

## Headline review

Bioenergetics

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Artur Osyczka

e-mail: [artur.osyczka@uj.edu.pl](mailto:artur.osyczka@uj.edu.pl)Distinct properties of semiquinone species detected at the ubiquinol oxidation  $Q_o$  site of cytochrome  $bc_1$  and their mechanistic implications

Rafał Pietras, Marcin Sarewicz and Artur Osyczka

Department of Molecular Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

RP, 0000-0001-8424-6590; AO, 0000-0002-1637-2365

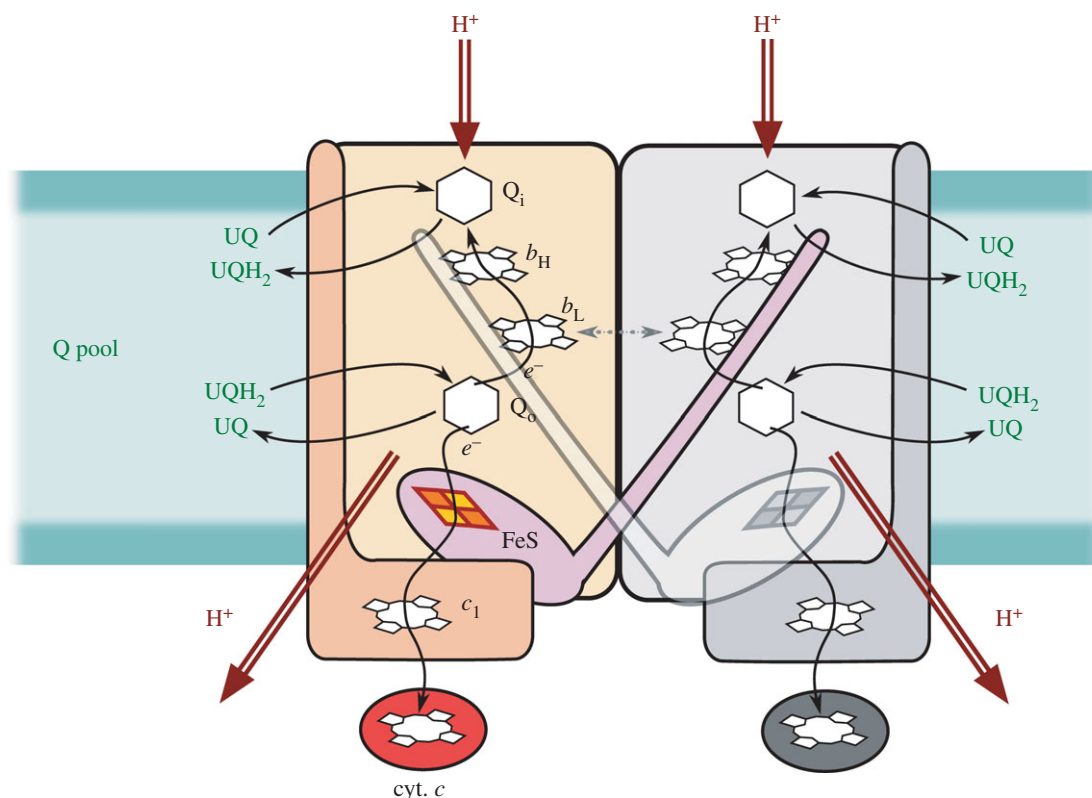
The two-electron ubiquinol oxidation or ubiquinone reduction typically involves semiquinone (SQ) intermediates. Natural engineering of ubiquinone binding sites of bioenergetic enzymes secures that SQ is sufficiently stabilized, so that it does not leave the site to membranous environment before full 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<sup>1</sup> across the membrane. Typically, the transfer of electrons from ubiquinol to cytochrome  $c$  contributes to generation of protonmotive 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  $Q_i$  site via two haems  $b$  (haem  $b_L$  and  $b_H$  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



**Figure 1.** Diagram of homodimeric cytochrome  $bc_1$  structure describing the general mechanism of enzymatic turnover. The ubiquinone binding sites  $Q_i$ ,  $Q_o$  together with haems  $b_L$  and  $b_H$  ( $b$ -chain) are embedded in cytochrome  $b$  subunit (light orange rectangle). The Rieske protein (light magenta) harbouring  $2\text{Fe}-2\text{S}$  ( $\text{FeS}$ ) iron–sulfur cluster and cytochrome  $c_1$  subunit (dark orange) with haem  $c_1$  transfer the electrons from  $Q_o$  site to cytochrome  $c$  (red). The proton uptake and release is indicated by red arrows. The intermonomer electron transfer at the level of two haems  $b_L$  [5,6] is indicated by dashed arrow. For clarity, the second monomer is shown in grey. Ubiquinone (UQ) and ubiquinol ( $\text{UQH}_2$ ) constitute the Q pool.

haem  $b$  reduction in the presence of antimycin (inhibitor of the  $Q_i$  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  $Q_o$  and  $Q_i$  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  $Q_i$  site both come from the same cofactor chain, leaving  $Q_o$  as the only site separating the route for two electrons upon catalysis.

