J. Biochem. **142,** 193–200 (2007)
doi:10.1093/jb/mvm112

Partially Folded Conformations of Bovine Liver Glutamate Dehydrogenase Induced by Mild Acidic Conditions

Hassan Rooki[1], Khosro Khajeh[2], Ali Mostafaie[3], Soheila Kashanian[4] and Sirous Ghobadi[1,*]

[1]Department of Biology, Faculty of Science, Razi University, 67149-67346, Kermanshah; [2]Department of Biochemistry, Faculty of Science, Tarbiat Modares University, Tehran; [3]Medical Biology Research Center, Kermanshah University of Medical Sciences; and [4]Department of Chemistry, Faculty of Science, Razi University, Kermanshah, Iran

Received February 21, 2007; accepted May 14, 2007; published online May 21, 2007

The acid-induced unfolding of bovine liver glutamate dehydrogenase (GDH) was studied using various spectroscopic methods such as far- and near-UV circular dichroism (CD), intrinsic and 1-anilino naphthalene-8-sulphonate (ANS) extrinsic fluorescence spectroscopy, light scattering and fluorescence quenching in 20 mM mixed buffer at various pHs. CD spectra show that at pH 3.5, GDH retains its secondary structure substantially, whereas its tertiary structure content is reduced considerably. Intrinsic fluorescence of GDH and ANS binding suggest that, at pH 3.5, the hydrophobic surface of enzyme is more exposed in comparison to the native form. Acrylamide quenching indicates more exposure of tryptophan residues of enzyme at pH 3.5 in comparison to pH 7.5. Another partially unfolded intermediate was detected at pH 5.0, which with its ANS binding capacity lies between the pH 3.5 intermediate and the native form of the enzyme. Gel filtration results revealed that the enzyme at pH 3.5 is dissociated into trimeric species whereas it exists as hexamer at pH 7.5 and 5.0. All the data taken together suggest the existence of two partially unfolded states of GDH at moderate acidic pHs which may be considered as molten and pre-molten globule-like states.

Key words: acid-induced intermediate, aggregation, glutamate dehydrogenase, molten globule, refolding.

Abbreviations: ANS, 1-Anilino naphthalene-8-sulphonate; GDH, glutamate dehydrogenase; NADH, nicotinamide adenine dinucleotide reduced; NAD^+, nicotinamide adenine dinucleotide.

Protein folding is the process by which the amino-acid sequence of a protein determines its three-dimensional conformation (*1*). Protein folding is considered as the translation of the second-half of the genetic code since it directs a disordered polypeptide chain to a specific native state (*2*). There are several reports concerning that polypeptide chains can adopt three-dimensional conformations which are different from the functional and native conformation of proteins (*3–7*). It is well known that protein folding proceeds through few intermediates which can accumulate during the folding process. For understanding the principles governing protein folding it is important to study these partially folded intermediates (*8*, *9*). A common intermediate, which populated during the early stage of protein folding, is referred as molten globule (*10*). According to Ptitsyn's (*11*) definition of the molten globule, the key characteristics of these intermediates are substantial secondary structure, a high compactness without a rigid packing inside the molecule and a substantial increase of fluctuations of side chains as well as of larger parts of the molecule. These partially folded intermediates can also be formed upon mild denaturing conditions, such as, moderately low or high pH, high temperatures or mild concentrations of chemical denaturants (*7*). Several studies are performed with monomeric proteins and results have improved our knowledge about the protein folding problem and folding intermediates (*12–16*). However, similar studies on oligomeric proteins are not so much. On the other hand, whereas some reports are concerning chemical (*17*), thermal (*18*) and mild denaturation (*19*) of bovine liver glutamate dehydrogenase (GDH), there is no report on acid-induced denaturation of this enzyme in the literature. So, this kind of study may provide insight regarding the tertiary and quaternary interactions as well as finding intermediate(s) during the probable protein disassembly and reconstitution. In addition, it may provide comparison between acid-induced unfolding of the bovine liver enzyme and previously reported thermostable counterpart (*20*).

In this study, protein folding/unfolding at acidic pH has been examined using bovine liver GDH [L-glutamate: $NAD\text{-}(p)^+$ oxidoreductase (deaminating), EC 1.4.1.3]. This enzyme is composed of six identical subunits with a molecular mass of 336 kDa (*21*) and catalyses the reversible reductive amination of α-ketoglutarate to L-glutamate using ammonia as the source of amine and NAD(P)H as the coenzyme (*22*). The results indicated the existence of an intermediate with molten globule-like properties at acidic pH with considerable secondary

*To whom correspondence should be addressed. Tel: +98-831-4274545, Fax: +98-831-4274545, E-mail: sirousghobadi@yahoo.com ghobadi@sci.razi.ac.ir

structure and relatively more exposed hydrophobic surface as judged by fluorescence, circular dichroism (CD) and **1-anilino naphthalene-8-sulphonate** (ANS) binding studies. Another intermediate has also been observed which may be considered as a pre-molten globule-like intermediate.

MATERIALS AND METHODS

Bovine liver GDH was purchased from Boehringer Manheim (Germany) as a solution in 50% glycerol (Type II). Nicotinamide adenine dinucleotide reduced (NADH), α-ketoglutarate, ANS and IgG were obtained from sigma (St Louis, MO, USA). All other chemicals were of analytical grade from Merck (Darmstadt, Germany) and solutions were prepared in double-distilled water. Mixed buffer (20 mM) containing glycine, acetate, phosphate and Tris at various pHs was used as the buffer. For all experiments, prior to use, the enzyme solution was dialysed at 4°C for 24 h with several changes of buffer, then centrifuged for 20 min at 15,000 r.p.m. to remove any precipitate. Enzyme and NADH concentration were measured spectrophotometrically using $\varepsilon_{280} = 0.97\ mg^{-1}\ ml\ cm^{-1}$ (*23*) and $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ (*24*), respectively. All experiments were carried out at 25°C.

Enzyme Assay—Activity of native, acid denatured and refolded GDH was determined from the decrement in the absorption at 340 nm following the oxidation of NADH at 25°C using a Cary-100 Bio (Varian) UV-Vis spectrophotometer with jacketed cell holders. The temperature was regulated by an external thermostated water circulator. The assays were carried out in the presence of 5.6 mM α-ketoglutarate, 55.5 mM NH_4Cl, 0.2 mM EDTA and 0.1 mM NADH in the mixed buffer pH 7.5. The final volume was 800 μl. The reaction started by adding enzyme to provide a final concentration of about 2×10^{-4} mg/ml. This condition provided a rate of 0.04 dA/min which remains linear for at least 1.5 min (*22*).

