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Hydrogen Exchange Kinetics of Bovine Pancreatic Trypsin Inhibitor β-Sheet Protons in Trypsin–Bovine Pancreatic Trypsin Inhibitor, Trypsinogen–Bovine Pancreatic Trypsin Inhibitor, and Trypsinogen–Isoleucylvaline–Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: Hydrogen exchange rates of six β-sheet peptide amide protons in bovine pancreatic trypsin inhibitor (BPTI) have been measured in free BPTI and in the complexes trypsinogen–BPTI, trypsinogen–Ile-Val–BPTI, bovine trypsin–BPTI, and porcine trypsin–BPTI. Exchange rates in the complexes are slower for Ile-18, Arg-20, Gln-31, Phe-33, Tyr-35, and Phe-45 NH, but the magnitude of the effect is highly variable. The ratio of the exchange rate constant in free BPTI to the exchange rate constant in the complex, k/k_{cplx}, ranges from 3 to $\gg 10^3$. Gln-31, Phe-45, and Phe-33 NH exchange rate constants are the same in each of the complexes. For Ile-18 and Tyr-35, k/k_{cplx} is $\gg 10^3$ for the trypsin complexes but is in the range 14–43 for the trypsinogen complexes. Only the Arg-20 NH exchange rate shows significant differences between trypsinogen–BPTI and trypsinogen–Ile-Val–BPTI and between porcine and bovine trypsin–BPTI.

Hydrogen isotope exchange kinetics of backbone amide protons are a measure of the dynamic structure of proteins. The perturbation of hydrogen exchange rates by ligand binding is a sensitive indicator of a shift in the conformational equilibria of interconverting substates that constitute the dynamic folded state of proteins. A decrease in exchange rates is taken as an indication of decreased conformational flexibility, and vice versa. The magnitude and direction of the ligand binding effect on hydrogen exchange kinetics vary with the system [cf. reviews of ligand effects on hydrogen exchange in Woodward

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and Hilton (1979), Woodward et al. (1982), Englander et al. (1972), and Barksdale and Rosenberg (1982)]. Small ligands may increase, decrease, or make no change in protein hydrogen exchange, although the most frequently observed effect is a reduction in exchange rates. The specificity of the method is demonstrated by the synergistic effects of multiple ligands for which the binding of one ligand has different effects depending on the presence of a second ligand. The sensitivity of the technique is illustrated by the observation that systems showing marked ligand-induced changes in hydrogen exchange kinetics often do not show detectable ligand-induced changes in UV, circular dichroism (CD), or fluorescence properties.

Most studies of ligand binding effects on hydrogen exchange kinetics have involved measurement of the average exchange of all labile sites. With the development of NMR[1] methods for measurement of exchange rates of individual H atoms, it is now possible to determine the effects of ligand binding on local mobility of atoms at and around specified sites in the protein. We have begun a study of the effect of a large ligand, bovine trypsin, on the hydrogen exchange kinetics of assigned NH groups in BPTI[1] (Simon et al., 1984). This report extends that work to include additional NH groups in BPTI and additional trypsin-related ligands, namely, porcine trypsin, trypsinogen, and trypsinogen–Ile-Val. This group of BPTI ligands comprises a well-characterized set of structural and functional analogues. All form 1:1 complexes with BPTI, but of differing stability. Trypsin–BPTI is among the most stable protein–protein complexes known, with an association constant of $\approx 10^{13}$ M^{-1}. The association constant of trypsinogen–BPTI is about half that, $\approx 10^{6}$ M^{-1}. For BPTI dissociation from trypsinogen–Ile-Val–BPTI, the dissociation constant is estimated at $< 6 \times 10^{-7}$ M (Antonini et al., 1983).

From comparison of the crystal structures of the free proteins and the various complexes in this system, Huber, Bode, and colleagues have proposed that the basis for the differences in binding constants resides in ligand binding effects on the dynamic structure of trypsin and trypsinogen. Proteolytic conversion of trypsinogen to trypsin leads to formation of a new N-terminus at Ile-16 and release of the N-terminal hexapeptide of the zymogen. In the crystal structure, about 85% of the trypsin molecule is identical with the corresponding residues of the trypsinogen molecule, but the activation domain is highly ordered in trypsin and disordered in trypsinogen (Bode & Schwager, 1975a; Bode et al., 1976; Fehlhammer et al., 1977). The activation domain contains the specificity site and a closely associated binding site for the two trypsin N-terminal residues, Ile-Val. The trypsinogen–BPTI complex more closely resembles trypsin, rather than trypsinogen, in the structure of its activation domain (Bode et al., 1978). An analogue for the trypsin N-terminus, free Ile-Val dipeptide, bindes to trypsinogen–BPTI, and except for the uncleaved peptide in the trypsinogen–Ile-Val–BPTI ternary complex, its crystal structure is identical with that of trypsin–BPTI (Bode et al., 1978). Huber, Bode, and colleagues have proposed that the binding of the trypsin N-terminal dipeptide, Ile-Val, is conformationally linked to the binding of specific ligands to the active site and that the reduced affinity of trypsinogen for BPTI is a consequence of the energy required to order the activation domain without the N-terminal Ile-Val binding in its specific pocket (Huber & Bennett, 1983). Consistent with this model, a number of experiments indicate that, in solution as well as in the crystal, trypsinogen structure in trypsinogen–BPTI and trypsinogen–Ile-Val–BPTI is trypsin-like (Bode & Huber, 1976; Bode, 1979; Perkins & Wüthrich, 1980; Wüthrich et al., 1981; Antonini et al., 1983). For example, the high-field regions of ^{1}H NMR spectra of trypsinogen–BPTI are more like trypsin than trypsinogen but still not the same as trypsinogen–Ile-Val–BPTI, whose NMR spectrum in the same region is indistinguishable from that of trypsin–BPTI (Perkins & Wüthrich, 1980; Wüthrich et al., 1981).

