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Pre-steady-state reduction kinetics of QH_2: cytochrome *c* oxidoreductase and the Q-pool: evidence for a special quinone not in rapid equilibrium with the Q-pool

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The pre-steady-state kinetics of the reduction of the prosthetic groups of QH_2: cytochrome *c* oxidoreductase in bovine heart submitochondrial particles were studied in relation to the kinetics of the Q-10 reduction, using duroquinol as substrate. The prosthetic groups, including semiquinone, were measured with EPR and low-temperature-diffuse reflectance spectroscopy, the samples being prepared with the rapid-freeze quench technique. For the determination of the redox state of ubiquinone in the pre-steady state the rapid chemical quench technique was used as an extension of the rapid-freeze quench technique, and Q-10 and QH_2-10 were measured with reversed-phase HPLC after extraction with petroleum ether. Ubiquinone was reduced biphasically, 8% of total Q-10 (equal to 1 mol Q-10/mol cytochrome c_1), being reduced within 5 ms, and the rest, the Q-pool, at a much lower rate. The initial rapid reduction of this special Q-10 was accompanied by rapid formation of Q_i^- and rapid reduction of a large part of the cytochrome *b*-562. Both semiquinone formation and reduction of *b*-562 showed transient kinetics due to a contribution of the reaction pathway via centre o when the iron-sulphur cluster and cytochrome c_1 were oxidised. The majority of the special quinol was located at centre i, probably bound, but also at centre o some bound quinol was formed. This was visible when antimycin was present, the antimycin-insensitive bound quinol being totally sensitive to myxothiazol. Myxothiazol alone accelerated the reduction of the Q-pool via centre i, but also the equilibration of cytochrome *b*-562 with the Q-pool. Antimycin drastically lowered the rate of reduction of the Q-pool and additionally seemed to block the rapid electron transfer from part of the Rieske iron-sulphur cluster to cytochrome c_1. It is concluded that, during the pre-steady-state, cytochrome *b*-562 is not in equilibrium with the Q-pool and that the rate of equilibration is probably determined by the rate of dissociation of the special bound quinol from centre i.

Abbreviations: Q-10, QH_2-10 (ubiquino(ne)(1)-10); Q-1, QH_2-1 (ubiquino(ne)(1)-1); DQ, DQH_2 (duroquino(ne)(1)); Q_i^-, semiquinone anion at centre i; Q_o^-, semiquinone anion at centre o; HMHQQ, *n*-heptadecylmercapto-6-hydroxy-5,8-quinolinequinone; TTFA, 2-thenoyltrifluoroacetone; British Anti-Lewisite, 2,3-dimercaptopropanol; Mops, 4-morpholinepropanesulfonic acid; $(Me)_2SO$, dimethylsulfoxide.

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Introduction

Quinol : ferricytochrome *c* oxidoreductase catalyses the transfer of electrons from the dehydrogenases to cytochrome *c* concomitantly with the translocation of protons across the mitochondrial membrane. According to the Q cycle, originally proposed by Mitchell [1], the protons are transferred by the trans-membrane diffusion of ubiquinol. As a consequence, the QH_2 : cytochrome *c* oxidoreductase is proposed to contain two different quinone reaction sites located on opposite sides of the membrane.

Support for the Q-cycle mechanism comes from (i) inhibitor studies with antimycin and myxothiazol [2], which independently inhibit the reactions at centre i and centre o, respectively, and with HMHQQ, which inhibits the reactions at both centres but with different affinities [3]; and (ii) the detection of two different semiquinone anions, Q_i^- [4–6] and Q_o^- [7,8]. A slightly different interpretation of these data is given by Rich and Wikström [9].

According to the Q-cycle, QH_2 at the myxothiazol-sensitive quinol oxidation centre o reduces the Rieske iron-sulphur cluster and cytochrome *b*-566 via Q_o^- as intermediate, resulting in branching of two electrons across the enzyme (according to the original proposal of Wikström and Berden [10]) and liberation of two protons at the P-side. The electron in the Rieske iron-sulphur cluster reduces cytochrome *c* via cytochrome c_1, while cytochrome *b*-566 is reoxidised by cytochrome *b*-562 which is located close to the antimycin-sensitive quinone reduction centre i. At centre i a quinone is reduced to the quinol in two turnovers with the formation of the semiquinone Q_i^- as the intermediate. The EPR spectrum of Q_i^- differs form that of Q_o^- with respect to the *g*-value, line shape and power-saturation behaviour [7]. Q_i^- is easily detected under equilibrium conditions as well as in the pre-steady state. Q_o^- has a very low stability, and has so far only been detected during oxidant-induced extra reduction of cytochrome *b*, i.e., when antimycin and oxidant are present [7,8].

A full catalytic cycle of the enzyme comprises two turnovers [11]. In the first turnover Q_i serves as the oxidant for cytochrome *b*-562. In the second turnover, when a new quinol is oxidised via centre o, Q_i^-, formed in the first turnover, serves as the oxidant for cytochrome *b*-562. The quinol dianion so formed associates with two protons from the N-side, yielding QH_2.

