The Q_o-site inhibitor DBMIB favours the proximal position of the chloroplast Rieske protein and induces a p*K*-shift of the redox-linked proton

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Abstract The interaction of the inhibitor 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) with the Rieske protein of the chloroplast b_6f complex has been studied by EPR. All three redox states of DBMIB were found to interact with the iron-sulphur cluster. The presence of the oxidised form of DBMIB altered the equilibrium distribution of the Rieske protein's conformational substates, strongly favouring the proximal position close to heme b_L. In addition to this conformational effect, DBMIB shifted the p*K*-value of the redox-linked proton involved in the iron-sulphur cluster's redox transition by about 1.5 pH units towards more acidic values. The implications of these results with respect to the interaction of the native quinone substrate and the Rieske cluster in cytochrome *bc* complexes are discussed.

Key words: Rieske; DBMIB; Redox-linked proton; Rieske protein's domain movement; EPR

1. Introduction

Dramatic progress in our understanding of cytochrome *bc* complexes has recently been achieved by the determination of the 3D-structure of the mitochondrial cytochrome bc_1 complex [1–3]. In addition to a detailed structural description of the enzyme, the X-ray work provided evidence for a long-range domain movement of the Rieske protein within the complex. This domain movement obviously allows the [2Fe-2S]-cluster of the Rieske protein to shuttle an electron from its substrate quinol towards cyt c_1 representing a novel mechanism for intra-complex electron transfer. EPR experiments on partially ordered samples have demonstrated that the conformational flexibility of the Rieske protein in cytochrome *bc* complexes is not unique to the mitochondrial enzyme [4–6].

Stimulated by the 3D-structures, detailed mechanistic models for quinol oxidation by cytochrome *bc* complexes [7–9] and more specifically for the role of the moving Rieske protein during turnover were proposed recently [10]. A crucial parameter in the model of Ugulava and Crofts [10] is the p*K*-value of the N^{ε}-proton on one of the cluster-ligating histidines. This p*K*-value indeed appears to be surprisingly well-conserved (at a value of close to pH 8) in all Rieske proteins from neutrophilic organisms [10,11]. Only the Rieske proteins from acidophilic organisms show significantly altered p*K*-values of the redox-linked protons [6,12].

Based on the sensitivity of the Rieske centre's EPR spectrum towards the redox state of the quinone occupying the Q_o-site, strong hydrogen interactions between the hydroxyl/phenoxy-functions on the quinol/ne and the N^{ε}-protons on the histidine ligand have been proposed several years ago [13]. These hypotheses are comforted by the positions of Q_o-site inhibitors as seen in the 3D-structures [2,3]. The structural model proposed by Ding et al. [13] suggests a possible influence of the protonation/redox state of the quinone on the p*K*-value of the proton on the histidine ligand. Such modifications of the p*K*-value on the histidine ligands depending on the redox state of the quinone may well be of functional significance but are difficult to study experimentally for the case of the physiological quinone substrate.

In this work, we show that the Rieske protein in cytochrome b_6f complex from spinach also displays conformational flexibility. Occupancy of the Q_o-site by the inhibitor DBMIB locks the majority of Rieske proteins in the proximal position (i.e. close to heme b_L) and leads to a substantial p*K*-shift of the redox-linked proton suggesting the possibility of comparable effects induced by the native quinone.

2. Materials and methods

Broken thylakoids were prepared from market spinach and cytochrome b_6f complex was purified according to [14].

EPR redox titrations were performed as described by Dutton [15] using the following redox mediators at 100 μM: *N*,*N*-dimethyl-*p*-phenylenediamine, 1,4-benzoquinone, *N*,*N*,*N*',*N*'-tetramethyl-*p*-phenylenediamine, diaminodurol, 2,6-dichlorophenolindophenol, 1,2-naphthoquinone, toluylene blue, phenazine methosulphate, phenazine ethosulphate, methylene blue, duroquinone. Fifty mM MES, MOPS and HEPES were present during titrations at pH 5.7, 6.3, 6.9, 7.1 and 8.0. Reductive and oxidative titrations were performed using dithionite and potassium ferricyanide, respectively.

EPR spectra were recorded on a Bruker ESP300e X-band spectrometer fitted with an Oxford Instrument He-cryostat and temperature control system.

