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Reversible folding of cysteine-rich metallothionein by an overcritical reaction path

Yi-Liang Liu,[a] Hui-Ting Lee,[a] Chia-Ching Chang,[a,1] and Lou-Sing Kan[b,*]

[a] *Department of Physics, National Dong Hwa University, Hualien 97401, Taiwan, ROC*
[b] *Institute of Chemistry, Academia Sinica, Nankang, Taipei 11529, Taiwan, ROC*

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Abstract

A first-order-like state transition is considered to be involved in the restoration of the activities of a few proteins by correctly folding the protein [Phys. Rev. E 66 (2002) 021903]. In order to understand the general applicability of this mechanism, we studied a metallothionein (MT) protein with an unconventional structure, i.e., without any α-helix or β-sheet. MT is a 61 amino-acid peptide. There are 6–7 Zn^{2+} ions, which bind avidly to 20 conserved cysteines (Cys) of MT. These properties indicate that the structure of MT is quite different from those of the other proteins. Similar to our previous findings, the denatured MT can be folded without any aggregation via a designated stepwise quasi-static process (an over-critical reaction path). The particle size of folded MT intermediates, determined by dynamic light scattering, shrank right after the first folding stage. It is consistent with a collapse-model. In addition, results from both atomic absorption and circular dichroism (CD) indicate that the stable intermediates may fold to the native conformation but with only partial Zn^{2+} binding, which in turn implies that those folding intermediates are in a molten globular state. These reversible unfolding and folding processes indicate that Cys-rich protein, MT, may also be folded by way of a first-order-like state transition mechanism. We suspect that this process may likely be involved in the reaction of the metal substitution process in metal containing enzymes.
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The folding of a protein has been considered, in general, to be a spontaneous process [1], or to occur in an alternative way [2]. Our previous study indicated that a general first-order-like state transition model can encompass these two processes without a conflict [3]. In this very model, the burst-phase transition intermediate [4] contains a finite boundary in which protein may be trapped and eventually aggregate into a colloidal form. A spontaneous folding is a degenerate case in which the burst-phase transition boundary is reduced and the protein has not been trapped in an unfolded form during a direct folding reaction. On the contrary, if a pre-designed refolding path (an over-critical path) is taken, then the reaction is detoured over the transition boundary and the protein can fold sequentially [4] without causing any aggregation. Moreover, those intermediates are in a molten globular state [5] and consistent with the collapse model [6]. According to the energy landscape funnels model [7], a quasi-static process is required [3] in order to avoid the diffusion limited aggregation of protein and to reach a steady state at each step during successive folding.

In general, a protein may precipitate in most high-concentration cases (conc. >0.1–0.3 mg/ml) of folding processes in vitro [8]. These precipitations may be caused by mis-linked disulfide bonds. To explore our stepwise model a bit further, a cysteine (Cys)-rich metallothionein (MT) was used in this study. MT is a 61 amino-acid polypeptide that has no α-helix or β-sheet, but wraps 6–7 divalent heavy metal ions at highly conserved Cys [9,10]. In addition to its unconventional structure, the avoidance of miss-linked disulfide bond formation during the folding process is also a challenge.

* Corresponding author. Fax: +886-2-2788-4184.
E-mail addresses: chiaching@mail.ndhu.edu.tw (C.-C. Chang), lskan@chem.sinica.edu.tw (L.-S. Kan).
[1] Also corresponding author. Fax: +886-3-866-2303.

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A stepwise quasi-static process [3,11] was used to follow the protein folding so that the intermediates and their functions could be monitored unambiguously. The rabbit native MT was unfolded and then refolded back into its functional form by a stepwise thermal equilibrium dialysis (TED) approach [3,11]. To ascertain the state of the MT intermediates, their metal-binding ratio and metal-binding configurations were examined by atomic absorption (AA) and circular dichroism (CD), respectively. The mechanism of the transitions among intermediates was eluded from the particle sizes at various stages and monitored by dynamic light scattering (DLS) analysis [12,13]. The reversibility of the refolding process under current study is consistent with our previously proposed stepwise quasi-static model (the over-critical refolding process). Through this work, the adaptability of this model to other systems is expected and discussed.

