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Effect of Zn^{2+} during Reactivation and Refolding of Urea-Denatured Creatine Kinase

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Abstract—The courses of refolding and reactivation of urea-denatured creatine kinase (CK) (ATP:creatine N-phosphotransferase, EC 2.7.3.2) have been studied in the absence and presence of zinc ions. The presence of Zn^{2+} at low concentrations blocks the reactivation and refolding of urea-denatured CK and keeps it in a partially folded state. The partially folded state proved to be a monomeric state which resembles the "molten globule" state in the CK folding pathway. During refolding in the presence of Zn^{2+}, creatine kinase forms aggregates with the aggregation dependent on zinc concentration and temperature. In the presence of EDTA, the partially folded creatine kinase can be reactivated and refolded following a biphasic course, suggesting the existence of a monomeric intermediate during the refolding of CK. The results also suggest that low concentrations of zinc ions might be toxic to some proteins such as creatine kinase by disrupting their proper folding.

Key words: creatine kinase, zinc ion, reactivation, refolding, partially folded state

Creatine kinase (ATP:creatine N-phosphotransferase, EC 2.7.3.2) is an important enzyme in energy metabolism that has been studied extensively [1, 2]. It catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine, leading to phosphocreatine and MgADP. In addition to its physiological role, creatine kinase is important in the diagnosis of some diseases such as myocardial infarction, cardiac hypertrophy, and muscular dystrophy.

Creatine kinase (CK) is completely unfolded after 1 h in either 6 M urea or 3 M guanidine hydrochloride (GuHCl) [3]. In both cases the unfolding of the enzyme molecules is complete in the sense that all the buried SH groups are exposed and the fluorescence and absorbance values both no longer change. The activity and conformational changes of CK in urea and guanidine hydrochloride have been well studied and it has been shown that the unfolding of CK is a multiple-step sequential process [4-6]. The refolding of guanidine- or urea-denatured CK has also been well studied [7, 8]. Under suitable conditions, the native conformation and the activity of the enzyme unfolded in chemical denaturants can be quantitatively recovered. Both the reactivation and the refolding processes are independent of enzyme concentration within a certain range (0-2.6 μM) [9]. When the CK concentration is higher than 2.6 μM, however, the CK activity recovery decreases as the concentration increases because of the formation of aggregates [10].

Various factors can block the refolding of creatine kinase. It has been reported that the binding of a monoclonal antibody inhibits the refolding of urea-denatured CK [11]. Wang et al. previously reported that, after being modified by thiol reagent in the denatured state, CK can not be refolded and remained in a partially folded state [12]. Our previous experiments proved that the molecular chaperone GroEL can bind the refolding intermediate of CK and block the refolding of CK [10].

Although there have been many studies on the unfolding and refolding of creatine kinase, there have been relatively few attempts to explore the effect of metal ions during its refolding. Park et al. recently studied the effect of Mg^{2+} during reactivation and refolding of guanidine-denatured CK and found that the reactivation of CK can be inhibited by Mg^{2+} and that Mg^{2+} can induce complex changes in the relative fluorescence intensity during refolding over a broad range of concentrations (0-100 mM) [13].

The present study investigates the courses of both the reactivation and the refolding of urea-denatured CK in the presence of low concentrations of zinc ions. The

Abbreviations: ANS) 8-anilino-1-naphthalene sulfonate; CK) creatine kinase; GuHCl) guanidine hydrochloride.

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results show that in the presence of very low concentrations of zinc ions (less than 12 μM), CK cannot be refolded but remained in a partially folded state. The effective molecular ratio of zinc to CK monomer was 3 : 1. The characteristics of the partially folded state of CK blocked by zinc measured in this experiment proved it to be a monomeric state which resembles that of the "molten globule" state in the CK folding pathway. When EDTA was used as a chelator to remove the Zn^{2+}, the partially folded CK could be reactivated and refolded following a biphasic course.

MATERIALS AND METHODS

Ultra pure grade urea and 8-anilino-1-naphthalene sulfonate (ANS) were from Sigma (USA); creatine and ATP were from Fluka (Switzerland); EDTA was from Promega (USA). All other chemicals were local products of analytical grade.

The preparation of rabbit muscle creatine kinase was as described earlier [14]. Purified CK was homogeneous on polyacrylamide gel electrophoresis in the presence and absence of SDS. The CK concentrations were determined using the absorption coefficient $A^{1\%}_{1\,cm} = 8.8$ at 280 nm [15]. The CK activity was determined by following proton generation during the reaction of ATP and creatine with the indicator Thymol Blue at 597 nm at 25°C [16].

The enzyme was denatured at 25°C in a solution containing 6 M urea in 30 mM Tris-HCl buffer (pH 8.0) for 1 h. In the refolding studies, the denatured enzyme was diluted 60-fold into the same buffer containing $ZnCl_2$ solutions of different concentrations without urea for activity, fluorescence, and circular dichroism measurements.

Fluorescence spectra measurements were made with a Hitachi 2500 spectrofluorometer (Japan) using an excitation wavelength of 280 nm. The excitation wavelength was 380 nm for the ANS-binding fluorescence spectra. Circular dichroism spectra were recorded on a Jasco 725 spectropolarimeter (Japan) with a sample cell path length of 22 mm. Size exclusion chromatography was carried out with a Superdex™ 200 HR 10/30 column on a Amersham Pharmacia Biotech. (USA) ÄKTA purifier. The activity was measured with a Perkin-Elmer Lambda Bio UV/VIS spectrophotometer (USA). All measurements were in 30 mM Tris-HCl buffer (pH 8.0).

