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Electrostatic complementarity within the substrate-binding pocket of trypsin

(site-directed mutagenesis/computer graphics/enzyme kinetics)

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ABSTRACT **The aspartic residue (Asp-189) at the base of the substrate-binding pocket of trypsin was replaced by serine (present in a similar position in chymotrypsin) through site-directed mutagenesis. The wild-type (with Asp-189 in the mature trypsin sequence) and mutant (Ser-189) trypsinogens were expressed in *Escherichia coli*, purified to homogeneity, activated by enterokinase, and tested with a series of fluorogenic tetrapeptide substrates with the general formula succinyl-Ala-Ala-Pro-Xaa-AMC, where AMC is 7-amino-4-methylcoumarin and Xaa is Lys, Arg, Tyr, Phe, Leu, or Trp. As compared to [Asp^{189}]trypsin, the activity of [Ser^{189}]trypsin on lysyl and arginyl substrates decreased by about 5 orders of magnitude while its K_m values increased only 2- to 6-fold. In contrast, [Ser^{189}]trypsin was 10–50 times more active on the less preferred, chymotrypsin-type substrates (tyrosyl, phenylalanyl, leucyl, and tryptophanyl). The activity of [Ser^{189}]trypsin on lysyl substrate was about 100-fold greater at pH 10.5 than at pH 7.0, indicating that the unprotonated lysine is preferred. Assuming the reaction mechanisms of the wild-type and mutant enzymes to be the same, we calculated the changes in the transition-state energies for various enzyme–substrate pairs to reflect electrostatic and hydrogen-bond interactions. The relative binding energies (E) in the transition state are as follows: $E_{II} > E_{PP} > E_{PA} > E_{IP} \approx E_{IA}$, where I = ionic, P = nonionic but polar, and A = apolar residues in the binding pocket. These side-chain interactions become prominent during the transition of the Michaelis complex to the tetrahedral transition-state complex.**

The binding of substrates or inhibitors to the specificity pocket of an enzyme involves a combination of chemical forces including hydrogen bonds and electrostatic, hydrophobic, and steric interactions. The complexity of the interactions involved in the substrate specificity of an enzyme is exemplified by trypsin. The three-dimensional structures of trypsin bound to pancreatic trypsin inhibitor (PTI) (1–4) or to the pseudosubstrate benzamidine (5, 6) suggest that the carboxylate of Asp-189, at the base of the trypsin binding pocket, is largely responsible for the specificity of binding of the enzyme to positively charged amino acid side chains.

The major role of electrostatic interactions in the trypsin binding pocket has been analyzed by measuring (7) and calculating (8) the stabilization energies of binding between a series of benzamidine analogs and trypsin. In addition, the high degree of structural similarity of the trypsin and chymotrypsin binding pockets (9, 10) is consistent with the experimental observations that aromatic side chains may form favorable hydrophobic interactions with the trypsin binding pocket (10–13).

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Progress in DNA technology has made it possible to analyze the structural bases of enzyme action and substrate specificity by directed replacement of residues of interest. In a previous study (10), Asp-189 of rat trypsin was replaced with lysine by site-directed mutagenesis, in an attempt to reverse the specificity of the enzyme for the charged residue at the scissile bond (10). [Lys^{189}]Trypsin, however, did not display specificity toward acidic substrates but instead showed an altered low activity to certain hydrophobic substrates. Possibly the positively charged ε-amino group of lysine is directed outside the substrate-binding pocket, so that the long aliphatic side chain distorts the dielectric constant and/or the topography of the binding pocket (10).

To analyze the forces contributing to the complementarity within the trypsin binding pocket, we have replaced Asp-189 with serine. Computer graphic modeling suggests that unlike the ε-amino group of [Lys^{189}]trypsin, the β-hydroxyl group of [Ser^{189}]trypsin would not interact with any neighboring atoms of the binding pocket. Thus the geometry of the binding pocket should remain unchanged. We have characterized the catalytic activity of [Asp^{189}]- and [Ser^{189}]trypsin by using a series of synthetic fluorogenic substrates with various amino acids in the C-terminal (P1) position in order to compare the electrostatic interactions of the different enzyme–substrate pairs.

