Registry No. Cu, 7440-50-8; L-ascorbic acid, 50-81-7; laccase, 80498-15-3.

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Effect of Trypsin Binding on the Hydrogen Exchange Kinetics of Bovine Pancreatic Trypsin Inhibitor β-Sheet NH's†

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ABSTRACT: The hydrogen–deuterium exchange rates of the slowest exchanging β-sheet NH's in the bovine pancreatic trypsin inhibitor (BPTI) have been determined in free BPTI and in the trypsin–BPTI complex at pH 9–10, 25–40 °C. Rate constants for individual protons have been measured from their assigned resonances in the ^{1}H NMR spectrum. Trypsin binding has a highly localized effect; the Tyr-35 NH exchange rate is slowed by a factor of $>10^3$ in the complex, while the other NH's measured are slowed by a factor of 3–15. In free BPTI, under conditions where the exchange rate constants have activation energies in the range 11–32 kcal/mol, the NH's of Tyr-21, Phe-22, and Tyr-23 are several orders of magnitude slower than the other β-sheet NH's.

The exchange of isotope between water hydrogens and labile protein hydrogens is a measure of the accessibility of solvent to the protein's internal sites. The largest group of exchangeable protons in proteins are peptide NH's. Exchange rate constants of NH's in extended polypeptides at pH 3 and 25 °C are around 10^{-1} min^{-1}, while exchange rate constants of NH's in folded proteins under the same conditions vary from about 10^{-1} to 10^{-10} min^{-1}. Although labile protons in folded proteins are clearly shielded from solvent, the fact that buried peptide NH's exchange with finite rates indicates that proteins undergo internal fluctuations that expose buried regions of the protein to solvent (Hvidt & Nielsen, 1966; Englander et al., 1972; Woodward & Hilton, 1979; Woodward et al., 1982; Barksdale & Rosenberg, 1982).

Traditionally, measurements of protein hydrogen exchange kinetics average over all exchangeable protons (Englander & Englander, 1978). More recently, assignments of average NH exchange rates to segments of the protein sequence have been made by rapid proteolysis methods (Rosa & Richards, 1981; Englander et al., 1980). Now, with the development of procedures for assigning NMR NH resonances (Dubs et al., 1979; Delpierre et al., 1982; Kuwajima & Baldwin, 1983), it is

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possible to determine individual rate constants for specific peptide protons.

The bovine pancreatic trypsin inhibitor (BPTI) contains a group of peptide NH's with extraordinarily slow hydrogen exchange rates. Their half-times at room temperature and neutral pH exceed months. These NH's, sometimes referred to as the β-core protons, are hydrogen bonded in the three strands of β-sheet of the small, globular protein. The chemical shifts of their ^1H NMR resonances are well downshifted from the main envelope of overlapping NH resonances around 7–8.5 ppm, a circumstance that facilitates the determination of their individual exchange rate constants.

Among the single NH's for which the exchange kinetics have been determined, the β-core protons of bovine pancreatic trypsin inhibitor (BPTI)[1] are the most extensively characterized (Woodward & Hilton, 1980; Wuthrich et al., 1980; Hilton et al., 1981; Wagner & Wuthrich, 1982). In this paper we report the effect of the binding of a large ligand, trypsin, on the hydrogen exchange kinetics of the BPTI β-core protons. In the course of these studies we have obtained further characterization of the exchange of these protons in uncomplexed BPTI.

Experimental Procedures

Hydrogen isotope exchange of NH's in free BPTI was measured by the decay of assigned resonances in the ^1H NMR spectrum of 4% BPTI (Novo Industries, Denmark) in ^2H_2O and 0.3 M KCl. Fourier transform spectra were recorded on a Nicolet 300-MHz spectrometer. Sequential spectra (900–18 000 transients) were recorded automatically over set time intervals while the sample remained in the probe. The intensity of the solvent ^1H band was reduced by presaturation. Exchange rate constants were obtained from nonlinear least-squares fits of peak height vs. time to a first-order rate equation.

