Proton NMR Studies of the Cobalt(II)–Metallothionein System

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Abstract: The binding of cobalt(II) ions to metallothionein (MT) from rabbit liver was followed through ^{1}H NMR spectroscopy of cysteine side-chain protons and magnetic susceptibility measurements at room temperature in solution. In the course of the titration of apo-MT with cobalt(II) no isotropically shifted ^{1}H NMR signals are observed up to about 3 equiv of metal. A few broad signals in the 300 to −50 ppm region are observed when 4 equiv is added, while more than 30 sharp signals in the same region are observed when 5 or more equiv is added, up to the full metal complement of 7 equiv. Parallel magnetic susceptibility measurements show that little magnetic coupling among metal ions occurs up to 3 equiv. Strong coupling is observed when the fourth equivalent is added, and a sizeable coupling is maintained, although with a smaller reduction of susceptibility value per metal ion, up to 7 equiv. The results are consistent with a metal binding scheme in which the first three metals do not share any cysteine sulfur donor, two metals possibly occupying the four-metal site and one the three-metal site. The fourth metal binds in the four-metal site, giving rise to a strongly coupled Co_3 cluster, and the fifth completes the four-metal cluster, yielding a well-resolved ^{1}H NMR spectrum. The sixth and seventh equivalents complete the three-metal cluster, where they are probably strongly coupled as well. We propose that the well-resolved ^{1}H NMR spectrum of the Co_7MT derivative essentially arises from the four-metal cluster.

Metallothioneins (MT) are a class of low molecular weight ($M_r \simeq$ 6–7 kDa) cysteine- and metal-rich proteins that are widely distributed in nature.[1] The best characterized forms are those isolated from vertebrate tissues. Mammalian proteins are composed of a single polypeptide chain of 61 or 62 amino acid residues out of which 20 are cysteines. These proteins usually contain 7 equiv of bivalent metal ions such as zinc and/or cadmium.[2] ^{113}Cd NMR[3] and Co^{II} EPR studies[4] on the respective metal derivatives established the existence of the tetrahedral tetrathiolate clusters in this protein. Evidence for the presence of two independent clusters of three and four metal ions, respectively, was first indicated by ^{113}Cd NMR studies[3] and subsequently supported by the enzymatic cleavage of the protein and isolation of the C-terminal cluster (α domain).[5] Recently both the crystal structure[6] and the solution NMR structure[7,8] have been solved. While both structures agree on the presence of two metal thiolate clusters, they differ in details of the sequence-specific metal cysteine connectivities.[9] The schemes of the clusters are reported in Figure 1.

To obtain more information on the structural features of the metal clusters and on the mechanism of their formation, we prepared the cobalt(II) derivatives of the protein, which had already been described,[4] and studied them through ^{1}H NMR spectroscopy and magnetic susceptibility measurements.

Indeed, cobalt(II) is an excellent spectroscopic probe for the analysis of the structure of zinc proteins.[10] Owing to its peculiar electronic properties, it is capable of inducing large isotropic shifts on the ^{1}H NMR signals of the neighboring protons without causing dramatic line broadening.[11] This allows extensive use of ^{1}H NMR spectroscopy for the investigation of the fine details of the metal binding site structure. The expected magnetic coupling between the cobalt(II) ions in each cluster is both a source of further information on the metal sites and a challenge to understand the hyperfine coupling in magnetically coupled polymetallic systems. Parallel measurements of magnetic susceptibility are performed to check the extent of magnetic coupling and the sequence of cluster formation. Finally, the strict similarity between the coordination chemistry of cobalt(II) and zinc(II) permits one to obtain cobalt(II) derivatives that closely mimic the native zinc systems.

