PROPERTIES OF A SEMIQUINONE ANION LOCATED IN THE QH_2:CYTOCHROME *c* OXIDOREDUCTASE SEGMENT OF THE MITOCHONDRIAL RESPIRATORY CHAIN

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1. Introduction

The way in which electrons are transferred from ubiquinol to cytochrome *c* is still under discussion. The results of potentiometric titrations and measurements of the pre-steady-state kinetics monitored optically, mainly giving information on the redox properties of the cytochromes, have led to proposals [1,2] for electron transfer through QH_2:cytochrome *c* oxidoreductase, in which the formation of a semiquinone is a prerequisite for electron transfer. The presence of a semiquinone in preparations of the respiratory chain has, indeed, been identified by EPR studies [3–10]. Ohnishi and Trumpower have detected two different populations of ubisemiquinone in isolated succinate:cytochrome *c* oxidoreductase [10], SQ_s and SQ_c, differing in relaxation time (see also [11]).

The results in this paper indicate the existence of a very stable semiquinone anion located in QH_2:cytochrome *c* oxidoreductase, presumably corresponding to SQ_c in [10]. Quantitation of the EPR signal of the semiquinone anion showed that the maximal concentration is 1/2 that of the cytochrome c_1. In order adequately to describe the effect of pH on the semiquinone anion concentration in the Nernst equation, a limited capacity of the binding site for the semiquinone anion must be taken into account.

Abbreviations and nomenclature: Q, ubiquinone; QH_2, ubiquinol; $Q^{\cdot}H$, (neutral) semiquinone; $Q^{\cdot-}$, semiquinone anion; $E(Q), E(QH_2), E(Q^{\cdot-})$, bound ubiquinone species; $E(Q^{\cdot-}_{max})$, maximal concentration of bound semiquinone anion

2. Materials and methods

Submitochondrial particles from beef-heart mitochondria were prepared essentially according to [12]. The succinate:Q oxidoreductase was activated as in [13]. EPR spectra were recorded on a Varian E-3 spectrometer. Experiments at 5°C, 20°C and 37°C were performed in an aqueous sample cell (47 mm × 3.5 mm × 0.5 mm). The temperature was controlled with a N_2-flow system and measured with a Cu-constantane thermocouple. EPR signals were quantitated by double integration and corrected for the *g*-value according to [14]. For quantitation at room temperatures a $CuSO_4$ standard was used, at low temperatures a $Cu(ClO_4)_2$ standard. The signal amplitudes were corrected for the contribution of the flavine semiquinone signal (see fig.1). The concentration of cytochrome c_1 was assumed to be equal to that of the antimycin-binding sites determined fluorometrically [15]. Since the semiquinone signal appeared to be very sensitive to ethanol, antimycin was added from a solution containing 100 mg bovine serum albumin/ml, 300 μM antimycin, 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 7.5). All potentials were calculated relative to the midpoint potential of the fumarate/succinate couple, E_{m7} = 24 mV [16].

3. Results and discussion

An intense signal at $g = 2.005$ is present in the EPR spectrum of submitochondrial particles, succinate:cytochrome *c* oxidoreductase and QH_2:cytochrome *c* oxidoreductase preparations at pH > 6.8, at 4.2 K–

