Alanine Point-Mutations in the Reactive Region of Bovine Pancreatic Trypsin Inhibitor: Effects on the Kinetics and Thermodynamics of Binding to β-Trypsin and α-Chymotrypsin†

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ABSTRACT: In an effort to relate structural, kinetic, and thermodynamic features in a model macromolecular recognition process, the amino acid residues in the reactive surface of bovine pancreatic trypsin inhibitor (BPTI) and surrounding residues were substituted individually by alanine, and the effects of the point-mutations on the kinetics and thermodynamics of inhibition by BPTI toward trypsin and chymotrypsin were investigated. Fifteen alanine mutants were produced. The majority of the BPTI mutants exhibited a binding affinity similar to that of the wild-type protein. The exceptions were the primary specificity site (P1) mutant and those mutants that seem to have nonlocal perturbations of structure, as revealed by circular dichroism and thermostability measurements. The mutation at the P1 site caused a reduction in the binding free energy of 10 and 1.8 kcal mol^{-1} for trypsin and chymotrypsin, respectively. The losses in binding affinity were determined almost exclusively by an increase in the dissociation rate constant. However, the rate of association of the P1 mutant, Lys-15-Ala, with trypsin was also drastically reduced (>200-fold). Calorimetric measurements of the heats of binding for the association of chymotrypsin with the wild-type inhibitor and its alanine mutants allowed determination of the relative contributions of the changes in enthalpy and entropy to the free energy of binding. Compensatory changes in the two parameters were observed in several cases, which were attributed to desolvation effects at the binding interface.

The evaluation of the parameters involved in the energetics of protein stability and binding to ligands, based upon the application of fixed rules to the known structures of proteins and protein/ligand complexes, is approaching saturation. Future knowledge of the relationship of protein structure to function and dynamics is more likely to come from correlations of structural information with experimental parameters. The number of macromolecular complex structures determined at high-resolution is increasing rapidly, including antibody/antigen, hormone/receptor, protease/inhibitor, and protein/nucleic acid complexes. However, the number of studies relating the structural features of these complexes with experimental kinetic and thermodynamic data is still relatively scarce (Ayala et al., 1995; Gómez & Freire, 1995; Ladbury et al., 1994; Murphy et al., 1993, 1995; Bhat et al., 1994; Ysern et al., 1994). These types of studies are an important contribution to the understanding of the molecular mechanisms involved in protein recognition and may provide valuable information for the rational design of molecules targeted at protein receptors. Serine proteases and their natural inhibitors are attractive systems for such studies, given the availability of high-resolution structural information. The serine protease inhibitor bovine pancreatic trypsin inhibitor (BPTI)[1] is among the best characterized proteins; its structural and physicochemical properties and mechanism of inhibition having been extensively documented. BPTI has also been shown to fold correctly in *Escherichia coli* into a functional molecule, indistinguishable from natural material (Marks et al., 1986). High-resolution crystal structures are available for BPTI (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987) and serine protease/BPTI complexes [for a review, see Bode and Huber (1991)]. Protease/BPTI systems are therefore particularly suitable to investigate the atomic details of the interactions determining the energetics of association processes. In the present study, using trypsin/BPTI and chymotrypsin/BPTI as model systems, we have investigated the effect of single alanine mutations (Cunningham & Wells, 1989) in the reactive surface of BPTI on the kinetics and thermodynamics of binding to β-trypsin and α-chymotrypsin.

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[1] Abbreviations: BAPA, *N*-α-benzoyl-L-arginine *p*-nitroanilide; BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; DMSO, dimethyl sulfoxide; *E*, enzyme concentration; E_o, initial enzyme concentration; *EI*, enzyme/inhibitor complex concentration; FPLC, fast-performance liquid chromatography; *I*, inhibitor concentration; I_o, initial inhibitor concentration; k_{cat}, catalytic rate constant; K_i, equilibrium dissociation constant; K_m, Michaelis−Menten constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate; P1, primary specificity site of protease substrate or inhibitor; SGGPPA, *N*-succinyl-Gly-Gly-Phe *p*-nitroanilide; wt, wild-type.

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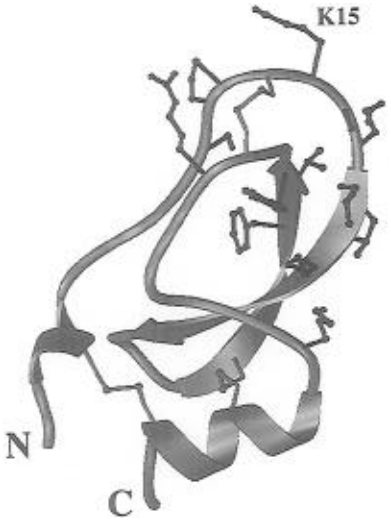

FIGURE 1: Tertiary structure of BPTI. The primary specificity site, P1, is occupied by a lysine. The side chains mutated to alanine and Ala-16 are represented.

BPTI is a basic 58 amino acid peptide with a molecular mass of 6.5 kDa (Kassell et al., 1963). It contains three disulfide bridges that contribute to a very compact tertiary structure and an extremely high stability against denaturing agents and proteolytic degradation (Kassell, 1970). The peptide has a broad inhibitory specificity, reacting tightly with several serine proteases [for a review, see Fritz and Wunderer (1983)], trypsin/BPTI being the most stable complex. The higher affinity toward trypsin is conferred by a lysine residue at the primary specificity site (P1). Common structural features shared with other serine protease/peptide inhibitor complexes are formation of a short antiparallel β-sheet between the enzyme and the N-terminal portion of the binding region of the inhibitor, hydrogen bonding between the carbonyl oxygen at the P1 residue and the amide groups of the reactive serine and a glycyl residue at the oxyanion binding site, and tight complementary of the binding surfaces, with water molecules excluded from the interface (Hubbard et al., 1991). Inhibition by BPTI is competitive and reversible (Engel et al., 1974; Finkenstadt et al., 1974; Quast et al., 1978a). The interaction between inhibitor and protease is characterized by a slow association rate, with formation of a loose complex, followed by a rate-limiting step that results in a more stable complex. For practical purposes, the cleavage of the reactive-site bond Lys-15−Ala-16 is negligible and the inhibition reaction is adequately expressed by the overall equation:

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \qquad (1)$$

Figure 1 shows the tertiary structure of BPTI. Alanine substitutions were made at the residues that directly contact or come in close proximity to the protease in the BPTI/trypsin complex (Rühlmann et al., 1973; Huber et al., 1974), except for the two cysteine residues that cross-link the binding loops (Cys-14 and Cys-38). The mutated residues were: Thr-11, Gly-12, Pro-13, Lys-15, Arg-17, Ile-18, Ile-19, Arg-20, Phe-33, Val-34, Tyr-35, Gly-36, Gly-37, Arg-39, and Lys-46. Positions 16 and 40 are occupied by alanines in the native inhibitor. The wild-type (wt) and the 15 alanine BPTI mutants were produced in a bacterial expression system. The binding affinities of the different peptides toward trypsin and chymotrypsin were determined by measuring the inhibition constants, K_i. The relative contributions of the rate constants of association, k_{on}, and dissociation, k_{off}, to the binding affinity were also determined. Heats of binding were determined for the chymotrypsin/BPTI system, allowing the estimation of the enthalpy and entropies of binding. Circular dichroism (CD) spectra of all the proteins were obtained, as well as thermostability measurements, for a more complete interpretation of the kinetic and thermodynamic data. The results are discussed in terms of the general principles governing protein/protein recognition and the atomic-resolution structures of BPTI and the trypsin/BPTI and chymotrypsin/BPTI complexes.

MATERIALS AND METHODS

Materials. Bovine pancreatic β-trypsin and α-chymotrypsin were purchased from Sigma. Both enzymes were purified further by FPLC through gel filtration, using a 16/60 Superdex 75 column (Pharmacia LKB Biotechnology), to eliminate autolysis products. The eluent was 1 mM HCl containing 150 mM NaCl, pH 3.0. Desalting was done on a FPLC fast-desalting column in 1 mM HCl, pH 3.0. The lyophilized material was dissolved in 1 mM HCl, 20 mM $CaCl_2$ at a concentration of around 0.1 mM and stored at −20 °C. The protease substrates used were *N*-α-benzoyl-L-arginine *p*-nitroanilide (BAPA; Boehringer Mannheim) for trypsin and *N*-succinyl-Gly-Gly-Phe *p*-nitroanilide (SGG-PPA; Sigma) for chymotrypsin. The active-site titrant *p*-nitrophenyl *p*′-guanidinobenzoate (NPGB) was from Sigma. All the chemicals were analytical or HPLC grade. FPLC columns used in the purification of BPTI were from Pharmacia LKB Biotechnology.

