Hydrophobic Interactions Control Zymogen Activation in the Trypsin Family of Serine Proteases†

Lizbeth Hedstrom,* Tiao-Yin Lin,‡ and Walter Fast§

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

Received August 16, 1995; Revised Manuscript Received January 24, 1996⊗

ABSTRACT: Trypsinogen is converted to trypsin by the removal of a peptide from the N terminus, which permits formation of a salt bridge between the new N-terminal Ile (residue 16) and Asp194. Formation of this salt bridge triggers a conformational change in the "activation domain" of trypsin, creating the S1 binding site and oxyanion hole. Thus, the activation of trypsinogen appears to represent an example of protein folding driven by electrostatic interactions. The following trypsin mutants have been constructed to explore this problem: Asp194Asn, Ile16Val, Ile16Ala, and Ile16Gly. The bovine pancreatic trypsin inhibitor (BPTI), benzamidine, and leupeptin affinities and activity and pH−rate profiles of these mutants have been measured. The changes in BPTI and benzamidine affinity measure destabilization of the activation domain. These experiments indicate that hydrophobic interactions of the Ile16 side chain provide 5 kcal/mol of stabilization energy to the activation domain while the salt bridge accounts for 3 kcal/mol. Thus, hydrophobic interactions provide the majority of stabilization energy for the trypsinogen to trypsin conversion. The pH−rate profiles of I16A and I16G are significantly different than the pH−rate profile of trypsin, further confirming that the activation domain has been destabilized. Moreover, these mutations decrease k_{cat}/K_m and leupeptin affinity in parallel with the decrease in stability of the activation domain. Acylation is selectively decreased, while substrate binding and deacylation are not affected. Together these observations indicate that the stability of protein structure is an important component of transition state stabilization in enzyme catalysis. These results also suggest that active zymogens can be created without providing a counterion for Asp194, and thus have important implications for the elucidation of the structural features which account for the zymogen activity of tissue plasminogen activator and urokinase.

Zymogens of the trypsin family of serine proteases are activated by proteolytic cleavage. The newly formed N terminus (Ile16 in chymotrypsinogen numbering) forms a salt bridge with Asp194, which triggers a conformational change creating active enzyme (Birktoft *et al*., 1976; Kossiakoff et al., 1977; Huber & Bode, 1978). Thus, zymogen activation appears to be an example of protein folding driven by electrostatic interactions. This observation contrasts with paradigms of protein folding which indicate that hydrophobic interactions are the primary forces determining protein stability (Alber, 1989; Dill, 1990). Moreover, recent work suggests that buried salt bridges such as Asp194−Ile16 are intrinsically destabilizing because a large amount of energy is required to desolvate two charged residues (Hendsch & Tidor, 1994; Waldburger *et al*., 1995). The present work reports experiments designed to explore these apparent contradictions, and to probe the mechanism of zymogen inactivity.

Approximately 85% of the structures of trypsinogen and trypsin are identical (Kossiakoff et al., 1977; Felhammer et al., 1977). However, the S1 binding site and the oxyanion hole of trypsinogen are unformed, which renders the zymogen inactive. Cleavage of the N terminus causes formation of the Ile16−Asp194 salt bridge (Figure 1); as a result, the S1 binding site and oxyanion hole are formed, and trypsin is active. This conformational change involves four peptide segments 16−19, 142−152, 184−194, and 216−223, termed the activation domain (Huber & Bode, 1978). These segments are disordered in trypsinogen. Bovine pancreatic trypsin inhibitor (BPTI)[1] can bind to trypsinogen and induce trypsinogen to form a conformation almost identical to that of trypsin, except that the N-terminal peptide is disordered, several residues display greater thermal motion, and a cavity exists where Ile16 is located in trypsin (Bode *et al*., 1978). This observation suggests that trypsinogen can exist in two conformations: an inactive conformation and an active, trypsin-like conformation. These two conformations are in equilibrium, with $K_{eq} = 10^8$ in favor of the inactive conformation for bovine trypsinogen. However, the magnitude of this conformational equilibrium is too large to be explained by formation of a single salt bridge.

† This work was supported by the following organizations: Lucille P. Markey Charitable Trust (to Brandeis University), Searle Scholars Program Grant 93-A-103, and NIH Grant HL50366 (L.H.). This work is Publication No. 1790 from the Graduate Department of Biochemistry, Brandeis University.

* Author to whom correspondence should be addressed.

‡ Present address: Institute of Biological Science and Technology, National Chiao Tung University, 75 Poai St., Hsinchu, Taiwan 300, R.O.C.

§ Present address: Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2153 Sheridan Rd., Evanston, IL 60208-0835.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1996.

[1] Abbreviations: I16V, I16A, and I16G, rat trypsin II with Ile16 changed to Val, Ala, and Gly, respectively; D194N, rat trypsin II with Asp194 changed to Asn; BPTI, bovine pancreatic trypsin inhibitor; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; Tos, tosyl; AMC, 7-amino-4-methylcoumarin; SBzl, thiobenzyl; Z, carbobenzoxy; tPA, tissue plasminogen activator.

0006-2960/96/0435-4515$12.00/0

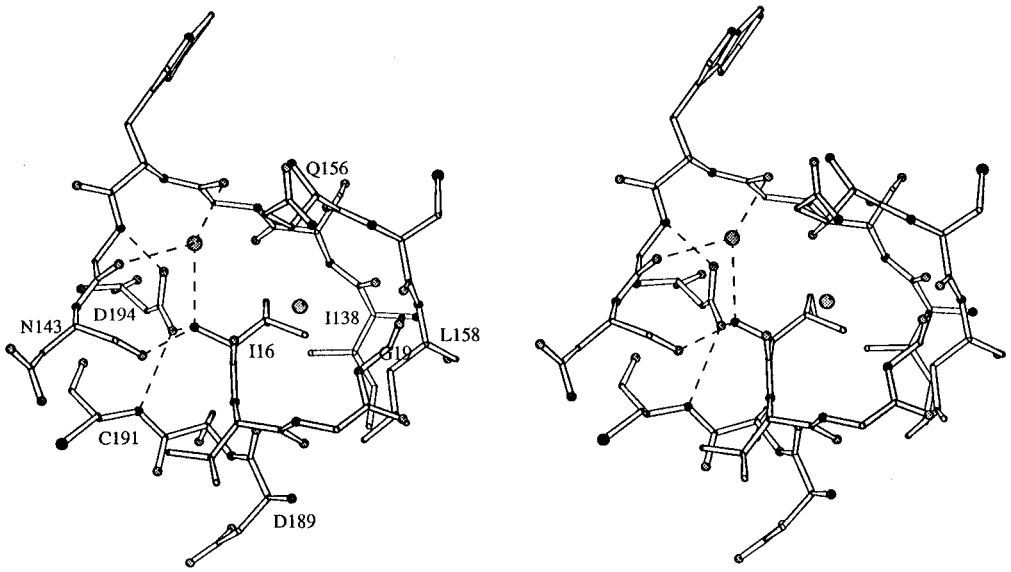

FIGURE 1: Structure of the Ile16−Asp194 salt bridge. Hydrogen bonds are denoted by the dotted lines, nitrogen atoms are denoted by dark spheres, and oxygen atoms are indicated by grey spheres. Coordinates are from the D102N rat trypsin−benzamidine complex, PDB file 1trm (Sprang et al., 1987). Visualization by MOLSCRIPT (Kraulis, 1991).

