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Hydrogen–Tritium Exchange Kinetics of Soybean Trypsin Inhibitor (Kunitz). Solvent Accessibility in the Folded Conformation[†]

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ABSTRACT: The hydrogen exchange kinetics of Kunitz soybean trypsin inhibitor (STI) has been studied at pH 2, 3, and 6.5. From the temperature dependence of proton exchange at low pH, the contribution of major, reversible protein unfolding to the hydrogen exchange kinetics has been determined. Exchange directly from the folded conformation is characterized by an apparent activation energy (E^*_{app}) of approximately 25 kcal/mol, close to that of the chemical exchange step. At pH 6.5 the protein is more temperature stable than at low pH, and exchange of all but $\simeq 8$ protons can be observed to exchange with $E^*_{\text{app}} \simeq 27$ kcal/mol. This implies that all but $\simeq 8$ protons are accessible to exchange with solvent in the solution structure of folded STI. Estimates can be made of the average number of water molecules per molecule of STI consistent with a solvent accessibility model of hydrogen exchange kinetics. These estimates indicate that very few water molecules within the protein matrix are necessary to explain the exchange data. Calculations are done for the STI hydrogen exchange kinetics at pH 3, 30°, approximating STI structure by a sphere of radius = 18 Å. These calculations indicate an average of $\simeq 4$ water molecules in the shell from 13 to 16 Å from the center of the molecule, while <1 water molecule is indicated in the innermost 13 Å. These calculations also suggest that there are $\simeq 190$ water molecules associated with the outermost 1.5–2 Å of the sphere. While these values are consistent with a hydrophobic region in the central protein matrix, they indicate more solvent accessibility in the outer 1/3 of the molecule than the static accessibility estimates made from X-ray coordinates. Our results suggest that any protein movements or fluctuations responsible for solvent accessibility in proton exchange processes are localized in the outer regions of the globular structure.

Solvent accessibility of buried groups in folded proteins is fundamentally related to the dynamic behavior of native proteins in solution and to the solvent contribution to the driving force of protein folding.

In protein tritium–hydrogen exchange experiments, the back exchange kinetics of tritium-labeled peptide amide protons with solvent is measured. It has long been recognized that proton exchange in native proteins is many orders of magnitude slower than that in the unfolded conformation (Linderstrom-Lang, 1955). In analyzing the processes responsible for the slowed exchange in the folded conformation, we have determined the contribution of major unfolding transitions to the overall exchange rates of native proteins (Woodward and Rosenberg, 1971a,b; Woodward et al., 1975a). From this, conditions for exchange only from the folded conformation have been characterized (Woodward et al., 1975a).

We have interpreted hydrogen exchange kinetics of proteins in the folded conformation in terms of solvent accessibility, rather than in terms of hydrogen bond breakage, per se (Woodward et al., 1975a). That is, the number of slowly exchanging protons in a folded protein is equal to the number with restricted solvent accessibility which may, or may not, be intramolecularly hydrogen bonded. The mechanism of solvent exposure may, and probably does, involve fluctuations of the protein, but these fluctuations need not involve H-bond breakage. It appears to us unlikely that the mechanism of protein fluctuations which accounts for solvent accessibility is a "breathing" process involving reversible, localized denaturations, accompanied by the breakage of several hydrogen bonds, because denaturants such as ethanol and urea do not affect the exchange rates (see below, and Woodward et al., 1975a,b).

We find that under conditions in which exchange occurs only from folded soybean trypsin inhibitor (STI),[1] all but

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[1] Abbreviation used is: STI, soybean trypsin inhibitor.

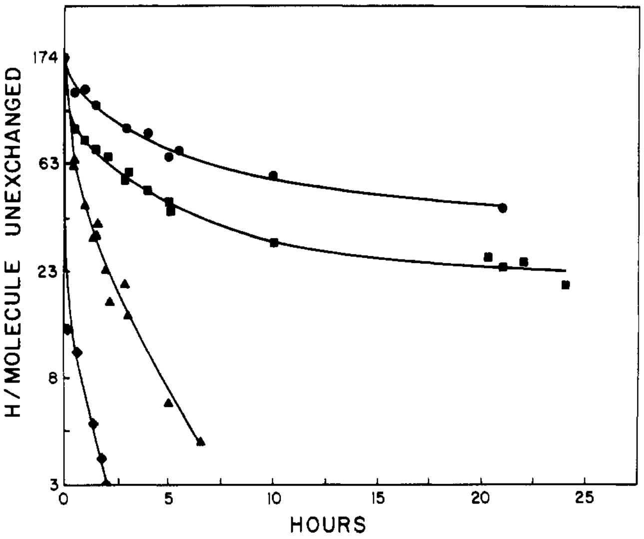

FIGURE 1: Hydrogen-tritium exchange kinetics of completely labeled STI (pH 2) at 20° (●), 30° (■), 40° (▲), and 50° (♦).