Preparation of the Acid Denatured Intermediates—Acid-induced unfolding of GDH was carried out in 20 mM solution of mixed buffer at various pHs. In order to assess the reversibility of acid denaturation, acid-induced denatured GDH at acidic pHs were diluted 30-fold by 20 mM mixed buffer, pH 7.5 and refolding of enzyme was studied by activity measurements and recording fluorescence emission spectrum.

Intrinsic and Extrinsic Fluorescence Measurements—Fluorescence measurements were performed on a Varian Cary Eclipse spectrofluorimeter with jacketed cell holders. The temperature was regulated by an external thermostated water circulator. GDH at pH 7.5 as well as acid-induced denatured forms were excited at 292 nm and the intrinsic emission spectra were recorded between 305 and 405 nm. The excitation and emission slit widths were set at 5 and 10 nm, respectively. In all the fluorescence experiments, the protein concentration was 0.02 mg/ml and recording the emission spectra was done after incubation of enzyme solutions in appropriate buffer at 25°C, otherwise stated.

Extrinsic fluorescence was measured using ANS as an extrinsic fluor. ANS binding was monitored by recording the ANS emission at various pHs between 410 and 590 nm which was excited at 380 nm. The concentration of protein was 0.06 μM and the final concentration of ANS in the enzyme solutions was 3 μM. The molar ratio of protein to ANS was 1 : 50. In fluorescence energy transfer measurements, GDH at various pHs was excited at 292 nm and the NADH emission peak intensity was recorded at 455 nm. The concentration of NADH was 0.1 mM.

Acrylamide Quenching—Fluorescence quenching experiments were carried out by addition of aliquots of acrylamide stock solution (10 M) to the protein solution (0.02 mg/ml) which previously incubated at pHs 7.5, 5.0 and 3.5. The enzyme was excited at 292 nm and the emission spectra were scanned between 310 and 410 nm. The decrease in fluorescence intensity at λ_{max} of emission was analysed according to the Stern–Volmer equation; $F_0/F = 1 + K_{sv}[Q]$ in which, F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of quencher, respectively, K_{sv} is the Stern–Volmer constant, and [Q] is the molar concentration of the quencher (*25*). The intrinsic protein fluorescence, F, was corrected for acrylamide inner filter effect, f, the later being defined as shown in the following equation, $f = 10^{-\varepsilon[Q]/2}$ using ε as the extinction coefficient for acrylamide at the measured wavelength.

Circular Dichroism Measurements—CD spectra were recorded on a JASCO (Tokyo, Japan) J-810 spectropolarimeter at protein concentration of 0.3 and 1.5 mg/ml for far-UV and near-UV, respectively. The results are expressed as molar ellipticity, [θ] (deg $cm^2\ dmol^{-1}$), based on a mean amino acid residue weight (MRW) assuming average weight of 111.0. The molar ellipticity was determined to be $[\theta]_\lambda = (\theta \times 100\ MRW)/(cl)$, where c is the protein concentration in milligrams per millilitre, l is the light pathlength in centimetre, and θ is the measured ellipticity in degrees at a given wavelength. The instrument was calibrated with (+)-10-camphorsulphonic acid, assuming $[\theta]_{291} = 7820\ deg\ cm^2\ dmol^{-1}$ (*26*), and with JASCO standard non-hygroscopic ammonium (+)-10-camphorsulphonate, assuming $[\theta]_{290.5} = 7910\ deg\ cm^2\ dmol^{-1}$ (*27*). Noise in the data was smoothed using JASCO J-810 software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without peak shape distortion.

Light Scattering Measurements—Rayleigh light scattering experiments were carried out on Varian Cary Eclipse spectrofluorimeter to follow protein aggregation. The partially folded enzyme at the concentration of 0.02 mg/ml in mixed buffer at pHs 3.5 and 5.0 as well as the enzyme sample at pH 7.5 were placed in the cuvette and the time-dependent change in scattering intensity was followed (*28*). The excitation and emission wavelengths were set at 450 and 455 nm, respectively and the excitation and emission slit widths were set at 5 and 10 nm, respectively.

Size Exclusion Chromatography—In order to study the probable subunit assembly changes in the enzyme, GDH (4 mg/ml) samples which previously incubated at pHs 7.5, 5.0 and 3.5 were applied onto a Sephacryl-S 200 gel chromatography column previously equilibrated with mixed buffer with appropriate pH. Runs were carried

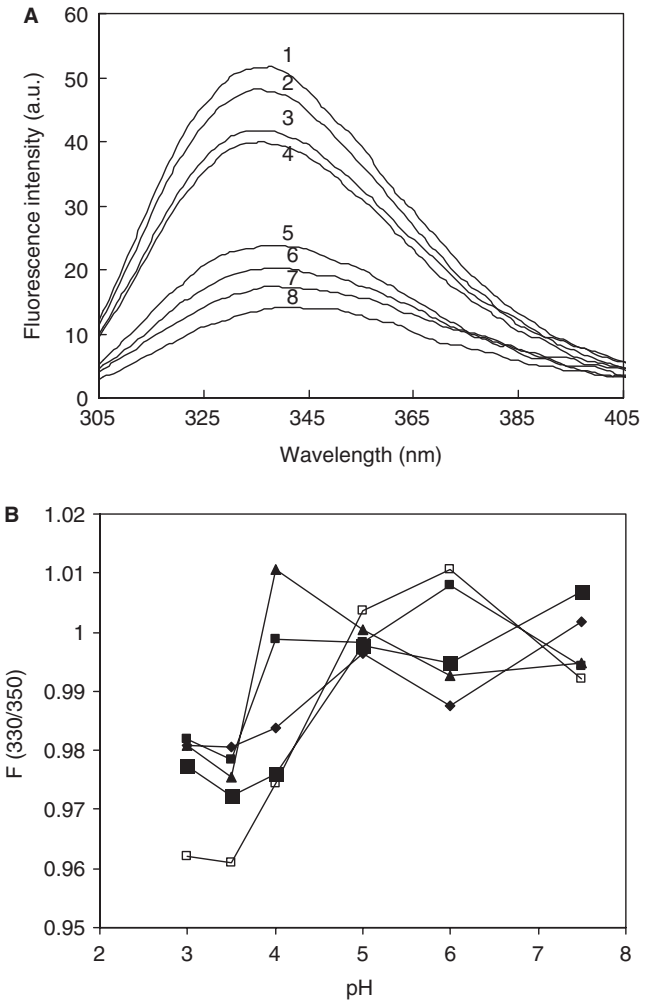

Fig. 1. **(A) Fluorescence emission spectra of GDH at different pHs**; pH 7.5 (1), pH 6.0 (2), pH 5.5 (3), pH 5.0 (4), pH 4.5 (5), pH 4.0 (6), pH 3.5 (7) and pH 3.0 (8). **(B)** The effect of pH upon the fluorescence emission ratio F330/F350 of GDH at different temperatures; 25°C (small filled square), 30°C (filled diamond), 40°C (filled triangle), 50°C (large filled square) and 60°C (open square). The enzyme was excited at 292 nm. (For more details please see MATERIALS AND METHODS).

