

HYDROGEN ISOTOPE EXCHANGE KINETICS OF SINGLE PROTONS IN BOVINE PANCREATIC TRYPSIN INHIBITOR

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ABSTRACT The exchange kinetics of the slowest exchanging BPTI β -sheet protons are complex compared to model peptides; the activation energy, E_a , and the pH dependence are temperature dependent. We have measured the exchange kinetics in the range pH 1–11, 33–71°C, particularly the temperature dependence. The data are fit to a model in which exchange of each proton is determined by two discrete dynamical processes, one with $E_a \sim 65$ kcal/mol and less than first order dependence on catalyst ion, and one with E_a 20–30 kcal/mol and approaching first order in catalyst ion. The low activation energy process is the mechanism of interest in the native conformation of globular proteins and involves low energy, small amplitude fluctuations; the high activation energy process involves major unfolding. The model is simple, has a precedent in the hydrogen exchange literature, and explains quantitatively the complex feature of the exchange kinetics of single protons in BPTI, including the following. For the slowest exchanging protons, in the range 36°–68°C, E_a is ~ 65 kcal/mol at pH ~ 4 , 20–30 kcal/mol at pH > 10 , and rises to ~ 65 kcal/mol with increasing temperature at pH 6–10; the Arrhenius plots converge around 70°C; the pH of minimum rate, pH_{\min} , is > 1 pH unit higher at 68°C than for model compounds; and at high pH, the pH-rate profiles shift to steeper slope; the exchange rates around pH_{\min} are correlated to the thermal unfolding temperature in BPTI derivatives (Wagner and Wüthrich, 1979, *J. Mol. Biol.* 130:31). For the more rapidly exchanging protons in BPTI the model accounts for the observation of normal pH_{\min} and E_a of 20–30 kcal/mol at all pH's. The important results of our analysis are (a) rates for exchange from the folded state of proteins are not correlated to thermal lability, as proposed by Wüthrich et al. (1979, *J. Mol. Biol.* 134:75); (b) the unfolding rate for the BPTI cooperative thermal transition is equal to the observed exchange rates of the slowest exchanging protons between pH 8.4–9.6, 51°C; (c) the rates for exchange of single protons from folded BPTI are consistent with our previous hydrogen-tritium exchange results and with a penetration model of the dynamic processes limiting hydrogen exchange.

INTRODUCTION

The hydrogen isotope exchange kinetics of peptide amide protons in proteins is a measure of the accessibility of solvent to the buried regions of the polypeptide backbone. Over twenty years ago Hvidt and Linderstrom-Lang noted that although the exchange in native proteins is many orders of magnitude slower than in unfolded polypeptides, most of the buried peptide amide protons in folded native proteins do exchange with finite rates (Hvidt and Linderstrom-Lang, 1954). They concluded that although the interior amide protons are shielded from solvent, the measurable exchange of buried backbone protons is due to protein conformational motility. This remains the qualitative conclusion of hydrogen isotope exchange kinetics; given the tight packing of interior atoms in folded proteins, the exposure of backbone atoms in the native

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protein matrix to solvent immediately implies internal motions involving equilibrium transitions between conformational substates of the folded protein. For over a decade hydrogen-isotope exchange in proteins was the only direct experimental evidence for the dynamic dimension of protein structure, but the observations remained peripheral to the dominate paradigm of a rigid protein conformation that accrued from the static x-ray crystal structures and oil-drop models.

This arose partly from the formidable analytical difficulties in the interpretation of the complicated kinetics of hydrogen isotope exchange experiments where all labile protons are measured simultaneously. Some of the difficulties have been overcome by the recent introduction of more sophisticated treatment of the data by distribution functions (Knox and Rosenberg, 1980) and by high precision partial labeling techniques (Rosa and Richards, 1979; Englander et al., this volume). Instead we have approached the problem by the study of individual protons by nuclear magnetic resonance (NMR) spectroscopy.

Analytical difficulties notwithstanding, studies of total hydrogen-tritium and hydrogen-deuterium exchange kinetics have provided a semiquantitative picture of solvent accessibility in globular proteins. The most important findings are that native proteins exchange with a distribution of exchange rates 4–8 orders of magnitude broader than the distribution of rates for the equivalent unfolded peptide, with the fastest rates on the order of those of unfolded protein; 85–90% of the amide protons exchange from the folded conformation with no contribution from unfolding; under certain conditions of pH and temperature, the major unfolding transition may contribute to, or dominate, the observed exchange kinetics; the activation energy for the conformational contributions to exchange in native proteins is typically 0–15 kcal/mol; denaturing cosolvents, at concentrations below those inducing unfolding, do not affect hydrogen exchange rates from the native conformation; ligand binding or covalent modification may increase, decrease, or not affect hydrogen exchange rates; protons at the protein-protein interface of protein complexes exchange with the same mechanism as protons in the buried regions of globular proteins; (Woodward and Hilton, 1979; Hilton and Woodward, 1979, and references therein). Recently, it has been shown that the exchange kinetics for lysozyme are the same for the crystalline protein as for the protein in solution (Tuchsen and Ottesen, 1979).

For the types of internal motions responsible for the accessibility of buried protein atoms to solvent, two limiting types of model have been proposed. In one type, we propose that the exchange event occurs inside the protein which is fluctuating between conformations that approximate, within tenths to several Å, the x-ray crystal structure. This, we propose, occurs after penetration of solvent species into the protein via numerous small atomic motions, each too small to accommodate solvent molecules or ions, but with a finite probability of collecting as holes or pathways for solvent (Woodward and Hilton, 1979, and references therein). A more specific mechanism of mobile defects, created by rearrangements of hydrogen bonds, has been proposed by Lumry and Rosenberg (Lumry and Rosenberg, 1975; Lumry, 1979). Richards (1979) has suggested that protein packing defects may contribute to such mobile defects. In the second type of model, exchange occurs in bulk solvent after the protein has undergone large amplitude motions that expose a segment of the protein to solvent (Englander, 1975; Hvidt, 1973; Nakanishi et al., 1973). The relative merits of these two types of model have recently been reviewed (Woodward and Hilton, 1979; Gurd and Rothgeb, 1979; McCammon and Karplus, 1980; Englander and Englander, 1978).