MATERIALS AND METHODS

Materials. Tetrapeptide substrates with the fluorogenic leaving group 7-amino-4-methylcoumarin (AMC) had the general formula Suc-Ala-Ala-Pro-Xaa-AMC, where Suc was succinyl (3-carboxypropanoyl) and Xaa (i.e., the P1 residue) was Lys, Arg, Tyr, Phe, Leu, or Trp. The peptides were synthesized by the conventional solution method with *t*-butyloxycarbonyl (Boc) and benzyl ester (OBzl) as protecting groups. Trifluoroacetic acid was used for deprotection. The Boc-protected C-terminal amino acid (Boc-Xaa-OH) was transformed into a mixed anhydride by isobutyl chloroformate in the presence of *N*-methylmorpholine and then reacted with AMC (Enzymes Systems Products, Livermore, CA) to yield Boc-Xaa-AMC. The protected tripeptide Boc-Ala-Ala-Pro-OBzl was synthesized from H-Pro-OBzl by the stepwise addition of the two alanines by the dicyclohexylcarbodiimide/1-hydroxybenztriazole method. The OBzl protecting group was removed by catalytic hydrogenation. The coumarin derivatives of the amino acids were acylated with Boc-Ala-Ala-Pro-OH by the dicyclohexylcarbodiimide/1-hydroxybenztriazole method. The deprotected tetrapeptide-coumarins were acylated by succinic anhydride. Homogeneity of each substrate was checked by thin-layer chromatog-

Abbreviations: PTI, pancreatic trypsin inhibitor; AMC, 7-amino-4-methylcoumarin; Suc, succinyl.

Table 1. Kinetic constants and comparative transition-state Gibbs energies of [Ser^{189}]trypsin and [Asp^{189}]trypsin

Substrate P1 residue	Enzyme residue 189	No. of experimental points	k_{cat}, min^{-1} (mean ± SEM)	K_m, mM (mean ± SEM)	k_{cat}/K_m, $min^{-1}·mM^{-1}$	(k_{cat}/K_m)Ser / (k_{cat}/K_m)Asp	ΔG,* kcal·mol^{-1}
Arg	Ser	18	0.229 ± 0.011	0.169 ± 0.017	1.35	1.73×10^{-5}	6.75
	Asp	17	5460 ± 285	0.0697 ± 0.0088	78,000		
Lys	Ser	18	0.487 ± 0.041	0.501 ± 0.060	0.97	1.33×10^{-5}	6.61
	Asp	18	7269 ± 90	0.099 ± 0.003	72,900		
Tyr	Ser	24	8.56 ± 0.29	0.819 ± 0.0360	10.5	54.12	−2.46
	Asp	18	0.038 ± 0.018	0.198 ± 0.0224	0.194		
Phe	Ser	24	2.56 ± 0.069	0.69 ± 0.026	3.7	7.7	−1.26
	Asp	12	0.378 ± 0.018	0.784 ± 0.056	0.48		
Leu	Ser	18	0.70 ± 0.05	1.7 ± 0.06	0.41	12.8	−1.51
	Asp		≈0.01	ND	0.032†		
Trp	Ser	17	0.059 ± 0.0025	0.259 ± 0.027	0.2		
	Asp		<0.01	ND			

ND, not determined.

*$\Delta G = -RT\ln[(k_{cat}/K_m)\text{Ser}/(k_{cat}/K_m)\text{Asp}]$ (ref. 20); 1 kcal = 4184 J.

†Determined from triplicate measurements at 0.05 mM substrate concentration ([*S*]) by applying the simplified form of Michaelis–Menten equation ($v = V_{max}/K_m \times [S]$).

raphy and amino acid and elemental analyses. Other reagents were obtained from commercial sources.

Site-Directed Mutagenesis. The oligodeoxynucleotide 5′ GAGGGAGGCAAGTCTTCCTGCCAGGGC 3′ was synthesized on an automatic DNA synthesizer and used to prime the synthesis of a DNA strand encoding a trypsinogen template with a serine codon (TCT) in place of the aspartic codon (GAT) at amino acid position 189 of the rat trypsin sequence. Mutagenesis was accomplished by the double-primer method of Zoller and Smith (14). The region that contains the mutated sequence was isolated by cleavage with the restriction endonucleases *Xho* I and *Sal* I, and this fragment was ligated into the corresponding sites of the expression vector (10).

