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Thermally induced changes in the structure and activity of yeast hexokinase B

Hassan Ramshini [a], Nasrollah Rezaei-Ghaleh [a], Azadeh Ebrahim-Habibi [a], Ali Akbar Saboury [a], Mohsen Nemat-Gorgani [a,b,*]

[a] Institute of Biochemistry and Biophysics, University of Tehran, Tehran 13145-1384, Iran
[b] Stanford Genome Technology Center, Stanford University, Palo Alto, CA, USA

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ABSTRACT

Yeast hexokinase has been poorly characterized in regard with its stability. In the present study, various spectroscopic techniques were employed to investigate thermal stability of the monomeric form of yeast hexokinase B (YHB). The enzyme underwent a conformational transition with a T_m of about 41.9 °C. The structural transition proved to be significantly reversible below 55 °C and irreversible at higher temperatures. Thermoinactivation studies revealed that enzymatic activity diminished significantly at high temperatures, with greater loss of activity observed above 55 °C. Release of ammonia upon deamidation of YHB obeyed a similar temperature-dependence pattern. Dynamic light scattering and size exclusion-HPLC indicated formation of stable aggregates. Taking various findings on the influence of osmolytes and chaperone-like agents on YHB thermal denaturation together, it is proposed that the purely conformational transition of YHB is reversible, and irreversibility is due to aggregation, as a major cause. Deamidation of a critical Asn or Gln residue(s) may also play an important role.

1. Introduction

Hexokinase (EC 2.7.1.1) is the first enzyme in the glycolytic pathway, catalyzing the transfer of a phosphoryl group from ATP to glucose to form glucose 6-phosphate and ADP. Hexokinases have been found in every organism checked, ranging from bacteria, yeast, and plants to humans and other vertebrates. They are categorized as actin fold proteins [1]. Four distinct mammalian isozymes designated as types I–IV have been characterized. While hexokinases types I and II bind to mitochondria through interaction with a porin known as voltage dependent anion channel (VDAC), type III and IV isozymes lack the hydrophobic N-terminal sequence which is required critically for enzyme binding to mitochondria [2]. The primary sequence alignment of a selection of proteins from the hexokinase family demonstrates extensive similarity between the N- and C-terminal halves of type I human hexokinase, rat hexokinase, and hexokinase from *S. mansoni* and between these and yeast hexokinase, consistent with the gene duplication-fusion concept proposed by Colowick [3].

There are two isoenzymes of hexokinase in yeast, A and B, with an overall homology in their amino acid sequences of about 76% [4]. They are structurally well characterized [4–7], showing a high degree of similarity in regard with tertiary structure [6,8,9]. Both of the isozymes share a similar α/β fold, and the polypeptide chain is distinctly folded into two domains of unequal size, the large and the small domain. These domains are separated by a large cleft forming the active site [10,11]. Each enzyme exists in monomer–dimer self-association equilibrium, with a dimer molecular weight of about 100 kDa. Dissociation is promoted by increases in pH, ionic strength, and temperature, and by a decrease in enzyme concentration [12,13]. The N-terminus of the protein is considered essential for its self-association [14]. Endogenous protease action during purification leads to the loss of 11 amino acids from the N-terminus, resulting in a predominantly monomeric form of about 50 kDa [15]. Yeast hexokinase B is the predominant hexose kinase in *S. cerevisiae* grown on glucose [16], involved in catabolite repression by glucose [17,18]. The enzyme exhibits regulatory properties at physiological pH values which include negative cooperativity with ATP, activation by citrate and some other anions [19,20].

Hexokinase malfunction has been implicated in a number of diseases in humans. For example, its activity has been reported to change significantly in patients with Alzheimer's disease [21,22] and markedly elevated in highly glycolytic, rapidly growing tumors [23,24]. Studies on protein stability is gaining more importance as the number of therapeutic protein products is increasing and protein stabilization is becoming more important due to their greater use under industrial conditions. As related to the present investigation, limited efforts have been directed toward elucidation of the mechanisms associated with thermal stability of the mammalian [25] and yeast [26,27] hexokinases.

In the present communication, we tried to elucidate the mechanisms involved in thermal denaturation of yeast hexokinase B. Various

* Corresponding author. Stanford Genome Technology Center, Stanford University, Palo Alto, CA, USA. Tel.: +1 650 812 1961; fax: +1 650 812 1975.
E-mail address: mohsenn@stanford.edu (M. Nemat-Gorgani).

