Effect of pH on the Stability and Structure of Yeast Hexokinase A

ACIDIC AMINO ACID RESIDUES IN THE CLEFT REGION ARE CRITICAL FOR THE OPENING AND THE CLOSING OF THE STRUCTURE*

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pH and salts have a marked effect on the stability, structure, and function of many globular proteins due to their ability to influence the electrostatic interactions. In this work, calorimetry, CD, and fluorimetric studies have been carried out to understand the pH-dependent conformational changes of the two-domain protein yeast hexokinase A. In conjunction with the crystal structural data available, the present results have enabled the complete characterization and analysis of the pH-dependent conformational changes of the enzyme that have strong implications in understanding its structure-function relationship. The calorimetric profiles show a single thermal transition in the acidic pH range, whereas two independent transitions were observed in the alkaline pH range, suggesting the structural merger of the domains at the acidic pH. Comparison of the thermal transitions at pH 8.5 studied by different techniques suggests that the first transition corresponds to the larger domain. The acid-denatured state of hexokinase A has high secondary structural content with little or no tertiary interactions and binds to the hydrophobic dye 8-anilinonaphthalene-1-sulfonic acid, suggesting that it is a molten globule-like state, whereas the alkali-denatured state is less structured than the acid-denatured state but more structured than the urea-denatured state, suggestive of a premolten globule-like state. Structural analysis using the published hexokinase B structure as well as the hexokinase A structure with the revised amino acid sequence in conjunction with the results obtained by us suggests that the ionization state of the acidic residues at the active site could regulate domain movements that are responsible for the opening and the closure of the cleft between the two domains and in turn affect the structure and function of the enzyme.

Hexokinase is a member of the kinase family of tissue-specific isozymes and 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 with the release of a proton. Its malfunction has been implicated in a number of diseases in humans. Reduction in the activity of hexokinase due to mutations at the active site in humans causes hereditary anaemia (1, 2) and cardiomyopathy (3). Type II hexokinase-mitochondrial interactions were observed to promote tumor cell growth, and hexokinase has been suggested as the ideal target for therapeutic intervention (4, 5). Modifications in the catalytic activity of hexokinase have been suggested to play a role in the pathogenesis of the Alzheimer’s disease (6). Recently, it has been shown that hexokinase plays a role in sensing and maintaining the glucose levels and in signal transduction to regulate the expression of genes in plants (7, 8).

In yeast, two isozymes named A and B (hexokinase PI and PII, respectively) are known, with 76% overall homology of the amino acid sequence (10). Hexokinase isozymes are known to exist as dimers of 100 kDa. Endogenous protease action during purification leads to the loss of 11 amino acids from the N terminus, resulting in a predominantly monomeric form of 50 kDa (11, 12). The crystal structures are available for both of the hexokinase isozymes, for hexokinase PI complexed with glucose (13, 14) and for hexokinase PII complexed with orthotolouylglucosamine, a competitive inhibitor (15). Both of these crystal structures, however, have missing residues, since at that time the amino acid sequence was not available, and the side chains were deduced from the electron density with only 30% of the amino acid sequence homology between the primary structure of the crystallographic models and the one obtained from cDNA sequence (10). Recently, the PII structure without any bound ligands and with a correct amino acid sequence has become available (10). Also the crystal structure of PI complexed with glucose has been elucidated with the correct amino acid sequence. 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. The large domain consists of residues 19–76 and 212–457, whereas the small domain consists of residues 77–211 and 458–486. Comparison of the two crystal structures of hexokinase with and without bound glucose reveals that the conformational changes involved upon binding of the substrate are due to the domain movements. These conformational changes have earlier been observed spectroscopically for various substrates (16–18).

In the era of proteomics, it is becoming increasingly important to understand the structure-function relationship of proteins using various biophysical tools (19). Despite the importance of hexokinase as an enzyme, relatively few biophysical studies have been carried out on this protein. From the structures with the correct amino acid sequence available now, it is clear that the interface between the two domains is rich in acidic residues. Therefore, it will be of importance to study the effect of pH on domain interactions and hence the structure

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and stability of the enzyme. We have selected yeast hexokinase A as a model protein for such studies and employed various biophysical techniques such as DSC, CD, and fluorescence spectroscopy. The results in conjunction with the new structural data available have been discussed in terms of domain-domain interactions and their relation to the catalytic activity as well as the stability of the enzyme and the formation of intermediates at extreme pH extremes of pH. The results are likely to throw further light on the role of electrostatic interactions in the structure-function aspects of the enzyme and can have far reaching implications in our understanding of the structural abnormalities caused by the mutations leading to several diseases.

