^{1}H NMR SPECTRA OF METALLOTHIONEINS

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1. Introduction

Metallothionein is a cysteine-rich protein which was first isolated from the equine renal cortex by Margoshes and Vallee in 1957 [1]. Subsequently proteins having similar properties were isolated from kidney, liver and other parenchymatous tissue of a wide variety of animal species and also from microorganisms [2]. They all contain between 6 and 11% metal, usually zinc and/or cadmium, and between 30 and 35% cysteinyl residues, per molecular weight of 6800 [2,3]. Tyrosinyl, tryptophanyl, phenylalanyl and histidnyl residues are always absent [2,3]. The protein accumulates in the same tissues on the administration of Zn, Cd or other heavy metals [4–6], implicating the protein in metal metabolism, homeostasis or detoxification [2]. These unusual properties have provoked much interest in the structure and function of this protein. Little information is presently available on the latter; the investigation of the former has involved the use of various spectroscopic methods. Previous ^{1}H NMR investigations led to the conclusion that the structure of the metallothionein was random coil [7]. If correct, this would have profound implications with respect to the function of this protein. The present investigations are concerned with a comparison of the structures of the metal-free protein and two different metallothioneins from equine liver and equine kidney.

2. Materials and methods

Equine liver metallothionein 1A (96% Zn; 2% Cd; 2% Cu) and two samples of equine kidney metallothionein 1A (one containing 62% Cd; 38% Zn and the other 55% Cd; 45% Zn) were isolated as previously described [3,8]. The amino acid compositions and sequences of these proteins have been reported [2]. Metallothionein samples were prepared for NMR experiments by dialysis against three changes of 1-mM phosphate buffer, pH 7.5, followed by freeze-drying. The protein was then taken up in 20-mM phosphate buffer, pH* 7.5, in either H_2O or ^2H_2O. (pH* is a direct meter reading uncorrected for the ^{2}H isotope in ^2H_2O solutions.)

The apo-thioneins were prepared by dissolving the freeze-dried metallothioneins in 60-mM HCl and dialysed against three changes of the same solution. The resulting apothioneins were stable at low pH values but polymerised rapidly at ~ pH 7. Therefore the preparation of the apothionein at neutral pH was carried out by using a glove box, purged with oxygen-free nitrogen. All solutions were thrice degassed on a vacuum line to remove oxygen and transferred to the glove box whilst still evacuated. Apothionein, freeze-dried from 60-mM HCl, was dissolved in the 100-mM phosphate buffer, pH 7.5, transferred to an argon-filled NMR tube, and sealed whilst still in the glove box. No precipitation occurred.

Pulsed Fourier transform ^{1}H NMR spectra were obtained at 270 MHz by using a modified Bruker HFX-90 console, an Oxford Instrument superconducting magnet and a Nicolet 1085 computer. Quadrature detection was used and 1024 transients were routinely accumulated with a pulse-to-pulse time of 0.6 s. The residual water signal was suppressed by applying a pulse at the appropriate frequency at all times except during data acquisition.

Convolution difference spectra [9] and spin-echo spectra [10] were obtained as described in the literature. All chemical shifts are reported downfield from 2,2-dimethyl-2-silapentane-5-sulphonate as internal standard.

3. Results and discussion

The ^1H NMR spectra of apothionein are shown in fig.1. There were essentially no differences between the spectra of samples at pH 7.5 and samples at pH 1.4. Fig.1a, a spectrum of equine liver apothionein dissolved in ^2H_2O, shows the absence of aromatic amino acid residues, which would normally occur to low field of the residual HO^2H. Peptide NH's, which have chemical shifts in the same region are also absent, having exchanged with solvent deuterons. When the apothionein is dissolved in H_2O (fig.1b) they are readily apparent. The range of chemical shifts for these peptide NH's is quite small and it is probable that the apothionein is mainly in the random coil form. This is borne out by a comparison of fig.1a with a spectrum constructed by using a mixture of amino acids having the same composition as the apothionein (fig.1c). The spectra are practically identical and this is consistent with each residue in the apothionein having an environment (chemical or magnetic) very similar to that of the free constituent amino acids dissolved in water. Whether the protein is in a random-coil form or simple sufficiently 'open' to solvent that each residue has an aqueous environment cannot be distinguished. There are a number of marked differences apparent in the ^1H NMR spectra of the Zn–liver metallothionein (fig.2). The peptide NH's (fig.2b) give rise to resonances which are quite different from those in fig.1b. This can only be explained by postulating a change in the chemical/magnetic environments of the NH's on binding Zn to the apothionein and suggests that, in contrast to the conclusions arrived at in previous work [7], the Zn metallo-

