Reactions of Electrophilic Reagents That Target the Thiolate Groups of Metallothionein Clusters: Preferential Reaction of the α-Domain with 5,5′-Dithio-bis(2-nitrobenzoate) (DTNB) and Aurothiomalate (AuSTm)

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Kinetic studies of the reactions of isolated α- and β-domains of rabbit liver MT-II with DTNB (5,5′-dithio-2,2′-dinitrobenzoic acid) and AuSTm (aurothiomalate, Myochrysine) were carried out in 5 mM Tris·HCl/0.1 M KCl at pH 7.4 and 25 °C. These results demonstrate that the kinetics of the DTNB reaction with the β-domain are monophasic, with observed rates similar to those of the slow step of the reaction of the holo-protein, Cd_7MT, which confirms previous findings that the α-domain is the site of the kinetically fast step. DTNB concentration dependence studies resulted in the following rate law, rate $= \{k_{1s} + k_{2s}[\text{DTNB}]\}[\text{MT}]$, that corresponds to two of the four terms in the holoprotein rate law, those of the slow step. The reaction of aurothiomalate with the β-domain is independent of the AuSTm concentration and described by a rate function with a single rate constant, rate $= k_{1s}[\text{MT}]$. The α-domain reaction with AuSTm, also AuSTm-concentration independent, involves slow and fast phases with rate $= \{k_{1s} + k_{1f}\}[\text{MT}]$. The latter dominates 87 ± 3% of the reaction. Comparison of these results with previous studies of other electrophiles demonstrate that the kinetically preferred reaction of MT with an electrophile may be localized in either the α- or the β-domain, depending on the specific attacking reagent.

Introduction

Metallothioneins (MT) are proteins found in many cells and tissues of humans, mammals and invertebrates.[1,2] Class I MTs are rich in cysteine residues (18−20 Cys among approximately 60 amino acids), all of which are in reduced form, conferring the ability to bind 7 divalent metal ions, such as Cd^{2+} and/or Zn^{2+}, per mole. The metal-free form (apo-MT) is a structureless random coil that upon addition of 7 Cd^{2+} or Zn^{2+} ions, folds into two domains generating two metal−thiolate clusters.[3−5] The β-cluster in the N-terminal domain includes 9 cysteine residues and binds 3 Cd^{2+} or Zn^{2+} ions; the α-cluster in the C-terminal domain forms with 11 cysteines and 4 Cd^{2+}or Zn^{2+} ions.[3−5]

MT is induced in response to a large variety of factors such as high metal levels, hormones and second messengers, growth factors, inflammatory agents and cytokines, tumor promoters, cytotoxic agents, and stress-producing conditions.[1,6] In addition to the well-studied role of MT in the detoxification of heavy metals[7] such as Hg^{2+} or Cd^{2+}, other putative roles include the regulation of zinc and copper metabolism, the donation of zinc to newly synthesized apoenzymes,[8,9] radical scavenging,[10,11] participation in the stress response[7,12] and detoxifying reactions with alkylating agents such as chlorambucil, $CCl_3^{\bullet}$ and metallodrugs such as *cis*-platinum and chrysotherapeutic gold complexes.[7,12−15] Studies of the structures and reactions of MT and model compounds by inorganic chemists have addressed metal binding, metal exchange, ligand substitution, and reactions targeting the sulfhydryl groups of MT have been described as a basis for considering the possible biological roles of this protein.[1,16,17a−t]

The study of Bernhard, Kägi and Vašák, which employed 20 min pulse reactions with [^{14}C]iodoacetamide to modify the thiolate groups of the cysteine residues, showed that β-domain of Cd_7MT reacts more rapidly than the α-domain with this reagent.[18] These results correlated well with the thermodynamic

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preference of the α-cluster for Cd^{2+} [19] and the more rapid movement of Cd^{2+} among sites of the *β*-domain than of the α-domain.[20] Together, they led to the commonly accepted assumption that the *β*-domain is generally the more labile of the two clusters in the various reactions of MT.[18]

The first biphasic reaction of MT, that with 5,5′-dithio-2,2′-dinitrobenzoate, DTNB, was reported in 1981.[21] Both, the fast and slow steps consist of DTNB-dependent and -independent components, giving rise to a four-term rate law (eq 1b):

$$M_7MT + 10\ DTNB \rightarrow (SS)_{10}MT + 20TNB + 7M^{2+} \quad (1a)$$

$$Rate = \{k_{2f}[DTNB] + k_{1f} + k_{2s}[DTNB] + k_{1s}\}[MT] \quad (1b)$$

where "f" and "s" denote fast and slow, respectively, and, "1" and "2" designate first-order and second-order, respectively.