The exchange rate constants of six buried NH groups in the β-sheet of BPTI have been compared in free BPTI and in each of the trypsin/trypsinogen complexes. These experiments are focused on ligand binding effects on the internal mobility of BPTI rather than of trypsin/trypsinogen. Ligand binding effects that are propagated through the molecule are monitored; none of the backbone NH groups are involved in contacts at the protein–protein interface of the complexes. The experiments test whether trypsinogen–Ile-Val–BPTI, known to be trypsin-like in its crystal and solution structures, is trypsin-like in its effects on local conformational flexibility in the BPTI β-sheet.

Materials and Methods

Proteins and Ile-Val dipeptide were purchased and used without further purification. BPTI was obtained from Novo Corporation, Copenhagen, Denmark, bovine trypsin (3× crystallized) and bovine trypsinogen (96% protein) were from Worthington, porcine trypsin was from Sigma, and Ile-Val dipeptide was from ICN Pharmaceuticals. The isoleucine and valine content of the dipeptide was confirmed by amino acid analysis. Trypsinogen purity was checked by discontinuous SDS–polyacrylamide (12.5%) electrophoresis (Laemmli, 1970). Overloaded gels show a single band of M_r 24 000 and <4% protein fragments (estimated from very faint Coomassie blue staining material at the ion front).

Exchange rates of BPTI β-sheet protons were determined from the change in intensity with time of assigned resonances in ^{1}H NMR spectra of protein dissolved in ^2H_2O. Spectral resolution of resonances was achieved by labeling the β-sheet protons with ^{1}H while all other BPTI exchangeable protons, and all exchangeable protons in trypsin/trypsinogen, are labeled with ^{2}H. The method is basically as described in Simon et al. (1984). BPTI is selectively labeled at specific amide protons by varying pH and temperature of back-exchange procedures. Trypsin and trypsinogen were deuteriated by reversibly unfolding the protein in ^2H_2O. Labeled BPTI and deuteriated trypsin and trypsinogen were mixed in a 1:1 molar ratio just prior to the beginning of the exchange experiment. The extent of NH hydrogen exchange from BPTI in the complexes as a function of time is followed by removing samples of the complex at specified times; further exchange is stopped by lowering the pH to 2.0. Exchange rates are determined from spectra obtained at pH 2 where the complexes are fully dissociated.

For deuteriation of bovine trypsin (15 mg/mL) and bovine trypsinogen (30 mg/mL), the protein was dissolved in ^{2}HCl–^2H_2O at pH 1.5 and then incubated for 15 min at 51 °C. Porcine trypsin (30 mg/mL) was dissolved in the same solvent and incubated for 15 min at 64 °C. The activity of deuteriated trypsin in a standard enzyme assay (Erlanger et al., 1961) is 20% less than trypsin never heated above 25 °C.

Two procedures were used to label BPTI. In one, BPTI (70 mg/mL) is dissolved in ^2H_2O and incubated at 40 °C, pH 8.0, for 90 min. After this, only the N^{1}H resonances of slowest exchanging β-sheet residues 20–23, 31, 33, and 45 are observed in the ^{1}H NMR spectrum since the overlapping resonances of faster exchanging protons are replaced by deuterons. A

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; *p*-GB–trypsin(ogen), *p*-guanidinobenzoate–trypsin(ogen); NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

second procedure labels Tyr-35 and Ile-18 peptide amide hydrogen with ^1H, while the seven slowest exchanging protons and the faster exchange protons are labeled with ^2H. All exchangeable sites are reversibly deuteriated by heating BPTI (70 mg/mL) in ^2H_2O at pH 6, 90 °C, for 10 min and then cooling to room temperature. To back-exchange with ^1H, deuteriated BPTI was passed through a Sephadex G-15 column (4 × 1.5 cm) equilibrated with ^1H_2O at pH 5, 21 °C. Pooled protein-containing fractions were adjusted to pH 8, incubated at 21 °C for 8 h, lyophilized, and stored. Just prior to useage, lyophilized BPTI was dissolved in ^2H_2O at pH 7.6, 21 °C, and incubated for 30 min in order to reexchange (with ^2H) proton sites that are faster exchanging than Tyr-35 and Ile-18.

