Non-inhibiting perturbation of the primary energy conversion site (Q_o site) in *Rhodobacter capsulatus* ubihydroquinone:cytochrome *c* oxidoreductase (cytochrome bc_1 complex)

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Abstract Ethanol added to *Rhodobacter capsulatus* chromatophore membranes containing the cytochrome bc_1 complex effectively *uncouples* the sensitivity of the [2Fe-2S] cluster EPR spectrum to the number and redox state of ubiquinone/ubihydroquinone within the Q_o site. Ethanol has no effect upon the rate of catalysis, leading to a *non-inhibiting perturbation* of cytochrome bc_1 function. We suggest that displacement occurs by ethanol acting from the aqueous phase to successfully compete with the Q_o site ubiquinones and water to hydrogen bond the $N_\varepsilon H$ atom(s) of the coordinating [2Fe-2S] cluster histidines.

Key words: Cytochrome bc_1 complex; [2Fe-2S] cluster; Q_o site; Alcohol; Ubiquinone

1. Introduction

Ubihydroquinone:cytochrome *c* oxidoreductase (cytochrome bc_1 complex in most organisms, cytochrome b_6f in chloroplasts) comprises the central portion of electron transfer chains in all energy transducing organelles. The Q_o site is the locus of the primary energy conversion steps within the cytochrome bc_1 complex. In prokaryotes, the Q_o site is located at the periplasmic face of the cytoplasmic membrane, where it catalyzes the two-electron oxidation of ubihydroquinone (QH_2) to ubiquinone (Q). This oxidation involves bifurcated electron transfer along both a high and low potential chain by the cooperation of two one-electron redox centers which flank the Q_o site, namely the [2Fe-2S] cluster and cytochrome b_L [1,2]. At present there is much to be resolved regarding the number of Q_o site ubiquinone occupants, their dynamics and the mechanism of the oxidation process. The advent of crystal structures of cytochrome bc_1 complexes from various species has assisted in defining the Q_o site locality, however no electron density ascribed to ubiquinone has so far been identified [3,4]. There are several plausible mechanisms for bifurcation of the electron transfer reaction in the Q_o site. These include: (a) a double Q occupancy variant model [5,6] invoking formation of a highly unstable ubisemiquinone transition state [6–8] or a quinhydrone like intermediate [6,9]; (b) a proton-gated charge transfer mechanism, where the activation barrier for the reaction is deprotonation of QH_2 [9,10]; (c) a proton-gated affinity change mechanism implicating a less unstable ubisemiquinone intermediate [11]; (d) formation of an unstable ubisemiquinone that diffuses within the Q_o site to facilitate bifurcated electron transfer by a catalytic switching movement [7,8]. The recent structural demonstration that the FeS cluster moves over a distance of 10 Å between the Q_o site and cytochrome c_1 adds another aspect to the possibilities for achieving efficient bifurcation of electron transfer [3,4].

A crucial aid in defining cytochrome bc_1 function has been the use of specific, tight binding inhibitors, classified according to whether they affect the Q_o or Q_i sites [12]. Another essential part of the progress into the Q_o site character has been provided by the electron paramagnetic resonance (EPR) spectral lineshape of the reduced, paramagnetic [2Fe-2S] cluster. This has proven to be highly sensitive to the degree and nature of the Q_o site occupants (Q/QH_2 or inhibitors) [5,6] and to provide a useful tool for dissecting cytochrome bc_1 function. Previously, we noted that a 1% addition of absolute ethanol to preparations of *Rhodobacter capsulatus* chromatophores modifies the [2Fe-2S] EPR spectrum [5]. Here we explore this effect at a higher level of quantitation and define a new functional state of the cytochrome bc_1 complex, where ethanol effectively *uncouples* the sensitivity of the [2Fe-2S] cluster to the Q_o site Q/QH_2, but has no effect upon catalysis [13]. This is novel, in that all previously reported exogenous effectors of the [2Fe-2S] cluster EPR spectrum induced by binding at the Q_o site also inhibit cytochrome bc_1 activity [5,6].

2. Materials and methods

Bacterial growth, harvesting and chromatophore preparation were as previously described [5,6,13]. EPR spectra [5] and flash-induced kinetics [13] were performed as previously described. Additional experimental details are reported in the appropriate figure legends.

