The effect of rate limitation by cytochrome c on the redox state of the ubiquinone pool in reconstituted NADH:cytochrome c reductase

Jacky S. REED and C. Ian RAGAN*
Department of Biochemistry, University of Southampton, Southampton SO1 3TU, U.K.

The kinetic model of Ragan & Cottingham [(1985) Biochim. Biophys. Acta 811, 13–31] for electron transport through a mobile pool of quinone predicts that, under certain conditions, the normal linear dependence of electron flow on the degree of reduction (or oxidation) of the quinone should no longer be found. These conditions can be met by reconstituted NADH:cytochrome c reductase (Complex I–III from bovine heart) when electron flow is rate-limited by a low concentration of cytochrome c. We show that, in such a system, the dependence of activity (varied by inhibition with rotenone) on the steady-state level of quinone reduction is indeed non-linear and very closely accounted for by the theory.

INTRODUCTION

The redox behaviour of the ubiquinone pool in bovine heart submitochondrial particles can be quantitatively accounted for by the observation that reduction of ubiquinone by dehydrogenases and oxidation of ubiquinol by the cytochrome bc1 complex are both first-order processes in the substrate [1,2]. This model has been found to hold in several systems, although deviations are also quite common [3–5]. The underlying causes of these deviations from first-order redoxreduction of a homogeneous quinone pool are not known with any certainty, although compartmentalization of the quinone pool is the most popular explanation [6]. Less attention has been focused on whether or not ubiquinone oxidoreduction is in general a simple first-order process. To explain first-order behaviour it has been proposed that ubiquinone and ubiquinol must be present at low, non-saturating, concentrations (see, e.g., [5]) and that the quinone reductase and quinol oxidase enzymes must be kept fully reduced and fully oxidized respectively (see, e.g., [6]). Since these conditions do not apply in natural membranes, which nevertheless do show apparent first-order oxidoreduction of ubiquinone, Ragan & Cottingham [4] devised a kinetic model that takes account of reversible binding of quinone and quinol to their enzymes. This model could give rise to first-order kinetics of oxidoreduction of ubiquinone even if the ubiquinone concentration were saturating. However, first-order oxidoreduction was not necessarily predicted if the dehydrogenase and cytochrome bc1 complexes were not maintained in the reduced and oxidized states by substrate and cytochrome c respectively. Consider, for example, the reduction of the bc1 complex by quinol:

\[ \text{QH}_2 + b-566^{\text{aq}} + (\text{Fe-S})_6 \rightarrow Q + b-566^{\text{aq}} + (\text{Fe-S})_6 \]

where the subscripts o and r refer to oxidized and reduced forms respectively. Q is ubiquinone and b-566 is cytochrome c-566. The equilibrium constant for this process varies from approx. 2 in chromatophores [7] to 70 in bovine heart mitochondria [8]. The latter high value implies that, at equilibrium, the bc1 complex is maintained highly reduced, even when the ubiquinone pool is quite oxidized. If the equilibrium condition is maintained by slow reoxidation of the complex by a low, limiting concentration of cytochrome c, the rate of overall electron transport will be largely insensitive to changes in ubiquinone redox state, i.e. first-order behaviour will be lost. The extent to which this occurs will depend on the degree of limitation of the rate by cytochrome c and the magnitude of the equilibrium constant.

In the present paper we have used NADH:cytochrome c oxidoreductase reconstituted from bovine heart NADH:ubiquinone oxidoreductase (Complex I) and ubiquinol:cytochrome c oxidoreductase (Complex III) to demonstrate this effect of cytochrome c limitation. We show that the kinetic model of Ragan & Cottingham [4] can quantitatively account for the magnitude of the effect, thereby providing support for the assumptions inherent in the analysis. Lastly we describe the implications of this type of behaviour for the control of electron flux in mitochondrial membranes.

EXPERIMENTAL

Preparations

Complexes I and III were prepared from bovine heart mitochondria by the methods of Hatefi & Rieske [8] and Rieske [9] respectively and stored at \(-70\) °C. A phospholipid fraction was obtained from crude soya-bean lipids as described by Ragan & Racker [10].