The volume of trypsin and BPTI solutions for a 1:1 molar mixture was determined for each experiment by titrating the enzymatic activity of the deuteriated trypsin solution against the BPTI solution (Simon et al., 1984). For the trypsinogen–BPTI experiments, the 1:1 molar volumes were determined from the absorbance of the deuteriated protein solutions at 280 nm, pH 3, by use of $E^{1\%} = 7.9$ for BPTI and 14.3 for trypsinogen (Laskowski & Laskowski, 1954). Immediately after the 1:1 molar solution was mixed, Ca^{2+} and buffer were added to give final concentrations of 25 mM $CaCl_2$ and 50 mM phosphate buffer (pH 7.8 or 8.0) or 0.1 M deuteriated glycine buffer (pH 9.4). In trypsinogen–Ile-Val–BPTI experiments, the peptide was added directly to the solution at a final concentration of 4.6 mM Ile-Val. The pH was then adjusted to the experimental pH and the solution equilibrated 15 min at the experimental temperature. The pH was continually checked and adjusted, if necessary, through the duration of the experiment. The final concentration is 1.6 mM for the trypsinogen complexes and 0.6 mM for the trypsin complexes, on the basis of A_{280} and by us of $E^{1\%} = 12.3$, reported for bovine trypsin–BPTI at pH 1 (Laskowski & Laskowski, 1954). The total volume for a complex exchange experiment was ≥5 mL, and solution temperatures were maintained by a thermostated water bath. Aliquots of 0.6 mL were removed at specified times, adjusted to pH 2, and stored for 1 h to several days at 4 °C, and their NMR spectra were recorded. Rate constants for BPTI protons exchanging from the complexes were determined from these spectra. Rate constants for exchange from free, uncomplexed BPTI were obtained from sequential spectra recorded while the protein sample remained in the thermostated probe.

NMR spectra (1200–15 000 transients) were obtained with a 300-MHz Nicolet spectrometer. The intensity of the $^1HO^2H$ peak is reduced by presaturation at its resonance frequency before transient data collection. Exchange rate constants are determined form the nonlinear least-squares fit of the log of peak height vs. time to the first-order rate equation. The BPTI resonance at 6.35 ppm, assigned to $C^\alpha H$ of Tyr-23, is used as an internal standard for chemical shift and resonance intensity.

Coordinates for crystal structures were obtained from the Brookhaven Protein Data Bank for BPTI (5PTI), bovine trypsin–BPTI (1TPA), trypsinogen–BPTI (2TGP), and trypsinogen–Ile-Val–BPTI (2TPI and 3TPI).

Results

Exchange rate constants of BPTI β-sheet peptide amide protons are obtained from the decrease with time of ^1H resonance intensity in sequential spectra taken while exchange is proceeding. Decay rates of BPTI N^1H resonances in spectra of trypsinogen and trypsin complexes, however, cannot be followed directly due to increased line broadening associated with the larger dimeric complex and the lack of resonance

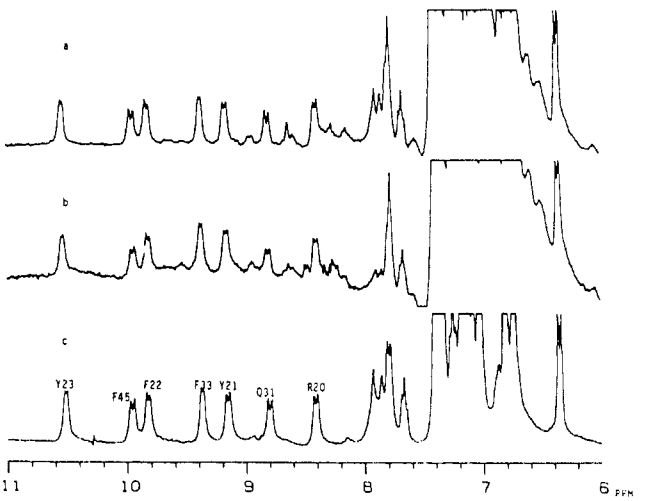

FIGURE 1: Downfield region of ^1H NMR spectra of (a) dissociated trypsinogen–BPTI and (b) dissociated trypsinogen–Ile-Val–BPTI. For comparison, the spectrum of free BPTI (c) is given with the slowest exchanging NH resonances labeled. Spectra were measured at pH 2.0 and 40 °C in ^2H_2O.

assignments for the complexes. Instead, BPTI N^1H resonance intensities for the complexes are obtained from spectra of aliquots removed from the exchange solution at timed intervals and adjusted to pH 2. The lower pH essentially stops NH exchange and dissociates the complex. Spectra of aliquots of complex at pH 2 are, then, composites of spectra of free BPTI and free deuteriated trypsin or trypsinogen.

