THE RECOGNITION AND REDOX PROPERTIES OF A COMPONENT, POSSIBLY A QUINONE, WHICH DETERMINES ELECTRON TRANSFER RATE IN UBIQUINONE-CYTOCHROME *c* OXIDOREDUCTASE OF MITOCHONDRIA

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1. Introduction

An important aspect of chemiosmotic views of photosynthetic and respiratory electron transport is the sequence of events that leads to the translocation of protons across the biological membrane. There seems to be little doubt that protons can move across the membrane coupled to electron transfer, but satisfactory recognition of the reactions, components, and structures that are directly involved has yet to be made.

Ubiquinone-cytochrome *c* (Q-*c*) oxidoreductase, comprises the second of the three major energy transducing sites in the mitochondrial inner membrane, and can be isolated as a multi-peptide complex (Complex III) from the membrane (see refs. 1–3 for review). Q-*c* oxidoreductase is considered to contain two cytochromes *b*, a cytochrome c_1 and a *g* 1.9 iron sulfur center (designated Rieska FeS or RFeS) as redox components. The active oxidoreductase also contains ubiquinone, but roles and characteristics of the quinone have not been clear in spite of the possible importance as a hydrogen carrier across the membrane as suggested by Mitchell [4,5].

In order to study the individual redox reactions of the mitochondrial Q-*c* oxidoreductase we constructed an in vitro system that permits a single turnover, light-activated electron transfer through the Q-*c* oxidoreductase [6]. This system, in detergent solution, consists of the mitochondrial Q-*c* oxidoreductase, mitochondrial cytochrome *c*, and the photochemical reaction center of *Rhodopseudomonas sphaeroides*. These three proteins form a cyclic electron transfer system which, under a variety of experimental conditions, displays parallel light-induced kinetic behavior to that found in vivo with the photosynthetic bacterial membranes of *Rps. sphaeroides*.

Light absorption by the photochemical reaction center results in a charge separation involving the transfer of an electron from bacteriochlorophyll dimer to bacteriopheophytin within the reaction center protein [7]. This charge separation is stabilized by two secondary electron transfer reactions: the oxidation of a cytochrome *c* and reduction of ubiquinone. The Q-*c* oxidoreductase catalyzes the transfer of the electron from the reduced ubiquinone to the oxidized cytochrome *c*. Thus far with the constructed system, flash activated electron transfer through the Q-*c* oxidoreductase has been shown to include cytochrome b_{562} [6].

Kinetic analysis of the flash-induced electron transfer in the native photosynthetic membranes of *Rps. sphaeroides* has revealed the role of an obligatory intermediate in electron transfer between cytochromes b_{560} (analogous to mitochondrial b_{562}) and c_2 [8–12]. This was deduced from experiments which showed that the rates of ferrocytochrome *b* oxidation and ferricytochrome c_2 reduction were obligatorily linked to the redox state of a component with an E_{m7} of 155 mV ($n = 2$). From quinone depletion and reconstitution experiments [10] this intermediate was determined to be a single ubiquinone associated with the Q-c_2 oxidoreductase, and thermodynamically distinct from the quinone pool; it was designated Q_z.

In this paper we report experiments that demonstrate that a two-electron ($n = 2$) and two-proton component, with an E_{m7} of 115 mV, similarly determines the electron transfer rate between the *b* and *c* cytochromes in the mitochondrial Q-*c* oxidoreductase. Although such a component is key in chemiosmotic models of respiration its existence has never been demonstrated both in functional and quantitative terms.

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2. Materials and methods

The Q-*c* oxidoreductase was isolated from beef heart mitochondria according to the method of Rieske [13]. Reaction center protein from *Rps. sphaeroides* was isolated and assayed as previously described [16]. Horse heart cytochrome *c* (Type III) was purchased from Sigma Chemical Co., St Louis, MO.

Flash-induced absorbance changes were measured in a Johnson Foundation dual wavelength spectrophotometer using a near-saturating 6 μs xenon flash lamp for actinic illumination. The flash-induced oxidation-reduction of cytochrome b_{562} and *c*-type cytochrome were measured spectrophotometrically as absorbance changes at 562 nm and 550 nm, respectively, using 540 nm as a reference wavelength. In the figures, a downward movement of the trace represents cytochrome oxidation. No attempt has been made to resolve the spectroscopic contributions of cytochrome c_1 and cytochrome *c*. Under the conditions used in the experiments presented here, contributions due to cytochrome b_{566} oxidation-reduction are not significant. Redox potentiometry was carried out as previously described [14].

