Appl Biochem Biotechnol (2010) 160:1309–1320
DOI 10.1007/s12010-009-8574-3

Kinetics of Zn^{2+}-induced Brain Type Creatine Kinase Unfolding and Aggregation

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Received: 3 January 2009 / Accepted: 17 February 2009 /
Published online: 11 March 2009

Abstract We studied the effect of Zn^{2+} on the folding and aggregation of brain creatine kinase (CK-BB). We developed a method to purify CK-BB from rabbit brain and conducted inhibition kinetics and unfolding studies of CK-BB. Zn^{2+} conspicuously aggregated and osmolytes, such as glycine and proline, were able to suppress the formation of aggregates and protect the enzymatic activity against Zn^{2+}. These results suggest that Zn^{2+} might act as a risk factor for CK-BB in the brain under certain conditions, and some osmolytes may help CK-BB to sustain the active state when Zn^{2+} is present. Our study provides useful

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information regarding the effect of Zn^{2+} on brain-derived metabolic enzymes, especially those that are putatively related to brain disease. Furthermore, our study suggests that although Zn^{2+} may induce CK-BB inactivation and misfolding, the ability of some abundant proteins and osmolytes to chelate Zn^{2+} nonspecifically may protect CK-BB and allow it to exist in the active form.

Keywords Creatine kinase · Brain-type · Zn^{2+} · Folding · Aggregation

Abbreviations

CK-BB	homodimer brain-type of creatine kinase
ANS	1-anilinonaphthalene-8-sulfonate
CD	circular dichroism

Introduction

Creatine kinase (CK) (ATP: creatine *N*-phosphotransferase, EC 2.7.3.2) catalyzes the reversible transfer of a phosphoryl group from the substrate MgATP to creatine. This results in the creation of 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 common, and three different isoenzymes including 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. Two mitochondrial types of CK, namely the ubiquitous and sarcomeric forms, are also found in tissues [1, 2]. Little, however, is known about the folding and aggregation behavior of the brain-type of CK (CK-BB).

CK-BB is associated with several pathologies, including neurodegenerative and age-related diseases. Recently, Chang et al. [3] reported an important role for CK-BB in osteoclast-mediated bone resorption found using a proteomics approach. They found that CK-BB is greatly increased during osteoclastogenesis and suggested that it represents a potential target for antiresorptive drug development. CK-BB interacts with the potassium chloride co-transporter 3, which is involved in the pathophysiology of hereditary motor and sensory neuropathy with agenesis of the corpus callosum [4]. Previous studies [5, 6] have reported that CK-BB is involved in Alzheimer's disease (AD) as an oxidatively modified protein. This suggests that oxidatively damaged CK-BB may be associated with aging and age-related neurodegenerative disorders such as AD. Zn^{2+} has many various physiological roles: It is a constituent of many enzymes and proteins, a transcriptional factor, a substrate for synaptic signaling, and it contributes to normal growth and development, immune functions, and wound healing [7–9]. However, regardless of its various roles, it is controversial whether excessive free Zn^{2+} is toxic [10]. A recent report showed a correlation between Zn^{2+} and AD and showed significant morphological evidence of Zn^{2+} and Zn^{2+} transporters being actively involved in the pathological processes that lead to plaque formation in AD [11]. Therefore, studies on the effect of Zn^{2+} on CK-BB are of great interest. In this study, we found that Zn^{2+} could induce CK-BB inactivation and unfolding, as well as conspicuous aggregation. We also found that some osmolytes and brain proteins protected against CK-BB aggregation induced by Zn^{2+}.

Materials and Methods

Materials

Creatine, ATP, dithiothreitol, magnesium acetate, thymol blue, zinc acetate, 1-anilino-8-naphthalene sulfonate (ANS), and 5,5′-dithiobis(2-nitrobenzoate) were all purchased from Sigma. All the other reagents were local products of pure analytical grade quality.

