On the Mechanism of Quinol Oxidation in the bc1 Complex

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Susanne Jünemann‡, Peter Heathcote§, and Peter R. Rich¶

From the ‡Glynn Laboratory of Bioenergetics, Department of Biology, University College London, Gower Street, London, WC1E 6BT, United Kingdom, and the §School of Biological Sciences, Queen Mary and Westfield College, Mile End Road, London, United Kingdom

The question of whether significant levels of a semiquinone can be generated in the Qo site of the bc1 complex under conditions of oxidant-induced reduction is relevant to the mechanism of bifurcation of electron transfer in this site. It has already been reported that beef heart submitochondrial particles under such conditions exhibit an EPR-detectable semiquinone, which is distinct from Q7 and which was attributed to a semiquinone in the Qo site (de Vries, S., Albracht, S. P. J., Berden, J. A., and Slater, E. C. (1981) J. Biol. Chem. 256, 11986–11988). However, we show here that this signal, which can be generated to a level of around 0.1 pmol bc1 monomer, is insensitive to the Qo site inhibitors myxothiazol, E-β-methoxyacrylate-stilbene, and stigmatellin, indicating that it does not arise from a Qo species. Based on sensitivities to inhibitors of other Q sites, up to 60% of the signal may arise from semiquinones of complexes I and II. We further show that the iron-sulfur center remains EPR silent under oxidant-induced reduction conditions. Overall, the results indicate that, under conditions of oxidant-induced reduction, the Qo site is occupied primarily by quinol with the iron-sulfur center oxidized, or, possibly, by an antiferromagnetically coupled semiquinone/reduced iron-sulfur center pair, which are EPR silent. This is discussed in relation to proposed mechanisms of quinol oxidation in the Qo site, and we describe a minimal intermediate-controlled bifurcation model based on rate constants by which bifurcated electron transfer at the Qo site might occur.

The Q-cycle (1) adequately describes the general mechanism of electron and proton transfers within the cytochrome bc1 complex. Quinol is oxidized and quinone reduced at two separate sites, the Qo and Qi sites, respectively. The oxidation of quinol at the Qo site entails an obligatory bifurcation of the two electrons. The first electron from the quinol is transferred to the iron-sulfur center and then to cytochrome c1. The electron on the semiquinone product is transferred to heme bL from where proceeds to the Qo site via heme bH.

This bifurcation is an integral part of the efficient coupling mechanism and seems to be obligatory; although the redox potential of the second electron transfer is much lower than that of the first, donation of both electrons to the iron-sulfur center cannot occur at a significant rate. This is most clearly illustrated under conditions where turnover is inhibited by antimycin A, a compound that binds at the Qo site (2). Two turnovers of the Qo site result in reduction of both hemes b. In this state, further full oxidation of quinol at the Qo site becomes extremely slow, resulting in the well known strong inhibition by antimycin A of the quinol-cytochrome c oxidoreductase activity.

The chemical basis for obligatory bifurcation of electron flow was not addressed explicitly in the original Q-cycle proposal. However, it should be stressed that, in principle, the basis could lie simply in the fact that the first reaction step of oxidation of quinol (QH2) to semiquinone (Q·) is thermodynamically very unfavorable. Hence, the first electron transfer cannot occur at a high net rate unless the unstable semiquinone product is removed rapidly by a second reaction, in this case reduction of ferric heme bL. More formally, the overall net rate constant k for the sequential reaction (Eq. 1, ignoring protonation changes for simplicity of treatment) is related to the microscopic rate constants by k = k1k2/k1 + k2.

\[
\begin{align*}
&\text{QH}_2\text{FeS} \rightarrow \text{QFeS} \rightarrow \text{bL} \rightarrow \text{bL}^- \rightarrow \text{QFeS} \\
&\text{bL}^- \rightarrow \text{bL}
\end{align*}
\]

(Eq. 1)

Hence if \( k_2 \gg k_1 \), which is the situation that we assume for the reaction above, then the overall rate constant, k, approaches \( k_1 \). However, if the reaction involves a second electron transfer to the iron-sulfur center, which is oxidized with a rate constant of \( k_3 \), as would be the case for the reaction in the antimycin-inhibited state (Eq. 2), then the overall rate constant, k, is instead given by k = k1k3/(k1 + k3).

