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Pitfalls in assigning heme axial coordination by EPR

c-Type cytochromes with atypical Met–His ligation

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Different monohemic *c*-type cytochromes were analyzed by visible, EPR and ^{1}H NMR spectroscopies. While the visible and NMR data show unambiguously that the heme iron has a Met–His heme axial coordination, the EPR data indicate an axial ligand field typical of that for a bis-histidinyl ligation. The validity of the widely used EPR methods for the determination of the heme iron axial coordination, based on the crystal field parameters (tetragonality and rhombicity), is questioned.

Heme protein; Axial ligand; EPR; NMR; Ligand field

1. INTRODUCTION

One of the key pieces of structural information required to understand the physico-chemical properties of heme proteins is the axial coordination of the heme iron, which plays a major role in modulating their reduction potentials, as well as their electronic structures [1]. Since 3D structures are not available for the large majority of heme proteins, several spectroscopic methods are commonly used to determine the axial ligation, namely visible, NMR, EPR and MCD [1]. The EPR method has been widely applied since Blumberg and Peisach [2,3] proposed that the crystal field parameters Δ/λ (tetragonal field) and V/λ (rhombic field), calculated directly from the principal values of the g-tensor measured by EPR, would allow the identification of the heme iron axial ligands. In a graphic representation of the rhombicity, V/Δ, vs. the tetragonal field for several proteins (the 'Truth Diagram'), the experimental data points associated with each type of axial coordination cluster in relatively well-defined regions of the diagram. While the rhombicity has a wide range of values within each group, the tetragonal field was thought to be typical for each ligand combination, reflecting both the non-equivalence between the equatorial (the four pyrrole nitrogens of the porphyrin ring) and the axial bonds, as well as their actual strength. In fact, a series of D values somewhat similar, but not identical, to the spectrochemical series for the usual ligands was found: thioether <imidazole<imidazolate<SH^-<OH^-<~ RS^- [4].

The Truth Diagram method has been routinely used when all the three g-values are directly obtained from the EPR spectra. In this paper we report several examples of *c*-type cytochromes with a Met–His axial ligation (as shown by NMR and visible data) which, although exhibiting 'normal' EPR spectra with the principal values of the g-tensor clearly defined and having a rhombic crystal field, yield crystal field parameters identical to those of bis-histidinyl coordination.

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2. MATERIALS AND METHODS

The soluble monohemic cytochromes c_L from *Methylophilus methylotrophus* (NCIB 10515) and *Methylobacterium extorquens* AM1 (NCIMB 9133) were purified to homogeneity as described by Anthony and co-workers [5,6], with some modifications. The cytochrome c_{551} from *Ectothiorhodospira halophila*, purified as described by Meyer [7], was a kind gift from Profs. I. Bertini and C. Luchinat. The EPR spectra were recorded on a Bruker ESP380 spectrometer, equipped with a ESR-900 continuous flow helium cryostat from Oxford Instruments, a Bruker Gaussmeter and a Hewlett-Packard 5350B microwave frequency meter. The ^{1}H NMR spectra were obtained using a Bruker AMX 500 spectrometer, operating at 500.13 MHz for protons. Chemical shifts are referenced to internal dioxane at 3.75 ppm, and are quoted relative to 3-trimethylsilyl-propane sulfonic acid (sodium salt).

3. RESULTS AND DISCUSSION

The spectroscopic data on *Methylophilus methylotrophus*, *Methylobacterium extorquens* and *Ectothiorhodospira halophila* cytochromes are presented in Figs. 1 and 2 and Table I. The EPR spectra exhibit a single

