Distinct properties of semiquinone species detected at the ubiquinol oxidation $Q_o$ site of cytochrome $bc_1$ and their mechanistic implications

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The two-electron ubiquinol oxidation or ubiquinone reduction typically involves semiquinone (SQ) intermediates. Natural engineering of ubiquinone binding sites of bioenergetic enzymes secures that SQ is sufficiently stabilized, so that it does not leave the site to membranous environment before full oxidation/reduction is completed. The ubiquinol oxidation $Q_o$ site of cytochrome $bc_1$ (mitochondrial complex III, cytochrome $b_6f$ in plants) has been considered an exception with catalytic reactions assumed to involve highly unstable SQ or not to involve any SQ intermediate. This view seemed consistent with long-standing difficulty in detecting any reaction intermediates at the $Q_o$ site. New perspective on this issue is now offered by recent, independent reports on detection of SQ in this site. Each of the described SQs seems to have different spectroscopic properties leaving space for various interpretations and mechanistic considerations. Here, we comparatively reflect on those properties and their consequences on the SQ stabilization, the involvement of SQ in catalytic reactions, including proton transfers, and the reactivity of SQ with oxygen associated with superoxide generation activity of the $Q_o$ site.

1. Introduction

Cytochrome $bc_1$ is one of the key enzymes of respiratory and photosynthetic electron transport chains. The enzyme couples electron transfer between ubiquinone/ubiquinol and cytochrome $c$ with proton translocation across the membrane. Typically, the transfer of electrons from ubiquinol to cytochrome $c$ contributes to generation of proton motive force used for adenosine triphosphate synthesis (for recent reviews, see [1,2]). However, in some cases, the direction of electron flow through cytochrome $bc_1$ can be reversed, leading to oxidation of cytochrome $c$ and reduction of ubiquinone [3,4].

The translocation of protons across the membrane involves two types of ubiquinone-binding sites facing opposite sides of the membrane: one site oxidizes ubiquinol, whereas the other reduces ubiquinone (figure 1). The joint action of these sites defines the basis of catalytic Q cycle. To secure energetic efficiency of this cycle, the ubiquinol oxidation site (the $Q_o$ site) directs electrons into two separate cofactor chains. One electron is used to reduce cytochrome $c_1$ via electron transfer through the Rieske cluster (FeS) and haem $c_1$ in one cofactor chain (the $c$-chain), whereas the other electron is transferred across the membrane to the $Q_o$ site via two haems $b$ (haem $b_1$ and $b_2$ of the $b$-chain).

The idea that oxidation of ubiquinol in complex III directs electrons into two separate chains, one involving cytochrome $b$ and the other cytochrome $c$, was introduced by Wikström & Berden in 1972 [7]. It emerged from a number of earlier observations documenting the intriguing effect of oxidant-induced
haem b reduction in the presence of antimycin (inhibitor of the Q site) (see [7] and references therein). This idea was preceded by a tentative scheme published in 1967 by Baum et al. [8], who also proposed two separate electron acceptors of ubiquinol, but in that work the connection between the two chains of cofactors was not yet understood. In 1975, Peter Mitchell adopted the idea of Wikström & Berden [7] and introduced the cyclic arrangement of electron transfer through the protonmotive Q cycle featuring two quinone binding sites (as we now know Qo and Qi sites), each standing at a divide of two cofactor chains [9,10]. In 1983, the Q cycle was modified by Crofts et al. [11], who realized that electrons for ubiquinone reduction at the Qi site both come from the same cofactor chain, leaving Qo as the only site separating the route for two electrons upon catalysis.

The reaction at the Qo site, often referred to as a bifurcation, is unusual in biology. Its mechanism is still a matter of intense debate. The lack of crystal structures containing native ubiquinone molecule bound in the Qo site [12] and a long-standing difficulty in spectroscopic identification of the intermediate states of the Qo site catalysis have left a high degree of freedom for mechanistic considerations [13–21]. Typically, because of the two-electron nature of ubiquinol oxidation or ubiquinone reduction, a semiquinone (SQ) species is expected to be formed as an intermediate of the reaction [22,23]. Indeed, such intermediates were detected by electron paramagnetic resonance (EPR) spectroscopy in several quinone binding sites, including the Qo site of cytochrome bc1 [24–26], the Qi site of photosynthetic reaction centre, and quinone sites of mitochondrial complex I and II (reviewed in [27–29]). All those sites are connected to a single chain of cofactors and, consequently, the two-electron oxidation/reduction of QH2/Q must proceed step-wise involving a relatively stable and manageable for experimental trapping SQ intermediate. However, the architecture of the Qo site creates distinctly different conditions for ubiquinol oxidation: the substrate binds in between the two chains of cofactors and thus can experience simultaneous presence of two redox centres (FeS cluster and haem b) ready to engage in electron transfers. In this case, the two-electron reaction does not need to proceed through the relatively long-lived SQ intermediate. With this simultaneous access to the two electron paths, a detection of SQ intermediate has proven difficult.