Experimental Section

Protein Isolation. Rabbit liver MT2 was isolated from rabbits injected subcutaneously 15 times in 2–3-day intervals with 1 mg of Cd^{2+}/kg of body weight in the form of the chloride salt.[12] The protein was purified as previously reported.[13] Each preparation was characterized by amino acid analysis (Durrum 500) and by metal analysis using atomic absorption spectroscopy (Instrumentation Laboratory, IL 157). The apo form of the protein was prepared by gel filtration of the native form at low pH. Protein concentration was determined spectrophotometrically by measuring the absorbance of apo-MT in 0.01 N HCl at 220 nm with an extinction coefficient of 48 200 M^{-1} cm^{-1}.[1]

Preparation of the Co_xMT Derivatives and of the Co_3Cd_4MT Derivative. All solutions used in the preparation of the cobalt(II) derivatives of the protein were degassed on a vacuum line prior to use; all the procedures were performed in an argon-purged glovebox.

A freshly prepared solution of 1.5 mM apo-MT in 0.1 N HCl was mixed with appropriate aliquots of a 50 mM $CoCl_2$ solution to give the desired metal-to-protein ratios. The solution mixture was then adjusted to pH 8.5 by addition of 0.5 M Tris base (TRIZMA from Sigma). Each measurement was performed on a separate sample. The metal-to-protein ratios were determined independently by using small aliquots of the sample.

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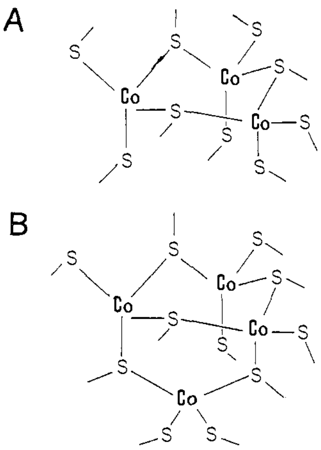

Figure 1. Schematic drawings of the three- (A) and the four- (B) metal clusters in MT.

The Co_3Cd_4MT derivative was prepared by adding 4 equiv of cadmium(II) to apo-MT at pH 7.5; then 3 equiv of cobalt(II) were added, and the pH was adjusted to 8.5. Selective filling of the four-metal cluster by cadmium(II) had been previously shown through analysis of the sample and ^{113}Cd NMR spectroscopy.[14] Formation of a three-cobalt(II) cluster in the β domain was checked by visible absorption spectra.[14]

^1H NMR Measurements. The ^1H NMR spectra of the protein samples in H_2O or D_2O were run at 90 MHz on a Bruker CXP 90 spectrometer. Solvent signal and diamagnetic signal suppression was accomplished by using the modified DEFT pulse sequence.[15,16] The spectra were obtained through block averaging of 10 spectra of 16000 scans each. Additional spectra were obtained at 300 and 200 MHz on Bruker CXP 300 and Bruker MSL 200 instruments, respectively.

The T_1 values were obtained by measuring the intensity of the signals as a function of the time delay between subsequent pulses of the modified DEFT sequence.[15] The T_2 values were determined by measuring the line width at signals half-height and using the relation $T_2^{-1} = \pi\Delta\nu_{1/2}$. Chemical shifts are reported in parts per million upfield and downfield from DSS.

Magnetic Measurements. Room-temperature susceptibility measurements were performed with the Evans method[17] on the Bruker MSL 200 spectrometer using DSS (4,4-dimethyl-4-silapentane-1-sulfonate) as inert standard. The accuracy of the measurements was ±0.1 Hz, over total bulk susceptibility shifts of 10–40 Hz.

Results

^1H NMR Titration of Apo-MT with Cobalt(II) and Magnetic Susceptibility Measurements. ^1H NMR spectra at increasing cobalt(II):apo-MT ratios have been recorded at 90 and 300 MHz in the spectral range between 400 and −300 ppm from TMS. The samples contained 0.9, 1.8, 3.0, 3.8, 4.7, 5.8, 6.2, and 7.0 equiv of cobalt(II) per protein as shown by analysis through atomic absorption spectroscopy.