Assays

Molar concentrations of Complex I and III solutions were determined from measurement of flavin [11] and cytochrome c (12) respectively. Phospholipid phos...
NADH:cytochrome c reductase was assayed by the method of Bartlett [13]. NADH:cytochrome c reductase was assayed by the decrease at 340 nm in a 1 ml final volume containing 20 μmol of potassium phosphate, pH 8.0, 0.1 μmol of NADH, 0.1 nmol of Complex I protein (previously reconstituted with Complex III) and either 20 nmol or 0.08 nmol of cytochrome c at 30 °C. The lower concentration was used for rate limitation by cytochrome c. Initially, purified bovine heart cytochrome oxidase was added to act as a regenerating system for oxidized cytochrome c. However, at the very low rates of electron flux encountered when using 80 nm-cytochrome c, trace contamination of Complex I by oxidase was found to be sufficient to maintain the rate. When present, myxothiazol was incubated with the enzyme in the cuvette for 2 min at 30 °C before addition of cytochrome c. After the establishment of a steady-state rate, the sample was quenched by rapid mixing with 5 ml of methanol/hexane (3:2, v/v). After centrifugation in a bench centrifuge, the upper hexane layer was removed. The aqueous layer was extracted three times with 1 ml of hexane, and the organic extracts were pooled and evaporated to dryness under vacuum. The residue was dissolved in 150 μl of ethanol, and 100 μl portions were analysed by h.p.l.c. Samples were applied to a Waters Resolve 5μm spherical C18 column as described by Takada et al. [14], except that the solvent used was ethanol/methanol (3:2, v/v). The HClO4 and NaClO4 used by the abovementioned authors for electrochemical detection of quinone caused degradation of the column packing and was not needed for effective separation of Q10 and Q10H2, as shown in Fig. 1. At a flow rate of 1.2 ml/min, retention times for Q10H2 and Q10 were 4.4 min and 8.3 min respectively. Detection was at 280 nm. At this wavelength, the absorption coefficients for Q10H2 and Q10 are 12000 M⁻¹·cm⁻¹ and 3300 M⁻¹·cm⁻¹ respectively [15]. Spectrophotometrically standardized Q10 in ethanol was used to provide a calibration curve that showed a linear relationship between peak area and amount of Q10 up to 800 pmol. Chemically reduced samples of Q10 were used to establish the linearity of the response to Q10H2 and the validity of the abovementioned absorption coefficients.

Hereafter Qr refers to the total concentration of redox-active quinone. Particularly in reconstituted systems supplemented with exogenous quinone, a large proportion of the quinone is not reducible by substrate (cf. [1]). Qr was therefore determined by the amount of quinone reducible by NADH in the absence of cytochrome c. The amount reduced in the steady state by NADH in the presence of cytochrome c is Qr, and Qr is the difference between Qr and Qc.

Reconstitution of NADH:cytochrome c reductase

Complex I (125 μl of a 30.7 μM solution) was mixed with Complex III (218 μl of a 137 μM solution). Because of contamination of the Complex I by Complex III [8], this gave a final molar ratio of complex III to complex I of 0.2. To the mixture, 125 μl of 5 mM soya-bean phospholipid (prepared by ultrasonic dispersion in water) and 5 μl of 2.98 mM-Q10 was added, thereby increasing the endogenous Q10 content by approx. 4 mol per mol of Complex I. Portions containing 0.1 nmol of Complex I were diluted to 1 ml for assay of NADH:cytochrome c reductase.

Rotenone titrations

After dilution of the sample in the assay cuvette, rotenone (from stock solutions in ethanol) was added to give final concentrations ranging from 0 to 5 mol/mmol of Complex I. The volume of ethanolic solution added was less than 10 μl. After 2 min at 30 °C, the assay was started by addition of NADH and cytochrome c.