\[
\begin{align*}
&\text{QH}_2\text{FeS} \rightarrow \text{QFeS} \rightarrow \text{bL} \rightarrow \text{[QFeS]bL} \rightarrow \text{QFeS} \\
&\text{bL} \rightarrow \text{bL}
\end{align*}
\]

(Eq. 2)

If \( k_3 \ll k_1 \), as we propose to be the case, then in the limit k becomes equal to \( k_1 k_3/k_1 \), and k is diminished by the ratio \( k_1/k_3 \). In this case, there would be no build up of semiquinone, and quinol oxidation at the Qo site would be inhibited.

Control by this simple intermediate-controlled bifurcation mechanism relies upon the exergonicity of the first step of quinol oxidation to semiquinone, a feature inherent in the original formulations (1, 3). A more quantitative assessment of this instability was made by Crofts and Wang (4), who considered the formation of semiquinone to be the rate-controlling step. However, Brandt (5) has challenged the tenet that the semiquinone formation is rate-controlling, suggesting instead a reaction controlled by the exergonic deprotonation of quinol to its anionic, active form and with semiquinone “more or less stabilized by the active site” (6).

Several further complications have arisen when considering the detailed chemistry of the Qo site, and the recent advances in structure determination of the bc1 complex (7, 8) have refo-
cused interest on these questions. Ding et al. (9) have raised the possibility that two quinone molecules may be required to be bound simultaneously at the $Q_\text{o}$ site for activity, arranged in a linear edge-to-edge or a stacked arrangement. This feature was included by Brandt (5) in his deprotonation-controlled mechanism, in which catalysis proceeds via a quinhydrone-like intermediate.

Brandt and von Jagow (10) also considered the possibility that bifurcation might require a protein conformational catalytic switch at the $Q_\text{o}$ site, in which the active reductant bound in the $Q_\text{o}$ site alternatively contacts the iron-sulfur center and the heme $b_2$. This switch was first thought to be controlled by the redox state of the iron-sulfur center (10), but control by the heme $b_2$ to heme $b_\text{H}$ electron transfer was also suggested (11). The recent crystal structures of the $bc_1$ complex have highlighted the possibility that the iron-sulfur center itself might have conformational flexibility as its headgroup has two quite different orientations in the two determined structures (7, 8), raising the possibility of long-range mobility of the iron-sulfur center during the catalytic cycle. This possibility has been included by Brandt (6) in his most recent model of $Q_\text{o}$ site function as the basis for the catalytic switch and, hence, as the basis for the mechanism of bifurcation itself.

Another distinct possibility for the $Q_\text{o}$ site bifurcation has been suggested by Link (12), based on the observation that binding of the $Q_\text{o}$ site inhibitor stigmatellin dramatically raises the midpoint potential of the Rieske center. If the semiquinone formed in the catalytic reaction were to mimic this effect, as suggested, then oxidation of the iron-sulfur center by cytochrome $c_1$ could not proceed until the semiquinone, which would be tightly bound to the iron-sulfur center, had reduced heme $b_2$. In this scheme, the semiquinone intermediate is extremely stable, and the reaction rate is controlled by quinol deprotonation, as in the Brandt model (5).