At cobalt(II) to apo-MT ratios up to about 4 only weak, broad, ill-resolved resonances are observed outside the diamagnetic region. For a cobalt(II) to apo-MT ratio of 3.8 a spectrum characterized by broad features is observed (Figure 2A).

Samples containing 4.7 or more equiv of cobalt(II) per protein generated extremely well-resolved ^1H NMR spectra with more than 30 isotropically shifted resonances spread over the 350 to −100 ppm range. Aside from a meaningful increase in the intensity, the latter spectra remained virtually unchanged on going from 5 to 7 cobalt(II) equiv. The ^1H NMR spectrum of the fully metallated derivative is shown in Figure 2B, and the spectral parameters are collected in Table II. In this form the seven cobalt(II) ions are positioned into two metal thiolate clusters of three and four metal ions.[1]

Figure 2C shows the ^1H NMR spectrum of the Co_3Cd_4MT derivative. In such a compound the cobalt(II) ions have been shown to be bound solely within the three-metal cluster.[14] The spectrum displays only a few rather broad isotropically shifted resonances at 215, 170, 140, 120, and 105 ppm. The line widths of the resonances arising from the three-metal cluster are thus by far larger than those of the final Co_7MT spectrum. No striking temperature effect on the shape of the resonances was observed on going from 298 to 318 K, nor was there an abnormal temperature dependence of the shifts.

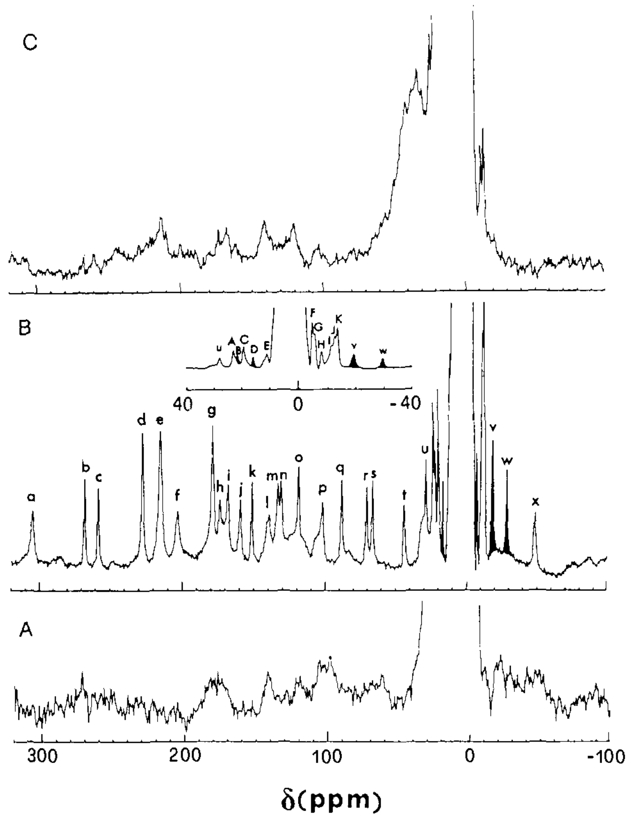

Figure 2. The 298 K 90-MHz ^1H NMR spectra of apo-MT solutions reacted with 3.8 (A) or 7 (B) equiv of cobalt(II), pH 8.5, Tris HCl 0.05 M. C: 298 K 90-MHz ^1H NMR spectrum of Co_3Cd_4MT. The filled signals in spectrum B disappear in D_2O.

