Interaction of Lysine Residues with the Metal Thiolate Clusters in Metallothionein†

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ABSTRACT: Metallothioneins are unique diamagnetic metal thiolate cluster proteins. Both vertebrate and invertebrate forms contain, besides their large cysteine content (30%), up to 14% lysine plus arginine. In the amino acid sequences, the basic residues are juxtaposed to cysteine residues and have been suggested to play a role in neutralizing the excess negative charge of the metal thiolate complexes [Kojima, Y., Berger, C., Vallee, B. L., & Kägi, J. H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3413–3417]. To document such a function, we compared the susceptibility of the lysine residues in cadmium and zinc metallothioneins and in the metal-free *S*-carboxamidomethyl derivative toward arylation by trinitrobenzenesulfonic acid. The results show an at least 20-fold lower initial rate of reaction with the metal-containing as opposed to that with the metal-free form, indicating a protective effect of metal complex formation on the lysine residues, the degree of protection being dependent on the nature of the metal. The modification of the lysine residues by trinitrobenzenesulfonic acid produces changes in the CD spectral features of the cadmium thiolate cluster structure. The lowered chemical reactivity of the lysine residues in the metal-containing form correlates with an upward displacement of their average pK_a's to 10.9 from 10.3 in the metal-free *S*-carboxamidomethyl derivative. We attribute these effects at least in part to hydrogen bonding of the positively charged ϵ-amino groups to the thiolate ligands of the negatively charged cadmium thiolate units.

Among the large class of extensively studied proteins, metallothionein (MT)[1] stands out as a rare example of a small, ubiquitous, and cysteine-rich protein of ambiguous function (Kägi & Nordberg, 1979). In spite of its presumable role in metal detoxification and its inducibility by various chemical agents, especially bivalent metal ions, no specific biological function has as yet been assigned to it. The quest for a function, unique or otherwise, has led however to a detailed examination of its structure and has yielded a wealth of information (Kägi et al., 1984; Winge & Nielsen, 1984; Hunt et al., 1984).

All mammalian MTs contain a single polypeptide chain with 20 cysteine residues ligated to a maximum of 7 bivalent metal ions. All seven metal ions participate in oligonuclear cluster formation (Otvos & Armitage, 1980), with an approximately tetrahedral geometry around each metal ion (Vašák & Kägi, 1981). While a multidirectional approach has yielded important insights into many aspects of the cluster structure of MT, little is understood about certain other essential structural features. One typical structural feature that originated this study is the curious juxtaposition in the sequence of cysteine residues and basic amino acids, particularly lysine residues, yielding a total of seven K-C or C-K units along the chain (Kägi et al., 1984). Attempts to understand this particular structural aspect have not been readily forthcoming. Recently, on the basis of chemical modification studies with cross-linking reagents, Templeton & Cherian (1984) concluded that the lysine residues do not play a major structural role in MT. In an earlier report, however, Kojima et al. (1976) were the first to envision a charge neutralizing role for the basic amino acids in MT. Recent ^{1}H NMR evidence from this laboratory (Vašák et al., 1985) is suggestive of an electrostatic interaction between the protonated lysine residues and the negatively charged metal thiolate clusters in the protein. Independent potentiometric measurements (Gilg, 1986) of the metal-free *S*-carboxamidomethyl derivative and the metal-containing protein lend further support to this postulate. In this report, we provide additional evidence that the juxtaposition of the lysine and cysteine residues in the polypeptide chain of MT is not merely coincidental but is an essential charge-balancing and structural element of the metal thiolate clusters in this protein.

Materials and Methods

Rabbit liver MT induced with either cadmium or zinc ions was extracted by the method of Kimura et al. (1979) and further purified into MT-1 and -2 by a modification of the method of Bühler & Kägi (1974). Native Zn_7-MT-1, as well as reconstituted Zn_7- and Cd_7-MT-1, was employed in this study. The in vitro reconstitution of apoMT-1 with zinc or cadmium ions to yield Cd_7-MT-1 or Zn_7-MT-1 was carried out as detailed earlier (Vašák & Kägi, 1983). TNBS was obtained from Fluka as the dihydrate and stored tightly closed in the dark at −20 °C. Prior to each experiment, the ϵ_{340} was determined in 5 mM borate buffer, pH 9.3. In several determinations over a period of months, the values of ϵ_{340} were close to the literature value of 600 L mol^{-1} cm^{-1} for the recrystallized material (Fields, 1971), and hence, no purification of TNBS was attempted. A fresh solution of the required molarity was prepared prior to each experiment in 5 mM borate buffer, pH 9.3. In experiments requiring a large molar excess of TNBS, the pH of the freshly prepared solution in borate buffer had to be readjusted to 9.3 with NaOH just prior