CK-BB Purification and Assay

CK-BB was purified as illustrated in Fig. 1a after pretreatment before loading of the columns. The equations we used for calculating V_B and V_C are $[(V_3 - \mathrm{MgAC_2}\text{ of used}) \times 0.6 + V_B]/[V_3 + V_B] = 0.36$ and $[V_4 \times 0.36 + V_C]/[V_4 + V_C] = 0.5$, respectively. The first loading column was filled with DEAE Sepharose Fast Flow (Amersham Biosciences, USA) and equilibrated by using 0.01 M Tris–HCl (pH 8.0) with a 16.5 ml/h flow speed. As a salt gradient, 0.8 M NaCl in the same buffer was used. After collecting the sample tubes, they were combined according to the activity assay and dialyzed before loading the subsequent column. Size elution chromatography was then performed using a Sephadex G-200 (Amersham Biosciences, USA) column equilibrated with 0.01 M Tris–HCl (pH 8.0) at a flow-speed of 5.7 ml/h. Finally, the active sample tubes were collected and analyzed by native polyacrylamide gel electrophoresis (PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The peptide sequences were matched to CK, brain (*Oryctolagus cuniculus*, gi|126723525; mass, 42636; score, 451; queries matched, 37; emPAI, 1.12).

CK activity was measured by following proton generation during the reaction of ATP and creatine at 597 nm at 25 °C using the indicator thymol blue. The substrate comprised 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 enzyme activity. Absorption was measured with a Perkin Elmer Lambda Bio U/V spectrophotometer.

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 at 25 °C. An excitation wavelength of 280 nm was used for the tryptophan fluorescence measurement, 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.

CD Spectroscopy

The circular dichroism (CD) spectra were recorded on a Jasco 725 Spectropolarimeter. The sample cell path length was 2 mm. The CD measurements were carried out according to the manufacturer's instructions at 25 °C.

Results

Purification of CK-BB from Rabbit Brain

We purified CK-BB from rabbit brain according to the established purification procedures (Fig. 1a). CK-BB protein that appeared as one band in sodium dodecyl sulfate and native

