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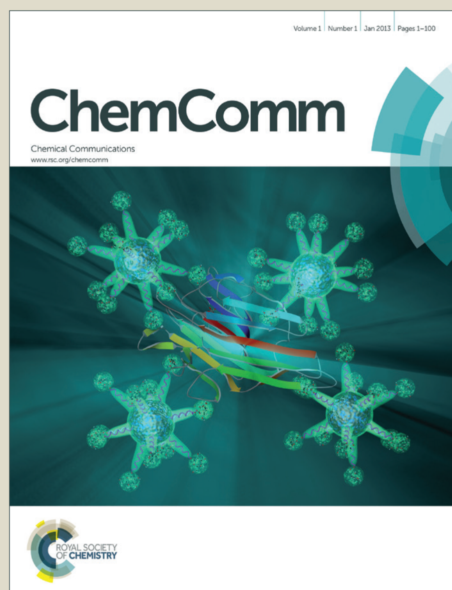

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COMMUNICATION

Artificial heme-proteins: determination of axial ligand orientations through paramagnetic NMR shift.

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An empirical equation, describing the relation between the porphyrin methyl hyperfine shifts and the position of the axial ligand(s), has been applied to an artificial heme-protein in order to obtain insight into the active site properties of heme-protein models.

The design of metalloprotein models, tailored to specific applications, is emerging as an important goal in protein chemistry. To date, several groups have made significant progress toward the design of proteins with similar functions and activities to those of natural enzymes. Metalloprotein mimetics have been developed through the introduction of novel metal-binding sites within the naturally occurring proteins as well as through *de novo* design. Such studies are stimulated by the ambition to shed light on the fundamental features for catalytic activity, and by the possibility to develop novel synthetic proteins for biotechnological applications. Tailoring synthetic models requires the development of sophisticated molecular architectures that distil the quintessential elements responsible for activities. Thus peptide-based metalloprotein models seem suitable candidates to mimic both the structural features and reactivity of the natural systems.[1-3]

This approach, has been successfully applied to different natural proteins involved in biological electron transfer, catalysis and gene regulation.[4-8] A protein family that has received considerable attention is the heme-protein family. Heme-proteins are involved in a wide range of biological process, and over the years, a large number of peptide and protein-based heme-protein mimetics have been developed, in order to determine how the protein matrix tunes the properties of the heme to evoke the wide variety of activities. Using a structure-based strategy we developed models for heme-proteins (mimochromes). These mimetics are composed by two peptide chains covalently linked to a deuteroporphyrins IX by a lysine side-chain (**Fig. 1**). Iron and cobalt mimochrome derivatives were extensively characterized by Uv-visibile spectroscopy (UV-vis) and circular dichroism (CD) spectroscopies.[9]

Mimochromes are able to support several reversible oxidation/reduction cycles, and the last born, Mimochrome VI, accommodating a peroxidase-like active site, exhibits catalytic performance similar to the natural counterpart horseradish peroxidase.[10]

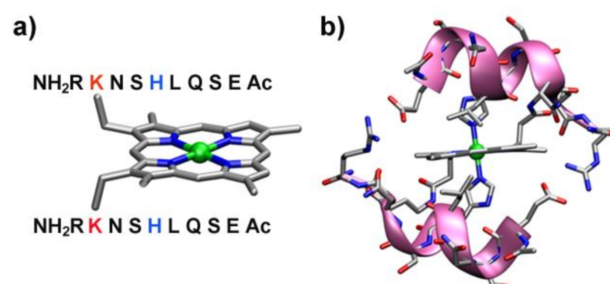

Fig. 1 Co(III)-mimochrome IV. **a)** amino acid sequence; the coordinating His and the covalently-linked Lys residues are highlighted in blue and red, respectively. **b)** Solution structure.