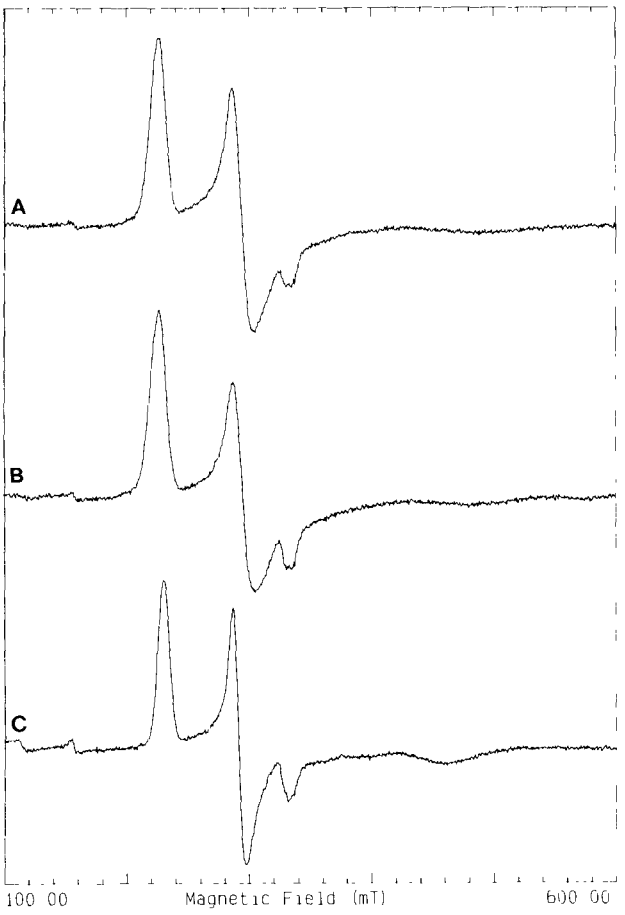

Fig. 1. EPR spectra of *Methylophilus methylotrophus* (A), *Methylobacterium extorquens* (B) and *Ectothiorhodospira halophila* (C) cytochromes, at pH ~ 7. Temperature 10 K, microwave power 2.4 mW, microwave frequency 9.42 GHz.

rhombic component, with g-values typical of low-spin hemes (Fig. 1). The crystal field parameters for these cytochromes, calculated according to the Blumberg and Peisach formalism [2], are also presented in the same Table. Clearly, both the g-tensor principal values and the tetragonal field values are typical of bis-histidinyl heme iron axial ligation. However, the visible spectra of these ferricytochromes display a band at 695 nm, a signature for the presence of a methionyl residue bound to the heme iron [1].

The ^{1}H NMR spectra of the ferrocytochromes in the low-frequency region show also unambiguously that methionine is the sixth ligand (Fig. 2). The three proton intensity singlet at ~-3 ppm has been known since the work of McDonald et al. [8] to come from the methyl group of the methionine ligand, which is shifted to low frequency by the heme ring current. The general pattern of methionine resonances, which includes a three-proton intensity peak at ~-3 ppm, and up to four resolved one-proton intensity peaks in the low-frequency region of the spectra (arising from the methionine β and γ protons) is now used routinely to identify methionine ligands in low-spin heme proteins [9], and is clearly present in the spectra shown in Fig. 2.

Another example of this kind of protein is cytochrome c_6 from *Monoraphidium braunii* [10]. The EPR spectra of samples at low pH (pH<6) exhibit a form with a tetragonality similar to that of a bis-histidinyl ligation. This form was shown to correspond to a Met–His coordination [10] by ^{1}H NMR and visible spectroscopies (Table I).

The data presented here clearly demonstrate that a Met–His coordinated heme iron can, in several cases, give rise to a tetragonal field similar to that of a bis-

Table I

Spectroscopic data and reduction potentials for *Ectothiorhodospira halophila, Methylophilus methylotrophus, Methylobacterium extorquens* and *Monoraphidium braunii* cytochromes

Cytochrome from	E_o mV (pH 7) [ref]	g-values			V/λ	Δ/λ	V/Δ	695 nm band	NMR***
		g_x	g_y	g_z					
Methylophilus methylotrophus	310 [5]	1.43	2.28	2.97	2.07	2.78	0.75	+	−3.06
Methylobacterium extorquens	256 [6]	1.38	2.29	2.98	1.85	2.62	0.71	+	−3.02
Ectothiorodospira halophila	58 [7]	1.45	2.32	2.92	1.83	2.78	0.66	+	−3.10
*Monoraphidium braunii**	350 [10]	1.43	2.29	2.95	1.93	2.74	0.70	+	−2.80
Met–His**						2.0–2.5			
His–His**						2.5–3.1			

+ Observed.
*Low pH form [10].
**Approximate range of the tetragonal field values for the two types of axial coordinations in the Truth Diagram.
***Chemical shift of the methionine ε-cH_3 in ^{1}H NMR spectra of ferrocytochromes (ppm).