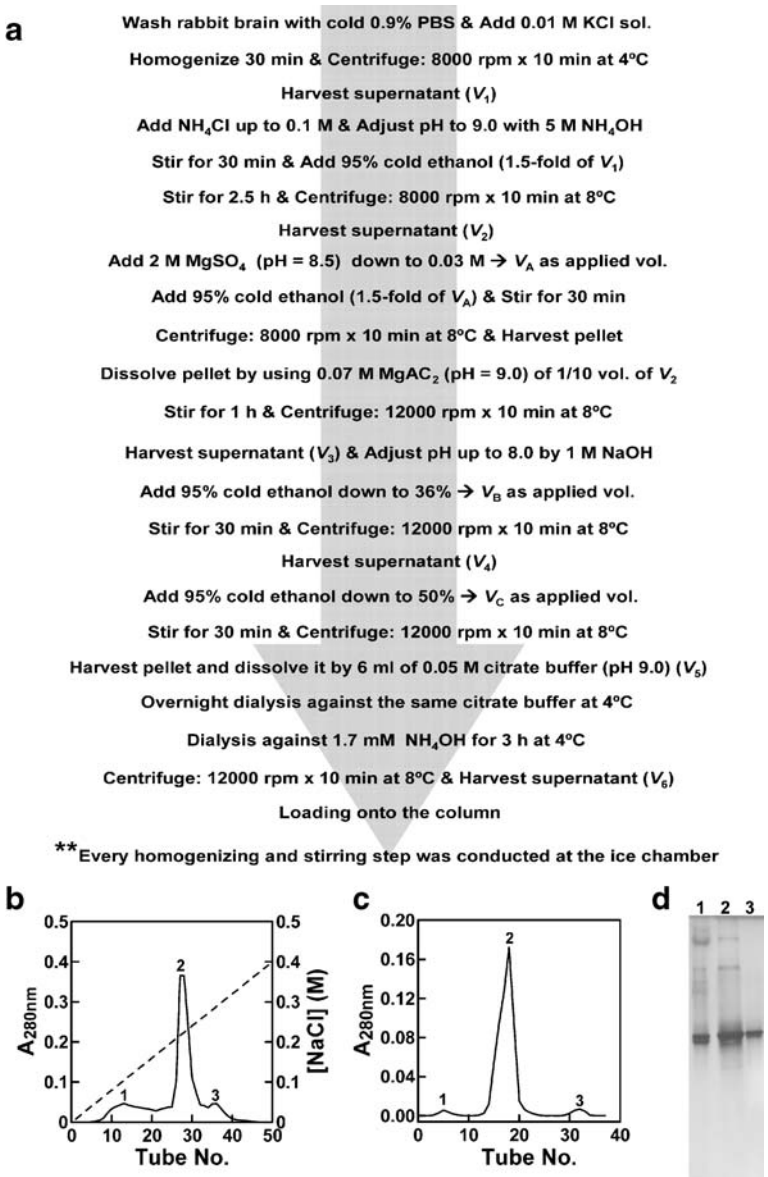


Fig. 1 Purification of CK-BB from rabbit brain. **a** Sample preparation procedures before loading columns. **b** Loading of DEAE Sepharose column and the salt gradient. **c** Size elution chromatography. **d** Native-PAGE. *Lanes 1–3* indicate samples from **a** to **c**, respectively

Table 1 Purification of CK-BB from rabbit brain.

Fraction	Volume (ml)	Protein concentration (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold
Homogenate	360	5,810.4	17361	2.988	1
Homogenate supernatant	255	1,693.2	8354	4.934	1.65
Supernatant with 58.8% ethanol	570	461.7	6445	13.96	4.67
Supernatant of Precipitate solution	44	84.04	3215	38.25	12.80
Citrate buffer dissolving sample	6.4	9.536	667	69.97	23.42
Sample after dialysis	8.2	5.822	657	112.8	37.75
Pooled active fraction from DEAE Sepharose Fast Flow	8.0	3.317	636	191.6	64.12
Pooled active fraction from Sephadex G200	11.4	2.982	612	205.1	68.64

PAGE gels was purified and confirmed by LC-MS/MS. The parameters of the purification procedure are summarized in Table 1.

Inhibition of CK-BB by Zn^{2+}

CK-BB activity was significantly inactivated by Zn^{2+}, which was determined after CK-BB was incubated with different concentrations of Zn^{2+} for 2 h at 25 °C (Fig. 2). When the Zn^{2+} concentration was 0.4 mM, CK-BB activity was completely abolished. The IC_{50} value was 0.15 mM. To determine the equilibrium inactivation conditions, time-interval measurements (kinetic time courses) were performed to determine the rate constants (Fig. 3). The results showed that the enzyme activity gradually decreased in a time-dependent manner as a first-order reaction (Fig. 3a). The inactivation rates were determined by a semilogarithmic plot (Fig. 3b), and it revealed that the inactivation process consisted of two phases: fast (k_1) and slow (k_2). These were further analyzed with different concentrations of Zn^{2+} and are summarized in Table 2. The fast (k_1) rate constant increased in a Zn^{2+} dose-dependent manner, while the slow (k_2) rate constant did not change significantly.

Effect of Zn^{2+} on the CK-BB Structure

Intrinsic fluorescence was used to monitor changes in the conformation of CK-BB at different Zn^{2+} concentrations. The maximum emission wavelength of CK-BB was detected

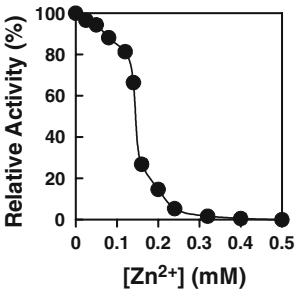


Fig. 2 The effect of Zn^{2+} on CK-BB activity. CK-BB was incubated with different Zn^{2+} concentrations at 25 °C for 2 h in 50 mM Tris–HCl buffer (pH 7.0), and then enzyme activity was measured. The final concentration of the enzyme was 2.0 μM

