ELSEVIER

Biophysical Chemistry 122 (2006) 58–65

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Comparison of the molten globule states of thermophilic and mesophilic α-amylases

Maryam Monsef Shokri [a], Khosro Khajeh [a,*], Jahan Alikhajeh [a], Ahmad Asoodeh [a], Bijan Ranjbar [a], Saman Hosseinkhani [a], Mehdi Sadeghi [b]

[a] *Department of Biochemistry, Faculty of Sciences, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran*
[b] *National Institute of Genetic Engineering and Biotechnology, Tehran, Iran*

Received 21 July 2005; received in revised form 17 December 2005; accepted 21 December 2005
Available online 3 March 2006

Abstract

In recent years great interest has been generated in the process of protein folding, and the formation of intermediates during the folding process has been proven with new experimental strategies. In the present work, we have examined the molten globule state of *Bacillus licheniformis* α-amylase (BLA) by intrinsic fluorescence and circular dichroism spectra, 1-anilino naphthalene-8-sulfonate (ANS) binding and proteolytic digestion by pepsin, for comparison to its mesophilic counterpart, *Bacillus amyloliquefaciens* α-amylase (BAA). At pH 4.0, both enzymes acquire partially folded state which show characteristics of molten globule state. They unfold in such a way that their hydrophobic surfaces are exposed to a greater extent compared to the native forms. Chemical denaturation studies by guanidine hydrochloride and proteolytic digestion with pepsin show that molten globule state of BLA is more stable than from BAA. Results from gel filtration indicate that BAA has the same compactness at pH 4.0 and 7.5. However, molten globule state of BLA is less compact than its native state. The effects of polyols such as trehalose, sorbitol and glycerol on refolding of enzymes from molten globule to native state were also studied. These polyols are effective on refolding of mesophilic α-amylase but only slightly effect on BLA refolding. In addition, the folding pathway and stability of intermediate state of the thermophilic and the mesophilic α-amylases are discussed.

Keywords: α-amylase; Molten globule states; Stability; Folding pathway; Aggregation

1. Introduction

The strategies which nature uses to stabilize the inventory of the cell, especially proteins, and under extreme conditions are still enigmatic despite 25 years of active research [1–4]. What has become clear is that proteins, independent of their mesophilic or extremophilic origin, consist exclusively of the canonical 20 natural amino acids, and if other protein constituents are found, they originate from covalent chemical modifications [1]. Thus, enhanced stability can come from only improved attractive forces: within the core, between domains and subunits, or from extrinsic protectants such as compatible solutes, conjugating components, and specific metabolites [1–4]. Based on the complete genome sequence of hyperthermophiles and systematic comparisons of their protein inventories with those of appropriate mesophilic counterparts, a wealth of data has accumulated indicating that stabilization involves all levels of protein structure hierarchy, i.e., secondary, supersecondary, tertiary, and quaternary interactions [5–9]. Moreover, it has recently been demonstrated that the folding of larger proteins often involves of partially folded states of protein [10–12]. Studies on the mechanism of protein folding are directly related to detection and the characterization of these intermediate states [13–16]. Evidence from physico-chemical analysis of increasing number of protein molecules supported the concept of the "molten globule" state. This state reflects a protein form which is compact and has extensive secondary structure but lacks some or all of the persistent tertiary

Abbreviations: ANS, 1-anilino naphthalene-8-sulfonate; BAA, α-amylase from *Bacillus amyloliquefaciens*; BLA, α-amylase from *Bacillus licheniformis*; CD, circular dichroism; Gdn–HCl, guanidine hydrochloride; MG, molten globule; SEC, size exclusion chromatography.

* Corresponding author. Tel./fax: +98 21 8009730.
E-mail address: khajeh@modares.ac.ir (K. Khajeh).

0301-4622/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2005.12.014

interactions typical of native globular proteins and is observed as a common intermediate in the folding process [10–16].

Since bacterial α-amylases are widely used in industrial processes of starch degradation [17] and often at high temperatures, a clear understanding of the molecular mechanism of their folding and structure–function–stability could be very useful [18–20]. In recent communications [21–23], we described our attempts in elucidating the structure–activity relationship of *Bacillus licheniformis* α-amylase (BLA) and *Bacillus amyloliquefaciens* α-amylase (BAA). Sequence alignment of BLA and BAA revealed a very high homology (81% identity, 88% similarity, see Ref. [24] but with a very different thermostability. For detailed understanding of how proteins achieve stability, it is not only important to study the native structures of these molecules, but also the intermediate forms such as molten globule states. In this study, we present comparative data on the characterization of molten globule states of BLA and BAA. Low pH induces a partially folded state in both enzymes which have lost most of their specific side-chain interactions but retained nearly all of their native secondary structures as deduced from circular dichroism (CD) and fluorescence-emission measurements. Here, we have examined the stability of molten globule state of thermophilic α-amylase and its mesophilic counterpart against chemical denaturation and proteolytic digestion. In addition, the refolding pathways of these enzymes will be discussed.

2. Materials and methods

Thermostable α-amylase from *B. licheniformis* (BLA) and mesophilic α-amylase from *B. amyloliquefaciens* (type II-A), pepsin, 3, 5-dinitrosalicylic acid (DNS), soluble potato starch, 1-anilino naphthalene-8-sulfonate (ANS) and Tris were purchased from Sigma (St. Louis, MO. USA). In order to eliminate the dye from BLA it was treated with activated charcoal 100 mg/ml and then it passed through Nylon Acrodisc syringe filter with size of 0.45 μm and after that BLA was subjected to ion chromatography using DEAE-Sepharose and eluted with a linear gradient of 0 to 500 mM NaCl in Tris buffer, pH 7.5. All other chemicals were from Merck (Darmstadt, Germany) and were reagent grade.

