CHAPTER 3

pH DEPENDENCE OF
DOMAIN TRANSITIONS
IN
YEAST HEXOKINASE A
INTRODUCTION

Ionizable groups play an important role in determining the protein structure (Perutz, 1978; Avbelji and Fele, 1998) and their functional activity (Sharp and Honig, 1990). These residues are also the source of the pH dependence of protein stability (Matthew and Gurd, 1986; Allewell and Oberoi, 1991; Yang and Honig, 1993, 1994; Antosiewicz et al., 1994). It is therefore essential that proteins maintain the appropriate side-chain ionization. The traditional view of acid denaturation considers the destabilizing effects of pH to be due to the large net charge accumulating on a protein far from its isoelectric point. This view is based on the Linderström-Lang model (Linderström-Lang, 1924) which treats a protein as a charged sphere wherein electrostatic free energy is proportional to the square of the net surface charge, and is thus repulsive at all pHs, except at the isoelectric point. The change in stability is thus, expected to vary rather uniformly as the net charge on the protein is varied: the greater the net charge, the lower should be the net stability (Hollecker and Creighton, 1982). Extensive studies on the pH dependence of the thermal stability of proteins have been carried out in the literature and the data correlated with the change in the electrostatic free energy of the system (Tanford, 1970; Privalov, 1979; Anderson et al., 1990; Yang and Honig, 1993; Khurana et al., 1995). However, there is paucity of data concerning the effect of pH on the thermal stability of multidomain and multimeric system in terms of understanding domain and monomer stability in the literature. Differential scanning calorimetry provides an excellent way of monitoring domain transitions in multidomain protein systems as the system is undergoing thermal denaturation and provides information not only of the transition temperatures but also yields valuable thermodynamic information on the enthalpy and heat capacity changes accompanying the unfolding process (Manly et al., 1985; Edge et al., 1985,1988; Freire et al., 1992; Lepock et al., 1992; Sanchez-Ruiz, 1995).

In this study we report the results on the pH dependence of thermal stability of the two domain protein yeast hexokinase A using Differential Scanning Calorimetry (DSC). Yeast hexokinase A (S-form) used by us exists as a monomer with a molecular weight of 50,000 daltons (Schmidt and Colowick, 1973a,b; Colowick, 1973) and the polypeptide chain folded into two distinct lobes (Bennett and Steitz, 1978, 1980a,b). However, it has been observed that close to its PI which is 5.1, it forms higher molecular weight oligomers (Schulze and Colowick, 1969). DSC profile of yeast hexokinase B at pH 8.5 shows two partially overlapped peaks which have been related to the presence of two structural domains.
in the native conformation of the enzyme that possess different thermal stabilities and are
denatured more or less independently (Takahashi et al., 1981; Catanzano et al., 1997). The
binding of D-glucose enhances the interactions between the two lobes, and results in a DSC
profile resembling that of a single domain protein (Catanzano et al., 1997). We report here
the results on the effect of pH 6.5, 7.5, and 8.5 on the domain transition temperatures (Tm) of
yeast hexokinase A and the calorimetric enthalpies obtained. Activity studies at different pHs
from 6.0 to 8.5 have shown an increase in activity till pH 8.0 followed by a sharp fall (Shill
and Neet, 1975). In this paper attempt has been made to correlate the thermal stability as
monitored by transition temperatures (Tm) with the activity of the enzyme retained after
incubating at various pH values for different intervals of time at 25 °C. To explore the effect
of pH on the secondary structure of the enzyme, Circular Dichroism (CD) studies have also
been carried out and the data presented and discussed in terms of the Tm and the activity of
the enzyme retained.