Table I. Magnetic Susceptibility Values for CoMT Samples

sample Co/MT ratio	$10^7\chi_M$ (total), $m^3\ mol^{-1}$	$10^7\chi_M$ (per metal), $m^3\ mol^{-1}$
0.8	1.2	1.5
1.5	2.0	1.3
2.1	2.5	1.2
3.3	3.4	1.0
3.9	2.0	0.5
4.1	2.7	0.6
4.2	3.1	0.7
4.9	3.9	0.8
5.3	4.3	0.8
5.6	4.3	0.8
6.7	5.0	0.7

Comparison of the spectrum of the Co_3Cd_4 MT with that of Co_7MT reveals that the broad signals at 215, 120, and 105 ppm are clearly seen also in the latter derivative. The remaining signals at 170 and 140 ppm could correspond to the signals h and l in Co_7MT respectively (Figure 2B). This result demonstrates that the major contribution to the well-resolved ^1H NMR spectrum developed above 4.7 Co(II) equiv comes from the four-metal cluster.

The magnetic susceptibility of several samples containing different Co^{II}:apo-MT ratios has been measured at 298 K by using the Evans method.[17] The results are shown in Table I and Figure 3. From inspection of Table I it appears that the magnetic susceptibility χ_M of the cobalt(II) complexes increases almost

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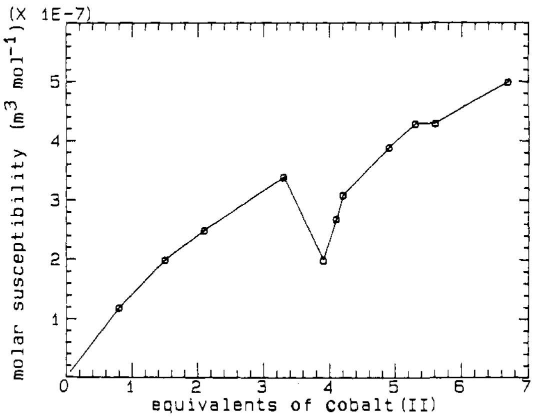

Figure 3. Magnetic susceptibility values of apo-MT in the presence of increasing amounts of cobalt(II) (conditions 298 K, pH 8.5). The values are corrected for the diamagnetic contribution.

linearly with increasing concentration of cobalt(II) up to a Co^{II}:apo-MT ratio of about 3.3, yielding susceptibility values not far from the sum of isolated cobalt sites. At 3.9 equiv of cobalt(II) a net decrease in the magnetic susceptibility is observed. Then the susceptibility increases again, although with a smaller slope.

Analysis of the ^1H NMR Spectra. As shown in Figure 2, isotropically shifted signals in the Co_7MT derivative are spread over a 400 ppm range. We assume that, as previously discussed, all signals labeled in Figure 2B, with the possible exception of resonances h and l, come from the Co_4 cluster. The T_1 values at 90 MHz of the signals that exhibit sizeable isotropic shifts range between 1.5 and 6 ms, thus being relatively short (Table II). As revealed by the T_1-to-T_2 ratio, which ranges between 1.5 and 3.0, the protons experience, besides dipolar, also some contact contribution (Table II).[11]

When the ^1H NMR spectra of Co(II)-MT samples containing 5–7 equivalents of Co(II) are recorded in D_2O, signals v, w, B, and D disappear. The former two are upfield and the other downfield shifted with respect to their diamagnetic position. They can be assigned to labile protons located in the immediate proximity of the paramagnetic centers. It should be noted that at least one nonexchangeable upfield shifted signal is also seen.

The temperature dependence of the isotropically shifted resonances of the Co_7MT sample has been investigated in the temperature range between 5 and 50 °C; while most signals follow a Curie like behavior, i.e., their isotropic shifts increase with decreasing temperature, there are at least eight signals with non-Curie or even anti-Curie behavior. Such an odd temperature dependence is sometimes found in strongly magnetic coupled systems and therefore provides independent support for the presence of magnetic coupling interactions in the four-metal cluster. The detailed investigation of this behavior is reported elsewhere.[18]

Unexpectedly, the ^1H NMR spectra of Co_3Cd_4MT do not reveal well-shaped signals. The effect of increasing temperature was also checked by recording a spectrum at 318 K. At high temperature the chemical shift values are shifted toward the diamagnetic region, although at a smaller extent than predicted on the ground of a Curie behavior, and the line widths are slightly larger.