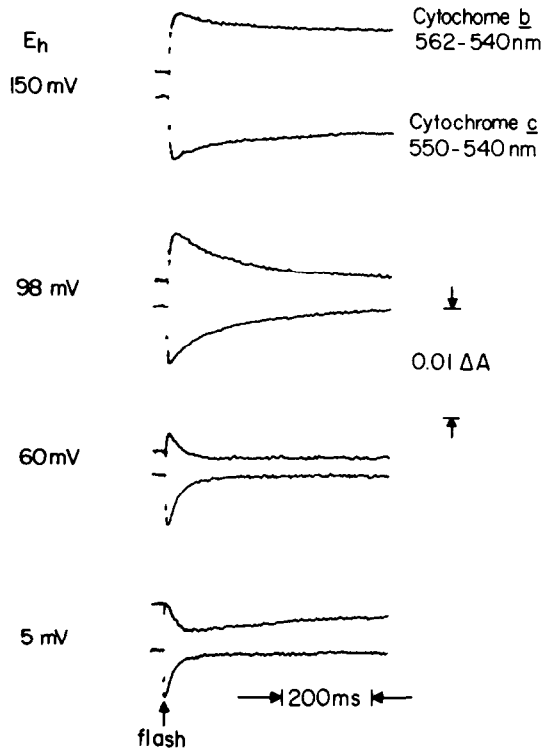

Fig.1. Flash-induced oxidation-reduction kinetics of cytochrome *b* and *c* in a mixture of mitochondrial Q-*c* oxidoreductase, cytochrome *c* and bacterial photochemical reaction center at various ambient redox potentials. 4 μM (in c_1) Q-*c* oxidoreductase, 6 μM cytochrome *c* and 0.6 μM RC were mixed in 10 mM Tris–HCL, pH 7.8. Redox mediators used were 0.1 mM Fe–EDTA (3.3 mM), 10 μM DAD and 10 μM pyocyanine.

3. Results

3.1. *Electron transfer kinetics between cytochromes* b *and* c

Fig.1 shows typical flash-activated oxidation-reduction kinetics of mitochondrial cytochrome c_1/c and cytochrome b_{562}. The kinetics were taken at pH 7.8 with the system poised at various ambient redox potentials (E_h) established before flash activation. At the higher E_h values (see trace taken at 150 mV) the flash-activated reaction center elicits ferrocytochrome c_1/c oxidation ($t_{1/2} < 1$ ms) and ferricytochrome b_{562} reduction ($t_{1/2} \simeq 5$ ms). This is followed by only a slow re-reduction of ferricytochrome c_1/c and a re-oxidation of ferrocytochrome *b* ($t_{1/2} > 500$ ms). However, as the E_h of the Q-*c* oxidoreductase is lowered (from 150 to 60 mV), the re-oxidation of flash generated ferrocytochrome *b* becomes faster, and there is matching behavior in the re-reduction of ferricytochrome c_1/c. At low E_h values where cytochrome b_{562} is reduced before activation (trace taken at 5 mV; cytochrome b_{562} ($E_{m7.8}$ 50 mV) 85% reduced), we observe net flash-induced oxidation of the ferrocytochrome *b*; again the oxidation halftime ($t_{1/2} \simeq 20$ ms) matches that of ferricytochrome c_1/c reduction.

Fig.2A compares the kinetics in more detail obtained with varying E_h. The E_h was varied using ferri/ferro-EDTA (E_{m7} 117 mV) as the principle redox buffer and mediator. In the latter role on its own the ferro/ferri-EDTA was slow in its reaction with the redox components of the Q-*c* oxidoreductase, taking many minutes before reasonably reproducible flash-induced kinetic behavior was obtained at any given E_h values; data typical for cytochromes *b* (△) and c_1/c (○) are shown in fig.2A. However, it was common to expedite the approach of redox equilibrium between electrode and protein bound redox components by adding 10 μM pyocyanine (E_{m7} −30 mV) and 2,3,5,6-tetramethylphenylenediamine (E_{m7} 240 mV). This yielded similar, but more reliable data (▲,●); obtained more