For the measurement of the relatively fast exchanging Tyr-35 NH in free BPTI, a procedure was developed by which Tyr-35 is ^1H labeled while the other β-sheet NH's, including the overlapping resonance of Phe-33, are deuterated. First, all exchangeable protons in BPTI were exchanged with ^2H by unfolding the protein at pH 5 at 90 °C for 10 min. Then, the more rapidly exchanging protons, including Tyr-35, were back-exchanged with ^1H by rapid transfer of the protein into ^1H_2O by chromatography on Sephadex G-15 (4 × 2.5 cm column), followed by lyophilization. For the exchange measurement, the lyophilized sample was dissolved in ^2H_2O.

The hydrogen exchange of BPTI protons in the trypsin–BPTI complex cannot be measured during exchange, as described above for free BPTI, due to the extensive line broadening in spectra of the 28 000 molecular weight complex. Instead, a procedure was developed in which the peak height measurement is taken after the complex is dissociated, and the exchange reaction quenched, by jumping the pH to 2, as follows. The exchangeable protons in 10 mL of 2% trypsin (Worthington, 3× crystallized) were deuterated by reversible unfolding at pH 2, 45 °C, and 18 h in deuterium (99.8%) in the presence of equimolar benzamidine. (Elimination of benzamidine at this step gave indistinguishable results.) After the trypsin solution was cooled to room temperature, its activity was titrated against a freshly made 8% BPTI solution in deuterium to determine equimolar volumes of the trypsin and BPTI solutions. For this, trypsin activity was monitored by the hydrolysis of N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (Erlanger et al., 1961) in a series of samples containing a constant volume of trypsin solution and varying volumes of BPTI solution. Equimolar volumes were determined by extrapolation of the BPTI solution volumes to zero trypsin activity.

[1] Abbreviation: BPTI, bovine pancreatic trypsin inhibitor.

Table I: Rate Constants (in 10^{-3} min^{-1}) for Hydrogen Exchange of BPTI NH's[a]

pH	temp (°C)	Arg-20	Gln-31	Phe-33	Phe-45
9.4	25	0.73	4.1	0.7	1.9
9.4	30	1.5	5.0	1.4	2.8
9.4	35	3.0	7.9	4.3	10
9.4	40	4.3	9.6	4.9	17
9.1	30	0.5	2	1	2
9.7	30	3	7	4	4
10	30	4	14	9	9

[a] Standard error is ±8% at pH 9.4 where exchange measurements were made on sequential spectra while the sample remained in the NMR probe. Standard error is ±30% at pHs 9.1, 9.7, and 10, where each time point was measured with a different aliquot that had been quenched at pH 2 (see Experimental Procedures).

After the BPTI and trypsin solutions were mixed at pH 2 in equimolar ratio, the pH was adjusted to 5 with $Ca(OH)_2$ dissolved in ^2H_2O. At this point either the experiment was continued or the sample was lyophilized overnight and redissolved in ^2H_2O at the time of the experiment. In either case, the pH was adjusted to the experimental pH at room temperature with slow additions of $Ca(OH)_2$ or NH_4OH with a controlled-delivery micropipet. Zero time for the exchange experiment was marked by the transfer of the sample to a constant-temperature bath. After the indicated exchange time intervals, a 0.7-mL aliquot was removed from the sample and its pH jumped to 2 by the addition of a small volume of ^2HCl. The NMR spectrum was obtained for the aliquot at pH 2, and the exchange rate constant was determined from the peak height of the BPTI downfield NH resonances vs. exchange time in the complex. The pHs reported are the pH meter readings with a standard glass electrode.

As a control, the exchange of free BPTI was measured by the aliquot method described for the complex. The rate constants obtained with these samples were essentially the same as with free BPTI measured in the probe, as described above. The exchange behavior of BPTI Tyr-35 in the complex was monitored both with BPTI dissolved directly in ^2H_2O and with BPTI having only Tyr-35 ^1H labeled by the procedure described above.

