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Hypothesis

A MECHANISM FOR THE REDUCTION OF CYTOCHROMES BY QUINOLS IN SOLUTION AND ITS RELEVANCE TO BIOLOGICAL ELECTRON TRANSFER REACTIONS

Peter R. RICH and Derek S. BENDALL
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

Received 8 June 1979
Revised version received 2 July 1979

1. Introduction

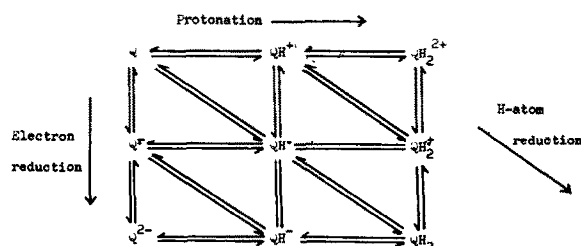
The mechanism by which reducing power is transferred from the biological quinols, ubiquinol and plastoquinol, to their respective acceptors, presumably cytochrome c_1 in mitochondria and cytochrome *f* of chloroplasts, is obscure. The difficulty arises from the fact that, at around neutral pH, the quinols are primarily two-equivalent hydrogen atom donors, whereas their acceptors are primarily one-equivalent electron acceptors. This immediately raises the question of the properties and possible fates of the intermediate semiquinone species which are inevitably involved. Furthermore, the feasibility of a significant reaction rate may be questioned when one considers the quinol/semiquinone couple which is operative in the first step of the reaction sequence – it has already been noted that such a couple would have an extremely high midpoint potential if the semiquinone species is highly unstable (see Clark [1] for a mathematical treatment of this).

These problems have become particularly relevant recently with the notion of a central role of quinone in the protonmotive function of site II of the mitochondrial respiratory chain and of the region of the chloroplast chain between the two photosystems [2]. A useful development has been the concept of bound quinone species which have altered properties, in particular such that the semiquinone species is stabilised and hence lowering the midpoint potential of the quinol/semiquinone couple [2] so that a reasonable reaction rate is possible. However, whereas support for such a system has been gained for the site of electron donation into the quinone pool in both mitochondria and chloroplasts [3–8], no such support has been found for the operation of a such a system at the quinol oxidation site.

The purpose of this paper is to suggest that the reaction between a quinol and an acceptor molecule such as a cytochrome may be treated as a bimolecular collision process. Thermodynamic analysis of the possible redox couples involved in the rate-limiting step of the reactions suggests a mechanism for the reactions which does not specifically require bound quinone or semiquinone intermediates. In addition the type of mechanism envisaged makes experimentally-testable predictions about the rates of reaction of such systems.

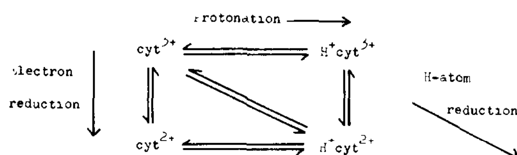
2. Nomenclature and abbreviations

When one considers the thermodynamics of the rate-limiting fundamental reaction, it is essential to consider not only the redox states but also the protonation states of the species involved. The nine major states of a quinone system may be written as follows:



This nomenclature will be followed for the states of the quinones discussed: BQ, benzoquinone; MQ, 2-methyl-1,4-naphthoquinone (menadione); UQ, ubiquinone; PQ, plastoquinone.

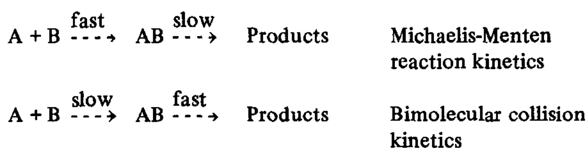
Cytochromes *c* and *f* have a p*K* on the oxidised carrier around 8.5 [9], attributed at least in cytochrome *c* to a lysine residue close to the heme [10]. The following nomenclature of major forms will therefore be used:



E_m denotes midpoint potential of the couple in question relative to a standard hydrogen electrode.