Despite considerable advances in understanding of structure and function of the $bc$ complexes, these questions of $Q_\text{o}$ site chemistry have remained unresolved. The initial quinol binding site is likely to involve residues on cytochrome $b$ and residues(s) (possibly a histidine ligand to an iron (13)) on the iron-sulfur center. However, the exact location of the site remains unresolved, as do the possibilities of two quinone sites or two sequential binding orientations for a single quinone (8). A critical point that can shed some light concerns the degree to which the $Q_\text{o}$ site can become loaded with a semiquinone under antimycin-inhibited conditions and in the presence of substrates. It is well known that both hemes $b$ are reduced and cytochrome $c_1$ is oxidized in this condition of oxidant-induced reduction (14, 15), as would be expected in all of the above models. However, a bifurcation mechanism based simply on an unstable semiquinone would predict quinol in the $Q_\text{o}$ site and an oxidized iron-sulfur center, whereas some other models would predict otherwise. This question of the redox poise of components in this condition was addressed using EPR by de Vries et al. (16), and a semiquinone signal was found that was sensitive to BAL (an inactivator of the iron-sulfur center (17)) and which was hence ascribed to a semiquinone in the $Q_\text{in}$ site. Because of the renewed interest in this question, we have further investigated the redox states of quinone bound in the $Q_\text{i}$ site and of the iron-sulfur center under conditions of oxidant-induced reduction. The results are discussed in terms of the possible mechanisms outlined above.

**MATERIALS AND METHODS**

Keilin-Hartree particles from beef heart were prepared as described (18) and were stored at 77 K until required. Protein concentrations were determined using a modified Biuret method, where 0.5 mM H$_2$O$_2$ and 0.5% sodium cholate are added during sample preparation (18). The concentration of $bc_1$ complex in the preparation was estimated from the succinate-reduced minus ferricyanide-oxidized difference spectrum using an extinction coefficient at 562 minus 575 nm of 562 minus 575 nm $\Delta\varepsilon = 28.5$ mm$^{-1}$ cm$^{-1}$ (10).

Optical spectra and multi-wavelength kinetics were monitored at room temperature in the same sample using a single-beam instrument built in house.

Continuous wave EPR spectra were recorded on a Jeol RE1X spectrometer fitted with an Oxford Instruments cryostat. Conditions of measurement are given in the figure legends. Spin intensities were quantitated by double integration and calibration with a known sample of photo-oxidized P700 in photosystem I particles from spinach, prepared using Triton X-100 as described previously (19). The concentration of P700 was determined optically from an ascorbate-reduced minus ferricyanide-oxidized difference spectrum using an extinction coefficient of 64 mm$^{-1}$ cm$^{-1}$ at 703 nm. P700$^+$ was generated by illuminating the sample in the EPR tube for 60 s and freezing in liquid nitrogen under illumination.

Inhibitors were added from ethanolic stock solutions, with final ethanol concentrations not exceeding 1% (v/v). Rolliniastatin-1 was a kind gift from Dr. Degli Esposti (Monash University, Australia).

**RESULTS**

**Oxidant-induced Reduction Monitored by Optical Spectroscopy**—Semiquinone in the $Q_\text{o}$ site can be expected to accumulate maximally when the $Q_\text{i}$ site is inhibited (e.g. by antimycin A) in the presence of substrates. It is well documented that this leads to an inhibited state in which both hemes $b$ are reduced and cytochrome $c_1$ is oxidized (15). A room temperature optical experiment illustrating this behavior is shown in Fig. 1. In the absence of antimycin A (panel A), addition of 5 mM succinate under aerobic conditions leads to a steady-state turnover with low levels of reduced hemes $b$ and $c_1/c_e$ followed, on anaerobiosis, by a sharp absorbance increase due to essentially full reduction of all hemes. In contrast, in the presence of antimycin A (panel B), addition of succinate induces substantial reduction of the $b$-type hemes in the aerobic state, whereas cytochrome $c_1/c_e$ remain oxidized. This inhibited state is stable for several minutes, even at the higher protein concentration used for the EPR samples described below, hence allowing samples to reach a steady state and be frozen for EPR measurements. The difference spectrum of the sample under steady-state antimycin
A-inhibited conditions versus the "as prepared" state (not shown) shows a single peak at 564 nm, indicating that both heme b-562 and heme b-566 contribute to the signal. Full reduction of the system with dithionite gives a further absorbance increase at 562 minus 580 nm (panel B). However, this additional change is caused by contaminating hemoglobin in the samples that, on anaerobiosis, undergoes an oxyferrous to ferrous transition resulting in the extra absorbance increase at 562 minus 580 nm induced by dithionite addition in panel B. This interpretation was confirmed by recording a dithionite-reduced minus succinate-reduced difference spectrum (not shown), which was characteristic of the deoxymyoglobin minus oxyhemoglobin difference spectrum and which agreed with the amount of contaminating hemoglobin quantitated from carbon monoxide binding spectra. Hence, we conclude that both b-type hemes were fully reduced in the aerobic steady state in the presence of antimycin A, as required for this investigation.