Results

Before the effect of trypsin binding on the exchange kinetics of BPTI β-sheet protons was determined, conditions were established for free BPTI under which exchange is limited by processes of the folded state. In the two process model (Woodward & Hilton, 1980; Hilton et al., 1981; Woodward et al., 1982), these are the conditions under which the apparent activation energy is in the range 20–40 kcal/mol. Since a higher activation energy process dominates the exchange of the β-sheet NH's at low pH, the temperature dependence at high pH was examined. Of the eight β-core protons, the NH's of Arg-20, Gln-31, Phe-33, and Phe-45 are measurable in the temperature range 25–40 °C at pH 9–10, Table I. The exchange rates of Tyr-21, Phe-22, and Tyr-23 are too slow ($\ll 10^{-5}$ min^{-1}) to measure under these conditions, while the NH of Tyr-35 is too fast. Since its exchange is base catalyzed at pH >4, Tyr-35 can be measured at lower pH, Table II. For Arg-20, Gln-31, and Phe-33 at pH 9.4, and for Tyr-35 at pH 7, the apparent $\Delta H^{\ddagger}$ in the interval 20–40 °C ranges between 11 and 32 kcal/mol, Table III. The pH dependence of the rate constants in Tables I and II reflects the well-known

Table II: Rate Constants (±8%) for Hydrogen Exchange of Tyr-35 at pH 7

temp (°C)	$k \times 10^3$ (min^{-1})
22	0.58
26	0.77
33	1.6
37	2.3
42	4.2

Table III: Apparent Activation Energies, $\Delta H^{\ddagger}$ (±15%), for Exchange of BPTI NH's over the Interval 20–40 °C[a]

NH	$\Delta H^{\ddagger}$ (kcal/mol)
Arg-20	22
Gln-31	11
Phe-33	25
Tyr-35	18
Phe-45	32

[a] Exchange rate constants were measured at pH 9.4 except for Tyr-35 at pH 7. $\Delta H^{\ddagger}$ is derived from a linear regression analysis of Arrhenius plots of the data in Tables I and II.

Table IV: Rate Constants (in 10^{-3} min^{-1}, ±30%) for NH Exchange of BPTI NH's in the Trypsin-BPTI Complex

pH	temp (°C)	Gln-31	Phe-33	Phe-45
9.4	25	1	<0.05	0.5
9.4	30	2	0.2	1
9.4	35	2	0.5	1
9.4	40	5	0.3	3
9.1	30	0.5	0.06	0.6
9.7	30	4	0.3	1
10	30	7	0.4	2

first-order base catalysis of NH hydrogen exchange.

BPTI and trypsin associate to form a tightly bound complex that has an association constant on the order 10^{14} at pH 8 and is fully dissociated at pH 2 (Vincent & Lazdunski, 1972). Complex association is maximal in the range pH 8.5–10. The exchange rate constants of the BPTI β-core NH's in the trypsin–BPTI complex, Table IV, were determined under the same conditions as in free BPTI. In these experiments, exchange takes place in the complex, and then the exchange reaction is stopped by jumping to pH 2. Peak heights as a function of exchange time were determined in spectra at low pH where the solution is a mixture of free BPTI and free, deuterated trypsin. The error is larger in these experiments because each time point is taken with a different aliquot of protein and because the BPTI concentration is limited by the solubility of the complex. While the error in the rate constants in Table IV does not warrant calculation of $\Delta H^{\ddagger}$, it is clear that they are not larger than 30 kcal/mol.

The ratios of the rate constants in free BPTI to the rate constants in the BPTI complex range from 3 to $>10^3$, Table V.