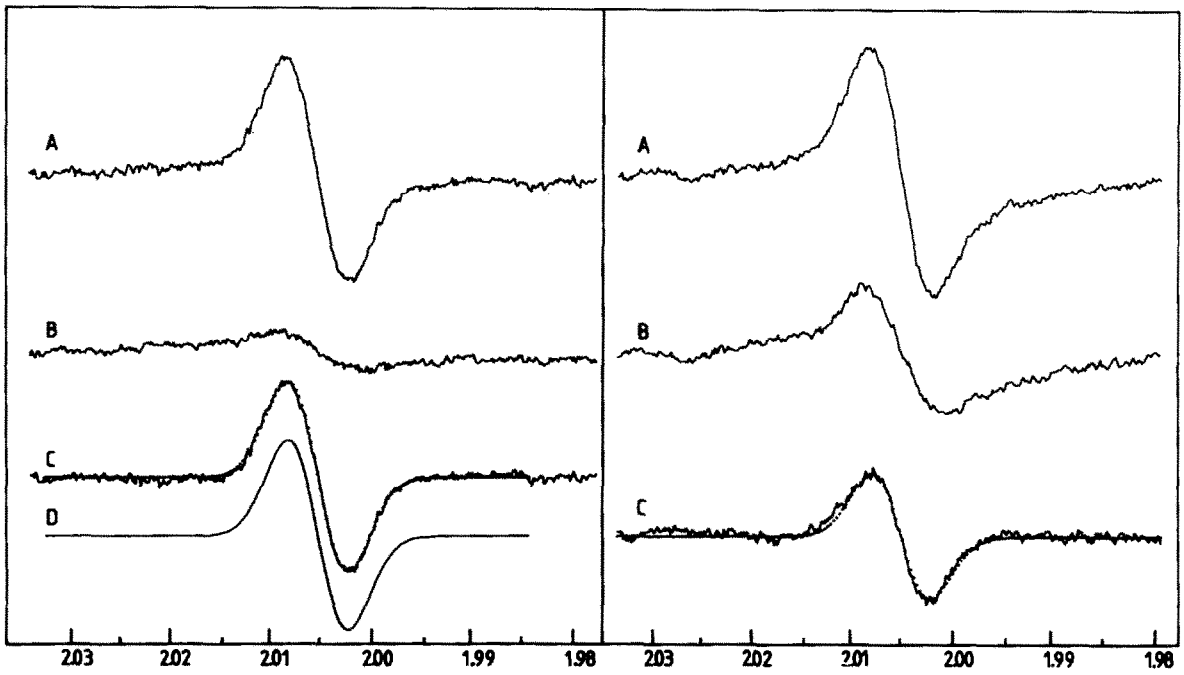


Fig.1. EPR spectra of submitochondrial particles (18 μM cytochrome c_1) in the presence of 0.25 mM sucrose, 50 mM Tris–HCl buffer (pH 8.4) and 4 mM KCN. Left: (A) after addition of 150 mM sodium fumarate and 1.5 mM sodium succinate (E_h = 0 mV) the particles were incubated for 5 min at room temperature; (B) same as (A) but 19 μM antimycin was also added; (C) difference A–B. Dotted line: simulation (from D); (D) Simulation of the semiquinone using a Gaussian line shape and g = 2.005, line width = 1 mT. Right: (A) after addition of 150 mM sodium fumarate and 9.3 mM sodium succinate (E_h = −22 mV); (B) same as (A) but 19 μM antimycin was also added; (C) difference A–B. Dotted line: the same simulation (corrected for the difference in signal amplitude) as in the left figure. EPR conditions: frequency (F), 9.13 GHz; modulation amplitude (MA), 1 mT; power (P), 3 mW; scanning rate (SR), 2.5 mT/min; temperature (T), 20°C.

37°C and at redox potentials of 0–100 mV maintained with a fumarate–succinate mixture (see fig.1). The signal is absent in pentane-extracted preparations, but present after reincorporation of ubiquinone (not shown) (cf. [3,4]). Titration with antimycin showed that the signal intensity declined to a constant value after addition of 1 mol antimycin/mol cytochrome c_1 (cf. [10]). The signal remaining (fig.1) is that of a flavine semiquinone, probably that of succinate:Q oxidoreductase. Ubisemiquinone bound to succinate dehydrogenase [10] is not detected at the low powers and relatively high temperatures used here. It is concluded that the antimycin-sensitive signal originates from a semiquinone of ubiquinone bound [17] to QH_2:cytochrome c oxidoreductase [10].