RESULTS

Effect of different Zn^{2+} concentrations on the courses of reactivation of urea-denatured creatine kinase. When creatine kinase denatured in 6 M urea for 1 h was diluted 60-fold into a 30 mM Tris-HCl buffer (pH 8.0) containing different Zn^{2+} concentrations at 25°C, the enzyme activity was restored to some extent. Figure 1 shows that the levels of activity recovery decreased with increasing Zn^{2+} concentrations. For Zn^{2+} concentration of 12 μM, the reactivation of urea-denatured CK was fully blocked. Tong et al. found that zinc concentrations less than 0.1 mM did not reduce the native CK activity but induced increased CK activity [17]. In our control experiments, the urea-denatured CK was diluted into the same buffer containing KCl, NaCl, $CaCl_2$, $MgCl_2$, or $MnCl_2$ at concentrations of 50 μM. The results (Fig. 1) show that there is no influence of the monovalent cations (K^+ and Na^+), while the addition of low concentrations of Ca^{2+}, Mg^{2+}, and Mn^{2+} resulted in only small decreases in the levels of CK activity recovery. Figure 2 shows the relationship between the CK and Zn^{2+} concentrations. At each CK concentration, the CK activity recovery decreased to nearly zero for Zn^{2+} to CK monomer molecular ratios of more than three.

Aggregation (turbidity) of creatine kinase while refolding in $ZnCl_2$ solutions with different concentrations was then measured by measuring the absorbance at 400 nm. The results (Fig. 3) show that CK is prone to aggregation during refolding in the presence of Zn^{2+}. Aggregation of CK refolding in excessive Zn^{2+} (20 μM) at different temperatures was also measured (Fig. 4). No aggregation took place at low temperatures (less than 8°C). At temperatures higher than 8°C, the aggregation increased quickly with temperature.

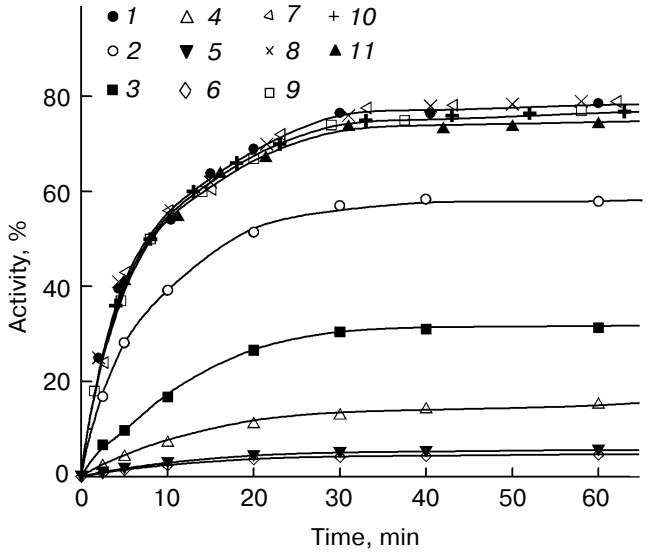


Fig. 1. Effect of Zn^{2+} and other metal ions on the course of reactivation of CK. The urea (6 M)-denatured CK was suitably diluted so that the final urea concentration was always 0.1 M. The CK concentration was 2.0 μM. The $ZnCl_2$ concentrations (μM) were: 0 (*1*), 3 (*2*), 6 (*3*), 9 (*4*), 12 (*5*), and 20 (*6*), respectively. The concentrations of other metal ions used were 50 μM NaCl (*7*), 50 μM KCl (*8*), 50 μM $MgCl_2$ (*9*), 50 μM $CaCl_2$ (*10*), and 50 μM $MnCl_2$ (*11*). The reactions occurred at 25°C.

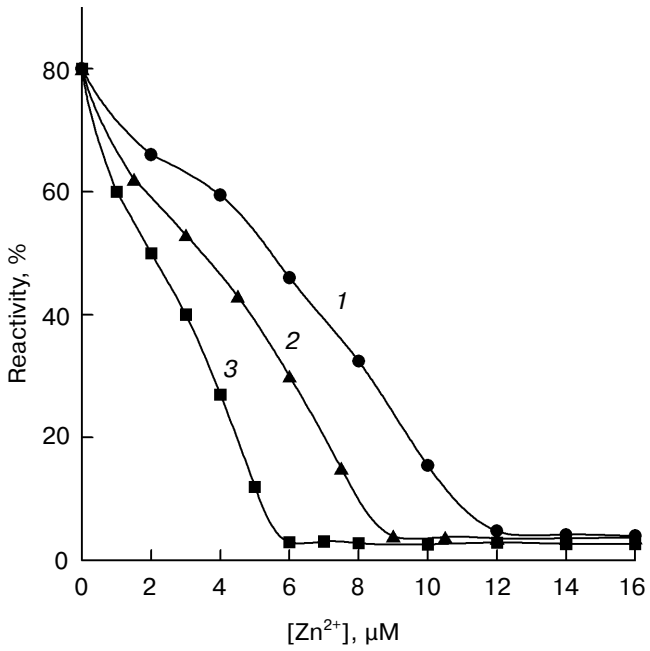


Fig. 2. Effect of different Zn^{2+} concentrations on the reactivity of urea-denatured CK. The urea-denatured enzyme was diluted with 30 mM Tris-HCl, pH 8.0, containing 0-16 μM Zn^{2+}. Other conditions were as for Fig. 1. The final enzyme concentrations (μM) were: 2 (*1*), 1.5 (*2*), and 1 (*3*), respectively.