Production of BPTI. Mutagenesis and expression were carried out using pEZZ vectors (Nilsson et al., 1991). BPTI was expressed as a secreted fusion protein that included a pair of synthetic IgG binding domains of staphylococcal protein A (ZZ). A short linker of five amino acids separated the two moieties, allowing specific cleavage of the fusion protein. Vectors carrying the different BPTI mutant genes were obtained by site-directed mutagenesis as described (Zoller & Smith, 1987) using a pEZZ single-stranded template containing a linker region that permitted site-specific cleavage of the expressed fusion protein by hydroxylamine. However, to avoid any possibility of chemical modification of BPTI during the hydroxylamine treatment and to preserve the natural N-terminus, the wt BPTI and alanine mutants were subsequently recloned in another pEZZ vector that had been engineered for efficient chymotrypsin cleavage of the fusion protein. A competing chymotrypsin cleavage site was eliminated in the linker region (Altman et al., 1991), by mutating a methionine to a serine, in order to facilitate purification. Cloning and DNA sequencing were done according to standard procedures.

The wt and the different Ala mutants of BPTI were expressed in *E. coli* grown in rich medium containing 250 mg/L ampicillin and supplemented with 1 g/L glucose and 100 mg/L thiamin. The fusion protein was recovered from the periplasmic space by osmotic shock as described (Nilsson et al., 1991). Cell debris were removed by centrifugation at 6000*g* for 20 min at 4 °C. After incubation with 0.5 mg/L of DNase *I* for 15 min at 4 °C, the periplasmic fraction was

filtered through a 0.45 μm filter and applied to a chymotrypsin affinity column (α-chymotrypsin immobilized onto Affi-Gel 10, Bio-Rad) preequilibrated with 0.1 M triethanolamine containing 0.3 M NaCl and 20 mM $CaCl_2$, pH 7.8. This purification step allowed the simultaneous cleavage of the fusion protein and recovery of the BPTI moiety. After the column was washed with 10 bed volumes of equilibration buffer, 50 μg of free chymotrypsin was added per 5 mL of gel, and the column was incubated with gentle rocking for 48 h at 4 °C. BPTI was eluted with 1 mM HCl containing 0.5 M KCl, pH 2.1 The eluate was diafiltered using a 3000 MW cutoff ultrafiltration membrane from Amicon. A contaminant of BPTI containing a 12 amino acid N-terminal extension (0−10% of the total protein) was removed by cation exchange FPLC using a Mono S column. The peptides were eluted with a 0−1 M NaCl gradient in 50 mM sodium phosphate buffer, pH 7.0, or pH 6.0 for the mutants that had one less positively-charged residue. In some cases, a gel filtration step was performed with the fraction containing the correct-size BPTI to eliminate residual amounts of the contaminating peptide, using a 16/60 Superdex 75 FPLC column, in 50 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.0. Although the difference in molecular weight between both BPTI molecules is only 1500, baseline resolution was possible. The fraction containing the main peak was desalted on an FPLC fast-desalting column in 50 mM ammonium bicarbonate. Due to the nonspecific binding of BPTI to dextran-based chromatographic media and consequent increased retention times, the salt concentration was monitored with an ion monitor. Completely salt-free BPTI was lyophilized and stored in water at concentrations higher than 2 mg/mL. For the wt BPTI, the final yield was around 1.5 mg of pure peptide per fermentation liter, which corresponds to ∼70% recovery. The final yields for the alanine mutants varied between 12% and 120% of the wt. The lowest yields were caused by degradation during expression, which seemed to be related to thermostability. The purity of the peptides was established by reverse phase HPLC, denaturing and nondenaturing PAGE, amino acid composition determination, and N-terminal sequencing. Homogeneity was >98%, in all the cases.

Determination of Active Protease and Inhibitor Concentrations. The concentrations of functional trypsin and chymotrypsin were determined with the burst titrant NPGB, according to Chase and Shaw (1967). In the case of chymotrypsin, since the titrant turnover is comparatively high, the determination obtained with NPGB was verified by titration with a known amount of wt BPTI. The two determinations differed by 5%, and the average value was taken. Protease stock solutions were not reused after thawing. The concentration of BPTI was obtained by titration with trypsin. A previously NPGB-titrated trypsin solution was incubated with half of the molar amount of inhibitor, estimated by UV absorption, under conditions that permitted stoichiometric binding (1 μM trypsin). The remaining trypsin activity allowed the accurate determination of the concentration of active inhibitor. Three measurements were taken per BPTI solution, and the differences between them were found to be smaller than 3%. The concentration of the Ala-15 BPTI mutant was determined by amino acid analysis, due to the lower binding affinities for trypsin and chymotrypsin. Determination of inhibitor concentration by amino acid analysis was found to be comparable to the concentration determined by titration with trypsin, for the wt and the rest of the mutants.

General Kinetic Methods. The kinetic parameters of inhibition, K_i and k_{on}, were determined using spectrophotometric assays, by monitoring the release of *p*-nitroanilide at 405 nm, resulting from the hydrolysis of the chromogenic substrate by the protease. Protease activity was correlated to protease concentration. A Kontron spectrophotometer was used, equipped with an automatic cell changer. The cuvette holder was connected to a thermostat-controlled water bath. Reference samples were not included since spontaneous hydrolysis of the synthetic substrates did not occur, even in the longest times required to measure enzymatic hydrolysis. Because of the very low concentration of protein present in most of the assays, only quartz cuvettes were used, which have been found to minimize the loss of protein by adsorption (Empie & Laskowski, 1982). Routinely, working stock solutions of protease and inhibitor were prepared, prior to performing the assays, in polypropylene vials treated with glycerol: 1 μM trypsin or chymotrypsin in 1 mM HCl/20 mM $CaCl_2$ and 1 μM BPTI in 0.005% Triton X-100. (At a concentration of 0.1 μM, BPTI absorption to polypropylene vials, even if glycerol-treated and in the presence of 0.005% Triton X-100, was found to be nonnegligible.) Stock solutions of 34 mM BAPA and 6 mM SGGPPA in dimethyl sulfoxide (DMSO) were prepared. The general assay conditions were 50 mM Tris-HCl containing 20 mM $CaCl_2$ and 0.005% Triton X-100, pH 8.2, 22 °C. The final assay volume was 1 mL. Reactions were started with the addition of 20 μL of substrate solution. The concentration of chromogenic substrate in the assay was below the K_m to avoid displacement of the inhibitor from the enzyme/inhibitor complex upon addition of the substrate. In the trypsin assays, the final concentration of BAPA was 0.67 mM (K_m = 0.91 mM) and in the chymotrypin assays, the final concentration of SGGPPA was 0.125 mM (K_m = 1.48 mM).

Equilibrium Dissociation Constant, K_i. The equilibrium dissociation constant, K_i, was determined by an adaptation of the method described by Green and Work (1953). Enzyme and inhibitor were incubated at different concentrations of inhibition. The incubation time was 10 half-lives of the association reaction ($t_{1/2} = 1/k_{on}I_o$), according to the rate constant of association, k_{on}, that had been determined previously. The residual protease activity allows the construction of inhibition curves. For trypsin, the concentration of protease was 5 nM, and the BPTI concentration was varied between 0 and 10 nM, except in the case of Ala-15, where the inhibitor concentration ranged from 0 to 6 μM. For chymotrypsin, the concentrations of protease and inhibitor were 0.1 μM and 0−1 μM, respectively. The data sets were fitted to the theoretical equation that defines the amount of free enzyme (E) as a function of the initial concentration of inhibitor (I_o), derived from eq 1, where E_o is the initial concentration of enzyme:

$$E = E_o - \frac{K_i + E_o + I_o - \sqrt{(K_i + E_o + I_o)^2 - 4E_oI_o}}{2} \quad (2)$$

A nonlinear regression analysis program was used (Leatherbarrow, 1987). This program was used in all the nonlinear regression analyses for the determination of kinetic constants. The standard error of the calculated variables was always less than 10%.

