Thermodynamics of the Binding of D-Glucose to Yeast Hexokinase†

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ABSTRACT: The binding of D-glucose to baker's yeast hexokinase (EC 2.7.1.1, ATP:D-hexose 6-phosphotransferase) was studied by isothermal and differential scanning calorimetry (DSC) and by fluorometric titration. The enthalpy and heat capacity changes associated with the binding of glucose were found to be nearly zero at both low and high ionic strengths over the temperature range from 7 to 29 °C. Thus, the free-energy change, amounting to −5.1 kcal mol^{-1} at 25 °C and high ionic strength, is nearly independent of the temperature and is primarily of entropic origin. DSC study of the thermal unfolding of the free enzyme at low ionic strength gave an excess heat capacity curve with two maxima. This result appears to reflect a difference in thermal stability of the two domains in the hexokinase molecule which are indicated by X-ray crystallography [Bennett, W. S., & Steitz, T. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4848–4852]. In contrast, the unfolding of free enzyme at high ionic strength was fully cooperative. The excess heat capacity curve for the unfolding of the glucose-bound enzyme had only one peak at both low and high ionic strengths. This is consistent with the X-ray result that the binding of glucose induces a conformational change in the enzyme which brings the two lobes into close proximity. It is interesting that such a significant, molecule-wide conformational change is accompanied by only very small net changes in enthalpy and heat capacity.

Many protein–ligand interactions are known to be accompanied by large changes in entropy and heat capacity. These changes are thought to be due, in large measure, to hydrophobic effects, although other effects undoubtedly make significant contributions (Sturtevant, 1977). The enzyme hexokinase from baker's yeast has been shown to undergo a large conformational change, both in the crystalline state (Bennett & Steitz, 1978) and in aqueous solution (McDonald et al., 1979), when the substrate D-glucose is bound, and it is of interest to learn how this conformational change is reflected in the thermodynamic parameters for the binding of glucose. In this paper, we report the results of calorimetric and equilibrium studies of this process, and also of the thermal unfolding of hexokinase in the presence and the absence of glucose.

According to the results of X-ray crystallography (Bennett & Steitz, 1978), the hexokinase monomer contains two domains separated by a cleft in which the substrate is bound, causing one of the domains to rotate 12° relative to the other, resulting in a movement of 8 Å in the polypeptide backbone and closing of the cleft between the two lobes. During this change, the surface area of hexokinase exposed to the solvent is significantly reduced, by about 80 Å^2 per molecule, thus decreasing the exposure of nonpolar groups to the solvent.

Materials and Methods

The B isozyme of yeast hexokinase, prepared by the method of Kaji et al. (1961), was purchased in crystalline suspension from Sigma Chemical Co. Lot numbers 59C-8085 and 96C-8610 were used in this work. This enzyme has lost by proteolysis 11 amino acid residues from the N-terminal end and exists as a monomer at alkaline pH and NaCl concentrations above 0.1 mM (Schmidt & Colowick, 1973; Steitz, et al., 1976). Glucose-6-phosphate dehydrogenase, NADP, and ATP were also purchased from Sigma Chemical Co. D-Glucose was obtained from Mallinckrodt Chemical Co. and was used without further purification. All other chemicals were of reagent grade. Doubly deionized water was used throughout.

The concentration of hexokinase used in isothermal calorimetry was determined by activity measurements performed on recovered solutions. Enzyme activity was assayed by the glucose-6-phosphate dehydrogenase coupled method (Schmidt & Colowick, 1973), the formation of NADPH being followed by the absorbance change at 320 nm. The assays were carried out at 25 °C, pH 8.5, in solutions containing 0.1 M glucose, 0.04 M tris(hydroxymethyl)aminomethane (Tris),[1] 0.01 M $MgCl_2$, 0.01 M EDTA, 0.5 mM NADP, 2.5 mM ATP, and 1 unit of glucose-6-phosphate dehydrogenase. The hexokinase concentrations in solutions used for DSC were determined by absorbance measurements, using a value of specific absorptivity of 0.947 cm^2 mg^{-1} (Schmidt & Colowick, 1973).

Enzyme solutions were prepared by exhaustive dialysis against 0.005 M Tris buffer, pH 8.5, of the crystalline suspension after dilution with the same buffer containing 2×10^{-5} M phenylmethanesulfonyl fluoride. For measurements at high ionic strength, the buffer contained 0.2 M NaCl. Glucose solutions were prepared by dissolving a weighed amount of glucose in the final enzyme dialysate.