Materials and methods

Materials and buffers. Native metallothionein was purchased from Sigma (St. Louis, MO). All other chemicals were obtained from Merck (Rahway, NJ). The denaturing/unfolding buffer (in pH 12) contained 4.5 M urea with 10 mM Tris–base, 0.1 M dithiothreitol (DTT), 0.1% mannitol, and 0.5 mM Pefabloc. There were five folding buffers employed in this study. Their compositions are summarized in Table 1. Thus, the folding buffers dialyzed away the urea and mannitol in the denaturing/unfolding buffer. In the meantime, the pH value was lowered from the basic to the neutral range.

The concentration measurement. MT contained no aromatic residues and the specific UV absorption OD_{254} of the metal-binding clusters changed during the folding stages. Thus, the concentration of MT could not be measured directly through the UV spectrophotometer [14]. The concentration of the unfolded (U), five intermediates (M_1–M_5), and natural form (N) were determined through the DC (detergent compatible) Protein Assay purchased from Bio-Rad Laboratories (Hercules, CA). The assay was similar to the Lowry assay [15]. The concentration of MT was determined by linear regression from a typical standard curve (e.g., lysozyme) in which the color absorption was measured at 750 nm with a microplate reader, Anthos 2010 (Anthos Labtec Instruments, Austria).

Folding of MT by quasi-static-like thermal equilibrium dialysis. The folding method of MT is by way of a quasi-static procedure that involves five consecutive thermal equilibrium dialysis (TED) steps. Each of the folding intermediates (M_1–M_5) is dialyzed against a particular folding buffer at 4 °C, as shown in Materials and buffers. All buffers contain 0.1 M $ZnCl_2$. The TED processes were carried out by saturating the dialysis buffer with nitrogen gas to prevent rapid oxidation. In order to avoid possible deterioration of the buffer during the dialysis, all refolding buffers were replaced by fresh ones midway through each dialysis.

Step 1. The unfolded MT (U) in the denature/unfolding buffer was dialyzed against folding buffer 1 for 72 h to dilute the urea concentration from 4.5 to 2 M (this produces intermediate 1 or M_1).

Step 2. M_2 was obtained by dialyzing M_1 against folding buffer 2 for 24 h to dilute the urea concentration to 1 M.

Step 3. M_3, an intermediate without denaturant (urea) in solution, was then obtained by dialyzing M_2 against folding buffer 3 for 24 h.

Step 4. M_3 was further dialyzed against folding buffer 4 for 24 h and the pH changed from 11 to 8.8 to produce M_4.

Step 5. Finally, the chemical chaperon mannitol was removed by dialyzing M_4 against the native buffer for 8 h to yield M_5.

CD Analysis of metal-binding configurations of MT. CD spectra in the UV region (290–210 nm) of U, M_{1-5}, and N were recorded on a Jasco J 720C spectropolarimeter at 20 °C. A cuvette with a 0.1-cm light-path was used to reduce the light scattering of the solution. Data are expressed in molar ellipticity (deg cm^2 $dmol^{-1}$), calculated based on a molecular weight of 6.8 kDa MT [16]. The measured CD spectrum of each individual sample was diluted with the same folding buffer without DTT of each stage to avoid noise. The solvent contribution on the CD was subtracted from each spectrum.

The CD profiles of U, M_{1-5}, and N are illustrated in Fig. 1. There are three significant absorptions in the CD data: 227 (relative positive), 239 (relative negative), and 259 nm (relative positive). These bands are correlated to the metal binding configuration [17,18].

Analysis of each metal-binding ratio of MT folding intermediates by atomic absorption. The metal-binding ratio was measured through the graphite furnace atomic absorption (AA) spectrophotometer (Z-8200, Hitachi, Japan) at 20 °C. Each Zn-binding content was recorded as ppm (mg/L). The ratio of metal to MT (n_{Zn}) of each dialyzing step was calculated by the volume of buffers, the molecular weight of MT

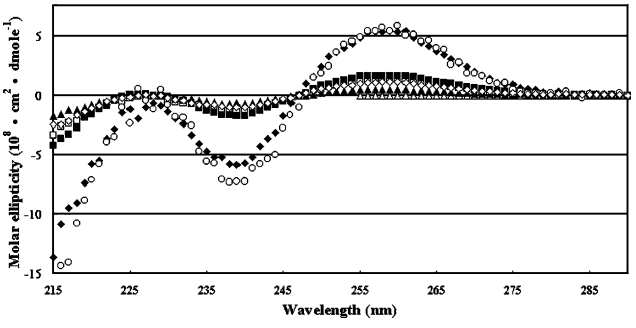

Fig. 1. Circular dichroism profiles of MT at 20 °C. The symbols open triangle, filled open square, filled square, open diamond, filled diamond, and open circle are represented U, M_1, M_2, M_3, M_4, M_5, and native form (N), respectively. The circular dichroism was taken at 0.1 nm datum point, but only 1 nm datum point is shown for clarity.