Association Rate Constant, k_{on}. The association rate constant, k_{on}, was determined by measuring the extent of protease inhibition after incubating equimolar amounts of enzyme and inhibitor (except for trypsin/K15A BPTI) for different times. The initial concentrations of protease and inhibitor were 5 nM in the trypsin assays, and 10 nM in the chymotrypsin assays. In the case of trypsin/K15A, the initial concentration of inhibitor was 0.6 μM. Nonlinear regression analysis was used to fit the data to a general equation that describes the variation of free enzyme with time, at any given ratio of E_o/I_o, according to a second-order model:

$$E = \{E_o - I_o + \sqrt{(I_o - E_o)^2 + 4E_oI_o/(1 + 2k_{on}t\sqrt{E_oI_o} + k_{on}{}^2t^2E_oI_o)}\}/2 \quad (3)$$

The fittings were done with either E_o or I_o as a variable in order to find the k_{on} value with the lowest standard error. Apart from fitting data obtained with nonequimolar amounts of enzyme and inhibitor, the nonlinear fitting allowed the use of data points obtained through the entire time-course of the reaction, which is not possible with typical linear fittings.

Dissociation Rate Constant, k_{off}. The published value for the k_{off} of the dissociation between wt BPTI and trypsin (Vincent & Lazdunski, 1972) was confirmed by following the time-course of exchange between isotopically ^{15}N-labeled trypsin-bound BPTI (Castro et al., submitted for publication) with unlabeled inhibitor, present at a 10-fold excess concentration with respect to the ^{15}N-labeled BPTI in the trypsin complex. Trypsin and ^{15}N-labeled BPTI were 0.4 mM. Samples were collected at appropriate time intervals over a period of 6 months, and the BPTI/trypsin complex was recovered by FPLC gel-filtration on a Superdex 75 column (Pharmacia LKB Biotechnology). Laser-desorption mass spectrometry analysis allowed the determination of the relative amounts of ^{15}N-labeled and unlabeled BPTI.

Heats of Binding. The heats of binding of the association of chymotrypsin with wt BPTI and alanine mutants were measured in a stopped-flow microcalorimeter (Mudd & Berger, 1988). Protein solutions were filtered and thoroughly degassed prior to use. The chymotrypsin (0.5 mM) and BPTI solutions (0.1 mM) were mixed with a 1:1 ratio, giving a reaction volume of 160 μL (80 μL + 80 μL). The variation in temperature in the mixing chamber was followed for 320 s, at a sampling rate of 2 per second. The heats measured (average of a minimum of 15 injections) upon mixing of the 2 solutions were corrected for the heats resulting from protease dilution, inhibitor dilution, and reaction medium mixture. The heat of buffer ionization arising from the release of protons upon BPTI binding to chymotrypsin was estimated to be negligible, based upon the comparison of the ΔH of binding obtained for the chymotrypsin/wt BPTI association with values of ΔH published previously for the same system using kinetic assays. The calorimetric ΔH for the chymotrypsin/wt BPTI system determined here is in good agreement with two measurements of ΔH for this system, obtained under similar pH conditions via kinetic assays (Vincent & Lazdunski, 1973; Quast et al., 1974). These authors determined the binding enthalpy from the difference between the energies of activation of the association and dissociation reactions (using Arrhenius plots for k_{on} and k_{off}), or from the temperature dependence of K_i. Possible cleavage of the inhibitor by chymotrypsin, or autolysis of chymotrypsin, was ruled out by inspection of the thermograms. Protein cleavage would result in incomplete relaxation of the heat signals to base line, and this was not observed. At the concentrations of protein used, the binding is stoichiometric, even in the case of chymotrypsin/K15A, for which the K_i is higher. Therefore, the binding enthalpy, ΔH, is equal to the symmetric of the total heat of binding divided by the number of active BPTI molecules present in the reaction chamber. The major source of error in the determination of the binding enthalpy is associated with the error in the determination of the BPTI concentration, which was estimated to be no greater than 5%. The measurements were carried out at 22 °C in the same reaction medium used for the determination of the kinetic constants. Chemical calibration with a 1:1 dilution of 10 mM NaCl was performed according to Mudd and Berger (1988).

Thermal Stability and Circular Dichroism (CD) Spectra. Thermal denaturation was monitored by circular dichroism (CD) using an AVIV 60DS spectropolarimeter. Ellipticity was recorded at 222 nm using a 1 mm path length cuvette (Helma) and thermoelectric control of temperature. Thermal denaturation profiles were obtained over the temperature interval 40−97 °C in 1 °C increments with a 1 min equilibration time at each temperature. The BPTI concentration was 0.2 mg/mL in 10 mM NaH_2PO_4, pH 3.0, adjusted with HCl. A two-state transition model was assumed. The melting temperature (T_m) and enthalpy change at the T_m (ΔH_m) were determined by fitting the data to the theoretical unfolding curves, using a nonlinear least-squares procedure. CD spectra were collected prior to each thermally-induced denaturation. Three spectra were collected per peptide, between 250 and 195 nm, in 1 nm steps and 2 s of averaging time, at 22 °C. An average spectrum of the solvent was subtracted for each peptide spectrum. Fractional helicity was calculated according to Chen et al. (1972).

RESULTS

Binding Parameters. Equilibrium binding constants were measured for the association of wt BPTI and its alanine variants with trypsin and chymotrypsin (Table 1). A lower limit of validity for the determination of the inhibition constants was set at $E_o/K_i \leq 100$, owing to poor definition of inhibition curves for higher values of the ratio E_o/K_i. The departure from linearity at the equivalence point ($E_o = I_o$) determines the accuracy of the determination of K_i. Under the experimental conditions used for the trypsin/BPTI system, such a limit corresponds to a K_i of 5×10^{-11} M. Most of the equilibrium dissociation constants measured were lower than this limit. Examples of inhibition curves are given in Figure 2. The published value of K_i for the association of trypsin with wt BPTI (Vincent & Lazdunski, 1972) was confirmed through the measurement of k_{on} and k_{off}, as described under Materials and Methods. The extremely slow dissociation rate constant of the trypsin/BPTI complex (half-life ~8 months), under the conditions used, precluded the direct measurement of k_{off} by spectrophotometric methods. The K_i determinations for the binding of wt BPTI to trypsin and chymotrypsin reported in Table 1 are in good agreement with previously reported K_i values obtained under similar conditions of pH and temperature (Vincent & Lazdunski, 1972, 1973; Quast et al., 1974). In the case of the chymotrypsin system, K_i was also determined through the

Table 1: Kinetic Constants of the Binding of Alanine Mutants of BPTI to Trypsin and Chymotrypsin at pH 8.2 and 22 °C[a]

