Activating a Zymogen without Proteolytic Processing: Mutation of Lys15 and Asn194 Activates Trypsinogen†

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Received April 27, 1998; Revised Manuscript Received September 22, 1998

ABSTRACT: The zymogen and mature enzyme forms of trypsin-like serine proteases exhibit a wide range of activities. The prototypical trypsinogen−trypsin system is an example of a minimally active zymogen and a maximally active mature protease. The present work identifies several features of trypsinogen which govern its activity. Our results indicate that rat trypsin is 10^8-fold more active than rat trypsinogen. Rat trypsinogen appears to be less active than bovine trypsinogen. His40 is believed to be an important determinant of zymogen activity. We are unable to verify this role for His40 in trypsinogen since the mutation of His40 to Phe appears to change the trypsin−substrate interface. Deletion of the N-terminal Ile16 from trypsin is expected to produce a trypsinogen-like protein since the Ile16−Asp194 salt bridge cannot form. Such mutants have higher activity and BPTI affinity than trypsinogen, which indicates that the activation peptide stabilizes the inactive trypsinogen conformation. The mutation of Lys15 to Ala increases the BPTI affinity and activity of trypsinogen to an even greater extent; thus, removal of Lys15 can account for the effect of the loss of the activation peptide. These results suggest that Lys15 is an important determinant of zymogen activity. The mutation of Asp194 to Asn also increases the BPTI affinity and activity of trypsinogen. This result suggests that in addition to stabilizing the active conformation of trypsin via the Ile16−Asp194 salt bridge, Asp194 also maintains the inactive conformation of trypsinogen. A correlation exists between the values of k_{cat}/K_m and BPTI affinity of mutant trypsinogens and trypsins. However, the slope of this correlation is 0.64, which indicates that different "active" conformations are involved in BPTI binding and substrate hydrolysis. ΔI16V17 trypsinogen is the lone outlier; its BPTI affinity is higher than would be expected based on the value of k_{cat}/K_m. We show that the rate of BPTI association is slower for ΔI16V17 trypsinogen than for a mutant trypsinogen with a similar BPTI affinity. This observation suggests that BPTI binds to an "active" trypsinogen conformation that is not kinetically accessible to substrates.

Serine proteases of the trypsin family are important elements in many physiological processes. The zymogen and mature protease forms possess varying degrees of activity depending on their regulatory functions. We would like to delineate the structural features which govern activity of zymogens and mature serine proteases. In this work we have uncovered two structural features that maintain trypsinogen in an inactive state.

Trypsinogen is activated by proteolytic cleavage of its N-terminus which releases the activation peptide (residues 8−15) (Figure 1). The α-amino group of the new N-terminus, Ile16, forms a salt bridge with the Asp194 side chain carboxylate group, triggering a conformational change which produces active enzyme. Similar proteolytic processing activates other members of the trypsin family, including the zymogens involved in blood coagulation and fibrinolysis. Approximately 85% of the structures of trypsinogen and trypsin are identical (*1*−*3*). However, the S1 binding site and the oxyanion hole of trypsinogen are unformed, which renders the zymogen inactive. The S1 binding site and oxyanion hole become structured when the Ile16−Asp194 salt bridge forms. This conformational change involves four segments: 16−19, 142−152, 184−194, and 216−223, termed the activation domain (*3*). These residues, with the exception of Asp194, are disordered in trypsinogen. Thus, the conversion of trypsinogen into trypsin is analogous to the folding of denatured protein.

In contrast to the rest of the activation domain, Asp194 appears to be ordered in trypsinogen, although two different positions have been observed. In one crystal structure, Asp194 is rotated 170° from its position in trypsin, where it interacts with His40 as part of the "zymogen triad" which includes Ser32 (*1, 4*). The interaction between His40 and Asp194 has also been observed in other zymogens, including chymotrypsinogen (*5*) and proproteinase E (*6*). However, Asp194 is found in a position similar to that in trypsin in another structure of trypsinogen. Here, Asp194 is unable to interact with His40, and instead forms hydrogen bonds to Ser190 and water molecules (*2*). NMR experiments have also failed to detect an interaction between His40 and Asp194

† This work was supported by NIH Grant HL50366 (L.H.) and a grant from the Lucille P. Markey Charitable Trust to Brandeis University. L.H. is a Searle Scholar and a Beckman Young Investigator.

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10.1021/bi980951d CCC: $15.00
Published on Web 10/26/1998

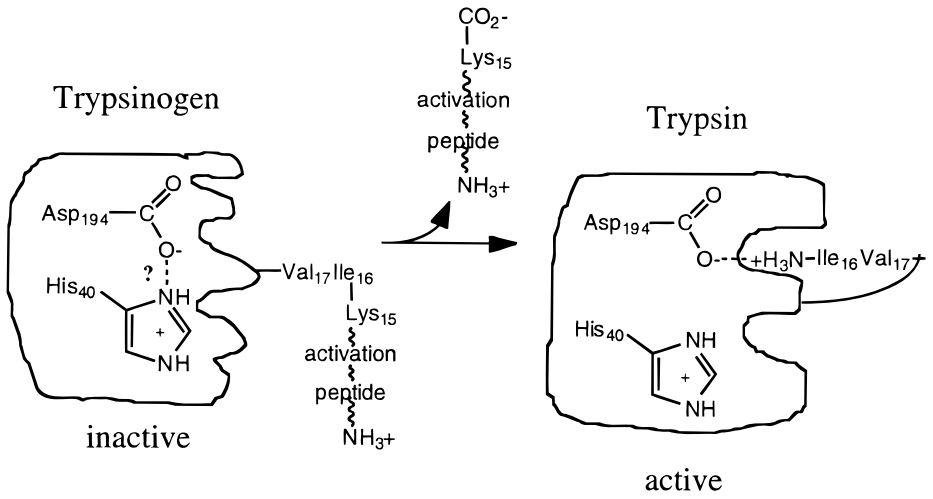

FIGURE 1: Conversion of trypsinogen into trypsin.

in trypsinogen, although such an interaction can be detected in the case of chymotrypsinogen (*7*, *8*).

Bovine pancreatic trypsin inhibitor (BPTI)[1] binds to trypsin with $K_i = 10^{-10}-10^{-13}$ M depending on the source (*9*, *10*). BPTI also binds to trypsinogen, although with much lower affinity than to trypsin ($K_i = 10^{-6}$ M for bovine trypsinogen) (*11*). Upon binding to BPTI, trypsinogen assumes a conformation almost identical to that of trypsin, except that the activation peptide is disordered, the residues of the activation domain display greater thermal motion, and a cavity exists where Ile16 is located in trypsin (*12*). 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 $K_{eq} = 10^7$ in favor of the inactive conformation for trypsinogen, as estimated from the difference in the BPTI affinities of bovine trypsin and trypsinogen (*12*). The magnitude of this free energy change between active and inactive conformations of trypsinogens can be accounted for in part by the absence of a compensating charge for Asp194 and the presence of a cavity where Ile16 binds (*10*, *12*, *13*). In addition, the inactive trypsinogen conformation may be stabilized by the interaction of Asp194 with His40 and by the presence of the activation peptide.