2.1. Determination of the enzymatic activity and protein concentration

α-Amylase was assayed in 20 mM Tris buffer, pH 7.5 containing 10 mM $CaCl_2$ at room temperature using potato starch as substrate. The concentration of reducing sugars obtained from the catalyzed reaction was measured by the dinitrosalicylic acid method according to Bernfeld [25]. Protein concentration was determined by the Lowry method [26].

2.2. Preparation of the intermediate form by acid denaturation

Acid-induced unfolding of BAA and BLA was carried out by incubation of each enzyme in mix buffer that contained glycine (20 mM), acetate (20 mM) and Tris (10 mM) at pH 4.0. In order to assess the reversibility of acid-induced unfolding, BAA and BLA at pH 4.0 in the presence and absence of additives such as trehalose, sorbitol and glycerol (13%, W/V or V/V) were diluted 30-fold by 20 mM Tris buffer, pH 7.5, containing 10 mM $CaCl_2$.

2.3. Size exclusion chromatography

Size exclusion chromatography (SEC) experiments were carried out with a Superdex 70 column on a Pharmacia FPLC system (Pharmacia, The Netherlands). BAA and BLA (0.7 mg/ml) were incubated in mix buffer at different pH values and loaded on to the column, equilibrated by the same buffer. The flow rate was set to 10 ml/h.

2.4. Circular dichroism studies

Circular dichroism (CD) measurements were conducted using a JASCO (Tokyo, Japan) J-715 spectropolarimeter equipped with a thermostatically controlled cell holder. The instrument was calibrated with (+)-10-camphorsulfonic acid. Far and near-UV CD spectra were measured at protein concentration of 0.3 and 1 mg ml^{-1}. Results are expressed as molar ellipticity, $[\Theta]$ (deg $cm^2 dmol^{-1}$), based on a mean amino acid residue weight (MWR) assuming average weights of 113.4. The molar ellipticity $[\Theta]$ was calculated from the formula $[\Theta]_\lambda = (\theta \times 100\ \mathrm{MRW})/(cl)$, where c is the protein concentration in mg/ml, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at wavelength λ.

2.5. ANS binding experiments

1-Anilino naphthalene-8-sulfonate (ANS) binding studies were performed in a Perkin Elmer luminescence spectrometer LS 50B. The spectra were measured in mix buffer, at different pH values. The final concentration of ANS in the enzyme solutions was 30 μM, and the molar ratio of protein to ANS was 1:50. The ANS emission was scanned between 400 and 500 nm with an excitation wavelength of 380 nm.

2.6. Proteolytic cleavage of BLA and BAA

Digestion of BLA and BAA were performed by incubation of each dissolved protein in 20 mM mix buffer, pH 4.0 containing 10 mM $CaCl_2$, with pepsin (substrate: protease ratio was 1:25, by mass) at 37 °C. At the end of incubation time, aliquots were removed from the reaction mixture, the pH changed to 6.5 and was immediately placed in sample solvent for SDS-polyacrylamide gel electrophoresis. SDS gel electrophoresis was performed using 15% acrylamide gel according to Laemmli [27]. The gels were stained with coomassie brilliant blue R-250.

2.7. Aggregation measurements

The partially folded enzyme at concentration of 0.1 mg/ml in mix buffer, pH 4 was placed in Perkin Elmer luminescence spectrometer LS 50B cuvette. The excitation and emission

monochromators were set at 350 and 355 nm with band pass of 1.5 nm and the extent of light scattering was monitored [28].

2.8. Stability measurements

The unfolding profiles of MG states of BAA and BLA were determined by intrinsic tryptophan (Trp) fluorescence measurements. The MG states were incubated for 15 min at 25 °C in different Gdn–HCl concentrations. The denaturation process can be monitored through the changes in the fluorescence intensity at 340 nm. The Gibbs free energy change ($\Delta G°$) is given by the following equation:

$$\Delta G° = -RT\ln K.$$

Where R is the universal gas constant, T is the absolute temperature and K is the equilibrium constant of the denaturation process. By plotting the $\Delta G°$ versus [Gdn–Hcl], $\Delta G°_{H2O}$, which is the Y-intercept, can be obtained [29].

2.9. Hydrophobicity calculation

Values for the hydrophobicity of the BAA and BLA protein sequences were calculated using a moving window averaging method that continuously determines the average hydrophobicity within a segment of predetermined length as it advances through the sequence [30]. The program (ProtScale program, from ExPAsy, Swiss Institute of Bioinformatics) assigns the appropriate hydropathy value to each residue, and then successively sums these values, starting at the N terminus, within overlapping segments displaced from each other by one residue. A segment window size of fifteen was used. For a window size n, $(n-1)/2$ neighboring residues on each side of residue i are used to compute the score for residue i; the first value therefore corresponds to the sum of the hydropathies of residues 1–15, and is plotted at location 8 [30]. The relative weight of the window edges was taken as 100%, i.e. all fifteen residues were taken equally to the sum.

Results presented in this paper are the mean from at least three repeated experiments in a typical run to confirm reproducibility.

