, this peak is somewhat broadened and that at $g = 3\,78$, belonging to either cytochrome *b*-566 or *b*-558 [19,25], is sharpened. Myxothiazol shifts the peak at $g = 3\,44$ to a higher field value and that at $g = 3.78$ is sharpened (trace C). Addition of both

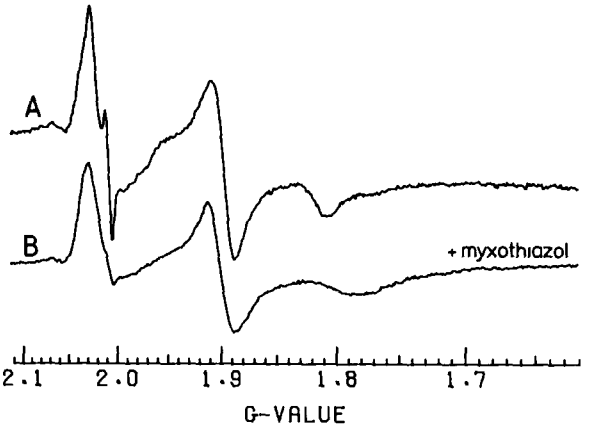


Fig 5 The effect of myxothiazol on the EPR spectrum of the [2Fe-2S] clusters of QH_2 cytochrome *c* oxidoreductase The enzyme was incubated for 15 s at 0°C with 1 mM ascorbate prior to freezing A, no further additions, B, 1 4 mol myxothiazol/mol cytochrome c_1 was added The final dimethyl sulphoxide concentration was 0 4% which did not change the spectrum EPR conditions microwave frequency, 9 24 GHz, microwave power, 2 mW, modulation amplitude, 0 63 mT, *T*, 36 K

antimycin and myxothiazol (trace D), the order of addition not being important, causes the peak at $g = 3.78$ to be very much intensified and sharpened However, the peak position of the resonances at $g = 3.44$ is the same as that in the absence of inhibitors (cf. traces A and D) Thus, the spectral shifts induced in this peak by antimycin and myxothiazol cancel each other but the sharpening of the resonance at $g = 3.78$ is additive. This latter phenomenon was, however, not observed in the fully oxidized enzyme (Fig. 4, inset) although the other effects were independent of the redox state (not shown).

We have shown before that the EPR spectrum of the [2Fe-2S] cluster of ascorbate-reduced QH_2 : cytochrome *c* oxidoreductase is in fact an overlap of two signals in a 1 · 1 weighted ratio in submitochondrial particles and that this ratio is somewhat variable in more purified preparations of the enzyme, owing to the variable and low ubiquinone content [19,25]. As shown in Fig. 5,