**EXPERIMENTAL PROCEDURES**

**Materials**—Yeast hexokinase A used in these experiments was obtained from Sigma, supplied as a mixture of isozymes PI and PII. The mixture of isozymes was separated by using the procedure of Womack et al., (9), which is based on the differential adsorption of hexokinase isozymes to hydroxyapatite. The PI fraction was eluted by 0.1 M potassium phosphate, pH 6.8. The specific activity determined using glucose and fructose as substrates to ascertain purity. The ratio of activities obtained for the substrates glucose/fructose was within the limits of 2.3–3.1. Tris, Hepes, Mops, ANS, NaCl, Ampso, and the pH standards used for calibrating the pH meter were procured from Sigma. Glycine, acetic acid, and sodium acetate were from Sisco Research Laboratories Pvt. Ltd. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate were from Merck. Either glass double-distilled or Milli-Q water from Millipore set-up was used to make the buffers. The pH of the buffer solutions was adjusted on a Control Dynamics pH meter model APX 175 by adding hydrochloric acid or sodium hydroxide solutions.

**Preparation of Solutions and DSC Measurements**—The following solutions were prepared for the pH-dependent studies: 20 mM glycine-HCl or NaOH, for pH 2.5, 3.0, 3.5, 10.5, 11.0, 11.5, 12.0, and 12.5; 20 mM acetate for pH 4.0, 4.5, and 5.0; 20 mM Hepes for pH 6.25; 20 mM Tris for pH 6.5–9.0; 20 mM Amso for pH 9.5 and 10.0; and 20 mM glycine, pH 3.5, containing 50, 100, and 200 mM NaCl for studies on the stability of hexokinase A carried out by DSC. The protein solutions were equilibrated with the corresponding buffers prior to their use in Centrikon ultrafiltration devices obtained from Amicon Inc.

The protein and the buffer solutions were degassed for 15 min prior to loading into the DSC cells. The instrument used for the calorimetric studies was either an MC-2D or a VP-DSC calorimeter from Microcal, LLC (Northampton, MA). The machines were calibrated with the temperature standards provided by the company. A scan rate of 1°C min⁻¹ was used, and the data were acquired through an in-built data translation board DT 2801 interfaced to a PC in the case of MC-2D or directly through an in-built data translation board DT 2801 interfaced to a PC in the case of VP-DSC using Origin™ software. The data were analyzed using the non-two-state model provided in the Origin™ software.

**CD and Fluorescence**—Circular dichroism spectra were recorded on a Jasco J-700 spectropolarimeter. The instrument was calibrated with (+)-10-camphor sulfonic acid. In general, an average of 4–6 scans were used, and the data were presented either as molar ellipticity or mean residue ellipticity expressed in degrees cm² dmol⁻¹. All of the spectra were obtained at an interval of 0.1 nm with a scanning speed of 50 nm/min at 18°C. Nitrogen gas was continuously flushed at the rate of 5 liters min⁻¹ through the instrument. 0.2- and 1-cm path length cuvettes were used in the far and near-UV regions, respectively. The protein concentrations for the far-UV and the near-UV spectra acquisition were 200 μM protein and 1.0 mg/ml, respectively. Thermal denaturation experiments were carried out at 222 nm on a Jasco J-810 spectropolarimeter with a Peltier attachment at a scan rate of 1°C min⁻¹ using a 0.2-cm path length cuvette. A molecular mass of 50 kDa and a total number of 468 residues for yeast hexokinase A (since crystal structure shows only 18–486 residues) were used in the calculation.

**RESULTS**

**Thermal Denaturation**—Thermal denaturation of yeast hexokinase A monitored by DSC in the pH range of 7.0–10.0 results in two independent thermal transitions, whereas only a single transition has been observed in the acidic pH range of 3.0–6.75 (Fig. 1). Thermal denaturation profiles of the protein monitored by ellipticity at 222 nm also match with the nature of transitions observed by DSC at acidic (pH 3.0) and alkaline (pH 8.5) pH that show monophasic and biphasic transitions, respectively (Fig. 2). A similar temperature dependence of ellipticity observed for the heat-denatured states of hexokinase A, both at the acidic (pH 3.0) and the alkaline pH (pH 8.5), suggests that the extent of unfolding is similar in both of the conditions. Thermal denaturation was observed to partially reversible between pH 8.5 and 10, whereas below pH 7.5, it was completely irreversible. Two independent transitions for thermal denaturation of yeast hexokinase A at pH 8.0 and 8.5 have been reported previously (23, 24). The two transitions have been assumed to result from the unfolding of the two domains, but these transitions have not been assigned to the individual domains previously. Thermal denaturation monitored by a decrease in the tryptophan fluorescence intensity at 330 nm at
Fig. 2. Temperature denaturation curves for hexokinase A at different pH values monitored by CD at 222 nm (1–4) and fluorescence intensity at 330 nm, excited at 280 nm (5). The numbers represent the pH values of buffers as follows. 1, pH 8.5; 2, pH 3.0; 3, pH 2.5; 4, pH 2.0; 5, pH 8.5.

pH 8.5 reveals only a single cooperative transition that shows correspondence to the first transition on the temperature axis with the biphasic transition observed by DSC and CD at 222 nm (Fig. 2). Yeast hexokinase A contains four tryptophans, three of which are buried in the hydrophobic core in the smaller domain, whereas the larger domain contains only a single partially exposed tryptophan. Hence, a comparison of the temperature denaturation curves of hexokinase A at pH 8.5 monitored by the different techniques suggests that the first transition is likely to be due to the melting of the smaller domain, whereas the second transition corresponds to the unfolding of the larger domain.

