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# KINETICS OF UBIQUINOL-1-CYTOCHROME *c* REDUCTASE IN BOVINE HEART MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

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**A kinetic study on ubiquinol-cytochrome *c* reductase (EC 1.10.2.2) has been undertaken either in situ in KCN-inhibited mitochondria and submitochondrial particles, or in the isolated cytochrome *b*-$c_1$ complex using ubiquinol-1 and exogenous cytochrome *c* as substrates. The steady-state two-substrate kinetics of the reductase appears to follow a general sequential mechanism, allowing calculation of a $K_m$ for ubiquinol-1 of 13.4 $\mu$M in mitochondria and of 24.6 $\mu$M in the isolated cytochrome *b*-$c_1$ complex. At low concentrations of cytochrome *c*, however, the titrations as a function of quinol concentration appear biphasic both in mitochondria and in submitochondrial particles containing trapped cytochrome *c* inside the vesicle space, fitting two apparent $K_m$ values for ubiquinol-1. Relatively high antimycin-sensitive rates of ubiquinol-1-cytochrome *c* reductase have been found in submitochondrial particles: both the $V_{max}$ and the $K_m$ for ubiquinol-1 are, however, affected by the overall orientation of the particle preparation, i.e., by the reactivity of cytochrome *c* with its proper site. The turnover numbers corrected for particle orientation with respect to cytochrome *c* interaction are at least 2-fold higher in submitochondrial particles than in mitochondria. This is particularly evident using inside-out particles containing trapped cytochrome *c* in the vesicle space (and therefore reacting with its physiological site). A diffusion step for the quinol substrate appears to be rate limiting in mitochondria and can be removed by addition of deoxycholate, suggesting that the oxidation site of ubiquinol may be more exposed to the matrix side of the inner mitochondrial membrane.**

## Introduction

The cytochrome *b*-$c_1$ segment (Complex III) of the mitochondrial respiratory chain catalyzes the oxidation of ubiquinol by ferricytochrome *c* coupled to proton pumping across the membrane [1]. The central role played by this multiprotein redox complex in oxidative phosphorylation has been extensively investigated, but its mechanism of action still remains a controversial matter (for reviews, see Ref. 2 and 3). Among the various reaction schemes proposed, the 'Q-cycle' postulated by Mitchell [4] is probably the most widely quoted and discussed. Elaborated versions of this mechanism have been advanced which can account for a great deal of experimental evidence [2,5,6], proposing a sidedness of the inner mitochondrial membrane with respect to the ubiquinol oxidation site, which should be located near to the cytoplasmic side [4].

In spite of the importance of the ubiquinol oxidation sidedness in the light of the above hypothesis, experimental evidence to this purpose mainly rests on few indirect physical [7] and immunological [8] data. On the other hand, other

Abbreviations: $Q_1H_2$, ubiquinol-1; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

studies do not confirm the above assumption, since they indicate a preferred exposure of the ubiquinol oxidation site to the matrix side of the membrane [9–13]. A detailed study of ubiquinol-cytochrome *c* reductase activity as a function of the membrane permeability barrier is therefore required to give more direct information about this problem.

There is another reason which renders desirable a detailed kinetic study of this enzyme. The kinetics of the isolated cytochrome $b$-$c_1$ complex have been recently investigated utilizing physiological long-chain ubiquinols as substrates in the presence of nonionic detergents to solubilize these water-insoluble compounds [14–16]. Such an experimental approach is not suitable for studying the in situ activity of the reductase, since the relatively high detergent concentrations used disrupt the membrane integrity, and may alter some lipid-protein interactions essential for the enzymatic function [17]. In fact, the maximal turnover numbers obtained in the presence of detergents for the cytochrome $b$-$c_1$ complex of beef heart [14,15] are at least one order of magnitude lower than the turnover calculated with endogenous ubiquinol [18] or the values usually found with short-chain ubiquinols as substrates in the absence of detergents [19–21]. Hydrophilic quinols can function also as excellent donors to Complex III either in mitochondria [22,23] or in submitochondrial particles [23–28], indicating that the reoxidation site of ubiquinol can be easily reached by quinols dissolved in an aqueous medium even in the intact mitochondrial membrane. In spite of their clear usefulness, detailed studies of the enzymatic activity of the cytochrome $b$-$c_1$ complex with short-chain ubiquinols are, however, still lacking.

We have investigated the ubiquinol-cytochrome *c* reductase activity using ubiquinol-1 as the electron donor substrate in different KCN-inhibited mitochondrial preparations and in the isolated enzyme. We have chosen this quinol for several reasons: it apparently displays the lowest $K_m$ for the enzymatic assay [13,14,23]; it has a relatively low partition coefficient in lipids [29] and in mitochondrial membranes [30] due to its remarkable water solubility (up to $10^{-3}$ M [29]); it presumably interacts with a physiological site in the mitochondrial membrane, since it supports phosphorylation and proton extrusion as does succinate [23]; on the other hand, its transmembrane mobility appears some orders of magnitude slower than the enzymatic rates [31], thus allowing investigations of the sidedness of the ubiquinol oxidation site in the intact mitochondrial membrane. We have found that the in situ kinetics of the cytochrome $b$-$c_1$ complex are comparable to those exhibited in the isolated form, but several kinetic indications outline that the site of ubiquinol-1 oxidation may be more accessible from the matrix side of the inner mitochondrial membrane.