One of the early attempts of detection of a semiquinone radical within the Qo site (SQo) by equilibrium redox titration failed to detect a radical signal in CW EPR spectra of redox-poised bacterial chromatophores [30]. In mitochondrial system, the first report of detection of SQo [31] was questioned in later work [32] which led to a commonly accepted view that detection of this species, if it exists, falls beyond the limits of EPR sensitivity. This has been considered as confirmatory of Mitchell’s original idea that the stability constant of SQo (Ks) must be less than unity. However, recently three groups reported a detection of a SQ at the Qo site [33–36]. Intriguingly, each of the described SQs seems to have different spectroscopic properties. Additionally, the conditions in which they were trapped and subsequently detected by EPR were different. Here, we summarize those reports focusing on comparison of SQ species with respect to their interactions with paramagnetic cofactors of cytochrome bc1.
and interaction with nearby magnetic nuclei of protein surroundings (tables 1 and 2). We reflect on new mechanistic perspectives offered by these discoveries.

2. First report of antimycin-insensitive semiquinone signal on submitochondrial particles

In 1981, de Vries et al. [31] reported the detection of a new SQ in antimycin-inhibited submitochondrial particles under conditions of oxidant-induced reduction of haems $b$ initiated by addition of fumarate/succinate to the membranes. This SQ signal was antimycin-insensitive but disappeared after addition of British anti-Lewisite—a thiol-containing compound that disrupts the Rieske cluster in cytochrome $bc_1$ and abolishes activity of the Q$_o$ site. Spectral properties of this SQ were different from the antimycin-sensitive SQ signal originating from the Q$_i$ site (SQ$_i$). This new SQ had clearly slower spin-lattice relaxation rate than SQ$_i$ and exhibited smaller linewidth; the reported values were 8.3 and 10 G for the new SQ and SQ$_i$, respectively. It should be noted that subsequent literature reported the linewidth of approximately 8.5 G for SQ$_i$ signal [24,38,39].

The possible sensitivity of the antimycin-insensitive SQ to specific inhibitors of the Q$_o$ site was not tested by the authors of the original report. However, the later work by Rich and co-workers [32] showed that under similar experimental conditions this SQ signal was not sensitive to inhibitors that block the activity of the Q$_o$ site (myxothiazol, MOA-stilbene or stigmatellin), but at the same time, it was at least partially sensitive to several inhibitors of complex I and II.

3. Light-induced transient semiquinone in photosynthetic membranes

In 2007, Dutton and co-workers [33] generated SQ$_i$ in chromatophore membranes of photosynthetic bacterium Rhodobacter (R.) capsulatus, which consisted of a complete cyclic electron transfer system that can be activated by light. In this system, cytochrome $bc_1$ is coupled to photosynthetic reaction centre via cytochrome $c_2$ and ubiquinone pool (figure 2a). The authors predicted that SQ$_i$ should be visible at high pH which lowers the redox midpoint values of the quinone couples provided that multiple flashes are delivered to mostly oxidized $c$-chain. The key to promoting SQ$_i$ was to use the haem $b_{11}$ knockout in which the $b$-chain can accept only one electron [14]. Indeed, with the help of these predictions, they detected flash-induced SQ$_i$ in this mutant which, based on its properties, was assigned as SQ$_i$. The radical signal at $g = 2.004$ was detected by EPR after freezing of the light-induced samples, and the amplitude of the signal was different depending on the time delay before freezing suggestive of its transient character. The signal was sensitive to stigmatellin, a potent inhibitor of the Q$_i$ site, but not to myxothiazol—another inhibitor of the Q$_o$ site. To explain the differential sensitivity to the two inhibitors, the authors assumed that in the case of myxothiazol, the inhibitor and ubiquinone bind simultaneously. In this mode, the residual activity of the Q$_o$ site (interaction of ubiquinone with Rieske cluster) can still generate SQ$_i$. The idea of a simultaneous presence of ubiquinone and myxothiazol within the Q$_o$ site is inspired from crystallographic data which show that inhibitors can bind to distinctly different domains of the Q$_o$ site: stigmatellin forms hydrogen bond with histidine ligand of FeS cluster while myxothiazol binds closer to haem $b_1$ [40]. Furthermore, simultaneous binding of ubiquinol and $\beta$-methoxyacrylate inhibitors or binding of two molecules of ubiquinol was implicated from biochemical work [41,42] and more recent NMR studies [43]. However, recent data obtained from molecular dynamics (MD) simulations of cytochrome $bc_1$ suggest that the Q$_o$ site is a rather compact cavity and binding of additional quinone-like molecule next to the ubiquinol is energetically unfavourable [44].