The hydrogen-deuterium exchange rates of single peptide amide protons of proteins can be followed by the decay of the proton NMR resonance in deuterium solvent. We began with

bovine pancreatic trypsin inhibitor (BPTI) which offers a number of advantages, e.g., well resolved peptide amide proton NMR resonances, an unusually wide range of pH and temperatures for the native conformation, and well characterized x-ray crystal structural (Huber et al., 1971) and NMR spectral (Wüthrich and Wagner, 1979) characteristics. Also, BPTI is the model protein for the dynamical calculations of Karplus, McCammon, and coworkers (McCammon and Karplus, 1980).

The slowest exchanging and most clearly resolved proton resonances in the BPTI NMR spectrum have been assigned to peptide protons in the β -sheet core (Dubs et al., 1979). The exchange rates of these protons over the temperature range 22°–79°C and pH range 1–12 have been reported (Hilton and Woodward, 1978; Richarz et al., 1979; Hilton and Woodward, 1979). The initial expectation that the measure of single protons would simplify the kinetics was not realized. Instead, the exchange kinetics of each of the slowest exchanging protons have complex pH and temperature dependencies.

We have advanced a simple model to explain these complex kinetics, that there are two exchange processes that differ in temperature and pH dependence (Hilton and Woodward, 1979). One process is proposed to correspond to low activation energy motions of the folded, native conformation; the other to major unfolding. Alternatively, Wagner and Wüthrich (1979a) have proposed a multistate model to explain the same data. This paper further examines the consistency of our model with the available data, the hypotheses generated for future experiments, and the implications for the physical nature of the two conformational processes.

THE MODEL

Exchange of each amide proton may proceed by either of two parallel processes, *a* or *b*, represented schematically in Fig. 1. For process *b*, the observed rate for exchange from the native protein, $k_{n,b} \equiv k_{exg}$. For process *a*, k_1 and k_2 are the unfolding/folding rate constants and k_{cx} is the chemical exchange rate for the amide freely exposed to solvent, and the observed rate for exchange from the native protein is $k_{n,a}$. When $k_{cx} \ll k_1 + k_2$, $k_{n,a} = k_1 \cdot k_{cx} / (k_1 + k_2 + k_{cx})$. When $k_{cx} \gg k_1 + k_2$, $k_{n,a} = k_1$ (Hvidt, 1973). The activation energy for $k_{n,b}$ ranges from 20–35 kcal/mol, while the activation energy for $k_{n,a}$ is ~60 kcal/mol. The pH dependence of $k_{n,b}$ approaches first order in $[\text{OH}^-]$ dependence while $k_{n,a}$ is significantly less than first order in $[\text{OH}^-]$ dependence. The pH and temperature dependence of the observed exchange rate, k_{obs} , for the native protein will depend on the relative magnitude of $k_{n,a}$ and $k_{n,b}$.

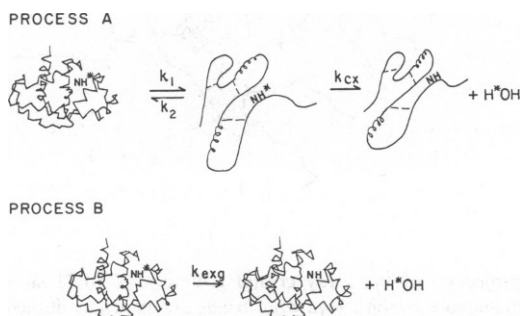


Figure 1 Illustration of the two pathways for exchange for a single proton in BPTI. See text for details.

under those conditions of pH and temperature, as

$$k_{obs} = k_{n,a} + k_{n,b} \quad (1)$$

THE FIT OF THE MODEL TO AVAILABLE DATA

Exchange rates for 13 individual, assigned peptide amide protons have been measured for BPTI as a function of pH and temperature (Richarz et al., 1979; Hilton and Woodward, 1978; Hilton and Woodward, 1979). The rates of the slowest exchanging protons group together at pH ~4; these are also the most down-shifted of the resonances in the PMR spectra. Their complete pH-rate profiles from 20–68°C have been reported. These include the NH's of Tyr 23, Phe 45, Phe 22, Phe 33, Tyr 21, Gln 31, Arg 20. For the purposes of this discussion, we refer to this group below as the "slowest exchanging protons." Some additional well-resolved NH resonances have been studied; they have faster exchange rates which vary over a larger range of times, and for which there are less complete data than the first group. These include the NH's of Tyr 35, Met 52, Gly 36, Cys 55, Ile 18, and an unassigned resonance at 8.31 ppm No. 13 (Richarz et al., 1979). We refer to this group below as the "more rapidly exchanging protons." A drawing of the β -sheet region of BPTI is shown in Fig. 2. The hydrogen bonds of most of the protons under study are shown by dashed lines, and each is labeled at the backbone-NH position.

Exchange of peptide amide protons is both acid and base catalyzed. For model amides, the exchange for the chemical step, k_{cx} , is given by

$$k_{cx} = k_H[H^+] + k_{OH}[OH^-] + k_{H_2O} \quad (2)$$

where k_H and k_{OH} are the rate constants for acid and base catalyzed exchange. These are sensitive to nearest neighbor inductive effects. k_{H_2O} is the rate for direct exchange with water, and generally is considered negligible. The pH- k_{cx} profile shows a characteristic pH of minimum rate, pH_{min} , on the basic side of which exchange is OH^- catalyzed, and vice versa. For polypeptides freely exposed to solvent, k_{cx} for each peptide NH can be calculated from empirical nearest neighbor rules (Molday et al., 1972).