	trypsin			chymotrypsin		
peptide	K_i (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})[b]	K_i (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})[b]
wt	5×10^{-14} [c]	$(9.9 \pm 2.5) \times 10^5$	5×10^{-8}	$(1.1 \pm 0.1) \times 10^{-8}$	$(1.7 \pm 0.2) \times 10^5$	1.8×10^{-3}
T11A	$<5 \times 10^{-11}$	$(3.5 \pm 0.2) \times 10^5$	$<1.7 \times 10^{-5}$	$(1.6 \pm 0.3) \times 10^{-8}$	$(1.4 \pm 0.1) \times 10^5$	2.3×10^{-3}
G12A	$(8.3 \pm 1.0) \times 10^{-11}$	$(2.3 \pm 0.3) \times 10^5$	1.9×10^{-5}	$(3.5 \pm 0.1) \times 10^{-8}$	$(5.8 \pm 0.7) \times 10^4$	2.0×10^{-3}
P13A	$<5 \times 10^{-11}$	$(6.8 \pm 1.9) \times 10^5$	$<3.4 \times 10^{-5}$	$(1.0 \pm 0.2) \times 10^{-8}$	$(2.3 \pm 0.9) \times 10^5$	2.3×10^{-3}
K15A	$(1.4 \pm 0.1) \times 10^{-6}$	$(4.3 \pm 0.6) \times 10^3$	4.2×10^{-5}	$(3.3 \pm 0.1) \times 10^{-7}$	$(6.0 \pm 0.8) \times 10^4$	2.0×10^{-2}
R17A	$<5 \times 10^{-11}$	$(1.1 \pm 0.2) \times 10^6$	$<5.6 \times 10^{-5}$	$(2.8 \pm 0.1) \times 10^{-8}$	$(1.8 \pm 0.4) \times 10^5$	4.9×10^{-3}
I18A	$(2.4 \pm 1.0) \times 10^{-10}$	$(3.9 \pm 0.3) \times 10^5$	9.2×10^{-5}	$(1.2 \pm 0.2) \times 10^{-7}$	$(1.5 \pm 0.3) \times 10^5$	1.7×10^{-2}
I19A	$<5 \times 10^{-11}$	$(5.5 \pm 1.6) \times 10^5$	$<5.5 \times 10^{-5}$	$(1.4 \pm 0.1) \times 10^{-8}$	$(1.8 \pm 0.8) \times 10^5$	2.5×10^{-3}
R20A	$<5 \times 10^{-11}$	$(1.1 \pm 0.5) \times 10^6$	$<5.6 \times 10^{-5}$	$(2.0 \pm 0.2) \times 10^{-8}$	$(2.0 \pm 0.5) \times 10^5$	3.9×10^{-3}
F33A	$<5 \times 10^{-11}$	$(4.7 \pm 0.9) \times 10^5$	$<2.4 \times 10^{-5}$	$(1.4 \pm 0.4) \times 10^{-8}$	$(1.8 \pm 0.3) \times 10^5$	2.4×10^{-3}
V34A	$<5 \times 10^{-11}$	$(7.0 \pm 0.6) \times 10^5$	$<7.0 \times 10^{-5}$	$(1.2 \pm 0.1) \times 10^{-8}$	$(2.4 \pm 0.2) \times 10^5$	2.8×10^{-3}
Y35A	$<5 \times 10^{-11}$	$(5.1 \pm 1.3) \times 10^5$	$<2.6 \times 10^{-5}$	$(4.9 \pm 0.2) \times 10^{-8}$	$(1.8 \pm 0.5) \times 10^5$	8.9×10^{-3}
G36A	$(2.1 \pm 0.7) \times 10^{-10}$	$(5.1 \pm 0.9) \times 10^5$	1.1×10^{-4}	$(5.6 \pm 0.7) \times 10^{-8}$	$(1.0 \pm 0.2) \times 10^5$	5.6×10^{-3}
G37A	$<5 \times 10^{-11}$	$(7.0 \pm 0.2) \times 10^5$	$<3.5 \times 10^{-5}$	$(4.4 \pm 1.3) \times 10^{-8}$	$(1.1 \pm 0.2) \times 10^5$	5.0×10^{-3}
R39A	$<5 \times 10^{-11}$	$(6.3 \pm 2.0) \times 10^5$	$<3.1 \times 10^{-5}$	$(1.6 \pm 0.1) \times 10^{-8}$	$(4.5 \pm 0.3) \times 10^5$	6.9×10^{-3}
K46A	$<5 \times 10^{-11}$	$(5.5 \pm 0.9) \times 10^5$	$<2.3 \times 10^{-5}$	$(1.4 \pm 0.1) \times 10^{-8}$	$(1.2 \pm 0.2) \times 10^5$	1.2×10^{-3}

[a] Values of K_i and k_{on} are mean ± SD of at least two independent experiments, except for the trypsin/wt BPTI K_i determination. [b] Obtained indirectly through the relationship $K_i = k_{off}/k_{on}$, except for the trypsin/wt BPTI determination. [c] Obtained indirectly through the relationship $K_i = k_{off}/k_{on}$.

measurement of k_{on} and k_{off} (results not shown), and both determinations of K_i proved to be consistent.

Figure 3 shows the difference in ΔG [$\Delta G = -RT \ln (1/K_i)$] relative to the wt for each of the BPTI mutants in the association with trypsin and chymotrypsin. In general, the mutations had a small effect on the free energy of binding, with variations in ΔG ($\Delta\Delta G$) of less than 1.5 kcal mol^{-1} for the chymotrypsin/BPTI system. A decrease in ΔG of more than 4.0 kcal mol^{-1} is observed for the association of four alanine mutants with trypsin, namely, Gly-12-Ala, Lys-15-Ala, Ile-18-Ala, and Gly-36-Ala. It is possible that other alanine mutants bind to trypsin with a significantly lower affinity, since K_i values below 5×10^{-11} M were not measurable, corresponding to a minimal detectable $\Delta\Delta G$ value of 4 kcal mol^{-1}. The drop in ΔG for the association of Lys-15-Ala with trypsin was extremely pronounced ($\Delta\Delta G \sim 10$ kcal mol^{-1}). Similar results have been reported in the literature for P1 mutants of BPTI obtained by semisynthetic engineering (Tschesche et al., 1987). The decrease in binding affinity observed with the Ile-18-Ala mutant for both the trypsin and the chymotrypsin systems could be related to the strong propensity of this peptide to aggregate in solution, as seen by light scattering, gel-filtration chromatography, and nondenaturing PAGE.

The rate constants of association of BPTI and its alanine mutants with trypsin and chymotrypsin were determined, thus allowing the evaluation of the relative contributions of k_{on} and k_{off} to the binding affinity ($K_i = k_{off}/k_{on}$). Typical curves for the time-course of the association of inhibitor and protease are shown in Figure 4. A second-order association mechanism was assumed. Table 1 lists the values of k_{on}, computed from the time-course of association, and of k_{off}, derived from the relationship between K_i and k_{on}. The values of k_{on} reported for the wt inhibitor are in good agreement with published values obtained with the same systems under comparable conditions (Vincent & Lazdunski, 1972, 1973; Quast et al., 1974).

None of the BPTI alanine mutations had a marked effect on the association rate of the inhibitor with trypsin or chymotrypsin, with the exception of the association of the Lys-15-Ala mutant with trypsin. Although the substitution of the charged side chain at the P1 site by a small hydrophobic side chain would be expected to cause a decrease in the association rate constant, a reduction over 2 orders of magnitude imposed by a single side chain substitution is comparatively high. This result suggests that the side chain of Lys-15 is important in orienting the inhibitor relative to the binding pocket of trypsin. Electrostatic interactions are likely to be involved in the initial stages of recognition, between the positively charged side chain of the lysine at P1 and the negatively charged aspartic acid side chain at the bottom of the specificity pocket of trypsin (Náray-Szabó, 1993). For the chymotrypsin/BPTI system, only two side chain substitutions, Lys-15-Ala and Gly-12-Ala, resulted in an appreciable (>2-fold) decrease in k_{on}. One mutant, Arg-39-Ala, associated with chymotrypsin at a rate around 3 times faster than that measured for the wt inhibitor.

The relative contributions of k_{on} and k_{off} to the change in binding affinity caused by each alanine mutation in both the trypsin and the chymotrypsin systems are expressed in the histograms shown in Figure 5. In all the cases, for both systems, the major determinant of the decrease in binding affinity is the increase in dissociation rate constant. The magnitude of the increase in k_{off} is at least twice as much as the magnitude of the decrease in k_{on}. The only exception is the case of chymotrypsin/G12A (Figure 5B), for which the change in k_{on} is larger that that of k_{off}. These results mimic the range of variation of k_{on} versus k_{off} observed in natural protein/ligand systems.

Thermodynamic Parameters (Chymotrypsin System). The enthalpic and entropic contributions to the energy of binding were determined for the association of chymotrypsin with wt BPTI and its alanine mutants. The heats of reaction for the association of protease and inhibitor were measured in a microcalorimeter, and the binding entropy was derived from the relationship between ΔG and ΔH ($\Delta G = \Delta H - T\Delta S$). The enthalpies of binding were not measured for the trypsin system, since for most of the alanine mutants it was not possible to calculate the ΔG of binding, and consequently the values of ΔS would not be known through the measurement of ΔH. The thermodynamic parameters ΔG, ΔH, and $-T\Delta S$ for the binding of wt BPTI and its alanine mutants to chymotrypsin are given in Table 2. The cases of BPTI mutants Ile-18-Ala and Phe-33-Ala were not investigated.