Discussion

Table II. NMR Parameters of the Isotropically Shifted ^1H Signals in Co–MT

signal[a]	δ,[b] ppm	T_1,[b] ms	$\Delta\nu_{1/2}$,[b] Hz	T_1/T_2
a	304.6	1.5	19	0.94
b	267.6	5.7	105	1.9
c	257.7	6.0	110	2.1
d	227.2	3.2	130	1.3
e	215.0	2.0	230	1.4
f	202.5	1.6	280	1.5
g	178.4	4.1	160	2.1
h	173.3			
i	167.9	4.1	196	2.5
j	159.4	2.9	140	1.3
k	150.9	6.3	100	1.9
l	139.4	4.1	300	3.6
m	133.3	4.4	140	1.9
n	130.9	3.6	140	1.5
o	118.7	3.9	120	1.5
p	102.1	2.9	230	2.1
q	88.2	4.1	125	1.6
r	70.2	5.6	115	2.0
s	66.2	4.8	115	1.7
t	44.1	3.3	140	1.3
u	28.5	3.7	88	1.0
v	−18.6	3.7	130	1.5
w	−28.8	6.3	90	1.8
x	−48.4	5.7	160	2.9

[a] Labeling as in Figure 2B. [b] At 298 K.

^1H NMR spectra of high-spin cobalt(II) protein complexes are relatively easy to observe; the short electronic relaxation times of the metal ion permit the observation of moderately sharp isotropically shifted proton resonances from the metal binding residues. Recently, resonances arising from the β-CH_2 and α-CH protons of coordinated cysteines have been observed in cobalt(II)-substituted liver alcohol dehydrogenase[16] (2 Cys + 1 His + 1 H_2O ligands, $M_r \simeq 90$ kDa) and cobalt(II)-substituted rubredoxin[19] (4 Cys ligands, $M_r \simeq 6$ kDa). The Cys β-CH_2 resonances are found in the 210–280 ppm range in the former and in a similar range in the latter; the spreading is probably due to the axial or equatorial nature of the geminal protons.[20]

No NMR spectrum is observed in the present case at Co(II) to apo-MT ratios up to 3. Owing to the similarity in geometry and ligand field experienced by cobalt thiolate complexes, we consider quite unlikely that the electronic relaxation times are now long and thus unfavorable for the ^1H NMR spectra. It is reasonable to assume that the cobalt ions are in equilibrium among several binding sites since the total number of thiolate ligands is higher than required for the formation of isolated CoS_4 chromophores. Fluxionality has been already considered in accounting for a broad ^{113}Cd NMR feature at low Cd:apo-MT ratios.[21]

The ^1H NMR spectra of Co(II)–MT as a function of increasing Co(II) to apo-MT ratios, together with the magnetic susceptibility measurements, allow a number of inferences concerning the mechanism of cluster formation to be drawn. Under the employed pH condition (pH = 8.5), a two-step process leading to cluster formation apparently exists. This is clearly shown by the magnetic susceptibility data which reveal virtually no important magnetic interactions up to a Co(II) to apo-MT ratio of 3.3. On the basis of the drop in magnetic susceptibility upon the addition of about 4 cobalt(II) equiv, the formation of cluster structures is inferred. On going from 4 to 7 cobalt(II) equiv, the susceptibility values increase again, strongly up to about 5 equiv and then with a smaller slope. The strong increase in susceptibility from 4 to 5 equivalents is paralleled by the occurrence of the well-resolved ^1H NMR spectrum characteristic of Co_7MT.

The simplest explanation for this behavior would seem to be the formation of the three-cobalt cluster before that of the four-cobalt cluster. If it were so, however, at Co(II) to apo-MT ratios of about 4, broad signals similar to those of the Co_3Cd_4MT form would be expected. It should also be noted that cadmium ions bind cooperatively to the four-metal cluster under similar experimental conditions. Furthermore, if the Co_3 cluster is formed first, then the ^1H NMR spectrum of the four-cobalt cluster should

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be observable only above 5 equiv and should increase dramatically in intensity up to 7 equiv. Instead, a relatively intense NMR spectrum is already observed at 5 equiv, and its intensity increases only moderately upon further addition of cobalt.