Isothermal Flow Calorimetry. The enthalpy of binding of glucose to hexokinase was determined at 7, 14, and 25 °C in the flow modification of the Beckman Model 190B microcalorimeter (Sturtevant & Lyons, 1969; Breslauer, 1973), with the platinum flow tubing replaced by Teflon tubing (Weber & Hinz, 1976). All measurements were made by using the stopped-flow procedure, the solutions being delivered at 3.63×10^{-3} mL s^{-1} for 60–150 s. The flow rate was frequently checked by weighing the effluent. The calorimeter was calibrated at each temperature by measurements of the heat of neutralization of 5 mM HCl by 10 mM NaOH (Grenthe et al., 1970).

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[1] Abbreviations used: DSC, differential scanning calorimetric (calorimetry); Tris, tris(hydroxymethyl)aminomethane.

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Table I: Enthalpy of Binding of Glucose to Hexokinase at pH 8.5[a]

NaCl concn (M)	temp (°C)	ΔH (kcal mol^{-1})
0	7	+0.46 ± 0.55 (8)
	14	−0.62 ± 0.96 (7)
	25	+0.17 ± 0.93 (8)
	25	+0.19 ± 0.73 (8)
0.2	7.2	+0.52 ± 1.40 (4)
	12.1	−1.78 ± 1.02 (6)
	20.0	+2.18 ± 1.15 (6)
	25.0	−2.25 ± 1.26 (7)
	29.3	−0.89 ± 3.26 (6)

[a] Uncertainties are standard errors. The numbers in parentheses indicate the number of measurements.

The concentration of enzyme after mixing was about 40 μM, and that of glucose was 50 mM. Since the dissociation constant of the glucose–enzyme complex is about 0.2 mM, the enzyme was saturated with glucose in all the flow calorimetric experiments. Corrections for the heat of dilution of glucose and for viscous heating were applied. The heat of dilution of the protein was found to be negligible. The correction terms were nearly as large as the heat of binding, a fact which seriously affected the precision of the latter.

Isothermal Batch Calorimetry. For some measurements, an LKB Model 10700 batch microcalorimeter with gold cells was employed. In the sample cell, 4 mL of glucose was mixed with 1 mL of enzyme solution, while in the reference cell, 4 mL of glucose was mixed with 1 mL of enzyme dialysate. The concentrations of glucose and enzyme after mixing were 5–8 mM and 7–22 μM, respectively. Corrections were applied for the amounts of enzyme remaining unreacted, using the equilibrium constants determined by fluorescence titration. The calorimeter was calibrated by electrical heating and, at 25 °C, by the heat of dilution of 0.12 M sucrose with an equal weight of water (Gucker et al., 1939).

Differential Scanning Calorimetry. The DASM-1M microcalorimeter described by Privalov et al. (1975), with some electronic modifications, was employed. The scan rate was 1 K min^{-1} throughout, and protein concentrations of 0.5–2.3 mg mL^{-1} were used. Calorimetric enthalpies were evaluated from the DSC recordings of excess heat capacity according to the method outlined by Privalov & Khechinashvili (1974).

Fluorometric Titrations. The spectrofluorometer used in these experiments was formed by replacing the cell assembly of a stopped-flow apparatus (Sturtevant, 1964) by a stirred and thermostated 1 × 1 cm quartz cuvette, with the transmitting and receiving monochromators at right angles to each other. Quenching of the fluorescence of hexokinase by glucose was observed at 330 nm with excitation at 300 nm (Feldman & Kramp, 1978). A hexokinase solution in the cuvette was titrated with a solution containing hexokinase at the same concentration and a large excess of glucose, so that the enzyme concentration remained constant throughout the titration. It was found that during exposure to the exciting light, the fluorescence emission of the enzyme decreased by about 1.6% min^{-1}. Care was taken to minimize such exposure, and it is believed that no serious error was introduced by photodecomposition.