Two previous studies are relevant to this problem. Fersht measured the equilibria between active and inactive conformations of δ-chymotrypsin at high and low pH, thus demonstrating that the Asp194−Ile16 salt bridge contributes 2.9 kcal/mol to the stability of δ-chymotrypsin (Fersht, 1972). Bode demonstrated that dipeptides resembling the N terminus of trypsinogen can bind to guanidinobenzoyltrypsinogen, and measured the dissociation of IleVal, ValVal, and AlaVal (among others) to this acyl enzyme (Bode, 1979). Bode's work clearly demonstrates that hydrophobic interactions of the Ile16 side chain are important determinants of dipeptide affinity for guanidinobenzoyltrypsinogen. However, it is difficult to translate these results into a quantitative measure of the contribution of hydrophobic interactions to the stability of the active conformation of trypsin due to the uncertainties inherent in comparing inter- and intramolecular processes. Indeed, the dissociation constant for AlaVal is 2×10^5-fold more than that of IleVal, representing a loss of 7 kcal/mol of binding energy. The magnitude of this energy loss is much greater than the 4 kcal/mol of stabilization energy that is typically lost when Ile is substituted with Ala (Shortle *et al*., 1990; Serrano *et al*., 1992; Jackson *et al*., 1993). In addition, the guanidinobenzoyl modification increases the affinity of the dipeptides, thus further complicating the comparison.

The results reported here demonstrate that hydrophobic interactions of the Ile16 side chain, rather than the electrostatic interactions of the Asp194−Ile16 salt bridge, are the primary force stabilizing the activation domain. Moreover, these experiments demonstrate that stability of the activation domain is an integral component of transition state stabilization in trypsin-catalyzed substrate hydrolysis.

MATERIALS AND METHODS

Materials. D-Val-Leu-Lys-AMC, Z-Lys-AMC, Z-Arg-AMC, Z-Arg-SBzl, and D-Val-Leu-Lys-SBzl were purchased from Enzyme Systems Products (Livermore, CA); Tos-Gly-Pro-Arg-AMC and Tos-Gly-Pro-Lys-AMC were purchased from Bachem Biosciences. Z-Lys-SBzl, benzamidine, and SBTI−Sepharose were obtained from Sigma Chemical Co. BPTI and leupeptin were obtained from Boehringer. Enterokinase was purchased from Biozyme.

Construction of Mutants. Site-directed mutagenesis was performed using the method of Kunkel as described previously (Kunkel, 1985; Hedstrom *et al*., 1992). All mutants were completely sequenced to confirm that only the desired mutations were introduced. The following oligonucleotides were used (mismatched bases are underlined): D194N, TGC-CAG-GGT-AAT-TCT-GGT-GGC-CCT; I16V, GAT-GAC-AAG-GTA-GTT-GGA-GGA-TAC-ACC-TGC; I16A, GAC-AAG-GCA-GTT-GGA-GGA-TAC; I16G GAT-GAC-AAG-GGA-GTT-GGA-GGA-TAC-ACC.

Expression and Purification of Mutant Trypsins. Recombinant rat trypsinogen II is produced as an α-factor fusion protein in a *Saccharomyces cerevisiae* expression system from the pYT plasmid as previously described (Hedstrom *et al*., 1992). Mutant trypsinogens are purified from the culture media by cation exchange chromatography using a Toyopearl 650M column (Suppelco). The trypsinogens are activated by addition of enterokinase and purified on soybean trypsin inhibitor resin. Trypsins are stored in 1 mM HCl. Enzyme concentration was determined by titration with 4-methylumbelliferyl *p*-guanidinobenzoate and the BioRad protein assay. I16G does not titrate with MUGB.

N-Terminal Sequencing. N-Terminal sequences were determined using Edman degradation with an Applied Biosystems 477A protein sequences at the peptide facility of the Department of Physiology, Tufts School of Medicine.

Activity of Mutant Trypsins. Substrate stock solutions were prepared in water or dimethylformamide. The final concentration of dimethylformamide in the assays was less than 4%. Assay mix contained 100 mM NaCl, 10 mM $CaCl_2$, and 50 mM Hepes, pH 8.0. The following buffers were substituted for Hepes to determine the pH dependence of enzyme activity: Mes, pH 5.5−6.5; Mops, pH 6.5−7.5; Taps, pH 7.5−8.5; Ches, pH 8.5−10.0. Hydrolysis of the AMC substrates was monitored fluorometrically, excitation at 380 nm and emission at 460 nm. Assays were performed using 2.0 mL of assay mix containing substrate (1 μM to 1 mM, depending on the mutant) in a stirred cell at 25 °C

Table 1: BPTI, Leupeptin, and Benzamidine Inhibition of Trypsin and Mutants[a]

	BPTI		benzamidine		leupeptin[b]	
enzyme	K_i[c] (nM)	$\Delta\Delta G_{BPTI}$ (kcal/mol)	K_i (μM)	$\Delta\Delta G_{Bz}$ (kcal/mol)	K_i (nM)	$\Delta\Delta G_{Lp}$ (kcal/mol)
trypsin	0.44 ± 0.15		6 ± 1		11 ± 2	
D194N	71 ± 15	3.0	1200 ± 500	3.1	2600 ± 100	3.2
I16V	0.55 ± 0.16	0.2	8 ± 1	0.2	16 ± 2	0.2
I16A	60 ± 23	2.9	1100 ± 100	3.1	2300 ± 500	3.2
I16G	390 ± 100	4.0	32000 ± 6000	5.1	169000 ± 15000	5.7

[a] K_i's determined as described under Materials and Methods. Assays were performed in 50 mM Hepes, pH 8.0, 100 mM NaCl, and 10 mM $CaCl_2$ at 25 °C. Values reported are the average of at least two independent experiments ± SEM. $\Delta\Delta G$ is calculated from the ratios of K_i's for wild type to mutant. [b] Leupeptin = *N*-acetyl-L-leucyl-L-leucyl-L-arginal. [c] ±SD.

with a Hitachi F2000 spectrofluorimeter. The hydrolysis of SBzl substrates was monitored spectrophotometrically at 324 nm using a Hitachi U2000 spectrophotometer. Assays were performed in 1.0 mL of assay mix containing substrate (2.0 μM to 2.0 mM, depending on the mutant) and 4,4′-dithiodipyridine (25 μM) ($\epsilon_{324} = 19.8\ mM^{-1}\ cm^{-1}$). Data were analyzed using KinetAsyst software, and the reported values are the average of at least two experiments.