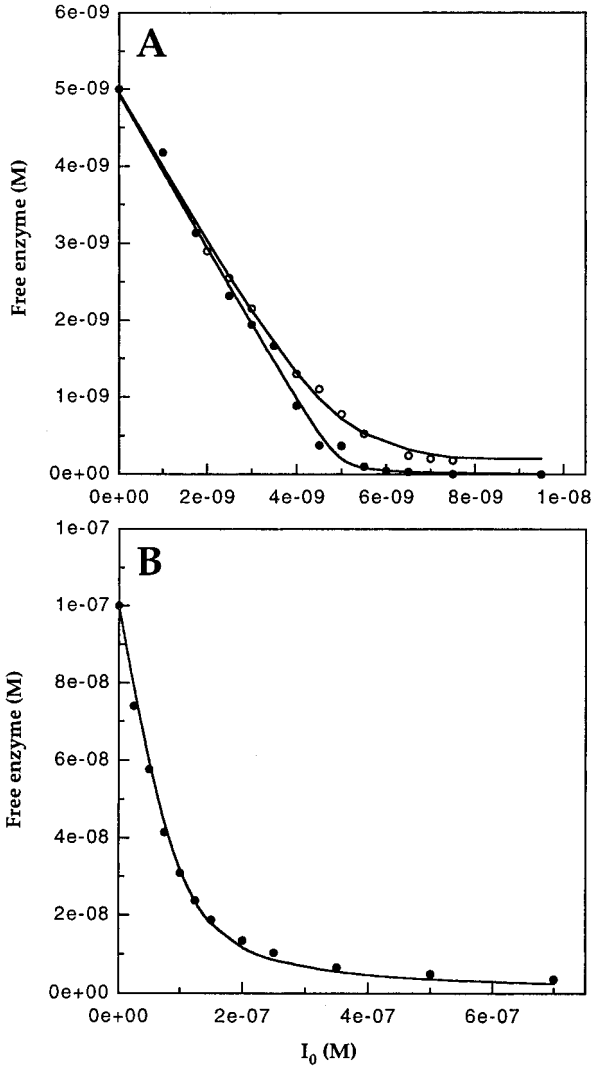

FIGURE 2: Typical inhibition curves, obtained at pH 8.2 and 22 °C. (A) Trypsin/BPTI system. The concentration of trypsin was 5 × 10^{-9} M in the cases represented: (●) trypsin/I19A and (○) trypsin/G36A. The K_i extracted from the trypsin/I19A curve was not considered valid (<5 × 10^{-11} M). (B) Chymotrypsin/BPTI system (chymotrypsin/R39A). The concentration of chymotrypsin was 10^{-7} M.

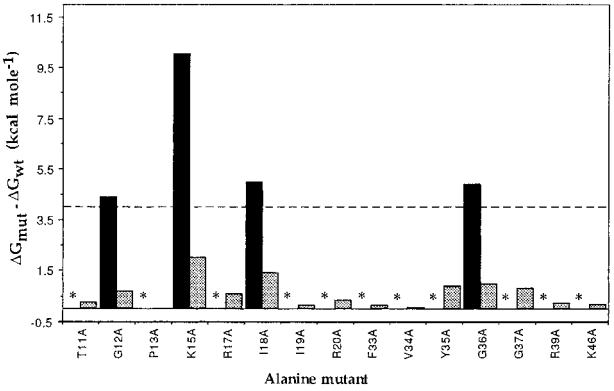

FIGURE 3: Effect of single alanine mutations in BPTI on the energy of association of the inhibitor with trypsin (solid bars) and chymotrypsin (stippled bars). The dashed line indicates the lower limit of detection for $\Delta\Delta G$ for the trypsin system in the cases where K_i was <5 × 10^{-11} M, indicated with an asterisk.

For the wt inhibitor, the binding enthalpy measured here for the association with chymotrypsin was 2.5 kcal mole^{-1}, a result that reproduces the values reported by Vincent and

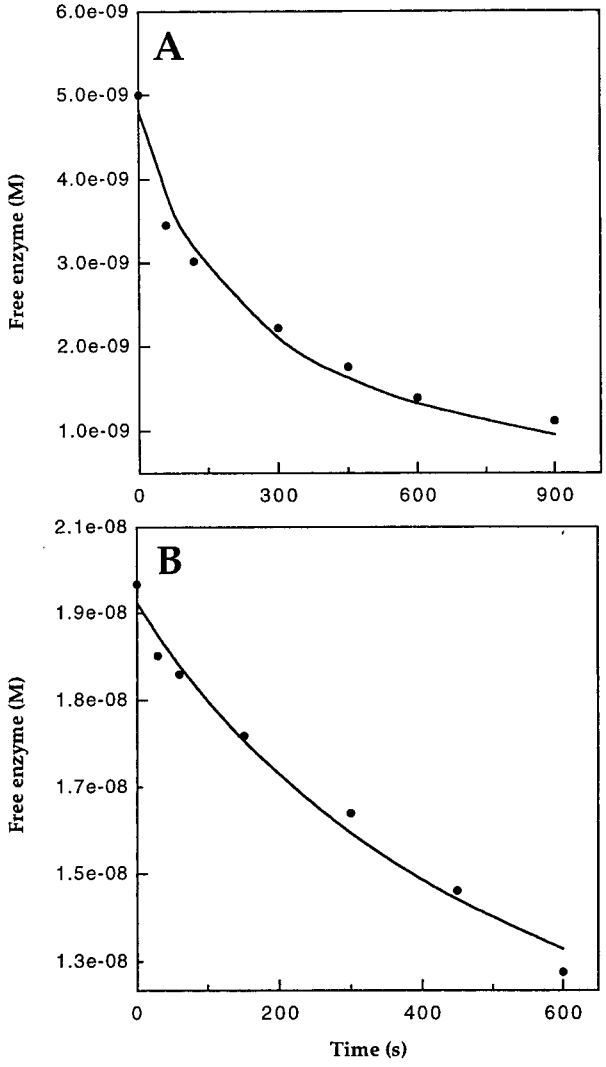

FIGURE 4: Typical time-courses of the association reactions, obtained at pH 8.2 and 22 °C. (A) Trypsin/BPTI system (trypsin/R39A). The concentrations of trypsin and inhibitor were 5 × 10^{-9} M. (B) Chymotrypsin/BPTI system (chymotrypsin/I19A). The concentrations of chymotrypsin and inhibitor were 10^{-8} M.

Lazdunski (1973) and Quast et al. (1974) of 3.0 and 2.4 kcal mol^{-1}, respectively. As seen in Table 2, the majority of the mutations did not have a significant effect on the enthalpy or entropy of binding to chymotrypsin. A graphical representation of the proportions of binding $\Delta\Delta G$ contributed by $\Delta\Delta H$ and $-T\Delta\Delta S$ is shown in Figure 6. For three of the mutants, namely, Gly-12-Ala, Val-34-Ala, and Tyr-35-Ala, a favorable decrease in binding enthalpy seems to be offset by a decrease in binding entropy of similar magnitude. In the first two cases, the change in the free energy of binding is very close to that observed for the wt BPTI. Inversions of relative contributions of ΔH and ΔS to the free energy of binding seem to occur in opposite directions for other mutants, although such trends may not be significant, due to the small quantities of energy involved. The association of chymotrypsin with Lys-15-Ala and Gly-36-Ala BPTI occurs with an unfavorable change in both the enthalpy and the entropy of binding as compared to the chymotrypsin/wt BPTI case.

Structural and Stability Parameters of BPTI Mutants. A comparison of structural and stability parameters of the alanine mutants of BPTI is given in Table 3. Differences with respect to the wt protein in helical content, melting

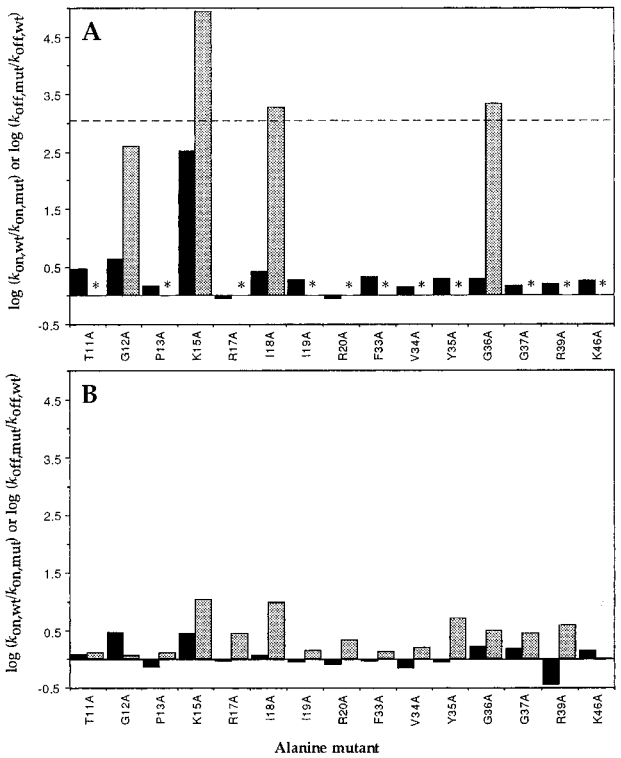

FIGURE 5: Relative contribution of k_{on} (solid bars) and k_{off} (stippled bars) to the change in binding affinity of BPTI alanine mutants. (A) Trypsin/BPTI system. The dashed line indicates the lower limit of detection of k_{off} in the cases where K_i is $<5 \times 10^{-11}$ M (asterisks), according to the values obtained for k_{on}. (B) Chymotrypsin/BPTI system.

