# Perturbing the Polar Environment of Asp102 in Trypsin: Consequences of Replacing Conserved Ser214[†,‡]

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*Received October 31, 1991*

ABSTRACT: Much of the catalytic power of trypsin is derived from the unusual buried, charged side chain of Asp102. A polar cave provides the stabilization for maintaining the buried charge, and it features the conserved amino acid Ser214 adjacent to Asp102. Ser214 has been replaced with Ala, Glu, and Lys in rat anionic trypsin, and the consequences of these changes have been determined. Three-dimensional structures of the Glu and Lys variant trypsins reveal that the new 214 side chains are buried. The 2.2-Å crystal structure ($R$ = 0.150) of trypsin S214K shows that Lys214 occupies the position held by Ser214 and a buried water molecule in the buried polar cave. Lys214-N$\zeta$ is solvent inaccessible and is less than 5 Å from the catalytic Asp102. The side chain of Glu214 (2.8 Å, $R$ = 0.168) in trypsin S214E shows two conformations. In the major one, the Glu carboxylate in S214E forms a hydrogen bond with Asp102. Analytical isoelectrofocusing results show that trypsin S214K has a significantly different isoelectric point than trypsin, corresponding to an additional positive charge. The kinetic parameter $k_{cat}$ demonstrates that, compared to trypsin, S214K has 1% of the catalytic activity on a tripeptide amide substrate and S214E is 44% as active. Electrostatic potential calculations provide corroboration of the charge on Lys214 and are consistent with the kinetic results, suggesting that the presence of Lys214 has disturbed the electrostatic potential of Asp102.

Catalysis in the serine proteases is attributed to a triad of amino acids which orchestrate an attack by serine (Ser195 in trypsin) on the substrate carbonyl carbon. The nucleophilicity of the serine is enhanced by an adjacent histidine (His57 in trypsin) which serves as a general base and subsequently mediates transfer of a proton to the leaving group. The buried aspartic acid (Asp102 in trypsin) forms a hydrogen bond to the histidine, and its negative charge likely stabilizes the charged imidazole of the histidine which is present in the transition state (Warshel & Russell, 1986). Replacement of this aspartic acid with a neutral amino acid in trypsin (Asn) (Craik et al., 1987) and subtilisin (Ala) (Carter & Wells, 1988) reduced $k_{cat}$ by approximately $10^4$ for both enzymes and provided evidence for the role of aspartic acid in the catalytic triad. In agreement with theory, these experiments suggested the importance of the negative charge on the aspartic acid. In the experiments reported here, we attempt to change the electric field at the active site of rat anionic trypsin (hereafter called trypsin) with minimal structural disruption of the catalytic triad.

Electrostatics are thought to play a prominent role in enzyme catalysis (Dao-pin et al., 1989; Hwang & Warshel, 1987; Pickersgill et al., 1988; Rao et al., 1987; Soman et al., 1989; Warshel et al., 1989b; Warwicker, 1986): appropriate electrostatic fields for transition state stabilization are established in the Michaelis complex of enzymes (Kraut, 1988) through the arrangement in space of charged and polar groups and by the exclusion of disordered bulk solvent (Warshel et al., 1989a). In trypsin, the Asp102 buried formal charge is stabilized by the polar environment, or cave, in which it is found. The polar cavity includes two buried water molecules and Ser214, as well as the backbone amides of Ala56 and His57 (Birktoft et al., 1970; Brayer et al., 1978; Warshel et al., 1989b). Ser214 appears to provide an important contribution to this environment. Of the approximately two hundred mammalian and bacterial trypsin-like serine proteases, all but three feature serine at position 214 (Bazan & Fletterick, 1990; Marquart et al., 1983; Ohara et al., 1989). Ser214 is solvent inaccessible, and Ser214-O$\gamma$ forms a hydrogen bond to the essential Asp102 of the catalytic triad in trypsin. Ser214 was replaced with Lys, Glu, and Ala to alter the polar environment, and consequently the electrostatic potential, of Asp102. Remarkably, trypsin S214A is more active than trypsin. The catalytic power of the other variants is compromised by a combination of structural and electrostatic perturbations.