To ascertain that the stigmatellin-sensitive signal originated from the Q$_o$ site but not from other ubiquinone reactive protein, the authors tested conditions where oxidizing power of high potential $c$-chain was severely limited by slowing the electron transfer through haem $c_1$ by orders of magnitude. As predicted, the light-induced SQ was not observed under those conditions, confirming that efficient outflow of electrons from Q$_o$ through the c-chain is necessary for SQ$_i$ generation.

The SQ$_i$ spectrum, having an EPR linewidth of 11.7 G, appeared broader than the spectrum of SQ formed at the Q$_i$ site (8.5 G). To explain the greater width of SQ$_i$ spectrum, the authors considered the possibility of magnetic interactions with reduced Rieske cluster. This should manifest itself in a difficulty to saturate the CW EPR signal of SQ$_i$ which, however, was not observed experimentally. Factors other than interaction with fast-relaxing paramagnetic centre that would explain the greater linewidth of the SQ$_i$ signal include greater $g$-tensor anisotropy [39] and/or hyperfine interactions with nearby magnetic nuclei [45] that are not resolved in CW EPR spectra at X-band.

4. Destabilized semiquinones in the Q$_o$ site detected in isolated cytochrome $bc_1$

Two publications by Kramer and co-workers [34,35] reported detection of SQ in the Q$_o$ site in isolated antimycin-inhibited bacterial and yeast cytochrome $bc_1$ under anaerobic conditions. In 2007, SQ was observed in the samples of R. capsulatus cytochrome $bc_1$ freeze-quenched 10 ms after mixing with ubiquinol analogue—decyubiquinol (DBH$_2$). Because cytochrome $c$ was absent (figure 2h) [34], to initiate the reaction at the Q$_o$ site, a significant fraction of Rieske cluster and cytochrome $c_1$ must have been in the oxidized state prior to mixing. This, however, is problematic given the relatively high redox midpoint potentials of these two cofactors and the fact that the experiments were carried out under anaerobic conditions. Native cytochrome $bc_1$ in this species, without any external oxidant added, typically shows 70–80% reduction level of cytochrome $c_1$ while significantly lower reduction levels may indicate some structural distortions or protein damage.

While the EPR radical signal was generally sensitive to stigmatellin, approximately 30% of the signal (SQ$_{res}$) still remained in the presence of this inhibitor. SQ$_{res}$ shared some of the characteristics of stigmatellin-sensitive signal which was assigned as SQ$_i$. Both SQ$_i$ and SQ$_{res}$ signals were broader than the signal of SQ$_i$ and both showed similar power-saturation profiles. On the other hand, addition of exogenous relaxation enhancer (Ni$^{2+}$ ions) suggested that the SQ$_{res}$ was more exposed to the aqueous phase. For that reason, SQ$_{res}$ was assigned to non-enzymatic oxidation of DBH$_2$ in solution. However, as the experiment was performed in the absence of...
Table 1. Comparison of spectroscopic features of different semiquinones reported for the Q<sub>i</sub> site. n.s., data not shown in the paper or experiment not performed; n.a., not applicable; SQ<sub>o</sub>, semiquinone in the Q<sub>i</sub> site; SQ<sub>res</sub>, stigmatellin-insensitive radical signal.