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[1] Abbreviations: MT, metallothionein; Cd_7-MT-1 and Zn_7-MT-1, metallothionein isoform 1 reconstituted with cadmium and zinc ions, respectively, and containing 7 mol/mol of either metal ion; CA-apoMT, carboxamidomethylated metal-free metallothionein; NMR, nuclear magnetic resonance; CD, circular dichroism; TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenyl; C, cysteine residue; K, lysine residue; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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to addition to the protein solution. On prolonged storage at room temperature, samples of TNBS developed a deep yellow color. These samples could not be crystallized according to the procedure of Fields (1971) and did not react reproducibly with amino groups. N^{α}-Acetyl-L-lysine and *N*-acetyl-L-cysteine were products of Serva. All other chemicals were reagent grade. For preparation of the apoprotein, quartz-distilled water and acid-washed glassware were used in order to minimize adventitious reconstitution by metal ions. The reaction of TNBS with protein was carried out by incubating the protein solution shielded from light at pH 9.3 with a required aliquot of TNBS solution at 50 °C for 6 min, followed by rapid quenching to 0 °C (Finkelstein et al., 1981). In experiments where the reaction was spectrally monitored, the contribution of the blank absorbance due to the reagent was constantly subtracted. The quenched reaction mixture was subjected to gel filtration over Sephadex G-25 equilibrated with 5 mM borate buffer at pH 9.3, and then to amino acid analysis as described below. The metal contents of the native and modified proteins were determined by atomic absorption spectroscopy on an Instrumentation Laboratory Model IL 157 atomic absorption spectrometer, and the extent of lysine modification was determined by amino acid analysis on a Durrum D-500 amino acid analyzer. The model compounds N^{α}-acetyllysine and *N*-acetylcysteine were modified with a 1:1 (mol/mol) ratio of TNBS under conditions identical with those for the protein. The products were subjected to performic acid oxidation and acid hydrolysis prior to amino acid analysis. The ϵ-TNP derivative of N^{α}-acetyllysine appeared in the region where glycine and alanine would elute in a normal protein hydrolysate. This overlap renders its quantitation difficult, and hence, the extent of modification in MT was estimated from the disappearance of unmodified lysine alone. Glycine and alanine could not be used as reference amino acids in MT due to the overlap from ϵ-TNP-lysine. Therefore, all amino acids have been calculated with reference to aspartic acid and proline. Under the experimental conditions, *S*-TNP-cysteine did not appear on the chromatogram.

The cysteine content of the native and modified holoproteins was determined by amino acid analysis as cysteic acid following performic acid oxidation (Hirs, 1956) and acid hydrolysis with 6 N HCl at 110 °C for 22 h. In the case of apoprotein, the cysteine content was estimated as the *S*-carboxymethyl derivative following *S*-carboxamidomethylation of the protein and acid hydrolysis. This was done essentially by the procedure of Jørnvall (1970) with slight modifications. Ten milligrams of MT-1 was dissolved in 1 mL of a thoroughly degassed buffer containing 6 M guanidine hydrochloride, 5 mM EDTA, and 0.5 M Tris, pH 8.6. A 40% molar excess (over protein SH groups) of dithioerythritol was then added and the mixture incubated at 37 °C for 2 h under nitrogen. Due to the predominance of thiol groups in this protein, the maintenance of an inert atmosphere throughout is essential. A 2–4-fold molar excess of iodoacetamide was added and the reaction mixture stirred at room temperature for 4 h, followed by gel filtration over Sephadex G-25 equilibrated with 0.01 M NH_4HCO_3, pH 8.6, to remove excess reagent. The nonprotein fractions were monitored for metal content, and the extent of carboxamidomethylation was determined by amino acid analysis. In general, the cysteine residues of the apoprotein were found to be better than 98% protected by this procedure.

All absorption spectra were recorded on a thermostated Perkin-Elmer Model 340 spectrophotometer, with 1.0-cm quartz cells. Protein concentrations were determined by using a molar absorption coefficient ϵ_{220} = 47 300 for the apoprotein at pH 1.7 (Bühler & Kägi, 1979). CD spectra were obtained on a JASCO Model J-500 spectropolarimeter at ambient temperature, and the molar ellipticity, $[\theta]$, was reported in terms of protein concentration as degrees centimeter squared per decimole.