Heterologous Expression of Wild-Type and Mutant Trypsinogens. The expression of trypsinogen in bacteria was accomplished by fusing the coding sequences of trypsinogen cDNA (15) to the DNA sequences encoding the signal peptide and regulatory regions of bacterial alkaline phosphatase (16) as described by Graf *et al.* (10). In the product encoded by this vector (for vector construction see figure 1 of ref. 10), the signal peptide of alkaline phosphatase replaces the native signal peptide of pretrypsinogen and ensures efficient secretion of the zymogen into the periplasmic space. *Escherichia coli* K-12 strain SM13 (F^-, *araD139*, Δ(*lac*)*U169*, *relA*, *rpsL*, *phoR*) was transformed with the vector to express constitutively and secrete the zymogen into the periplasmic space.

Protein Purification and Chemistry. The recombinant wild-type and mutant trypsinogens were isolated from the periplasmic protein fraction as described (10). Trypsinogen was activated by purified porcine enterokinase (10) (enzyme/substrate ratio, 1:50, wt/wt) in 50 mM 2-(*N*-morpholino)-ethanesulfonate (Mes), pH 6.0/10 mM $CaCl_2$ for 1 hr at room temperature (mutant trypsinogen) or overnight at 4°C (wild-type trypsinogen). Concentration of activated trypsin was determined by UV absorption ($\varepsilon_{280} = 38{,}000\ M^{-1}·cm^{-1}$) (17) and by active-site titration (18). 4-Methylumbelliferyl *p*-guanidinobenzoate and 4-methylumbelliferyl *p*-trimethylammonium cinnamate chloride were used for the active-site titration of wild-type ([Asp^{189}]-) and mutant ([Ser^{189}]-) trypsin, respectively.

Enzyme Kinetics. Enzyme assays were carried out in 50 mM Tris·HCl, pH 8.0/10 mM $CaCl_2$ at 37°C. The concentrations of fluorogenic substrates (Suc-Ala-Ala-Pro-Xaa-AMC) were 0.025, 0.05, 0.075, 0.1, 0.2, 0.4, and 0.6 mM. [Asp^{189}]Trypsin concentrations determined by active-site titration were 20–50 pM in the case of substrates with Xaa = Arg or Lys and were 300–900 nM in the case of the other substrates (Xaa = Phe, Tyr, Leu, or Trp). [Ser^{189}]Trypsin concentrations employed were in the range 65–450 nM.

For fluorescence measurements, excitation and emission wavelengths were 366 and 440 nm, respectively. Fluorescence values were converted to product concentrations by using standard solutions of AMC; calibration curves of the fluorescence intensities were linear for AMC concentrations in the range 0.05–1 μM in a 1-cm rectangular fluorescence cell.

When the pH dependence of the rate constants (k_{obs}) for [Asp^{189}]trypsin- and [Ser^{189}]trypsin-catalyzed hydrolyses of various substrates was measured, Tris·HCl buffers of pH 8.0 and 8.8 and glycine·NaOH buffers of pH 9.5, 10.0, and 10.5 containing 10 mM $CaCl_2$ were used. The stabilities of the substrates, the product (AMC), and the enzymes were satisfactory at each pH. The activities of [Asp^{189}]trypsin and [Ser^{189}]trypsin using Suc-Ala-Ala-Pro-Lys-AMC as a substrate at pH 10.5 were linear with time for up to 1 hr.

Computer Graphics. To model the active site of [Ser^{189}]-trypsin, we used the coordinates of the bovine trypsin–PTI complex¶ [IBM PC minicomputer program PC Model (19)], omitted the carboxyl oxygens (OD1 and OD2) of the Asp-189 side chain, and manually adjusted the orientation of the tyrosine side chain superimposed on Lys-15 of the inhibitor.

RESULTS

Comparison of the Catalytic Efficiencies of [Ser^{189}]Trypsin and [Asp^{189}]Trypsin on Various Substrates. Table 1 summarizes the k_{cat} and K_m values and the catalytic efficiencies (k_{cat}/K_m) for recombinant rat [Ser^{189}]trypsin (mutant) and [Asp^{189}]trypsin (wild type) with six different substrates. The relative specificities of the enzymes [(k_{cat}/K_m)Ser/(k_{cat}/K_m)Asp] for each substrate and the differential contributions to the binding energy (ΔG) of the side chains of serine and aspartic acid were also calculated by using the equation $\Delta G = RT\ln[(k_{cat}/K_m)\text{Ser}/(k_{cat}/K_m)\text{Asp}]$ (20). The K_m values for the substrates are relatively similar for the two enzymes (variations <10-fold), but the k_{cat} values vary widely (by factors $>10^5$).