Several trypsin-like zymogens possess considerable activity. For example, the zymogen form of t-PA has at least 10% of the activity of the mature form (*14*), and the zymogen of u-PA has 1% of the activity of the mature form (*15*). These observations demonstrate that alternative mechanisms exist to stabilize the active zymogen conformation in the absence of a free N terminus. In t-PA, Lys156[2] forms a salt bridge with Asp194 in the same manner as observed in the structure of the trypsinogen−PSTI complex (*16*−*20*), and in u-PA, Lys143 is believed to play this role (*21*). In addition, residue 40 is Phe in t-PA, which would favor the active zymogen conformation by eliminating the interaction between His40 and Asp194. Replacement of residue 40 with His in t-PA selectively decreases the activity of the zymogen (*4*, *22*). This observation suggests that the interaction between Asp194 and His40 can stabilize the inactive zymogen conformation.

In this work, we probe the importance of Asp194, His40, and the activation peptide in maintaining the inactive conformation of trypsinogen. The equilibrium between active and inactive conformations of mutant trypsinogens and trypsins was determined by monitoring BPTI affinity and activity. Our results suggest that both Asp194 and Lys15, but not His40, stabilize the inactive conformation of trypsin.

MATERIALS AND METHODS

Materials. 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 and SBTI−Sepharose were obtained from Sigma Chemical Co. BPTI was obtained from Boehringer. N-Terminal sequencing was performed by the Tufts Medical School Protein Sequencing Facility.

Construction of Trypsinogen Mutants. Site-directed mutagenesis was performed using the method of Kunkel as described previously (*23*, *24*) to make the mutants S195A trypsinogen, ΔI16 trypsinogen, ΔI16V17 trypsinogen, D194N trypsinogen, H40F trypsinogen, ΔI16V17/H40F trypsinogen, K15A trypsinogen, and I16G trypsinogen. ΔI16V17/S195A trypsinogen, ΔI16V17/D194N trypsinogen, ΔI16/D194N trypsinogen, and I16G/D194N trypsinogen were constructed by combining *Bam*HI/*Bal*I fragments of appropriate single mutants. Mutants were completely sequenced to ensure that only the desired mutations were introduced.

Expression and Purification of Trypsinogen and Trypsin Mutants. Recombinant rat trypsinogen II was produced as an α-factor fusion protein in a *Saccharomyces cerevisiae* expression system from the pYT plasmid as previously described (*24*). Trypsinogens were purified from the culture media by cation exchange chromatography using a Toyopearl 650M column (Suppelco), and were further purified by an HQ anion exchange column on a PerSeptive Biosystems Biocad Sprint perfusion chromatography workstation (Framingham, MA). Trypsinogens were stored in 1 mM HCl. Trypsinogen concentration was determined by the

[1] Abbreviations: AMC, 7-amino-4-methylcoumarin; SBzl, thiobenzyl; Z, carbobenzoxy; pNP, *p*-nitrophenyl; BPTI, bovine pancreatic trypsin inhibitor; PSTI, porcine pancreatic secretory trypsin inhibitor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

[2] Chymotrypsinogen numbering is used throughout.

absorbance at 280 nm ($\epsilon = 34\,800$ M^{-1} cm^{-1}). Mutant trypsins were obtained by activating the appropriate trypsinogen with enterokinase and purified by affinity chromatography on SBTI resin as previously described (*24*). Mutant trypsins that did not bind to SBTI resin were purified by anion exchange chromatography on an HQ column (PerSeptive Biosystems).

Activity of Trypsinogens. Substrate stock solutions were prepared in water or dimethylformamide. The final concentration of dimethylformamide in the assays was less than 2%, except in the case of substrate D-Val-Leu-Lys-SBzl in which the concentration was 4%. Assay mix contained 100 mM NaCl, 10 mM $CaCl_2$, and 50 mM Hepes, pH 8.0. Hydrolysis of the AMC substrates was monitored fluorimetrically, with excitation at 380 nm and emission at 460 nm. Assays were performed in 2.0 mL of assay mix containing substrate in a stirred cell at 25 °C with a Hitachi F2000 spectrofluorimeter. The values of k_{cat}, K_m, and k_{cat}/K_m were determined from seven substrate concentrations between 5 and 570 μM as appropriate for the particular mutant trypsinogen. The hydrolysis of SBzl substrates was monitored spectrophotometrically at 324 nm using a Hitachi U2000 spectrophotometer. Assays were performed in 0.5 or 1.0 mL of assay mix containing 0.03−1.5 mM substrate 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.

Determination of the BPTI Affinity of Trypsinogen and Trypsin Mutants. Inhibition of ΔI16V17 trypsinogen, ΔI16V17/S195A trypsinogen, S195A trypsinogen, and ΔI16V17/D194N trypsinogen was studied using a Microcal Omega titration calorimeter from Microcal, Inc. (Northampton, MA) (*25*). Trypsinogen and BPTI solutions were dialyzed in the same beaker against 50 mM Hepes, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0, and degassed under vacuum. Ten microliter injections of 2−7.4 mM BPTI from a 250 μL syringe were added to a 100−390 μM solution of trypsinogen in a 1.4 mL sample cell. Injections of 15 s duration were made at 4 min intervals for ΔI16V17/D194N trypsinogen, but at 10 min intervals for the other mutants. ΔI16V17 trypsinogen and ΔI16V17/S195A trypsinogen required 10 min intervals in order for the binding reaction to reach equilibrium. Heats of reaction were obtained by integration of the peaks. The control heat of dilution of BPTI into the sample cell containing only buffer was measured and then subtracted from the heats of the reaction between trypsinogen and BPTI. The stoichiometry constant n was fixed to 1.00, and the binding constant K_d and enthalpy of binding ΔH were obtained from the curve fit to the integrated data using the equation for a ligand binding to a macromolecule in one site using the nonlinear least-squares algorithm included in the ORIGIN software accompanying the instrument (*25*). The affinity of BPTI for ΔI16V17/D194N trypsinogen was also determined by measuring the inhibition of substrate hydrolysis, as was the affinity of H40F trypsin and ΔI16 trypsin (*10*). The binding constants from these two methods are in agreement.

Rate Constants of BPTI Binding to Trypsinogen Mutants. Trypsinogen (5 μM) and BPTI (50−400 μM) were rapidly mixed on a BioSX.17M sequential stopped-flow spectrofluorimeter (Applied Photophysics). The change in fluorescence with time was recorded in volts using a 320 nm cutoff emission filter and an excitation wavelength of 280 nm. Data were fit using SX17.MV Kinetic Spectrometer Workstation Software (Applied Photophysics). The data for ΔI16V17 trypsinogen were fit to a single exponential of the form: $F = F_o + F_1\, e^{-k_{obs}t}$. The data for ΔI16V17, D194N trypsinogen did not fit a single exponential, but rather a double exponential of the form: $F = F_0 + F_1\, e^{-k_{obs1}t} + F_2\, e^{-k_{obs2}t}$.

RESULTS

Characterization of Recombinant Trypsinogens. The experiments described here utilize recombinant anionic rat trypsinogen II, which is expressed as a fusion protein to the α-factor leader sequence in *S. cerevisiae* (*24*). Amino acid sequencing revealed that the recombinant trypsinogen contained two additional N-terminal residues, Glu-Ala, before the trypsinogen sequence. These added residues arise from incomplete processing of the α-factor leader sequence by dipeptidyl aminopeptidase (*26*). The trypsinogen preparation appeared homogeneous by N-terminal sequencing (<5% N-terminal Phe, the N-terminus of completely processed trypsinogen). Trypsins were obtained after processing with enterokinase. The N-terminal sequences of ΔI16V17 trypsin and ΔI16 trypsin were confirmed by N-terminal sequencing.