Structural data on mimochrome active sites came from solution characterization, by nuclear magnetic resonances (NMR), of the diamagnetic Co(III) complexes. The presence of the paramagnetic iron(III) ion in mimochromes complicates the structure determination.[11]

A paramagnetic ion modifies the properties of the nuclear spins coupled to it, and dramatically alters the NMR spectra. The coupling can occur through both chemical bonds and space, and affects chemical shifts and relaxation rates. In particular, the relaxation rates increase, due to coupling with unpaired electron(s), and this phenomenon induces, in a distance-dependent manner, line broadening of vicinal nuclear spins, even beyond signal detection. Nevertheless, the paramagnetic effects are a precious source of structural data, because they offer distance and geometric information about the environment of the paramagnetic center in metalloproteins.[12,13]

Focusing on heme-proteins, several methodologies have been developed. They combine experimental NMR data with theoretical approaches, in order to obtain detailed information about the geometry of the metal binding site. In their essence, these methodologies shed light on the relationship between the electronic structure of the heme, inferred by the paramagnetic shifts of its nuclei, and the orientation of the axial ligand(s). The heme of the low-spin heme-proteins has been characterized extensively, and semi-empirical and empirical equations have been developed to correlate the hyperfine shifts of the heme methyl resonances with the orientation of the iron axial ligand(s).[14-17]

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With the aim to extend these methods to artificial metalloproteins, and to fully characterize the active site structures of mimochromes, we report here the NMR characterization of Fe(III)-mimochrome IV complex. By combining an empirical equation, proposed for bis-histidine ferriheme-proteins, with experimental data obtained on Fe(III)-mimochrome IV, we obtained information about histidine orientations. Within the family of mimochromes, mimochrome IV is a simple, structurally defined heme-protein model, made up by two helical nonapeptides, both containing a histidine residue, which acts as axial ligand to the metal ion. This molecule was completely characterized and the three-dimensional structure of its Co(III) complex was determined by NMR and x-ray diffraction.

These studies revealed that Co(III)-mimochrome IV structures, both in solution and in the solid state, share numerous common features and conform well to the design, adopting the designed sandwich structure, with the Λ configuration of the hexa-coordinated Co(III) ion.[11,18] The simple and structurally defined mimochrome IV molecule can be an useful tool for exploring the subtle mechanisms that control the heme functions, such as redox potential. The heme redox potential of mimochrome IV falls within the range observed for natural cytochromes, which present a bis-His coordination.[11] Thus, this molecule may be a valuable system in the development of electrochemical biosensors. Unfortunately, we were unable to structurally characterize the active site geometry of the Fe(III) complex. Thus, the methodology herein reported can help to elucidate the axial ligand orientations which has been demonstrated to modulate the heme redox potential.[19]

Therefore, we undertook the NMR analysis of Fe(III)-mimochrome IV. Proton 1D and 2D nuclear overhauser spectra were collected at 298 K on a Bruker Avance II 500 MHz spectrometer. Details on data acquisition are reported in ESI.

A preliminary analysis was carried out in aqueous solution (phosphate buffer 10 mM, pH = 6.5). Although broad resonances were observed, four signals (at 21.5 ppm, 19.9 ppm, 14.5 ppm and 10.7 ppm) could be reasonably assigned to the heme methyl protons (See Fig. S1 in ESI). The spread of these resonances is 10.8 ppm, still indicative of a preferred orientation of axial ligands (~5 ppm is the spread observed for freely rotating axial ligands).[20] In order to improve the NMR spectral quality, the analysis was performed in phosphate buffer (10 mM, pH 6.5)/TFE/DMSO solution (60/20/20, v/v/v). TFE is a well-known helix-inducing solvent, and the addition of a small amount of TFE to the Fe(III)-mimochrome IV solution resulted in an increase of helical content.[11] The folding induced by TFE causes the two helical peptides to enwrap the heme faces, thus positioning the imidazole rings correctly with respect to the deuteroporphyrin plane. The correct folding assumed in mixed water/TFE solvent is relevant for catalytic mimochromes. In fact, the presence of an organic co-solvent does not inactivate this class of artificial enzymes, as often observed for natural enzymes.[10] This finding is very important for the catalysis of substrates with limited aqueous solubility.

