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Effect of Ubiquinone Extraction on the Reaction of the Mitochondrial bc_1 Complex with Ferricyanide

Petronio Pasquali,[1,3] Mauro Degli Esposti,[2] Laura Landi,[1] Luciana Cabrini,[1] and Giorgio Lenaz[2]

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Abstract

Depletion of endogenous ubiquinone by pentane extraction of mitochondrial membranes lowered succinate-ferricyanide reductase activity, whereas quinone reincorporation restored the enzymatic activity as well as antimycin sensitivity. The oxidant-induced cytochrome *b* extrareduction, normally found upon ferricyanide pulse in intact mitochondria in the presence of antimycin, was lost in ubiquinone-depleted membranes, even if cytochrome *c* was added. Readdition of ubiquinone-2 restored the oxidant-induced extrareduction with an apparent half saturation at 1 mol/mol bc_1 complex saturating at about 5 mol/mol. These findings demonstrate a requirement for the ubiquinone pool of the cytochrome *b* extrareduction. Since the initial rates of cytochrome *b* reoxidation upon ferricyanide addition, in the presence of antimycin, did not saturate by any ferricyanide concentration in ubiquinone-depleted mitochondria, a direct chemical reaction between ferricyanide and reduced cytochrome *b* was postulated. The fact that such direct reaction is much faster in ubiquinone-depleted mitochondria may explain the lower antimycin sensitivity of the succinate ferricyanide reductase activity after removal of endogenous ubiquinone.

Key Words: Ubiquinone-depleted mitochondria; succinate-ferricyanide reductase; bc_1 complex; cytochrome *b*.

Introduction

Ferricyanide as an artificial electron acceptor was used for abbreviated phosphorylating electron path (Lee *et al.*, 1967) and for detecting the sidedness of membrane-bound dehydrogenases in mitochondria (Klingenberg, 1977;

[1] Istituto di Chimica Biologica, Universitá di Bologna, Bologna, Italy.
[2] Istituto Botanico, Universitá di Bologna, Bologna, Italy.
[3] Author to whom correspondence should be addressed: Dr. Petronio Pasquali, Istituto di Chimica Biologica, via Irnerio, 48, Bologna 40126, Italy.

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Harmon *et al.*, 1974). In fact it was shown to be impermeant through the inner mitochondrial membrane and to react readily with an electron donor such as reduced cytochrome *c* owing to its relatively high redox potential (E_h = 430 mV). Hence, in intact mitochondria, succinate reduces ferricyanide in an antimycin-sensitive fashion, via cytochrome *c*.

We previously studied the effect of extraction and reincorporation of ubiquinone[4] (UQ) on mitochrondrial succinate dehydrogenase measured with ferricyanide as electron acceptor in the presence of antimycin. This activity was drastically diminished upon UQ extraction, and was completely restored when UQ was reincorporated into the membranes (Landi *et al.*, 1982). In order to ascertain whether ubiquinol in the membrane can be oxidized by ferricyanide, we incorporated into phospholipid vesicles either ubiquinol-3 or ubiquinol-9. In this model system the initial rates of ubiquinol oxidation by external ferricyanide were almost two orders of magnitude lower than the rates of succinate-ferricyanide reductase in mitochondria (Landi *et al.*, 1982). These findings indicate that some redox components in the succinate cytochrome *c* span of the respiratory chain are able to feed directly electrons to ferricyanide, besides cytochrome *c*. The most likely candidate appears to be the bc_1 complex, a multiprotein redox complex containing four prosthetic groups: two cytochromes *b*, cytochrome c_1, and a high-potential iron–sulfur center (Rieske, 1976). In accord with this idea are the findings of Harmon and Crane (1973) and De Pierre and Ernster (1979) showing an antimycin-sensitive succinate-ferricyanide reductase in both mitochondria and electron transport particles. Since cytochrome *c* is sequestered within the membrane vesicle in the latter, the ferricyanide reactive site was suggested to be either cytochrome c_1 or the Fe–S centre of the bc_1 complex.

Due to the spectral overlapping of cytochrome c_1 with cytochrome *c* and to the inability to follow the redox changes of the iron–sulfur protein by spectrophotometric techniques (Rieske, 1976), we have initially chosen to follow cytochrome *b* rapid reoxidation by ferricyanide in different mitochondrial preparations. Such reaction should be only an indirect proof of the ferricyanide interaction with the hydrophilic redox groups of the bc_1 complex, mainly cyt c_1 and/or the Fe–S protein, since it is well known that cytochromes *b* are deeply buried in the membrane and do not react easily with hydrophilic mediators such as ferricyanide (Rieske, 1976).