Inhibition of Trypsin Mutants by BPTI. Enzyme concentrations were determined by titration with pNGB. BPTI concentrations were determined by titrating with bovine trypsin. Reactions were performed in assay mix containing 0.1% Triton X-100 to stabilize the enzyme. BPTI is a slow binding inhibitor; therefore, it is necessary to follow these reactions over several hours to ensure that equilibrium has been attained. Enzyme and inhibitor were incubated at 25 °C, and aliquots were removed and assayed versus Z-Lys-SBzl at appropriate intervals over a minimum of 2 h. Enzymes were stable over this time period. Inhibition did not depend on Z-Lys-SBzl concentration, which indicates that BPTI dissociation is slow relative to the assay time. The dissociation constant was calculated from:

$$K_d = [E]_f[I]_f/[E\cdot I]$$

$$[E]_f = [E]_0 a_i/a_0$$

$$[E\cdot I] = [E]_0(1 - a_i/a_0)$$

$$[I]_f = [I]_0 - [E\cdot I]$$

where $[E]_f$ and $[I]_f$ are the concentrations of free enzyme and inhibitor, respectively, $[E]_0$ and $[I]_0$ are the total concentrations of enzyme and inhibitor, respectively, $[E\cdot I]$ is the concentration of enzyme−inhibitor complex, a_0 is the activity of the enzyme in the absence of BPTI, and a_i is the activity in the presence of BPTI after equilibrium is established. The following concentrations of enzyme and inhibitors were used: rat trypsin, 0.3 and 3.0 nM enzyme and 0.16−1.6 nM BPTI, inhibition monitored over 2 h, reaction complete within 1 h; I16V, 2.5 nM enzyme and 0.32−8.0 nM BPTI, inhibition monitored over 2 h, reaction complete within 1 h; I16A, 42 nM enzyme and 46−920 nM BPTI, reaction monitored over 3 h, complete within 1 h; I16G, 1.7 μM enzyme and 1.15−10.6 μM BPTI, reaction monitored over 7 h, complete in 3 h; D194N, 38 nM enzyme and 4.6−23 nM BPTI, reaction monitored over 2.5 h, complete within 30 min.

Inhibition of Trypsin Mutants by Benzamidine and Leupeptin. Assay mix contained varying amounts of Z-Lys-SBzl and inhibitor, and were initiated by addition of enzyme. K_i was calculated for competitive inhibition using KinetAsyst software.

RESULTS

Characterization of Trypsin and Mutant Trypsins. The experiments described here utilize recombinant rat trypsin II and I16V, I16A, I16G, and D194N mutants. The N-terminal residues of the mutant trypsins were confirmed by N-terminal sequencing. The enzyme preparations contained a single protein as determined by Coomassie blue stained SDS−PAGE. Enzyme concentrations were determined by active site titration with MUGB for all enzymes except I16G, which was determined by the BioRad protein assay against a bovine trypsin standard. Ligand binding affinity (Table 1), activity (Tables 2 and 3), and pH−rate profiles (Table 4) have been characterized. Several observations indicate that the enzyme activities reported in Table 2 are the properties of the mutant trypsins rather than the result of a contaminating protease: (a) The ligand affinities of the mutant enzymes (with the exception of I16V) are different from wild type (Table 1), which indicates that activity does not result from the presence of wild-type trypsin originating from miss-translation during protein synthesis or contamination during enzyme purification. (b) The mutant trypsins are at least 10^3-fold more active than trypsinogen (A. Pasternak and L. Hedstrom, data not shown)—this observation indicates that no contaminating protease activity originates in the trypsinogen preparation. (c) A second potential source of contaminating protease activity is the enterokinase used to activate the mutant trypsinogens. The activities of the mutant trypsins are at least 80-fold greater than that of a trypsin mutant lacking the N-terminal Ile16 (T.-Y. Lin and L. Hedstrom, data not shown). This observation indicates that no significant protease contamination arose during the activation process. (d) At least two different preparations of each enzyme have identical specific activity.

BPTI Affinity of Mutant Trypsins. The K_i's for BPTI inhibition of wild-type and mutant trypsins are shown in Table 1. The K_i for wild-type rat trypsin is substantially higher than that determined for bovine trypsin [$K_i = 4.4 \times 10^{-10}$ M versus 10^{-13} M (Vincent & Lazdunski, (1972)], as well as that previously reported for rat trypsin ($K_i < 10^{-11}$ M) (Hanlon & Lienher, 1986). However, this comparatively low affinity of rat trypsin for BPTI is consistent with crystallographic determinations of the structures of the D189G/G226D rat trypsin−C5A/C55A BPTI complex and the D189S rat trypsin/BPTI/acetate complex (Perona *et al.*, 1993, 1994). These structures show that 157 Å^2 less surface

Table 2: Activity of Trypsin and Mutants[a]

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	specificity (Arg/Lys)	$\Delta\Delta G^{\ddagger}$ (kcal/mol)
			Trypsin		
Z-Arg-SBzl	71 ± 1	4 ± 1	$(1.8 \pm 0.4) \times 10^7$	1.3	
Z-Lys-SBzl	128 ± 4	9.5 ± 0.7	$(1.4 \pm 0.1) \times 10^7$		
D-Val-Leu-Lys-SBzl	18 ± 1	5.5 ± 0.7	$(3.6 \pm 0.3) \times 10^6$		
Tos-Gly-Pro-Arg-AMC	29 ± 1	3.5 ± 0.5	$(9 \pm 1) \times 10^6$	6.4	
D-Val-Leu-Arg-AMC	17 ± 2	39 ± 6	$(4.2 \pm 0.5) \times 10^5$	13	
Z-Arg-AMC	0.53 ± 0.02	55 ± 3	$(9.6 \pm 0.2) \times 10^3$	6.4	
Tos-Gly-Pro-Lys-AMC	16.9 ± 0.8	12.3 ± 1.2	$(1.36 \pm 0.05) \times 10^6$		
D-Val-Leu-Lys-AMC	13 ± 2	395 ± 30	$(3.1 \pm 0.1) \times 10^4$		
Z-Lys-AMC	0.32 ± 0.05	220 ± 50	$(1.50 \pm 0.1) \times 10^3$		
			D194N[b]		
Z-Arg-SBzl	21 ± 2	21 ± 3	$(1.1 \pm 0.1) \times 10^6$	1.8	1.6
Z-Lys-SBzl	29 ± 3	47 ± 8	$(6.2 \pm 0.5) \times 10^5$		1.8
D-Val-Leu-Lys-SBzl	4.9 ± 0.3	24 ± 5	$(2.2 \pm 0.4) \times 10^5$		1.6
					(avg 1.7 ± 0.1)
Tos-Gly-Pro-Arg-AMC	5.8 ± 0.5	100 ± 20	$(5.4 \pm 0.4) \times 10^4$	4	3.0
D-Val-Leu-Arg-AMC	0.3 ± 0.1	170 ± 80	$(2.1 \pm 0.3) \times 10^3$	19	3.1
Z-Arg-AMC	0.016 ± 0.002	280 ± 60	54 ± 8	3.6	3.1
Tos-Gly-Pro-Lys-AMC	1.9 ± 0.2	146 ± 30	$(1.3 \pm 0.1) \times 10^4$		2.7
D-Val-Leu-Lys-AMC	0.09 ± 0.02	900 ± 200	104 ± 6		3.4
Z-Lys-AMC	0.005 ± 0.002	300 ± 200	15 ± 3		2.7
					(avg 3.0 ± 0.3)
			I16V		
Z-Arg-SBzl	165 ± 5	4.5 ± 0.5	$(4 \pm 1) \times 10^7$	1.3	−0.5
Z-Lys-SBzl	240 ± 10	8.1 ± 0.7	$(3.0 \pm 0.2) \times 10^7$		−0.5
D-Val-Leu-Lys-SBzl	42 ± 1	6.5 ± 0.8	$(6.6 \pm 0.6) \times 10^6$		−0.6
					(avg −0.5 ± 0.1)
Tos-Gly-Pro-Arg-AMC	36 ± 3	2.5 ± 0.5	$(1.4 \pm 0.2) \times 10^7$	2.4	−0.3
D-Val-Leu-Arg-AMC	nd	nd	nd		
Z-Arg-AMC	0.74 ± 0.04	40 ± 5	$(1.8 \pm 0.2) \times 10^4$	6.4	−0.4
Tos-Gly-Pro-Lys-AMC	30 ± 5	6.4 ± 0.2	$(4.8 \pm 0.9) \times 10^6$		−0.7
D-Val-Leu-Lys-AMC	34 ± 4	670 ± 120	$(5.2 \pm 0.3) \times 10^4$		−0.3
Z-Lys-AMC	0.39 ± 0.03	140 ± 30	$(2.8 \pm 0.2) \times 10^3$		−0.4
					(avg −0.4 ± 0.2)
			I16A		
Z-Arg-SBzl	76 ± 3	81 ± 2	$(9.5 \pm 0.5) \times 10^6$		1.6
Z-Lys-SBzl	72 ± 2	320 ± 20	$(2.3 \pm 0.2) \times 10^6$		2.4
D-Val-Leu-Lys-SBzl	33 ± 3	315 ± 5	$(1.06 \pm 0.04) \times 10^6$		2.1
					(avg 2.0 ± 0.4)
Tos-Gly-Pro-Arg-AMC	40 ± 10	400 ± 100	$(1.11 \pm 0.01) \times 10^5$	5.2	2.6
D-Val-Leu-Arg-AMC	nd	nd	nd		
Z-Arg-AMC	0.11 ± 0.02	1400 ± 400	79 ± 8	4.4	2.8
Tos-Gly-Pro-Lys-AMC	5 ± 1	260 ± 90	$(2.1 \pm 0.2) \times 10^4$		2.5
D-Val-Leu-Lys-AMC	1.1 ± 0.1	2700 ± 300	420 ± 40		2.5
Z-Lys-AMC	≥0.02	≥1000	18 ± 4		2.6
					(avg 2.6 ± 0.1)
			I16G		
Z-Arg-SBzl	1.93 ± 0.07	735 ± 5	2600 ± 100	1.5	5.2
Z-Lys-SBzl	6.1 ± 0.3	3500 ± 200	1750 ± 20		5.3
D-Val-Leu-Lys-SBzl	0.62 ± 0.03	640 ± 50	966 ± 2		4.9
					(avg 5.1 ± 0.2)
Tos-Gly-Pro-Arg-AMC	0.39 ± 0.05	700 ± 70	562 ± 7	4.9	5.7
D-Val-Leu-Arg-AMC	nd	nd	nd		
Z-Arg-AMC	nd	nd	nd		
Tos-Gly-Pro-Lys-AMC	>0.1	>1300	114 ± 7		5.5
D-Val-Leu-Lys-AMC	0.0036 ± 0.0002	3050 ± 200	1.18 ± 0.005		6.0
Z-Lys-AMC	nd	nd	nd		
					(avg 5.7 ± 0.4)