$\simeq 8$ protons are accessible for solvent exchange within observable time limits. We suggest that the broad distribution of exchange rates in the folded STI molecule reflects the distribution of dynamic solvent accessibilities in the solution structure of the native proteins. With that assumption, we have estimated the average number of water molecules as a function of distance from the center of the STI globular structure.

STI is a protein of molecular weight 21,500 and is composed of a single polypeptide chain. STI binds specifically to the active site of trypsin, inhibiting its proteolytic activity. STI literature has recently been reviewed by Laskowski and Sealock (1971).

Materials and Methods

Soybean trypsin inhibitor (Kunitz) was purchased from Worthington Biochemical Corp. (SI) and used without further purification.

The procedure for the tritium-hydrogen exchange experiments, based upon the method introduced by Englander (1963), has been described previously (Woodward and Rosenberg, 1971a; Woodward et al., 1975a). STI, 15-20 mg/ml, was labeled by reversible unfolding at 60°, 5 min, in 0.02 *M* phosphate buffer (pH 6.5) with 0.3 *M* KCl. Under these buffer conditions, 65° is the midpoint of the unfolding transition (Wu and Scherage, 1962). After heating, the protein solution was quickly cooled to 0°. For the experiments carried out at pH 2 and pH 3, the solution was acidified to the appropriate pH before the zero time filtration (Sephadex G-25, 2.5 × 13 cm column) which separates the excess label from the labeled, folded protein. The first 2/3 of the protein peak eluted in the zero time filtration was then pooled and immediately placed in a controlled temperature bath. Subsequent out-exchange kinetics were measured by rapid filtration on Sephadex G-25 of aliquots of this pooled material. The Sephadex columns used for the filtrations were equilibrated with 0.05 *M* glycine-HCl (pH 2 or pH 3), or with 0.02 *M* phosphate-0.3 *M* KCl (pH 6.5). The temperature of the exchange solutions was regulated to ±0.5° by a mineral oil bath in the cavity of a thermoregulated aluminum block.

Table I: Apparent Activation Energies for Exchange from STI at pH 2, pH 3, and pH 6.5.

pH	Temp Interval (°C)	E^*_{app} (kcal/mol)[d]
2[a]	20–30	24 ± 3
	30–40	30 ± 5
	40–50	36 ± 7
3[b]	10–20	20 ± 2
	20–30	25 ± 2
	30–40	28 ± 3
	40–45	35 ± 3
	45–50	45 ± 6
6.5[c]	20–30	26 ± 3
	30–40	28 ± 4
	40–50	32 ± 7

[a] Calculated from data in Figure 1. [b] Calculated from data in Figure 2. [c] Calculated from data in Figure 3. [d] Activation energies are calculated as described previously (Woodward and Rosenberg, 1971a). The error indicates the range of values for different values of H_{rem}.

The urea dependence of exchange of partially tritiated STI was determined in the following way. STI, 25 mg/ml, in 0.04 *M* phosphate buffer-0.6 *M* KCl (pH 6.5) was cooled to 0° for 1 hr. The solution was then diluted 1:1 with tritiated water, 1 mCi/ml, precooled to 0°. After 18 hr at 3°, the zero time filtration was carried out at 3°, at pH 6.5, and the eluted protein peak divided into two parts. One part was diluted 1:1 with 8 *M* urea in buffer, and the second part was diluted 1:1 with buffer only. The subsequent exchange of both solutions was then measured.

The molar extinction coefficient for STI at 280 nm, ϵ 2.05×10^{-4}, was used for calculations of the number of hydrogens remaining unexchanged per protein molecule, H_{rem}, at a given time (Woodward et al., 1975a).

Results

The temperature dependence of hydrogen exchange kinetics of tritium-labeled STI at pH 2, pH 3, and pH 6.5 is shown in Figures 1–3.

From these data the apparent activation energy (E^*_{app}) of the overall exchange process can be estimated (Table I) (Rosenberg and Chakravarti, 1968; Woodward and Rosenberg, 1971a; Woodward et al., 1975a). At low pH's, E^*_{app} increases from $\simeq 20$ to $\simeq 45$ kcal/mol in the temperature interval 30-50°. This is reflected in the decreasing curvature of the first-order plots at higher temperatures (Figures 1 and 2). At pH 6.5, $E^*_{app} \simeq 27$ kcal/mol at all temperatures. At pH 6.5, all but $\simeq 8$ protons exchange with the lower E^*_{app} (Figure 3 and Table I).

