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^{1}H Nuclear Magnetic Resonance Studies on Metallothionein from *Scylla serrata*†

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The ^{1}H NMR spectrum of $^{113}Cd_6$ *Scylla serrata* metallothionein isoform 1 (MT-1) is presented and analyzed in considerable detail by using two-dimensional chemical shift correlated (COSY) methods. Using these methods, it has been possible to associate most ^{1}H resonances with amino acid type. The ^{1}H chemical shifts for all 18 cysteine residues present in the protein have been identified, as has the range of heteronuclear ^{1}H–^{113}Cd scalar coupling between the ^{113}Cd ions and the cysteine β-CH_2 protons, 10 Hz < $^3J_{^{113}Cd-^1H}$ < 65 Hz. The relationship of these ^{1}H NMR data to the proposed cluster structures derived from ^{113}Cd NMR is discussed with some emphasis being placed on the identification of bridging cysteine residues, required by the proposed cluster structures, via the analysis of the heteronuclear scalar coupling present in the cysteine $\alpha\beta$ cross-peaks in the ^{1}H COSY spectrum. It is concluded that, in the absence of a calibrated Karplus equation for $^3J_{^{113}Cd-^1H}$ and in the presence of short ^{113}Cd T_1 values, identification of the ^{1}H resonances arising from bridging cysteine residues using heteronuclear scalar coupling data alone is not readily achievable.

Introduction

Metallothioneins (MTs) are low molecular weight (≤6100 daltons) cysteine-rich proteins that are believed to play an important, although as yet undefined, role in zinc and copper metabolism (for reviews see ref 1–4). In addition, the inducibility of this protein at the transcriptional level by heavy metals[5] suggests that it also functions to provide a protective mechanism against a toxic overload of these agents.[4,6,7] Metallothioneins are ubiquitous in nature, existing principally in kidney and liver tissue of higher vertebrates and in invertebrates, plants, and both eukaryotic and procaryotic microorganisms.[1,4] Features that characterize all metallothioneins are their high metal and cysteine content and, in all sequenced mammalian and invertebrate species at least, an absence of aromatic residues.[1,4] There are no disulfides or free sulfhydryls present, and all the cysteines are coordinated to metal ions via metal–thiolate bonds.[1-3] Metallothioneins have been studied extensively by conventional spectroscopic methods, e.g. UV, CD, and MCD spectroscopy, both to determine the protein's metal-binding affinities[2] and to elucidate the nature of metal ion coordination. However, overlap of the numerous metal–thiolate charge-transfer transitions in these forms of spectroscopy has only allowed a derivation of the general properties of the metal sites.[8] Furthermore, EPR methods are unsuitable for probing the structure of the sites since the metal ions contained in the native form of the protein, Zn^{2+}, Cd^{2+}, and Cu^+, are EPR silent.[9]

Several years ago, we initiated a comprehensive study of the metal binding sites in metallothioneins from different sources using ^{113}Cd NMR as the structural probe.[10] When $^{113}Cd^{2+}$ was substituted for the native metals, one resonance was observed for each bound metal ion. These ^{113}Cd resonances occurred over a relatively narrow chemical shift range, 670–610 ppm, compared to the possible chemical shift range of 800 to −100 ppm, which is observed for ^{113}Cd bound to specific sites on metalloproteins.[11] The range of ^{113}Cd chemical shift observed in metallothioneins compared well with that observed in model complexes where the Cd^{2+} ion was bound tetrahedrally, in clusters, by a mixture of terminal and bridging sulfhydryl ligands.[12,13] Each metal resonance in metallothionein showed ^{113}Cd–^{113}Cd homonuclear scalar coupling, in addition to ^{1}H–^{113}Cd heteronuclear scalar coupling. Using ^{113}Cd NMR homonuclear decoupling methods, it was possible to derive the ^{113}Cd coupling patterns for both the seven-metal-containing (mammalian) and the six-metal-containing (invertebrate) metallothioneins.[11,14] For the protein from mud crab, *Scylla serrata*, which forms the subject of this paper, two distinct sets of coupled resonances were found that both contained three $^{113}Cd^{2+}$ resonances.[14] Since both crab MT-1 and MT-2 contain 18 cysteine residues, it was proposed that two similar metal clusters were present in both isoforms, each containing 3 metals coordinated in a pseudotetrahedral fashion by 9 cysteine residues. The proposed cluster structures are shown in Figure 1.[14]

The next stage in the refinement of this protein's structure involves the determination of how the protein backbone is arranged around these metal cluster structures. Structural refinement to this level is feasible by either X-ray crystallography of ^{1}H NMR methods. We have initiated the refinement using the latter course and describe herein our general strategy and initial results.

The frequent occurrence of cysteine residues in the amino acid sequence of both crab MT isoforms, every two or three amino acid residues (Figure 1),[15] suggests that a determination of which cysteine residues are bound to a particular metal ion will provide an initial description of the protein's fold. This fold can then be refined via the use of distance geometry calculations[16] based on nuclear Overhauser data.[17] The first stage in this process is the assignment of the resonances in the ^{1}H NMR spectrum of the protein, first to amino acid type, and then to specific residues. In this paper, we report the assignments of numerous resonances in the ^{1}H NMR spectrum of crab MT-1 using two-dimensional (2D) methods. In particular, chemical shifts for the α and β proton resonances arising from the 18 cysteine residues present are deduced, and the range of $^3J_{^{113}Cd-^1H}$ is reported and discussed.