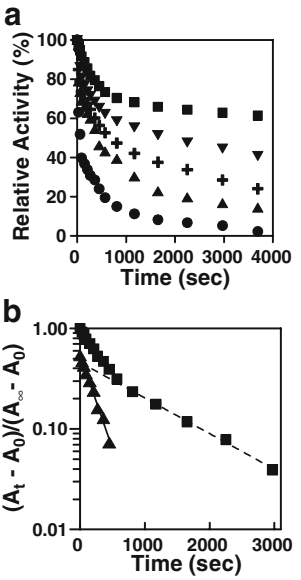


Fig. 3 Time interval measurements of CK-BB inactivation. **a** Inactivation kinetics. The enzyme solutions were mixed with various concentrations of Zn^{2+}, 0.08 (*square*), 0.14 (*inverted triangle*), 0.16 (*plus symbol*), 0.2 (*triangle*), and 0.4 mM (*circle*), and aliquots were taken at the indicated time points. **b** Semilogarithmic plot. *Square* Experimental points. *Triangle* Points obtained by subtracting the contribution of the slow phase from the data in the curve (*line*). The final concentration of the enzyme was 2.0 μM

as 335 nm, and it did not change when the Zn^{2+} concentration was increased; however, the maximum fluorescence intensity increased in a dose-dependent manner (Fig. 4). When the Zn^{2+} concentration was 0.4 mM, the maximum intensity increased almost twofold compared to the native state. *N*-Acetyl-tryptophan was used as a control, and the data showed that the maximum intensity did not change with Zn^{2+} interference.

Next, the effect of Zn^{2+} on exposure of the CK-BB hydrophobic surfaces was probed by measuring the ANS-binding fluorescence spectra. The results showed that Zn^{2+} binding to the enzyme produced a strong increase in ANS fluorescence in a dose-dependent manner, indicating that hydrophobic disruption of CK-BB occurred via Zn^{2+} (Fig. 5). Compared to the native state of CK-BB, the ANS-fluorescence intensity was increased 50% at 0.4 mM Zn^{2+}. This indicates that Zn^{2+} induced tertiary structural changes in CK-BB in terms of both its overall structure and its hydrophobic surfaces directly. Exposed hydrophobic surfaces are known to be prone to misfolding and aggregation under certain conditions. To evaluate if this is true for CK-BB, we evaluated protein aggregation in the presence of Zn^{2+}.

Table 2 Microscopic inactivation rate constants of CK-BB in the presence of Zn^{2+}.

Zn^{2+} (mM)	Inactivation rate constants ($x10^{-3}$ s^{-1})[a]	
	k_1	k_2
0.08	4.4±0.4	0.82±0.01
0.14	6.4±0.5	0.72±0.02
0.16	12.3±2.1	0.74±0.05
0.2	19.1±2.3	1.07±0.03
0.4	24.7±4.3	0.84±0.14

[a] Data were calculated using semilogarithmic plots as shown in Fig. 3. k_1 and k_2 are the first-order rate constants for the fast and slow phases, respectively