At acidic pH, thermal transitions consist of a single peak, indicating the merger of the two domains. Between pH 4.0 and 6.75, the $\Delta H_{\text{cd}}/\Delta H_{\text{d}}$ (data not shown) is close to unity, suggesting a two-state unfolding with both the domains melting cooperatively. However, between pH 3.0 and 4.0, the $\Delta H_{\text{cd}}/\Delta H_{\text{d}}$ ratio is less than unity, possibly due to the existence of considerable amounts of the partially denatured species as suggested by near-UV CD data (Fig. 5). The $T_m$ values have been observed to decrease at the extremes of pH in general, whereas $T_m$, corresponding to the smaller domain, decreases drastically compared with $T_m$, corresponding to the larger domain in the alkaline pH range (Fig. 3), suggesting that the interdomain interactions are stabilizing the smaller domain to a greater extent than the larger domain. This is in agreement with the model of Brandts et al. (25), which suggests that in multidomain proteins, the interdomain interactions stabilize the domain with a lower $T_m$ to a greater extent. At pH 2.5, we did not observe any transition, suggesting that the pH 2.5 state is an acid-denatured state. Since pH affects the stability and structure of hexokinase A, it is important to probe whether the buffer components make any contribution to the structure and stability at any given pH. We used Hapes, Tris, Mops, and phosphate buffers at 20 mM concentrations in the pH region 6.5–8.5 and did not observe any effect on stability as monitored by DSC and structure as monitored by CD and fluorescence for the protein at a given pH using the various buffer systems.

In order to probe the contribution of electrostatic interactions to domain merger, thermal denaturation at pH 3.5 was carried out in the presence of 50–200 mM NaCl (Fig. 4). pH 3.5 condition was selected, since minimal aggregation was observed in the presence of salt at this pH compared with higher pH values in the acidic pH range, where a single transition has been observed. At pH 3.5, the DSC profile shows a single transition with a $T_m$ of 38.9°C in the absence of salt, whereas the addition of the salt leads to a slight destabilization. Interestingly, the transition peak observed was asymmetric toward the pretransition zone, and the whole transition could be deconvoluted into two transitions. The deconvoluted thermogram shows that the first transition is getting more destabilized than the second transition. However, enthalpy changes are not very dependent on salt concentration (data not shown). Salt-induced destabilization with no enthalpy change has also been observed in a fragment representing the leucine zipper of GCN4 at low salt concentrations, whereas there was stabilization at higher salt concentrations (26). The destabilizing effect is saturating at 100 mM salt, and a further increase to 200 mM has essentially the same effect (Fig. 4). Irreversibility, strong aggregation, and weak calorimetric signal at pH 3.5 restrained us from carrying out detailed studies on the salt effect.

Near-UV CD—The near-UV CD spectrum of the native state of hexokinase A reveals three negative ellipticity peaks at 268, 277, and 286 nm and a positive one at 291 nm (Fig. 5). In acidic conditions (Fig. 5a), the ellipticity values at these wavelengths respond differently to changes in the pH. Whereas there is a decrease in the intensity as a result of decrease in the pH at 268, 277, and 286 nm, the intensity of the peak at 291 nm initially increases with a decrease in the pH, reaching a maximum at pH 3.5 followed by a decrease below this pH (Fig. 5a, inset). The positive ellipticity at 291 nm could be attributed to tryptophan’s environment, since tryptophan absorbs in this region. The differential changes observed at 291 nm might, thus, be related to a change in the tryptophan environment as a result of the loss of some tertiary interactions. In alkaline
conditions (Fig. 5b), the native spectrum of hexokinase A is nearly preserved up to pH 10.0, whereas at pH 11.0, the protein loses most of its tertiary interactions. At 258 nm, there is a considerable increase in the ellipticity at pH 11.0. This could be due to the abrupt exposure of phenylalanine residues or due to the formation of disulfides in the extreme alkaline pH region.

**Far-UV CD**—In the far-UV region, the CD spectra of the native hexokinase A show characteristic double minima at 208 and 222 nm (Fig. 6). Yeast hexokinase A belongs to a \( \alpha/\beta \) class of proteins (10), and the secondary structure content determined from the crystal structure consists of 39.7% helices and 16.6% \( \beta \)-strands. The calculated \( \alpha \)-helical contents as a function of pH using the method of Chen et al. (27), which is based on far-UV CD data at 222 nm, are listed in Table I. In the native pH conditions, helical content is about 35.7%, which is in close agreement with that derived from the crystal structure data.