Materials and Methods

Protein Preparation. ^{113}Cd-labeled MT-1 was obtained from *Scylla serrata* hepatopancreas as described previously.[18] Crab MT-1 samples

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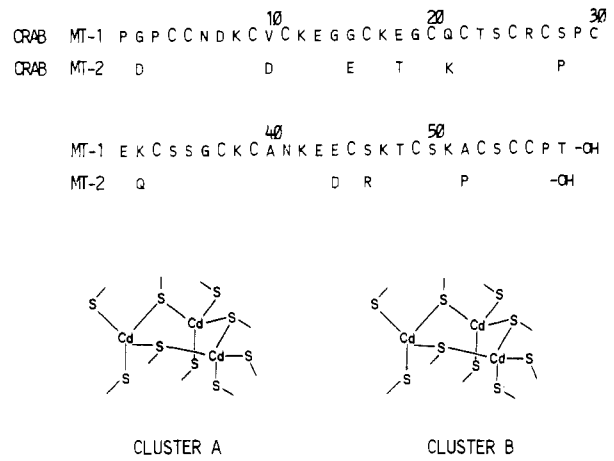

Figure 1. Amino acid sequence of *Scylla serrata* MT-1 and MT-2[15] using the single-letter code for the amino acids and indicating only the amino acid substitutions between MT-1 and MT-2 in the latter case. The proposed three-metal cluster structures, two of which occur in both isoforms, are also shown.

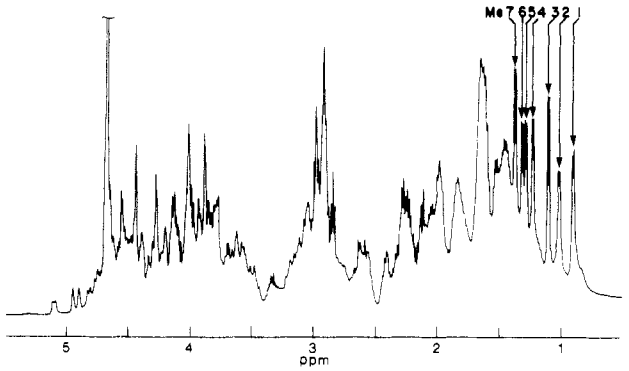

Figure 2. 500-MHz ^{1}H NMR spectrum of *Scylla serrata* MT-1 dissolved in D_2O. Only resonances from N–H protons lie outside this spectral region. The large peak at 4.7 ppm arises from the residual HDO present in the sample. Acquisition parameters: sweep width, 2800 Hz; pulse width, 12 μs (90°); number of scans, 64; recycle delay, 1 s; line broadening, 0.5 Hz.

were prepared for ^{1}H NMR studies by dialysis against 10 mM NH_4HCO_3 buffer, lyophilization, and addition of 0.4 mL of D_2O or D_2O/H_2O mixtures. The solution pH was then checked and adjusted to pH 7.5, if necessary. For the two-dimensional NMR study, the protein samples were taken up twice in 5 mL of D_2O and lyophilized in order to further reduce the residual water (HDO) concentration. The final concentration of MT-1 was 8 mM for the two-dimensional experiments and 4 mM for the one-dimensional (1D) spectra.

NMR Methods. ^{1}H NMR spectra were recorded at 500.13 MHz on a Bruker WM-500 instrument. All measurements were made at a temperature of 303 K. The spectrum recorded in 85:15 H_2O/D_2O was acquired by using the (45°–τ–45°) pulse sequence of Kime and Moore.[19] The absolute value chemical shift correlated (COSY)[20] datasets were acquired by using ~64 scans/t_1 point and a total of 512 t_1 points. These data were transferred to a VAX 11/750 and processed by using the FTNMR software of Dr. Denis Hare. Skewed sine-bell window functions were used in both dimensions, and the data were symmetrized after processing.[21]

Results

The ^{1}H NMR spectrum of *Scylla serrata* crab MT-1 dissolved in D_2O is presented in Figure 2. Despite the small size of the protein, 58 amino acids, it is apparent that considerable overlap of resonances occurs, apart from in the most upfield and downfield regions of the spectrum. This overlap reflects the limited variety of amino acids present (i.e. principally cysteine, lysine, serine, glycine, and proline) and the lack of aromatic side chains resulting in the absence of ring-current shifts on side-chain resonances. Nevertheless, despite this reduced side-chain dispersion, it is clear that the protein does possess a well-defined fold since the α-CH region of the spectrum is well dispersed compared to a predicted random-coil spectrum,[22] with some resonances occurring 0.4 ppm downfield of water (HDO) at 4.7 ppm. The principal source of this α-CH secondary shift is the orientation of the neighboring C=O group, an arrangement that is dictated by the backbone fold.[23]