Partially ordered samples were obtained from purified cytochrome b_6f complex as reported previously [16–18].

DBMIB and analogs were kindly provided by Prof. Walter Oettmeier Bochum, Germany.

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The inhibitors were reduced in ethyleneglycol by addition of sodium borohydride followed by chemical decomposition of the reductant via acidification.

The structure depicted in Fig. 5 is partly based on the X-ray structure of the mitochondrial cytochrome bc_1 complex [2] (pdb-entry 3bcc).

3. Results and discussion

Halogenated quinone analogs and in particular the dibromo-derivative DBMIB [19–21] have been described as potent inhibitors of the chloroplast cytochrome b_6f complex [22–26] and with much lesser efficiency of cytochrome bc_1 complexes from mitochondria [27] and purple bacteria [28]. DBMIB was shown to bind at the Q_o-site of the enzyme and to strongly alter the EPR spectrum of the Rieske cluster (in particular, inducing a shift of the g_y-line from 1.89 to 1.94) [24].

Based on an EPR redox titration at pH 8.0 of DBMIB-treated thylakoids, the existence of four paramagnetically differing states of the complex between DBMIB and the b_6f Rieske protein had been proposed (see Scheme 1) [23]. According to this model, the inhibitor-induced spectral alterations (i.e. appearance of the g = 1.94 signal) are only observed in the presence of the oxidised form of DBMIB (state 2), whereas the singly reduced semiquinone was proposed to couple antiferromagnetically to the paramagnetic centre of the Rieske cluster yielding the EPR-silent state 3 [23]. In the fully reduced state 4 of the inhibitor ($DBMIBH_2$), the $g_y = 1.89$ line was observed to reappear suggesting that either $DBMIBH_2$ does not bind to the enzyme or that the binding is modified in a way abolishing the characteristic strong interaction between the inhibitor and the Rieske centre [23].

Fig. 1a shows EPR spectra obtained on purified cytochrome b_6f complex reduced by ascorbate in the presence (continuous line) and absence (dotted line) of $DBMIBH_2$. Whereas a g = 1.94 signal was present at very minor amounts (most probably due to incomplete chemical prereduction of the inhibitor), the EPR-signal in the presence of reduced DBMIB differed measurably from that of the control sample, both with respect to the g_y-line and the g_x-trough. This demonstrated that $DBMIBH_2$ in fact bound to the Q_o-site in the vicinity of the Rieske cluster. The results depicted in Fig. 1b corroborated this result by showing that reduced DBMIB was able to displace oxidised DBMIB from its binding site yielding a decrease of the g = 1.94 signal and an increase of the g = 1.89 line. The high concentrations of cytochrome b_6f complex used for the spectra in Fig. 1 required amounts of DBMIB and $DBMIBH_2$ that entailed problems related to inequivalent solubilities. Titrating away the 1.94 signal by addition of $DBMIBH_2$ in significantly less concentrated samples allowed us to estimate that the affinity of the reduced form of the inhibitor for the Q_o-site is about 500-fold lower than that of the oxidised form. These results furthermore indicated that the binding sites for oxidised DBMIB and reduced DBMIB were at least overlapping if not similar.

As evidenced by these data, fully reduced DBMIB binds to the Q_o-site and interacts with the [2Fe-2S]-cluster of the Rieske protein. The dramatic spectral changes induced by the oxidised form of DBMIB, however, are absent in the presence of $DBMIBH_2$ and the modifications of the EPR spectrum rather resemble those observed for the case of other inhibitors such as stigmatellin or UHDBT [18].