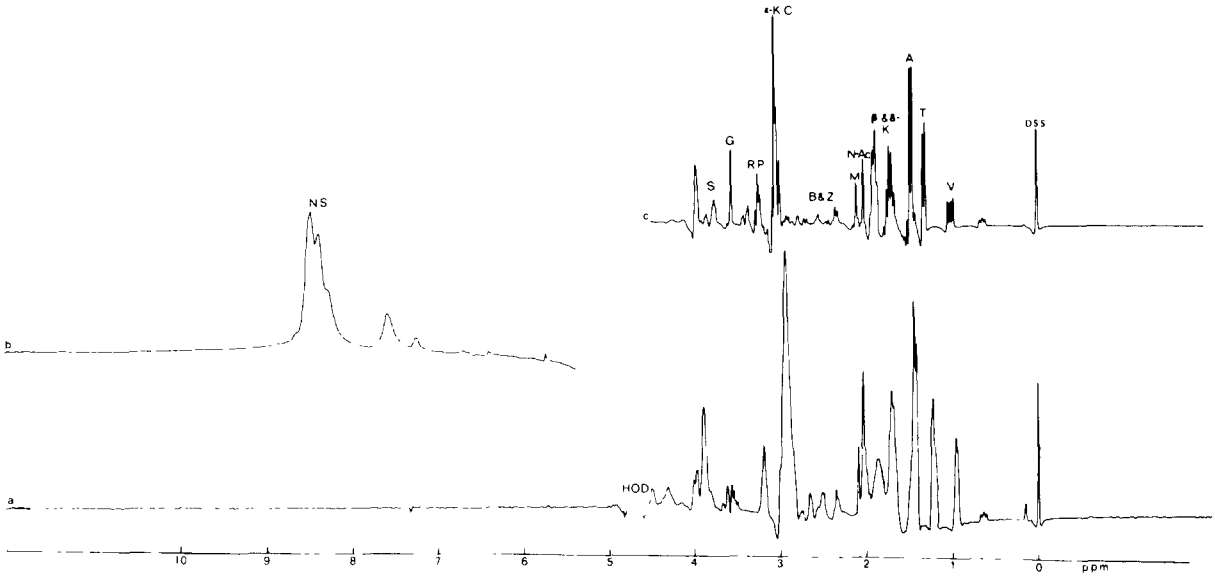

Fig.1. (a) Convolution difference ^1H NMR spectrum at 270 MHz of equine liver apothionein 1A in phosphate (100 mM) buffer in ^2H_2O, pH* 7.5. The resonance marked HOD is due to the residual H_2O signal; (b) Conventional ^1H NMR spectrum, low-field region, of equine liver apothionein 1A in HCl, 60 mM, pH 1.4. The resonances marked N are due to the amide protons; (c) Convolution differences ^1H NMR spectrum, high-field region, at 270 MHz of an amino acid mixture having the same composition as equine liver apothionein 1A in phosphate buffer (20 mM) in ^2H_2O, pH* 7.4. The resonance marked DSS is from the internal standard, while the other labels refer to the main amino acid residues contributing to the spectrum. The resonance, NAc, is from the N-terminal acetyl group.