The pH rate profile for seven of the slowest exchanging protons is shown in Fig. 3. At 68°C, rates that span the pH_{min} are observable. The dashed lines in Fig. 3 show the pH-rate profile

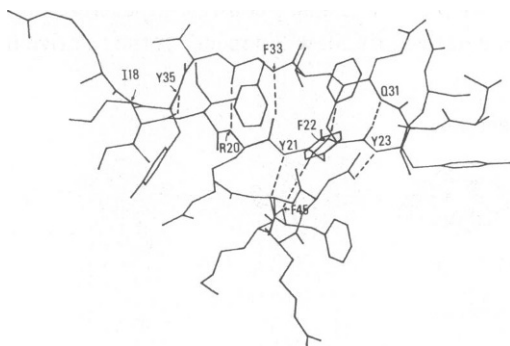


Figure 2 Computer projection of the x-ray crystal structure of BPTI showing the β -sheet region containing assigned exchangeable protons. Hydrogen bonds are shown as dashed lines. Assigned peptide amide protons are indicated at the backbone -NH position. (Drawing provided by Dr. Richard Feldmann, National Institutes of Health.)

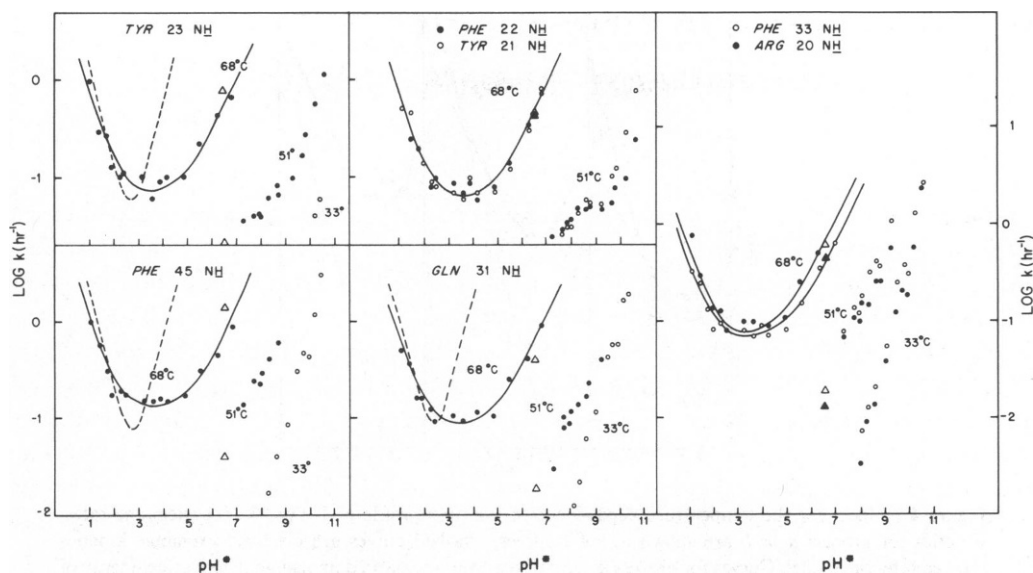


Figure 3 The pH dependence of exchange rates of single protons in BPTI. The assignments are from Dubs et al. (1979). The circles are data of Hilton and Woodward; triangles are from Wüthrich and Wagner (1979) and Brown et al. (1978) extrapolated from 55°C or from 60°C using activation energies determined at $\text{pH}^* 7.7$. The solid curves are fits of the pH^* profile at 68°C to the equation $k = k_1[\text{H}^+]_0^{.51} + k_2[\text{H}^+]^{-0.51} + k_3$. Dashed lines are calculated model compound rates at 68°C ($\times 10^{-4}$) from the rules of Molday et al. (1972). (Reprinted from Hilton and Woodward, 1979, with permission from *Biochemistry*).

calculated for Tyr 23, Phe 45 NH, and Gln 31 NH, assuming no configurational shielding (Molday et al., 1972). The unusual features of these data are: (a) the rates at 68°C in the region of the pH_{\min} are about the same for all these protons; (b) the pH_{\min} is ~ 1.2 pH units higher than for model compounds; (c) the slope of the base catalyzed limb of the pH -log k profile is <1 at high temperature and ~ 1 at low temperature; (d) the exchange rates of Phe 22 NH and Tyr 21 NH at 51°C are pH independent between pH 8.4 and 9.6.

The model is suggested by the variation of the pH dependence with temperature, Fig. 3, and by the striking Arrhenius plots for exchange at $\text{pH}^* 7.8$ which are curved and which extrapolate to a common rate at high temperature (Hilton and Woodward, 1979). The fit of the model to the data can be tested by calculation of the pH rate profile for $k_{n,a}$ and $k_{n,b}$ for each proton at constant temperature, Fig. 4. The expected rate at specified pH and temperature, dashed line, is determined by the relative magnitude of $k_{n,a}$ and $k_{n,b}$, Fig. 4. The pH-rate profile for $k_{n,a}$ is generated from the data at 68°C using the activation energy for process *a* for each proton listed in Table I and the pH dependence observed at 68°C. Similarly, the pH-rate profile for $k_{n,b}$ is generated from the experimental data at low temperature using an activation energy of process *b* for each proton listed in Table I and the pH dependence observed at low temperature. The calculated pH-rate profiles fit the data from our laboratory (circles) as well as from Wüthrich and coworkers (triangles), Fig. 4 and Hilton and Woodward (1979).