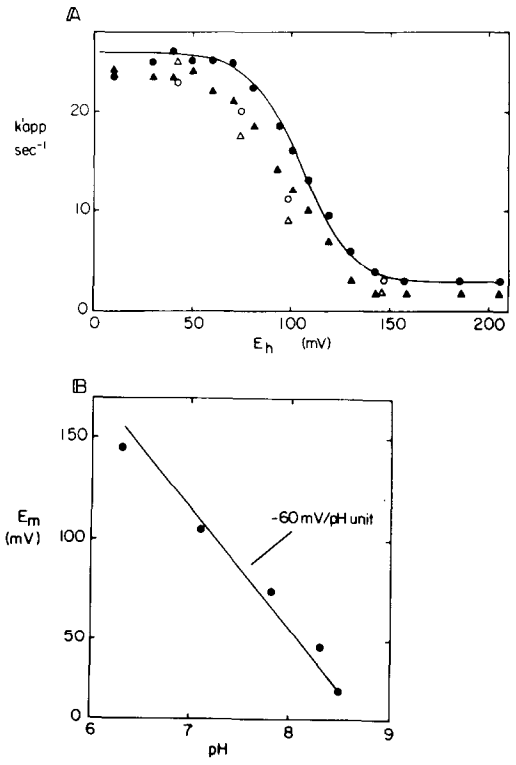

Fig.2. Redox potential dependence of electron transfer through Q-*c* oxidoreductase (A) and pH dependence of the E_m (B). A: The ratio of initial rate and extent of ferrocytochrome *b* oxidation (△,▲) and ferricytochrome *c* reduction (○,●) are plotted as $k'_{apparent}$ from similar kinetic traces to fig.1. The buffer used was 10 mM MOPS–NaOH, pH 7.1. Other conditions were the same as in fig.1 except that DAD and pyocyanine were excluded in the open symbols (△,○). The curve drawn is a Nernst curve for E_m of 105 mV with *n* value of 2. B: E_m values obtained as in fig.2A were plotted against various pH values. The buffer used was 10 mM Tris–HCl (pH > 7.5) or 10 mM MOPS–NaOH (pH < 7.5).

rapidly but without significant interference from the dyes to the flash-activated kinetics. The E_h dependency of the rates of ferrycytochrome *b* oxidation and ferricytochrome c_1/c reduction were similar. The small difference that is observed may be caused by a systematic error in measurement, due to the fact that for most of the E_h range studied the time period for flash induced cytochrome *b* reduction is overlapping with that of its re-oxidation (see for example the trace at E_h 60 mV in fig.1). Thus the cytochrome c_1/c data (●) are considered most reliable. In fig.2A, these described an $n = 2$ Nernst curve with an $E_{m7.1}$ value at 105 mV. Repeating experiments at different pH values revealed the E_m value to have a –60 mV/pH unit dependency between pH 6.3 and 8.5 as shown in fig.2B. The E_{m7} value is 115 ± 10 mV.

3.2. *Action of antimycin and 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT)*

Fig.3 shows kinetics of cytochrome c_1/c oxidation reduction under low E_h conditions, optimal for electron transfer from cytochrome *b* to c_1/c (no additions, top trace). Antimycin, the inhibitor of electron transfer between cytochromes *b* and *c* dramatically slows ferrocytochrome *b* oxidation (not shown; see ref. 6) and similarly affects the course of a part of ferricytochrome c_1/c reduction fig.3, center trace). (The partial re-reduction of cytochrome *c* may be related to cytochrome *b* reduction, and is discussed elsewhere [15].) UHDBT has been shown to inhibit electron transfer between Rieske FeS cluster and cytochrome c_1/c [16, 17]. Consistent with this, fig.3 (lower trace) shows that when UHDBT is added after antimycin all rapid reduction of flash oxidized cytochrome c_1/c is severely blocked. The same result (not shown) is obtained without antimycin.

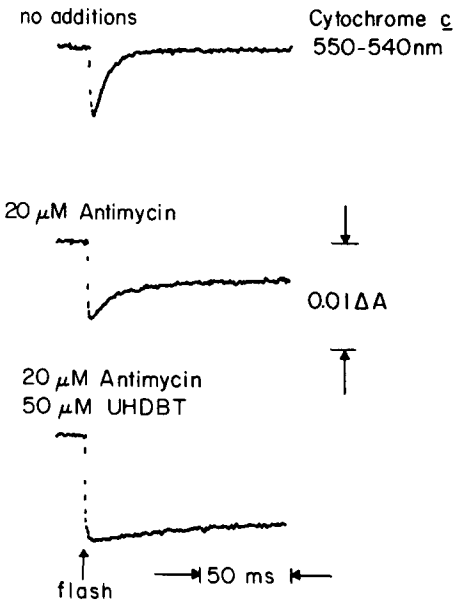

Fig.3. Inhibition of cytochrome *c* re-reduction after flash-induced oxidation by antimycin and UHDBT. E_h was adjusted to 40 mV at pH 8.0. Other conditions are as in fig.1.