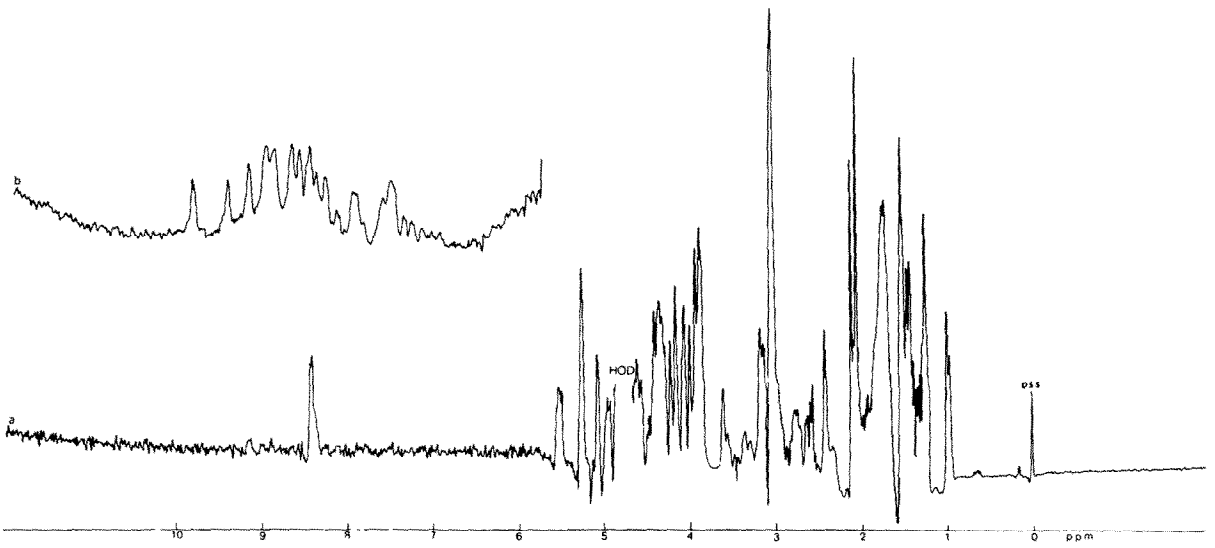

Fig.2. The 270 MHz ^1H NMR spectra of equine liver metallothionein 1A in phosphate buffer (20 mM), pH* 7.5. (a) Convolution difference spectrum in ^2H_2O, (b) conventional spectrum of the low field region in H_2O. The vertical scale of the low-field region is expanded X 8 in both spectra.

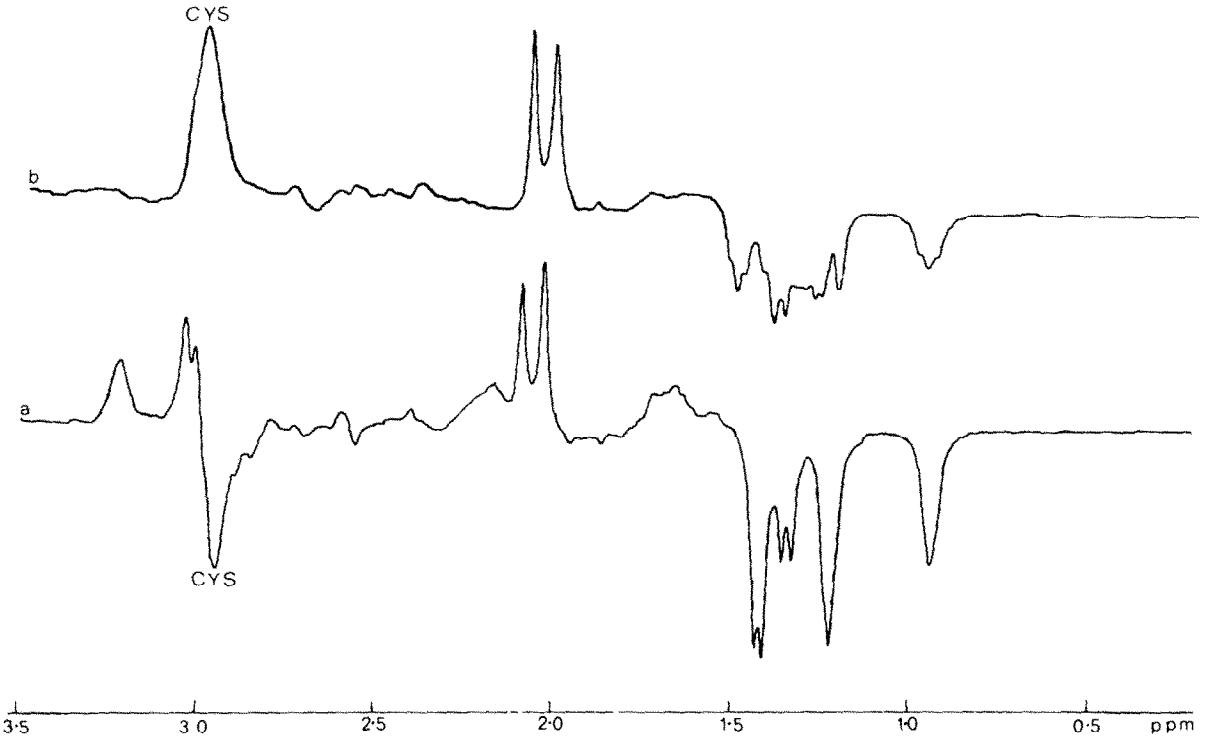

Fig.3. The 270 MHz ^1H NMR spin-echo spectra of equine liver metallothionein 1A. The pulse sequence employed is 90-τ-180-τ (collect), with τ = 71 ms. (a) Apothionein in phosphate (100 mM) in ^2H_2O pH* 7.5; (b) Holothionein (96% Zn) in phosphate (20 mM) in ^2H_2O, pH* 7.3.