Spectra obtained by this procedure for trypsinogen–BPTI and trypsinogen–Ile-Val–BPTI are shown in Figure 1a,b. For comparison, a spectrum of BPTI is given in Figure 1c. Resonances originating from BPTI β-sheet protons at 8.2–10.6 ppm in Figure 1a,b have narrower line widths due to the size difference between the two proteins. Resonances at 6–8 ppm are nonexchangeable aromatic protons of BPTI and trypsinogen. Broad downfield resonances arising from trypsinogen at 8.25, 8.63, and 8.95 ppm are seen in Figure 1a,b. An equivalent series of spectra for the bovine and porcine trypsin–BPTI complexes (data not shown) are like Figure 1, except for the nonexchangeable broad resonance(s) at 8.63 ppm arising from deuteriated trypsin (e.g., Figure 2c). From spectra like Figure 1, BPTI amide proton exchange rate constants for Arg-20 (8.4 ppm), Gln-31 (8.8 ppm), Phe-33 (9.38 ppm), and Phe-45 (9.95 ppm) were measured in each of the four complexes. NH resonances of residues 21–23, also H bonded in the β-sheet, are clearly observed in Figure 1, but their exchange rates are too slow to measure under these conditions [cf. Simon et al. (1984)].

Exchange rate constants of Tyr-35 and Ile-18 NH's were also followed in the four complexes. Representative spectra for experiments with bovine and porcine trypsin complexes are shown in Figure 2. BPTI is labeled to give clearly resolved resonances of the amide protons of Tyr-35 and Ile-18 (Figure 2d). Other proton resonances, Gly-36 NH (8.6 ppm) and at least two others at 7.9 and 7.85 ppm, are also present. The exchange rate for Gly-36 in the complexes, Figure 2a,b, cannot be measured because of overlap with the broad, nonexchangeable peak at 8.6 ppm from trypsin (and trypsinogen), Figure 2c.

The choice of pH and temperature for comparison of BPTI NH exchange rates in the free vs. complexed forms was made on the basis of three considerations, optimal binding of BPTI to trypsin/trypsinogen, optimal binding of Ile-Val to trypsinogen, and exclusion of contributions to exchange from global unfolding. Binding of BPTI to trypsinogen and trypsin is

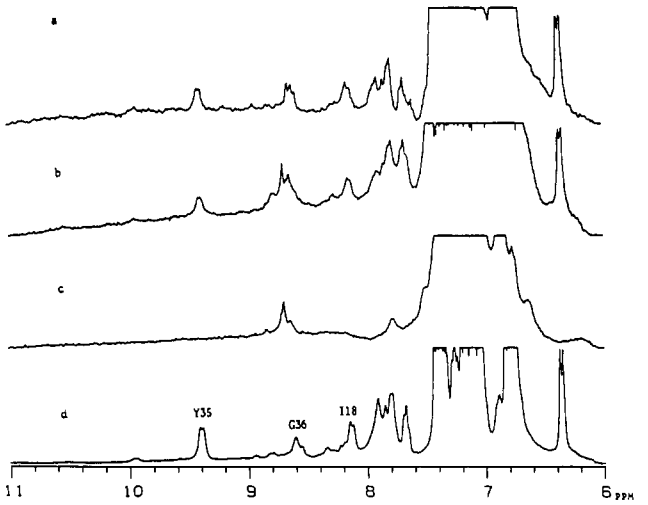

FIGURE 2: Downfield region of ^1H NMR spectra of (a) dissociated bovine trypsin–BPTI, (b) dissociated porcine trypsin–BPTI, (c) porcine trypsin with exchangeable hydrogens deuteriated, and (d) free BPTI. Samples for spectra a, b, and d were made with BPTI selectively ^1H labeled at Tyr-35 and Ile-18 NH. Spectra were measured at pH 2.0 and 40 °C in ^2H_2O.

highest at pH 8–10 (Vincent & Lazdunski, 1976; Laskowski & Sealock, 1971). However, the K_a for Ile-Val binding to *p*-guanidinobenzoate–trypsinogen reaches a maximum ($\sim 5 \times 10^4$) between pH 6.5 and pH 8.0 (Bode & Huber, 1976). At pH >8, the N-terminus of the dipeptide is deprotonated and dissociates from trypsinogen. Thus the optimal pH for the trypsinogen complex experiments is around 8. The trypsin–BPTI complex may be studied over a broader pH range, as its K_a is still at a maximum at pH 9.4 (Laskowski & Sealock, 1971). The higher pH for the trypsin complexes was chosen because the slower exchange rates are more easily measured over several days at the higher catalyst (OH^-) concentration. Exceptions to this are Gln-31 and Phe-45, which exchange too fast in the porcine trypsin complex to measure at pH 9.4. Their exchange rates were determined at pH 8.

