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Reaction Center and UQH_2:Cyt c_2 Oxidoreductase Act as Independent Enzymes in *Rps. sphaeroides*

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Abstract

Turnover of the ubiquinol oxidizing site of the UQH_2:cyt c_2 oxidoreductase (b/c_1 complex) of *Rps. sphaeroides* can be assayed by measuring the rate of reduction of cyt b_{561} in the presence of antimycin (AA). Oxidation of ubiquinol is a second-order process, with a value of k_2 of about $3 \times 10^5 \mathrm{M}^{-1}$. The reaction shows saturation at high quinol concentrations, with an apparent K_m of about 6–8 mM (with respect to the concentration of quinol in the membrane). When the quinone pool is oxidized before illumination, reduction of the complex shows a substantial lag (about 1 ms) after a flash, indicating that the quinol produced as a result of the photochemical reactions is not immediately available to the complex. We have suggested that the lag may be due to several factors, including the leaving time of the quinol from the reaction center, the diffusion time to the complex, and the time for the head group to cross the membrane. We have suggested a *minimal* value for the diffusion coefficient of ubiquinone in the membrane (assuming that the lag is due entirely to diffusion) of about $10^{-9} \mathrm{cm}^{-2} \mathrm{sec}^{-1}$. The lag is reduced to about 100 μsec when the pool is significantly reduced, showing that quinol from the pool is more rapidly available to the complex than that from the reaction center. With the pool oxidized, similar kinetics are seen when the reduction of cyt b_{561} occurs through the AA-sensitive site (with reactions at the quinol oxidizing site blocked by myxothiazol). These results show that there is no preferential reaction pathway for transfer of reducing equivalents from reaction center to b/c_1 complex. Oxidation of cyt b_{561} through the AA-sensitive site can be assayed from the slow phase of the carotenoid electrochromic change, and by comparison with the kinetics of cyt b_{561}. As long as the quinone pool is significantly oxidized, the reaction is not rate-determining for the electrogenic process. On reduction of the pool below 1 quinone per complex, a slowing of the electrogenic process occurs, which could reflect a dependence on the concentration of quinone. If the process is second-order, the rate constant must be about 2–5 times greater than that for quinol oxidation, since the effect on rate is relatively small compared with the effect seen at the quinol oxidizing site when the quinol concentration is changed over the E_h range where the first few quinols are produced on reductive titration. When the quinone pool is extracted (experiments in collaboration with G. Venturoli and B. A. Melandri), the slowing of the electrochromic change on reduction of the pool

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is not enhanced; we assume that this is due to the fact that a minimum of one quinone per active complex is produced by turnover of the quinol oxidizing site. Two lines of research lead us to revise our previous estimate for the minimal value of the quinone diffusion coefficient. These relate to the relation between the diffusion coefficient and the rate constants for processes involving the quinones: (a) The estimated rate constant for reaction of quinone at the AA-site approaches the calculated diffusion limited rate constant, implying an improbably efficient reaction. (b) From a preliminary set of experiments, the activation energy determined by measuring the variation of the rate constant for quinol oxidation with temperature, is about 8 kcal mol^{-1}. Although we do not know the contribution of entropic terms to the pre-exponential factor, the result is consistent with a considerably larger value for the diffusion coefficient than that previously suggested.

Key Words: Ubiquinol: cytochrome c_2 oxidoreductase; *Rhodopseudomonas sphaeroides*; electron transfer; chromatophores; Q-cycle; antimycin; ubiquinone diffusion; cytochrome b_{561}; cytochrome b_{566}.