We have studied cytochrome *b* oxidation also in the presence of antimycin, the best known inhibitor of the bc_1 complex, which induces an enhanced reduction of cytochrome *b* by substrates and, upon ferricyanide addition, the so-called oxidant-induced cytochrome *b* extrareduction

[4]UQ, ubiquinone; UQ_1, ubiquinone-1; UQ_2, ubiquinone-2; UQ_7, ubiquinone-7; UQ_{10}, ubiquinone- 10; BHM, beef heart mitochondria; TTFA, thenoyltrifluoroacetone.

(Rieske, 1976). In this investigation we show that the latter effect is dependent upon the UQ pool of mitochondria.

Materials and Methods

Membrane Preparations

Beef heart mitochondria (BHM) were a kind gift from the late Prof. D. E. Green from the Institute of Enzyme Research, Madison, Wisconsin.

UQ-depleted mitochondria were obtained by pentane extraction of lyophilized BHM (Szarkowska, 1966). Since mitochondria depleted with this method still showed residual endogenous UQ ranging from 7 to 10%, an additional extraction with pentane + 10% acetone was used (Norling *et al.*, 1974).

The UQ-depleted mitochondria were reconstituted with exogenous UQ_{10} as described by Ernster *et al.*, (1969).

Spectrophotometric Determinations

The amount of UQ incorporated in mitochondria was determined according to Kröger (1978). Succinate-ferricyanide reductase activity was assayed by the method of Klingenberg (1979) following the disappearance of ferricyanide at 420 nm (the molar extinction coefficient of ferricyanide is $1 \times 10^3\,cm^{-1}$). The isotonic incubation medium contained 0.25 M sucrose, 20 mM KCl, 10 mM Tris-HCl buffer, pH 7.2, 1 mM EDTA, 0.66 mM $K_3Fe(CN)_6$, 1 mM KCN, rotenone ($2\,\mu g \cdot mg^{-1}$ protein), and membrane preparations (1 mg protein) in a final volume of 3 ml at room temperature.

Antimycin and thenoyltrifluoroacetone, when added, were $1\,\mu g \cdot mg^{-1}$ protein and 1 mM, respectively.

The rapid reoxidation of cytochrome *b* was monitored at 562–575 nm in a dual-wavelength Sigma ZWS II spectrophotometer equipped with a rapid mixing apparatus in the same medium at room temperature with 5–6 mg protein $\cdot$ ml^{-1}. The extinction coefficient was $25\,mM^{-1}$. At these wavelengths we have verified very small spectral interferences by *c*-cytochromes. The mixing time of the apparatus was below 0.4 sec. The preparation was reduced with 5 mM succinate and, after the equilibration of the redox level of cyt *b*, treated with 5 mM malonate to decrease the electron input into the bc_1 complex (see Fig. 1).

Since the reoxidation of cytochrome *b* by ferricyanide was transient, and the steady-state level of reduction was reached again within a few minutes, it was possible to perform repetitive pulses of different ferricyanide concentrations in the same cuvette. The rates of cyt *b* reoxidation in this system or in separate experiments were found to be identical.

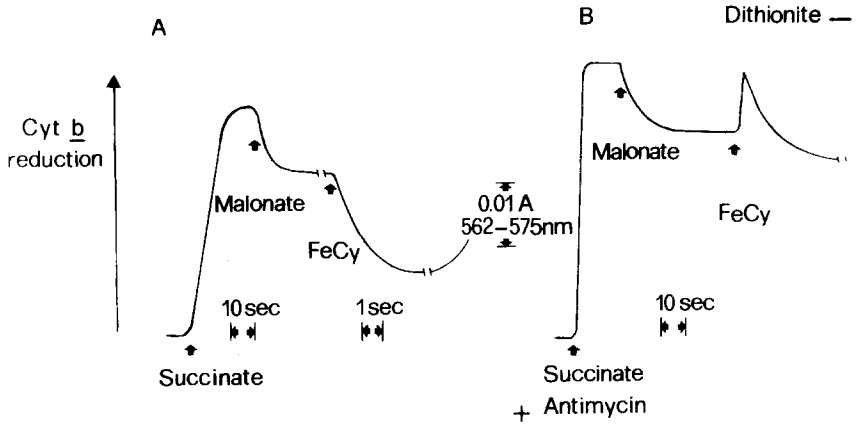

Fig. 1. Kinetic of cytochrome *b* reduction by succinate and rapid reoxidation by 100 μM ferricyanide (FeCy) in intact mitochondria in the presence of cyanide and rotenone (A); the same conditions plus antimycin (1 $\mu g \cdot mg^{-1}$ protein) (B). The assay was performed as described under Materials and Methods.