The reaction at the  $Q_o$  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  $Q_o$  site [12] and a long-standing difficulty in spectroscopic identification of the intermediate states of the  $Q_o$  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  $Q_i$  site of cytochrome  $bc_1$  [24–26], the  $Q_B$  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  $\text{QH}_2/\text{Q}$  must proceed step-wise involving a relatively stable and manageable for experimental trapping SQ intermediate. However, the architecture of the  $Q_o$  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 ( $\text{FeS}$  cluster and haem  $b_L$ ) 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  $Q_o$  site ( $\text{SQ}_o$ ) 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  $\text{SQ}_o$  [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  $\text{SQ}_o$  ( $K_s$ ) must be less than unity. However, recently three groups reported a detection of a SQ at the  $Q_o$  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  $bc_1$

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*<sub>1</sub> and abolishes activity of the Q<sub>o</sub> site. Spectral properties of this SQ were different from the antimycin-sensitive SQ signal originating from the Q<sub>i</sub> site (SQ<sub>i</sub>). This new SQ had clearly slower spin-lattice relaxation rate than SQ<sub>i</sub> and exhibited smaller linewidth; the reported values were 8.3 and 10 G for the new SQ and SQ<sub>i</sub>, respectively. It should be noted that subsequent literature reported the linewidth of approximately 8.5 G for SQ<sub>i</sub> signal [24,38,39].

The possible sensitivity of the antimycin-insensitive SQ to specific inhibitors of the Q<sub>o</sub> 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<sub>o</sub> 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<sub>o</sub> 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*<sub>1</sub> is coupled to photosynthetic reaction centre via cytochrome *c*<sub>2</sub> and ubiquinone pool (figure 2a). The authors predicted that SQ<sub>o</sub> 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<sub>o</sub> was to use the haem *b*<sub>H</sub> knockout in which the *b*-chain can accept only one electron [14]. Indeed, with the help of these predictions, they detected flash-induced SQ in this mutant which, based on its properties, was assigned as SQ<sub>o</sub>. 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<sub>o</sub> site, but not to myxothiazol—another inhibitor of the Q<sub>o</sub> 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<sub>o</sub> site (interaction of ubiquinone with Rieske cluster) can still generate SQ<sub>o</sub>. The idea of a simultaneous presence of ubiquinone and myxothiazol within the Q<sub>o</sub> site is inspired from

crystallographic data which show that inhibitors can bind to distinctly different domains of the Q<sub>o</sub> site: stigmatellin forms hydrogen bond with histidine ligand of FeS cluster while myxothiazol binds closer to haem *b*<sub>L</sub> [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*<sub>1</sub> suggest that the Q<sub>o</sub> 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<sub>o</sub> 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*<sub>1</sub> by orders of magnitude. As predicted, the light-induced SQ was not observed under those conditions, confirming that efficient outflow of electrons from Q<sub>o</sub> through the *c*-chain is necessary for SQ<sub>o</sub> generation.

The SQ<sub>o</sub> spectrum, having an EPR linewidth of 11.7 G, appeared broader than the spectrum of SQ formed at the Q<sub>i</sub> site (8.5 G). To explain the greater width of SQ<sub>o</sub> 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<sub>o</sub>, which, however, was not observed experimentally. Factors other than interaction with fast-relaxing paramagnetic centre that would explain the greater linewidth of the SQ<sub>o</sub> 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<sub>o</sub> site detected in isolated cytochrome *bc*<sub>1</sub>

Two publications by Kramer and co-workers [34,35] reported detection of SQ in the Q<sub>o</sub> site in isolated antimycin-inhibited bacterial and yeast cytochrome *bc*<sub>1</sub> under anaerobic conditions. In 2007, SQ was observed in the samples of *R. capsulatus* cytochrome *bc*<sub>1</sub> freeze-quenched 10 ms after mixing with ubiquinol analogue—decylubiquinol (DBH<sub>2</sub>). Because cytochrome *c* was absent (figure 2b) [34], to initiate the reaction at the Q<sub>o</sub> site, a significant fraction of Rieske cluster and cytochrome *c*<sub>1</sub> 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*<sub>1</sub> in this species, without any external oxidant added, typically shows 70–80% reduction level of cytochrome *c*<sub>1</sub> 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<sub>res</sub>) still remained in the presence of this inhibitor. SQ<sub>res</sub> shared some of the characteristics of stigmatellin-sensitive signal which was assigned as SQ<sub>o</sub>. Both SQ<sub>o</sub> and SQ<sub>res</sub> signals were broader than the signal of SQ<sub>i</sub> and both showed similar power-saturation profiles. On the other hand, addition of exogenous relaxation enhancer (Ni<sup>2+</sup> ions) suggested that the SQ<sub>res</sub> was more exposed to the aqueous phase. For that reason, SQ<sub>res</sub> was assigned to non-enzymatic oxidation of DBH<sub>2</sub> in solution. However, as the experiment was performed in the absence of