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spectroscopic techniques were employed to investigate his process for the monomeric form of this enzyme, and elucidate the effects of osmolytes and chaperone-like agents on prevention of its thermal inactivation.

2. Experimental

2.1. Materials

Yeast hexokinase used in these experiments was the B isoenzyme (YHB), fraction II of the Kaji et al. preparation [28], obtained from Sigma Chemical Co. (St. Louis, MO, USA) as crystalline suspensions in ammonium sulfate. After re-suspension of the enzyme in the buffer used in the reaction medium, it was dialyzed at 4 °C for 24 h. To avoid the presence of dimeric form in the solution, the experiments were performed at pH 8.0, where the protein is known to exist predominantly as the monomeric form [12,13]. 8-Anilinonaphthalene-1-sulfonic acid (ANS) and di-potassium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). α, β and γ-cyclodextrin, α and β-casein and 3-[*N*-Morpholino] propane-sulfonic acid (MOPS) were obtained from Sigma Chemical Co. Solutions were made in 50 mM MOPS buffer (pH 8.0). Protein concentration was determined by 280-nm absorbance measurements, using an extinction coefficient of 9.47($E^{1\%}_{280}$) [15].

2.2. Turbidity measurements

Turbidity measurements were made at specified wavelengths, essentially as described earlier [29,30] to follow protein aggregation. A Cary-100 Bio VARIAN spectrophotometer was used and temperatures were controlled to within ±0.1 °C by a Cary temperature controller.

2.3. Centrifugation

100-µl aliquots of the sample were centrifuged at 13,000 rpm (9000 ×g) for 20 min using the Beckman AvantiTM 30 centrifuge. The supernatant was removed, mixed with 900 µl buffer, and protein concentration was determined by measuring absorbance at 280 nm.

2.4. Circular dichroism

Circular dichroism (CD) spectra in the far-UV (190–260 nm) region were obtained using an AVIV 215 spectropolarimeter and a 1-mm path cell, at 25 °C. Protein concentration was fixed at 0.15 mg/ml. Thermal denaturation experiments were carried out by monitoring the temperature-dependent alterations of 222-nm ellipticity with a scan rate of 1 K min^{-1}.

2.5. Fluorescence spectroscopy

Fluorescence experiments were performed on a Cary Eclipse VARIAN fluorescence spectrophotometer, with temperature controlled within ±0.1 °C by a Cary temperature controller. The intrinsic emission spectra were obtained at a protein concentration of 0.05 mg/ml. The excitation wavelength was 280 nm and the emission spectra were collected between 290 and 450 nm. The excitation and emission slit widths were both set at 5 nm. Thermal denaturation experiments were performed by following the temperature-dependent changes of 335-nm emission intensity with a scan rate of 1 K min^{-1}. ANS fluorescence studies were measured in MOPS buffer, using a final ANS concentration of 250 µM, while the molar ratio of protein to ANS was 1:50. ANS fluorescence emission was scanned between 400 and 700 nm with an excitation wavelength of 380 nm.

2.6. Polyacrylamide gel electrophoresis

SDS-PAGE analysis was performed on 12.5% polyacrylamide slab gel as described by Laemmeli [31].

2.7. Enzyme assay

Hexokinase activity was measured spectrophotometrically by coupling the catalyzed reaction with that of Glucose-6-phosphate dehydrogenase (G6PDH) and following the disappearance of NADP at 340 nm, as described in [15].

2.8. Thermoinactivation studies

Thermoinactivation studies were performed by incubating the enzyme in 50 mM MOPS buffer, pH 8.0 at various temperatures for specified durations. The remaining enzymatic activity was then measured after removal of samples at regular intervals, and cooling on ice. Activity of the enzyme solution kept on ice was considered as the control (100%).

2.9. Determination of deamidation

Production of ammonia was determined after a 20-minute incubation of the protein samples at different temperatures. The amount of dissolved ammonia was determined enzymatically using glutamate dehydrogenase [32].