## Methods

*Production of Recombinant Trypsin.* Variant trypsins S214K, S214E, and S214A, in which Ser214 is replaced with Lys, Glu, and Ala, respectively, were made using oligonucleotide-directed site-specific mutagenesis as previously described for other mutants (Evnin & Craik, 1988). The mutant trypsins are expressed into the periplasm of *Escherichia coli* (Vâsquez et al., 1989) and purified according to published protocol (Higaki et al., 1989).

*Crystallography.* Crystals of trypsins S214K and S214E were grown as previously described for other trypsin mutants (McGrath et al., 1989). X-ray diffraction data were measured from single crystals to 2.2 Å for trypsin S214K and to 2.7 Å for the smaller trypsin S214E crystal (Table I) using mono-

[†] This work was supported by NIH Grant DK 39304 to R.J.F., NIH Grant GM 30518 to B.H., NSF Grant GM DMB 8904956 to C.S.C., and a University of California President's Dissertation Year Fellowship to J.R.V.

[‡] The crystallographic coordinates have been deposited in the Brookhaven Protein Data Bank.

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0006-2960/92/0431-3059$03.00/0 

Table I: Crystal and Diffraction Data for Trypsins S214K and S214E

| | S214K | S214E |
|---|---|---|
| space group | I23 | |
| cell dimensions | $a$ = 124.4 | |
| molecules per asymmetric unit | 1 | |
| data measurement | Xentronics | |
| resolution (Å) | 2.2 | 2.6 |
| total observations | 52900 | 33300 |
| observations >2$\sigma$ | 47100 | 21100 |
| unique reflections | 17000 | 9600 |
| $R_{sym}$[a] | 0.091 | 0.130 |
| Hendrickson–Konnert refinement (cycles) | 100 | 55 |
| $R_{cryst}$[b] | 0.154 | 0.167 |
| resolution (Å) | 7.0–2.2 | 7.0–2.8 |
| rms bond differences | 0.012 | 0.02 |
| rms angle differences | 3.1 | 2.4 |
| no. of inhibitor molecules/protein | 2 | 2 |
| no. of putative solvent molecules | 159 | 147 |

[a] $R_{sym} = \{h_i[I(h)] - I_i(h)\}/h_iI_i(h)$, where $[I(h)]$ is the mean of all measurements of reflections $h$ and $I_i(h)$ is the $i$th measurement of reflection $h$. [b] $R_{cryst} = \sum|F_o - F_c|/\sum F_o$.

chromatic Cu K$\alpha$ radiation and a Xentronics area detector. The XENGEN software package from Nicolet Instrument Co. was used for data reduction. Difference Fourier analysis using trypsin D102N (Sprang et al., 1987) as the starting model was used to determine the structures. Initially, the three catalytic residues and amino acid 214 were replaced with alanines in the atomic coordinates and refined for four cycles using the program of Hendrickson and Konnert (HKREF) (Hendrickson & Konnert, 1980). The $F_o - F_c$ maps calculated from that refined model allowed fitting of the four omitted side chains to electron density (Figure 1a). The new models, with Lys or Glu at position 214, were refined for a total of 100 and 64 cycles, respectively. This process included occupancy refinement of alternative conformations of two side chains for trypsin S214E. Placement of solvent molecules and adjustments of protein side chains during refinement were facilitated by molecular graphics on the Evans & Sutherland PS330 and PS390 using the program FRODO (Jones, 1978). INSIGHT (Biosym, Inc) was used for structure analysis.

*Enzyme Kinetics.* The concentration of active sites for trypsin and trypsins S214K, S214E, and S214A was determined from burst kinetics of the active site titrant 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973).