The reduction kinetics of the prosthetic groups in QH_2 : cytochrome *c* oxidoreductase with duroquinol as electron donor are complex [8,12]. Cytochrome *b* reduction kinetics revealed that part of cytochrome *b*-562 is rapidly reduced, followed by a lag and a slow reduction phase. Concomitantly with the fast phase of *b*-562 reduction, a semiquinone was formed in substoichiometric amounts [8,12]. Also the slow phase of *b*-562 reduction was accompanied by formation of an extra semiquinone. After extraction of Q-10, all *b*-562 is reduced by duroquinol within 5 ms [12]. Also Linke and coworkers [13] studied the Q-dependent reduction kinetics of cytochrome *b*. The rapid *b*-reduction in their Q-deficient QH_2 : cytochrome *c* oxidoreductase was inhibited upon addition of Q-10.

The kinetic behaviour of cytochrome *b* is thus dependent on the Q-content of the preparation, suggesting equilibration with the Q-pool [14]. It is therefore important to know whether all cytochrome *b*-562 really equilibrates with the Q-pool in the pre-steady-state and during steady-state turnover. For this reason we used a recently developed technique to determine the ox/red ratio of the Q-pool [15] in submitochondrial particles. With tools such as rapid freeze quench, rapid chemical quench and a combination of low-temperature diffuse-reflectance spectroscopy, EPR and HPLC we have investigated the kinetic behaviour of the cytochromes, the Rieske iron-sulphur cluster and the semiquinone anions in relation to that of the Q-pool. New data on the function of the Q-cycle are obtained by studying the effects of the inhibitors antimycin and myxothiazol.

Materials and Methods

Bovine heart mitochondria were prepared as in Ref. 16, cytochrome *c*-depleted mitochondria as in Ref. 17, and EDTA particles according to Ref. 15. Particles were suspended in 0.25 M sucrose, 50 mM Tris-HCl (pH 8.8) or 50 mM Mops-KOH (pH 6.6) to a final protein concentration of 70–80 mg/ml and mixed in a Ballou mixer [8,15] with an

equal volume of 0.25 M sucrose, 1 mM acetic acid containing 2 mM DQH_2 and then quenched at various times using two different methods in parallel experiments. The first type of experiment was performed by rapid freezing as in Ref. 8 and after reaction the samples were analysed with EPR and low-temperature diffuse-reflectance spectroscopy [19]. For the second type of experiment the equipment was extended with a second Ballou mixer and a third syringe with a 10-times larger volume, so that the reaction could be quenched chemically with 0.2 M $HClO_4$ in methanol [20] and subsequently extracted with petroleum ether. The extracts were evaporated to dryness with an argon flush. Under these conditions, the samples could even be stored at room temperature until analysis without any alterations in the redox state of Q-10. Analysis was performed with a reversed-phase HPLC RP TSK-ODS-12OT column (size i.d., 4.6 × 250 mm, 5 μm particle size). The column was equilibrated with 50 mM $NaClO_4 \cdot H_2O$, 7 mM $HClO_4$ in ethanol according to Ikenoya and coworkers [21] and this ethanolic buffer (water content, 0.10–0.13%) was used as the mobile phase. The dried samples were dissolved in 30 μl argon-purged mobile phase and an aliquot of 20 μl was injected via a Rheodyn injection loop. The column was protected with a Brownlee disposable aquapore RP ODS (particle size, 7 μm) guard column, the flow-rate was 1 ml/min. Detection of the quinones was performed at the isosbestic point of Q-10 and QH_2-10 at 292.5 nm [22]. The home-built flow cell (i.d., 1 mm; path length, 1 cm) was connected to a PM Q II Zeiss spectrophotometer via a light pipe. The amounts of Q-10 and QH_2-10 were calculated from the peak heights in chromatograms (see also Results). The maximum amount of quinone or quinol applied to the column did not exceed 20 nmol to prevent the effect of size exclusion, causing line broadening which gives rise to errors in the determination of the amount of Q-10 and QH_2-10. Protein was determined by the Biuret method [23]. Antimycin, myxothiazol (Boehringer), rotenone and TTFA (analytical grade) were dissolved in 90% dimethylsulfoxide, DQH_2 and QH_2-1 in 90% dimethylsulfoxide and 100 mM acetic acid. All other chemicals were of analytical grade.

Results

Determination of the redox state of quinones

When a mixture of Q-10 and QH_2-10 was injected in a column as described in Materials and Methods, Q-10 and QH_2-10 showed different retention times (Fig. 1A). The ratio of the retention times was determined as $t_R(\text{Q-10})/t_R(QH_2\text{-10}) = 4/3$. Increase of the water content of the mobile phase up to 1.0% did not affect this ratio (not shown). Upon further increase of the water content the retention times of Q-10 and QH_2-10 became longer, exceeding 20 min, and the recovery of the quinone and quinol became gradually lower. The number of theoretical plates was found to be the same for Q-10 and QH_2-10, indicating that the diffusion coefficients of both forms are equal. As a consequence, the tangents of the calibration curves (Fig. 1B), which are linear up to 20 nmol, also displayed a ratio of 4/3.