The model also fits the pH dependence of the activation energies of all of the exchange rates measured. Over the temperature range 22°-68°C for slowest exchanging protons, process *a* will dominate around the pH_{\min} , and process *b* will dominate at pH's far away from pH_{\min} . For these, at pH ~ 4 , the activation energy should be constant and ~ 60 kcal/mol; at pH > 10

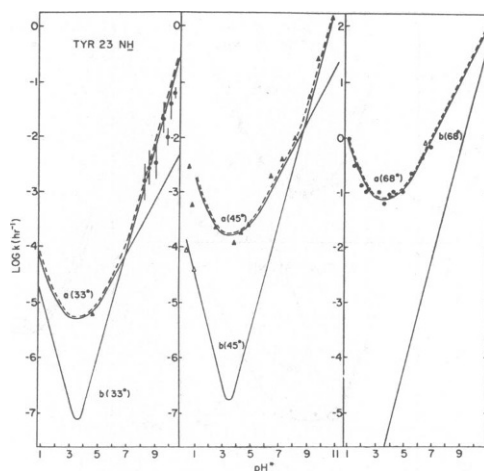


Figure 4 Model for the temperature dependence of the pH* profile of 1 Tyr 23 NH exchange rates. Kinetics for process *a* or *b* are shown as solid curves; dashed curves are observed exchange kinetics predicted by the model. Curves for process *a* are derived from the 68°C data using an activation energy of 57 kcal/mol; curves for process *b* are derived from a linear fit to the data at 33°C and an activation energy of 30 kcal/mol. Circles are data from Hilton and Woodward; triangles are from Wüthrich and Wagner (1979) and Brown et al. (1978). For the latter the 33°C and 68°C points were extrapolated from 36°C and 60°C data using an activation energy of 57 kcal/mol. (Reprinted from Hilton and Woodward, 1979, with permission from *Biochemistry*).

the activation energy should be constant and 20–30 kcal/mol; and at pH ~8 the Arrhenius plots will be curved. There is quantitative agreement between the expected and the observed pH dependence of the activation energies of the slowest exchanging protons, Fig. 5 and Hilton and Woodward (1979).

A further prediction is that at 45°C around pH_{min}, the activation energy of the more rapidly exchanging protons should be constant and 20–30 kcal/mol. This is because for these protons $k_{n,b} \gg k_{n,a}$ at all temperatures <60°C. This is borne out; the exchange rates of the more rapidly exchanging protons for which the observed rates are faster than the calculated

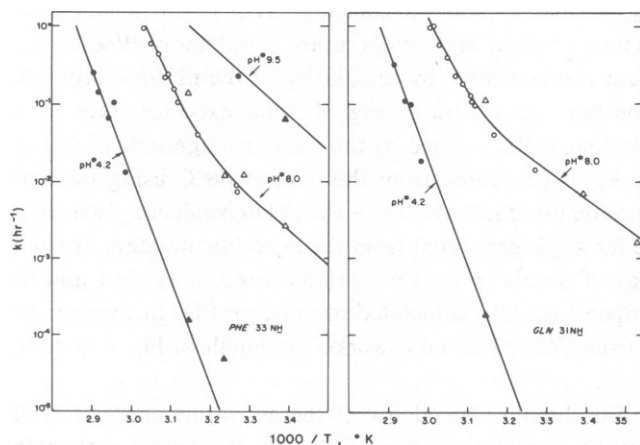


Figure 5 The temperature dependence of exchange rates of BPTI amide protons Phe 33 and Gln 31. Circles are data of Hilton and Woodward (1978; 1979). Triangles are data of Richarz et al. (1979). Data in some instances are extrapolated over small intervals of pH and temperature.

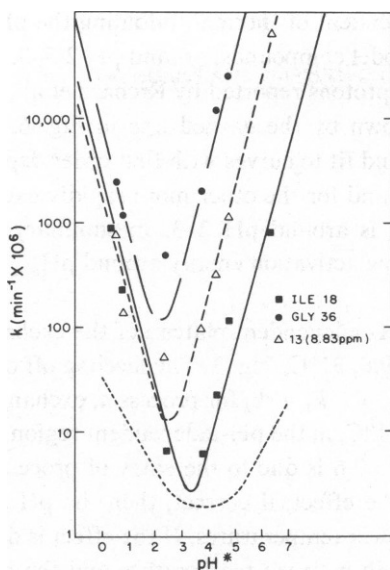


Figure 6 pH dependence of three more rapidly exchanging protons of BPTI at 45°C. Data are from Richarz et al. (1979). Lines are first order dependencies in $[\text{OH}^-]$ and $[\text{H}^+]$. The lower dashed line is the predicted curve for process *a* using 60 kcal/mol and the data at 68°C.

$k_{n,a}$ at temperatures $>20^\circ\text{C}$, Fig. 6. For these the activation energy between 22°C and 45°C is 17–38 kcal/mol over the range pH 1–7, Table I.

Our model explains why, in a number of derivatives and homologues of BPTI, the exchange rates of the slowest exchanging protons in each protein are correlated with the thermal stability of that protein (Wagner and Wüthrich, 1979). At temperatures $>20^\circ\text{C}$, in the region of the pH_{\min} , the slowest exchanging protons exchange by process *a*, which involves thermal unfolding. Likewise, the model predicts that exchange rates will not be correlated with thermal stability under conditions where exchange is dominated by process *b*, i.e., at high pH for the slowest exchanging protons and at all pH's for the more rapidly exchanging protons. Also urea or other denaturants are expected to accelerate exchange rates when process *a* dominates, and to have little or no effect when process *b* dominates. This is presently being tested.

The model is consistent with the value of the pH_{\min} for the slowest exchanging protons at >1 pH unit higher than that of model compounds, Fig. 2 and Table I. If in the native protein the acid catalysis term is greater than the base catalysis term, as compared to the relative values of the acid and base catalysis terms of the model compounds, Eq. 2, then this has the effect of raising the pH at which the acid and base catalysis terms are equal, i.e., of raising the pH_{\min} in the native protein as compared to model compounds. Thermal unfolding studies for BPTI¹, show that between pH 3 and 4, the temperature of the midpoint of the unfolding increases $\sim 10^\circ\text{C}$. Thus the model predicts that for process *a*, the acid catalyzed exchange will be accelerated relative to the base catalyzed exchange due to increased thermal lability, and the pH_{\min} will be shifted to higher pH's, as observed for the slowest exchanging protons, Fig. 2.

¹Thermal unfolding of BPTI has been measured by the change in ellipticity at 225 nm in the circular dichroism spectra in 0.3 M KCl and in 8 M urea (Hilton, et al., manuscript in preparation). In both solvents, the transition midpoint temperature increases $\sim 10^\circ\text{C}$ between pH 3 and 4. In 0.3 M KCl, unfolding is not detected at temperature $<80^\circ\text{C}$, and only a portion of the melting curve is observed; in urea the entire unfolding curve can be measured.