<table>
<thead>
<tr>
<th>properties of the signal/signals</th>
<th>de Vries et al. [31]</th>
<th>Zhang et al. [33]</th>
<th>Cape et al. [34]</th>
<th>Vennam et al. [35]</th>
<th>SQ&lt;sub&gt;o&lt;/sub&gt; uncoupled</th>
<th>SQ&lt;sub&gt;o&lt;/sub&gt; –FeS spin-coupled</th>
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<tbody>
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<td>transience of signal</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>sensitivity to specific Q&lt;sub&gt;i&lt;/sub&gt;-site inhibitors:</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>stigmatellin</td>
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<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
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<td>yes</td>
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<td>n.s.</td>
<td>n.s.</td>
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<td>yes</td>
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<td>n.a.</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>g-factor of central line of SQ&lt;sub&gt;o&lt;/sub&gt; [G]</td>
<td>2.005</td>
<td>2.0040</td>
<td>2.0054</td>
<td>2.0044&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.005</td>
<td>1.94</td>
</tr>
<tr>
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<td>11.7</td>
<td>11.9</td>
<td>11.6</td>
<td>14.2</td>
<td>n.a.</td>
</tr>
<tr>
<td>microwave power saturation</td>
<td>saturable; slower relaxation than SQ&lt;sub&gt;i&lt;/sub&gt;</td>
<td>saturable at 130 K; similar to SQ&lt;sub&gt;i&lt;/sub&gt;</td>
<td>saturable at 77 K; similar to SQ&lt;sub&gt;o&lt;/sub&gt;</td>
<td>n.s.</td>
<td>non-saturable at 200 K</td>
<td></td>
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<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>anti-Curie behaviour</td>
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<tr>
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<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
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<tr>
<td>dipole–dipole interactions with:</td>
<td>reduced FeS</td>
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<td>no</td>
<td>no</td>
<td>possibly yes</td>
<td>n.a.</td>
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<td>oxidized haem b&lt;sub&gt;L&lt;/sub&gt;</td>
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<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>interaction with magnetic nuclei</td>
<td>hydrogen</td>
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<td>n.s.</td>
<td>deprotonated</td>
<td>deprotonated</td>
<td>n.s.</td>
</tr>
<tr>
<td>nitro</td>
<td>n.s.</td>
<td>n.s.</td>
<td>deprotonated</td>
<td>deprotonated</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>&lt;sup&gt;a&lt;/sup&gt;Reported for yeast cytochrome bc&lt;sub&gt;1&lt;/sub&gt;.</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>&lt;sup&gt;b&lt;/sup&gt;Interaction between SQ&lt;sub&gt;o&lt;/sub&gt; –FeS coupled centre and oxidized haem b&lt;sub&gt;L&lt;/sub&gt; is inferred from pulse EPR measurements [37].</td>
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</table>
Myxothiazol (M) did not preclude the SQo trapping in (a). For simplicity, the second cytochrome initiated by light activation of reaction centre (RC), while in (b), the authors speculated that SQo is formed in the vicinity of myxothiazol binding site. In (c), dotted green lines denote the possible dipole–dipole interaction of SQo with haems what lead to paramagnetic relaxation enhancement (PRE) of SQo. The analysis of PRE resulted in assigning two possible locations of for SQo within the Qo site. For proton electron nuclear double resonance (ENDOR) spectrum from the chemically produced SQchem. Both types of SQs (i.e. SQo, SQres and SQchem) were in the anionic form. This was inferred from the observation that hyperfine coupling constant of five-methyl group to the SQ electron spin was reported to have indistinguishable CW EPR spectrum from the SQ chemically induced in buffer (SQchem). At the same time, SQo signal was reported to have indistinguishable CW EPR spectrum from the SQchem which was taken as indication that SQo and SQres showed decreased amplitudes (greater than 10-fold) in the presence of molecular oxygen. The signals were not seen in the bc1 subcomplex (a complex of cytochromes b and c1 but lacking FeS subunit [46]).

Analysis of proton ENDOR spectra indicated that all three types of SQs (i.e. SQo, SQres and SQchem) were in the anionic form. This was inferred from the observation that hyperfine coupling constant of five-methyl group to the SQ electron spin in all three cases was different from the values characteristic for protonated/neutral SQs. A contribution of central line in proton electron nuclear double resonance (ENDOR) spectrum was different from that found in the spectrum of SQchem which was taken as indication that both SQo and SQres are located in the environment of lower proton concentration comparing with the aqueous phase of the SQchem environment. Electron spin echo envelope modulation (ESEEM) spectra showed no indications that SQs form hydrogen bonds with amide group of polypeptide chain nor histidine residues. Importantly, the properties of SQo, including magnetic interactions between SQo and neighbouring paramagnetic cofactors of the Qo site, such as reduced FeS or oxidized haem b1. This, together with the confusing, in our view, properties of SQo and SQres and problematic initial state of the enzyme raise concern about the origin of the signals.

In 2013, Kramer and co-workers [35] described SQo trapped using a method similar to that described previously [34], except that this time cytochrome c was added to provide oxidizing power to the c-chain and initiate the reactions in the Qo site (figure 2c). While the width of new EPR signal of SQo was similar to that described previously, the relaxation properties were clearly different. The spin echo of SQs decayed (2p-ESEEM experiment) much faster in comparison with SQchem signal in the SQchem environment. Electron spin echo envelope modulation (ESEEM) spectra showed no indications that SQs form hydrogen bonds with amide group of polypeptide chain nor histidine residues. Importantly, the properties of SQo, including magnetic interactions between SQo and neighbouring paramagnetic cofactors of the Qo site, such as reduced FeS or oxidized haem b1. This, together with the confusing, in our view, properties of SQo and SQres and problematic initial state of the enzyme raise concern about the origin of the signals.

Table 2. Comparison of the experimental conditions of trapping and measurements of semiquinone in the Qo site. n.s., not shown or not performed; SMP, submitochondrial particles; cyt., cytochrome; R. caps., Rhodobacter capsulatus; S. cerev., Saccharomyces cerevisiae.