The effect of 4 *M* urea on the exchange of partially labeled STI at pH 6.5 is shown in Figure 4. STI was partially labeled by incubating the protein-tritium solution at 3°, pH 6.5, for 18 hr before the zero filtration (see Materials and Methods). In the presence of 4 *M* urea there is no detectable change in the exchange rate. STI is not denatured by 4 *M* urea under these conditions (Edelhoch and Steiner, 1963) and exchange kinetics are not complicated by urea-induced unfolding.

Similarly in RNase A, urea concentrations below those inducing unfolding do not affect low E^*_{app} exchange rates (Woodward and Rosenberg, 1971b).

Discussion

Soybean trypsin inhibitor undergoes reversible unfolding at pH 3, with midpoint around 45° (Kunitz, 1948). From an analysis of the temperature dependence of hydrogen exchange kinetics at pH $\simeq 2$-3 vs. that around neutrality, the

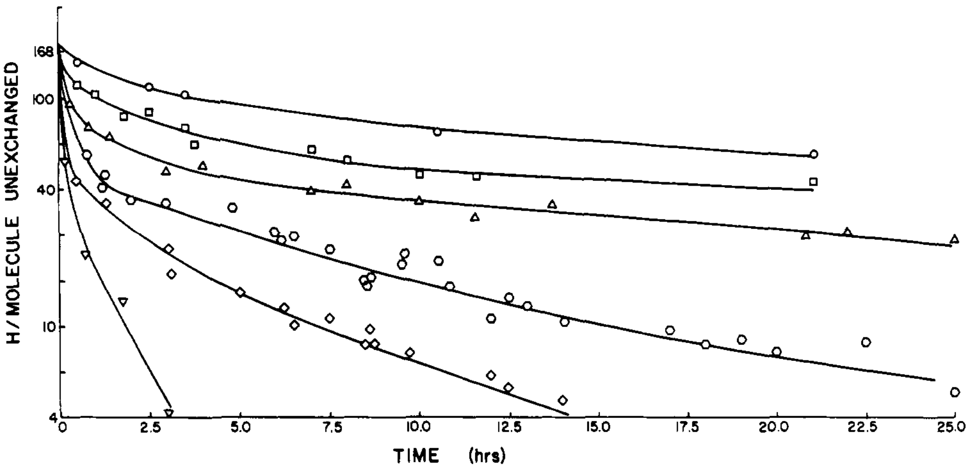

FIGURE 2: Hydrogen-tritium exchange kinetics of completely labeled STI (pH 3) at 10° (○), 20° (□), 30° (△), 40° (○), 45° (◇), and 50° (▽).

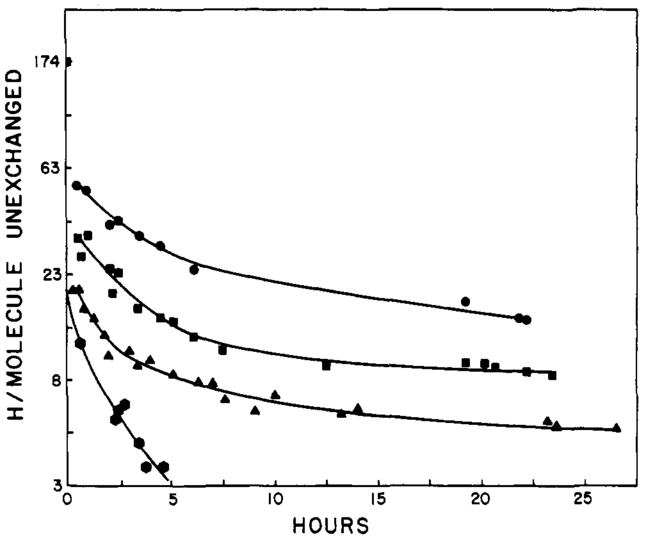

FIGURE 3: Hydrogen-tritium exchange kinetics of completely labeled STI (pH 6.5) at 20° (●), 30° (■), 40° (▲), and 50°, (●).

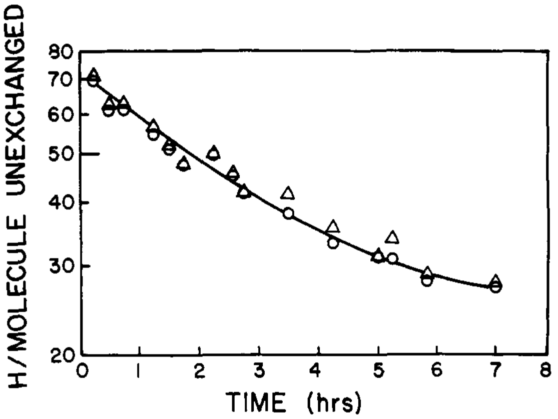

FIGURE 4: Effect of 4 *M* urea on hydrogen-tritium exchange kinetics of partially labeled STI (pH 6.5), 3° (△); without 4 *M* urea (○). The curve is hand drawn through the circles. STI was partially labeled by incubation in tritium solution at 3°, pH 6.5 for 18 hr, as described under Materials and Methods.