2.10. Dynamic light scattering (DLS)

DLS experiments were conducted at 60 °C, using the nanosizer-ZS (Mastersizer, Malvern Instruments, Worcestershire, UK, version 40.2) equipped with a Helium–Neon laser (633 nm). The protein samples (0.1 mg/ml in MOPS buffer pH=8) were heated to 60 °C, and the DLS experiments were then performed at 5-minute intervals. To remove large dusts, samples were filtered twice by Whatman filter papers before the experiments. The scattered intensity was measured at right angle, and the autocorrelation function was calculated to analyze the fluctuations of scattered intensity and compute the average diffusion coefficients. The average hydrodynamic radius of the protein particles was then estimated according to Stokes–Einstein formula.

2.11. Size exclusion chromatography (SEC-HPLC)

Size exclusion chromatography (Lc-6A, Shimadzu, Japan) was carried out on a 7.9 mm (ID)×25 cm shim-pack diol-300 column

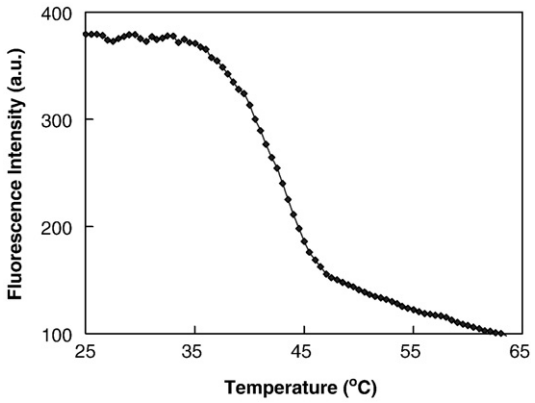

Fig. 1. Thermal denaturation curves of yeast hexokinase B, obtained through monitoring temperature-dependent changes of 335-nm fluorescence emission intensity of the protein. The excitation wavelength was 280 nm. See Materials for further details.

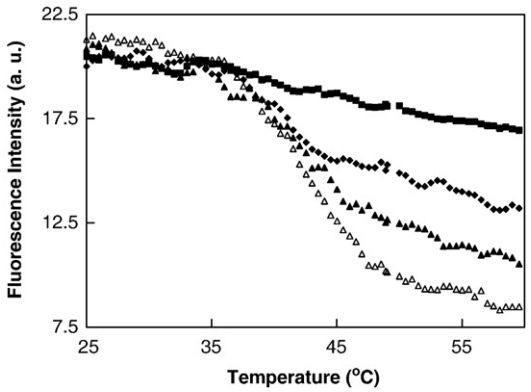

Fig. 2. Conformational reversibility of yeast hexokinase B thermal unfolding through monitoring temperature-dependent changes of 335-nm fluorescence emission intensity after excitation at 280 nm. The protein samples were first heated up to the specified temperatures (Δ: 47.5, ▲: 50, ♦: 55; ■: 60 °C), then cooled at 25 °C for 15 min, followed by reheating to 60 °C at a rate of 1 °C/min. The displayed spectra are those obtained in the reheating phase. Protein concentration was 7 μg/ml.

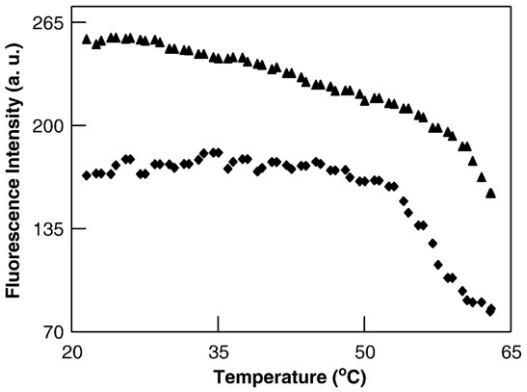

Fig. 4. Effect of 1.5 M sorbitol (♦) or glucose (▲) on conformational reversibility of yeast hexokinase B thermal unfolding. The protein samples were first heated to 65 °C then cooled to 25 °C and after 15 min, they were reheated to 65 °C at a rate of 1 °C/min. The displayed spectra are those obtained in the reheating phase.

(Shimadzu, Japan, part No.228-14776-92) as the stationary phase. The protein concentration was 0.1 mg/ml. The experiments were conducted by fixing the flow rate at 0.5 ml/min. The column was calibrated using bovine serum albumin (BSA), lysozyme and glutamate dehydrogenase (GDH).

