Appl Biochem Biotechnol (2009) 152:15–28
DOI 10.1007/s12010-008-8282-4

Effect of Cysteine Modification on Creatine Kinase Aggregation

He-Chang Zou · Zhi-Rong Lü · Ye-Jing Wang · Ying-Mei Zhang · Fei Zou · Yong-Doo Park

Received: 1 November 2007 / Accepted: 6 May 2008 /
Published online: 12 June 2008

Abstract We studied the effect of cysteine modification on creatine kinase (CK) aggregation as well as the kinetics of the process. We found that CK aggregation was modulated by different pH conditions in the presence of Zn^{2+}, which is a CK aggregation trigger. The CK aggregation followed first-order kinetics, and this was effectively suppressed in acidic conditions. Even under the acidic condition, cysteine modification at the active site with using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) induced conspicuous aggregation in a dose-dependent manner. This aggregation process is directly related with decreasing the change of transition free-energy ($\Delta\Delta G_{AG}$). When dithiothreitol (DTT) was applied to the reaction system, the aggregates were significantly reduced: DTT treatment can fully reactivate (higher than 80%) the inactive CK that was separated from CK aggregates, whereas CK was completely inactivated by Zn^{2+} and DTNB. Some added osmolytes such as glycine and proline were able to successfully block CK aggregation by increasing the $\Delta\Delta G_{AG}$ as well as by suppressing the hydrophobic CK surface. Our study suggests the effect of cysteine modification on the unfavorable aggregation of CK and on the aggregation process that followed first-order kinetics with the accompanying changes of transitional free energy and disruptions of the hydrophobic surface. We also demonstrate the successful protocol to block the aggregation.

Keywords Creatine kinase · Aggregation · Cysteine modification · Active site · Osmolytes

H.-C. Zou
Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, People's Republic of China

Z.-R. Lü · F. Zou · Y.-D. Park (✉)
Department of Environmental Health, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, People's Republic of China
e-mail: parkyd@hotmail.com

Y.-J. Wang · Y.-M. Zhang
School of Life Science, Lanzhou University, Lanzhou 730000, People's Republic of China

Y.-D. Park
Yangtze Delta Region Institute of Tsinghua University, Jiaxing 314050, People's Republic of China

Abbreviations

CK	muscle type creatine kinase
DTNB	5,5′-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
MES	2-[N-morpholino]ethanesulfonic acid
ANS	1-anilinonaphthalene-8-sulfonate

Introduction

Creatine kinase (CK; ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine, and this results in phosphocreatine and MgADP. Therefore, CK is critically involved in energy metabolism as well as homeostasis. It has been shown that several types of CK are expressed in various tissues: the muscle and brain types of CK are the most common, and three different isoenzymes that include CK-MM (the muscle type homodimer), CK-BB (the brain type homodimer), and CK-MB (the muscle plus brain type heterodimer) originate from these two common types. Besides that, two mitochondrial types of CK such as the ubiquitous and sarcomeric forms are also found in tissues [1, 2]. Among the different types of CK, the muscle type CK is a good model to use for studying protein folding and aggregation because of several of its characteristics: (1) it is a dimer consisting of two identical subunits (the N-terminal domain with about 100 residues and the C-terminal domain with about 250 residues) [3], (2) extensively denatured CK can be spontaneously refolded with restoration of its enzymatic activity in the absence of any external assistance [4], (3) several intermediates are involved in its folding pathway [5–7], (4) the tertiary structure can be easily measured by monitoring the activity change [8], and (5) denatured CK acts as a binding partner for studying protein–protein interactions during refolding and aggregation [9–11].

It has generally been recognized that the CK active site is very flexible, and this property is the basis for the theory of active site flexibility [12–15]. CK contains one cysteine at the active site per subunit, and a simple cysteine modification at the native state of CK by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) leads to complete inactivation of the enzyme without the use of any inhibitors [16]. Among the free forms of cysteine, only one form with a specific active site is essential for catalytic activity. However, the relationship between active site flexibility and the role of the cysteine residue in the active site pocket on CK aggregation has never been clarified. CK aggregation is a phenomenon that is competitive between different states of correct folding and misfolding. Tong et al. [17] previously reported that Zn^{2+} induced the inactivation and aggregation of CK by exposing the hydrophobic surface with the accompanying conformational changes, and this occurred in a dose-dependent and temperature-dependent manner. This study implied that Zn^{2+} could be used to trigger aggregation to the native state of CK.

In this study, we determined the relationship between aggregation and the nature of the flexible CK active site. As the active site of CK is the biggest and the most important part to the overall structure, studying the responses of the flexible active site to outer stimulation is important, in addition to determining its enzymatic catalysis. To conduct this study, we set up proper reaction conditions by using Zn^{2+} as well as modulating the pH condition before performing cysteine modification by DTNB. We found that CK aggregation was further promoted by DTNB-mediated cysteine modification at the active site in the presence of Zn^{2+} due to the increased hydrophobic surface, and this suggests that flexibility at the active

site might play a key role for aggregation. To confirm these results, dithiothreitol (DTT) and osmolytes such as glycine and proline were applied to block aggregation, and we found that suppression of CK aggregation is directly related with the changes of transitional free-energy ($\Delta\Delta G_{AG}$) as well as being related to the hydrophobicity of the CK structure. We provide here new information regarding the relationship between enzyme aggregation and a flexible active site; further, the aggregation followed first-order kinetic behavior, which is involved with the enzyme's stability changes, as indicated by $\Delta\Delta G_{AG}$.

Materials and Methods

Materials

Rabbit muscle CK was purified as described previously [18]. Creatine, ATP, DTT, magnesium acetate, thymol blue, zinc acetate, ANS, and DTNB were all purchased from Sigma in the USA. All the other reagents were local products of a pure analytical grade.

CK Assay

The CK activity was measured by following the proton generation during the reaction of ATP and creatine at 597 nm at 25 °C using the indicator thymol blue. The substrate was composed of 24 mM creatine, 4 mM ATP, 8 mM magnesium acetate, 0.01% thymol blue, and 5 mM glycine–NaOH buffer, pH 9.0. The reaction volume was 1 ml, and 10 μl of enzyme solution was added to the substrate system to measure the activity. Absorption was measured with a Perkin Elmer Lambda Bio U/V spectrophotometer. The enzyme concentration was determined using the absorption coefficient, $A^{1\%}_{1\,\mathrm{cm}} = 8.8$ and the Bradford method.