As can be estimated from trace A, the succinate-sustained turnover of the bc1 complex is comparatively low, around 10 s
-1 under these high pH conditions. The experiments were also repeated under identical conditions, but with 5 mM NADH as substrate instead of succinate. In this case, the uninhibited turnover number of the cytochrome bc1 complex was around 100 s
-1. However, the antimycin-inhibited aerobic steady state of cytochromes was identical to the succinate condition (not shown).

As expected, when Q site antagonists such as myxothiazol or stigmatellin are added in addition to antimycin A in the above experiments, reduction of the b-type hemes does not occur in the aerobic steady state (not shown).

Associated EPR Signal of Semiquinones—The antimycin A-inhibited conditions described above were used to set up EPR samples. The only difference was an increased concentration of Keilin-Hartree particles required for the EPR measurements. Even at these concentrations, at least 1 min was available to establish and freeze an aerobic steady-state condition, and it was not deemed necessary to work at 0 °C with short (2 s) incubation times as described (16). The magnitude of the EPR signal of oxidized heme a of cytochrome oxidase near g = 3 (cf. Ref. 20) was used to confirm that samples remained in the aerobic state. Samples thus prepared do indeed give rise to an EPR signal of 10 G linewidth at g = 2.0048 (cf. g = 2.006 and 8.3 G linewidth in Ref. 16), which can be attributed to a semiquinone (Fig. 2A). At a spin concentration of 1.6 ± 0.1 μM in a sample 16 μM in bc1, the occupancy is comparable with the value of 0.12 given by de Vries et al. (16). The signal can also be generated using NADH as electron donor, although at about 20% lower occupancy than with succinate (Table I).

To obtain for comparison an internal standard of semiquinone bound in the Q site, Keilin-Hartree particles were reoxidized with succinate/fumarate at alkaline pH under aerobic conditions in the presence of cyanide. A maximum occupancy of about 0.5 Q1
- in bc1 is reached at a succinate/fumarate ratio of 1:20 (pH 8.5) when heme b565 is about 75% reduced (21). In the present experiments, these conditions give rise to a semiquinone signal of 4.95 μM spin in a sample approximately 15–16 μM in bc1. The signal at g = 2.0048 (Fig. 2C) has a linewidth of 10 G (cf. g = 2.005 and 10 G linewidth in Ref. 16) and is sensitive to antimycin A (Fig. 2D).

The semiquinone signal generated under conditions of oxidant-induced reduction is distinct from Q1
- (cf. Fig. 3A). Values for P1/2 can be estimated to be 117 μW for Q1
- and 287 μW for the second species, at 45 K. These values are comparable with those reported previously (16) of 105 and 276 μW, respectively, at 50 K.

The pH dependence of the semiquinone signal induced under conditions of oxidant-induced reduction is given in Fig. 3B. It shows a decrease of signal intensity by a factor of 2 at low pH with an apparent pK near 7.7, again similar to the properties of the semiquinone signal described by de Vries et al. (16), which has been attributed to a semiquinone in the Qo site.

Effects of Inhibitors—In the previous study (16), the disruption of the iron-sulfur center by treatment with BAL abolished the semiquinone ascribed to Q1
-. In the present experiments, we have used myxothiazol, MOA-stilbene, and stigmatellin to test sensitivity of the signal to Qo site inhibitors. From Fig. 2B and Table I, it can be seen that these compounds do not affect

![EPR spectra of different species of semiquinone under conditions of equilibrium redox poise and oxidant-induced reduction of cytochrome b.](image_url)