Reactivation of partially folded creatine kinase by EDTA. One hour after the urea-denatured creatine kinase was diluted into the $ZnCl_2$ solutions, EDTA was added to remove the Zn^{2+} with the enzyme activity then measured over time. The results showed that the partially folded CK blocked by Zn^{2+} was refolded in the presence of EDTA to more than 80% of its initial activity. Figure 5 gives a semilogarithmic plot of the reactivation course of the partially folded CK at different EDTA concentrations. The reactivation course is a biphasic course whose rate constants can be calculated from the data. The fast and slow phase rate constants were calculated as $k_1 = 4.3 \cdot 10^{-3}$ sec^{-1} and $k_2 = 0.75 \cdot 10^{-3}$ sec^{-1} which are smaller than those for the self reactivation of urea-denatured CK ($k_1 = 9.8 \cdot 10^{-3}$ sec^{-1} and $k_2 = 0.99 \cdot 10^{-3}$ sec^{-1}) [9]. The results also showed that the EDTA concentration has little effect on the reactivation rate within a limited range, indicating that the removal of Zn^{2+} is not the rate limiting step of the reactivation.

Effect of Zn^{2+} on refolding of urea-denatured creatine kinase and refolding of partially folded CK by EDTA. The refolding of creatine kinase unfolded in 6 M urea was followed by changes in the intrinsic fluorescence of the molecules. As the molecules gradually refolded, the emission intensity decreased accompanied by a blue shift of the emission maximum, which agrees with the previous study

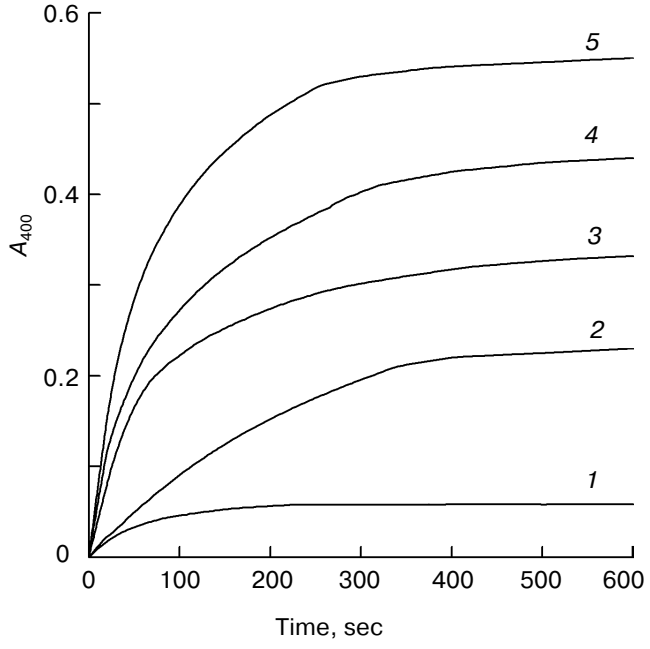


Fig. 3. Effect of different Zn^{2+} concentrations on aggregation of partially folded CK. The urea-denatured enzyme was diluted 60-fold into 30 mM Tris-HCl, pH 8.0, containing Zn^{2+} of different concentrations. Other conditions were as for Fig. 1. The final enzyme concentration was 2.0 μM. For curves *1-5*, the Zn^{2+} concentrations (μM) were 0, 3, 6, 9, and 12, respectively.

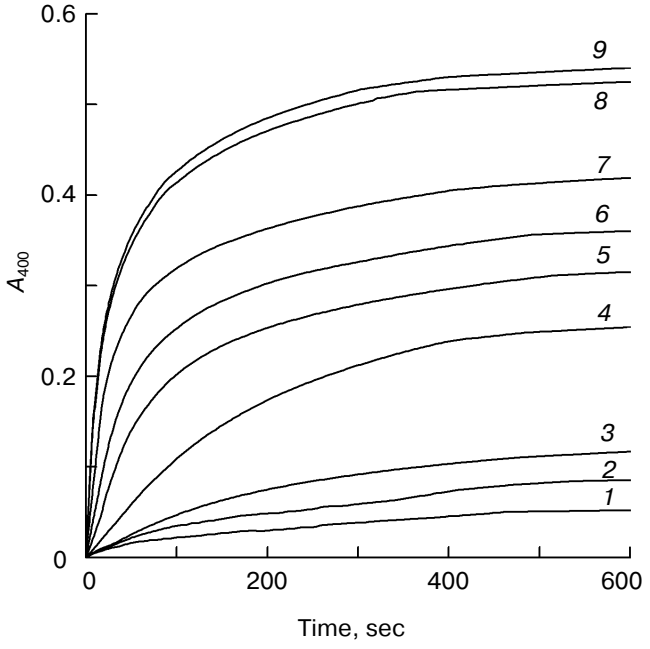


Fig. 4. Effect of temperature on aggregation of partially folded CK. The urea-denatured enzyme was diluted 60-fold into 30 mM Tris-HCl, pH 8.0, containing 20 μM Zn^{2+}. The final enzyme concentration was 2.0 μM. The temperatures for the curves *1-9* were 4, 6, 8, 10, 12, 16, 20, 25, and 37°C, respectively.