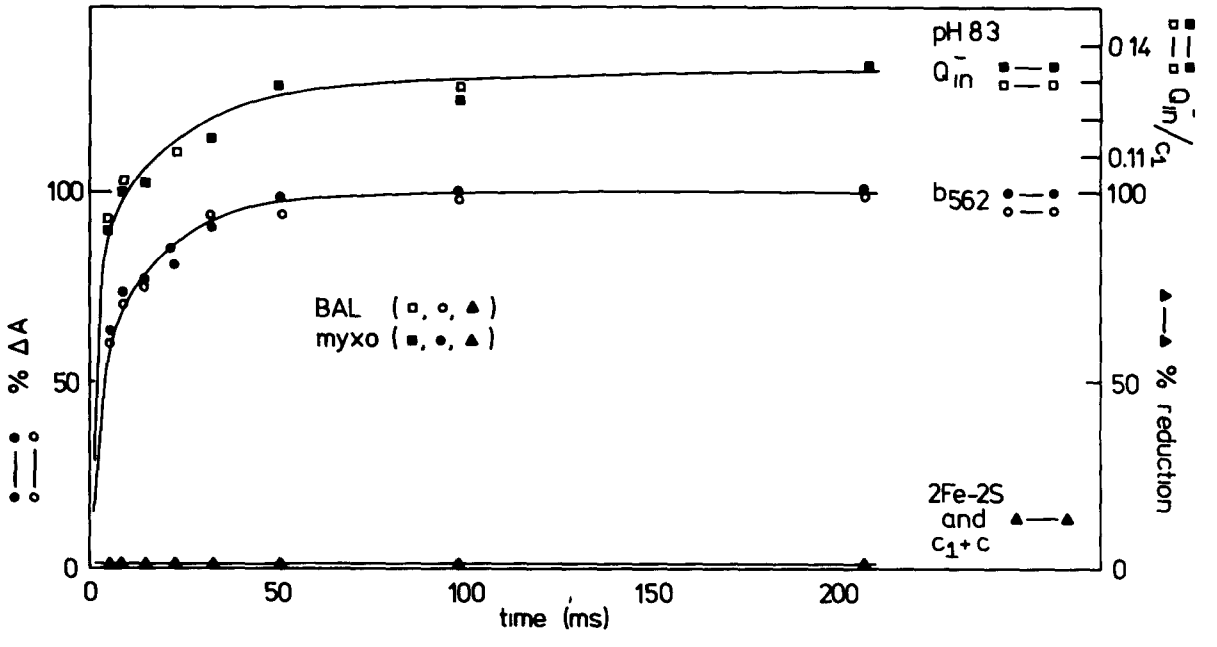


Fig 6 The effect of myxothiazol or BAL ($+O_2$) treatment on the reduction kinetics of the prosthetic groups and the formation of $Q^-_{in(II)}$ of QH_2 cytochrome *c* oxidoreductase as present in submitochondrial particles Experimental conditions as in Fig 1 The pH after mixing was 8 3 Open symbols refer to experiment with myxothiazol (3 mol/mol cytochrome c_1) and closed symbols to that of BAL ($+O_2$) treated particles (succinate oxidation was inhibited by 95%) In the presence of myxothiazol the [2Fe-2S] clusters and cytochromes $c + c_1$ remain oxidized BAL treatment destroys the [2Fe-2S] clusters [6] and prevents reduction of cytochrome $c + c_1$ [33] Note that in the first 5 ms about 60% of the total amount of cytochrome *b*-562 is reduced, i e , low-potential cytochrome *b*-562

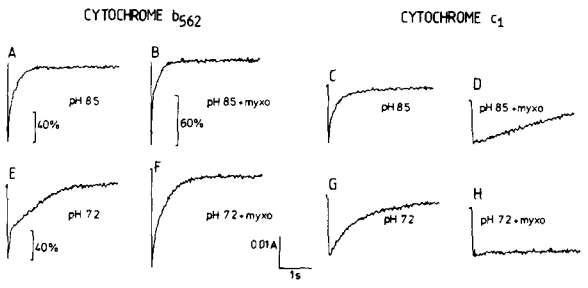


Fig 7 The effect of myxothiazol on the reduction behaviour of cytochromes *b*-562 and c_1 as measured by stopped flow One syringe contained 0.25 M sucrose, 100 mM NaCl, 1 mM acetic acid, 200 μM DQH_2, pH 4, the other 0 25 M sucrose, 100 mM NaCl, 1% sodium cholate, 0 8 mg/ml succinate cytochrome *c* oxidoreductase (1 5 μM c_1) and 50 mM Mops-Tris buffer Traces A–D, pH 8 5, traces E–H, pH 7 2, Myxothiazol (4 5 μM) was present in traces B, D, F and H

myxothiazol alters the EPR spectrum most markedly in the $g_x = 1.80$ resonance belonging to cluster 1, broadening and shifting it to a lower *g* value, but the g_z and g_y peaks are also affected The EPR spectrum after addition of myxothiazol is quite similar to that obtained after Q depletion [19] or reduction by succinate [19,25], in which cases the EPR parameters of cluster 1 and 2 are indiscernible. However, in the latter two cases the g_y resonance is symmetrical around the zero-crossing in contrast to the asymmetrical line shape in the presence of myxothiazol (trace B)