[a] Michaelis−Menten parameters are listed below for the hydrolysis of substrates by wild-type and mutant trypsins. Values reported are the average of at least two independent experiments ± SEM. Assays were performed in 50 mM Hepes, pH 8.0, 100 mM NaCl, and 10 mM $CaCl_2$ at 25 °C as described under Materials and Methods. Specificity is the ratio of k_{cat}/K_m of a substrate containing P1 Arg to the analogous substrate containing P1 Lys. $\Delta\Delta G$ is calculated from the ratios of k_{cat}/K_m for mutant to wild type. nd, no data. [b] Assays in 50 mM Ches, pH 9.0, 100 mM NaCl, and 10 mM $CaCl_2$.

area is buried in the BPTI complex with the rat trypsin mutants than in the complex with bovine trypsin. This decrease in buried surface area signals a decrease in BPTI affinity for rat trypsin relative to bovine trypsin. Further, several interactions between BPTI and rat trypsin appear less favorable than those between BPTI and bovine trypsin. The discrepancy with the earlier study of rat trypsin might be explained by the use of a different isozyme in the previous work [at least six isozymes of rat trypsin exist (Rypniewski et al., 1994)].

The BPTI affinity of the Ile16 mutants decreases as the size of the side chain of residue 16 decreases; no change for I16V, 140-fold decrease for I16A, and 900-fold for I16G, while the affinity of D194N is 100-fold less than that of wild type. Table 1 also lists $\Delta\Delta G_{BPTI}$ for changes in BPTI binding as measured by the ratio of the K_i of trypsin to the K_i of the mutant.

Benzamidine and Leupeptin Affinities of Mutant Trypsins. The K_i's for benzamidine and leupeptin inhibition of trypsin and mutants were measured versus Z-Lys-SBzl (Table 1). The inhibition is competitive in all cases. The K_i for benzamidine inhibition of wild type agrees with that reported previously [literature $K_i = 12\ \mu$M (Wilke *et al*., 1991)]. The K_i of benzamidine also increases for the Ile16 mutants as the size of the side chain of residue 16 decreases: no change for I16V, 180-fold for I16A, and 5300-fold for I16G. The K_i of benzamidine is increased 200-fold for D194N. Table 1 also lists $\Delta\Delta G_{Bz}$ for changes in benzamidine affinity as measured by the ratio of the K_i of trypsin to the K_i of the mutant enzyme. $\Delta\Delta G_{Bz}$ is similar to the $\Delta\Delta G_{BPTI}$ for all of the mutant enzymes except I16G. For I16G, BPTI affinity is decreased 900-fold while benzamidine affinity is decreased 5300-fold, which corresponds to a 1.1 kcal/mol difference between $\Delta\Delta G_{Bz}$ and $\Delta\Delta G_{BPTI}$.

The K_i for leupeptin inhibition of rat trypsin is similar to that reported previously for bovine trypsin [literature $K_i =$ 13 nM (Kuramochi *et al*., 1979)]. The K_i of leupeptin also increases for the Ile16 mutants as the size of the side chain of residue 16 decreases: 1.4-fold for I16V, 210-fold for I16A, and 15 000-fold for I16G. The K_i of leupeptin is increased 240-fold for D194N. Table 1 lists $\Delta\Delta G_{Lp}$ for changes in leupeptin affinity as measured by the ratio of the K_i of trypsin to the K_i of the mutant enzyme. $\Delta\Delta G_{Lp}$ is equivalent to $\Delta\Delta G_{Bz}$ for all of the mutations.

Enzymatic Activity. The activity of the trypsin mutants was measured by assaying the hydrolysis of nine substrates chosen to probe changes in Arg/Lys specificity; S1, S2, and S3 binding site function; and amide/ester hydrolysis (Table 2). None of the mutations caused a significant change in the specificity for P1 Arg versus P1 Lys containing substrates as evidenced by the ratio of k_{cat}/K_m's for hydrolysis of Tos-Gly-Pro-Arg-AMC/Tos-Gly-Pro-Lys-AMC, D-Val-Leu-Arg-AMC/D-Val-Leu-Lys-AMC, Z-Arg-AMC/Z-Lys-AMC, or Z-Arg-SBzl/Z-Lys-SBzl. In addition, both tripeptide and single-residue substrates are affected equally by the mutations as measured by relative k_{cat}/K_m's. The mutation of Asp194 to Asn causes an ~100-fold decrease in k_{cat}/K_m for amide hydrolysis, but only a 10-fold decrease for ester hydrolysis; both a decrease in k_{cat} and an increase in K_m are observed. In contrast, the Ile16 mutations have similar effects on the hydrolysis of amides and esters. The mutation of Ile16 to Val does not have a significant effect on hydrolysis of any of the substrates assayed. The k_{cat}/K_m's for substrate hydrolysis by the other mutations at Ile16 decrease with the decrease in size of the side chain of residue 16: ~100-fold for I16A, and $\sim 10^4$-fold for I16G. Both k_{cat} and K_m are affected by the mutations. Table 2 also reports $\Delta\Delta G^{\ddagger}$'s as measured by the ratio of k_{cat}/K_m's for the mutant enzymes relative to wild-type trypsin. For the Ile16 mutations, $\Delta\Delta G^{\ddagger}$ is equivalent for ester and amide hydrolysis and similar to $\Delta\Delta G_{BPTI}$, $\Delta\Delta G_{Bz}$, and $\Delta\Delta G_{Lp}$. However, for D194N, $\Delta\Delta G^{\ddagger}$ for ester hydrolysis is smaller than $\Delta\Delta G^{\ddagger}$ for amide hydrolysis. Interestingly, for D194N, $\Delta\Delta G^{\ddagger}$ for amide hydrolysis is similar to $\Delta\Delta G_{BPTI}$, $\Delta\Delta G_{Bz}$, and $\Delta\Delta G_{Lp}$.

