Appl Biochem Biotechnol (2011) 165:476–484
DOI 10.1007/s12010-011-9266-3

Trehalose Has a Protective Effect on Human Brain-Type Creatine Kinase During Thermal Denaturation

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Received: 24 December 2010 / Accepted: 11 April 2011 /
Published online: 26 April 2011

Abstract We investigated the effects of trehalose on thermal inactivation and aggregation of human brain-type creatine kinase (hBBCK) in this study. In the presence of 1.0 M trehalose, the midpoint temperature of thermal inactivation (T_m) of hBBCK increased by 4.6 °C, and the activation energy (E_a) for thermal inactivation increased from 29.7 to 41.1 kJ mol^{-1}. Intrinsic fluorescence spectra also showed an increase in the apparent transition temperature ($T_{1/2}$) of hBBCK from 43.0 °C to 46.5 °C, 47.7 °C, and 49.9 °C in 0, 0.6, 0.8, and 1.2 M trehalose, respectively. In addition, trehalose significantly blocked the aggregation of hBBCK during thermal denaturation. Our results indicate that trehalose has potential applications as a thermal stabilizer and may aid in the folding of other enzymes in addition to hBBCK.

Keywords Creatine kinase · Trehalose · Thermal denaturation · Folding · Kinetics

Jiang-Liu Yang and Hang Mu contributed equally to this study.

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Abbreviations

CK	Creatine kinase
hBBCK	Recombinant human brain-type CK
T_m	Midpoint temperature of thermal inactivation
E_a	Activation energy
$T_{1/2}$	Apparent transition temperature of thermal denaturation
λ_{max}	Maximum emission wavelength

Introduction

Osmolytes are small solutes that are ubiquitous in cells. They facilitate cellular responses to abiotic stress and fine-tune the molecular functions of cells [1]. Through nonspecific and weak interactions, osmolytes can modulate protein folding and unfolding [2], interact with lipid membranes [3], DNA [4], and control cell volume [5]. α,α-Trehalose, a nonreducing disaccharide, is a naturally occurring osmolyte that is quite common in fungi, bacteria, and invertebrates [6]. Previous studies have demonstrated that this sugar plays an important role in stabilizing proteins [7–9] and inhibiting protein dehydration and desiccation [10, 11]. In solution, trehalose stabilizes the folded structures of proteins as a result of greater preferential hydration of the unfolded state than the native state [8, 12]. As a kosmotrope (hydro-structure) maker, trehalose is excluded from the protein surface, thus facilitating interactions between the protein and water molecules. It has been proposed that the osmophobic effect, which results from unfavorable interactions between the peptide backbone and the protecting osmolyte, is the main force driving proteins from an unfolded to a folded state [13]. Trehalose also acts as a chemical chaperone by binding and stabilizing partially folded intermediates in the folding process and preventing the formation of aggregates [14]. Both in vivo and in vitro studies have demonstrated that trehalose is the disaccharide that is most effective at inhibiting the formation of protein aggregates [15].

Creatine kinase (CK, EC 2.7.3.2) isoenzymes are members of the phosphate kinase family and catalyze the reversible transfer of a phosphate group between ATP and creatine [16]; CK isoenzymes play significant roles in energy homeostasis. There are two types of CKs in vertebrates according to their subcellular location: dimeric cytosolic isoforms of CK and mitochondrial CK forms that exists as dimers or octamers. Muscle-type (MMCK) and brain-type (BBCK) are cytosolic CKs that share high sequence and structural homology to one another. MMCK has a two-state irreversible thermal denaturation curve that is accompanied by sequential aggregation [17]. The pre-transitional conformational change in the active site and the C-terminal domain is thought to be responsible for MMCK thermal aggregation [18]. Recent data revealed that thermal denaturation and aggregation of human BBCK (hBBCK) are sequential events in contrast to MMCK; furthermore, the thermal stability of hBBCK is much lower than that of human MMCK. The thermal inactivation of hBBCK is also partially reversible at temperatures below 55 °C [19].

In this study, we investigated the effects of trehalose on hBBCK thermal inactivation and aggregation. Our results indicate that trehalose increases hBBCK thermal stability by increasing the midpoint temperature of thermal inactivation (T_m) and the apparent transition temperature of thermal denaturation ($T_{1/2}$). Furthermore, Arrhenius plots revealed that trehalose increased the activation energy (E_a) of hBBCK. Trehalose also effectively inhibited hBBCK aggregation during thermal denaturation in a concentration-dependent manner.