3. Hypothesis

It will be demonstrated that, in our model system, quinol to cytochrome electron transfer may be considered to be a simple bimolecular collision reaction between appropriately protonated species. This is to say, formation of the bimolecular complex is the rate-limiting event and once an appropriate collision has occurred then the electron transfer process is rapid. In this way the reaction may be distinguished from one exhibiting classical Michaelis-Menten kinetics where substrate saturation may be acheived and the rate-limiting step becomes some event after the formation of the complex. Thus:



AB represents an activated bimolecular complex

In our particular case of electron transfer from fully-reduced quinol to cytochrome, one must consider the couples ($QH_2/QH_2^{\cdot+}$), ($QH^-/QH^{\cdot}$) or ($Q^{2-}/Q^{\cdot-}$) reducing either (cyt^{3+}/cyt^{2+}) or ($H^+ cyt^{3+}/H^+ cyt^{2+}$), since these are the possible couples which may be involved in the rate-limiting step. Alternatively, if one is to consider the process as one involving H-atom transfer then the couples ($QH^-/Q^{\cdot-}$) or ($QH_2/QH^{\cdot}$) reducing the couple ($cyt^{3+}/H^+ cyt^{2+}$) should be invoked.

The forward velocity of the reaction will be given by:

Rate = k [donor] [acceptor]

If the products of this reaction are rapidly removed, such that the back reaction becomes negligible, then this forward rate will control the overall maximal rate of the reaction. Hence a consideration of the factors which will influence such a rate is of relevence.

It has already been observed and theoretically predicted that the rate constant, k, of an outer sphere electron transfer reaction is related to the overall difference in midpoint potentials of the fundamental donor and acceptor couples involved [11–13]. In general, for a like series of donors donating to a given acceptor, the rate constant will increase as the E_m of the donor couple decreases. This is not necessarily a strict relation since other factors such as molecular shape and environment may also alter the rate constant and distort this relation.

Since the protonation states of donor and acceptor are of key importance, it may also be predicted that the observed rates of such reactions are pH-dependent. The effective concentrations of relevant species involved at any given pH will be determined by the p*K* values of the ionisable groups involved. Hence, the profile of reaction rate versus pH for any given reaction will be easily predictable and will vary considerably for many of the reactions which may be considered as possible routes of electron (or H-atom) transfer. Such variation allows experimental testing of the hypothesis.

4. An experimental example

By way of an illustration of the application of such an hypothesis to a quinol to cytochrome reaction, we have chosen to study the kinetics of reduction of horse heart cytochrome *c* by 2-methyl-1,4-naphtoquinol (menadiol). The experiments were performed anaerobically under an atmosphere of nitrogen in a

buffer containing 100 mM 2-(*N*-morpholino) ethanesulfonate and 2 mM EDTA at 25°C. A pH range of 4–6 was found to give workable reaction rates. An initial cytochrome *c* concentration of ~7.5 μM was used. Menadiol was prepared from the quinone as in [14] and a stock solution was made up in acidic ethanol. This stock solution was stable for several days at –20°C. The reaction rate was followed by monitoring $\Delta A_{550-575}$ and using an extinction coefficient, $E_{\text{mM}^{-1}\text{ cm}^{-1}}$, of 19 [15].

Figure 1 illustrates the dependence of the reaction rate, d[cyt c^{3+}]/dt, on the concentration of menadiol. A first-order dependency was found up to the limits of concentration experimentally testable. No stimulation of the rate was found on addition of menadione, and neither was the rate autocatalytic, indicating that the reductant was indeed a form of the quinol and not a semiquinone species. In fig.2, the dependence of reaction on cytochrome c^{3+} concentration is illustrated. A linear plot of $-\ln(A_\infty - A_t)$ demonstrates the first-order dependence. This is in contrast to the reduction of cytochrome *c* by catechol [21], where the reaction is second-order with respect to cytochrome c^{3+} and hence a plot of $(A_\infty - A_t)^{-1}$ is linear. This is clearly not the case in our system (fig.2).

Figure 3 illustrates the pH dependency of the reaction at fixed quinol and cytochrome concentrations.