contribution of this unfolding transition to the overall proton exchange in native proteins can be determined (Woodward et al., 1975a; Woodward and Rosenberg, 1971a,b). We propose that each proton may exchange by one of two mechanisms, either directly from the folded, globular structure, or via a reversible, major unfolding transition, with exchange then taking place from the unfolded conformation. The observed number of hydrogens/molecule retaining label, H_{rem}, at any time t is

$$H_{\text{rem}} = \sum_{i=1}^{n} A_i e^{-(k_{\text{L},i} + k_{\text{U},i})t} \qquad (1)$$

where $k_{\text{L},i}$ is the rate constant of the exchange of the ith proton from the folded conformation and $k_{\text{U},i}$ is the rate constant of the exchange of the same proton via reversible unfolding. A_i is the number of peptide residues of type i.

The observed exchange at a given temperature and pH will depend upon the relative values of $k_{\text{L},i}$ and $k_{\text{U},i}$. The value of $k_{\text{U},i}$ is governed by k_f and k_b, the forward and back rate constants of the reversible unfolding,

$$k_{\text{U},i} = k_f k_{\text{cx},i}/(k_f + k_b + k_{\text{cx},i}) \qquad (2)$$

where $k_{\text{cx},i}$ is the chemical exchange rate constant for the proton transfer step. k_{U} has a large activation energy in the range 45–60 kcal/mol due to the large temperature dependence of k_f. k_{L} has a lower activation energy ($\simeq$20–25 kcal/mol) approximately equal to that of the chemical exchange step (Woodward et al., 1975a). Values of k_f and k_b at several temperatures, taken from Kunitz (1948), are shown in Table II.

Table II: Temperature Dependence of the Rate Constants for Unfolding, k_f, and Refolding, k_b, for STI, pH 3. Taken from Kunitz (1948).

Temp (°C)	k_f (min^{-1})	k_b (min^{-1})
10	4.7×10^{-8}	1.8×10^{-3}
20	1.8×10^{-6}	3.2×10^{-3}
30	6.0×10^{-5}	5.4×10^{-3}
40	8.3×10^{-4}	3.8×10^{-3}
45	2.5×10^{-3}	2.9×10^{-3}
50	1.0×10^{-2}	2.3×10^{-3}

Hydrogen exchange from a protein always occurs with a distribution of rates. Randomly coiled proteins exchange with a distribution of $\simeq$2 orders of magnitude (Woodward and Rosenberg, 1970; Molday et al., 1972). The range of k_{L} is 6–8 orders of magnitude. The distribution of k_{U} is $\simeq$1 order of magnitude, or less, depending on the specific values of k_f and k_b for that protein, eq 2. For STI at pH 3, the

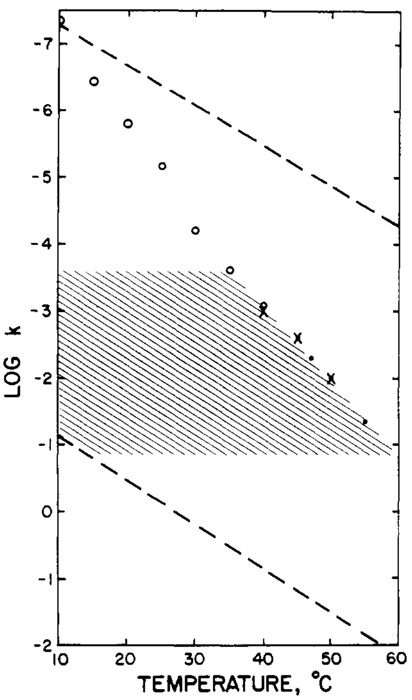

FIGURE 5: The temperature dependence of the relative values of k_U and k_L for STI (pH 3) within the experimental time limits. The dashed lines indicate the approximate upper and lower limits of the k_L distribution as a function of temperature, estimated from Figure 2. The upper and lower limits of the calculated distribution of $k_{U,i}$, eq 2, are indicated by the circles in the same figure. The slowest rates observed at each temperature, equal to the slope of the tangent to the slowest portion of the first-order plots, Figure 2, is shown by X. See text for discussion.

values of k_f and k_b taken from Kunitz (1948) (Table II) are used for calculation of k_U.

The relative values of k_L and k_U for STI, as a function of temperature at pH 3, are shown in Figure 5. The estimated upper and lower bounds of the distribution of $k_{L,i}$ are shown by the dashed lines in this figure.