out at 4°C with a flow rate of 0.3 ml/min with monitoring the absorption at 280 nm. The size of column was 30 cm × 0.9 cm in height and diameter, respectively. Catalase (230 kDa), IgG (168 kDa) and ovalbumin (45 kDa) at concentrations of 0.5, 1.5 and 1.5 mg/ml, respectively were used as molecular mass standards for detecting the subunit assembly states of GDH at various pHs.

RESULTS

Fluorescence Measurement of pH-induced Denatured GDH—The intrinsic tryptophan fluorescence spectra of GDH at various pH values are presented in Fig. 1A. As can be seen, with lowering of pH, intrinsic fluorescence of GDH decreases. In proteins, the intrinsic fluorescence of tryptophan is highly sensitive to the polarity of its environment. Each subunit of bovine liver GDH contains three tryptophan residues (*29*). The emission spectrum of GDH at pH 7.5 shows a maximum at 338 nm which suggests non-polar surrounding environment of buried tryptophans of the enzyme. The ratio of the fluorescence intensity of GDH at 330 and 350 nm was measured at different pH values to detect small changes in the emission maximum as well as the effect of temperature and the results are shown in Fig. 1B. A decrease in F330/350 ratio is indicative of a red shift whereas an increase in the ratio signifies a blue shift (*30*). With lowering of pH there is a decrease in the ratio, suggesting that at acidic pH, there are some changes in the tertiary (or quaternary) structure of enzyme resulting in the exposure of the buried tryptophans to the polar solvent. It is noteworthy that at pHs 3.5 and 5.0, no significant change in the F330/350 ratio of enzyme is observed in the temperature range of 25–60°C (with the exception of 60°C at acidic pHs), revealing that at these two pH values the enzyme exists as stable intermediate states in the N↔D transition. Similar results are reported previously for glucose/xylose isomerase upon lowering the pH at different temperatures (*31*). It has been proposed that upon acid-titration of proteins, intramolecular charge repulsions are the driving force for the partial unfolding of the molecule (*32*). Similar results have been reported for acidic titration of apomyoglobin (*33*), glucose/xylose isomerase (*31*), stem bromelain (*34*), bovine growth hormone (*35*) and *Bacillus amyloliquefaciens* α-amylase (*36*).

ANS and NADH Binding—The binding of ANS to GDH at various pHs was investigated by spectrofluorimetry, as illustrated in Fig. 2A. ANS itself has a very weak fluorescence in aqueous solutions, but its fluorescence intensity increases in a hydrophobic environment. Thus, ANS binding study is a convenient way to show the existence of the surface hydrophobic patches as well as to detect non-native, intermediate conformations of globular proteins (*19*, *36*, *37*). The fluorescence emission spectrum of ANS displays an increase in the emission intensity and a blue shift of the maximum emission wavelength (from 522 nm to 470 nm) as the pH decreases. Variation of ANS maximum emission upon binding to enzyme at various pHs is shown in Fig. 2B. As can be seen, the surface hydrophobicity of the enzyme must be increased in acidic pHs which results in higher ANS binding and subsequent more intense fluorescence. Note that the emission intensity of ANS-protein at pH 3.5 is greater than its intensity at pHs 3.0 and 4.0 as well as all other pHs. These observations suggest the presence of a larger number of solvent-accessible non-polar clusters in GDH at pH 3.5 as more ANS molecules bind to its hydrophobic surface.

To explore the effect of pH on the NADH binding properties of GDH, fluorescence energy transfer experiments were carried out by exciting GDH and recording the NADH fluorescence emission spectra. It is known that the emission of NADH increases upon binding to proteins. Figure. 2C shows the NADH fluorescence intensity decreases by lowering the pH and indicates that at acidic pHs, the conformation of enzyme is changed and NADH binding site became disturbed.