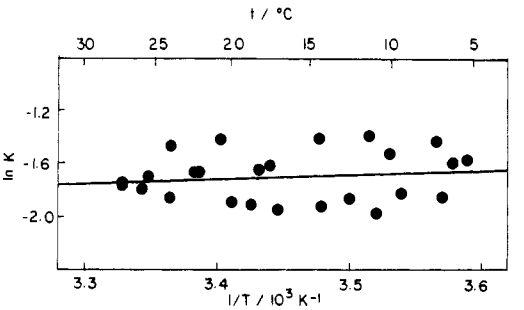

FIGURE 1: van't Hoff plot of the equilibrium constants, determined by fluorescence titration, for the dissociation of the glucose–hexokinase complex in the presence of 0.2 M NaCl. The line was determined by linear least squaring.

Results and Discussion

Change in Protonation on Binding Glucose. Excess glucose was added to an unbuffered solution of enzyme in 0.2 M NaCl at pH 8.5 to give a final concentration of 16 μM. From the change in pH, it was concluded that $\Delta[H^+] = 1.4 \times 10^{-9}$ M, indicating a negligible change in protonation of the enzyme. Thus, no corrections for buffer ionization heats were necessary.

Enthalpy Change on Binding Glucose. The values obtained for the enthalpy of binding of glucose to hexokinase are listed in Table I. When the standard errors associated with these values, which amounted to about ±0.04 mcal in each calorimetric experiment, are considered, it is evident that the enthalpy change in this process is indistinguishable from zero, over the entire temperature range covered at both low and high ionic strengths. Thus, the heat capacity change is also indistinguishable from zero and expressed in specific heat terms cannot amount to more than ±0.001 cal K^{-1} g^{-1}. This figure is much smaller than that which is usually found for protein–ligand reactions (Sturtevant, 1977).

It is surprising that a process involving a very substantial conformational change in the protein is accompanied by a vanishingly small change in enthalpy. This must indicate the fortuitous cancellation of a number of exothermic and endothermic effects.

Free-Energy Change on Binding Glucose. Fluorometric titrations utilizing the partial quenching of the protein fluorescence by glucose were performed in the presence of 0.2 M NaCl at temperatures in the range 5–28 °C. The observed fluoresence quenching followed closely the variation with glucose concentration expected for simple, single-site binding. Dissociation constants for the enzyme–glucose complex were evaluated as described by Feldman & Kramp (1978) and are shown in Figure 1 in the form of a van't Hoff plot. In agreement with the calorimetric results, the dissociation constants are very nearly independent of temperature. The least-squared line in Figure 1 has a slope corresponding to the value $\Delta H_{vH} = +0.55 \pm 0.90$ kcal mol^{-1}.

The thermodynamic parameters for the binding of glucose to hexose in 0.2 M NaCl at 10, 25, and 30 °C are given in Table II, the values for the dissociation constant, K, and the enthalpy being read from the van't Hoff plot of Figure 1 and

Table II: Thermodynamic Parameters for the Binding of D-Glucose to Hexokinase at pH 8.5 in 0.2 M NaCl

temp (°C)	K^a (mM)	$\Delta G°$ (kcal mol^{-1})	ΔH^b (kcal mol^{-1})	$\Delta S°$ (entropy unit)	ΔC_P (cal mol^{-1} K^{-1})
10	0.186 ± 0.010	−4.83 ± 0.03	−0.05 ± 0.90	+16.9 ± 3.2	
25	0.177 ± 0.011	−5.12 ± 0.04	−0.73 ± 0.90	+14.7 ± 3.1	50 ± 110
30	0.174 ± 0.014	−5.21 ± 0.05	−0.96 ± 0.90	+14.0 ± 3.1	

[a] Calculated by least-squares analysis of the data given in Figure 1. [b] Calculated by least-squares analysis of the data given in Table I.

Table III: Summary of DSC Measurements on Hexokinase[a]

figure no./curve	NaCl concn (M)	glucose concn (mM)	enzyme concn (mg mL^{-1})	T_{max} (°C)	calorimetric enthalpy (cal g^{-1})	van't Hoff enthalpy (kcal mol^{-1})
	0	0	1.46	41.0, 47.5	1.50	
2/A	0	0	2.35	41.0, 48.0	1.51	
	0	0	2.49	42.3, 49.5	1.74	
					mean[b] 1.60 ± 0.15	
	0	20	0.51	50.5	2.63	130
	0	20	0.93	50.8	2.78	120
	0	20	1.46	50.0	2.93	110
	0	20	2.22	52.5	2.77	120
2/C	0	20	2.39	52.8	3.72	130
					mean[b] 3.09 ± 0.40	
	0.2	0	0.49	41.3	1.60	80
	0.2	0	0.71	42.5	1.55	60
2/B	0.2	0	1.46	42.5	1.50	90
					mean[b] 1.53 ± 0.05	
3/A	0.2	20	0.53	51.0	2.75	110
3/B	0.2	20	0.94	50.0	3.03	120
3/C	0.2	20	1.46	50.0	2.92	120
					mean[b] 2.90 ± 0.10	

[a] Scan rate 1 K min^{-1}. [b] Mean value ± standard error.