The native secondary structure is preserved up to pH 3.5 in the acidic conditions (Fig. 6a). However, at pH 2.5, the protein loses 38% helicity with no minimum observed at 222 nm in CD. At pH 2.0, there is a slight increase in the helicity, thus suggesting that the pH 2.5 state is an acid-denatured state. In the alkaline conditions, hexokinase A loses secondary structure rapidly at pH >10.0 and reaches a plateau at pH 12.0 (Fig. 6b, *inset*). As compared with the acid-denatured state, the alkali-denatured state is more unstructured, and it loses ~77% helicity. In both the acidic and the alkaline pH range, the spectra show an isodichroic point at 207 nm, suggesting that the acid and alkali denaturation is a two-state process.

**Intrinsic Fluorescence**—Hexokinase A has 16 tyrosine residues distributed all over the protein and four tryptophan residues, of which three are located in the small domain and one in the large domain. The accessibility of Trp ranges from being completely buried to being partially solvent-exposed. Fluorescence emission was measured either upon excitation at 295 nm to follow the changes in the environment of the Trp residues specifically or upon excitation at 280 nm, in which case the emission arises both from Tyr and Trp residues as well as due to the result of energy transfer from Tyr to Trp residues. Thus, the excitation wavelength of 280 nm links the Tyr probes distributed throughout the protein with fluorescence emission from the four Trp residues.

Emission spectra of the native state have an emission maximum of 330 nm, suggesting that the tryptophan residues are buried in a nonpolar environment. The emission maximum of the acid-denatured protein is red-shifted to 340 nm, and that of the alkali-denatured protein is shifted to 350 nm (Fig. 7), indicating that the solvent exposure of tryptophans in the acid and the alkali-denatured protein takes place to varying extents. These data are in accordance with the far-UV CD data, which shows more secondary structure content for the acid-denatured protein as compared with the alkali-denatured protein. The emission intensity of the protein also decreases upon deviation from the native state due to the solvent quenching of tryptophan fluorescence.

**ANS Fluorescence**—ANS has been shown to bind to hydrophobic regions of partially unfolded proteins that become exposed to solvent (28). The results of ANS binding to hexokinase A are shown in Fig. 8. Titration of the protein with ANS as a function of pH ranging from 2.5 to 5.0 and at pH 12.5 suggests binding of ANS in the acidic region, whereas no binding was observed at pH 12.5. ANS binding was also saturating at concentrations of >25 \( \mu M \) (Fig. 8a). ANS binding in the acidic region led to an increase in the emission intensity as well as a concomitant shift in the emission maximum, indicating the presence of exposed hydrophobic groups (Fig. 8b). These studies also support the near-UV CD absorption data at pH 3.5 that show partial loss of tertiary interactions without any secondary structure loss. The lack of binding of ANS to the alkali-denatured protein suggests complete unfolding as evidenced by far-UV CD and by wavelength of emission maximum at pH

### Table I

| pH  | Percentage of \( \alpha \)-helix | pH  | Percentage of \( \alpha \)-helix |
|-----|-------------------------------|-----|-------------------------------|
| 7.5 | 35.7                          | 8.5 | 35.7                          |
| 6.5 | 35.8                          | 9.0 | 35.1                          |
| 5.0 | 35.7                          | 9.5 | 34.1                          |
| 4.5 | 35.7                          | 10.0| 32.4                          |
| 4.0 | 36.0                          | 10.5| 26.8                          |
| 3.5 | 35.4                          | 11.0| 19.3                          |
| 3.0 | 28.4                          | 11.5| 8.8                           |
| 2.5 | 22.2                          | 12.0| 6.7                           |
| 2.0 | 23.7                          | 12.5| 8.0                           |
pH 12.5.

4 each other just as in the presence of glucose at pH 8.5. pH, the two domains could be structurally interacting with 

contraction of ANS binding to hexokinase A; b, fluorescence emission spectra of hexokinase A excited at 485 nm plotted against the ANS concentration; c, fluorescence intensity at 485 nm plotted against pH of the buffer. The symbols , and pH 12.5 ( ), pH 2.0 ( ), pH 3.5 ( ), pH 4.0 ( ), pH 8.5 ( ), pH 10.5 ( ).

In alkaline pH range (b), the numbers represent pH of the buffers as follows. 1, pH 8.5; 2, pH 9.0; 3, pH 9.5; 4, pH 10.0; 5, pH 10.5; 6, pH 11.0; 7, pH 11.5; 8, pH 12.0; 9, pH 12.5.

12.5. However, in addition to the alkali-induced conformational changes responsible for the effect of pH, repulsive electrostatic interactions at alkaline pH between ANS and the exposed protein surface could also prevent ANS from having any binding effect.