Fig. 2 shows the results of a control experiment in which 1 mM QH_2-1 was added to particles inhibited by antimycin, myxothiazol, rotenone and TTFA. After various periods of time the reaction was quenched with neutral methanol/petroleum ether. Using QH_2-1 both the exogenous redox potential of the Q-1/QH_2-1 couple and that of the Q-10 pool could be determined, since Q-1 and

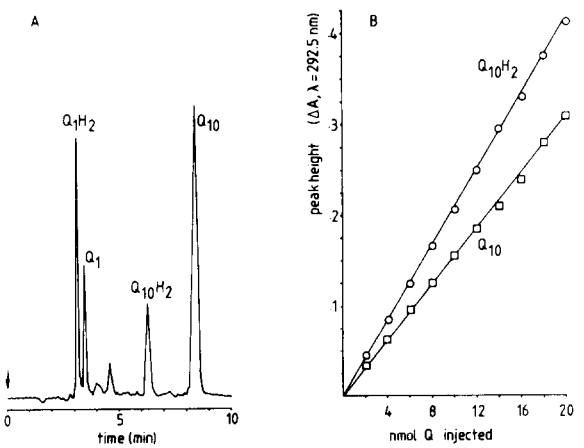

Fig. 1. Analysis of the redox state of ubiquinone. Typical recorder trace (A) and HPLC response concentration curves (B) for Q-10 (□———□) and QH_2-10 (○———○). (A) At $t = 0$ min, a 20 μl sample was injected. Note the separation of Q-1 and QH_2-1. For further details see Materials and Methods.

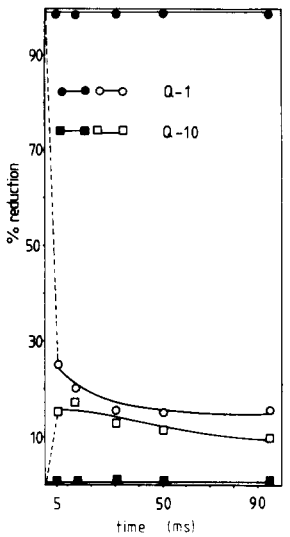

Fig. 2. Reduction of endogenous Q-10 (≈ 0.35 mM) with QH_2-1 (1 mM) in submitochondrial particles (70–80 mg/ml) inhibited by 90 μM antimycin, 90 μM myxothiazol, 25 μM rotenone and 25 μM TTFA. Measurements were performed with the HPLC method, using the chemical-quench technique. The quench solution consisted of methanol and petroleum ether as in Ref. 12: Q-1 (○———○), Q-10 (□———□). The quench solution consisted of 0.2 M $HClO_4$ in methanol: Q-1 (●———●), Q-10 (■———■).

QH_2-1 were extracted together with the Q-10 and QH_2-10. It is seen that part of the Q-pool was rapidly reduced, and concomitantly QH_2-1 was rapidly oxidised. The amount of QH_2-1 oxidised was, however, much larger than the amount of Q-10 reduced. The equilibration between QH_2-1 and Q-10 and the (auto)oxidation of QH_2-1 or/ and QH_2-10 occurred in the methanol phase, after quenching, since the same results were obtained (data not shown) with pure Q-10 or with particles first denatured with methanol and subsequently mixed with QH_2-1 followed by extraction with petroleum ether. After acidification of the methanol with 0.2 M $HClO_4$, the equilibration between OH_2-1 and Q-10 in the quenching medium was prevented (Fig. 2). The Q-10 remained fully oxidised and fully reduced QH_2-1 remained reduced. In addition, QH_2-10 did not (auto)oxidise in this medium (not shown).

These results indicate that the use of neutral methanol as quenching solvent [24] in the presence of exogenous quinones/quinols leads to erroneous results. The use of an acid-quenching solvent has the following advantages: it rapidly denatures the protein, it prevents auto-oxidation of quinols and it inhibits equilibration between different quinones. Furthermore, in the subsequent extraction with petroleum ether only quinones are extracted, whereas with neutral methanol as quenching solvent also other substances are extracted (Ref. 15; see also Van Hoek, A.N., unpublished results), giving rise to a chromatogram displaying large absorptions both at low and at high retention times. Using the acidified methanol as quenching medium, it is possible to quantify the amount of short-chain quinones such as Q-1 and QH_2-1 in the same chromatogram (Fig. 1), irrespective of a possible overlap of their absorption spectrum. The amounts of DQ and DQH_2 could, however, not be determined due to the low partitioning of DQ and DQH_2 between petroleum ether and acidic methanol/H_2O.

The reduction kinetics of the redox groups in QH_2: cytochrome c oxidoreductase

Fig. 3 shows the reduction kinetics of cytochrome ($c_1 + c$), the Rieske iron-sulphur cluster, cytochrome *b* and Q-10 and the formation of Q_i^- as measured in submitochondrial particles largely depleted of cytochrome *c*, after mixing with duroquinol in the presence of KCN at two different pH values. The rate of cytochrome ($c_1 + c$) reduction and the rate of the Rieske iron-sulphur cluster reduction increased with increasing pH (cf. Ref. 12). At high pH values, both cytochrome ($c_1 + c$) and the iron-sulphur cluster became reduced with a half-reduction time of 15 ms. At low pH the rate of cytochrome ($c_1 + c$) reduction lagged behind that of the iron-sulphur cluster: the iron-sulphur cluster became reduced with a half-reduction time of 25 ms, while cytochrome ($c_1 + c$) had a half-reduction time of 100 ms. This lag in the rate of cytochrome ($c_1 + c$) reduction is due to the higher midpoint potential of the Rieske iron-sulphur cluster than that of cytochrome ($c_1 + c$). At pH 8.8, this difference in E_m was almost zero, confirming earlier reports [10,25].

