270 MHz PROTON MAGNETIC RESONANCE SPECTRA OF METALLOTHIONEIN

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Received 22 January 1974

1. Introduction

The existence of a specific Zn, Cd and Hg binding protein in human and animal tissues is now quite established [1–10]. The protein was called metallothionein attributable to its extraordinary metal binding capacity (up to 8 g atoms per 12 000 g of protein) and the high content of cysteine residues (approx. one third of the total amino acid residues). Due to these properties this metalloprotein excited great interest on both its structure and its biochemical function. While the latter one still remains obscure some progress has been made on the evaluation of several physicochemical parameters [1–4, 10].

From CD measurements [10] strong indication was obtained that metallothionein is a random coil polymer in aqueous solution and the contribution of the metal ions Zn^{2+} and Cd^{2+} to the overall structure of the metalloprotein could be seen. X-ray photoelectron spectroscopy revealed the exclusive binding of Zn^{2+} and Cd^{2+} with the sulphur of the cysteine residues [10].

In this context proton magnetic resonance measurements employing high resolution PMR spectroscopy at 270 MHz promised to shed more light upon the conformation of metallothionein, the metal binding and the detection of aromatic amino acid residues. The present pmr study revealed that throughout the pD regions from 1.3–6.5 the protein actually remained in the random coil form and no sign of aggregation was detectable. The aromatic amino acid residues phenylalanine, tryptophan and tyrosine appeared to be virtually absent. Cd^{2+} and Zn^{2+} binding started to appear at pD2. As in the case of the glutathione Cd and Zn complexes and pmr signals of the cysteine–methylene protons at 3.02 ppm were markedly broadened using native metallothionein. This phenomenon suggested that in the metal binding centres a somewhat rigid structure can be assumed.

2. Materials and methods

D_2O (99.9%) was obtained from Merck, Darmstadt, $Cd(NO_3)_2$ and $Zn(NO_3)_2$ were of spectroscopic purity. Metallothionein was prepared from liver tissues of chicken which were pretreated each with 50 μmol Cd/kg for 4 weeks. The isolation procedure was essentially the same as described elsewhere [9, 10].

270 MHz proton magnetic spectroscopy was performed using a Bruker WH-270 pmr spectrometer. Chemical shifts are referred to the proton resonance position of 2,2,3,3-tetradeutero-3 (trimethylsilyl)propionic acid, sodium salt, as an internal standard. 60 MHz pmr spectra were recorded using a Varian A60-PMR spectrometer. All solutions contained tetramethylsilane as the internal standard. Prior to the recording of pmr spectra metallothionein (35 mg/ml) was incubated in D_2O at 25°C for 70 hr and lyophilised to achieve deuteration of exchangeable protons. The computed spectrum of a random coil polymer was obtained using the method developed by McDonald and Phillips [11].

3. Results and discussion

High resolution nuclear magnetic resonance spectroscopy proved most appropriate to elucidate both the

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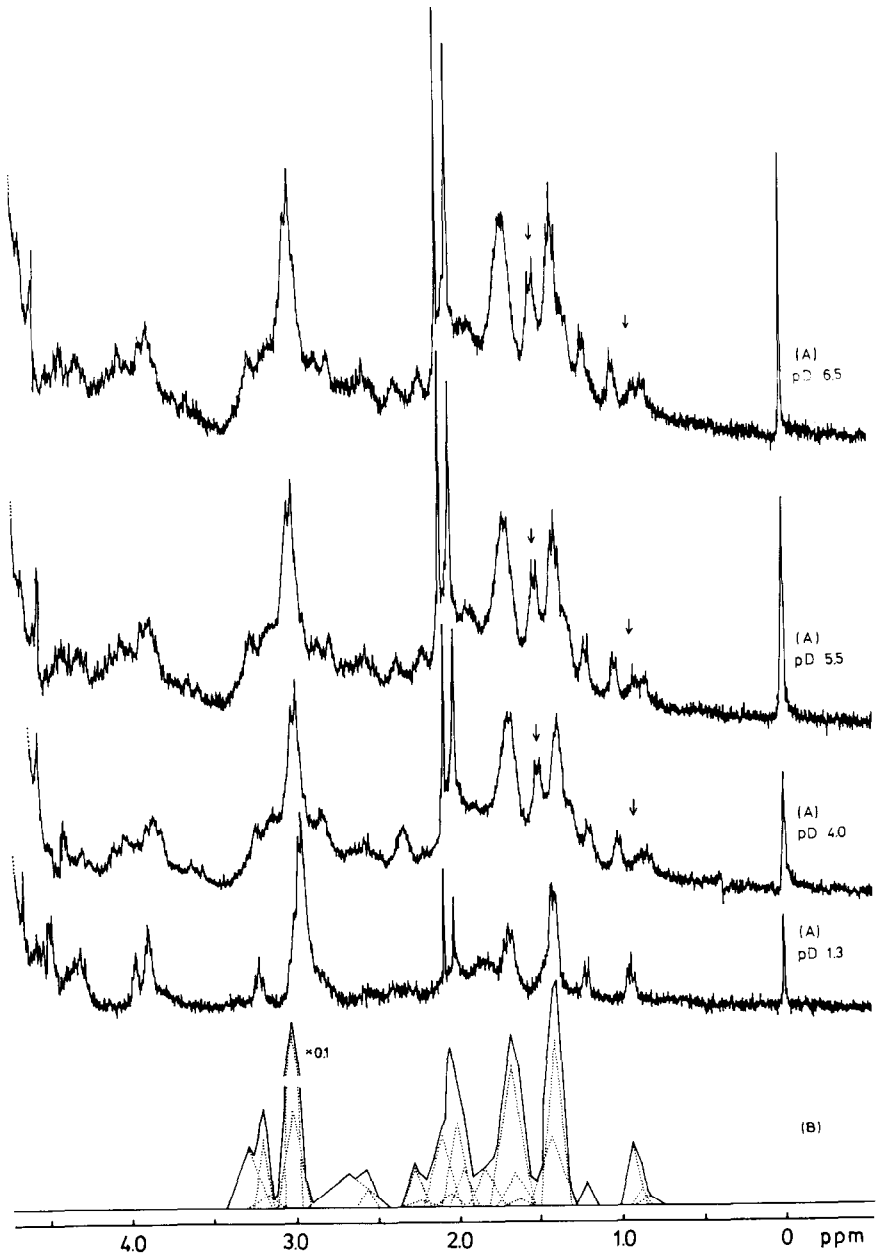