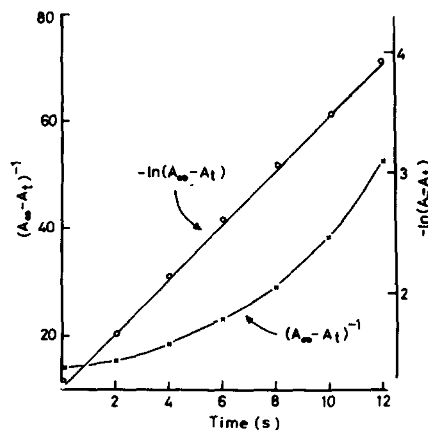


Fig.2. Dependence of cytochrome *c* reduction rate on ferric cytochrome concentration. Menadiol, 400 μM, was added to an anaerobic solution of 7.5 μM ferric cytochrome *c*. The reaction rate was measured as a tangent to the curve at appropriate ferric cytochrome concentrations. The linear plot of $-\ln(A_\infty - A_t)$ demonstrates first-order decay (c.f., linear plot of $(A_\infty - A_t)^{-1}$ for the second-order decay of ferric cytochrome in [21]). Buffer, as in fig.1; pH 5.99; temp. 25°C; A_∞, maximal absorbance change; A_t, absorbance change at time *t*.

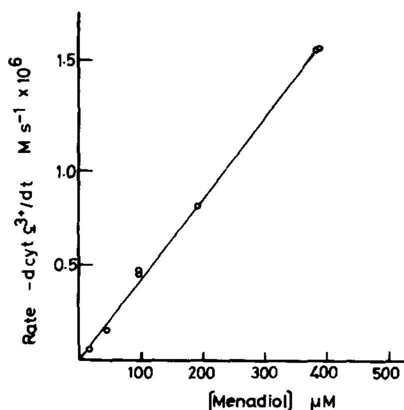


Fig.1. Dependence of the rate of cytochrome *c* reduction on menadiol concentration. The experiment was performed anaerobically as described in the text. In each case, the reaction rate was measured as a tangent to the curve at a fixed ferric cytochrome concentration. Buffer: 100 mM 2-(*N*-morpholino)ethanesulfonate plus 2 mM EDTA; pH 5.99; temp. 25°C.

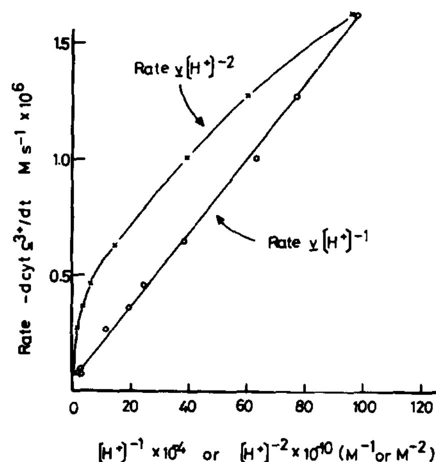
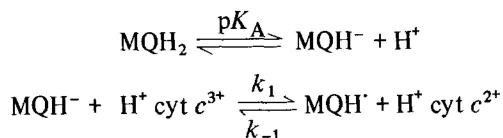


Fig.3. pH dependency of menadiol reduction of cytochrome *c*. The reaction was carried out as in fig.2, except that the pH of the reaction mixture was varied between 4 and 6. Rates were measured as a tangent to the curve at 5 μM cytochrome *c* remaining. Buffer, as in fig.1; temp. 25°C; initial menadiol 400 μM.

An excellent experimental fit of the data with rate proportional to $[H^+]^{-1}$ was obtained over the range of pH values tested (pH 4–6).

The conclusion from these experiments is that the reductant is the anionic form of the quinol, MQH^-, and that this species reduces the dominant H^+ cyt^{3+} species of the cytochrome by an electron transfer:



$$MQH_2 \underset{}{\overset{pK_A}{\rightleftharpoons}} MQH^- + H^+$$

$$MQH^- + H^+ \text{ cyt } c^{3+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MQH^{\cdot} + H^+ \text{ cyt } c^{2+}$$

If we assume that removal of the $MQH^{\cdot}$ species is rapid (routes include deprotonation, dismutation and reduction of a second cytochrome *c*), then:

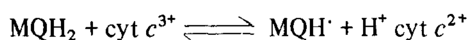
the observed rate = forward rate of reaction

$$= k_1 [MQH^-] [H^+ \text{ cyt } c^{3+}]$$

and k_1 may be calculated (although the value obtained may actually be $2 \times k_1$ if the semiquinone also reduces a cytochrome molecule).