Kinetic Analysis

Equations were adopted from the previously reported methods for the kinetic analysis of CK aggregation [19, 20].

$$\Delta\mathrm{AG} = \mathrm{AG}_\infty - \mathrm{AG}_t \tag{1}$$

where AG_∞ is the absorbance at the end of the aggregation reaction before reaching the precipitation state and AG_t is the absorbance at time t during aggregation. The experimental data were fitted to first-order expressions as:

$$\Delta\mathrm{AG} = \exp\left(-k_{\mathrm{AG}}/t\right) \tag{2}$$

$$\Delta\mathrm{AG} = P_1 \exp\left(-k_{\mathrm{AG1}}/t\right) + P_2 \exp\left(-k_{\mathrm{AG2}}/t\right) \tag{3}$$

where k_{AG} is the rate constant for a monophasic reaction (Eq. 2). P_1 and P_2 indicate the fractions reacting with the rate constants k_{AG1} and k_{AG2}, respectively, for a biphasic reaction (Eq. 3). The change of transition free energy during aggregation in the presence of an additive is expressed as:

$$\Delta\Delta G_{\mathrm{AG}} = RT \ln\left(k_{\mathrm{AG,none}}/k_{\mathrm{AG,additive}}\right) \tag{4}$$

Intrinsic and ANS Binding Fluorescence Measurements

The intrinsic fluorescence emission spectra were measured with an F-2500 fluorescence spectrophotometer using a 1-cm path length cuvette. An excitation wavelength of 280 nm was used for measuring the tryptophan fluorescence, and the emission wavelengths ranged between 300 and 400 nm. To probe the hydrophobic surface changes, the fluorescence spectra changes were studied by labeling with 40 μM ANS for 30 min before measurement. An excitation wavelength of 390 nm was used for the ANS binding fluorescence, and the emission wavelength ranged from 400 to 600 nm.

Results

The Effect of pH on CK Aggregation in the Presence of Zn^{2+}

In addition to the previous results [17], we found that CK aggregation by Zn^{2+} is closely related with the pH condition: CK aggregation was conspicuously suppressed under the condition of pH 6.5 (Fig. 1A). CK was aggregated quickly at the initial phase at a pH of 7.0 and 7.2 compared to the more acidic conditions, indicating that Zn^{2+} is preferably reactive to CK with increasing pH values and this reaction was more favorable at a neutral pH condition than at slightly acidic conditions. To determine the tertiary structural changes of CK at pH 6.0 and 6.5 in the presence of 5 mM Zn^{2+}, we measured the intrinsic fluorescence spectra (Fig. 1B). The results showed that the intrinsic fluorescence spectra were changed with Zn^{2+} treatment both at pH 6.0 and 6.5 compared to the native state: the maximum fluorescence intensities were increased (59% for pH 6.0; 30% for pH 6.5), and the maximum wavelengths were red-shifted 3 nm for both the pH values, respectively.

Our subsequent kinetic analyses showed that CK had a tendency to follow a kinetic time course (Fig. 2A). Semi-logarithmic plot analysis showed that two kinds of first-order kinetics were observed: biphasic kinetics was noted at pH 7.2 and monophasic kinetics was noted at pH 7.0 to 6.5 (Fig. 2B and C). The CK aggregation rate constants showed exponent increases with the increasing pH values (Fig. 2D).

The Effect of DTNB Modification on CK Aggregation in the Presence of Zn^{2+}

Under the condition of pH 6.5 where CK aggregation was efficiently suppressed even in the presence of 5 mM Zn^{2+}, we found that cysteine modification at the active site could significantly promote CK aggregation (Fig. 3). This implied that the cysteine modification at the active sites might trigger and/or promote CK aggregation if an additional factor is involved such as Zn^{2+}. As observed in Fig. 3A, the sample pretreated with DTNB was conspicuously aggregated in the presence of Zn^{2+} in a dose-dependent manner. The kinetic analysis also showed that this reaction followed the monophasic kinetic order (Fig. 3B). Increasing the cysteine residue modification via DTNB was accompanied by decreasing the transient free-energy changes at a fixed concentration of Zn^{2+} (Fig. 3C). Thus, the CK stability was drastically decreased, and this resulted in the inner binding of CK molecules. The above results suggested that a combination of cysteine modification and Zn^{2+}, even at an acidic buffer condition, could effectively promote CK aggregation.