The freeze-quench experiments of Fig 6 show that in the presence of myxothiazol the [2Fe-2S] clusters and cytochrome c_1 remain oxidized. Cytochrome *b*-562 is, however, very rapidly reduced in two phases. The first phase ($t_{1/2} < 5$ ms) comprises about 60% of the total absorbance change and probably corresponds to the reduction of low-potential cytochrome *b*-562 and in the second phase ($t_{1/2} = 15$ ms) high-potential cytochrome *b*-562 becomes reduced. The kinetics of the formation of $Q^{-}_{in(II)}$ correlate with those of the two cytochromes *b*-562. This reduction behaviour of the two cytochromes *b*-562 is confirmed in stopped-flow experiments with succinate · cytochrome *c* oxidoreductase, i.e., in the presence of myxothiazol the rapid reduction phase contributes 60% to the total and in the absence of the inhibitor about 40%, corresponding to low- and high-potential cytochrome *b*-562, respectively (Fig 7) Myxothiazol inhibits the reduction of cytochrome c_1 (Fig 7) at both pH 7 2 (98.5%) and pH 8.5 (95%). Note that in the presence of myxothiazol or after BAL + O_2 treatment the lag time between the reduction of the two cytochromes *b*-562 is absent

Discussion

The rate of reduction of the prosthetic groups of QH_2 . cytochrome *c* oxidoreductase by DQH_2 increases with increasing pH. Although the effect of pH could in principle be described in the same manner as Rich and Bendall [37,38] did for the reaction between quinols and cytochrome *c*, such an analysis would be premature for the moment because of the complexity of QH_2 : cytochrome *c* oxidoreductase Complicating factors are, for instance· (i) the reduction by DQH_2 of its immediate acceptor may not be purely a bimolecular collision reaction; (ii) the midpoint potentials of possible acceptors, e.g., cytochrome *b* and the [2Fe-2S] clusters, are pH dependent; (iii) the pK_a of bound quinol is unknown; protonation-deprotonation reactions on the enzyme are not a priori more rapid than electron-transfer reactions

Between pH 7 2 and 8 3 the two [2Fe-2S] clusters are reduced in apparently a single phase, the half-times of reduction increasing with decreasing pH At pH 6 6, however, the reduction is biphasic. The first half follows a single exponential with a $t_{1/2}$ of about 18 ms and the second half is reduced much more slowly.

Since the $t_{1/2}$ of the rapid phase at pH 6 6 is very similar to that of both [2Fe-2S] clusters at pH 8.3, it is likely that the rate of reduction of one of the two [2Fe-2S] clusters is independent of pH and that of the other dependent. If this is the case, the traces of the [2Fe-2S]clusters at pH 7.2 and 7 6 (Fig. 1C and D) would be a sum of two exponentials, with equal contributions, and with slightly different values of $t_{1/2}$, but at pH 6.6, the difference between the two values of $t_{1/2}$ is large enough to detect two separate phases The different effect of pH on the reduction rate of the two [2Fe-2S] clusters cannot be described by the finding that above pH 8 the [2Fe-2S] clusters have pH-dependent midpoint potentials [39], since this p*K* value applies to both [2Fe-2S] clusters The nature of the reducing quinol couple must also be

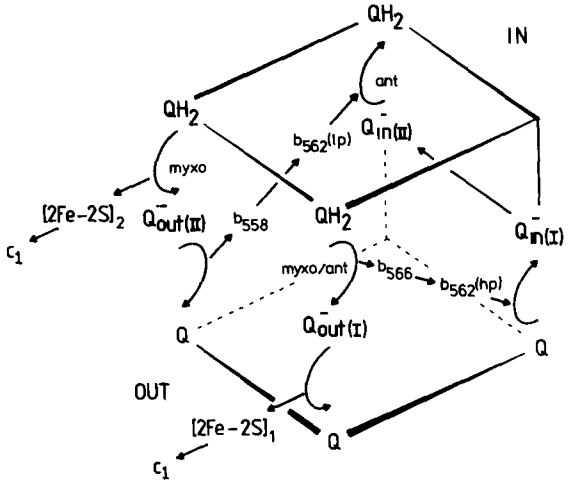


Fig 8 Diagram showing a three-dimensional representation of a double Q-cycle that describes electron transfer in a dimeric QH_2 cytochrome *c* oxidoreductase Curved arrows represent chemical equations, straight full arrows the pathway of electron transfer Q and QH_2 are freely diffusible, the semiquinone 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 Subscripts I or II indicate that Q^- 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 $[2Fe\text{-}2S]_1$ and $[2Fe\text{-}2S]_2$ The cytochrome c_1 in protomer I is reduced via an antimycin-sensitive pathway A possible direct electron transfer between the two cytochrome *b* polypeptides is not shown in the diagram Antimycin-binding sites are represented by ant, and those of myxothiazol by myxo hp, high-potential, lp, low-potential