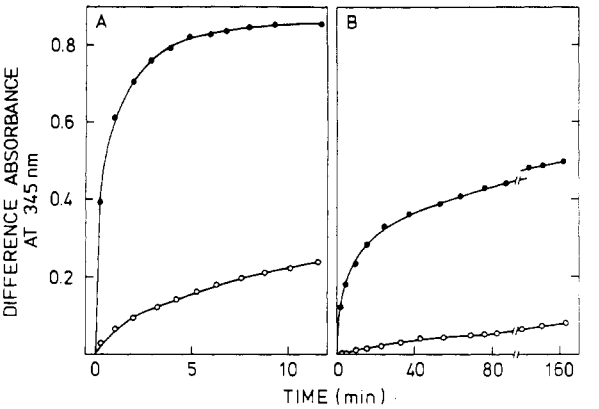

FIGURE 1: Time course of the reaction of carboxamidomethylated apoMT (●) and Cd_7-MT (○) at 50 (A) and 10 °C (B) with TNBS. The reaction is monitored by the change in the difference absorbance at 345 nm. Protein concentrations: [apoMT] = 1.5×10^{-5} M (50 °C) and 1.3×10^{-5} M (10 °C), [Cd_7-MT] = 0.9×10^{-5} M (50 °C) and 1.6×10^{-5} M (10 °C).

Table I: Effect of TNBS on Lysine and Cysteine Residues of Cd_7-MT and CA-apoMT[a]

protein	amino acid	reaction time (min) 0	6[b]	12[b]	180[c]
Cd_7-MT	TNP-lysine	0.0	2.6 (1.7)	3.3 (2.4)	3.0 (0.4)
CA-apoMT	TNP-lysine	0.0	5.6 (4.7)	5.5 (4.8)	4.6 (3.1)
Cd_7-MT	cysteine[d]	20.0	19.6	15.2	14.8
CA-apoMT	*S*-(carboxamidomethyl)cysteine[e]	20.0	15.5	8.0	10.0

[a] TNP-lysine was estimated from the reduction in lysine content as measured by amino acid analysis, i.e., 7 (native control) minus residual lysine content (see Materials and Methods for details of sample preparation). The values in parentheses represent spectrophotometric estimates obtained directly in the reaction mixtures from the difference absorbance at 345 nm (Figure 1) on the basis of $\Delta\epsilon_{345} = 1.2 \times 10^4$ M^{-1} cm^{-1} (Goldfarb, 1966a). [b] Reaction carried out at 50 °C. [c] Reaction carried out at 10 °C. [d] Measured as cysteic acid following performic acid oxidation. [e] Measured as *S*-(carboxymethyl)cysteine.

RESULTS

Figure 1 compares the reaction of Cd_7-MT and CA-apoMT with a 9-fold molar excess of TNBS over lysine residues as a function of time, at temperatures of 50 (A) and 10 °C (B). With Cd_7-MT, there is only a relatively slow generation of difference absorption, ΔA_{345}, signaling TNP-lysine formation (Goldfarb, 1966a). In contrast, the CA-apoMT displays a rapid development of ΔA_{345}, with initial rates that are about 20- and 60-fold higher than those for the metal-containing form at 50 and 10 °C, respectively. There are also large differences in the extent of modification attained within the duration of the experiment.

Estimates by amino acid analysis (see Materials and Methods) of the number of lysine residues modified at different times of reaction are shown in Table I. They indicate that in CA-apoMT nearly six out of a total of seven lysine residues have reacted within 6 min at 50 °C and no further change occurs at longer reaction time, whereas in Cd_7-MT less than half as many have reacted at 6 min. Also included in Table I are estimates of the number of the modified lysine residues

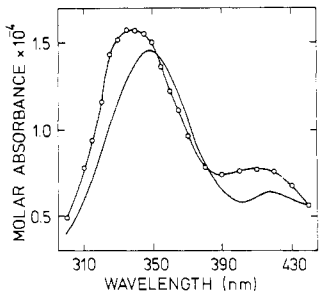

FIGURE 2: Absorption spectra of the TNP derivatives of Cd_7-MT (○) and N^{α}-acetyllysine (—). The spectrum of TNP-Cd_7-MT was obtained in 5 mM borate buffer, pH 9.3, and represents the product with 4.8 lysine residues modified after removal of excess reagent by gel filtration. The molar absorbance is based on the concentration of TNP-lysine residues measured by amino acid analysis. The spectrum of TNP-N^{α}-acetyllysine was adapted from Goldfarb (1966a).