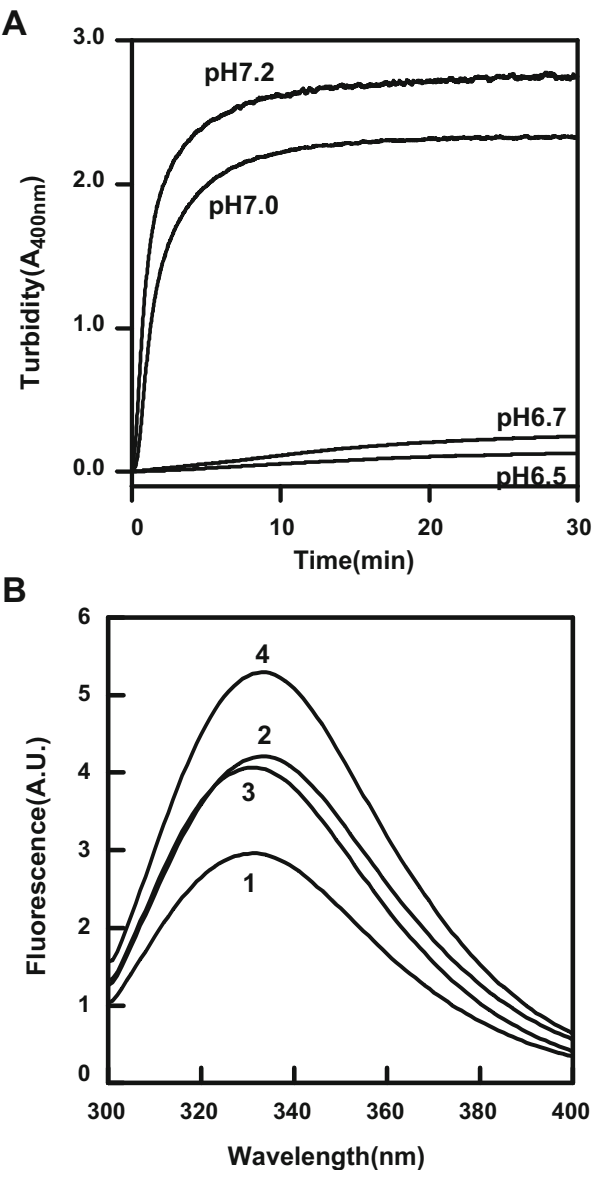

Fig. 1 CK aggregation and the conformation changes in the presence of Zn^{2+} at the various pH conditions. **A** The aggregation experiments were conducted at different pHs; these experiments were done using 50 mM Tris–HCl buffers labeled as 7.2 and 7.0 and using 50 mM MES buffers labeled as 6.7 and 6.5 on the curves, respectively. The final concentrations for CK and Zn^{2+} were 10 μM and 5.0 mM, respectively. The incubating temperature was 37 °C. **B** The intrinsic fluorescence spectra of CK were measured with an excitation wavelength of 280 nm. Label *1*: native CK in 50 mM MES buffer pH 6.0, label *2*: 5 mM of the Zn^{2+}-treated CK in sample 1, label *3*: native CK in 50 mM MES buffer pH 6.5, and label *4*: 5 mM of the Zn^{2+}-treated CK in sample 3. The final enzyme concentrations were 2.0 μM

In addition, the number of free thiol groups (–SH) of CK in the presence of 5 mM Zn^{2+} at pH 6.5 was detected according to the method by Okonjo et al. [21]. The results showed that two –SH groups for the native CK and eight –SH groups for the fully denatured CK using 3 M guanidine hydrochloride at pH 6.5 were detected, while four –SH groups were detected for the 5 mM Zn^{2+}-treated CK at pH 6.5. These results implied that the partial conformational changes and exposure of the –SH groups of CK in the presence of Zn^{2+} were induced, and these exposed –SH groups could be easily interacted with DTNB, resulting in the stability changes of CK.

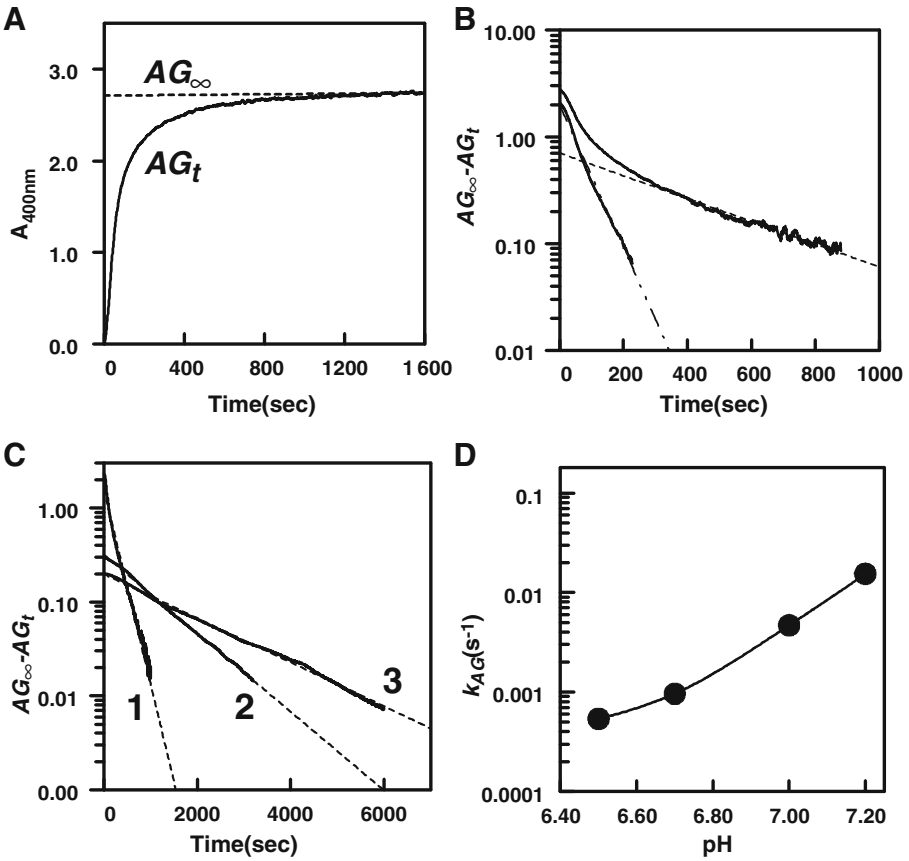

Fig. 2 Kinetic analysis of CK aggregation in the presence of 5 mM Zn^{2+}. **A** Kinetic analysis of CK aggregation at pH 7.2 according to Eq. 1. **B** A semi-logarithmic plot of CK aggregation at pH 7.2. The biphasic rate constants for k_{AG1} (fast, *dashed line*) and k_{AG2} (slow, *solid-dashed line*) were calculated from the data (**A**). **C** Semi-logarithmic plots of CK aggregations at pH 7.0 (label *1*), 6.7 (label *2*), and 6.5 (label *3*), respectively. The monophasic rate constants were calculated in a fashion similar to **A**. **D** Plot of the rate constant (k_{AG}) versus the pH values. The data was collected from **B** and **C**

The Effect of Zn^{2+} on the DTNB-Modified CK

To further verify the relationship between Zn^{2+} and the DTNB modification, we next conducted cysteine modification at a fixed DTNB concentration (75 μM), and then we added different concentrations of Zn^{2+} to the reaction solution. The result showed that the DTNB-modified CK was significantly aggregated in a Zn^{2+} dose-dependent manner (Fig. 4A). This result indicated that even a low concentration of Zn^{2+} (less than 1 mM) acted as an aggregation trigger compared to the relatively high concentration in which 5 mM Zn^{2+} at pH 6.5 hardly induced aggregation (Fig. 1). When the enzyme had its cysteine residues modified at the active site, then the aggregation was more susceptible to the added Zn^{2+}. Similar to Fig. 3, the kinetic parameters were suitably calculated, and we deduced the transition free-energy changes (Fig. 4B). Zn^{2+} also decreased the CK stability, as was monitored by decreasing the change of transition free energy. These results