Initial velocities for the hydrolysis of the amide substrate succinyl-Ala-Phe-Lys-aminomethylcoumarin (Suc-AFK-AMC) were performed as described previously (Higaki et al., 1989). The enzyme concentration was 2.5 or 250 nM in 100 mM NaCl, 20 mM $CaCl_2$, and 50 mM Tris, pH 8.0, with 1% DMF. The parameters $k_{cat}$ and $K_m$ were calculated from Eadie–Hofstee plots. The equations of Cornish-Bowden and Wharton (1988) were also used to determine the apparent second-order rate constant $k_{cat}/K_m$.

*Electrostatic Calculations.* Electrostatic calculations employed the program DelPhi (Nicholls & Honig, 1991) which uses a finite difference algorithm to solve the Poisson–Boltzmann equation (FDPB). As described previously (Soman et al., 1989), this method places the protein and its associated solvent shell on a grid where charges are assigned to each atom which contributes to the electrostatic potential. The actual contribution of each charged atom to the electrostatic potential at a specific site (taken to be a point charge) in the protein is given by the product of the magnitude of the charge times the potential induced by the specific site on the charged atom. The solvent is described as a continuum of dielectric constant 80, while the protein provides a medium of dielectric constant 4 (Rogers & Sligar, 1991).

Table II: Relevant Interatomic Distances for Trypsin S214K and S214E

| amino acid residue | atoms | distance (Å) S214K | S214E | trypsin |
|---|---|---|---|---|
| H57 | Nε2–S195 Oγ | 3.7 | 3.9 | 3.0 |
| H57 | Nδ1–D102 Oδ2 | 2.6 | 2.6 | 2.9 |
| D102 | Oδ2–K/E214 Nζ/Oε1 | 4.8 | 2.9 | |
| D102 | Oδ2–S/K/E214 Oγ/Cγ/Cγ | 3.5 | 4.2 | 2.8 |
| Wat or K/E214 | Nζ/Oε2–Wat241 | 2.6 | 2.9 | 2.6 |
| Wat or K/E214 | Nζ/Oε1–L99 O | 2.9 | 2.6 | 2.9 |
| Wat or K/E214 | Nζ/Oε1–T229 Oγ | 3.2 | 3.0, 3.0[a] | 3.2 |
| W215 | Cδ–G216N | 3.1 | 3.3 | >5.0 |

[a] Both conformers of E214 in trypsin S214E form hydrogen bonds with Thr229, and so both distances are given.