Table V: Ratios of BPTI NH Exchange Rate Constants in Free BPTI (k_{BPTI}) to Their Rate Constants in the Trypsin-BPTI Complex (k_{cplx})

NH	k_{BPTI}/k_{cplx}[a]
Gln-31	3 ± 1
Phe-33	15 ± 6
Tyr-35	$>10^3$
Phe-45	5 ± 3

[a] Values are the mean and standard deviation of the ratios calculated at each of the conditions of pH and temperature listed in Table IV.

Discussion

β-Core Protons in Uncomplexed BPTI. The data in Table I are consistent with the two-process model of protein hydrogen exchange in which the variation in the temperature coefficient of the β-core protons of BPTI with pH and temperature is explained by two parallel pathways (Woodward & Hilton, 1980; Hilton et al., 1981; Woodward et al., 1982). One involves fluctuations of the folded state, and the rate constant for exchange by that process, k_L, has a lower apparent activation energy, usually in the range 20–35 kcal/mol. Rates for exchange by the low activation energy mechanism are not correlated with thermal stability. The second pathway involves major, cooperative unfolding, and the rate constant for exchange by that process, k_H, has a higher apparent activation energy (~65 kcal/mol for BPTI), on the order of the enthalpy of unfolding. Rates for exchange by the high activation energy mechanism are correlated with thermal stability. The two process model explains the data for NH exchange in BPTI (Hilton et al., 1981) and in lysozyme (Wedin et al., 1982; Delepierre et al., 1983).

The two-process model does not predict that the relative rates for individual neighboring protons will necessarily be dominated by the intrinsic exchange rates, as has been suggested (Wagner & Wuthrich, 1982). This would be true if, when one proton exchanges by the high activation energy mechanism, its neighboring NH also does. However, in the model, at a given pH and temperature, the exchange of one NH may be determined by the low activation energy mechanism while the exchange of the next NH is determined by the high activation energy mechanism. For example, Ile-19 NH is on the surface of BPTI and its low activation energy exchange rate is fast, while Arg-20 is in the β-sheet and its exchange rate is >6 orders of magnitude slower at room temperature. At high temperatures, it is likely that for Ile-19 $k_L \gg k_H$ while for Arg-20 $k_H \gg k_L$.

The two-process model predicts that at constant pH and temperature, the k_H values for all NH's are about the same, while k_L for different NH's may vary over orders of magnitude. This is consistent with the exchange behavior of the BPTI β-core NH's. It is already known that at 68 °C, pH 2–7, the seven slowest exchanging NH's in BPTI have very similar rate constants and that they have similar high activation energies, ~65 kcal/mol (Woodward & Hilton, 1980; Hilton et al., 1981). Table I shows that at pH 9–10 and 25–40 °C, conditions for low activation energy exchange, Tyr-21, Phe-22, and Tyr-23 have exchange rate constants that are ≫2 orders of magnitude smaller than the other four.

The important new conclusion is that Tyr-21, Phe-22, and Tyr-23 are qualitatively more shielded from solvent in the folded state than the other β-core protons.

It has long been speculated that folded proteins contain an "unexchangeable core" of NH's that do not exchange unless the protein unfolds. Suggestions have been made that the unexchangeable core might constitute a nucleation unit around which the protein condenses during folding, and/or the section of the protein that is the last to unfold. Although NH's of residues 21–23 apparently do have measurable low activation energy rate constants at pH 10 (Hilton & Woodward, 1979), it is clear that they are qualitatively less accessible to solvent than all other NH's in the molecule.

While these protons are on sequential residues, they are not adjacent in the the same secondary structure. Tyr-21 is H bonded to a different strand of β-sheet than is Phe-22, and Tyr-23 is H bonded to the side chain oxygen of Asn-43, which also participates in two other H bonds between its side chain

amide NH's and the main chain carbonyl oxygens of Glu-7 and Tyr-23 (Levitt, 1981).