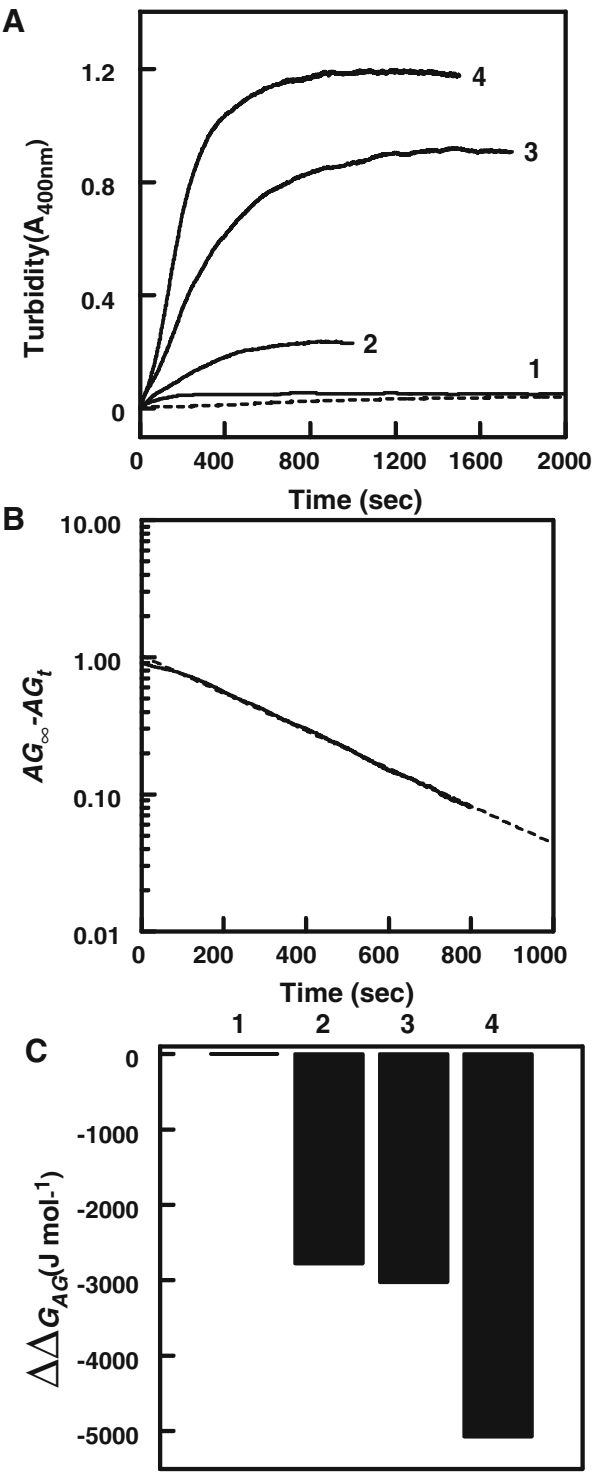

Fig. 3 Effect of DTNB on CK aggregation in a fixed Zn^{2+} concentration. **A** Cysteine modifications at the active site with different concentrations of DTNB were conducted for 1 h before the enzyme sample was added to the reaction mixture. The Zn^{2+} concentration (5 mM) was fixed in all the reaction buffers (50 mM MES buffer, pH 6.5). Labels *1* to *4* indicate the DTNB concentrations as 10, 50, 75 and 100 μM, respectively. The curve (*dashed line*) shows the CK state with incubation with 5 mM Zn^{2+} and without DTNB modification. The final CK concentration was 10 μM. The incubating temperature was 37 °C. **B** A semi-logarithmic plot of the DTNB-modified CK aggregation at pH 6.5 in the presence of Zn^{2+}. The concentrations of DTNB and Zn^{2+} were 75 μM and 5 mM, respectively. The data were obtained from **A**. **C** The change of transition free energy during CK aggregation. The labels (*1–4*) indicate the enzyme samples that were differently modified with 10, 50, 75, and 100 μM DTNB, respectively

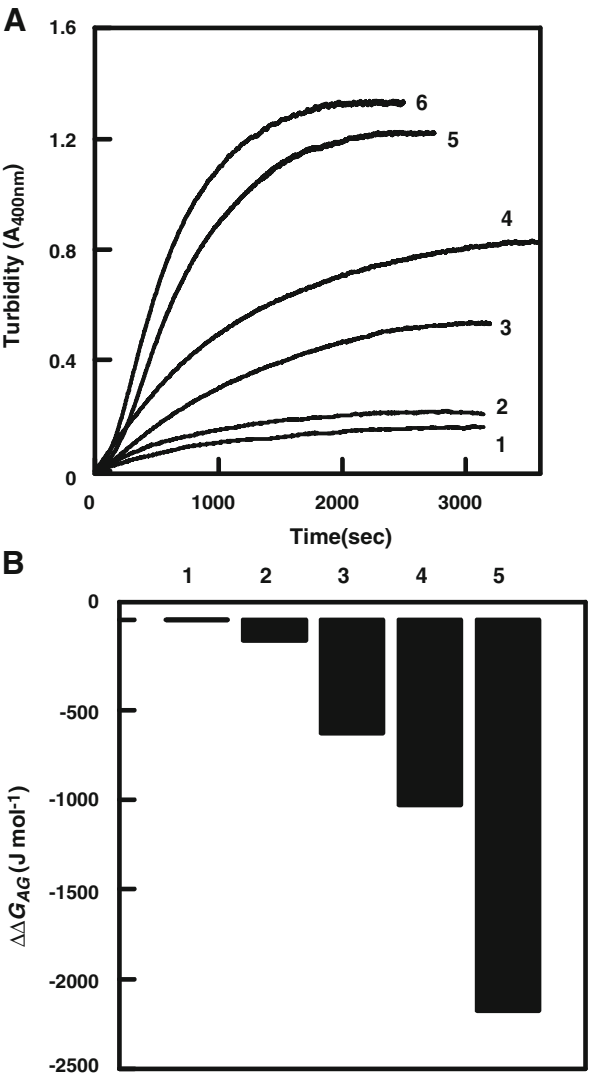

Fig. 4 Effect of Zn^{2+} on the CK aggregation in a fixed DTNB concentration. **A** The CK sample was modified with 75 μM DTNB for 1 h before applying it to the reaction mixture that contained different concentrations of Zn^{2+}. The other conditions were the same as for Fig. 3. Labels *1* to *6* indicate the Zn^{2+} concentrations as 0, 0.1, 0.2, 0.3, 0.5, and 1.0 mM, respectively. **B** The change of transition free energy during CK aggregation. Kinetic analyses for calculating the rate constants (k_{AG}) were conducted in the same fashion as for Fig. 3. The labels (*1–5*) indicate the DTNB-modified enzyme samples that were differently treated with 0.1, 0.2, 0.3, 0.5, and 1.0 mM Zn^{2+}, respectively

supported the observation that DTNB-mediated active site modification was a factor for inducing aggregation.