The oxidized MT, $(SS)_{10}MT$, containing principally monomers with intraprotein disulfide bonds and relatively fewer mixed disulfide bonds to TNB and interprotein disulfide bonds was described previously.[22] The DTNB reaction with the isolated α-domain was studied to explore the presumption that the biphasic character of the reaction of MT with DTNB arises from differential reactivities with the two domain structures rather than differences in reactivity among various terminal and bridging thiolates.[23] The finding of a monophasic reaction with a rate law having first- and second-order terms,

$$Rate_\alpha = \{k_{2f}[DTNB] + k_{1f}\}[MT] \quad (2)$$

confirmed the linkage of the two reaction phases with the two domains.[23] Totally unexpected was the fact that the α-cluster reacted with the kinetics comparable to that of the fast step, not the slow step, since it contradicted prevailing expectations about the relative reactivity of the two clusters.[2,18] A similar conclusion about the relative reactivity of the clusters ($\alpha > \beta$) was drawn from the observation of the preferential alkylation of Cys33 and Cys48 when reacting with the electrophilic agents Melphalan[24] and chlorambucil.[25] Both findings are consistent with the crystallographic results that the α-cluster has more surface-accessible thiolate sulfur atoms than the *β*-cluster.[5]

Aurothiomalate, AuSTm, is a clinically used, gold-based antiarthritic agent that reacts with liver and kidney MT in animal studies and in vitro.[26−28] It, too, shows a biphasic reaction with Zn_7MT and Cd_7MT:

$$Rate = \{k_{1f} + k_{1s}\}[MT] \quad (3)$$

Under the conditions of the kinetic studies, the product is (TmSAuS)∼20MT, characterized by retention of radiolabeled thiomalate, gold-coordination number of two, Au−S bond distances of 230 pm, expanded molar volume and total displacement of Zn^{2+} or Cd^{2+}.[26−28] The kinetics of AuSTm and MT have been extensively reviewed elsewhere.[13,29] One can hypothesize that the fast and slow phases are associated with independent reactions of the two domains, whether in the sense of DTNB (α more reactive than *β*) or iodoacetamide (*β* more reactive than α). To explore further the differential reactivity of the metal clusters of MT, we carried out kinetic studies of reaction of the *β*-domain with DTNB and the α- and *β*-domains with AuSTm.

The kinetics of the AuSTm reactions can be studied using the chromophoric probe method developed for examination of MT reactions that are otherwise spectroscopically opaque. A chromophoric dye which acts as a reporter of the reaction has the properties that it (1) reacts with the Cd^{2+} or Zn^{2+} ions released from MT and (2) is kinetically or thermodynamically incompetent to independently remove these ions.[27,29] The appearance of the metal−ligand chromophore is monitored at a visible wavelength beyond the peptide backbone and metal−thiolate absorbances. For example, using PAR as a chromophoric ligand for Zn^{2+} or Cd^{2+}, the overall reaction can be described as follows:

$$AuSTm + M_7MT \xrightarrow{slow} (TmSAuS)_{20}MT + 7M^{2+} \quad (4a)$$

$$M^{2+} + n\text{PAR} \xrightarrow{fast} M(PAR)_n \quad (4b)$$

Experimental Procedures

Materials. Subtilisin (peptidase type VIII), Trizma base, and Sephadex G50, G25, A25 DEAE were obtained from Sigma Biochemicals (St. Louis, MO). Tetrakis(acetonitrile)copper(I) hexafluorophosphate, PAR, DTNB, and AuSTm from Aldrich Chemical Co. Rabbit liver Zn_7MT was isolated and purified as previously described.[1,6]

Isolation of $Zn_4\alpha$ and $Cd_4\alpha$ Domains. The $Zn_4\alpha$ and $Cd_4\alpha$ domains were prepared from rabbit liver Zn_7MT according to procedures previously described.[30] $Cd_4\alpha$ and $Zn_4\alpha$ were obtained by limited

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proteolysis with subtilisin at pH 7.5 of apo-MT that was partially reconstituted with 4.5 equiv of either Cd^{2+} or Zn^{2+}.[31]

