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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.

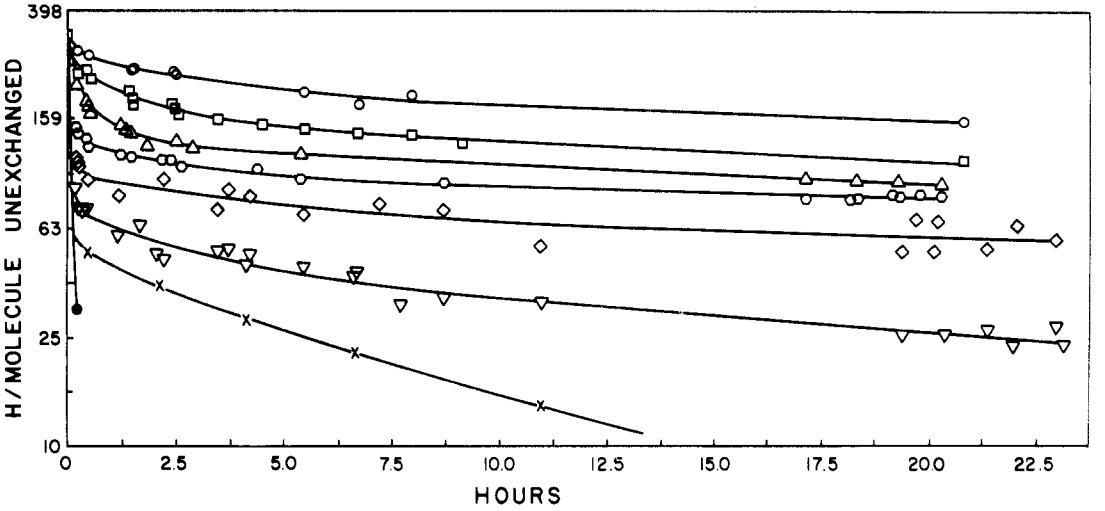

FIGURE 1: Out-exchange kinetics of STI-trypsin complex, pH 6.5, at 10°, (○), 20° (□), 30° (△), 40° (○), 50° (◇), 60° (▽), 65° (x), and 70° (●).

Table I: Apparent Activation Energies for Exchange from Trypsin–STI Complex, pH 6.5, Figure 1.

Temp Interval (°C)	E^*_{app} (kcal/mol)[a]
10–20	27 ± 3
20–30	25 ± 3
30–40	29 ± 3
40–50	45 ± 4
50–60	58 ± 6
60–65	61 ± 5

[a] Activation energies are estimated as described (Woodward and Rosenberg, 1971). The error indicates the range of values for different values of H_{rem}.

Benzamidine hydrochloride was purchased from Eastman Co.

Hydrogen–tritium exchange experiments were carried out as previously described (Woodward et al., 1975; Ellis et al., 1974). Most work was carried out in 0.05 *M* sodium phosphate–0.3 *M* KCl (pH 6.5) called phosphate–KCl buffer. The tritiation of the trypsin–STI complex was accomplished by first tritiating equimolar amounts of trypsin and STI alone, then combining them just before the zero time filtration. Trypsin, 20 mg, was dissolved in 2 ml of tritiated water, 0.5 mCi/ml, at pH 2.0. The solution was heated for 3 hr at 45°, then cooled to 0° for 30 min (Woodward et al., 1975). STI, 24 mg, was dissolved in 2 ml of phosphate-KCl buffer (pH 6.5) made with tritiated water, 0.5 mCi/ml. Then the solution was heated for 5 min at 60° and cooled to 0° for 30 min. The cooled, tritiated trypsin solution was mixed with the cooled, tritiated STI solution. After 2–3 min, the zero time filtration (3°) was begun on a column equilibrated with phosphate–KCl buffer (pH 6.5). Subsequent out-exchange was measured as described before (Woodward et al., 1975). In the calculation of the number of hydrogens remaining unexchanged, H_{rem}, the molar extinction coefficient at 280 nm, ϵ 5.9 × 10^4, for the STI-trypsin complex was used.