obtained from the difference absorbance increments of Figure 1 and the molar difference absorption coefficent $\Delta\epsilon_{345} = 1.2 \times 10^4$ (Goldfarb, 1966a). The low and varied values obtained indicate that such direct spectral monitoring of the reaction mixture containing, in addition to the arylated protein, excess reagent and the sulfite byproduct does not afford an accurate measure of lysine modification. As shown below, the spectra can, however, yield a reliable estimate of the number of modified lysine residues, when excess reagent and sulfite ions are removed.

Table I also shows the consequence of the reaction of TNBS with Cd_7-MT and CA-apoMT on the respective cysteine and (carboxamidomethyl)cysteine contents determined by amino acid analysis. Until 6-min reaction time at 50 °C, the cysteine content of Cd_7-MT remains unchanged, followed by a time-dependent decrease. In contrast, the CA-apoMT, prepared to protect the cysteine residues from unwanted reactions, shows a substantial loss of the (carboxamidomethyl)cysteine residues even at 6 min.

The reaction product of Cd_7-MT and TNBS when freed from excess reagent and sulfite byproducts by gel filtration displays the spectral features resembling that of TNP-N^{α}-acetyllysine (Figure 2). The representative sample shown has been modified under standard conditions (see Materials and Methods) at a protein concentration of 3.5×10^{-6} M and a 12-fold molar excess of reagent over lysine residues. This sample contained 4.8 modified lysine residues as determined by amino acid analysis. The molar absorption coefficient based on the effective concentration of modified lysine residues has a maximum of 1.58×10^4 at 338 nm. The corresponding absorption spectrum of the model compound TNP-N^{α}-acetyllysine, also shown in Figure 2 in the absence of sulfite ions, has a maximum at 348 nm with a molar absorption coefficient of 1.45×10^4 (Goldfarb, 1966a). A comparable blue shift occurs in the low-energy band from 418 nm in the model compound to 412 nm in the protein derivative. Parallel spectral shifts have been observed in the TNP derivatives of small peptides, when compared with those of simple amino acids (Goldfarb, 1966a). There is a near-constancy of the ratio of the molar absorption coefficient of the two bands; i.e., $\epsilon_{338}/\epsilon_{412} = 2.0$ for TNP-Cd_7-MT and $\epsilon_{348}/\epsilon_{418} = 2.3$ for the model compound.

The marked difference in reactivity of TNBS with metal-containing protein and the metal-free carboxamidomethylated apoprotein is also clearly demonstrated when the concentration of the reagent is varied at constant reaction time (Figure 3). While at a reagent to lysine ratio of 5 the carboxamidomethylated apoprotein attains modification of about five lysine residues, in Cd_7-MT less than two are reacted. This discrimination is minimized at reagent ratios above 15, where in CA-apoMT nearly six and in Cd_7-MT close to five lysines out of a total of seven are arylated. Thus, one to two lysine residues appear to resist modification even at 100-fold reagent ratios and above (data now shown). It is noteworthy that Zn_7-MT and native Cd_5Zn_2-MT (J. Pande, unpublished results) react more readily with TNBS than Cd_7-MT.

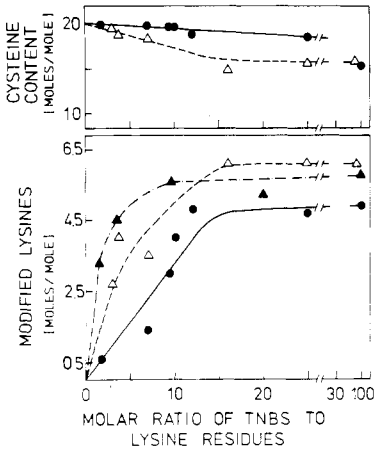

FIGURE 3: Chemical modification of carboxamidomethylated apoMT (▲), Zn_7-MT (△), and Cd_7-MT (●) by TNBS. (Bottom) Modification of lysine residues as a function of increasing TNBS/lysine ratio. The extent of lysine residue modification was determined by amino acid analysis by subtracting the residual lysine content from a total of seven lysines in the unmodified protein. The data points are averages of four determinations. (Top) Effect of modification on cysteine content.