Scheme 1: Mechanism of Substrate Hydrolysis by Trypsin

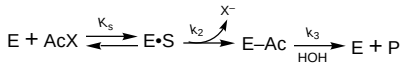

Table 3: Mechanistic Kinetic Parameters for the Hydrolysis of Amide Substrates by Mutant Trypsins[a]

enzyme	K_s (M), $\Delta\Delta G_S$ (*kcal/mol*)	k_2 (s^{-1}), $\Delta\Delta G^{\ddagger}_2$ (*kcal/mol*)	k_3 (s^{-1}), $\Delta\Delta G^{\ddagger}_3$ (*kcal/mol*)
		D-Val-Leu-Lys-AMC	
trypsin	6500	280	18
D194N	900	0.09	4.9
	−1.2	*4.7*	*0.8*
I16V	4200	220	42
	−0.2	*0.1*	*−0.5*
I16A	2700	1.1	33
	−0.5	*3.3*	*−0.4*
I16G	3050	0.0036	0.62
	−0.5	*6.6*	*2.0*
		Z-Lys-AMC	
trypsin	220	0.32	128
D194N	300	0.005	29
	0.2	*2.5*	*0.9*
I16V	140	0.39	240
	−0.3	*−0.1*	*−0.4*
I16A	≥1000	≥0.02	72
	≥0.9	*≥1.6*	*0.3*
		Z-Arg-AMC	
trypsin	55	0.53	71
D194N	280	0.16	21
	1.0	*2.0*	*0.7*
I16V	40	0.74	165
	−0.2	*−0.2*	*−0.5*
I16A	1400	0.11	76
	1.9	*0.9*	*0*

[a] The mechanistic kinetic parameters for the hydrolysis of D-Val-Leu-Lys-AMC, Z-Lys-AMC, and Z-Arg-AMC are derived from the steady-state kinetic constants of Table 2. Assuming the deacylation is completely rate-determining for the hydrolysis of the analogous ester, then K_s, k_2, and k_3 for amide hydrolysis can be determined from the following equations: $k_{cat,ester} = k_3$; $K_m = K_s[k_{cat,ester}/(k_2 + k_{cat,ester})]$; $k_{cat} = k_2 k_{cat,ester}/(k_2 + k_{cat,ester})$. $\Delta\Delta G_S$ denotes the difference in free energy of substrate binding for trypsin and mutant as calculated from the ratio of K_s of trypsin to K_s of mutant. $\Delta\Delta G^{\ddagger}_2$ and $\Delta\Delta G^{\ddagger}_3$ similarly denote the change in free energy of the transition state for acylation and deacylation, respectively, as calculated from the ratio of the pertinent rate constants of mutant to trypsin.

Mechanistic Rate Constants. Trypsin catalyzes substrate hydrolysis via a three-step mechanism involving substrate binding (K_s), acylation of Ser195 (k_2), and hydrolysis of the acylenzyme intermediate (k_3) (Scheme 1). The mechanistic rate constants for the hydrolysis of D-Val-Leu-Lys-AMC, Z-Arg-AMC, and Z-Lys-AMC can be calculated from the steady-state kinetic constants for the hydrolysis of these compounds and the analogous thiobenzyl esters and are shown in Table 3 (Zerner *et al*., 1964). This calculation assumes that deacylation is completely rate-determining for ester hydrolysis. Although generally accepted, this assumption has not been demonstrated for the mutant enzymes. Therefore, k_{cat} for ester hydrolysis is more properly a lower limit for k_3. Interestingly, k_3 is the rate-determining step for the hydrolysis of D-Val-Leu-Lys-AMC by wild-type trypsin and I16V, while k_2 is rate-determining for the hydrolysis of Z-Arg-AMC and Z-Lys-AMC. The mutation of Ile16 to Val does not have a significant effect on the mechanistic rate constants. The other mutations have the greatest effect on

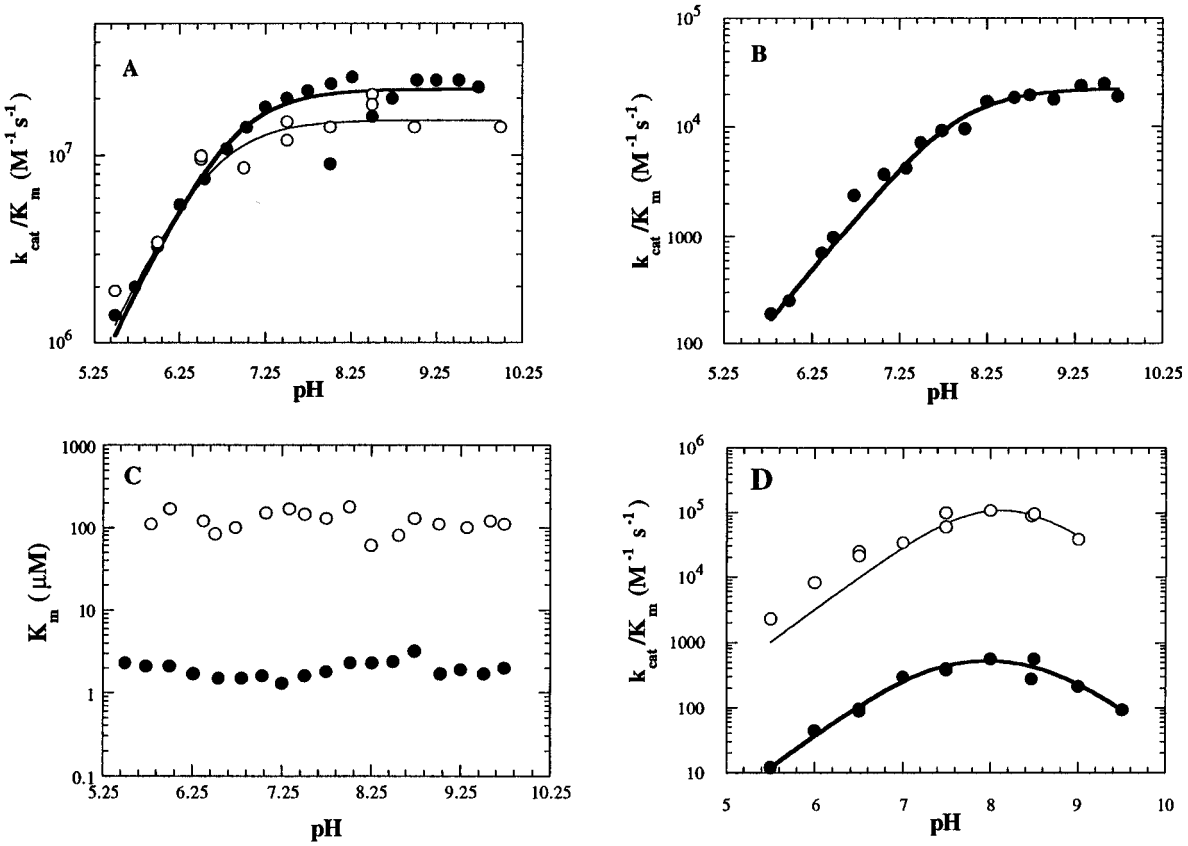

FIGURE 2: pH−rate profiles for the hydrolysis of TosGlyProArg-AMC by trypsin mutants. Conditions as described under Materials and Methods. (A) (●) Wild-type trypsin; (○) D16V. (B) (●) D194N. (C) (●) Wild-type trypsin; (○) D194N. (D) (○) I16A; (●) I16G.

