Selectivity of Methylation of Metal-Bound Cysteinates and Its Consequences

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Abstract: Alkylation of metal-bound cysteinate residues forms an integral step in both the activation of the DNA-damage sensing Ada protein from *E. coli* and the reaction mechanisms of several zinc-dependent enzymes. The roles of metal ions and the protein structure in regulating the reactivity of bound cysteinate residues is not well-understood. Variants of a consensus zinc finger peptide were used to determine the effects of alkylation of cysteine residues on both metal binding and stability of the peptide structure. The ability of thioethers to act as ligands was probed through the direct synthesis of peptides with methionine or *S*-methylcysteine replacing the second histidine within the zinc finger framework. This position can be substituted with cysteine with no significant loss of structure or stability. Two-dimensional ^{1}H NMR studies and water exchange experiments of the peptide with *S*-methylcysteine in this position showed that methylation affected the structure of the peptide−zinc complex in the last turn of the helix, adjacent to the site of methylation, without disrupting the rest of the structure. Titrations with cobalt revealed that the peptides with methionine or *S*-methylcysteine do not bind metal ions as tightly as do peptides with histidine or cysteine in this position. Similar to peptides lacking a fourth ligand, these thioether containing peptides form two-to-one peptide-to-cobalt complexes at low metal concentrations. Alkylation of the cobalt complex of the peptide with cysteine as the fourth ligand with dimethyl sulfate in aqueous solution yielded a product with absorption spectral features essentially identical with those of the *S*-methylcysteine derivative. Methylation of either of the other two cysteine residues within this peptide resulted in the loss of detectable metal binding. The carboxyl terminal cysteine was alkylated at a rate approximately 5-fold higher than the other cysteine residues, potentially due to the relative accessibility of this cysteine sulfur compared with the others which are shielded by peptide amide to sulfur hydrogen bonds. Other studies suggest that all of the cysteine residues in this peptide are less prone to alkylation in the cobalt complex than they are in the unfolded, metal-free form under similar solution conditions. These results indicate that thioether residues have a significantly lower affinity for cobalt(II) and zinc(II) than cysteine or histidine. Thus, significant modulation of metal-bound cysteinate reactivity can be achieved through the position of the cysteinate within the three-dimensional structure of a metal−peptide complex.

Introduction

Several zinc proteins such as the *E. coli* DNA repair protein Ada, and cobalamin-dependent and independent methionine synthases use zinc-associated thiolates as nucleophiles that accept alkyl groups from appropriate donors.[1] The Ada protein, for example, accepts a methyl group from a phosphotriester in the backbone of alkylated DNA onto a zinc-bound cysteinate.[2] The methylated protein then binds DNA in a sequence-specific fashion leading to an increase in the rate of transcription of the Ada gene as well as other genes in the methylation repair pathway.[3,4] Similarly, the active site zinc of *E.coli* cobalamin-independent methionine synthase binds the sulfur of homocysteine during the transfer of a methyl group from methyl tetrahydrofolate.[5]

What role does zinc ion play in activating cysteine toward nucleophilic attack? How does the protein structure control the reactivity of specific cysteine residues when more that one is bound to the same zinc ion? How does the alkylation of cysteine or other thiolate ligands affect their metal binding properties? Limited information regarding these questions is available from previous studies. Myers et al. hypothesized that bound zinc in the Ada protein activates the methyl-accepting cysteine by deprotonating it, making it more nucleophilic and thus capable of attacking the methyl group of the methylphosphotriester.[2] The same mechanism is thought to mediate the increased nucleophilicity of homocysteine by the *E. coli* methionine synthase.[5] Studies of simple coordination complexes showed that thiolate ligands in zinc centers can be readily alkylated. Additionally, these studies indicate that zinc tetrathiolate centers have an intrinsically higher nucleophilicity than free thiols or the thiolates in mixed thiolate−imidazole complexes in non-aqueous solution.[6,7] In these complexes, alkylation of a thiolate ligand resulted in its loss from the coordination sphere of the zinc ion. In contrast, the methylated cysteine residue of the Ada