Unfortunately, rat trypsinogen rapidly autoactivates at neutral pH; autoactivation is also observed under the acidic conditions used for storage. This autoactivation can be attributed to the presence of trypsin—once formed, trypsin will rapidly convert trypsinogen to trypsin. Therefore, we were unable to characterize wild-type trypsinogen directly, and instead have characterized S195A trypsinogen, K15A trypsinogen, I16G trypsinogen, and ΔI16V17 trypsinogen as models for wild-type trypsinogen. Ser195 is the catalytic serine; its mutation will inactivate the enzyme, preventing autoactivation. The mutation of Lys15 changes the propeptide cleavage site so that it is no longer recognized by trypsin, thus preventing autoactivation. The mutation and/or deletion of Ile16 also inhibits autoactivation because the trypsin forms of such mutants are at least 10^4-fold less active than the wild-type trypsin [Table 2; (*10*)]. Thus, even if the mature enzyme forms, it will not catalyze further activation as does wild-type trypsin.

BPTI Affinity of Mutant Trypsinogens and Trypsins. The formation of a complex between BPTI and bovine trypsinogen induces trypsinogen to adopt a conformation resembling trypsin (*3*, *12*). Since substrate hydrolysis by trypsinogen presumably also occurs via a trypsin-like conformation, the affinity of trypsinogen for BPTI should correlate with activity. Such a correlation is observed for trypsin mutants containing substitutions at Ile16 (*10*). Therefore, we measured the affinity of the mutant trypsinogens and trypsins for BPTI. Two kinds of measurements were undertaken: (A) Isothermal titration calorimetry. This method does not rely on the activity of the mutant trypsinogens and can therefore be used to measure the BPTI affinity of trypsinogens containing the S195A mutation. Figure 2 shows data from the calorimetric titration of BPTI binding to ΔI16V17/D194N trypsinogen as an example. From the binding isotherm (Figure 2b), the value of the dissociation constant (K_d), the number of binding sites per trypsinogen molecule (n), and the enthalpy (ΔH) of binding can be obtained (*25*). In these experiments, n is fixed at 1.0 since the stoichiometry

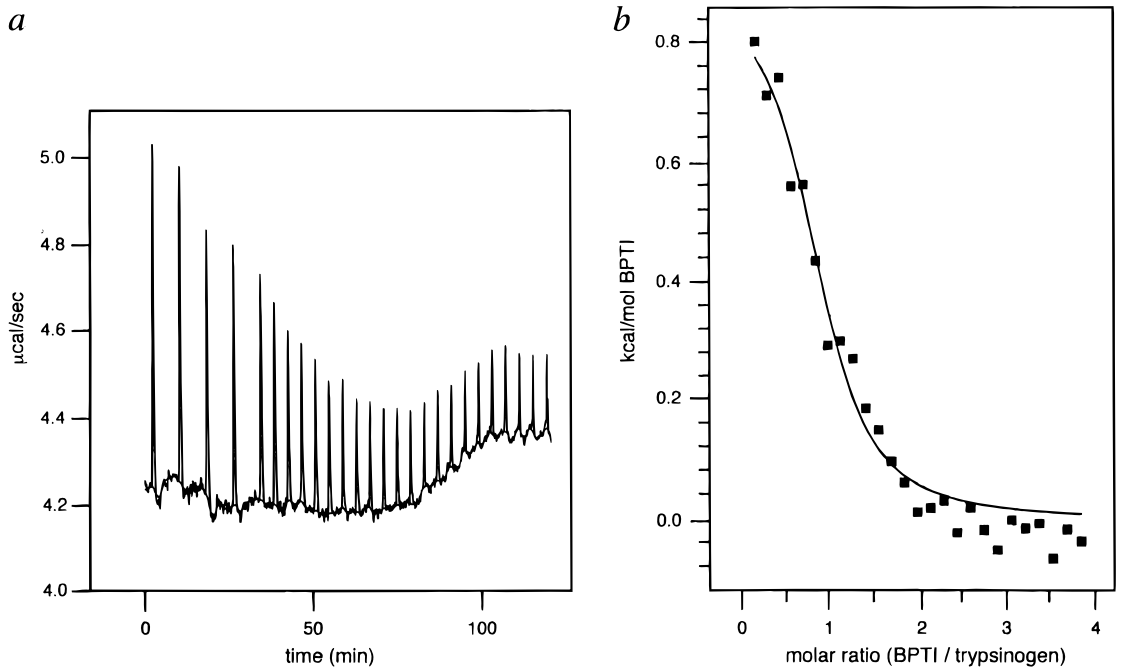

FIGURE 2: Microcalorimetric titration of ΔI16V17/D194N trypsinogen with BPTI. (*a*) Raw data; each peak corresponds to one 10 μL injection of 2.15 mM BPTI into 120 μM ΔI16V17/D194N trypsinogen. The first five injections were performed at 8 min intervals; the following injections were performed at 4 min intervals. (*b*) Integrated data from (*a*), fitted to the titration curve using the ORIGIN program.

Table 1: BPTI Binding to Mutant Trypsinogens[a]

enzyme	K_d (μM)	ΔH (kcal/mol)	relative K_d (mutant/trypsin)
S195A trypsinogen	$\geq 300^a$	≥ 3	7×10^5
K15A trypsinogen	12 ± 1^b	na[d]	3×10^4
I16G trypsinogen	110 ± 30^a	3.1 ± 0.3	2×10^5
ΔI16V17 trypsinogen	9 ± 8^a	1.04 ± 0.12	2×10^4
ΔI16V17/S195A trypsinogen	33 ± 3^a	1.70 ± 0.07	6×10^4
D194N/S195A trypsinogen	93 ± 9^a	2.8 ± 0.1	2×10^5
ΔI16V17/D194N trypsinogen	12 ± 3^a	0.85 ± 0.07	3×10^4
	29 ± 2^b	na	6×10^4
ΔI16 trypsin	33 ± 6^b	na	6×10^4
H40F trypsin	$\leq 0.00001^b$	na	≤ 0.02
D194N trypsin	$0.071^{b,c}$	na	160
I16G trypsin	$0.39^{b,c}$	na	890
trypsin	$0.00044^{b,c}$	na	−

[a,b] The K_d for dissociation of the following BPTI complexes were determined either by (*a*) isothermal titration calorimetry or by (*b*) monitoring the inhibition of enzyme activity as described under Materials and Methods. [c] Data from Hedstrom et al. (1996) (*10*). [d] Not applicable.

of BPTI binding to trypsinogen is known. ΔH and K_d for BPTI binding to trypsinogen mutants are listed in Table 1. ΔH is positive for all of the mutant trypsinogens; therefore, BPTI binding is driven by a positive ΔS. The binding of bovine trypsinogen to PSTI is also entropy-driven (*27*). (B) Competitive inhibition experiment. This method was used to measure the BPTI affinity of mutant enzymes with substantial activity, such as ΔI16 trypsin and ΔI16V17/D194N trypsinogen. The values of K_d determined by different methods are in good agreement (Table 1).