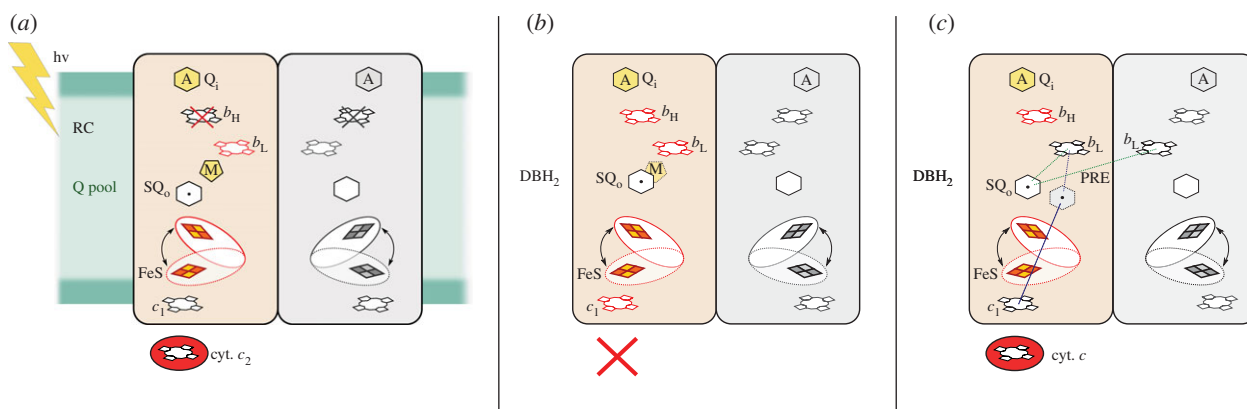
**Table 1.** Comparison of spectroscopic features of different semiquinones reported for the  $Q_0$  site. n.s., data not shown in the paper or experiment not performed; n.a., not applicable;  $SQ_{0p}$ , semiquinone in the  $Q_0$  site;  $SQ_i$ , semiquinone in the  $Q_i$  site;  $SQ_{res}$ , stigmatellin-insensitive radical signal.

properties of the signal/signals	$SQ_{0p}$ reported by				
	de Vries <i>et al.</i> [31]	Zhang <i>et al.</i> [33]	Cape <i>et al.</i> [34]	Vennam <i>et al.</i> [35]	Sarewicz <i>et al.</i> [36]
transience of signal	n.s.	yes	n.s.	n.s.	yes
sensitivity to specific $Q_0$ -site inhibitors:					
stigmatellin	n.s.	yes	yes	yes	yes
myxothiazol	n.s.	no	n.s.	n.s.	yes
strobilurins	n.s.	n.s.	n.s.	n.s.	yes
presence of stigmatellin-insensitive residual signal	n.a.	n.a.	yes	yes	no
$g$ -factor of central line	2.005	2.0040	2.0054	2.0044 <sup>a</sup>	2.005
linewidth of X-band spectrum of $SQ_0$ [G]	8.3	11.7	11.9	11.6	14.2
microwave power saturation	saturable; slower relaxation than $SQ_i$	saturable at 130 K; similar to $SQ_i$	saturable at 77 K; similar to $SQ_{res}$	n.s.	non-saturable at 200 K
temperature-dependence of the EPR spectrum amplitude	n.s.	n.s.	n.s.	n.s.	anti-Curie behaviour
spin – spin exchange interactions	no	no	no	no	no
dipole – dipole interactions with:					
reduced FeS	no	no	no	no	possibly yes
oxidized haem $b_L$	no	no	no	yes	yes
$c_1$					yes <sup>b</sup>
interaction with magnetic nuclei	n.s.	n.s.	deprotonated	deprotonated	n.s.
hydrogen					
nitrogen	n.s.	n.s.	no	no	n.s.

<sup>a</sup>Reported for yeast cytochrome  $b_{c_1}$ .

<sup>b</sup>Interaction between  $SQ_{0p}$  – FeS coupled centre and oxidized haem  $b_L$  is inferred from pulse EPR measurements [37].