The total cytochrome $b_{566} + b_{562}$ content was determined after the addition of excess solid sodium dithionite. Fraction S_1 (crude succinate-cytochrome *c* reductase) and isolated bc_1 complex were prepared by the method of Rieske (1967).

Protein was assayed by a biuret method (Gornall *et al.*, 1949).

Ubiquinones were stored as ethanolic solutions at $-20°$ C at concentrations ranging between 10 and 30 mM as determined spectrophotometrically from the decrease in absorption at 275 nm observed upon the reduction of the chromophore with $NaBH_4$ (5 $mg \cdot ml^{-1}$). The extinction coefficient used was 12.25 mM^{-1}. Antimycin, rotenone, and TTFA were purchased from Sigma Chemical Co., St Louis, Missouri. Other reagents were of analytical grade.

Results

Depletion of endogenous UQ by pentane extraction of lyophilized mitochondrial membranes lowered succinate ferricyanide reductase in a reversible way (Table I). All activities studied were sensitive to TTFA, the specific inhibitor of the succinate-UQ reductase. Antimycin sensitivity of this enzymatic activity was diminished upon extraction of UQ (65% insensitive rate) and was restored when UQ_{10} was reincorporated into the membrane.

When succinate-ferricyanide reductase activity was assayed in different mitochondrial preparations preincubated in the presence of a short-chain quinone, such as UQ_1, ferricyanide reduction was stimulated but the activity

Table I. Effect of UQ Extraction on Succinate-Ferricyanide Reductase Activity[a]

Mitochondria	Specific activity (nmol ferricyanide reduced · min^{-1} mg^{-1} protein)			
	–	+ Antimycin	+ TTFA	+ Antimycin and TTFA
Control	772	114	144	114
Lyophilized	750	99	171	99
UQ-depleted	268	174	41	75
UQ_{10}-reconstituted	810	123	87	111

[a]The succinate-ferricyanide reductase activity was tested spectrophotometrically following the decrease in absorption of ferricyanide. UQ-depleted mitochondria were prepared after the method of Szarkowska (1966). UQ-reconstituted BHM were prepared according to the method of Ernster *et al.* (1969). For further details see Materials and Methods.

was essentially antimycin-insensitive. On the contrary, preincubation with a more physiological long-chain quinone homolog, such as UQ_7, restored the ferricyanide reduction in UQ-depleted membranes, as well as its sensitivity to antimycin (Table II).

Therefore, long-chain ubiquinones are able to modulate the effect of antimycin on ferricyanide interaction with the bc_1 complex, whereas a direct equilibrium between ubiquinol-1 and the external ferricyanide, bypassing the bc_1 complex, appears responsible for the high ferricyanide reductase activity.

We then studied the responses of the bc_1 complex by monitoring the redox changes of prereduced cyt *b* upon ferricyanide addition.

In intact mitochondria, as shown in Fig. 1, cyt *b* is about 60% reduced by succinate in the presence of rotenone and KCN, and undergoes a partial oxidation upon the addition of malonate, necessary to slow down the electron input into the bc_1 complex from succinate dehydrogenase. Ferricyanide addition induces a transient, rapid reoxidation of cyt *b*, whose rate and extent are dependent on ferricyanide concentration.

Table II. Succinate-Ferricyanide Reductase Activity in Mitochondria Preincubated with Ubiquinones Having Different Side-Chain Length[a]

Mitochondria	Specific activity (nmol ferricyanide reduced · min^{-1} mg^{-1} protein)					
	+ UQ_1 (40 μM)			+ UQ_7 (40 μM)		
	–	+ Antimycin	+ TTFA	–	+ Antimycin	+ TTFA
Control	1500	1350	240	607	120	48
Lyophilized	1590	1320	162	816	126	141
UQ-depleted	1560	1600	150	588	204	108
UQ_{10}-reconstituted	1500	1350	195	690	120	105

[a]For experimental details, see Table I.