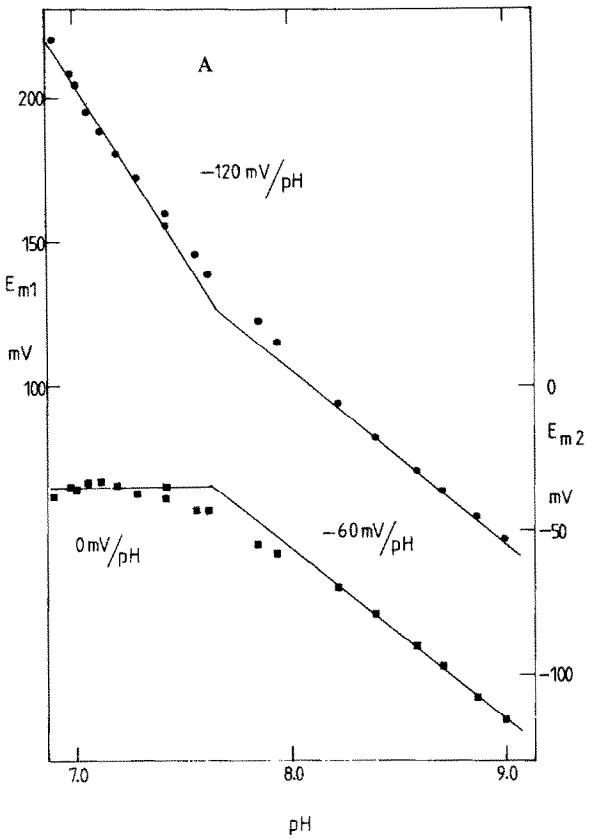
The results of potentiometric titrations are summarized in table 1. The midpoint potential of the bound quinone-quinol couple is given by the potential at which the amount of semiquinone is maximal [16]. Since this was found at the same fumarate/succinate ratio, independent of pH at 6.8–9.0, it follows that the E_m of the bound quinone–quinol couple changes by −60 mV/pH within this range of pH, as is the case for free ubiquinone [18]. This excludes a pK of 8.0 for the quinol, as proposed in [10].

In fig.2A,B the results of experiments performed at a fixed fumarate/succinate ratio, but variable pH, are shown. We conclude, in agreement with [10], that since the concentration of semiquinone strongly increases with increasing pH, the semiquinone anion $Q^{\cdot -}$, and not the neutral $Q^{\cdot}H$, is bound, at least at pH > 6.8.

Although below pH 7.6 the E_m values vary with pH in the manner to be expected on the basis of the equilibria:

$$QH_2 \rightleftharpoons Q^{\cdot -} + 2\,H^+ + e^- \qquad E_{m1}\ (-120\ \text{mV/pH})$$

$$Q^{\cdot -} \rightleftharpoons Q + e^- \qquad E_{m2}\ (0\ \text{mV/pH})$$



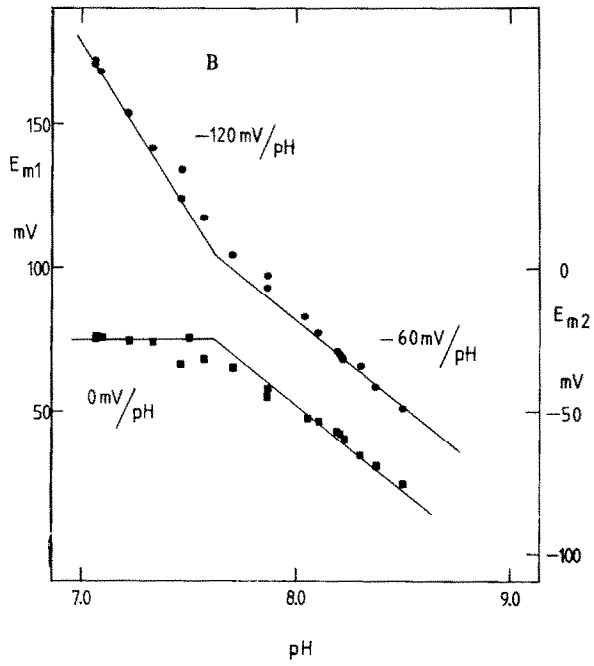


Table 1
Midpoint potentials of various ubiquinone couples

Redox couple	Temp.	E_{m7} (mV)	Slope (mV/pH)
$E(Q)/E(QH_2)$	20°C	84	−60
	77 K	77	−60
$Q^{\cdot-}/QH_{2tot}$ (E_{m1})	20°C	204	−120 (pH < 7.6); −60 (pH > 7.6)
	77 K	184	−120 (pH < 7.6); −60 (pH > 7.6)
$Q_{tot}/Q^{\cdot-}$ (E_{m2})	20°C	−36	0 (pH < 7.6); −60 (pH > 7.6)
	77 K	−30	0 (pH < 7.6); −60 (pH > 7.6)
$E(Q^{\cdot-})/QH_{2tot}$ (E'_{m1})	20°C	233	−129
	77 K	226	−130
$Q_{tot}/E(Q^{\cdot-})$ (E'_{m2})	20°C	−65	+9
	77 K	−71	+10