Fig. 2 shows an experiment where the semireduced form of

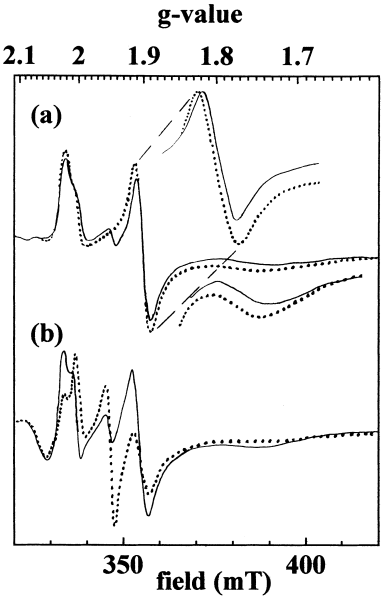


Fig. 1. EPR spectra of the Rieske [2Fe-2S]-cluster in purified cytochrome b_6f complex (10 μM in cytochrome *f*) from spinach chloroplasts in the presence and absence of reduced and oxidised DBMIB. (a) The dotted spectrum was taken on the ascorbate reduced protein in the absence of DBMIB. After addition of 100 μM $DBMIBH_2$, the continuous spectrum was recorded. The lower traces showing the g_x-trough are expanded 4-fold with respect to the main spectra. The g_y-lines are furthermore depicted on a wider magnetic field scale (expanded by a factor of 2.5) in order to visualise the spectral changes induced by $DBMIBH_2$. (b) When oxidised DBMIB (50 μM) rather than the reduced form was added to the ascorbate-reduced enzyme, the dotted spectrum was obtained. Subsequent addition of 15 mM $DBMIBH_2$ resulted in conversion into the continuous spectrum. Instrument settings: temperature, 20 K; microwave frequency, 9.44 GHz; modulation amplitude, 1.6 mT; microwave power, 6.3 mW.

DBMIB has been generated by turnover of the cytochrome b_6f complex rather than by equilibrium titrations as performed by Malkin [23]. A slight excess of chemically reduced DBMIB was added to fully oxidised (Fig. 2a vs. b) cytochrome b_6f complex. A small 1.89 signal (about 30% of the total chemically reduced amplitude represented by the dotted spectrum) was induced by this addition of $DBMIBH_2$ (Fig. 2b; for a discussion of the nature of this signal, see below). Subsequent addition of a 10-fold excess of oxidised DBMIB (i.e. no further reducing equivalents were injected into the system) resulted in the appearance of a g = 1.94 line together with a slight increase of the g = 1.89 signal. This shows that in spectrum 2b, a significant fraction of reduced Rieske clusters were in an EPR-silent state. Further addition of oxidised DBMIB increased the signal amplitude of the $g_y = 1.94$ line (not shown) indicating that the binding affinity for the semiquinone form of DBMIB to the Q_o-site exceeded those of both the fully oxidised and the fully reduced forms. An exact value for the relative binding affinities, however, could not be obtained due to the fact that the required high concentrations of DBMIB in the sample tended to denature the complex.

Our results thus confirm Malkin's hypothesis of an EPR-silent state formed by the DBMIB semiquinone and the reduced Rieske cluster. Since the data shown in Fig. 1 furthermore demonstrated that the fully reduced quinol form of DBMIB still bound to the Q_o-site and affected the EPR spec-

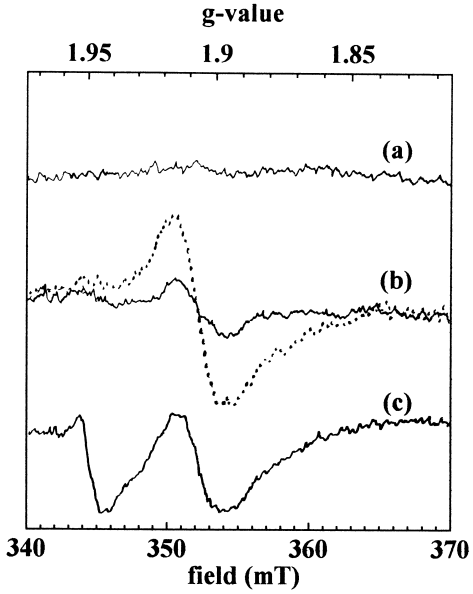


Fig. 2. 2 μM of $DBMIBH_2$ were added (b) to purified cytochrome b_6f complex (1.5 μM in cytochrome *f*) in which the Rieske protein was poised in the oxidised state (a), followed by additional treatment with 15 μM oxidised DBMIB (c). The dotted curve in (b) represents the full signal of the Rieske cluster obtained by reduction with 5 mM ascorbate. Instrument settings, see Fig. 1.