## Materials and Methods

*Preparations.* Heavy beef heart mitochondria were prepared by a large-scale procedure [32]. Cytochrome *c*-depleted mitochondria were obtained by hypotonic-isotonic treatment of mitochondria with KCl [33]. They routinely contained less than 0.1 nmol cytochrome *c* per mg protein and had only low succinate or NADH oxidase activities largely stimulated by exogenous cytochrome *c*. Submitochondrial particles were obtained either by sonic irradiation [34] or by alkaline treatment of mitochondria [35]. Submitochondrial particles containing trapped cytochrome *c* were prepared as described in Ref. 36; unless otherwise stated, the particles were washed by centrifugation at $105000 \times g$ in 0.15 M KCl, and then passed through a Sephadex G-75 column at a flux rate of 1 ml per min. Using this procedure, about 90% of the overall cytochrome *c* of the resulting preparation is not accessible to ascorbate reduction.

The orientation of mitochondria and submitochondrial particles was checked by different independent tests: (i) affinity chromatography on a Sepharose-cytochrome *c* column [37]; (ii) stimulation by exogenous cytochrome *c* of NADH oxidation [38]; (iii) stimulation by deoxycholate treatment of the rates of succinate-cytochrome *c* reductase and ferrocytochrome *c* oxidase activity [38,39]. All the functional tests have generally given converging indications that cytochrome *c*-depleted mitochondria are over 80–85% right-side out and submitochondrial particles usually 60–75% inside out. For routine evaluation of the orientation we have employed the formula: %inside out = NADH oxidase without cytochrome *c*/NADH oxidase

stimulated by exogenous cytochrome *c* (10–15 μM) [38]. The cytochrome *c* affinity column has been found to overestimate the inside-out particle orientation in comparison with the functional tests.

Ubiquinone-depleted and ubiquinone-reconstituted mitochondria were prepared as previously described [40]. Purified cytochrome *b*-$c_1$ complex was prepared by the method of Rieske [19]. It contained 3.4 nmol/mg protein of cytochrome $c_1$ and was kept at −70°C in small aliquots. Reduced cytochrome *c* was prepared as described in Ref. 39.

*Spectroscopic determinations.* All determinations were performed in a Cary 15 or Perkin-Elmer 559 spectrophotometer. The cytochrome $c_1$ concentration was determined by the dithionite reduced minus ferricyanide oxidized spectrum in the presence of 1% deoxycholate using an extinction coefficient of 20 $mM^{-1}$ at 553–540 nm or 17.5 $mM^{-1}$ at 552–547 nm [18].

Ubiquinone-1 (a kind gift from Eisai Co., Tokyo, Japan) was reduced using the method of Rieske [19], suspended in a methanolic solution slightly acidified with HCl to retard autooxidation, and stored at −20°C. Its concentration was determined after addition of KOH in ethanol, using an extinction coefficient of 12 $mM^{-1}$ at 275 nm [29], or directly in the reduced state at 290 nm using an extinction coefficient of 4 $mM^{-1}$.

Protein was determined by the biuret method [41].

*Enzyme assays.* Ubiquinol-cytochrome *c* reductase activity of mitochondrial particles was assayed at room temperature (22°C) in a mixture containing 200 mM sucrose, 10 mM potassium malonate, 20 mM Tris, 2.5 mM EDTA, 0.1 μg/ml each of rotenone and valinomycin, 1 mM KCN, pH 7.3, with 0.03–0.3 mg/ml of mitochondrial protein, and different amounts of cytochrome *c* (horse heart, type III, Sigma) to a final volume of 2 ml. The reductase activity of the isolated cytochrome *b*-$c_1$ complex was assayed as described in Ref. 42; the buffer used was sodium phosphate, pH 7.4, containing 1 mM EDTA. The reaction was started by a rapid pulse of ubiquinol-1 (the final methanol concentration never exceeding 2%), and the reduction of cytochrome *c* was monitored at 550 nm in a Cary 15 spectrophotometer against a reference cuvette containing the same assay mixture, using an extinction coefficient of 18.7 $mM^{-1}$ [33]. The sample cuvette was equipped with a rapid-mixing apparatus completing mixing in less than 0.2 s. To evaluate the chemical reduction of cytochrome *c* by the quinol, each assay was repeated in the presence of antimycin A (usually 0.25 μg/ml); the enzymatic rate, in fact, was generally over 95% antimycin sensitive. Conditions were selected in which the chemical rates were normally lower than 10% of the overall rates. Occasionally, when very low cytochrome *c* concentration had to be measured, its reduction was monitored at 418 nm, using an extinction coefficient of 40.7 $mM^{-1}$, as verified experimentally. The oxidation of ubiquinol was followed at 274 nm, using an extinction coefficient of 12.5 $mM^{-1}$. Succinate-cytochrome *c* reductase was assayed in the same system, with 20 mM KCl replacing malonate in the assay medium.

NADH oxidase activity was assayed spectrophotometrically at 340 nm with 0.25 mM NADH and 0.03–0.05 mg/ml particle suspensions, whereas ubiquinol-oxidase and ferrocytochrome *c* oxidase activities were measured at 274 and 550 nm, respectively; the buffer was 0.2 M sucrose, 10 mM Tris-HCl, 20 mM KCl and 2 mM EDTA, pH 7.3, containing 0.1 μg/ml valinomycin.

The initial rates were elaborated by a least-squares regression analysis, whereas the time courses were as described in Ref. 43.

*Materials.* All the reagents were the most pure commercially available.

## Results

### *Reductase kinetics in cytochrome c-depleted mitochondria*

Fresh preparations of cytochrome *c*-depleted mitochondria exhibit a ubiquinol-1-cytochrome *c* reductase activity that is stimulated by ionophores like valinomycin and FCCP, and thus is under coupling control. After freeze-thawing, however, the reductase activity usually increases about 2-fold and is not further stimulated by ionophores. To avoid complications due to coupling control, we have performed the enzymatic assays under uncoupling conditions. ADP and ATP stimulate the reductase activity in an oligomycin-insensitive fashion, and the effect is mimicked by EDTA.