The third consideration in the choice of pH and temperature is to assure that exchange occurs from the folded state, and not by cooperative unfolding (Woodward et al., 1982; Tüchsen & Woodward, 1987). Exchange from the folded state is characterized by a wider range (15–40 kcal/mol) of apparent activation energies that are significantly lower than the enthalpy of unfolding and wide variation in the exchange rates of individual amide protons. Exchange rate constants for β-sheet protons in free BPTI determined in these experiments are given in Table I, along with values from other reports (Simon et al., 1984; Richarz et al., 1979). From these data, estimates of the apparent activation energies can be made. For Ile-18, Arg-20, Gln-31, Phe-33, and Tyr-35 NH's, Arrhenius plots of the pH 8 data in Table I are approximately linear and E_a values are in the range 15–33 kcal/mol (Table II), well within the limits for the low activation energy process. Comparable values were observed for residues 20, 31, 33, and 45 at pH 9.4 (Simon et al., 1984). Arrhenius plots of the Phe-45 data at pH 8 in Table I show a break; at temperatures >40 °C, $E_a \approx 64$ kcal/mol, while in the interval 21–40 °C, $E_a \approx 24$ kcal/mol. Thus at pH 7.8–9.4, at temperatures ≤35 °C the protons listed in Table I exchange by the low activation energy process in free BPTI. If binding of trypsin or trypsinogen affects the global unfolding transition of BPTI, it will be to stabilize the folded protein, and therefore exchange of these protons in the complex is reasonably assumed also to be from the folded state.

Table I: Exchange Rate Constants k (in 10^{-4} min^{-1}) for BPTI NH

NH	pH	T (°C)	k^a	ref
Ile-18	7.8	21	14	
	7.8	40	400	
	8.0	30	170	
Arg-20	8.0	22	0.4	*b*
	8.0	32	1.7	*b*
	8.0	35	2.7	
	8.0	40	5.4	
	8.0	45	15	*b*
	9.4	35	30	*c*
Gln-31	8.0	10	0.3	*b*
	8.0	22	3.7	*b*
	8.0	30	4.2	
	8.0	35	7.9	
	8.0	40	13	
	8.0	45	20	*b*
	9.4	35	79	*c*
Phe-33	8.0	22	0.4	*b*
	8.0	32	2.0	*b*
	8.0	35	3.5	
	8.0	40	8.5	
	8.0	45	23	*b*
	9.4	35	43	*c*
Tyr-35	7.8	21	30	
	7.8	40	670	
	8.0	30	260	
Phe-45	8.0	22	1.3	*b*
	8.0	30	3.6	
	8.0	32	3.8	*b*
	8.0	35	9.2	
	8.0	40	12	
	8.0	45	60	*b*
	9.4	35	100	*c*

[a] Rate constants from this work (5% standard error) are given along with literature values. [b] Richarz et al. (1979), standard error 15%. [c] Simon et al. (1984), standard error 8%.

Table II: Apparent Activation Energies (±15%) for Exchange of BPTI NH[a]

NH	E_a (kcal/mol)	NH	E_a (kcal/mol)
Ile-18	32	Phe-33	33
Arg-20	29	Tyr-35	30
Gln-31	15	Phe-45	24

[a] Activation energies, E_a, are obtained from the pH 7.8–8.0 rate constants in Table I from least-squares fits of the data to the Arrhenius equation. Data obtained at pH 8.0 were used for residues 20, 31, 33, and 45. For residues 18 and 35, data were obtained at pH 7.8 or extrapolated from pH 8.0 to pH 7.8.

In summary, the conditions chosen for the comparison of exchange rate constants in free BPTI and in the complexes are pH ~8 for the trypsinogen complexes and pH ~9.4 for the trypsin complexes, except Gln-31 and Phe-45 for the porcine complexes. The exchange temperature was 35 °C, except for residues 35 and 18 in the trypsinogen complexes. These were measured at 21 °C because they exchange too rapidly at the higher temperature to be measured at pH 8. Rate constants for peptide protons of residues 18, 20, 31, 33, 35, and 45 for trypsinogen, trypsinogen–Ile-Val, and trypsin complexes are given in Table III.

The relative exchange rates of these NH's in the free protein and the three complexes, expressed as k/k_{cplx}, the ratio of the rate constant for exchange from free BPTI to the rate constant of the same proton in complexed BPTI, are given in Table IV. Values of k and k_{cplx} used to determine the ratios in Table IV were determined at the pH and temperature given for the complexes in Table III, except for Tyr-35 and Ile-18 in the trypsin complexes. At pH 9.4 Tyr-35 and Ile-18 NH exchange too fast to measure in free BPTI, and k was measured at pH 8, 30 °C, and extrapolated to pH 9.4, 35 °C, assuming first-order base catalysis and activation energies of 30 and 32

Table III: Exchange Rate Constants k_{cplx} (in 10^{-4} min^{-1}) of BPTI Peptide NH in Trypsinogen–BPTI (Tg–BPTI), Trypsinogen–Ile-Val–BPTI (Tg–IV–BPTI), and Bovine and Porcine Trypsin–BPTI (Trp–BPTI)[a]