3. Results

The acid denaturation of BAA and BLA was compared over a pH range of 2 to 7. BAA and BLA contain 17 tryptophan residues, which are distributed in the buried interior and solvent accessible surface of the protein [31]. As the intrinsic fluorophore tryptophan is highly sensitive to the polarity of its surrounding environment, the pH-dependent changes in the conformation of α-amylase were followed using fluorescence spectroscopy. As shown in Fig. 1a, on lowering the pH, the tryptophan fluorescence of BAA and BLA progressively decreases to become more or less constant, indicative of the presence of a non-native stable intermediate at low pH. ANS is essentially non-fluorescent in aqueous solution whereas its emission intensity increases in a hydrophobic environment [32]. This property of ANS was used to study the acid unfolding of BAA and BLA. As shown in Fig. 1b and c, at acidic pH, BAA and BLA show a marked increase in ANS fluorescence intensity as compared to the native protein at pH 7.0. In addition, this effect was accompanied by a blue shift in the ANS fluorescence spectrum at low pH. The changes in the secondary structures of BAA and BLA at different pH values were monitored by far-UV circular dichroism. Since Tris strongly absorbs at wavelengths below 200 nm, the CD spectra have been recorded in the 200–250 nm range (Fig. 2a and b). The ellipticity of a polypeptide chain at 222 nm is usually considered as an index of its α-helix secondary structure. These measurements showed that the ellipticity at 222 nm changes slightly between pH 7.0 and pH 4.0, indicating that even at low pH a native-like secondary structure persists for BAA and BLA. Also, near UV CD spectra for both enzymes showed gradual loss of tertiary structure content as the pH values were decreased (Fig. 2c and d). Therefore, the acid-denatured enzymes retain pronounced secondary structure,

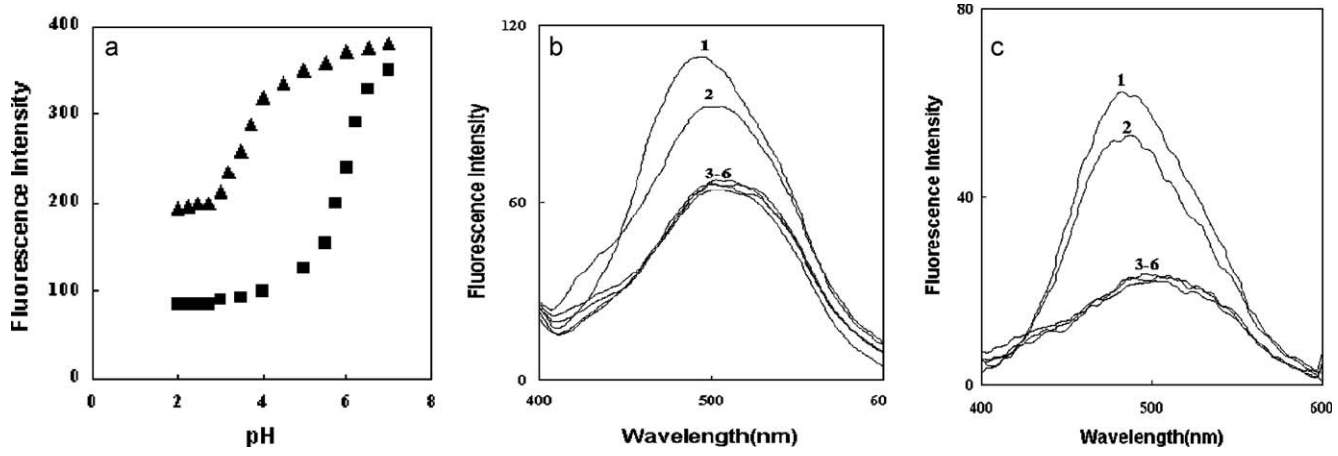

Fig. 1. Characterization of BAA and BLA at acidic pHs. a) Fluorescence intensities of BAA (■) and BLA (▲) at 340 nm upon incubation at different pHs. The excitation wavelength was 280 nm. Fluorescence spectra of 30 μM ANS in the presence of BAA (b) and BLA (c) at (1) pH 4.0; (2) pH 4.5; (3) pH 5; (4) pH 5.5; (5) pH 6.0 and (6) pH 7.0. For more details please see Materials and methods section.

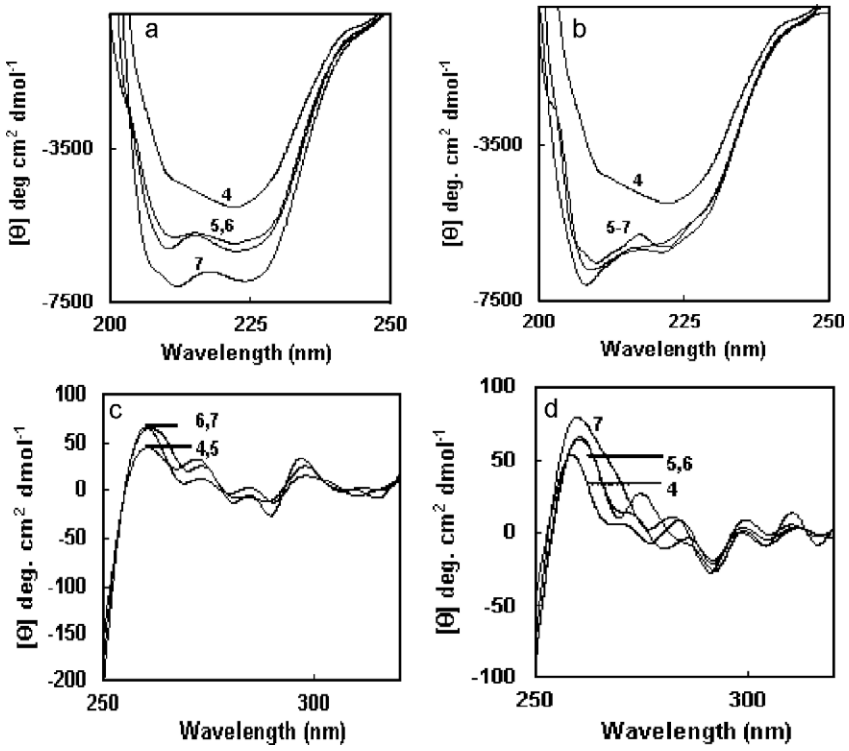

Fig. 2. Far- and near-UV CD spectra of BAA (a and c) and BLA (b and d) at different pH values. Each curve is labeled by corresponding pH value. For more details please see Materials and methods section.

while the near-UV CD spectrum and intrinsic fluorescence of the acid-induced partially folded states are essentially featureless. Goto et al. have proposed that upon acid titration, intramolecular charge repulsions are the driving force for partial unfolding of the protein molecule [33]. Similar results have been reported for the intermediate states of bromelain [34], glucose/xylose isomerase [35], bovine growth hormone [36], apomyoglobin [37] and cellular retinol-binding protein type I [38].

