THE COORDINATION ENVIRONMENT OF MITOCHONDRIAL CYTOCHROMES *b*

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1. Introduction

It is generally believed that the cytochromes *b* of the mitochondrial electron-transport chain are structurally related to the more thoroughly characterized *b*-type hemes such as that present in microsomal cytochrome b_5. Consequently, the amino acids which function as heme ligands are presumed to be a pair of histidine residues.

However, the electron paramagnetic resonance (EPR) spectra of cytochromes *b*-561 and *b*-566 for both yeast [1] and heart [2] mitochondria are unusual in that the *g*-values (g_z) associated with the low-field absorption feature are atypically large (3.4–3.8 in specific cases). By contrast, the g_z in cytochrome b_5 [3] and other bis-imidazole species [4] is ~3.0.

Based on this difference, and the demonstration of comparably large *g*-values in amine derivatives of protoheme [5], it might be expected that the lysine–histidine pair and/or bis-lysine exist as the heme ligands in the mitochondrial *b*-type cytochromes. However, this proposal undermines the conclusion of Beychok [6], where a detailed comparison of the amino acid sequence of cytochromes *b* of both mitochondrial and other origin showed homology among these proteins, especially in the neighborhood of the heme binding domain.

Here, we present the results of some EPR and magnetic circular dichroism (MCD) measurements on several pertinent model compounds which make it clear that the unusual EPR features of the mitochondrial cytochromes *b* can be explained by the traditional bis-imidazole coordination subjected to steric strain.

2. Materials and methods

Hemin chloride was obtained from Calbiochem-Behring Corp. 1-Methylimidazole (1-MeIm), 2-methylimidazole (2-MeIm), and 1,2-dimethylimidazole (1,2-diMeIm) were obtained from Aldrich Chemical Co. Solutions of protoheme chloride in dimethyl sulfoxide (DMSO) were made 0.6 M in the appropriate imidazole for spectroscopy.

Complex III from yeast mitochondria was isolated as in [1]. Just prior to MCD spectroscopy, complex III was oxidized with ferricyanide and passed through a Biogel P-6 column (1.5 cm × 7 cm) equilibrated with 0.1 M potassium phosphate (pH 7.4) containing 0.1% deoxycholate and 0.1% Triton QS-30. Cytochrome c_1 was isolated from yeast complex III which had been treated with dithiothreitol, and was subsequently purified using DEAE cellulose (Whatman DE52) and Sephadex G-100. Cytochrome *b* was solubilized from yeast complex III using tetrahydrophthalic anhydride and purified by chromatography on hydroxylapatite. Cytochrome c_1 was oxidized prior to MCD spectroscopy with ferricyanide and passed through a Biogel P-6 column (1.5 cm × 7 cm) equilibrated with 0.1 M Tris–HCl (pH 7.6) containing 0.1% deoxycholate and 0.1% cholate. Cytochrome *b* was dissolved in 0.1 M potassium phosphate (pH 7.4) containing 0.5% cholate.

Heme concentration was measured by the pyridine–hemochromogen method [7] or by using extinction coefficients [1].

EPR spectroscopy was performed on a Varian E-6 EPR spectrometer and MCD spectra were recorded on a Jasco J-500C spectropolarimeter equipped with an electromagnet (1.4 Tesla) and a Jasco DP-500 data processor for data accumulation, signal averaging, and manipulation. Both instruments were interfaced to the laboratory data system for storage, manipulation, and presentation of the data.

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3. Results and discussion

In fig.1 we present the EPR spectra of ferric protoheme coordinated with several imidazole derivatives. With 1-methylimidazole a typical rhombic low-spin heme spectrum is obtained (fig.1A) with $g_x = 1.51$, $g_y = 2.27$ and $g_z = 2.97$. These values are quite typical of those found in well-characterized proteins such as cytochrome b_5 [3].

With imidazoles alkylated at the 2-position, quite different spectra were obtained. The most homogeneous spectrum was obtained with 1,2-dimethylimidazole which exhibited the low spin g_z peak at 3.48 (1900 G) with a feature reasonably interpreted as g_y at 2.067; g_x is not observed. The spectrum also exhibited a contribution from an axially symmetric high-spin heme with $g_\perp = 6.0$ and $g_\parallel$ clearly visible at 2.0. There was also a signal from iron at $g = 4.3$.