Table 4: pH Dependence of k_{cat}/K_m for Hydrolysis of Tos-GlyProArg-AMC by Trypsin Mutants[a]

enzyme	pK_{a_1}	pK_{a_2}
trypsin	6.8	>10
D194N	7.8	>10
I16V	6.4	>10
I16A	7.2	8.8
I16G	7.3	8.6

[a] Experimental conditions as described under Materials and Methods.

k_2, which is decreased as much as 10^5-fold. In contrast, the maximum change in K_s or k_3 is 30-fold. Indeed, the mutations either improve or have no effect on the binding of D-Val-Leu-Lys-AMC. Table 3 also lists $\Delta\Delta G_s$, $\Delta\Delta G^{\ddagger}_2$, and $\Delta\Delta G^{\ddagger}_3$ for changes in K_s, k_2, and k_3.

pH−Rate Profiles. Figure 2 shows the pH−rate profiles of the hydrolysis of Tos-Gly-Pro-Arg-AMC by wild-type and mutant trypsins. k_{cat}/K_m for wild-type trypsin is pH-independent above pH 7, as previously observed (Craik *et al*., 1987). The pH−rate profile of k_{cat}/K_m for I16V is essentially identical to that of wild type, while the profiles for I16A and I16G are bell-shaped. The pH−rate profile for k_{cat}/K_m of D194N is hyperbolic like that of wild type, although the pK_a is shifted from 6.8 to 7.8. K_m's of both wild-type and D194N trypsin are pH-independent. The pK_a's are listed in Table 4.

DISCUSSION

Hydrophobic Interactions Provide ~5 kcal/mol of Stabilization Energy for the Conformational Change from Inactive Trypsinogen to Active Trypsin. Trypsinogen assumes a trypsin-like conformation upon BPTI binding (Bode *et al*., 1978). Thus, the low affinity of BPTI for trypsinogen is a consequence of the energetic cost of ordering the activation domain. Therefore, the decrease in BPTI affinity will measure the destabilization of trypsin's activation domain caused by the mutations of Ile16 and Asp194. BPTI binding involves many interactions with trypsin beyond the S1 site. In contrast, benzamidine interacts only with the S1 site of trypsin (Kreiger *et al*., 1974; Bode & Schwager, 1975). Thus, benzamidine affinity is a probe of the integrity of the S1 site alone. $\Delta\Delta G_{Bz}$ and $\Delta\Delta G_{BPTI}$ are equivalent for all of the enzymes except I16G, where $\Delta\Delta G_{Bz}$ is 1.1 kcal/mol greater than $\Delta\Delta G_{BPTI}$.

As measured by BPTI and benzamidine affinity, removal of the Ile16 side chain by mutation of Ile16 to Gly destabilizes the activation domain by 4−5 kcal/mol (Table 1). This destabilization energy is similar to the 4.7 ± 1.8 kcal/mol destabilization observed for Ile to Gly mutations of staphylococcal nuclease as measured by guanidine hydrochloride denaturation (Shortle *et al*., 1990). Similarly, the mutation of Ile16 to Ala destabilizes the activation domain by 3 kcal/mol. This destabilization energy is similar to the 4.4 ± 1.4 kcal/mol that is observed for Ile to Ala mutations in staphylococcal nuclease, gene V protein, barnase, and chymotrypsin inhibitor 2, as measured by guanidine hydrochloride denaturation (Shortle *et al*., 1990; Serrano *et al*., 1992; Jackson *et al*., 1993). These observations suggest that the activation domain of trypsin is a good general model for protein stability. The mutation of Ile16 to Val has little effect on the stability of the activation domain. This result contrasts with the ~1 kcal/mol loss of stabilization energy usually observed for Ile to Val mutations measured by guanidine denaturation (Shortle *et al*., 1990,

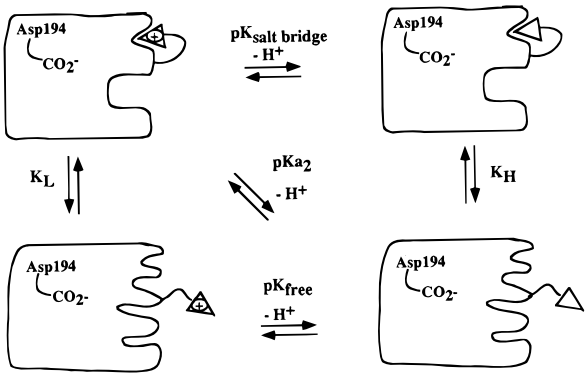

FIGURE 3: Conformational equilibrium and ionization of trypsin [after Fersht (1972)].

Sandberg & Terwillinger, 1989; Serrano *et al*., 1992; Jackson *et al*., 1993). Interestingly, Val is the only residue other than Ile commonly found at the N terminus of trypsin-like serine proteases (Greer, 1990).

Bode measured the binding of IleVal, ValVal, and AlaVal dipeptides to guanidinobenzoyltrypsinogen (Bode, 1979), demonstrating that the substitution of Ile by Val and Ala resulted in losses of 2.0 and 7.0 kcal/mol of binding energy, respectively. These values are much greater than those observed for the mutation of Ile16 to Val or Ala (0.2 and 3.0 kcal/mol, respectively). The greater penalty for substitution of the Ile in the dipeptide most likely results from the greater entropic penalty associated with intermolecular reactions. The difference between $\Delta\Delta G$ for dipeptide binding and $\Delta\Delta G$ for the N-terminal mutations reflects the effective concentration of the N terminus of trypsin (e.g., $2.0 - 0.2 = 1.8$ kcal/mol for Ile to Val substitution and 4.0 kcal/mol for Ile to Gly substitution). Intriguingly, the effective concentration of the Ala N terminus appears to be greater than that of the Val N terminus. Further experiments are required to substantiate this result.

Destabilization of the Activation Domain Perturbs the pH−Rate Profile of Trypsin. The enzymatic activity of chymotrypsin displays a bell-shaped pH dependence; $pK_{a_1} = 6.5$ results from deprotonation of His57, while $pK_{a_2} = 8.8$ results from deprotonation of Ile16 (Hess *et al*., 1970). pK_{a_2} is a product of both the ionization of Ile16 and the conformational equilibrium between active and inactive conformations (Figure 3). Fersht has measured these equilibria for δ-chymotrypsin ($K_L = 0.055$ and $K_H = 7.06$) and calculated $pK_{free} = 7.9$ (which is the expected value for an N-terminal isoleucine) and $pK_{salt\ bridge} = 9.96$ (Fersht, 1972). In contrast to δ-chymotrypsin, pK_{a_2} must be greater than 10 for rat trypsin. Therefore, if $pK_{free} = 7.9$, K_L must be less than 0.01 for rat trypsin.