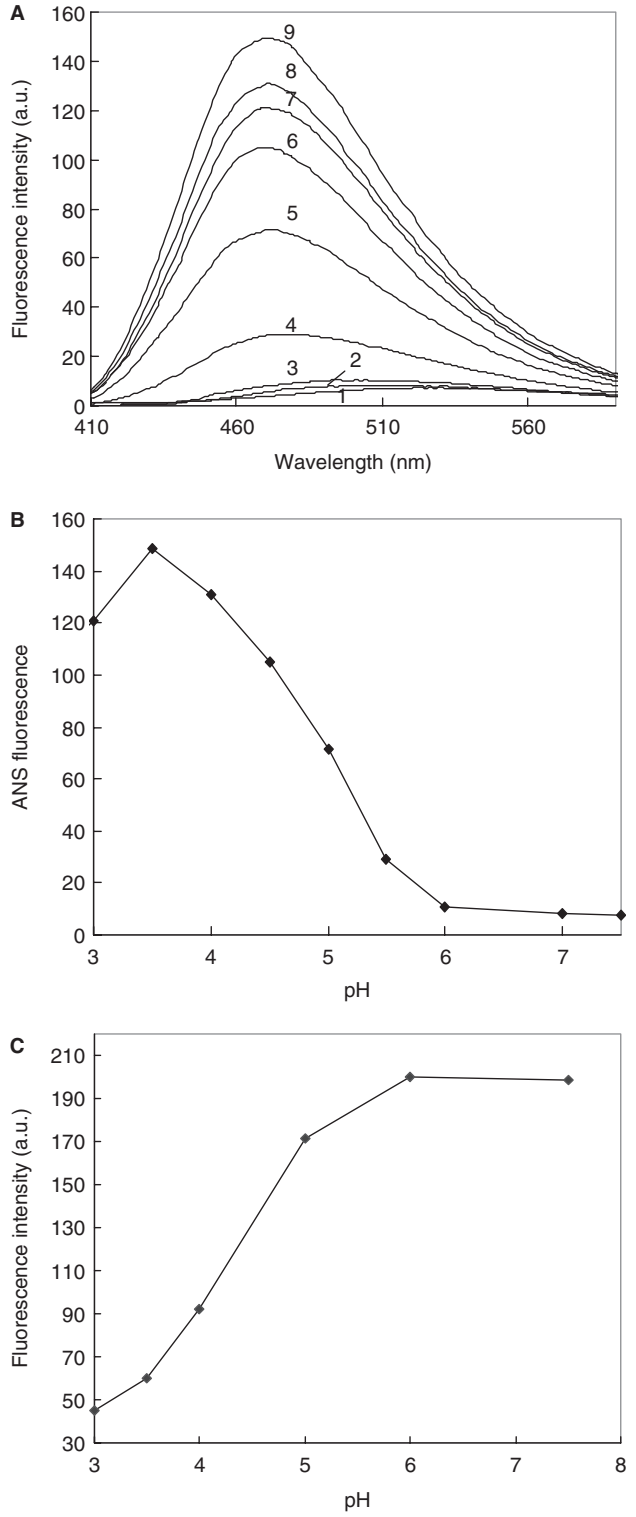

Fig. 2. **(A) The fluorescence emission spectra**; of 3 μM ANS alone (1) and in the presence of 0.06 μM GDH at pH 7.5 (2), pH 6.0 (3), pH 5.5 (4), pH 5.0 (5), pH 4.5 (6), pH 3.0 (7), pH 4.0 (8) and pH 3.5 (9). (B) The effect of pH on the ANS fluorescence intensity which bound to GDH. (C) The effect of pH on the NADH fluorescence energy transfer. Studies were carried out by excitation the enzyme at 292 nm and recording NADH emission at the maximum emission value. (For more details please see MATERIALS AND METHODS).

Circular dichroism Measurements—For monitoring the effects of pH on the secondary and tertiary structures of enzyme, CD measurements were performed in the far- and near-UV regions, respectively. Since Tris has a high absorption of UV wavelengths below 200 nm, the CD spectra have been recorded in the 200–250 nm range. The far-UV CD spectra of GDH at various pHs are shown in Fig. 3A. As can be seen, far-UV CD spectrum of GDH at pH 7.5 exhibited double minima at 208 and 222 nm, typical of a protein with α-helical structure. The far-UV CD changes observed over pH 7.5–3.0 are mainly characterized by decrease of negative ellipticities at 208 nm and 222 nm, where the latter is more pronounced. These results show that in acidic conditions, in spite of some loss of α-helix content, GDH retains its secondary structure substantially. The variation of $[\theta]_{222}$ upon pH alteration is depicted in Fig. 3B. It is obvious that loss of secondary structure proceeds through a cooperative transition, as judged by its sigmoidal curve, with pH 5.0 as the midpoint of the transition. The near-UV CD spectrum, which characterizes the tertiary structure of GDH, at pH 7.5 (as the native form), pH 5.0 (the form which exists as the secondary structure transition midpoint) and pH 3.5 (the most ANS-bound acidic form) are shown in Fig. 3C. The spectrum of GDH in the near-UV region which is determined by the conformation of aromatic residues has marked differences upon pH changing. The CD signals in the near-UV region at pH 3.5 and 5.0 are lower than the measured one at pH 7.5, suggesting that decreasing the pH induces a looser and more flexible environment nearby the aromatic residues (*38*). Therefore, acid-denatured GDH retains pronounced secondary structure, while the near-UV CD spectrum and intrinsic fluorescence of acid-induced partially folded states are essentially featureless. The above results as well as the ANS binding results indicate that at low pH (3.5), GDH adopts, as do many other proteins (*31–36*), the features of the molten globule state which characterized as a structural intermediate; it has a substantial secondary structure, but its tertiary structure is less organized and fluctuating in respect to the native state (*39*).

Fluorescence Quenching by Acrylamide—Acrylamide is an effective quencher of tryptophan fluorescence and due to its relatively polar nature, it cannot quench the fluorescence of completely buried tryptophan residues, but quenches the surface exposed and partially buried tryptophan residues (*36*, *40*). This property of acrylamide enables us to assess the relative solvent exposure of different types of tryptophans. Figure 4 illustrates acrylamide quenching of intrinsic fluorescence of GDH in pHs 3.5 and 5.0 in comparison to pH 7.5 as judged by Stern–Volmer plot. The quenching constants (K_{sv} values) calculated for pHs 7.5, 5.0 and 3.5 were 2.6, 3.2 and 5.7 (M^{-1}), respectively. The Stern–Volmer plot indicates that the aromatic amino acids at pHs 3.5 and 5.0 are more exposed in comparison to the native folded conformation at pH 7.5 and the extent of aromatic residues exposure at pH 3.5 is substantially more than pH 5.0. So, the extent of tryptophan fluorescence quenching by acrylamide at pH 3.5 is more than pHs 5.0 and 7.5.