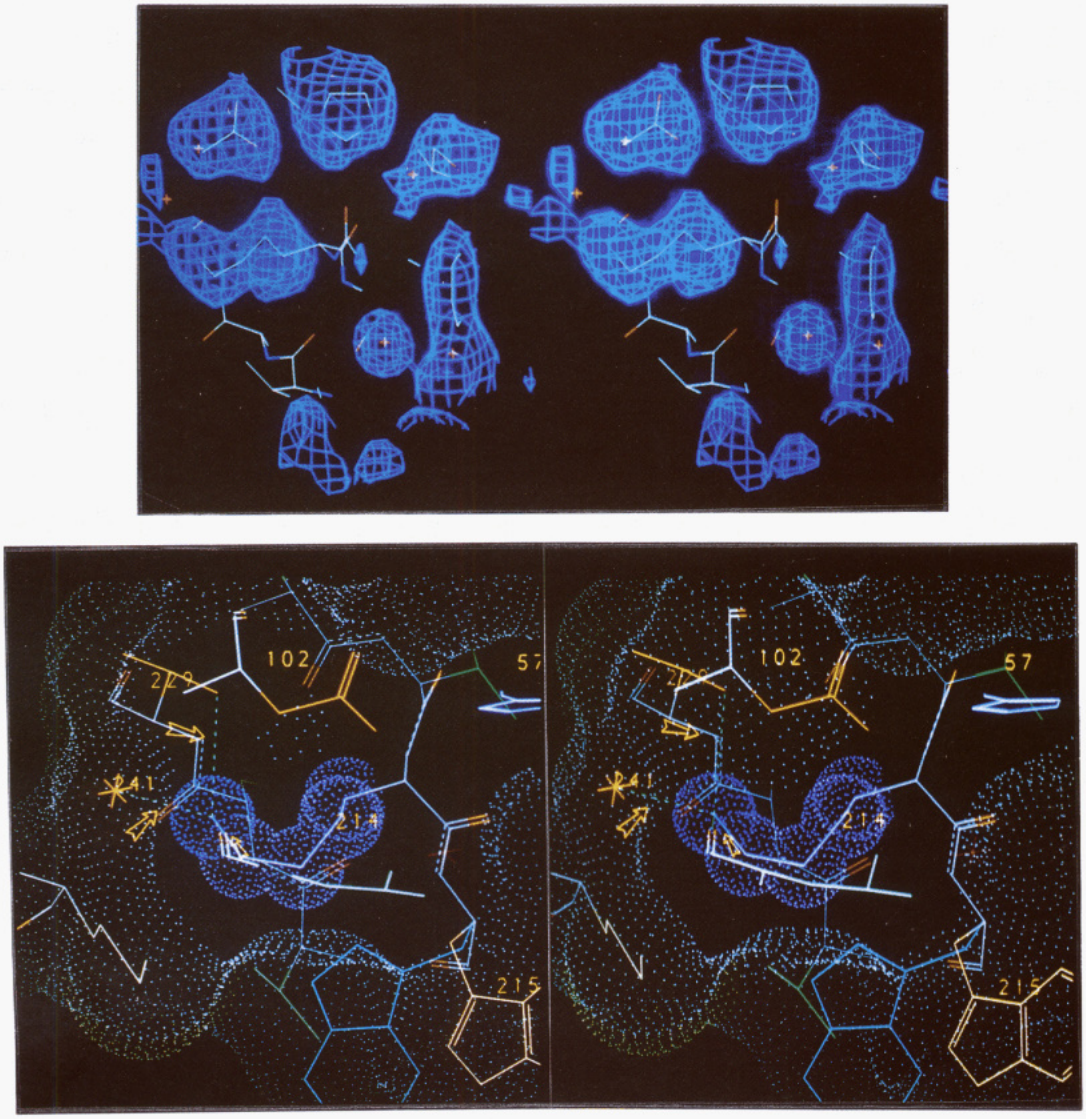
## Results and Discussion

*Crystal Structures of Trypsins S214K and S214E.* The structure of trypsin is mostly unperturbed by replacement of serine with Lys or Glu at position 214 (rms deviation from rat trypsin = 0.17 Å). However, a localized disruption causes displacement of the side chain of Trp215.

*(1) S214K.* In trypsin S214K, equivalent side-chain atoms of Ser214 and Lys214 adopt different positions. Ser214-Oγ forms a 2.8-Å hydrogen bond to Asp102-Oδ2 while Lys214-Cγ is 3.5 Å from this atom. The lysine side chain dips down and away from Asp102 at this point to fit deep into the polar cave or water channel (Meyer et al., 1988) (Figure 1b) where it makes the same hydrogen bonds as the buried water molecule (Birktoft & Blow, 1972; Bode & Schwager, 1975) it replaces (Table II). Specifically, Lys214-Nζ is 4.8 Å from Asp102-Oδ2 and also forms hydrogen bonds with three oxygen atoms, including Wat241. Thus, although not solvated, Lys214-Nζ is in an unusually polar environment. Lys214-Cγ closely approaches the pyrrole ring of Tryp215 which forms part of the extended substrate binding site (Janin & Chothia, 1976). The Trp215 side chain has rotated 134° around Cα–Cβ such that it is closer to the substrate binding pocket (Figure 1b) (thereby displacing a water molecule). The adjustment in position has alleviated the close contacts to Lys214-Cγ but has resulted in the Trp215 side chain occluding the main-chain atoms of amino acids 215 and 216. This could compromise the ability of the enzyme to bind substrates containing more than one peptide bond since the occluded atoms form hydrogen bonds with substrate amino acids P2 and P3. The refined structures of trypsins S214E and S214K at the active site are shown in Figure 1c.