The reduction kinetics of the iron-sulphur cluster and cytochrome ($c_1 + c$) were affected by the presence of KCN at low pH values, but not at high pH values. At low pH in the absence of KCN

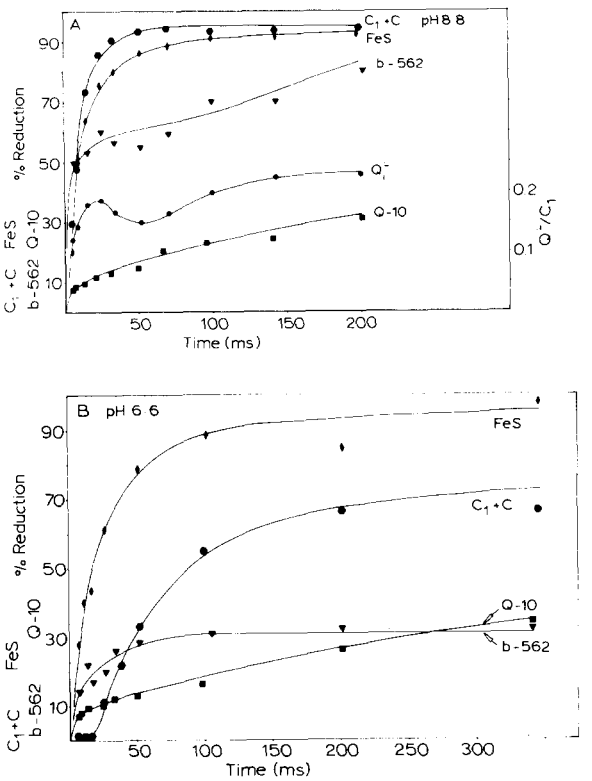

Fig. 3. Kinetics of reduction of the [2Fe-2S] cluster, cytochrome ($c_1 + c$) cytochrome *b*, Q-10 and formation of Q_i^- in submitochondrial particles, largely depleted of cytochrome *c* in the presence of 5 mM KCN. EPR was used to detect [2Fe-2S] and Q_i^- and low-temperature diffuse reflectance spectroscopy to detect cytochrome ($c_1 + c$) and cytochrome *b*, using the freeze-quench technique. The reduction of Q-10 was measured with the reversed-phase HPLC method as depicted in Fig. 1 and described in Materials and Methods, using the chemical quench technique as an extension of the freeze-quench method. The reaction was performed at room temperature. One syringe contained 70–80 mg/ml particles suspended in 0.25 M sucrose, 50 mM Tris-HCl (pH 8.8) (A) or 50 mM Mops-KOH (pH 6.6) (B). The other syringe contained 0.25 M sucrose, 1 mM acetic acid and 2 mM DQH_2. The DQH_2 solubilisation was facilitated with the help of hot tap water. After mixing, the DQH_2 concentration was 1 mM. Diffuse-reflectance spectra were recorded at 110 K with a slit width of 1 nm. Cytochrome ($c_1 + c$) was measured in the reduced-minus-oxidised spectrum as 552 nm minus 542 nm. Cytochrome *b*-562 was measured in the reduced-minus-oxidised spectrum as 559 nm minus 570 nm. The EPR measurements were performed at 9.25 GHz; $T = 36$ K; $P = 6.3$ mW; $MA = 1.25$ mT. The amount of reduced [2Fe-2S] cluster was determined with the g_y amplitude. Semiquinone formation was measured at 9.25 GHz; $T = 36$ K; $P = 10$ μW; $MA = 0.5$ mT; 100% reduction of cytochrome ($c_1 + c$) and [2Fe-2S] cluster was set with samples reduced by ascorbate + TMPD (pH 8.8 and pH 6.6). 100% reduction of cytochrome *b*-562 was set with samples reduced by succinate at pH 8.8 and pH 6.6. ◆———◆, reduction of [2Fe-2S] cluster; ●———●, reduction of cytochrome ($c_1 + c$); ▼———▼, reduction of cytochrome *b*-562; ■———■, reduction of Q-10; ●———●, formation of Q_i^-. Samples at zero time, using only buffer in the substrate syringe, showed that the prosthetic groups were fully oxidised and no semiquinone was present. (A) $t_{1/2}$ of iron-sulphur cluster and cytochrome ($c_1 + c$) equals 15 ms, $t_{1/2}$ of rapidly reduced cytochrome *b* equals less than 2.5 ms. (B): $t_{1/2}$ of iron-sulphur cluster equals 25 ms, $t_{1/2}$ of cytochrome ($c_1 + c$) equals 100 ms, $t_{1/2}$ of rapidly reduced cytochrome *b* equals 15 ms. The particles contained 12 mol of Q-10 per mole of cytochrome c_1.

(not shown), the rates of reduction of both prosthetic groups were strongly decreased and reached a steady-state level. Since a low amount of cytochrome *c* was present (0.1 mol of cytochrome *c* per mol of cytochrome c_1) in these cytochrome *c*-depleted particles, this KCN effect at low pH compared to no effect at high pH, is most likely due to the differential effect of pH on the turnover rates of QH_2 : cytochrome *c* oxidoreductase and cytochrome *c* oxidase. The rate of turnover of cytochrome *c* oxidase increases with decreasing pH, whereas that of QH_2 : cytochrome *c* oxidoreductase decreases, so that at low pH electron transfer from cytochrome c_1 via cytochrome *c* (and cytochrome *c* oxidase) to oxygen is faster relative to the rate of oxidation of QH_2 by the Rieske iron-sulphur cluster.