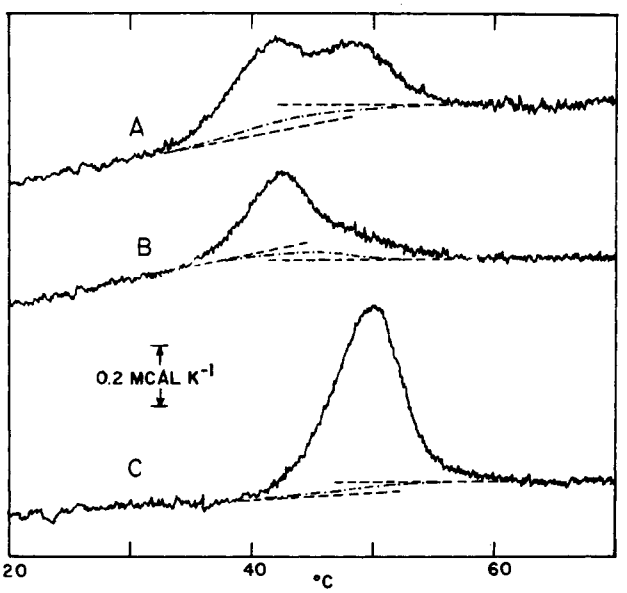

FIGURE 2: Representative DSC traces obtained with hexokinase at a scan rate of 1 K min^{-1}. (A) No added glucose or NaCl, 2.35 mg of protein per mL; (B) 0.2 M NaCl, 1.46 mg of protein per mL; (C) 20 mM glucose, 2.39 mg of protein per mL.

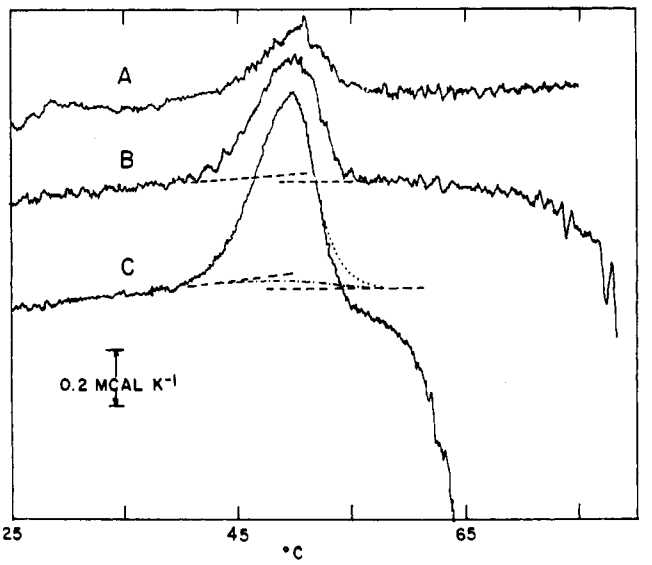

FIGURE 3: DSC scans obtained with hexokinase in the presence of 0.2 M NaCl and 20 mM glucose. Scan rate, 1 K min^{-1}. Protein concentrations: (A) 0.53 mg mL^{-1}; (B) 0.94 mg/mL^{-1}; (C) 1.46 mg mL^{-1}.

a least-squares analysis of the pertinent data in Table I, respectively.