taken into account. The finding that the two [2Fe-2S] clusters respond differently to a variation of pH indicates that the reducing quinol couples for $[2Fe\text{-}2S]_1$ and $[2Fe\text{-}2S]_2$ are not the same. This is, indeed, the case in the scheme of Fig. 8, but it is very well possible that the protonation states of the various quinol and semiquinone intermediates differ from those depicted in the scheme in Fig. 8. Following the proposal by Rich and Bendall [37,38] that the anionic quinol, QH^-, is a much better reductant than QH_2, it is understandable that both the [2Fe-2S] clusters and cytochrome *b*, but not cytochrome c_1, would have pH-dependent midpoint potentials, i.e., a redox-linked acid-base group, because with the help of this acid-base group the QH_2 can be deprotonated enzymatically to QH^- The sequence of reactions could thus be visualized by:

$$QH_2\ [2Fe\text{-}2S]^{2+} \rightleftharpoons QH^-\ [2Fe\text{-}2S]^{2+}\ H^+$$

$$\rightarrow Q^{\cdot}H\ [2Fe\text{-}2S]^{1+}\ H^+$$

and similarly for cytochrome *b*. According to this interpretation the pH dependence of the midpoint potentials of cytochrome *b* and the [2Fe-2S] clusters is not necessarily related to a proton-pump function [13,40,41], but merely serves to create a proper reductant

Although at pH 7 6 and 8.3 the reduction kinetics of the [2Fe-2S] clusters and cytochromes ($c + c_1$) are identical, there is a large discrepancy at pH 6.6, 7.2 and 9 3. This difference in the rates of reduction can in principle be explained by the pH-dependent midpoint potential of the [2Fe-2S] clusters At pH values far below the pK of 8 the difference between the midpoint potentials of the [2Fe-2S] clusters and cytochromes ($c + c_1$) is about 50–60 mV. This means that at the point where the [2Fe-2S] clusters are about 90% reduced, cytochromes ($c + c_1$) are only 50% reduced, if the equilibration between the prosthetic groups is rapid. At pH values around and somewhat above the pK (7.6 and 8.3) the difference in redox state between the [2Fe-2S] clusters and cytochromes ($c + c_1$) is too small to be detected experimentally. Finally, at pH values far above the pK, the midpoint potentials of the [2Fe-2S] clusters are so much lower than those of cytochromes ($c + c_1$) that the cytochromes would become reduced first, as is indeed found at pH 9.3 (Fig 1F). It is not clear, however, why at this pH only 75–80% of the [2Fe-2S] clusters are rapidly reducible.

It is, in principle, possible to calculate the difference in midpoint potentials of the [2Fe-2S] clusters and cytochrome c_1 * from the kinetic traces, again assuming rapid equilibration. A rough calcu-

* Although the sum of cytochromes *c* and c_1 was measured at 552 nm minus 542 nm, the preparation used contained about 10-times as much cytochrome c_1 as cytochrome *c* (see Ref 19), so that the effects measured are predominantly due to cytochrome c_1

lation shows that at pH 7 the midpoint potential of the [2Fe-2S] clusters is about 40 mV higher, than that of cytochrome c_1 (230 mV [42]), i.e., about 270 mV (cf Refs. 43 and 39). At pH 9 3, the midpoint potentials of the [2Fe-2S] clusters are about 45 mV lower than that of cytochromes ($c + c_1$), indicating a pK of 7 9, which is in good agreement with the value of 8 reported by Prince and Dutton [39].

Cytochrome b-562 is reduced in two phases separated by a lag time. The lag time decreases with increasing pH. We showed in our previous communication [19] that the rapid formation of $Q^-_{in(II)}$ parallels the rapid reduction of high-potential cytochrome b-562. From Fig. 1 it is clear that also the formation of $Q^-_{in(II)}$ shows two phases, and that the second slow phase follows, kinetically, the reduction of low-potential cytochrome b-562 The amount of $Q^-_{in(II)}$ formed both in the first and the second phase increases with increasing pH as is to be expected from the potentiometric titrations [15,16]. The second phase of the formation of $Q^-_{in(II)}$ and the reduction of low-potential cytochrome b-562 probably proceed via reversal of the antimycin-sensitive reaction (Ip, low-potential).