4. Discussion

4.1. *Single electron transfer from cytochrome* b_{562} *to Rieske FeS cluster via a quinone component,* Q_z

This report describes the recognition of a redox component that is obligatory for the primary function of the mitochondrial Q-*c* oxidoreductase. The difference in rate of electron transfer between cytochromes b_{562} and c_1/c observed when the component is oxidized or reduced has enabled us to obtain information about its equilibrium redox properties. We have, as yet, no direct evidence for the chemical identity of the component. However, a similar component has been described in vivo with the photosynthetic bacterial Q-c_2 oxidoreductase [8–12], and depletion/reconstitution experiments [10] have identified it to be ubiquinone [Q_z]. The equilibrium redox properties of this Q_z corresponds to eqn 1 over the pH range 5–11 (E_{m7} 155 mV). By analogy we tentatively identify:

$$Q_z + 2e^- + 2H^+ = Q_zH_2 \qquad (1)$$

the mitochondrial component as a Q_z-like quinone; its equilibrium properties also corresponds to eqn 1 over the pH range 6.3–8.5 with an E_{m7} 115 ± 10 mV.

The functional position of Q_z *between* cytochrome b_{562} and c_1/c is supported by the action of antimycin. Evidence that it interacts on a redox basis with the cytochromes c_1/c only indirectly via the Rieske FeS cluster (RFeS) is supported by the action of UHDBT (see [16,17]). Thus it is reasonable to consider that the Q_zH_2/Q_z couple represents a rate-controlling step for the passage of electrons from cytochrome b_{562} to what is probably the Rieske FeS cluster, which is in rapid equilibrium with the roughly isopotential cytochromes c_1 and c.

4.2. *The determination of the electron transfer rate by the redox state of* Q_z

The passage of electrons is slow when at the time of activation Q_z is present (i.e., oxidized) and is stimulated 10–100 fold (pH dependent; unpublished results) when Q_zH_2 is present. We can currently only speculate on the factors that may contribute to the control of electron transfer. However, since cytochrome *b* and RFeS are single-electron donors and acceptors, it is natural to consider that one source of the control could reside in the single electron redox reactions of Q_zH_2/Q_z that involve the semiquinone. When Q_zH_2 is present, an electron may go rapidly through the quinone component using the $Q_zH_2/Q_z\cdot^-$ (or $Q_z \cdot H$) couple [15,18]. However, when Q_z is present electron transfer through the quinone using the $Q_z\cdot^-$ ($Q_z \cdot H$)/Q_z couple may for simple thermodynamic reasons be less probable, and this may be the reason for the much slower rate under these conditions. Other possible contributions to the rate may come from events coupled to the oxidation and reduction of Q_z such as proton release and binding or protein conformational changes of the Q-*c* oxidoreductase. One such possibility may be rationalized with observations by Rieske et al. [19]. They have shown that the redox state of a noncytochrome component (X) has a 5–6 fold effect on the rate of cleavage and inactivation of the Q-*c* oxidoreductase (Complex III [19]) by a taurocholate–ammonium sulfate mixture; the effect suggests that the oxidation and reduction of X is coupled to a protein conformational change. Comparison of X and Q_z can be made: (a) the E_{m7} of Q_zH_2/Q_z is in the range of that for X, estimated to be between 60 and 150 mV; (b) when X is oxidized the enzyme is susceptible to attack from the detergent–salt mixture; when Q_z is oxidized the rate of electron transfer is impeded; (c) antimycin stabilizes the enzyme independent of the redox state of X; antimycin interferes with the reactions closely associated with Q_z. The work of Rieske et al. [19] warrants further attention.

4.3. *Quinone binding to proteins*

It is becoming clear that proteins play a major role in conferring upon quinones distinct thermodynamic and kinetic properties. The quinone–protein interactions that yield quinones capable of serving in different catalytic roles have been manifested experimentally as differences in their E_m values, pK values and semiquinone stability constants. In several cases the interaction leads to the stabilization of the semiquinone form of the quinone which has facilitated their detection by use of EPR spectrometry [7,20–28].