Further, the addition of DMSO, an high viscosity solvent (η = 2 mPa•s at T = 298 K), to an aqueous solution, increases the medium viscosity, modifies the relaxation rates, thus affecting the spectral lines.

The 1D spectrum (see **Fig. 2a**) is characteristic of low-spin *S* = 1/2 bis-His ferriheme-proteins. It shows several peaks shifted out of the diamagnetic region, due to the effect of the paramagnetic ferric ion. In particular, four resonances in the region spanning from 5 to 30 ppm can be attributed to the heme methyl substituents. The methyl groups have a mean shift of 18.5 ppm and a spread of *ca.* 20 ppm, indicative of fixed orientation of the heme axial ligands. This is typical of natural heme-proteins, where the axial ligands are held in fixed orientation by interaction with the protein matrix. In contrast, several small heme protein models are characterized by axial ligands that rotate very rapidly (on the order of hundreds of thousands to a million times per second), and an average hyperfine shift is detected for each heme substituent.[20,21] In mimochrome IV, the presence of the peptide chains constrains the histidines to be uniquely oriented. The assignment was performed starting from the 5-meso proton at -1.06 ppm: it is the only proton in dipolar contact with the two methyl groups, 7-CH_3 and 3-CH_3 at 26.1 ppm and 16.9 ppm, respectively (**Fig.2b**). The methyl resonance at 24.5 ppm exhibits dipolar connectivities to a signal at -0.77 (15-meso) and to two protons at about 3 ppm (reasonably attributed to the propionic methylene protons), and therefore it was attributed to the methyl groups at positions 17. Finally, the resonance at 6.67 was attributed to methyl at position 12.

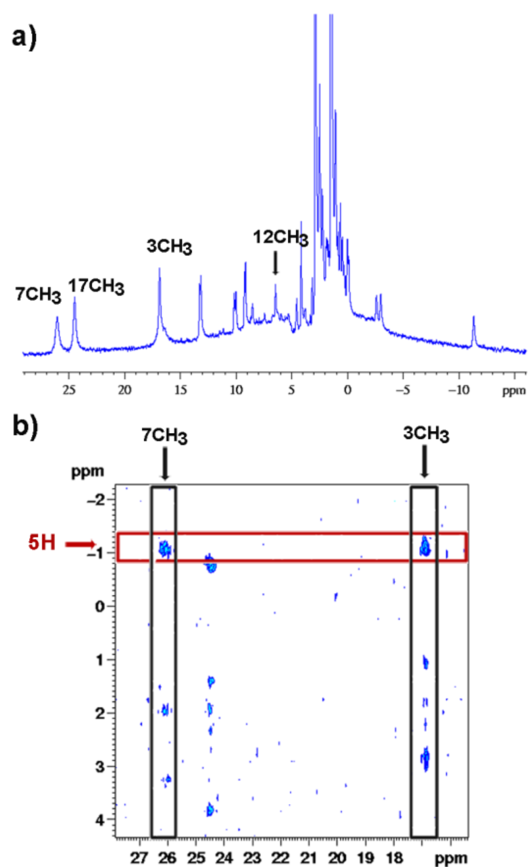

Fig. 2 a) Fe(III)-mimochrome IV 1D ^{1}H spectrum. **b)** Details of the NOESY spectrum.

In order to determine the axial ligand orientations of Fe(III)-mimochrome IV, the methyl resonances measured at 298 K were introduced into the empirical equation proposed by Turner:[15]

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$$\delta_i(\text{ppm}) = \cos\beta[38.0 \sin^2(\theta_i-\phi)-4.1 \cos^2(\theta_i+\phi)-15.9]+13.8 \quad (1)$$

where δ_i is the hyperfine shift of the *i*th methyl, θ_i is the angle between the metal-*i*th methyl direction and the metal-N_{23} nitrogen axis, β is the acute angle between the two histidine planes, and ϕ is the angle between the bisector of the angle β and the metal-N23 direction (see **Fig. 3** for nomenclature). Eqn. (1) provided β and ϕ values of 51° and 34°, respectively.