**DISCUSSION**

*pH Dependence of Domain Interactions*—From Fig. 1, it is evident that yeast hexokinase A exists in two alternative conformations below and above pH 6.75. Hexokinase A crystal structure reveals two domains, and its calorimetric profile at pH 8.5 consists of two transitions that have been attributed to the independent unfolding of the two domains (23). At acidic pH (pH 4.0–6.75), the thermal transitions consist of a single peak with ΔHcal/ΔHv ratio of unity. This is similar to the results obtained at pH 8.5 in the presence of glucose, where the thermal transition consists of a single cooperative peak with a ΔHcal/ΔHv value close to unity (23). This suggests that at low pH, the two domains could be structurally interacting with each other just as in the presence of glucose at pH 8.5.

FIG. 7. Fluorescence emission spectra of hexokinase A excited at 280 nm as a function of pH at 18 °C. In both a and b, the upper insets show the emission intensity at 330 nm, excited at 280 nm ( ) and at 295 nm (Δ), whereas the lower insets show the emission maximum wavelength plotted against pH. In acidic pH range (a), the numbers represent pH of the buffers as follows. 1, pH 5.0; 2, pH 4.5; 3, pH 4.0; 4, pH 3.5; 5, pH 3.0; 6, pH 2.5; 7, pH 2.0. In alkaline pH range (b), the numbers represent pH of the buffers as follows. 1, pH 8.5; 2, pH 9.0; 3, pH 9.5; 4, pH 10.0; 5, pH 10.5; 6, pH 11.0; 7, pH 11.5; 8, pH 12.0; 9, pH 12.5.

FIG. 8. Fluorescence of ANS binding to hexokinase A as a function of pH, excited at 400 nm at 18 °C. a, Fluorescence intensity at 485 nm plotted against the ANS concentration; b, fluorescence spectra of ANS binding to hexokinase A; c, fluorescence intensity at 485 nm plotted against pH of the buffer. The symbols represent the pH values of buffers as follows. 1, pH 5.0 ( ); 2, pH 4.5 ( ); 3, pH 4.0 ( ); 4, pH 3.5 ( ); 5, pH 3.0 ( ); 6, pH 2.5 ( ); 7, pH 2.0 ( ); and pH 12.5 (dashed line).

The pH optimum for the activity of hexokinase is 7.5, and it rapidly loses its activity below pH 6.5 with no enzymatic activity obtained at pH 4.0 (29). These results correlate very well with the domain merger as a function of pH observed by DSC (Fig. 1). At pH 6.5, where only one transition has been observed, the enzyme has 80% of its activity compared with that at pH 7.5 where two transitions are observed. Above pH 8.0, the transitions are broader and well separated, with the first transition getting considerably destabilized (Fig. 1b). This could be related to the rapid loss in the enzymatic activity at pH >8.5. However, using near- and far-UV CD, we did not observe any significant conformational changes between pH 8.5 and 4.0. Although pH 6.5 falls in the region of the pK values of the histidyl residues, Grousselle et al. (30) showed that histidyl residues have no role in binding to the substrate or in catalysis. Viola and Cleland (31) have used the pH variation of the enzyme kinetic parameters, Vmax and K, to elucidate the chemical mechanism of the enzyme action. From the pH dependence profiles of Vmax and K for glucose binding, they observed cooperative protonation of at least five carboxylate acid residues that cause a loss of activity below pH 7.0. The calculated pK values were in the range of 6.87–6.44 and 6.59–6.05, depending on temperature, for Vmax and K profiles, respectively. Also, the calculated ionization enthalpy was higher than for simple protonation of the carboxylic acid group. They attributed the high ionization enthalpy to conformational changes associated with the protonation and the elevated pK values observed for carboxyl residues to their location in the deep cleft with limited accessibility to the solvent.

Analysis of the recently available crystal structures with the correct amino acid sequence of hexokinase B (PII) in the absence of glucose (10) and hexokinase A (PI) in the presence of glucose reveals the presence of seven acidic residues (Asp86, Asp211, Glu457, Glu502, Asp417, Glu457, Asp515) in the interdomain region of the active site cleft that are conserved among different species. Of these residues, Asp211, Glu502, and Glu502 are close (<6 Å) to the basic residues, Arg172 and Lys176, respectively in the liganded form, probably stabilizing the closed conformation by hydrogen bonding to the substrate glucose (see Table II for distances between these residues and accessible surface area values). These basic residues are located in the small domain and stay far apart from the acidic residues in the unliganded or open conformation (crystal structure of PII). Also, Asp211, Glu502, and Glu502 show significant decrease in the accessible surface area values in the closed conformation, suggesting that some of these residues may probably be undergoing protonation in the closed conformation, as

**TABLE II**

| Residue | COO | Fraction ASA |
|---------|-----|--------------|
|         |     | <6 Å | <8 Å |
| Asp86   | 0.244/0.225 | 1/1 | ←/← |
| Asp211  | 0.182/0.292 | 1/1 | ←/← |
| Glu457  | 0.063/0.401 | 1/1 | ←/← |
| Glu502  | 0.042/0.155 | 1/1 | ←/← |
| Asp417  | 0.312/0.327 | ←/← | ←/← |
| Glu457  | 0.423/0.387 | ←/← | ←/← |
| Asp515  | 0.352/0.418 | 1/1 | ←/← |

These residues are located at the active site cleft between the two domains.