Materials and Methods

Materials

Creatine, ATP, magnesium acetate, thymol blue, and guanidine-HCl were purchased from Sigma. The other chemicals were local products of analytical grade. The purification of hBBCK was carried out as reported previously [20]. SDS-PAGE and FPLC analyses confirmed that the purified hBBCK was homogeneous.

CK Assay

CK activity was determined by the pH-colorimetry method [21]. One milliliter of reaction mixture (pH 9.0) contained 24 mM creatine, 4 mM ATP, 5 mM magnesium acetate, and 0.01% thymol blue in 5 mM glycine–NaOH buffer. Proton generation was followed by monitoring the absorbance change of the indicator at 597 nm at 25 °C. Enzyme concentrations were measured using the absorption coefficient of the 8.8 value.

Activity Measurements of Thermal Inactivation Transitions

hBBCK thermal inactivation was assessed in various concentrations of trehalose in 10 mM HEPES–NaOH buffer (pH 8.0). The samples (final enzyme concentration of 1 μM) were incubated for 10 min at different temperatures ranging from 25 °C to 55 °C. The aliquots (10 μl) of samples were withdrawn at specific times, and the residual activity was measured immediately. T_m (the temperature at which the enzyme lost 50% of its activity) was calculated from the plot of residual activity percentage versus temperature.

Activation Energy of hBBCK

The thermal stability of hBBCK in the absence or presence of trehalose was determined by examining inactivation rate constants (k) from 43 °C to 50 °C. Inactivation rate constants (k) were calculated from the slope of a semi-logarithmic plot of residual activity versus time. The activation energy (E_a) for the thermal inactivation of hBBCK in the different concentrations of trehalose was determined from the slope of the Arrhenius plot.

Intrinsic Fluorescence Measurements

To evaluate the effects of trehalose on hBBCK conformations, the enzyme (final concentration of 2 μM) was diluted in buffer containing different concentrations of trehalose and was incubated at 25 °C for 5 h. For thermal unfolding tests, the samples were kept in a constant temperature bath at a given temperature ranging from 30 °C to 69 °C in the absence or presence of trehalose 10 min, and then equilibrated for 1 min. The intrinsic fluorescence of hBBCK was measured on a Hitachi F-2500 fluorospectrometer using a 2×10 mm path length cuvette. The excitation wavelengths of 280 and 295 nm were used, and the emission spectra range was from 300 to 400 nm. The value of the parameter $A=I_{320}/I_{365}$, where I_{320} and I_{365} are fluorescence intensities at $\lambda=320$ and 365 nm, respectively, was used to characterize the fluorescence spectra positions [22]. $T_{1/2}$ (with the amount of folded protein) was calculated from the plot of percentage unfolding (I_{320}/I_{365} value change) versus temperature.

