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^{1}H resonances of proximal histidine in CO complexes of hemoglobins provide a sensitive probe of coordination geometry

Claudio Dalvit, Linda Tennant and Peter E. Wright

Department of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

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A straightforward strategy for assignment of the $C^{\varepsilon}H$, $C^{\delta}H$ and $N^{\delta}H$ proton resonances of the proximal histidine ligand in diamagnetic complexes of monomeric hemoglobins and myoglobins is reported. These resonances are subject to large ring current shifts and are highly sensitive to coordination geometry. There are no significant differences between the CO complexes of myoglobin, leghemoglobin or hemoglobin α-subunits in proximal His coordination geometry or hydrogen bonding to the backbone at Leu F4. Ring current calculations show that the His F8 coordination geometry in the CO complexes of myoglobin and hemoglobin α-subunits is very similar in crystal and solution.

^{1}H NMR; Proximal histidine; Coordination geometry; Myoglobin; Leghemoglobin; Hemoglobin α-subunit

1. INTRODUCTION

The molecular mechanisms by which the CO and O_2 affinities of hemoglobins are controlled have been the subject of intensive study for many years. Ever since Perutz's [1,2] original proposal of proximal base strain, the proximal histidine-heme interaction has occupied a central place in theories of hemoglobin reactivities. The FeN^{ε} (histidine) bond has been subjected to detailed study by many techniques including X-ray diffraction, EXAFS, XANES and resonance Raman spectroscopy. These studies have encompassed both tetrameric hemoglobins and a variety of monomeric hemoglobins and myoglobins from different species. Despite the potential of NMR to investigate the structure of proteins in solution, only a few studies of proximal histidine ligation have been reported. Most of those focus on deoxy and cyanide complexes [3–5], for which large paramagnetic resonance shifts occur which are difficult to interpret in terms of coordination geometry. In the present paper we describe a simple strategy for the assignment of proximal histidine resonances in diamagnetic complexes of monomeric hemoglobins. These resonances are subject to large ring current shifts from the heme which are easily interpreted in terms of proximal histidine-heme distances. The present NMR technique thus provides a highly sensitive approach to investigate the role of the proximal histidine ligand(s) in diamagnetic heme proteins which is competitive with other technologies. We show that there are no significant differences in proximal His coordination geometry between the carbon monoxide complexes of sperm whale myoglobin (Mb), soybean leghemoglobin (Lb) and isolated α-subunits of human hemoglobin (Hb^{α}).

Correspondence address: P.E. Wright, Dept of Molecular Biology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

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2. MATERIALS AND METHODS

Sperm whale myoglobin (Sigma, type II), human hemoglobin α^{SH} subunits and soybean leghemoglobin *a* were purified according to earlier procedures [6–9]. Samples of the CO complexes for NMR measurements [6,10] were prepared by elution from a column of Sephadex G-15 equilibrated with CO-saturated potassium phosphate buffer (50 mM, pH 5.2) in ^1H_2O. A small amount (10%) of ^2H_2O was added for the spectrometer lock.

^1H NMR spectra were recorded at 36°C on a Bruker AM500 spectrometer equipped with a 16 bit digitizer. Two-dimensional phase-sensitive double-quantum spectra were recorded with excitation periods τ = 30, 40 and 80 ms as described [11]. Phase-sensitive NOESY spectra were acquired using the standard pulse sequence [12]. The mixing times (τ_m) were sufficiently short (100–250 ms) to avoid problems of spin diffusion.

3. RESULTS AND DISCUSSION

The proximal histidine, His F8, is covalently bound to the heme iron atom through its N^{ε} atom; its $C^{\delta}H$ and $C^{\varepsilon}H$ resonances are therefore subject to extremely large upfield ring current shifts. The backbone amide and $C^{\alpha}H$ and the imidazole side chain proton resonances have been unambiguously assigned for MbCO and LbCO (Dalvit and Wright, manuscript in preparation) using sequential assignment procedures [13]. However, this approach is arduous and very lengthy for proteins of such high molecular mass. We report here on a simple and straightforward method for identification of the $C^{\varepsilon}H$, $C^{\delta}H$ and $N^{\delta}H$ resonances by the combined use of phase-sensitive double-quantum and NOESY spectra recorded in ^1H_2O. This strategy can be used even for monomeric hemoglobins for which complete amino acid sequences are not available.

