Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 217-225

^{1}H N.M.R. INVESTIGATIONS ON THE STRUCTURE OF SHEEP METALLOTHIONEINS

Alphonse Galdes and H. Allen O. Hill*
Inorganic Chemistry Laboratory, South Parks Road,
Oxford OX1 3QR, U.K.

and

Ian Bremner and Brian W. Young
Rowett Research Institute, Greenburn Road, Bucksburn,
Aberdeen AB2 9SB, Scotland, U.K.

Received August 29, 1978

SUMMARY The 270 MHz ^{1}H n.m.r. spectra of sheep metallothioneins I and II have been measured. It is concluded that, though the structures of the metal-free proteins are such that each residue is exposed to solvent, the metalloforms have well-defined tertiary structures.

INTRODUCTION Metallothionein is a protein which was first isolated (1) from the equine renal cortex. Since its discovery, it has been identified (2) in the liver and kidney of a wide variety of animal species and also in microorganisms. All metallothioneins contain (2,3) 7-10 g. atoms of metal ions, depending on the metal ion, and between 30 and 35% of half-cystinyl residues per molecular weight of 6800, while aromatic amino-acids are always absent. They occur in several distinct types of similar chain length but of differing amino acid composition. Metallothionein synthesis may be

0006-291X/78/0851-0217$01.00/0

induced (4-7) by the administration of heavy metals implicating this protein in metal metabolism, homeostasis or detoxification. This has provoked much interest in the structure and function of this protein.

The results from previous ^{1}H n.m.r. investigations were interpreted (8) as showing that both thionein and metallothioneins have random coil structures. This seemed difficult to reconcile with other (2) physical properties and, if true, would mean that metallothionein was unique amongst metalloproteins in lacking a well-defined tertiary structure.

EXPERIMENTAL Metallothionein was isolated (6,9,10) from the liver of zinc-supplemented lambs and purified, by a combination of gel filtration and anion exchange chromatography, according to established procedures. As is customary (6,11) in the purification of metallothionein two isomers were obtained, I and II. They were both homogeneous by disc electrophoresis on a 10% polyacrylamide gel. Amino acid analyses were carried out on samples hydrolysed at 100°C in 6M-HCl for 24 hr after performic acid oxidation. Metal determinations were made by atomic absorption by using a Techtron AA5 atomic absorption spectrometer. All reagents were analytical grade and buffers were purified from trace metals either by passage through Chelex-100 columns or by extraction (12) with dithiozone in carbon tetrachloride. Spectropor membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, USA), mol. wt. cut-off ca. 3500, was used for the dialysis of metallothionein samples.

Metallothionein samples were prepared for n.m.r. experiments by dialysis against three changes of 1 mM-sodium phosphate buffer, pH 7.5, followed by freeze-drying. The

protein was then taken up in 20 mM-sodium phosphate buffer, pH* 7.5, in either H_2O or ^2H_2O (pH* is a direct meter reading, uncorrected for the ^2H isotope effect in ^2H_2O solutions).

The thioneins (the apo-proteins) were prepared by dissolving the freeze-dried metallothioneins in 60 mM-HCl and dialysed against three changes of the same solution.

Pulsed Fourier transform ^1H n.m.r. spectra were obtained at 270 MHz by using a modified Bruker HFX-90 console, and Oxford Instruments superconducting magnet and a Nicolet 1085 computer. Quadrature detection was used and 1024 transients were routinely accumulated, with a pulse-to-pulse time of 0.6s. The residual water signal was suppressed by applying a pulse at the appropriate frequency at all times except during data acquisition. Convolution difference spectra were obtained as described in the literature (13). All chemical shifts are reported downfield from 2,2-dimethyl 2-silapentane-5 sulphonate as internal standard. Solutions for n.m.r. experiments contained 2mM protein in the indicated buffer. For the experiments done in H_2O as a solvent, 10% ^2H_2O was present to provide an internal magnetic-field lock.

RESULTS AND DISCUSSION The amino-acid compositions and metal content of sheep metallothioneins I and II are given in the Table. In common with all metallothioneins so far isolated, they are characterized (2) by a high content of cysteinyl residues and the complete absence of aromatic amino-acids. As can be seen from the Table, the amino acid compositions, and the metal contents, are very similar in sheep metallothioneins I and II, the main difference being the higher lysine content of the II isomer.