**Figure 2.** Schematic of the SQ intermediate trapped in the Q<sub>0</sub> site with the corresponding enzyme state as reported in (a) [33], (b) [34] and (c) [35]. The redox states of cytochrome bc<sub>1</sub> cofactors (FeS and haems) were either reduced or oxidized (red or black contour, respectively). In all cases, the Q<sub>1</sub> site was occupied by antimycin (A). Myxothiazol (M) did not preclude the SQ<sub>0</sub> trapping in (a). In (b), the authors speculated that SQ<sub>0</sub> is formed in the vicinity of myxothiazol binding site. In (c), dotted green lines denote the possible dipole–dipole interaction of SQ<sub>0</sub> with haems what lead to paramagnetic relaxation enhancement (PRE) of SQ<sub>0</sub>. The analysis of PRE resulted in assigning two possible locations of for SQ<sub>0</sub> within the Q<sub>0</sub> site. For simplicity, the second cytochrome bc<sub>1</sub> monomer was shaded. In (a) the reaction was initiated by light activation of reaction centre (RC), while in (b) and (c) the reaction was initiated by injection of the reduced ubiquinone analogue (DBH<sub>2</sub>).

**Table 2.** Comparison of the experimental conditions of trapping and measurements of semiquinone in the Q<sub>0</sub> site. n.s., not shown or not performed; SMP, submitochondrial particles; cyt., cytochrome; *R. caps.*, *Rhodobacter capsulatus*; *S. cerev.*, *Saccharomyces cerevisiae*.

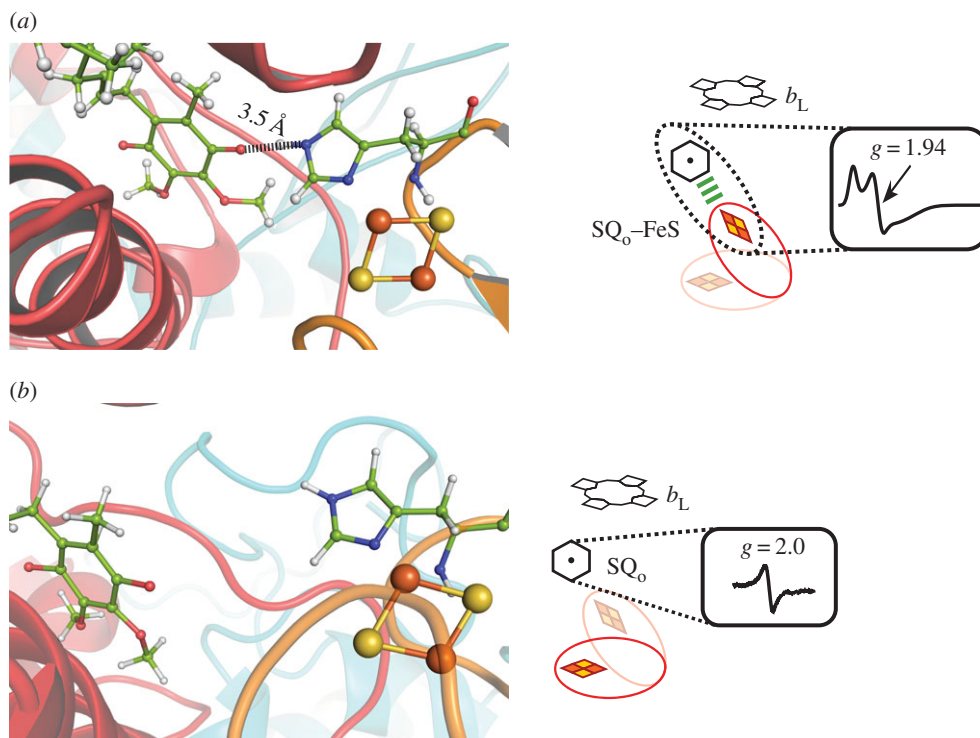
		de Vries <i>et al.</i> [31]	Zhang <i>et al.</i> [33]	Cape <i>et al.</i> [34]	Vennam <i>et al.</i> [35]	Sarewicz <i>et al.</i> [36] SQ <sub>0</sub> uncoupled	SQ <sub>0</sub> –FeS spin-coupled
aerobic (A) or anaerobic (AN)		A	AN	AN	AN	A	A
external oxidant		cyt. <i>c</i>	cyt. <i>c</i> <sub>2</sub>	none	cyt. <i>c</i>	cyt. <i>c</i>	cyt. <i>c</i>
isolated protein (I) or membranes (M)		M (SMP)	M	I	I	I	I
source/organism		beef heart	<i>R. caps.</i>	<i>R. caps.</i>	<i>R. caps.</i> and <i>S. cerev.</i>	<i>R. caps.</i>	<i>R. caps.</i>
temperature of detection (K)	CW EPR	50	130	77	77	105–210	20
	pulsed EPR	n.s.	n.s.	60	10–100	n.s.	10, 20
Q <sub>1</sub> site blocked by antimycin		yes	yes	yes	yes	yes	yes