The circles show the calculated upper and lower limits of the k_U distribution. Values of k_U for each peptide proton are calculated as previously described (Woodward et al., 1975a) from eq 2, using k_{cx} of the primary sequence position of that proton (Molday et al., 1972) and k_f and k_b from Kunitz (1948). The values of k_U for all protons fall within the distribution indicated by the height of the circles. The apparent break in the temperature dependence of k_U between 25 and 30° is probably artifactual since values of k_f below 30° were obtained by extrapolation of the tabulated data (Kunitz, 1948). For STI, the distribution of $k_{U,i}$ is much narrower than that for trypsin (Woodward et al., 1975a).

The approximate time limits of observation in these experiments are shown in the hatched area. The range of rates predicted by the model at any temperature is given by a vertical line through the hatched region at that temperature.

At pH 3, below 38°, all values of k_U are too small to measure. All observed exchange will be determined by k_L, although only a portion of the distribution of k_L will be observed at temperatures below 38°. Above 38°, many values of k_U fall within the observable time, and overall exchange, eq 1, will begin to have a larger and larger contribution from k_U. In this temperature region the distribution of observed rates should narrow, the apparent activation energy (E^*_{app}) should increase from $\simeq$20–25 to $\simeq$45–60 kcal/mol, and the value of the slowest rates observed should be equal to those at the lower end of the $k_{U,i}$ distribution (Woodward et al., 1975a).

All of these predictions are met with STI. The distribution of rates at pH 3 clearly decreases above 38° (Figure 2). The increase in E^*_{app} is indicated in Table I. The slowest rates measured at 40, 45, and 50°, shown by X symbols in Figure 5, are very close to the calculated values of $k_{U,i}$. Similar results have been obtained for trypsin (Woodward et al., 1975a), except that the distribution of $k_{U,i}$ is much broader for trypsin than for STI.

Our analysis of the contribution of thermal unfolding to the overall exchange kinetics circumvents some of the difficulties of dealing with broad distributions of rates, and it provides a criterion for exchange from the folded conformation, namely, low E^*_{app}. If the $E^*_{app} \simeq 25$ kcal/mol, then exchange is from the folded state.

By this criterion, the exchange experiments at pH 6.5 indicate that all but a very few protons can exchange from the protein while it is folded (Figure 3 and Table I). At pH 6.5 the protein is stable enough to allow the temperature to be raised without elevating k_U to a value approaching k_L, eq 1.

Our interpretation of the temperature dependence of STI proton exchange kinetics differs from that of Nakanishi and Tsuboi (1974). In studies of the deuterium–hydrogen exchange of STI, they report that at pH 2.7 and pH 6.5, the slowly exchanging protons show kinetics characterized by a single first-order decay constant, and that for temperatures between 25 and 55° the lines extrapolated from the linear portion of the first-order plots all intersect at a common value of H_{rem}. They assume that this value of H_{rem} is equivalent to the number of protons involved in intramolecular hydrogen bonding.[2] At pH 2.7, they resolved the observed kinetics into two kinetic classes by a graphical subtraction method. At pH 6.5, the kinetics were resolved into three kinetic classes by the same procedure. Attempts were made to assign the classes to structural segments of the protein (Nakanishi and Tsuboi, 1974).

We find no indication of a common intersection of tangents to the slow portions of first-order exchange plots for STI (Figures 1–4). A possible origin of this discrepancy is that Nakanishi and Tsuboi (1974) did not measure exchange beyond 6–8 hr.

The same authors report similar findings for lysozyme (Nakanishi et al., 1972, 1973). In careful hydrogen–tritium exchange studies with lysozyme, Wickett et al. (1974) did not observe simple first-order kinetics under any conditions.

Furthermore, the graphical method of successive subtraction for the resolution of a linear combination of exponentials is not applicable to complex kinetics such as hydrogen exchange kinetics, especially given the imprecision of the data. Laiken and Printz (1970) have shown that serious interpretative errors can result from an uncritical use of this method in hydrogen exchange kinetics.

Nakanishi and Tsuboi (1974) concluded that the slow ex-

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[2] In the determination of the actual number of protons assumed to be involved in intramolecular hydrogen bonding, the number of protons corresponding to a given value of the infrared spectral amide I/amide II band ratio is obtained by extrapolating to zero time the linear region of the first-order exchange plot at 4° (Nakanishi and Tsuboi, 1974). This procedure is based on the assumption that at 4° all protons exchange with a first-order rate constant. However, at 4° there is a broad distribution of exchange rates for heteropolymers, resulting in curved first-order plots. The curvature is most evident at the faster times, for which no data at 4° are shown (Nakanishi and Tsuboi, 1974).

change in native STI is governed only by the major thermal transition, i.e., that the hydrogen-bonded peptide groups are unexchangable in the folded, native conformation and exchangable in the thermally unfolded conformation.