Since EDTA does not alter the apparent $K_m$ for ubiquinol-1 (result not shown) and exerts a stimulating effect also in the isolated cytochrome $b$-$c_1$ complex [19], we have routinely added it throughout the assays in order to obtain maximal rates.

As exogenous cytochrome $c$ added back to cytochrome $c$-depleted mitochondria in the presence of rotenone, KCN and malonate to functionally isolate the cytochrome $b$-$c_1$ complex should not differ from the endogenous one in its rapid redox turnovers [22], we have undertaken a two-substrate titration of the reductase activity varying ubiquinol-1 at different fixed levels of the electron acceptor. The resulting enzymatic activity was almost completely antimycin-sensitive and proportional to the mitochondrial concentration. The reduction pattern of cytochrome $c$ is first order up to 30 $\mu$M cytochrome $c$; at higher concentrations it becomes mixed zero-first order. By increasing the cytochrome $c$ level, the first-order rate constant progressively decays to lower values (Fig. 1), in agreement with previous findings with succinate-cytochrome $c$ reductase [39,43], whereas the initial rates increase in a hyperbolic fashion. The stoichiometry of the reaction calculated from the initial rates of cytochrome $c$ reduction and ubiquinol oxidation has been found to be one redox equivalent from the quinol to one redox equivalent accepted from cytochrome $c$, at different substrate concentrations, indicating that we are following the steady-state kinetics of the enzyme.

Fig. 2 shows double-reciprocal plots of the antimycin-sensitive initial rates of the reductase activity as a function of ubiquinol-1 concentration; the curves intersect each other at about the same level on the abscissa, corresponding to an apparent $K_m$ of 13 $\mu$M. The curves obtained by plotting the same data as a function of the cytochrome $c$ concentration appear linear at any fixed level of $Q_1H_2$, extrapolating to an apparent $K_m$ for cytochrome $c$ that increases (from 7 to 16 $\mu$M) by increasing the fixed quinol concentration. These kinetic features have been confirmed by several separate experiments. The replots of the intercepts of the curves with $Q_1H_2$ as the variable substrate are shown in Fig. 3; for the nonlinear curves at low cytochrome $c$ levels, only the linear portions at

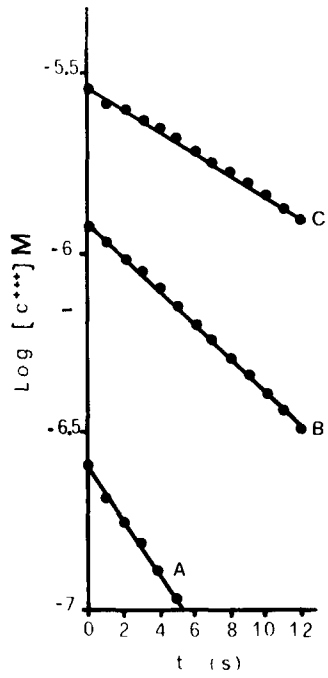


Fig. 1. Effect of increasing cytochrome $c$ concentrations on the rate constants of ubiquinol-1-cytochrome $c$ reductase activity in cytochrome $c$-depleted mitochondria (0.049 mg/ml). $Q_1H_2$ concentration was 100 $\mu$M and those of cytochrome $c$ were 2.4, 11.8 and 28.9 $\mu$M in A, B and C, respectively.

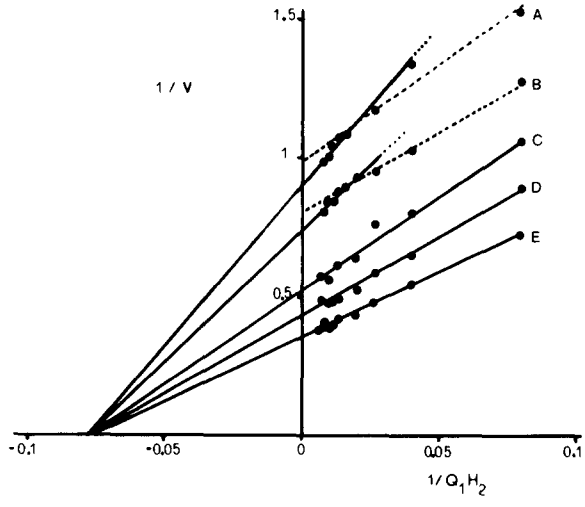


Fig. 2. Double-reciprocal plots of the antimycin-sensitive initial rates of the reductase activity in cytochrome $c$-depleted mitochondria (0.049 mg/ml) with ubiquinol-1 as the variable substrate. The cytochrome $c$ concentrations were: curve A, 8.22 $\mu$M; B, 11.8 $\mu$M; C, 28.9 $\mu$M; D, 47 $\mu$M; E, 94 $\mu$M. The values of $V$ are expressed in $\mu$mol cytochrome $c$ reduced/min per mg protein, and those of ubiquinol-1 concentration in $\mu$M.