1. Introduction

The UQH_2:cyt c_2 oxidoreductase (b/c_1 complex) of *Rps. sphaeroides* has many similarities with the equivalent complex from mitochondria, including the same set of redox centers, a similar function and mechanism, and a high degree of homology in amino acid sequences of common protein subunits (Crofts, 1985; Crofts and Wraight, 1983; Hauska *et al.*, 1983; Gabellini and Sebald, 1986; Widger *et al.*, 1984). The kinetics of the complex can be studied *in situ* by flash activation, with a time resolution in the microsecond range which is unavailable in studies of the mitochondrial complexes. In addition, because turnover of the complex following flash activation of chromatophores does not require addition of substrate, the electron transfer chain can be poised in a known redox state before activation. We have proposed a modified Q-cycle mechanism for the complex, described in detail in Crofts and Wraight (1983), Crofts (1985), Crofts *et al.* (1983), and Glaser and Crofts (1984). The mechanism provides for the function of the complex as an independent enzyme, reacting by second-order processes with substrates cytochrome c_2, QH_2, and Q at three independent catalytic sites. These are a cyt c_2 reductase site, a quinol oxidizing site (Q_Z site), and a quinone reductase site (Q_C site).

The Quinol Oxidizing Site

Turnover of the ubiquinol oxidizing site of the b/c_1 complex can be assayed by measuring the rate of reduction of cyt b_{561} in the presence of antimycin (Crofts and Wraight, 1983; Crofts, 1985; Crofts *et al.* 1983). Oxidation of ubiquinol is a second-order process, with a value for k_2 of about

$3 \times 10^5 M^{-1} sec^{-1}$ (Crofts *et al.* 1983). The reaction shows saturation at high quinol concentrations, with an apparent K_m of about 6–8 mM (with respect to the concentration of quinol in the membrane; Crofts *et al.*, 1983; Glaser and Crofts, 1984; Snozzi and Crofts, 1984; Venturoli *et al.* 1986). These characteristics are similar to those computed for the mitochondrial complex (Ragan and Cottingham, 1985). When the quinone pool is oxidized before illumination, reduction of the complex shows a substantial lag (about 1 msec) after a flash, indicating that the quinol produced as a result of the photochemical reactions is not immediately available to the complex (Crofts and Wraight, 1983; Crofts *et al.*, 1983; Glaser and Crofts, 1984; Snozzi and Crofts, 1984; Venturoli *et al.*, 1986). We have suggested that the lag may be due to several factors, including the leaving time of the quinol from the reaction center, the diffusion time to the complex, and the time for the head group to cross the membrane. We have suggested a *minimal* value for the diffusion coefficient of ubiquinone in the membrane (assuming that the lag is due entirely to diffusion) of about $10^{-9} cm^2 sec^{-1}$. The lag is reduced to about 100 μsec when the pool is significantly reduced, showing that quinol from the pool is more rapidly available to the complex than that from the reaction center. The residual lag is at least partly accounted for by the time taken to oxidize the Rieske-type FeS of the complex. With the pool oxidized, similar kinetics are seen when the reduction of cyt b_{561} occurs through the antimycin-sensitive site (with reactions at the quinol oxidizing site blocked by myxothiazol) (Glaser *et al.*, 1984). These results show that there is no preferential reaction pathway for transfer of reducing equivalents from reaction center to b/c_1 complex.

The Antimycin-Sensitive Site

Although there is substantial agreement that the Q_Z site operates through the "concerted" reaction of the Q-cycle, the mechanism of the second quinone reactive site (Q_C site) through which cyt b_{561} is oxidized is much more controversial. Most workers favor a mechanism which allows for the independent operation of the complex, but several different modifications of the original Q-cycle have been proposed which allow for this. These fall into five classes:

(i) The semiquinone formed by one-electron reduction of quinone disproportionates to form quinol and regenerate quinone (Slater, 1981).

(ii) Reduction of quinone occurs at a site formed by dimeric interaction of two complexes (DeVries, 1983; DeVries *et al.*, 1982; Meinhardt and Crofts, 1983).

(iii) Reduction of quinone occurs by a "two-electron gate" mechanism, in which electrons are delivered sequentially to the quinone from a serial *b*-cytochrome chain, with formation of an intermediate semiquinone species. This mechanism requires the delivery of two electrons to the *b*-cytochrome chain by two successive turnovers of the quinol oxidizing site of the complex (Crofts and Wraight, 1983; Crofts, 1985; Crofts *et al.*, 1983; Glaser and Crofts, 1984; Glaser *et al.*, 1984; Garland *et al.*, 1975).