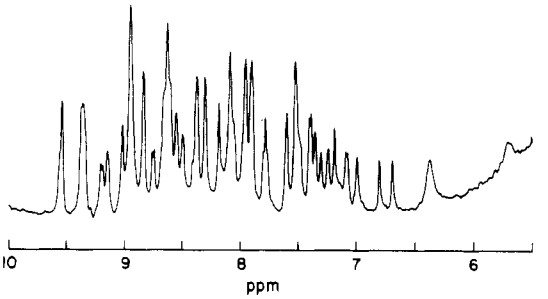

Figure 3. Fully relaxed 500-MHz ^{1}H NMR spectrum of *Scylla serrata* MT-1 recorded in 85:15 H_2O/D_2O showing only the spectral region downfield of the H_2O resonance. A 45°–τ–45° excitation sequence was employed for suppression of the H_2O resonance. Acquisition parameters: sweep width, 13 157 Hz; pulse width, 6 μs (45°); τ, 119.3 μs; number of scans, 64; recycle delay, 5 s; line broadening, 0.5 Hz.

Figure 3 shows the amide region of the ^{1}H NMR spectrum of crab MT-1 recorded in 85:15 H_2O/D_2O. Backbone NH resonances are observed between 9.57 and 6.69 ppm. In comparison with the amide chemical shifts observed in a random-coil peptide of 8.35 ± 0.26 ppm,[22] these data then indicate a maximum magnitude of amide secondary shift of approximately 1 ppm. The resonances that occur at 6.4 and 5.7 ppm can be assigned to exchange-broadened side-chain NH resonances. If a fully-relaxed amide spectrum is used, integration of the resonances between 9.5 and 6.7 ppm results in an integrated intensity of 46 ± 5 protons arising from the 29 resolved resonances in this region, which should be compared with the value of 53 amide protons calculated from the amino acid composition (Figure 1). The slight discrepancy between the calculated and experimental values may well be due to the nonuniform excitation of the resonances over even this restricted spectral window with the particular water suppression pulse sequences (45°–τ–45°) that was used to acquire this spectrum.

It is clear from the 1D ^{1}H NMR data presented above that it is necessary to resort to 2D methodology to disperse the spectrum sufficiently for assignment purposes, and we will now describe these datasets for crab MT-1. A 2D contour plot of a ^{1}H NMR COSY dataset obtained for crab MT-1 in D_2O is shown in Figure 4. The initial step is to identify each resonance (cross-peak) to amino acid type. Subsequently, assignments to specific residues in the primary sequence can be made by using the sequential assignment methods developed by Wüthrich and co-workers.[24]

Crab MT-1 contains one valine residue (Val-10), three threonine residues (Thr-23, -48 and -58) and two alanine residues (Ala-40 and -52); thus, we anticipate seven methyl doublet resonances to occur in the spectrum. These are all clearly observed even in the 1D spectrum (Figure 2) between 0.90 and 1.37 ppm and are labeled Me1–Me7. Figure 4 indicates that the two most upfield methyl resonances, Me1 and Me2, are both coupled to a resonance at 2.63 ppm and can therefore be assigned to the γ-CH_3 protons of Val-10 on the basis of this coupling pattern and their chemical shifts.[22] The resonance at 2.63 ppm is then the β-CH proton of

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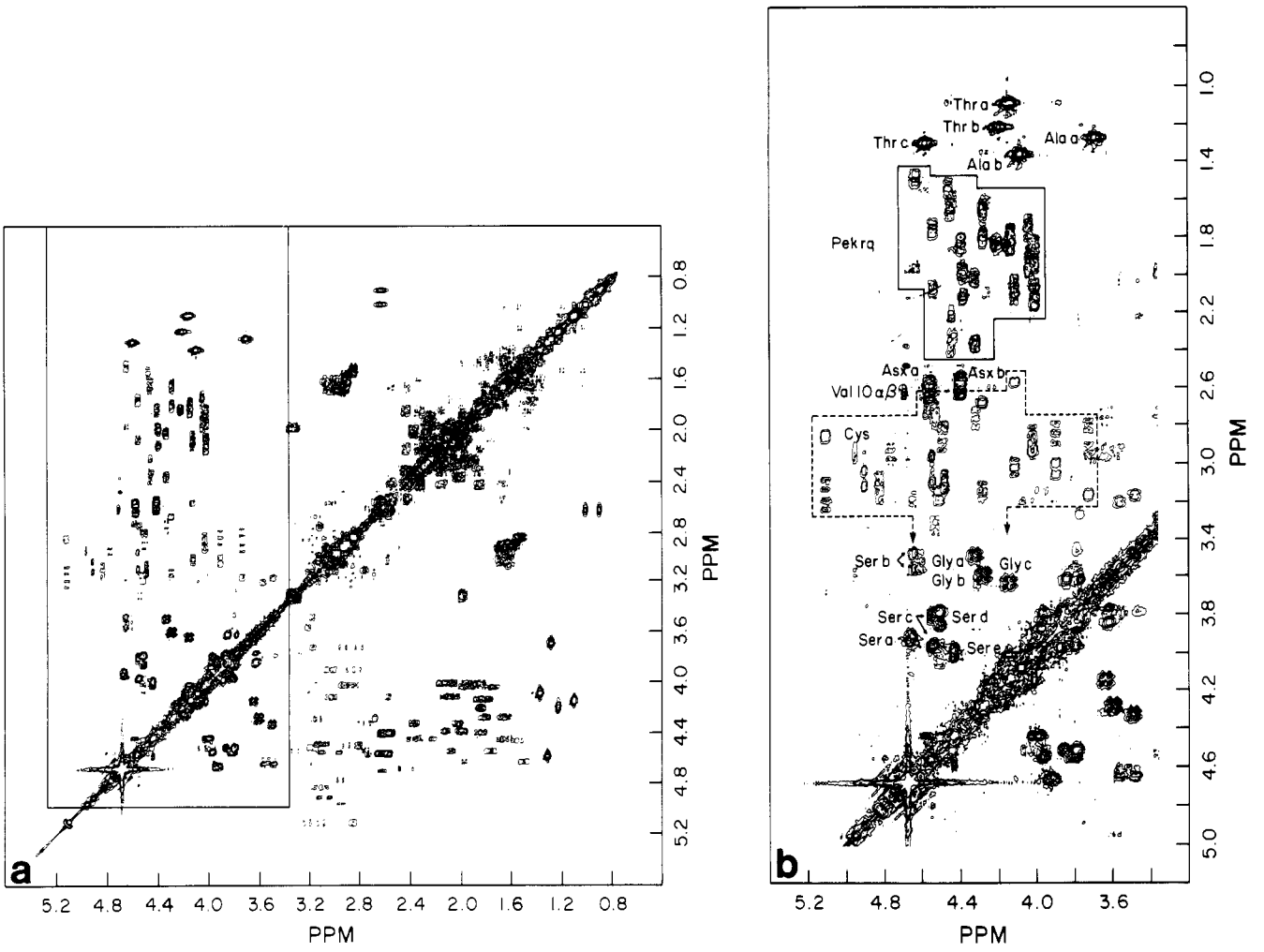