**Table I**

| Experimental conditions | Signal % |
|-------------------------|---------|
| Succinate (5 mM)/antimycin A (20 μM) | 100 |
| + Myxothiazol (16 μM) | 93–105 |
| + Stigmatellin (20 μM) | 102 |
| + MOA-stilbene (16 μM) | 106 |
| + Rotenone (15 μM), roliniastatin I (9 μM) | 75–86 |
| + Stigmatellin (20 μM), rotenone (15 μM), roliniastatin I (9 μM) | 90 |
| NADH (5 mM)/antimycin A (20 μM) | 82 |
| + Myxothiazol (16 μM) | 104 |
| + Carboxin (20 μM) or TTFA (0.25 mM) | 63–64 |
| + Stigmatellin (20 μM), TTFA (0.25 mM) | 61 |
| Ascorbate-reduced (10 mM) | 47–53 |
the semiquinone signal. Yet, in an analogous optical experiment similar to that shown in Fig. 1, oxidant-induced reduction of the 6-type hemes was abolished in the presence of the above inhibitors (not shown). When NADH was used as electron donor, addition of myxothiazol even leads to an increase in signal size (Table I).

With succinate as electron donor in the presence of antimycin A, a combination of the complex I inhibitors rotenone and rolliniastatin-1 gave a signal loss of 10–20% (Table I). Similarly, with NADH as electron donor (in the presence of antimycin A), addition of the succinate dehydrogenase inhibitors carb oxin or 2-thenoyltrifluoroacetone (TTFA) led to a 40% decrease of the semiquinone signal. A semiquinone signal could also be seen in an ascorbate reduced sample in the absence of antimycin A. This was of unknown origin and may in part arise from the ascorbate itself. Overall, from these data we conclude that the signal of Fig. 2A cannot be attributed to a \( \text{Q}^- \) species and instead arises from species in complexes I and II and other unknown sources.

Redox State of the Iron-Sulfur Center—The same EPR samples used to monitor the semiquinone radical under conditions of oxidant-induced reduction were subsequently used to determine the redox state of the iron-sulfur center. In a control sample reduced with ascorbate in the presence of cyanide, the iron-sulfur center was essentially fully reduced, resulting in a characteristic signal with \( g_x = 1.90 \) and \( g_y = 1.79 \) (Fig. 4D). In contrast, under conditions of oxidant-induced reduction, only a very small signal of the iron-sulfur center could be seen (Fig. 4A). Further addition of myxothiazol (Fig. 4B) or MOA-stilbene (not shown) abolished all residual EPR signal attributable to reduced iron-sulfur center. A special case was encountered when stigmatellin was added as a Qo site inhibitor. This compound raises the midpoint potential of the Rieske center by about 250 mV to nearly 600 mV (22), resulting in a reduced iron-sulfur center signal with \( g \) values characteristically shifted (Fig. 4C, cf. Ref. 23).

**DISCUSSION**

de Vries et al. first reported (16) a semiquinone species in beef heart submitochondrial particles, which can be generated under conditions of oxidant-induced reduction of the cytochrome bc1 complex and which was assigned at the time to a \( \text{Q}^- \) species, partly on the basis of its sensitivity to BAL, a known effector of the iron-sulfur center (17). This possibility of a \( \text{Q}^- \) species has gained importance in the light of the crystal structure information and the recent ideas on the bifurcation mechanism, and these have prompted us to reinvestigate the question further. The availability of newer specific Qo site inhibitors and the use of room temperature conditions to establish steady-state conditions have aided this task.

**Characterization of the EPR Signal Associated with the Second Semiquinone Species**—On the basis of stoichiometry relative to the amount of bc1 complex, pH dependence of amplitude, and power saturation behavior relative to that of \( \text{Q}^- \), we may conclude that the signal shown in Fig. 2A is equivalent to that described previously (16). However, the lack of significant effects of any of the Qo site inhibitors myxothiazol, MOA-stilbene, or stigmatellin argues compellingly against this species being located in the Qo site. Previously, it had been shown (16) that the signal can be abolished by aerobic treatment with BAL, a reaction known to disrupt the iron-sulfur center (17). However, this treatment requires extreme conditions and may well also have secondary effects on submitochondrial particles, accounting for the difference of effect in comparison with the more specific compounds that we have used. Effects of inhibitors of complexes I and II suggest that at least part of the signal may arise from semiquinones in other complexes. de Vries et al. (16) noted that the signal was still observed in such cross-inhibitor experiments, but the absence of quantitative data does not
allow direct comparison. The power saturation behavior of the signal in Fig. 2A confirms that it is equivalent to that described in Ref. 16. It is unlike that of the semiquinone signals associated with succinate dehydrogenase, which are not saturated until \( \geq 100 \) mW (24), and this is consistent with the indications from inhibitor sensitivities that it arises from a mixture of species.