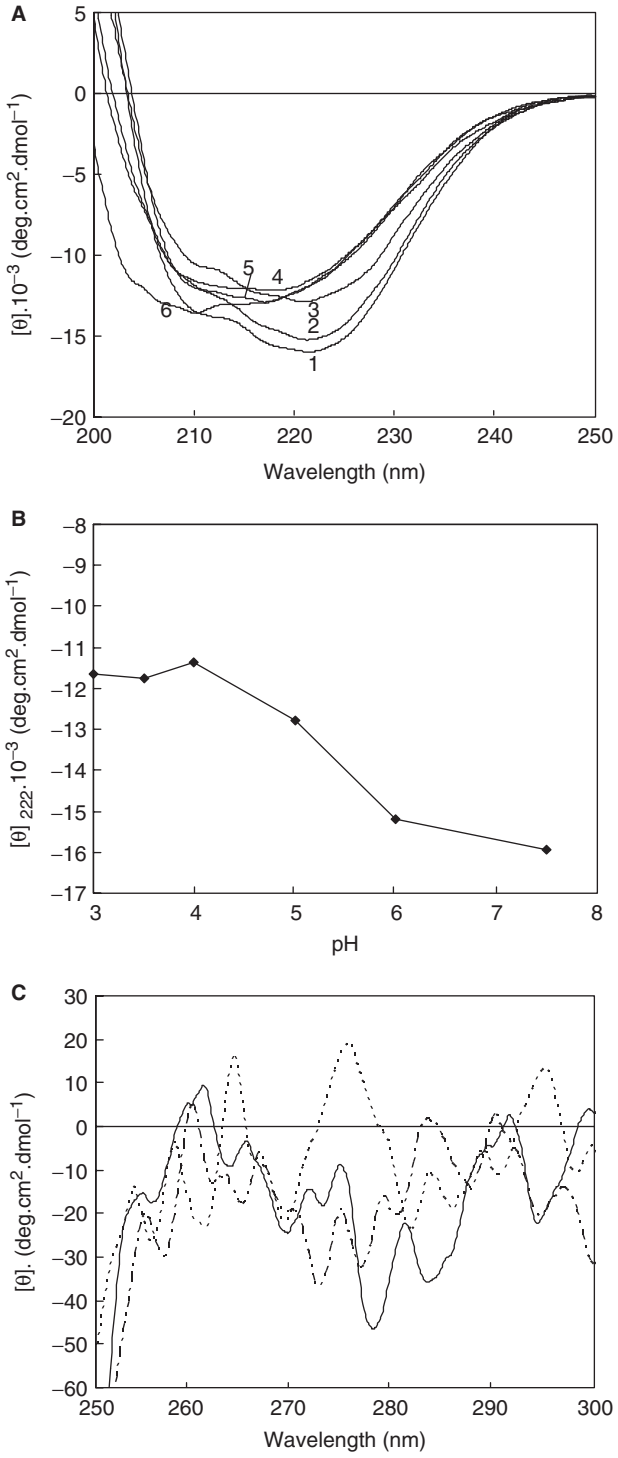

Fig. 3. **(A) Far-UV CD spectra of GDH (0.3 mg/ml) at different pHs**; pH 7.5 (1), pH 6.0 (2), pH 5.0 (3.0), pH 4.0 (4), pH 3.5 (5) and pH 3.0 (6).(B) Variation of $[\theta]_{222}$ at various pH values. (C) Near-UV CD spectra of GDH (1.5 mg/ml) at different pHs; pH 7.5 (–), pH 5.0 (---) and pH 3.5 (···). (For more details please see MATERIALS AND METHODS).

Size Exclusion Chromatography—To characterize the structural changes induced by mild acidic conditions, previously incubated GDH samples at pHs 7.5, 5.0 and 3.5 were applied onto a Sephacryl-S 200 gel chromatography column. The column was equilibrated with appropriate mixed buffer, previously. The results of gel filtration are illustrated in Fig. 5. Figure. 5A shows the chromatogram of GDH at pH 7.5. As can be seen, there are three peaks, a major peak (which is located at about 8 ml of elution volume) and two minor peaks (which are located at about 15 and 17 ml of elution volume, respectively) in the chromatogram of enzyme at pH 7.5. These three peaks (denoted as I, II and III) can be ascribed to hexameric (336 kDa), trimeric (168 kDa) and monomeric (56 kDa) forms of GDH, respectively, as judged by the position of the elution volumes of catalase (230 kDa), IgG (168 kDa) and ovalbumin (45 kDa) which are denoted as peaks I (8.5 ml), II (15 ml) and III (17.5 ml), respectively and represented in Fig. 5B. However, due to limitations of Sephacryl-S 200 for detection of molecular masses >250 kDa, peak I may comprise of some GDH aggregates with higher molecular weights, in addition to the hexameric form which we are interested in. Based on the size exclusion chromatography results, it seems that the predominant form of GDH at pH 7.5 is the hexameric form of the enzyme (Fig. 5A). However, chromatogram of GDH at pH 3.5 (Fig. 5C) indicates decrement of peak I and increment of peak II. We can infer that at pH 3.5, the predominant form of enzyme is its trimeric form as its peak coincides to the peak of IgG (Fig. 5B) with the same molecular weight. The chromatogram of GDH at pH 5.0 is essentially the same as its chromatogram at pH 7.5 (data not shown), which implies no dissociation of the hexameric form occurs at this pH. Note that the position of the peak II, the predominant form of enzyme at pH 3.5, is somewhat shifted to less elution volumes in respect to its position at pH 7.5. This shift can be ascribed to the more extended tertiary structure of enzyme at pH 3.5, a result which also has been obtained in ANS binding, acrylamide quenching as well as CD measurements.

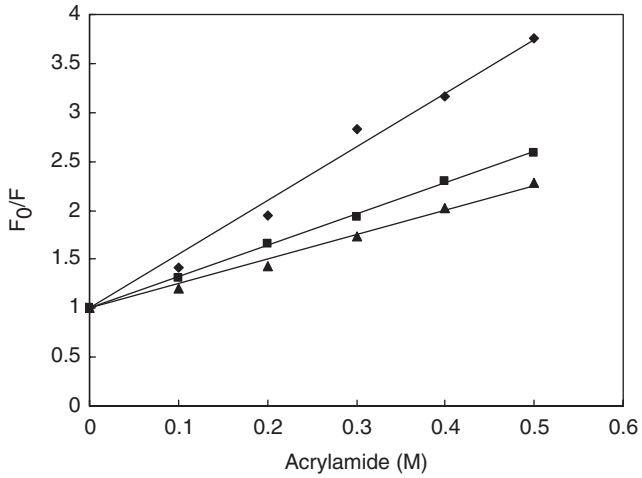

Fig. 4. **Stern–Volmer plot of GDH fluorescence quenching by acrylamide**. Fluorescence quenching at pH 7.5 (filled triangle), pH 5.0 (filled square) and pH 3.5 (filled diamond). (For more details please see MATERIALS AND METHODS).

Aggregation Measurements—Rayleigh light scattering measurements were carried out to determine the aggregation status of intermediates in the acid-induced