The replacement of Asp-189, which lies at the bottom of the trypsin binding pocket, with serine results in a dramatic (5 orders of magnitude) decrease of the catalytic efficiency of the enzyme toward both arginyl and lysyl substrates. In contrast, the Ser-189 mutant is more active than wild-type

¶Brookhaven National Laboratory (1987) Brookhaven Protein Data Bank (Brookhaven Natl. Lab., Upton, NY), File 2PTC.

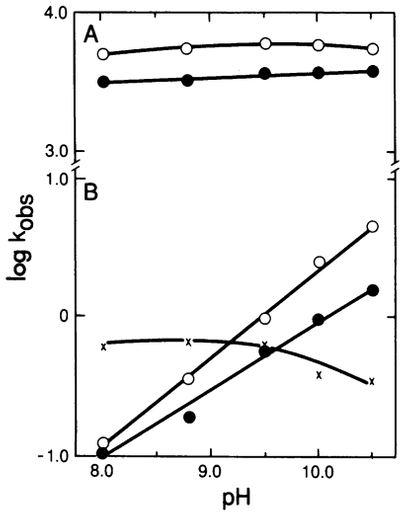

FIG. 1. pH dependence of the rate constants (k_{obs}, min^{-1}) for the hydrolysis of various substrates by [Asp^{189}]trypsin (*A*) and by [Ser^{189}]trypsin (*B*). Enzyme concentration was 0.05 nM (*A*) or 100 nM (*B*); substrates were 0.2 mM Suc-Ala-Ala-Pro-Lys-AMC (○), 0.2 mM Suc-Ala-Ala-Pro-Arg-AMC (●), and 0.2 mM Suc-Ala-Ala-Pro-Phe-AMC (×).

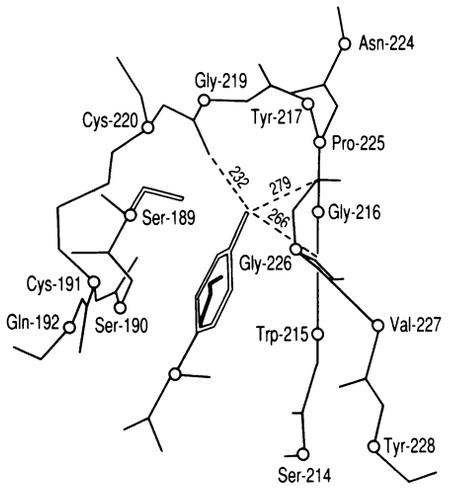

FIG. 2. Computer graphics representation of the substrate-binding pocket of [Ser^{189}]trypsin with bound substrate side chains. α-Carbon atoms are represented by circles. The Ser-189 and tyrosyl-substrate side chains are drawn as double lines; heavy lines represent the lysine side chain of PTI.

trypsin on tyrosyl, phenylalanyl, and leucyl substrates. However, the activity of the [Ser^{189}]trypsin toward these substrates is much lower than that of the wild-type enzyme on the typical basic substrates. Further, [Ser^{189}]trypsin exhibits some activity on the tryptophanyl substrate, whereas trypsin is inactive on the same substrate.

Comparison of the Catalytic Efficiencies of [Ser^{189}]Trypsin on Protonated and Deprotonated Lysyl and Arginyl Substrates. To explore the contribution of an unpaired charged residue to substrate binding and catalysis in the binding pocket of [Ser^{189}]trypsin, we studied how deprotonation of the ε-amino and δ-guanidino groups of the lysyl and arginyl substrates affects catalytic efficiency. As shown in Fig. 1*A*, the activity of [Asp^{189}]trypsin on lysyl and arginyl substrates is not affected by pH within the range 8–10.5. This is consistent with the alkaline stability of rat trypsin (17). Hydrolysis of the phenylalanyl substrate by the [Ser^{189}]trypsin mutant also is not altered significantly within this pH range (Fig. 1*B*). In contrast, the rate of hydrolysis (k_{obs}) of the lysyl and arginyl substrates by [Ser^{189}]trypsin steadily increases as a function of pH (Fig. 1*B*). There is a linear correlation between log k_{obs} and pH, with a slope of 0.5. This suggests that the activity is qualitatively influenced by the charge of the ε-amino and δ-guanidino groups in the two substrates. Thus the deprotonated lysyl and arginyl substrates are hydrolyzed at a higher rate than their charged forms. Kinetic analysis of the [Ser^{189}]trypsin-catalyzed hydrolysis of the lysyl substrate was performed at pH 7.0 (at which practically 100% of the ε-amino groups are protonated) and at pH 10.5 (at which about 50% of the ε-amino groups are protonated) (Table 2). [Ser^{189}]Trypsin hydrolyzes the deprotonated lysyl substrate about 100 times more efficiently than it does the protonated one. Studies with commercially available chymotrypsin also indicated that the deprotonated lysyl substrate is preferred over the protonated one (data not shown), but quantitation was difficult due to the instability of chymotrypsin at higher pH values.

