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Metals as a probe of 'Rieske' ISP domain movements

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Introduction

Cytochrome (cyt) b_6f complex, analogous to the mitochondrial cyt bc_1 complex, is the central enzyme in linear photosynthetic electron transport in the chloroplast, serving to shuttle electrons between Photosystem II and Photosystem I, and to pump protons into the thylakoid lumen for ATP synthesis (for review see Hauska,1996; Berry,2000). This complex is comprised of 4 catalytic subunits: cytochrome b_6 and subunit IV with two b-type cytochromes, cytochrome f subunit with a cytochrome f heme and the iron-sulfur protein (ISP) with an iron-sulfur (2Fe2S) cluster. Electron transfer through the complex occurs through a bifurcated process, known as the Q-cycle, where two protons are pumped into the thylakoid lumen for every electron that is transferred (Mitchell,1975; Crofts,1985). An accurate structural explanation of this process was an enigma, until the recent x-ray crystal structures of the cyt bc_1 complex revealed that the ISP moves 16-22 Å and rotates 57^o-63^o during catalysis between at least two positions including, one close to the cytochrome b_L or ISP_B and one close to the cytochrome c_1/f or ISP_C. These movements are thought essential for gating electron transfer, forcing the Q-cycle to shuttle protons into the thylakoid lumen (Iwata,1998; Berry,2000). It has been known for some time that Zn^{2+} and other metal ions inhibit cyt *bc* complexes (Lorusso,1991; Link,1995) and previously we showed that Cu^{2+} inhibits turnover of the cyt b_6f complex through long range interactions, most likely binding to cytochrome f (Rao B. K.,1999). We proposed that metals interact with the Q_o site by changing the conformation of the ISP through cyt f (Rao B. K.,1999).

Here, through EPR spectroscopy of mylar-oriented cyt b_6f complex, we show that Cu^{2+}, Cu^{1+}, and Zn^{2+} alter the conformation of the ISP and thus represent a specific inhibitor of ISP domain movements in the cyt b_6f complex and represents strong support for a critical role for ISP domain movements in Q_o site catalysis.

Materials and Methods

Spinach thylakoids were prepared by a modified procedure from (Kramer,1993). Cyt b_6f complex was isolated from spinach thylakoids as essentially described in (Hauska,1986) with some minor modifications. Partially ordered samples on mylar sheets were obtained from purified cyt b_6f complex as reported previously (Riedel,1991). The samples were partially dehydrated and ordered with metals present throughout and were subsequently dipped in 10 mM ascorbate or 5 mM ferricyanide to reduce or oxidize the samples, respectively. Electron paramagnetic resonance (EPR) spectra were obtained using a Bruker 200tt EPR spectrometer, using a GFS-300 transfer tube, ESR-900 helium cryostat, and the model ITC4 temperature controller. The EPR parameters used in the experiments are found in the figure legends.

Results and Discussion

Figure 1 shows the effects of Q_o site inhibitors, DBMIB and stigmatellin, on the EPR spectra of Cu^{2+}-bound to the cyt b_6f complex. The $g_\perp$ transition (g=2.04, line width = 140 G, and amplitude) showed no significant changes with addition of these inhibitors. However, the low field $g_\parallel$ transitions were significantly altered. In the control, a $g_\parallel$ transition is found at g=

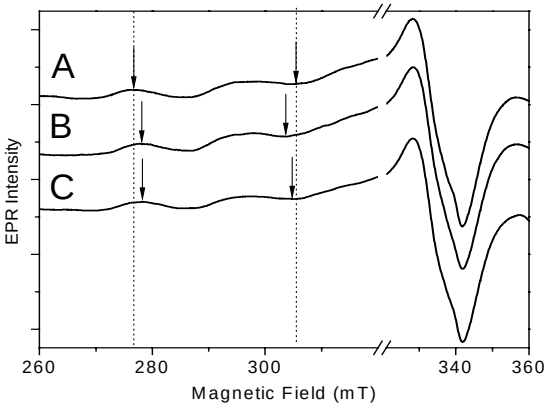

Figure 1. Effect of Q_o site inhibitors on the Cu^{2+}-cyt b_6f spectra A) without inhibitors, B) with DBMIB, and C) stigmatellin. Arrows and dotted lines are used to emphasize the difference between the spectra. EPR parameters were: microwave frequency = ~9.429 GHz; microwave power = 6.32 mW, time constant = 1000 ms, modulation amplitude = 20 G, center field = 3100 G, sweep width = 2000G, sweep time = 2 min. and temperature = 70K.