trum of the Rieske cluster, all three reduction states of DBMIB therefore strongly interact with the [2Fe-2S]-centre of the Rieske protein. In the mitochondrial cytochrome bc_1 complex, the Rieske protein was shown to pivot between a 'proximal' state close to the Q_o-site and a 'distal' state close to cytochrome c_1 [2,3]. The observed strong interactions of all redox states of DBMIB with the [2Fe-2S]-cluster implied that in the presence of the inhibitor the *bf* Rieske protein had to be in a conformation equivalent to the proximal position. An examination of the orientations of the Rieske cluster's magnetic axes in two-dimensionally ordered samples (Fig. 3) indeed provided evidence for substantially differing conformational equilibria in the presence and absence of the inhibitor. Fig. 3b shows the orientation-dependences of the Rieske cluster's g_z-, g_y- and g_x-signals (see spectra in Fig. 3a) with respect to the membrane. The polar plots of g_z and g_y were characterised by a poor anisotropy as already reported [18,29,30]. We have previously shown that the g_y-signal actually consisted of two or more components differing in EPR relaxation properties [18]. In our previous work, we have discussed the possibility that the observed effects may reflect differing orientations of the Rieske cluster but strongly favoured a purely physical interpretation of the observed phenomena [18]. The recent demonstration of a domain movement of the Rieske protein in mitochondrial cytochrome bc_1 complex [2], however, showed that the interpretation invoking conformational heterogeneity of the Rieske protein, discarded in [18], was in fact correct. A detailed reinterpretation of the respective data in the light of the Rieske protein's conformational flexibility will be presented elsewhere.

When the sample used for the experiments of Fig. 3a, b was sprayed with a solution containing DBMIB followed by redrying under a stream of argon, the spectra shown in Fig. 3a were converted into those depicted in Fig. 3c, featuring signals characteristic of the Rieske protein in the presence of DBMIB. In contrast to the uninhibited centre, the principal g-directions were well-ordered parallel (g_z and g_y) and perpendicular (g_x) to the membrane in the presence of DBMIB. It is of note that the signal at about $g = 1.89$, which arises from g_y in the uninhibited Rieske centre (Fig. 3a), mainly reflected the g_x-direction of the inhibitor-induced spectrum (Fig. 3c). This is evident from the facts that (a) g_z and g_x of the uninhibited centre have dramatically diminished upon addition of DBMIB whereas still more than half of the $g = 1.89$ signal parallel to the membrane can be observed (Fig. 3c) and that (b) the two g-tensor orientations $g_z = 2.01$ and $g_y = 1.94$ parallel to the membrane require a third orthonormal g_x-direction for which no other candidate than the $g = 1.89$ signal is detectable in the spectrum. The attribution of the $g = 1.89$ line as the g_x-trough of the rhombic DBMIB-induced spectrum of the Rieske cluster is in line with the fact that we detected this signal even under conditions where high concentrations of DBMIB were present (e.g. see Fig. 1b). Respective observations have been reported previously for thylakoid membranes [29,30] and other cytochrome *bc* complexes [4]. By contrast, Malkin has reported that the $g = 1.89$ signal can be fully interconverted into the $g = 1.94$ signal using saturating concentrations of DBMIB [23,24]. At present, we have no explanation for these differences.

As stated above, the Rieske protein is in the proximal position in the presence of DBMIB. In this state, the Fe-Fe-axis of the cluster of the X-ray structure [2] is seen to be perpendicular to the plane of the membrane. The fact that the same orientation was observed for the g_x-axis (Fig. 3d) demonstrates that g_x is collinear with the Fe-Fe-axis, in line with independent evidence obtained on a purple bacterial complex [5].

Addition of DBMIB therefore displaces the equilibrium distribution of the Rieske protein from a rather heterogeneous population (Fig. 3b) towards a new equilibrium strongly favouring the proximal position (Fig. 3d). This demonstrates the presence of a conformational flexibility of the Rieske protein in the plastidic cytochrome b_6f complex analogous to that seen in the X-ray structures of the mitochondrial cytochrome bc_1 complex [2,3]. Independent evidence for this conformational flexibility of the plastidic Rieske protein has also been obtained by the experimental procedure described in [5] (Baymann, Brugna, Wollman and Nitschke, unpublished).