Fractional accessible surface area (ASA) of the acidic residues listed in the left column in both the closed (C) and open (O) conformations were calculated using a probe size of 1.4 Å.
the burial of the charged residues would be energetically unfavorable (32). The above mentioned interactions along with additional interactions from the active site cavity being occupied by glucose might be favoring interactions between the two domains and consequently resulting in the single transition observed by DSC at pH 8.5 in the presence of glucose as reported by Takahashi et al. (23). In the open conformation, the two acidic residues, Asp$^{117}$ and Asp$^{458}$, are closer to each other (<6 Å) than in the closed conformation and might be resulting in net repulsive interactions. Also, the overall negative potential created by the close proximity of these seven acidic residues to one another in the interdomain region could be responsible for the separation of the domains, thus resulting in two separate transitions observed by DSC in the alkaline pH range (Fig. 1b).

Gerstein et al. (33) have classified the domain motions in hexokinase to be of shear type in which three moving layers x, b, and a (small domain) move against three static layers, X, B, and A (large domain). The a and A layers participate in major shear motion and consist of two helices oriented perpendicularly. The above mentioned seven acidic residues (Table II) are located within a, A, b, B, and X layers, which suggests that the protonation status of these residues could be influencing the domain movements. In the light of results obtained by Viola and Cleland (31) and the model for domain movement suggested by Gerstein et al. (33), one of the reasons for observing a single peak in the calorimetric profiles at acidic pH could be due to the protonation of these acidic residues, which would eliminate the repulsive forces between the domains, leading to the merger of the two domains. Such a merger might be responsible for the single cooperative conformation transition observed at pH 6.5 by calorimetry, which coincides well with the observation of Viola and Cleland (31) of the elevated pK$_a$ values in the range of 6.5 for carboxylic acids in hexokinase active site.

The binding enthalpy of glucose to hexokinase has been reported to be zero with an indistinguishable heat capacity change, and the process is entropy-driven. The relatively indistinguishable $\Delta C_p$ change upon binding of glucose has been attributed to the cancellation effect of burial of nonpolar and charged groups (23). It has also been reported that hexokinase undergoes large dehydration/rehydration reactions upon binding of glucose, which implies a significant contribution of solvation to the energetics of the conformational changes (34, 35). The contact surface area upon the formation of enzyme-glucose ($E$-$G$) complex is reduced, and the compaction has been assumed to be due to the hydrophobic effect, which predicts that the active (closed) conformation of hexokinase would form in the absence of glucose, but that would create an empty cavity, which might destabilize the closed conformation (36). In the present context, thus, the absence of repulsive forces at pH $\leq$6.75 due to the protonation of acidic residues at the base of the cleft might favor the closed conformation with an empty cavity due to the hydrophobic effect, as hypothesized by Bennett and Steitz (36). It thus appears that the domain-domain interactions in hexokinase are regulated by a fine balance of hydrophobic and charge-charge interactions. Whereas the hydrophobic interactions favor the closed conformation, the ionization status of the acidic residues at the active site could act as a switch between the closed and open conformations to facilitate domain movements necessary for the catalytic activity of the enzyme.

Takahashi et al. (23) have observed that in 0.2 M NaCl at pH 8.5, the $T_m$ of the high temperature melting domain ($T_{m2}$) of hexokinase A shifts toward the $T_m$ of the low temperature melting domain ($T_{m1}$) to give rise to a single transition, which is opposite to that of the glucose binding effect. In the present work at pH 3.5, the less cooperative transition ($T_{m1}$) was destabilized to a larger extent than the sharper transition ($T_{m2}$) with increasing concentration of NaCl (Fig. 4). This suggests that the salt has a destabilizing effect for one of the two domains probably caused by disruption of the ionic interactions. Salts are known to modulate the pK$_a$ values of charged amino acids such that with increasing salt concentration the pK$_a$ shifts observed in a folded protein drift toward the intrinsic value (37–39). It could thus be possible that the addition of NaCl both affects the ionization of the acidic residues and leads to the disruption of the ionic interactions, consequently affecting the domain-domain interactions.