(iv) Reduction of quinone occurs by two-electron transfer from a parallel *b*-cytochrome chain. This mechanism also requires delivery of two electrons to the *b*-cytochromes by successive turnovers of the Q_Z site (Malviya *et al.*, 1980; Velthuys, 1980).

(v) Cyt b_{561} is oxidized by the semiquinone anion which is formed at the quinol oxidizing site by electron transfer to FeS, and is then transfered to the Q_C site in an electrogenic process (Wikstrom and Saraste, 1984; Rich and Wikstrom, 1986; P. Mitchell, personal communication). In this mechanism, quinone is not a normal reactant at the Q_C site, and the Q_Z site is required to operate in two different modes. The major advantage of the mechanism is that it provides a pathway for turnover on oxidation of the fully reduced complex.

We have discussed elsewhere the kinetic parameters involved in electron transfer through the *b*-cytochrome chain, and the electrogenic processes coupled to these reactions (Glaser and Crofts, 1984). We demonstrated that, at least when the membrane potential is low (i.e., following the first flash from the dark state), the electrogenic process of the complex occurs at a rate determined by the turnover of the Q_Z site. The electron transfers to and from cyt b_{561} were both rapid, so that cyt b_{561} showed only a transient reduction. The amplitude of the transient varied from zero to about 20% of the full extent of reduction observed in the presence of antimycin, depending on ambient redox potential. The electrogenic process in the absence of antimycin extended for about twice the period required for reduction of cyt b_{561} in the presence of antimycin, indicating that two successive turnovers of the Q_Z site occur during the full turnover of the complex. In these experiments there was no indication of either a transient complete reduction of cyt b_{561}, or a delay in the onset of the transient, as may have been expected from mechanisms such as those in (iv) or (v) above. In the absence of antimycin, but under uncoupled conditions, when cyt b_{561} was reduced before flash activation, a rapid transient reduction of cyt b_{566} occurred; cyt b_{561} oxidation was observed only after a lag of 500 μsec when the reduction of cyt b_{566} had reached a maximal value (Meinhardt and Crofts, 1983). We interepreted these data as showing a two-electron gate mechanism [see (iii) above]. Although we cannot exclude any of the other types of mechanism, the data are not readily explained by these without *ad hoc* postulates.

Oxidation of cyt b_{561} through the antimycin-sensitive site can be assayed from the slow phase of the carotenoid electrochromic change, and by comparison with the kinetics of cyt b_{561} (Crofts *et al.*, 1983; Glaser and Crofts, 1984). When the slow phase of the carotenoid change was measured as a function of redox potential in order to vary the concentration of quinone by reduction of the pool, we found that the rate decreased more rapidly than the extent (Glaser and Crofts, 1984). The extent was dependent on the fraction of complexes turning over, as determined by the fraction of reaction centers in which the primary acceptor, Q_A, was oxidized before the flash, since this determined the delivery of oxidizing equivalents to the complex. The more rapid decline in rate could then be interpreted as indicating that the process was dependent on a second-order reaction in which the concentration of an additional reactant (presumably quinone) became rate-determining. However, the effect of lowering the preflash concentration of quinone to less than one per complex was relatively small compared to the effect seen at the Q_Z site; the corrected rate was 50% slower than the maximal rate, whereas for the quinol oxidizing site, the rate with only one quinol per complex was only 10–20% of the maximal rate. A possible interpretation is that the second-order rate constant for quinone reduction is 2–5 times greater than that for quinol oxidation. As a consequence, as long as the quinone pool is significantly oxidized, the reaction is not rate determining for the electrogenic process. When the quinone pool was partly or completely extracted (experiments in collaboration with G. Venturoli and B. A. Melandri), the slowing of the electrochromic change on reduction of the pool was not enhanced; we assume that this is due to the fact that a minimum of one quinone per active complex is produced by turnover of the Q_Z site, even in the extracted preparations.