DTT-Mediated Suppression of CK Aggregation

DTT is known to be a strong reducing agent as well as a chelator for Zn^{2+} and Cu^{2+} ions. Therefore, the additive DTT can reduce the DTNB from the CK molecule and it can simultaneously chelate Zn^{2+} from the reaction solution. As a result, DTT might efficiently suppress CK aggregation. The result confirmed our idea that DTT not only suppressed CK aggregation in a dose-dependent manner but it also fully reactivated the CK activity from the aggregation (Fig. 5). This result reaffirmed that cysteine modification of CK was directly involved with aggregation, and its reduction suppressed the occurrence of aggregation.

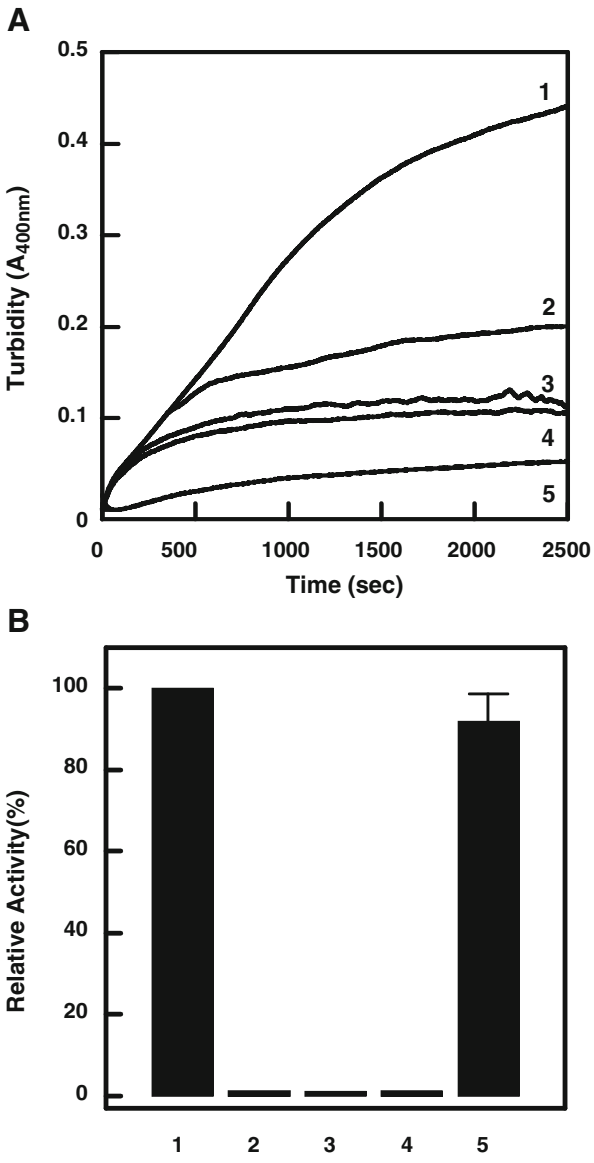

Fig. 5 Preventing CK aggregation and recovering CK activity by DTT. **A** Blocking CK aggregation by DTT. Aggregation was done in the presence of 0.2 mM Zn^{2+} by applying 75 μM of DTNB-modified CK (curve *1*). Curves *2* to *5* show the DTT treatment at 0.55, 1.1, 3.3, and 20 mM, respectively, to the incubation solutions. **B** Recovery of CK activity in the presence of DTT. Label *1*: native CK control, label *2*: 5 mM Zn^{2+}-treated CK, label *3*: 75 μM DTNB-treated CK, label *4*: 5 mM Zn^{2+} plus 75 μM DTNB-treated CK, and label *5*: 20 mM of the DTT-treated CK sample in label *4*. The data are given as mean values (n=3)

Preventing CK Aggregation with the Aid of Osmolytes Such As Glycine and Proline

It is well known that some osmolytes effectively blocked CK aggregation during refolding [22, 23]. To observe the enzyme stability changes via the changes of transitional free energy, we added osmolytes such as glycine and proline to the incubation solutions. The results showed that CK aggregation was effectively suppressed by glycine (Fig. 6) and proline (Fig. 7). Subsequent analyses showed that glycine decreased the aggregation rate constant (Fig. 6B); thus, this resulted in increases of the transitional free-energy changes in a dose-dependent manner (Fig. 6C). The ANS-binding fluorescence changes showed that glycine significantly