The assignment strategy is illustrated for LbCO in fig.1. In the double-quantum spectrum (fig.1A) direct connectivities are observed between an exchangeable proton resonance (histidine $N^{\delta}H$) at 9.40 ppm and resonances at 1.34 and 0.62 ppm. These latter resonances arise from the $C^{\varepsilon}H$ and $C^{\delta}H$ protons and can be assigned from the NOESY spectrum; a strong NOE is observed between the

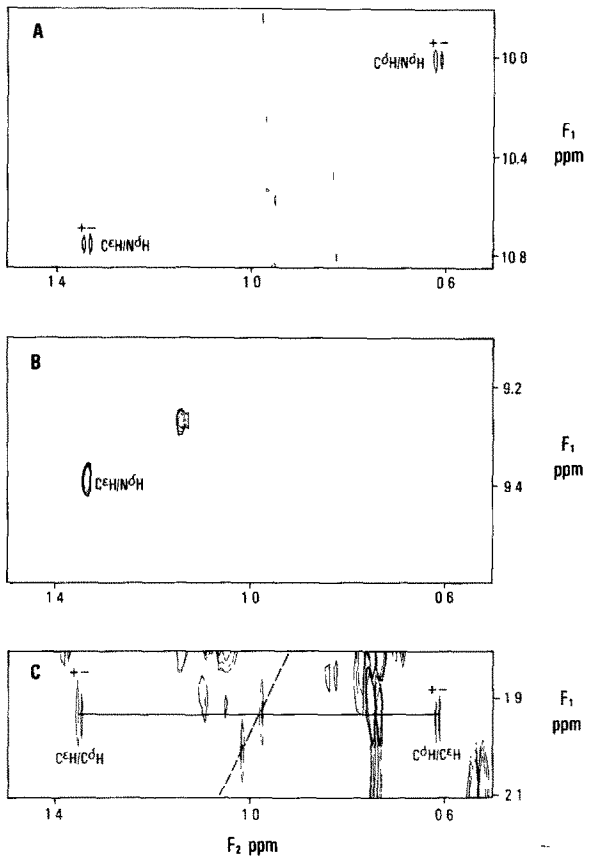

Fig.1. (A) Region of a phase-sensitive double-quantum spectrum (τ = 40 ms) of LbCO in 90% ^1H_2O and 10% ^2H_2O showing connectivities between the $N^{\delta}H$ and the $C^{\varepsilon}H$ and $C^{\delta}H$ resonances of the proximal histidine (His F8). Both positive and negative contour levels are drawn. (B) Region of a phase-sensitive NOESY spectrum (τ_m = 150 ms) of LbCO in 90% ^1H_2O and 10% ^2H_2O showing the NOE connectivity between the $N^{\delta}H$ and the $C^{\varepsilon}H$ resonances of the proximal histidine. (C) Region of a phase-sensitive double-quantum spectrum (τ = 80 ms) of LbCO in ^2H_2O (pH 7.0) showing direct connectivities between the $C^{\varepsilon}H$ and $C^{\delta}H$ resonances of His F8.

$N^{\delta}H$ resonance and the resonance at 1.34 ppm (fig.1B), unambiguously identifying this resonance as that of the spatially adjacent $C^{\varepsilon}H$ proton. The assignments of the proximal His resonances are further supported by observation of (i) direct double-quantum connectivities between the $C^{\delta}H$ and $C^{\varepsilon}H$ resonances (fig.1C), (ii) weak NOEs between the His $C^{\varepsilon}H$ and $C^{\delta}H$ and the heme δ-meso and β-meso proton resonances respectively (fig.2),

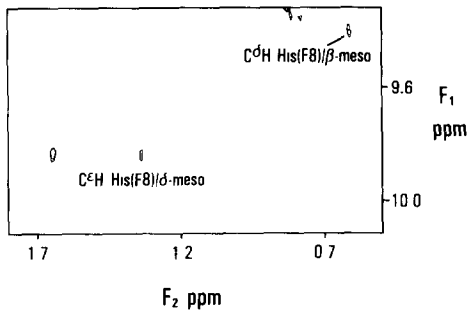

Fig.2. Region of a phase-sensitive NOESY spectrum (τ = 250 ms) of LbCO in ^2H_2O (pH 7.0) showing the cross peaks between the resonances of proximal histidine and the δ and β heme meso proton resonances.

and (iii) strong NOEs between the His N$^{\delta}$H and C$^{\varepsilon}$H resonances and both C$^{\beta}$H$_3$ resonances of Leu F4. The assignments of Leu F4 and the NOEs to the C$^{\varepsilon}$H resonance have been reported [14]. The assignments of His F8 and other heme pocket residues in MbCO and Hb$^{\alpha}$CO will be described in detail elsewhere [10]. For Hb$^{\alpha}$CO the proximal His resonance assignments are based solely on NOE data [10] since double-quantum spectra are of rather poor quality due to the tendency of the isolated α-subunits to aggregate. The chemical shifts of the proximal histidine resonances for all three heme proteins are summarized in table 1.

The present NMR experiments provide several significant results pertinent to the role of the proximal histidine in control of reactions of hemoglobins and myoglobins with ligands.