Mutation of Lys15 Increases the BPTI Affinity of Trypsinogen. The value of K_d for the BPTI complex with S195A trypsinogen is too high to measure; a lower limit of 300 μM is estimated from the binding isotherm (data not shown) (Table 1). To determine the effect of the S195A mutation on the BPTI affinity of trypsinogen, the value of K_d for the BPTI complex with ΔI16V17/S195A trypsinogen was measured; this mutation decreases BPTI affinity by 3-fold relative to ΔI16V17 trypsinogen, which indicates that the mutation of Ser195 has little effect on BPTI affinity. Assuming that the mutation of Ser195 would have a similar effect on wild-type trypsinogen, a value of $K_d = 100$ μM can be estimated. This value is most similar to the K_d of I16G trypsinogen, which suggests that this mutant is a good model for wild-type trypsinogen. In contrast, the values of K_d for complexes with both K15A trypsinogen and ΔI16V17 trypsinogen are substantially lower than 100 μM. Therefore, neither of these frameworks is a good model of trypsinogen. The increased affinity of K15A suggests that Lys15 is a structural determinant of zymogenicity.

Mutation of Asp194 Increases the BPTI Affinity of Trypsinogen. To probe the role of Asp194 in maintaining the inactive conformation of trypsinogen, we measured the BPTI affinity of D194N/S195A trypsinogen and ΔI16V17/D194N trypsinogen. The S195A framework was chosen as the most similar to wild-type trypsinogen, while the ΔI16V17 framework was chosen because the value of K_d of BPTI for this mutant is low enough to be readily measured. The value of K_d for the BPTI complex with D194N/S195A trypsinogen is at least 3-fold lower than that of S195A trypsinogen. This observation suggests that Asp194 is also involved in maintaining the inactive conformation of trypsinogen. However, the addition of D194N to the ΔI16V17 framework had no effect on BPTI affinity. We show below that the BPTI affinity of ΔI16V17/D194N trypsinogen correlates with activity, while the BPTI affinity of ΔI16V17 trypsinogen is anomalous.

The Presence of the Activation Peptide Stabilizes the Inactive Conformation of Trypsinogen. To assess the importance of the activation peptide in maintaining the inactive conformation of trypsinogen, the BPTI affinity of ΔI16 trypsin was measured. This mutant trypsin should have a trypsinogen-like conformation because the Ile16−Asp194

Table 2: Hydrolysis of Amide Substrates by Mutant Trypsinogens and Trypsins[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	rel k_{cat}/K_m
		Tos-Gly-Pro-Arg-AMC		
I16G trypsinogen	$\geq 5 \times 10^{-5}$	$\geq$1400	0.069 ± 0.011	1×10^8
K15A trypsinogen	$(5 \pm 2) \times 10^{-4}$	1200 ± 400	4.4	2×10^6
ΔI16 trypsinogen	$\geq 3 \times 10^{-5}$	$\geq$1100	$\leq$0.033	$\geq 3 \times 10^8$
ΔI16V17 trypsinogen	$(2.8 \pm 0.6) \times 10^{-5}$	1200 ± 500	0.025 ± 0.003	4×10^8
I16G/D194N trypsinogen	$(5.7 \pm 0.2) \times 10^{-5}$	300 ± 20	0.19 ± 0.01	5×10^7
ΔI16V17/D194N trypsinogen	$(7.1 \pm 0.1) \times 10^{-5}$	18 ± 2	3.9 ± 0.1	2×10^6
ΔI16V17/H40F trypsinogen	$(8 \pm 1) \times 10^{-5}$	900 ± 300	0.084 ± 0.007	1×10^8
ΔI16 trypsin	$\geq$0.006	$\geq$3000	1.9 ± 0.1	5×10^6
ΔI16V17 trypsin	0.0011 ± 0.0003	1400 ± 200	0.8 ± 0.1	1×10^7
H40F trypsin	38 ± 3	1.6 ± 0.3	$(2.5 \pm 0.2) \times 10^7$	0.4
I16G trypsin[c]	0.39 ± 0.05	700 ± 70	562 ± 7	2×10^4
D194N trypsin[c]	5.8 ± 0.5	100 ± 20	$(5.4 \pm 0.4) \times 10^4$	200
trypsin[c]	29 ± 1	3.5 ± 0.5	$(9 \pm 1) \times 10^6$	–
		Tos-Gly-Pro-Lys-AMC		
I16G trypsinogen	nd	nd	$\leq$0.06	$\geq 10^7$
K15A trypsinogen	$(1.3 \pm 0.3) \times 10^{-3}$	4000 ± 2000	0.34 ± 0.07	4×10^6
ΔI16 trypsinogen	nd	nd	nd	nd
ΔI16V17 trypsinogen	$(3.0 \pm 0.2) \times 10^{-6}$	160 ± 20	0.019 ± 0.003	7×10^7
I16G/D194N trypsinogen	$(1.3 \pm 0.2) \times 10^{-5}$	300 ± 80	0.044 ± 0.004	3×10^7
ΔI16V17/ D194N trypsinogen	$(1.2 \pm 0.1) \times 10^{-5}$	51 ± 5	0.24 ± 0.02	6×10^6
ΔI16V17/ H40F trypsinogen	$(8 \pm 2) \times 10^{-6}$	270 ± 60	0.031 ± 0.008	4×10^7
ΔI16 trypsin	$\geq$0.003	$\geq$3000	1.3 ± 0.3	1×10^6
ΔI16V17 trypsin	$\geq$0.007	$\geq$1000	0.67 ± 0.01	2×10^6
H40F trypsin	49 ± 1	15 ± 1	$(3.2 \pm 0.2) \times 10^6$	0.4
D194N trypsin	1.9 ± 0.2	146 ± 30	$(1.3 \pm 0.1) \times 10^4$	100
I16G trypsin	$>$0.1	$>$1.3	114 ± 7	1×10^4
trypsin[c]	16.9 ± 0.8	12.3 ± 1.2	$(1.36 \pm 0.05) \times 10^6$	–

[a] Michaelis–Menten parameters are listed below for wild-type and mutant trypsinogens. 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. "rel k_{cat}/K_m" is calculated from the ratio of the k_{cat}/K_m of wild-type trypsin to that of the mutant. [b] nd, no data. [c] Data are from Hedstrom et al. (1996) (*10*).

salt bridge cannot form. The value of K_d for the BPTI complex with ΔI16 trypsin is at least 3-fold lower than that estimated for trypsinogen. This observation indicates that the presence of the activation peptide favors the inactive conformation of trypsinogen. However, this effect is smaller than the decrease in K_d upon mutation of Lys15.

The Mutation of His40 to Phe Increases the BPTI Affinity of Trypsin. To assess the importance of His40 in determining the zymogenicity of trypsinogen, we constructed H40F trypsin. The value of K_d for the BPTI complex with this mutant is at least 40-fold less than that of wild-type trypsin. Such an increase in BPTI affinity cannot be attributed to a change in zymogenicity; i.e., the conformation of trypsin cannot become more trypsin-like. His40 is located near the S1′–S3′ sites of trypsin; we believe that this mutation has changed the structure of these sites such that they are more complementary to BPTI. This observation suggests that mutations of His40 will also perturb the enzyme–substrate interface. Therefore, the effects of substitutions of His40 on the zymogenicity of trypsinogen will be difficult to interpret.