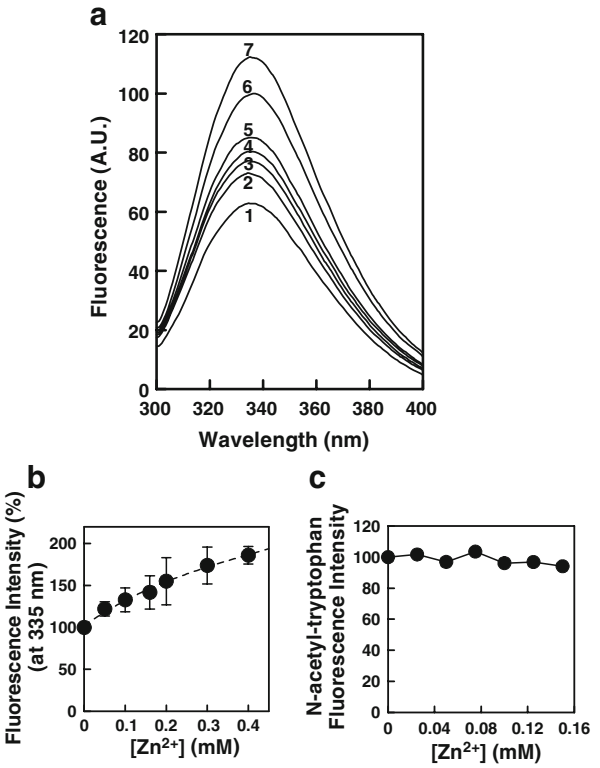


Fig. 4 Intrinsic fluorescence changes in CK-BB induced by Zn^{2+}. **a** Fluorescence emission spectra were measured with excitation at 295 nm and emission in the range of 300–400 nm after CK-BB was incubated with different concentrations of Zn^{2+} for 2 h. The final concentration for the enzyme was 2.0 μM. *Labels 1–7* indicate 0, 0.05, 0.1, 0.15, 0.2, 0.3, and 0.4 mM Zn^{2+}, respectively. **b** Plot of the maximum fluorescence intensity versus [Zn^{2+}]. The data was obtained from **a**. **c** *N*-Acetyl-tryptophan was used for the control experiment

CK Aggregation Induced by Zn^{2+}

When CK-BB was incubated in the presence of Zn^{2+} under suitable conditions, we successfully captured the aggregation process (Fig. 6a). We found that CK-BB aggregation was dependent on Zn^{2+}; as the Zn^{2+} concentration increased from 0.5 to 1.5 mM, marked aggregation occurred. Aggregation was also dependent on temperature in the presence of 1.5 mM Zn^{2+} (Fig. 6b), with higher temperatures increasing the rate of aggregation. Aggregation also occurred, even at 25 °C, when the enzyme concentration was increased (Fig. 6c).

Zn^{2+}-Induced Secondary Structural Changes in CK-BB

To gain more insight into the structural changes, we measured the far-UV CD spectra of CK-BB treated with various concentrations of Zn^{2+}. The results showed that the spectra did not change with increasing Zn^{2+} concentration (Fig. 7), indicating that the helical structure of CK-BB did not unfold with Zn^{2+} binding, even though the enzyme was completely inactivated (Fig. 2). The overall secondary structure of CK-BB, in contrast to the tertiary structure and activity of the enzyme, is resistant to Zn^{2+}.

Preventing CK-BB Aggregation with the Aid of Glycine or Proline and Reactivation of Enzymatic Activity

Some osmolytes have been shown to be able to effectively block or suppress enzyme aggregation. Therefore, we tested the effects of glycine and proline on the aggregation of

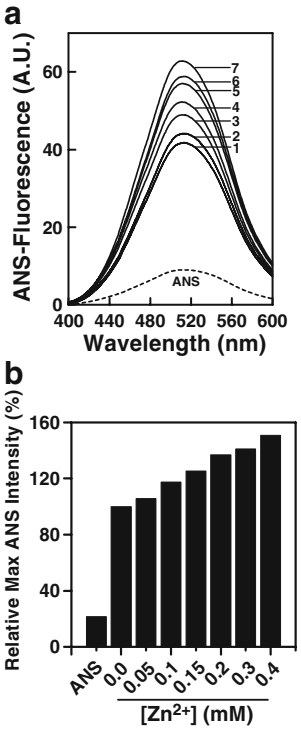


Fig. 5 Zn^{2+}-Induced ANS-binding fluorescence changes in CK-BB. **a** CK-BB was incubated with different concentrations of Zn^{2+} at 25 °C for 2 h and then ANS was incubated for 30 min to label the hydrophobic surface of CK-BB before measurement. The fluorescence was measured with an excitation of 380 nm and emission in the wavelength range of 400–600 nm. The final enzyme concentration was 2.0 μM. *Labels 1–7* indicate 0, 0.05, 0.1, 0.15, 0.2, 0.3, and 0.4 mM Zn^{2+}, respectively. **b** Maximum ANS-fluorescence intensity versus [Zn^{2+}]. The data was obtained from **a**