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Table 1. The Sequences of the Different Zinc Finger Peptides Used in These Studies Shown Using the One-Letter Amino Acid Code[a]

	4 7	20 24
CP-1	PYK**C**PE**C**GKSFSQKSALVK**H**QRT**H**TG
CP(H24C)	PYK**C**PE**C**GKSFSQKSALVK**H**QRT**C**TG
CP(H24M)	PYK**C**PE**C**GKSFSQKSALVK**H**QRT**M**TG
CP(H24X)	PYK**C**PE**C**GKSFSQKSALVK**H**QRT**X**TG
CP(C4X)	PYK**X**PE**C**GKSFSQKSALVK**H**QRT**H**TG
CP(C7X)	PYK**C**PE**X**GKSFSQKSALVK**H**QRT**H**TG
CP-C4	PYK**C**PE**X**GKSFSQKSALVK**H**QR

[a] Potential metal liganding residues are shown in bold. *S*-methylcysteine is denoted by X.

protein has been shown to remain bound to either Zn^{2+} or Cd^{2+} after alkylation.[8,9] This may be facilitated by the binding of the methylated protein to DNA in these studies.

To address questions of the fundamental properties of metal-coordinating cysteine residues within peptide complexes in aqueous solution, we used modified consensus zinc finger peptides. These peptides represent well-characterized model systems for studies of the metal binding and structural properties of zinc finger domains.[10−12] This allowed us to investigate the intrinsic reactivity of metal-coordinated cysteine residues, the role of three-dimensional structure on cysteinate reactivity, and the quantitative effects of methylation on the metal binding properties of cysteinate ligands. While this model system has many advantages, the peptides contain four lysine residues that were also methylated under the conditions utilized. This precluded product analysis by HPLC and detailed stoichiometry and kinetic studies.

Experimental Section

Synthesis and Purification of Peptides. Seven variants of the consensus zinc finger peptide CP-1[11] were used in these studies. These peptides vary with respect to the identity of potential metal binding ligands as shown in Table 1. Peptides were synthesized with the use of a Milligen Biosearch 9050 peptide synthesizer, reduced with excess dithiothreitol, and purified by reversed phase HPLC as described previously.[11] Peptide purity and identity were confirmed by mass spectrometry. All peptides were stored in an atmosphere of 2−5% dihydrogen in dinitrogen to minimize cysteine oxidation. The concentration of peptide used in each experiment was estimated with the use of an extinction coefficient of 1420 M^{-1} cm^{-1} at 275 nm.

Fmoc-*S*-methylcysteine was synthesized by using an adaptation of a published protocol for Fmoc-methionine.[13] A 0.0152 mol sample of *l*-*S*-methylcysteine was dissolved together with 1.62 g of Na_2CO_3 in 19.82 mL of H_2O. Fluorenylmethylsuccinimidyl carbonate (0.0152 mol) dissolved in 19.82 mL of acetone was added in portions over a period of 45 min. The stirred solution was kept at pH 9−10 by addition of 1 M Na_2CO_3. After being stirred overnight, 45.7 mL of ethyl acetate was added and the mixture was acidified to pH 6.5 with concentrated HCl. After addition of 55 mL of H_2O and 55 mL of ethyl acetate, the mixture was acidified to pH 1−2 with concentrated HCl. The ethyl acetate layer was separated, washed with water, dried over anhydrous magnesium sulfate, and evaporated to one-third of its volume. The product crystallized at 4 °C on addition of 5 mL of petroleum ether. Purity was confirmed by thin-layer chromatography and NMR. The identity of the product was confirmed by mass spectrometry.