Isolation of $Zn_3\beta$ and $Cd_3\beta$ Domains. $Zn_3\beta$ and $Cd_3\beta$ domains were isolated from rabbit liver MT-II by using slight modifications of the method previously described.[32] The apo-MT obtained by acidification of Zn_7MT was reconstituted with 6 equiv of Cu(I) at pH 7 under anaerobic atmosphere and in the presence of 2-mercaptoethanol.[31] Then, after limited proteolysis with subtilisin was performed, the copper was removed from the β-domain by gel-filtration chromatography at pH < 1.[32] The apo-β domain obtained was then reconstituted with 3 equiv of either Cd^{2+} or Zn^{2+}.

Kinetic Studies with DTNB. The solutions of $Cd_3\beta$ and DTNB were mixed at time zero in UV cuvettes. Immediately, the absorbance change at 412 nm (λ_{max} of 5-thio-2-nitrobenzoate, a product of the reaction) was measured over time in a Perkin-Elmer Lambda 6 spectrophotometer, against a blank containing the same concentration of all reagents, excluding the protein. All reactions were carried out under pseudo-first-order conditions for DTNB in 5 mM Tris-HCl/0.1 M KCl (pH 7.4) at 25 °C.

DTNB concentrations ranged from 1 to 8 mM, and $Cd_3\beta$ was kept constant at 2.9 μM protein, measured as Cd^{2+}. The values for k_1 and k_2 were calculated for each of the two steps observed, according to standard mathematical algorithms,[33] by plotting $\ln(A_\infty - A_t)$ vs time.

Kinetic Studies with AuSTm. The reactions were monitored spectrophotometrically by using the method developed by Shaw et al.,[26,27] in which the metallochromic agent PAR ((pyridylazo)resorcinol) is used to verify the Zn^{2+} displacement form MT. The absorbance change at 485 nm due to the $Zn(PAR)_2$ complex formed upon Zn^{2+} displacement from MT by AuSTm ($\epsilon = 3200\ M^{-1}\ cm^{-1}$) was measured over time in a Perkin-Elmer Lambda 6 spectrophotometer. Each sample was analyzed against a blank containing the same concentration of all reagents, excluding the protein. All reactions were carried out under pseudo-first-order conditions for AuSTm in 5 mM Tris-HCl/0.1 M KCl (pH 7.4) at 25 °C.

AuSTm concentrations ranged from 25 to 500 μM, Zn-domain concentration was kept constant at 5 μM Zn^{2+}, and PAR at 500 μM. The values for k_1 and k_2 were calculated for each of the two steps observed, according to standard mathematical algorithms,[33] by plotting $\ln(A_\infty - A_t)$ vs time.

Results

The α- and β-domains of MT were isolated as $Cd_4\alpha$ and $Cu_3\beta$ or $Cu_6\beta$ from rabbit liver Zn_7MT according to the procedures described in the literature.[34,35] They were characterized by UV−vis spectroscopy, gel filtration, chromatography, and their SH/Cd ratios. The desired metal loadings were effected by reducing the pH to 2.0 and 0.5 for $Cd_4\alpha$ and $Cu_3\beta$ or $Cu_6\beta$, respectively, chromatographically purified the apo-domains and reconstituted with the desired metal ions.

DTNB Kinetic Studies. When the isolated β-domain ($Cd_3\beta$) reacts with excess of DTNB under pseudo-first-order conditions, the reactions go to completion after 180 min for the highest concentrations, yielding 9 ± 1 TNB per mole of domain. Typical absorbance and first-order kinetics for three reactions of $Cd_3\beta$ domain with DTNB ranging from 1 to 8 mM are shown in Figure 1. Figure 2 compares the $(A_\infty - A_t)$ plots for holoprotein, α-domain, and β-domain. These plots revealed that the reaction of β-domain with DTNB, like that of the α-domain, occurred in a single reaction phase that corresponded to the slow step of the biphasic reaction of the holo-protein.