$$Q^-_{in(II)} + \text{cytochrome } b^{2+}\text{-562(lp)} + 2H^+_{in} \rightarrow QH_2 + \text{cytochrome } b^{3+}\text{-562(lp)} \quad (1)$$

since by the time that the reduction of low-potential cytochrome b-562 starts the [2Fe-2S] clusters and cytochromes ($c + c_1$) are highly reduced. The equilibration between DQH_2, Q of the pool, low-potential cytochrome b-562 and $Q^-_{in(II)}$ is rather slow. This may be because DQH_2 cannot reduce Q of the pool directly [44], the large electron-accepting capacity of the Q pool or the fact that only sub-stoicheiometric amounts of $Q^-_{in(II)}$ are found at equilibrium [15,16]. The equilibration of low-potential cytochrome b-562 and $Q^-_{in(II)}$ with the Q pool is very rapid, within 5 ms, as demonstrated by the pH-jump experiment of Fig. 2. Under these conditions the ratio Q/QH_2 hardly changes and the reaction is given by the reversal of Eqn. 1 in which QH_2 is the electron donor. The amount of $Q^-_{in(II)}$ increases because the Q/QH_2 and $Q^-_{in(II)}/QH_2$ couples have different pH dependencies. The amount of extra $Q^-_{in(II)}$ formed is equal to the amount of extra reduced low-potential cytochrome b-562 (cf Fig 2) The latter finding and the close correlation between the kinetics of $Q^-_{in(II)}$ and low-potential cytochrome b-562 both suggest that $Q^-_{in(II)}$ is indeed formed by the reversal of Eqn. 1 and not by a reversed dismutation reaction [11,12].

The interpretation of the experimental results with Q-depleted particles is always somewhat hampered by the question as to whether or not all Q has been extracted. Although we could not detect Q chemically [45], an antimycin-sensitive signal equal to about one-fifteenth of the concentration in normal particles could be detected. It is probably the $Q^-_{in(II)}$ species formed from residual Q in the preparation.

The most important structural difference between Q and DQ is the hydrophobic isoprenoid chain. It is proposed that due to this hydrophobic chain the binding affinity of Q relative to that of DQ is increased and that as a consequence the ubisemiquinone (anion) can be stabilized, but not the durosemiquinone This proposal is in apparent disagreement with the finding that the time required for half reduction of cytochrome b in the presence of antimycin is the same in Q-depleted and normal particles, although the kinetics differ (cf Fig. 3B and Fig. 10 of Ref. 19), which suggests that $DQ^-_{out(II)}$, the product of the oxidation of DQH_2 by $[2Fe\text{-}2S]_2$ (see Fig. 8), exists as an intermediate. Since in normal particles the kinetics of $Q^-_{out(II)}$ and all cytochromes b are identical [19] all these species equilibrate very rapidly. If the equilibration time is shorter or the rate of electron transfer from $(D)Q^-_{out(II)}$ to cytochrome b-558 faster than the dissociation and dismutation of $DQ^-_{out(II)}$, this intermediate would exist sufficiently long to reduce cytochrome b-558. The fact, however, that no radical signal is observed in Q-depleted particles with antimycin, implies that $Q^-_{out(II)}$ in normal particles is a ubisemiquinone anion and not a durosemiquinone anion. It follows that the [2Fe-2S] is reduced in Q-containing particles by ubiquinol formed by reduction of ubiquinone by duroquinol

In Q-depleted particles both high- and low-potential cytochrome b-562 become reduced within 5 ms (Fig 3A). Since this rapid reduction is sensi-

tive to antimycin, it follows on the basis of the scheme of Fig. 8 that these cytochromes *b* become reduced via the QH_2-oxidation site of protomer I, the reversal of Eqn. 1 or both pathways. The fact that only a very small amount of $Q^{\cdot-}_{in(II)}$ is formed means that low-potential cytochrome *b*-562, and consequently all other cytochromes *b*, can only be oxidized at a low rate. Since the $[2Fe\text{-}2S]_2$ is reduced at the normal rate (Fig. 3A) (see also below), the situation is similar to that when antimycin is present in normal particles and it is not surprising that cytochromes *b*-566 and *b*-558 become reduced (see Fig. 3A) That the reduction of these cytochromes is slow and incomplete may be the result of the greater activity of the reaction in which low-potential cytochrome *b*-562 is oxidized via residual $Q^{\cdot-}_{in(II)}$ in Q-depleted particles, than in normal particles inhibited by antimycin