Fig. 1. (A) Proton nmr spectra recorded at 270 MHz of metallothionein in D_2O at different pD values. The temperature was 25°C. A Bruker WH-270 pmr spectrometer was used. The employed protein concentration was 35 mg/ml. (B) Computed spectrum of a random coil metallothionein in neutral D_2O at 40°C. The peak at 3.02 ppm is depicted in a reduced scale using a factor of 1:10. Computation of the spectrum was carried out essentially as given in [11]. The data of the amino acid analyses for chicken metallothionein were taken from [10].

structure and interactions of small molecules in the liquid state [12]. Changes in the tertiary structure of a protein produce marked influences on the pmr chemical shifts. The computed spectrum (B) using the method of McDonald and Phillips [11] for a random coil metallothionein fitted rather nicely the experi-

mentally obtained data (fig. 1). Indeed, in some cases the resolution was much more distinct compared to the computed spectrum. The pmr signal at 3.02 could be assigned for the presence of cysteine residues of metallothionein. Due to the powerful 270 MHz pmr measurements the resolution of the spectra was of exceptionally good quality. A much smaller half width (up to 10 Hz) of the different signals resulted in a dramatic rise of the signal height. Even spin–spin splitting may be seen at 1.23 ppm attributable to the CH_3 residue of threonine [11]. Due to the constancy of the recorded spectra it was of special interest to conclude that in the pD regions between 3 and 7 no marked conformational changes occurred. Unlike earlier observations during electrophoretic separation using polyacrylamide-gel in the presence of sodium dodecylsulphate (10) there was no sign of aggregated metallothionein molecules. Even the apoprotein prepared by DCl treatment to pD 1.3 did not show any measurable tendency to form higher molecular weight species.

Between 5.6 and 9.0 ppm no signals were detected indicating the virtual absence of the aromatic amino acid residues such as phenylalanine, tryptophan and tyrosine. These data agree well with earlier chemical analyses obtained from this laboratory (10).

As in the case of previous metal binding studies employing CD-measurements [10] it is evident that upon displacement of Cd^{2+} and Zn^{2+} by D^+ several distinct changes in the pmr spectrum occurred. First of all the signal at 2.95 ppm is well resolved. This is not the case in the spectra recorded form pD 4.0–6.5. The split signal at 1.52 ppm is completely leveled off and from the two signals at 1.03 and 0.87 ppm only one signal at 0.94 ppm can be seen. A decision cannot be made whether or not the signal at 1.03 ppm is shifted to 0.94 ppm or the signal at 0.87 ppm is shifted downfield to 0.94 ppm. In either case one signal is abolished. Nevertheless it is attractive to assign most of these spectral changes to the binding of Cd^{2+} and Zn^{2+} with the apometallothionein. Substantial support of this conclusion was obtained after recording the pmr spectra of Cd^{2+} and Zn^{2+} complexes of glutathione at 60 MHz (figs. 2 and 3). These glutathione–Cd^{2+} and Zn^{3+} complexes served as convenient models since strong evidence was presented in a ^{13}C-nmr spectroscopic study [13] that the metal ions were bound with the cysteine sulphur.