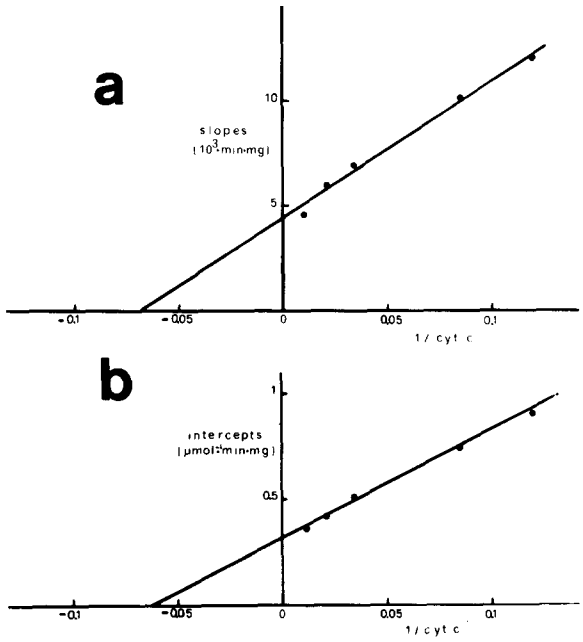


Fig. 3. Replots of the slopes (a) and of the intercepts (b) of the curves in Fig. 2 as a function of cytochrome *c* concentration in μM.

high ubiquinol concentrations have been considered. From these replots and those derived from the titrations with cytochrome *c* as the variable substrate, we have calculated the kinetic parameters of the reductase assuming a sequential mechanism, since all the curves intersect each other [44] (Table I).

Using cytochrome *c* concentrations below 10 μM the titrations as a function of ubiquinol-1 concentration tend to be nonlinear (cf. Fig. 2, lines A and B), appearing clearly biphasic when cytochrome *c* is kept at about 1–3 μM. Under these conditions, the data fit two apparent $K_m$ values for ubiquinol-1 of about 1–2 and 5–13 μM, respectively [45]. This kinetic feature is maintained in ubiquinone-depleted mitochondria with respect to ubiquinone-reconstituted mitochondria, and in a number of mitochondrial subfractions, including submitochondrial particles with trapped cytochrome *c* [45]. Moreover, ubiquinol-2 also presents the same phenomenon (unpublished observations), and therefore such biphasic behavior could be a real kinetic feature of ubiquinol-cytochrome *c* reductase when the cytochrome *c* concentration is well below saturating levels.

TABLE I

KINETIC PARAMETERS OF UBIQUINOL-1-CYTOCHROME *c* REDUCTASE IN CYTOCHROME *c*-DEPLETED MITOCHONDRIA AT 22°C.

The data were calculated by a least-squares regression analysis according to Ref. 44, assuming a sequential mechanism.

| | | |
|---|---|---|
| $K_m$ | ubiquinol-1 | 13.4 μM |
| $K_D$ | ubiquinol-1 | 13.5 μM |
| $K_m$ | cytochrome *c* | 14.5 μM |
| $K_D$ | cytochrome *c* | 12.3 μM |
| $V_m$ | | 3.08 μmol/min per mg protein |
| Maximal turnover number | | 223 $s^{-1}$ |

*Reductase kinetics in the isolated cytochrome b-$c_1$ complex*

The reductase activity of the isolated cytochrome *b*-$c_1$ complex decreases to about 30–40% of the original values after freeze-thawing, and follows first- or mixed zero-first-order kinetics with a progressive decrease in the rate constants with increasing cytochrome *c* concentration, as found in intact mitochondria. The ubiquinol-1 titrations at any fixed level of cytochrome *c* are generally linear, as shown in Fig. 4. The kinetic parameters calculated from the replots of the slopes and the

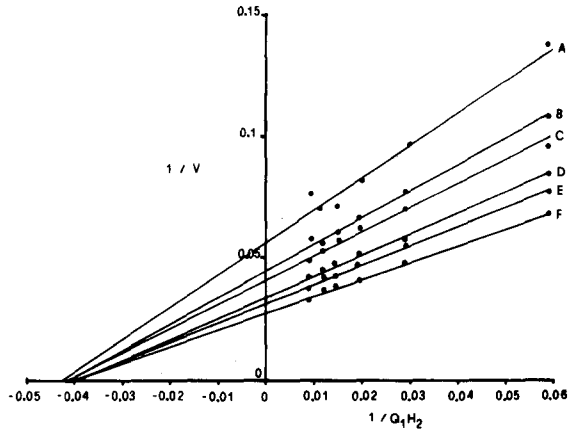


Fig. 4. Double-reciprocal plots of the antimycin-sensitive initial rates of the reductase activity of the purified cytochrome *b*-$c_1$ complex (0.68 μg/ml) with ubiquinol-1 as the variable substrate at different fixed levels of cytochrome *c*, which were: curve A, 2.68 μM; B, 3.14 μM; C, 4.91 μM; D, 7.49 μM; E, 12.04 μM; F, 24.08 μM. The values of *V* and $Q_1H_2$ concentration are expressed as in Fig. 2.

TABLE II

KINETIC PARAMETERS OF UBIQUINOL-1-CYTOCHROME *c* REDUCTASE IN THE ISOLATED CYTOCHROME *b*-$c_1$ COMPLEX

The data were obtained as in Table I.

| | |
|---|---|
| $K_m$ ubiquinol-1 | 24.6 μM |
| $K_D$ ubiquinol-1 | 21.3 μM |
| $K_m$ cytochrome *c* | 3.3 μM |
| $K_D$ cytochrome *c* | 3.0 μM |
| $V_m$ | 40.69 μmol/min per mg protein |
| Maximal turnover number | 200 $s^{-1}$ |

intercepts of the curves obtained with either ubiquinol-1 or cytochrome *c* as the variable substrate are listed in Table II, assuming again a sequential mechanism [44]. The maximal turnover number is 200 $s^{-1}$, apparently lower than that found in the in situ titrations, but considering the partial enzyme inactivation upon freeze-thawing, the real maximal turnover can be estimated to be about 300 $s^{-1}$.