We measured a k_1 of $1.1 \pm 0.3 \times 10^6$ M^{-1} s^{-1} at 25°C for the electron-transfer reaction, assuming a pK_A (MQH_2/MQH^-) of around 9.45 (this figure was surmised by analogy with the pK_A of 9.45 reported for 1,4-naphthoquinol [16]. It is likely that the methyl group of menadiol will cause the pK_A of the molecule to rise only slightly above this value).

As noted [17,18], a simple treatment such as this cannot exclude the possibility of a H-atom transfer mechanism from the uncharged quinol to the deprotonated cytochrome as the dominant reaction:



$$MQH_2 + \text{cyt } c^{3+} \rightleftharpoons MQH^{\cdot} + H^+ \text{ cyt } c^{2+}$$

although arguments favouring the electron transfer process have been invoked [17–19].

5. Thermodynamic considerations

The preceeding experiment is a demonstration of a model reaction for electron transfer from a fully reduced quinol to a cytochrome via a bimolecular collision process. The mechanism is in contrast to that described for the reduction of cytochrome *c* by hydroquinone [20] or by catechol [21]. The dominant reductant of the hydroquinone system is the deprotonated semiquinone formed by dismutation with the quinone [20] whereas catechol reduction requires the formation of a trimolecular complex with two cytochrome *c* molecules [21]. The mechanism of menadiol reduction of cytochrome *c* is clearly more analogous to the biological events which also occur via the quinol as the initial reductant and so the thermodynamics of such systems will be considered.

If one considers the reaction as an electron transfer, then the redox couples involved in the rate-limiting step of this reaction are $QH^-/QH^{\cdot}$ and H^+ cyt c^{3+}/H^+ cyt c^{2+}. In order to calculate the midpoint potential of the theoretical $QH^-/QH^{\cdot}$ couple for menadione, a thermodynamic profile of the menadione system has been constructed from the following information (fig.4): E_0 (MQH_2/MQ) = +415 mV (an average value taken from the figures given by Clark [1]);

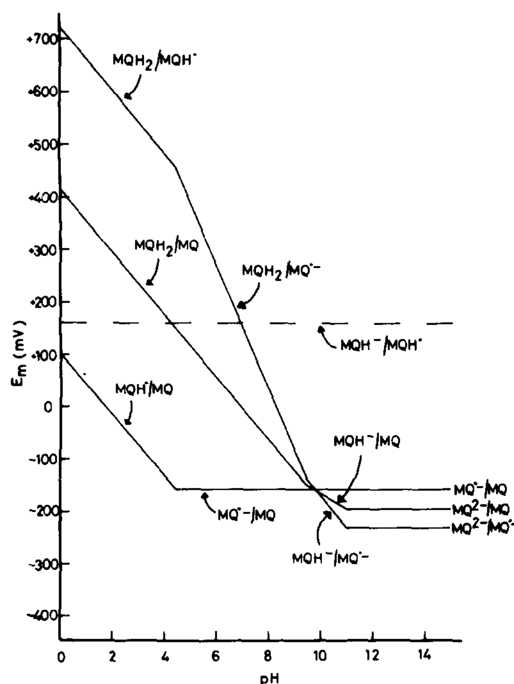


Fig.4. A thermodynamic profile of the dominant redox couples of the menadione system. The figure was constructed from data given in the text.

pK_A (MQH_2/MQH^-) and pK_B (MQH^-/MQ^{2-}) = 9.45 and 11.0 (by analogy with the figures given for 1,4-naphthoquinol [16] – these will be only a slight underestimate of the values for the menadiol system); $pK(MQH^{\cdot}/MQ^{\cdot-})$ = 4.4 [22]; K_s ($2\ MQH^{\cdot} \rightleftharpoons MQH_2 + MQ$) = 1.36 × 10^5 at pH 7.0 (an average calculated from the data of Ohnishi et al. [23] using $K_s = k_f/k_d$). The pH-independent midpoint potential of MQH^-/$MQH^{\cdot}$ is then given by:

$$E_m(MQH^-/MQH^{\cdot}) = E_o(MQH_2/MQH^{\cdot}) - \frac{2.303RT}{nF} pK_A(MQH_2/MQH^-)$$