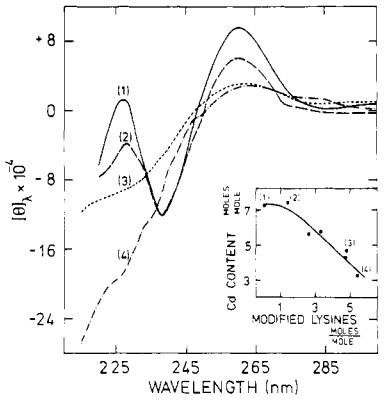

FIGURE 4: CD spectra of Cd_7-MT and its TNP derivatives after gel filtration in 5 mM sodium borate buffer, pH 9.3. Unmodified control (—); dervatives with the following number of residues modified: 1.4 (– –), 4.8 (···), and 5.4 (–·–). The samples showed no significant alteration in cysteine content measured as cysteic acid. The molar ellipticity, $[\theta]$, is based on the protein concentration. Inset: Effect of lysine modification on cadmium ion binding. Numbers 1–4 refer to samples whose CD spectra are shown.

The effect of increasing reagent concentration on the cysteine content of Cd_7-MT and Zn_7-MT is also depicted in Figure 3. Up to reagent to protein ratios of 10, there is virtually no loss of cysteine residues in Cd_7-MT, and even at higher ratios, there is only a moderate progressive loss of this amino acid. The recovery of cysteine residues in modified Zn_7-MT is, in contrast, lowered already at reagent to lysine ratios of about 3 to 5 and drops to about 80% above a ratio of 15. We have observed that increasing reagent ratios also affect the recovery of *S*-(carboxamidomethyl)cysteine from CA-apoMT to about the same degree as in the case of Zn_7-MT (data not shown).

The covalent modification of the lysine residues in Cd_7-MT has pronounced effects on some of its structural features as reflected in the CD spectrum (Figure 4). While the un-

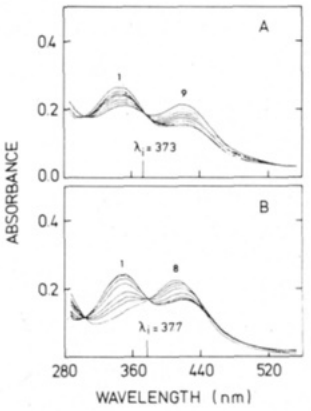

FIGURE 5: Effect of EDTA and *N*-acetylcysteine on the absorption spectrum of TNP-Cd_7-MT with approximately three lysine residues modified. (A) Repetitive scans of TNP-Cd_7-MT (5.2×10^{-6} M) following addition of EDTA (9.6×10^{-4} M) in 5 mM borate buffer, pH 9.3. Scans 1–9 were recorded at varying intervals over a period of 1 h. (B) Spectra of TNP-Cd_7-MT with increasing concentrations of *N*-acetylcysteine in the same buffer. Scans 1–8 were recorded in the presence of 0–16.4 mM mercaptan.

modified control displays the characteristic spectrum of the cadmium thiolate chromophore with ellipticity maxima at 260 and 225 nm and a minimum at 237 nm, even a moderate modification of only about 1.4 lysine residues causes a significant perturbation of this profile. At a higher modification (4.8–5.2 lysine residues modified per mole of protein), the characteristic spectral features are virtually obliterated. As is apparent from the inset, these changes are accompanied by partial loss of cadmium ions from the protein (Figure 4, inset).

Despite its effect on the CD spectral features of the metal thiolate chromophore, moderate modification with TNBS does not measurably alter the stability of the metal complexes. This is shown by a comparative study in which modified Cd-MT and unmodified Cd-MT were exposed to EDTA. Previous measurements on native Cd_7-MT have shown that at neutral pH incubation with EDTA removes preferentially one to two cadmium ions with corresponding changes in the cadmium thiolate absorption profile near 250 nm (J. K. Nicholson, P. J. Sadler, and M. Vašák, unpublished results). A spectral change of comparable magnitude is observed at pH 9.3 in the presence of 1 mM EDTA, both in native Cd_7-MT and in the TNP-lysine-modified derivative. Unexpectedly in the latter, metal ion removal by EDTA is also accompanied by very distinctive time-dependent spectral changes in the region where the TNP chromophore absorbs. This effect of EDTA on a representative sample with 2.6 lysine residues modified is shown in Figure 5A. The spectral changes that manifest themselves following incubation with a 180-fold molar excess of EDTA, over a period of 2 h at room temperature, are characterized by progressive increase in extinction and a blue shift of the shoulder around 420 nm and a simultaneous decrease in extinction and a red shift of the band around 340 nm. These changes are also accompanied by the development of distinct isosbestic points at 373 and 303 nm. All of these changes are reversed by acidification to pH 1.5. Remarkably, when the TNP-lysine derivative was incubated with the same concentration of EDTA at pH 6.9, the spectral changes observed at pH 9.3 did not occur.