3. Results and discussion

3.1. Thermal denaturation of yeast hexokinase B

Thermal denaturation of yeast hexokinase B was investigated using fluorescence and CD spectroscopic techniques. When the enzyme solution was heated to 65 °C, its fluorescence emission showed a characteristic structural unfolding transition; with an apparent T_m of 41.9 °C (Fig. 1). This was independent of protein concentration in the range of 3–60 μg/ml. Upon cooling and reheating to 65 °C, no apparent transition was manifested, suggesting that the process was irreversible. However, as displayed in Fig. 2, when the initial heating process was stopped at temperatures below 55 °C, reheating could lead to relatively similar sigmoid-like changes in fluorescence emission intensity. These observations were confirmed by CD (Fig. 3). In contrast to the present study, two transitions have been observed for hexokinase A [33], presumably due to the structural differences between the two protein molecules and differences in the conditions utilized. These results could be taken to propose that the event causing the irreversible behavior of hexokinase denaturation may occur predominantly at temperatures above 55 °C, and the pure unfolding of the protein is probably reversible. This is consistent with the previous results obtained by a calorimetric method [26].

To further confirm that the initial unfolding of YHB molecule is essential for progression of the irreversible step, possible stabilization of the process was tested. It was found that polyols (sorbitol or glucose, at, 1.5 M concentration) could elevate the T_m of YHB thermal denaturation up to 10–20 °C (Fig. 4). In the presence of these polyols, the heat-induced unfolding of the protein remained reversible even at 65 °C, but reversibility was lost when the heating process was continued up to 70 °C (Fig. 5).

Based on the obtained results, the Lumry–Eyring model is used to describe thermal unfolding of YHB, according to the following scheme:

$$N \leftrightarrow U \rightarrow I$$

Where N is the native state, U is the unfolded state and I represents the final irreversible denatured state, which is unable to find its correct fold after being cooled [34–36]. According to our findings and the earlier reports that the urea-induced unfolding of YHB at room temperature is reversible [37], it is suggested that the pure unfolding of YHB may be reversible and the irreversible conversion of U to I species occurs predominantly at high temperatures, where most of the YHB molecules are unfolded.

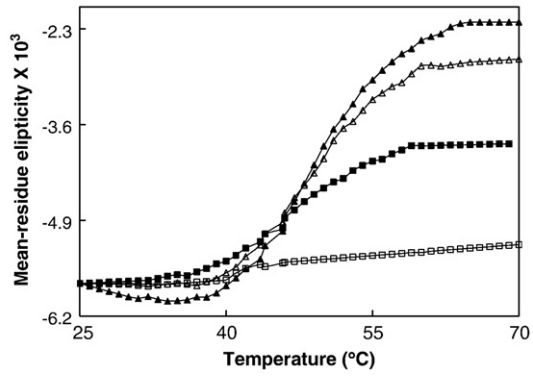

Fig. 3. Conformational reversibility of yeast hexokinase B thermal unfolding through monitoring temperature-dependent changes of 222-nm Far UV CD. The protein samples were first heated up to the specified temperatures (▲: 45, Δ: 50, ■: 55, □: 60), then cooled to 25 °C for 15 min, followed by reheating to 70 °C at a rate of 1 °C/min. The displayed spectra are those obtained in the reheating phase. Protein concentration was 0.15 mg/ml.

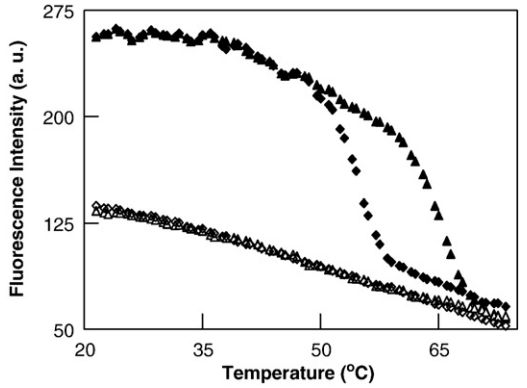

Fig. 5. Thermal denaturation curves of yeast hexokinase B. The protein samples were first heated to 70 °C in the presence of sorbitol (♦) or glucose (▲) then cooled to 25 °C for 15 min, followed by reheating to 70 °C at a rate of 1 °C/min. The reheated curves were obtained in the presence of sorbitol (◊) or glucose (Δ).