CK-BB in the presence of Zn^{2+}. We found that aggregation of CK-BB induced by Zn^{2+} was gradually blocked by adding glycine and proline. Both 85 mM glycine and 200 mM proline can completely prevent CK-BB aggregation in the presence of Zn^{2+} (Fig. 8a, b). After incubation of CK-BB with 1.5 mM Zn^{2+}, different concentrations of glycine or proline were incubated with the fully inactivated enzyme for 2 h, and then the activity of the sample was measured (Fig. 8c). The results showed that the activity of CK-BB recovered significantly with increasing glycine concentrations (Fig. 8d). When the concentration of glycine was higher than 10 mM, CK-BB activity recovered to almost 80%. Similarly, when the proline concentration was higher than 200 mM, CK-BB was reactivated. Glycine and proline therefore act as stabilizers for CK-BB and are able to suppress aggregation in the presence of Zn^{2+}. Glycine and proline may reduce disruption of the hydrophobic surface and restore the active conformations of CK-BB. To affirm this hypothesis, we measured changes in the ANS-fluorescence intensity of the protein after incubation with glycine or proline.

The Effects of Glycine and Proline on Recovery of the Tertiary Structure of CK-BB

Neither glycine nor proline influenced the ANS-binding fluorescence of native CK-BB. However, both glycine and proline reduced the 1.5 mM Zn^{2+}-induced exposure of CK-BB hydrophobic surfaces in a dose-dependent manner (Fig. 9). These results show that both glycine and proline acted as stabilizers by restoring the tertiary structure of CK-BB and prevent the Zn^{2+}-induced aggregation of CK-BB by reducing exposure of the hydrophobic surfaces of CK-BB. We also tested other osmolytes such as glycerol, dimethyl sulfoxide, and sucrose. These osmolytes did not reactivate Zn^{2+}-inactivated CK-BB nor did they prevent CK-BB aggregation in the presence of 1.5 mM Zn^{2+} (data not shown).

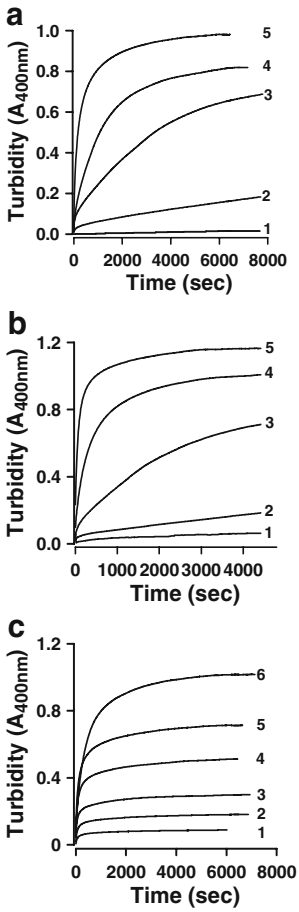


Fig. 6 Zn^{2+}-Induced CK-BB aggregation. **a** The final concentrations of Zn^{2+} (labeled *1–5*) were 0.5, 0.8, 1.0, 1.25, and 1.5 mM, respectively. The reaction temperature was 25 °C, and the final concentration of CK-BB was 10 μM. **b** The effect of temperature on CK-BB aggregation. The temperatures labeled *1–5* correspond to 10, 15, 20, 25, and 30 °C, respectively. The final concentrations of Zn^{2+} and CK-BB were 1.5 mM and 10 μM, respectively. **c** The effect of the concentration of CK-BB on aggregation. The final concentrations of CK-BB labeled *1–6* are 1.0, 2.0, 3.0, 5.0, 7.0, and 10.0 μM, respectively. The reaction temperature was 25 °C and the final concentration of Zn^{2+} was 1.5 mM

