The Reaction of Metallothionein-3 with DTNB

Qi Zheng, Bin Cai, Dong Chen, Wen-Hao Yu, Yun-Hua Wang, and Zhong-Xian Huang*
Chemical Biology Laboratory, Department of Chemistry, Fudan University, Shanghai 200433, P. R. China

(Received April 1, 2003; CL-030279)

MT3 reacts with DTNB by a biphasic process, which roots from the difference in reactions between two domains with DTNB. The β-domain reacts with DTNB faster, implying that the CPCP motif distorts the peptide and leads to the Cd_3S_9 cluster more accessible to solvent. This reaction is a S_N2 process according to the activation entropy.

Metallothionein-3 (MT3) is a brain-specific isoform of MTs. Resembling other mammalian MTs in containing 20 cysteines in conserved C-X-C and C-X-X-C sequence motifs, the polypeptide of MT3 also wraps around the two metal-thiolate (Cd^{2+} or Zn^{2+}) clusters forming two domains. The N-terminal β-domain consists of 30 residues and the C-terminal α-domain contains the other 35 residues. The KKS fragment (residues 31–33) is the linker. It was reported that MT3 inhibits the elevated neurotrophic activity of Alzheimer's disease (AD) brain extracts,[1] a function is not shared by MT1/2. The functional difference between MT3 and MT1/2 implies the uniqueness of MT3 in structure and property. Here we investigate the reaction of MT3 with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).

Human MT3 cDNA was prepared from cells by reverse transcription followed by polymerase chain reaction (PCR), then cloned into vector pGEX-4T-2 as a BamH I/EcoR I fragment. The expression and purification procedures were carried out as described by Amersham Pharmacia Biotech with a slight modification.[2] To clarify the reaction between MT3 and DTNB, we prepared several different proteins with PCR and ligation, including the wild-type MT3, single α-domain, single β-domain and β(MT3)–β(MT3) protein. All of these samples were characterized by DNA sequencing and ESI-MS, and the results were entirely consistent with what expected. The reaction of protein with DTNB was performed according to the method of Shaw[3] and the conditions were listed below: 3 μM protein in 10 mM Tris-HCl buffer (pH 7.5) with 100 mM KCl. The DTNB concentration was 0.8 mM and the reaction temperature was 25 °C. Unless stated, all the proteins used here were cadmium-reconstituted.

Under pseudo-first-order condition, the reaction of MT with DTNB is a biphasic process. At the same condition, the single α-domain, the single β-domain and β(MT3)–β(MT3) reacted with DTNB monophasicly (Figure 1). Obviously, this reaction is a domain-relative process, especially in the case of β(MT3)–β(MT3). By plotting the $\ln[A_\infty - A_t]$ vs time, we can obtain the pseudo-first-order rates, k_f and k_s, which are listed in Table 1. The observed rate constants of Cd_7-MT3 ($k_f = 8.0 \pm 0.6 \times 10^{-3}$, $k_s = 3.4 \pm 1.2 \times 10^{-3}$) are nearly equal to those of Zn_7-MT3 under the same conditions ($k_f = 8.5 \pm 0.1 \times 10^{-3}$, $k_s = 3.5 \pm 0.1 \times 10^{-3}$), which prove the hypothesis that the reaction of MTs with DTNB reflects the accessibility of clusters by solvent but not the stability of clusters. It seems that, in MT3, the β-domain reacts with DTNB faster than α-domain. This result is not coincident with the results reported in some literatures about MT2.[4,5] According to Savas's report,[4] in the case of MT2, the faster and the slower phases correspond to the reactions of DTNB with the α-domain and β-domain, respectively. This difference reflects the uniqueness in the structure of MT3. In MT3, the continual proline residues in the C(6)-P-C-P(9) motif bend the peptide intensely, which makes the Cd_3S_9 cluster more accessible to solvent. In our opinion, this more accessible metal-thiolate cluster structure is crucial to the properties and the functions of MT3.

The rate constant of the reaction with DTNB is actually DTNB concentration-dependant. In the case of Cd_7-MT3, the constant of the fast phase is in direct proportion to the concentration of DTNB (Figure 2) and the rate equation for this phase can be expressed as $k_{obs} = -2.77 \times 10^{-3} + 15.38[\text{DTNB}]$. This result argues the Li's conclusion that Cd–thiol bond breaks before the formation of MT–DTNB complexes.[6] The data here suggest that compared to the DTNB-dependent portion, the DTNB-independent portion can be ignored, which implies an association reaction in the rate-determining step. The rate con-

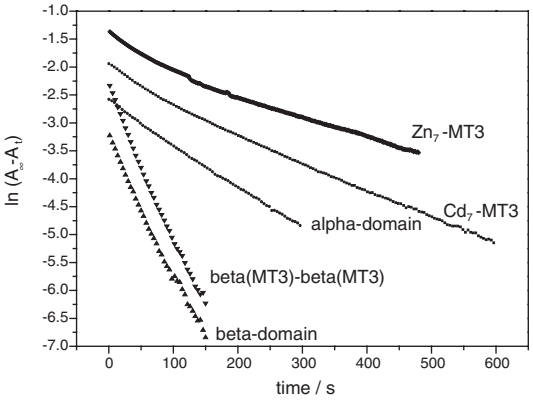

Figure 1. The logarithmic plots of the reaction rates of DTNB with Cd_7-MT3 (■), α-domain (●), β-domain (▲), β(MT3)-β(MT3) (▼) and Zn_7-MT3 (◆). Concentration changes of product were measured through absorbance at 412 nm (A_t).