3. Results

Fig. 1 shows the lineshapes of the cytochrome bc_1 [2Fe-2S] cluster EPR spectra in *R. capsulatus* chromatophores. Spectrum A was acquired from chromatophores poised at a redox potential of 200 mV which establishes the Q_{pool} oxidized and yields a prominent EPR g_x resonance at 1.800, characteristic of a cytochrome bc_1 complex with the Q_o site fully occupied by Q [5,13]. Upon addition of 1% (v/v) ethanol (170 mM) to the chromatophore suspension the g_x resonance at 1.800 shifts to 1.773 and broadens (Fig. 1B). The g_y and g_z [2Fe-2S] cluster EPR resonances are also altered upon addition of ethanol, however, in this report we will focus upon changes in the g_x resonance. When a slight molar excess of the Q_o site specific

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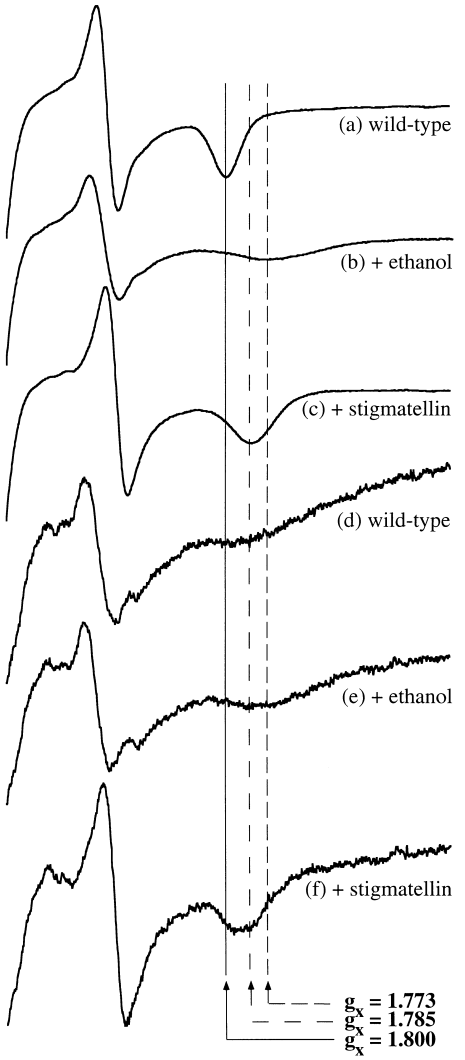

Fig. 1. Effect of ethanol upon the cytochrome bc_1 [2Fe-2S] cluster EPR spectra in *R. capsulatus* chromatophores. All samples were suspended to a reaction center (RC) concentration of 10 μM and poised at 200 mV. Each spectrum is an addition of five successive scans. Spectra A–C were obtained with chromatophores containing wild-type levels of ubiquinone (20 per RC). Spectra D–F were obtained with chromatophores extensively extracted of ubiquinone (1 per RC). Spectrum A, unextracted chromatophores suspended in buffer solution (50 mM MOPS, 100 mM KCl, pH 7.0); B, unextracted+170 mM ethanol; C, unextracted+10 μM stigmatellin; D, extracted chromatophores suspended in buffer solution; E, extracted+170 mM ethanol; F, extracted+10 μM stigmatellin. EPR conditions: sample temperature, 20 K; microwave frequency, 9.474 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 19.8 G; time constant, 164 ms.

inhibitor stigmatellin is added to cytochrome bc_1 chromatophores suspended in buffer plus 170 mM ethanol, the EPR spectrum displays the characteristic stigmatellin-induced resonance with a g_x at 1.785, implying that the ethanol effect is overridden by the presence of stoichiometric amount of stigmatellin. When Q-extracted chromatophores were used, rendering the Q_o site devoid of Q (Fig. 1D, acquired under identical conditions to Fig. 1A), the [2Fe-2S] cluster EPR spectrum displays a very broad g_x resonance at approximately 1.765. Upon addition of 170 mM ethanol to Q-extracted chromatophores, the ethanol-induced g_x resonance at 1.773 is observed (Fig. 1E), implying that this effect is independent of the Q_o site occupancy (compare Fig. 1B with Fig. 1E). Addition of stigmatellin to the Q-extracted chromatophores with ethanol again produces a spectrum similar to Fig. 1C, demonstrating that Q-extraction has not destroyed the integrity of the Q_o site.