**Acid Denaturation**—The calorimetric profiles show a single transition at low pH with a $\Delta H_{m}/\Delta H_{c}$ ratio close to unity at pH 4.5 and 4.0, suggesting the cooperative melting of the two domains in a two-state fashion. No change was observed in the spectroscopic properties, indicating that the protein is in the native state at these pH values. However, at pH 3.5, the near-UV CD shows a sudden enhancement of the positive peak at 291 nm along with a reduced negative ellipticity, whereas there is no change in the far-UV CD spectrum. The loosening of the tertiary interactions might be exposing the hydrophobic groups, which is manifested by ANS binding (Fig. 8). Intrinsic fluorescence results at pH 3.5 show a decrease in the intensity at 330 nm without any change in the emission maximum, suggesting the quenching of the tryptophan fluorescence due to altered tertiary interactions. At pH 3.0, the near-UV CD shows no change in the negative ellipticity compared with pH 3.5 state but a complete loss of the positive peak at 291 nm and enhanced ANS binding, which is associated with the partial loss of secondary structure as shown by the far-UV CD spectrum. At pH 2.5, the protein loses most of the tertiary interactions, and it shows no transition upon heating monitored by DSC (data not shown) or CD at 222 nm (Fig. 2), suggesting that the pH 2.5 state is an acid unfolded state. This suggests that the loss of the structure due to a decrease in the pH from 4.0 to 3.0 involves only the breakdown of tertiary interactions, whereas a further decrease in the pH leads to the cooperative loss of both the tertiary and the secondary interactions.

Acid denaturation of hexokinase A in low ionic strength buffers was incomplete and involves a conversion from the native state to a partially collapsed state, which has high secondary structure content as shown by far-UV CD, and it has an emission maximum of 340 nm, suggesting incomplete exposure of tryptophans to the aqueous solvent. ANS binding as measured by fluorescence intensity at 480 nm is maximum for this state, suggesting that it could be a molten globule-like state. Acid denaturation of yeast hexokinase A is akin to the type II proteins such as $\alpha$-lactalbumin and carbonic anhydrase that directly transform from native state to the molten globule state without any detectable unfolded species, and the transformation is characterized by a low amplitude change in the ellipticity at 222 nm, whereas the proteins lose complete tertiary interactions (40).

**Alkali Denaturation**—The base-induced transition begins around pH 10.0, which is close to the pK$_a$ of tyrosine ionization. Far-UV CD of alkali-denatured protein also shows less helicity compared with the acid-denatured state but more helicity compared with the 8 M urea-denatured protein. The emission maximum shifts to 350 nm for the alkali-denatured protein compared with 340 nm for the acid-denatured state, suggesting that the alkali-denatured protein is more unfolded than the acid-denatured protein. These results suggest that the alkali-denatured protein is similar to a pre-molten globule form observed in other proteins (41, 42) in the sense that it is less ordered than the molten globule form and more structured
than the 8 M urea unfolded protein. Since no enhancement of ANS fluorescence was observed during alkali denaturation, it suggests that each domain apparently unfolds in a two-state manner without populating any intermediates. Alkali denaturation appears to be a two-state process, since the conformational changes monitored by different probes coincide well with each other with an apparent midpoint of pH 10.8 (Fig. 9).

In conclusion, the results of the studies carried out show that domain-domain interactions in hexokinase are significantly influenced by the pH and appear to be critically controlled by the acidic residues located at the base of the active site, consequently altering the structure as well as the stability of the domains. These studies have implications in understanding the catalytic mechanism of hexokinase in the sense that the ionization state of these acidic residues might be critical to domain movements associated with substrate binding and the release of products. The acid-denatured state of yeast hexokinase A has properties similar to that of the molten globule state, whereas the alkali-denatured protein is less structured than the acid-denatured protein and probably represents a pre-molten globule form. Further studies involving mutagenesis of these acidic residues and determining their role in domain-domain interactions would be useful in understanding the catalytic mechanism of hexokinase thoroughly.