Optical Titrations of Cobalt(II) Binding. Optically monitored cobalt(II) titrations of peptides were performed over a range of 200−820 nm on a Perkin-Elmer Lambda 9 spectrophotometer at 25 °C. Spectra in the region from 500 to 820 nm were utilized for the spectral analysis. Titrations were performed with peptide concentrations of 70, 100, 330, and 500 μM in 100 mM HEPES, pH 7.0. During these titrations, the cobalt concentration was raised from 0 to 50 times that of the peptide in a stepwise manner.

The results from the titration of the peptides with *S*-methylcysteine and methionine were analyzed by factor analysis as described previously.[14] The original data matrix ($\mathbf{D}_{obs}$) was generated from the data of multiple titrations, with the column indices corresponding to wavelength values and the row indices running over the titration points at different cobalt(II) concentrations. Only two basis spectra were needed to describe the data within the limits of experimental error, showing that there are two distinct absorbing species. Coefficients representing the contributions of the two basis spectra to the data set at each cobalt concentration i are denoted $f_1o(i)$ and $f_2o(i)$.

To describe the chemical components of these cobalt titrations, the data were fit to a model in which both one-to-one and two-to-one peptide-to-metal complexes can form as shown below:

$$P + M \overset{K_{d1}}{\rightleftharpoons} PM$$

$$2P + M \overset{K_{d2}}{\rightleftharpoons} P_2M$$

The absorption spectra of the cobalt(II) complexes were estimated as linear combinations of the basis vectors obtained from the eigenvalue analysis above. Subsequently the contribution of each basis spectrum to the observed data was calculated as follows:

$$f_1c(i) = c_{11}[PM](i) + c_{12}[P_2M](i)$$

$$f_2c(i) = c_{21}[PM](i) + c_{22}[P_2M](i)$$

where [PM] is the concentration of the one-to-one peptide−cobalt complex, [P_2M] is the concentration of the two-to-one peptide to cobalt complex, and the index i runs over all the data points at different cobalt(II) concentrations.

The quantity $F = \sum_i((f_1o(i) - f_1c(i))^2 + (f_2o(i) - f_2c(i))^2)$ was then minimized by using the simplex method with respect to the coefficients $c_{ij}(i,j = 1,2)$, K_{d1}, K_{d2}, and scaling factors accounting for the precise concentration of active peptide. After completion of the minimization, absorption spectra for pure components (one-to-one and two-to-one peptide−cobalt(II) complexes) were calculated and values for the equilibrium constants were obtained. Standard deviations for the equilibrium constants were estimated from the sensitivity of the agreement factor F to changes in the individual parameters.

Titration of Co(II)−Peptide Complexes with Exogenous Thiol and Imidazole. Titrations of peptide−cobalt complexes with exogenous ligands were performed in 50 mM HEPES, 50 mM NaCl at pH 8.0 and monitored by visible spectroscopy on a Perkin-Elmer Lambda 9 spectrophotometer at 25 °C. Peptides at concentrations of 110−150 μM were 80% saturated with $CoCl_2$, and these peptide−cobalt complexes were treated with either β-mercaptoethanol or *N*-methylimidazole from 0 to 50 molar equiv. The apparent equilibrium constants for the binding of these ligands were determined by Scatchard analysis.

Alkylation of Zinc Finger Peptides. Alkylation of the peptide−Co^{2+} complexes with dimethyl sulfate was performed in 450 mM borate

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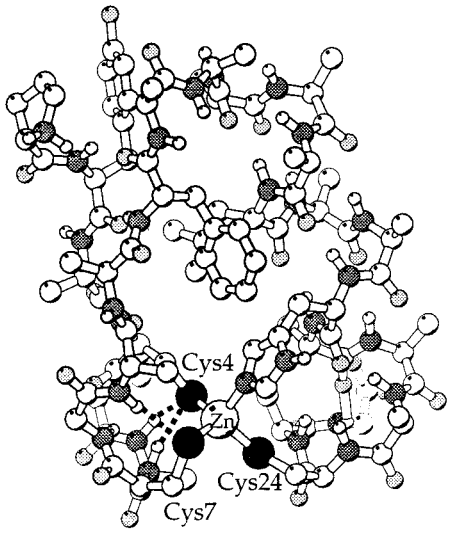

