IDENTIFICATION OF THE SIXTH LIGAND OF METHIONINE SULFOXIDE CYTOCHROME c

Kathryn M. Ivanetich, Jean J. Bradshaw and G. Victor Fazakerley

Department of Physiology and Medical Biochemistry, University of Cape Town Medical School, Observatory, C.P., and Department of Chemistry, University of Cape Town, Rondebosch, C.P., South Africa.

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SUMMARY: Relevant portions of the proton n.m.r. spectra of ferri and ferro methionine sulfoxide cytochrome c are reported. At neutral pH, resonances attributable to the methyl group of methionine sulfoxide-80 are located at -5.3 and -2.45 ppm relative to DSS for ferri and ferro methionine sulfoxide cytochrome c, respectively. No such resonances are observable in the ferric compound at pH 4.5 where the derivative is in the high spin form. It is concluded that methionine sulfoxide-80 provides the sixth ligand in methionine sulfoxide cytochrome c. The liganding is definitely via the sulfur atom in the ferro derivative, and is probably also via this atom in the ferric compound.

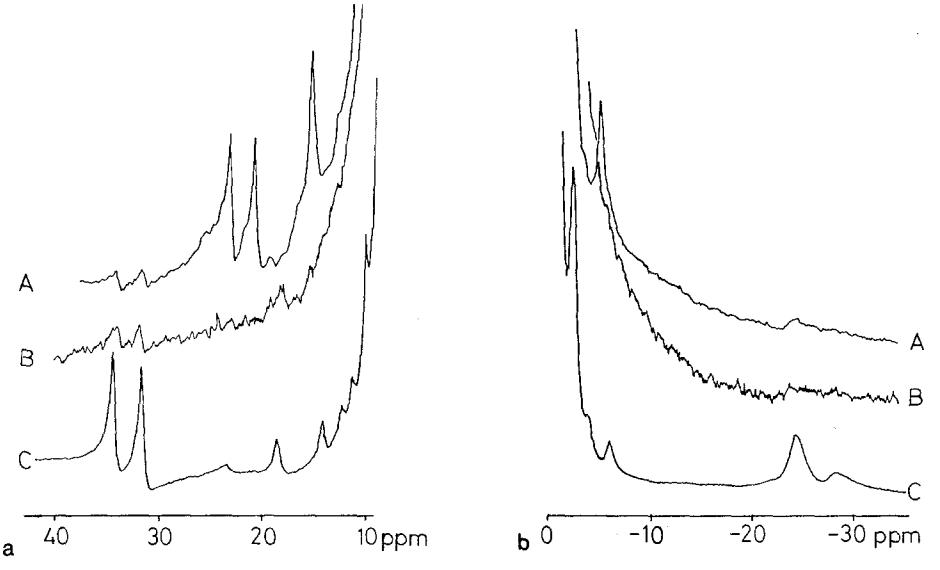
Methionine sulfoxide cytochrome c is a derivative of horse heart cytochrome c in which the methionine residues at positions 65 and 80 are converted to methionine sulfoxide residues. Methionine sulfoxide cytochrome c is unique in that it is the only active derivative of cytochrome c in which the sixth axial ligand, methionine-80, is chemically modified (1). It has been proposed that methionine sulfoxide-80 provides the sixth (axial) ligand to the heme iron ion in methionine sulfoxide cytochrome c (1). The identity of the sixth ligand, however, remains to be firmly established.

Proton magnetic resonance studies have been very successful in elucidating the nature of the sixth axial ligand in both oxidation states of cytochrome c and derivatives thereof (2-6). We have, therefore, attempted to identify the sixth ligand of ferri and ferro methionine sulfoxide cytochrome c using this technique.

MATERIALS AND METHODS: Methionine sulfoxide cytochrome c was prepared and purified according to the method of Ivanetich et al. (1). The derivative contained less than 7% unmodified cytochrome c by analysis of the 695 band content. This analysis was confirmed by observing resonances of the unmodified compound in the proton magnetic resonance spectra of methionine sulfoxide cytochrome c. Circular dichroism spectra were performed on a Jasco JA40B recording spectropolarimeter.