Table Amino acid composition and metal content of sheep metallothioneins I and II

	(Number of residues/mol.wt. of 6800)	
	Metallothionein I	Metallothionein II
Cysteine*	18.3	19.7
Aspartic acid	3.4	3.8
Methionine+	1.2	1.1
Threonine	2.6	2.2
Serine	9.0	7.5
Glutamic acid	1.3	2.1
Glycine	7.0	5.5
Alanine	6.2	7.5
Isoleucine	0.4	-
Lysine	7.9	10.2
Valine	1.9	2.1
Proline	-	-
Arginine	-	-
TOTAL	59	62
Zinc (% of total metal)	79	82
Copper (% of total metal)	21	18

* Measured as cysteic acid

+ Measured as methionine sulphone

The ^{1}H n.m.r. spectra of sheep thionein I are shown in Fig. 1. The resonances occurring in the high-field region (Fig. 1a) are due to the methyl, methylene and methine protons of the constituent amino acids, while the resonances observed in the low-field region of the sample dissolved in H_2O (Fig. 1b) can be assigned to the peptide NH's. The spectrum of thionein dissolved in $^{2}H_2O$, (Fig. 1a), shows

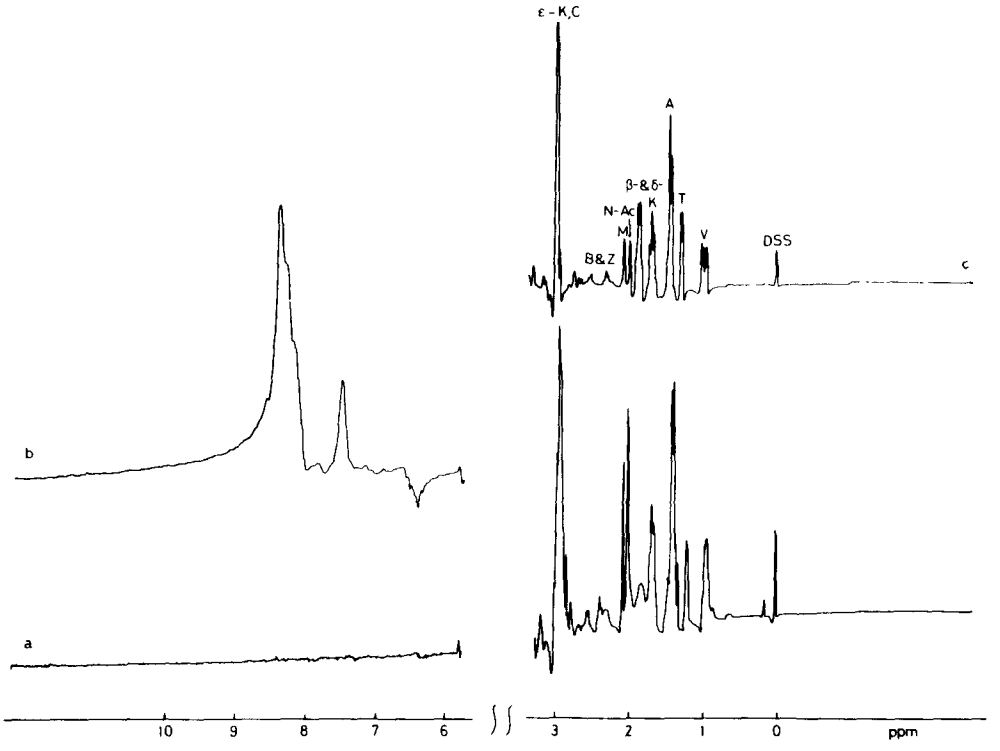

Figure 1

(a) Convolution difference ^1H n.m.r. spectrum at 270 MHz of sheep thionein I in 60 mM-DCl.
(b) Conventional ^1H n.m.r. spectrum, low-field region, of sheep thionein I in 60 mM-HCl.
(c) Convolution difference ^1H n.m.r. spectrum, high-field region, of an animo-acid mixture having the same composition as sheep thionein I in phosphate buffer (20 mM) in ^2H_2O, pH* 7.5. The resonance marked DSS is from the internal standard, while the other labels refer to the main amino acid residues contributing to the spectrum.

that these resonances are absent, the peptide NH's having exchanged with solvent deuterons. (The complete lack of resonances in this region also confirms the absence of any aromatic amino-acid residues.) The resonances of the peptide NH protons occur over a very small chemical shift span, centred around 8.30 ppm, with a much smaller band (comprising approx. 8% of the amide protons) occurring at 7.51 ppm. Hence most of the amide protons in thionein experience an almost identical

chemical/magnetic environment, consistent with the absence of a well-defined tertiary structure. This is borne out by a comparison of the high field region of thionein (Fig. 1a) with a spectrum of a mixture of the constituent amino-acids (Fig. 1c) having the same composition as the apothionein. As can be seen these spectra are practically identical. This would result from each residue in thionein having a similar environment to that of the free constituent amino-acids, unperturbed by shifts arising from polypeptide chain folding. Whether the structure of the apothionein is purely random-coil, or sufficiently loose such that each residue experiences an aqueous environment, cannot at present be distinguished.