It appears that, over the temperature range employed, the binding of glucose to hexokinase is entropically driven. As mentioned earlier, X-ray data (Bennett & Steitz, 1978) indicate that a significant decrease in the surface area of the protein which is exposed to the solvent is caused by the binding of glucose. It might thus be argued that the entropy drive is primarily the result of a hydrophobic effect resulting from withdrawal of nonpolar groups from exposure to the solvent. However, according to this view, there should also have been a definite decrease in heat capacity (Sturtevant, 1977), whereas little if any change was observed. On the other hand, if the decrease in surface area caused a decrease in the exposure of charged groups to solvent, presumably with formation of one or more close ion pairs or salt bridges within the protein, one would expect increases in both entropy and heat capacity. Since the primary structure of hexokinase is not yet available, most of the groups in the cleft region have not been identified, and it is therefore not possible to cite structural support for a possible compensation between these heat capacity increasing and heat capacity decreasing effects. A third possible contribution to the entropy and heat capacity changes is a decrease in the number of easily excitable internal vibrational modes (Sturtevant, 1977) which might reasonably be expected to accompany the tightening of the protein structure resulting from the coming together of the two lobes of the protein. Such a decrease would lead to decreases in both the entropy and the heat capacity, an effect opposite to that resulting from the formation of internal ion pairs.

DSC Measurements. Hexokinase was heated in the scanning calorimeter at low and high ionic strengths and in the absence and presence of 20 mM glucose, with the results summarized in Table III. Typical scans obtained under each of these operating conditions are shown in Figures 2 and 3. In view of the large heat of ionization of Tris, it must be assumed that the pH dropped by almost 1 unit in the temperature interval 25–60 °C.

In these experiments, as in most DSC experiments, a major problem is the definition of base lines. We have followed a procedure which is strictly correct only for two-state processes.

The pre- and posttransitional base lines were extrapolated, and a curved base line was calculated on the assumption that the change between the two extrapolated base lines was proportional to the fractional heat absorption, as indicated in the figures.

The pretransitional base lines in the absence of glucose had slopes corresponding to unusually large temperature dependencies of the apparent specific heat of the protein, amounting to 0.0035–0.006 cal K^{-2} g^{-1}, whereas the usual value for globular proteins is approximately 0.002 cal K^{-2} g^{-1}. The absolute apparent specific heat of the protein in the absence of glucose, approximately 0.45 cal K^{-1} g^{-1} at 15 °C, is also unusually large, being nearly 50% larger than the value reported for several globular proteins (Privalov, 1979).

If protein which had been scanned to 60 °C was cooled and rescanned, a thermogram showing a much reduced area was obtained. It appears that the thermal unfolding of hexokinase, which may be reversible, is accompanied by considerable irreversible denaturation under the conditions of the DSC experiments. As seen in Table III, we have nevertheless applied reversible thermodynamics to some of the DSC curves, to the extent of estimating van't Hoff enthalpies, ΔH_{vH}, according to the equation (Velicelebi & Sturtevant, 1979)

$$\Delta H_{vH} = 4RT_{1/2}^2 \frac{C_{ex(max)}}{\Delta H_{cal}} \quad (1)$$

where $C_{ex(max)}$ is the maximal value of the excess heat capacity, ΔH_{cal} is the calorimetric enthalpy obtained by integration of the DSC curves, and $T_{1/2}$ is the temperature of half-completion of the transition. The quantities in eq 1 can be expressed in any consistent units such that the ratio $C_{ex(max)}/\Delta H_{cal}$ has the dimension T^{-1}.

An interesting feature of the DSC data is the appearance of a double-peaked endotherm for the unfolding of hexokinase at low ionic strength in the absence of glucose, with maxima in the excess heat capacity at 41 and 48 °C (Figure 2, curve A). The fact that addition of glucose changes this to a single-peaked endotherm with a maximum at 51 °C (Figure 2, curve C) suggests that the two peaks in the absence of glucose result from independent unfolding of the two domains of the native enzyme which appear in the X-ray structure.

According to this view, the addition of 0.2 M NaCl destabilizes the more stable of the two domains to a level about equal to that of the less stable domain so that they both unfold in the same temperature range (Figure 2, curve B). The fact that the van't Hoff enthalpy for the transition is approximately equal to the calorimetric enthalpy indicates that the two domains unfold as a single fully cooperative unit in the presence of NaCl.

Double-peaked transitions have previously been observed for Bence-Jones protein (Zav'yalov et al., 1977), aspartate transcarbamylase (Vickers et al., 1978), and the λ repressor protein (Pabo et al., 1979) and have been interpreted as showing the existence within the molecule of two or more structural domains which unfold independently. On the other hand, there are proteins which show single-peaked transition curves in spite of having two domains separated by a cleft. Two of these, parvalbumin (Filimonov et al., 1978) and lysozyme (Pfeil & Privalov, 1976; Velicelebi & Sturtevant, 1979), unfold with molecule-wide cooperation, as indicated by equality in each case of the van't Hoff and calorimetric enthalpies. A third protein, papain (Tiktopulo & Privalov, 1978), has a van't Hoff enthalpy considerably smaller than its calorimetric enthalpy, which suggests that although the two domains unfold in the same temperature range, they do so with some degree of independence. This seems to be the case also with hexokinase in the presence of glucose.