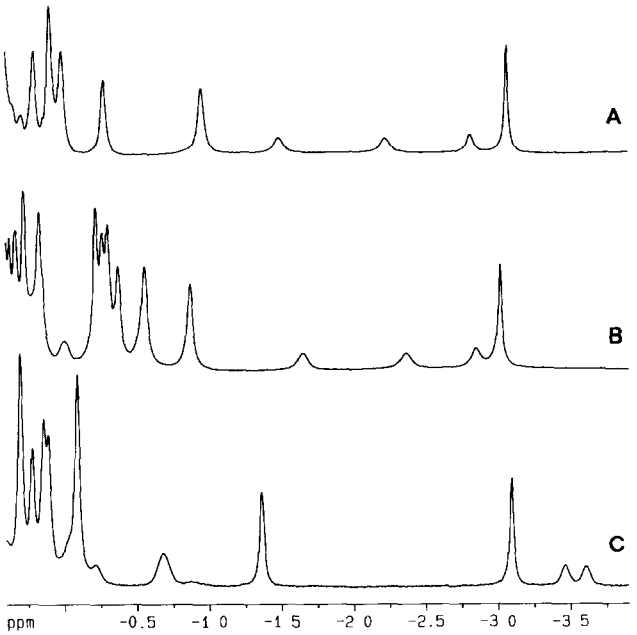

Fig. 2. Low-frequency region of the 500 MHz ^{1}H NMR spectra of *Methylophilus methylotrophus* (A), *Methylobacterium extorquens* (B) and *Ectothiorhodospira halophila* (C) cytochromes at pH ~ 7. Temperature 298 K.

histidinyl coordination. It is worth stressing that the amino acid sequence and analysis of cytochrome c_{551} from *E. halophila* show that it contains only one histidinyl residue [7].

The structural basis for the higher value of the axial ligand field, in relation to the usual values of Met–His ligation, is presently unknown. These cytochromes have different primary structures and reduction potentials (Table I) and, while *M. braunii* c_6 has a quadrupole splitting of 2.5 mm/s at 200 K [10], *Ectothiorhodospira halophila* c_{551} has the usual splitting of 2.2 mm/s [11,12] (our unpublished results). Furthermore, in the case of the cytochromes c_L, the pattern of the methionine ligand proton resonances is unusual insofar as none of the methylene protons resonate at a lower frequency than the ε-CH_3 group. This shows that the methionine configuration is quite unlike that found either in mammalian cytochrome *c*'s or in small bacterial cytochromes, which are often exemplified by horse cytochrome *c* [13] and *Pseudomonas aeruginosa* cytochrome c_{551} [14], respectively.

The major conclusion at present is that the Truth Diagram does not provide an unambiguous distinction between a Met–His and a His–His heme iron axial coordination in heme proteins even when, as in the present examples, the EPR spectrum has the three principal values of the g-tensor clearly defined (rhombic crystal field). MCD spectroscopy has been used for the identification of the axial ligation, based on the value of the energy of the charge-transfer transition (E_{CT}) in the near infrared MCD spectra [4,15,16]. However, as shown by Thomson and co-workers [4], this approach and the Truth Diagram method are interrelated since the value of E_{CT} is linearly correlated with the energy of the d_{yz} orbital in relation to the t_{2g} barycenter, and hence with the crystal field parameters, Δ and V. Thus, assuming the general validity of the equations in [4], the estimated E_{CT} for the cytochromes studied in the present work is ~6,200 cm^{-1} (~1,600 nm), which is in the typical region for bis-histidinyl ligation. However, it should be noticed that such correlation may be limited to closely related heme proteins, as shown by the recent MCD studies on bacterioferritin hemes [17,18].

In summary, the present work shows that methods based on a crystal field analysis may lead to erroneous conclusions, and that different spectroscopic tools based on a distinct approach must be used to unambiguously identify the heme iron axial ligation. ^{1}H NMR seems to remain the most reliable spectroscopic technique for the identification of methionine coordination in low-spin heme proteins. Indeed, although the detection of the absorption band at about 695 nm in the visible spectra of ferricytochromes is a fingerprint for Met–His coordination, it is not always observed [1]. Also, the fact that cytochromes with very distinct reduction potentials, which are within the normal range for cytochromes with Met–His coordinated hemes [1], may have crystal field parameters typical for His–His ligation, shows that the heme reduction potentials are not directly correlated with the axial and rhombic distortions of the crystal field. This is in contrast to earlier suggestions [16] and indicates that such a correlation may be fortuitous, or valid only for proteins with very similar structures.

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