The above results indicate that at low pH, BAA (also see Ref. [39]) and BLA adopt, as do many other proteins [33–37], the features of a partially denatured state, characterized as a "molten globule" state by the properties reported in the following three sections.

3.1. Molten globule state of BAA is less resistant to unfolding and proteolysis than BLA

Fluorescence emission data show that in the acid-denatured state of BAA and BLA, tryptophan residues are not completely

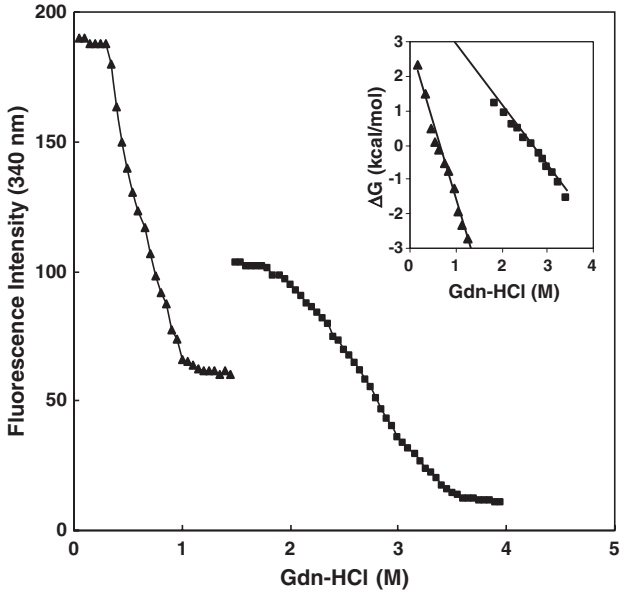

Fig. 3. Chemical denaturation of molten globule states of BAA and BLA (at pH 4.0) by Gdn–HCl. Global unfolding measured through the changes in the fluorescence intensity at 340 nm for BAA (▲) and BLA (■) following excitation at 280 nm. Inset shows the free energy versus [Gdn–HCl] plot for BAA (▲) and BLA (■).

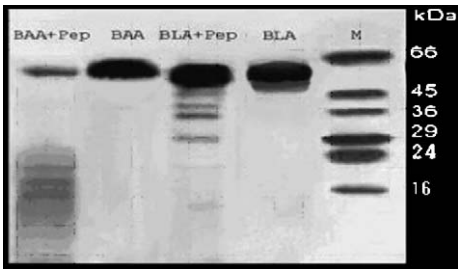

Fig. 4. Proteolysis of BAA and BLA by pepsin (pep) at pH 4.0. BAA and BLA were digested by incubation of each protein dissolved in 20 mM mix buffer, pH 4.0 with pepsin (protein : substrate ratio 1 : 25). After 60 min reaction at 37 °C, an aliquot of the proteolysis mixture was analyzed by SDS-PAGE. The molecular weights of marker (M) are indicated.

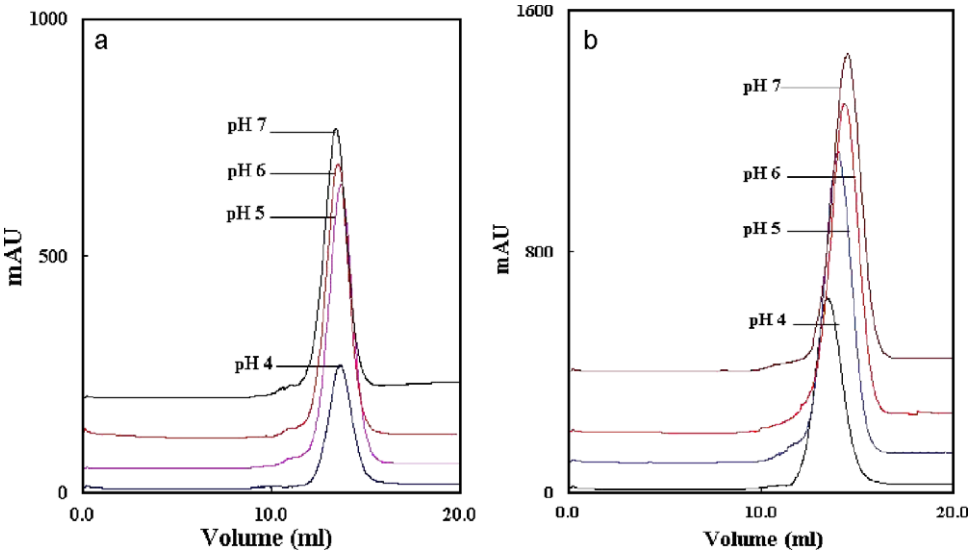

Fig. 5. SEC elution profiles of BAA (a) and BLA (b) at pH 7.0, 6.0, 5.0 and 4.0. Two hundred microliters of enzyme solution was loaded on to a FPLC Superdex 70 column. For more details please see Materials and methods.

exposed to the solvent. In fact, the maximum emission is observed at a wavelength significantly lower than in 6 M Gdn–HCl, a medium in which the protein has lost both secondary and tertiary structures. The unfolding of the molten globules of BAA and BLA at pH 4 and 25 °C as a function of Gdn–HCl concentration was followed by monitoring wavelength fluorescence emission at 340 (Fig. 3) and the inset indicates the free energy versus [Gdn–HCl] plot for the two enzymes at pH 4. Both proteins seem to be unfolded in a two-state process. The apparent midpoint concentration of unfolding (C_m) of BAA in Gdn–HCl is 0.7 M, while that of BLA is 2.7 M. The conformational stabilities in the absence of denaturant (ΔG_{H2O}) at pH 4 and 25 °C are 2.81 and 4.46 kcal/mol for BAA and BLA, respectively. The results show that the molten globule state of a thermophilic enzyme has higher stability than the molten globule state of its mesophilic counterparts.