For the menadione system, we obtain a value of +160 mV for E_m ($MQH^-/MQH^{\cdot}$). The reaction is therefore thermodynamically favourable (E_m (H^+ cyt c^{3+}/H^+ cyt c^{2+}) in solution = +254 mV [24]) and the electron transfer is able to proceed at a reasonable rate.

The same considerations have been applied to the benzoquinone system. Figures used were E_o (BQH_2/BQ) = +700 mV [1]; pK_A (BQH_2/BQH^-) and pK_B (BQH^-/BQ^{2-}) = 9.85 and 11.4 [25]; K_s (high pH) = 4.2 [25]; pK ($BQH^{\cdot}/BQ^{\cdot-}$) = 4.1 [22]. From these figures we obtain a value of +482 mV for E_m ($BQH^-/BQH^{\cdot}$). Thus, the reaction:

$$BQH^- + H^+ \text{ cyt } c^{3+} \rightleftharpoons BQH^{\cdot} + H^+ \text{ cyt } c^{2+}$$

is thermodynamically unfavourable and must be driven by the rapid removal of the product, $BQH^{\cdot}$. The value of k_1 is therefore expected to be lower than that for the menadiol system (c.f., Marcus theory [11,12]) and furthermore back reactions will rapidly become significant, making the observed rate of reaction by this mechanism rather low. Instead, the dominant reaction for the reduction of cytochrome c is via the more reducing $BQ^{\cdot-}/BQ$ couple [20] (from the figures given above for the benzoquinone system one may calculate the pH-independent E_m ($BQ^{\cdot-}/BQ$) to be at +81 mV). Such a reaction is dependent on the presence of quinone and so is an autocatalytic process and in practice is probably limited by the rate of dismutation to the semiquinone reductant [26].

An alternative approach thermodynamically would be to consider a H-atom transfer process to the deprotonated ferric cytochrome, and similar calculations may be performed to calculate the midpoint potentials of the appropriate couples which might be involved. The overall outcome of these considerations would lead to the same general result and so at present we are unable to distinguish such a H-atom transfer from an electron transfer mechanism.

6. Relevence to biological systems

It has already been recognised that the one-equivalent reduction of an acceptor by ubiquinol or plastoquinol in biological systems presents a problem since the potential of the $UQH_2/UQH^{\cdot}$ or of the $PQH_2/PQH^{\cdot}$ couple is high compared to the acceptor potential [2] and mechanisms involving quinone binding sites have been invoked as a possible solution. However, what we wish to emphasise in this communication is that when one considers the potentials of the actual couples involved in the rate-limiting step of the reaction, then feasible mechanisms of electron transfer may be envisaged simply as bimolecular collision processes. This may be true even if the rate-limiting step is thermodynamically unfavourable, provided that the product(s) is rapidly removed. To some extent we may estimate the feasibility of such a reaction by comparison of the dependence of the forward rate constant, k_1, on the midpoint potential difference of the fundamental couples involved in the rate-limiting step, for a series of benzoquinones of varying thermodynamic properties to a fixed acceptor. These values of k_1 may then be used to estimate k_1 values for the biological systems which may then be compared with the observed kinetics and thermodynamics. Such considerations will be reported separately. It may be noted in this context that the maximal rate of such a process in biological systems may be increased by a 'directive' influence of protein side groups or of the hydrophobicity of the membrane such that k_1 is increased above its value in simple systems. Such a mechanism might account for the catalytic influence of isolated complexes, e.g., the bc_1 [27] and b_6f [28] complexes, on quinol oxidations.

Acknowledgements

We are indebted to the Science Research Council for financial support and would like to thank Dr P. M. Wood, University of Bristol, for his useful comments.

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