In Q-depleted particles, in the absence of antimycin (Fig. 3A), the [2Fe-2S] clusters are reduced in two phases, each consisting of about 50% of the total amount. The half-time of the first phase is about 12 ms, i.e , the same as in normal particles, and that of the second phase is much longer. In the presence of antimycin (Fig. 3B) only one reduction phase is visible in the first 200 ms with a $t_{1/2}$ of 15 ms and again only 50% of the total amount of [2Fe-2S] is reduced rapidly. These findings suggest that, according to the scheme of Fig. 8, $[2Fe\text{-}2S]_2$ is reduced rapidly both in the presence and absence of antimycin, and that the slow reduction of $[2Fe\text{-}2S]_1$ is inhibited by antimycin.

The fact that the rate of reduction of $[2Fe\text{-}2S]_2$ is independent of the presence of Q but that $[2Fe\text{-}2S]_1$ reduction is greatly inhibited by extraction of Q fits nicely with the proposal of Fig. 8 in which $[2Fe\text{-}2S]_1$ becomes reduced via a semiquinone anion. Apparently, this species cannot exist long enough in Q-depleted particles to reduce $[2Fe\text{-}2S]_1$ It is puzzling, however, that in Q-depleted particles in the presence of antimycin, $[2Fe\text{-}2S]_2$ is completely reduced while cytochromes $(c + c_1)$ are fully oxidized, whereas in the absence of antimycin the redox states of the [2Fe-2S] clusters and cytochromes $(c + c_1)$ are indistinguishable. Moreover, in normal particles in the presence of antimycin, $[2Fe\text{-}2S]_2$ becomes, in part, transiently reduced (see Fig. 10 of Ref 19) while in Q-depleted particles the oxidation phase is apparently absent (see Fig. 3B). In these cases the discrepancy between the redox state of the [2Fe-2S] clusters and cytochromes $(c + c_1)$ cannot be ascribed to the pH-dependent midpoint potentials of the [2Fe-2S] clusters. It is possible that antimycin alone or the combination of addition of antimycin and Q-depletion results in an increase of the midpoint potentials of the [2Fe-2S] clusters in a fashion similar to the effect of some Q analogues [20,46].

It can be concluded from the EPR spectra of Fig 4 that both antimycin and myxothiazol affect the two resonances at $g = 3.44$ and that at $g = 3.78$ In addition, these inhibitors seem to decrease the intensity of the broad resonance at $g = 3.71$ (cf. trace A with traces B–D and Ref. 25). The observation that the EPR spectral shifts induced by antimycin or myxothiazol are of the same order of magnitude, although of opposite direction, on the resonance at $g = 3.44$, and that both inhibitors sharpen the $g = 3.78$ resonance in a similar way, is in apparent contrast to the preferential shifts on cytochrome *b*-566 and *b*-562 by myxothiazol or antimycin, respectively, in the optical spectra The fact that the EPR spectra mainly reflect the properties of the Fe(III) and the optical spectra those of the haem moiety may be relevant in this context, but the fact that in the EPR spectra the cytochromes are studied in the oxidized state and in the optical spectra in the reduced state may also be important. This is illustrated, for instance, by the finding that antimycin and/or myxothiazol sharpen the $g = 3\ 78$ resonance in the ascorbate-reduced enzyme but not in the oxidized enzyme (Fig. 4, inset). This suggests that reduction of the [2Fe-2S] clusters and/or cytochrome c_1 has a profound influence on the configuration of the enzyme [47]. A similar conclusion can be drawn from the observation that some inhibitory Q analogues bind much more firmly to the (ascorbate-) reduced enzyme than to the oxidized one [20,46].