Figure 1. A model of the structure of CP(H24C) based on the crystal structure of a protein containing consensus zinc finger domains. Side chains are only shown to the β-carbon except for highly conserved or proline residues. Nitrogen atoms are shown in dark gray, oxygen atoms in light gray, and sulfur in black. Peptide NH to sulfur hydrogen bonds are shown as dotted lines.

buffer at pH 7.0. Dimethyl sulfate was chosen as a methyl donor since conditions for its use in aqueous solution have been well-established for DNA alkylation. The reactions were quenched at the desired time point by the addition of 100 equiv of β-mercaptoethanol. Reactions were monitored by optical spectroscopy.

NMR Studies. Purified CP(H24X) (X = *S*-methylcysteine) was resuspended in 7.3 mM Tris pH 6.5, 24 mM $ZnCl_2$ at a concentration of 2 mM. The mixture was then filtered through a prewashed 0.22 μm filter to remove precipitated material which reduced the peptide concentration to approximately 0.5−0.8 mM. Measurements were made at 25 °C. The pH of D_2O samples was adjusted for isotope effects. Total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), double quantum filtered correlation spectroscopy (dqfCOSY), and pulse excitation correlation spectroscopy (P.E.COSY)[15] spectra were recorded in both D_2O and H_2O on a Varian UNITY Plus 500 MHz spectrometer. The peptide backbone and side chain residues were assigned using standard methods. Hydrogen exchange experiments were performed on the zinc complex of CP(H24X) (X = *S*-methylcysteine) at pH 6.5 and 25 °C by using the water exchange filter sequence WEX-2 at time intervals between 5 and 60 ms.[16,17] The integrated volumes beneath the peaks of the exchanging protons in these experiments were fit to yield hydrogen exchange rates.

Results

Studies were performed with CP(H24C), a peptide that binds zinc(II) and cobalt(II) within a three-cysteine, one-histidine site. The cysteine in position 24 was added since this cysteine is expected to be more solvent accessible that the two cysteines within the Cys−X−X−Cys loop as shown in Figure 1. Methylation of the cysteine residues within this peptide will yield peptides with *S*-methylcysteine in place of cysteine. Such peptides were directly synthesized and characterized. A peptide with methionine in position 24 was also synthesized for comparison. Substitution of *S*-methylcysteine in place of cysteine at positions 4 or 7 resulted in peptides which do not show absorption spectra characteristic of tetrahedral cobalt(II) complexes, even in the presence of large excesses of cobalt(II).

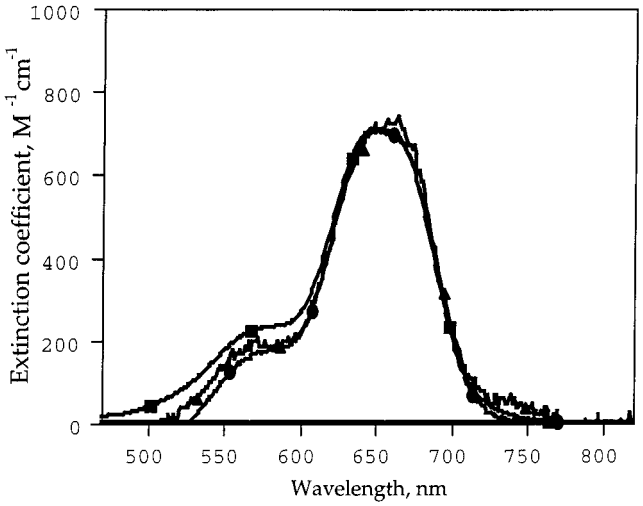

Figure 2. The absorption spectra of the cobalt(II) complexes of CP(H24X) (X = *S*-methylcysteine) (squares), CP(H24M) (circles), and CP-C4 (triangles). These spectra were collected in the presence of saturating excesses of cobalt(II).