In the presence of antimycin, upon the ferricyanide pulse, the oxidant-induced cyt *b* extrareduction is usually found (Fig. 1B). After such transient reduction cyt *b* undergoes a slower oxidation.

We note that the latter phenomenon is apparently lost in lyophilized mitochondria. This could be mostly due to the removal of the endogenous cyt *c*, since the lyophilization procedure was accomplished after washing with 0.15 M KCl, which is well known to detach most cyt *c* (Lenaz and MacLennan, 1966). In fact, when low concentrations of cyt *c* were added to lyophilized mitochondria the oxidant-induced extrareduction of cyt *b* was completely restored. However, after pentane extraction of lyophilized mitochondria, the phenomenon was lost and was not restored by exogenous cyt *c*.

On the contrary, in mitochondria that were pentane extracted and then reconstituted with UQ_{10}, the extrareduction was recovered (Fig. 2). The loss of the extrareduction by pentane extraction can not be due to the pentane treatment itself, since reconstituted mitochondria were subjected to the same procedure as UQ-depleted mitochondria. The residual UQ not removed by pentane extraction (see Discussion) is able, in the presence of antimycin, to feed electrons from succinate to cyt *b* but at a slower rate (Fig. 2).

In order to evaluate the dependence of the restoration of cyt *b* extra-reduction on the concentration of exogenous UQ, the experiments shown in Fig. 3 were performed in UQ-depleted mitochondria. The amount of UQ_2 able to restore 50% of the maximal cyt *b* extrareduction has been found to be 1 mol UQ_2 per mole of bc_1 complex, but a remarkable excess of UQ_2 is needed for the full restoration of the phenomenon.

The decrease of the reoxidation rate of cyt *b* after extrareduction has a similar titer and it is similarly dependent on a large overstoichiometric excess of exogenous UQ_2 for maximal change. This suggests again that the UQ pool function of the quinone is mainly responsible for both phenomena.

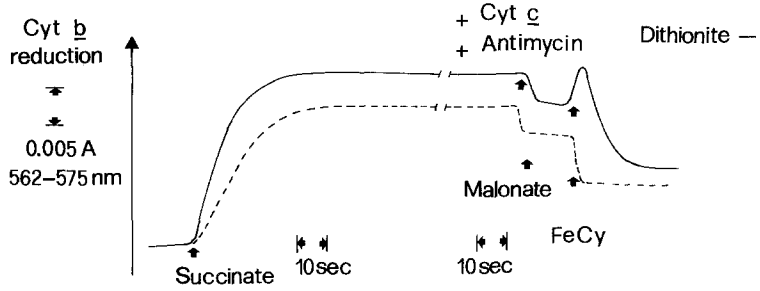

Fig. 2. Kinetic of cytochrome *b* reduction by succinate and reoxidation by 100 μM ferricyanide (FeCy) in UQ-depleted (dashed line) and UQ_{10}-reconstituted mitochondria (solid line). Reoxidation was monitored in the presence of 2 μM cytochrome *c* and antimycin (1 $\mu g \cdot mg^{-1}$ protein). The residual UQ in UQ-depleted mitochondria was 0.3 nmol $\cdot$ mg^{-1} protein. The amount of UQ_{10} in UQ_{10}-reconstituted mitrochondria was 6.3 nmol $\cdot$ mg^{-1} protein.