Table 1
Chemical compositions of refolding buffers

	Tris–base (mM)	pH	Urea (M)	DTT (mM)	Mannitol (%)	Pefabloc (μM)	Zinc chloride (M)
Folding buffer 1	10	11	2	0.1	0.1	0.5	0.1
Folding buffer 2	10	11	1	0.1	0.1	0.5	0.1
Folding buffer 3	10	11		0.1	0.1	0.5	0.1
Folding buffer 4	10	8.8		0.1	0.1	0.5	0.1
Folding buffer 5 (the native buffer)	10	8.8		0.1		0.5	0.1

Table 2
The Zn content of each folding intermediate

Folding state	Ratio of Zn/MT
U	0.35 ± 0.02
M_1	1.15 ± 0.03
M_2	1.29 ± 0.03
M_3	1.17 ± 0.15
M_4	1.14 ± 0.06
M_5	6.01 ± 0.21
N	6.34 ± 0.32

(6800 Da) and the atomic weight of Zn (MW_{Zn}) (65.4 Da), as described in the following equation:

$$n_{Zn} = \frac{ppm_{Zn}}{MW_{Zn}M_{MT}}. \quad (1)$$

The results are summarized in Table 2.

Analysis of size distribution of refolding MT in steps 1–5 by dynamic light scattering. The light scattering apparatus was equipped with an argon ion laser-light source (4880 Å) (Ion Laser Tech., UT). The scattered light was collected at 90° by a goniometer from Brookhaven Instruments (BIC, Holtsville, NY). The chamber temperature was controlled by a water circulator. The ACF (auto-correlation function) was computed using a digital correlator (BI9000) and then analyzed with the non-negatively constrained least squares (NNLS) method [19]. The instrument performance was calibrated by measuring the DLS of standard suspensions of polystyrene beads ($R_H = 68$ nm) (Polysciences, Warrington, PA). Concentration of MT in each dialyzing step was 0.2 mg/ml and all experiments were conducted at 20 °C.

Results and discussion

The refolding of MT by stepwise quasi-static process

Monitored by CD

Fig. 1 shows the CD of native MT from wavelengths of 210–290 nm. There are two peaks and a trough at 227, 259, and 239 nm, respectively. The characteristics of the CD of MT are different from those of the proteins with α-helix or β-sheet secondary structures, because MT contains many metal chelating sites through its Cys residues. Thus, the peaks and trough will be markers for the structural refolding as well as function recovery. On the contrary, the CD of unfolded MT (U) showed no meaningful spectrum in the same range of wavelength. After 72 h TED processing of the first folding step, in which the urea concentration changed gradually from 4.5 to 2 M, the optical characteristics of the first intermediate (M_1) were partially restored when compared to the CD profile of the native MT (N), except in smaller amplitudes. Subsequent quasi-static-like studies showed that the CD profiles of M_2, M_3, and M_4 in 227, 259, and 239 nm, which are absorptions of metal-binding configurations, were strikingly similar to that of M_1. This indicated that the metal-binding content of these intermediates was in the same level as M_1. Also shown in Fig. 1, the CD of M_5, the product of the last step of dialysis for depletion of mannitol, is strikingly similar to that of the native MT. The molar ellipticity absorption at 259 and 239 nm rose up to that of the native MT (N). This is strong evidence that the refolding of MT is a three-stage process. Namely, the structure of MT is relaxed in 4.5 M urea and in a basic solution (pH 12). Then the secondary structure is resumed when the urea concentration is as high as 2 M and the pH of the solution is changed to 11. The structure and function were re-natured by dialysis of mannitol. Mannitol, in general, played the role of chemical chaperon, thus protecting side chains of proteins from being unexpectedly modified by a highly reductive/oxidative environment, and it did not affect the formation of secondary structures and only slightly affected the tertiary structure, as determined by examination with a thermal denaturation assay of growth hormone [6]. Moreover, mannitol protects the SH-enzyme from the damage of the hydroxyl radical [20]. In general, the protection mechanism of mannitol may come from its direct interaction with the affecting factors of the environment; or, with the functional groups of protein, to prevent unexpected modifications from taking place. However, in this study, the molar ellipticity of the metal-binding configurations at 227, 239, and 259 nm by CD profile (Fig. 1) indicated that the Zn binding ability was affected by mannitol.