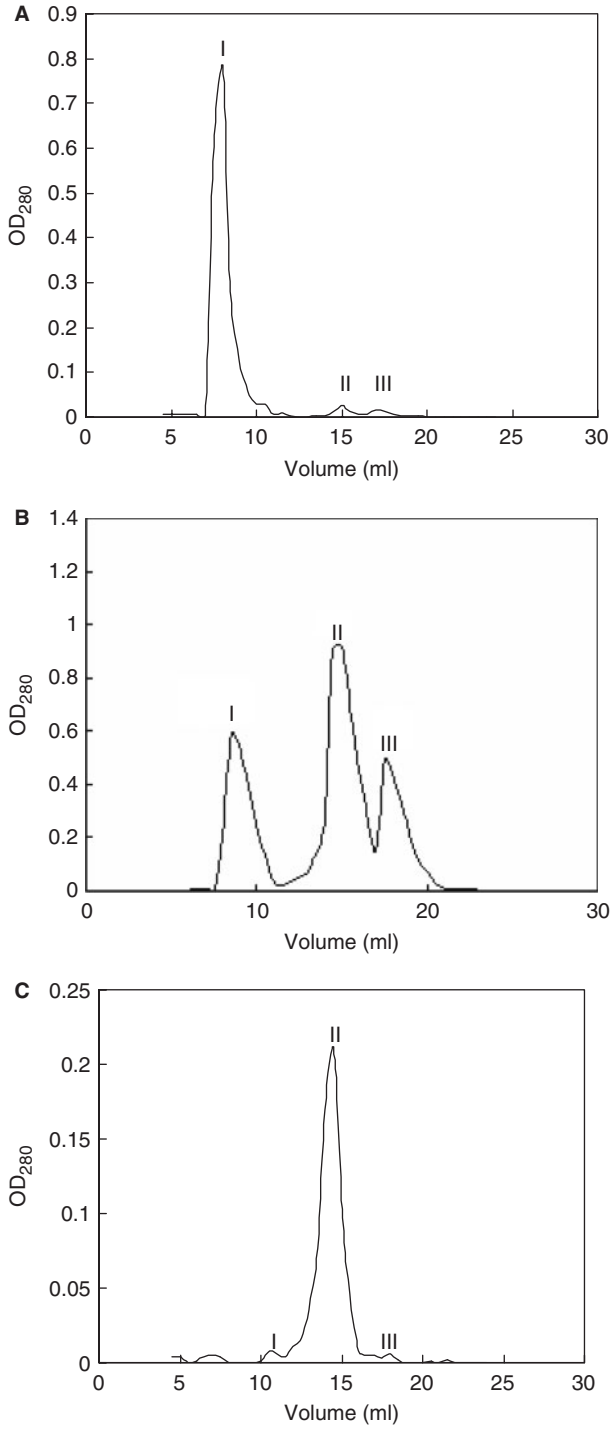

Fig. 5. **(A) Chromatograms of gel filtration of GDH incubated at pH 7.5**, (B) Catalase, IgG and ovalbumin as the mass standard markers and (C) GDH at pH 3.5. (For more details please see MATERIALS AND METHODS).

unfolding of GDH as well as the native form. As depicted in Fig. 6, GDH at pH 5.0 showed a marked increase in scattering suggesting existence of aggregation at this pH, whereas no significant scattering occurred in GDH solutions at pHs 7.5 and 3.5.

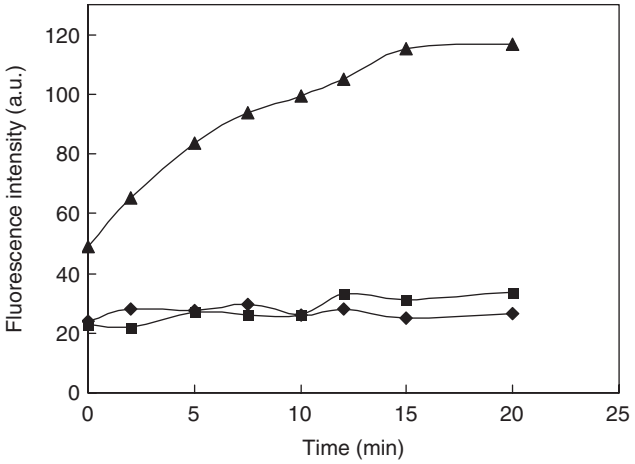

Fig. 6. **Light scattering profile of GDH as a function of time at different pHs**; pH 7.5 (filled diamond), pH 5.0 (filled triangle) and pH 3.5 (filled square). (For more details please see MATERIALS AND METHODS).

Refolding of Enzyme—In order to deduce whether or not, partially unfolded acidic-intermediates of enzyme can be refolded back to the native form (at pH 7.5), refolding studies were carried out. Aliquots of GDH which has been incubated at pH 3.5 over a 60-min period, was up taken at appropriate time intervals and its residual activity was measured. The results are shown in Fig. 7A. It is obvious that re-activation of enzyme is time-dependent and its dependency is proportional to the length of incubation time at pH 3.5, inversely (Fig. 7A). Within a short range of time (1–5 min), a refolding yield >70% was measured whereas on longer incubation periods (>5 min) a substantial decrease in the refolding yield was observed probably due to irreversible misfolding of partially unfolded molecules. Similar results were obtained for GDH which incubated at pH 5.0 (data not shown). Intrinsic fluorescence emission spectra of refolded enzyme are shown in Fig.7B. Fluorescence emission spectra were recorded after diluting the previously incubated enzyme at pHs 5.0 and 3.5 for 5 min. It is obvious that enzyme regains its structure to a high extent, as can be inferred from fluorescence intensity of the refolded enzyme especially for pH 5.0 in which only refolding process occurs. Refolding of the enzyme which incubated at pH 3.5 involves refolding the subunits accompanied by their reassembling to the hexameric array of enzyme.

DISCUSSION

Protein denaturation can be performed upon denaturing conditions such as low or high temperatures, low or high pH values, using high concentrations of chemical denaturants and so on. It has been shown that whereas chemical denaturants such as guanidine hydrochloride or urea disrupts the three-dimensional structure of proteins completely (*17*, *41*), acid-induced denaturation cannot do so and leaves some residual structures (*3–6*). On the other hand, the behaviour of various proteins upon acid-induced denaturation is different. Some of them are