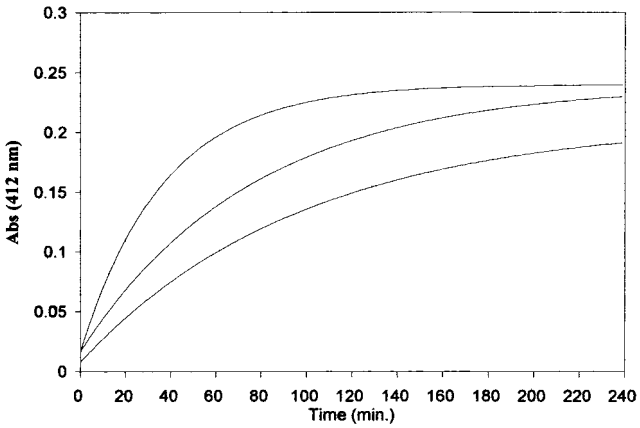

Figure 1. Time dependence of TNB formation during the reaction of DTNB with $Cd_3\beta$-domain in Tris·HCl (pH 7.4) buffer with 100 mM KCl (25.0 ± 0.1 °C). Absorbance at 412 nm vs time plots for (a) 5 mM, (b) 2 mM, and (c) 1 mM DTNB.

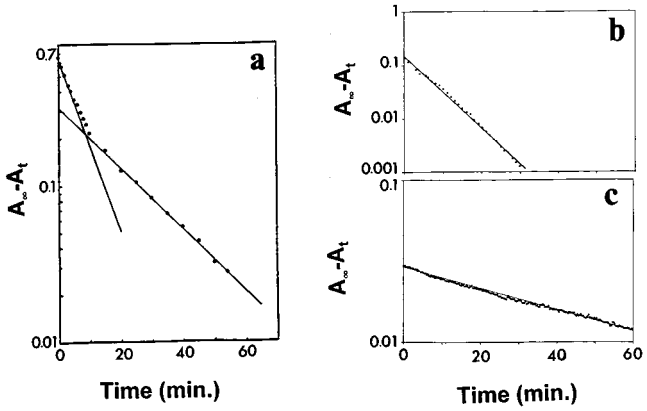

Figure 2. First-order plots, $\ln(A_\infty - A_t)$ vs time, for reactions of (a) holoprotein[21] (b) α-domain,[23] and (c) β-domain (this study). Conditions: [DTNB] = 1.0 mM; $[Cd]_{MT}$ = 20 μM; pH 7.4 in Tris·HCl buffer with 100 mM KCl (25.0 ± 0.1 °C).

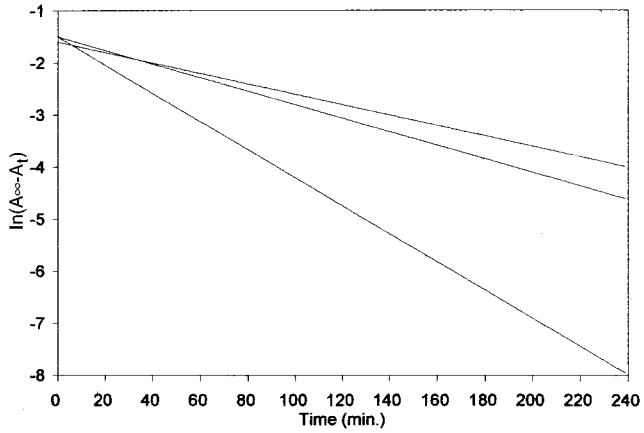

Figure 3. Plots of the pseudo-first-order rate constants vs [DTNB] for the β-domain. Conditions as in Figure 1 except for variation of [DTNB]. The intercept and slope imply a two-term rate law.

When the β-domain pseudo-first-order rate constants were plotted vs DTNB concentration, the data yielded a straight line with a finite intercept ($k_{1s} = 0.13 \times 10^{-3}\ s^{-1}$) and slope ($k_{2s} = 0.062\ M^{-1}\ s^{-1}$) (Figure 3) indicating that there are both DTNB-dependent and DTNB-independent components in the reaction. Thus, the rate law for the reaction of the $Cd_3\beta$ cluster is

$$\text{Rate}_\beta = \{k_{2s}[\text{DTNB}] + k_{1s}\}[\text{MT}] \qquad (5)$$

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Table 1. Comparison of the DTNB Rate Constants for MT and Its Domains[a]

MT	k_{2f}/M·s	$k_{1f} \times 10^3$/s	k_{2s}/M·s	$k_{1s} \times 10^3$/s
Cd_7MT[21,23]	1.75	1.26	0.12	0.42
$Cd_4\alpha$[23]	1.12	0.64	–	–
$Cd_3\beta$	–	–	0.062	0.14

[a] k_{1f} and k_{1s}, first-order rate constants for the fast and slow steps and k_{2f} and k_{2s}, second-order rate constants for the fast and slow steps, respectively.