Computer Graphics Modeling of the Binding Pocket of [Ser^{189}]Trypsin with a Tyrosine Side Chain in the Pocket. The structure of the [Ser^{189}]trypsin binding pocket was modeled with the Lys-15 side chain of PTI (1–4) and with a tyrosine side chain substituted for Lys-15 of PTI (Fig. 2). According to this rigid model, a close contact exists between the hydroxyl group of tyrosine and the α carbon of Gly-216 (O–C distance, 241 pm). Presumably, the binding pocket of rat trypsin may adjust somewhat to accommodate the tyrosine side chain. Similarly, the very short (232 pm) hydrogen-bond distance between the hydroxyl of tyrosine and the oxygen of Gly-219 may also relax. Other hydrogen-bond distances, between the tyrosine hydroxyl and the oxygen of Gly-216 and between the tyrosine hydroxyl and the nitrogen of Gly-216, are 279 and 266 pm, respectively.

DISCUSSION

The serine proteases trypsin and chymotrypsin have very different substrate specificities (9), yet the substrate-binding pockets, formed by residues 214–220, 189–192, and 224–228, exhibit a high degree of structural similarity (9, 21). When an aromatic group of the substrate occupies the chymotrypsin specificity pocket, it is sandwiched between the peptide bonds of residues 190–192 on the one side and of residues 215–216 on the other. The hydrophobic group presumably can make several hydrophobic contacts involving Ser-190, Cys-191 Cys-220, Val-213, Trp-215, and Tyr-228 (22), residues

Table 2. Catalytic efficiencies and comparative transition-state Gibbs energies of [Ser^{189}]trypsin with deprotonated (Lys) and protonated (Lys^+) lysyl substrates

Substrate P1	No. of experimental points	k_{cat}, min^{-1} (mean ± SEM)	K_m, mM (mean ± SEM)	k_{cat}/K_m, $min^{-1} \cdot mM^{-1}$	$\frac{(k_{cat}/K_m)Lys}{(k_{cat}/K_m)Lys^+}$	ΔG,* kcal·mol^{-1}
Lys (pH 10.5)†	18	9.507 ± 0.18	0.152 ± 0.01	62.54	84.5	−2.72
Lys^+ (pH 7.0)‡	12	0.095 ± 0.006	0.129 ± 0.02	0.74		

*$\Delta G = -RT\ln[(k_{cat}/K_m)Lys/(k_{cat}/K_m)Lys^+]$ (ref. 20).
†Determined at pH 10.5 (50% of the applied actual substrate concentration, the deprotonated fraction, was taken for calculation).
‡Determined at pH 7.0 (100% of the substrate is protonated).

that are identical in bovine trypsin, rat chymotrypsin, and rat trypsin. It has been conjectured that the Asp→Ser replacement at position 189 at the bottom of the substrate-binding pocket accounts primarily for the different substrate specificities of trypsin and chymotrypsin. If so, then replacement of Asp-189 with serine in trypsin might enhance its inherent low-level chymotryptic activity (10, 11). The activity of recombinant rat [Ser^{189}]trypsin on lysyl and arginyl synthetic substrates is very low as compared to that of recombinant rat wild-type trypsin (Table 1). Thus Asp-189 appears to be crucial for activity against these basic substrates. On the other hand, [Ser^{189}]trypsin displays higher activity than wild-type trypsin toward the tyrosyl, phenylalanyl, and leucyl substrates (Table 1), but this is still low compared to the activity of chymotrypsin on these substrates and low compared to the activity of trypsin on its preferred (basic) substrates. This confirms the assumption (10) that a number of changes in the structure of the binding pocket will be required to alter the substrate specificity.