It is significant that changes strikingly similar to those elicited in the presence of EDTA at pH 9.3 are also effected by the incremental addition of the mercaptan N^{α}-acetylcysteine to the TNP derivative of Cd_7-MT with a comparable lysine content (3.3 lysine residues modified, Figure 5B). The set of spectra obtained shows the same opposing variation in the 348- and 420-nm bands. However, compared to the isosbestic point occurring with EDTA at about 373 nm, that generated by *N*-acetylcysteine is red shifted slightly to around 377 nm. The second isosbestic point at 303 nm is identical with the set of spectra shown in Figure 5. Entirely comparable spectral variations were also induced in TNP-modified CA-apoMT (not shown) by the addition of incremental amounts of *N*-acetylcysteine. Again, acidification to a pH of about 2 reverted the spectrum to that observed prior to mercaptan addition.

Discussion

The chemical modification of amino acid residues by group-specific reagents has been effective in implicating invariant residues in structural and functional roles in proteins (Glazer, 1976; Lundblad & Noyes, 1984). TNBS, a well-established arylating agent, was selected for modifying the lysine residues in MT due to its intensely colored chromophore which enables spectrophotometric monitoring of the reaction (Goldfarb, 1966a,b; Fields, 1971). Furthermore, the TNP-lysine derivatives are known to be stable to acid hydrolysis and thus amenable to estimation by amino acid analysis (Glazer, 1976). Means et al. (1972) have shown that trinitrophenylation of primary amino groups proceeds optimally just below their pK_a in solutions of low ionic strength. For MT, the most effective reaction pH would therefore lie between 10 and 10.5, as deduced from ^1H NMR and potentiometric titration data in the alkaline region (Vašák et al., 1985; Gilg, 1986). However, since in strongly alkaline solutions the solvent-mediated hydrolysis of reagent competes with the arylation reaction (Means et al., 1972), we carried out the reaction at a lower pH, 9.3, in 5 mM sodium borate buffer essentially by the procedure of Finkelstein et al. (1981). As TNBS is known to react preferentially with free sulfhydryl groups (Fields, 1971), the metal-free apoprotein was not deemed suitable for comparison. It was replaced, therefore, by the *S*-carboxamidomethyl derivative as an appropriate metal-free control.

One of the complications of the TNBS reaction with amino groups is the formation of an adduct with the trinitrophenyl ring by the sulfite ions released as a byproduct of the reaction. This nucleophile adds to the meta position of the TNP ring, yielding a mono- or disulfite adduct with corresponding changes in the difference spectrum (Goldfarb, 1966a; Means et al., 1972). This difficulty has been circumvented by some investigators by monitoring the reaction product in the presence of excess sulfite ions either at 420 nm, the wavelength at which the TNP-lysine sulfite adduct absorbs (Fields, 1971), or at 367 nm, the isosbestic point of the TNP complex of N^{α}-acetyllysine and its monosulfite adduct (Goldfarb, 1966a; Plapp et al., 1971). However, in our hands under these conditions, the estimated number of modified lysine residues was even lower than those measured at the difference absorbance maximum at 345 nm, and we therefore monitored the reaction at this wavelength (Figure 1). The reasons for the still low estimates from the spectrophotometric measurements as compared to those from amino acid analysis (Table I) are not immediately obvious and could stem from a variety of reasons. In our case, the discrepancies are clearly related to the presence of excess reagent and sulfite ions, since after their removal by gel filtration there is reasonable agreement between spectral data and amino acid analysis (Figure 2).