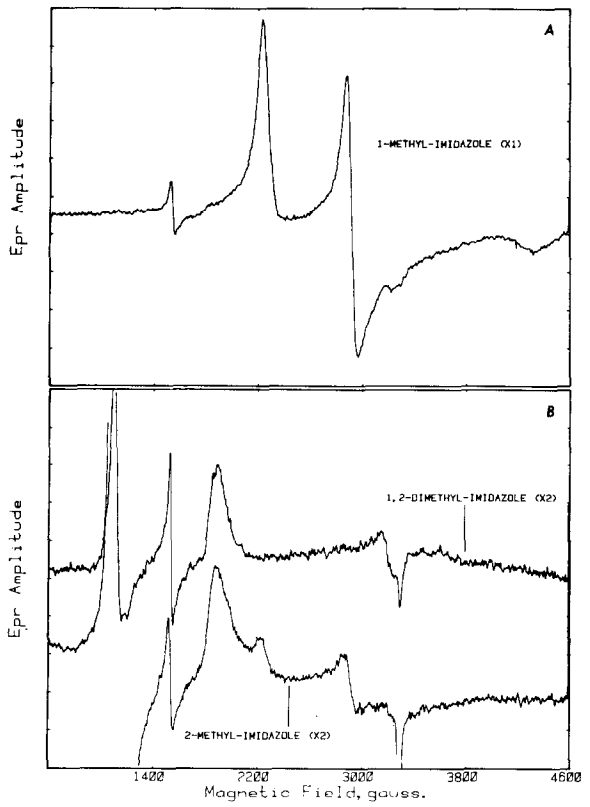

Fig.1. EPR spectra of ferric protoheme–imidazole complexes in DMSO recorded under the following conditions: 4 mW power at 6.9 K and 9.243 GHz with 10 G modulation; protoheme was 440 μM.

The same features are present in the spectrum of the 2-methylimidazole complex. However, with this derivative the contribution of the high-spin species is much larger and a second minority low-spin species with $g_z = 2.96$ and $g_y = 2.26$ was clearly evident.

A crude attempt to quantitate the low-spin species using Albracht's approximate method [8] showed that the same amount of heme was present in each case, that is, the $g = 3.48$ resonance observed in the complexes with the imidazoles methylated at position 2 are not minority species. It should be noted that it is a characteristic of the $g = 6$ high-spin resonance to dominate an EPR spectrum even when it represents a minor component in solution.

The most important conclusion to be drawn from the results is that the unusual EPR of mitochondrial *b* cytochromes can be reproduced using sterically hindered imidazoles.

The question then arises whether these derivatives are still low-spin at room temperature or whether this low-spin behavior is a consequence of the low temperature of the EPR measurements. This question is addressed in fig.2 which shows the Soret and visible MCD spectra of the model compounds. The analogous MCD spectra of complex III and its component cytochromes are shown in fig.3.

It can be seen from fig.2 that the low-spin character of the 2-methylimidazole–protoheme complex substantially persists at room temperature, whereas the 1,2-dimethylimidazole protoheme derivative has much more high-spin character. In drawing these conclusions we have used the 1-methylimidazole derivative as representative of a low-spin standard and used the trough at 418 nm as the benchmark. The high-spin reference was provided by the bis-DMSO–protoheme complex with its characteristic trough in the near-infrared at 633 nm. Note that this trough is absent in the 1-methylimidazole derivative and slightly and substantially developed in the 2-methylimidazole and 1,2-dimethylimidazole complexes, respectively. The converse behavior is observed in the Soret region MCD spectra.

The heme proteins of complex III appear to be completely low-spin at room temperature as gauged by the substantial Soret amplitudes and by the absence of any high-spin feature in the near-infrared region. The shape and intensity of the MCD spectrum of cytochrome *b* in the visible region are very similar to those of other bis-imidazole heme proteins [9].