RESULTS

Differential Scanning Calorimetry

Differential Scanning Calorimetric (DSC) studies were carried out on yeast hexokinase A
at different pHs. From Figure 1a,b it is evident that hexokinase A at pH 8.5 unfolds into two
partially overlapped transitions similar to what has been reported in the literature for
hexokinase B (Takahashi et al., 1981; Catanzano et al., 1997). The first transition
corresponding to one of the two domains of hexokinase (designated as domain 1) has lower
enthalpy and is smaller compared to the second transition due to the other domain (domain 2)
(Figure 1a,b) with Tm1 of 35.7 °C and Tm2 of 47.3 °C, respectively (Table I). The peak
(transition) 2 is higher and broader than peak 1 (Figure 1a,b). These transitions can be
ascribed to the presence of two structural domains in the native conformation of hexokinase
A, that possess different thermal stabilities and are denatured more or less independently.

At pH 7.5, yeast hexokinase A unfolds with increased overlap of the two peaks due to
the two domains. The enthalpy of denaturation due to the individual domains is nearly
identical (Table I). The transition temperature of domain 1, Tm1 is 41.1 °C and is much
higher compared to that at pH 8.5 (Figure 1c,d; Table I). However, there is no increase in
the Tm2 value relative to pH 8.5. The overall transition zone of the protein denaturation was
observed to be decreased indicative of cooperative unfolding. The enthalpy due to domain 1,
Figure 1. Effect of pH on the thermal stability of hexokinase A monitored by Differential Scanning Calorimetry. a, c, and e show raw data after concentration normalization, while b, d, and f are the analyzed curves after baseline subtraction. The sudden drop in c, and e indicate protein aggregation.
**TABLE I:** Thermodynamic parameters for hexokinase A stability as a function of pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>( \Delta C_p ) (kcal K(^{-1}) mol(^{-1}))</th>
<th>( T_m (\degree C) )</th>
<th>( \Delta H_{cal}(\text{kcal mol}^{-1}) )</th>
<th>( \Delta H_{vH}(\text{kcal mol}^{-1}) )</th>
<th>( \frac{\Delta H_{cal}}{\Delta H_{vH}} )</th>
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<tbody>
<tr>
<td>8.5</td>
<td>9.1 ± 1.1</td>
<td>35.7 ± 0.41</td>
<td>47.3 ± 0.21</td>
<td>44.6 ± 4.2</td>
<td>113 ± 6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47.2 ± 0.07</td>
<td>118 ± 6.7</td>
<td>127 ± 24</td>
<td>86.7 ± 2.7</td>
</tr>
<tr>
<td>7.5</td>
<td>----</td>
<td>41.1 ± 0.21</td>
<td>118 ± 6.7</td>
<td>127 ± 24</td>
<td>86.7 ± 2.7</td>
</tr>
<tr>
<td>6.5</td>
<td>----</td>
<td>47.9 ± 0.02</td>
<td>190 ± 5</td>
<td>185 ± 2</td>
<td>1.02</td>
</tr>
</tbody>
</table>
ΔH_1 increases sharply at pH 7.5 compared to that at pH 8.5, with very little change in the enthalpy of domain 2, ΔH_2. The protein also undergoes aggregation at 84 °C indicated by the sudden drop in C_p^ex value at pH 7.5 (Figure 1c). The ratio of calorimetric (ΔH_cal) and van't Hoff (ΔH_vH) enthalpies for the two domains designated R_1 and R_2 obtained at pH 8.5 and 7.5 indicate low cooperativity at pH 8.5 compared to 7.5. A very high value of 9.1 kcal K^{-1} mol^{-1} for the heat capacity of denaturation (ΔC_p) has been obtained at pH 8.5. This could be due to large accessible surface area of the protein exposed on denaturation as has been observed with other proteins (Myers et al., 1995). At pH 7.5 and 6.5 ΔC_p values could not be determined due to the aggregation of the protein immediately after denaturation (Figure 1c,e).