Table 1
Residual activity of yeast hexokinase B after treatment at the specified temperatures for 2, 4, 6, 8 and 10 min

	Time (min)	2	4	6	8	10
Temperature (°C)	35	75.9±3.4	61.06±3.7	44±4.24	41.7±6.9	37.7±4.4
	40	33.17±3.2	25.2±4.1	20.18±3.6	18.6±3.9	16±2.31
	45	19.53±1.5	18.8±0.106	17.±0.25	12.5±1.4	12.6±2
	50	17.5±2	11.5±3	9.1±2.5	7.7±2.5	3.05±2
	55	8.44±1.2	8.2±2	3±1.5	0.35±0.12	0.21±0.1
	60	5.7±0.7	2.3±0.64	2.25±0.57	1.35±0.29	101±0.21

Activity measurements were performed after cooling the enzyme samples for 30 min. % remaining activities are provided.

3.2. Thermoinactivation of yeast hexokinase B

Thermoinactivation study of YHB revealed a significant loss of enzymatic activity after a 10-minute incubation at various temperatures (from 25 to 65 °C). As shown in Table 1, remaining enzymatic activity decreased from 35 to 65 °C, with a much greater extent of inactivation at temperatures above 55 °C, in accord with previous observations [38]. T_m of YHB denaturation was raised by 10 and 20 °C due to the presence of sorbitol or glucose, respectively, and the irreversibility of thermal unfolding was deferred to temperatures as high as 70 °C. Glucose was shown to protect YHB activity at temperatures up to 55 °C, while sorbitol was less effective (Fig. 6). Since the extent of YHB thermoinactivation appeared to change in parallel with the emergence of the irreversibility in various conditions, it was suggested that the accelerated rate of thermoinactivation was related to events causing irreversible thermal unfolding.

3.3. Possible mechanisms of "irreversible" thermal denaturation of YHB

As proposed in Scheme 1, the conversion of U to I is responsible for the irreversible character of YHB thermal denaturation. This irreversible step may constitute chemical reactions including fragmentation/cleavage of the polypeptide chain, cysteine oxidation, and Asn/Gln deamidation. Alternatively, this step may involve protein aggregation, which is frequently considered to be responsible for irreversible denaturation [39,40]. To investigate this further, we examined a number of possibilities as outlined below:

3.4. Cysteine oxidation and fragmentation/cleavage of the polypeptide chain

YHB has four cysteine residues per identical monomer, all present in free thiol form [41], which may undergo oxidation when the protein molecule is unfolded at high temperatures, leading to irreversible denaturation. To examine this possibility, thermal denaturation of YHB was followed in the presence and absence of dithiothreitol (DTT, 5 mM). Thermally induced conformational transition of YHB remained irreversible irrespective of the presence or absence of DTT (data not shown), suggesting that cysteine oxidation could not be a mechanism involved here.

Polypeptides are known to be susceptible to peptide bond hydrolysis at high temperatures, especially after aspartate residues. Upon incubation at 60 °C for 30 min with or without DTT, followed by SDS SDS-PAGE analysis, no cleavage/fragmentation was detectable (results not shown), thus ruling out this possibility.

Table 2
Deamidation of amide residues of yeast hexokinase B, measured as the amount of liberated ammonia after 20-minute incubation at various temperatures

Temperature (°C)	25	35	45	50	55	60	65
Ammonia liberated (μM)	N.D.	10±1.5	11±1.25	14±1.21	27±2.23	28±2.5	30±1.85

The protein concentration was 0.1 mg/ml (1.9 μM).
Details are described in Materials section (N.D. not detectable).

3.5. Deamidation

As revealed by thermoinactivation studies, the rate of irreversible loss of YHB function is greatly enhanced above 55 °C, where the conformational unfolding transition of this protein progressively manifests irreversibility. Yeast hexokinase B contains 21 glutamine and 19 asparagine residues, among which asparagines 91 and 237, glutamines 109, 122, 145, 163, 251, 324 and 444 are especially susceptible to deamidation [4]. Asn/Gln deamidation, is frequently considered responsible for thermal loss of enzymatic functions [42]. As demonstrated in Table 2, deamidation occurred at all temperatures between 35 and 65 °C, but the released ammonia level was significantly higher at temperatures above 55 °C. We also measured the time course of ammonia release at 60 °C, and observed that the ammonia level was elevated in a time-dependent manner (data not shown). The accelerated rate of deamidation above 55 °C may be originated from de-protection of a susceptible Asn/Gln side chain upon thermal unfolding. In relation with this hypothesis, it was shown that the two osmolytes tested afforded protection (inset to Fig. 6).