In contrast, we include the unfolding–refolding rates in the calculation of k_U, eq 2, to estimate the involvement of unfolding in the overall kinetics, eq 1. Our analysis indicates that exchange from the folded conformation occurs with E^*_{app} 3–8 kcal/mol larger than that of the chemical exchange step. Under certain conditions, e.g., at pH around neutrality, exchange of almost all protons occurs without contribution from the thermal unfolding (Figure 3). That is, the process(es) responsible for slow exchange in native proteins is not equivalent to a major unfolding transition, exchange does occur from the folded conformation, and under certain conditions almost all protons can exchange when the protein is folded, but with a broad distribution of exchange rates.

We suggest that the distribution of exchange rates with low E^*_{app} reflects a similar distribution of solvent accessibilities, only a certain portion of which are measured at a given temperature. In folded proteins, the exchange rate of a proton depends upon its access to hydrogen bonding with water and upon the chemical exchange rate constant for that proton. Since the latter has a high temperature coefficient, raising the temperature will shift the entire distribution of rates. Because the experimental time interval remains the same, the exchange of different protons is measured at each temperature even though the solvent accessibility in the molecule remains constant. The result is a series of nearly parallel curves, leveling off to an immeasurably slow rate at a lower value of H_{rem} with each increment of temperature, as in Figure 3. However, this value of H_{rem} does not correspond to a kinetic "class" attributable to a structural element of the molecule. For further discussion of the characteristic step-like first-order exchange plots obtained by changes in temperature, pH, and/or ligand concentration, see Woodward et al. (1975a).

In view of our finding that in folded STI most of the labile protons can exchange with solvent, it is interesting to note the $H_2{}^{18}O$ exchange studies with chymotrypsinogen by Weber et al. (1974). Although ten immobilized water molecules per chymotrypsinogen molecule are indicated by X-ray crystallographic studies, no nonexchangable waters are observed for chymotrypsin in solution (Weber et al., 1974).

We propose that the broad distribution of exchange rates is a manifestation of a continuum of fractional solvent accessibilities throughout the molecule, and that this constitutes the dynamic solvent accessibility of folded proteins in solution.

The static accessibilities of several crystalline proteins have been computed from their atomic coordinates, obtained from X-ray crystallography, using a rigid sphere atomic model (Lee and Richards, 1971; Shrake and Rupley, 1973). In this approach, accessible surface area is determined by computer modeling with a sphere the size of a water molecule tested for van der Waal's contact with one atom without overlap of other atoms. By this criterion, atoms are accessible or not accessible. Partial accessibility due to movement or flexibility of the protein cannot be evaluated. The density of packing of side chains is not uniform (Lee and Richards, 1971; Shrake and Rupley, 1973) and in some proteins internal cavities are indicated (Lee and Richards, 1971). The potential contribution of nonuniform packing to protein flexibility cannot be determined at present.

In the treatment of Lee and Richards (1971) the relative accessibilities of main chain nitrogen are 0.14–0.23, based upon fully extended chains as unity. This value corresponds roughly to the percentage of protons that exchange in native proteins with rates comparable to those for random coil form of the protein.

In native proteins, the protons that exchange with rates slower than random coil rates may correspond to protons that are inaccessible in the static analysis but which actually have a distribution of fractional accessibilities in the molecule in solution. That is, we propose that the "slowly" exchanging protons, determined by the intersection at the ordinate by a tangent to the slow region of the first-order exchange plots, are not equivalent to intramolecular hydrogen bonded protons. The relationship of solvent accessibility to intramolecular hydrogen bonding in exchange processes of folded proteins is considered in detail elsewhere (Woodward et al., 1975a).

A crude estimate of the dynamic solvent accessibility can be made from $k_{L,i}$, the exchange rate of a proton from the folded conformation,

$$k_{L,i} = [OH^-]_{(i)}k_{OH,i} + [H^+]_{(i)}k_{H,i} + [H_2O]_{(i)}k_{H_2O,i} \tag{3}$$

where $[OH^-]_{(i)}$, $[H^+]_{(i)}$, and $[H_2O]_{(i)}$ are the local concentrations of hydroxyl ion, hydrogen ion, and water in the region of the ith proton, and $k_{OH,i}$, $k_{H,i}$, and $k_{H_2O,i}$ are the base catalyzed, acid catalyzed, and water exchange constants of the ith proton. The exchange rate for the same proton in the random coil form of the protein, $k_{cx,i}$, is

$$k_{cx,i} = [OH^-]_{(o)}k_{OH,i} + [H^+]_{(o)}k_{H,i} + [H_2O]_{(o)}k_{H_2O,i} \tag{4}$$

where the subscript (o) denotes the concentrations in bulk solvent.