The Hydrolysis of Amide Substrates by Trypsinogen. Table 2 summarizes the steady-state kinetic data for the hydrolysis of Tos-Gly-Pro-Arg-AMC and Tos-Gly-Pro-Lys-AMC by the mutant trypsinogens, ΔI16 trypsin, ΔI16V17 trypsin, and H40F trypsin. The parameters for trypsin, D194N trypsin, and I16G trypsin are included for comparison. Tos-Gly-Pro-Arg-AMC is the best amide substrate of trypsin, and the fluorometric AMC assay is extraordinarily sensitive. Nonetheless, high concentrations of trypsinogen (as much as 85 μM) were required in order to measure the activity of the trypsinogen mutants. Under these conditions, less than one turnover of substrate hydrolysis was observed. Serine proteases hydrolyze substrates via an acylenzyme intermediate. If formation of the acylenzyme intermediate is slow relative to the deacylation, then steady-state rates of AMC release will be attained during the first turnover. However, if deacylation is the slow step, then a burst of AMC production will be observed in the first turnover, followed by a slower steady-state rate; in this case, the values of Table 2 for the hydrolysis of Tos-Gly-Pro-AMC will represent upper limits for the activity of the trypsinogen mutants. It is likely that these values do represent steady-state velocities. Multiple turnovers are observed during the hydrolysis of Z-Arg-SBzl and similar thiobenzyl esters by the trypsinogen mutants; therefore, steady-state rates can be measured with confidence for these substrates (Table 3). The value of k_{cat} for the hydrolysis of Tos-Gly-Pro-Arg-AMC by ΔI16V17 trypsinogen is much less than the value of k_{cat} for the

Table 3: Hydrolysis of Ester Substrates by ΔI16V17 Trypsinogen[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	rel k_{cat}/K_m
		Z-Arg-SBzl		
ΔI16V17 trypsinogen	$\geq$0.1	$\geq$8000	1.6 ± 0.4	1×10^7
trypsin[b]	71 ± 1	4 ± 1	$(1.8 \pm 0.4) \times 10^7$	–
		Z-Lys-SBzl		
ΔI16V17 trypsinogen	$\geq$0.1	$\geq$8000	1.6 ± 0.4	1×10^7
trypsin[b]	128 ± 4	9.5 ± 0.7	$(1.4 \pm 0.1) \times 10^7$	–
		D-Val-Leu-Lys-SBzl		
ΔI16V17 trypsinogen	$\geq$0.07	$\geq$10000	7.2 ± 0.5	5×10^5
trypsin[b]	18 ± 1	5.5 ± 0.7	$(3.6 \pm 0.3) \times 10^6$	–

[a] Conditions as in Table 2. [b] Data are from Hedstrom et al. (1996) (*10*).

hydrolysis of Z-Arg-SBzl (10^{-5} s^{-1} versus 0.1 s^{-1}; see Table 3). This observation suggests that the acylation step is rate-determining (if the deacylation step were rate-determining, the values of k_{cat} for amide and ester hydrolysis would be similar; for example, compare the values of k_{cat} for trypsin). Therefore, it is likely that these experiments measure steady-state rates of substrate hydrolysis by trypsinogen.

It is important to substantiate that the activities reported in Table 2 are the properties of the mutant trypsinogens rather than the result of a contaminating protease. Unfortunately, this possibility can never be completely eliminated. Two sources of contaminating activity can be considered: fortuitous proteases and undesired activation of trypsinogen. Several observations indicate that the enzyme activities reported in Table 2 are not due to fortuitous proteases: (A) If a fortuitous protease originated from copurification of a yeast enzyme, similar amounts would be present in all of the mutant trypsinogens. However, the activity of a trypsinogen mutant containing ΔI16V17/C191A/C220A is at least 10-fold less active than the trypsinogen mutants reported here (X. Liu and L. Hedstrom, data not shown). Cys191 and Cys220 form a disulfide bridge which cross-links the S1 binding site of trypsin. This mutant is purified by the same methods as the other trypsinogen mutants; if a yeast protease copurified with trypsinogen, it should also be present in the preparation of ΔI16V17/C191A/C220A trypsinogen. Since this mutant is less active than the other mutant trypsinogens, it is unlikely that a yeast protease copurifies with the trypsinogen. (B) If a fortuitous protease originated from contaminants in the yeast culture (e.g., bacteria), the activities of a given mutant trypsinogen would vary from one preparation to another. Such variation is not observed. The presence of undesired activation to trypsin is more difficult to eliminate. A 0.01% contamination of I16G trypsin is sufficient to account for the activity of I16G trypsinogen. However, 3% contamination of ΔI16 trypsin is required to account for the activity of ΔI16 trypsinogen, and 10% contamination of ΔI16V17 trypsin is required to account for the activity of ΔI16V17 trypsinogen. While it is impossible to observe these amounts of activated trypsinogen by SDS−PAGE or similar techniques, it is extremely unlikely that more activation will be observed in the ΔI16 trypsinogen and ΔI16V17 trypsinogen preparations than in the I16G trypsinogen preparation. Further, the similarity of the activities of the three mutant trypsinogens and the reproducibility of activity between different preparations of mutant trypsinogens argue strongly that the observed activities are the properties of the mutant trypsinogens.

The Zymogenicity of Trypsinogen Is ~10^8. Zymogenicity has been defined as the ratio of the values of k_{cat}/K_m of mature enzyme and zymogen (*4*). We estimated the zymogenicity of trypsinogen from the values of k_{cat}/K_m for the hydrolysis of Tos-Gly-Pro-Arg-AMC. The activities of I16G trypsinogen, ΔI16V17 trypsinogen, and ΔI16 trypsinogen are similar to each other (Table 2). Assuming that I16G trypsinogen is the best model for wild-type trypsinogen, the zymogenicity (ratio of k_{cat}/K_m of mature form to zymogen) is ~10^8. The values of k_{cat} are ~10^6-fold less and values of K_m are ~400-fold greater than trypsin. Thus, trypsinogen is approximately 100-fold less active than a mutant trypsin containing Ala substitutions in the catalytic triad (*28*). This observation illustrates the importance of the substrate binding site and oxyanion hole in controlling the activity of trypsin. Similar results are obtained when the hydrolysis of Tos-Gly-Pro-Lys-AMC is monitored (Table 2).

Hydrolysis of Thiobenzyl Ester Substrates by ΔI16V17 Trypsinogen. Previous reports indicate that bovine trypsinogen is 10^5-fold less active than bovine trypsin as measured by of the hydrolysis of *p*-nitrophenyl esters (*29*−*31*). Since rat trypsin and bovine trypsin have similar activities on both ester and amide substrates, this result suggests that rat trypsinogen may be intrinsically less active than bovine trypsinogen. However, it also seemed likely that this discrepancy results from the greater intrinsic reactivity of ester substrates. We have previously observed that mutations of trypsin usually have a smaller effect on ester hydrolysis than amide hydrolysis (*10*, *32*). Therefore, we assayed the hydrolysis of thiobenzyl ester substrates by ΔI16V17 trypsinogen (Table 3). This mutant was chosen because its activity was similar to I16G trypsinogen and larger quantities of this enzyme were available. While ΔI16V17 trypsinogen is ~10^8-fold less active than trypsin as measured by amide hydrolysis, it is 10^7-fold less active than trypsin as measured by the hydrolysis of Z-Lys-SBzl and Z-Arg-SBzl, and only 10^6-fold less active as measured by the hydrolysis of D-Val-Leu-Lys-SBzl. These results indicate that the zymogenicity of rat trypsinogen is decreased to 10^6−10^7 when esterase activity is monitored. Nevertheless, the zymogenicity of rat trypsinogen remains greater than that of bovine trypsinogen. This observation suggests that rat trypsinogen is intrinsically less active than bovine trypsinogen.