Recent investigations with mitochondrial preparations, mainly using EPR have revealed several signals that have been considered to originate in protein bound semiquinones [21–25, and 26 for a review]. E_{m7} values of 100 mV [23] and 84 mV [25] have been reported for one of them that is associated with the Q-*c* oxidoreductase. EPR yielded a value for the semiquinone stability constant of this component ($\sim 10^{-2}$) and thereby the information shown in eqns 2 and 3:

$$Q + e^- = Q\cdot^- \qquad E_{m7}^{\frac{1}{2}} = 30\ \text{mV} \qquad (2)$$

$$Q\cdot^- + e^- + 2H^+ = QH_2 \quad E_{m7}^2 = 170\ mV \qquad (3)$$

It has been shown under equilibrium conditions that antimycin rendered the semiquinone signal undetectable, probably due to a decreased stability constant. This interference by antimycin together with reasonably close similarities of the E_{m7} values of the QH_2/Q couple suggests that the EPR signal might come from the same quinone component as that revealed functionally in this study as Q_z. However, the similarity does not appear to extend to the high semiquinone stability constant: a previous EPR study on Q_z in photosynthetic membranes from *Rps. sphaeroides* did not detect a semiquinone signal [27], implying an obscured or modified signal or a very much lower ($<10^{-7}$) stability constant.

Another EPR study has yielded another candidate for comparison with the functionally recognized Q_z. DeVries et al. [28] observed the line shape of the Rieske FeS cluster to change depending on the redox state of a component which has an E_{m7} of about 100 mV and may conform to redox eqn 1 from pH 6.5 to 9.0; they suggested that the component may be a quinone. Again from these similar equilibrium properties and the recognition that Q_z interacts on a redox basis with the Rieske FeS, it is worth considering that Q_z and the component observed indirectly by DeVries et al. [28] are one and the same.

Clearly experimental work is needed that is directed toward testing whether Q_z is identifiable with either of the two EPR observed species and the component X of Rieske [19]. Results relevant to this goal are already emerging: Bowyer, J. R., Trumpower, B. L. and Ohnishi, T. (unpublished observations) have evidence that suggest that the semiquinone signal and the RFeS spectral change arise from quinone binding to two distincts sites.

4.4. *Dynamics of quinone-protein interaction*

A major question now is how permanent are the quinone-protein associations with respect to the time scale of function. An example of an effectively permanent association is ubiquinone-10 and the photochemical reaction center protein of *Rps. sphaeroides* which together form the primary quinone (Q_I; see [7,20]); Q_I is very resistant to removal from its binding site [20], but functions vary rapidly, undergoing reduction with a halftime of 150 ps and re-oxidation with a halftime of 100 μs [7]. In comparison, the Q_z of the Q-c_2 oxidoreductase of *Rps. sphaeroides* has been shown to be bound much weaker than the Q_I of the reaction center [10] and to function, as also shown here for mitochondria, on the much longer timescale of milliseconds [8–10]. As such there is the increased possibility that 'Q_z' could be representative of a ubiquinone molecule occupying a specific binding site in the protein but which nevertheless is exhanging with the molecules of the ubiquinone 'pool' on a timescale of function. This possibility is consistent with the view of Mitchell [5] for his Q-cycle model, a view that Rich [29] has shown to be feasible at least with benzoquinones devoid of the poly-isoprene side chain. However, without the poly-isoprene side chain many quinones appear to be exchanging on the millisecond time range even with the Q_I binding site (Gunner, M. R. and Dutton, P. L.; unpublished observations). Thus the possibility remains that the Q_z, like Q_I, may effectively be a permanent or restricted occupant of a Q_z binding site or pocket. It is worth considering at this point that if Q_z is able to exchange with pool quinone or to move between more than one site in the oxidoreductase in a functional timescale, the E_m value in this paper may represent an average value expressing its existence in several states.

4.5. *Possible role of Q_z in proton translocation*

The foundations of chemiosmotic models of energy coupling in Q-*c* oxidoreductases have been based on the character of the ubiquinone pool. Charge movement across the membrane is commonly seen as originating in electron transfer while the members of the ubiquinone pool serve to transport protons electroneutrally. From the work presented in this report we believe an additional possibility is worth considering: that quinone in association with protein may be involved in both charge and proton movement concurrently. The possibility rests firstly on (a) the recognition of Q_z as a quinone species electrochemically distinct from the quinone pool and (b) its vital role in electron and proton transfer and its demonstrated requirement for the electrogenic reaction in *Rps. sphaeroides* [8,10]. The possibility also rests more speculatively on comparisons made between Q_z and other redox species identified with the mitochondrial Q-*c* oxidoreductase. If Q_z is identifiable with the component X of Rieske et al. [19] and if the functional redox couple is $Q_zH_2/Q_z\cdot^-$ (redox eqn 3), then it is possible that in electron transfer from cytochrome *b* to *c*, Q_z is a key element of an electrogenic proton

pump [30,31] in which gating of the proton channel is controlled by oxidation and reduction of Q_z linked to a protein conformational change.

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