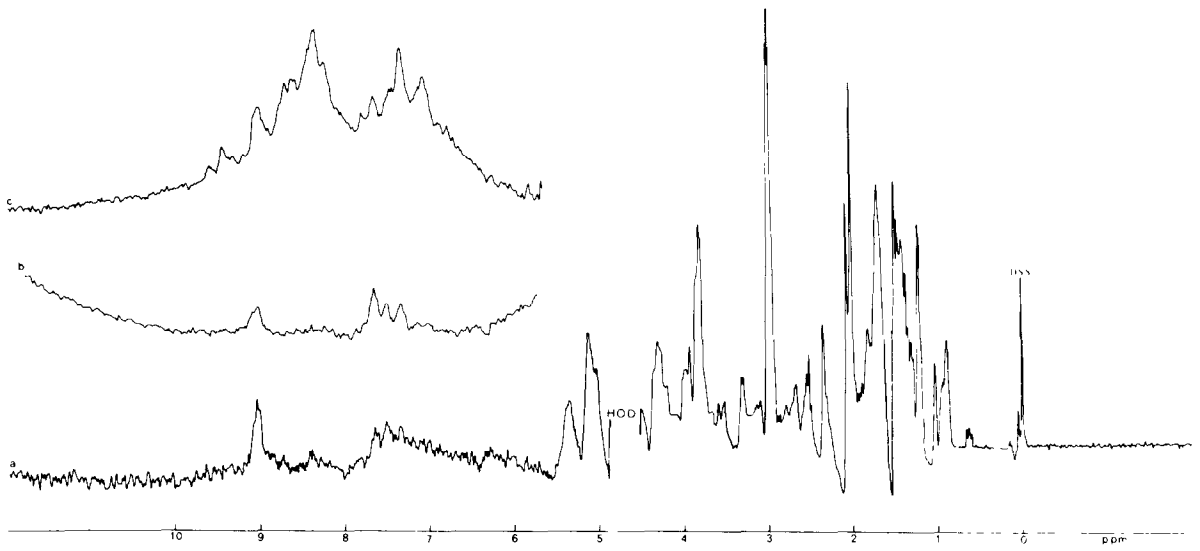

Fig.4. The 270 MHz ^1H NMR spectra of equine kidney metallothionein 1A in phosphate buffer (20 mM), pH* 7.5. (a) Convolution difference spectrum of a sample containing 55% Cd and 45% Zn in ^2H_2O: (b) Conventional spectrum of the low-field region of a sample containing 62% Cd, 38% Zn in H_2O; (c) Conventional spectrum of low-field region of sample as in (a) in H_2O. The vertical scale of the low-field region is expanded ×8 in each case.

thionein has a well-defined tertiary structure. This is borne by the observation of resonances associated with unexchanged peptide NH's in ^2H_2O (fig.2a) which implies that some part of the Zn metallothionein is not readily accessible to solvent. The spectrum of the high field region is related to, but not identical to, the apothionein (fig.1a) or the constructed spectrum (fig.2c). For example, although the threoninyl residues seem little different, implying perhaps that they are still exposed to solvent, the alanyl residues are quite differentiated. The most stiking difference is apparent in an examination of the spin-echo spectra (fig.3). The spectrum of the apothionein (fig.3a) is just that expected from the constituent amino acid residues freely exposed to solvent. Most resonances in the spin-echo spectrum of the Zn metallothionein (fig.3b) are slightly altered but the resonances of the cysteinyl residues dramatically so, consistent with their accepted role as ligands to the zinc ions.

The spectra (fig.4) of the Cd–Zn kidney metallothionein in the low field region show both in ^2H_2O and in H_2O, marked differences from the spectrum Zn metallothionein with no coincidences of the resonances of the unexchanged peptide NH's. Thus the Zn–Cd protein does not correspond to a mixture of Zn metallothionein and a Cd metallothionein. Differences in the high field region of the spectra are not marked. Only the alanyl residues appear to be further perturbed, the threoninyl residues remaining unchanged.

In conclusion, these investigations suggest that the apothionein is a loose open structure with all residues solvated. The metallo-forms have well-defined structure, parts of which are inaccessible to solvent, any minor differences between the different metal forms being of the order expected from the consequence of the different size and/or coordination preferences of Zn and Cd.

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