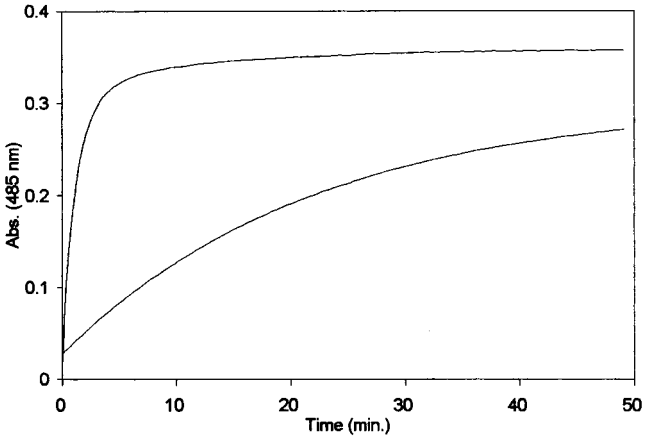

Figure 4. Time dependence of the formation of the Zn·$(PAR)_2$ complex formed during the reaction of AuSTm and either (a) Zn_4-α or (b) Zn_3-β domains in 5 mM Tris·HCl buffer with 100 mM KCl and 500 μM PAR, at pH 7.4 (25.0 ± 0.1 °C).

Previous studies have determined that the $Cd_4\alpha$ reacts according to the same rate law, but with larger rate constants (Table 1). This reaction is monophasic, independent of the metal bound to it, as demonstrated when $Zn_4\alpha$ reacted with DTNB (results not shown). Thus, the holoprotein reaction kinetics (eq 1) can be expressed as the sum of the contributions of the α-domain (eq 2) and the β-domain half-reactions (eq 5), which correspond to the faster and slower phases, respectively. This result confirms the conclusion, drawn earlier,[23] that the relative reactivity of the MT clusters toward DTNB is greater for the α- than for the β-domain.

AuSTm Kinetic Studies. The isolated $Zn_4\alpha$ and $Zn_3\beta$ fragments were allowed to react with excess AuSTm under the conditions used previously for the Zn_7-MT holoprotein (5 μM Zn, 25−500 μM AuSTm). The chromophoric probe (pyridylazo)resorcinol (PAR) was used to monitor the rate of Zn^{2+} displacement by AuSTm. Figure 4 shows the absorbance changes at 485 nm vs time for the reactions of $Zn_4\alpha$ and $Zn_3\beta$ domains with AuSTm, corresponding to the formation of the Zn^{2+}·PAR complex.

The reaction of the β-domains is cleanly monophasic and exhibits pseudo-first-order rate constants (k_{1s} = 7.6(±1.3) × 10^{-4} s^{-1}) that correspond to the slower phase of the holoprotein reactions. However, the reaction of the α-domain is more complex. Typically 87 ± 3% of the reaction occurs according to a pseudo-first-order rate constant that is more rapid than the β-domain reaction, with about 13% following the slow phase kinetics (k_{1f} = 1.0(±0.8) × 10^{-2} s^{-1}, k_{2s} = 9.3(±2.2) × 10^{-4} s^{-1}). Similar responses were observed when $Cd_4\alpha$ and $Cd_3\beta$ reacted with AuSTm with rate constants falling in the same range as those observed for the Zn-derivatives (k_f = (3.7 ± 0.2) × 10^{-2} s^{-1} and k_s = (11.0 ± 0.3) × 10^{-4} s^{-1} with 18 ± 2%), confirming that the reaction is metal independent as previously indicated for the holo-protein.[27] Figure 5 shows the

