HEAVY METAL BINDING TO BIOLOGICAL MOLECULES

Identification of ligands by observation of $^{199}Hg-^{1}H$ coupling

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1. Introduction

Many biological molecules bind metal ions either at specific sites as a prerequisite to the expression of function, or at suitable sites elsewhere in the molecules [1,2]. There have been many approaches towards elucidating the nature and the geometry of the immediate coordination sphere around the metal ion, and although X-ray diffraction methods yield the most unequivocal answer, many proteins have not yet succumed to this technique.

Recently we have used high resolution nuclear magnetic resonance (NMR) spectroscopy to study the coordination in a variety of metalloproteins [3,4]. Although NMR spectroscopy has proved an extremely powerful method when the coordinating groups include histidyl residues, the distinction between coordinated and non-coordinated residues is generally based upon a comparison of the apo- and the holo-protein, and may thus be open to the objection that the observed differences are due to indirect effects of metal binding, rather than to coordination per se.

An ideal probe of metal coordination would involve the use of a metal ion which perturbs the ligands by a direct, through bond, effect and have a blank as near identical as possible. A metal ion with a nuclear spin, I, of 1/2 is expected to be such a probe, for it should give rise to spin–spin splitting in the NMR spectrum of coupled, ligand, nuclei. The use of an isotope of the same metal with $I = 0$ would provide an ideal blank. The only example of metal–ligand spin–spin coupling of which we are aware is $^{203,205}Tl-^{13}C$ coupling in thallium valinomycin [5]. We describe in the following work the substitution of mercury into the metal chelating antitumour drug bleomycin and the metallo-enzyme superoxide dismutase (EC 1.15.1.1.) and the observation of coupling between the ^{199}Hg nucleus ($I = 1/2$) and the protons on the coordinating histidyl groups.

2. Materials and methods

Bleomycin was a gift from Lunbeck Ltd, Luton, Bedfordshire, supplied metal-free, as for injection. Superoxide dismutase from bovine erythrocytes was a gift from Dr J. V. Bannister of the University of Malta. The apoenzyme was prepared by the method in [6] and excess EDTA removed by the method in [7].

^{199}Hg (70% enriched) was from the Oak Ridge National Laboratory, Tennessee. It was obtained as the oxide, which was dissolved in 1 M sulphuric acid, freeze dried and taken up in $^{2}H_2O$ before use. All other reagents were of analytical grade.

Pulsed Fourier-transform ^{1}H NMR spectra were obtained at 270 MHz on a modified Bruker spectrometer fitted with quadrature detection. Routinely 1024 transients were accumulated with a pulse-to-pulse time of 0.6 s. Convolution difference spectra were obtained as in [8]. Chemical shift values are quoted in parts per million (ppm) downfield of the internal standard sodium 2,2-dimethyl-2-silapentane-

5-sulphonate. pH measurements were made with a Pye Ingold micro-electrode and a Radiometer pH 26 meter. The pH is quoted as pH*, the direct meter reading, uncorrected for the ^2H isotope effect.

Solutions of superoxide dismutase were prepared for NMR spectroscopy in ^2H_2O containing 1 M NaCl and 20 mM sodium phosphate buffer, pH* 6.5. Bleomycin solutions were prepared in ^2H_2O containing 20 mM sodium cacodylate buffer, pH* 6.8 as in [9].

Mercury-substituted bleomycin was prepared by the addition of a stoichiometric amount of a solution of the metal sulphate to the bleomycin solution.

Mercury superoxide dismutase was prepared by the addition of one equivalent of Hg(II)/subunit+ to a solution of the apo-enzyme in the NMR tube and incubating the solution at 40°C for 15 min at pH* 6.5.