For process *b*, which is independent of thermal unfolding the pH_{\min} is expected to be normal and approximately that of model compounds, around pH 2.5–3. The exchange rates for all of the more rapidly exchanging protons reported by Richarz et al., (1979) lie above the pH rate profile calculated for k_{na} shown by the dashed line in Fig. 6. The data for three of these protons are shown in Fig. 6, and fit to curves with first order dependence on $[\text{OH}^-]$ and $[\text{H}^+]$ ion. As seen, for these three, and for the other more rapidly exchanging protons reported by Richarz et al., (1979) pH_{\min} is around pH 2–3. In summary, as predicted by the model, protons that exchange with low activation energy around pH_{\min} have normal values of pH_{\min} , Table I.

The model explains the pH-independent plateau of the exchange rates of Phe 22 NH and Tyr 21 NH between pH 8.4–9.6, 51°C, Fig. 2. The leveling off of the rates at pH 8.4, 51°C is because at these conditions $k_{ex} \gg k_1 + k_2$ for process *a*, exchange is limited by the unfolding rate, and $k_{obs} = k_{na} = k_1$. At 51°C, in the pH-independent region, $k_{obs} = 5 \times 10^{-2} \text{ h}^{-1}$. The rise in the pH-rate profile at pH >9.6 is due to the entry of process *b*. A test of this idea is the temperature dependence of the effect; if correct, then the pH at which the pH-rate profile levels off would be lower at lower temperatures. If the effect is due to titration of an ionizable group, the opposite relationship between temperature and the break in the pH-rate curve is expected. Exchange data at 45°C support the model (Hilton and Woodward, 1979). Similarly, we expect that if urea is added to the protein at 51°C, the curve for process *a* will be

TABLE I
APPARENT ACTIVATION ENERGIES, E_a , AND pH^* OF MINIMUM RATE, pH^*_{\min} , FOR SINGLE AMIDE PROTONS IN BPT1

Resonance No.*	-NH assignment‡	E_a , process a§ ($\pm 10\%$)	E_a , process b ($\pm 10\%$)	pH^*_{\min} ¶ observed	pH^*_{\min} † calculated
1	Tyr-23	60 kcal/mol	30 kcal/mol	3.7	2.7
2	Phe-45	51	20	3.7	2.7
3	Phe-22	65	38	3.6	2.8
4	Phe-33	63	20	3.3	2.6
5	Tyr-35	—	24	2.9	3.0
6	Tyr-21	62	29	3.8	2.6
7	Gln-31	65	17	3.6	2.5
9	Gly-36	—	28	2.4	2.5
10	Met-52	—	30	2.0	2.6
11	Arg-20	64	23	3.2	2.7
13	—	—	24	2.8	—
15	Cys-55	—	<20	2.2	2.2
16	Ile-18	—	19	3.3	2.7

*Resonances are numbered as in the spectra shown in Richarz et al. (1979). The resonance assigned to Arg 20 is numbered 11 in Richarz et al. (1979) and 9 in Hilton and Woodward (1978, 1979).

‡Assignments are those of Dubs et al. (1979).

§Process *a* apparent activation energies of the slowest exchanging protons are calculated from the data at $\text{pH}^* 4.2$, 22–68°C (Hilton and Woodward, 1979).

||For resonances 1, 3, 4, and 6, process *b* apparent activation energies are calculated from the data at $\text{pH}^* 10.6$, 22–51°C; for resonances 2, 7, and 11, the values listed are the low activation energy terms of a two-term fit to Arrhenius plots at $\text{pH}^* 8$, 22–51°C; for resonances 5, 9, 10, 13, 15, and 16, apparent activation energies are calculated from data at 22° and 45°C over the range $\text{pH}^* 1$ –7.

¶From data of Hilton and Woodward (1979) and Richarz et al. (1979). The pH^*_{\min} listed for resonances 1, 2, 3, 4, 6, 7, and 11 are observed at 68°C, and for resonances 5, 9, 10, 13, 15, and 16 at 45°C.

† pH^*_{\min} values are calculated from the amino acid sequence position of the resonances and the rules of Molday et al. (1972).

effectively raised to higher rates, the pH at which $k_{cx} \gg k_2$ will be higher, and the pH-rate profile will level off at higher pH's. At high concentrations of urea, there may be no pH independent region observable before onset of high pH denaturation. The effect of urea on the pH dependence at 51°C for the Phe 22 NH and Tyr 21 NH is being tested.

PROCESS *a* IS THERMAL UNFOLDING

All the data suggest that process *a* involves thermal unfolding. The apparent enthalpy, assuming 20 kcal/mol activation energy for the chemical step, is on the order of 40 kcal/mol, as compared to 47 kcal/mol for the carboxamidomethyl derivative of BPTI (Vincent et al., 1971) and 45 kcal/mol for BPTI in 8 M urea (B. Hilton, K. Russell, and C. Woodward, manuscript in preparation). A similar contribution from thermal unfolding to hydrogen-tritium exchange kinetics is observed for other globular proteins (Woodward et al., 1975; Ellis et al., 1975).