Destabilization of the activation domain is expected to be expressed by changes in K_L, and therefore in pK_{a_2}. Like wild-type trypsin, the pH−rate profile of I16V displays a pK_{a_2} greater than 10. This observation suggests that the mutation of Ile16 to Val does not destabilize the activation domain, in agreement with the previous assays. In contrast, the pH−rate profiles of I16A and I16G are bell-shaped, with pK_{a_2} of 8.8 and 8.6, respectively. This decrease in pK_2 must result from an increase in K_L: pK_{free} values of N-terminal alanine and valine residues are expected to be *higher* than that of an N-terminal Ile (by at most 0.3 pH unit; Jencks & Regenstein, 1976). pK_{a_2} is not observed in the pH−rate profile of D194N. This result is anticipated because the predominant active conformation of D194N is expected to contain neutral Ile16.

The value of pK_{a_1} is also affected by the mutations at Asp194 and Ile16. Interestingly, pK_{a_1} is lower for I16V than in wild-type trypsin (6.4 versus 6.8); the reason for this apparent change in the pK_a of His57 is unclear, although it may result from changes in the microscopic rate constant that are not evident in the steady-state kinetic parameters. pK_{a_1} is higher in I16A and I16G than in wild-type trypsin (7.2 versus 6.8). This observation is consistent with previous work which demonstrates that the pK_a of His57 is higher in zymogens than in active enzymes (Markley & Ibanez, 1978); thus, the increase in pK_{a_1} observed for I16A and I16G further confirms a shift toward an inactive, zymogen-like conformation in these enzymes. For D194N, $pK_{a_1} = 7.9$ most likely results from the deprotonation of Ile16.

Electrostatic Interactions between Asp194 and Ile16 Stabilize the Activation Domain by No More than 3 kcal/mol. The mutation of Asp194 to Asn replaces a salt bridge with two neutral isosteric residues (Asn and neutral N terminus).[2] This substitution decreases the stability of the activation domain by 3.0 kcal/mol as determined by BPTI and benzamidine affinity. This value is similar to the 2.9 kcal/mol of stabilization energy attributed to the Asp194−Ile16 salt bridge of δ-chymotrypsin by measuring the conformational equilibria between active and inactive forms at low pH (Asp-$CO_2^-\cdots+H_3N$-Ile16) and high pH (Asp-$CO_2^-\cdots H_2N$-Ile16) (Fersht, 1972). The similar contributions of the Asp194−Ile16 salt bridge to the stability of rat trypsin and δ-chymotrypsin are surprising given the very different pH dependence of these two enzymes. Together these observations suggest that there is no additional penalty for an uncompensated negative charge at Asp194 beyond the loss of the electrostatic interactions with Ile16, although further experiments are required to substantiate this conclusion. Similar results have been obtained for the disruption of an Asp−His salt bridge in T4 lysozyme (Anderson *et al*., 1990). In this work, mutants containing single replacements of either the Asp or the His were as stable as a mutant with the replacement of both residues, again indicating that the decrease in stability results from the loss of an electrostatic interaction and not from an uncompensated charge.

The Stability of the Activation Domain Provides Transition State Stabilization for the Enzymatic Reaction. The activation domain includes the S1 site and the oxyanion hole; therefore, it is not surprising that the stability of the activation domain controls trypsin activity. The mutations selectively decrease k_2, which indicates that the destabilization of the activation domain is primarily expressed as a loss of catalytic energy (Table 3). Amazingly, K_s for D-Val-Leu-Lys-AMC is not increased in any of the mutant enzymes, and only modest increases in K_s are observed for Z-Lys-AMC and Z-Arg-AMC. Indeed, the mutation of the Ile to Gly causes a 10^5-fold decrease in k_2 for hydrolysis of D-Val-Leu-Lys-AMC, but has no effect on substrate binding. Thus, protein stability can be an important component of transition state stabilization. Since the mutations have negligible effects on substrate binding, it is tempting to postulate that the mutations destabilize the oxyanion hole rather than the S1 binding site. However, these mutations clearly destabilize

[2] These assays were performed at pH 9.0 where the N terminus would be deprotonated.

the S1 site as evidenced by decreases in benzamidine affinity. Thus, substrate interactions in the S1 site are primarily utilized in transition state stabilization, not ground state stabilization.

This conclusion is further supported by the effect of the mutations on leupeptin affinity. Leupeptin is considered a transition state analog of trypsin (Ortiz *et al*., 1991). Transition state analogy can be confirmed by demonstrating that the K_i for a series of inhibitors correlates with k_{cat}/K_m for the analogous series of substrates (Wolfenden, 1976; Bartlett & Marlowe, 1983). The correlation of K_i with k_{cat}/K_m for a single inhibitor/substrate pair in a set of mutant enzymes has also been used to demonstrate transition state analogy, and to confirm the role of a given enzymatic residue in transition state stabilization (Phillips *et al*., 1992; Hedstrom *et al*., 1994). The changes in leupeptin affinity correlate with changes in k_{cat}/K_m for amide hydrolysis, as expected for a transition state analog (see Tables 1 and 2), and again demonstrate that destabilization of the activation domain also destabilizes the transition state of trypsin reaction.

BPTI and Benzamidine Are Transition State Analogs. The correlation of K_i with k_{cat}/K_m indicates that both BPTI and benzamidine are transition state analogs although neither of these compounds form tetrahedral adducts with Ser195 like leupeptin (Tables 1 and 2). The idea that BPTI is a transition state analog is somewhat counter-intuitive: it is easy to assume that BPTI is a ground state analog. The transition state analogy of BPTI is more apparent if one considers that the transition state structure of a protease reaction includes extensive enzyme−substrate interactions beyond the immediate vicinicty of the scissile peptide bond. BPTI, aldehyde, and boronic acid transition state analogs bind in identical extended conformations to trypsin-like proteases, which suggests that the substrate must be constrained into this conformation for reaction to occur (Polgar, 1989). Thus, the rigid conformation of the reaction loop of BPTI provides transition state analogy. Moreover, interactions at the S and S′ binding sites are known to accelerate peptide hydrolysis in trypsin-like proteases (Thompson & Blout, 1970; Bauer, 1976; Stein *et al*. 1987; Hedstrom *et al*., 1992). These observations indicate that these extended enzyme−substrate interactions stabilize the transition state. Thus, BPTI recapitulates many transition state-like interactions even though a tetrahedral adduct is not formed at the scissile bond.

Likewise, the proposition that benzamidine is a transition state analog is surprising because it only interacts at the S1 site. Benzamidine binding retains the favorable interactions of the substrate at the S1 site, while avoiding the destabilizing interactions at the scissile bond and much of the conformational constraint required for substrate binding; therefore, benzamidine also behaves as a transition state analog. These results suggest a new strategy for the design of potent enzyme inhibitors: compounds which reproduce the substrate interactions that favor binding, while avoiding the interactions that destabilize the substrate and promote catalysis, will be transition state analogs.