2.220 with the peaks split 214 G ($A_\parallel$ = 2.21 x 10^{-2} cm^{-1}). With DBMIB, this is changed to $g_\parallel$ = ~2.235 with hyperfine splitting of 191G ($A_\parallel$ = 1.98 x 10^{-2} cm^{-1}) (see Figure 1B). Finally with stigmatellin, the transition was found at $g_\parallel$ = ~2.225 with a hyperfine splitting of 196G ($A_\parallel$= 2.04 x 10^{-2} cm^{-1}). The distortion of $A_\parallel$ and $g_\parallel$ confirm long range interactions initiated by the Cu^{2+} on the Q_o site (see Rao B. K.,1999). Furthermore, the axial spectra from this figure are characteristic of type-II or "normal" Cu^{2+} ligand geometry, possessing pure or distorted tetragonal symmetry (Pilbrow,1990).

Figure 2 shows the effect of Cu^{2+} and Zn^{2+} on the EPR spectrum of the 'Rieske' 2Fe2S cluster in the presence of 2 equivalents (equiv.) of DBMIB and 2 equiv. Cu^{2+} (Figure 2C, solid line) or in the presence of 10 equiv Zn^{2+} (Figure 2E). The g_y-transition in the presence of 2 equiv. DBMIB in the absence of metals is clearly shifted from g=1.89 to g=1.94 as has been previously been shown (Malkin,1981; Schoepp,1999) (Figure 2A and 2D). In contrast,

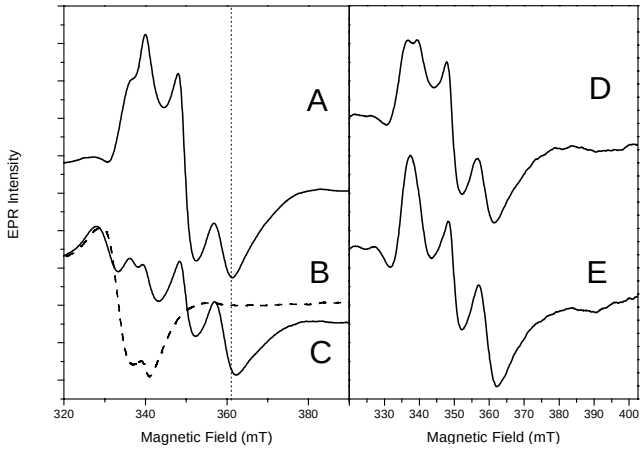

Figure 2: Effect of metals, Cu^{2+} and Zn^{2+}, on the DBMIB-induced shift in the 2Fe2S cluster. A) 2 equiv. DBMIB, B) 2 equiv. Cu^{2+} and 2 equiv. DBMIB (ISP oxidized, dotted line), C) 2 equiv. DBMIB and 2 equiv. Cu^{2+} (ISP reduced, solid line), D) 2 equiv. DBMIB, E) 2 equiv. DBMIB and 10 equiv. Zn^{2+}. EPR parameters are the same as Figure 1 except: center field = 3700 G and temperature = 20K

in the presence of 2 equiv. DBMIB and 2 equiv. Cu^{2+}, there are some significant changes in the DBMIB-induced shift of the ISP (see Figure 2C, solid line) including, apparent changes in the g=1.89 transition with an increase in line width to 85 G from 70G and a 50% increase in the amplitude. Unfortunately, there is significant overlap of the g_z-transition (g=2.03) and g=1.94 transition with the Cu^{2+} spectra, making conclusions from these peaks somewhat

speculative (see Figure 2B, dotted line). Experiments with Cu^{1+} produced similar results as shown in Figure 2 (data not shown). In the presence of 10 equiv. Zn^{2+} (Figure 2E), the amplitude of the transition at g=1.89 increases 50% from Figure 2D, and the line width increases to 85G. All the metals show line shapes that are characteristic of the displacement of DBMIB (Malkin,1982), but this is inconclusive because at concentrations of excess metal (i.e. 100 fold) there is never complete displacement of the DBMIB-induced shift in the 2Fe2S spectrum. Furthermore, in figure 1, we see simultaneous binding with Cu^{2+} and the Q_o site inhibitors, implying compatible interaction with these inhibitors and Cu^{2+}. We concluded long-range interactions do indeed occur between the Q_o site and the metals, although the binding of Q_o site inhibitors may be significantly different than quinol.

Kinetic assays of the cyt b_6f complex with Zn^{2+} (data not shown) demonstrate that Zn^{2+} is competitive with plastoquinol and non-competitive with cyt c, as we found earlier for Cu^{2+} (Rao B. K.,1999). Moreover, copper bound to cyt b_6f complex is more reducible by ascorbate, than in free solution suggesting a thermodynamic relationship with Cu^{1+} and at high concentrations Cu^{1+}, we can eliminate the Cu^{2+}-bound EPR signal, implying overlapping binding sites (data not shown). Zn^{2+} and Cu^{1+} also had effects on the DBMIB-induced shifts of the 2Fe2S cluster (see above), leading us to suggest that they bind to similar sites as Cu^{2+}.