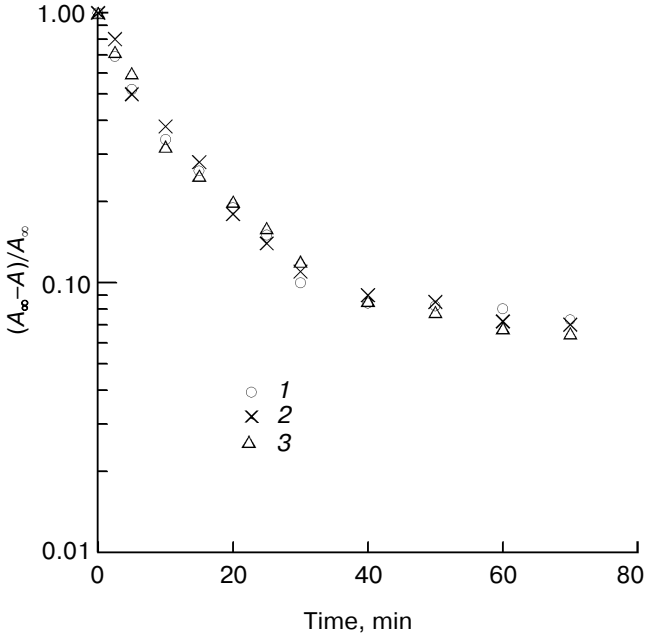


Fig. 5. Reactivation kinetics of the partially folded CK in the presence of EDTA different concentrations. The reactions were made at 25°C. EDTA concentrations were 0.05 (*1*), 0.1 (*2*), and 0.2 mM (*3*). The enzyme concentration was 2.0 μM in 30 mM Tris-HCl, pH 8.0. Enzyme activity was determined in aliquots taken at suitable time intervals.

[9]. The effect is the reverse of the fluorescence changes during denaturation in denaturant solutions [5, 6]. However, for CK refolding in the presence of Zn^{2+}, the conformational changes were only partially recovered. The results in Fig. 6 showed that after refolding of CK in the absence of Zn^{2+}, the fluorescence emission spectrum was recovered to the same intensity and the maximum emission level was recovered to the same level as the native enzyme. However, the zinc-bound enzyme was only partially recovered. Compared with the native enzyme, the fluorescence emission maximum of partially refolded zinc-bound enzyme red shifted about 5 nm, indicating that the partially refolded zinc-bound enzyme was much looser than the native enzyme. The fluorescence emission maximum of the partially refolded zinc-bound enzyme is quite close to that of the unfolding intermediate (ranging from 338 to 340 nm) [4, 6, 18, 19]. The maximum is also very similar to that of a genetically engineered fragment of the C-terminal of chicken mitochondrial CK which includes amino acid residues 168 to 380 [20]. The study using CD produced similar results (Fig. 7). The partially folded CK seems to remain at an intermediate state which also occurs in the unfolding equilibrium and initial of refolding processes. This implies that the existence of Zn^{2+} blocks further folding of CK.

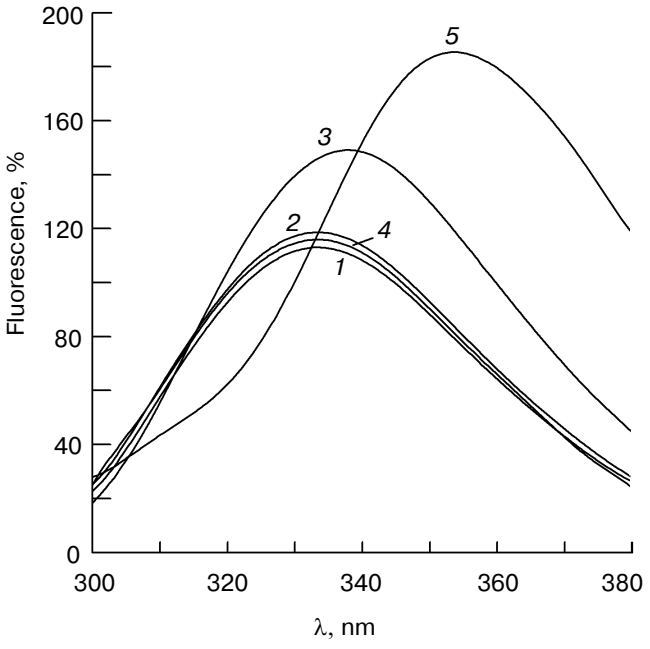


Fig. 6. Fluorescence spectra of creatine kinase treated under various conditions. The enzyme concentration was 2.0 μM in 30 mM Tris-HCl, pH 8.0. The excitation wavelength was 280 nm. Lanes: *1*) native CK; *2*) refolded CK; *3*) partially refolded CK bound by Zn^{2+}; *4*) refolded zinc-bound CK treated with EDTA; *5*) CK denatured in 6 M urea.