The Diffusion Coefficient for Quinone in the Membrane

Two lines of evidence lead us to question our previous estimate for the minimal value of the quinone diffusion coefficient. These relate to the relation between the diffusion coefficient, the collision-limited rate constant, the activation energies, and the rate constants for processes involving the oxidation or reduction of quinones. In the Arrhenius equation

$$k = A \exp(-E_a/RT)$$

the rate constant k approaches the preexponential factor A as the activation energy E_a approaches zero. The preexponential factor is then the rate constant for the process in which there is no activation barrier, or in which all

collisions are effective for forward reaction—the collision-limited rate constant. The value of A is directly related to the diffusion coefficient by the Smoluchowski equation (von Smoluchowski, 1917).

$$A = \frac{4(r_m + r_n)(D_m + D_n)N_0}{1000}$$

where r and D are the effective radii and diffusion coefficient, respectively, of the reacting molecules M and N (denoted by subscripts m, n), and N_0 is Avogadro's number. Both these equations are oversimplified for the case in point (Hardt, 1979), but serve to illustrate the argument.

From the minimal value for diffusion coefficient of quinone (about $10^{-9}\,\mathrm{cm^2\,sec^{-1}}$) estimated as above, the value for the diffusion-limited rate constant for reaction with a catalytic site on a membrane-bound protein is in the range $k_{\mathrm{diff}} = 10^6\,\mathrm{M^{-1}\,sec^{-1}}$. The second-order rate constant measured for the oxidation of quinol at the Q_Z site (a value of $3 \times 10^{-5}\,\mathrm{M^{-1}\,sec^{-1}}$) was uncomfortably close to this diffusion-limited value, implying that the process should have a small activation energy. We have recently measured the activation energy for the reaction at the Q_Z site, and found a value of $8\,\mathrm{kcal\,mol^{-1}}$ (Zhenngan Wang and A. R. Crofts, unpublished results). Although we do not know the contribution of entropic terms to the pre-exponential factor, the result is consistent with a considerably larger value for the diffusion coefficient than that we had previously suggested.

The second line of evidence favoring a higher value for the diffusion coefficient comes from the experiments on turnover of the Q_C site discussed above. We have interpreted the experiments as indicating a two-electron gate mechanism, which implies a second-order reaction with quinone (Crofts and Wraight, 1983; Crofts, 1985; Crofts *et al.*, 1983; Glaser and Crofts, 1984). Additional support for a reaction in which quinone supplies the oxidant for cytochrome b_{561} comes from the observation of von Jagow *et al.* (1984) that fumarate addition (at low succinate concentration) induces oxidation of cyt b_{562} in myxothiazol-inhibited succinate:cyt c oxidoreductase preparations in an antimycin-sensitive reaction, presumably through the intermediate oxidation of endogenous quinone by reversal of the succinate dehydrogenase reaction. In addition, DeVries and Dutton (1985) have observed a strong dependence on quinone concentration of the rate of oxidation of cyt b_{562} following a flash in a hybrid system containing mitochondrial b/c_1 complex, cyt c, and *Rps. sphaeroides* reaction center. In our own experiments, the estimated rate constant for reaction of quinone at the antimycin-site was two- to fivefold greater than that for oxidation of quinol, giving a value of about $10^6\,\mathrm{M^{-1}\,sec^{-1}}$. This is the same as the diffusion-limited rate constant calculated using our earlier estimate of a minimal value for the diffusion

coefficient of quinol. Although reaction at the diffusion limit is not impossible, in view of the difficulty of lining up the somewhat unwieldy quinone molecule with its catalytic configuration, it seems unlikely that the process has the high efficiency implied. We would therefore expect that the collision limit must be considerably higher, implying a higher diffusion coefficient than that previously estimated.

In contrast with the above experiments which suggest an upward revision in value for the diffusion coefficient, we note the results of experiments in which we have observed the lag in reduction of cyt b_{561} (following flash activation of antimycin-inhibited chromatophores poised with the quinone pool oxidized) in chromatophores in which the pool had been diluted by fusion with liposomes. The increase in the lag was consistent with a substantial contribution of diffusion to the lag (Snozzi and Crofts, 1984). We have observed a similar apparent increase in the lag on reconstitution of excess ubiquinone into lyophilized chromatophores (Venturoli *et al.*, 1986). It seems possible that the discrepancy between these conclusions relates more to the difficulty of estimating the lag with accuracy when the reduction kinetics are slowed than to any fundamental paradox.