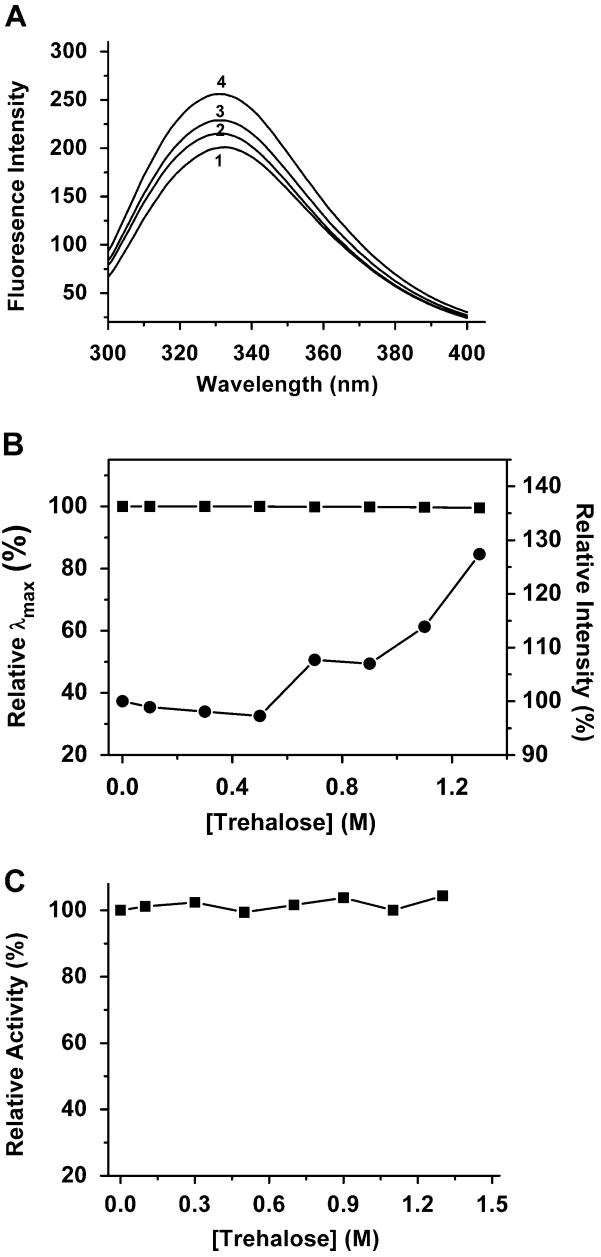

Fig. 1 Effects of trehalose on the tertiary structures of hBBCK. **a** Intrinsic fluorescence spectra. The trehalose concentration for curves 1–5 was 0, 0.3, 0.9, 1.1, and 1.3 M, respectively. The enzyme samples were equilibrated at room temperature for 5 h with different concentrations of trehalose before measurements were taken. The intrinsic fluorescence spectra were measured using an excitation wavelength of 280 nm. The final enzyme concentration was 2 μM. **b** Changes in λ_{max} (*black square*) and relative fluorescence (*black circle*). **c** Effect of trehalose on the activity of native hBBCK

Aggregation of hBBCK

hBBCK (at a final concentration of 56 μM) was completely denatured in 3 M guanidine-HCl at 25 °C for 2 h. The denatured enzyme was diluted in 10 mM Tris–HCl buffer (pH 8.0) in the presence or the absence of trehalose. The aggregation was followed by monitoring the turbidity at 400 nm with a Thermo UV spectrophotometer at 25 °C.

Results

Effect of Trehalose on the Activity and Structure of hBBCK

As shown in Fig. 1a, addition of trehalose resulted in minor changes to the maximum peak wavelength of the intrinsic fluorescence of hBBCK. In the presence of 1.3 M trehalose, λ_{max} blue shifted about 1.5 nm, while the fluorescence intensity increased about 30% compared with that of the control native sample (Fig. 1b). These results suggest that trehalose did not induce conformational changes in the native state of hBBCK, but may have increased the hydrophobicity of the microenvironments of the tryptophan residues of hBBCK. These changes did not affect enzyme activity, as shown in Fig. 1c.

Effect of Trehalose on the Thermal Stability of hBBCK

The residual activity of hBBCK at different temperatures in the presence or the absence of trehalose is indicated in Fig. 2. Trehalose effectively enhanced the thermal stability of hBBCK; the T_m of hBBCK shifted from 41.9 °C to 46.5 °C when the trehalose concentration was increased from 0 to 1.0 M, respectively. There was a linear relationship between T_m and trehalose concentration. When the temperature reached 53 °C, the enzyme was inactivated completely, regardless of the presence of trehalose. Consistent with our results, a recent study reported that 30% glycerol increased the T_m of MMCK by 4.6 °C during thermal denaturation [23].

Thermodynamic Parameters of hBBCK Thermal Inactivation

To gain further insight into the effects of trehalose on hBBCK stability, we investigated the thermal inactivation kinetics of hBBCK. The thermal inactivation curve of hBBCK showed exponential decay for all experimental temperatures. The semi-logarithmic plots (Fig. 3a–d) revealed that the thermal inactivation kinetics followed the first-order reactions and that the inactivation rate slowed down gradually in the presence of trehalose. The activation energy