Arguments that exchange cannot involve major unfolding concern the relative values of k_2 and k_{cx} under conditions where the exchange is pH dependent (Wagner and Wüthrich, 1979a). To recapitulate their argument, if the pH dependence of the observed exchange is approximately that of k_{cx} for model compounds, then k_{cx} enters the overall rate expression, and $k_1 + k_2 \gg k_{cx}$. Under native conditions, $k_1 \ll k_2$ and the crucial relationship is k_2 vs. k_{cx} . The statement that "exchange follows an EX_2 mechanism" means that exchange shows acid and base catalysis and/or that base catalyzed exchange is approximately first order in $[\text{OH}^-]$. The maximum value of k_1 and k_2 under native conditions can be estimated from the folded/unfolded interconversion rate at the midpoint in the unfolding transition, T_m , where $K_{eq} = k_1/k_2 = 1$. At pH 1, $T_m = 86^\circ\text{C}$, the interconversion rate $= k_1 = k_2 \leq 10 \text{ s}^{-1}$ (Wüthrich et al., 1978). At lower temperatures, k_1 and k_2 are expected to be slower (Pohl, 1969). At pH 11, 45°C , the extreme of pH and temperature for the rates of Wagner and Wüthrich (1979a) $k_{cx} = 10^6 \text{ s}^{-1}$, i.e., $k_{cx} \gg k_2$. They conclude that, since exchange follows EX_2 over the entire range of pH and temperatures measured, major unfolding rates do not enter the overall expression for the observed exchange. However, these relative values of k_{cx} and k_2 are quite consistent with our model, including major unfolding for process *a* as follows. At high pH, 45°C , exchange is via process *b* which is not affected by thermal unfolding. At the same pH and temperature, process *a* is limited by k_1 and the pH-rate profile levels off. This is observed at 51°C for Phe 22 and Tyr 21 before exchange by process *b* takes over. That is, for each proton a pH-independent plateau is expected for process *a*, but whether it is observed depends on the relative exchange rates by process *b*.

A further consequence of our model is that for process *a* at pH's below the pH-independent region, $k_{obs} \approx K_{eq} \cdot k_{cx}$, and the value and pH dependence of K_{eq} can be estimated from the exchange kinetics. Then the deviation of process *a* from first order dependence $[\text{OH}^-]$ arises from the pH dependence of K_{eq} . Over the range pH 4-7, K_{eq} decreases from 5×10^{-4} to 8×10^{-6} .

COMPARISON TO THE MODEL OF WAGNER AND WUTHRICH

Wagner and Wüthrich (1979a) have proposed a "global, multi-state model" to explain the BPTI exchange data. Their model consists of two parts. First they present a general, formal solution to the exchange kinetics of a proton in a protein in dynamic equilibrium between conformational forms with exchange possible from each form. If exchange from different forms have different pH dependencies, for example from the variation of the amide group pK

in different conformations, one can generate a pH dependence for a single proton that is different from first order in $[\text{OH}^-]$ for the overall exchange of that proton. Once the pH dependence of exchange is allowed to vary with the conformational form, there are enough parameters to fit any conceivable hydrogen exchange data.

The formal treatment of simultaneous exchange from interconverting substrates does not speak to the nature of the interconversion transitions. With regard to this, their argument rests on the observations that (a) proton exchange is correlated with the thermal stability of the protein, with the rank-order of exchange rates for different amide protons preserved, while (b) the rotational motions of the aromatic rings are not correlated with the stability of the protein. Concluding that a penetration model cannot account for this data, they propose that the transitions consist of intramolecular translational and rotational motions of the intact hydrophobic clusters relative to each other.

In our model, the correlation of exchange with thermal stability will be observed for conditions where process *a* dominates, but this does not reflect internal motions responsible for exchange from the folded conformation (process *b*). The conditions under which Wagner and Wüthrich (1979) measured the correlation with thermal stability, around pH_{min} , 36°C, for eight derivatives and pH 6.5, 55°C, for two derivatives, are conditions where process *a* is expected to dominate for all the resonances they report except Tyr 35 and Met 52, Table I. For the latter two resonances the correlation is far less clear than for the other resonances (Fig. 3 of Wagner and Wüthrich, 1979). The preservation of rank order is expected for all protons exchanging by process *a* if the major change is in the relative value of k_1 and k_2 , Fig. 1. Thus, any modification, regardless of its location in the protein, that has the effect of increasing the thermal lability of the protein will affect exchange by process *a* without necessarily having an effect on the internal motions of the folded macrostate.

In summary, in our analysis, there is not a correlation between hydrogen exchange rates and thermal lability unless exchange is measured under conditions of temperature and pH where the major cooperative thermal transition enters the overall exchange expression. When exchange is limited by thermal unfolding, it is not surprising that exchange rates are correlated to thermal stability. The motions of interest in a consideration of the dynamic structure of the folded state of proteins, process *b*, are not correlated to thermal unfolding.

THE NATURE OF PROCESS *B*

The presently available exchange data for single protons in BPTI corroborate what was surmised from tritium-hydrogen exchange experiments. (a) The exchange mechanisms for exchange from the folded conformation is low energy. Assuming 20 kcal/mol for the chemical exchange step, the activation energies for different protons vary over the range 0–15 kcal/mol for the conformational process. The same range was found for a number of proteins from hydrogen-tritium studies (Woodward et al., 1975; Ellis et al., 1975; Woodward and Hilton, 1979). In general, it appears that the slower exchanging protons have higher activation energies (Wickett et al., 1974; Woodward, unpublished results). Apparently the activation energy for a single proton is temperature independent. (b) Exchange from the folded state is not accelerated by high concentrations of denaturants. At pH 6.5, 35°C, 8 M urea does not affect the tritium-hydrogen exchange rates of uniformly tritiated BPTI (Woodward, unpublished results). Other denaturants also have no effect on low activation energy exchange of globular proteins (Woodward et al., 1975a; Woodward and Hilton, 1979). These together with the observation that 95% of the amide protons in crystals of myoglobin exchange with deuterium after perfusion of the crystals with deuterium solvent (Schonborne et al., 1978),

that protons at the protein-protein interface of the trypsin-trypsin inhibitor exchange with pH and temperature dependence characteristic for globular proteins (Woodward, 1977) and that the exchange kinetics of lysozyme are identical in the crystal and in solution (Tuchsen and Ottesen, 1979) constitute the strongest argument for our proposal that exchange involves penetration of solvent species by pathways provided by small amplitude, low energy motions. We suggest that these data rule unlikely large amplitude motions, such as local unfolding (Englander, 1975) because such motions would be expected to be high energy, correlated to unfolding, accelerated by denaturants, and absent at the protein-protein interface and in the crystal lattice. A penetration model requires that charged solvent species migrate to interior regions of the protein matrix, a process not intuitively compatible with standard conceptualizations of the protein interior. It has been suggested (Richards, 1979) that this may be accomplished by OH⁻ transfer through H-bonded water networks.