As can be seen from Fig. 3c, the g_z- and g_x-signals of the uninhibited Rieske centre, although substantially diminished, were still visible, even after repeated treatment with DBMIB (not shown). These residual signals from the uninhibited centre can be due to (a) incomplete penetration of the inhibitor solution into the oriented multilayer, (b) presence of Rieske proteins detached from the complex or (c) presence of a population of complexes where the Rieske protein is in the distal state, i.e. too far away from the Q_o-site to interact with the inhibitor. While the g_x-signal at $g = 1.78$ was too small to be evaluated and while the g_y-signal at 1.89 was strongly overlapped by the g_x-trough of the inhibitor-affected spectrum, the intensity of the g_z-peak at $g = 2.03$ could still be determined with sufficient precision yielding the orientation-dependence depicted in Fig. 3d (open triangles). The signal was found to still be orientationally anisotropic arguing against the rationalisation invoking detached Rieske protein. The angular dependence, however, differed from that observed in the uninhibited state and showed a maximum at

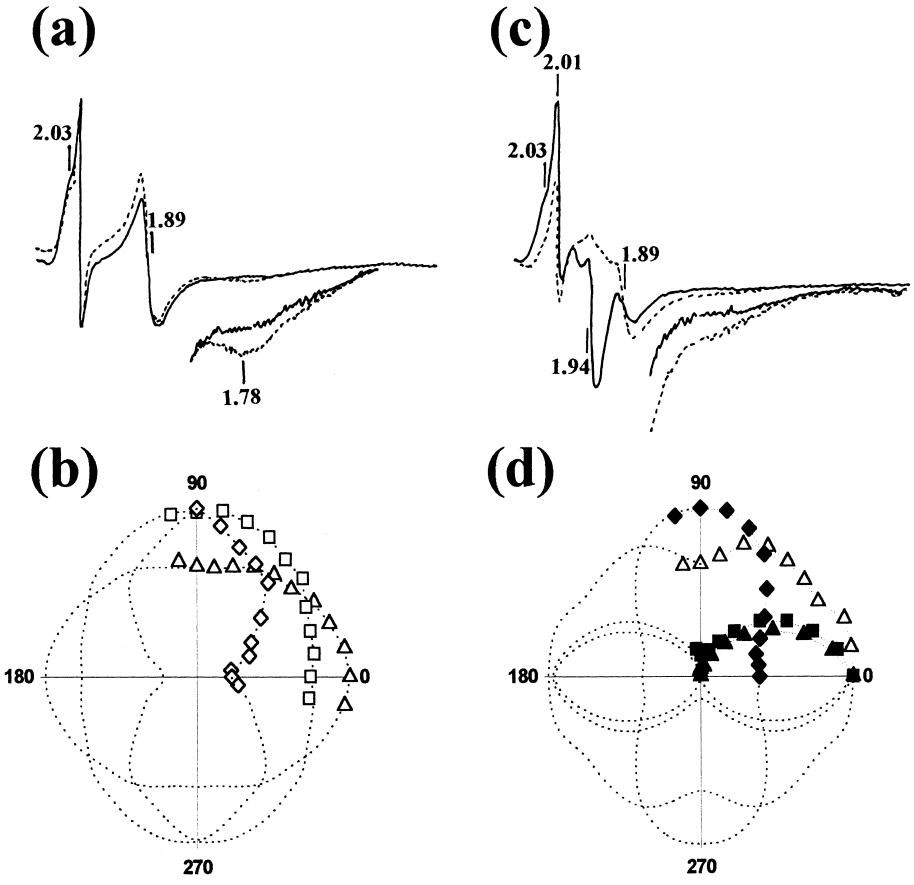
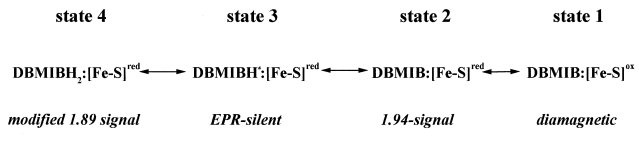