If we assume[3] that the relative concentrations of OH^-, H^+, and H_2O are the same in the vicinity of exchanging protons in the folded conformation and in the extended conformation, then

$$\frac{k_{L,i}}{k_{cx,i}} = \frac{[OH^-]_{(i)}}{[OH^-]_{(o)}} = \frac{[H^+]_{(i)}}{[H^+]_{(o)}} = \frac{[H_2O]_{(i)}}{[H_2O]_{(o)}} \tag{5}$$

As discussed above, individual values of $k_{L,i}$ cannot be extracted from the observed distribution of rates. However, the average rate constant, $\langle k_L \rangle_{H_{\text{rem}}}$, of the protons remaining unexchanged at each H_{rem} can be obtained from the first time derivative at that value of H_{rem}. Likewise, $\langle k_{cx} \rangle_{H_{\text{rem}}}$ can be obtained from the distribution of rate constants constructed from the data of Molday et al. (1972). Then at a given value of H_{rem}

$$[H_2O]_{(i)} = 55.5\frac{k_{L,i}}{k_{cx,i}} \approx 55.5\frac{\langle k_L \rangle_{H_{\text{rem}}}}{\langle k_{cx} \rangle_{H_{\text{rem}}}} \tag{6}$$

and the local water concentration, $[H_2O]_{(i)}$, can be obtained as a function of H_{rem}. A small difficulty arises because exchange from the random coil is not in the same rank order[4] as that from the folded protein (Woodward and

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[3] While this assumption raises questions concerning the penetration of ions into the protein matrix, a more complicated assumption would be arbitrary in view of the evidence presently available.

[4] Rank order refers to the relative values of the first-order rate constants for each proton within the distribution.

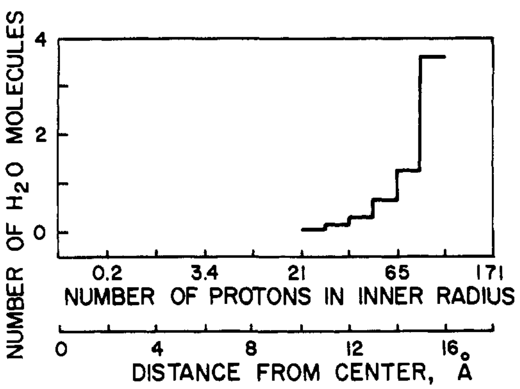

FIGURE 6: The average number of water molecules per 1-Å shell, estimated from the hydrogen exchange kinetics of STI, pH 3, 30°. Abcissa, lower scale: the radial distance from the center of a 18-Å sphere. Abcissa, upper scale: The number of peptide amide protons contained in the sphere with that radius, assuming uniform distribution of protons throughout the sphere. The average number of water molecules per molecule of protein was calculated from the exchange kinetics as described in the text.

Rosenberg, 1971b). However, the calculated value of $[H_2O]_{(i)}$ is not greatly influenced by this uncertainty because of the large differences between the value and distribution of k_{cx} and k_L.

If it is assumed that all exchangable protons are uniformly distributed through a spherical protein domain, and that exchange rate is determined by solvent accessibility, then $[H_2O]_{(i)}$ as a function of the distance from the center of the sphere can be calculated. From X-ray analysis, STI has a radius of about 18 Å (Sweet et al., 1974). If that sphere is divided into concentric shells, each 1 Å deep, then a number of exchangeable protons in each shell volume can be calculated. This is indicated on the abscissa of Figure 6, showing two scales, one with distance from the center of the sphere, and the other with the number of exchangeable protons within that radius. The ordinate in Figure 6 is the average number of water molecules in each 1-Å shell, calculated as $([H_2O]_{(i)}N/1000) \times 10^{24}\ \text{Å}^3/\text{ml} \times (4\pi/3)(R_i^3 - R_{i-1}^3)$ where N is Avogadro's number and R_i and R_{i-1} are the outer and inner radii of the shell.

The numbers of Figure 6 were obtained for STI at pH 3, 30°. Under these conditions, the observed exchange kinetics have no contribution from protein unfolding. Also, at pH 3, the values of k_{cx}, calculated from the rules of Molday et al. (1972), are the most accurate.

Although quantitative conclusions cannot be drawn from this treatment, the results in Figure 6 suggest that the average number of water molecules accessible to exchanging protons in the folded protein drops rapidly within the outer 5-6 Å.

About 36 protons exchange with rates equal to those in the random coil (Figure 2). In this treatment, these would be located in the outermost 1.5-Å shell, containing $\simeq$0.2 of the total volume. If solvent accessibility of these protons is equal to that in bulk solvent, about 190 water molecules are in the volume of the outer 1.5-Å shell. This is interesting in view of the discrepancies between the volume (Richards, 1974) and densities (Kauzmann et al., 1974) of proteins in solution and those of crystalline proteins obtained from the atomic coordinates. These discrepancies suggest that there may be more water associated with the protein than is indicated by the X-ray coordinates, but this point remains unclear (Kauzmann et al., 1974).