The spectral effects on the *b*-type cytochromes induced by antimycin or myxothiazol cannot be related unequivocally to the way in which they exert their inhibitory action. However, the effect of myxothiazol on the EPR spectrum of cluster 1, which is not necessarily identical to $[2Fe\text{-}2S]_1$ of Fig. 8 (see for a discussion on this point Ref. 19), suggests that this inhibitor displaces the ubiquinone bound in the vicinity of cluster 1 (see also Refs 9,

19 and 20) and that, consequently, QH_2 and $Q^{\cdot-}_{out}$ cannot bind to this site, so that the [2Fe-2S] clusters and cytochrome c_1 remain oxidized. Since neither [2Fe-2S] cluster can be reduced if myxothiazol is present, we have to postulate that myxothiazol binds to the QH_2-binding domains near both $[2Fe\text{-}2S]_I$ and $[2Fe\text{-}2S]_2$ on protomer I and II, respectively. This proposal is corroborated by the finding that the initial rapid reduction of high-potential cytochrome *b*-562 is inhibited by myxothiazol (Figs. 6 and 7). According to the scheme of Fig. 8 this is the result of the inhibition of the oxidation of QH_2 by cytochrome *b*-566 in protomer I. Thus, in the presence of myxothiazol or after BAL + O_2 treatment the two cytochromes *b*-562 can only become reduced via reversal of the antimycin-sensitive reaction of Eqn 1. This reaction is very rapid (cf. Figs 2, 6 and 7). The observation that low-potential cytochrome *b*-562 is reduced first is in agreement with the scheme of Fig. 8 and shows that electron transfer between the two cytochromes *b*-562, mediated by $Q^{\cdot-}_{in(I)}$ and $Q^{\cdot-}_{in(II)}$, is slower than the reduction of low-potential cytochrome *b*-562 by QH_2, i.e., $t_{1/2} = 15$ ms vs less than 5 ms at pH 8.5 The fact that at pH 7.2 the two reduction phases of the cytochromes *b*-562 are not clearly visible (Fig 7F) may be due to the different pH dependencies of the two reactions. Our explanation for the lag time between the reduction of high- and low-potential cytochrome *b*-562 in the absence of inhibitors as given previously [19] is consistent with the findings that this lag time is not observed in the presence of myxothiazol (or antimycin) and that the initial rapid reduction of high-potential cytochrome *b*-562 is inhibited by antimycin and/or myxothiazol

These observations, particularly those in which the [2Fe-2S] clusters are reduced biphasically, all sustain and corroborate the proposal of the double Q-cycle of Fig. 8 and are absolutely irreconcilable with the schemes of a monomeric Q-cycle [5,11,12]. The effect of pH on the reduction kinetics of the prosthetic groups permits several refinements as to the protonation states of the various quinol/semiquinone and semiquinone/quinone oxidation-reduction couples, but at the moment any assignment in more detail would be too speculative.

Acknowledgements

We are grateful to Messrs C.G.G Jasper and F.P A Mol for their expert assistance and advice during the development and design of the freeze-quench apparatus, the stopped-flow and the integrating sphere. We also like to thank Mr H. Gerritsen, Mr B van Swol and Dr J.H Meijling for their assistance in linking the stopped-flow apparatus to the computer and Drs G von Jagow and W Trowitzch for the kind gift of myxothiazol This work was supported in part by a grant from the Netherlands Organization for the Advancement of Pure Research (Z W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.)

References

1 Hatefi, Y , Haavik, A G and Griffiths, D E (1962) J Biol Chem 237, 1681–1685
2 Rieske, J S , Hansen, R E and Zaugg, W S (1964) J Biol Chem 239, 3017–3022
3 Wikstrom, M K F and Berden, J A (1972) Biochim Biophys Acta 283, 403–420
4 Deul, D H and Thorn, M B (1962) Biochim Biophys Acta 59, 426–436
5 Trumpower, B L (1976) Biochem Biophys Res Commun 70, 73–80
6 Slater, E C and De Vries, S (1980) Nature 288, 717–718
7 Von Jagow, G and Engel, W D (1981) FEBS Lett 136, 19–24
8 Edwards, C A , Bowyer, J R and Trumpower, B L (1982) J Biol Chem 257, 3705–3713
9 Marres, C A M , De Vries, S and Slater, E C (1982) Biochim Biophys Acta 681, 323–326
10 Mitchell, P (1976) J Theor Biol 62, 327–367
11 Van Ark, G , Raap, A K , Berden, J A and Slater, E C (1981) Biochim Biophys Acta 637, 34–42
12 Slater, E C (1981) in Chemiosmotic Proton Circuits in Biological Membranes (Skulachev, V P and Hinkle, P , eds), pp 69–104, Addison-Wesley, Reading
13 Wikstrom, M and Krab, K (1980) Curr Top Bioenerg 10, 51–101
14 Konstantinov, A A and Ruuge, E K (1977) FEBS Lett 81, 137–141
15 Ohnishi, T and Trumpower, B L (1980) J Biol Chem 255, 3278–3284
16 De Vries, S , Berden, J A and Slater, E C (1980) FEBS Lett 122, 143–148
17 Wei, Y -H , Scholes, C P and King, T E (1981) Biochem Biophys Res Commun 99, 1411–1419
18 De Vries, S , Albracht, S P J , Berden, J A and Slater, E C (1981) J Biol Chem 256, 11996–11998