(i) The ring current shifts experienced by the C$^{\varepsilon}$H and C$^{\delta}$H resonances are very similar for MbCO, Hb$^{\alpha}$CO and LbCO. This is strong evidence for a highly conserved heme-proximal histidine interaction in the CO complexes of these three proteins.

(ii) For each of the proteins, the C$^{\varepsilon}$H and C$^{\delta}$H resonances experience similar upfield ring current shifts. This indicates that these two protons are at approximately the same distance from the heme plane and implies that the heme-proximal histidine linkage is nearly symmetric.

(iii) The chemical shift of the N$^{\delta}$H resonance of His F8 is influenced both by the heme ring current and by formation of a hydrogen bond to the backbone carbonyl of residue F4. This resonance has almost identical chemical shifts in the spectra of the three proteins, suggesting that not only the coordination geometry but also the hydrogen bond to the backbone is highly conserved.

Comparison of the proximal histidine coordination geometry in MbCO and Hb$^{\alpha}$CO in solution and in crystals is possible by calculation of ring current shifts from the crystal structure coordinates. The calculations were carried out using the eight-loop Johnson-Bovey model [15] with the calibrations of Cross and Wright [16]. Although all aromatic rings were included in the calculations, the ring current contributions of all but the porphyrin ring are negligibly small. The results are summarized in table 2. For MbCO there is reasonable agreement between the experimental ring current shifts and those calculated from the

Table 1

Chemical shifts of proximal histidine resonances

Proton	MbCO		Hb$^{\alpha}$CO		LbCO	
	δ^a	$\Delta\delta_{rc}{}^b$	δ^a	$\Delta\delta_{rc}{}^b$	δ^a	$\Delta\delta_{rc}{}^b$
C$^{\varepsilon}$H	1.66	−6.95	1.45	−7.16	1.34	−7.27
C$^{\delta}$H	1.13	−6.22	0.98	−6.37	0.62	−6.73
N$^{\delta}$H	9.36	[c]	9.33	[c]	9.40	[c]

[a] Chemical shift relative to dioxane at 3.75 ppm

[b] Experimental ring current shift calculated using the random-coil chemical shifts of Bundi and Wüthrich [20] for protonated histidine [16]

[c] The N$^{\delta}$H resonances experience a large upfield shift due to the heme ring current and hydrogen bonding to the backbone at residue F4

Table 2

Comparison of experimental and calculated[a] ring current shifts (ppm) for the proximal histidine in MbCO and Hb$^{\alpha}$CO

	$C^{\varepsilon}H$	$C^{\delta}H$
MbCO		
Experimental shift[b]	−6.95	−6.22
X-ray structure [17]	−6.10	−6.50
Neutron structure [18]	−8.53	−4.73
Hb$^{\alpha}$CO		
Experimental shift[b]	−7.16	−6.37
X-ray: real-space-refined [19]	−10.21	−5.08
X-ray: energy-refined [19]	−6.86	−6.48

[a] Calculated from crystal structure data using the Johnson-Bovey 8-loop model

[b] From table 1

refined 1.5 Å resolution X-ray structure [17]. Agreement with the neutron structure [18] is very poor. For Hb$^{\alpha}$CO, ring current shifts were calculated using both the real-space-refined and energy-refined coordinates of the α-subunit in the 2.7 Å resolution X-ray structure of the human carbonmonoxy hemoglobin tetramer [19]. The experimental ring current shifts do not agree well with those calculated from the real-space-refined structure but after energy-refinement the agreement is excellent. The sensitivity of the ring current shifts to changes in proximal histidine coordination geometry can be judged from a comparison of these structures. In the real-space-refined structure the Fe-N^{ε} bond is tilted ~10° from the heme normal such that the $C^{\varepsilon}H$ proton is closer than the $C^{\delta}H$ proton to the heme plane [19]. In the process of energy refinement the proximal histidine adopts a nearly symmetrical coordination geometry such that the $C^{\varepsilon}H$ and $C^{\delta}H$ proton resonances would experience very similar ring current shifts. The changes in tilt angle (~10°) and distance from N^{ε} to the mean heme plane (~0.05 Å) translate to calculated differences in ring current shift of >3 ppm for the $C^{\varepsilon}H$ resonance (table 2).

The strategy described in this paper for assignment of proximal histidine proton resonances in diamagnetic complexes of heme proteins leads to a highly sensitive technique for comparison of histidine coordination geometry. It should be generally applicable to proteins of molecular mass up to ~20000 Da. In the case of hemoglobins and myoglobins, the method is sensitive enough to reveal any subtle differences in heme-proximal histidine interaction which might contribute to differences in the O_2 and CO affinities of proteins from different species.

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