The midpoint potential of the bound quinone–quinol couple ($E(Q)/E(QH_2)$) was taken to be equal to the potential of the succinate–fumarate couple at which the amount of $Q^{\cdot-}$ is maximal. E_{m1} and E_{m2} were calculated from the amount of $Q^{\cdot-}$ found by EPR and the total amount of ubiquinone measured in the particles, assuming that the potential of $E(Q)/E(QH_2)$ is the same as that of Q_{tot}/QH_{2tot}. See text for calculation of E'_{m1} and E'_{m2}

above pH 7.6 the slope of the curves becomes −60 mV/pH in both cases. In the case of E_{m1} this could be explained by binding of QH_2 to a site in such a way that protons are dissociated from QH_2 with a pK of 7.6, thus:

$$E(QH_2) \rightleftharpoons H^+ + E(QH^-) \rightleftharpoons E(Q^{\cdot-}) + e^- + H^+$$

However in this case the E_m of the bound quinone–quinol couple would have a slope of −30 mV above pH 7.6. This is not the case (see table 1 and fig.6 of [10]). Moreover, dissociation of a proton from QH_2 cannot explain the slope of −60 mV in E_{m2}. A redox equilibrium with cytochrome b, for example:

Fig.2. E_m–pH plots [(•) E_{m1}; (▪) E_{m2}] constructed from the measured semiquinone anion concentration in submitochondrial particles. (A) Incubated with 4 mM KCN, 150 mM sodium fumarate, 1.5 mM succinate (E_h = 82 mV, pH 7) at different pHs. EPR conditions as in fig.1. (B) Incubated in the presence of 4 mM KCN, 150 mM sodium fumarate, 1.9 mM sodium succinate (E_h = 79 mV, pH 7). EPR conditions: F, 9.14 GHz; MA, 0.63 mT; P, 5 μW; SR, 2.5 mT/min; T, 77 K.

$$Q^{\cdot -} + H^+ + b^{2+}H \rightleftharpoons QH_2 + {}^{-}b^{3+}$$

could explain a slope of −60 mV but the pK is rather far from that reported to be involved in the redox reaction of cytochrome b, namely 6.9 [18].

The curves in fig.3 suggest that the amount of $Q^{\cdot -}$ that can be bound becomes limiting at high pH. The maximum amount of semiquinone bound is dependent upon the temperature and the preparation used (table 2). The highest amount found in submitochondrial particles is equal to 1/2 of the concentration of cytochrome c_1. This is much more than that reported in [10] using isolated succinate:cytochrome c oxidoreductase (cf. table 2).

In order to give a complete description of the experimental data including binding of the three ubiquinone species to a specific site, the following equations are needed:

$$E(QH_2) \overset{K_r}{\rightleftharpoons} E + QH_2 \qquad (1)$$

$$K_r = \frac{[E] \,.\, [QH_2]}{[E(QH_2)]}$$

$$E(QH_2) \rightleftharpoons E(Q^{\cdot -}) + 2\,H^+ + e^- \qquad (2)$$

$$E(Q^{\cdot -}) \overset{K_s}{\rightleftharpoons} E + Q^{\cdot -} \qquad (3)$$

$$K_s = \frac{[E]\ [Q^{\cdot -}]}{[E(Q^{\cdot -})]}$$

$$E(Q^{\cdot -}) \rightleftharpoons E(Q) + e^- \qquad (4)$$

$$E(Q) \overset{K_o}{\rightleftharpoons} E + Q \qquad (5)$$

$$K_o = \frac{[E]\ [Q]}{[E(Q)]}$$

$$[E] = e_{tot} - [E(QH_2)] - [E(Q)] - [E(Q^{\cdot -})] \qquad (6)$$

The derivation of the Nernst equation is straightforward [19]:

$$E_h = E'_{m1} + 60 \log E(Q^{\cdot -})/QH_{2\ tot} - 60 \log \{E(Q^{\cdot -}{}_{max}) - E(Q^{\cdot -})\} - 120\,(pH - 7) \qquad (7)$$