The $K_m$ for ubiquinol-1 of 24.6 μM appears almost double that found in mitochondria, but the most relevant kinetic differences consist in the remarkable decrease in both the dissociation constant and the $K_m$ for cytochrome *c* in the cytochrome *b*-$c_1$ complex.

We have found that the kinetics of ubiquinol-1-cytochrome *c* reductase at low (below 5 μM) cytochrome *c* concentrations appear again markedly biphasic if lipids or bovine serum albumin are added to the assay mixture [45].

*Studies with submitochondrial particles*

Our preparations of submitochondrial particles exhibit high antimycin-sensitive ubiquinol-1-cytochrome *c* reductase rates. Since the physiological site of cytochrome *c* interaction has been proven to be at the cytoplasmic side of the inner mitochondrial membrane [38], and thus should not be available to completely inside-out submitochondrial vesicles, such findings suggest that our particle suspensions are not homogeneous with respect to their membrane orientation. Employing a number of functional criteria based on the reactivity of the preparation to exogenous cytochrome *c* [38], we have verified that the rates of ubiquinol-1-cytochrome *c* reductase are proportional to the extent of particles which are not inside out, as shown in Table III. Such noninside-out particles are presumably broken membrane fragments and not really right-side-out vesicles, since there is no stimulation of NADH oxidation upon detergent (deoxycholate) addition, indicating that also those membranes which are not inside out are accessible to NADH. The submitochondrial preparations appear therefore to be a mixture of sealed inside-out particles and of unsealed membranes. This point is extremely important for the results presented below. In order to calculate the turnover numbers of

TABLE III

UBIQUINOL-1-CYTOCHROME *c* REDUCTASE ACTIVITY IN DIFFERENT MITOCHONDRIAL PREPARATIONS

Ubiquinol and cytochrome *c* concentrations were 58 and 15 μM, respectively. The orientation of the preparations was determined as described in Materials and Methods by the extent of stimulation by cytochrome *c* on NADH oxidase activity. The reductase rates are expressed as turnover numbers on the basis of the cytochrome $c_1$ content of the particles. The apparent turnovers obtained experimentally were corrected by the extents of right-side-out particles with respect to the reactivity toward cytochrome *c* (corrected turnover = apparent turnover/fraction of noninside-out particles). DOC, deoxycholate.

| Preparation | Apparent turnover ($s^{-1}$) | | Corrected turnover ($s^{-1}$) |
|---|---|---|---|
| | −DOC | +DOC [a] | |
| Cytochrome *c*-depleted mitochondria (82% right-side out) | 67.8 | 168.8 | 82.7 |
| Alkaline particles (75% inside out) | 43.8 | 114.3 | 175.3 |
| Sonicated particles (70% inside out) | 48.5 | 169.6 | 161.5 |
| Sonicated particles (55% inside out) | 95.3 | 206.2 | 211.0 |

[a] The particle suspension was previously treated with 0.3 mg/mg protein of deoxycholate.

the enzyme in situ, we must consider the percentage of particles which can normally react with cytochrome *c*. This has been obtained by dividing the apparent experimental turnover numbers of the reductase by the percentage of noninside-out particles (i.e., those functionally reacting with exogenous cytochrome *c*); such calculations yield turnover numbers very close to those found in deoxycholate-treated submitochondrial particles, which should totally react with cytochrome *c* [38] (cf. Table III).

In some submitochondrial particle preparations, particularly after many freeze-thaw cycles, displaying only a 2-fold stimulation by deoxycholate of both succinate reductase and ferrocytochrome *c* oxidase activities, the ubiquinol-1 titrations fit an apparent $K_m$ for the quinol which resembles the values found in the isolated cytochrome *b*-$c_1$ complex, ranging between 20 and 30 $\mu$M, with maximal rates higher than in mitochondria. Only if the particle suspension is over 70% inside out, as is usually the case in alkaline particles [38], is the apparent $K_m$ for ubiquinol-1 closer to the value found in cytochrome *c*-depleted mitochondria (13–16 $\mu$M).

Thus, the kinetic parameters of the reductase titrations are highly sensitive to the overall membrane orientation of the mitochondrial vesicles, i.e., they appear to be related to the ability of cytochrome *c* to react with its proper site in the cytochrome *b*-$c_1$ complex located at the cytoplasmic side [38,46]. An interesting observation is, however, that considering the extent of noninside-out particles, the real turnover number of both ubiquinol-1 oxidase and ubiquinol-cytochrome *c* reductase activities are at least 2-fold higher in submitochondrial particles than in mitochondria (Tables III and IV).

In order to elucidate this observation, we have used two experimental approaches. First, we have investigated the effect of deoxycholate treatment on ubiquinol-1-cytochrome *c* reductase activity in both mitochondria and submitochondrial particles, and secondly we have employed submitochondrial particles containing trapped cytochrome *c*, where the problems of the correct reactivity of cytochrome *c* with its proper site are overcome.

*Effect of deoxycholate on the reductase*

Deoxycholate added at different concentrations (0.3–1 mg per mg protein to the concentrated preparations) enhances by 2–4-fold the rates of ubiquinol-1-cytochrome *c* reductase activity in cytochrome *c*-depleted mitochondria, depending on their integrity and overall orientation. This effect is not due to greater reactivity of cytochrome *c*, since both succinate-cytochrome *c* reductase and ferrocytochrome *c* oxidase are only slightly stimulated by the detergent (Table V). Moreover, this stimulation of ubiquinol-cytochrome *c* reductase is analogous to that exhibited by NADH-cytochrome *c* reductase, which is usually 3–5-fold increased by deoxycholate under such conditions [47].