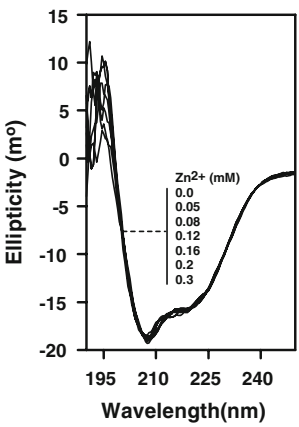


Fig. 7 CD spectra of CK-BB treated with various concentrations of Zn^{2+}. CK-BB was incubated with different concentrations of Zn^{2+}, and then the CD spectra were measured from 250 to 190 nm. The final enzyme concentration was 2.0 μM. The labels show the various concentrations of Zn^{2+}

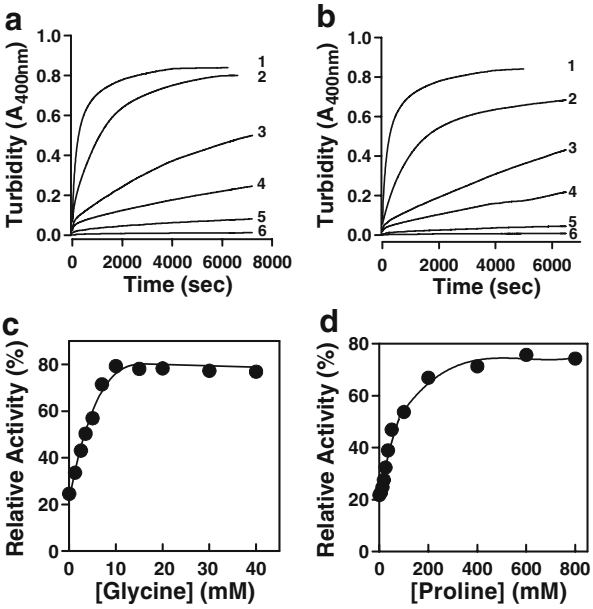


Fig. 8 The effects of glycine and proline on the reactivation of CK-BB. **a** The effect of glycine on aggregation. The final concentration of glycine labeled *1–6* was 0, 2.1, 5, 7.5, 10, and 85 mM, respectively. The final concentration of Zn^{2+} and CK-BB was 1.5 mM and 10 μM, respectively. The reaction was conducted at 25 °C. **b** The effect of proline on aggregation. The final concentration of proline labeled 1 to 6 was 0, 6, 12, 20, 80, and 200 mM, respectively. Other conditions were as for **a**. **c** CK-BB partially inactivated by 0.2 mM Zn^{2+} was incubated with different concentrations of glycine for 2 h, and then enzyme activity was measured. The final concentration of CK-BB was 2.0 μM. **d** Fully inactivated CK-BB was incubated with various concentrations of proline. Other conditions were as for **c**

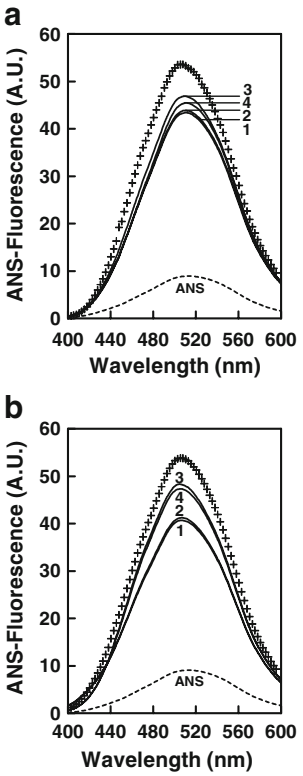


Fig. 9 The effects of glycine and proline on the hydrophobic surfaces of CK-BB. **a** The effect of glycine on CK-BB treated with 0.2 mM Zn^{2+} at 25 °C for 2 h. The curve (*plus symbol*) represents the 0.2 mM Zn^{2+}-treated CK-BB. *Labels 1* and *2* indicate 2.0 μM CK-BB with and without 40 mM glycine, respectively. *Labels 3* and *4* indicate 10 and 40 mM glycine-treated samples, respectively. **b** The effect of proline on 0.2 mM Zn^{2+}-treated CK-BB. *Labels 1* and *2* indicate 2.0 μM CK-BB with and without 400 mM proline, respectively. Labels 4 and 5 indicate 100 and 400 mM glycine-treated samples, respectively