RESULTS

In the original experiments of Kröger & Klingenberg [1], submitochondrial particles were titrated with rotenone to provide progressive inhibition of NADH oxidase activity. Measurement of the steady-state level of reduction of quinone by NADH showed a progressive oxidation as rotenone was increased, and there was a linear relationship between the rate of NADH oxidation and the proportion of quinone in the reduced state, plotted as Qr/Qc. The advantages of using a reconstituted system were, firstly, that the ratio of Complex III to Complex I could be manipulated so as to give an initial high level of quinone reduction and, secondly, that the cytochrome c concentration could be more readily controlled. In several previous papers [16-19] we have described the properties of the reconstituted NADH:cytochrome c reductase. In the presence of ubiquinone and low concentrations of exogenous phospholipid, the interaction between Complex I and Complex III behaves exactly as expected for first-order kinetics of quinone oxidoreduction, as shown, for example, by the dependence of velocity on the molar ratio of the two Complexes [16]. The reconstituted membranes do not present a permeability barrier to either NADH or cytochrome c, and the kinetic properties of the system are consistent with all molecules of the complexes having access to a single quinone pool. The reconstituted system consists apparently of vesicular membranes (despite the absence of a permeability barrier) in which the protein molecules are randomly distributed and in rapid motion [19].

In the experiment of Fig. 2, reconstitution was
Ubiquinone pool kinetics in NADH:cytochrome c reductase

Fig. 2. Dependence of NADH:cytochrome c reductase on quinone redox state in the presence of excess cytochrome c

Assays were performed as described in the Experimental section on reconstituted Complex I–III pretreated with between 0 and 5 mol of rotenone/mol of Complex I to reduce both v and Qr/Qt. V0′ was determined from the slope to be 0.42 μmol/min per mg of Complex I.

performed with a low molar ratio of Complex III to Complex I to ensure substantial reduction of the quinone pool. Activity was then decreased by addition of various amounts of rotenone, which also caused oxidation of quinone. When measurement of rate was made with an excess of cytochrome c as acceptor, the dependence of velocity on Qr/Qt was linear, in agreement with the results of Kroger & Klingenberg [1] and in accord with expectations derived from previous work with this system [16,17].

The same experiment was then repeated either in the presence of myxothiazol or using much lower concentrations of cytochrome c. In either instance, the maximum rate was reduced by approximately one order of magnitude. The effect on rotenone titration curves is shown in Fig. 3. As anticipated, more rotenone is required to achieve a given degree of inhibition when activity through Complex III is severely curtailed either by myxothiazol or by cytochrome c limitation.

Curve A of Fig. 4 shows the dependence of rate on the redox state of the quinone in the presence of myxothiazol or limitation by cytochrome c. The effect of the former treatment is to decrease the slope of the line without greatly affecting its shape. This was expected, since myxothiazol decreases the maximum velocity of the cytochrome bc1 complex (kcat as defined in the Appendix) without affecting the rate constant for interaction with cytochrome c (ksub as defined in the Appendix). On the other hand, when velocity was reduced by cytochrome c limitation to a similar extent (Fig. 4, B), the dependence of rate on Qr/Qt became distinctly non-linear. The more extreme the limitation the more marked the non-linearity became. Indeed, in the experiment of Fig. 4(C), reduction of the quinone pool could not be detected until the rate

Fig. 3. Rotenone inhibition of NADH:cytochrome c reductase

Activity was measured with 20 μM- (A), with 80 nM- (B) or with 20 μM- (C) cytochrome c in the presence of 0.55 mol of myxothiazol/mol of Complex III. Maximum rates were, respectively, 0.40, 0.058 and 0.072 μmol/min per mg of Complex I protein.

of electron flux was an appreciable proportion of its maximum.

The continuous curved lines of Fig. 4 were obtained by fitting to a theoretical equation derived in the Appendix [eqn. (7)]:

\[ v = \frac{\alpha \cdot V_0' \cdot Q_r/Q_t}{(\alpha + \beta) + (1 - \beta)Q_r/Q_t} \]

In this equation, v is the velocity, \( \alpha \) is a measure of the degree of limitation by substrate (a low value depicting more extreme limitation), \( V_0' \) is the maximum rate of electron flux when \( Q_r = Q_t \) and \( \alpha \) is very high (determined from the slope of the line in experiments such as Fig. 2), and \( \beta \) is the reciprocal of the equilibrium constant for the reaction of quinol with the cytochrome bc1 complex and is taken as 1/70 or 0.0143 in the present work.