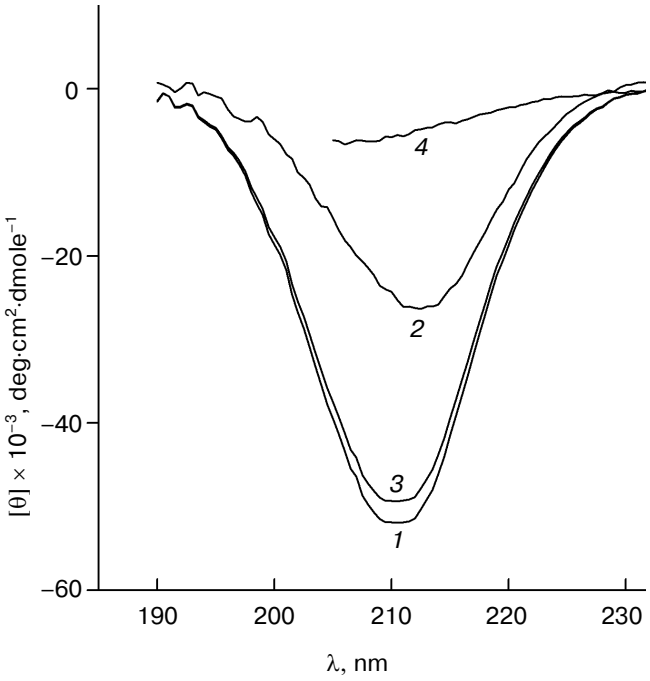


Fig. 7. CD spectra of creatine kinase treated under various conditions. The enzyme concentration was 3 μM in 30 mM Tris-HCl, pH 8.0. Lanes: *1*) native CK; *2*) the partially refolded CK bound by Zn^{2+}; *3*) refolded zinc-bound CK treated with EDTA; *4*) CK denatured in 6 M urea.

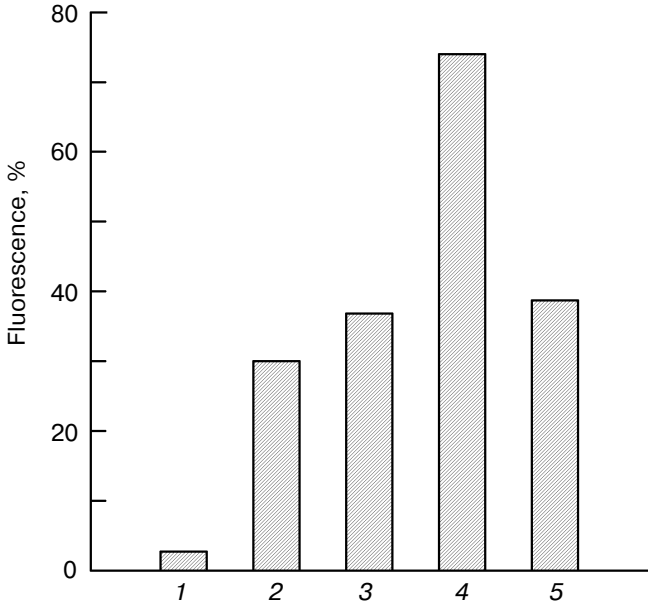


Fig. 8. ANS fluorescence of creatine kinase treated under various conditions. Various forms of creatine kinase were incubated for 20 min at 25°C with a 20-fold molar excess of ANS. The fluorescence emission intensities were recorded at 480 nm for: *1*) ANS (control); *2*) ANS + native CK; *3*) ANS + refolded CK; *4*) ANS + the partially refolded CK bound by Zn^{2+}; *5*) ANS + refolded zinc-bound creatine kinase treated with EDTA. The concentrations of the various forms of CK were all 2.0 μM.

The fluorescence emission of ANS is known to increase when the dye binds to the hydrophobic regions of a protein [21]. Figure 8 shows the ANS fluorescence of CK refolding in the absence and in the presence of Zn^{2+}. The partially folded CK in the presence of Zn^{2+} shows strong ANS fluorescence compared with the native and self-folded CK in the absence of Zn^{2+}, indicating that the partially folded creatine kinase contains much more hydrophobic surface which is one of the properties of the "molten globule" state. Size exclusion chromatography, used to measure the quaternary structure of the partially folded CK, suggested that it was monomeric (Fig. 9).

The results in Fig. 6 showed that after treating the partially refolded zinc-bound enzyme with EDTA, the fluorescence emission spectrum was recovered to the same intensity level and peak position as the native enzyme. The CD spectra studies showed that the secondary structure of the partially folded CK was also recovered (Fig. 7). The results in Figs. 8 and 9 also show that the CK conformation was recovered in conjunction with the reactivation. Figure 10 shows the intrinsic fluorescence emission intensity at 333 nm measured at different times after EDTA was added to the partially folded CK. A semilogarithmic plot of the fluorescence intensity versus time gives a curve which can be resolved into two straight lines, indicating that the refolding course is a biphasic course. The fast and slow phase rate constants were calculated as $k_1 = 2.6 \cdot 10^{-3}$ sec^{-1} and $k_2 = 0.43 \cdot 10^{-3}$ sec^{-1}, respectively.