The profound effect of NaCl on the interaction between the two domains in the hexokinase molecule is not accompanied by any significant change in the calorimetric enthalpy. Two unusual features of the transition curves observed in the presence of 0.2 M NaCl are the decrease in the apparent heat capacity accompanying the denaturation and the large temperature dependence of this quantity. In sharp contrast, although the addition of glucose does not lead to as strong an interaction between the domains, as judged by the fact that the van't Hoff denaturational enthalpy in its presence is smaller than the calorimetric enthalpy, it does lead to a doubling of the enthalpy of unfolding and to normal values for the temperature variation of the apparent heat capacity below the transition and for the denaturational change in heat capacity. The large increase in enthalpy is especially difficult to understand in view of the negligible enthalpy of binding of glucose to the native protein observed in our isothermal calorimetric experiments. The denaturational changes in heat capacity evident in the DSC curves in the presence and absence of glucose are of the wrong signs and of insufficient magnitudes to account for the difference in denaturational enthalpies. This difference could be accounted for if glucose interacts with unfolded hexokinase with a large absorption of heat. This unlikely possibility is difficult to check by means of isothermal calorimetry because of the irreversible denaturation which accompanies or follows the unfolding of the protein.

Another pronounced effect of glucose, which appeared only when NaCl was also present, was to increase the tendency of the thermally denatured protein to undergo an exothermic aggregation. The DSC traces in Figure 3 show the expected effect of protein concentration on the temperature of initiation of this rate-limited process.

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References

Bennett, W. S., & Steitz, T. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4848–4852.

Breslauer, K. (1973) Ph.D. Thesis, Yale University.

Feldman, I., & Kramp, D. C. (1978) *Biochemistry 17*, 1541–1547.

Filimonov, V. V., Pfeil, W., Tsalkova, T. N., & Privalov, P. L. (1978) *Biophys. Chem. 8*, 117–122.

Grenthe, I., Ots, H., & Ginstrup, O. (1970) *Acta Chem. Scand. 24*, 1067–1080.

Gucker, F. T., Jr., Pickard, H. B., & Planck, R. W. (1939) *J. Am. Chem. Soc. 61*, 459–470.

Kaji, A., Trayser, K., & Colowick, S. P. (1961) *Ann. N.Y. Acad. Sci. 94*, 798–810.

McDonald, R. C., Steitz, T. A., & Engelman, D. M. (1979) *Biochemistry 18*, 338–342.

Pabo, C. O., Sauer, R. T., Sturtevant, J. M., & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1608–1612.

Pfeil, W., & Privalov, P. L. (1976) *Biophys. Chem. 4*, 23–50.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.

Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665–684.

Privalov, P. L., Plotnikov, V. V., & Filimonov, V. V. (1975) *J. Chem. Thermodyn. 7*, 41–47.

Schmidt, J. J., & Colowick, S. P. (1973) *Arch. Biochem. Biophys. 158*, 458–470.

Steitz, T. A., Fletterick, R. J., Anderson, W. F., & Anderson, C. M. (1976) *J. Mol. Biol. 104*, 197–222.
Sturtevant, J. M. (1964) in *Rapid Mixing and Sampling Techniques in Biochemistry* (Chance, B., Eisenhardt, R. H., Gibson, Q. H., & Lonberg-Holm, K. K., Eds.) pp 89–102, Academic Press, New York.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Sturtevant, J. M., & Lyons, P. A. (1969) *J. Chem. Thermodyn. 1*, 201–209.
Tiktopulo, E. I., & Privalov, P. L. (1978) *FEBS Lett. 91*, 57–58.
Velicelebi, G., & Sturtevant, J. M. (1979) *Biochemistry 18*, 1180–1186.
Vickers, L. P., Donovan, J. W., & Schachman, H. K. (1978) *J. Biol. Chem. 253*, 8493–8498.
Weber, K. W., & Hinz, H.-J. (1976) *Rev. Sci. Instrum. 47*, 592–594.
Zav'yalov, V. P., Troitsky, G. V., Khechinashvili, N. N., & Privalov, P L. (1977) *Biochim. Biophys. Acta 492*, 102–111.