For the exchange of trypsin in excess benzamidine, trypsin, 10 mg/ml, in tritiated water, 0.5 mCi/ml, was heated at 45°, pH 2.0, for 3 hr, then cooled to 0°. This solution was applied to the zero time filtration column (3°) equilibrated with 0.05 *M* benzamidine hydrochloride in 0.1 *M* acetate buffer (pH 6.5). The eluted protein peak was quickly transferred to a temperature regulated bath for the subsequent timed filtrations, which were done on columns equilibrated with 0.05 *M* glycine-HCl (pH 2). During the second filtrations at pH 2, benzamidine eluted after the trypsin, preventing interference with the uv absorbance readings. pH 2 was chosen to eliminate trypsin autolysis after separation from benzamidine.

Results

The temperature dependence of the proton exchange of tritium-labeled trypsin–STI complex is shown in Figure 1. The apparent activation energy of exchange, E^*_{app}, estimated as before (Woodward and Rosenberg, 1971; Woodward et al., 1975) is given in Table I. E^*_{app} remains around 27 kcal/mol until the temperature is raised above 40°, where the dissociation of the complex and/or the unfolding of the component proteins enters the overall exchange process.

The exchange kinetics of STI at pH 6.5 have been reported (Ellis et al., 1975). To compare the observed exchange kinetics of the STI–trypsin complex to the sum of the kinetics of STI and trypsin, the exchange of trypsin at pH 6.5 must also be determined. Since, at this pH, trypsin undergoes autolysis, its exchange was measured in the presence of a 100-fold molar excess of benzamidine-HCl, a potent inhibitor of trypsin (Mares-Guia and Shaw, 1965). Figure 2 shows the exchange kinetics of the trypsin–benzamidine complex, pH 6.5, at 30 and 40°. With these data and the analogous STI data (Ellis et al., 1975) the kinetics predicted for a 1:1 complex, assuming simple additive kinetics, is easily calculated. These are shown in Figure 3, along with the observed exchange kinetics for the complex at the same temperature, taken from Figure 1. There is a substantial difference between the simple sum of the kinetics of STI and trypsin measured separately and those actually observed for the complex (Figure 3).

This difference is due to the hydrogen exchange process, provided there are no artifacts in the separate measurements of the trypsin and STI exchange kinetics. The most obvious source of artifacts is trypsin autolysis at pH 6.5. If limited autolysis in 100-fold molar excess of benzamidine were to occur, the apparent exchange kinetics would be faster, due to the more rapid exchange from the protein

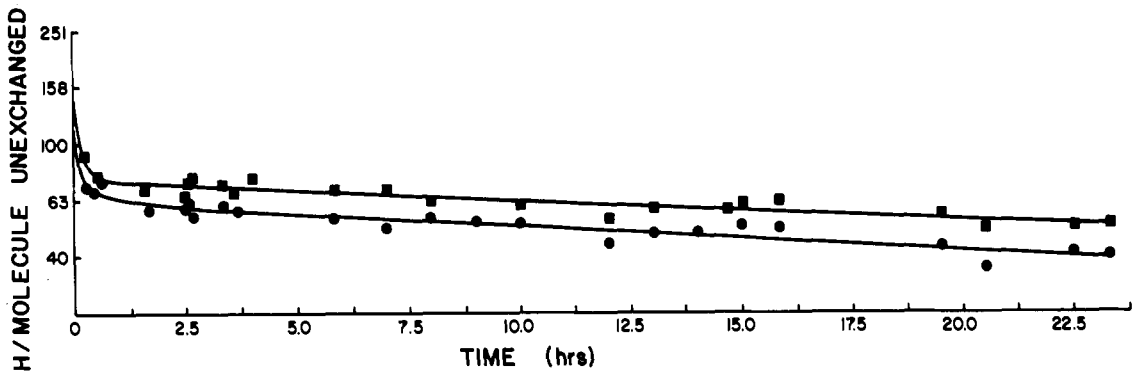

FIGURE 2: Out-exchange kinetics of trypsin in the presence of 100 molar excess benzamidine hydrochloride (pH 6.5) at 30° (■), and 40° (●).