Previous studies have shown that specific or preferential fission of peptide bond(s) by limited proteolysis is a useful method to probe structural properties of proteins [40–42]. In this study, limited proteolysis was performed by incubating BLA and BAA at pH 4 with pepsin. The SDS-PAGE analysis of the proteolysis mixture after 60 min reaction at 37 °C reveals that the digestion was more extensive with MG state of BAA compared to BLA (Fig. 4).

3.2. Compactness of acid-induced molten globule states of BAA and BLA

In acid-denatured states of BAA and BLA, tryptophan residues are not completely exposed to the solvent (Figs. 1 and 2). This finding is in accordant to the idea that acid-denatured BAA and BLA, similar to other proteins, retain compactness. The compactness of the acid-denatured mesophilic α-amylase

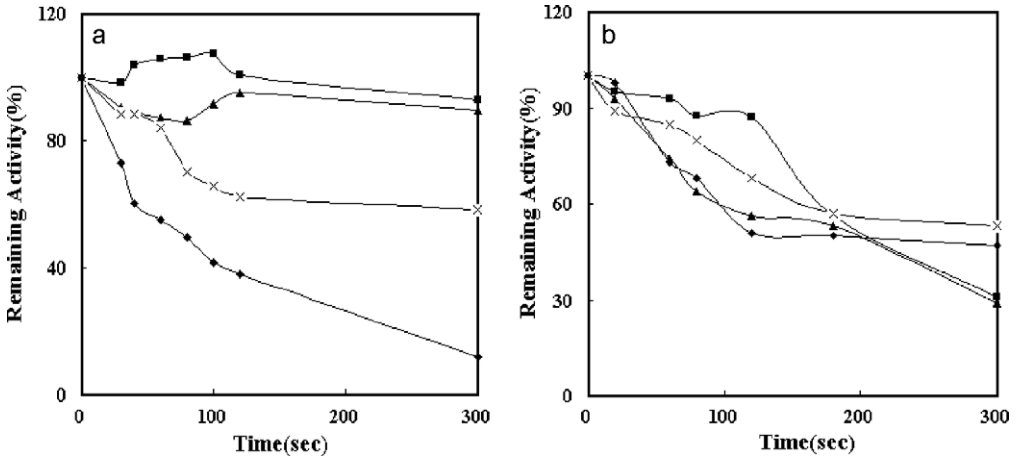

Fig. 6. Reactivation of BAA (a) and BLA (b) in the absence (♦) or presence of trehalose (▲), sorbitol (x) and glycerol (■) (13%, W/V or V/V) as a function of incubation time at pH 4.0. The enzymes were incubated at pH 4.0, in the absence or presence of polyols, and were diluted 30-fold in 20 mM Tris buffer, pH 7.5 containing 10 mM $CaCl_2$ and remaining activities were determined.

and its thermophilic counterpart can be demonstrated by the highly efficient size-exclusion chromatography [10,13,15]. Fig. 5a and b shows elution profiles for acid-induced unfolding of these proteins at 25 °C. At pH 4, a single elution peak is observed for BAA at the position which essentially coincides with that of the native proteins, but the elution peaks of acid-denatured BLA shift to smaller elution volumes with the decrease in pH. These results indicate that the molecular shape of BLA becomes less compact at low pH compared to that of native enzyme, which may correspond to a more expanded state of BLA at acidic pH.

3.3. Refolding studies and aggregation measurements of BAA and BLA at low pH

BAA and BLA previously incubated in mix buffer, pH 4, were diluted 30-fold in 20 mM Tris buffer, pH 7.5 containing 10 mM $CaCl_2$. Enzyme activities of refolded BAA and BLA were recorded 30 min after the dilution (Fig. 6). Results indicated that the reactivation of both enzymes depends on the incubation time at acidic pH. For BLA incubation period at pH 4, after which the enzyme regains 50% of its activity in the reactivation process, was calculated to be 130 s compared to 80 s for BAA indicating more reactivation for BLA. In addition, effect of certain polyols such as sorbitol, trehalose and glycerol on the refolding of BAA and BLA was studied. The enzyme in mix buffer, pH 4, was treated with 13% (W/V or V/V) of each polyol and following various incubation periods, samples were diluted. As shown in Fig. 6 BAA showed higher reactivity in the presence of polyols; however, BLA was affected to a less degree. The observed decrease in intrinsic fluorescence for both enzymes in the presence and absence of polyols at low pH was the same (data not shown). This observation indicates that these additives probably do not prevent the disruption of the tertiary structure in acidic pH.

Light scattering methods provide a sensitive measure of aggregate formation in protein solutions [28]. Fig. 7a and b shows the light scattering profile of BAA and BLA (0.1 mg/ml) as a function of time at pH 4. As is evident in Fig. 7, BAA has a tendency to aggregation in pH 4 while no aggregation is observed for BLA. Furthermore, polyols such as trehalose prevent aggregation and the cohesive force of sugars responsible for the increase in the surface tension of water is also suggested to be an important factor [43]. Accordingly, preferential interaction of proteins with solvent components, in aqueous sugar systems, may take place concomitant with enhancement in refolding process [44].