Table 2: Thermodynamic Parameters of the Binding of wt BPTI and Its Alanine Mutants to Chymotrypsin at pH 8.2 and 22 °C (in kcal mol^{-1})

peptide	ΔG	ΔH	$-T\Delta S$	$\Delta\Delta G^a$	$\Delta\Delta H^a$	$-T\Delta\Delta S^a$
wt	−10.7	2.5	−13.2	–	–	–
T11A	−10.5	2.8	−13.3	0.2	0.3	−0.1
G12A	−10.1	−0.6	−9.5	0.6	−3.1	3.7
P13A	−10.8	2.6	−13.4	−0.1	0.1	−0.2
K15A	−8.7	3.4	−12.1	2.0	0.9	1.1
R17A	−10.2	2.8	−13.1	0.5	0.3	0.1
I19A	−10.6	2.5	−13.1	0.1	0.0	0.1
R20A	−10.4	2.5	−12.9	0.3	0.0	0.3
V34A	−10.7	−2.7	−8.0	0.0	−5.2	5.2
Y35A	−9.9	1.8	−11.7	0.8	−0.7	1.5
G36A	−9.8	3.0	−12.8	0.9	0.5	0.4
G37A	−9.9	3.6	−13.5	0.8	1.1	−0.3
R39A	−10.5	3.1	−13.6	0.2	0.6	−0.4
K46A	−10.6	2.3	−12.9	0.1	−0.2	0.3

[a] mut-wt.

temperature, and variation of enthalpy at the T_m are represented. These parameters, although reflecting perturbations in structure or in thermostability, should be considered apparent. The calculated changes in helical content found for some of the mutants may be overestimated if the chromophores of the side chains of tyrosines and phenylalanines have contributed to the observed perturbations in the far-UV spectra (Manning & Woody, 1989). The thermostability measurements should be considered approximate since the unfolding of the protein may not be completely reversible (Makhatadze et al., 1993). Partial refolding may lead to lower T_m values as well as sharper transitions. It is clear from the analysis of Table 3 that there is a strong correlation between the parameters considered. The observed

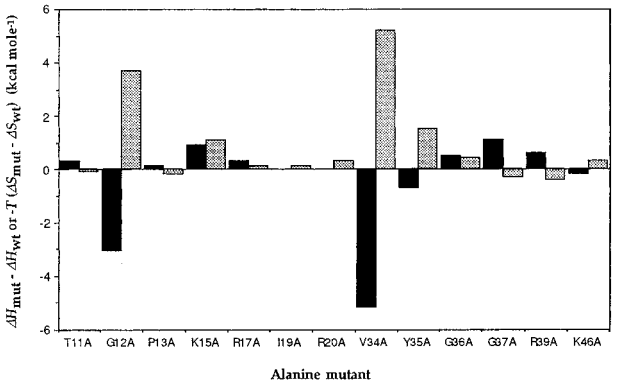

FIGURE 6: Relative contribution of $\Delta\Delta H$ (solid bars) and $-T\Delta\Delta S$ (stippled bars) to the change in ΔG for the association of chymotrypsin with alanine mutants of BPTI.

Table 3: Comparison of Structural and Stability Parameters of the BPTI Alanine Mutants[a]

peptide	gain/loss in helical content (%)[b]	ΔT_m (°C)[c]	$\Delta\Delta H_m$ (kcal mol^{-1})[c]
I18A[d]	+44	0.2	e
F33A	−29	−16.5	−64.4
G12A	−35	−16.1	−35.4
Y35A	−28	−16.2	−31.7
G37A	−26	−4.2	−65.9
G36A	−14	−11.7	−14.5
K15A	−18	−4.2	−0.5
R17A	−13	−4.7	2.1
I19A	−18	−4.8	−1.3
T11A	−11	−0.3	−4.2
P13A	−10	e	e
R20A	−11	e	e
V34A	−4	e	e
R39A	−20	0	0.4
K46A	−3	e	e

[a] Percent wt or mut-wt. Obtained at pH 3.0. These are apparent measurements, as explained under Results. [b] Helical content calculated according to Chen et al. (1972). [c] T_m, wt = 94.2 °C; ΔH_m, wt = 84.3 kcal mol^{-1}). [d] Aggregates in solution. [e] Value not determined accurately but similar to that obtained for wt BPTI.

perturbations of structure and/or decrease in thermal stability also correlate well with the degree of proteolytic degradation during expression (results not shown).

Ile-18-Ala is the only mutant that aggregates strongly, and this may explain the pronounced perturbations in the far-UV spectrum. Interestingly, position 18, whose side chain is only partially exposed to the solvent, is preferably occupied by large hydrophobic side chains in proteins homologous to BPTI (Creighton & Charles, 1987). Five mutants, Phe-33-Ala, Gly-12-Ala, Tyr-35-Ala, Gly-36-Ala, and Gly-37-Ala, are considerably less stable than the wt peptide, with parallel loss in helical content. This can be explained by perturbation of the packing of the molecule for Phe-33-Ala and Tyr-35-Ala, which have buried side chains, and by distortions of the main chain conformation for the Gly-12-Ala, Gly-36-Ala, and Gly-37-Ala variants. The mutants Lys-15-Ala, Arg-17-Ala, and Ile-19-Ala exhibit slight structural perturbations and decreases in stability. The remaining mutants do not exhibit significant differences with respect to wt BPTI for any of the parameters shown in Table 3, with the exception of Arg-39-Ala, which has a larger loss in helical content.

Although surface single side chain substitutions rarely have significant destabilizing effects in proteins, a decrease in stability upon mutating surface residues has been observed

in cases where electrostatic interactions on the surface of the protein are disrupted (Akke & Forsén, 1990; Hammen et al., 1995), or when interior hydrophobic groups become more exposed to the solvent (Hurley et al., 1995). Minor rearrangements of a side chain can also cause subtle changes in the main chain structure (Smith et al., 1986). These destabilizing effects introduced by side chain substitutions are more likely to occur when glycine is the residue replaced, due to steric hindrance restrictions caused by the presence of a C_β-atom. Indeed, the three glycines replaced by alanine in the binding epitope of BPTI (positions 12, 36, and 37) all have ϕ and ψ angles (Deisenhofer & Steigemann, 1975; Wlodawer et al., 1984, 1987) that in general are sterically not allowed for side chains with a C_β-atom (Ramachandran & Sasisekharan, 1968), and all are highly conserved among protein sequences homologous to BPTI (Creighton & Charles, 1987).

DISCUSSION

Binding Affinity. Overall, the effect of alanine single mutations in the reactive region of BPTI on the binding affinity of the inhibitor toward trypsin and chymotryosin was relatively modest (Figure 3). In the case of trypsin, the analysis was limited by the small number of mutants for which the absolute value of the inhibition constant was obtained (a reduction in binding energy of less than 4 kcal mol^{-1} was undetectable by the methods used). This lack of effect is explained in part by the reduced contact between inhibitor and protease at the periphery of the binding epitope and by the fact that most intermolecular hydrogen bonds are mediated by main chain atoms.