Hydrophobic Interactions May Also Be Important in "Active" Zymogens. Unlike trypsinogen, which is 10^7-fold less active than trypsin (Antonini et al., 1984; A. Pasternak and L. Hedstrom, unpublished experiments), several trypsin-like zymogens possess significant proteolytic activity. The single-chain form of tissue plasminogen activator (tPA) is only 7-fold less active than the two-chain form of tPA, prourokinase is 250-fold less active than urokinase, and factor XII is 4000-fold less active than factor XIIa (Boose *et al*., 1989; Petersen *et al*., 1988; Silverberg & Kaplan, 1982). These observations suggest that the equilibrium between active and inactive conformations of tPA, prourokinase, and facotr XII is shifted to favor the active conformation. The structural features which stabilize these active zymogens are unknown, although one possibility is that alternate counterions for Asp194 may exist in these zymogens (Nienaber *et al*., 1992; Petersen *et al*., 1990). However, the results reported here indicate that the presence of an uncompensated charge at Asp194 is less destabilizing than the cavity created by the absence of the Ile16 side chain. Therefore, additional hydrophobic interactions can be sufficient to stabilize the activation domain of these zymogens.

ACKNOWLEDGMENT

We thank Annette Pasternak for helpful discussions and critical reading of the manuscript and Ben Kunz and Erza Peisach for the molecular graphics.

REFERENCES

Alber, T. (1989) *Annu. Rev. Biochem. 58*, 765−798.
Anderson, D. E., Becktel, W. J., & Dahlquist, R. W. (1990) *Biochemistry 29*, 2403−2408.
Antonini, E., Ascenzi, P., Bolognesi, M., Guarneri, M., & Menegatti, E. (1984) *Mol. Cell. Biochem. 60*, 163−181.
Bartlett, P. A., & Marlowe, C. K. (1983) *Biochemistry 22*, 4618−4624.
Bauer, C.-A. (1976) *Biochem. Biophys. Acta 438*, 495−502.
Birktoft, J., Kraut, J., & Freer, S. (1976) *Biochemistry 15*, 4481−4485.
Bode, W. (1979) *J. Mol. Biol. 127*, 357−374.
Bode, W., & Schwager, P. (1975) *J. Mol. Biol. 98*, 693−717.
Bode, W., Schwager, P., & Huber, R. (1978) *J. Mol. Biol. 118*, 99−112.
Boose, J. A., Kuismanen, E., Gerard, R., Sambrook, J., & Gething, M.-J. (1989) *Biochemistry 28*, 635−643.
Craik, C. S., Roczniak, S., Largman, C., & Rutter, W. J. (1987) *Science 237*, 909−913.
Dill, K. A. (1990) *Biochemistry 29*, 7133−7155.
Felhammer, H., Bode, W., & Huber, R. (1977) *J. Mol. Biol. 111*, 415−438.
Fersht, A. R. (1972) *J. Mol. Biol. 64*, 497−509.
Greer, J. (1990) *Proteins: Struct., Funct., Genet. 7*, 317−334.
Hanlon, M. H., & Lienher, I. E. (1986) *Comp. Biochem. Physiol. 84B*, 53−57.
Hedstrom, L., Szilagyi, L., & Rutter, W. J. (1992) *Science 255*, 1249−1253.
Hedstrom, L., Farr-Jones, S., Kettner, C., & Rutter, W. J. (1994) *Biochemistry 33*, 8764−8769.
Hendsch, Z. S., & Tidor, B. (1994) *Protein Sci. 3*, 211−226.
Hess, G. P., McConn, J., Ku, E., & McConkey, G. (1970) *Philos. Trans. R. Soc. London, B 257*, 89−104.
Huber, H., & Bode, W. (1978) *Acc. Chem. Res. 11*, 114−122.
Jackson, S. E., Moracci, M., elMasry, N., Johnson, C. M., & Fersht, A. R. (1993) *Biochemistry 32*, 11259−11269.
Jencks, W. P., & Regenstein, J. (1976) in *CRC Handbook of Biochemistry and Molecular Biology* (Fasman, G. D., Ed.) 3rd ed., Vol. 1, pp 305−351, CRC Press, Cleveland, OH.
Kossiakoff, A. A., Chambers, J. L., Kay, L. M., & Stroud, R. M. (1977) *Biochemistry 16*, 654−664.
Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.
Kreiger, M., Kay, L. M., & Stroud, R. M. (1974) *J. Mol. Biol. 83*, 209−230.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Kuramochi, H., Nakata, H., & Ishii, S.-I. (1979) *J. Biochem. 86*, 1403−1410.

Markley, J. L., & Ibanez, I. B. (1978) *Biochemistry 17*, 4627−4640.
Nienaber, V. L., Young, S. L., Birktoft, J. J., Higgins, D. L., & Berliner, L. J. (1992) *Biochemistry 31*, 3852−3861.
Ortiz, C., Tellier, C., Williams, H., Stolowich, N. J., & Scott, A. I. (1991) *Biochemistry 30*, 10026−10034.
Perona, J. J., Hsu, C. A., Craik, C. S., & Fletterick, R. J. (1993) *J. Mol. Biol. 230*, 919−933.
Perona, J. J., Hedstrom, L., Wagner, R. L., Rutter, W. J., Craik, C. S., & Fletterick, R. J. (1994) *Biochemistry 33*, 3252−3259.
Petersen, L. C., Lund, L. R., Dano, K., Nielsen, L. S., & Skriver, L. (1988) *J. Biol. Chem. 263*, 11189−11195.
Petersen, L. C., Boel, E., Johannessen, M., & Foster, D. (1990) *Biochemistry 29*, 3451.
Phillips, M. A., Kaplan, A. P., Rutter, W. J., & Bartlett, P. A. (1992) *Biochemistry 31*, 959−963.
Polgar, L. (1989) *Mechanisms of Protease Action*, CRC Press, Inc., Boca Raton, FL.
Rypniewski, W. R., Perrakis, A., Vorgias, C. E., & Wilson, K. S. (1994) *Prot. Eng. 7*, 57−64.
Sandberg, W. S., & Terwillinger, T. C. (1989) *Science 245*, 54−57.
Serrano, L., Kellis, J. T., Jr., Cann, P., Matouschek, A., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 783−804.
Shortle, D., Stites, W. E., & Meeker, A. K. (1990) *Biochemistry 29*, 8033−8041.
Silverberg, M., & Kaplan, A. P. (1982) *Blood 60*, 64.
Sprang, S., Standing, T., Fletterick, R. J., Stroud, R. M., & Finer-Moore, J., Xuong, N.-H., Hamlin, R., Rutter, W. J., & Craik, C. S. (1987) *Science 237*, 905−909.
Stein, R. L., Strimpler, A. M., Hori, H., & Powers, J. C. (1987) *Biochemistry 26*, 1301−1305.
Thompson, R. C. (1973) *Biochemistry 12*, 47−51.
Thompson, R. C., & Blout, E. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1734−1740.
Vincent, J. P., & Lazdunski, M. (1972) *Biochemistry 11*, 2967−2977.
Vincent, J. P., & Lazdunski, M. (1976) *FEBS Lett. 63*, 240−244.
Waldburger, C. D., Schildbach, J. F., & Sauer, R. T. (1995) *Nat. Struct. Biol. 2*, 122−128.
Wilke, M. E., Higaki, J. N., Craik, C. S., & Fletterick, R. J. (1991) *J. Mol. Biol. 219*, 525−532.
Wolfenden, R. (1976) *Annu. Rev. Biochem. Bioeng. 5*, 271−306.
Zerner, B., Bond, R. P. M., & Bender, M. L. (1964) *J. Am. Chem. Soc. 86*, 3674−3679.

BI951928K