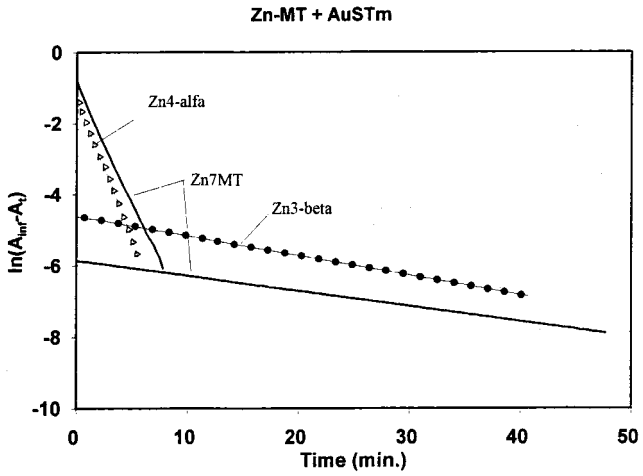

Figure 5. First-order plots, $\ln(A_\infty - A_t)$ vs time, for the displacement of Zn^{2+} from Zn_7-MT (—); Zn_3-β (···); and Zn_4-α (△), showing the correspondence of the holoprotein reactions to the domain contributions. $[Zn]_{MT}$ = 5 μM; [AuSTm] = 125 μM; [PAR] = 500 μM; in Tris·HCl with 100 mM KCl at pH 7.4 (25.0 ± 0.01 °C).

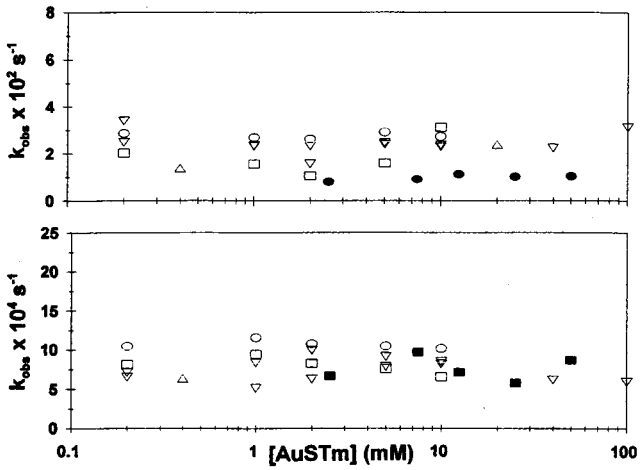

Figure 6. Comparison of the α-domain (●) and the β-domain (■) rate constants with rate constants for various holoprotein reactions with AuSTm (▽, Zn-MT + PAR; ○, Zn-MT +ZI; □, Zn,Cd,Cu-MT + PAR; △, Zn,Cd-MT + ZI); conditions as in Figure 4. These plots demonstrate the [AuSTm] independence of the rates and the association of the fast step with the α-domain and the slow step with the β-domain.

first-order kinetics for the holoprotein, the $Zn_4\alpha$ and $Zn_3\beta$ domain reactions.

The rate constants for both domains are independent of the AuSTm concentrations (Figure 6), which implies simple first-order reactions dependent only on the MT concentration:

$$\text{Rate}_\alpha = \{k_{1f} + k_{1s}\}[\text{MT}] \quad (6a)$$

$$\text{Rate}_\beta = k_{1s}[\text{MT}] \quad (6b)$$

Evidently, direct reaction between cluster sulfhydryl groups and AuSTm is precluded, and the rate-determining steps are localized in the domains.

Comparison of the rate constants obtained for the domains to those of the holoprotein showed that the fast, dominant rate constant of the α-domain falls within the range to those corresponding to the fast step of the holo-protein. The rate constant for the β-domain are in the range of the rate constants of the slow step of the holo-protein reaction (Table 2). Therefore, the results indicate that the fast phase of the holoprotein reaction occurs in the α-domain and the slow step in primarily in the β-domain.

Table 2. Comparison of the AuSTm Rate Constants for MT and Its Domains[a]

MT	$k_{1f} \times 10^2$/s	$k_{1s} \times 10^4$/s
Zn_7MT[26,27]	2.4 ± 0.5 (50 ± 5)%	8.3 ± 1.7 (50 ± 5)%
$Zn_4\alpha$	1.0 ± 0.8 (87 ± 5)%	9.3 ± 2.2 (13 ± 2)%
$Zn_3\beta$	−	7.6 ± 1.3 (100)%

[a] k_{1f} and k_{1s}, first-order rate constants for the fast and slow steps, respectively.

Table 3. Comparison of the Reactivity Toward the α- and β-Domains

α > β	β > α
DTNB[23, this work]	iodoacetamide[18]
AuSTm[this work]	EDTA[42]
NTA[20]	Cd lability[19]
melphalan[a 24]	Cd interdomain exchange[19,38]
chlorambucil[a 25]	
mechlorethamine[a 40]	
thiol solvent accessibility[4]	

[a] Only first alkylation was considered.