TABLE IV

UBIQUINOL-1-CYTOCHROME *c* REDUCTASE AND UBIQUINOL-1 OXIDASE ACTIVITIES IN MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

Experimental conditions as in Table III, with 15 $\mu$M cytochrome *c* also in ubiquinol oxidase assays. The corrected turnovers were calculated dividing the apparent turnovers of the reductase or the oxidase rates by the extent of noninside-out particles (i.e., normally reacting with exogenous cytochrome *c*), which was 82% for cytochrome *c*-depleted mitochondria and 25% for alkaline submitochondrial particles.

| Activity | Cytochrome *c*-depleted mitochondria | | Alkaline particles | |
|---|---|---|---|---|
| | Apparent turnover ($s^{-1}$) | Corrected turnover ($s^{-1}$) | Apparent turnover ($s^{-1}$) | Corrected turnover ($s^{-1}$) |
| $Q_1H_2$ oxidase | 35.0 | 44.0 | 31.2 | 125.0 |
| $Q_1H_2$-cytochrome *c* reductase [a] | 72.5 | 86.0 | 55.6 | 222.0 |

[a] Maximal turnover extrapolated to infinite ubiquinol-1 concentrations at a fixed level of 15 $\mu$M cytochrome *c*.

TABLE V

EFFECT OF DEOXYCHOLATE TREATMENT ON VARIOUS REDOX ACTIVITIES INVOLVING CYTOCHROME *c*

Deoxycholate (DOC) (0.5 mg/mg protein) was added to the concentrated suspension of mitochondrial particles. The extent of noninside-out particles was 82% for mitochondria and 70% for sonicated submitochondrial particles.

| Activity | Initial rates (μmol/min per mg protein) | | | | | |
|---|---|---|---|---|---|---|
| | Cytochrome *c*-depleted mitochondria | | | Submitochondrial particles | | |
| | −DOC | +DOC | +DOC/−DOC | −DOC | +DOC | +DOC/−DOC |
| Succinate-cytochrome *c* reductase [a] | 0.111 | 0.138 | 1.2 | 0.045 | 0.130 | 2.9 |
| Cytochrome *c* oxidase [b] | 0.590 | 0.670 | 1.1 | 0.980 | 3.90 | 4.0 |
| $Q_1H_2$-cytochrome *c* reductase [c] | 1.220 | 3.040 | 2.5 | 0.770 | 2.70 | 3.5 |

[a] With 15 μM cytochrome *c* and 5 mM succinate.
[b] With 20 μM ferrocytochrome *c*.
[c] With 15 μM cytochrome *c*.

Deoxycholate also changes, in parallel to the maximal rate, the apparent $K_m$ for ubiquinol-1 from 13 to 26 μM, i.e., to a value closer to that found in the isolated cytochrome *b*-$c_1$ complex (cf. Table II). The maximal turnover numbers at saturating cytochrome *c* concentration obtained upon deoxycholate treatment are practically identical in mitochondria and submitochondrial particles, reaching values higher than 400 $s^{-1}$. It should be noted that we have checked that deoxycholate is not able per se to enhance the reductase activity of the isolated cytochrome *b*-$c_1$ complex.

*Studies with submitochondrial particles containing trapped cytochrome c*

Submitochondrial particles with trapped cytochrome *c*, after KCl washing, present only 20–30% of cytochrome *c* reducible by ascorbate (a well known impermeable reagent); the remaining cytochrome *c* (usually 5–10 nmol/mg protein) is reducible after deoxycholate treatment. Using any quinol concentration up to 100 μM the reductase reaction appears to be of second-order kinetics with respect to cytochrome *c*, at least in the first few seconds. With unwashed particles the reaction, is however, first order as in mitochondria, but becomes second order after ascorbate reduction of the externally bound cytochrome *c* (Fig. 5). Titration of the reductase activity as a function of ubiquinol-1 concentration in KCl-washed particles with trapped cytochrome *c*, in comparison with that obtained in cytochrome *c*-depleted mitochon-

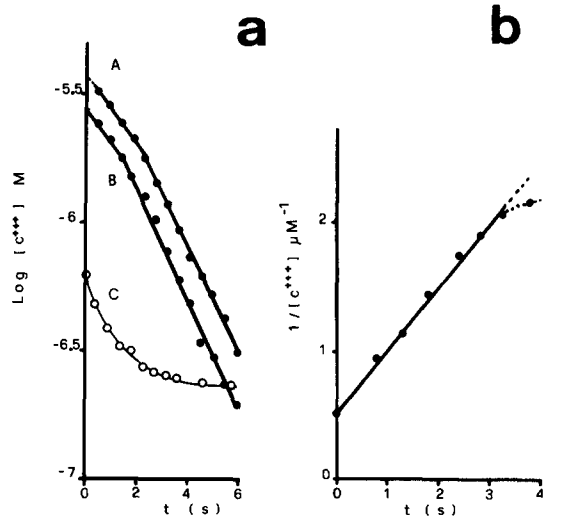


Fig. 5. (a) Semilogarithmic plots of ferricytochrome *c* reduction with 100 μM $Q_1H_2$ in submitochondrial particles with trapped cytochrome *c* (0.226 mg/ml) and cytochrome *c*-depleted mitochondria (0.206 mg/ml) at the same concentration of externally added cytochrome *c* (about 21 nmol/mg protein). Curve A, mitochondria; B, submitochondrial particles unwashed with KCl; C, submitochondrial particles unwashed in the presence of 2.5 mM ascorbate to reduce externally-bound cytochrome *c* (○———○). (b) Second-order plot of the antimycin-sensitive cytochrome *c* reduction with the same submitochondrial preparation which was subsequently washed with KCl. 0.207 mg/ml of particles containing about 10 nmol/mg of trapped cytochrome *c*; other conditions as in a.