oxygen, this oxidation could not have been associated with O<sub>2</sub>. Rather, one can envisage that SQ<sub>res</sub> formation might have been a result of a comproportionation. SQ<sub>res</sub> exhibited different proton electron nuclear double resonance (ENDOR) spectrum from the SQ chemically induced in buffer (SQ<sub>chem</sub>). At the same time, SQ<sub>0</sub> signal was reported to have indistinguishable CW EPR spectrum from the chemically produced SQ<sub>chem</sub>. Both SQ<sub>0</sub> and SQ<sub>res</sub> showed decreased amplitudes (greater than 10-fold) in the presence of molecular oxygen. The signals were not seen in the bc<sub>1</sub> subcomplex (a complex of cytochromes *b* and *c*<sub>1</sub> but lacking FeS subunit [46]).

Analysis of proton ENDOR spectra indicated that all three types of SQs (i.e. SQ<sub>0</sub>, SQ<sub>res</sub> and SQ<sub>chem</sub>) 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 SQ<sub>0</sub> and SQ<sub>res</sub> ENDOR spectra was different from that found in the spectrum of SQ<sub>chem</sub> which was taken as indication that both SQ<sub>0</sub> and SQ<sub>res</sub> are located in the environment of lower

proton concentration comparing with the aqueous phase of the SQ<sub>chem</sub> 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 SQ<sub>0</sub>, including power saturation behaviour, did not reveal signs of dipolar magnetic interactions between SQ<sub>0</sub> and neighbouring paramagnetic cofactors of the Q<sub>0</sub> site, such as reduced FeS or oxidized haem *b*<sub>L</sub>. This, together with the confusing, in our view, properties of SQ<sub>0</sub> versus SQ<sub>res</sub> and problematic initial state of the enzyme raise concern about the origin of the signals.

In 2013, Kramer and co-workers [35] described SQ<sub>0</sub> 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 Q<sub>0</sub> site (figure 2c). While the width of new EPR signal of SQ<sub>0</sub> was similar to that reported previously, the relaxation properties were clearly different. The spin echo of SQ<sub>0</sub> decayed (2p-ESEEM experiment) much faster in comparison with SQ<sub>chem</sub> signal in buffer which indicated that this time, unlike the previous case,



**Figure 3.** Structural model explaining the existence of two populations of  $SQ_0$  detected by CW EPR [36]. (a) Reduced FeS cluster is in the  $Q_0$  site and putative hydrogen bond between  $SQ_0$  and the cluster liganding histidine 156 (*R. capsulatus* numbering) facilitates spin–spin exchange interaction. (b) Increasing the distance between  $SQ_0$  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  $SQ_0$  and neighbouring fast-relaxing metal centres are possible. Structures shown in left panel are based on MD simulations [44].

the  $SQ_0$  interacted with fast-relaxing paramagnetic species. The authors concluded that the paramagnetic species that affect  $SQ_0$  are haems nearest to  $SQ_0$ , 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  $Q_0$  site inhibitors was not reported and it was not shown whether this new  $SQ$  signal disappears in the control mutants with inactive  $Q_0$  site. The overall shape of  $SQ_0$  proton ENDOR spectrum was similar to those reported previously for  $SQ_0$  and  $SQ_{res}$  indicating that  $SQ_0$  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  $SQ_0$ 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  $SQ_0$  was not hydrogen-bonded to the protein. Comparison of bacterial and yeast cytochrome  $bc_1$  did not reveal any spectral differences which indicated that  $SQ_0$  in both cases is the same chemical species trapped in similar environment.

The properties of  $SQ_0$  that emerged from ESEEM and ENDOR data led the authors to propose a model of ‘electrostatic cage’ trapping deprotonated  $SQ_0$ . 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  $SQ_0$  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  $SQ_0$  is proposed to conserve sufficient redox energy to reduce haem  $b_L$  which seems difficult to reconcile with the statement that  $SQ_0$  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  $Q_0$  site [36]. Those transitions revealed the presence of two distinct populations of  $SQ_0$  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  $SQ_0$  and the other from the reduced Rieske cluster (figure 3a). The second transition near  $g = 2.0$  corresponded to the population of  $SQ_0$  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,