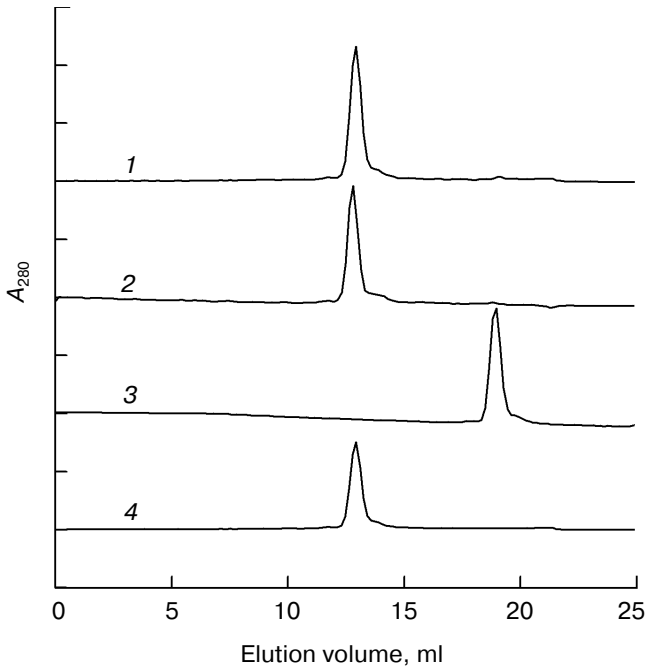


Fig. 9. Size exclusion chromatography elution profiles of creatine kinase treated under various conditions. CK, 100 μl 2.0 μM solution was loaded on a Superdex^{TM} 200 HR 10/30 column at 8°C in 30 mM Tris-HCl buffer, pH 8.0. Curves *1-4* are native CK, refolded CK, the partially refolded CK bound by Zn^{2+}, and refolded zinc-bound CK treated with EDTA, respectively.

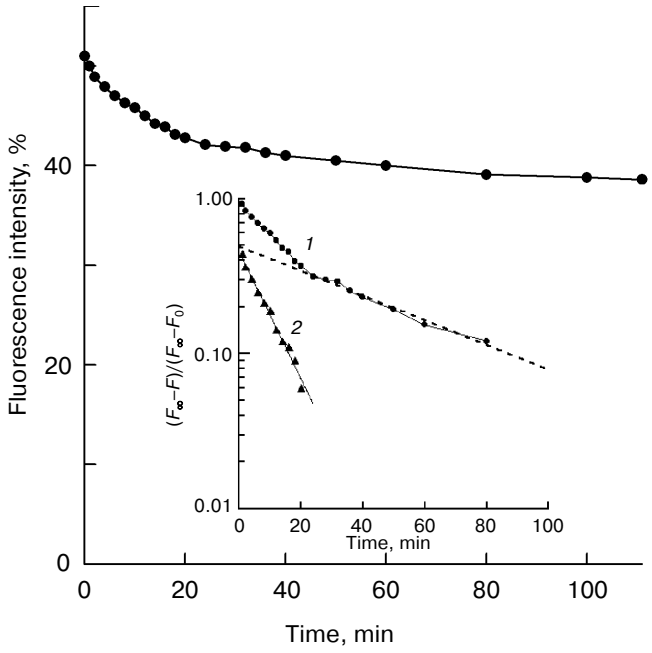
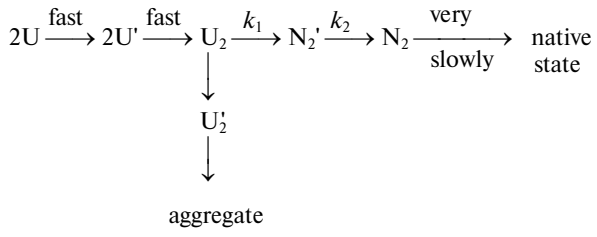


Fig. 10. Kinetic course of fluorescence emission intensity during refolding of newly prepared partially refolded CK. Experimental conditions were as for Fig. 5. The final concentrations were 2.0 μM for the enzyme and 0.2 mM for EDTA. Emission wavelength was 333 nm. The inset shows a semilogarithmic plot: *1*) experimental data; *2*) points obtained by subtracting the contribution of the slow phase from the original curve.

DISCUSSION

Zinc is an essential metal element in physiology and is an integral component of a large number of proteins involved in a multiplicity of vital processes accounting for its essentiality in metabolism, transmission of the genetic message, growth, and development [22, 23]. In addition to its roles in catalysis and gene expression, zinc stabilizes the structures of proteins and nucleic acids, preserves the integrity of subcellular organelles, participates in transport processes, and plays important roles in viral and immune phenomena. Its nutritional essentiality has focused attention on the pathology and clinical consequences of both its deficiency and toxicity. Its chemical stability, stereochemical flexibility and amphoteric properties may contribute to its importance in life [24]. In the past, zinc was thought to be nontoxic. However, recent *in vivo* studies revealed the toxicity of zinc in living organisms [25, 26]; but the detailed mechanism is not yet well established. It has been reported that zinc can induce aggregation and/or hydrophobic exposure of many proteins, including GroEL [27], calregulin [28], aminoacylase [29], myelin basic-protein [30], and calsequestrin [31]. Experiments have shown that zinc can induce aggregation of soluble amyloid β-peptide at pH 7.4 *in vitro* and that this reaction is totally reversible with chelation [32]. Zinc may also play a role in the formation of senile plaques which contain amyloid β-peptide in Alzheimer's disease [33]. Tong et al. found that zinc over 0.1 mM denatured and inactivated rabbit muscle creatine kinase and zinc over 0.05 mM induced CK aggregation at 37°C which may be the reason for the CK abnormality in Alzheimer's disease [17].