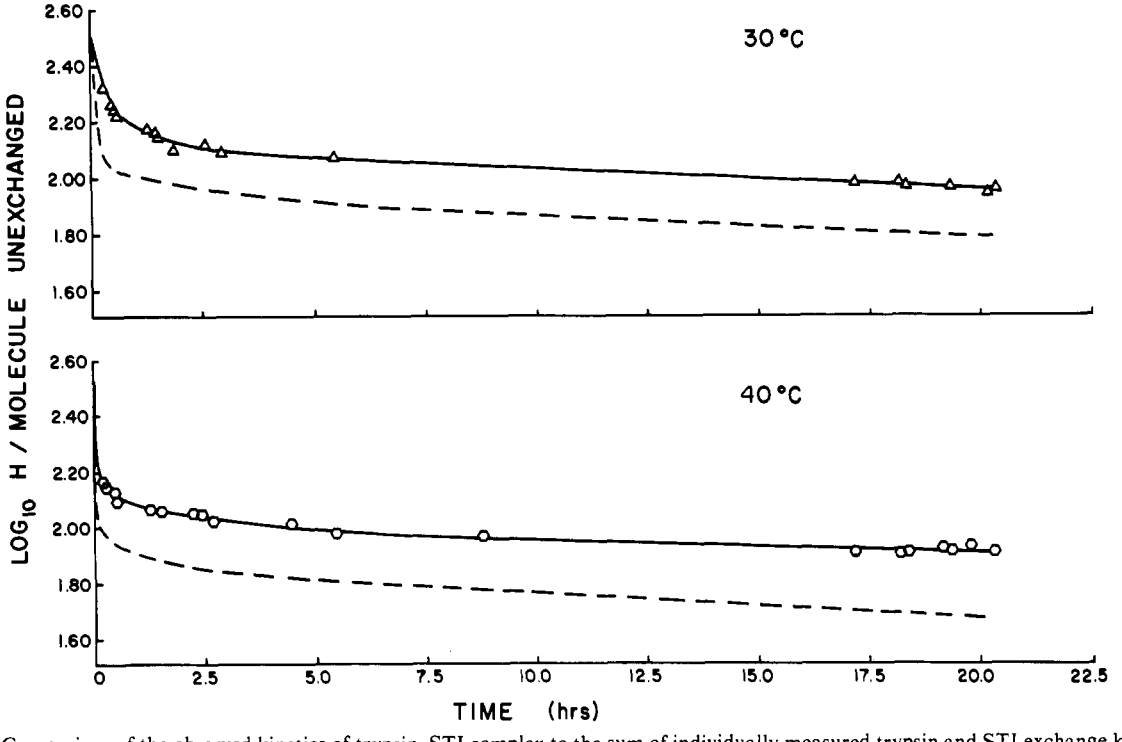

FIGURE 3: Comparison of the observed kinetics of trypsin–STI complex to the sum of individually measured trypsin and STI exchange kinetics, pH 6.5. The dashed line is the sum of the exchange curve of trypsin, Figure 2, plus STI (Woodward et al., 1975). The solid curves and data points are from Figure 1.

fragments. To test for this we have assayed for proteolytic activity of the trypsin benzamidine solutions with benzoylarginine *p*-nitroanalide assay (Erlanger et al., 1961). Less than 1% of the trypsin activity was expressed during the course of our experiments with trypsin and benzamidine at 30 and 40° (Figure 2).