Monitored by AA

The metal (Zn) content of each quasi-static refolding stage can be monitored by AA. The results are shown in Table 2. According to a previous report [21], each active MT avidly binds to 6–7 divalent metal ions through its conserved 20 Cys. Current AA results showed similar results, in which the MT molecule at the N state has an average 6.34 Zn ions bound to it. On the other hand, the U lost both activity and folding due to the depletion of Zn ions entirely (average 0.35 Zn ion to one MT). It is interesting to note that M_{1-4} have similar Zn ion binding numbers, around one ion per MT molecule. This may mean that at least one Zn ion binds to MT. Combining the CD results in the previous section, this Zn ion may equilibrate at its preferential metal-binding sites, such as the third site of the MT β domain or the first/sixth site of MT α domain [10], and keep the structure of MT in its gross shape. In other words, MT starts its folding mechanism as soon as there is one Zn ion present, and the metal-binding structure of MT is resumed by partial Zn ion bindings. The number of Zn ion bindings of M_5 increases sharply to six (6.01), as in the absence (by dialysis) of mannitol, as described in the previous section. There is a slight possibility that M_5 has one Zn ion less than that of N. The depletion of one metal ion may be caused by the lower binding energy of Cys clusters in the fourth metal-binding site of the MT β domain and tend to dissociate [10,21,22]. Thus, in any case, we may conclude that M_5 is similar to the native MT. The evidences from AA, complementary to CD, proved that MT could be correctly refolded by quasi-static-like

dialysis. At the same time, a two-step function restoration is observed.

DLS studies on MT intermediates (U, N, and M_{1-5})

In solution, dynamic light scattering is one method for measuring particle size distribution (PSD) in the submicron range [13]. As shown in Fig. 2, the DLS measurements indicated that the size distributions of all folding intermediates are uniform in single distribution. The uniformity of the diameters indicated that the TED process reached equilibrium and that the intermediates were in a uniform state rather than a mixture of 'folded' and 'unfolded' states. The effective diameters (R_H) of U, M_{1-5}, and N measured were 5.76 (±0.39%), 2.87 (±0.13%), 2.89 (±0.36%), 2.89 (±0.34%), 2.89 (±0.44%), 2.89 (±0.51%), and 2.89 (±0.47%) nm, respectively, at 20 °C. These measurements were calibrated against reference polystyrene beads with $R_H = 68$ nm. However, the measured standard suspensions of polystyrene beads gave an average diameter of 72 (±0.14%) nm. The error range of the instrument performance is thus around 6%.

In comparison, the effective diameter of the rabbit MT obtained from X-ray crystallography, around 2.85 nm [23], is very close to that of M_{1-5} and N. However, the diameter of unfolded MT is 5.76 nm, which is twice the linear dimension of the native MT (2.89 nm). It indicated that the unfolded MT was not fully extended and this is consistent with previous studies [3,24].

Quasi-static like TED folding process is an over-critical reaction path

Rapid dilution of a high concentration of unfolded protein by the native buffer caused precipitation, possibly due to protein trapping in a sub-global state or a "burst-phase" [25,26]. However, in this study, denatured MT has been refolded stepwise, and there is no aggregation taking place. Based on the first-order-like state transition model of protein folding [3], this folding process is an over-critical path. Namely, the reaction path detoured around the reaction to avoid being trapped into the burst-phase and subsequently forming aggregates [4]. Those stable folding intermediates of the MT intermediates on this path can be considered to be in a "super-state" with respect to ordinary state and ordinary path. Although M_1, M_2, M_3, and M_4 contained similar amounts of Zn, CD profile CD, and size by DLS, they may still be different. We have directly dialyzed MT from U to the M_4 buffer and found that 88.3% of the MT aggregated. This indicated that this buffer changing process might be too harsh for the "burst-phase" intermediate to take place. Therefore, a stepwise process (M_1–M_4) is necessary for an over-critical path.