A comparison between the experimental and calculated values of ^{1}H methyl chemical shifts is schematically illustrated in **Fig. 4**, which shows the good agreement between the pattern of distribution of the experimental shifts and the theoretical fit.

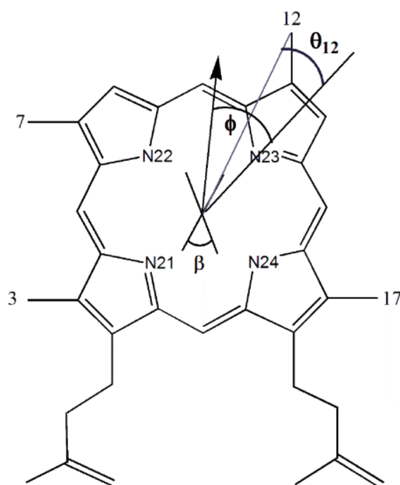

Fig. 3 Schematic representation of the heme moiety. The reference axis is taken along the metal-N23 direction. The β angle defines the acute angle between the two histidine planes and the ϕ angle defines the average orientation of the His planes, projected on the heme plane, with respect to the N_{21}-N_{23} direction.

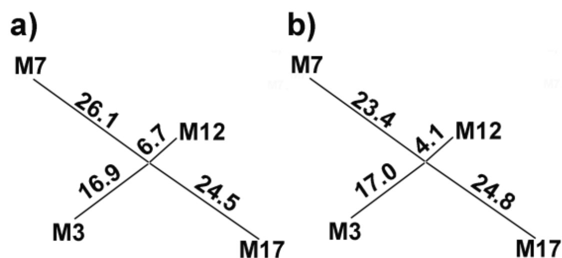

Fig. 4 Diagram showing **a)** experimental and **b)** calculated chemical shifts of the heme methyls. Lines have the iron at the origin and are directed towards each substituent, with a length proportional to the shift. The implicit orientation of the heme is as represented in **Fig. 3**.

Table 1 reports the β and ϕ values calculated for the Fe(III)-mimochrome IV complex compared with those observed in Co(III)-mimochrome IV crystal and NMR structures.

Table 1 Comparison of β and ϕ in mimochrome IV complexes

	β	ϕ
Fe(III)-mimochrome IV	51°	34°
Co(III)-mimochrome IV (x-ray)	57°	47°
Co(III)-mimochrome IV (NMR)	52°	33°

The orientation of axial histidines (**Fig. 4**) in Fe(III)-mimochrome IV is almost identical to that observed in the solution structure of its cobalt form, with the imidazole planes nearly eclipsing the Np-Co-Np bonds.[11] On the contrary, the active site shows a different geometry in solid state, where the histidine planes are almost perpendicular.[18] These data highlight that the main factor in determining the histidine orientation in mimochrome IV is the direction of the two helices with respect to the porphyrin plane, which in the Co(III)-mimochrome IV crystal is affected by crystal packing interactions.

In summary, we have shown that it is possible to determine the histidine orientations in artificial peptide-based heme-protein models by taking advantage of an empirical methodology previously used for natural ferriheme-proteins. To the best of our knowledge, this powerful approach has been applied for the first time to the active site characterization of paramagnetic peptide-based heme-protein mimetics.

This represents an important result, as it allows to quickly determine the axial ligand orientations, which finely tune the heme functional properties, i.e. redox potential and reactivity.[19-22] As a consequence, an iterative process of redesign and characterization, through the methodology herein reported, may provide artificial heme-enzymes, able to selectively catalyze the oxidation of different substrates.

Notes and references

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