Therefore, we favor an entirely different interpretation that is consistent with the combination of NMR and magnetic susceptibility measurements. At the very beginning of cobalt titration, the metal tends to occupy both binding domains, possibly with a preference for the four-metal domain. At 3 equiv of cobalt we propose that two metal ions are in the four-metal domain and one is in the three-metal domain. All of them are isolated, fluxional, cobalt tetrathiolate complexes. The fourth equivalent of cobalt selectively goes as the third metal into the four-metal domain; strong magnetic coupling occurs and a cluster geometry similar to the final arrangement of the three-metal cluster is formed. This causes a drop in magnetic susceptibility, which would correspond to the sum of one isolated cobalt site in the three-metal cluster domain and a Co_3 cluster in the four-metal cluster domain. Indeed, the broad NMR signals observed at this point of the titration are similar in shape to those of the Co_3Cd_4MT derivative but with different chemical shifts. Addition of the fifth equivalent of cobalt completes the four-metal-cluster, giving rise to a well-resolved ^{1}H NMR spectrum. The strong increase in susceptibility can be explained by an overall weaker coupling among the four metal ions with respect to the three-metal cluster arrangement. Addition of the sixth and seventh equivalents of cobalt causes the progressive filling of the three-metal cluster. The increase in susceptibility is again smaller because a new coupling sets in, and the ^{1}H NMR spectrum shows evidence of additional broad signals from the three-metal cluster of the fully metal-occupied Co_7MT form.

Overall, the proposed pathway of cluster formation in CoMT does not differ much from that suggested on the basis of the spectral changes in the ^{113}Cd NMR spectra of CdMT as a function of increasing Cd:apo-MT ratios. Under similar experimental conditions, below a Cd:apo-MT ratio of 4 a broad NMR feature at 695 ppm has been seen, presumably due to chemical exchange.[14] Exactly with 4 cadmium equiv, ^{113}Cd NMR signals of the four-metal cluster occurred. Additional cadmium ions generated the three-metal cluster in a cooperative manner. We detect an intermediate Co_3 cluster in the Co_4 cluster domain with some (<1) cobalt in the Co_3 cluster domain. The possible reason for the differences in cluster formation between Cd(II)-MT and Co(II)-MT can be the overall larger thermodynamic stability of the Cd_4 cluster (ca. 10^6-fold). The observed magnetic behavior is similar to the pattern of a titration study of apo-MT with cobalt(II) where the intensity changes of the EPR signal had been followed. The latter studies revealed a similar linear intensity increase up to about 3.3 cobalt(II) equiv followed by an amplitude loss at 5 or more cobalt(II) equiv.[4] In the latter case magnetic coupling occurs and the EPR signal intensity decreases.

Although all the seven cobalt(II) ions in both clusters are bound in a comparable distorted tetrahedral tetrathiolate environment and would be expected to give rise to similar proton NMR patterns, it appears that the resonances from the three-metal cluster are dramatically broader than those originating from the four-metal cluster. As discussed before, we consider the possibility of alteration of the electronic relaxation time τ_S rather unlikely. On the other hand, in the recent ^{113}Cd NMR saturation transfer experiments the existence of an intramolecular metal-exchange process within the three-metal cluster of ^{113}Cd(II)-MT was clearly documented.[22] Furthermore, the preservation of the well-resolved ^{113}Cd-^{113}Cd coupling patterns with increasing temperature led the authors to postulate a still unknown dynamic process in the three-metal cluster domain.[23] Hence it is possible that a similar dynamic process is also responsible for the broadening of the ^{1}H NMR lines of the $Co(II)_3$ cluster. The fact that the line widths of the Co_3Cd_4-MT derivative slightly increase with increasing temperature supports the presence of important dynamic processes in the three-metal cluster. Such processes occur on the NMR time scale and cause dramatic line-width broadening.