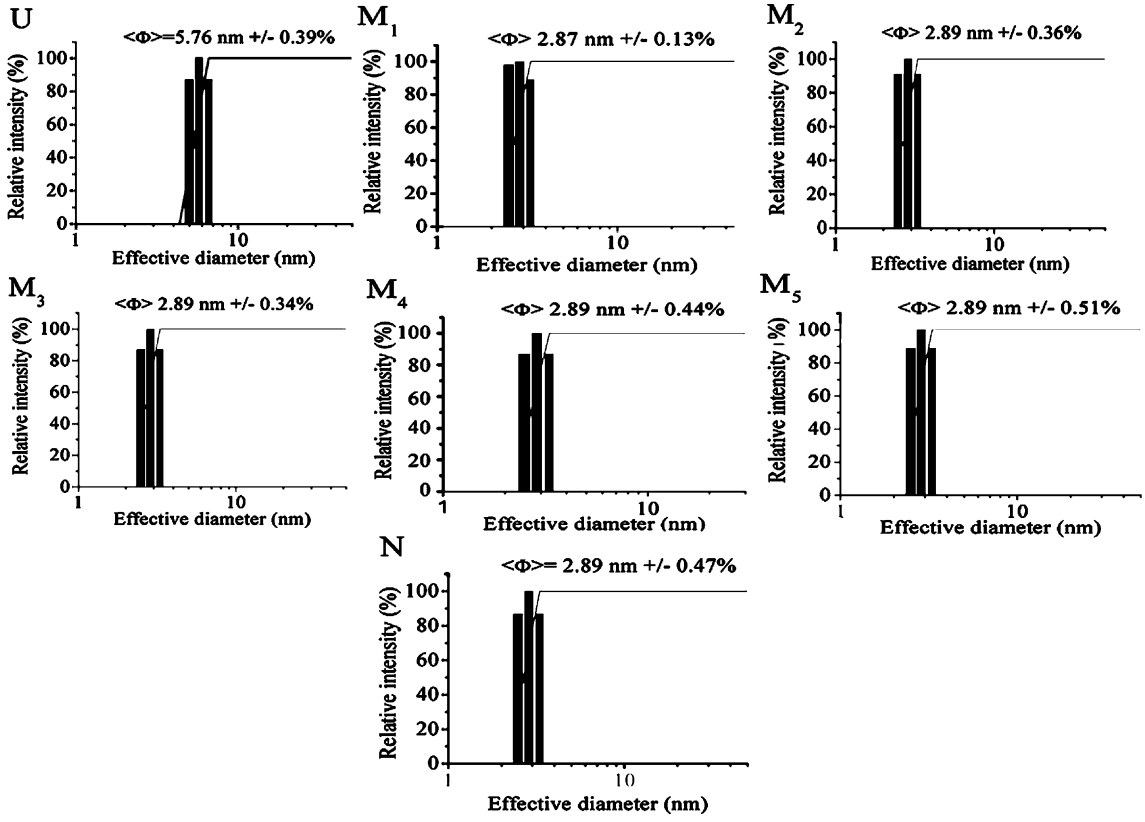

Fig. 2. Particle size distribution of variant stages of U, M_{1-5}, and N of MT. The solid lines indicate accumulation percentage of particles (see text). (Those portions with particle size larger than 50 nm may be artifact and are negligible.)

Protein folding on an over-critical path is consistent with the collapse model and intermediates are in molten globule-like forms

As described in the above section, the R_H shrinks from 5.75 nm of U to 2.89 nm of all the "super-state" intermediates and the native form. The phenomenon of the proteins' collapse in particle sizes is consistent with the collapse model of protein folding [6]. Both CD (Fig. 1) and DLS (Fig. 2) showed that the four "super-state" intermediates might be classified as molten globule-like forms.

Conclusion

Compared with our previous studies [3,11], the growth hormone could be unfolded and refolded reversibly by our TED process. Through similar processes, the metal-binding protein MT could also be refolded to the native protein according to the CD, AA, and DLS data. It suggested that not only the normal protein could be folded through our process, but also the specific highly Cys containing protein, MT. The results are consistent with our previous studies [3,11]. The folding reaction of MT follows a first-order-like state transition process, which implies that the folding path of protein is not unique, i.e., if a correct path is carefully chosen, a protein can always be folded. Namely, an unfolded protein which refolds back through a carefully designed stepwise quasi-static-like process may experience a gradual change in its environment and thus will not be trapped in a burst-phase nor subsequently form an aggregation. In addition to the protein folding, this process may also be adapted to the reaction of metal substitution of metal-containing enzymes.

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