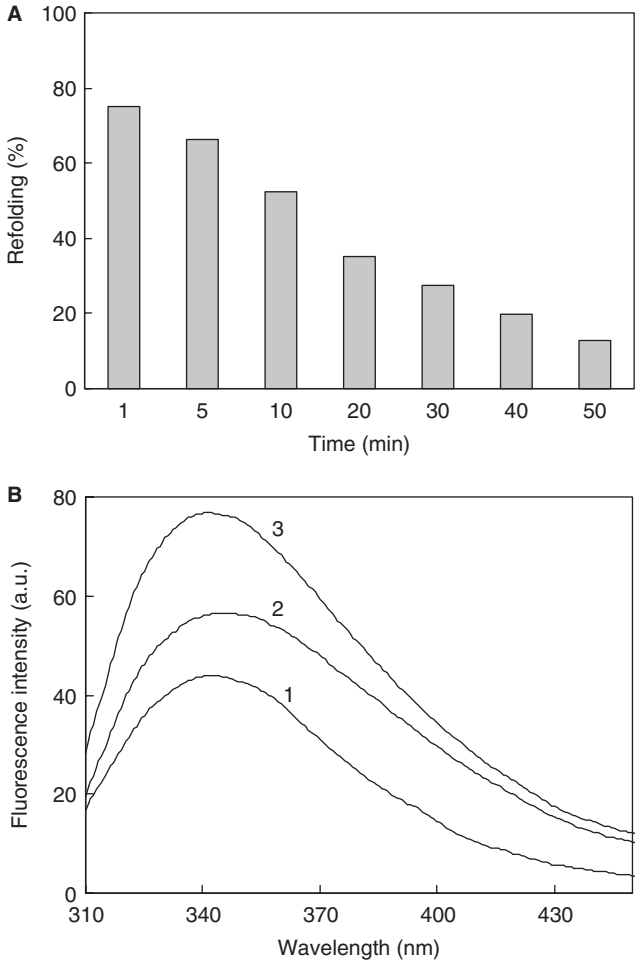

Fig. 7. **(A) Effect of buffer dilution (pH 7.5) on reactivation of GDH which previously incubated at pH 3.5 as a function of time**. (B) Fluorescence spectra of refolded enzyme. GDH previously incubated for 5 min at pH 3.5 (1) and pH 5.0 (2) were diluted by the mixed buffer, pH 7.5. Fluorescence spectra of the native enzyme at pH 7.5 (3). (For more details please see MATERIALS AND METHODS).

resistant to acid-induced denaturation, whereas some others undergo transition to an intermediate structure which is known as molten globule state. The third group first adopts an extended conformation and then undergoes a transition to a molten globule state (*42*). Interestingly, there is not a unique molten globule state upon acid-titration of various proteins, and various molten globule states are reported in which the extent of organization depends mainly on the protein (*3*, *11*).

Our results indicate the formation of two partially unfolded intermediates at pHs 5.0 and 3.5 while the latter appears to have the characteristics of the molten globule state. CD results revealed that this intermediate has a high content of secondary structure whereas most of its tertiary structure has been lost. As ANS binding to this partially unfolded state is maximal in the pH range of 3.0–7.5, it seems that this intermediate has the highest surface hydrophobicity in this pH range. Acrylamide quenching of the intermediate at pH 3.5 is also higher than the other pHs, suggesting the most exposure of tryptophans in this pH. Size-exclusion chromatography analysis clearly shows the existence of trimers as the predominant form of the enzyme at pH 3.5, in spite of the presence of enzyme as a hexameric assembly of subunits at pHs 7.5 and 5.0. Other reports on thermal denaturation of bovine liver GDH (*18*) and mild denaturation using non-ionic detergent (octyl glucoside) (*19*), have been emphasized on a hexameric assembly of GDH at the molten globule state as well as acid-induced denaturation report on hyperthermophilic archaeon *Pyrococcus furiosus* GDH (*20*). Dissociation of hexamer to monomers occurs only below pH 2.0 in the latter case, which can be ascribed to higher stability of the hyperthermophilic enzyme in comparison to its mesophilic counterpart.

Another partially unfolded state was characterized on the midpoint of transition from the native form to the molten globule state as well as from monitoring the effect of pH upon the F330/F350 ratio of the enzyme at different temperatures. This state retains its secondary and tertiary structure considerably, in comparison to the molten globule state. Its ANS binding capacity is lower than the molten globule state but is higher than the native enzyme. Furthermore, its tryptophans exposure lies between the native's and the molten globule's states, as judged by acrylamide quenching and is able to aggregate. These findings led us to consider the pH 5.0 partially unfolded state as an intermediate between the native and the molten globule states of the enzyme. Since catalytic activity of enzyme is strictly related to the presence of the hexameric assembly, refolding studies confirm the reversibility of acid-induced denaturation of GDH, at least for short incubation periods.

All the above data together let us conclude that while in the thermal denaturation (*18*) and non-ionic detergent (octyl glucoside) mild-denaturation (*19*) of bovine liver GDH, the formation of the molten globule state is straightforward; in mild-acidic denaturation, a partially unfolded intermediate is formed (prior to molten globule formation) which may be considered as a pre-molten globule-like state, a situation which has been observed previously for some proteins such as glucose/xylose isomerase (*31*).

We thank Mr A. Taravati for CD technical assistance. The financial support from the Research Council of Razi University is gratefully acknowledged.

REFERENCES

1. Anfinsen, C.B., Harber, E., Sela, M., and While, F.H. Jr. (1961) The amino acid sequence of a protein determines its tertiary structure. *Proc. Natl Acad. Sci. USA* **67**, 1309–1314
2. Jenicke, R. (1991) Protein folding: local structures, domains, subunits, and assemblies. *Biochemistry* **30**, 3147–3161
3. Nath, D. and Rao, M. (2001) Acid-induced partly folded conformation resembling a molten globule state of xylanase from an alkalothermophilic Bacillus sp. *Biochem. Biophys. Res. Commun.* **288**, 1218–1222
4. Dong, A. and Lam, T. (2005) Equilibrium titrations of acid-induced unfolding-refolding and salt-induced molten globule of cytochrome c by FT-IR spectroscopy. *Arch. Biochem. Biophys.* **436**, 154–160