In addition, other considerations lend confidence to the exchange kinetics shown in Figure 2. For trypsin at pH 2, first-order plots of the low E^*_{app} exchange kinetics level off around 45 H_{rem} at 20° (Woodward et al., 1975). At pH 2, 40°, exchange is very rapid due to thermal unfolding, and therefore cannot be used for comparison. Although it is difficult to extrapolate from pH 2, 20°, to pH 6.5, 40°, one does not expect the exchange at pH 6.5, 40°, to be slower than that at pH 2, 20°, since the proton transfer step is base catalyzed above pH 3 and has a high positive temperature coefficient. Also, Lenz and Bryan (1969), using hydrogen–deuterium exchange, have reported that at pH $\simeq$6–7, trypsin exchange curves level off at $\simeq$40 H_{rem} at 23°. At 40°, that value of H_{rem} should be even lower.

Although trypsin does not autolyze at pH 2, comparisons at that pH could not be made since the stability of the STI–trypsin complex is pH dependent, reaching a maximum stability at pH $\simeq$6.5. The complex is dissociated at pH 2 (Laskowski and Sealock, 1971).

Discussion

There is a large difference between the observed hydrogen exchange kinetics of the STI–trypsin (bovine) complex and the calculated sum of the kinetics of STI and trypsin measured individually (Figure 3). A priori, the two most probable factors contributing to this effect are an increase in the number of inter- or intramolecular hydrogen bonds, or a reduction in solvent accessibility, in the complex vs. in the separate proteins. Consideration of other physical measurements of the complex indicate that hydrogen bonding is not the primary factor.

A substantial increase in the number of intramolecular hydrogen bonds should be reflected in changes in the secondary structure of STI and trypsin upon complex formation.

The only evidence of a small conformational change in trypsin with binding of STI is equivocal, and there is no demonstration of any change in STI (Laskowski and Sealock, 1971).

Intermolecular hydrogen bonding of protons at the dimer contact region, which then become immeasurably slowly exchanging, cannot, alone, explain the results in Figure 3, since that would require placing $\simeq$35 protons in that category. The X-ray crystal structure of the STI–trypsin (porcine) complex indicates that only a very small part of the component proteins are involved in the association region (Sweet et al., 1974). In the complex, there are $\simeq$18 possible intermolecular hydrogen bonds, 7 of which involve peptide amide NH (Sweet et al., 1974).

In a recent study of hydrogen–tritium exchange of synthetic polypeptides, Welch and Fasman (1974) argue against an emphasis on solvent accessibility in the mechanism of slow exchange in native proteins. These authors point out that if solvent accessibility were important, then it would be expected that proteins of high molecular weight (or greater molecular diameter) ought to exchange, in general, more slowly than those of lower molecular weight or with a smaller radius, assuming the same general amount of internal structure (Welch and Fasman, 1974). As an indication that this does not occur, they refer to a report of Willumson (1971) that there is no correlation with the mass, and therefore presumably with the diameter, of globular proteins and their slowly exchanging protons.

Willumson also found no correlation between the helical content (assumed to be some measure of the intramolecular hydrogen bonding) and the slowly exchanging protons. A more important consideration, however, is that Willumson (1971) employs poly-D,L-alanine as the random coil reference in the plotting parameters as well as in the calculation of the free energy of "transconformational" reactions, the basis for his comparison of the various sized proteins. It has become apparent that poly-D,L-alanine is not an appropriate standard for the exchange rate of the random conformation of proteins (Woodward and Rosenberg, 1970; Molday et al., 1972; Woodword et al., 1975).

On the other hand, the STI–trypsin complex is an excellent model for a test of the assumption that, if solvent accessibility is an important contribution to the exchange rate of labile protons, then proteins of smaller molecular diameter will exchange more rapidly than those with larger diameter, but with about the same degree of internal structure.

We propose that the attenuation of the hydrogen exchange kinetics upon association of the trypsin–STI complex is due primarily to shifts in the distribution of exchange rates resulting from a change in solvent accessibility in STI and trypsin. A smaller contribution from intermolecular hydrogen bonding cannot be ruled out.