**Redox Poise of the Iron-Sulfur Center**—The reduced form of the iron-sulfur center has a characteristic and strong EPR signal (25). However, only a very small signal attributable to reduced iron-sulfur center could be detected under conditions of oxidant-induced reduction, a result most simply interpreted as indicating that it is mostly in the oxidized state. Any small reduced fraction is most likely due to some residual electron transfer. Complete oxidation of the iron-sulfur center occurs when myxothiazol is added as a second inhibitor in addition to antimycin A (although without affecting the semiquinone signal).

It might also be noted that the ubiquinone pool (\( E_{\text{ub}} \) of +30 mV) will tend to reach equilibrium with the succinate/fumarate couple (\( E_{\text{ub}} \) of \( -3.5 \) mV (26)) in the antimycin-inhibited aerobic condition. Because no more than 10% of the succinate could have been oxidized to fumarate, we may conclude that the ubiquinone pool is practically fully reduced under these conditions.

**Mechanistic Implications**—The simplest conclusion from our data is that the oxidant-induced reduction steady state is characterized by a quinol, rather than a semiquinone, in the Q site and an oxidized state of the iron-sulfur center. Hence, quinol oxidation does not occur because the net formation of semiquinone by the first step does not occur to any significant extent rather than because there is a barrier to the reaction between semiquinone and oxidized iron-sulfur center.

This situation is as expected in the simple intermediate-controlled bifurcation model described in the Introduction, in which a very rapid second reaction is required to allow significant net reaction through the unstable intermediate. However, as outlined in the Introduction, other models for the obligatory bifurcation of electron transfer from the \( Q \) site are also possible, and our data may also be considered in relation to their predictions.

An oxidant-induced reduction situation in which the site is occupied by quinol and with the iron-sulfur center oxidized is generally consistent with any model involving a thermodynamically unstable semiquinone at the Q site, such as those described by Crofts and Wang (4) and Ding et al. (9). However, it does rule out models in which semiquinone should accumulate. In the catalytic switch model in its original form (10), a change does rule out models in which semiquinone should accumulate. This is not supported by our data. However, it has been pointed out by Link (12) that the two redox centers may be antiferromagnetically coupled in such a way that neither species could be detected by EPR spectroscopy. Some precedence comes from studies of the interaction of the quinone analogue 2,5-dibromo-3-methyl-6-isopropylbenzoquinone with the iron-sulfur center of the homologous plant cytochrome \( b \) complex, where redox titrations indicate that generation of the semiquinone form of 2,5-dibromo-3-methyl-6-isopropylbenzoquinone, while bound to the iron-sulfur center, results in loss of the iron-sulfur EPR signal (27). It was suggested that this arose from the antiferromagnetic coupling of the two species, although the corresponding semiquinone signal was not analyzed to confirm this point. In any case, the possibility that we observe neither semiquinone nor iron-sulfur center because they are strongly antiferromagnetically coupled cannot be excluded with our present measurements and may require an alternative spectroscopic technique to resolve the issue.

In summary, our data support a model of control of bifurcation governed simply by the instability of the intermediate semiquinone state that, in simplest form, may be described by the intermediate-controlled bifurcation model described in the Introduction. Models in which the semiquinone is stabilized are ruled out, except for the special case in which semiquinone may be strongly antiferromagnetically coupled to the reduced iron-sulfur center (12), a possibility that requires further investigation. Although other complications remain unresolved, the likely redox states of Q site semiquinone and of the iron-sulfur center under oxidant-induced reduction conditions, as established here, are key observations in limiting the mechanistic possibilities at this site.

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