The Effect of Mutations of the Activation Peptide and Lys15 on Zymogenicity. Zymogenicity can be decreased by two mechanisms: an increase in the activity of the zymogen or a decrease in the activity of the mature enzyme. We are only interested in mutations which increase the activity of the trypsinogen. We expect that these will correspond to the mutations that increase BPTI affinity.

To assess the importance of the activation peptide in determining zymogenicity, the activity of two N-terminal truncated trypsin mutants was determined. Although ΔI16 trypsin and ΔI16V17 trypsin are approximately 10^7-fold less active than trypsin as measured by the values of k_{cat}/K_m for amide hydrolysis, they are nevertheless at least 10-fold more active than I16G trypsinogen as measured by k_{cat}/K_m (Table 2). As in the case of BPTI affinity, this effect can be attributed to loss of Lys15. The value of k_{cat}/K_m for K15A trypsinogen is 64-fold greater than that of I16G trypsinogen. Thus, ΔI16 trypsin and ΔI16V17 trypsin are less active than K15A trypsinogen. These observations indicate that Lys15 is an important determinant of zymogenicity.

Asp194 Stabilizes the Inactive Conformation of Trypsinogen. Both ΔI16V17/D194N trypsinogen and I16G/D194N trypsinogen are more active than their counterparts containing Asp194 (Table 2). The value of k_{cat}/K_m for the hydrolysis of Tos-Gly-Pro-Arg-AMC by I16G/D194N trypsinogen is 3-fold greater than I16G trypsinogen while the value of k_{cat}/K_m for the hydrolysis of Tos-Gly-Pro-Arg-AMC by ΔI16V17/D194N trypsinogen is 160-fold greater than that of ΔI16V17 trypsinogen. Thus, Asp194 is also a structural determinant of zymogenicity. These results contrast with the 170-fold decrease in activity when Asp194 is changed to Asn in trypsin (*10*). Interestingly, only a 12-fold increase in k_{cat}/K_m is observed for the hydrolysis of Tos-Gly-Pro-Lys-AMC

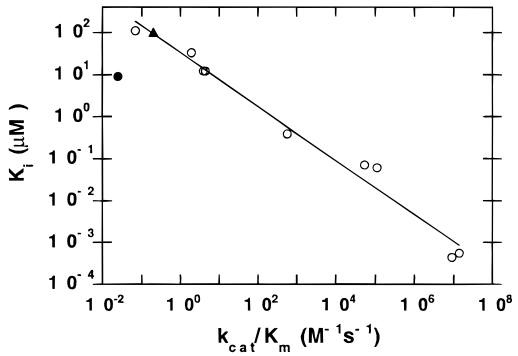

FIGURE 3: Correlation between BPTI affinity and activity of the mutant trypsinogens and trypsins. Data from Tables 1 and 2. The solid line is the fit to the open circles, slope = −0.64, $r = 0.98$. The data point for ΔI16V17 trypsinogen is represented by the closed circle. The extrapolation of the fit to $K_d = 300$ μM, the lower limit of BPTI affinity of S195A trypsinogen, is denoted by the triangle.

when the Asp194Asn mutation is placed in the ΔI16V17 trypsinogen framework. This observation suggests that the mutation of Asp194 to Asn specifically increases the hydrolysis of Arg-containing substrates.

His40 Does Not Appear To Control the Zymogenicity of Trypsinogen. In contrast to Lys15 and Asp194, the mutation of His40 to Phe appears to have little effect on zymogenicity. This mutation increases the value of k_{cat}/K_m by 2-fold in the ΔI16V17 trypsinogen framework. However, the value of k_{cat}/K_m also increases 2-fold in trypsin; zymogenicity is therefore unchanged. This modest increase in activity of both trypsinogen and trypsin can probably be attributed to changes in the enzyme−substrate interface as discussed above. While the ΔI16V17 trypsinogen framework is probably not the best for drawing conclusions about effects on zymogenicity, the ambiguity introduced by the changes in the enzyme−substrate interface indicates that further construction and characterization of His40 mutants is unlikely to yield more interpretable results.

Correlation between BPTI Affinity and Activity. As expected, a correlation is observed between the value of log (k_{cat}/K_m) for the hydrolysis of Tos-Gly-Pro-Arg-AMC and log K_d for the BPTI complexes with the mutant trypsinogens and trypsins (Figure 3). The slope of this plot is 0.64, with $r = 0.98$, and extends over at least 6 orders of magnitude on both axes. Assuming that the value of K_d for the BPTI complex with wild-type trypsinogen is not less than 100 μM, the value of k_{cat}/K_m for the hydrolysis of Tos-Gly-Pro-Arg-AMC will not be more than 0.2 M^{-1} s^{-1}, which is consistent with the value estimated from the activity of I16G trypsinogen. Since the mutation of Asp194 to Asn increases the BPTI affinity of S195A trypsinogen by at least 3-fold, the value of k_{cat}/K_m for D194N trypsinogen will be at least 6-fold greater than wild-type trypsinogen. This observation suggests that the mutation of Asp194 to Asn will also decrease the zymogenicity of wild-type trypsinogen.

Association of BPTI with ΔI16V17/ D194N Trypsinogen and ΔI16V17 Trypsinogen. One outlier is evident in the correlation between BPTI affinity and k_{cat}/K_m shown in Figure 3. The value of K_d for the complex of BPTI with ΔI16V17 trypsinogen is much lower than would be predicted from the activity of this mutant trypsinogen. Indeed, the BPTI affinity of this mutant trypsinogen is equivalent to that of ΔI16V17/D194N trypsinogen although these two enzymes

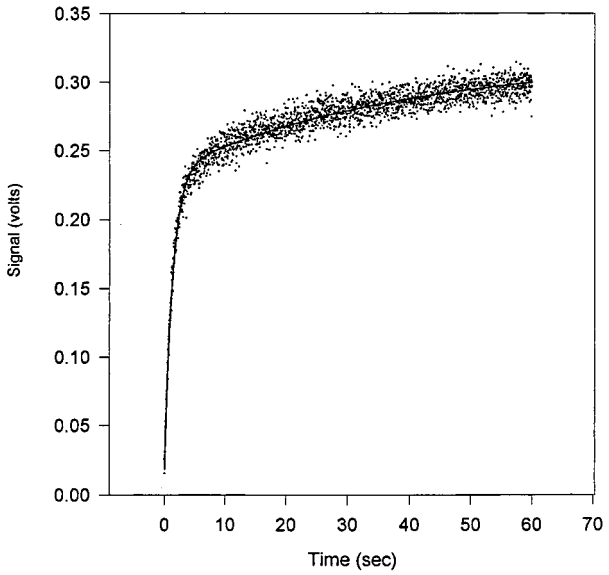

FIGURE 4: Stopped-flow fluorescence scan of the formation of the complex between BPTI and ΔI16V17/D194N trypsinogen. Trypsinogen and BPTI solutions were mixed 1:1 to attain final concentrations of 5.2 μM ΔI16V17/D194N trypsinogen and 150 μM BPTI. Data were acquired over 200 s (only 60 s is shown for clarity). The solid line is the fit to the equation: $F = F_0 + F_1 e^{-k_{obs1}t} + F_2 e^{-k_{obs2}t}$.

differ in activity by 100-fold. During the course of the titration calorimetry experiment, we noticed that both ΔI16V17 trypsinogen and ΔI16V17/S195A trypsinogen complexes required more time to equilibrate than either S195A trypsinogen or ΔI16V17/D194N trypsinogen. It seemed likely that the rate of complex formation might be a clue to the unusual BPTI affinity of ΔI16V17 trypsinogen. Therefore, we measured the rate of BPTI association to ΔI16V17 trypsinogen and ΔI16V17/D194N trypsinogen. The rates of formation of the BPTI complexes with the mutant trypsinogens were measured at varying concentrations of BPTI by stopped-flow spectroscopy by monitoring the change in intrinsic protein fluorescence (Figure 4).