The ^{1}H n.m.r. spectra of thionein II are virtually identical to those of thionein I, as would be expected from their close amino acid compositions.

The ^{1}H n.m.r. spectra for metallothioneins I and II are shown in Figures 2 and 3 respectively. A comparison of these spectra with those of thionein (Fig. 1) shows many marked differences. In the low-field region, in contrast to the apothionein (Fig. 1a) in the peptide NH's of the metallo-thioneins (Figs. 2b and 3b) give rise to numerous resonances over a wide chemical-shift range (ca. 3ppm). This situation can only arise if the amides experience many different chemical/magnetic environments, depending on their positions within the molecule, indicating that the metallothioneins have well-defined tertiary structures. The high field region of the metallothionein spectra (Figs. 2a and 3a) also differs from that of thionein (Fig. 1a) and from that of the constituent amino-acid mixture (Fig. 1c). Most of the residues give rise to resonances over a wider region in the

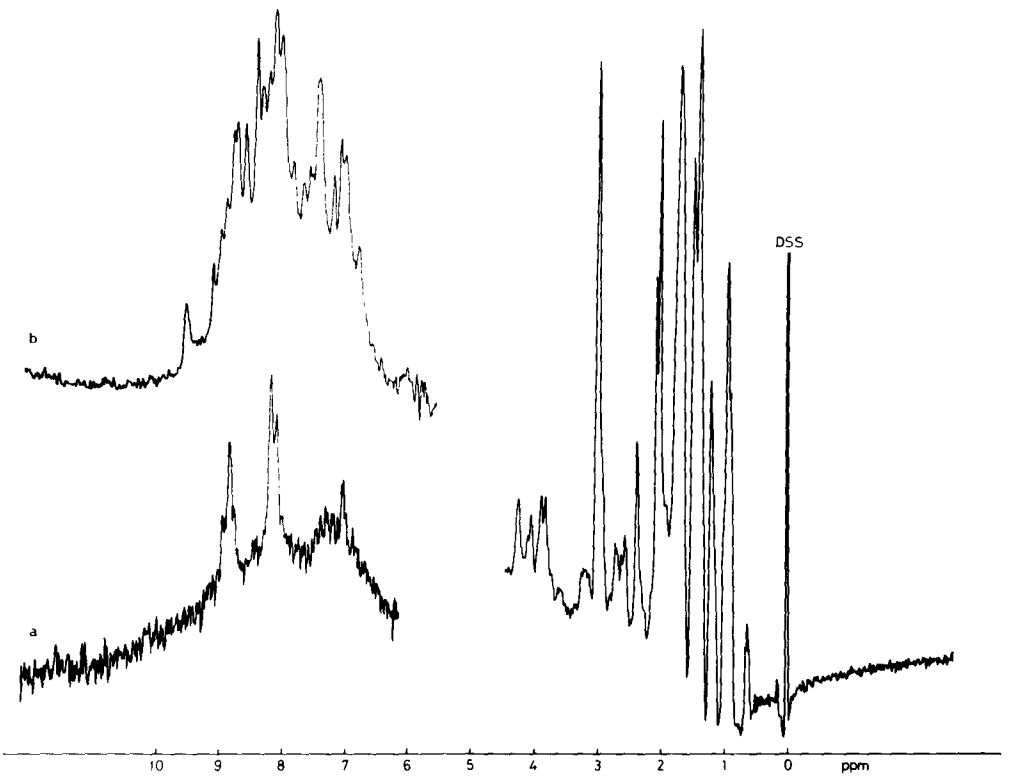

Figure 2

The 270 MHz ^{1}H n.m.r. spectra of sheep metallothionein I in phosphate buffer (20 mM), pH* 7.5.

(a) convolution difference spectrum in $^{2}H_2O$,

(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.

metalloth ioneins, the difference being particularly striking for the alanine residues. This again suggests that the metallothioneins possess well-defined tertiary structures. Furthermore some of the peptide NH's in the metallothioneins are slow to exchange in $^{2}H_2O$ (Figs. 2a and 3a); in the thioneins all the peptide NH's undergo rapid deuterium exchange with solvent deuterons (Fig. 1b). This implies that in the metallothioneins, but not in the thioneins,