Thus with \( V_0' \) determined from a parallel experiment with an excess of cytochrome c, only \( \alpha \) is unknown. The line of Fig. 4(A) is the result of a non-linear least-squares fit giving \( \alpha = 0.109 \pm 0.010, \text{s.e.m.} \). The data could be fitted equally well if \( \beta \) was decreased to zero, but the fit deteriorated at values of \( \beta \) greater than 0.05, i.e. the results are consistent with value of the equilibrium constant in excess of 20, but cannot distinguish between higher values (see the Appendix). The data of Fig. 4(C) were fitted with \( \alpha = 0.058 \pm 0.004, \text{s.e.m.} \), i.e. a more severe degree of limitation. The results in Figs. 2 and 4(A) are much better fitted as straight lines. When \( \alpha \) is high, as in these experiments, the above equation reduces to:

\[ v = V_0' \cdot Q_r/Q_t \]

d.p. \( v \) is linearly related to \( Q_r/Q_t \) (see the Appendix).

DISCUSSION

The observation that rate limitation by cytochrome c makes ubiquinol oxidation no longer a first-order process
is not particularly surprising, although the dependence of the effect on the equilibrium constant for electron transfer between quinol and enzyme is less obvious. Nevertheless, in previous studies of the function of quinones, the possibility that limitation of rate by the availability of the dehydrogenase substrate or oxidized cytochrome c could cause a deviation from the simple kinetics of Kroger & Klingenberg [1] has been completely ignored. Certainly, in experiments with intact mitochondria, limitation of flux by the rate of substrate permeability is a very strong possibility. Even if this occurs, however, the extent of deviation from simple first-order behaviour will depend on the equilibrium constant for oxidation of quinol by its oxidase enzyme, and this is not necessarily as large as the value found for bovine heart mitochondria.

Another reason why the effect of substrate limitation has not been previously reported is that measurements of quinone redox state as a function of electron flux are rarely undertaken, and most workers rely on the shape of inhibitor titrations (see, e.g., [20]) or on the extent of competition between substrates (see, e.g., [21,22]) to assess the workings of the quinone pool. The problem with these approaches is that first-order quinone oxidation is invariably assumed, and minor deviations from expected behaviour may then be interpreted as indicating heterogeneity of the quinone pool. For example, consider a branched respiratory chain containing several types of quinone reductases linked to a single quinol oxidase. Suppose that one of these quinone reductases has a terminal redox group of relatively low
midpoint potential so that reduction of ubiquinone is essentially irreversible. Fig. 4 shows that, when the flux through the enzyme is controlled by its substrate, electron transfer becomes largely insensitive to ubiquinone redox state. Thus oxidation of the substrate would not be affected by simultaneous oxidation of other substrates, the competition between branches of the system would not be apparent, and it would be concluded that there are multiple ubiquinone pools present in the membrane. This example illustrates that marked deviations from expected behaviour can arise without any change in the underlying mechanism or in the existence of a homogeneous ubiquinone pool.

Lastly, the ubiquinone pool model of Ragan & Cottingham [4] was designed to provide a mechanism in which first-order oxidoreduction of quinone would be anticipated over the wide range of conditions employed experimentally. The fact that the same model can quantitatively account for deviations from first-order oxidoreduction provides strong support for the basic correctness of the model and the assumptions made in the kinetic analysis.

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REFERENCES


APPENDIX

Theoretical analysis

For reduction of the cytochrome bc complex (bc) by quinol, we consider the following steps:

\[ bc_o + Q_r \rightleftharpoons bc_r + Q_o \]
\[ bc_o + Q_r \rightleftharpoons bc_r + Q_o \]
\[ bc_o + Q_r \rightleftharpoons bc_r + Q_o \]
\[ bc_r + Q_o \rightleftharpoons bc_r + Q_o \]
\[ bc_r + Q_o \rightleftharpoons bc_r + Q_o \]

where \( bc_o \) and \( bc_r \) are the oxidized and reduced forms of the enzyme, \( Q_o \) and \( Q_r \) the oxidized and reduced forms of quinone, \( k_{+1} \) and \( k_{-1} \) are rate constants governing the reversible formation of enzyme-quinone complexes and assumed to apply to all four binding reactions, \( k_{+2} \) and \( k_{-2} \) are rate constants governing the reversible electron transfer from \( Q_o \) to the enzyme, and \( k_{-sub} \) is a pseudo-first-order rate constant covering binding of cytochrome c and its reduction by the reduced enzyme. The most obvious simplification inherent in this scheme is the consideration of events occurring only at the quinol oxidase site. However, it seems that the quinone reductase site of the bc complex is always effectively saturated with \( Q_o \) even at low \( Q_o \) concentrations, and therefore electron flux is modulated by changes in the pool redox state only through the quinol oxidase site [1].