From these data we conclude that the unusual

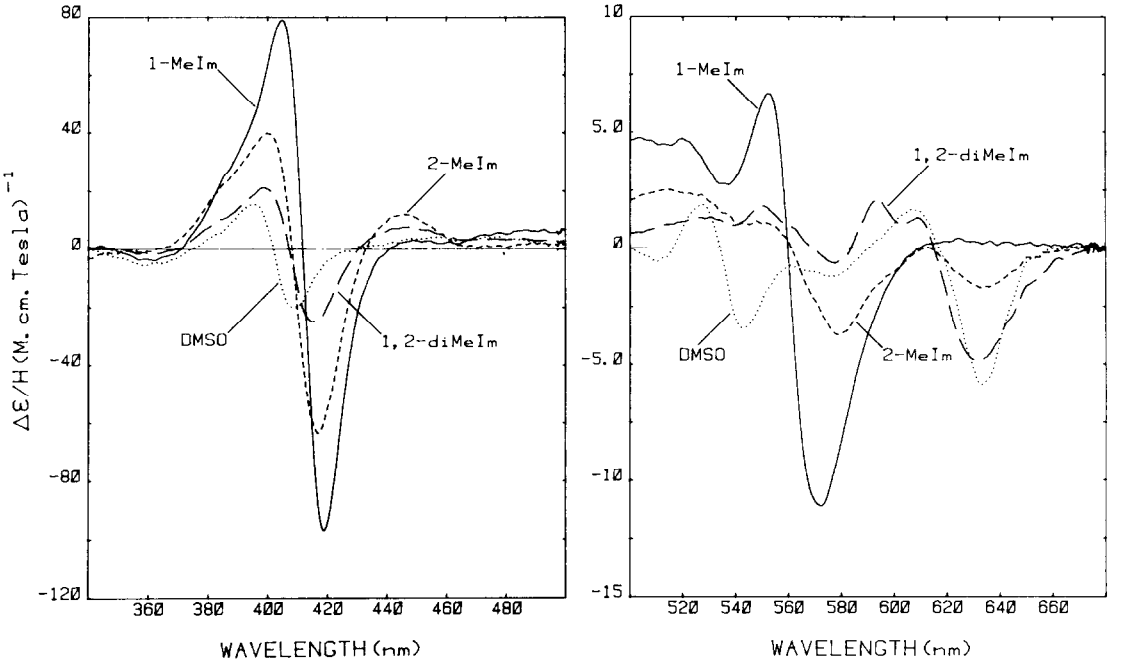

Fig.2. MCD spectra of ferric protoheme complexes with DMSO and imidazoles taken at 20°C; protoheme was 5.0 μM in a 1 cm pathlength cell for the Soret region and 88 μM in a 0.2 cm pathlength cell for the visible region. Eight passes were averaged per spectrum for the Soret region and 16 passes for the visible region with a time constant of 0.5 s and a scan speed of 50 nm/min.

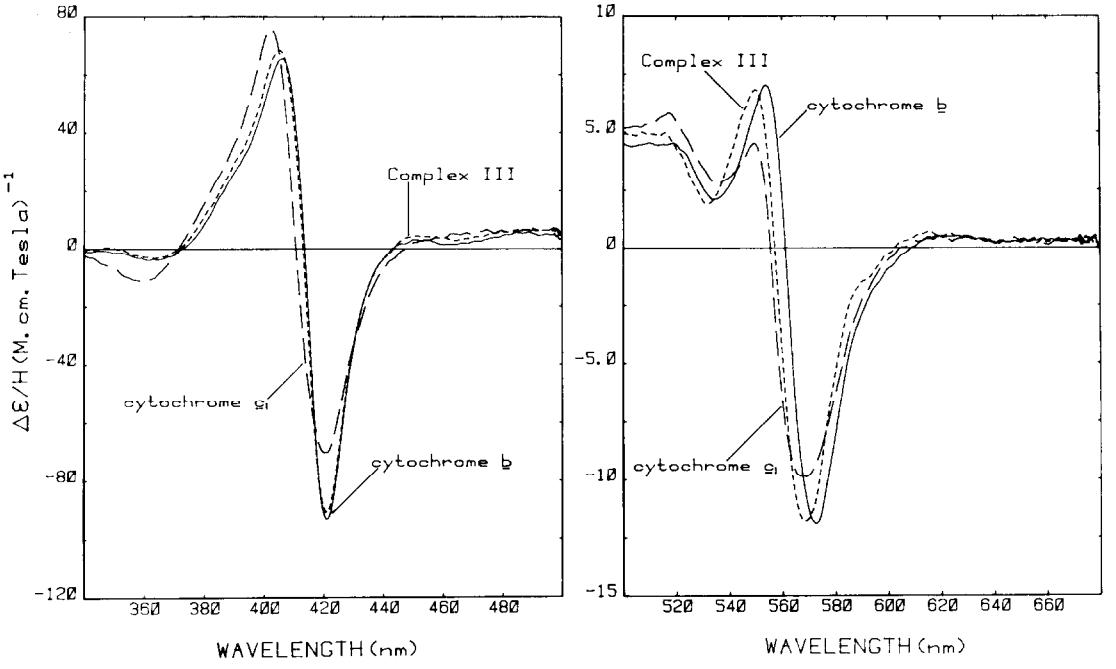

Fig.3. MCD spectra of oxidized complex III and isolated cytochromes *b* and c_1 taken at 10°C. The heme concentrations for complex III, cytochrome *b* and cytochrome c_1 were: for the Soret region, 7.9 μM, 7.0 μM and 6.9 μM, respectively; for the visible region, 63 μM, 70 μM and 41 μM, respectively, all in a 1 cm pathlength cell. Other conditions were as in fig.2.

magnetic resonance properties of mitochondrial cytochromes *b* can be approached in 'strained' complexes of heme with sterically hindered imidazoles. These structures are on the border line of stability and the equilibrium between the high-spin and low-spin configurations can be modulated by temperature, a circumstance which, by analogy, may provide an explanation for the relative ease with which the mitochondrial *b*-type cytochromes acquire reactivity with CO [10].

Finally, we conclude that the available EPR data do not contradict the deductions [6] drawn from sequence homologies between mitochondrial and other cytochromes *b*.

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