Proton magnetic resonance spectra were obtained with a Bruker 90 MHz fourier transform nuclear magnetic resonance spectrophotometer using a 10 mm probe. For initial experiments, 36 mg of cytochrome c or methionine sulfoxide cytochrome c (1.94 mM) were dissolved in 1.5 ml of D_2O. For final results, 300 mg of cytochrome c or methionine sulfoxide cytochrome c (16.1 mM) were dissolved in 1.5 ml of D_2O. pD was adjusted with DCl or NaOD. Chemical shifts are reported relative to DSS. Samples were reduced by the addition of a 3-fold excess of sodium dithionite after bubbling with oxygen-free nitrogen for 10 min. The tubes were then sealed under nitrogen and spectra of the reduced compounds were obtained within 30 min of reduction. There was no sign of oxidation of the samples at the end of these experiments.

RESULTS: Proton magnetic resonance spectra of ferricytochrome c (16.1 mM), pD 7.2 and ferri methionine sulfoxide cytochrome c



1. Proton magnetic resonance spectra of ferricytochrome c and ferri methionine sulfoxide cytochrome c. Room temperature; protein concentration, 16.2 mM. (A) ferri methionine sulfoxide cytochrome c, pD 7.5; (B) ferri methionine sulfoxide cytochrome c. pD 4.5; (C) ferri cytochrome c, pD 7.2.

(16.1 mM), pD 4.5 and 7.5 are presented in Figure 1. The resonances at +35.5, +32.8 and -24.2 p.p.m. in the spectrum of ferricytochrome c appear to be shifted in the spectrum of the ferri methionine sulfoxide c toward the peak representing the majority of unshifted protons of the protein plus the water peak. In the spectrum of the derivative at pD 7.5, analogous resonances appear at 23.3, 20.9 and -5.3, respectively, whereas at pD 4.5, all shifted resonances have collapsed to under the main protein/ water peak, and are not observable.

The proton magnetic resonance spectra of ferro methionine sulfoxide cytochrome c at neutral pH is presented in Figure 2. The unsplit resonance assigned to the methyl group of methionine-80 in ferrocytochrome c is located at -3.3 p.p.m. in the native protein (3,4) and appears to be shifted to -2.54 p.p.m. in the

2. Proton magnetic resonance spectrum of ferro methionine sulfoxide cytochrome c. Room temperature; protein concentration 16.2 mM, pD 7.2.

derivative. Proton magnetic resonance spectra of dilute solutions of ferri and ferro methionine sulfoxide cytochrome c (1.94 mM) exhibited identical peaks as described above for concentrated (16.1 mM) samples.

Circular dichroism spectra of 0.13 and 2 mM ferri methionine sulfoxide cytochrome c in 0.02M sodium phosphate buffer, pH 7.5, are identical to published spectra (1) indicating that at concentrations up to 2mM, the conformation of the derivative was not altered.

Table I. Shifted resonances of cytochrome c and methionine sulfoxide cytochrome c.[a]

Compound	pD	Resonances (p.p.m.)		
		Porphyrin Methyls	Unresolved Methyls	Met(sulfoxide) -80
Fe^{+3} cyt c	7.5	35.5, 33		-24.2
Fe^{+3} methionine sulfoxide cyt c	7.5	23.3,20.9 (35.5,33)[b]		-5.3 (-24.2)[b]
Fe^{+3} methionine sulfoxide cyt c	4.5	n.o. (35.5,33)[b]		n.o. (n.o.)[b]
Fe^{+2} cyt c[c]	6.4		-0.7	-3.3
Fe^{+2} methionine sulfoxide cyt c	7.2		-0.7	-2.45 (-3.3)[b]

[a] Abbreviations: cyt c, cytochrome c; n.o., not observable.

[b] Values in parentheses are resonances arising from cytochrome c contamination. The intensities of these resonances are approximately 7-10% of comparable resonances of methionine sulfoxide cytochrome c.

[c] From reference 4.