Figure 3 shows the magnetic field orientation-dependence of EPR signals in mylar-oriented cyt b_6f complex in the presence of Zn^{2+} and Cu^{1+}. In uninhibited cyt b_6f complex (Figure 3A),

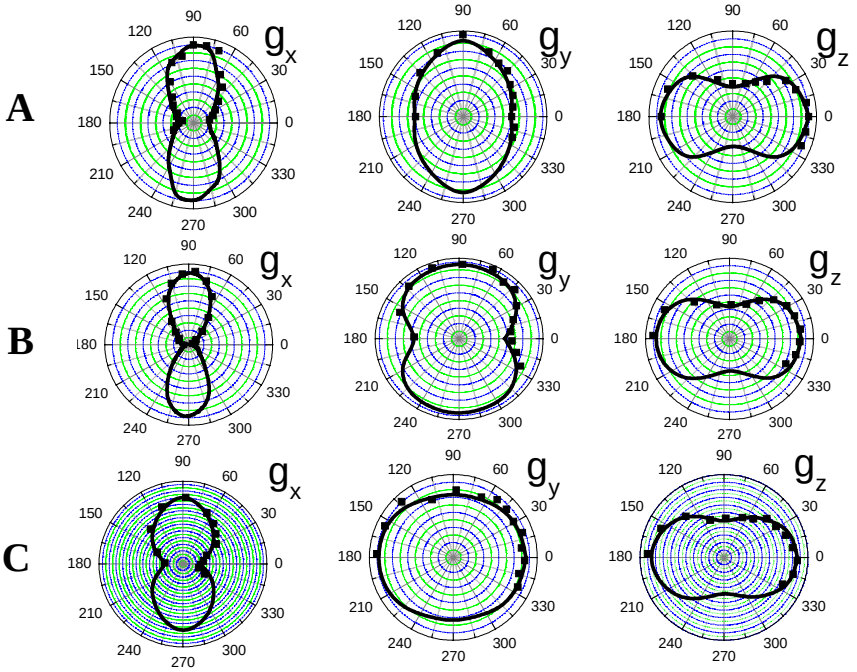

Figure 3: Orientation dependence of the g_x, g_y and g_z transitions in A) uninhibited b6f complex, B) Cu^{2+}-inhibited cyt b_6f complex and C) Zn^{2+}-inhibited b_6f complex. EPR spectrometer parameters are the same as in Figure 2.

maximal g_x, g_y and g_z transitions were found at ~85°, 90° and ~10° with respect to membrane plane, as previously found (Schoepp,1999). Cu^{1+} inhibited cyt b_6f complex (Figure 3B) shows significantly altered g_x and g_y-transition orientations of 90° and 20° with respect to the membrane plane, respectively and an unchanged g_z-transition. On the other hand, Zn^{2+}-inhibited cyt b_6f (Figure 3C) shows similar shifts to the ISP as Q_o site inhibitors DBMIB (Schoepp,1999) and stigmatellin (Riedel,1991), shifting the g_y transition ~80° away from the membrane normal. The g_x transition also showed a shift to 20°, while the g_z transition appeared to be shifted to about ~25°. Furthermore, the effects of these metals on the ISP cannot be ascribed to large-scale disordering since the orientation of the cyt b g_z-transitions were found to be identical in all the samples that were tested (data not shown), implying that

the orientation of the transmembrane helices which surround the cyt b hemes are also the same.

From the similarities of the orientation of the g-transitions of the 2Fe2S cluster in the presence of Q_o site inhibitors and metals with the expected structure of the cyt b_6f complex (see Rao B. K.,1999; Schoepp,1999; Soriano,1999) and the cyt bc_1 structure with Q_o site occupants (Zhang,1998), we propose that metals shift the ISP into a position intermediate between the proximal (ISP_B) and (ISP_C) distal positions, perhaps similar to that observed by (see Iwata,1998) for the mitochondrial cyt bc_1 complex. Because of disorder within the membrane plane, we cannot rule out that the 2Fe2S cluster is in the opposite orientation, but this would place the cluster in a structurally dubious position. In addition, consistent with a Cu^{2+} binding site on cyt *f*, as proposed in (Rao B. K.,1999), the orientation of the g_z transition of ferricytochrome was shifted by 5^o upon addition of Cu^{2+} (not shown).

Unlike inhibition by Q_o site inhibitors or Q_i site inhibitors, metals, such as Zn^{2+} and Cu^{2+}, represent a novel mechanism of inhibition, binding at sites that are distant from the Q_o site and Q_i site. As indicated by EPR spectroscopy of oriented samples, metal inhibition represents a potential specific inhibitor of ISP motions. By shifting the Rieske ISP into an intermediate position, metal inhibition strongly supports the role of ISP movements in Q_o site catalysis and the interconvertibility of the ISP conformations. Thus metals can serve as tools for studying the thermodynamics and kinetics of ISP motion.

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