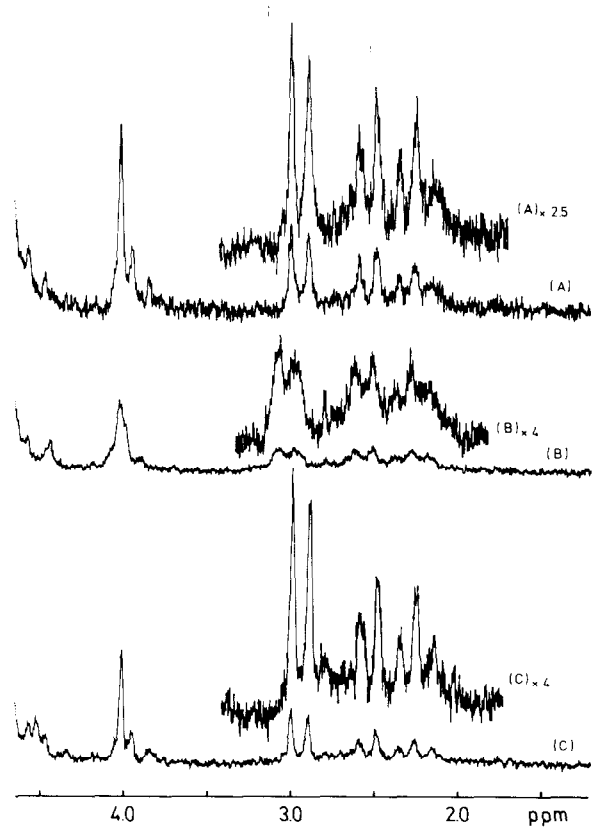

Fig. 2. Proton nmr spectra at 60 MHz of glutathione recorded on a Varian A-60 pmr spectrometer. (A) 0.3 M glutathione in D_2O, pD 1.90; (B) 0.3 M glutathione and 0.15 M $Cd(NO_3)_2$ in D_2O, pD 1.60; (C) 0.3 M glutathione and 0.15 M $Zn(NO_3)_2$ in D_2O, pD 185.

Rather similar to the pmr spectrum of metallothionein the pmr measurements of glutathione revealed pmr doublet signals at 2.94 ppm attributable to cysteine–methylene protons. It was interesting to note that already at pD 1.60 Cd^{2+} started to bind with the cysteine sulphur. This was seen by the broadening of the pmr signals. Upon the addition of Zn^{2+} a much higher pD-value was required to produce a similar broadening of the pmr signals. At pD 4.0 there was also no doubt that Zn^{2+} formed a complex with glutathione. Whenever the metal ions were bound with the cysteine residue a downfield shift of the cysteine methylene protons could be observed.

The broadening of the pmr signals in the pmr spectrum of metallothionein at 3.02 ppm (fig. 1) would indicate that in the cysteine region the random coil structure of the protein is somewhat disturbed and a more

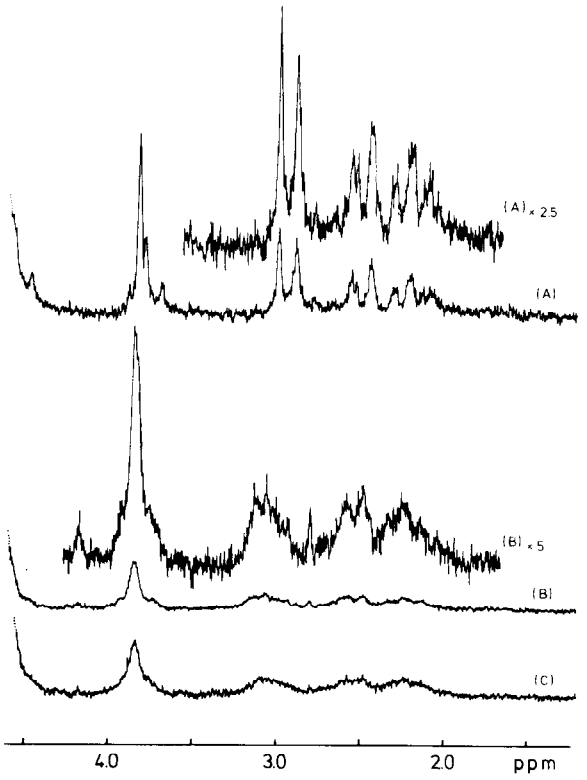

Fig. 3. Proton nmr spectra at 60 MHz. (A) 0.3 M glutathione in D_2O, pD 4.00; (B) 0.3 M glutathione and 0.15 M Cd $(NO_3)_2$ in D_2O, pD 3.90; (C) 0.3 M glutathione and 0.15 M $Zn(NO_3)_2$ in D_2O, pD 4.06.

rigid structure attributable to the metal binding has to be taken into consideration. All these pmr data presented here support rather nicely our earlier conclusions which were drawn from Cd–Zn binding studies employing circular dichroism measurements and X-ray photoelectron spectroscopy.

Acknowledgements

This study was supported by a DFG grant No. We401/9 awarded to U.W. and in part by the Fonds der Chemischen Industrie. H. Rupp is a recipient of a predoctoral grant of the Stiftung Villigst. Special thanks go to Prof. R.J.P. Williams, Oxford, for his stimulation and encouragement. The expert technical help of Miss K. Siemers, B. Mayer and Dr. Schneiders (Bruker Physik Ag, 75 Karlsruhe-Forchheim, Silberstreifen) is greatly acknowledged.

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