Figure 4. Two-dimensional contour plots of the absolute value COSY[20] dataset of *Scylla serrata* MT-1 in D_2O. In A, the plot spans the entire high-field region of the protein's spectrum, whereas in B, only the α-CH region is shown in the f_1 dimension. The region depicted in B is enclosed by a box in A. In B, a number of resonances discussed in the text are labeled, and the region associated with the $\alpha\beta$ cross-peaks of proline, arginine, lysine, glutamic acid, and glutamine ("Pekrq") is enclosed by the box with solid lines. The cysteine $\alpha\beta$ cross-peak region is only partially enclosed with dashed lines, since several of the downfield cysteine $\alpha\beta$ cross-peaks overlap with cross-peaks from other resonances. Acquisition parameters: sweep width, 3333.33 Hz; pulse width 12 μs (90°); t_1 increment, 300 μs; 512 fids; 64 scans; recycle delay, 1.1 s. Skewed sine-bell window functions were used in both dimensions. The data were symmetrized after processing.[21]

this residue, which is scalar coupled to the α-CH proton of Val-10 at 4.69 ppm. Me3–Me7 are then the methyl protons of the alanine and threonine resonances present in the protein. Identification of $\alpha\beta$ cross-peaks for Me3, Me4, and Me6, which are absent for Me5 and Me7, in the COSY dataset presented in Figure 4 enables us to assign the former set of resonances to threonine methyls and the latter set to alanine methyls. In support of this assignment is the magnitude of the coupling constant observed in the methyl resonances since, from model peptides,[22] $^3J_{\alpha\beta}$ = 7.0 Hz for alanine and $^3J_{\beta\gamma}$ = 6.3 Hz for threonine. In order to determine the methyl resonance coupling constants with the required accuracy from the protein spectrum, we recorded a 2D J-resolved spectrum[25] of crab MT-1 (not shown) from which we measured the following coupling constants: Me3, J = 6.4 Hz; Me4, J = 6.1 Hz; Me5, J = 7.2 Hz; Me6, J = 6.6 Hz; Me7, J = 7.4 Hz. With all the alanine, threonine, and valine resonances assigned, the secondary shifts present for these residues could be calculated. Analysis of these secondary shifts indicates that, for these residues, α-CH protons experienced shifts of up to 0.6 ppm in either direction, and β-CH protons experienced slightly smaller shifts, again in either direction, whereas γ-CH protons experienced much smaller secondary shifts (±0.1 ppm). Our assignments of other cross-peaks to amino acid type were aided by a knowledge of the approximate range of secondary shifts to be expected.

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Crab MT-1 contains five glycine residues, and three of these produce cross-peaks that are readily assignable on the basis of their chemical shifts and large geminal coupling constant.[22] These are labeled Gly-a–Gly-c (see Table I). The other two glycine residues must have less inequivalent α-CH chemical shifts since their cross-peaks are practically obscured in the diagonal. Tentative assignments of the resonance positions for Gly-d and Gly-e are shown in parentheses in Table I. Seven serine residues are present in MT-1, and of these five residues, Ser-a–Ser-e, give rise to $\alpha\beta$ cross-peaks, assignable on the basis of chemical shifts,[22] which are well separated from the diagonal. Assignment of resonances from the remaining two serine residues is much less firm since their cross-peaks lie much closer to the diagonal and, therefore, Ser-f–Ser-g assignments are reported with brackets in Table I. Resonances from other amino acid types in crab MT-1 (four Pro, eight Lys, one Arg, five Glu, and one Gln) were assigned in the same way. However, all of these amino acid types produce side-chain cross-peaks that lie in very similar positions, thereby making it difficult in most instances to clearly identify complete spin systems and, hence, make assignments to specific amino acid type. Nonetheless, it is still possible to identify 18 of the total of 19 pairs of $\alpha\beta$ cross-peaks expected for these residues. In Table I, we have recorded 17 of these pairs of $\alpha\beta$ cross-peaks under the heading "Pekrq" since they have not yet been unequivocally assigned to amino acid type. For one $\alpha\beta$ cross-peak pair in this region, it is possible to trace cross-peaks as far as 2.85 ppm, clearly the ϵ proton resonance of a lysine residue, which thus identifies