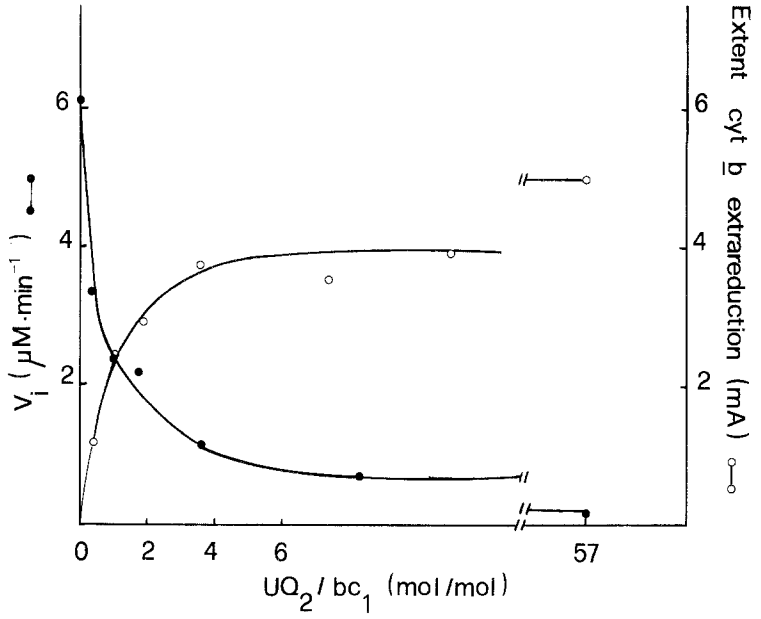

Fig. 3. Extents of cyt *b* extrareduction (○) in the presence of increasing amounts of UQ_2 in UQ-depleted mitochondria treated with antimycin. Initial rates of reoxidation of cyt *b* when the rapid extrareduction is completed (●). In fact, after cyt *b* extrareduction the cytochrome normally undergoes a slower reoxidation, as shown in Figs. 1 and 2. Determination of bc_1 complex was made considering 2 : 1 ratio cyt *b* per bc_1 corresponding to 0.37 nmol · mg^{-1} protein. The residual UQ_{10} was 0.3 nmol · mg^{-1} protein corresponding to 0.8 mol UQ_{10} per mole bc_1 complex.

We have confirmed the above data also in a crude preparation of succinate-cytochrome *c* reductase after UQ depletion by pentane plus acetone extraction (not shown). Due to the dramatic denaturation induced by this treatment in the bc_1 complex, we could not perform similar experiments with the purified enzyme.

We have also titrated the rates of cyt *b* reoxidation in UQ_{10}-reincorporated mitochondria in a wide range of ferricyanide concentrations.

As shown in Fig. 4, the initial rates were somewhat higher in UQ_{10}-reincorporated mitochondria than in UQ-depleted ones, particularly at high ferricyanide concentrations.

The rates tended to saturate with the oxidant concentration in an apparently biphasic fashion.

However we have verified that in intact mitochondria or submitochondrial particles (not shown), the ferricyanide saturation was almost monotonic under the same conditions. In the presence of antimycin, the initial rates did not saturate by any ferricyanide concentration in UQ-depleted mitochondria

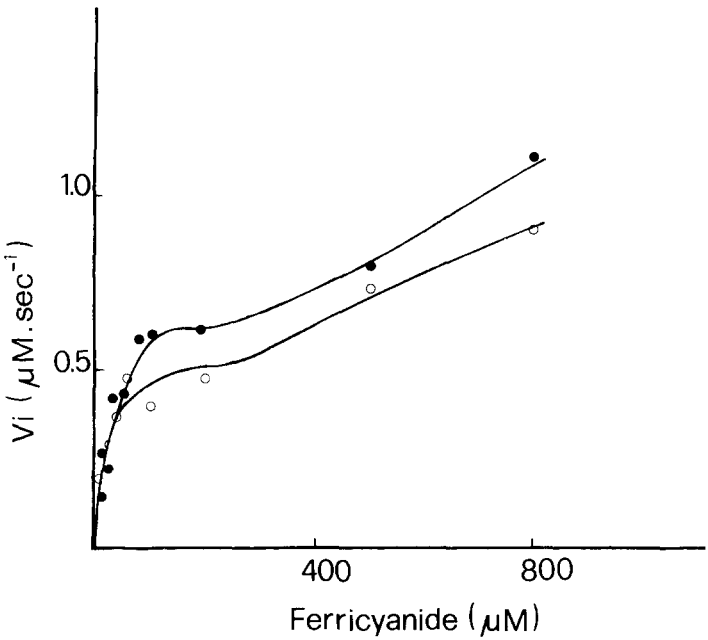

Fig. 4. Initial rates of cytochrome *b* reoxidation by increasing ferricyanide concentrations in UQ-depleted mitochondria (○) and in UQ_{10}-reconstituted mitochondria (●) in the absence of antimycin. For experimental details see Materials and Methods.

(Fig. 5). The same behaviour has not been found in UQ_{10}-reincorporated mitochondria, where the rates were much slower than in pentane-extracted mitochondria (cf. Fig. 5).

Also in intact mitochondria the cyt *b* reoxidation in the presence of antimycin was extremely slow, and practically independent of the oxidant concentration (not shown).