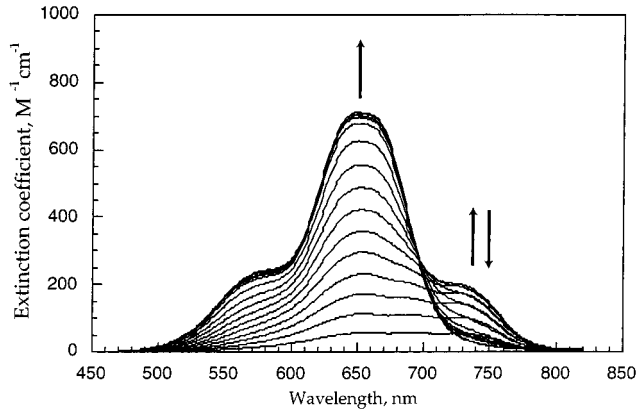

Figure 3. A titration of CP(H24X) (X = *S*-methylcysteine) (510 μM) with cobalt(II).

These observations suggest that methylation of either of these residues dramatically affects the metal binding properties of the consensus zinc finger peptide. Amino acid substitutions in position 24 within the consensus peptide sequence have been shown previously to have significant effects on the positions of the absorption bands in the visible region for complexes of these peptides with cobalt(II) while still retaining spectra characteristic of tetrahedral complexes.[11,12] The spectra in this region for the peptides CP(H24M) and CP(H24X) (X = *S*-methylcysteine) in the presence of excess cobalt(II) are essentially identical to one another and to that from a previously characterized peptide that completely lacks a fourth peptide-derived ligand[12] as shown in Figure 2. The similarity of these spectra suggests that the thioether ligands may not be bound to the cobalt(II). Instead, water likely serves as the fourth ligand in these complexes.

Metal Binding by Thioether-Containing Peptides. To more fully characterize the metal binding properties of the peptides that include thioethers as potential ligands, the CP(H24X) (X = *S*-methylcysteine) and CP(H24M) peptides were titrated with $CoCl_2$ (Figure 3). The shape of the absorption envelopes in the visible region changed during these titrations as observed earlier for peptides, including the truncated peptide CP-C4.[12] This behavior has been shown to be due to the formation of two peptide to one cobalt(II) complexes at the initial stages of the titration when the peptide-to-cobalt(II) ratio is high. To more

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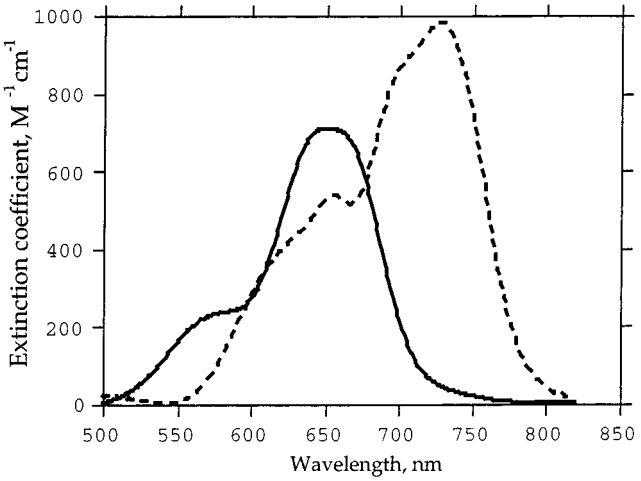

Figure 4. Spectra for the one-to-one (solid) and two-to-one peptide (dashed) to cobalt complexes for CP(H24X) (X = *S*-methylcysteine) derived from factor analysis.