Table I. ^{1}H NMR Chemical Shift Positions for $^{113}Cd_6$ Crab MT-1

	proton chemical shift, ppm						
amino acid	α	β	β'	γ	γ'	δ	ϵ
Val-10	4.69	2.63		1.02 (Me2)	0.90 (Me1)		
Thr-a	4.01	4.14		1.10 (Me3)			
Thr-b	4.12	4.21		1.23 (Me4)			
Thr-c	4.43	4.58		1.31 (Me6)			
Ala-a	3.69			1.28 (Me5)			
Ala-b	4.08			1.37 (Me7)			
Gly-a	4.32						
	3.49						
Gly-b	4.28						
	3.59						
Gly-c	4.14						
	3.64						
(Gly-d	4.17)[a]						
	4.08)						
(Gly-e	4.25)						
	4.18)						
Ser-a	4.65	3.93	3.93				
Ser-b	4.64	3.56	3.48				
Ser-c	4.54	3.96	3.80				
Ser-d	4.51	3.86	3.79				
Ser-e	4.43	4.00	4.00				
(Ser-f	3.98	3.88	...)				
(Ser-g	3.62	3.77	3.85)				
Cys-a	5.12	3.16	2.86				
Cys-b	4.95	2.94	2.94				
(Cys-c	4.90	3.12	3.03)				
Cys-d	4.84	3.17	3.07				
Cys-e	4.77	2.95	...				
Cys-f	4.64	3.51	3.18				
Cys-g	4.60	3.52	...				
(Cys-h	4.55	3.10	2.97)				
Cys-i	4.53	3.33	2.76				
Cys-j	4.51	3.93	3.17				
Cys-k	4.49	2.81	...				
(Cys-l	4.48	3.13	3.06)				
Cys-m	4.30	3.61	2.76				
Cys-n	4.26	3.15	2.67				
Cys-o	4.12	3.02	2.57				
Cys-p	4.02	2.92	2.83				
Cys-q	3.90	3.04	2.84				
Cys-r	3.73	3.17	2.87				
(Asx-a	4.65	2.65	2.58)				
(Asx-b	4.40	2.60	2.56)				
(Asx-c	4.57	2.73	2.66)				
Lys-a	4.27	1.81	1.65	1.21	1.04	1.54	2.85
Pekrq-a[b]	4.63	1.97	1.55				
Pekrq-b	4.54	2.08	1.76				
Pekrq-c	4.46	1.66	1.55				
Pekrq-d	4.46	2.40	1.67				
Pekrq-e	4.45	1.65	...				
Pekrq-f	4.43	2.22	...				
Pekrq-g	4.42	2.33	1.61				
Pekrq-h	4.39	1.98	1.84				
Pekrq-i	4.38	2.12	2.00				
Pekrq-j	4.32	2.37	2.02				
Pekrq-k	4.21	1.82	...				
Pekrq-l	4.13	1.86	1.78				
Pekrq-m	4.11	2.12	2.04				
Pekrq-n	4.03	1.82	1.73				
Pekrq-o	4.03	1.97	1.88				
Pekrq-p	4.01	2.15	2.06				
Pekrq-q	4.00	1.95	1.85				

[a] Resonances whose assignment to amino acid type are less certain are enclosed by parentheses. [b] The abbreviation "Pekrq" refers to those resonances that arise from the amino acids proline, glutamic acid, lysine, arginine, and glutamine.

these cross-peaks to a lysine residue. The chemical shifts of all the protons belonging to this lysine residue are identified as Lys-a in Table I.

We turn now to the identification of the $\alpha\beta$ cross-peaks arising from the 18 cysteine residues present in crab MT-1. Previous chemical and ^{113}Cd NMR studies have shown that all the cysteine residues are coordinated to either one or two Cd^{2+} ions.[1,3] Thus, since this protein contains its full complement of six isotopically enriched $^{113}Cd^{2+}$ metal ions, in addition to the usual homonuclear scalar coupling between the α, β, and β' protons, one expects to observe ^{113}Cd–^{1}H three-bond heteronuclear scalar coupling between the spin-$^1/_2$ ^{113}Cd metal ions and the β-CH_2 protons of the cysteine residues. The presence of this heteronuclear scalar coupling therefore provides a convenient way of identifying cross-peaks arising from cysteine residues. Fifteen pairs of $\alpha\beta$ cross-peaks were found that clearly showed ^{113}Cd–^{1}H coupling in either one or both cross-peaks, thus identifying them as cysteine residues. An additional three pairs of $\alpha\beta$ cross-peaks occurred