The time course of TNBS modification of MT monitored at 345 nm (Figure 1) dramatically illustrates the relative susceptibility of lysine residues to arylation in the presence and absence of bound cadmium ions. The considerable lowering of the initial rate of the reaction of the holoprotein at both

10 and 50 °C clearly suggests that the presence of the full complement of metal exerts a stabilizing influence over the protonated lysine residues. Another indication for such a stabilizing effect of the metal is the elevated pK_a values of the lysine residues which ranged between 10.6 and 10.9, as measured by the chemical shift pH titration of ϵ-CH_2 lysine resonances by ^1H NMR (Vašák et al., 1985). The same effect was verified by potentiometric measurements which showed for the lysine residues an average pK_a of 10.8 in the metal-containing protein vs. 10.2 in the carboxamidomethylated apoprotein (Gilg, 1986). This increase in the pK_a by about 0.6 unit could be brought about by an electrostatic interaction of the protonated lysine residues with the two 3-fold negatively charged metal thiolate clusters. It would correspond to an average stabilization of nearly 1 kcal/mol, an energy difference leading to a 4-fold lowering of the initial rate. Thus, a part of the protection afforded to the lysine residues in the metal-containing protein seems to be a consequence of the pK_a elevation. Another likely factor contributing to the lowered reactivity is steric or electrostatic shielding of the lysine residues from the reagent. The resistance of the last one to two lysine residues to modification in the time domain examined favors this explanation. Since the same fraction of lysine residues resists modification even in CA-apoMT, it would appear that this protection is not dependent on an interaction of lysine residues with the metal thiolate sites (Figure 3).

The present study attests to the feasibility of lysine modification in MT by TNBS in the presence of a large number of metal-bound thiolate groups. The similarity of the absorption spectra of the TNBS-modified Cd-MT following gel filtration to that of pure N^{α}-acetyllysine (Figure 2) is a good indication that the most likely product of modification is the TNP-lysine derivative. Under these conditions, any sulfite adducts formed on exposure to TNBS (vide supra) are dissociated (Goldfarb, 1966b; Means et al., 1972). The blue shifts in the two major bands in the derivatized protein parallel those observed in the TNP derivatives of di- and tripeptides with respect to those of simple amino acids (Goldfarb, 1966a). In addition, the ratio of the two absorption peaks in the derivatized protein after dissociation of the sulfite adduct ($\epsilon_{338}/\epsilon_{412} = 2$) compares favorably with that in the model compound ($\epsilon_{345}/\epsilon_{418} = 2.3$). These factors taken together document that essentially only the lysine residues have been derivatized by TNBS. This is also supported by the almost complete recovery of cysteine residues by amino acid analysis in the modified holoprotein at short reaction times and low reagent to protein mole ratios.

At reagent concentrations exceeding a 12-fold molar excess with respect to lysine residues, or at incubation times longer than 6 min, the recovery of cysteine residues is also affected (Figure 3). This is not surprising in view of the finite stability of the cadmium thiolate complexes (Vašák & Kägi, 1983) and the known high reactivity of TNBS with uncomplexed cysteine side chains (Fields, 1971). Since *S*-TNP-cysteine derivatives are known to be unstable (Kotaki et al., 1964; Wallenfels & Streffer, 1966; Glazer, 1976), it is not surprising that following performic acid oxidation and acid hydrolysis (see Materials and Methods) no derivative of cysteine was identified in the amino acid chromatogram.

Somewhat more unexpected was the incomplete recovery of *S*-(carboxymethyl)cysteine in the acid hydrolysates of the TNP derivatives of CA-apoMT. The constant loss of four to five (carboxamidomethyl)cysteine residues and its independence on reagent concentration negate the formation of S-TNP derivatives of the *S*-(carboxamidomethyl)cysteine residues. However, it is conceivable that the destruction of these residues is the result of selective removal of the protecting group by hydrolysis (Webb, 1966), followed by secondary reactions (Clark, 1932; Bailey, 1957), or by intramolecular attack by a juxtaposed TNP-lysine group on the thioether. The restriction of the loss of *S*-(carboxamidomethyl)cysteine to about 4 of the 20 cysteine residues suggests that the reaction is conditioned by local steric effects in the polypeptide chain.

The higher reactivity of the lysine residues of Zn_7-MT with TNBS documented in Figure 3 is probably an indirect consequence of the lower stability of the zinc thiolate clusters as compared to the cadmium thiolate clusters (Vašák & Kägi, 1983). As the Zn–S bond is about 10 times weaker than the Cd–S bond (Gurd & Wilcox, 1956), the thiolate groups in Zn_7-MT are more accessible to the electrophilic reagent. As a consequence, the metal may be released and lysine stabilization abolished.