The location of the three slowest exchanging NH's in BPTI is pertinent to proposals for the molecular mechanisms that may govern exchange from the folded state (Sheridan et al., 1983; Salemme, 1982). Molecular dynamics simulations of BPTI (Karplus & McCammon, 1979; McCammon et al., 1976) indicate that folded proteins simultaneously undergo two types of rapid internal motions. One type, on the subpicosecond time scale, is more localized, with average root mean square position fluctuations of 0.5–1 Å and with the larger fluctuations nearer the surface. The second type, in the 1–10-ps time scale, involves collective motions of groups of atoms. It has been suggested the regional differences in the amplitude of the more local fluctuations arise primarily from low-frequency collective modes (Levy et al., 1982; Morgan et al., 1983; Swaminathan et al., 1982) that may also determine hydrogen exchange rates (Sheridan et al., 1983). The qualitatively slower exchange rates of the NH's of residues 21–23 seem consistent with this. Normal mode analysis of BPTI indicates that the backbone atoms with the lowest root mean square fluctuation are in residues 21–23 and 43–45 (Brooks & Karplus, 1983). Also, in a calculation of normal modes in a simplified BPTI model (Levy et al., 1983), residues 21–23 and 43 are among those that deviate least from the crystal structure and are also the closest to the molecular center of mass for the molecule (Levy et al., 1983).

Salamme (1982) has proposed that the conformational flexibility and hydrogen-bond stability of the β-sheet structure in BPTI arise from the continuous interconversion between numerous flat and twisted β-sheet configurations via concerted motions that involve considerable excursions from the average crystal structure. The observation that hydrogen exchange rates of NH's at the end of the sheet are much faster that those in the middle is cited as supporting evidence (Salemme, 1982). If this model is to explain the qualitatively slower exchange of residues 21–23, then it should also provide for concerted motions that involve atoms in three strands of β-sheet and a side chain. Further, it should explain how exchange of Tyr-35 NH, located at the end of the sheet, is slowed by >3 orders of magnitude by binding of trypsin, while exchange of those in the middle of the sheet is relatively unperturbed.

β-Core Protons in the BPTI–Trypsin Complex. BPTI and trypsin associate to form the most tightly bound protein–protein complex known (Huber & Bode, 1978). The interface is composed of highly complementary surfaces in which the charged side chain of BPTI Lys-15, located at the narrow end of the pear-sharped molecule, is buried almost completely in the specificity pocket of the trypsin active site (Ruhlmann et al., 1973; Huber et al., 1974). The interface of the crystal structure has a packing density like those of protein interiors and crystals of small organic molecules (Janin & Chothia, 1976).

The eight β-core (slowest exchanging) NH's are H bonded in two long strands of twisted β-sheet that run along the long axis of BPTI and in a third short strand located near the middle [for example, see Figure 2 in Woodward & Hilton (1980) and Figure 1 in Salemme (1982)]. The long antiparallel strands are connected by a β-turn at one end, and at the other end one strand terminates in the trypsin binding segment, residues 11–18. Comparison of the X-ray structures of free and complexed inhibitor shows a marked conformational change at the inhibitor binding segment (Huber et al., 1974; Bode et al., 1976). None of the atoms of the residues containing the eight core NH's are involved in intramolecular contacts at the dimer interface, except for a small region of the accessible surface area of Arg-20 buried at the interface (Janin & Chothia, 1976). However, Tyr-35 is located in a weak contact segment of residues 34–39 in which the surface of residues 34 and 37–39 are ≥50% buried in the interface. The difference in main chain dihedral angles in the free vs. the complexed inhibitor for Tyr-35 is ~15°, considerably larger than for Gln-31, Phe-33, and Phe-45, 5° (Huber et al., 1974; Bode et al., 1976).

The outstanding result of the experiments testing the effect of trypsin binding on the exchange rates of β-core NH's is that the exchange rate of Tyr-35 in the complex is slowed >3 orders of magnitude more than the other NH's measured, Table V. The conformational difference in the crystal structures of free and complexed BPTI, as well as the fact that Tyr-35 is closer to the trypsin binding region than the other β-core NH's, is consistent with this but does not explain the magnitude of the effect.