	Tg–BPTI		Tg–IV–BPTI		Trp–BPTI (bovine)		Trp–BPTI (porcine)	
NH	pH	k_{cplx}	pH	k_{cplx}	pH	k_{cplx}	pH	k_{cplx}
Ile-18	7.8	1.0 ± 0.2	7.8	0.7 ± 0.08	9.4	<3[b]	9.4	<3[b]
Arg-20	8.0	0.2 ± 0.02	8.0	<0.1[c]	9.4	<2[b]	9.4	2.0 ± 0.3
Gln-31	8.0	3.7 ± 0.4	8.0	2.9 ± 0.2	9.4	20 ± 6	8.0	3.2 ± 0.7
Phe-33	8.0	0.2 ± 0.03	8.0	0.1 ± 0.02	9.4	2.0 ± 0.2	9.4	2.7 ± 0.5
Tyr-35	7.8	1.0 ± 0.2	7.8	0.7 ± 0.07	9.4	<3[b]	9.4	<3[b]
Phe-45	8.0	1.0 ± 0.09	8.0	0.9 ± 0.04	9.4	10 ± 3	8.0	1.0 ± 0.2

[a] Exchange rate, constants, k_{cplx} (±standard deviation), were measured at 35 °C, except Tyr-35 and Ile-18 in Tg–BPTI and Tg–IV–BPTI, measured at 21 °C. [b] No detectable exchange after 2 days. [c] No detectable exchange after 2 weeks.

Table IV: Ratio of BPTI NH Exchange Rate Constants in Free BPTI, k, to Exchange Rate Constants in Trypsinogen and Trypsin Complexes, k_{cplx}[a]

	k/k_{cplx}			
NH	Tg–BPTI	Tg–IV–BPTI	Trp–BPTI (bovine)	Trp–BPTI (porcine)
Gln-31	2 ± 0.2	3 ± 0.2	4 ± 1	3 ± 1
Phe-45	10 ± 1	10 ± 0.4	10 ± 3	10 ± 2
Phe-33	18 ± 3	29 ± 5	21 ± 2	17 ± 3
Arg-20	16 ± 1	≫30[b]	≫20[c]	15 ± 2
Tyr-35	30 ± 5	43 ± 4	≫10^3	≫10^3
Ile-18	14 ± 3	20 ± 2	≫10^3	≫10^3

[a] For calculation of k/k_{clpx}, values of k_{cplx} are taken from Table III and values of k are taken from Table I at the pH and the temperature listed for k_{cplx} in Table III, except for Tyr-35 and Ile-18 in the trypsin complexes (see text). The error listed gives the range of values of k/k_{cplx} for values of k and k_{clpx} plus and minus their standard error. [b] No detectable exchange in the complex after 2 weeks at pH 8, 35 °C. [c] No detectable exchange in the complex after 2 days at pH 9.4, 35 °C.

kcal/mol, respectively. Lower limits of k/k_{cplx} are given for cases where exchange in the complex could not be detected for the duration of the experiment, 2 days for trypsin complexes and 2 weeks for trypsinogen complexes. After these periods, the solutions of the complexes become cloudy, and therefore the length of the experiments were not extended. The limits are estimated by dividing the value of k for that proton by the upper limit of k_{clpx}. Thus the actual ratios may be very much larger than the limits given in Table IV.

The concentration of Ile-Val used in these experiments is limited by its solubility. Saturated Ile-Val is in 4-fold excess over trypsinogen. The NMR solution results of Perkins and Wüthrich (1980) indicate that trypsinogen–Ile-Val–BPTI is similar to trypsin–BPTI when a 1.5-fold higher concentration of Ile-Val over trypsinogen is used. A 0.1 M solution of Ile-Val was added to crystals of trypsinogen–BPTI for conversion of the crystal structure to trypsinogen–Ile-Val–BPTI. An effective concentration of the Ile-Val N-terminus of trypsin is estimated to be around 10^4 on the basis of the K_a of Ile-Val binding to *p*-guanidinobenzoate–trypsinogen (*p*-GB-trypsinogen). However, complete conversion of *p*-GB–trypsinogen to a structure having a CD spectrum resembling that of *p*-GB-trypsin requires a 26-fold greater concentration of Ile-Val over *p*-GB-trypsinogen (Bode & Huber, 1976). Also, an Ile-Val concentration at least 1000-fold greater than trypsinogen was used in experiments determining catalytic rate constants and dissociation constants for cationic substrates in the binary adduct trypsinogen–Ile-Val (Antonini et al., 1984).

Discussion

Trypsin/trypsinogen binding effects on exchange rates of six BPTI backbone NH groups are given in Table IV. Five are located in the twisted, antiparallel β-sheet of residues 16–36 that traverses the long axis of BPTI, and one, Phe-45 NH, is in the third, short strand of β-sheet (Figure 3). Two pairs of residues, Ile-18–Tyr-35 and Phe-33–Arg-20, have reciprocal hydrogen bonds from the amide NH of one residue to the carbonyl O of the other. A number of other NH groups are also hydrogen bonded in the twisted β-sheet (Wlodawer et al., 1984), but their exchange is too fast or too slow to measure in these experiments.