<table>
<thead>
<tr>
<th>Source/Organism</th>
<th>de Vries et al. [31]</th>
<th>Zhang et al. [33]</th>
<th>Cape et al. [34]</th>
<th>Vennam et al. [35]</th>
<th>Sarewicz et al. [36]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic (A) or Anaerobic (AN)</td>
<td>A</td>
<td>AN</td>
<td>AN</td>
<td>AN</td>
<td>A</td>
</tr>
<tr>
<td>External Oxidant</td>
<td>cyt. c</td>
<td>cyt. c</td>
<td>none</td>
<td>cyt. c</td>
<td>cyt. c</td>
</tr>
<tr>
<td>Isolated Protein (I) or Membranes (M)</td>
<td>M (SMP)</td>
<td>M</td>
<td>I</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Temperature of Detection (K)</td>
<td>CW EPR</td>
<td>50</td>
<td>130</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Detection (K)</td>
<td>Pulsed</td>
<td>n.s.</td>
<td>n.s.</td>
<td>60</td>
<td>10–100</td>
</tr>
<tr>
<td>Reaction Time (s)</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
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</table>

Oxygen, this oxidation could not have been associated with O2. Rather, one can envisage that SQres formation might have been a result of a disproportionation. SQres exhibited different proton electron nuclear double resonance (ENDOR) spectrum from the SQ chemically induced in buffer (SQchem). At the same time, SQo signal was reported to have indistinguishable CW EPR spectrum from the chemically produced SQchem. Both SQo and SQres showed decreased amplitudes (greater than 10-fold) in the presence of molecular oxygen. The signals were not seen in the bc1 subcomplex (a complex of cytochromes b and c1 but lacking FeS subunit [46]).
the SQ$_o$ interacted with fast-relaxing paramagnetic species. The authors concluded that the paramagnetic species that affect SQ$_o$ are haems nearest to SQ$_o$ which, based on simulations, were proposed to be either two haems $b_L$ (each coming from individual monomers of cytochrome $bc_1$ dimer) or haem $b_L$ and haem $c_1$ (both coming from the same monomer; figure 2c). However, no spectroscopic data verifying the oxidation state of haems were provided, nor relaxation rates for haems used in simulations, which are crucial parameters in determining distances by the use of relaxation enhancement [47,48]. The FeS cluster was excluded because of its slow relaxation when compared with haems at the temperature used in the experiments.

While the new SQ signal was generally sensitive to stigmatellin, around 30% of the signal was still observed in CW EPR spectra in the presence of substoichiometric concentration of this inhibitor. The sensitivity to other Qo site inhibitors was not reported and it was not shown whether this new SQ signal disappears in the control mutants with inactive Qo site. The overall shape of SQ$_o$ proton ENDOR spectrum was similar to those reported previously for SQ$_o$ and SQ$_{res}$ indicating that SQ$_o$ was deprotonated. Nevertheless, the splitting of doublet signals flanking the distant protons peak in ENDOR spectra was clearly larger than previously reported [34] implying that the detected SQ$_o$s were in different environments.

The analysis of $4p$-ESEEM spectra combined with the lack of the signal of nitrogen in $2p$-ESEEM indicated that SQ$_o$ was not hydrogen-bonded to the protein. Comparison of bacterial and yeast cytochrome $bc_1$ did not reveal any spectral differences which indicated that SQ$_o$ in both cases is the same chemical species trapped in similar environment.

The properties of SQ$_o$ that emerged from ESEEM and ENDOR data led the authors to propose a model of ‘electrostatic cage’ trapping deprotonated SQ$_o$. In this model, SQ is destabilized by lack of specific binding through hydrogen bonds or salt bridges. Insulating dielectric cage blocks the proton uptake back to SQ$_o$ which secures that it does not leave the site. At the same time, the cage is supposed to prevent escape of any superoxide anion (or SQ) formed in the site. However, the destabilized SQ$_o$ is proposed to conserve sufficient redox energy to reduce haem $b_L$ which seems difficult to reconcile with the statement that SQ$_o$ interacts paramagnetically with the oxidized haem $b_L$. Furthermore, it is important to bear in mind that in photosynthetic reaction centres a similar concept of low dielectric gate around the SQ binding site was introduced to rationalize high stability of SQ because the contributions from electrostatic energy and hydrogen bonds were not enough to explain SQ stabilization [49].