At pH 6.5, domain 1 is stabilized by a significant extent, resulting in only one sharp transition, with a T_m of 47.9 °C (Figure 1e,f; Table I). At this pH both the domains merge and co-melt as a single domain. The overall enthalpy due to the transition is higher than the total enthalpy at pH 8.5 but lower than that at pH 7.5 (Table I). At pH 6.5 the total transition zone narrows down considerably and the endothermic peak is very sharp resembling a 2 state transition as indicated by the ratio of the calorimetric (ΔH_cal) and van’t Hoff (ΔH_vH) enthalpies, which is ~ 1. However, it has been observed that the temperature onset of aggregation shifts to a much lower temperature of 62 °C at pH 6.5 (Figure 1e). Figure 2
shows a plot of the dependence of $T_{m1}$ with pH. A linear dependence of $T_{m1}$ with respect to pH is obtained and it has been observed that as the pH is increased the $T_{m1}$ shifts to a lower temperature and vice versa.

To check for the reversibility of the thermal transitions at different pH values, reheating of the protein samples after first heating scan followed by cooling was carried out. The heat and reheat cycles of yeast hexokinase A at pH 8.5 showed partial reversibility of the two transitions (Figure 3). At pH 7.5 and 6.5, yeast hexokinase A did not show any appreciable recovery of transitions on heating and reheating owing to aggregation followed by denaturation (data not shown). DSC transitions have been shown to be independent of scan rate at pH 8.5 (Takahashi et al., 1981; Catanzano et al., 1997). At pH 6.5 it shows decrease in $T_m$ by 2 °C with no appreciable change in ΔH values at lower scan rate (30 °C/hr.) (data not shown).

**Circular Dichroism Spectroscopy**

Far UV circular dichroism studies were carried out at 25 °C at pH 8.5, 7.5, and 6.5 in 20 mM Tris-HCl buffer in the 184-260 nm range. The double minima and a maxima in the spectra indicate the presence of the secondary elements like $\alpha$-helix and $\beta$-sheet etc. However, there is negligible difference in the spectra obtained at the three pH values (Figure 4). It is thus evident from the DSC and CD studies that although change in the pH does not

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**Figure 4.** CD curves for hexokinase A at pH 6.5, 7.5, and 8.5 in 20mM Tris-HCl buffer at 25°C.

**Figure 5.** Activity of hexokinase A after incubation at 25 °C at pH 6.5, 7.5, and pH 8.5 for different time periods at a concentration of 2.5 µg/ml. The activity measurements were carried out at pH 7.8, 25 °C.
affect the secondary structure content yet it affects the overall tertiary interactions in the protein leading to the varied thermal stabilities.

**Activity Measurements**

Activity assay of yeast hexokinase A was carried out at pH 7.8 and 25 °C after incubating the enzyme at 25 °C, at pH 6.5, 7.5, and 8.5 for different intervals of time (Figure 5). From the plot of percent activity versus time it can be seen that at pH 6.5 the enzyme was most stable. Incubation at pH 7.5 leads to a gradual fall in activity, while there was a sudden drop in activity at pH 8.5. In the case of protein incubated at pH 8.5, activity drops down to 3-5% of the native in 11-12 hr. For the protein incubated at pH 7.5 the decrease in activity is not sharp, with the activity falling to a level of 10% in 30 hr. The protein incubated at pH 6.5 showed maximal stability with retention of almost 85% activity for 32 hr. followed by a gradual decrease in activity to ~ 48% in 70 hr. (Figure 5).

![Graph showing activity measurements](image)

**Figure 6.** Effect of dilutions done at different time intervals on the activity of hexokinase incubated at 5°C at pH 8.5. The dilutions (100 fold) were carried out from the stock (250 µg/ml) at a time interval of 0 hour (-■-), 47 hours (-○-), 96 hours (-▲-), and 191 hours (-△-), followed by the measurement of activity as a function of time. The activities were measured at 25 °C, pH 7.8.