Previous reports of the average exchange kinetics of free and trypsin-complexed BPTI (Pershina & Hvidt, 1974) and soybean trypsin inhibitor (Woodward, 1977) indicate that the number of NH's with slowed exchange rates in the complexed inhibitors exceeds the number of amino acid residues at the active site. These results open the question of whether the effect of protein–protein association might be a global effect distributed throughout the molecules rather than a localized effect at the binding site. The exchange results in Table IV indicate that the damping of fluctuations governing exchange of the β-core NH's in folded BPTI is very highly localized.

One plausible explanation for the selective slowing of Tyr-35 in the complex is that one, or a few, low-frequency collective modes are responsible for Tyr-35 NH exchange in free BPTI and these are eliminated in the complex.

Registry No. BPTI, 12407-79-3; trypsin, 9002-07-7; hydrogen, 1333-74-0.

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NADH- and Oxygen-Dependent Multiple Turnovers of Cytochrome P-450-CAM without Putidaredoxin and Putidaredoxin Reductase†

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ABSTRACT: Phenazine methosulfate (PMS) has been successfully used to mediate electron transfer from NADH to cytochrome P-450-CAM in the absence of putidaredoxin and putidaredoxin reductase under aerobic conditions. Identification and quantitation of *exo*-5-hydroxycamphor, the only product, has been accomplished by gas chromatography. In the absence of cytochrome P-450-CAM, or when other heme proteins (hemoglobin, myoglobin, horseradish peroxidase) are substituted for P-450-CAM, no *exo*-5-hydroxycamphor is detected. Product formation is not inhibited by the addition of catalase, superoxide dismutase, or hydroxyl radical scavengers; however, significant inhibition is observed with carbon monoxide and metyrapone, known inhibitors of the fully reconstituted P-450 system. Addition of 2,3-dimercaptopropanol to the NADH/PMS/P-450 system leads to a 4-fold increase in product formation; when putidaredoxin is added (without dimercaptopropanol), a 20-fold increase in product formation is observed. Constant bubbling with oxygen results in a further increase in the amount of product (150-fold increase overall). Our results show that PMS can substitute for the electron-transfer proteins putidaredoxin and putidaredoxin reductase in the transfer of electrons from NADH to P-450-CAM, resulting in multiple turnovers. Molecular oxygen dependent multiple turnovers of cytochrome P-450 have not been previously observed without the fully reconstituted, three-protein system.

Cytochrome P-450, unlike most other cytochromes, does not function merely as an electron carrier but is also an enzyme capable of catalyzing oxygenation reactions. This heme-containing mono-oxygenase activates molecular oxygen for insertion of one oxygen atom into organic substrates with concomitant reduction of the other oxygen atom to water. Pyridine nucleotides serve as the ultimate source of reducing equivalents. Since the hemoprotein itself cannot react directly with NAD(P)H, an electron-transfer system is required (Figure 1) (Gunsalus & Sligar, 1978; Ullrich, 1979; White & Coon, 1980).

The bacterial cytochrome P-450 isolated from camphor-grown *Pseudomonas putida* (P-450-CAM)[1] utilizes molecular oxygen and NADH to hydroxylate camphor at the exo-5 position and initiate camphor degradation (Katagiri et al., 1968). Figure 1 summarizes the current understanding of the reaction cycle of P-450 (Gunsalus & Sligar, 1978). Electrons

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[1] Abbreviations: P-450-CAM, the camphor-hydroxylating cytochrome P-450 isolated from *Pseudomonas putida* grown on camphor; CAM, camphor; fp, the flavoprotein (putidaredoxin reductase) that accepts electrons from NADH; Pd, the iron–sulfur protein (putidaredoxin) that accepts electrons from fp and delivers them to P-450-CAM; PMS, 5-methylphenazinium methyl sulfate (phenazine methosulfate); NMR, nuclear magnetic resonance.

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