Welch and Fasman (1974) attribute the slow exchange of poly(Glu) to hydrogen bonding. At pH 4.1, poly(Glu) exchanges with a distribution of rates from $\simeq 10^{-3}$ to $\simeq 7 \times 10^{-6}$ min^{-1} at 25° in 0.01 *M* NACl (Welch and Fasman, 1974). From the tangent drawn to the slow portion of the curved first-order plot, the number of "slowly" exchanging protons was determined by the intersection of this line on the ordinate. The number thus obtained is $\simeq$60% of the exchangable protons in the polymer. Sedimentation velocity studies at the same ionic strength, pH, and temperature indicate that $\simeq$60% of the material is in an aggregated form. It is assumed that $\simeq$75% of the protons are accessible to bulk solvent in the aggregate. Solvent accessibility is eliminated as a factor in the slow exchange process because the number of proteins in the slow kinetic class corresponds to the amount of material in the aggregated form.[2] Slow exchange is attributed to the stabilization of the closed peptide hydrogen bond due to thermodynamic factors involved in the aggregation process (Welch and Fasman, 1974). In the same study, an analysis of the contribution of β structure to the slow exchange of a random copolymer of L-glutamic acid and L-valine is also made, based upon the number of slowly exchanging protons obtained by the same type of extrapolation (Welch and Fasman, 1974).

However, determination of the number of protons having slow exchange rates, from graphical resolution of linear exponentials by successive subtraction, is a method suitable only for very high precision data of no more than two exponential terms (Lanczos, 1956). The pitfalls of this approach to complex kinetics, such as those of protein hydrogen exchange, have been well stated by Laiken and Printz (1970). The resolution of two exponential terms by graphical subtraction does not prima-facie indicate that there are only two kinetic classes, each corresponding to a segment of polypeptide structure.

On the average, protons located on or near the "surface" of the folded conformation are expected to have greater solvent accessibility than those located deep in the interior of the structure. But solvent accessibility is not determined only by considerations of the surfact topology of a macromolecule delineating an outer, accessible, and an inner, inaccessible, region of the molecule. Local steric arrangement of neighboring atoms determine the area within a protein available for van der Waals contact with a water molecule (Lee and Richards, 1971; Chothia, 1974; Schrake and Rupley, 1973). Participation in a hydrogen bond is one, but not the only, steric arrangement that can restrict the solvent accessibility of an exchangeable proton in a protein. Instances of attenuated exchange rates have been reported for nonhydrogen bonded amide protons in fairly extended conformations, compared to folded proteins (Brewster and Bovey, 1971; Scarpa et al., 1967). For other considerations of the relationship of hydrogen bonding to solvent accessibility see Woodward et al. (1975), and Ellis et al. (1975).

In a sense, the formation of the STI–trypsin complex is equivalent to the binding of a large ligand to each of the two proteins. The resulting shift in the distribution of exchange rates is much larger than that observed for small ligands. The addition of small active site ligands usually causes a shift in the curved first-order exchange plots of native proteins. Most often the change is to slower rates, (e.g., Wickett et al., 1974; Schechter et al., 1968), but faster rates have also been observed (e.g., Stryker and Parker, 1970). Such a shift is often attributed to the burying or exposure of a small number of protons accompanying ligand binding. However, this type of kinetic behavior can also result from a shift in the entire distribution of rates, only a portion of which are observed at a single set of conditions (Woodward et al., 1975; Ellis et al., 1975). In studies of hemoglobin, Benson et al. (1973) proposed that the shifts in exchange kinetics upon the addition of ligand could be due to the shift of the entire spectrum rather than to the exposure of a few protons. Recently, in high precision studies of lysozyme, Wickett et al. (1974) have shown that the entire distribu-

[2] No correlation was found between the number of slowly exchanging protons and the percentage of aggregated material in 0.1 *M* NaCl at the same pH and temperature (Welch and Fasman, 1974).

tion of exchange rates, and their activation energies, is shifted by the addition of a small active site inhibitor. Since the trypsin–benzamidine complex was used for calculation of the sum of the undissociated proteins, any changes in trypsin in the trypsin–STI complex must be over and above those due to small ligand binding.

Studies are presently being undertaken to determine whether the protons having slower rates in the complex are limited to the region in and around the contact area, or are more widely spread throughout the STI and trypsin molecules.

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