As we envision them, small amplitude motions, and associated solvent accessibility, may well be perturbed by events distant in a tightly packed protein structure. Generalized ligand and covalent modification effects on hydrogen exchange rates are well known (Woodward and Hilton, 1979). Qualitative examination of the x-ray crystal structure shows that exchange rates by process *b* may be identical for two protons hydrogen bonded in adjacent rungs of pleated sheet (Phe 33 NH and arg 20 NH), or may differ by a factor of ~10 for another pair of protons hydrogen bonded across the same pleated sheet (Phe 22 NH and Gln 31 NH), or may be identical for hydrogen bonds that are on two successive peptide amide-NH's but oriented in opposite directions to two different strands of pleated sheet (Phe 22 NH and Tyr 21 NH). On the face of it, these data make local unfolding of peptide segments unlikely. Any interpretation in terms of a penetration model would have to include consideration of the packing and dynamical potential of surrounding atoms.

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DISCUSSION

Session Chairman: Hans Frauenfelder *Scribe:* Joseph J. Rosa

WÜTHRICH: Am I correct that for the amide proton of Tyr-23, your mechanism *b* determines the rate at pH > 6? If so, you would predict that the exchange rate at pH > 6 would be unaffected by variation of the denaturation temperature. Is that correct?

WOODWARD: We predict that for any NH proton under conditions where the exchange rate has an activation energy of 20-30 kcal, exchange is by mechanism *b* and there is not a correlation of exchange rate with thermal unfolding temperature.

WÜTHRICH: And that would be the case for the amide proton of Tyr-23?

WOODWARD: I cannot say without looking at the temperature dependence. At pH 6 the activation energy for Tyr-23 varies with temperature; this reflects a change in mechanism.

WÜTHRICH: After we read your paper in *Biochemistry*, we recalled that we once spent 6 months measuring the pH dependence of the exchange in one of the reduced forms of BPTI. This modified protein has a denaturation

temperature of ~65°C, as compared to 95°C for native BPTI. The exchange data, collected for 6 amide protons including Tyr-23 in both the native and reduced proteins, show that the difference in thermal stability is clearly manifested all the way up to pH 10. This clearly shows that for the one proton specifically treated, namely, Tyr-23, your mechanism *b*, is definitely correlated with the thermal denaturation temperature of the protein up to pH 10.

WOODWARD: This experiment is not a test of our model. Process *a* involves thermal unfolding and therefore exchange rates by this mechanism are a function of the thermal stability of the protein. Given a decrease in thermal stability of 30°C it is likely that exchange rates for process *a* are greatly accelerated relative to exchange rates for process *b* (exchange from the folded conformation). If this is so the curves for Fig. 4 *a* will be raised while those for *b* stay the same. Then exchange by unfolding occurs at higher pH's in the derivative as compared to the native BPTI. The critical experiment is to determine whether exchange from the disulfide reduce derivative at high pH is still with low activation energy and if so whether these exchange rates are correlated with thermal unfolding temperature.

I should add that we have looked at the urea dependence of the tritium exchange of the rapidly exchanging protons of BPTI. We find that 8 M urea does not accelerate on exchange rates even though 8 M urea lowers the thermal unfolding temperature of BPTI by 20°C.

Lastly, let me say that there are several protons for which Kurt Wüthrich has reported exchange rates that do not correlate with thermal unfolding around pH 4. Among these are Met 52, Tyr 35, and Ile 18. These are precisely the ones that we would expect to exchange without contribution from the major unfolding process. That is, they exchange with low activation energy around the pH minimum.

VON HIPPEL: The questions I raised in reviewing the papers of both Clare Woodward and Walter Englander had to do with clarifying and identifying the differences between models *a* and *b*, as they are called in this paper. They seem to be approaching one another a bit. We can all imagine model *a* in one form or another. Although it shouldn't necessarily require a global unfolding, it could certainly be an unfolding which involves a small segment and therefore may have different thermodynamic parameters. It remains to define the solvent penetration model specifically in molecular terms. I know that is a hard question. If it were easy it would have been answered long ago.

Two routes that might be pursued are: 1) the use of the fluctuations discussed earlier in this meeting by the crystallographers and Dr. Karplus in terms of a set of conditional probabilities of channel formation that leads to solvent penetration within the bounds of the observed kinetics and thermodynamics, and 2) the variation in pH dependencies to define local environmental effects on acid and base catalysis which should reflect the specific pathways into the protein.

F. RICHARDS: I would like to make a specific response to Peter von Hippel. We have, in fact, made calculations regarding the fluctuations of cavities of just the kind you are talking about. If you make reasonable estimates of the physical properties of a protein and use its crystal structure, you can "predict" the probabilities of channel formation and from there the hydrogen exchange behavior of BPTI and various other proteins. The theory is not good, but it does show that local fluctuations, without macro-unfolding, are perfectly capable of explaining entry into the protein to the extent necessary to explain exchange behavior.

VON HIPPEL: Can you say anything about the enthalpy of activation of these processes?

F. RICHARDS: No, nothing at all. It's not that kind of a calculation.

KARPLUS: This is to supplement Fred Richards' point in terms of the question of channel formation within BPTI as a function of specific local motions. Although the results have not been analyzed in detail, we've looked at the fluctuations that occur on the picosecond time scale in the N-O distance of all the main chain H-bonds. What one finds is that there is a good correlation between the amide hydrogen exchange kinetics and the very short time fluctuation in their r.m.s. H bond lengths. Obviously, this only gives you some idea of what the local force constant might be and how it varies. Extrapolating to the very large fluctuations that are needed for exchange is difficult. But one can hope to be able to understand why certain protons are slow to exchange and others fast, in various proteins.