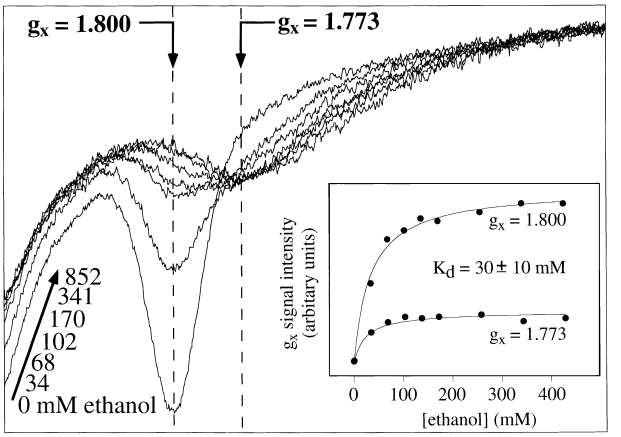

Fig. 2. Titration of ethanol-induced cytochrome bc_1 [2Fe-2S] cluster EPR spectrum in chromatophores containing wild-type levels of ubiquinone. The numbers refer to the concentration of added ethanol, resulting in the loss of the g_x resonance at 1.800 and the appearance of the g_x signal at 1.773 upon saturation with ethanol. The inset shows a standard two state hyperbolic binding isotherm for the ethanol effect. The redox poise and EPR conditions were as reported in Fig. 1.

The concentration dependence of the ethanol effect upon the cytochrome bc_1 [2Fe-2S] cluster EPR lineshape is illustrated in Fig. 2. This is clearly not a solvent effect as it reaches saturation between 150 and 200 mM ethanol. The loss of the g_x resonance at 1.800 and the gain in the g_x resonance at 1.773 appear to be adequately described by a two state process, as there is a well defined isosbestic point at about $g_x = 1.780$. The inset to the figure shows a hyperbolic binding isotherm fit to either the loss of intensity at $g_x = 1.800$ or gain in intensity of the 1.773 resonance. The 50% saturation point, I_{50}, was the same at both g_x values (30 ± 10 mM).

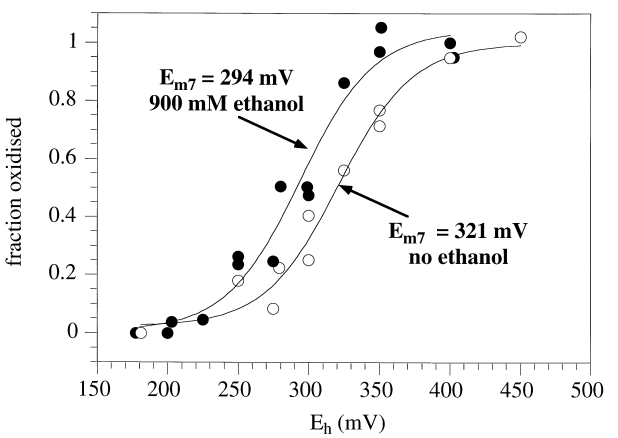

Fig. 3. Nernst plot of cytochrome bc_1 [2Fe-2S] cluster redox midpoint potential in the absence (○) and presence of 900 mM ethanol (●). The fraction reduced was determined from the peak to trough amplitude difference of the g_y resonance and the data were fit to the Nernst equation for a one electron couple, with the indicated midpoint potentials (standard error ±10 mV). EPR conditions are as reported in Fig. 1.

The effect of excess ethanol (900 mM) on the cytochrome bc_1 [2Fe-2S] cluster redox midpoint potential is illustrated in Fig. 3. The equilibrium redox midpoint potential of the one electron $[2Fe\text{-}2S]^{2+/+}$ couple is slightly lowered by about 25 mV in the presence of ethanol, which is not significantly different to the potential obtained in buffer alone [14]. This implies that the redox state of the [2Fe-2S] cluster is practically independent of the ethanol effect.

Single and multiple flash-induced cytochrome bc_1 turnover kinetics were monitored in wild-type *R. capsulatus* chromatophores and a typical experiment is illustrated in Fig. 4. Each experiment was performed in triplicate on fresh samples to ensure reproducibility and all the traces were fit to exponential decay profiles (fits omitted for clarity). Interestingly, no significant differences were observed for ubihydroquinone (QH_2) oxidation at the Q_o site in the presence of 900 mM ethanol, compared to that in buffer alone. These observations were both independent of the redox poise of the Q_{pool} and the presence of the Q_i site inhibitor, antimycin.