Figure 6 shows the effect of dilution done at different time intervals on the activity of hexokinase A incubated at 5°C at pH 8.5. The results indicate dilution dependent inactivation of the enzyme. Dilution dependent inactivation has been observed in several oligomeric enzymes in the literature (Fan et al., 1975; Attwood et al., 1993; Velasco-Garcia et al., 1999). In all these cases dilution induces monomer formation which is inactive. We have observed a reduction in the activity of the stock solution (250µg/ml) of hexokinase A as a function of time. In about 8 days the % activity reduces to ~ 65%. Upon dilution of the stock the activity loss is much rapid as observed in Figure 6. Williams and Jones (1976) have observed dilution dependent inactivation of yeast hexokinase A and ascribed it to a monomer-dimer equilibrium. However, in the case of S-form of hexokinase A under the pH conditions
studied by us, wherein the enzyme exists as a monomer, the dilution dependent inactivation can be ascribed to the inherent thermolability of the enzyme which can enhance further due to intermolecular collisions upon dilution.

DISCUSSION

The calorimetric transitions of hexokinase A at pH 8.5, 7.5, and 6.5 are different from each other indicative of the importance of the surface charge and electrostatic interactions in the thermal stability and domain transitions of the enzyme. The two transitions observed for the thermal denaturation of hexokinase A at pH 8.5 and 7.5 indicate the unfolding of two independent structural domains of the enzyme, the large and the small lobe (Figure 1a,b). It is evident from the crystal structure of hexokinase A that the binding of glucose takes place in the cleft of the large and the small lobe and the active site is formed by the residues of the small lobe (Bennett & Steitz, 1978, 1980a,b). The large lobe is formed by the residues 2-58 and 187-458, and the small lobe is formed by the residues 59-186 (Bennett & Steitz, 1980b).

The smaller lobe consists of 2 α-helices and 2 small β-strands forming a sheet and few β- and γ-turns (Chapter 1: Figure 1). There are also large stretches of random coil regions present in it. The larger lobe has 3 helices from the residues 2-58 and 11 helices from the residues 187-458, with several β-turns, γ-turns, and 5 β-strands forming two sheets. Several interchain helix interactions take place within the large lobe which keeps the N-terminal and C-terminal ends close to each other. Thus, overall, the large lobe appears to be better structured with far more number of stabilizing interactions and secondary structural elements present in it than the small lobe. It has been observed that domain 1 has lower $T_m$ and calorimetric enthalpy values compared to domain 2 and is thus less stable (Figure 1a,b; Table I). It is, therefore, quite evident that the domains 1 and 2 observed calorimetrically are the ‘small lobe’ and the ‘large lobe’ of hexokinase respectively.

At pH 7.5, yeast hexokinase A unfolds with an increased overlap of the two transitions. The two transitions appear very close to each other and on deconvolution lead to almost the same enthalpic contribution. There is a marked shift in $T_m_1$ with almost no shift in $T_m_2$ (Figure 1c,d; Table I). This leads to the narrowing of the transition indicating increased overall cooperativity in the folding of the protein (Table I). Interestingly, at pH 6.5 the protein unfolds as a single domain due to the merger of the two peaks leading to a single coupled transition (Figure 1e,f; Table I). The overall transition zone also becomes much
smaller with a sharp single peak indicative of increased cooperativity in the unfolding of the protein. The variations in the thermal stability of the two domains as well as the coupling of the domain transitions appear to be critically controlled by variations in the electrostatic interactions among the charged groups upon changing the pH of the medium. It has been observed that the stability of a protein is maximal near its PI when the charges are located on the surface, and maximal away from PI when some charged groups are buried (Stigter and Dill, 1990). It has also been suggested that when electrostatic interactions in proteins are of a general coulombic nature, the change in the stability should vary rather uniformly as the net charge on the protein is varied; the greater the net charge, the lower should be the net stability (Hollecker and Creighton, 1982). Hence a pH condition far away from PI of the protein will lead to its destabilization. This has been observed in a wide variety of proteins (Privalov, 1979). Recently Pace et al. (1990) have also observed maximum free energy of stabilization for RNase A and RNase T\textsubscript{1} at the PI of proteins based on pH dependence of the urea and guanidinium hydrochloride based denaturation. The PI of yeast hexokinase A is 5.1 and based on the net charge prediction using software ‘CHARGPRO’ available in the “PC GENE” package (Intell Genetics Inc., Geneva, Switzerland) it was found that net charge on yeast hexokinase A at pH 6.5, 7.5, and 8.5 is (-) 12, (-)14, and (-) 17 respectively. It is thus possible that at pH 8.5 the large net negative charge on the protein molecule may lead to its destabilization with a lowering in the transition temperatures, relative to pH 7.5 and 6.5. It is evident from the $T_{\text{m}}$ values for the protein observed at the three pH conditions studied (Table I) that $T_{\text{m1}}$ increases by a large extent as we decrease the pH, while $T_{\text{m2}}$ does not show any change. Similar trend has been observed for the $\Delta H$ values as well. It can, thus, be concluded that increase in the electrostatic interactions in domain 1 leads to increase in the thermal stability as the pH is lowered to 6.5.