5. Semiquinone uncoupled and spin–spin coupled to Rieske cluster in isolated cytochrome $bc_1$

In 2013, our group reported a discovery of two EPR transitions associated with the activity of the Qo site [36]. Those transitions revealed the presence of two distinct populations of SQ$_o$, formed at this site. The first signal at $g = 1.94$ was assigned as one of the transitions originating from the spin–spin exchange of two unpaired electron spins: one coming from SQ$_o$ and the other from the reduced Rieske cluster (figure 3a). The second transition near $g = 2.0$ corresponded to the population of SQ$_o$ for which the spin–spin exchange did not exist or was too weak to be resolved (figure 3b). Both populations were observed in samples of isolated, antimycin-inhibited cytochrome $bc_1$ of R. capsulatus exposed to substrates.

Figure 3. Structural model explaining the existence of two populations of SQ$_o$, detected by CW EPR [36]. (a) Reduced FeS cluster is in the Qo site and putative hydrogen bond between SQ$_o$ and the cluster liganding histidine 156 (R. capsulatus numbering) facilitates spin–spin exchange interaction. (b) Increasing the distance between SQ$_o$ and the FeS cluster owing to the dynamics of the FeS head domain and/or semiquinone within the site abolish the spin–spin exchange interaction and only dipole–dipole magnetic interactions between SQ$_o$ and neighbouring fast-relaxing metal centres are possible. Structures shown in left panel are based on MD simulations [44].
DBH₂ and oxidized cytochrome c, under aerobic conditions. The changes in the amplitudes for these two signals (radical at g = 2.0 and SQo–FeS spin-coupled centre at g = 1.94) during the catalytic turnover can be divided into two time regions. In the first (earlier) region, the amplitudes increase until they reach maximum, whereas in the second (later) region, the amplitudes progressively decrease to zero at the time point when the system reaches equilibrium.

Both signals were sensitive to stigmatellin and several other Qₐ-site-specific inhibitors (including myxothiazol and various synthetic strobilurins). Both signals were not observed in specific mutants that disabled activity of the Qₐ site (such as cyt b–G158 W) [42,50] or the bc-subcomplex [46]. Moreover, in the presence of these inhibitors or mutations, no residual radical signals were detected. On the other hand, in +2Ala mutant (a mutation that makes the FeS head domain stay at Qₐ site for prolonged time), the signal amplitude was higher compared with the native protein. More recent experiments indicate that both signals can also be generated under anaerobic conditions and that the characteristic g = 1.94 can be observed in native chromatophore membranes of R. capsulatus [37].

We proposed that the two populations of SQo reflect two configurations of the Qₐ site. The spin–spin exchange (g = 1.94) by its nature has a clear distance constraint and can take place only when SQo and Rieske cluster are in proximity, as shown in figure 3e. In this configuration, a formation of a hydrogen bond between histidine residue coordinating Rieske cluster and ubiquinone molecule is possible. At larger distances (figure 3b) or upon breaking the putative hydrogen bond between SQo and histidine ligand, spin–spin exchange disappears and SQo becomes detected as a separate free radical species having a signal near g = 2.0. Nevertheless, in this case, SQo exhibited unusually fast relaxation compared with the relaxation of chemically generated SQ in buffer (by auto-oxidation of DBH₂ in alkaline pH), which was expected given that the SQo is located in proximity to fast-relaxing paramagnetic metal centres of the Qₐ site: oxidized haem b₁ [51] and/or reduced FeS [32]. The fast spin-lattice relaxation of SQo manifested itself in significant homogeneous broadening of the EPR lines (both at X and Q band), the inability to saturate it with microwave power, and the presence of a Leigh effect (decrease in amplitude without apparent line broadening upon decrease of temperature). All these specific properties differentiated this SQo from the radical signals described in [33–35].

The two populations of SQo were incorporated to the model of electronic bifurcation of the Qₐ site. We envisaged that the SQo–FeS (g = 1.94) form might represent an initial step of ubiquinol oxidation when oxidized FeS withdraws an electron from ubiquinol. This state evolves into the state where SQo, and reduced FeS exist as separate identities (distinguished by separate spectra, one of which is radical g = 2.0) before reduction of haem b₁ by SQo takes place to complete the oxidation of QH₂ at this site.

6. Semiquinone intermediates in relation to proton management of the Q₀ site

The process of uptake and release of protons is an inherent part of redox chemistry of ubiquinones. As the energy of the SQH₂⁺ (double protonated SQ) is very high [53], at least one proton needs to be released during oxidation of QH₂ to make transfer of the first electron possible. Accordingly, in the ubiquinol oxidation at the Qₐ site, a release of one or two protons is often considered to be a step initiating the entire reaction [15,54,55]. While the proton paths are largely unknown for the Qₐ site, the detected SQo intermediates offer interesting new insights into this issue.