in the same spectral region, which were also assigned to cysteine on the basis of only their chemical shift since ^{113}Cd–^{1}H coupling was not clearly visible in these $\alpha\beta$ cross-peaks. The assignment of these three cysteine residues (Cys-c, -h, -l) is therefore tentative since the absence of ^{113}Cd–^{1}H coupling in their cross-peaks does not distinguish them from the three-spin systems of the two Asp and one Asn residues. Proton chemical shifts for all the assigned cysteine residues of crab MT-1 are given in Table I with those for Cys-c, -h, and -l enclosed in brackets. Although the data were recorded in the absolute value mode, it is still possible to measure, with reasonable accuracy, the range of the ^{113}Cd–^{1}H coupling from inspection of contour plots and submatrix rows. The largest observed $^3J_{^{113}Cd-^1H}$ heteronuclear coupling is approximately 65 Hz while some cysteine residue cross-peaks do not exhibit any resolved heteronuclear splitting, implying that it must be considerably smaller than the cross-peak width, i.e. much less than 20 Hz.

Only two further amino acid types occur; aspartate (two Asp) and asparagine (one Asn). Two distinct and one less distinct sets of $\alpha\beta$ cross-peaks occur close to the cysteine region, which we have assigned to these residue types. Parentheses are again used in Table I to denote the uncertainty in these assignments with the three cysteine residues discussed above.

Discussion

The spectral dispersion of both amide and α-CH ^{1}H NMR resonances in crab MT-1 supports the contention that this protein possesses a well-defined tertiary structure, consistent with the involvement of its numerous cysteine residues in Cd^{2+} coordination[1,3] as determined in our previous ^{113}Cd NMR studies.[14] In this paper, we have extended the earlier ^{113}Cd NMR structural studies by the application of 2D ^{1}H NMR methods to identify the chemical shift positions of a number of amino acid types in crab MT-1. In the case of the cysteine residues, this initial assignment process was facilitated by the appearance of ^{113}Cd–^{1}H scalar coupling into the β-CH_2 protons of these residues. In similar 2D ^{1}H NMR studies on rabbit metallothionein, identification of cysteine $\alpha\beta$ cross-peaks was achieved by comparing cross-peak structure in COSY datasets run with the protein saturated with $^{112}Cd^{2+}$ ($I = 0$) or $^{113}Cd^{2+}$ ($I = {}^1/_2$).[26] In the case of the crab MT-1 dataset presented herein, this isotopic substitution method was unnecessary since the appearance of heteronuclear coupling could be clearly distinguished in 15 (out of 18) cysteine $\alpha\beta$ cross-peak pairs directly by inspection. Furthermore, since all the cross-peaks can be accounted for in the cysteine $\alpha\beta$ region of the spectrum, it is unlikely that a cysteine spin system with ^{1}H–^{113}Cd J coupling in the crab protein has been confused with two AMX spin systems with degenerate α-proton resonances. The Cys assignments are labeled as Cys-a–Cys-r in Table I. The cysteine α-CH protons span a range of 5.12–3.73 ppm, implying a secondary shift range of –0.49 to +0.90 ppm,[22] and the β protons span a range of 3.93–2.57 ppm, indicating a secondary shift range of –0.65 ppm to +0.39 ppm.[22] For the cysteine β protons, 30 of the 36 protons lie between 3.4 and 2.6 ppm or within a 300-Hz range at a field of 11.7 T. This range of cysteine β-proton chemical shifts agrees precisely with that determined from the heteronuclear COSY experiments that we have acquired on this protein using proton-detected multiple-quantum-coherence methods.[27] The range of the three-bond ^{113}Cd–^{1}H scalar-coupling values observed for the cysteine β-CH_2 protons is approximately 10–65 Hz. This range can be compared to that observed for rabbit MT, $^3J_{^{113}Cd-^1H}$ = 0–80 Hz, by Neuhaus et al.[26]

The ultimate goal of these ^{1}H NMR studies is to provide a refined model for the metal binding sites in this protein, and, thus, it seems reasonable at this stage to discuss the implications that the ^{1}H NMR data presented above have upon the model derived for the metal cluster structure in this laboratory by using ^{113}Cd NMR.[14] To briefly summarize the results from our ^{113}Cd NMR studies, ^{113}Cd homonuclear decoupling experiments indicated that the six $^{113}Cd^{2+}$ metal ions bound to both crab MT-1 and MT-2 were divided into two sets of ^{113}Cd–^{113}Cd homonuclear scalar-coupled resonances, with coupling constants ranging from 24 to 40 Hz, each containing three metal ions.[14] The ^{113}Cd chemical shifts of these resonances indicated that tetrahedral thiolate coordination was involved, with a mixture of bridging and nonbridging thiolate ligands, and the relatively narrow ^{113}Cd NMR chemical shift range (~50 ppm) suggested that the coordination geometry of all the metal ions are very similar. Furthermore, the size of the smallest $^2J_{^{113}Cd-^{113}Cd}$ coupling observed in these clusters, 24 Hz, indicated that the lifetime of the ^{113}Cd spin state must be longer than ~40 ms.[28] From the ^{113}Cd NMR data and the fact that both crab MT-1 and MT-2 contains 18 cysteine residues, it was suggested that each Cd^{2+} ion was tetrahedrally coordinated to two nonbridging and two bridging cysteine thiolate ligands and thus each cluster contained three bridging trivalent thiolate ligands and six nonbridging divalent thiolate ligands.