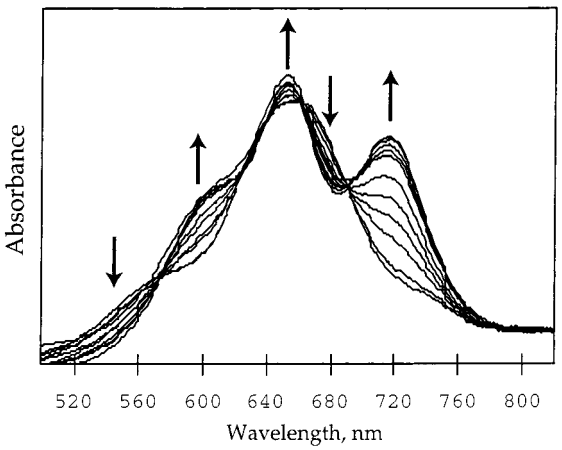

Figure 5. The results of titration of the cobalt complex of CP(H24X) (X = *S*-methylcysteine) (155 μM) with β-mercaptoethanol.

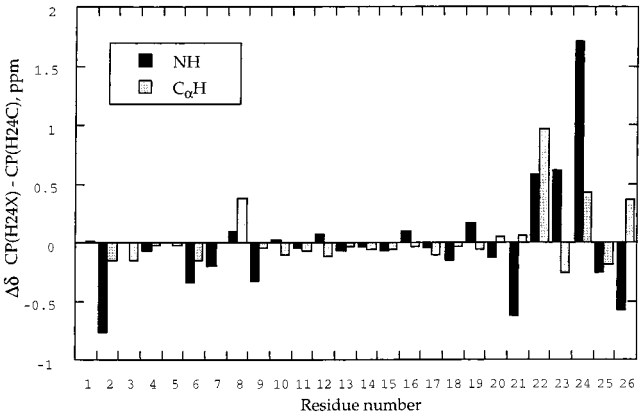

Figure 6. A comparison of the NH and C_αH proton chemical shifts for the zinc complexes of CP(H24C) and CP(H24X) (X = *S*-methylcysteine).

precisely determine the equilibrium constants for the two-to-one and one-to-one peptide-to-cobalt complexes, titrations were performed at four different peptide concentrations. The data from these titrations were analyzed using factor analysis and could be fully described with use of two basis vectors, indicating that there are at least two optically active species in these titrations. Fitting these data yielded $K_{d1} = (2.6 \pm 1.4) \times 10^{-7}$ M and $K_{d2} = (6.0 \pm 1.4) \times 10^{-11}$ M^2. These fall within the range of the values previously determined for the truncated peptide.[12] The

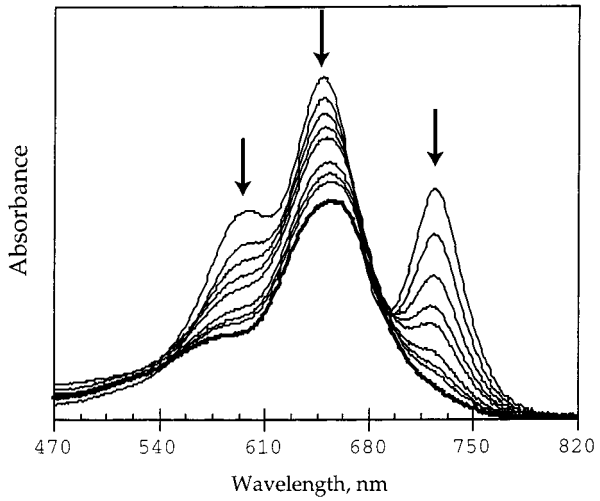

Figure 7. The results of treatment of the cobalt complex of CP(H24C) with 40 equiv of dimethyl sulfate followed over the course of 60 min.

dissociation constant for the one-to-one complex is larger than that for the complexes of CP-1 and CP(H24C),[18] 6.3×10^{-8} M, indicating weaker cobalt(II) binding by the *S*-methylcysteine containing peptide. The deconvoluted optical spectra of the individual cobalt binding species are consistent with the two-to-one peptide-to-cobalt(II) involving four cysteinate ligands (two from each peptide) and the one-to-one complex involving a two cysteinate, one histidine, one H_2O binding site (Figure 4).