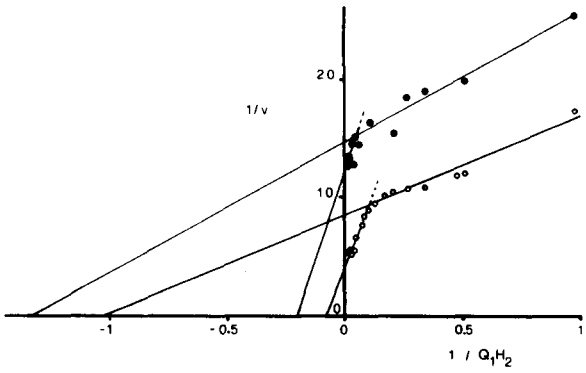


Fig. 6. Double-reciprocal plots of the antimycin-sensitive initial rates of the reductase activity in cytochrome *c*-depleted mitochondria (●———●) and submitochondrial particles with trapped cytochrome *c* washed with KCl (○———○) at the same ratio of 19 between exogenous cytochrome *c* and endogenous cytochrome $c_1$. Cytochrome *c* reduction was monitored at 418 nm.

dria at the same ratio of 19:1 of cytochrome *c* to cytochrome $c_1$, yields a similar biphasic pattern, but the maximal rates of both kinetic phases are higher in submitochondrial particles (Fig. 6). It is interesting to note that the curves tend to be parallel, since also the apparent $K_m$ values for ubiquinol-1 are higher in submitochondrial particles, similar to that found by treating cytochrome *c*-depleted mitochondria with deoxycholate.

In order to undertake a more exact comparison between the rates in mitochondria and in cytochrome *c*-enriched submitochondrial particles, we have passed through a Sephadex G-75 column a preparation of sonicated particles plus cytochrome *c* previously washed in KCl. The first 2–3 ml of the eluate are largely homogeneous particles (over 85% inside out) and contain only about 10% of ascorbate-reducible cytochrome *c*; the subsequent fractions contain particles with less inside-out content and with more ascorbate-reducible cytochrome *c*. Using a fixed, saturating ubiquinol-1 concentration and the same ratio of 19:1 of cytochrome *c* to cytochrome $c_1$, we have compared the turnover numbers of the reductase in mitochondria to those found in the gel-filtered particles. As shown in Table VI, it is clear that the ubiquinol-1-cytochrome *c* reductase activity is 3.7-fold higher in mitochondria upon deoxycholate treatment, reaching practically the same turnover value found in submitochondrial particles with cytochrome *c* inside the vesicle space.

## Discussion

This study reports the first detailed kinetic analysis of ubiquinol-cytochrome *c* reductase in intact mitochondrial membranes. The possibility to add from the aqueous medium at varying levels both the electron donor (ubiquinol-1) and the electron acceptor (ferricytochrome *c*) allows an interpretation of the enzyme kinetics in terms of the mechanism of action of the cytochrome *b*-$c_1$ complex in situ. In this respect it is important to recall that oxidation of ubiquinol-1 is fully antimycin sensitive and is under coupling control, as it supports phosphorylation [23], indicating that the path of electrons in the complex should be the same as

TABLE VI

COMPARISON OF THE UBIQUINOL-1-CYTOCHROME *c* REDUCTASE ACTIVITY IN CYTOCHROME *c*-DEPLETED MITOCHONDRIA AND IN SUBMITOCHONDRIAL PARTICLES WITH TRAPPED CYTOCHROME *c*

The ratio between exogenous cytochrome *c* and endogenous cytochrome $c_1$ was 19 for both preparations. The orientation of cytochrome *c*-depleted mitochondria was 85% right-side out and that of submitochondrial particles, washed with KCl and then passed through a Sephadex G-75 column (see Materials and Methods), 86% inside-out. Ubiquinol-1 concentration was 87 μM.

| Preparation | Cytochrome $c_1$ (nM) | $V$ (μM/s) | Apparent turnover number ($s^{-1}$) |
|---|---|---|---|
| Cytochrome *c*-depleted mitochondria | 57.5 | 0.214 | 3.7 |
| Cytochrome *c*-depleted mitochondria plus 0.5 mg/mg deoxycholate | 57.0 | 0.803 | 13.9 |
| Submitochondrial particles with trapped cytochrome *c* | 90.0 | 1.210 | 13.5 |

with endogenous ubiquinone. This is confirmed by the high reductase rates found in ubiquinone-depleted mitochondria and by the fact that the apparent $K_m$ for $Q_1H_2$ does not significantly change upon ubiquinone extraction [40,45].

In cytochrome *c*-depleted mitochondria, the two-substrate steady-state kinetics of ubiquinol-1-cytochrome *c* reductase appear to conform to a general sequential mechanism as with the isolated cytochrome *b*-$c_1$ complex, in contrast to that found in the cytochrome *b*-$c_1$ complex from yeast mitochondria [48]. In fact, all the lines obtained varying one substrate at a fixed level of the other in double-reciprocal plots intersect each other, generally below the abscissa. Our data cannot provide further information about the mechanism of action of the enzyme. Nevertheless, the present study constitutes a necessary starting point to investigate the presteady-state kinetics of redox interconversions occurring within the cytochrome *b*-$c_1$ complex which are now underway in our laboratory. One important observation is that the enzymatic kinetics of the reductase in situ and in the isolated cytochrome *b*-$c_1$ complex are comparable. Some features, however, appear to differentiate the kinetic responses of the complex when it is integrated in the native mitochondrial membrane, particularly with respect to the interaction with cytochrome *c*. This could be due to the presence of low-affinity binding sites for cytochrome *c* in the mitochondrial membrane which have an apparent dissociation constant of 16–20 $\mu$M [18,46], i.e., very close to the value found in our study (cf. Table I). The $K_D$ for cytochrome *c* is much lower (approx. 3 $\mu$M) in the isolated enzyme; it is thus conceivable that the reductase activity in the intact membrane involves both the high-affinity and low-affinity binding sites for the cytochrome [46].