Fig. 3. Orientation of the Rieske cluster's g-tensor in the presence (c, d) and absence (a, b) of DBMIB. EPR spectra of the Rieske centre were recorded on oriented samples in the absence of DBMIB (a). The samples were subsequently treated with DBMIB until saturation of the g = 1.94 signal had been achieved (c). Continuous and dotted spectra in (a) and (c) were taken with the magnetic field parallel and perpendicular to the plane of the mylar sheets, respectively. (b) and (d) represent polar plots of the signals observed at g = 2.03 (open triangles), 2.01 (filled triangles), 1.94 (filled squares), 1.89 (open squares in (b) and filled diamonds in (d)) and 1.78 (open diamonds). Open and filled symbols denote spectral features arising from the uninhibited and DBMIB-treated samples, respectively. Instrument settings, see Fig. 1.

about 60° to the membrane in addition to that observed at 0°. We would therefore hesitate to exclude the possibility that, even in the presence of DBMIB, a fraction of Rieske clusters were in a conformation differing from the proximal state and thus insensitive to spectral alterations induced by DBMIB. This could also provide a rationale for the g = 1.89 signal observed in Fig. 2b. A more detailed investigation of these centres is presently underway in our laboratories.

In the EPR redox titration at pH 8.0, Malkin [23] observed the electrochemical transition of the Rieske cluster in the presence of oxidised DBMIB (i.e. between states 1 and 2, see Scheme 1) at $E_m = 180$ mV, i.e. about 140 mV lower than what was found in the uninhibited enzyme. DBMIB-binding was therefore considered to alter the redox potential of the Rieske protein as also observed for other inhibitors, such as stigmatellin [31,32] or UHDBT [33]. In this EPR titration, the DBMIB-induced g = 1.94 signal of the Rieske cluster was seen to disappear at lower potentials with an apparent E_m-value of +20 mV. As mentioned above, the loss of the Rieske spectrum must be attributed to antiferromagnetic interaction between the DBMIB semiquinone and the Rieske cluster (state 3).

We have performed EPR redox titrations similar to that reported by Malkin [23], however, covering a range of pH-values from 5.6 to 8.0. Unlike the remaining data reported in this work, these pH-dependent redox titrations were performed on spinach thylakoids since purified cytochrome b_6f complex tended to denature in the presence of DBMIB during the several hours of an EPR redox titration. The obtained data (Fig. 4) demonstrated that, rather than inducing a strong absolute shift of about 140 mV in the Rieske cluster's E_m-value (as reported in [23]), the E_m vs. pH curve was shifted by 1.5 pH units towards more acidic values as compared to that of the uninhibited enzyme [34] together with a relatively small effect of about 40–50 mV on the E_m-value in the pH-independent region. The E_m-value for the formation of the EPR-silent Rieske/semireduced DBMIB state at lower potentials (open triangles) was also pH-dependent (about −60 mV/pH, dotted line), but did not show a p*K*-value within the examined pH-range. This implies that the single electron reduction of DBMIB involves a protonation step and we have therefore denoted the semiquinone species as 'DBMIBH·' in Scheme 1 (state 3) in contrast to Malkin's original proposal [23]. Zhang et al. [35] have recently reported a p*K*-value for



Scheme 1.

the b_6f Rieske protein (obtained by an optical titration at room temperature on the isolated soluble fragment) that deviated from that determined by EPR on thylakoids at cryogenic temperatures [34] and have concluded that the discrepancy is due to a temperature-dependence of this pK-value. This appears highly unlikely to us since in the other cytochrome bc complexes studied so far, no major difference was observed between the optical (RT) and EPR measurements (e.g. [10], for a more detailed discussion of this problem, see [6]).

The results of the pH-dependent redox titration show that the principal effect of DBMIB on the electrochemical properties of the Rieske cluster lies in a destabilisation of the redox-linked proton. This proton is generally believed to correspond to the N^{ε}-proton of one of the cluster-ligating histidine residues [36–38]. The hypothetical structural model of DBMIB in the Q_o-site of cytochrome b_6f complex depicted in Fig. 5 (inspired by the X-ray structure of the mitochondrial cytochrome bc_1 complex) visualises possible molecular bases for the observed effects. Either the bromide substituent (due to its negative partial charge) or the oxygen atom adjacent to the Rieske cluster or both may form a hydrogen bridge with the N^{ε}-proton of one of the histidine ligands. Such a hydrogen bond is likely to stabilise the Rieske protein in the proximal conformation. On the other side, these interactions must be expected to destabilise the bond strength of the N^{ε}-proton, i.e. to lower its pK-value.