The radicals with typical g = 2.0 are believed to be in a deprotonated/anionic form. Thus, it is plausible to expect that these SQs are relevant to a state having the two protons already released (here we consider the direction of ubiquinol oxidation; figure 4a). However, the spin–spin exchange state (g = 1.94), which most likely involves the hydrogen bond between histidine ligand of Rieske cluster and ubiquinone molecule, could represent a state before the proton release. For this state, at least two scenarios are possible.

The first scenario would accommodate an early model of proton pathway which proposed that initially deprotonated histidine ligand of Rieske undergoes protonation upon formation of hydrogen bond with ubiquinone molecule to subsequently withdraw the first proton from ubiquinone [55,56]. This hydrogen bond could be a good candidate for an element of the spin–spin exchange configuration (g = 1.94; figure 4b). This model, however, requires that histidine residue is maintained by the enzyme in a deprotonated form before it reacts with ubiquinol, which, as discussed in [44,57], is disputable.

The attractive alternative emerges from recent MD simulations which indicate that water molecules in the Qₐ site can directly accept protons from ubiquinol upon its oxidation [44]. In this scenario, water molecules form hydrogen bonds with ubiquinol molecule. While protonated waters are short-lived, they may form an easy path for protons out of the protein through the cavity filled with water molecules. Hydrogen bond is also formed between histidine residue (protonated) and ubiquinol molecule but this bond is not involved in proton transfers from ubiquinol to the aqueous phase. This hydrogen bond may also serve as an inherent part of the configuration supporting spin–spin exchange between SQo and FeS cluster (figure 4c). Unlike the first scenario, this model allows the hydrogen-bonded configuration for spin–spin exchange to be assembled independently of ubiquinol proton stripping events.

7. Emerging questions about stability of SQo and its reactivity with oxygen

Quinones in solution under equilibrium undergo compoprtionation (reverse of disproportionation) reaction according to the scheme [23,58]:

\[ Q + QH₂ = 2SQ⁻ + 2H⁺. \]

The equilibrium constant Kₛ for this reaction is often referred to as the stability constant for SQ which depends on the redox potentials of Q/SQ and SQ/QH₂ pairs (EQ/SQ and ESQ/QH₂, respectively):

\[ Kₛ = \frac{[SQ]^{-2} \cdot [H⁺]^2}{[Q]^{-1} \cdot [QH₂]} = 10^{[E_{SQ/QH₂}(mV) – E_{SQ/Q}(mV)]/591}. \]

For ubiquinone-10, the redox potentials of EQ/SQ and ESQ/QH₂ couples at pH 7 are −230 and +190 mV in bulk solution [23], respectively, which means that Kₛ is around 10⁻⁸. When
concentrations of both Q and QH2 are, for example 100 μM, then equilibrium concentration of SQ− is approximately 30 nM, which is at the lower limit of concentration needed to detect this species by EPR spectroscopy.

The $K_s$ has a strict sense when considering comproportionation/disproportionation of Q/SQ/QH2 triad under equilibrium in solution but it is often used to describe the stability of SQ that can be formed within the Qo sites of cytochrome $bc_1$ even though species formed in the catalytic sites are insulated from bulk solution and they are unable to disproportionate directly [59]. Since original Mitchell’s description of the Q cycle, SQo, has been considered as highly unstable with the low stability constant $K_s$ of the order of $10^{-7}$ or less [13,30,32,59,60]. This however remains an open question in the light of the SQo detections which report signals in the range from 1% up to 17% of the total Qo sites. Cape et al. reported that SQo occupies 0.01–0.1 Qo sites per monomer [34]. Given the total concentration of 10 μM for both cytochrome $bc_1$ and QH2, it is possible to calculate the value of $K_s$ around $10^{-2}$. Similar calculations performed by Sarewicz et al. give the estimated $K_s$ of the order of $10^{-2.6}$ [36]. These values are in agreement with measured concentration of radicals reported for chemically modified SQs in solutions (10 μM of chloride-substituted quinone produces 260 nM of SQ with $K_s$ that is larger than $10^{-7}$) [61]. Such relatively large values of $K_s$ suggest some kind of stabilization of SQo in comparison with bulk solutions. We emphasize, however, a potential difficulty in describing stability of SQo using the $K_s$ parameter, because all reported SQo signals in cytochrome $bc_1$ were detected under non-equilibrium conditions for which the $K_s$ parameter defining thermodynamic equilibrium may not be valid.