The drastic changes in the CD spectrum upon arylation (Figure 4) imply a structural role for the lysine residues. Even at a low degree of modification (one to two lysine residues per mole, curve 2), where virtually no metal was lost (Figure 4, inset), the ellipticity near 260 nm is reduced by almost 30%. Since the CD profile above 220 nm arises from the various metal thiolate transitions and is related to cluster formation (H. Willner, M. Vašák, and J. H. R. Kägi, unpublished results), this loss of ellipticity signals a change in the native cadmium thiolate cluster structure. Upon more extensive lysine modification, the cadmium thiolate ellipticity is still further reduced, but the interpretation of these changes is complicated by the incident loss of metal and subsequent thiol oxidation (Figure 4, curves 3 and 4, inset). Nonetheless, the sensitivity of the spectropolarimetric features to lysine modification lends support for a close spatial relationship of these residues to the metal thiolate clusters.

Indirect evidence for the proximity of the lysine residues and the metal thiolate centers comes also from the time-dependent spectral changes brought about by EDTA addition (Figure 5A). The incubation of a sample of modified Cd-MT with 1 mM EDTA at pH 9.3 generates spectra with an isosbestic point around 373 nm (Figure 5A). These changes are curiously reminiscent of the addition of sulfite ions to the meta position of the phenyl ring in the model compound TNP-N^{α}-acetyllysine, producing a spectral shift with an isosbestic point at 367 nm (Goldfarb, 1966a). The TNP derivatives of Cd_7-MT prepared in this study being essentially sulfite free (see discussion above), the observed spectral effects must have an alternative origin. A likely logical extrapolation would be the complexation with the TNP ring of an internal protein group of comparable nucleophilicity to sulfite ions. We propose that the intrinsic thiolate residues freed from metal binding due to the complexation of metal ions by EDTA would, by virtue of their proximity, be the most likely candidates for addition to the TNP ring. The reversal of the observed spectral pattern upon acidification [see also Goldfarb (1966a)] and its complete absence when the experiment was conducted at pH 7 reinforce this suggestion. The premise that complexation with the ring is due to an RS^- species is further strengthened by the production of similar spectral effects by the addition of an extrinsic thiol, *N*-acetylcysteine, at pH 9.3 (Figure 5B). The remote possibility that this reagent, like EDTA, may function indirectly as a metal chelating agent is effectively ruled out by the slight difference in isosbestic point and the generation of similar spectra upon addition of *N*-acetylcysteine to the metal-free, TNP-modified CA-apoMT. Thus, the effect of EDTA would be to unanchor some of the cysteine residues

for intramolecular addition to the favorably located aromatic ring of TNP-lysine. The origin of these spectral changes from intermolecular adduct formation is unlikely in view of the rather high dissociation constant for the monoadduct of the analogous sulfite complex (Means et al., 1972; Goldfarb, 1966b). Recent evidence from this laboratory (W. R. Bernhard, M. Vašák, and J. H. R. Kägi, unpublished results) indicates that the binding of cadmium ions to cysteine residues is weakest in the segment of the sequence between amino acids 20 and 30 [see Kägi et al. (1984) for the published sequence of MTs]. This suggests that EDTA would be most effective in preferentially labilizing the metal ions in this region. Since this segment of the peptide backbone is also rich in lysine residues, the formation of the intramolecular complexes described above with the relatively labile cysteine residues is a distinct possibilty.

To summarize, these chemical modification data emphasize that the reactivity of the lysine residues in MT is conditioned by the metal component. Since the position of the basic residues is also largely preserved in the known invertebrate MTs (Kägi et al., 1984), its positively charged side chains are likely to be essential elements of the structure and function of these proteins. One obvious role of these residues suggested earlier was an electrostatic outer sphere stabilization of the highly negatively charged group 12 metal thiolate clusters (Kojima et al., 1976). This stabilization could occur in the form of hydrogen bonds between a sulfur atom of the negatively charged cadmium thiolate units and the positively charged ϵ-amino groups of the lysine residues. An analogous situation has been shown to exist for the ferredoxins (Carter, 1977), where the backbone amide hydrogen is bonded to the cysteine sulfur in the negatively charged iron–sulfur cluster. Hydrogen bonds between charged groups have recently been shown to be particularly stable (Fersht et al., 1985). In MT, this bond would be disrupted through modification of lysine residues by charge neutralization, leading to secondary changes within the cluster. Work is in progress to substantiate this claim.

More dynamic functions for the lysine residues such as gating in metal uptake and transfer to intracellular acceptors can also be envisioned. It also remains to be seen to what extent the integrity of the lysine residues defines the domain structure of these proteins.

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