These data indicate that only a direct chemical reaction between ferricyanide and reduced cyt *b* can occur in UQ-depleted and antimycin-treated mitochondria.

Discussion

The major finding of the present work is that the pool of ubiquinone seems to be required for the ferricyanide-induced cyt *b* extrareduction in the presence of antimycin (cf. Fig. 2). Such phenomenon is considered a peculiar feature of the mechanism of electron transfer of the native bc_1 complex (Rieske, 1976), and can be observed also in the detergent-solubilized purified enzyme (Rieske, 1976). Probably the oxidant interacts mainly with

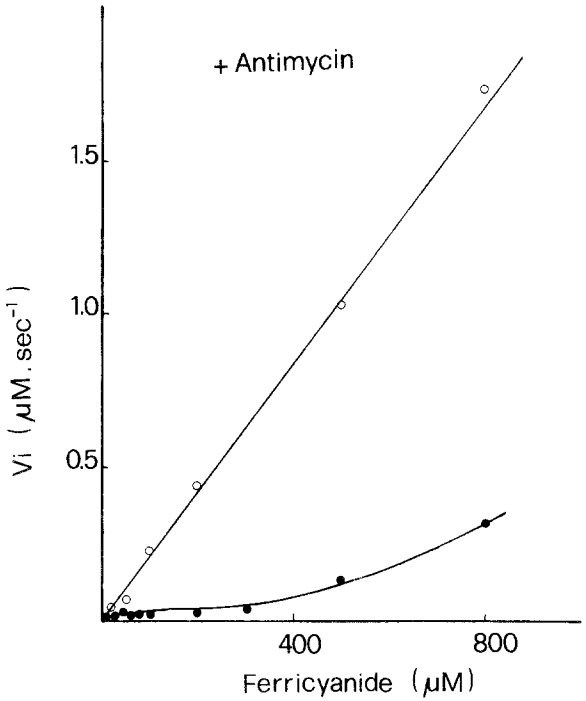

Fig. 5. Initial rates of cytochrome *b* reoxidation by increasing ferricyanide concentrations in UQ-depleted mitochondria (○) and in UQ_{10}-reconstituted mitochondria (●) in the presence of antimycin (1 μg · mg^{-1} protein). The rate of cytochrome *b* reoxidation in UQ_{10}-reconstituted mitochondria was taken after the extrareduction phase (cf. Fig. 2). For experimental details see Materials and Methods.

cyt c_1 in the isolated bc_1 complex (Tsai *et al.*, 1983). However, in intact mitochondrial particles, the main site of ferricyanide interaction is cyt *c* (Ernster *et al.*, 1969).

When this component is lacking, the bc_1 complex could hardly react *in situ* with the membrane-impermeant reagent, probably because of physical constraints which are removed during the detergent treatment used to isolate the complex. The requirement for cyt *c* to observe a large extrareduction of cyt *b* has been noted previously (Erecinska *et al.*, 1972).

With solvent extraction, we have previously verified that it is practically impossible to completely remove the endogenous ubiquinone from mitochondria; a residual amount in the range of the bc_1 concentration (i.e., 0.2–0.5 nmol per milligram of protein corresponding to 0.5–1.4 mol UQ_{10}/cyt c_1) is always retained (Cabrini *et al.*, 1981). However, many redox complexes of the mitochondrial respiratory chain presumably contain high-affinity binding sites for UQ, i.e., Complex I, Complex II, and α-glycerophosphate

dehydrogenase. The total amount of such UQ reaction partners largely exceeds the bc_1 complex concentration in the membrane (Capaldi, 1982). Therefore, in pentane-extracted mitochondria the residual UQ should be fractionated among its various binding sites, so that its actual concentration bound or close to the bc_1 complex should be substoichiometric. At least one ubiquinol per complex is necessary for the complete development of the cyt *b* extrareduction according to Mitchell's "Q-cycle," presently the most widely accepted scheme for the electron transfer in the bc_1 complex (Mitchell, 1975, 1985). In such model, when ferricyanide oxidines the high-potential components of the enzyme, constituted by cyt c_1 in redox equilibrium with the Rieske iron–sulfur center (Tsai *et al.* 1983; Rich, 1984), a new molecule of ubiquinol can be oxidized again, even in the presence of antimycin. Since the antibiotic prevents the subsequent reoxidation of cyt *b* (Mitchell, 1975), the ubisemiquinone thus formed completely reduces the *b* cytochromes.