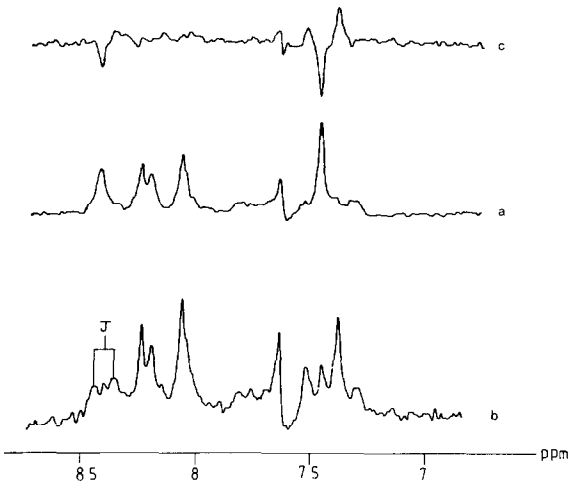

Fig.1. ^1H NMR spectrum of bleomycin substituted with: (a) native Hg(II); (b) 70% enriched ^{199}Hg(II); (c) the difference (b) – (a).

3. Results and discussion

3.1. *Bleomycin*

When Hg(II) is added to bleomycin, the C2(H) and C4(H) resonances of the β-histidyl group (which is thought to be one of the metal-binding ligands in zinc bleomycin [9,10]) are shifted from their positions in the spectrum of the metal free form. The ^1H NMR spectrum (fig.1a) of the derivative substituted with ^{199}Hg shows distinct difference from that of the derivative containing native mercury (17% ^{199}Hg). In the former the C2(H) and C4(H) resonances of the histidyl group are doublets, due presumably to ^{199}Hg–^1H coupling with coupling constants $J_{Hg-C(2)H}$ = 21 Hz and $J_{Hg-C(4)H}$ = 38 Hz. The intensities of the lines in the ^{199}Hg-coupled proton resonances are consistent with the abundance of the various isotopic species: $I = 0$ (^{198}Hg 10%, ^{200}Hg 23%, ^{202}Hg 30%, ^{204}Hg 7%), $I = 1/2$ (^{199}Hg 17%) and $I = 3/2$ (^{201}Hg 13%). As the $I = 0$ nuclei have no nuclear magnetic moment they will not spin–spin couple with proton nuclei; the $I = 3/2$ nuclei have short spin-lattice relaxation times due to their quadrupole moment and the spin–spin

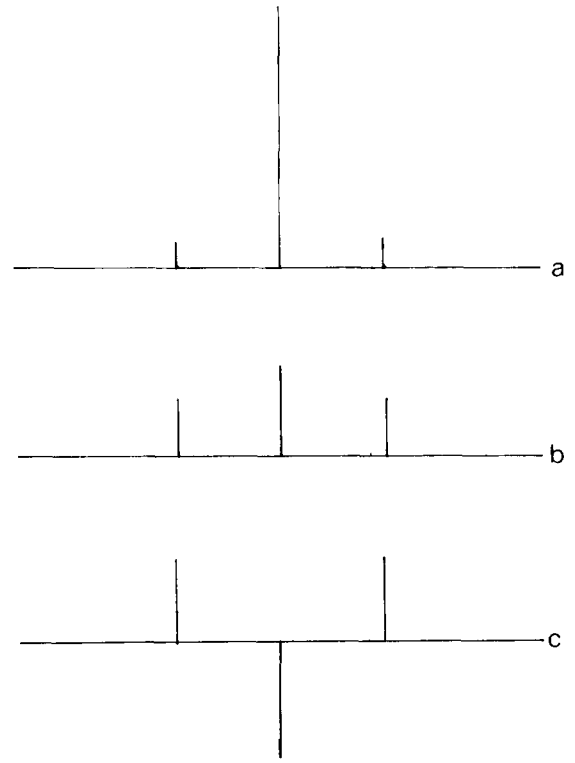

Fig.2. Expected intensities for a single proton split by coupling to: (a) native Hg (17%); (b) 70% enriched ^{199}Hg; (c) the difference (b) – (a).

+ Bovine erythrocyte superoxide dismutase consists of two identical subunits each containing one Cu(II) and one Zn(II) ion

interaction is effectively decoupled yielding a single line at the resonance position of the uncoupled nucleus [11]. This means that a sample containing $x\%$ ^{199}Hg will show a pattern of lines with intensities $1 : 2\,(100 - x)/x : 1$. This expected pattern is shown diagrammatically in fig.2 and can be compared with the actual spectra obtained in fig.1,3.