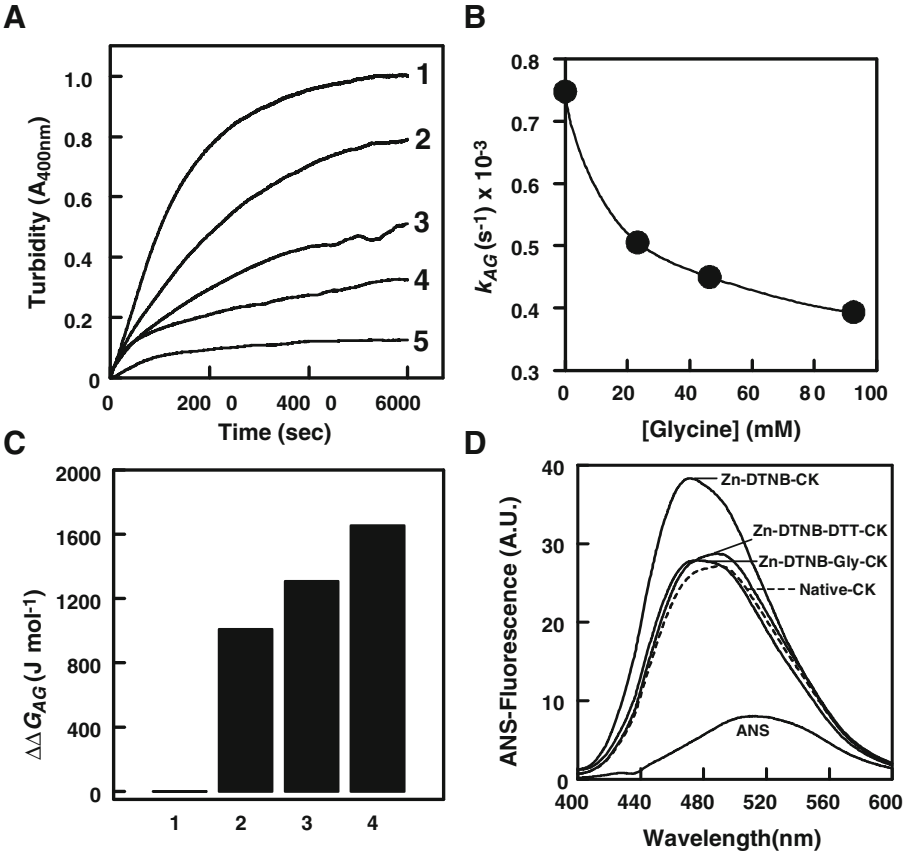

Fig. 6 Effect of glycine on CK aggregation. **A** Blocking CK aggregation by glycine. Different concentrations of glycine were applied (labels *1* to *5*) as 0, 23.2, 46.3, 92.5, and 185 mM, respectively. **B** Plot of the aggregation rate constant (k_{AG}) versus [glycine]. **C** The change of transition free energy during CK aggregation in the presence of glycine. The data were collected from **C**, and it was appropriately evaluated according to Eq. 4. The glycine concentrations used for the experiments labeled *1* to *4* were 0, 23.2, 46.3, and 92.5 mM, respectively. **D** The ANS-binding fluorescence changes. ANS (40 μM) was labeled for 30 min before measurement. An excitation wavelength of 390 nm was used for the ANS-binding fluorescence. The final concentrations for CK, DTNB, Zn^{2+}, DTT, and glycine were 2 μM, 5 μM, 0.5 mM, 20 mM, and 185 mM, respectively

suppressed the hydrophobic surfaces of CK to the native state (Fig. 6D). The additive DTT also suppressed the hydrophobic surfaces. These results indicated that CK aggregation was caused by exposure of its hydrophobic surfaces, as was triggered by Zn^{2+} and cysteine modification. Similar to glycine, proline also effectively blocked CK aggregation (Fig. 7A): it decreased the aggregation rate constant (Fig. 7B), and this resulted in increases of the transitional free-energy changes (Fig. 7C). The ANS-binding fluorescence changes confirmed that the suppression of CK's hydrophobic surface, by exposure to Zn^{2+} and DTNB modification, was directly involved with preventing aggregation (Fig. 7D). Interestingly, we additionally tested glycerol, dimethyl sulfoxide (DMSO) and sucrose, which are all known to be effective osmolytes that block CK aggregation during refolding; however, those osmolytes did not effectively suppress the CK aggregation that was due to the Zn^{2+} and cysteine modification even at high concentrations: 1.5 M for glycerol, 1.4 M

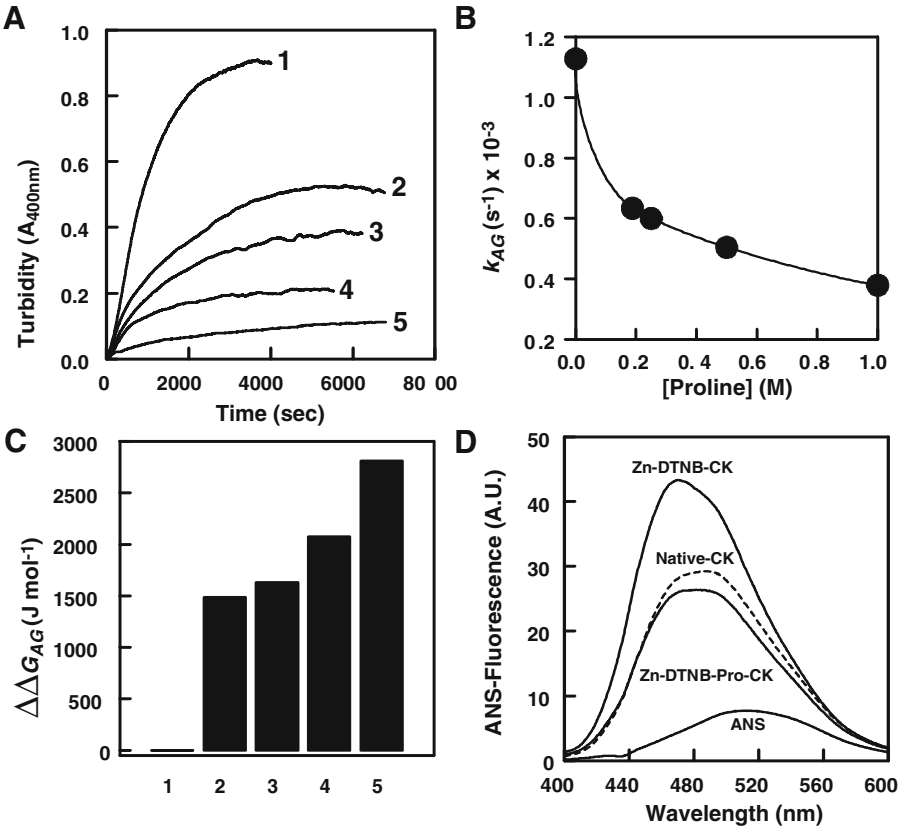

Fig. 7 Effect of proline on CK aggregation. **A** Blocking CK aggregation by proline. Different concentrations of proline were applied (labels *1* to *5*) as 0, 0.188, 0.25, 0.5, and 1.0 M, respectively. **B** Plot of the aggregation rate constant (k_{AG}) versus [proline]. **C** The change of transition free energy during CK aggregation in the presence of proline. The data were collected from **C**, and it was appropriately evaluated according to Eq. 4. The proline concentrations used for the experiments (labels *1* to *5*) were 0, 0.188, 0.25, 0.5, and 1.0 M, respectively. **D** The ANS-binding fluorescence changes. The experimental conditions were the same as for Fig. 6D except that the final concentration of proline was 0.5 M

for DMSO, and 1.2 M for sucrose (the data are not shown). Among the osmolytes we tested, glycine and proline effectively blocked CK aggregation.