According to empirical estimates, a decrease in binding energy of 4 kcal mol^{-1} corresponds to the loss of the contribution of up to five methyl groups to the energy of binding (Andrews et al., 1984), or to the disruption of two uncharged hydrogen bonds or one charged hydrogen bond (Fersht et al., 1985; Shirley et al., 1992; Byrne et al., 1995). Analysis of the X-ray structures of free BPTI (PDB entry 5PTI) and of the complexes formed with trypsin (PDB entry 2PTC) and chymotrypsin (coordinates supplied by T. Hynes and A. A. Kossiakoff) reveals that the reduction of surface area upon complex formation of BPTI side chain atoms beyond the C_β-atom is around 100 Å^2 for Lys-15, Arg-17 (trypsin and chymotrypsin complexes), and Arg-39 (trypsin complex), and significantly less than that for all the other side chains in both complexes. Each of these three side chains mediates two intermolecular hydrogen bonds in the case of the trypsin/BPTI complex (excluding the salt bridge between the charged amino group of Lys-15 and Asp-189 of trypsin). In the chymotrypsin/BPTI complex, the amino group of Lys-15 forms two intermolecular hydrogen bonds, and the guanidino groups of Arg-17 and Arg-39 form one hydrogen bond each. The recent elucidation of the structure of the chymotrypsin/BPTI complex by X-ray diffraction studies (T. Hynes, and A. A. Kossiakoff, unpublished results) has revealed that the Lys-15 side chain does not pack fully into the hydrophobic specificity pocket of chymotrypsin, but rather makes a sharp turn with the charged amino group, reaching the surface of the contact region where it makes two hydrogen bonds (Kossiakoff et al., 1993).

Partial exposure to the solvent of groups involved in intermolecular hydrogen bonding is also likely to minimize energy perturbations resulting from alanine substitutions, if water molecules substitute for the donor groups removed. The guanidino groups of Arg-17 and Arg-39 of BPTI are partially exposed to the solvent in both the trypsin and chymotrypsin complexes, as well as the amino group of Lys-15 of BPTI in the chymotrypsin complex. The very limited impact of the alanine substitutions at positions Lys-15, Arg-17, and Arg-39 on the binding affinity of BPTI toward chymotrypsin is striking, and could reflect local favorable rearrangements in the structures of the protein molecules and solvent.

Predictions of individual residue contributions to the free energy of stabilization of the trypsin/BPTI complex using an empirical energy function indicated that none of the binding epitope residues of BPTI contributed more than 4.0 kcal mol^{-1} to the binding energy, with the exception of Lys-15, which had an estimated contribution of 11.6 kcal mol^{-1} (Krystek et al., 1993). The mutation at the P1 site of BPTI caused a pronounced drop in the energy of binding to trypsin ($\sim$10 kcal mol^{-1}) . In this complex, even if water molecules in the protease binding pocket can act as hydrogen bond partners, the charged group of Asp-189 will not be neutralized, which by itself can cause a pronounced decrease in the free energy of association, of up to 6$-$10 kcal mol^{-1} (Andrews et al., 1984; Fersht, 1985). Molecular electrostatic potential (MEP) maps of the trypsin specificity pocket and Lys-15 side chain reveal an almost perfect electrostatic complementarity (Náray-Szabó, 1993). In addition, the loss of three methylene groups in the P1 side chain with the Lys-15-Ala mutation means an appreciable loss in hydrophobic energy (0.8$-$1.5 kcal mol^{-1} per methylene group removed) (Andrews et al., 1984; Kellis et al., 1989).

It is unlikely that the reduction in the binding free energy of the BPTI mutant Ile-18-Ala with trypsin and chymotrypsin, of nearly 5 and 0.5 kcal mol^{-1}, respectively, is attributable exclusively to reduction in the aliphatic binding surface, since part of the side chain of Ile-18 is already partially buried in free BPTI. The aggregation of the peptide molecule probably contributes to the observed loss in binding affinity.

Distortions in the main chain are likely to be the cause of the reduction in binding affinity observed for the glycine to alanine variants of BPTI, and for the Tyr-35-Ala mutant (Figure 3), based on the structural and thermostability properties of these mutants (Table 3). A Tyr-35-Gly mutant of BPTI (Housset et al., 1991) has shown significant main chain perturbations, especially in the binding loops. These effects are probably magnified in the case of the trypsin system (Gly-12-Ala and Gly-36-Ala mutants) given the optimal positioning of the side chain of Lys-15 of the wt protein with respect to the negatively charged side chain of Asp-189 in the binding pocket of trypsin. A difference in distance between two charges of 0.1 Å can translate into a difference in interaction energy of 1.3 kcal mol^{-1} (Weber, 1992). Steric hindrance caused by the addition of one methyl group could also contribute to the decrease in binding affinity observed here for the glycine to alanine mutants. A binding cavity that is configured for a hydrogen atom, when occupied by a methyl group, can impose an energy penalty of $>$7.5 kcal mol^{-1} (Fersht, 1985).

The results obtained in the present work are in agreement with the general findings of alanine-scanning mutagenesis studies, where only a few side chains dominate a functional

epitope in the contribution to the energy of association, and these side chains tend to be charged or have aromatic groups (Cunningham & Wells, 1989; Jin et al., 1992; Nuss et al., 1993; Dembowski & Kantrowitz, 1994; Clackson & Wells, 1995; Jendeberg et al., in press). The dominance of these side chains in protein binding epitopes confirms their role in conferring specificity to binding processes, as happens in protein folding processes (Barlow & Thorton, 1983). On the other hand, disruption of intermolecular hydrogen bonds and/or an appreciable reduction in intermolecular van der Waals contacts (Arg-17-Ala and Arg-39-Ala mutations) did not produce substantial effects in the ΔG, at least in the case of the interaction with chymotrypsin, for which the binding affinities of all the mutants were quantified (Figure 3). The data obtained for the Ile-18-Ala mutant are inconclusive because of the aggregation of this peptide in solution.

Contribution of k_{on} and k_{off} to the Binding Affinity. In naturally occurring protein/ligand complexes, the affinity of binding is determined primarily by the dissociation rate constant. Dissociation rate constants for protein/ligand complexes can vary over a wide range (10^{-8} s^{-1} < k_{off} < 10^{6} s^{-1}), the fastest dissociation rate constants being around $10^{3}-10^{5}$-fold lower than the diffusion-controlled rates (Fersht, 1985), whereas association rates vary over a much narrower range, to within only a few orders of magnitude of the diffusion-limited rate (10^{4} M^{-1} s^{-1} < k_{on} < 10^{8} M^{-1} s^{-1}). In protease/inhibitor systems, the rate-limiting step of the dissociation reaction is the transition from stable to loose complex, and the rate constant of this initial step can be considered equal to the overall dissociation rate constant, k_{off} (Engel et al., 1974; Finkenstadt et al., 1974; Quast et al., 1978a,b). In this work, the decrease in the dissociation rate constants was the predominant cause for the reduction in affinity of the alanine BPTI mutants toward trypsin and chymotrypsin (Figure 5). Therefore, the results obtained are in accordance with the natural variation of rate constants for protein/ligand systems and previous findings in other mutagenesis studies (Jin & Wells, 1995; Jendeberg et al., in press). It is the stability of the protein/ligand complex that determines most of the binding affinity and not its rate of formation.

The alanine substitutions had very little effect on the rate of formation of the trypsin/PBTI and chymotrypsin/BPTI complexes, with only one notable exception: the association of trypsin with the P1 mutant (Table 1 and Figure 5). The decrease in the association rate constant for the trypsin/K15A complex was 230-fold, which corresponds to a loss in binding free energy of around 3 kcal mol^{-1}. This is a pronounced drop in the rate constant of association, for a single side chain substitution, and it suggests that changes in electrostatic interactions are involved. Isolated cases been observed in mutant studies where k_{on} is drastically reduced (Jendeberg et al., in press). *In vivo*, ligands that associate slowly with target receptors will probably be selected against, in order to maintain a rapid response to physiological conditions.

In the two-step reaction that characterizes the association of protease and inhibitor, the overall association rate constant, k_{on}, is determined by the equilibrium constant of the initial reaction (formation of loose complex), and by the rate constant of the conversion from loose to stable complex. Obviously, both these constants influence the overall association rate constant. It is likely that the decrease in k_{on} observed for the association of trypsin with the Lys-15-Ala BPTI mutant is imposed by an increase in the dissociation binding constant of the loose complex, because of a reduction in the number of collisions and/or a decrease in the stability of the loose complex. It seems that the charged amino group of the side chain of Lys-15 of BPTI is crucial in the initial recognition steps of the association with trypsin, probably steering the BPTI molecule into productive orientations during diffusional encounters with the protease. There are no effects on the association rate constants with the replacement by alanine of other positively charged side chains of BPTI (Arg-17, Arg-20, and Arg-39), suggesting that these additional charges do not contribute to promoting productive encounters between the inhibitor and trypsin, as seems to be the case with Lys-15. Determinations of association rate constants under different ionic strengths (results not shown) indicated that the presence of formal positive charges in the vicinity of the P1 site can have a counter-productive effect in the absence of the Lys-15 side chain, especially in the association with trypsin. This would occur if any of the positively-charged side chains in the vicinity of P1 oriented the BPTI molecule in a less favorable manner with respect to the protease.