If we assume that \( Q_o \) and \( Q_r \) are greater than the concentration of bc complex and \( k_{-2} \) is greater than \( k_{+2} \), we can derive the following equation for the dependence of velocity \( (v) \) on \( Q_o \) and \( Q_r \):

\[
v = \frac{k_2 k_{-sub} [bc] Q_r}{k_{sub} (K_s + Q_o) + k_{+2} Q_o + k_{+2} Q_r}
\]

(1)

where \( K_s = k_{-1}/k_{+1} \) and \( Q_o = Q_o + Q_r \). To simplify further analysis, we assume that quinone is saturating \( (K_s \ll Q_o) \) and obtain:

\[
v = \frac{\alpha k_2 [bc] Q_o}{(\alpha + \beta) Q_o + (1 - \beta) Q_r}
\]

(2)

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when $\alpha = k_{\text{sub}}/k_{+2}$ and $\beta = k_{-2}/k_{+2}$. The maximum
velocity, $V_0$, is obtained when all quinone is reduced ($Q_r = Q_t$), giving:

$$V_0 = \frac{\alpha k_{+2}[bc]}{(\alpha + 1)} \quad (3)$$

Combining eqns. (2) and (3), we obtain:

$$v = \frac{(\alpha + 1)V_0 \cdot Q_r}{(\alpha + \beta)Q_t + (1 - \beta)Q_r} \quad (4)$$

The corresponding expression expected for simple first-order dependence of $v$ on $Q_r$ is:

$$v = \frac{V_0 \cdot Q_r}{Q_t} \quad (5)$$

as originally derived by Kröger & Klingenberg [2]. Eqn. (4) collapses to eqn. (5) if $\beta = 1$ or if $\alpha$ is very large. The first of these conditions requires that $k_{-2} = k_{+2}$, which appears not to be the case for bovine heart mitochondria, where $\beta$ is 1/70 or 0.0143 [3]. The second condition requires that electron transfer should not be limited by cytochrome $c$, a state which is commonly established in work on quinone pools.

The behaviour of eqn. (4) is illustrated in Fig. 5 of the main paper, where deviation from a linear dependence of $v/V_0$ on $Q_r/Q_t$ becomes more marked as $\beta$ is increased or decreased from 1 (Fig. 5a, main paper) or if $\alpha$ is decreased (Fig. 2, main paper). Note that even if $\beta$ is zero, i.e. electron transfer is irreversible, values of $\alpha$ in excess of one produce an almost linear dependence of $v/V_0$ on $Q_r/Q_t$.

As it stands, eqn. (4) contains two unknown quantities, $\alpha$ and $V_0$, the velocity when $Q_r = Q_t$. However, these two are not independent variables, since when $\alpha$ is very large, $V_0$ becomes constant [$k_{+2}[bc]$ from eqn. (3)] and can be determined from the slope of the linear dependence of $v$ on $Q_r/Q_t$ in the presence of excess cytochrome $c$ (e.g. as in Fig. 2 of the main paper). If we denote this quantity as $V_0'$, eqn. (3) gives:

$$V_0 = \frac{\alpha \cdot V_0'}{1 + \alpha} \quad (6)$$

and substituting eqn. (6) into eqn. (4) we obtain:

$$v = \frac{\alpha \cdot V_0' \cdot Q_r/Q_t}{(\alpha + \beta)Q_t + (1 - \beta)Q_r} \quad (7)$$

If we take $\beta = 0.0143$, then there is only one unknown parameter, $\alpha$, to be fitted. Alternatively, the fit can be made with various values of $\beta$ to test the robustness of the dependence of the relationship between $v$ and $Q_r/Q_t$ on $\beta$.

REFERENCES

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