The effect of increasing the alkyl chain length of primary alcohols and benzyl alcohol upon the cytochrome bc_1 [2Fe-2S] cluster EPR lineshape is illustrated in Fig. 5. All the alcohols were added to 5% (v/v) (between 500 and 2000 mM), which in the case of ethanol completely abolishes the prominent g_x resonance at 1.800 and replaces it with a much shallower feature at 1.773 (Fig. 2). Primary alcohols with increasing alkyl chain length from methanol to butanol all cause very similar lineshape changes in the [2Fe-2S] cluster EPR spectrum, shifting the g_x at 1.800 to 1.773, as does benzyl alcohol. However, primary alcohols with alkyl groups longer than five carbon units have no effect upon the [2Fe-2S] cluster EPR spectrum. The effect of alcohols upon the [2Fe-2S] cluster EPR spectra is correlated with their solubility in aqueous solution, as primary alcohols larger than pentanol are insoluble. This implies that the alcohols are eliciting their effect upon the [2Fe-2S] cluster EPR spectra by acting from the aqueous phase.

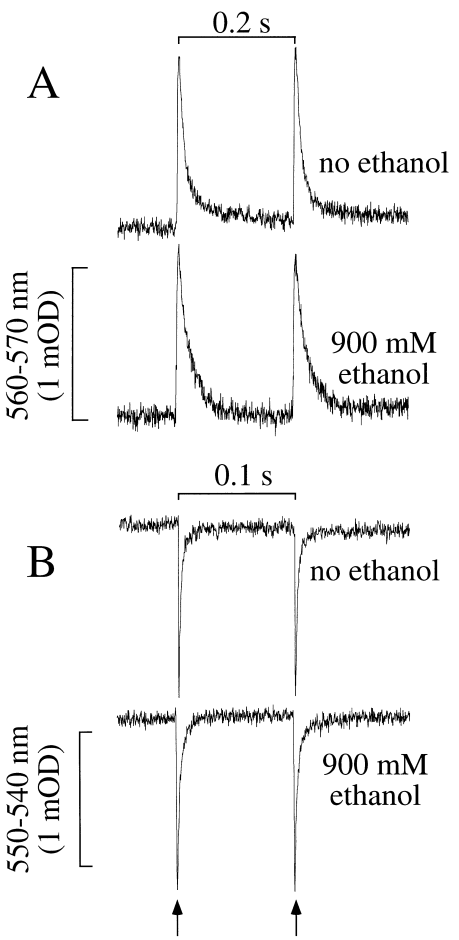

Fig. 4. Effect of ethanol upon cytochrome bc_1 Q_O site function. A: Flash-activated cytochrome *b*. B: Cytochrome *c* absorption changes in wild-type chromatophores in the absence and presence of 900 mM ethanol. The chromatophores were suspended to 0.2 μM RC concentration and poised at a potential of 100 mV, to render the ubiquinone half reduced. 5 μM valinomycin was added as an uncoupler, but no inhibitors were present, so the system could support multiple flash-induced turnover. The arrows indicate the time of the light flashes.

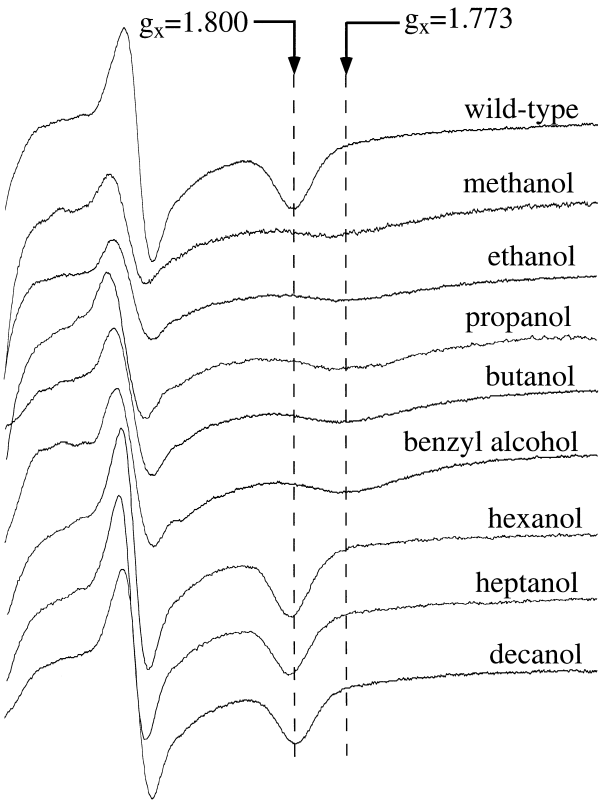

Fig. 5. Stack plots illustrating the effect of increasing chain length alcohols upon cytochrome bc_1 [2Fe-2S] cluster EPR spectra in *R. capsulatus* chromatophores. Experimental conditions are the same as Fig. 1.