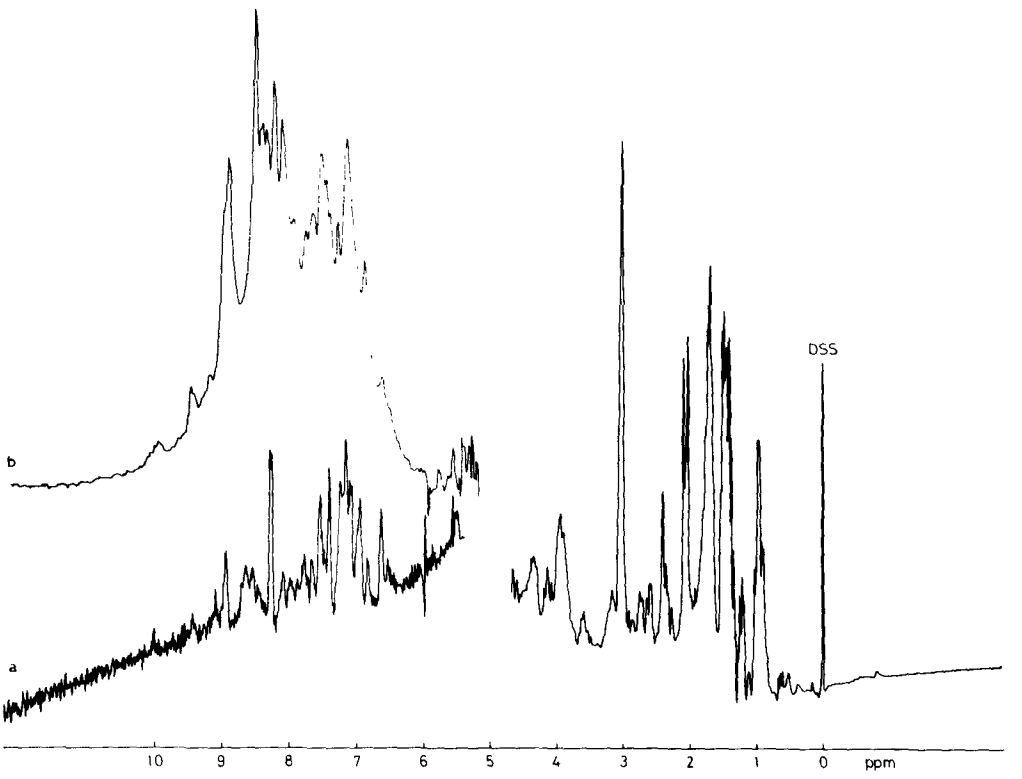

Figure 3

The 270 MHz ^{1}H n.m.r. spectra of sheep metallothionein II in phosphate buffer (20 mM), pH* 7.5.

(a) convolution difference spectrum in $^{2}H_2O$
(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.

some of the amide protons are not readily accessible to solvent, which is consistent with the conclusion arrived at above.

Although the spectra of metallothionein I (Fig. 2) and metallothionein II (Fig. 3) are similar, they are not identical. In particular the two metallothionein isomers show differences in the number and position of slowly exchanging protons in $^{2}H_2O$ (Figs. 2b and 3b). This suggests that the overall tertiary structures of these two isomers are not identical.

In conclusion, the present work demonstrates that while it is possible that thioneins have random-coil structures, the metallothioneins possess characteristic and well-defined tertiary structures.

ACKNOWLEDGEMENTS We thank the Rhodes Trust for a scholarship to A.G. H.A.O.H. is a member of the Oxford Enzyme Group.

References

1. Margoshes, M. and Vallee, B.L. (1957) J.Am.Chem.Soc. 79, 4813-1814
2. Kojima, Y. and Kägi, J.H.R. (1978) Trends in Biochem. Sci., 90-93
3. Kägi, J.H.R., Himmelhoch, S.R., Whanger, P.D., Bethune, J.L. and Vallee, B.L. (1974) J.Biol.Chem., 249, 3537-3542
4. Piscator, M. (1964) Nord.Hyg.Tidshr. 45, 76-82
5. Piotrowski, J.K., Trojanowska, B., Wieniewska-Knypl, J.M. and Bolanowska, W. (1974) Toxicol.Appl.Pharmacol. 27, 11-19
6. Bremner, I. and Davies, N.T. (1975) Biochem.J. 149, 733-738
7. Bremner, I. and Young, B.W. (1976) Biochem.J. 157, 517-520
8. Rupp, H., Voelter, W. and Weser, U. (1974) FEBS Lett. 40, 176-179
9. Bremner, I. and Marshall, R.B. (1974) Brit.J.Nutr. 32, 293-300
10. Bremner, I., Williams, R.B., and Young, B.W. (1977) Brit.J.Nutr. 38, 287-292
11. Bühler, R.H.O. and Kägi, J.H.R. (1974) FEBS Lett. 39, 229-234
12. Thiers, R.E. (1957) Methods Biochem.Anal. 5, 273-335
13. Campbell, I.D., Dobson, C.M., Williams, R.J.P. and Xavier, A.V. (1973) J.Magn.Reson. 11, 172-181