4. Discussion

How the stabilization of thermophilic proteins affects their molten globule state stability and folding rates may provide insight into the relationship between structure, stability, and folding. There are a relatively small number of comparative studies on the partially denatured states and on the folding pathways of thermophilic and mesophilic homologues in the literature [6,45–47]. We have showed the existence of an intermediate state at low pH for a thermophilic α-amylase (BLA) and its mesophilic counterpart (BAA). The following results were obtained: (1) Far-UV CD spectra of these intermediates for BLA and BAA are very pronounced, which suggest that these intermediates have high content of native-like secondary structure. Not only the main retention of secondary structure in acidic pH but also the remarkable loss of tertiary structure content as a result of decreasing pH values propose a partially folded state known as molten globular like state for both enzymes. (2) The intrinsic fluorescence of tryptophan residues was decreased in BAA and BLA as accompanied by red shift. These results show that at acidic pH, there are changes in the tertiary structure of BAA and BLA resulting in exposure of the buried tryptophan residues to polar solvents. (3) ANS fluorescence intensity in the presence of BAA and BLA increases with a decrease in pH. This shows that BAA and BLA strongly bind ANS in the acidic pH, which is a specific test for molten globule states [13,38]. (4) Size-exclusion chromatography results indicated that the main elution peak of the intermediate state of BAA at low pH practically coincides with that of the native state. In the case of thermophilic enzyme (BLA), the compactness of intermediate state is lower than the native state but is higher than the denatured form of enzyme at

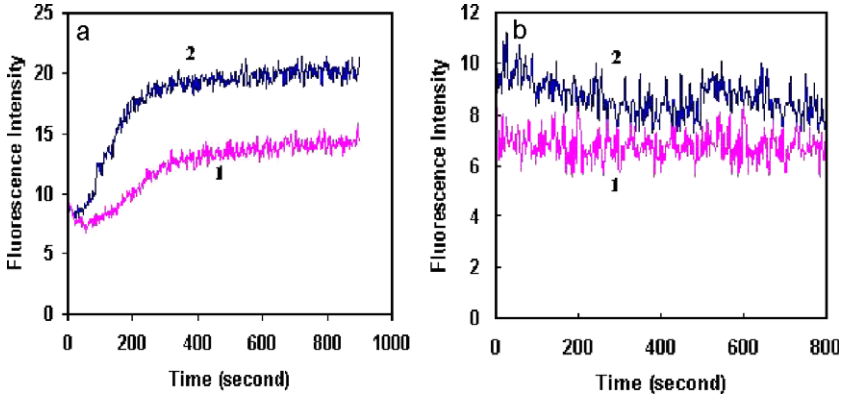

Fig. 7. Aggregation of the molten globule state of BAA (a) and BLA (b) at pH 4.0 in the presence (1) and absence (2) of trehalose (13%, W/V). The enzymes' concentration was 0.1 mg/ml. The excitation and emission monochromators were set at 350 and 355 nm. Standard deviations were within 5% of the experimental values. For more detail please see Materials and methods.

6 M Gdn–HCl (data not shown). All these observations suggest that at acidic pH, BAA and BLA have a compact structure with molten globule-like characteristics. "Molten globule" is partially structured protein folding intermediate that adopts a native-like overall backbone topology in the absence of extensive detectable tertiary interactions. As known from many studies, thermostable proteins often exhibit a high resistance against unfolding induced by chemical denaturants such as urea and Gdn–HCl [6,45,48]. Our results demonstrated that in the intermediate state, also the thermophilic enzyme was highly resistant to chemical denaturant compared to its mesophilic counterparts, as indicated by obtained ΔG_{H2O} values. Resistance was also observed when thermophilic enzyme, in the intermediate state, was subjected to pepsin treatment (Fig. 4). The difference in stability observed for the core residues of the molten globule states of BLA and BAA may be rationalized by the difference in their hydrophobicities. An analysis of the hydrophobic nature of the sequences of the two proteins was carried out using the hydrophobicity scale devised by Kyte and Doolittle [30]; the variations in the hydrophobicities as a function of the sequence are shown in Fig. 8. The β-sheet 3 (Bβ3, His133–His140) in domain B, and α-helix 5 (Aα5, Leu267–Lys276) and β-sheet 2 (Aβ2, Ala39–Trp41) in domain A of BLA can be seen to be significantly more hydrophobic than those of BAA. In addition, the internal elements average hydrophobicity units of the α-amylase secondary structure $(\beta/\alpha)_8$-barrel in BLA is higher than BAA [49]. These observations suggest that the hydrophobic interactions are particularly important in stabilizing the molten globule state of BLA. These results and available data on adapted enzymes to high temperatures can be integrated in a model based on the "new view" using the folding funnel which is described by D'Amico et al. [48]. A decrease in free energy level is observed as the enzyme polypeptide folds when it passes intermediate states and reaches local minima of energy. D'Amico et al. have shown that these intermediate states account for the ruggedness of the funnel slopes in the folding pathway [48]. We have demonstrated that the ruggedness of the funnel which depicts the stability of the intermediate state (such as the molten globule states), is different when thermophilic and mesophilic α-amylases are compared.

Another point warranting the discussion is reactivation of BAA as compared to BLA. Results indicate that BAA, in the MG state, tends to aggregate (Fig. 7a) while it is not observed for BLA. It could be suggested that polyols such as trehalose prevents aggregation (Fig. 7a) and consequently, in the case of BAA increases reactivation (Fig. 6a). When BLA is incubated for 30 min at pH 4, treated with 4 M Gdn–HCl, and dialyzed against Tris pH 7.5 containing 10 mM $CaCl_2$, retrieves almost 90% of its initial activity (data not shown). These results indicate that most probably, in pH 4.0 and following the formation of the intermediate state, BLA tends to adopt a scrambled or misfolded conformation. Gdn–HCl appears to unfold this misfolded structure and following dialysis, at pH 7.5, BLA regains its former activity. Therefore, acid-induced conformational changes in bacterial α-amylases, promote the formation of the aggregation-prone molten globule state in BAA and an intermediate state in BLA which has a tendency to make misfolded or scrambled structure.