We have previously shown [9] that when zinc(II) binds to bleomycin, in addition to the effect upon the C2(H) and C4(H) histidyl resonances, the pyrimidine methyl resonance is also shifted consistent with the proposed role of the pyrimidine group as a metal ligand in bleomycin [9,10]. However no such shift is observed on mercury binding, and the pyrimidine methyl protons resonance is not split by the ^{199}Hg nucleus. It therefore appears that, in contrast to zinc(II) binding, the pyrimidine group is not involved in mercury(II) binding.

The other two suggested metal-binding groups in bleomycin, an α-amino group and a carbamoyl group, are not observed in ^{1}H NMR spectra run in $^{2}H_2O$ [9]. A molecular model of bleomycin suggests that the four coordinating groups are oriented such that the histidyl moiety must be *cis* to the pyrimidine group. We therefore suggest that it is not possible for mercury to satisfy its known preference for linear coordination by using both the pyrimidine and histidyl groups as ligands and that the ligand, additional to the histidyl group, is the α-amino moiety.

3.2. *Superoxide dismutase*

When mercury(II) is added to apo superoxide dismutase resonances previously assigned to C2 protons of histidines in the metal binding sites [3] are shifted. A well-defined splitting of one of these C2(H) resonances can be observed in the ^{199}Hg derivative (fig.3) and the coupling constant, 27 Hz, is comparable to those previously seen in ^{199}Hg–bleomycin. Interestingly we find that the mercury derivatives will not bind zinc(II) although they will bind Cu(II). We therefore suggest that in this derivative the mercury(II) is found in the zinc site. Unfortunately attempts to obtain a spectrum of the Cu(I)–Hg(II) enzyme resulted in precipitation of the elemental mercury upon addition of a reducing agent. The observation of only one C2(H) resonance split by ^{199}Hg again suggests that the Hg(II) is coordinated to two groups, a histidyl residue and perhaps aspartyl 1 i 8 (see [12] for details of the metal-binding sites as revealed by X-ray diffraction).

In conclusion, we have shown that when ^{199}Hg is bound to bleomycin and superoxide dismutase the protons on coordinating histidyl groups give rise to doublet resonances due to ^{199}Hg–^{1}H coupling. This coupling is characterized by $J_{^{199}Hg-^{1}H} = 20$–40 Hz. The observation of such metal–ligand coupling should prove to be a useful method in assigning coordinating groups both with ^{199}Hg and with other nuclei such as ^{113}Cd, ^{195}Pt, ^{203,205}Tl and ^{207}Pb.

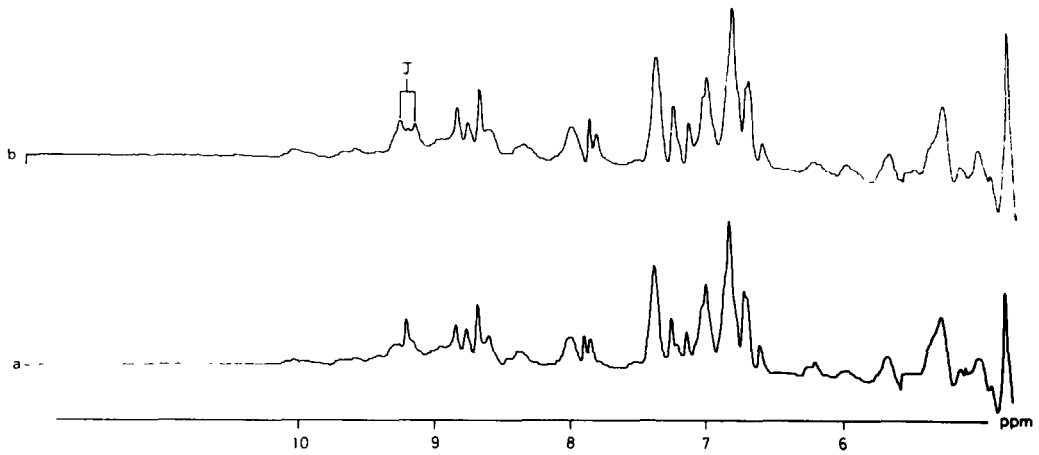

Fig.3. ^{1}H NMR spectrum of the aromatic region of superoxide dismutase coordinating: (a) native Hg(II); (b) 70% enriched ^{199}Hg(II).

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