Figure 4. Different possibilities of proton involvement in the interactions of SQo (green) with the cluster liganding histidine (black) and/or water (blue). (a) SQo anion does not form a hydrogen bond with the histidine that reversibly exchange proton (red) with water molecules (arrows represent the reversibility of the reaction). (b) Protonated SQo reversibly donates proton to histidine which results in formation of hydrogen bond between this histidine and SQo anion. (c) Neutral SQo forms hydrogen bond with protonated histidine, whereas the proton originating from SQo is exchanged with water molecule. All three cases (a–c) may exist in an equilibrium but only in cases (b, right-hand panel) and (c, both panels) is a relatively strong spin–spin exchange interaction expected between SQo and the Fe5 cluster (magenta shadow shows the possible paths for the electron spin exchange).
Considering the properties and conditions of trapping (table 1 and 2, respectively) the SQo intermediates summarized above it appears as if different SQ species for the Qo site have been reported. A feature that unites all these reports is the observation that SQo cannot be detected in cytochrome bc1 unless the Q site is inhibited by antimycin or haem h11 is knocked-out by mutation. This effectively impedes oxidation of haem h1 through the path involving haem b11/Q. It thus appears that the state with reduced haem h1 is required as condition for increasing probability of trapping SQo. In reversibly operating Qo site, SQo can, in principle, be formed in two ways and both indeed require reduced haem h1 as an initiation [62–66]. In a semiforward reaction, reduced haem h1 prevents electron transfer from SQo to haem h1 after oxidized FeS initially withdraws one electron from QH2 forming SQo. In a semireverse reaction, reduced haem h1 initiates SQo formation by electron transfer to Q. In this context, the properties of SQo in [33,34] suggest that SQo was formed along with reduced haem h1 pointing towards the semiforward reaction scheme. On the other hand, the properties of SQo in [36] and [35] indicate that it was trapped along with oxidized haem h1 which points towards the semireverse reaction scheme. This mechanism is also supported by the observation that the rate of superoxide generation has a bell-shaped dependence on Q/QH2 ratio [67,68].

Interestingly, the semireverse reaction has recently been considered as the one leading to formation of SQo that can interact with oxygen and thus is responsible for generation of superoxide by the Qo site [63–65]. In this scheme, unlike in a semiforward scheme, SQo can be formed in the configuration of the Qo site that misses the second cofactor necessary to complete the reaction. The missing cofactor is the FeS cluster embedded in the head domain which during the catalytic cycle naturally undergoes movement between the Qo site and haem c1 (outermost cofactor of the c-chain) [69]. It is thus possible that Q is reduced by haem h1 at the time when FeS cluster occupies positions remote from the Qo site, and is unable to immediately engage in electron transfer reaction with SQo. This increases the probability of reaction of SQo with oxygen (if all electron transfers compete kinetically), as indeed implicated experimentally [64,65,68].

The presumed high reactivity of SQo with oxygen implies that anaerobic conditions should promote trapping SQo. The reports of detection of SQo signals under anaerobic condition follow this expectation [33–35]. In one of these cases, it was additionally recognized that SQo could not have been observed under aerobic conditions [34]. There was also another report of failure to detect SQo in the presence of molecular oxygen, but those experiments were performed using freeze-quenched samples of cytochrome bc1 non-inhibited by antimycin [20]. However, the report of detection of two populations of SQo (g = 1.94 and g = 2.0) concerned aerobic conditions [36]. The relatively large quantities of SQo (spin–spin coupled to the Rieske cluster) detected under these conditions suggest that SQo is not as highly reactive with oxygen as commonly presumed at least in the presence of the spin exchange. In addition, high levels of SQo were observed in +2Ala mutant, which does not produce any detectable superoxide [64], implying that conditions of spin–spin coupling between SQo and FeS (g = 1.94) might be protective against superoxide generation.

8. Concluding remark
The assumption about extremely low $K_s$ of SQo, has traditionally been used to explain the long-standing difficulty in experimental detection of SQo. We now seem to face the opposite situation where several seemingly different SQo intermediates have been exposed. The differences concern both the properties of SQo species and the experimental conditions used to trap the SQo intermediates. In our view, this certainly does not make it easier for a general reader to follow the progress in understanding the mechanism of ubiquinol oxidation at the Qo site as it leaves space for various interpretations and mechanistic considerations that at this stage do not seem to converge into one generally accepted model of action. It remains to be seen whether the detected SQo signals represent the same intermediate of the Qo site, or rather reflect different states of the reaction scheme. Are all of them truly associated with the operation of the Qo site? What is the role of haem h1 in the formation of SQo and superoxide production? How does the intermonomer electron transfer between the two haems h1 influence these reactions? The available set of data on SQo provides now a framework for further studies in which various hypotheses can be critically examined and verified. Hopefully, this will lead to the formulation of the integrated model of the Qo site catalysis and its involvement in superoxide generation.

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Endnote
1. We use the ‘proton translocation’ term in the context of cytochrome bc1 catalysis to describe creation of proton gradient by coupled oxidation/reduction of ubiquinol/ubiquinone taking place at the opposite sides of the membrane which is a different mechanism from active ‘proton pumping’ via specific proton channels within protein interiors.

References
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