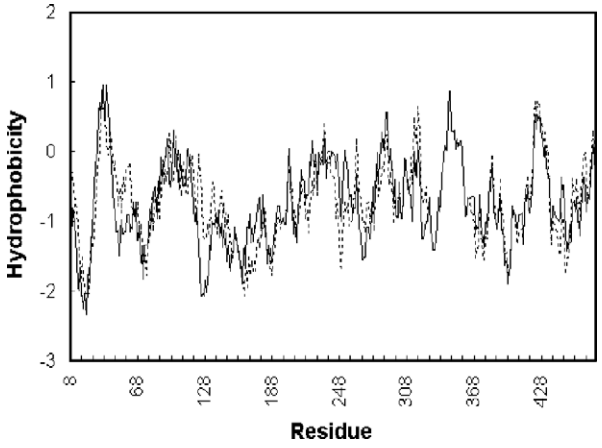

Fig. 8. The hydrophobicity profile for the sequences of BAA (solid) and BLA (dotted). A hydrophobicity value is assigned to each residue and the values summed successively, starting at the N terminus, within overlapping segments displaced from each other by one residue. A segment size of fifteen residues was used and a given sum is plotted above the middle residue of the segment involved.

In summary, we prepared the folding intermediate products (the molten globule states) of two α-amylase homologues, which were confirmed by intrinsic fluorescence, CD spectra and ANS-binding measurement. Characterization of these products demonstrated that the "MG" state of BLA is more stable than that of BAA in terms of resistance to denaturant (Gdn–HCl) and proteolytic digestion. Size exclusion chromatography showed that the compactness of BLA might change with environmental pH whereas BAA remains relatively stable. Effects of polyols on the refolding of the enzyme were also studied. Results indicated that polyols are more effective on refolding of mesophilic α-amylase than thermophilic enzyme.

Acknowledgment

Financial support of this work was provided by the Research Council, Tarbiat Modares University.

References

[1] R. Jaenicke, Do ultrastable proteins from hyperthermophiles have high or low conformational rigidity? Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 2962–2964.
[2] K.O. Stetter, Extremophiles and their adaptation to hot environments, FEBS Lett. 452 (1999) 22–25.
[3] R. Scandurra, V. Consalvi, R. Chiaraluce, L. Politi, P.C. Engel, Protein thermostability in extremophiles, Biochimie 80 (1998) 933–941.
[4] R. Das, M. Gerstein, The stability of thermophilic proteins: a study based on comprehensive genome comparison, Funct. Integr. Genomics 1 (2000) 76–88.
[5] C. Vieille, G. Zeikus, Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability, Microbiol. Mol. Biol. Rev. 65 (2001) 1–43.
[6] J. Fitter, S. Haber-Pohlmeier, Structural stability and unfolding properties of thermostable bacterial α-amylases: a comparative study of homologous enzymes, Biochemistry 43 (2004) 9589–9599.