If an Asn/Gln residue is critically involved in the binding/active site of the enzyme and also plays a major conformational role, its deamidation at high temperatures would be expected to cause irreversible inactivation and thermal unfolding. Asn 237 is strictly conserved among all of the hexokinase sequence comparisons (suggesting its critical functional and/or structural role), and its role in the binding site of the enzyme has been previously proposed

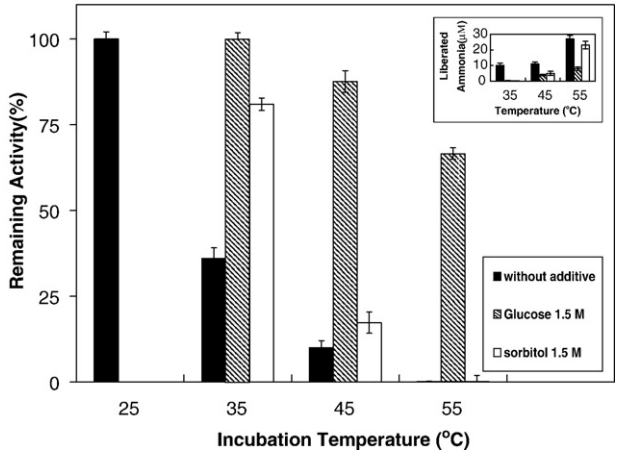

Fig. 6. Irreversible thermoinactivation of YHB in control (■) and in the presence of 1.5 M glucose (▧) or sorbitol (□). Inset: deamidation of amide residues of YHB in control (■) and in the presence of 1.5 M glucose (▧) or sorbitol (□). Ammonia liberated was determined at the indicated temperatures (35–55 °C).

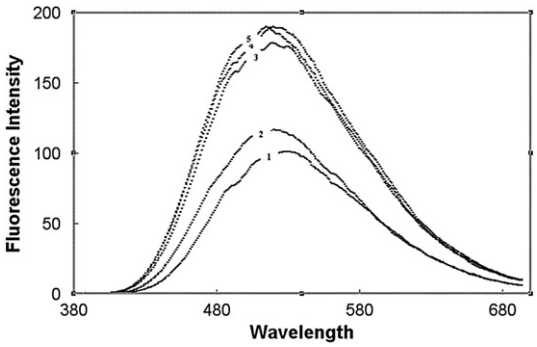

Fig. 7. ANS fluorescence in the absence (1) or presence of yeast hexokinase B, at 25 °C (2), 45 °C (3), 50 °C (4), 60 °C (5). The excitation wavelength was 380 nm and the emission spectra were taken between 400 and 700 nm. See Materials for additional details.