Shill and Neet (1975) have carried out activity assays of yeast hexokinase A and B as a function of pH. They have shown that at pH 6.0 the activity is very low increasing marginally at pH 6.5. The activity of the protein increases rapidly till pH 8 and then again starts declining. A very low activity observed at pH 6.5 may be due to the closure of the cleft between the two domains at low pH as observed by us calorimetrically (Figure 1f) leading to the masking of the active site for glucose binding. This may be the reason why yeast hexokinase A shows enhanced activity at higher pH, as the two lobes of the enzyme at high pH are likely to be well separated leading to an easy access for the binding by glucose. This
is evident from the independent melting of the two domains at pH 8.5. At pH higher than 8.5, there is a fall in activity of the protein due perhaps to its denaturation as a result of the decrease in the stability of domain 1 (smaller lobe) indicated by a very low $T_{m1}$ value at pH 8.5 as evident from Figure 2. These results also suggest that at lower pH values the enhanced electrostatic interactions in domain 1 could also be leading to slight conformational changes in the active site leading to the closure of the cleft. Upon subjecting yeast hexokinase A to a heat and a reheat cycle more reversibility and recovery of the peaks was observed at pH 8.5 compared to that at pH 7.5 and 6.5. The higher reversibility at pH 8.5 could be due to high net negative charge on the chain preventing the unfolded molecules to aggregate.

Circular dichroism studies of hexokinase A carried out at pH 8.5, 7.5, and 6.5 did not show any perceptible difference in the secondary structure content of the protein at these pH values (Figure 4). These results in conjunction with our DSC data and activity data available in the literature suggest that changing the pH leads to change in the tertiary interactions, as observed by a loss of activity and an increase in the $T_m$ and enthalpy values as we go from pH 8.5 to 6.5, while maintaining the secondary interactions intact. Thermal stability data obtained from DSC studies correlate very well with the data on the stabilization of the activity of the enzyme upon incubation at 25 °C for different periods of time (Figure 5). At pH 6.5 the onset of denaturation is shifted to a much higher temperature being ~42 °C relative to pH 7.5 and 8.5. At 25 °C, thus, the protein would be essentially in the native state and is less amenable to inactivation due to kinetically controlled processes.

In conclusion, it has been observed that hexokinase A is more stable at pH 6.5 compared to higher pH values owing to the shift in the $T_m$ values to a higher temperature. The decrease in the activity at pH 6.5 is essentially due to the closure of the cleft between the two domains as observed in the DSC profile showing a single cooperative transition. A change in pH from 6.5 to 8.5 does not lead to a change in the secondary structure but alters tertiary interactions leading to varied thermal stabilities.