4. Discussion

We have built on the earlier observation that addition of 1% ethanol (v/v) to cytochrome bc_1 chromatophores affects the [2Fe-2S] cluster EPR spectrum [5]. The prominent EPR resonance characteristic of a [2Fe-2S] cluster interaction with Q in the Q_o (g_x at 1.800) site is completely abolished and replaced by a spectrum with a much broader g_x resonance at 1.773. Similarly, after extraction of the Q_{pool} the resonance indicative of a Q_o site devoid of Q (g_x at 1.765) is replaced by the same resonance at 1.773, suggesting that this spectrum is characteristic of an ethanol interaction with the [2Fe-2S] clus-

ter (Fig. 1). Furthermore, a series of alcohols induce a similar [2Fe-2S] cluster EPR spectral lineshape, including native QH_2 (g_x at 1.777). It seems likely that the [2Fe-2S] EPR spectrum is influenced by the hydroxy functionalities displayed by these molecules, presumably by the lone pairs of electrons on the oxygen atom acting as a hydrogen bond acceptor from the non-iron liganding $N_\varepsilon H$ atom(s) of the histidine ligands to the cluster. Thus, ethanol effectively *uncouples* the sensitivity of the reduced [2Fe-2S] cluster EPR spectrum from the presence and stoichiometry of Q/QH_2 within the Q_o site. The I_{50} for this effect is manifested at ethanol concentrations approximately 5000-fold higher than the cytochrome bc_1 concentration within the chromatophores (Fig. 2), implying that ethanol is not binding to the Q_o site in an analogous manner to Q_o site inhibitors, which at the cytochrome bc_1 concentrations used here would bind stoichiometrically [11]. Increasing the length of the alkyl chain in primary alcohols does not appear to significantly increase the binding affinity (Fig. 5) in sharp contrast to the Q_o site-specific *ortho*-hydroxy-naphthoquinone series of inhibitors, which clearly act from the membrane phase and the Q_o site and not the aqueous phase [15]. This implies that ethanol interacts with the [2Fe-2S] cluster directly from the aqueous phase and not via the Q_o site membrane phase. Addition of stigmatellin to chromatophores in the presence of ethanol produces the characteristic stigmatellin-induced [2Fe-2S] EPR spectrum, effectively masking the ethanol effect and *re-coupling* the [2Fe-2S] cluster to the Q_o site. Under a variety of experimental conditions ethanol consistently had no effect upon Q_o site catalysis within the cytochrome bc_1 complex (Fig. 4), despite the fact that it clearly affects the [2Fe-2S] cluster EPR signature, illustrating that exchange of one hydrogen bonding state on the liganding histidines for another, does not impede Q_o site catalysis. We term this phenomenon *non-inhibiting perturbation* of the Q_o site, since it is apparent that the site can accommodate a full complement of Q/QH_2 for catalysis and also have ethanol present interacting with the [2Fe-2S] cluster histidines.

These non-inhibiting alcohols clarify the utility of [2Fe-2S] cluster EPR spectral lineshape as an effective monitor of the hydrogen bonding interactions of the exposed cluster histidine ligands with the immediate environment and with the Q_o site occupants. Water, of course, is another alcohol which is apparently free to interact with the [2Fe-2S] cluster histidines when other competing hydrogen bond acceptors have been removed (g_x at 1.765 for fully ubiquinone extracted chromatophores), as we have previously suggested [5]. However, the access of water to these histidines in the intact cytochrome bc_1 complex is probably constrained and the presence of small hydrophobic alkyl groups on water soluble alcohols increase their effective local concentration and enables them to displace water or Q/QH_2 as hydrogen bond acceptors to the [2Fe-2S] cluster histidine ligand. Simple hydrophobicity alone is not sufficient, as longer chain alcohols partition elsewhere. Appropriately structured hydrophobic alcohols, including QH_2, compete even more effectively as hydrogen bond acceptors, while classic Q_o site inhibitors such as stigmatellin and *ortho*-hydroxy naphthoquinone analogues compete away all others.

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