The observed results in our experiments showed that the reactivation and refolding processes of rabbit muscle creatine kinase can be blocked by zinc at very low concentrations (less than 12 μM). As a result, the CK remained in a partially folded state. Rabbit muscle CK is a perfect model to study the reactivation and refolding of proteins. Under suitable conditions, both the native conformation and the activity of the enzyme unfolded in chemical denaturants can be quantitatively recovered. The model for the refolding of CK has been well established based on previous studies [9, 34, 35]:



Here U is an extensively unfolded CK subunit; U′ is an inactive, monomeric folding intermediate; U_2 is an inactive dimeric folding intermediate; U_2' is a misfolded dimer; N_2' is a partially active dimeric folding intermediate; and N_2 is an active dimer. Our recent experiments showed that both GroEL, a molecular chaperon, and casein, which is believed to be a protein with high exposed hydrophobic regions content can block the refolding of CK. A dimeric folding intermediate can bind with GroEL [10] and a monomeric intermediate can bind with casein (unpublished data). Wang et al. reported that CK, after being modified by thiol reagent in the denatured state, cannot be refolded but remained in a partially folded state [12]. The characterizations of the partially refolded state by Yang et al. suggested that the conformation of the partially folded modified CK resembled that of a monomeric intermediate in the refolding of CK [36]. The characteristics of the partially folded state of CK blocked by zinc were measured in this experiment. The partially folded state was shown to differ from the native state and from the urea fully denatured state. The maximum emission wavelengths for the native CK, urea fully denatured CK and the partially folded state are 333, 355, and 338 nm, respectively. The significant decrease of ellipticity showed that the secondary structure of the partially folded state also differs from that of native CK. The ANS fluorescence results showed that the partially folded state has more hydrophobic surface exposure than the native enzyme. The molten globule state, which has been observed in the refolding of a number of proteins as both a kinetic and equilibrium intermediate [37], has the following characteristic conformational properties: it contains all or part of the secondary structure of the protein in conjunction with a relatively compact, but flexible tertiary structure with more hydrophobic surface exposure. Comparing these characteristics to those of the partially folded CK suggests that the conformation of the partially folded CK blocked by zinc resembles that of a molten globule state in the CK folding pathway. In addition, the result of size exclusion chromatography suggested that the partially folded state of CK is monomeric.

Recently, the model for the formation and structure of protein aggregate was well reviewed that specific intermolecular interactions between hydrophobic surfaces of structural subunit in partially folded intermediates are responsible for the aggregation [38]. Brazil et al. suggested that aggregation of GroEL was possibly due to the increased hydrophobic surface or a change in the electrostatic repulsion. Their study revealed a strong exposure of hydrophobic surface as measured by ANS fluorescence [27]. Similar results were also observed in our study. Therefore, we propose a possible explanation for aggregation of CK refolding in the presence of zinc. Zinc blocked the refolding process of urea-denatured CK and the interaction between the hydrophobic clusters of the partially folded CK is then the main force for

aggregation. At the same time, the positive charges of the CK-bound zinc might counteract the negative charges of CK to minimize the electrostatic repulsion. We also found that the aggregation of partially folded zinc-bound CK was very temperature dependent. Aggregation cannot take place at temperatures below 8°C, which is much lower than the physiological temperature. Temperatures above 8°C promote strong aggregation. These phenomena are due to the increased collision possibility and the increased hydrophobic driving force [39].

The mechanism by which zinc blocks the refolding process of urea-denatured CK is still not clear. We propose that zinc can be bound by some reactive groups exposed to the solvent in the unfolded state which results in the blockage of refolding. The refolding course of CK was fully blocked when the molecular ratio of zinc ions to CK monomers was more than three, showing that each CK monomer can be bound by three zinc ions. The results of fluorescence emission spectra, CD spectra, size exclusion chromatography, and activity measurements all show that after chelation of zinc by EDTA, the partially folded CK can be reactivated and refolded into the native state. The rate of reactivation is independent of the EDTA concentrations. Therefore, the binding of zinc ions by unfolded CK is reversible and the removal of the bound zinc ions is not a rate limiting step of the refolding process. The refolding and reactivation courses of zinc-blocked CK are quite similar to those observed in the refolding of urea-denatured CK. It was previously reported that the refolding process and the initial course of reactivation both consist of two first-order reactions [9]. The results in our experiments (Figs. 5 and 10) show that the refolding and reactivation of the partially folded CK follow a biphasic course. The fast and slow phase rate constants of the reactivation and refolding process calculated from the data in Figs. 5 and 10 are a little slower than those of reactivation and refolding of urea-denatured CK.

The effect of zinc on CK was unique compared with other monovalent cations (K^+ and Na^+) and bivalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) that play important roles in living organisms. The zinc concentration range used in this experiment is much less than that used by other investigators to study the physiological effects of zinc (the zinc concentration commonly used in studies of amyloid β-peptide aggregation is 100 μM [32, 33], the zinc concentration used by Tong et al. was over 100 μM, too [17]). Our results show that the existence of zinc ions at very low concentrations might be toxic to some proteins such as CK and that the toxicity may result from the disruption of their proper folding. On the other hand, molecular chaperones and foldases such as peptidyl-prolyl-*cis-trans*-isomerase and protein disulfide isomerase existing in cells may interact with these proteins to protect their folding processes from disruption by zinc.