DISCUSSION: Cytochrome c has been extensively investigated using the technique of proton magnetic resonance (see for example, 2-6). Because of the electronic nature of the heme moiety, the resonances of protons in close proximity to the heme group are drastically shifted from their normal positions. Resonances upfield and/or downfield from DSS are clearly dissociated from the complex, heavily overlapping region of the spectrum (ca. 0-10 p.p.m.) associated with the normal protein resonances. For ferricytochrome c, markedly shifted resonances have been assigned unequivocally to the protons of the heme ring (i.e. +32.8, +35.4 p.p.m.) and those of the sixth axial ligand of cytochrome c, methionine-80 (-24.2 p.p.m.) (6).

We have taken advantage of the shift in the position of the resonances associated with methionine-80 to investigate the nature of the sixth ligand in a derivative of cytochrome c in which the normal sixth ligand, methionine-80 is chemically modified to methionine sulfoxide-80. This derivative has been extensively characterized, and the sixth ligand has been proposed to be methionine sulfoxide-80 bound via its sulfur atom (1). Direct evidence for this proposal, however, is lacking. The proton magnetic resonance studies of methionine sulfoxide cytochrome c discussed below indicate that methionine sulfoxide-80 is coordinated to the central iron atom of ferri and ferro methionine sulfoxide cytochrome c.

In the p.m.r. spectra of ferri and ferrocytochrome c prominent unsplit resonances appear shifted far upfield from a DSS standard. These resonances have been assigned to the unsplit methyl group of methionine 80, and their large shift can only be explained as arising from their extremely close proximity to the heme group. Less prominent, broadened re-

sonances assigned to the β and γ methylene protons of methionine-80, visible at high resolution, are also shifted greatly in both ferri and ferrocytochrome c (2-4). The high-field methyl resonance of methionine-80 appears at -3.3 p.p.m. and -24.2 p.p.m. in ferro and ferricytochrome c, respectively (3,6).

For ferro and ferri methionine sulfoxide cytochrome c, analogous unsplit sharp signals occur at -2.5 p.p.m. and -5.3 p.p.m., respectively. The unsplit sharp nature of these peaks strongly suggests methyl resonances. The large shift upfield of these peaks is most likely explained by the close proximity or liganding of an atom adjacent to this group to the heme iron. In ferri methionine sulfoxide cytochrome c, the shifted resonance is equal in intensity to that of each of the two methyl resonances of the porphyrin ring, substantiating the assignment as a methyl group. Of possible strong field ligands such as lysine, histidine or methionine, only methionine could give rise to an unsplit methyl resonance. The resonances of the single β and γ methylene protons which could further support this proposal are not clearly discernible in ferrocytochrome c or methionine sulfoxide ferrocytochrome c under the conditions of our experiments. For methionine sulfoxide ferricytochrome c, the large magnitude of the shift of peaks assigned as the methyl resonance of methionine sulfoxide-80 (-5.3 p.p.m.) and the 2 methyl resonances of the porphyrin ring (23.3 and 20.9 p.p.m.) compared to ferricytochrome c may be due to a drastic alteration in the environment of the heme moiety accompanying the coordination of methionine sulfoxide as the sixth ligand. It has been reported that the heme crevice of this derivative is weakened relative to cytochrome c (1), and thus the environment of the heme crevice must be altered compared to cytochrome c. Similar shifts in the positions

of the resonances assigned to the methyl groups of the porphyrin ring (22.9, 21.1) have been reported for cyanoferricytochrome c (3). In this derivative cyanide has displaced methionine-80 as the sixth ligand and the heme crevice is weakened relative to ferricytochrome c.

The similar positions of the methyl resonances of methionine-80 in ferrocytochrome c (-3.3 p.p.m.) and of methionine sulfoxide-80 in methionine sulfoxide ferrocytochrome c (-2.45 p.p.m.) indicate that the liganding is via the sulfur atom of methionine sulfoxide-80 Were the liganding via the oxygen atom, a much smaller shift than observed might be expected in view of the greater distance between the methyl of methionine sulfoxide-80 and heme moiety. It seems likely that the liganding is also via the sulfur in methionine sulfoxide ferricytochrome c for reasons described earlier (1); however, our data does not unequivocally support this position.

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