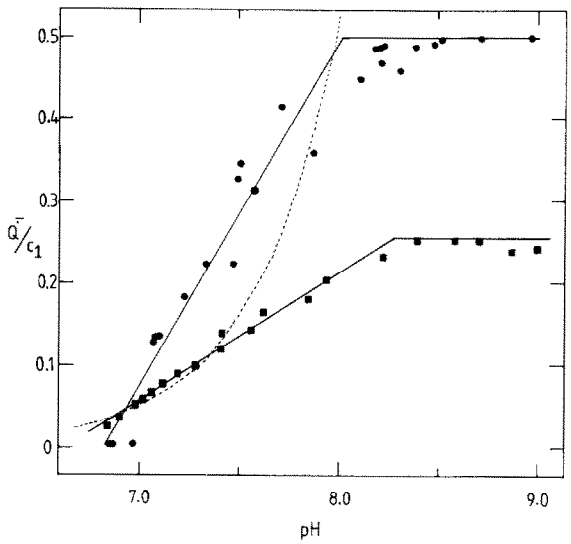


Fig.3. Variation with pH at constant fumarate/succinate ratio of the semiquinone anion concentration/cytochrome c_1. The data were taken from fig.2A (■) and fig.2B (●). Dotted line: Theoretical semiquinone anion concentration, based on measurement at pH 7 and assumption of infinite number of binding sites. (In this case at pH 9, ~1/2 of the total ubiquinone content would be in the semiquinone anion form.)

$$E'_{m1} = E_m\,(E(Q^{\cdot -})/E(QH_2)) + 60 \log K_r$$

$$E_h = E'_{m2} + 60 \log Q_{tot}/E(Q^{\cdot -}) + 60 \log \{E(Q^{\cdot -}{}_{max}) - E(Q^{\cdot -})\} \qquad (8)$$

$$E'_{m2} = E_m\,(E(Q)/E(Q^{\cdot -})) - 60 \log K_o$$

In the derivation of eq. (7) and (8) it was assumed that all three forms of ubiquinone bind with a high affinity,

Table 2
Maximal concentration of semiquinone anion under different conditions

Preparation	Q/c_1	Temp.	$(Q^{\cdot -}{}_{max})/c_1$	$(E(Q)/E(QH_2))$ (mV)
Submitochondrial particles	11	77 K	0.5	77
		5°C	0.35	82
		20°C	0.26	84
		37°C	0.13	84
Succinate or QH_2: cytochrome c oxidoreductase	1-2	77 K	0.3	75
		20°C	0.15	80