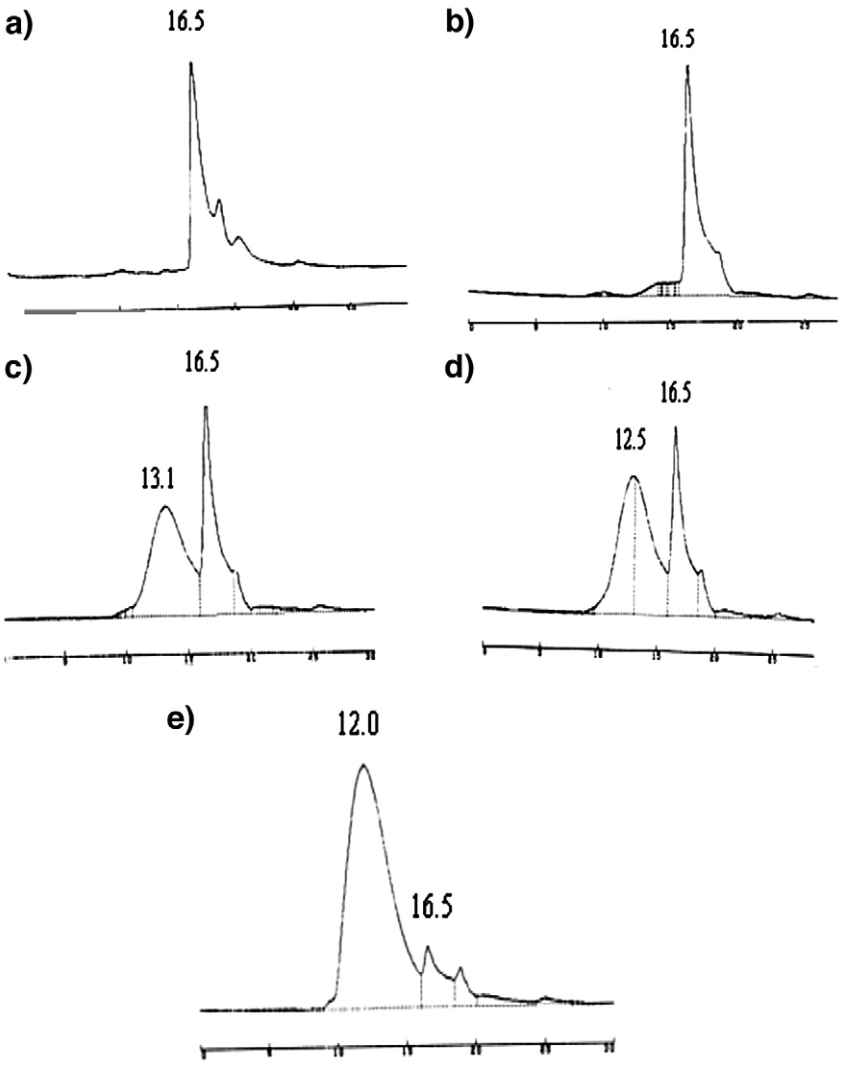

Fig. 8. Representative size exclusion chromatography of yeast hexokinase B. Protein samples were incubated at 60 °C for 0 min (a), 15 min (b), 30 min (c), 60 min (d) and 90 min (e), then loaded on the column. Protein concentration was 0.1 mg/ml.

[4,43,44]. The residue which immediately follows Asn 237 is Gly 238 (which renders Asn 237 strongly susceptible to deamidation [42]), this residue may be a probable candidate for thermal deamidation responsible for thermoinactivation, and irreversibility of thermal unfolding [45].

3.6. Protein aggregation

Since aggregation has been frequently blamed for irreversible thermal denaturation of proteins [40,46], it was explored as a possible mechanism leading to YHB denaturation. Thermal aggregation of YHB was studied at various protein concentrations, in a range of 0.05–0.25 mg/ml in 50 mM MOPS buffer pH 8.0, following the relative 350-nm turbidity for 1 h [29]. However, heating at various temperatures higher than the T_m led to no detectable turbidity. To further examine possible formation of large aggregates, various concentrations of the enzyme (0.05–0.25 mg/ml in 50 mM MOPS, pH 8.0) were incubated for 30 min at 60 °C, followed by centrifugation (9000 ×g for 20 min) and protein concentrations of the supernatants determined. The protein concentrations were identical for the control and heated protein samples, suggesting that all of the protein remained within the supernatant and no large protein aggregates were formed at this temperature. However, as depicted in Fig. 7, ANS emission spectrum was significantly enhanced and blue-shifted when it was exposed to YHB treated at temperatures above 45 °C. This indicated that the unfolded YHB molecule exposes relatively large hydrophobic patches over its surface, making it susceptible to aggregation. The mean

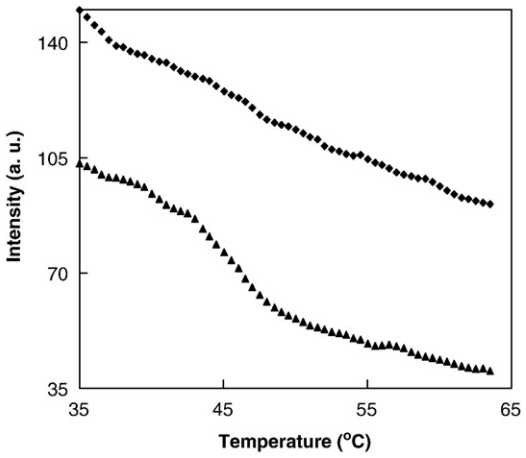

Fig. 9. Thermal denaturation curves of monomeric (▲) and small aggregate fraction (♦), eluted at 16.5 and 12.5 min, respectively. The excitation wavelength was 280 nm and the emission intensity was measured at 335 nm.