From simple structural considerations and computer graphic modeling it appears that the Asp→Ser replacement at position 189 does not have deleterious steric consequences. We presume that the backbone structure of the trypsin binding pocket remains unaltered. However, more subtle structural changes, such as the presence or absence of the water molecules, cannot be eliminated. The difference between the catalytic activities of [Asp^{189}]trypsin and [Ser^{189}]trypsin on the same substrate might be reasonably accounted for by the altered electrostatic interaction between the side chains of residue 189 and the P1 residue of the substrate. The differential action of the enzyme on a set of substrates in which only the P1 residue is varied should reflect changes in the electrostatic complementarity between the interacting side chains, provided that the structural differences between the P1 side chains do not significantly affect their hydrophobic and steric complementarity with the binding pocket. Phenylalanine vs. tyrosine residues and protonated vs. deprotonated lysine residues appear to satisfy this criterion.

If we assume that [Asp^{189}]trypsin and [Ser^{189}]trypsin have the same mechanism of action, the kinetic data allow a comparison of the transition-state activation energies between the various enzyme–substrate pairs (Fig. 3). If the electrostatic interaction between Asp-189 of trypsin and the ε-amino group of the lysyl substrate is the basic condition, then replacement of either interacting charged group with a neutral one (that is, assaying phenylalanyl or tyrosyl substrate against [Asp^{189}]trypsin or the lysyl substrate against [Ser^{189}]trypsin) increases the transition-state activation energy by about 7 kcal/mol. Thus, an unpaired charged residue in the binding pocket of trypsin will have the most unfavorable effect on the catalytic efficiency of the enzyme. Quiocho *et al.* (22) have shown that isolated ionic groups in proteins can be stabilized by polarized peptide units. That an unpaired charged residue in the trypsin binding pocket affects the catalytic activity most unfavorably (Fig. 3) points to the lack of such a stabilizing hydrogen-bond array in our case. Replacement of the unpaired charged group with either a polar or an apolar group apparently stabilizes the enzyme transition-state complex to an extent dependent upon the nature of the other group. Deprotonation of the lysyl substrate, when assayed with [Ser^{189}]trypsin, resulted in a 2.7-kcal/mol decrease in the transition-state activation energy. With the tyrosyl substrate, a similar improvement in catalytic efficiency (decrease of 2.46 kcal/mol in the transition-state activation energy) was observed with [Ser^{189}]trypsin as compared to [Asp^{189}]trypsin. The transition-state activation energy was also decreased (by 1.3 kcal/mol) for the [Ser^{189}]trypsin–phenylalanyl substrate pair as compared to the [Asp^{189}]trypsin–phenylalanyl substrate pair (Fig. 3).