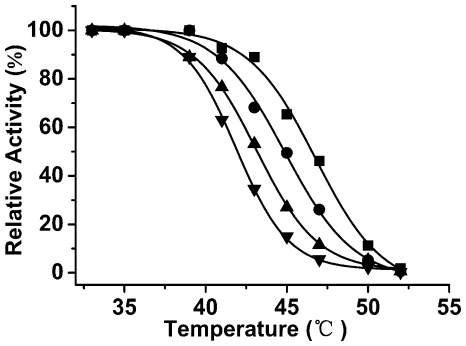

Fig. 2 Effect of trehalose on the thermal inactivation of hBBCK. Thermal transition curves of residual activity versus temperature. The enzyme (final concentration of 2 μM) was dissolved in 10 mM HEPES–NaOH buffer (pH 8.0), containing different concentrations of trehalose, and was assayed after incubation in a water bath for 10 min at the desired temperature. The aliquots (10 μl) of samples were withdrawn and the residual activity was measured immediately. Residual activity was expressed relative to that of the control (unheated) sample. The trehalose concentrations were 0 M (*filled inverted triangle*), 0.3 M (*filled triangle*), 0.6 M (*filled circle*), and 1.0 M (*filled square*)

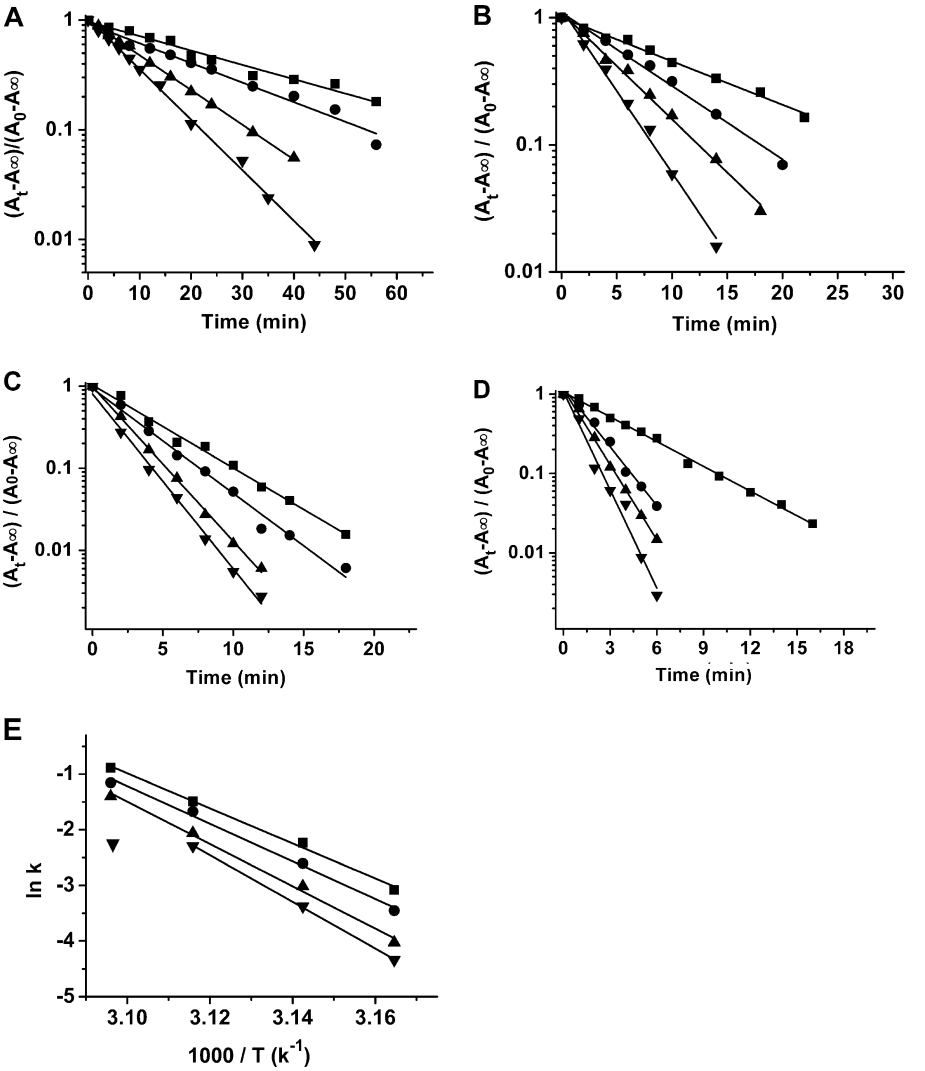

Fig. 3 Kinetics of thermal stability of hBBCK according to different trehalose concentrations after incubation at various temperatures. Semi-logarithmic plots of the activities of thermal denatured hBBCK at **a** 43 °C, **b** 45 °C, **c** 48 °C, and **d** 50 °C. The trehalose concentration was 0 M (*filled inverted triangle*), 0.3 M (*filled triangle*), 0.6 M (*filled circle*), or 1.0 M (*filled square*). Enzyme residual activity was measured at suitable time intervals. The final enzyme concentration was 2 μM. **e** Arrhenius plot for hBBCK

(E_a; Fig. 3e) of thermal inactivation showed an obvious increase when trehalose was added: E_a increased from 29.7 to 41.1 kJ mol^{-1} when the trehalose concentration was increased from 0 to 1.0 M, respectively, indicating that trehalose increases the thermal stability of hBBCK.

Intrinsic Fluorescence Measurements

Because of the relative small change in λ_{max} (less than 5 nm) during thermal denaturation, the effect of trehalose on the temperature of thermal unfolding of hBBCK was

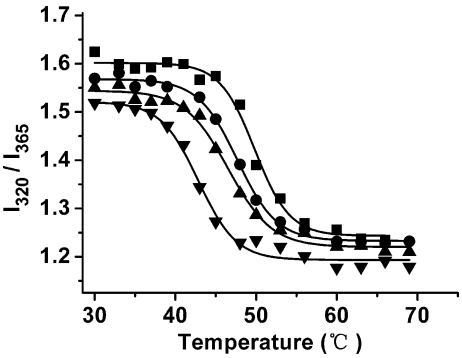