Hydrogen Exchange Rates in Pancreatic Trypsin Inhibitor Are Not Correlated to Thermal Stability in Urea†

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ABSTRACT: The hydrogen–isotope exchange rates of single, assigned peptide amide NH protons have been reported for bovine pancreatic trypsin inhibitor (BPTI). We have interpreted the complex pH and temperature dependence of the single proton exchange rates of BPTI as arising from changes in the mechanism between two processes that differ in temperature dependence [Hilton, B. D., & Woodward, C. K. (1979) *Biochemistry 18*, 5834; Woodward, C. K., & Hilton, B. D. (1980) *Biophys. J. 32*, 561]. One process, characterized by an activation energy of 20–35 kcal/mol, involves motions of the folded state that allow exchange of interior protons. The second process, characterized by an activation energy of ~65 kcal/mol, corresponds to major, cooperative unfolding. This two-process model explains all of the unusual features of the pH and temperature dependence of the kinetics of the slowest exchanging protons and differs fundamentally from the interpretation of the same data by Wuthrich & Wagner (1979) [Wuthrich, K., & Wagner, G. (1979) *J. Mol. Biol. 130*, 1]. For example, in the two-process model, characteristics of the high activation energy exchange rates cannot be ascribed to internal motions that limit exchange from the folded state. The model predicts that high activation energy exchange rates correlate with thermal stability of the protein, while low activation energy exchange rates may not. As a further test of the model, we have measured the pH and urea dependence of the major BPTI thermal unfolding transition and the effect of 8 M urea on low activation energy exchange rates. In support of the two-process interpretation of the BPTI single proton exchange data, we find the following: (1) The enthalpy of unfolding in 8 M urea, 45 ± 5 kcal/mol, agrees well with the value of ~45 kcal/mol obtained from the proton exchange analysis. (2) BPTI low activation energy exchange rates are not correlated to thermal stability in 8 M urea. (3) Between pH 4 and 2.5, BPTI titrates between two isomers of the folded state that differ in thermal stability. This transition, and the two-process model, explains the unusual pH dependence of the single proton exchange data, particularly the shallowness of the pH–rate curve and the abnormally high pH of the minimum rate, pH_{min}.

For folded proteins, the hydrogen–isotope exchange kinetics of peptide amide NH protons with solvent measure internal motions of the tightly packed conformation. Exchange rates for NH's within a single protein may vary over a range of >8 orders of magnitude, with the rates at the fast end of the distribution on the order of those of randomly coiled polypeptides. Exchange kinetics of folded globular proteins demonstrate that although interior NH atoms are shielded from solvent, there are conformational motions by which most buried backbone NH protons are rendered accessible to solvent OH^- and H^+ ions and H_2O. Understanding the nature of these motions is the object of hydrogen exchange studies (Woodward & Hilton, 1979, 1980; Englander & Englander, 1978; Englander et al., 1980).

Hydrogen–isotope exchange in proteins has most often been reported as total exchange, the average exchange kinetics of all labile protons measured simultaneously (Englander & Englander, 1978; Hvidt & Pedersen, 1974; Nakanishi & Tsuboi, 1974; Englander et al., 1979). With these kinds of data, there is the drawback that one cannot derive individual rate constants nor assign specific rates to individual NH groups. Advances in the analysis of total NH exchange kinetics have been made in the application of a distribution function (Knox & Rosenberg, 1980), and in the assignment of exchange rates to known peptide sequences (Rosa & Richards, 1979, 1981). However, the most specific approach to the quantitative analysis of hydrogen exchange kinetics is to measure the exchange rates of single protons by NMR spectroscopy. Extensive single proton exchange data have been reported for one set of protons, the eight slowest exchanging NH protons in bovine pancreatic trypsin inhibitor (BPTI) (Hilton & Woodward, 1978, 1979; Wuthrich & Wagner, 1979; Richarz et al., 1979), and in BPTI homologues and derivatives (Wagner & Wuthrich, 1979a). Assignments of these NH's have been made (Dubs et al., 1979). Our interpretation of these single proton exchange data differs fundamentally from the interpretation of Wuthrich and co-workers.

We have proposed that the observed exchange rate of each NH proton is the sum of its rates for exchange by two processes that differ widely in temperature dependence. One

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