DBH<sub>2</sub> and oxidized cytochrome *c*, under aerobic conditions. The changes in the amplitudes for these two signals (radical at  $g = 2.0$  and SQ<sub>o</sub>-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 Q<sub>o</sub>-site-specific inhibitors (including myxothiazol and various synthetic strobilurins). Both signals were not observed in specific mutants that disabled activity of the Q<sub>o</sub> site (such as *cytb*: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 Q<sub>o</sub> 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 SQ<sub>o</sub> reflect two configurations of the Q<sub>o</sub> site. The spin-spin exchange ( $g = 1.94$ ) by its nature has a clear distance constraint and can take place only when SQ<sub>o</sub> and Rieske cluster are in proximity, as shown in figure 3*a*. In this configuration, a formation of a hydrogen bond between histidine residue coordinating Rieske cluster and ubiquinone molecule is possible. At larger distances (figure 3*b*) or upon breaking the putative hydrogen bond between SQ<sub>o</sub> and histidine ligand, spin-spin exchange disappears and SQ<sub>o</sub> becomes detected as a separate free radical species having a signal near  $g = 2.0$ . Nevertheless, in this case, SQ<sub>o</sub> exhibited unusually fast relaxation compared with the relaxation of chemically generated SQ in buffer (by auto-oxidation of DBH<sub>2</sub> in alkaline pH), which was expected given that the SQ<sub>o</sub> is located in proximity to fast-relaxing paramagnetic metal centres of the Q<sub>o</sub> site: oxidized haem *b*<sub>L</sub> [51] and/or reduced FeS [52]. The fast spin-lattice relaxation of SQ<sub>o</sub> 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 SQ<sub>o</sub> from the radical signals described in [33–35].

The two populations of SQ<sub>o</sub> were incorporated to the model of electronic bifurcation of the Q<sub>o</sub> site. We envisaged that the SQ<sub>o</sub>-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 SQ<sub>o</sub> and reduced FeS exist as separate identities (distinguished by separate spectra, one of which is radical  $g = 2.0$ ) before reduction of haem *b*<sub>L</sub> by SQ<sub>o</sub> takes place to complete the oxidation of QH<sub>2</sub> at this site.

## 6. Semiquinone intermediates in relation to proton management of the Q<sub>o</sub> site

The process of uptake and release of protons is an inherent part of redox chemistry of ubiquinones. As the energy of the SQH<sub>2</sub><sup>+</sup> (double protonated SQ) is very high [53], at least

one proton needs to be released during oxidation of QH<sub>2</sub> to make transfer of the first electron possible. Accordingly, in the ubiquinol oxidation at the Q<sub>o</sub> 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 Q<sub>o</sub> site, the detected SQ<sub>o</sub> intermediates offer interesting new insights into this issue.

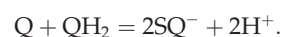
The radicals with typical  $g = 2.0$  are believed to be in a deprotonated/anionic form. Thus, it is plausible to expect that these SQs are relevant to a state having the two protons already released (here we consider the direction of ubiquinol oxidation; figure 4*a*). However, the spin-spin exchange 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 4*b*). 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 Q<sub>o</sub> 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 SQ<sub>o</sub> and FeS cluster (figure 4*c*). 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 SQ<sub>o</sub> and its reactivity with oxygen