Identification of resonances in the ^{1}H NMR spectrum belonging to these bridging and nonbridging cysteines would obviously provide additional strong support for the proposed cluster structures. Two possibilities exist for identifying the bridging cysteines: ^{1}H NMR chemical shift criteria for these cysteine β-CH_2 protons and/or an analysis of the heteronuclear coupling in the cysteine $\alpha\beta$, $\alpha\beta'$, and $\beta\beta'$ cross-peaks. A consideration of the magnitude of the shift of the ϵ protons of lysine upon ionization of the side-chain amino group, ~0.58 ppm,[22] suggests that any shifts of the cysteine β-CH_2 protons arising from the trivalent nature of its sulfur would likely be indistinguishable from shifts arising from other sources. Indeed, inspection of the cysteine resonance positions in crab MT-1 does not indicate any regular pattern that would be consistent with the number of bridging and nonbridging cysteine residues. The same conclusion appears to apply to the cysteine resonance positions reported for rabbit liver MT-2.[26] On the other hand, the analysis of the cysteine cross-peak structures can in theory provide unequivocal evidence for bridging cysteine resonances. Unfortunately, however, while the largest observed coupling can be measured with some confidence, the value of the smallest coupling constant is much less certain. Presumably a Karplus dependence[29] will exist for $^3J_{^{113}Cd-^1H}$; however, such a relationship has not yet been derived from measurements on a series of rigid Cd complexes of known structures, and thus, the minimum value of this coupling constant is uncertain. Even if this were known, the minimum observable heteronuclear coupling constant in metallothionein will most likely be dictated by the longitudinal relaxation time (T_1) of the metal nucleus, as opposed to exchange effects, which further complicates such an analysis. We have determined the field dependence of the ^{113}Cd T_1 values and the ^{113}Cd{^{1}H} nuclear Overhauser effect for the ^{113}Cd resonances of crab MT-1 and have concluded that, at the higher field (11.7 T), the principal relaxation mechanism arises from chemical shift anisotropy (CSA).[30] At a field of 11.7 T (500 MHz for ^{1}H NMR) the ^{113}Cd T_1 values for MT-1 lie between 0.1s and 0.8s. For the more rapidly relaxing ^{113}Cd resonances, this would predict a "spin-decoupling" effect for values of $J \lesssim 10$ Hz. This effect, together with the lack of a defined Karplus dependence to indicate whether certain observed heteronuclear coupling constants are geometrically possible from a single ^{113}Cd ion, may explain the apparent paucity of bridging cysteines detected by an analysis of the $J_{^{113}Cd-^1H}$ in this work and in rabbit liver MT.[26] While such a "spin-decoupling" effect on heteronuclear scalar couplings is common for spin-$^1/_2$ nuclei coupled to quadrupolar nuclei, to our knowledge, these high-field ^{113}Cd and ^{1}H NMR studies on MT provide the first reported situation where this effect may prevail for protons coupled to the spin-$^1/_2$ isotope of cadmium. A previous example of a case where this effect was observed for scalar-coupled spin-$^1/_2$ nuclei was for the proton–platinum heteronuclear couplings in square-planar complexes of ^{195}Pt ($I = {}^1/_2$) with organic

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and nucleotide ligands studied at relatively low-field strengths.[31]

The discussion above infers that, thus far, ^{1}H NMR studies on MT alone have provided no additional evidence that either substantiates or refutes the proposed model for the cluster structures derived on the basis of the ^{113}Cd NMR studies.[14] Indeed, the ^{113}Cd homonuclear decoupling experiments still provide the most definitive evidence for the proposed metal cluster structures in MT from *Scylla serrata*.

It is possible that the arrangement of the cysteine residues in these metal clusters may be more clearly defined by using ^{113}Cd-^{1}H chemical shift correlation experiments to correlate cysteine β-proton resonances with ^{113}Cd resonances. The experimental means we have adopted to pursue this possibility utilizes the indirect detection of ^{113}Cd via ^{1}H-^{113}Cd multiple quantum coherences.[27,32] Both the execution of these experiments and the analysis of the datasets are critically dependent on the ^{1}H NMR analysis presented in this paper.

In summary, in this paper we have presented the first detailed analysis of the ^{1}H NMR spectrum of crab MT-1 where we have identified numerous cross-peaks to amino acid type, in particular those arising from its 18 cysteine residues. The range of observed heteronuclear coupling constants ($^3J_{^{113}Cd-^1H}$) has been deduced, and the consequences of this range of coupling constants on the structure of the metal binding sites has been discussed. Finally, evidence is presented for the presence of a T_1-induced "spin decoupling" of the ^{113}Cd-^{1}H heteronuclear coupling in the 500-MHz ^{1}H NMR spectrum of ^{113}Cd crab metallothionein, which could account for the paucity of bridging cysteines identified and required in the proposed structures of the metal thiolate clusters.