As a consequence of the above reasoning the majority of the signals discerned in the spectrum of Co_7MT originate from the four-metal cluster. Consequently, the downfield shifted resonances are attributed to the β-CH_2 groups of 11 cysteine residues. Thus one would expect a total of 22 isotropically shifted signals. The corresponding 11 α-CH resonances should lie closer to the diamagnetic region, in agreement with their larger distance from the paramagnetic center. The magnetic susceptibility is smaller than expected for isolated ions. This suggests that the magnetic coupling is of the order of 10–100 cm^{-1} if all or most coupling constants are equal. This causes the isotropic shifts to be smaller than expected for isolated ions since the shift is proportional to the magnetic susceptibility. The fitting of the data with a theoretical model is beyond the scope of this report and is the subject of a second paper.[18]

A comment is due, however, on the isotropically shifted signals that disappear when the spectrum is recorded in D_2O and therefore cannot be due to the cysteine side chains. They can be attributed to the exchangeable protons that are hydrogen bonded to the sulfur atoms of the coordinating cysteine residues. The rather high thermodynamic stability of these bonds is revealed by the absence of appreciable effects of the increased ionic strength (0.2 M KCl) on the overall ^{1}H NMR spectrum of Co-MT (data not shown). Possible candidates are the NH groups of the peptide backbone and/or exchangeable groups of the aminoacid side chains, e.g., Lys, Asp, etc. Recently, in the preliminary crystallographic refinement of the $Zn(II)_2Cd(II)_5$-MT at 1.9-Å resolution two such hydrogen bonds—namely, those between Lys 43 and Cys 26 and between Thr 27 and Cys 59—were recognized.[24] The existence of such unusual hydrogen bonds has been encountered previously in a number of iron proteins, e.g., ferredoxin,[25,26] HiPiP[27] protein, and rubredoxin.[28] It is generally believed that they are involved in modulation of their redox potential. In view of the still elusive function of MTs, diamagnetic Zn(II) and/or Cd(II) proteins, the existence of such hydrogen bonds is peculiar and deserves further studies.

Concluding Remarks

The proton NMR spectra of the cobalt(II) derivatives of metallothionein provide a direct insight into the fine details of the cluster formation. Titration of the apoprotein with cobalt(II) indicates that well-resolved proton NMR spectra can be observed at metal-to-protein ratios higher than 4. Dynamic processes could be responsible for the undetectability of the isotropically shifted NMR resonances at low metal/protein ratios.

The contributions of the two clusters to the overall spectrum have been separated. Whereas the spectrum of the three-cobalt cluster (and that of a three-metal cluster that is postulated to be an intermediate in the formation of the four-metal cluster) are characterized by a few broad lines and are scarcely informative, the spectrum generated by the four-metal cluster is extremely well resolved, exhibiting more than 30 relatively narrow signals spread over a 400 ppm interval.

Parallel magnetic susceptibility measurements have permitted us to describe the magnetic interactions that take place inside the two clusters and to identify a possible mechanism for cluster

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formation; such mechanism includes the formation of a Co$_3$ cluster intermediate in the four-metal domain that eventually transforms into the final Co$_4$ cluster.

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Proton NMR Spectra of the Co$_4$S$_{11}$ Cluster in Metallothioneins: A Theoretical Model

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Abstract: The theory for the electron–nucleus interaction in exchange-coupled dimetallic systems is extended to the case of a four-metal cluster to develop a theoretical model for the ^{1}H NMR spectra of the Co$_4$S$_{11}$ cluster in Co$^{II}_7$ reconstituted metallothionein (MT-2) from rabbit liver. A model is obtained that qualitatively rationalizes the isotropic shifts pattern and their temperature dependence in terms of five large and one essentially zero J values for the six exchange coupling constants operative in the four-metal cluster. This finding is consistent with the results from the X-ray and solution structures of the protein. A discussion is presented that shows under what conditions the experimental features are met. Also, the ^{1}H NMR spectra of the already reported Co$_4$S$_{10}$ cluster, present in the model system [Co$_4$(SEt)$_{10}$]$^{2-}$, are discussed.