19 De Vries, S, Albracht, S P J, Berden, J A and Slater, E C (1982) Biochim Biophys Acta 681, 41–53
20 Zhu, Q S, Berden, J A, De Vries, S, Folkers, K, Porter, T and Slater, E C (1982) Biochim Biophys Acta, 682, 160–167
21 Berden, J A, Opperdoes, F R and Slater, E C (1972) Biochim Biophys Acta 256, 594–599
22 Dutton, P L and Jackson, J B (1972) Eur J Biochem 30, 495–510
23 Wikström, M K F (1973) Biochim Biophys Acta 301, 155–193
24 Higuti, T, Mizuno, S and Muraoka, S (1975) Biochim Biophys Acta 396, 36–47
25 De Vries, S, Albracht, S P J and Leeuwerik, F J (1979) Biochim Biophys Acta 546, 316–333
26 Von Jagow, G, Schagger, H, Riccio, P, Klingenberg, M and Kolb, H J (1977) Biochim Biophys Acta 462, 549–558
27 Weiss, H and Kolb, H J (1979) Eur J Biochem 99, 139–149
28 Wingfield, P, Arad, T, Leonard, K and Weiss, H (1979) Nature 280, 696–697
29 Jin, Y-Z, Tang, H-L, Li, S-L and Tsou, C-L (1981) Biochim Biophys Acta 637, 551–554
30 Fessenden, J M and Racker, E (1966) J Biol Chem 241, 2483–2489
31 Engel, W D., Schägger, H and Von Jagow, G (1980) Biochim Biophys Acta 592, 211–222
32 Norling, B, Glazek, E, Nelson, B D and Ernster, L (1974) Eur J Biochem 47, 475–482
33 Slater, E C (1949) Biochem J 45, 14–30
34 De Vries, S, Albracht, S P J, Den Bakker, C W, Berden, J A and Slater, E C (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F, Quagliariello, E, Siliprandi, N and Slater, E C, eds), pp 173–177, Elsevier/North-Holland, Amsterdam
35 Thierbach, G and Reichenbach, H, (1981) Biochim Biophys Acta 638, 282–289
36 Dervertanian, D V, Albracht, S P J, Berden, J A, Van Gelder, B F and Slater, E C (1973) Biochim Biophys Acta 292, 496–501
37 Rich, P R and Bendall, D S (1979) FEBS Lett 105, 189–194
38 Rich, P R and Bendall, D S (1980) Biochim Biophys Acta 592, 506–518
39 Prince, R C and Dutton, P L (1976) FEBS Lett 65, 117–119
40 Von Jagow, G, Engel, W D and Schägger, H (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F, Quagliariello, E, Siliprandi, N and Slater, E C, eds), pp 149–161, Elsevier/North-Holland, Amsterdam
41 Guerrieri, F, Izzo, G, Maida, I and Papa, S. (1981) FEBS Lett 125, 261–265
42 Dutton, P L, Wilson, D F and Lee, C-P (1970) Biochemistry 9, 5077–5082
43 Leigh, J S and Erecinska, M (1975) Biochim Biophys Acta 387, 95–106
44 Von Jagow, G and Bohrer, C (1975) Biochim Biophys Acta 387, 409–424
45 Kröger, A and Klingenberg, M (1966) Biochem Z 344, 317–336
46 Bowyer, J R, Dutton, P L, Prince, R C and Crofts, A R (1980) Biochim Biophys Acta 592, 445–460
47 Sidhu, A and Beattie, D S (1982) J Biol Chem 257, 7879–7886