The fact that the described phenomena can be observed in the presence of DBMIB suggests the possibility that the reduction state (oxidised, reduced or semireduced) of the native substrate quinone may also influence the redox potential of the Rieske cluster and/or the pK-value of the redox-linked proton. $DBMIBH_2$ in fact resembles the physiological substrate plastoquinol in that it is a good electron donor to the

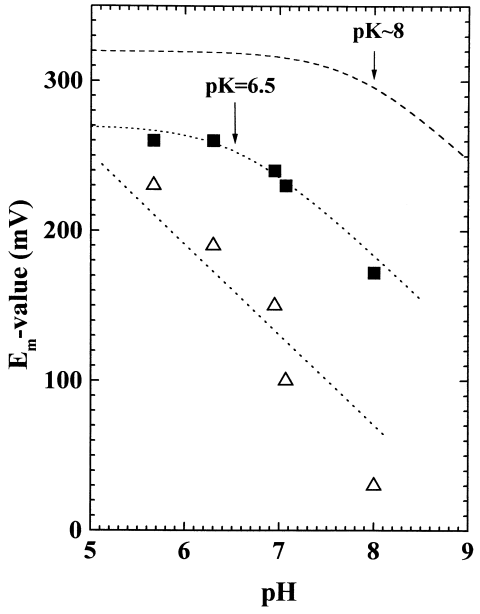


Fig. 4. pH-dependence of the E_m-values of the appearance (filled squares) and disappearance (open triangles) of the g = 1.94 signal. The redox titrations on DBMIB-inhibited thylakoid membranes were performed using 5 μM cytochrome b_6f complex and 100 μM DBMIB. The dashed line represents the E_m vs. pH curve of the uninhibited Rieske cluster as determined in [34].

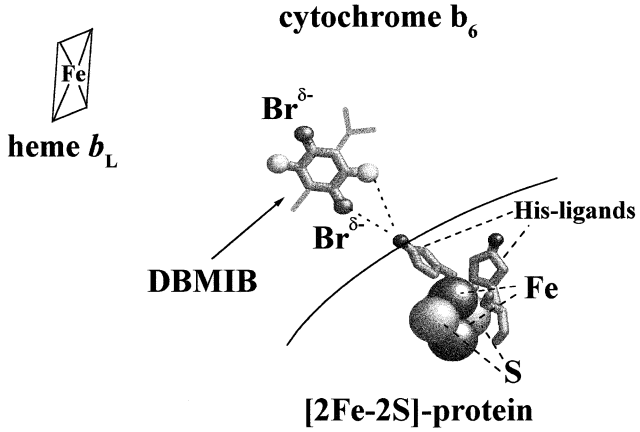


Fig. 5. Schematic model of the interaction of DBMIB in the Q_o-site with the [2Fe-2S]-cluster of the Rieske protein. Dotted lines represent possible hydrogen bridge interactions between the bromide substituents and/or one of the redox-active oxygens of DBMIB's ring moiety. The positions of heme b_L, the Rieske cluster and the inhibitor were inspired by the 3D-structure of the mitochondrial cytochrome bc_1 complex (pdb-entry 3bcc [39,40]).

Rieske protein. The inhibitory action mainly arises from the fact that the semiquinone state of DBMIB formed after electron donation to the Rieske protein is not sufficiently reducing to allow electron transfer towards heme b_L.

In a recent study addressing the question of a possible accumulation of a Q_o-site semiquinone during turnover of the cytochrome bc_1 complex [9], neither a signal from semiquinone nor from reduced Rieske cluster could be observed under conditions of oxidant-induced reduction. In this work, the authors mention the possibility that an antiferromagnetically coupled pair formed by semiquinone and the reduced Rieske cluster might explain the experimental results. The above described EPR-silent pair DBMIB:$[2Fe\text{-}2S]^{red}$ substantiates this possibility and is likely to provide a promising model system for the study of such a state during normal turnover of the complex.

It is of note that the detailed geometry of DBMIB and in particular the position of the bromide atoms with respect to the Rieske cluster is only one of a set of different possibilities. We expect that an examination of the range of DBMIB-derivatives described previously [21] with respect to the phenomena reported in this work will provide a more detailed picture of the interaction between quinones and quinone analogs in the Q_o-site and the Rieske cluster.

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