Fig. 4 Effect of trehalose on the thermal equilibrium during the unfolding of hBBCK. The enzyme (final concentration of 4 μM) was incubated in 10 mM HEPES–NaOH buffer (pH 8.0) in the presence of different concentrations of trehalose: 0 M (*filled inverted triangle*), 0.8 M (*filled triangle*),1.0 M (*filled circle*), or 1.2 M (*filled square*). Thermal unfolding of hBBCK was followed in the temperature range of 30–69 °C. Unfolding was recorded by intrinsic fluorescence spectroscopy at an excitation wavelength of 295 nm

measured by parameter A. hBBCK thermal denaturation followed a typical two-state process, and an excess molar ratio of trehalose did not change this process (Fig. 4). Trehalose greatly attenuated the temperature-induced unfolding of hBBCK: the apparent transition temperature $T_{1/2}$ of hBBCK changed from 43.0 °C to 49.9 °C when the trehalose concentration was increased from 0 to 1.2 M, respectively (Table 1). In addition, the value of parameter A value increased in trehalose solution. This phenomenon is consistent with the observed fluorescence intensity changes.

Effect of Trehalose on hBBCK Aggregation

A previous study demonstrated that trehalose, as a chemical chaperone, promotes the stabilization of folded proteins and can be used as an aggregation suppressor [16]. When guanidine-HCl denatured hBBCK was diluted in refolding buffer at 25 °C, significant aggregation occurred immediately, but this aggregation was effectively blocked by trehalose in a dose-dependent manner. When the trehalose concentration reached 1.0 M, the aggregation was almost completely suppressed (Fig. 5).

Discussion

Many organisms accumulate trehalose to enhance thermotolerance and reduce aggregation of denatured proteins during heat shock and stationary phase. In yeast cells, the trehalose

Table 1 Thermodynamic parameters of hBBCK in the presence of trehalose during thermal denaturation

Trehalose (M)	T_m (°C)	ΔT_m (°C)	$T_{1/2}$ (°C)	E_a (kJ mol^{-1})
0	41.9	0	43.0	29.7±0.6
0.3	43.2	1.3	–	32.6±0.5
0.6	44.9	3.0	46.5	36.6±0.7
0.8	–	–	47.7	–
1.0	46.5	4.6	–	41.1±0.4
1.2	–	–	49.9	–