Effect of Glycine and Proline on the Recovery of CK Activity in the Presence of Zn^{2+}

CK activity was inactivated by Zn^{2+} in a dose-dependent manner (Fig. 8A). To observe whether glycine and proline can prevent CK inactivation from Zn^{2+}, the various concentrations of glycine and proline were pre-incubated with CK in the presence of Zn^{2+} before assaying the activity. The results showed that the CK activity could be restored by adding glycine or proline (Fig. 8B and C). The CK activity was reactivated up to 80–100% even in the presence of 5 or 2.5 mM Zn^{2+} for which the enzyme activity was completely or partially inactivated (Fig. 8A). These results implied that a high concentration of glycine or proline may directly chelate Zn^{2+}.

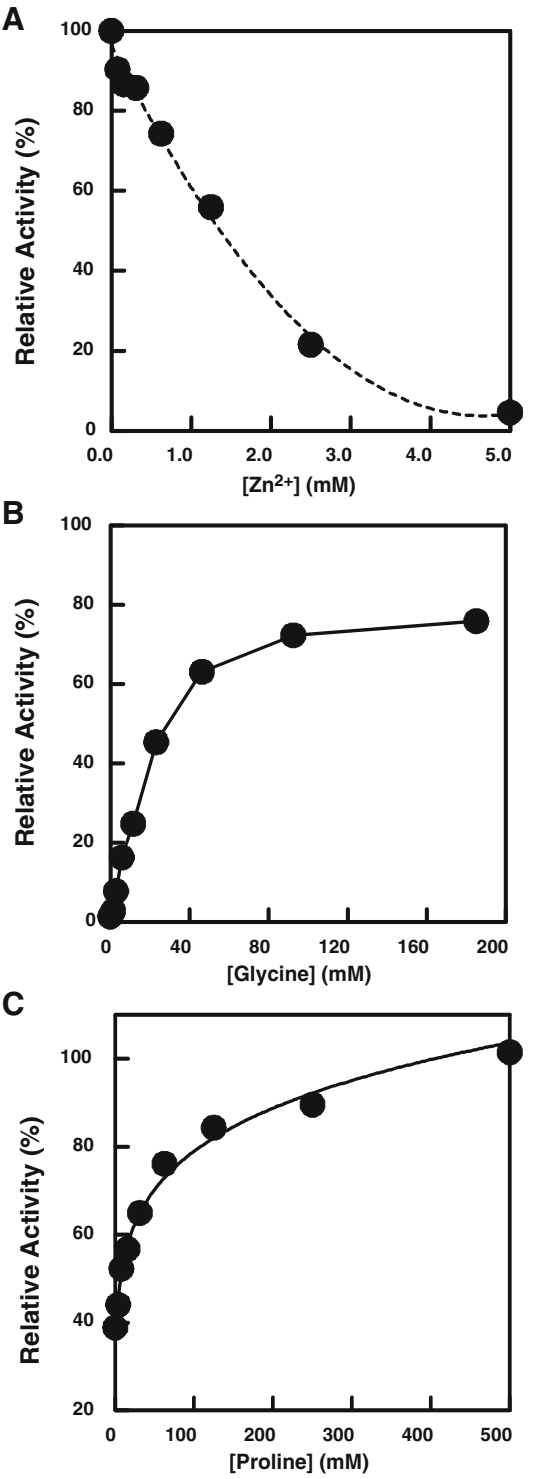

Fig. 8 Effects of glycine and proline on CK activity in the presence of Zn^{2+}. **A** The inhibitory effect of Zn^{2+} on the activity of CK. **B** Reactivating CK with glycine in the presence of 5 mM Zn^{2+}. **C** Reactivating CK with proline in the presence of 2.5 mM Zn^{2+}. Glycine and proline were pre-incubated with CK for 3 h before assay. The final concentration of CK was 2.0 μM

Discussion

CK aggregation is a frequently observed phenomenon in the presence of an inactivator such as Zn^{2+} or trifluoroethanol [17, 24] and also during thermal inactivation [24, 25], in addition to this phenomenon being observed in the folding pathways [10, 11, 20, 24, 26]. Therefore, this aggregation problem with the structural changes is very important, and the relationship between the enzyme's structure and aggregation is being gradually revealed. As the CK active site is known to be flexible [12, 13], insight on the responses of the active site to environmental factors can provide useful information in addition to providing information on catalysis and folding. In this regard, we set up the proper conditions using a CK aggregation inducer such as Zn^{2+} in the reaction solutions to test whether aggregation is affected by active site modification. If the flexibility of the active site is directly related with aggregation, then it might be dependent on the DTNB concentration at a fixed Zn^{2+} condition or it can synergically induce aggregation in the presence of Zn^{2+} with the help of DTNB modification. To prove our ideas, we adjusted and controlled the buffer condition for directly aggregating CK by Zn^{2+} via changing the pH condition. When reevaluating the Zn^{2+}-induced CK aggregation, we found that the additive Zn^{2+}-induced CK aggregation is directly affected by the pH conditions: Zn^{2+} did not induce conspicuous aggregation in the acidic conditions as compared to that in the medium buffer conditions. With this condition, the DTNB modification triggered aggregation in a dose-dependent manner with a fixed concentration of Zn^{2+}. In addition to this observation, Zn^{2+} also induced aggregation of DTNB-modified CK even under the acidic condition, implying that active site modification of CK is related to inducing aggregation.

From our studies on kinetics and thermodynamics, we found that the aggregation processes followed first-order kinetics with changes of the transitional free energy ($\Delta\Delta G_{AG}$) in the presence of two additive factors such as DTNB and Zn^{2+}. These results supported the fact that aggregation was related to the structural stability of the enzyme, and in our experiments, the instability was caused by the flexible active site. First-order kinetics has also been observed for other enzymes [27].