Table 1. Rate constants of reaction for MT3 and its domains with DTNB

	k_f/s^{-1a}	k_s/s^{-1b}
Cd_7-MT3	$8.0 \pm 0.6 \times 10^{-3}$	$3.4 \pm 1.2 \times 10^{-3}$
Zn_7-MT3	$8.5 \pm 0.1 \times 10^{-3}$	$3.5 \pm 0.1 \times 10^{-3}$
α-domain	$7.5 \pm 0.3 \times 10^{-3}$	—
β-domain	$22.8 \pm 1.0 \times 10^{-3}$	—
β(MT3)–β(MT3)	$24.6 \pm 1.1 \times 10^{-3}$	—

[a]The rate constant of the fast reaction phase. [b]The rate constant of the slow reaction phase.

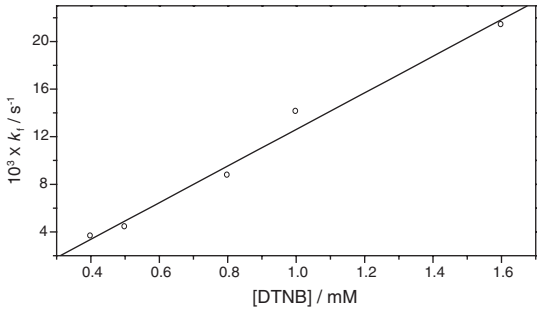

Figure 2. Plots of the fast phase observed rate constants vs [DTNB] for reactions of Cd_7-MT3. Conditions: [DTNB] = 0.4–1.6 mM, [Cd_7-MT3] = 3 μM, and pH 7.5 in 10 mM Tris-HCl buffer with 100 mM KCl at 25 °C.

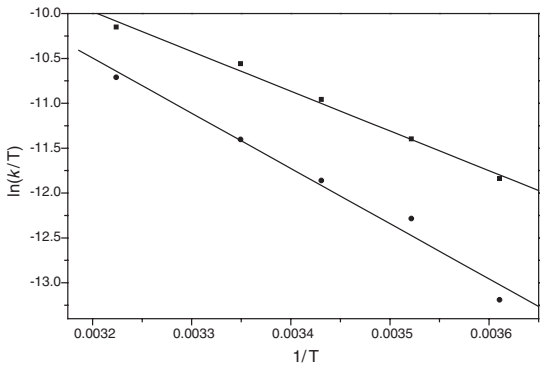

Figure 3. Plots of the $\ln(k/T)$ vs $1/T$ for the reaction of Cd_7-MT3 with DTNB: k_f (■); k_s (●). Conditions: CD_7-MT3 = 3 μM, DTNB = 0.8 mM, and pH 7.5 in 10 mM Tris-HCl buffer with 100 mM KCl at a temperature range 4-37 °C.

stants of the slow phase are also in direct proportion to the DTNB concentration (data not shown).

The temperature-dependence of each reaction phase of Cd_7-MT3 with DTNB is shown in Table 2. According to the equation,

$$\ln(k/T) = -\Delta H^{\neq}/RT + (\Delta S^{\neq}/R + 23.76)$$

we can obtain the activation enthalpies and activation entropies of each phase by plotting the $\ln(k/T)$ vs $1/T$ (Figure 3). The activation entropies are −162.7 J/mol·K and −121.1 J/mol·K for k_f and k_s, respectively, suggesting a S_N2 process in the rate-determining step. That is, the reaction undergoes an association process. The activation enthalpies for the MT3-DTNB first-order reaction components are 36.8 KJ/mol and 51.2 KJ/mol for k_f and k_s, respectively. The lower activation enthalpy in the fast phase is consistent with that in the faster rate constant.

We acknowledge the financial support of National Foundation of Science in China.

References

1 Y. Uchida, K. Takio, K. Titani, Y. Ihara, and M. Tomonaga, *Neuron*, **7**, 337 (1991).
2 W.-H. Yu, B. Cai, Y. Gao, Y. Xie, and Z.-X. Huang, *J. Protein Chem.*, **21**, 177 (2002).
3 C. F. Shaw, III, M. M. Savas, and D. H. Petering, *Methods Enzymol.*, **205**, 401 (1991).
4 M. M. Savas, D. H. Petering, and C. F. Shaw, III, *Inorg. Chem.*, **30**, 581 (1991).
5 A. Munoz, D. H. Petering, and C. F. Shaw, III, *Inorg. Chem.*, **38**, 5655 (1999).
6 T.-Y. Li, D. T. Minkel, C. F. Shaw, III, and D. H. Petering, *Biochem. J.*, **193**, 441 (1981).

Published on the web (Advance View) June 17, 2003; DOI 10.1246/cl.2003.616