Clearly, if the ubiquinol concentration able to rapidly react with the bc_1 complex is only a fraction of the minimal 1 : 1 ratio, a corresponding lower extent of cyt *b* extrareduction is to be expected.

Nevertheless, we have found that the amount of UQ_2 required to restore the maximal extent of cyt *b* extrareduction is severalfold the concentration of the bc_1 complex. Since the partition of UQ_2 in the mitochondrial membrane is very high (Degli Esposti *et al.*, 1981), under the conditions employed most UQ would be partitioned in the lipid phase. Moreover the specificity of such UQ homolog is extremely high also at steady state, excluding that this quinone could interact less efficiently and/or at different sites in the complex than the natural UQ_{10} (Lenaz *et al.*, 1985). We conclude therefore that the overstoichiometric requirement for UQ results from a real need of the UQ pool, possibly to maintain a sufficient electron input via ubiquinol and/or ubisemiquinone.

The second type of evidence found in this investigation concerns the direct chemical reaction of reduced cyt *b* with ferricyanide in UQ-depleted mitochondria in the presence of antimycin. The fact that such a reaction is much faster in UQ-depleted mitochondria may explain the lower antimycin sensistivity of the succinate-ferricyanide reductase activity after removal of the endogenous UQ (cf. Table I). In the presence of antimycin, in fact, also the reduced cyt *b* could directly deliver electrons to ferricyanide. Clearly, endogenous UQ prevents this direct chemical equilibration (cf. Fig. 5), accounting for the restoration of the antimycin sensitivity of the succinate-ferricyanide reductase activity (cf. Tables I and II).

The possibility of a direct interaction of cyt *b* with ferricyanide under certain experimental conditions (cf. also Kunz *et. al.*, 1984) should be

considered whenever ferricyanide is used as an artificial electron acceptor from the bc_1 complex.

The enhanced rate of chemical reaction between cyt *b* and ferricyanide may be due to the removal of UQ, whose redox state normally exerts a redox buffer on the reduction level of cyt *b* (Rich, 1984). Also the striking conformational changes induced by antimycin in the structure of the bc_1 complex (Baum *et al.*, 1967) could contribute to the above reaction. The antibiotic may induce the cytochrome *b* hemes, normally embedded in a hydrophobic environment (Rieske, 1976), to be more exposed to the water surface. This may enable them to react more easily with the water-soluble ferricyanide and, on the other hand, to communicate less easily with the hydrophobic ubiquinone.

In conclusion, the present data can be explained on the basis of the available knowledge of the bc_1 complex. In particular, our findings agree with the recent discussions of Rich (1984) and Crofts *et al.* (1983) excluding the presence of a special "bound" UQ in the function of the mitochondrial bc_1 complex. The concept of a tightly bound, or Qz quinone, was introduced to account for potentiometric, kinetic, and solvent extraction results (Prince and Dutton, 1977). From these data it was concluded that a special form of ubiquinone, tightly bound to the bc_1, was involved in the electron transfer within the redox components of the enzyme, including the *b* extrareduction in the presence of antimycin. Such Qz was shown to possess a midpoint potential somewhat higher than that of the UQ pool, and not to be easily extracted by organic solvents (Prince and Dutton, 1977). However, Rich (1984) has recently demonstrated that such specific features of a hypothetical tightly bound UQ can be explained simply on the basis of the reaction between an ubiquinol coming from the pool and the oxidized complex. Such reaction, involving a binding step to the complex, shifts the apparent midpoint potential of the quinol to a higher value than that of the UQ pool, without implying the presence of a Qz form previously inserted in the complex (Rich, 1984).

Our data confirm the interpretation of Rich (1984) and the similar one proposed by Crofts *et al.* (1983) in the sense that when a limited amount of UQ is left in the mitochondria, the phenomena, such as cyt *b* extrareduction, possibly linked to the presence of a tightly bound UQ, are lost (cf. Fig. 2). Addition of stoichiometric UQ concentrations to the bc_1 complex is not able to fully restore these reactions (cf. Fig. 4), but larger amounts of UQ are required for this purpose. This seems to exclude that a special form of UQ (i.e., Qz) is functionally operative in the mitochondrial bc_1 complex, and that the pre-steady-state reduction of the enzyme is mainly governed by its interaction with the UQ pool.

Acknowledgments

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