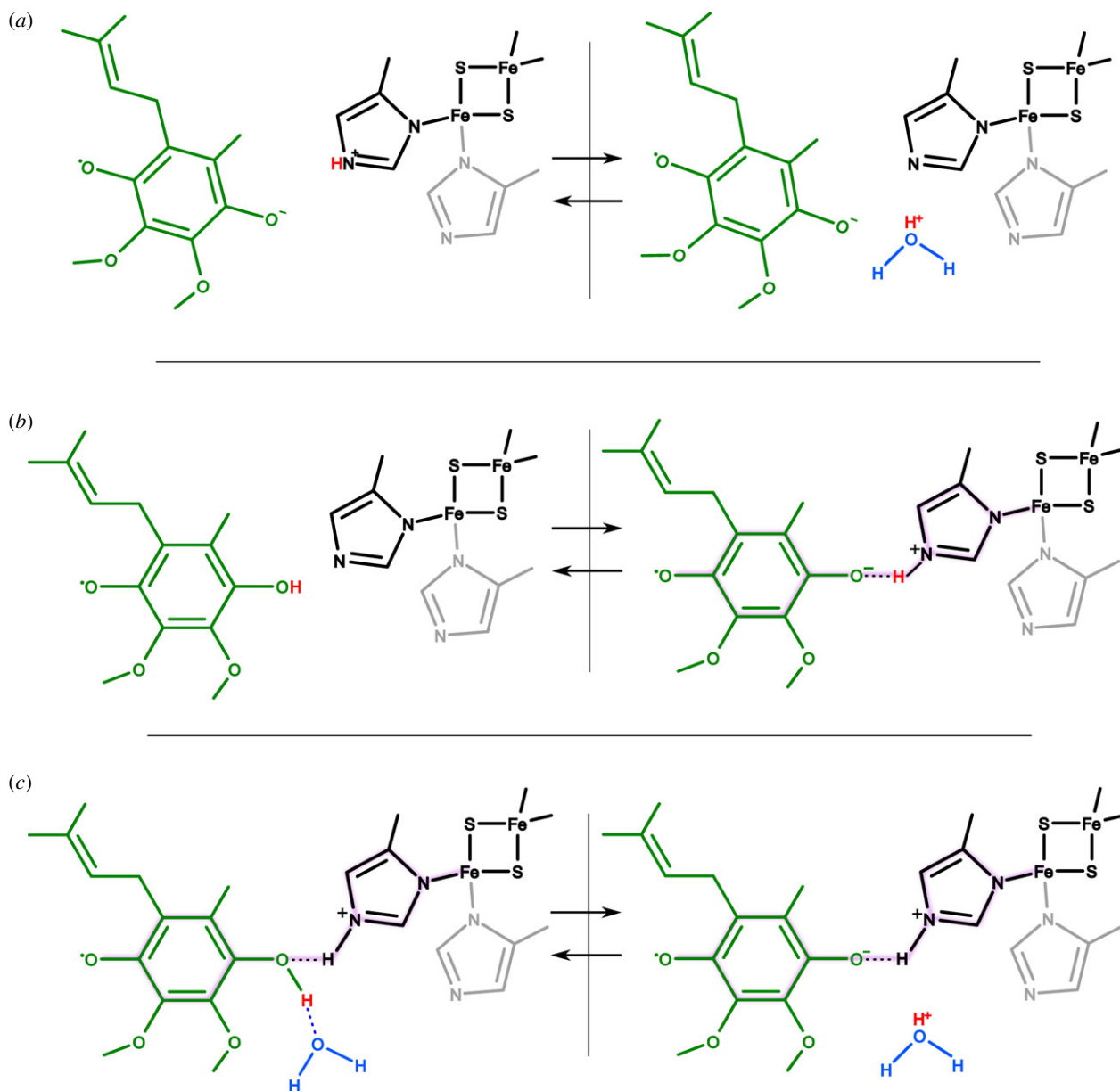
Quinones in solution under equilibrium undergo comproportionation (reverse of disproportionation) reaction according to the scheme [23,58]:



The equilibrium constant  $K_s$  for this reaction is often referred to as the stability constant for SQ which depends on the redox potentials of Q/SQ and SQ/QH<sub>2</sub> pairs ( $E_{Q/SQ}$  and  $E_{SQ/QH_2}$ , respectively):

$$K_s = \frac{[SQ^-]^2 \cdot [H^+]^2}{[Q] \cdot [QH_2]} = 10^{[E'_{Q/SQ}(\text{mV}) - E'_{SQ/QH_2}(\text{mV})]/59.1}$$

For ubiquinone-10, the redox potentials of  $E_{Q/SQ}$  and  $E_{SQ/QH_2}$  couples at pH 7 are -230 and +190 mV in bulk solution [23], respectively, which means that  $K_s$  is around  $10^{-8}$ . When



**Figure 4.** Different possibilities of proton involvement in the interactions of  $SQ_0$  (green) with the cluster liganding histidine (black) and/or water (blue). (a)  $SQ_0$  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  $SQ_0$  reversibly donates proton to histidine which results in formation of hydrogen bond between this histidine and  $SQ_0$  anion. (c) Neutral  $SQ_0$  forms hydrogen bond with protonated histidine, whereas the proton originating from  $SQ_0$  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  $SQ_0$  and the FeS cluster (magenta shadow shows the possible paths for the electron spin exchange).

concentrations of both Q and  $QH_2$  are, for example 100  $\mu\text{M}$ , 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/ $QH_2$  triad under equilibrium in solution but it is often used to describe the stability of SQ that can be formed within the  $Q_0$  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,  $SQ_0$  has been considered as highly unstable with the low stability constant  $K_s$  of the order of  $10^{-7}$  or less [13,30,32,42,59,60]. This however remains an open question in the light of the  $SQ_0$  detections which report signals in the range from 1% up to 17% of the total  $Q_0$  sites. Cape *et al.*

reported that  $SQ_0$  occupies 0.01–0.1  $Q_0$  sites per monomer [34]. Given the total concentration of 10  $\mu\text{M}$  for both cytochrome  $bc_1$  and  $QH_2$ , 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  $\mu\text{M}$  of chloride-substituted quinone produces 260 nM of SQ with  $K_s$  that is larger than  $10^{-2}$ ) [61]. Such relatively large values of  $K_s$  suggest some kind of stabilization of  $SQ_0$  in comparison with bulk solutions. We emphasize, however, a potential difficulty in describing stability of  $SQ_0$  using the  $K_s$  parameter, because all reported  $SQ_0$  signals in cytochrome  $bc_1$  were detected under non-equilibrium conditions for which the  $K_s$  parameter defining thermodynamic equilibrium may not be valid.