Physical constraints, such as the presence of an outer membrane or aggregation phenomena of the particles, should not limit the reactivity of cytochrome *c* with the reductase, since addition of detergents like deoxycholate to cytochrome *c*-depleted mitochondria slightly enhances succinate-cytochrome *c* reductase or cytochrome oxidase activities (cf. Table V).

It is, however, likely that the physical state of the cytochrome *b*-$c_1$ complex is involved in the biphasic character of ubiquinol titrations found at low cytochrome *c* concentrations (cf. Fig. 6). This biphasic behavior appears to be a real kinetic feature of the enzyme, as it has been reported elsewhere [45], and it is present also in the isolated cytochrome *b*-$c_1$ complex when reconstituted into liposomes (unpublished observations). The fact that the nonlinear titrations are clearly found only at low cytochrome *c* concentrations could also indicate some kind of modulation by the electron acceptor substrate on the redox mechanism of ubiquinol-cytochrome *c* reductase.

*Sidedness of ubiquinol-cytochrome c reductase*

Although the reductase kinetics are extremely sensitive to the membrane orientation of both mitochondria and submitochondrial preparations, it is always found that the real turnovers of the cytochrome *b*-$c_1$ complex are 2–4-fold higher in submitochondrial particles (cf. Tables III–VI). It is not possible to determine whether there are also antimycin-sensitive electron pathways to cytochrome *c* at the matrix side of the membrane accounting for such high rates found in submitochondrial particles. There are some indications in the literature which could support this possibility [27,46,49]. The clearcut correspondence between the extent of noninside-out particles and the reductase turnover after deoxycholate treatment (Table III), however, suggests that the high reductase activity found in submitochondrial vesicles is due to a better accessibility of the quinol substrate to its oxidation site when added from the matrix side of the membrane, in accordance with previous findings [9,10,13].

The stimulating effect of deoxycholate on ubiquinol-1-cytochrome *c* reductase in cytochrome *c*-depleted mitochondria strongly supports such an idea, indicating that the overall maximal turnover number is identical in both detergent-treated mitochondria and submitochondrial particles (cf. Table III), but constant rate-limiting steps are present when the quinol substrate is added to intact mitochondria, i.e., from the cytoplasmic side of the membrane. These steps are likely to be represented by diffusion across the mitochondrial membrane, since they can be removed by detergents and appear analogous to the kinetic responses of NADH-cytochrome *c* reductase, the

site of NADH oxidation being at the matrix side of the membrane [38]. Such physical constraints also modify the apparent $K_m$ for ubiquinol-1, which assumes a value typical of the isolated enzyme only after deoxycholate treatment of cytochrome *c*-depleted mitochondria.

Further indications in support of the above view arise from the data obtained with submitochondrial particles containing trapped cytochrome *c* (cf. Fig. 6 and Table VI). Such particles appear to have the correct orientation for both the electron donor (ubiquinol-1) and the electron acceptor (cytochrome *c*) to obtain maximal turnovers of the reductase, and therefore should be most usefully employed for sidedness studies on the function of the cytochrome *b*-$c_1$ complex. In fact, at the same ratio between exogenous cytochrome *c* to the enzyme, both the turnover numbers and the rate constants of the reductase activity are clearly higher in the particles than in mitochondria. Furthermore, the highest second-order rate constants found in such particles, in the region of 3 $\mu$M/s, appear very close to the values reported for the fast redox reaction between cytochrome $c_1^{2+}$ and cytochrome $c^{3+}$ of 3.3 $\mu$M/s [50], indicating that the real reaction followed in submitochondrial particles with trapped cytochrome *c* is the redox equilibration between the reduced cytochrome *b*-$c_1$ complex and the electron acceptor, similar to that found with endogenous cytochrome *c* by stopped-flow techniques [13]. This has not been verified in mitochondria under identical assay conditions (cf. Fig. 5).

The comparison between the deoxycholate effect on various reactions involving cytochrome *c* (cf. Table V) clearly suggests that the ubiquinol-1-cytochrome *c* reductase is limited not only by the ability of the electron acceptor to react with its proper site located at the cytoplasmic side of the membrane, but also by the diffusion of the relatively hydrophilic ubiquinol-1 across the membrane to reach its oxidation site. As it has been demonstrated that exogenous quinols share the same pathway as endogenous ubiquinol for feeding electrons to the proton-extrusion device of the cytochrome *b*-$c_1$ segment of the respiratory chain [23,51], the finding of a preferred interaction of ubiquinol-1 at the matrix side of the membrane appears in contrast with the mechanisms based on the proton-motive Q-cycle hypothesis [2,4]. The picture of a functional asymmetry of the membrane would be, in fact, opposite to that postulated by such models, which assume that ubiquinol oxidation should take place more close to the cytoplasmic side of the membrane.

Furthermore, it is not possible to speculate further about the mechanism of coupling at the level of the cytochrome *b*-$c_1$ complex on the basis of our kinetic data; they can give only functional indications on the steady-state activity of ubiquinol-cytochrome *c* reductase.

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