Binding of Exogenous Ligands. One of the differences between the parent zinc finger peptide and the truncated peptide (CP-C4) is the ability of exogenous imidazole or thiolate to interact directly with the cobalt(II). Previous studies have revealed that exogenous imidazole or thiolate ligands can bind to the cobalt(II) within the complex of the truncated peptide, presumably displacing bound water.[12] The dissociation constants for complexes with *N*-methylimidazole and β-mercaptoethanol are 2×10^{-4} M and 8×10^{-4} M, respectively, at pH 8.0. Titrations of the cobalt(II) complex of CP(H24X) (X = *S*-methylcysteine) with β-mercaptoethanol were performed to determine how effectively exogenous thiolate ligands can compete with the potential thioether residue. Upon addition of β-mercaptoethanol, spectral changes were observed consistent with thiolate binding (Figure 5). These spectra changes could be fit to yield a dissociation constant of 2×10^{-4} M for the thiolate complex, identical to the value obtained for the truncated peptide.[12] For comparison, the cobalt(II) complex of CP(H24C) was titrated with *N*-methylimidazole. No appreciable spectral changes were observed.

NMR Studies of the Zinc Complex of CP(H24X) (X = *S*-Methylcysteine). As a final probe of the ability of *S*-methylcysteine to act as a ligand, the zinc complex of the *S*-methylcysteine-containing peptide was studied by NMR methods. Full assignments of the amide and side chain protons were made for each amino acid residue although no peak due to the methyl group in *S*-methylcysteine could be unambiguously located. Proton chemical shifts of this complex were found to differ most from those for the CP(H24C) complex for residues 22 to 26 (Figure 6). This indicates that the substitution of *S*-methylcysteine disrupts the structure only in the region surrounding the site of methylation. Hydrogen exchange studies revealed that the NH group of the *S*-methylcysteine residue

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exchanges with a rate constant of 5.7 s^{-1}, which is relatively rapid when compared with a rate constant of 0.5 s^{-1} for the exchange rate of the NH group of histidine in the parent peptide complex. For comparison, exposed NH groups show exchange rates near 50 s^{-1} under these conditions.[17]

Alkylation of Zinc Finger Peptides. To explore the susceptibility of metal bound cysteinates to alkylation in aqueous solution, the cobalt complexes of CP(H24C) and CP-1 were treated with dimethyl sulfate (DMS). Addition of 40−60 equiv of DMS to the cobalt complex of CP(H24C) in borate buffer resulted in changes in the absorption spectrum leading to a final spectrum resembling that of the complex of CP(H24X) (X = *S*-methylcysteine) over 45−60 min (Figure 7). The overall intensity of the absorption spectrum also decreased somewhat over this period. The cobalt complex of the parent CP-1 peptide was also treated with DMS under the same conditions. A similar decrease in the intensity of the spectrum was observed, but no change in the shape of the spectrum occurred (data not shown). For comparison, the CP(H24C) peptide without added cobalt was treated with DMS. Cobalt was added to this reaction mixture after 60 min. No spectral features indicating of the formation of tetrahedral complexes were observed. Attempts to analyze the products of these alkylation reactions by HPLC were thwarted by the presence of four lysine residues in each peptide which are presumably methylated by DMS treatment leading to the formation of many products differing by the mass of a methyl group. This precluded more detailed kinetic analysis.