Fig. 3 also shows the triphasic reduction kinetics of cytochrome *b* as earlier observed by Jin and coworkers [26]. The rates of the first fast phase and the third slow phase increased with increasing pH and the lag between these two phases, called the second phase, decreased as reported earlier [12]. At pH 8.8 also the multiphasic formation of Q_i^- was reproduced [12].

In the initial phase of 5 ms (Fig. 3A) already 8% (±1) of Q-10, equivalent to 1 mol Q/mol c_1, was rapidly reduced concomitantly with the rapid reduction of cytochrome *b*-562 and the rapid formation of Q_i^-. After this phase the Q-10 was monophasically further reduced with a lower rate, allowing the quantitative determination of the extent of the initial rapid phase. The first phase of cytochrome *b* reduction was followed by a lag period in which no net reduction of cytochrome *b* was observed. In this period, from 5 to 50 ms,

transient extra formation of semiquinone took place. After 50 ms the rates of cytochrome *b*-562 reduction and formation of Q_i^- matched the rate of the reduction of the Q-pool. The final level of reduction of Q-10 obtained in the presence of KCN was 90%. Also at low pH (Fig. 3B), the Q-10 was biphasically reduced and the rates of reduction in the two phases were almost the same as the rates found at high pH. In the kinetics of cytochrome *b* reduction only two phases could be seen, since the second phase lasted more than 300 ms. It is clearly seen that during the second phase of cytochrome *b* reduction the Q-10 was further reduced. The reduction of cytochrome *b* and Q-10 and the formation of Q_i^- were not influenced by the presence or absence of KCN within the first 200 ms (pH 8.8) or the first 300 ms (pH 6.6). It should be noted that the relative reduction levels of the Q-pool and cytochrome *b* are dependent on pH [27,28].

The effects of myxothiazol and antimycin

Myxothiazol (Fig. 4) completely blocked the reduction of the Rieske iron-sulphur cluster and cytochrome $(c_1 + c)$ (not shown), as might be expected for an inhibitor at centre o [2]. In the presence of myxothiazol (Fig. 4A) Q-10 was reduced faster than in the absence of myxothiazol. The reduction kinetics, however, were still biphasic, 9% of Q-10 being reduced in the first 5 ms. Myxothiazol had hardly any effect on the kinetics of reduction of cytochrome *b*-562, only slightly decreasing the rate of reduction (and rate of formation of the semiquinone) in the first phase. The amount of the semiquinone formed was about half that in the absence of myxothiazol, and no transient kinetics were observed. At low pH values (Fig. 4B), a similar biphasic reduction of the Q-10 was observed as that found at high pH values. The second phase of cytochrome *b* reduction, however, was not present, the first phase (not affected) being immediately followed by the third phase. The biphasic reduction of cytochrome *b*-562 was in perfect match with the biphasic reduction of the Q-10. At high pH values cytochrome *b* was, also under these conditions, more reduced relative to the Q-pool than at low pH.

In contrast with the effect of myxothiazol, antimycin abolished (Fig. 5) the rapid reduction of the

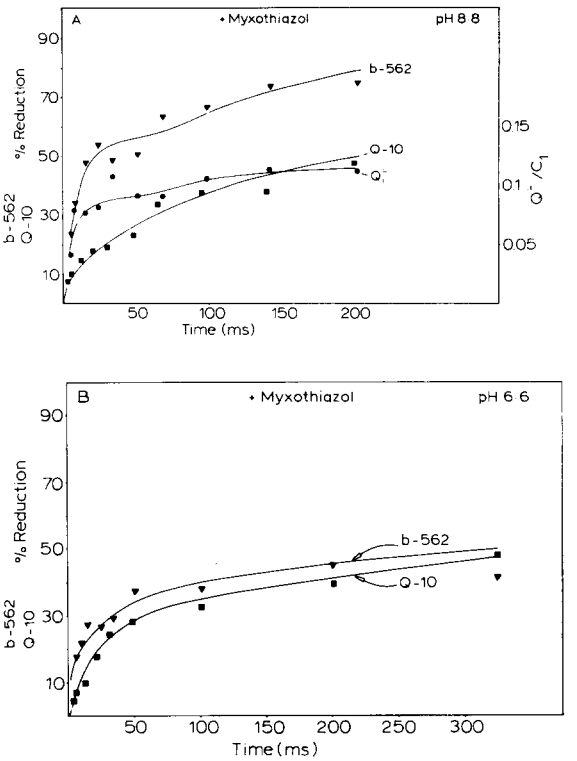

Fig. 4. Reduction kinetics of cytochrome *b* and Q-10 and formation of semiquinone in the presence of 90 μM myxothiazol at pH 8.8 (A) and at pH 6.6 (B), as measured in submitochondrial particles largely depleted of cytochrome *c*. Reduction of cytochrome *b* and formation of semiquinone were measured with low temperature diffuse reflectance spectroscopy and EPR, using the freeze-quench technique, and reduction of Q-10 with reversed phase HPLC, using the chemical quench technique as described in Fig. 3. ▼———▼, reduction of cytochrome *b*-562; ●———●, formation of semiquinone; ■———■, reduction of Q-10. Samples at zero time, prepared by mixing with buffer, showed that all components were fully oxidised. (A) $t_{1/2}$ of the first phase of cytochrome *b*-562 reduction equals 5 ms.