Discussion

The results shown here demonstrate the utility of examining the reactivity of the isolated α- and β-domains for comparison with that of the holoprotein. For the DTNB reaction, they establish unequivocally that the slow and fast phases of the holoprotein reaction are associated with the β- and α-domain, respectively. This solidifies the conclusion drawn previously from the α-domain reaction.[23] Furthermore, the properties of the holoprotein reactions are the sum of the independent reactions of the two domains. The latter finding is not surprising given the clear separation of the two metal−thiolate clusters in the X-ray structure[4] and the absence of inter-domain NOEs in the NMR spectra of mammalian MTs.[36]

The biphasic reaction of AuSTm was also successfully decomposed into component reactions that sum to yield the overall reaction. The fast step is exclusively in the α-domain, and the slow step predominantly in the β-domain with a small contribution from the α-domain.

In both reactions there is a simplicity in the rate laws for the domains that indicates single, initial reactions are rate limiting for reaction of the entire cluster. Thus, with DTNB there can be either a bimolecular reaction of DTNB with one of the SH groups or a unimolecular process within the domain which controls subsequent non-rate-limiting reaction of the entire cluster with DTNB.

Perusal of the three-dimensional structures of both domains shows that solvent accessibility to the interior of the clusters and their sulfhydryl components in the two domains is severely structurally hindered by amino acid side chains except in a crevice located in each domain. Thus, bimolecular reaction or unimolecular reorganization of the domain is hypothesized to focus on these crevice regions.

A key difference in the reactions of DTNB and AuSTm with the domains is that the bimolecular pathway is precluded for the gold complex. Steric effects may account for the difference in kinetic pattern observed in the case of AuSTm. While DTNB is a monomeric structure, X-ray studies show that aurothiomalate is a polymeric structure with gold−sulfur chains forming two interpenetrating spirals with approximate 4-fold screw symmetry (units with $a = b = 18.767(2)$ Å, $c = 4.798(2)$ Å).[37] This structure is maintained in solution as demonstrated by WAXS/DAS (differential anomalous scattering).[38] Because of its large structure, direct bimolecular reaction is precluded between the drug and sulfhydryl groups in the domain crevices.

The general expectation that the β-domain should be more labile than the α-domain has been based on several well-founded studies showing that the ^{111}Cd-intramolecular exchange rates for $Cd_3\beta$ are approximately 3000 times as fast as those for $Cd_4\alpha$,[20] that the α-domain has greater affinity for Cd^{2+},[19,20,39] and that $Cd_3\beta$ is three times more accessible than $Cd_4\alpha$ to alkylation with (^{14}C)iodoacetamide.[18] There are, however, an accumulating number of examples where the pattern of reactivity is reversed. These include oxidation by DTNB[23] and metal ion extraction by NTA[20] determined using isolated domains and alkylation with melphalan,[24] chlorambucil,[25] and mechlorethamine[40] determinated by mass spectrometric methods (summarized in Table 3).

Most MT reactions have unique rate laws, which indicates that the transition states and mechanisms are specific to each cluster−reagent combination. Nevertheless, the smaller, rate constant for the reaction with AuSTm (first-order in MT)[41] is similar in magnitude to the first-order steps observed for reactions of DTNB, EDTA, *cis*-platinum and other reagents with metallothionein, suggesting a common rate-limiting step that has been attributed to rearrangement of the protein.[21,23,42]

The differential reactivity and metal-binding affinity of the two clusters of MT have suggested that the two domains play different cellular roles.[43] For example, the α-domain binds toxic metal ions such as Cd^{2+}, whereas the β-domain participates in the essential metal metabolism of Zn^{2+}.[43] However, the expanding studies of the electrophilic reaction of MT with iodoacetamide, *cis*-platinum, chlorambucil, melphalan, MNNG, and NNU suggest an alternative view. With the recognition that, depending on the reaction, α- or β-domain clusters may be more reactive, it is hypothesized that the presence of two metal−thiolate clusters and domains within the protein markedly expands the possibilities for relatively rapid, efficient reaction of a variety of toxic agents with MT. In this view, it is the presence of two structurally different sites of reaction in the protein which contributes to the apparent participation of MT in a broad array of cellular reactions.

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