Discussion and Conclusions

Three conclusions emerge from these studies. First, cysteinate residues within these peptide complexes can be methylated by an appropriate methyl donor in aqueous solution. This is demonstrated by the formation of cobalt complexes which have absorption spectral features that are essentially identical with those of complexes of *S*-methylcysteine-containing peptides prepared by direct synthesis. Interestingly, methylation of the peptide in the absence of metal ions results in complete methylation of the cysteine residues as indicated by the complete loss of detectable metal binding activity upon treatment of the metal free peptide with DMS for 1 h; methylation of the cysteine residues in the cobalt complexes appears to occur more slowly. These peptides do not adopt a well-defined structure in the absence of bound metal ion. The effect of burying cysteine residues during peptide folding appears to overcome the increased nucleophilicity expected due to the deprotonation of the cysteine residues that occurs upon metal binding.

Second, *S*-methylcysteine is a much worse ligand for zinc and cobalt than is cysteine. Properties of the *S*-methylcysteine-containing peptide complexed with cobalt are consistent with this side chain either not binding to the metal ion at all or rapidly exchanging with water. Several lines of evidence support this conclusion. First, the cobalt binding behavior of this peptide closely resembles, both qualitatively and quantitatively, that of a previously characterized peptide that entirely lacks a fourth peptide-derived ligand. In both cases, two-to-one peptide-to-metal complexes form under conditions of high peptide-to-metal ratios and equilibrium constants for the formation of these complexes are essentially equivalent for the *S*-methylcysteine-containing and truncated peptides. Furthermore, if the *S*-methylcysteine is bound to the cobalt, it can be readily displaced by exogenous ligands such as *N*-methylimidazole and the thiolate form of β-mercaptoethanol. Again, the behavior is quite similar to that for the truncated peptide and such displacement reactions do not occur for the complexes of peptides with cysteine or histidine in this position. Finally, direct NMR studies are consistent with the disruption of the last turn of helix that includes the carboxyl terminal metal binding residue upon methylation of cysteine. The NMR properties of the zinc complex of this peptide suggest that this region of the *S*-methylcysteine-containing peptide is either not involved in metal binding and is disordered or is participating in an exchange process between metal-bound and -unbound forms. These results make the observation that the cysteine residue in the Ada protein remains metal bound upon methylation even more surprising. The observation that the Ada protein precipitates rapidly upon methylation in the absence of DNA suggests that the *S*-methylcysteine residue in this protein may be a poor ligand but remains bound to the metal due to structural effects associated with DNA-complex formation.[8,9]

Third, different cysteine residues within the complex show different behavior with regard to both their rates of alkylation and the effects of methylation upon the metal binding properties of the peptide. Comparison of the rates of changes in UV−visible spectral shape and spectral intensity upon treatment of the cobalt complex of CP(H24C) with DMS indicates that the carboxyl terminal cysteine is at least five times more susceptible to attack by an DMS than are the other two cysteines. As noted above, methylation of this cysteine residue does not result in the complete loss of structure within the peptide complex. In contrast, methylation of either of the other two cysteine residues led to the loss of the ability of the peptide to form tetrahedral complexes with cobalt. These differences in reactivity and in structural importance are consistent with known structural properties of zinc finger domains. Cysteines within the Cys−X−X−Cys loop are rather buried and are involved in characteristic NH-to-sulfur hydrogen bonds. The cysteine residue substituted at position 24 is more accessible to reagents in solution. Additionally, this location has more space available to accommodate structural changes as indicated by the lack of substantial differences in metal binding properties following substitution of histidine or cysteine at this position. Finally, treatment of the peptide with DMS in the absence of bound metal ion for 1 h resulted in the loss of detectable cobalt binding activity, suggesting that methylation of the cysteines may be faster for the free peptide than for the metal-bound forms.

Characterization of the differences in reactivity between metal binding cysteine residues in different contexts may be useful in the design and evaluation of antiviral drugs. Recently, compounds that inactivate retroviral nucleocapsid proteins by reacting with metal-binding cysteine residues have been described.[19] The relative sensitivity of particular cysteine residues within these zinc binding domains may be crucial in providing a therapeutic role for these agents.

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