In the 15-16-Å shell about 3.5 water molecules are indicated, while for the entire inner 15 Å, enclosing $\simeq$80 of the most slowly exchanging protons, $\simeq$1.5 water molecules is indicated (Figure 6). The inner radius of $\simeq$8 Å contains the nine most slowly exchanging protons.

The values in Figure 6 do not indicate the actual number of water molecules inside the protein. They do show that, given the validity of the simplifying assumptions, very few water molecules in the outer region of a globular protein are sufficient to account for proton exchange rates in terms of solvent accessibility.

Refinements of this treatment are being undertaken. At present the results in Figure 6 are consistent with a very hydrophobic inner region of the folded protein conformation, but they also imply more accessibility to solvent in the outer regions of the molecule than indicated by static accessibility calculations (Lee and Richards, 1971; Shrake and Rupley, 1973, Richards, 1974).

The conception of hydrogen exchange kinetics as a measure of gross solvent accessibility does not necessitate a model of water diffusion or penetration into a rigid, inflexible protein matrix. Protein movements or fluctuations are not precluded as long as they are low energy and unaffected by 4 *M* urea (Figure 4) and 20% ethanol and other organic cosolvents (Woodward et al., 1975a,b). The fact that denaturants have no effect of the low E^*_{app} argues against an exchange mechanism involving localized, reversible unfolding in the native conformation (Englander and Rolfe, 1973). Exchange by such a mechanism would be accelerated by urea and other denaturants (Woodward et al., 1975b).

Figure 6 suggests that the protein flexibility required to account for dynamic solvent accessibility is localized in the surface regions of the folded conformation. This is consistent with the findings of Tulinsky and coworkers (Tulinsky et al., 1973; Vandlen and Tulinsky, 1973; Mavridis et al., 1974). In high resolution studies of the α-chymotrypsin dimer, they have shown that the structure of the two monomer proteins are not equivalent. Difference electron density maps show that all of the variability between the structures of the proteins in the dimer is located in the outer regions of the globular structure, while the inner regions are constant. The structure of chymotrypsin approximates a sphere of $\simeq$28 Å, the outer 6-7 Å of which contains the variability.

While most X-ray crystal structures solved at $\geq$2.8 Å do not indicate solvent water in the protein matrix, surface variability in the solution structure, of similar magnitude (1-2 Å) to that detected in the α-chymotrypsin dimer studies (Vandlen and Tulinsky, 1973), might account for the dynamic solvent accessibility indicated by our hydrogen exchange results.

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Hydrogen Exchange Kinetics Changes upon Formation of the Soybean Trypsin Inhibitor–Trypsin Complex[†]

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ABSTRACT: The hydrogen exchange kinetics of the complex of trypsin–soybean trypsin inhibitor (Kunitz) have been compared to the calculated sum of the exchange kinetics for the inhibitor and trypsin measured separately. The exchange rates observed for the complex are substantially less than the sum of the exchange rates in the two individual proteins. These results cannot be accounted for by changes in intermolecular or intramolecular hydrogen bonding. The decrease in exchange rates in the complex are ascribed to changes in solvent accessibility in the component proteins.

We have proposed that hydrogen exchange kinetics in native proteins reflects the gross solvent accessibility of the folded conformation (Woodward et al., 1975; Ellis et al., 1975). It follows that larger molecules should have diminished exchange rates compared to smaller ones with the same relative amount of internal structure. Comparison of the peptide NH hydrogen exchange kinetics of soybean trypsin inhibitor (STI)[1] and trypsin to the STI–trypsin complex is an ideal experimental model for a test of this prediction.

The trypsin–STI complex is a highly stable 1:1 molar complex with an association constant of $\simeq 10^9$ at pH 6.5 (Laskowski and Sealock, 1971). Each protein contributes about half of the mass to the complex. The molecular weights of trypsin and STI are respectively 23,900 and 21,500 (Keil, 1971; Frattali and Steiner, 1968). There is no substantial change in the internal structure of STI and trypsin induced by complex formation. Upon association, there may be a very small conformational change in trypsin, but no change whatever in STI is detectable with standard physical techniques (Laskowski and Sealock, 1971). The X-ray crystal structure of the STI–trypsin (porcine) complex indicates that the intermolecular contact region is small, with <10 possible hydrogen bonds involving peptide NH (Sweet et al., 1974).

In this paper, the exchange kinetics of the STI–trypsin (bovine) complex are compared to those of STI and trypsin measured separately. The observed exchange of the complex is much slower than that indicated by the sum of the exchange kinetics of the two component proteins, measured in the undissociated state. We propose that the large attenuation of exchange rates is primarily due to a reduction of solvent accessibility in the two proteins within the complex.

Materials and Methods

STI (SI) and trypsin (TRL) were purchased from Worthington Co. and used without further purification.

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[1] Abbreviation used is: STI, Soybean trypsin inhibitor.