REFERENCES

1. Watts, D. C. (1973) *Enzymes*, **8**, 383-445.
2. Jacobus, W. E. (1985) *Annu. Rev. Physiol.*, **47**, 707-725.
3. Yao, Q. Z., Tian, M., and Tsou, C. L. (1984) *Biochemistry*, **23**, 2740-2744.
4. Couthon, F., Clottes, E., Ebel, C., and Vial, C. (1995) *Eur. J. Biochem.*, **234**, 160-170.
5. Clotts, E., Leydier, C., Couthon, F., Marcillat, O., and Christian, V. (1997) *Biochim. Biophys. Acta*, **1338**, 37-46.
6. Gross, M., Lustig, A., Wallimann, T., and Furter, R. (1995) *Biochemistry*, **34**, 10350-10357.
7. Hou, L. X., Zhou, H. M., Yao, Q. Z., and Tsou, C. L. (1983) *Acta Biochim. Biophys. Sin.*, **15**, 393-397.
8. Grossman, S. H. (1984) *Biochim. Biophys. Acta*, **785**, 61-67.
9. Zhou, H. M., and Tsou, C. L. (1986) *Biochim. Biophys. Acta*, **869**, 69-74.
10. Li, S., Bai, J. H., Park, Y. D., and Zhou, H. M. (2001) *Int. J. Biochem. Cell Biol.*, **33**, 279-286.
11. Morris, G. E., Frost, L. C., Newport, P. A., and Hudson, N. (1987) *Biochem. J.*, **248**, 53-59.
12. Wang, H. R., Bai, J. H., Zheng, S. Y., Wang, Z. X., and Zhou, H. M. (1996) *Biochem. Biophys. Res. Commun.*, **221**, 174-180.
13. Park, Y. D., and Zhou, H. M. (2000) *J. Protein Chem.*, **19**, 193-198.
14. Yao, Q. Z., Zhou, H. M., Hou, L. X., and Tsou, C. L. (1982) *Sci. Sin.*, **25B**, 1296-1302.
15. Noda, L., Kuby, S. A., and Lardy, H. (1954) *Meth. Enzymol.*, **2**, 605-610.
16. Yao, Q. Z., Hou, L. X., Zhou, H. M., and Tsou, C. L. (1982) *Sci. Sin.*, **25B**, 1186-1198.
17. Tong, X. F., Zeng, X. G., and Zhou, H. M. (2000) *J. Protein Chem.*, **19**, 551-560.
18. Raimbault, C., Couthon, F., Vial, C., and Buthet, R. (1995) *Eur. J. Biochem.*, **234**, 570-578.
19. Fan, Y. X., Zhou, J. M., Kihara, H., and Tsou, C. L. (1997) *FASEB J.*, **11**, 254.
20. Gross, M., Wyss, M., Furter-Graves, E. M., Wallimann, T., and Furter, R. (1996) *Protein Sci.*, **5**, 320-330.
21. Stryer, L. (1965) *J. Mol. Biol.*, **13**, 482-495.
22. Vallee, B. L., and Auld, D. S. (1990) *Biochemistry*, **29**, 5647-5659.
23. Vallee, B. L., and Auld, D. S. (1993) *Biochemistry*, **32**, 6493-6500.
24. Vallee, B. L., and Auld, D. S. (1995) *EXS*, **73**, 259-277.
25. Schmalz, G., Arenholtbindslev, D., Hiller, K. A., and Schweikl, H. (1997) *Eur. J. Oral Sci.*, **105**, 86-91.
26. Chaoui, A., Mazhoudi, S., Ghorbal, M. H., and Elferjani, E. (1997) *Plant Sci.*, **127**, 139-147.
27. Brazil, T., Ybarra, J., and Horowitz, P. M. (1998) *J. Biol. Chem.*, **273**, 3257-3263.
28. Khanna, N. C., Tokuda, M., and Waisman, D. M. (1986) *J. Biol. Chem.*, **261**, 8883-8887.
29. Wu, H. B., and Tsou, C. L. (1993) *Biochem. J.*, **296**, 435-441.
30. Cavatorta, P., Giovanelli, S., Bobba A., Riccio, P., Szabo, A. G., and Quagliariello, E. (1994) *Biophys. J.*, **66**, 1174-1179.
31. Baksh, S., Spamer, C., Oikawa, K., McCubbin, W. D., Heilmann, C., Kay, C. M., and Michalak, M. (1995) *Biochem. Biophys. Res. Commun.*, **209**, 310-315.

32. Atwood, C. S., Moir, R. D., Huang, X., Scarpa, R. C., Bacarra, N. M. E., Romano, D. M., Hartshorn, M. A., Tanzi, R. E., and Bush, A. I. (1998) *J. Biol. Chem.*, **273**, 12817-12826.
33. Brown, A. M., Tummolo, D. M., Rhodes, K. J., Hofmann, J. R., Jacobsen, J. S., and Sonnenberg-Reines, J. (1997) *J. Neurochem.*, **69**, 1204-1212.
34. Yang, H. P., Zhong, H. N., and Zhou, H. M. (1997) *Biochim. Biophys. Acta*, **1338**, 147-150.
35. Wang, Z. F., Yang, Y., and Zhou, H. M. (1995) *Biochimie*, **77**, 953-956.
36. Yang, Y., Park, Y. D., Yu, T. W., and Zhou, H. M. (1999) *Biochem. Biophys. Res. Commun.*, **259**, 450-454.
37. Jorg, M., Thomas, L., Raina, B., Andrea, S., Arthur, L. H., and Hartl, F. U. (1991) *Nature*, **352**, 36-42.
38. Fink, A. L. (1998) *Fold. Des.*, **3**, 9-23.
39. Zettlmeissl, G., Rudolph, R., and Jaenicke, R. (1979) *Biochemistry*, **18**, 5567-5571.