first 8% of the Q-10, but biphasic reduction was still observed, showing a rapid reduction of only 2% during the first phase, followed by a very slow further reduction of the Q-10. Both these two phases were sensitive to the additional presence of myxothiazol (see Fig. 6). The triphasic reduction kinetics of cytochrome *b*-562 was not observed under these conditions and also cytochrome *b*-566/558 was reduced. The rate of reduction of cytochrome *b*-562 was one order of magnitude lower than in the absence of antimycin. The pref-

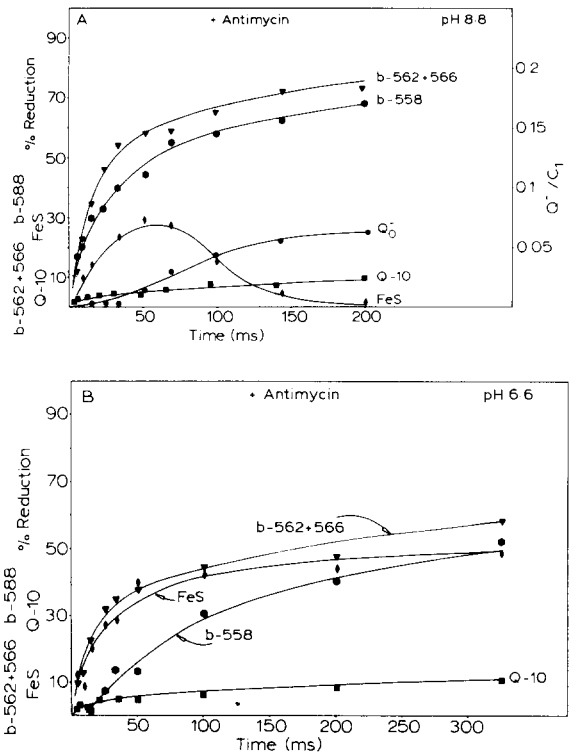

Fig. 5. Reduction of cytochrome *b*, [2Fe-2S]cluster, Q-10 and formation of semiquinone in the presence of 90 μM antimycin at pH 8.8 (A), and at pH 6.6 (B), as measured in submitochondrial particles largely depleted of cytochrome *c*. Reduction of cytochrome *b* and the [2Fe-2S]cluster and formation of semiquinone were measured with low temperature diffuse reflectance spectroscopy and EPR, using the freeze-quench technique as in Fig. 3. Reduction of Q-10 was measured with reversed phase HPLC, using the chemical quench technique as in Fig. 3. Cytochrome (*b*-562 + *b*-566) was measured in the reduced-minus-oxidised spectrum as 560 minus 570 nm. Reduced cytochrome ($c_1 + c$) was not detected by inspection of its β-absorption band, therefore cytochrome *b*-558 could be measured in the reduced-minus-oxidised spectrum as 554 minus 544 nm. 100% reduction was set with dithionite added to samples at pH 6.6 and pH 8.8. All other components were measured as in Fig. 3. ▼——▼, reduction of cytochrome *b*-562 + *b*-566; ●——●, reduction of cytochrome *b*-558; ◆——◆, reduction of [2Fe-2S]cluster; ●——●, formation of semiquinone; ■——■, reduction of Q-10. Samples at zero time, prepared by mixing with buffer, showed that all components were fully oxidised. $t_{1/2}$ of cytochrome *b*-562 + cytochrome *b*-566 and $t_{1/2}$ of [2Fe-2S] cluster equals about 25 ms in both (A) and (B).

erential reduction of cytochrome *b*-562, hardly seen at high pH (Fig. 5A, cf. Ref. 8), became pronounced at low pH (Fig. 5B), under which conditions reduction of cytochrome *b*-558 even showed a lag period. Since *b*-566 could not be measured distinctly from *b*-562, the trace of *b*-562 + *b*-566 contains two contributions, of which the contribution of *b*-566 is supposed to have the same kinetics as *b*-558. At high pH (Fig. 5A), formation of the semiquinone at centre o was detected after a lag period in which the reduction of cytochrome *b* was nearly completed.

With antimycin no reduction of cytochrome ($c_1 + c$) (not shown) was detected, since only one turnover is possible under these conditions and the presence of cytochrome *c* and cytochrome *c* oxidase caused rapid oxidation of the reduced cytochrome c_1. The kinetics of reduction of the Rieske iron-sulphur cluster were different with respect to those of cytochrome ($c_1 + c$). At high pH values the iron-sulphur cluster was reduced to a level of 30% after 50 ms (cf. Ref. 12). After longer reaction times the cluster was oxidised again. At low pH the Rieske iron-sulphur cluster did not show this reoxidation. After 50 ms a final level of 50% reduction was reached.