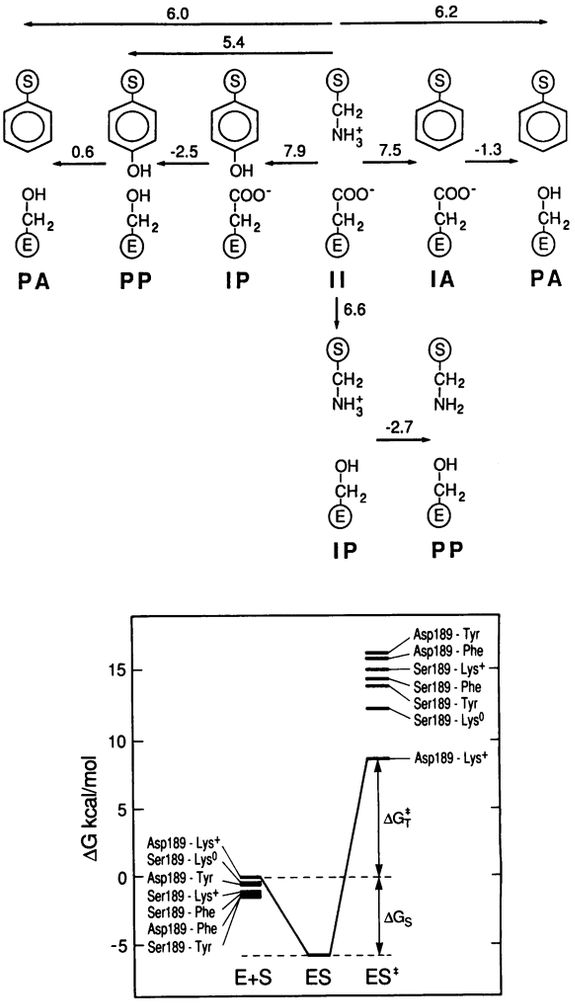

FIG. 3. (*Upper*) Side-chain interactions and Gibbs energy differences between the corresponding enzyme–transition-state complexes (Δ*G*, kcal/mol). E, enzyme; S, substrate. Nature of each enzyme–substrate pair is indicated by two letters characterizing enzyme residue 189 (first letter) and substrate residue P1 (second letter) as polar but nonionic (P), apolar (A), or ionic (I). (*Lower*) Gibbs energy diagram of [Asp^{189}]- and [Ser^{189}]trypsin with different substrates, normalized to the level of ES (Michaelis) complex. For [Asp^{189}]trypsin with protonated lysyl (Lys$^+$) substrate, ΔG_S and $\Delta G_T^{\ddagger}$ are −5.7 and +8.6 kcal/mol, respectively.

Thus, for trypsins, the electrostatic interactions between side chains of residue 189 (in the enzyme) and P1 (in the substrate) at the base of the specificity pocket contribute significantly to the binding of the substrate in the transition state. The relative binding energies (*E*) in the transition state are as follows: $E_{II} > E_{PP} > E_{PA} > E_{IP} \approx E_{IA}$, where I = ionic, P = nonionic but polar, and A = apolar residues in the binding pocket. Ionic–ionic and polar–polar interactions contribute more to the transition-state stabilization than interactions between other pairs. The *similis simili gaudet* principle appears to hold here: groups producing electrostatic potentials and fields of similar magnitude around them tend to interact more strongly.

For the interpretion of this phenomenon, it is significant that the K_m values for all the enzyme–substrate pairs remain relatively similar (Tables 1 and 2); thus, the stability of the Michaelis complex does not seem to be greatly affected by the electrostatic forces described above. Thus, P1 side-chain pairing becomes significant during the transition of the Michaelis complex to the tetrahedral transition-state complex (9). Recently, two studies (23, 24) have been carried out

on electrostatic interactions between different subtilisin mutants and substrates. The charged residues mutated by Russel *et al.* (23) are not closely involved in substrate binding, whereas Wells *et al.* (24) have directly addressed the issue of electrostatic complementarity between substrates and charged amino acids at two different sites of the binding cleft. In their study (24), the average Gibbs energies for enzyme–substrate pair interactions were calculated to be about -2.0 kcal/mol, and the observed changes in k_{cat}/K_m were dominated by changes in the $1/K_m$ term. This contrasts with the results obtained in our experiments with trypsin: the ratio of K_m values for wild-type trypsin and [Ser^{189}]trypsin with the lysyl substrate is 0.41, whereas the ratio of k_{cat} values is 5.8×10^5. Thus the mechanisms for interacting with the substrates to form the putative tetrahedral transition-state complexes may be different in the cases of subtilisin and trypsin. This may in part explain the difference between the numerical values of transition-state Gibbs energies obtained by us and Wells *et al.* (24). Our data do not allow direct calculation of the Gibbs energies for enzyme–substrate ion-pair interactions. The increase of the transition-state activation energy by 6.6 kcal/mol as a consequence of replacing Asp-189 with serine (lysyl substrate) does not represent the Gibbs energy of the enzyme–substrate ion-pair interaction, since in the case of [Ser^{189}]trypsin interacting with the lysyl substrate, for example, an unpaired charged residue is left in the substrate binding cleft. Fersht *et al.* (20) estimated that this may weaken binding by about 3 kcal/mol, a value that closely approximates our calculated increase of about 2.7 kcal/mol (Table 2) in the transition-state binding energy on deprotonation of the ϵ-amino group of lysine. Thus, we estimate the Gibbs energy for enzyme–substrate ion-pair interactions in trypsin to be about 4 kcal/mol, about 1 kcal/mol higher than the value for a buried ion pair in chymotrypsin (25). However, differences in the ΔG values can be accounted for by differences in the ion-pair separations, formal charges, side-chain torsional energies, and dielectric constants. The involvement of these factors in the interaction of trypsin with its substrates may be further explored by kinetic and structural studies of appropriate trypsin mutants.

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