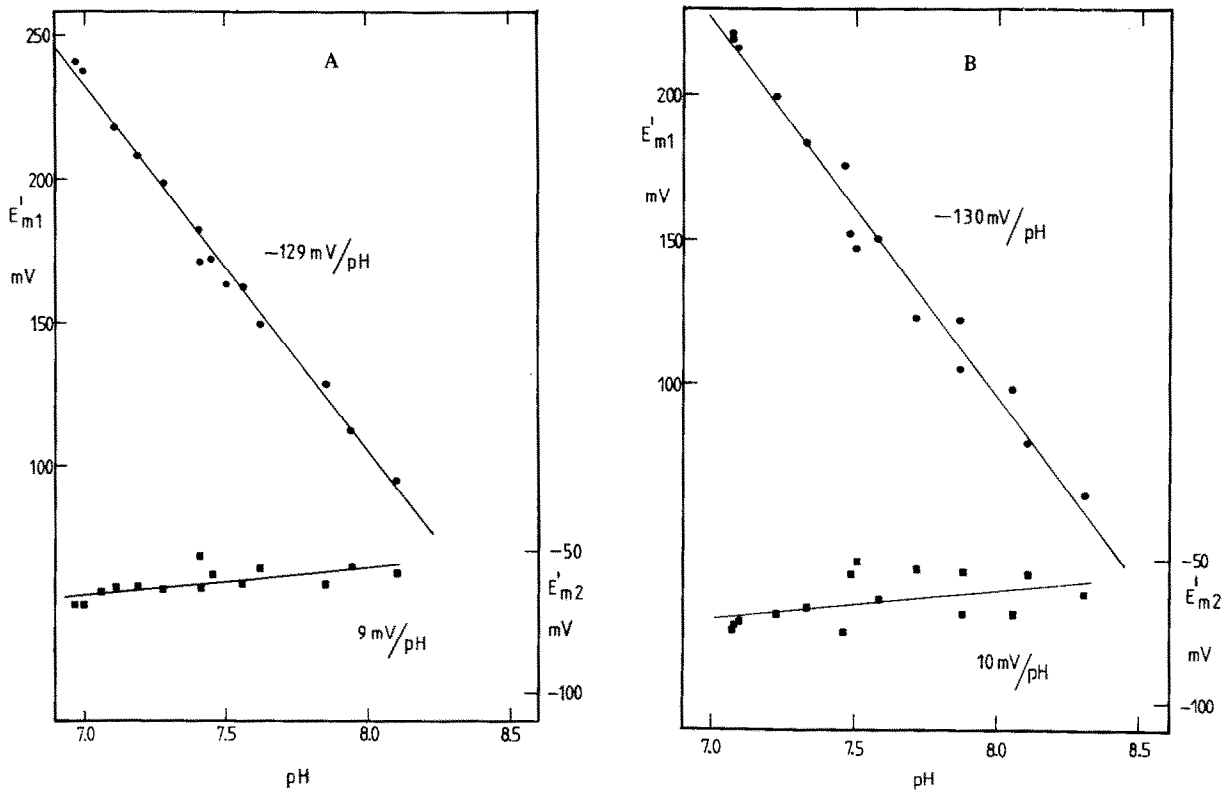


Fig.4. E_m–pH plots correcting for limited binding sites for semiquinone anion, using eq. (7) and (8). The lines drawn are least-square fits. (A) Points corresponding to fig.2A; (B) points corresponding to fig.2B.

but the semiquinone anion is bound preferentially, and that no measurable free semiquinone anion exists. Consequently:

$$K_o \approx K_r >> E >> K_s \approx 0; Q^{\cdot -}{}_{tot} = E(Q^{\cdot -})$$

In fig.4A and 4B the values are shown of E'_{m1} and E'_{m2} calculated from eq. (7) and (8) using the values of $E(Q^{\cdot -}{}_{max})$ for 20°C and 77 K, respectively, from fig.3. The Nernst plots now show no break at pH 7.6 and the slopes are close to the expected values of −120 mV/pH and zero for E'_{m1} and E'_{m2}, respectively. Indeed, as a consequence of normalizing to maximal $Q^{\cdot -}$, the plots for E'_{m1} and E'_{m2} are very similar at the two temperatures, in contrast to significant differences in the case of E_{m1} and E_{m2} (see fig.2A,B and table 1). That the maximum amount of semiquinone differs at the two temperatures is not completely understood.

4. Conclusions

The experiments show, in agreement with [10], that QH_2:cytochrome *c* oxidoreductase has a specific binding site for an antimycin-sensitive semiquinone anion. This site might be on a Q-binding protein [20]. The maximal amount of semiquinone anion is 1/2 the cytochrome c_1 concentration in submitochondrial particles, which might be significant in connection with the evidence that the active enzymic unit in the membrane is a dimer [21–23] and the proposal that in only one of the two monomers (inside or outside) a stable semiquinone anion can be formed [2].

The anomalous redox behaviour of the semiquinone anion can be readily described with a model that includes strong binding of all three forms of ubiquinone, but preferential binding of the semiquinone anion, and a limited capacity of the binding site. It is not necessary to assume a p*K* of 8.0 for the quinol as has been done in [10].

The calculated E_m for bound ubiquinone is 84 mV (pH 7). In [18] 65 mV (pH 7) was measured for free ubiquinone. If this difference is not due to a difference in experimental approach, this implies that QH_2 binds ~4-times more firmly than Q. The calculated values of E'_{m1} and E'_{m2} (table 1) suggest that the $Q/E(Q^{\cdot -})$ couple has the lower potential and is therefore a suitable electron donor for cytochrome *b*. However, since the actual redox pressure on cytochrome *b* might originate from the bound forms of the various ubiquinone species, one cannot decide from these experiments which of the two redox couples is, in fact, the electron donor for cytochrome *b*.

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