The progress curve for ΔI16V17 trypsinogen fit a single exponential (data not shown). The values of k_{obs} increase linearly with BPTI concentration (Figure 5a). The values of both k_{on} and k_{off} could be determined from this plot: $k_{on} = 61 \pm 9$ M^{-1} s^{-1} and $k_{off} = (4.4 \pm 1.9) \times 10^{-3}$ s^{-1}. If BPTI associates with ΔI16V17 trypsinogen via a simple one-step binding mechanism, then the ratio of k_{off}/k_{on} will equal K_d. However, the ratio of k_{off}/k_{on} is 72 μM, which is ∼8-fold higher than the value of K_d measured by titration calorimetry. This observation indicates that the association of BPTI and ΔI16V17 trypsinogen involves at least one more step that cannot be observed by monitoring fluorescence.

The progress curve for the complex with ΔI16V17/D194N trypsinogen has two phases as shown in Figure 4. The values of both k_{obs1} and k_{obs2} increase linearly with BPTI concentration (Figure 5b,c, respectively). These data are not consistent with either a two-step or a three-step binding mechanism. Two possible explanations are consistent with the data: (1) the two phases represent BPTI binding to two different forms of ΔI16V17/D194N trypsinogen; or (2) one of the phases is an artifact of some kind, possibly aggregation. The k_{on} and k_{off} rates determined from the plots in Figure 5b,c are $k_{on1} = 2600 \pm 600$ M^{-1} s^{-1}, $k_{off1} = 0.63 \pm 0.15$ s^{-1}, $k_{on2} = 110 \pm$

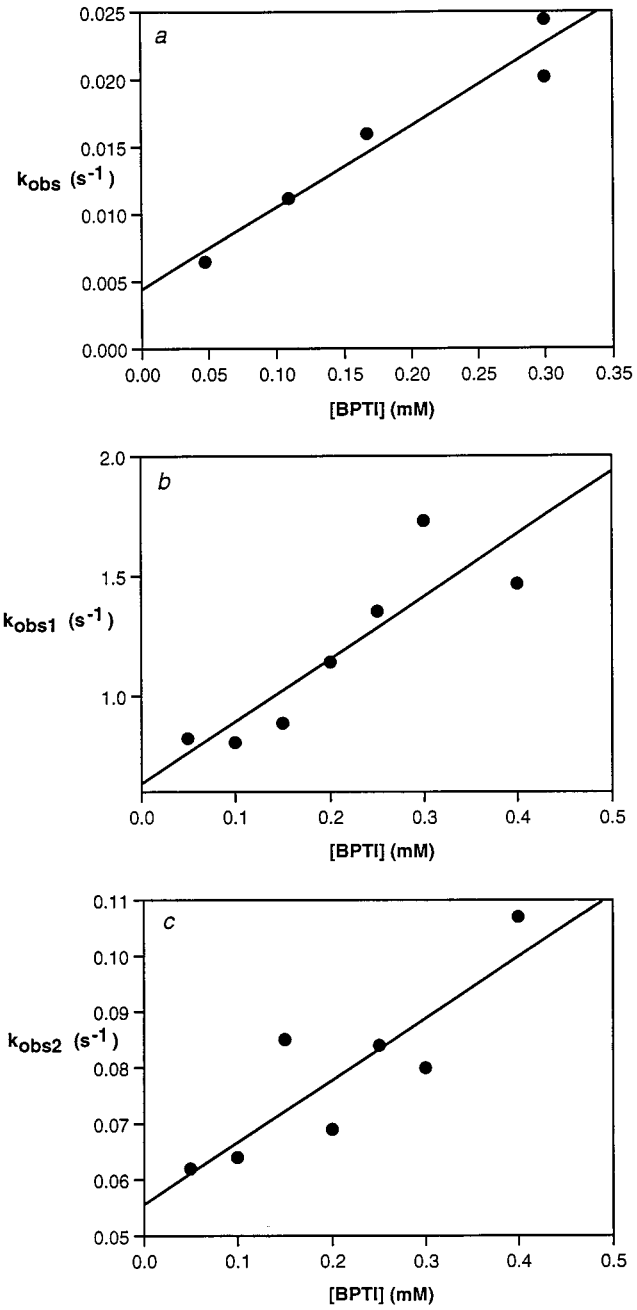

FIGURE 5: Observed rates of ΔI16V17 trypsinogen and ΔI16V17/D194N trypsinogen binding to BPTI as a function of [BPTI]. (*a*) The observed rate constants for the association of ΔI16V17 trypsinogen (4.9 μM) with BPTI; this reaction is a single-exponential process. (*b*) The observed rate constants for the faster phase of the association of ΔI16V17/D194N trypsinogen with BPTI. This reaction is a double exponential process as shown in Figure 4. (*c*) The observed rate constants for the slower phase of the association of ΔI16V17/D194N trypsinogen with BPTI.

30 M^{-1} s^{-1}, and $k_{off2} = 0.057 \pm 0.005$ s^{-1}. Values of K_d calculated from these rate constants are $K_1 = 240 \pm 80$ μM and $K_2 = 510 \pm 150$ μM. These values are 20- and 40-fold larger than those measured by titration calorimetry. Thus, as in the case of ΔI16V17 trypsinogen, the binding of BPTI to ΔI16V17/D194N trypsinogen involves at least one additional step. Regardless of the mechanism of BPTI binding, the values of k_{on} and k_{off} for the binding of BPTI to ΔI16V17/D194N trypsinogen for both phases are faster than those of ΔI16V17 trypsinogen.

DISCUSSION

Activity of Rat Trypsinogen and Equilibrium between Inactive and Active Conformations. Although the activity of rat trypsinogen could not be measured directly, we estimate that the activity is not more than 0.2 M^{-1} s^{-1} as measured by hydrolysis of Tos-Gly-Pro-Arg-AMC. In contrast, trypsin catalyzes the hydrolysis of this substrate with a value of $k_{cat}/K_m = 9 \times 10^6$ M^{-1} s^{-1}. Thus, trypsinogen is $\sim 10^8$-fold less active than trypsin. Assuming that trypsinogen must attain a conformation similar to trypsin in order to catalyze substrate hydrolysis, the equilibrium between the inactive and active conformations of trypsinogen must be on the order of 10^{-8}, representing a free energy change of more than 10 kcal/mol.

In contrast, if the hydrolysis of thiobenzyl ester substrates is monitored, trypsinogen is 10^6-10^7-fold less active than trypsin. This difference most likely results from the greater intrinsic reactivity of thiobenzyl esters, which allows the hydrolysis of ester substrates to be less structurally demanding than the hydrolysis of amide substrates. This conclusion is consistent with the observation that trypsin catalyzes the hydrolysis of ester substrates with much less discrimination than amide substrates (*24*).