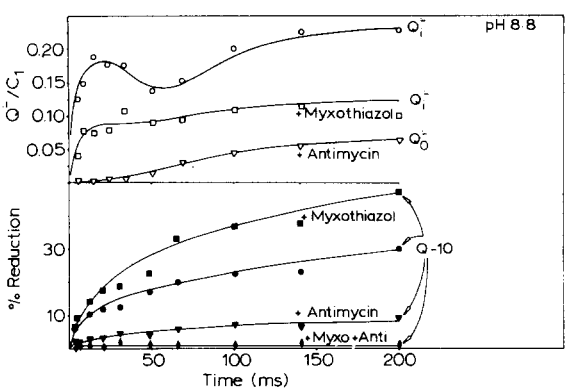

Fig. 6. Reduction of Q-10 and formation of semiquinones in the presence of KCN, myoxthiazol, antimycin, or myxothiazol plus antimycin, measured as described in Figs. 3–5. Open symbols, formation of semiquinone; filled symbols, reduction of Q-10; circles, in the presence of KCN; squares, in the presence of myxothiazol; triangles, in the presence of antimycin; rhombuses, in the presence of both myxothiazol and antimycin. After long incubation the degree of reduction of Q-10 was increased till 90%, irrespective of the inhibitor present. The amount of Q_i^- in the presence of myxothiazol was after long incubation $Q_i^-/c_1 = 0$, the amount of Q_i^- in the presence of KCN was $Q_i^-/c_1 = 0.20$, and the amount of Q_o^- in the presence of antimycin was $Q_0^-/c_1 = 0$.

Discussion

The data reported in this paper confirm and extend the observations previously reported by De Vries and coworkers [12]. With the introduction of the use of the reversed-phase HPLC technique to measure the redox state of the Q-pool, a more complete picture can be given of the function of the prosthetic groups in QH_2 : cytochrome *c* oxidoreductase. Using acidified methanol as quenching solvent, equilibration of oxidised and reduced quinones after quenching was fully inhibited and clean chromatograms could be obtained. The determination of the amounts of Q-10 and QH_2-10 from the peak heights displayed in the chromatogram is justified, due to a constant width at half height of the peaks as long as less than 20 nmol is injected.

Biphasic kinetics of reduction of the Q-pool: demonstration of a special ubiquinone

In Fig. 6 a summary of the reduction kinetics of Q-10 and of the formation of the semiquinones is shown. In the absence or presence of antimycin or myxothiazol the Q-10 became biphasically reduced. Addition of both antimycin and myxothiazol inhibited all reduction of Q-10. In uninhibited particles, 8% of the Q-10 was rapidly reduced in the first phase. Due to the fact that the sample at zero time was prepared by mixing with plain buffer, this 8% reduction within 5 ms, followed by a low rate of reduction, can be regarded as a quantitative value. This fast reduction of only 8% of the Q-10 indicates then that one mol of QH_2-10 per cytochrome c_1 was rapidly formed because in the particles 12 Q-10 per cytochrome c_1 were present. This rapidly reduced quinol was mainly formed at centre i, because antimycin abolished the majority of the rapid QH_2-10 formation, the residual formation being sensitive to myxothiazol. Myxothiazol alone did not inhibit the rapid formation of QH_2-10, which confirms that the kinetic appearance of most of the fast QH_2-10 occurred at centre i. The semiquinone, Q_i^- (antimycin-sensitive), is probably part of this special ubiquinone, since the formation of Q_i^- and the rapid reduction of the special ubiquinone occurred at the same time.

One may wonder whether Q^- shows up in the chromatogram as Q, QH_2 or $(Q + QH_2)$. Since we found that at pH 6.6 and at pH 8.8 the amount of QH_2 formed after 5 ms was the same whereas the amount of Q^-/c_1 was 0 and 0.15, respectively, we believe that Q_i^- is measured as Q. However, a more direct experiment is needed to settle this question.

The rapid reduction of a special ubiquinone followed by a much slower reduction of the rest of the Q-10, the Q-pool, suggests that reduction of the Q-pool by duroquinol occurs via bound QH_2. We should like to propose that the rate-limiting step in the reduction of the Q-pool is the dissociation of bound QH_2, although it remains possible that cytochrome *b* reoxidation after the initial rapid reduction becomes rate-limiting. The rate of the rate-limiting step is increased in particles inhibited by myxothiazol. It should be noted that also at centre o a small amount of rapidly reduced QH_2-10 was formed, as can be deduced from the biphasic kinetics of reduction of Q-10 in the presence of antimycin, which showed that about 2% of the total Q-10 was reduced faster than the rest. This reduction was sensitive to myxothiazol. The trace of Q-10 reduction at pH 8.8 can be superimposed on the trace obtained at low pH both in the presence and in the absence of inhibitors. This finding of pH independence of the rate of reduction of the Q-pool by duroquinol is consistent with the proposal (see above) that the rate-limiting step in the reduction of the Q-pool is the dissociation of the (neutral) QH_2 from the Q-binding site.

The formation and concentration of Q_i^-

In submitochondrial particles the amount of Q_i^- under equilibrium conditions (with the fumarate/succinate couple) was not changed when inhibitors of centre o were present (Ref. 6; see also Van Hoek, A.N., unpublished results). Differences are observed under pre-steady-state conditions. The amount of Q_i^- detected was substantially decreased in particles inhibited by myxothiazol (see Fig. 6 and Ref. 12), or in particles treated with British Anti-Lewisite ($+O_2$) with regard to the amount of Q_i^- detected in uninhibited particles. This difference was not due to additional presence of Q_o^- in the uninhibited enzyme: EPR difference spectra of uninhibited minus myxothiazol-inhibited particles did not

show the Q_o^- characteristics. This is not surprising, since cytochrome *b* (*b*-562 + *b*-566) in the uninhibited enzyme is not highly reduced.