To correct unfavorable aggregation, which might further support our ideas, DTT and some other osmolytes such as glycine and proline were applied to block the aggregation. We found that CK aggregation was effectively suppressed by DTT, and this was accompanied by recovery of the enzyme's activity. DTT has a dual effect in our reaction conditions, that is, it can directly reduce the DTNB agent from the active site and it can also directly chelate Zn^{2+}, and so the enzyme activity was reactivated along with the suppression of aggregation. Disruption of the covalent bond that bridged between DTNB and cysteine occurred very quickly with the chelation of Zn^{2+} in the presence of DTT, and then it stopped CK from aggregating in a dose-dependent manner. In similar ways, we found that glycine and proline specifically suppressed CK aggregation in a dose-dependent manner, which was directly related with the increases of $\Delta\Delta G_{AG}$ that were observed from the subsequent kinetics. These osmolytes stabilized the enzyme, to a normal level, from embedding of the exposed hydrophobic surfaces, which was measured from the ANS-binding fluorescence spectra. The kinetic studies of aggregation provided some insights on preventing aggregation: the osmolytes could stabilize the intermediates that were very susceptible to the misfolding pathway, and this also led to the formation of aggregates in the presence of Zn^{2+} and DTNB. Glycine and proline probably not only stabilized the CK protein intermediate surface but they also enhanced the ionic strength of the buffer solution so as to adjust the random binding between the unstable partially folded CK.

Taken together, our thermodynamic and kinetic studies of CK aggregation, as well as the prevention strategies, provide new information regarding the relationship between aggregation and the flexible CK active site. The site reacted with other factors and this resulted in increased flexibility, and so this might be a trigger for inducing aggregation.

Acknowledgments This work was supported by funds from the Natural Science Foundation of China (Grant 30221003), from the Key Technologies R & D Program of Zhejiang Province (No. 2005C22053) and from the Science and Technology Bureau of Jiaxing, Zhejiang. Dr. Fei Zou was supported by a grant from the National Basic Research Program of China (No. 2006CB504100).

References

1. McLeish, M. J., & Kenyon, G. L. (2005). *Critical Reviews in Biochemistry and Molecular Biology*, *40*, 1–20.
2. Schlattner, U., Tokarska-Schlattner, M., & Wallimann, T. (2006). *Biochimica et Biophysica Acta*, *1762*, 164–180.
3. Rao, J. K., Bujacz, G., & Wlodawer, A. (1998). *FEBS Letters*, *439*, 133–137.
4. Zhou, H. M., & Tsou, C. L. (1986). *Biochimica et Biophysica Acta*, *869*, 69–74.
5. Zhang, Y. L., Fan, Y. X., Huang, G. C., Zhou, J. X., & Zhou, J. M. (1998). *Biochemical and Biophysical Research Communications*, *246*, 609–612.
6. Leydier, C., Clottes, E., Couthon, F., Marcillat, O., Ebel, C., & Vial, C. (1998). *Biochemistry*, *37*, 17579–17589.
7. Li, S., Bai, J. H., Park, Y. D., & Zhou, H. M. (2006). *Protein Science*, *15*, 171–181.
8. Park, Y. D., Ou, W. B., Yu, T. W., & Zhou, H. M. (2001). *Biochemistry and Cell Biology*, *79*, 479–487.
9. Zhao, T. J., Ou, W. B., Xie, Q., Liu, Y., Yan, Y. B., & Zhou, H. M. (2005). *Journal of Biological Chemistry*, *280*, 13470–13476.
10. Ou, W. B., Luo, W., Park, Y. D., & Zhou, H. M. (2001). *Protein Science*, *10*, 2346–2353.
11. Li, S., Bai, J. H., Park, Y. D., & Zhou, H. M. (2001). *International Journal of Biochemistry & Cell Biology*, *33*, 279–286.
12. Tsou, C. L. (1998). *Annals of the New York Academy of Sciences*, *864*, 1–8.
13. Tsou, C. L. (1993). *Science*, *262*, 380–381.
14. Grossman, S. H., & Sellers, D. S. (1998). *Biochimica et Biophysica Acta*, *1387*, 447–453.
15. Wang, Z. F., Huang, M. Q., Zou, X. M., & Zhou, H. M. (1995). *Biochimica et Biophysica Acta*, *1251*, 109–114.
16. Wang, H. R., Bai, J. H., Zheng, S. Y., Wang, Z. X., & Zhou, H. M. (1996). *Biochemical and Biophysical Research Communications*, *221*, 174–180.
17. Tong, X. F., Zeng, X. G., & Zhou, H. M. (2000). *Journal of Protein Chemistry*, *19*, 553–562.
18. Yao, Q. Z., Zhou, H. M., Hou, L. X., & Zhou, C. L. (1982). Scientia Sinica (B), *25*, 1296–1302.
19. Tams, J. W., & Welinder, K. G. (1996). *Biochemistry*, *35*, 7573–7579.
20. Hahn, H. S., Park, Y. D., Lee, J. R., Park, K. H., Kim, T. J., Yang, J. M., & Hahn, M. J. (2003). *Journal of Protein Chemistry*, *22*, 563–570.
21. Okonjo, K. O., & Adejoro, I. A. (1993). *Journal of Protein Chemistry*, *12*, 33–37.
22. Meng, F. G., Park, Y. D., & Zhou, H. M. (2001). *International Journal of Biochemistry & Cell Biology*, *33*, 701–709.
23. Ou, W. B., Park, Y. D., & Zhou, H. M. (2002). *International Journal of Biochemistry & Cell Biology*, *34*, 136–147.
24. Huang, K., Park, Y. D., Cao, Z. F., & Zhou, H. M. (2001). *Biochimica et Biophysica Acta*, *1545*, 305–313.
25. He, H. W., Zhang, J., Zhou, H. M., & Yan, Y. B. (2005). *Biophysical Journal*, *89*, 2650–2658.
26. Fedurkina, N. V., Belousova, L. V., Mitskevich, L. G., Zhou, H. M., Chang, Z., & Kurganov, B. I. (2006). *Biochemistry (Mosc)*, *71*, 325–331.
27. Kurganov, B. I. (2002). *Biochemistry (Mosc)*, *67*, 409–422.