5. Fink, A.L., Calciano, L.J., Goto, Y., Nishimura, M., and Swedberg, S.A. (1993) Characterization of the stable, acid-induced, molten globule-like state of staphylococcal nuclease. *Protein Sci.* **2**, 1155–1160
6. Naeem, A. and Khan, R.H. (2004) 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**, 2281–2292
7. Vassilenko, K.S. and Uversky, V.N. (2002) Native-like secondary structure of molten globules. *Biochim. Biophys. Acta* **1594**, 168–177
8. Uversky, V.N. (2002) Cracking the folding code. Why do some proteins adopt partially folded conformations, whereas other don't? *FEBS Lett.* **514**, 181–183
9. Mårtensson, L.G. and Jonsson, B.H. (1993) Characterization of folding intermediates of human carbonic anhydrase II: probing substructures by chemical labeling of SH groups introduced by site-directed mutagenesis. *Biochemistry* **32**, 224–231
10. Matthews, C.R. (1993) Pathway of protein folding. *Ann. Rev. Biochem.* **62**, 653–683
11. Ptitsyn, O.B. (1995) Molten globule and protein folding. *Adv. Protein Chem.* **47**, 83–229
12. Radford, S.E., Dobson, C.M., and Evans, P.A. (1992) The folding of hen lysozyme involves partially structured intermediates and multiple pathways. *Nature* **358**, 302–307
13. Redfield, C., Smith, R.A.G., and Dobson, C.M. (1994) Structural characterization of a highly-ordered 'molten globule' at low pH. *Nat. Struc. Biol.* **1**, 23–29
14. Muzammil, S., Kumar, Y., and Tayyab, S. (1999) Molten globule-like state of human serum albumin at low pH. *Eur. J. Biochem.* **266**, 26–33
15. Naseem, F., Ahmad, B., Ashraf, M.T., and Khan, R.H. (2004) Molten globule-like folding intermediate of asialofetuin at acidic pH. *Biochim. Biophys. Acta* **1699**, 191–199
16. Ahmad, B., Kamal, M.Z., and Khan, R.H. (2004) Alkali-induced conformational transition in different domains of bovine serum albumin. *Protein Pept. Lett.* **11**, 307–315
17. Fukushima, K., Inoue, T., Oda, C., and Shimozawa, R. (1985) Denaturation of bovine liver glutamate dehydrogenase by guanidine hydrochloride. Correlation between enzymatic activity and molecular state. *J. Biochem.* **97**, 1111–1117
18. Singh, N., Liui, Z., and Fisher, H.F. (1996) The existence of a hexameric intermediate with molten-globule-like properties in the thermal denaturation of bovine-liver glutamate dehydrogenase. *Biophys. Chem.* **63**, 27–36
19. Ghobadi, S., Safarian, S., Moosavi-Movahedi, A.A., and Ranjbar, B. (2001) Octyl glucoside induced formation of the molten globule-like state of glutamate dehydrogenase. *J. Biochem.* **130**, 571–577
20. Chiaraluce, R., Schwerdtfeger, R.M., Scandurra, R., Antranikian, G., and Consalvi, V. (1997) Acid-induced disassembly of glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem.* **247**, 224–230
21. Moon, K., Piszkiewicz, D., and Smith, E. (1972) Glutamate dehydrogenase amino-acid sequence of the bovine enzyme and comparison with that from chicken liver. *Pro. Natl Acad. Sci. USA* **69**, 1380–1383
22. Fisher, H.F. (1985) L-glutamate dehydrogenase from bovine liver. *Methods Enzymol.* **113**, 16–27
23. Olson, J.A. and Anfinsen, C.B. (1952) The crystallization and characterization of L-glutamic acid dehydrogenase. *J. Biol. Chem.* **197**, 67–79
24. Chalabi, P., Maniscalo, S., Cohn, L.E., and Fisher, H.F. (1987) The effects of an acetate-sensitive anion binding site on NADH binding in glutamate dehydrogenase. *Biochim. Biophys. Acta* **913**, 103–110
25. Eftink, M.R. and Ghiron, C.A. (1976) Exposure of tryptophanyl residues and protein dynamics. *Biochemistry* **16**, 5546–5551
26. Schippers, P.H. and Dekkers, H.P.J.M. (1981) Direct determination of absolute cicular dichroism data and calibration of commercial instrument. *Anal. Chem.* **53**, 778–788
27. Protasevich, I., Ranjbar, B., Lobachov, V., Makarov, A., Gilli, R., Briand, C., Lafitte, D., and Haiech, J. (1997) Conformation and thermal denaturation of apocalmodulin: role of electrostatic mutations. *Biochemistry* **36**, 2017–2024
28. Rajaraman, K., Raman, B., and Rao, C.M. (1996) Molten-globule state of carbonic anhydrase binds to the chaperone-like α-crystallin. *J. Biol. Chem.* **271**, 27595–27600
29. Piszkiewicz, D., London, M., and Smith, E.L. (1973) Sequence of bovine liver glutamate dehydrogenase. *J. Biol. Chem.* **248**, 3067–3081
30. Jiang, J.X. and London, E.R. (1990) Involvement of denaturation-like changes in *Pseudomonas* exotoxin A hydrophobicity and membrane penetration determined by characterization of pH and thermal transitions. Roles of two distinct conformationally altered states. *J. Biol. Chem.* **265**, 8636–8641
31. Pawar, S.A. and Deshpande, V.V. (2000) Characterization of acid-induced unfolding intermediates of glucose/xylose isomerase. *Eur. J. Biochem.* **267**, 6331–6338
32. Goto, Y., Takahashi, N., and Fink, A.L. (1990) Mechanism of acid-induced folding of proteins. *Biochemistry* **29**, 3480–3488
33. Nishii, I., Kataoka, M., and Goto, Y. (1995) Thermodynamic stability of the molten globule states of apomyoglobin. *J. Mol. Biol.* **250**, 223–238
34. Haq, S.K., Rasheedi, S., and Khan, R.H. (2002) Characterization of a partially folded intermediate of stem bromelain at low pH. *Eur. J. Biochem.* **269**, 47–52
35. Holzman, T.F., Dougherty Jr, J.J., Brems, D.N., and Mackenzie, N.E. (1990) pH-induced conformational states of bovine growth hormone. *Biochemistry* **29**, 1255–1261
36. Asghari, S.M., Khajeh, K., Moradian, F., Ranjbar, B., and Naderi-Manesh, H. (2004) Acid-induced conformational changes in *Bacillus amyloliquefaciens* α-amylase: appearance of a molten globule like state. *Enzyme Microb. Technol.* **35**, 51–57
37. Semistonov, G.V., Rodinonova, N.A., Razgulyaev, O.I., Uversky, V.N., Gripas, A.F., and Gilmanshin, R.I. (1991) Study of the 'molten globule' intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers* **31**, 119–128
38. Strickland, E.H. (1974) Aromatic contributions to circular dichroism spectra of proteins. *CRC Crit. Rev. Biochem.* **2**, 113–175
39. Borén, K., Andersson, P., Larsson, M., and Carlsson, U. (1999) Characterization of a molten globule state of bovine carbonic anhydrase III: loss of asymmetrical environment of aromatic residues has a profound effect on both the near- and far-UV spectrum. *Biochem. Biophys. Acta* **1430**, 111–118
40. Varley, P.G. and Pain, R.H. (1991) Relation between stability, dynamics and enzyme activity in 3-phosphoglycerate kinases from yeast and *Thermus thermophilus*. *J. Mol. Biol.* **220**, 531–538
41. Zhou, H.M. and Tsou, C.L. (1986) Comparison of activity and conformation changes during refolding of urea-denatured creatine kinase. *Biochem. Biophys. Acta* **830**, 59–63
42. Fink, A.L., Calcinano, L.J., Goto, Y., Kurotsu, T., and Palleros, D.R. (1994) Classification of acid denaturation of proteins: Intermediates and unfolded states. *Biochemistry* **33**, 12504–12511