A broad range in variation of k_{on} rate constants for homologous protein ligands adds to the limitations of empirical methods to calculate binding free energies, which do not take into account differences in association rates. Electrostatic effects are extremely difficult to characterize, especially if conformational fluctuations of ligand and/or receptor molecules are taken into account. Interestingly, complexes that form with similar activation energies can associate with very different rates (Riggs et al., 1970). This suggests that adverse electrostatics (reduction of total number of encounters or of productive encounters between the two molecules) can be more limiting to the overall rate of association than conformational and solvation effects. It is reasonable to say that molecular recognition begins before actual contact between ligand and receptor occurs.

Thermodynamic Parameters of Binding (Chymotrypsin/BPTI System). A comparison of the thermodynamic parameters for the association of chymotrypsin with wt BPTI and its alanine mutants shows that changes in entropy tend to be compensated by changes in enthalpy (Figure 6). This is particularly noticeable for the mutants Gly-12-Ala, Val-34-Ala, and Tyr-35-Ala. For the Lys-15-Ala and Gly-36-Ala variants, there is a small unfavorable change in both entropy and enthalpy. As observed before for the effect of the alanine mutations on the kinetic parameters of inhibition, differences in the thermodynamic parameters occur predominantly for those mutants that exhibit structural perturbations and for the P1 mutant. The range in ΔG variation caused by the alanine mutations (2 kcal mol^{-1}) was significantly less than the range in variation for ΔH or $T\Delta S$ (5.2 kcal mol^{-1} for both parameters). Therefore, $\Delta\Delta H$ measurements may be more sensitive than $\Delta\Delta$G measurements (compare Figures 3 and 6) in mapping binding epitopes by alanine-scanning mutagenesis.

The compensating changes in enthalpy and entropy, in the cases of Gly-12-Ala and Tyr-35-Ala, could be related to desolvation effects. For these mutants, assuming that even small distortions of the loop structures can create room for more water molecules at the binding interface, binding entropy would be lost by a reduction in the number of water molecules displaced from the interacting surfaces upon

binding, while favorable electrostatic interactions could be preserved. The Val-34-Ala mutation caused the largest changes in ΔH and ΔS. This mutant showed identical structural properties to the wt peptide, and its stability and kinetics of inhibition were also identical. For the desolvation argument to hold in this case, it is necessary to postulate that the interactions of the side chain of Val-34 of BPTI with chymotrypsin are not optimal, and that the removal of two methyl groups could create room for water in the chymotrypsin/V34A complex. Indeed, inspection of the X-ray structures of the chymotrypsin/BPTI and trypsin/BPTI complexes revealed that the Val-34 side chain of BPTI is close to two aromatic rings of the protease (5.8−8.0 Å) in the complex with chymotrypsin, a situation that does not occur in the trypsin/BPTI complex, and the ϵ-nitrogen of the Arg-17 of the inhibitor is pushed 1 Å closer (4.8 Å) toward the γ-CH_3 of Val-34. In future work, we plan to determine the heat capacities of binding to investigate possible solvation effects.

Consistent compensatory changes in the enthalpy and entropy of binding have been observed in other protein systems, namely, protease/inhibitor (Amiconi et al., 1987), antigen/antibody (Szewczuk & Mukkur, 1977; Oss et al., 1982; Herron et al., 1986; Siguskjøld et al., 1991; Siguskjøld & Bundle, 1992; Brummell et al., 1993; Ito et al., 1993), and enzyme/substrate associations (Berland et al., 1995). Such entropy/enthalpy correlations have also been interpreted as evidence for the involvement of water molecules in the association process and are observed in a wide variety of reactions in aqueous solution (Leffler & Grunwald, 1963; Lumry & Rajender, 1970; Hindsgaul et al., 1985). These enthalpy/entropy compensation effects may limit efforts to engineer the specificity of binding in protein/ligand associations.

A prominent result obtained in this thermodynamic study is that the binding enthalpies of the association of chymotrypsin with the BPTI alanine mutants are always positive, except in two cases (Table 2), contrary to what is generally observed in the association of proteins with their ligands. However, a positive enthalpy of binding for the interaction of Kunitz inhibitors with their cognate proteases seems to be the rule rather than the exception, judging by the thermodynamic parameters reported in the literature for these protease/inhibitor systems (Laskowski & Laskowski, 1954; Vincent & Lazdunski, 1972, 1973; Finkenstadt et al., 1974; Quast et al., 1974; Amiconi et al., 1987; Ascenzi et al., 1988, 1990a,b). Although the information available on the thermodynamics of protein/ligand interactions is currently still scarce, as compared to structural and kinetic information, and especially for protease/inhibitor associations, these data suggest that the association of Kunitz-type inhibitors has a particular thermodynamic profile that contrasts with the customary negative-change-in-enthalpy and positive-change-in-entropy observed in protein/ligand association processes. It is possible that this characteristic of Kunitz inhibitors is shared by other serine protease inhibitor groups.

Several explanations can be advanced to rationalize this unusual thermodynamic behavior. It could be hypothesized that exclusion of water from the contact interface is a determinant of a highly favorable binding entropy and unfavorable enthalpy, arising from suboptimal van der Waals contacts and electrostatic interactions. However, removal of water from binding interfaces is a general occurrence in protein/protein associations (Janin & Chothia, 1990). Specific desolvation effects could be in part related to the balance between the enthalpy and entropy of binding, given the variation in the enthalpy and entropy of hydration for aliphatic, aromatic, and polar groups (Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993). The percentage of aromatic and charged residues in the binding interfaces of protease/inhibitor complexes is significantly less than in antibody/antigen complexes, which can have more than half of the contact interfaces composed of these residues (Kabat et al., 1987; Janin & Chothia, 1990; Padlan, 1990; Krystek et al., 1993; Nuss et al., 1993). Since polar surfaces have more unfavorable enthalpies of desolvation than apolar surfaces (Novotny & Sharp, 1992; Makhatadze & Privalov, 1993), at a first analysis, this factor does not seem to be the determinant for the particular thermodynamics of the formation of protease/Kunitz inhibitor complexes. The relative rigidity of the binding loops of small protein inhibitors, in particular BPTI, may also contribute to the large gain in entropy upon binding to the protease. Binding surfaces of several inhibitors and enzymes were found to be rich in amino acids with side chains that incur no conformational entropy penalty on complex formation (Krystek et al., 1993). The loss of one torsional degree of freedom can result in the loss of 0.6−0.8 kcal mol^{-1} of binding energy (Andrews et al., 1984; Krystek et al., 1993). In BPTI, in addition to two of these residues being present at the binding epitope (Pro-13 and Ala-16), three other side chains are totally immobilized by covalent cross-linking or burial in the structure (Cys-14, Cys-38, and Tyr-35), and three residues are glycines (Gly-12, Gly-36, and Gly-37). Ile-18 is also partially inaccessible to solvent.

Perhaps the positive change in enthalpy and large positive change in entropy, which seems to characterize the association of at least some small inhibitors with their cognate proteases, are the natural consequence of the selective pressures under which these inhibitors have evolved: the rigidity of the binding loops (presence of a particular conformation) is used to raise the energy of the transition state of the hydrolysis of the P1−P1′ bond by the protease (Longstaff et al., 1990). The fact that most of the intermolecular hydrogen bonds in protease/Kunitz inhibitor complexes that are contributed by the inhibitor involve main chain atoms adds to the requirement for maintaining the backbone conformation of the inhibitor. Increased mobility of the binding loops could mean a decrease in internal energy and more favorable intermolecular contacts, as well as possibility for cleavage of the P1−P1′ bond. Increased flexibility of the inhibitor binding loops has been pointed out as the reason for cleavage of BPTI selectively reduced at the Cys-14/Cys-38 disulfide bridge (Vincent & Lazdunski, 1972) and of the *Cucurbita maxima* trypsin inhibitor-V (Cai et al., 1995).

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