In the complexes, one end of the pear-shaped inhibitor molecule interacts with trypsin or trypsinogen, with its Lys-15 side chain bound in the specificity pocket of trypsin(ogen) (Figure 3). Intermolecular contacts involve 14 out of 58 amino acids in BPTI and 24 out of 224 in trypsin. None of the backbone atoms of the six residues reported here are involved in protein–protein contacts, and all have zero static accessibility to solvent in free BPTI and in the complexes. Of their side chains, only Ile-18 has significant interactions at the interface, with 84% of its available surface area buried in the complex (Janin & Chothia, 1976). The Arg-20 side chain has <10 Å^2 of surface area at the interface. 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 complex.

For the six amide protons studied, exchange is slower in the complex than in free BPTI, but the extent to which exchange is retarded varies for each. The residues in Table IV are listed in order of their distance from the interface. Gln-31, the furthest from the intermolecular contact region, is slowed about 3-fold in each of the four complexes. Phe-45 and Phe-33 are slowed 10-fold and 20-fold, respectively, in each of the complexes. Ligand effects on exchange are more complicated for residues closest to the interface, Tyr-35, Ile-18 and Arg-20. The hydrogen-bonded pair Tyr-35–Ile-18 show similar responses to ligand binding. Their exchange is about 1.5 times slower in the trypsinogen–Ile-Val–BPTI complex than in the trypsinogen–BPTI complex. However, in the trypsin complexes, Tyr-35 and Ile-18 show no measurable exchange in 2 days (the duration of the experiment).

Further down the β-sheet, Arg-20 NH exchange shows a strong response to addition of Ile-Val to the trypsinogen–BPTI complex. In trypsinogen–BPTI, exchange of Arg-20 NH is slowed 16-fold, but in trypsinogen–Ile-Val–BPTI no exchange is observed after 2 weeks. Arg-20 NH is also the only group to show a difference in the effect of bovine vs. porcine trypsin (Table IV). In porcine trypsin–BPTI, k/k_{cplx}, is about the same as in trypsinogen–BPTI, but in bovine trypsin–BPTI exchange of Arg-20 NH is blocked. The quenching of Arg-20 NH exchange in trypsinogen–Ile-Val–BPTI and in bovine trypsin–BPTI, but not in trypsinogen–BPTI and porcine trypsin–BPTI, indicates a highly localized sensitivity of the dynamic structure around Arg-20 NH to small alterations in ligand structure. The localization of the effect is emphasized by the qualitatively different behavior of Phe-33 NH, the other residue forming a hydrogen-bonded pair with Arg-20, (Figure 3).