In addition, when the Q-10 pool reached 90% reduction (Fig. 6) Q_i^- was still detected in uninhibited particles (0.20 Q_i^-/c_1), whereas in myxothiazol-inhibited particles no semiquinone was detected (Fig. 6), as one would expect when the Q-pool is highly reduced. Thus, we conclude that a fundamental difference exists between the Q_i^- in the uninhibited enzyme and the Q_i^- in the enzyme inhibited by myxothiazol: in the uninhibited enzyme the special quinone never equilibrates with the Q-pool, whereas myxothiazol affects the special quinone in such a way that it can equilibrate with the redox state of the Q-pool.

The triphasic reduction kinetics of cytochrome b

The triphasic reduction kinetics of cytochrome *b*-562 seems to be an intrinsic property of the bc_1 complex, regardless which kind of substrate is added. With DQH_2 or succinate the same pattern is observed [8,26]. A difference, however, is the nature of the rate-limiting step in the process of equilibration. Using succinate, the turnover rate of succinate : Q oxidoreductase controls the rate at which electrons enter the QH_2 : cytochrome *c* oxidoreductase. Using DQH_2, the rate-limiting step is due to dissociation of the special Q. Chen and Zhu [29] reported that the reduction kinetics of the Q-pool is in perfect match with the kinetics of reduction of cytochrome *b*-562, when succinate is the substrate. With duroquinol (Fig. 3) only the third phase of cytochrome *b* reduction kinetics matched the reduction of the Q-pool. This matching does not have to be due to real equilibrium, but it indicates that the slow reduction of cytochrome *b*-562 reflects the concomitant reduction of the Q-pool.

A second illustration of the fundamental difference between the use of DQH_2 and succinate is the demonstration of the preferential use of centre o when succinate is used as substrate. The initial phase of cytochrome *b* reduction was completely sensitive to myxothiazol and insensitive to antimycin [33]. With DQH_2 as substrate, however, electrons enter QH_2-cytochrome *c* oxidoreductase via both centres, cytochrome *b*-562 being rapidly reduced via centre i, mediated by the special quinone, but also via centre o as can be deduced from the transient extra formation of Q_i^- which is sensitive to myxothiazol [8,12,30].

The difference in extent of reduced cytochrome *b*-562 at the two pH values, while the redox state of the Q-pool was independent of pH, is probably due to a lower value of the pH dependency of the E_m of cytochrome *b*-562 than that of the Q-pool (45 mV vs. 60 mV per pH unit, Ref. 30).

The extra reduction of cytochrome b and formation of Q_o^-

The triphasic reduction of cytochrome *b*-562 was lost in the presence of antimycin and no equilibration of cytochrome *b* with the Q-pool was observed (Fig. 5). In the absence of KCN, also the well-known extra reduction of cytochrome *b* was observed. At high pH values, cytochrome *b*-562 and cytochrome *b*-566/cytochrome *b*-558 were almost monophasically reduced. The time for half-reduction of cytochrome *b* was comparable to that of the transiently reduced Rieske cluster (half-reduction in this case was 15% at 15 ms). At low pH values cytochrome *b* was biphasically reduced. The two phases were well resolved and the first phase was totally due to cytochrome *b*-562. No contribution of *b*-566 was involved during the first 20 ms, as can be concluded from the reduction pattern of *b*-558. Such a lag in the reduction of *b*-558 was not observed at high pH values, suggesting that the rate of the second turnover in which *b*-566 and *b*-558 were reduced was increased at high pH values. Thus, a higher input of electrons would decrease the lag period and no clear distinction could be made between the rate of *b*-562 reduction and the rate of *b*-566/*b*-558 reduction (cf. Refs. 8 and 34).

Prior to the formation of Q_o^- (high pH) a lag period was observed. At first sight, this result is in contrast with the data in Ref. 8, but in our case the reduction of cytochrome *b* was not monophasic as was the case in Ref. 8. Due to the low stability of Q_o^-, the midpoint potentials of the half couples of Q/QH_2 are strongly different: Q_o^- can only be detected when cytochrome *b*-566 is reduced. The estimated E_m value of the Q^-/Q couple, then, is slightly lower than that of cytochrome *b*-566/*b*-558.

Kinetics of the Rieske iron-sulphur cluster and cytochrome c_1

The data of Fig. 3 show that the Rieske iron-sulphur cluster was reduced in a single phase, not only at high pH but also at low pH. The biphasic reduction of this cluster at low pH in the absence of KCN [12] was interpreted as being due to the presence of two different species of the cluster, one of them displaying a rate of reduction dependent on pH. The absence of any clear inhomogeneity of the iron-sulphur cluster in the presence of KCN indicates that the inhomogeneity in the absence of KCN can be explained by the presence of a low amount of cytochrome *c*, enough to allow electron transfer to cytochrome *c* oxidase and oxygen. The large effect of pH on the rate of reduction of cytochrome c_1 and the small effect on the Rieske Fe-S cluster is in agreement with the known pH-dependency of the E_m values of cytochrome c_1 and the Fe-S cluster [25].

In the presence of antimycin and in the absence of KCN, variation of pH caused a different behaviour of the reduction kinetics of the iron-sulphur cluster, while cytochrome c_1 remained fully oxidised. At high pH values the cluster was transiently reduced, with a maximal level of reduction of 30% after 50 ms, but at low pH values the reduction level increased to 50% after 50 ms, without further changes. The observed differences between the reduction of the Rieske iron-sulphur cluster and cytochrome ($c + c_1$) in the presence of antimycin indicate that some barrier exists between the cluster and cytochrome c_1 [34,35]. This block is not necessarily induced by antimycin, although it is most clearly observed when antimycin is present.

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