Discussion

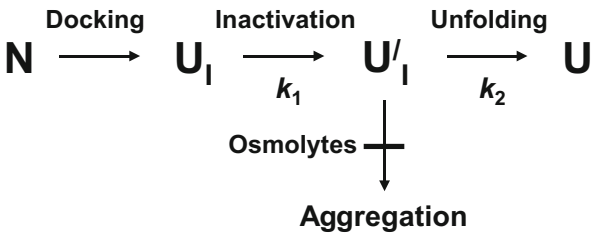
The phenomenon of CK-BB biphasic inhibition implies that transient intermediates exist during Zn^{2+}-induced inactivation. The Zn^{2+}-induced inactivation was probably caused by the accumulation of intermediates with increasing Zn^{2+} concentrations. The disruption of the active site conformation by Zn^{2+} occurred very rapidly and was accompanied by the loss of most of CK-BB's activity and then underwent a slow phase. The partially unfolded states of the transient intermediates underwent slow inactivation until they became completely unfolded. When CK-BB passed the intermediate states, it misfolded easily, resulting in aggregation. We propose a Zn^{2+}-induced unfolding pathway for CK-BB as follows (Scheme 1).

N is the native state; U_I and U'_I are the partially inactivated and unfolded intermediate states, respectively. U is an extensively unfolded state, and k_1 and k_2 are the kinetic first-order rate constants for the inactivation processes. The kinetic real-time analyses for inactivation and aggregation reflect the presence of transient intermediate states, U_I and U'_I caused by increasing Zn^{2+} concentrations. Aggregation is probably caused by the accumulation of these intermediates with exposed hydrophobic surfaces that compete with the normal protein for unfolding. At this point, certain osmolytes such as glycine and proline can effectively suppress aggregation via stabilization of the folding intermediates of CK-BB. It has been shown that certain misfolded proteins are likely to aggregate, thereby causing several neurodegenerative diseases. To investigate a possible relationship between human brain diseases and the aggregation of CK-BB, we first studied the aggregation of CK-BB under in vitro conditions, and then we studied Zn^{2+}-induced CK-BB aggregation under physiological conditions. Interestingly, we found in vitro that both glycine and proline can prevent aggregation and recover the activity of CK-BB. These results have therapeutic implications.

Abnormal regulation of Zn^2 under physiological conditions can induce some problems [11]. If Zn^{2+} is concentrated in certain regions, this ion might induce aggregation in proteins such as CK-BB. However, there are many cellular factors that protect enzyme activities. We therefore hypothesized that CK-BB, like other abundant proteins, might be protected in vivo against Zn^{2+} by proteins that non-specifically chelate Zn^{2+}.

Taken together, our thermodynamic and kinetic studies of CK aggregation inform our understanding of the relationship between aggregation and the flexible CK active site. This site reacted with other factors, which resulted in increased flexibility, which is a key factor for inducing aggregation.

In conclusion, our study provided insight into the inhibitory effects of Zn^{2+} on CK-BB and its potential docking site. Furthermore, our findings provide additional support for the hypothesis that Zn^{2+} may be toxic to some brain-derived metabolic enzymes.



Scheme 1 Zn^{2+}-induced unfolding pathway for CK-BB

Acknowledgments Dr. Fei Zou was supported by a grant from the National Basic Research Program of China (no. 2006CB504100). Dr. Jong Bhak was supported by a grant from the KRIBB Research Initiative Program of Korea. Dr. Yong-Doo Park was supported by a fund from the Science and Technology Planning Project of Jiaxing (no. 2008AZ1024). Dr. Jun-Mo Yang was supported by the grants of the Korea Health 21 R&D Project (Ministry of Health, Welfare and Family Affairs, Republic of Korea, 01-PJ3-PG6-01GN12-0001 and A030003).

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