MATTHEW: I would like to raise the question of the pH dependence of hydrogen exchange. Unlike model compounds, proteins often fail to obey first-order dependence on hydroxyl ion. We must at least attempt to explain this in terms of the properties of the protein. As is well-known, the transition temperature of proteins is very pH dependent. In terms of solvent penetration, not only must a hydroxide ion get to the amide, but the exchange process *per se* involves a negatively charged intermediate. I would suggest two things: (*a*) the electrostatic field around and within the protein will clearly vary unsymmetrically with pH, such that approach and migration of hydroxyl ions in a given path will either be enhanced or hindered. (*b*) the electrostatic potential generated by the formal charge array can also affect the stability of the charged intermediate required for exchange. The magnitude of these effects can be quite large despite the use of a dielectric constant of 40 within the protein.

These effects would be in addition to the pH-induced conformational fluctuations or electrostatic modulation of substrate population.

BARKSDALE: One of the problems that those of us working with hydrogen exchange have been concerned about is the correlation between the specific behavior of the very highly protected amides as seen by Woodward and Wüthrich using n.m.r. and the global exchange properties of proteins observed in the more traditional tritium exchange or IR experiments. Comparison of the overall exchange data by Hvidt and Pedersen (1974) obtained by deuterium infrared measurements with a summation of Wüthrich's specific amide exchange data on BPTI indicates that the very slowly exchanging amides monitored by Woodward and Wüthrich are in fact a microcosm accurately reflecting the overall exchange behavior of BPTI. Interestingly, the distribution of rate constants for BPTI follows a log-normal distribution. By comparison, rate constants for many other proteins, such as lysozyme and myoglobin, are distributed according to a power law. It may be that the distribution function for these large proteins are the sum of several log-normal distributions over the domains of multi-domain proteins.

ENGLANDER: For outsiders this discussion must seem terribly fragmented. I would like to say some things which may give a picture of what is going on. Looking at the structure of BPTI and the location of the very slowly exchanging protons in the β sheet, we can easily see that the small size of BPTI precludes the necessity of penetration by solvent molecules. In fact, two of these very slow protons are on the surface of the molecule, in contact with water. There is no issue of penetration or process *b* for these protons.

With respect to what Jim Matthew said about electrostatic effects on hydrogen exchange, the consequences for hydroxyl ion penetration should be pointed out. The pH dependence for small molecule H-exchange shows acid-base catalysis with a minimum \sim pH 3 for peptide hydrogens. In the case of a protein exchanging via solvent penetration, as the pH is lowered the surface positive charge generally increases. The effect, for exchange dominated by a solvent penetration mechanism, should be to promote hydroxyl ion penetration, decrease proton penetration and so move the pH minimum to more acid values. Such behavior has never been observed for any protein I know about. If any changes in the pH minimum are seen, the shift is generally to higher pH, as would be expected for exchange dominated by an unfolding mechanism.

The other issue has to do with the temperature dependence of hydroxyl ion penetration into the protein as required by process *b*. Hydroxyl ion, initially in water, with a rather large hydration sphere, can only be transferred into the protein by stripping off the coordination sphere. This costs on the order of 50 kcal/mol in enthalpy. This means that the temperature dependence of hydrogen exchange via a solvent penetration mechanism should show an apparent activation energy which includes 50 kcal/mol for that step alone plus 20 kcal/mol for the exchange step plus other terms. The sum approaches 100 kcal/mol and is never observed.

MATTHEW: The behavior predicted by Dr. Englander for an electrostatically induced shift of the pH minimum assumes a uniform charge distribution and would apply only if hydroxide migration in the protein's electric field is the only pH dependent property of the protein solvent interactions.

WÜTHRICH: I would like to make one comment and ask one question. The comment regards what Dr. Matthew just said. We have recently published a theoretical interpretation of the exchange data for BPTI which is based largely on considerations of the effect of electrostatic force on the stability. We have measured all the pK values for the ionizable groups in BPTI, so we were able to treat this in a reasonably accurate manner. There was no problem in fitting all the pH dependencies that have been observed using only the equilibria between the variously protonated forms.

I have a question for Fred Richards. Our reference to global fluctuations definitely does not imply a denaturing fluctuation. We do not require the denatured form of the protein, all of the motions are within the conformation space of the globular form. I do not, however, believe that the very high frequency motions of the type encountered in the picosecond range would give rise to sufficiently large fluctuations to allow amide proton exchange. Do you think that what you see as possible channel-forming fluctuations would be fluctuations that can be related to the stability of the protein? That is the crux of the matter. We see internal fluctuations in the native protein which are correlated with stability but which do not promote transitions to the denatured state at low temperature.

F. RICHARDS: Our own calculations are not able to address that question. They are based on the assumption that you can characterize a protein by an isothermal compressibility. We then assume that the cavities in the protein, which already exist in the x-ray structure, fluctuate. We calculate what these fluctuations ought to be, then assume combined probabilities, the way Peter von Hippel suggested, to calculate the probability of forming a channel of the necessary size. The time scale is imposed on this thermodynamic calculation simply by the hydrogen exchange kinetics since this is the only time scale available and everything is scaled on this basis. Doing this, it is perfectly possible to fit the hydrogen exchange data with a single parameter.

The results indicate that small fluctuations are adequate to explain accessibility. With regard to Walter Englander's question, it is not necessary to get hydroxide ion inside the protein, and I don't think anybody believes

that it ever happens. My own prejudice is based entirely on Bill Jencks' suggestion that a cavity is formed within the protein and is filled with a chain of neutral water molecules. Having established that channel, with very low probability in many cases, we now can bring up a hydroxide ion at the protein-solvent interface. With an ice-like proton shift, the hydroxide "appears" in the protein, the charge is transferred, distributed over the peptide, and leaves the way it came. I don't see any problem here. Forming the water channels is a relatively low energy process since we're not desolvating an ion.

ENGLANDER: The process you describe generates, it seems to me, an internal hydroxide ion intermediate that is largely desolvated. As we all know, the equilibrium thermodynamic problem cannot be side-stepped merely by utilizing a clever pathway to achieve this state.