Turnover of the Complex from the Fully Reduced State

A perennial criticism of the Q-cycle has been that it provides no mechanism by which the *b*-cytochromes of the electron transport chain can become oxidized on introduction of oxidant to the fully reduced b/c_1 complex. The introduction of the *b*-cycle (Wilkstrom and Saraste, 1984) and its most recent varient, the SQ-cycle (Rich and Wikstrom, 1986; Mitchell, P., personal communication), by Wikstrom and colleagues represents an attempt to overcome this problem by allowing a semiquinone species produced at the Q_Z site to act as the oxidant at the Q_C site. In a recent paper, Rich and Wikstrom (1986) have claimed to exclude the possibility that a conventional Q-cycle can provide the oxidant, on the basis of kinetic experiments in which ferricyanide was added to dithionite or succinate-reduced mitochondria, or to isolated succinate: cyt *c* oxidoreductase, in a hand-mixing experiment. They showed that the kinetics (within the limitations of mixing artifact and response time of the apparatus) were the same when the *b*-cytochromes were partially reduced with succinate, or completely reduced by dithionite. They also measured the turnover of the complex in the antimycin-inhibited steady state, to arrive at a maximal rate of production of oxidant at the Q_Z site of the inhibited complex, and claimed that the steady-state rate was not sufficient to support the rate of oxidation after mixing. Their result is in contrast to the previously published result of DeVries (1983), which showed,

using rapid mixing techniques, a significantly lag (of about 50 msec) in oxidation of cytochrome *b* when the complex was fully reduced by dithionite. The discrepancy between these results may be attributed to some deficiencies in the protocol used by Rich and Wikstrom. Although their experiments were performed at $-7°C$, it seems probable that the kinetic resolution of the Rich–Wikstrom experiments was not sufficient to resolve the lag seen by DeVries, since the mixing time was about 200 msec, and stirring artifacts obscured the kinetics till about 350 msec after mixing. A further criticism of their conclusions arises from their use of steady-state rates to compare with transient kinetic data. The turnover number of the isolated complex in the antimycin-inhibited steady state was about $1.6\,sec^{-1}$. The authors do not quote the turnover number of their uninhibited complex, but values around $100–200\,sec^{-1}$ are found in the literature; the rates of oxidation measured showed a half-time "significantly faster than the mixing half-time of about 200 msec." The authors concluded that the turnover of the inhibited complex was not sufficiently rapid to account for the kinetics observed. However, they failed to take account of the fact that, under the uninhibited conditions of their kinetic experiment, production of oxidant at the Q_Z site would be an autocatalytic process, since each equivalent of oxidant produced would generate two equivalents by turnover of the uninhibited complex. Even if only 1% of centers produced oxidant on the first turnover, the complex could be fully oxidized after the time needed for eight turnovers. Clearly, in future experiments, it will be necessary to resolve the kinetics on a faster timescale, and closer to the mixing, if these questions are to be unambiguously answered.

One further point with respect to the SQ-cycle mechanism is worth making. Wikstom and colleagues have assumed that the mechanism they propose requires a special structure within the complex to allow rapid transfer of semiquinone between the two catalytic sites, and this would seem reasonable if this were the only means of turnover of the complex (the normal mechanisms). However, if the SQ-cycle mechanism is proposed as operating only when the complex is fully reduced, it seems more reasonable to consider an alternative possibility—that the semiquinone produced at the Q_Z site migrates to the Q_C site through the lipid phase of the membrane. As discussed by Crofts and Wraight (1983), the stability of the semiquinone is closely related to its dissociation constant. It seems likely from the thermodynamic properties that the Q_Z species is not strongly stabilized, and may therefore be able to dissociate quite readily from its catalytic site. If this were to happen with a value for k of $10\,sec^{-1}$, the SQ-cycle would provide a mechanism for oxidation of the fully reduced complex within the context of the normal Q-cycle.

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