Metallothioneins (MT) are a class of low molecular weight ($M_w \simeq$ 6–7 kDa), single-chain proteins capable of binding seven bivalent metal ions[1] such as zinc and cadmium.[2] They are bound in two independent clusters of three and four metal ions, respectively.[3–5] The Co$^{II}_7$ derivative of rabbit liver MT-2 has recently been reported;[6,7] it is proposed that the seven cobalt(II) ions are arranged in the same way as the native metals, i.e., three cobalt(II) ions in the β-domain and four cobalt(II) ions in the α-domain with the cluster structure substantially retained (Figure 1).

It is shown in the preceding paper[7] that the Co$^{II}_7$ derivative gives a surprisingly well-resolved ^{1}H NMR spectrum characterized by more than 20 isotropically shifted signals spread over a region spanning between 300 and −100 ppm (Figure 2).[6] The signals have been unequivocally assigned to the Co$_4$ cluster, by comparing the spectra of the Co$_7$ derivative and the Cd$_4$Co$_3$ derivative; the Co$_3$ cluster in the Cd$_4$Co$_3$ derivative does not give sharp isotropically shifted signals.[7] It has been suggested that the Co$_3$ cluster experiences some fluxional character that broadens the lines beyond detection.[7] Most or all of the isotropically shifted ^{1}H NMR signals of the Co$_4$ cluster in the Co$_7$ derivative arise from the β-CH$_2$ protons of the 11 cysteines involved in metal coordination; some of the less shifted signals, both up and down field, might be assigned to Cys α-CH protons. Two upfield signals (v and w) belong to exchangeable protons. Signals d, e, and g seem to have double intensity.

The cobalt(II) ions in the Co$_4$ cluster are magnetically coupled with an average magnetic susceptibility per cobalt ion equal to about 50% that of the isolated ion.[7] This result has been evaluated by subtracting the magnetic susceptibility of the Cd$_4$Co$_3$ derivative from the total magnetic susceptibility of the Co$_7$ derivative. The many new levels arising from exchange coupling can account for the decreased susceptibility as well as for the short electron relaxation rates and sharp NMR lines (Figure 2).[7]

^{1}H NMR spectra of paramagnetic species exhibiting such an extraordinary spectral dispersion and resolution contain in principle a wealth of structural information. The isotropic shifts can be contact or dipolar in origin.[8] Since the geometry around each of the metal ions is pseudotetrahedral, it is likely that the shifts are predominantly contact in origin. The contact hyperfine coupling for protons of methylene groups bound to the metal-coordinated sulfurs S are expected to depend on the dihedral angle ϕ between the M–S–C and S–C–H planes[9] according to the following equation:[9–11]

$$A = \beta_0 + B_2 \cos^2 \phi \qquad (1)$$

where $B_2 \cos^2 \phi$ is related to the spin density on the proton resulting from hyperconjugation and β_0 is related to spin density resulting from other delocalization mechanisms. For alkylamines β_0 is negligible,[12] whereas no estimates are available for alkyl thiolates. In principle, the spreading of the signals could be accounted for on the basis of the above equation, but the nonequivalence of the four CoS$_4$ moieties may also contribute heavily to the signal spreading.

Finally, it has been observed that the shifts of a few signals increase with increasing temperature (anti-Curie behavior), whereas for most signals the pattern is normal (Curie behavior). The observation of both Curie and anti-Curie behavior in the same molecule is rather peculiar. In exchange-coupled dimetallic

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