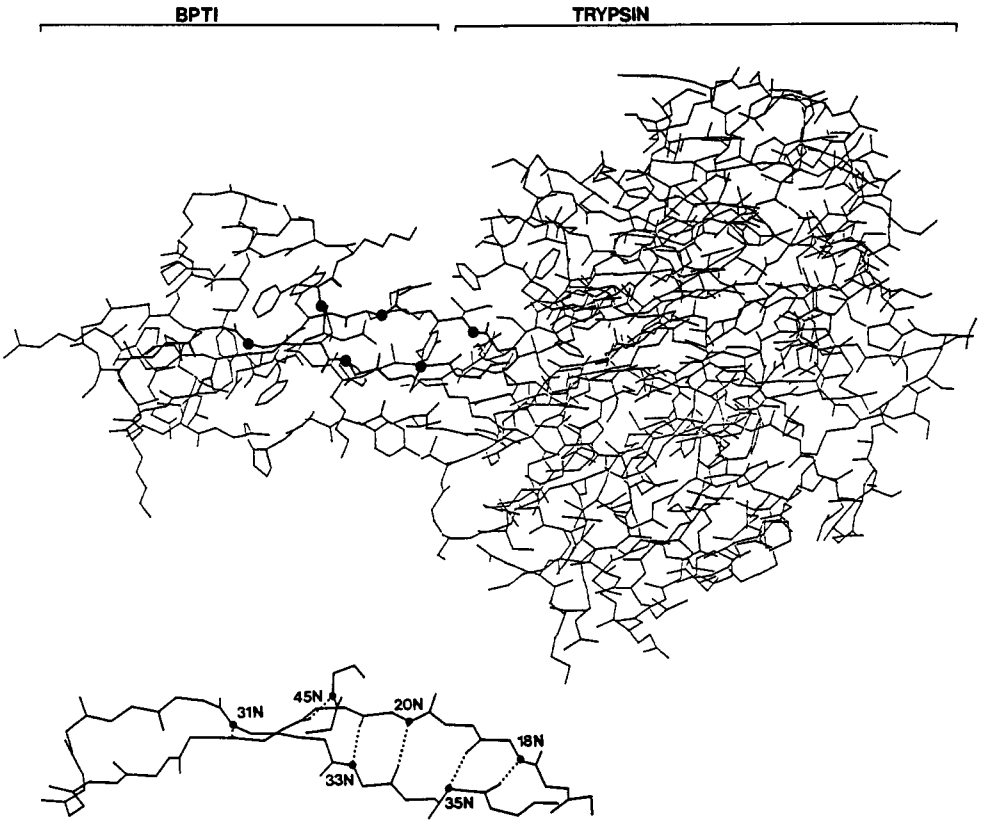

FIGURE 3: Bovine trypsin–BPTI complex and the twisted β-sheet of BPTI. The backbone atoms of the β-sheet consisting of residues 16–36 and 44–45 are highlighted by darker lines in the complex (above) and given in the enlarged fragment (below) from the same orientation. The N atoms of the NH groups whose exchange rate constants are reported here are indicated with closed circles, and their H-bonding pattern is indicated in the fragment. The drawing is made from coordinates in the Brookhaven Protein Data Bank file 1TPA.

A general damping of BPTI internal motions may be expected for the end of the inhibitor in contact with such a large ligand, and the decrease in regional flexibility around Ile-18, Tyr-35, and Arg-20 NH may be linked to the rigidification of the trypsin activation domain associated with binding at the trypsin active site. But this does not explain why Ile-18 and Tyr-35 NH exchange is quenched in the trypsin complexes and not in the trypsinogen complexes nor why Arg-20 NH exchange is blocked by addition of Ile-Val to the trypsinogen complex. These striking differences between the trypsin and trypsinogen complexes are also not explained by comparison of their crystal structures. When the BPTI molecules in the crystal structures of free inhibitor, bovine trypsin–BPTI, trypsinogen–BPTI, and trypsinogen–Ile-Val–BPTI are aligned by least-squares fit to backbone atoms in the β-sheet, the atoms of the backbone and of the buried side chains in the region of residues 18, 35, and 20 are virtually superimposable, with a root mean square distance of 0.07–0.3 Å. Crystallographic temperature factors give no indication of differences between the trypsin and trypsinogen complexes in flexibility around NH atoms of 18, 35, and 20. The temperature factors are basically the same in free BPTI and in the complexes for backbone atoms of residues 18, 35, and 20, and for buried atoms in their immediate vicinity, with a slight trend to somewhat larger temperature factors for these atoms in the complexes as compared to free BPTI. (The only significant difference in BPTI β-sheet backbone atoms in free BPTI vs. the complexes is for Gln-31 backbone atoms, which have smaller temperature factors in trypsin–BPTI and trypsinogen–BPTI.) With regard to porcine vs. bovine trypsin, there is 82% sequence homology, including the active site residues (Hermodson et al., 1973). Interactions made by soybean trypsin inhibitor with porcine trypsin are quite similar to interactions observed in bovine trypsin–BPTI; the positions of the active site residues are identical, and the Ile-Val N-terminus binds in its specificity pocket as in bovine trypsin (Sweet et al., 1974). Naray-Szabo et al. (1985) have suggested that electrostatic interactions with Asp-102 of trypsin may account for the quenching of Tyr-35 NH exchange in trypsin–BPTI reported in Simon et al. (1984). Such electrostatic interactions may affect exchange rates, but they cannot be the dominant contribution to the slow exchange of the NH's of 18, 35, and 20 observed here because similar effects would then be seen for all trypsinogen and trypsin complexes where the residues of the "charge relay" system, including Asp-102, are identically placed (Bode et al., 1978).

The data in Table IV provide a test of whether trypsinogen–BPTI and trypsinogen–Ile-Vale–BPTI are trypsin-like in their effect on local motions of BPTI. Clearly they are not. Although the exchange rates of Tyr-35 and Ile-18 NH in the trypsinogen complexes are significantly reduced compared to that of free BPTI, they are still qualitatively higher than in the trypsin complexes. For Arg-20 NH, however, Ile-Val binding to trypsinogen–BPTI stops exchange (on the week time scale). Apparently, bovine trypsin binding induces decreased flexibility in BPTI around Ile-18, Tyr-35, and Arg-20 NH groups which is different from the restriction of motion in BPTI around Arg-20 NH induced by the binding of Ile-Val to trypsinogen–BPTI. In this regard, perturbation of hydrogen exchange is a more sensitive indicator of ligand effects than crystal or spectral solution measurements.

In summary, ligand-induced changes in local flexibility at six sites in the β-sheet of BPTI have been assayed by hydrogen exchange in four trypsin/trypsinogen–BPTI complexes. Binding of trypsinogen, trypsinogen–Ile-Val, and trypsin decreases the internal motions in the β-sheet, with the largest

decrease observed for NH's closes to the intermolecular interface. However, the effects are highly localized and are not propagated uniformly along the sheet. Large, specific differences in hydrogen exchange rates of Ile-18, Tyr-35, and Arg-20 NH in the various complexes reflect differences in the dynamic solution structures that are not apparent in the static crystal structures.

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