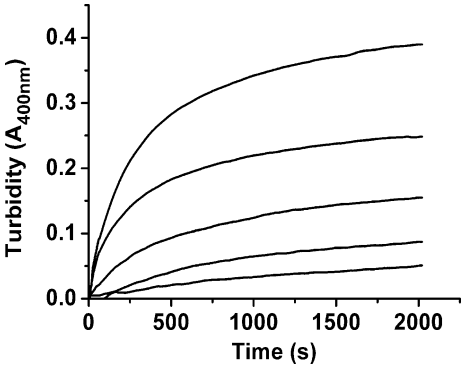

Fig. 5 Effect of trehalose on aggregation of guanidine-HCl denatured hBBCK at 25 °C. hBBCK was completely denatured in 3 M GdnHCl at 25 °C for 2 h and refolded by dilution into 10 mM HEPES–NaOH buffer (pH 8.0). The final concentration of CK was 56 μM. The final concentration of trehalose from *top* to *bottom* was 0, 0.1, 0.2, 0.3, and 0.5 M, respectively

concentration can reach up to 500 mM during stress, and trehalose has been shown to prevent proteins from thermal inactivation [24]. Compared with other sugar osmolytes found in nature, trehalose appears to be the most effective protective osmolyte for counteracting environmental stress because it induces maximal increases in the transition temperatures (ΔT_m) of enzymes [7]. We found that 1.0 M trehalose increased the ΔT_m of hBBCK by 4.6 °C. The trehalose-induced increase in the E_a of hBBCK indicates that trehalose stabilized hBBCK during thermal denaturation.

hBBCK thermal denaturation is a typical two-state process ($T_{1/2}$=43.0 °C) and hBBCK is not fully unfolded during this process; the trehalose-saturated sample exhibited a higher $T_{1/2}$ than the sample without trehalose. In the absence of trehalose, $T_{1/2}$ was higher than T_m (in the presence of 0.6 M trehalose, $T_{1/2}$ was also higher than T_m), suggesting that the active site of hBBCK is flexible and that its structure changes prior to the overall conformational change.

Osmolyte-driven stabilization of protein folding is due to the solvophobic effect of the osmolyte on the peptide backbone [13]. Both λ_{max} and fluorescence intensity measurements revealed that trehalose moved hydrophobic amino acids into a more non-polar environment (Fig. 1). As shown in Fig. 4, the presence of trehalose only increased parameter A of hBBCK. These results suggest that trehalose shifts the native state toward a more compact structure and that the solvophobic effect may increase thermal stability and promote enzyme folding, thereby changing $T_{1/2}$. Based on the crystal structure of hBBCK, this enzyme contains two domains in each subunit and the active site is located in the cleft between the two domains. Domain motion is thought to play a role in CK catalysis [25]. We found that enzyme activity was not inhibited by increasing trehalose concentrations. This may be because the domain motions of the subunits were not disturbed by trehalose.

Loss-of-function of mutations CK can lead to several diseases. Cytosolic BBCK is significantly inactivated by oxidation in Alzheimer's disease patients [26]. Abnormal CK activity is associated with several diseases such as myocardial infarction, cardiac hypertrophy, and muscular dystrophy [27]. Trehalose has already been shown to be useful in protecting against oxidative stress; this disaccharide inhibited the aggregation of β-amyloid in amyloid plaques, which are implicated in Alzheimer's disease [28], and was also able to inhibit polyglutamine-mediated protein aggregation in a mouse model of Huntington's disease [29]. Our results indicate that trehalose is a useful hBBCK

aggregation suppressor during refolding. One molar trehalose completely inhibited aggregation during hBBCK refolding. Taken together, our thermodynamic and kinetic studies of hBBCK inactivation and aggregation suggest that trehalose can stabilize the structure of hBBCK, maintain enzyme activity, and inhibit its aggregation.

Acknowledgments This work was supported by grants of Key Science and Technology Innovation Teams of Zhejiang Province (grant nos. 2009R50031 and 2009R50031-1) from the Science and Technology Department of Zhejiang Province and the Zhejiang Provincial Top Key Discipline of Modern Microbiology and Application (grant nos. KF2010006 and KF2010007). Dr. Zi-Ping Zhang was supported by grant 30970635 of the China National Science Foundation, grant Y2101329 of the Zhejiang Provincial Science Foundation, and the grant of the Scientific and Technological Research Programs of Ning Xia Hui Autonomous Region.

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