[7] N. Declerck, M. Machius, G. Wiegand, R. Huber, C. Gaillardin, Probing structural determinants specifying high thermostability in *Bacillus licheniformis* α-amylase, J. Mol. Biol. 301 (2000) 1041–1057.
[8] A. Fontana, Analysis and modulation of protein stability, Curr. Opin. Biotechnol. 2 (1991) 551–560.
[9] J.N. Panasik, J.E. Brenchley, G.K. Farber, Distributions of structural features contributing to thermostability in mesophilic and thermophilic α/β barrel glycosyl hydrolases, Biochim. Biophys. Acta 1543 (2000) 189–201.
[10] Q. Xie, T. Guo, J. Lu, H.M. Zhou, The guanidine like effects of arginine on aminoacylase and salt-induced molten globule state, Int. J. Biochem. Cell Biol. 36 (2004) 296–306.
[11] A. Naeem, K.A. Khan, R.H. Khan, Characterization of a partially folded intermediate of papain induced by fluorinated alcohols at low pH, Arch. Biochem. Biophys. 432 (2004) 79–87.
[12] A. Naeem, R.H. Khan, Characterization of molten globule state of cytochrome *c* at alkaline, native and acidic pH induced by butanol and SDS, Int. J. Biochem. Cell Biol. 36 (2004) 2281–2292.
[13] V.N. Uversky, R.H. Semisotnov, R.H. Pain, O.B. Ptitsyn, 'All-or-none' mechanism of the molten globule unfolding, FEBS Lett. 314 (1992) 89–92.
[14] J.K. Myers, T.G. Oas, Preorganized secondary structure as an important determinant of fast protein folding, Nat. Struct. Biol. 8 (2001) 552–558.
[15] K. Huang, Y.D. Park, Z.F. Cao, H.M. Zhou, Reactivation and refolding of rabbit muscle creatine kinase denatured in 2,2,2-trifluoroethanol solutions, Biochim. Biophys. Acta 1545 (2001) 305–313.
[16] K. Borén, H. Grankvist, P. Hammarström, U. Carlsson, Reshaping the folding energy landscape by chloride salt: impact on molten-globule formation and aggregation behavior of carbonic anhydrase, FEBS Lett. 566 (2004) 95–99.
[17] A. Pandey, P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh, R. Mohan, Advances in microbial amylases, Biotechnol. Appl. Biochem. 31 (2000) 135–152.
[18] M. Machius, N. Declerck, R. Huber, G. Wiegand, Activation of *Bacillus licheniformis* α-amylase through a disorder→order transition of the substrate-binding site mediated by a calcium–sodium–calcium metal triad, Structure 6 (1998) 281–292.
[19] N. Declerck, M. Machius, R. Chambert, G. Wiegand, R. Huber, C. Gaillardin, Hyperthermostable mutants of *Bacillus licheniformis* α-amylase: thermodynamic studies and structural interpretation, Protein Eng. 10 (1997) 541–549.
[20] G. Feller, F. Payan, F. Theys, M. Qian, R. Haser, C. Gerday, Stability and structural analysis of α-amylase from the antarctic psychrophile *Alteromonas haloplanctis* A23, Eur. J. Biochem. 222 (1994) 441–447.
[21] K. Khajeh, H. Naderi-Manesh, B. Ranjbar, A.A. Moosavi-Movahedi, M. Nemat-Gorgani, Chemical modification of lysine residues in *Bacillus* α-amylases: effect on activity and stability, Enzyme Microb. Technol. 28 (2001) 543–549.
[22] K. Khajeh, B. Ranjbar, H. Naderi-Manesh, A. Ebrahim-Habibi, M. Nemat-Gorgani, Chemical modification of bacterial α-amylases: changes in tertiary structures and the effect of additional calcium, Biochim. Biophys. Acta 1548 (2001) 229–237.
[23] S.M. Asghari, K. Khajeh, B. Ranjbar, R.H. Sajedi, H. Naderi-Manesh, Comparative studies on trifluoroethanol (TFE) state of a thermophilic α-amylase and its mesophilic counterpart: limited proteolysis, conformational analysis, aggregation and reactivation of the enzymes, Int. J. Biol. Macromol. 34 (2004) 173–179.
[24] N. Declerck, M. Machius, P. Joyet, G. Wiegand, R. Huber, C. Gaillardin, Engineering the thermostability of *Bacillus licheniformis* α-amylase, Biologia 57 (2002) 203–211.
[25] P. Bernfeld, Amylase, α and β, Methods Enzymol. 1 (1955) 149–151.
[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[27] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[28] K. Rajaraman, B. Raman, C.M. Rao, Molten-globule state of carbonic anhydrase binds to the chaperone-like α-crytallin, J. Biol. Chem. 271 (1996) 27595–27600.
[29] M.M. Santoro, D.W. Bolen, Unfolding free energy changes determined by the linear extrapolation method: 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants, Biochemistry 27 (1988) 8063–8068.
[30] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.
[31] M. Machius, G. Wiegand, R. Huber, Crystal structure of calcium-depleted *Bacillus licheniformis* α-amylase at 2.2 Å resolution, J. Mol. Biol. 246 (1995) 545–559.
[32] G.V. Semisotnov, N.A. Rodionova, O.I. Razgulyaev, V.N. Uversky, A.F. Gripacæ, R.I. Gilmanshin, Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe, Biopolymers 31 (1991) 119–128.
[33] Y. Goto, N. Takahashi, A.L. Fink, Mechanism of acid-induced folding of proteins, Biochemistry 29 (1990) 3480–3488.
[34] S.K. Haq, S. Rasheedi, R.H. Khan, Characterization of a partially folded intermediate of stem bromelain at low pH, Eur. J. Biochem. 269 (2002) 47–52.
[35] S.A. Pawar, V.V. Deshpande, Characterization of acid-induced unfolding intermediates of glucose/xylose isomerase, Eur. J. Biochem. 267 (2000) 6331–6338.
[36] T.F. Holzman, J.J. Dougherty Jr., D.N. Brems, N.E. MacKenzie, pH-induced conformational states of bovine growth hormone, Biochemistry 29 (1990) 1255–1261.
[37] I. Nishii, M. Kataoka, Y. Goto, Thermodynamic stability of the molten globule states of apomyoglobin, J. Mol. Biol. 250 (1995) 223–238.
[38] F. Torta, A.E. Dyuysekina, D. Cavazzini, A. Fantuzzi, V.E. Bychkova, G. L. Rossi, Solvent-induced ligand dissociation and conformational states of cellular retinol-binding protein type I, Biochim. Biophys. Acta 1703 (2004) 21–29.
[39] S.M. Asghari, K. Khajeh, F. Moradian, B. Ranjbar, H. Naderi-Manesh, Acid-induced conformational changes in *Bacillus amyloliquefaciens* α-amylase: appearance of a molten globule like state, Enzyme Mirob. Technol. 35 (2004) 51–57.
[40] S.J. Hubbard, The structural aspects of limited proteolysis of native proteins, Biochim. Biophys. Acta 1382 (1998) 191–206.
[41] A. Fontana, P.P. de Laureto, B. Spolaore, E. Frare, P. Picotti, M. Zambonin, Probing protein structure by limited proteolysis, Acta Biochim. Pol. 51 (2004) 299–321.
[42] K. Khajeh, S. Khezre-Barati, M. Nemat-Gorgani, Proteolysis of mesophilic and thermophilic alpha-amylases: a comparative study, Appl. Biochem. Biotechnol. 94 (2001) 97–109.
[43] J.F. Back, D. Oakenfull, M.B. Smith, Increased thermal stability of proteins in the presence of sugars and polyols, Biochemistry 18 (1979) 5191–5196.
[44] T. Arakawa, S.N. Timasheff, Stabilization of protein structure by sugars, Biochemistry 21 (1982) 6536–6544.
[45] J. Hollien, S. Marqusee, Comparison of the folding processes of *T. thermophilus* and *E. coli* ribonucleases H, J. Mol. Biol. 316 (2002) 327–340.
[46] R. Wijesinha-Bettoni, C.M. Dobson, C. Redfield, Comparison of the denaturant-induced unfolding of the bovine and human α-lactalbumin molten globules, J. Mol. Biol. 312 (2001) 261–273.
[47] F.A. Chowdhury, D.P. Raleigh, A comparative study of the α-subdomains of bovine and human α-lactalbumin reveals key differences that correlate with molten globule stability, Protein Sci. 14 (2005) 89–96.
[48] S. D'Amico, J.C. Marx, C. Gerday, G. Feller, Activity–stability relationships in extremophilic enzymes, J. Biol. Chem. 278 (2003) 7891–7896.
[49] Š. Janeček, Does the increased hydrophobicity of the interior and hydrophilicity of the exterior of an enzyme structure reflect its increased thermostability? Int. J. Biol. Macromol. (1993) 317–318.