Rat trypsinogen appears to be less active than bovine trypsinogen. The value of k_{cat}/K_m for the hydrolysis of Z-Lys-pNP by bovine trypsinogen is 36 M^{-1} s^{-1} (*29, 30*), which is 20-fold higher than that for the hydrolysis of Z-Lys-SBzl reported here. This difference might reflect the greater reactivity of *p*-nitrophenyl esters, the presence of a contaminating protease in the bovine trypsinogen preparation, or a difference in the structure of rat and bovine trypsinogens. Intriguingly, bovine trypsinogen has a Lys at residue 156, whereas rat trypsinogen has a Gln at this position. Lys156 can interact with Asp194 as shown in the structure of bovine trypsinogen with PSTI (*19*). This interaction is believed to account for the comparatively high activity of the zymogen of tissue plasminogen activator (*16*−*18*, *20*). Thus, the presence of Lys156 may account for the increased activity of bovine trypsinogen.

His40 Does Not Appear To Control the Zymogenicity of Trypsinogen. The role of His40 in maintaining the inactive conformation of trypsinogen is controversial. His40 appears to interact with Asp194 in one crystal structure of bovine trypsinogen and in chymotrypsinogen (*33*, *34*), and the substitution of His at position 40 in t-PA increases zymogenicity by 5-fold (*4*). However, the His40−Asp194 interaction is not observed in another crystal structure of bovine trypsinogen, nor in the ^{1}H NMR spectrum of porcine trypsinogen (*2*, *7*). Our results suggest that the His40Phe mutation has no effect on the zymogenicity of trypsinogen, although this mutation does appear to change the enzyme−substrate interface. This result indicates that the effects of mutations at position 40 in trypsinogen will be difficult to interpret.

Asp194 Stabilizes the Inactive Conformation of Trypsinogen. Trypsinogen exists in a predominantly inactive conformation where the S1 site and the oxyanion hole are deformed. While trypsinogen can attain an active, trypsin-like conformation as demonstrated by the structure of the BPTI−trypsinogen complex, this conformation is disfavored by the absence of a compensating charge for Asp194 (*3*, *10*). Asp194 was changed to Asn in order to probe the importance

of the uncompensated charge in stabilizing the inactive conformation of trypsinogen. This mutation would be expected to remove the uncompensated charge in the active conformation. As expected, the Asp194Asn mutation increases both the BPTI affinity and the activity of trypsinogen, although the magnitude of these effects depends on the framework of the mutation. In the case of S195A trypsinogen, BPTI affinity increases by at least 3-fold when Asp194 is replaced with Asn. Using the correlation of BPTI affinity with activity, the corresponding value of k_{cat}/K_m is predicted to increase by at least 6-fold. These values are lower limits because the affinity of BPTI for S195A trypsinogen is too low to measure. This observation indicates that Asp194 stabilizes the inactive conformation of trypsinogen as well as stabilizes the active conformation of trypsin via interaction with Ile16.

Analogous Asp→Asn mutations of coagulation factors underlie several genetic blood clotting disorders (*35, 36*). These defects are generally attributed to a loss of function in the active protease form. Indeed, this mutation causes a 200−2000-fold decrease in the activity of trypsin and plasmin (*10, 37*). However, the above results suggest that a gain of function in zymogen activity could also contribute to a disease phenotype. To our knowledge, u-PA is the only other example of the characterization of an Asp194Asn mutation in a zymogen framework (*38*). However, the zymogen of u-PA is only 100-fold less active than the mature enzyme form. The active form of the u-PA zymogen is believed to be stabilized by the formation of a salt bridge between Asp194 and Lys143 (*21*). Consequently, the Asp194Asn mutation in u-PA causes a 270-fold decrease in zymogen activity.

The activation peptide stabilizes the inactive conformation of trypsinogen. We probed the role of the activation peptide (residues 6−15 in recombinant trypsinogen) by deleting the Ile16 and Ile16Val17 from trypsin. These results indicate that the activation peptide also stabilizes the inactive conformation of trypsinogen. Interestingly, the activation peptide has been shown to possess a high degree of flexibility. The NMR signals assigned to the activation peptide are in random coil positions and have narrow line widths, indicating more flexibility in these residues relative to the bulk of the protein (*39*). In addition, these residues are disordered in one trypsinogen structure (*3*), and only weakly ordered in the other (*2*). The *B* factors of Val17 and Gly18 decrease upon cooling, which indicates that the disorder in the activation peptide is due to thermal motion (*40*). These observations suggest that the activation peptide does not make a specific interaction with the remainder of trypsinogen. Rather, it seems likely that the presence of the activation peptide sterically prevents the formation of the active conformation, or that the formation of the active conformation requires the activation peptide to be constrained.

Interestingly, the mutation of Lys15 to Ala has a larger effect on the BPTI affinity and activity of trypsinogen than does the deletion of the activation peptide. We estimate that this mutation increases the activity of trypsinogen by 50-fold. This observation suggests that Lys15 is an important determinant of zymogenicity. Analogous mutations are commonly used to create noncleavable zymogen forms of trypsin-like enzymes. Our results suggests that such mutations can have a large effect on the function of the zymogen.

Substrate Hydrolysis and BPTI Binding Utilize Different "Active" Conformations of Trypsinogen. Since BPTI binding induces trypsinogen to form a trypsin-like conformation, the affinity of the mutant trypsins and trypsinogens for BPTI is expected to correlate with their activity. Such a correlation has been demonstrated for a series of mutant trypsins (*10*). Here we extend this correlation as shown in Figure 3. These data show that the mutations have a less pronounced effect on BPTI affinity than on activity: whereas trypsinogen has 10^8-fold less activity than trypsin, its affinity for BPTI has decreased by only 10^7-fold. This result is expected: although the structure of the trypsinogen−BPTI complex resembles the trypsin−BPTI complex, the temperature factors of the trypsinogen are higher than those of trypsin (*12*). A more ordered conformation appears to be required for substrate hydrolysis. Thus, different "active" conformations are required for inhibitor binding and amide hydrolysis.

ΔI16V17 Trypsinogen Binds BPTI Anomalously. The BPTI affinity of ΔI16V17 trypsinogen is higher than would be expected from its activity. The rate of formation of the BPTI complex of this mutant trypsinogen is also slower than that observed for ΔI16V17/D194N trypsinogen. This result suggests that an anomalous complex forms between ΔI16V17 trypsinogen and BPTI. An anomalous complex is indeed observed in the crystal structure of the ΔI16V17 trypsinogen−BPTI complex (A. Pasternak, D. Ringe, and L. Hedstrom, unpublished results). This structure shows that the side chain of Lys15 (now occupying position 17 after the deletion of Ile16Val17) interacts with Asp194, binding in the pocket occupied by Ile16 in trypsin. It seems likely that the slow step in formation of the BPTI complex for this mutant is the insertion of Lys15 into the pocket. Perhaps this structural rearrangement is too slow to be a factor in substrate hydrolysis.

ACKNOWLEDGMENT

Xiaowu Zhang made the initial measurement of ΔI16V17/D194N activity, and Marguerite Cahoon purified some of the mutant trypsinogens. We acknowledge the excellent technical support of Becky Meyers for DNA sequencing. We thank MicroCal, Inc., Dr. John Blanchard (Albert Einstein College of Medicine), and Dr. Robert Weis (University of Massachusetts) for use of titration calorimeters.

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BI980951D