Considering the properties and conditions of trapping (table 1 and 2, respectively) the  $SQ_o$  intermediates summarized above it appears as if different  $SQ$  species for the  $Q_o$  site have been reported. A feature that unites all these reports is the observation that  $SQ_o$  cannot be detected in cytochrome  $bc_1$  unless the  $Q_i$  site is inhibited by antimycin or haem  $b_H$  is knocked-out by mutation. This effectively impedes re-oxidation of haem  $b_L$  through the path involving haem  $b_H/Q_i$ . It thus appears that the state with reduced haem  $b_L$  is required as condition for increasing probability of trapping  $SQ_o$ . In reversibly operating  $Q_o$  site,  $SQ_o$  can, in principle, be formed in two ways and both indeed require reduced haem  $b_L$  as an initiation [62–66]. In a semiforward reaction, reduced haem  $b_L$  prevents electron transfer from  $SQ_o$  to haem  $b_L$  after oxidized FeS initially withdraws one electron from  $QH_2$  forming  $SQ_o$ . In a semireverse reaction, reduced haem  $b_L$  initiates  $SQ_o$  formation by electron transfer to  $Q$ . In this context, the properties of  $SQ_o$  in [33,34] suggest that  $SQ_o$  was formed along with reduced haem  $b_L$  pointing towards the semiforward reaction scheme. On the other hand, the properties of  $SQ_o$  in [36] and [35] indicate that it was trapped along with oxidized haem  $b_L$  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  $Q/QH_2$  ratio [67,68].

Interestingly, the semireverse reaction has recently been considered as the one leading to formation of  $SQ_o$  that can interact with oxygen and thus is responsible for generation of superoxide by the  $Q_o$  site [63–65]. In this scheme, unlike in a semiforward scheme,  $SQ_o$  can be formed in the configuration of the  $Q_o$  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  $Q_o$  site and haem  $c_1$  (outermost cofactor of the c-chain) [69]. It is thus possible that  $Q$  is reduced by haem  $b_L$  at the time when FeS cluster occupies positions remote from the  $Q_o$  site and is unable to immediately engage in electron transfer reaction with  $SQ_o$ . This increases the probability of reaction of  $SQ_o$  with oxygen (if all electron transfers compete kinetically), as indeed implicated experimentally [64,65,68].

The presumed high reactivity of  $SQ_o$  with oxygen implies that anaerobic conditions should promote trapping  $SQ_o$ . The reports of detection of  $SQ_o$  signals under anaerobic condition follow this expectation [33–35]. In one of these cases, it was additionally recognized that  $SQ_o$  could not have been observed under aerobic conditions [34]. There was also another report of failure to detect  $SQ_o$  in the presence of molecular oxygen, but those experiments were performed using freeze-quenched samples of cytochrome  $bc_1$  non-inhibited by antimycin [20]. However, the report of detection of two populations of  $SQ$  ( $g = 1.94$  and  $g = 2.0$ ) concerned aerobic conditions [36].

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The relatively large quantities of  $SQ_o$  (spin–spin coupled to the Rieske cluster) detected under these conditions suggest that  $SQ_o$  is not as highly reactive with oxygen as commonly presumed at least in the presence of the spin exchange. In addition, high levels of  $SQ_o$  were observed in +2Ala mutant, which does not produce any detectable superoxide [64], implying that conditions of spin–spin coupling between  $SQ_o$  and FeS ( $g = 1.94$ ) might be protective against superoxide generation.

## 8. Concluding remark

The assumption about extremely low  $K_s$  of  $SQ_o$  has traditionally been used to explain the long-standing difficulty in experimental detection of  $SQ_o$ . We now seem to face the opposite situation where several seemingly different  $SQ_o$  intermediates have been exposed. The differences concern both the properties of  $SQ_o$  species and the experimental conditions used to trap the  $SQ_o$  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  $Q_o$  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  $SQ_o$  signals represent the same intermediate of the  $Q_o$  site, or rather reflect different states of the reaction scheme. Are all of them truly associated with the operation of the  $Q_o$  site? What is the role of haem  $b_L$  in the formation of  $SQ_o$  and superoxide production? How does the intermonomer electron transfer between the two haems  $b_L$  influence these reactions? The available set of data on  $SQ$ s 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  $Q_o$  site catalysis and its involvement in superoxide generation.

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**Competing interests.** We declare we have no competing interests.

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## Endnote

<sup>1</sup>We use the 'proton *translocation*' term in the context of cytochrome  $bc_1$  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.

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