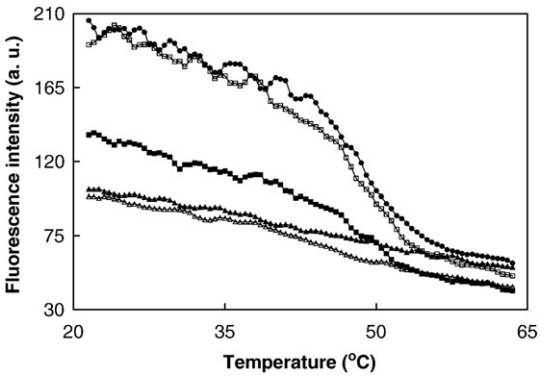

Fig. 10. Effect of various concentrations of γ-cyclodextrin on conformational reversibility of yeast hexokinase B thermal unfolding in the presence of 0.05 mg/ml β-casein. The protein samples and β-casein were first heated to 60 °C and then cooled to 25 °C. γ-cyclodextrin was added after a waiting time of 30 min. Reheating of the samples to 60 °C was followed at a rate of 1 °C/min. The displayed spectra are those obtained in the reheating phase. YHB concentration was 0.05 mg/ml. (Δ: 0.5 mM γ-CD without β-casein, ▲: β-casein without γ-CD, ■: 0.5 mM γ-CD and β-casein, ◊: 1 mM γ-CD and β-casein, ◆: 2 mM γ-CD and β-casein).

hydrodynamic radius of YHB particles (incubated at 60 °C for 5 min) was estimated as 20.025 ± 1 nm by dynamic light scattering. The size of YHB particles remained nearly constant during an incubation period of 30 min confirming that YHB forms small and relatively-stable aggregates at this high temperature. The method was further used to measure the hydrodynamic radius of YHB particles in 50 mM phosphate buffer, in which turbidimetric experiments had indicated formation of large YHB aggregates at 60 °C. The mean hydrodynamic radius of YHB particles in this buffer was estimated as 240 ± 2.02 nm. SEC-HPLC was also employed to confirm thermally-induced formation of small YHB aggregates and separate them from the protein monomers.

The protein sample was loaded on a SEC-HPLC column after various times of heat treatment at 60 °C. As illustrated in Fig. 8, concomitant with a gradual decrease of the characteristic native peak at 16.5 min, a wide peak around 12.0–13.1 min gradually emerges. Based on the observed elution time and its comparison with those of chicken egg white lysozyme (M.W. = 14.38 kDa), bovine serum albumin (M.W. = 66 kDa) and mammalian glutamate dehydrogenase (M.W. = 336 kDa), the new peak is estimated to represent a trimeric or hexameric YHB, approximately. The results provided by dynamic light scattering and SEC-HPLC confirmed that YHB forms small aggregates upon heating at 60 °C. The eluted fractions were separately collected and tested for possible thermal denaturation. Only the monomeric fraction (eluted at 16.5 min similar to the native form) underwent the characteristic unfolding transition (Fig. 9).

Caseins are known to prevent thermal aggregation through their chaperone-like activities [47]. To examine possible protection by casein, heating (at 60 °C) was carried out in the presence of β-casein. Thereafter, the heated protein sample was cooled to 25 °C and treated with γ-CD (known to cause dissociation of protein–casein complexes [48–50]) for 30 min. These additives were only effective in combination as neither of them afforded protection when included alone (Fig. 10). It is possible that the combined effect of these chaperone-like molecules involves a mechanism similar to the "chaperone-assisted refolding" reported earlier [50]. This is a further evidence to support the causative relationship between small aggregates of YHB and its "irreversible" thermal denaturation.

4. Conclusions

In conclusion, the irreversible character of thermal denaturation of yeast hexokinase B was studied in some detail and possible involved mechanisms were explored. It is clear that the process is reversible at temperatures lower than 55 °C. Furthermore, protein fragmentation and cysteine oxidation did not appear to contribute to the mechanism of irreversible thermal denaturation. Taking all of the observations into account, it is suggested that aggregation may be responsible. Formation of small soluble aggregates was confirmed by dynamic light scattering and size exclusion exclusion-HPLC. The combined presence of chaperon -like molecules such as β-casein and γ-CD was effective in affording protection. Deamidation of a critical asparagine residue may also play an important role.

Acknowledgements

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