Registry No. Cys, 52-90-4; ^{113}Cd, 14336-66-4.

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Assembly of $[Fe_nS_n(SPh)_4]^{2-}$ (n = 2, 4) and Their Iron-Thiolate Precursors in Aqueous Media

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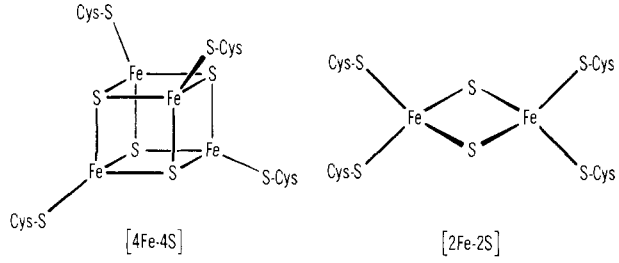
Tetraalkylammonium salts of $[Fe_nS_n(SPh)_4]^{2-}$ (n = 2, 4), which are synthetic analogues of [2Fe-2S] and [4Fe-4S] sites in proteins, can be prepared in >80% yields from 4/1/1 PhSH/$FeCl_3$/S in a medium consisting of 90/5/5 vol % (pH ~8) aqueous buffer/CH_3CN/Triton X-100. For this system the sole assembly pathway appears to be $[Fe(SPh)_4]^{2-} \rightarrow [Fe_2S_2(SPh)_4]^{2-} \rightarrow [Fe_4S_4(SPh)_4]^{2-}$, which suggests an analogous assembly pathway for [4Fe-4S] sites in ferredoxins. At lower PhSH/Fe mole ratios or at lower pH the $[Fe_4(SPh)_{10}]^{2-} \rightarrow [Fe_4S_4(SPh)_4]^{2-}$ pathway appears to occur, but to a lesser extent in aqueous Triton than in organic solvents. High yields of the precursors $[Fe(SPh)_4]^{2-}$ and $[Fe_4(SPh)_{10}]^{2-}$ are also achieved in the aqueous-based detergent medium. Evidence from ^{1}H and ^{19}F NMR indicates that the equilibrium $[Fe_4(SPh)_{10}]^{2-} + 6PhS^- \rightleftharpoons 4[Fe(SPh)_4]^{2-}$ is established prior to addition of S. The two most prominent unique features in the 90/5/5 medium are (i) the ability of detergent aggregates in the presence of relatively hydrophobic R_4N^+ to shield $[Fe_2S_2(SPh)_4]^{2-}$ from water, thereby inhibiting the rate of conversion to $[Fe_4S_4(SPh)_4]^{2-}$, and (ii) the facility with which the $[Fe_4(SPh)_{10}]^{2-} \rightleftharpoons [Fe(SPh)_4]^{2-}$ equilibrium can be shifted. Unique feature i leads to the ability to achieve essentially quantitative production of either $[Fe_2S_2(SPh)_4]^{2-}$ or $[Fe_4S_4(SPh)_4]^{2-}$ from the same 4/1/1 PhSH/$FeCl_3$/S reagent ratio. Unique feature ii leads to the production of substantial proportions of $[Fe_2S_2(SPh)_4]^{2-}$ upon reaction of $[Fe_4(SPh)_{10}]^{2-}$ with S, due to a shift of the above equilibrium to the right. Unique feature ii also leads to larger proportions of $[Fe_4S_4(SPh)_4]^{2-}$ at pHs below 6 (below the pK_a of PhSH), apparently due to a shift of the above equilibrium to the left. These two unique features suggest means by which ferredoxins could exert both kinetic and thermodynamic control over assembly of [2Fe-2S] and [4Fe-4S] sites.

Introduction

Iron-sulfur clusters usually function as electron carriers in proteins from diverse bacterial, plant, and mammalian sources. The [2Fe-2S] and [4Fe-4S] centers represent those biological



[4Fe-4S] [2Fe-2S]

clusters containing both iron and inorganic sulfide for which synthetic analogues currently exist.[1] The title clusters exemplify the latter, having PhS^- in place of Cys-S^-. Although the structural and redox properties of these centers have been thoroughly studied, almost nothing is known about their assembly in vivo, even for the smallest iron-sulfur proteins, generically termed ferredoxins. In fact, until our recent success in aqueous-based media,[2] no one had reported the assembly in and isolation from predominantly aqueous media of synthetic clusters containing the Fe_2S_2 and Fe_4S_4 cores. Previous published results in aqueous solutions containing iron salts, sulfide, and thiols are limited to visible absorption and EPR spectra, which at the time were difficult to interpret in terms of structure.[3,4] However, during subsequent years the stage has been set for reexaminations of these aqueous mixtures by results obtained in the laboratories of Holm and others.[1] For the simple reaction system ≥3.5/1/1 $PhS^-/FeCl_3$/S in methanol or acetonitrile, Hagen et al.[5] have identified two pathways leading to formation of $[Fe_4S_4(SPh)_4]^{2-}$. At 3.5/1 $PhS^-/FeCl_3$, $[Fe_4(SPh)_{10}]^{2-}$, containing an adamantane-like Fe-S framework, is

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