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REFERENCES
1. Magnani, M., Stoechi, Y., Curchiarii, L., Novelli, G., Lodi, S., Isa, L., and Formani, G. (1985) Blood 66, 690–697
2. Peters, L. L., Lane, P. W., Andersen, S. G., Gwynn, B., Barker, J. E., and Beutler, E. (2001) Blood Cells Mol. Dis. 27, 850–860
3. Barrie, S. E., Saad, E. A., Uhabuha, S., Da Silva Laza, P., and Harris, P. (1979) Res. Commun. Chem. Pathol. Pharmacol. 23, 375–381
4. Smith, T. A. (2000) Br. J. Biol. Chem. 57, 170–178
5. Pedersen, P. L., Mathyapala, S., Rempele, A., Geschwind, J. F., and Ko, Y. H. (2002) Biochim. Biophys. Acta 1555, 14–20
6. Sorbi, S., Morilla, M., Piacentini, S., Tonini, S., and Amaduzzi, L. (1990) Neurou. Lett. 117, 165–168
7. Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W. H., Liu, Y. X., Hawing, I., Jones, T., and Sheen, J. (2003) Science 300, 332–336
8. Frommer, W. B., Schuler, W. X., and Lahonde, S. (2003) Science 300, 261–263
9. Womack, F. C., Welch, M. K., Nielsen, J., and Cowan, V. P. S. (1973) Arch. Biochem. Biophys. 158, 451–457
10. Kuser, P. R., Krauchence, S., Antunes, O. A., and Polikarpov, I. (2000) J. Biol. Chem. 275, 20814–20821
11. Cowoicz, S. P. (1973) In The Enzymes (Beyer, P. D., ed) Vol. 9, pp. 1–48, Academic Press, New York
12. Schmidt, J. J., and Cowoicz, S. P. (1973) Arch. Biochim. Biophys. 158, 458–470
13. Bennett, W. S. J., and Steitz, T. A. (1980a) J. Mol. Biol. 140, 183–209
14. Bennett, W. S. J., and Steitz, T. A. (1980b) J. Mol. Biol. 140, 211–230
15. Steitz, T. A. (197) J. Mol. Biol. 61, 695–709
16. Feldman, I., and Kramp, D. C. (1978) Biochemistry 17, 1541–1547
17. Peters, B. A., and Neet, K. E. (1978) J. Biol. Chem. 253, 6826–6831
18. Ohning, G. V., and Neet, K. E. (1983) Biochemistry 22, 2986–2992
19. Neet, K. E., and Lee, J. C. (2002) Mol. Cell. Proteomics 1, 415–420
20. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., Kluwer Academic/Plenum Publishers, New York
21. Lee, B., and Richards, F. M. (1971) J. Mol. Biol. 55, 379–400
22. Shrake, A., Rupley, J. A. (1973) J. Mol. Biol. 79, 351–371
23. Takabashi, K., Casey, J. L., and Sturtevant, J. M. (1983) Biochemistry 20, 4693–4697
24. Catanzano, F., Gambuti, A., Graziano, G., and Barone, G. (1997) J. Biochem. (Tokyo) 121, 568–577
25. Brandts, J. F., Hu, C.Q., Lin, L. N., and Mos, M. T. (1989) Biochemistry 28, 8588–8595
26. Kenar, K. T., Garcia-Bovero, M., and Freire, E. (1995) Protein Sci. 4, 1954–1958
27. Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) Biochemistry 11, 4120–4131
28. Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F., and Gilmanshin, R. I. (1991) Biopolymers 31, 119–128
29. Sols, A., De La Fuente, G., Villar-Palasi, C., and Asensio, C. (1985) Biochim. Biophys. Acta 780, 93–101
30. Grouselle, M., Thiam, A. A., and Pudles, J. D. (1973) Eur. J. Biochem. 39, 431–441
31. Vinits, R. E., and Cleland, W. W. (1978) Biochemistry 17, 4111–4117
32. Miller, S., Janin, J., Leck, A. M., and Chothia, C. (1987) J. Mol. Biol. 196, 641–656
33. Gerstein, M., Leck, A. M., and Chothia, C. (1994) Biochemistry 33, 6739–6749
34. Sand, R. P., Fuller, N. L., Butko, P., Francis, G., and Nicholls, P. (1990) Biochemistry 32, 5925–5929
35. Filfil, R., and Chalikian, T. V. (2003) FEBS Lett. 554, 351–356
36. Bennett, W. S. J., and Steitz, T. A. (1979) Tid. Natl. Acad. Sci. U.S.A. 75, 4848–4852
37. Antoniewicz, J., McCammon, A. J., and Gilson, M. K. (1994) J. Mol. Biol. 238, 415–426
38. Abe, Y., Ueda, T., Iwashita, H., Hashimoto, Y., Motoshima, H., Tanaka, Y., and Imoto, T. (1995) J. Biochem. (Tokyo) 118, 946–952
39. Garcia-Bovero, M. E. (1995) Methods Enzymol. 259, 512–540
40. Pink, A. L., Calziano, L. J., Goto, Y., Kurotsu, T., and Palleros, D. R. (1994) Biochemistry 33, 12504–12511
41. Pitzay, O. B. (1995) Adv. Protein Chem. 47, 83–229
42. Rami, R. R., and Udgaonkar, J. B. (2002) Biochemistry 41, 1710–1716

FIG. 9. Normalized spectroscopic data represented as fraction of protein unfolded as a function of pH. The symbols represent ellipticity at 222 nm (●), emission intensity at 330 nm excited at 280 nm (△), emission intensity at 330 nm excited at 285 nm (●), and emission wavelength maximum (●). The continuous line is the sigmoid fit to the data.
Effect of pH on the Stability and Structure of Yeast Hexokinase A: ACIDIC AMINO ACID RESIDUES IN THE CLEFT REGION ARE CRITICAL FOR THE OPENING AND THE CLOSING OF THE STRUCTURE

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