*(2) S214E.* In trypsin S214E, electron density indicated two positions for the Glu214 side chain. The first position (refined to 40% occupancy) is very similar to that of Lys214 in trypsin S214K (Figure 1c). A water molecule is displaced, and Glu214-Oε2 forms a hydrogen bond (2.7 Å) to the remaining buried Wat241, located 0.6 Å from where it is found in trypsin S214K. Consequently, in trypsin S214E, Wat241 forms a hydrogen bond to Leu99-O, while it bonds to Asn101-N in trypsin S214K. Glu214-Oε1 forms hydrogen bonds with Thr229-Oγ1 and Leu99-O (Table II).

The second position (60% occupancy) allows the side chain of Glu214 to form hydrogen bonds with Thr229-Oγ1 and Asp102-Oδ2 (Table II). The Trp215 side chain is rotationally displaced by an additional 27° (about $X_1$) in trypsin S214E. Electron density shows only 30% of full occupancy for the displaced indole ring and 70% occupancy for an almost normal position. Thus, the bifurcated Glu side chain is buried and



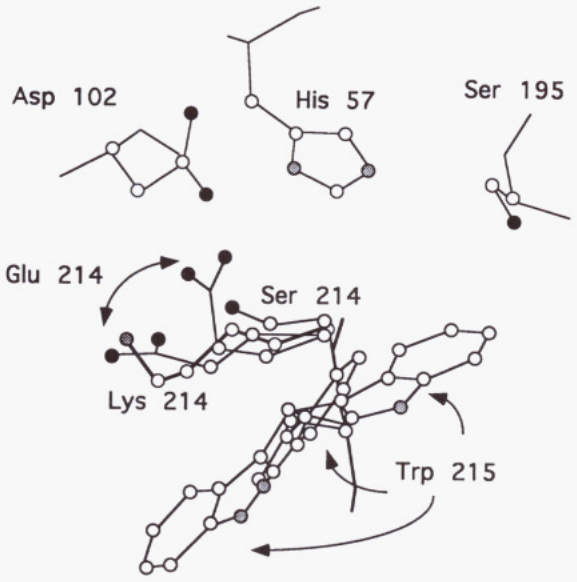




FIGURE 1: (a, top) Stereo photo of the electron density at the active site. Diffraction data were measured using a Xentronics area detector. The Xengen software package from Nicolet Instrument Co. was used for data reduction. Molecular replacement with the trypsin D102N coordinates (Sprang et al., 1987) provided initial phase information for determination of the Lys214 position. This $F_o - F_c$ difference map shows contours only for atoms not included in the phase calculation. A lysine side chain was fitted to the density at position 214. The three cage contours across the top correspond to the three catalytic amino acids Asp102, His57, and Ser195. The electron density for the inhibitor benzamidine (omitted from the phase calculation) is seen at the lower right. The (+) symbols represent water molecules that were also left out of the phase calculation. Alanine was used as residue 214 and for the three catalytic residues in the first four cycles of Hendrickson–Konnert refinement in order to determine the position of the lysine side chain without phase bias. (b, middle) Stereo photo of the environment of the buried lysine. The structure is shown after 84 cycles of HKREF with the side-chain atoms of Lys214 highlighted as spheres. Lys214-N$\zeta$ (blue sphere) is stabilized by three hydrogen bonds (arrows) to a buried water molecule (Wat241), to the side-chain hydroxyl of Thr229, and to the backbone carbonyl (red double bond) of Leu99 (unlabeled in foreground). Lys214-N$\zeta$ occupies the position held by a conserved, buried water molecule in trypsin. A solvent-accessible surface (represented by dots) is shown around the buried Asp102. The side chain of Trp215 is shown twice: blue represents the normal trypsin position while yellow shows the location of the side chain in trypsin S214K. (c, bottom) Refined coordinates are shown for the active site of trypsin S214E and for positions 214 and 215 of trypsin, trypsin S214K, and trypsin S214E. The two positions of the Glu214 side chain are shown for trypsin 214E. There are three positions shown for Trp215. The middle position is that refined for trypsin S214K, while the flanking orientations are seen in trypsin S214E.

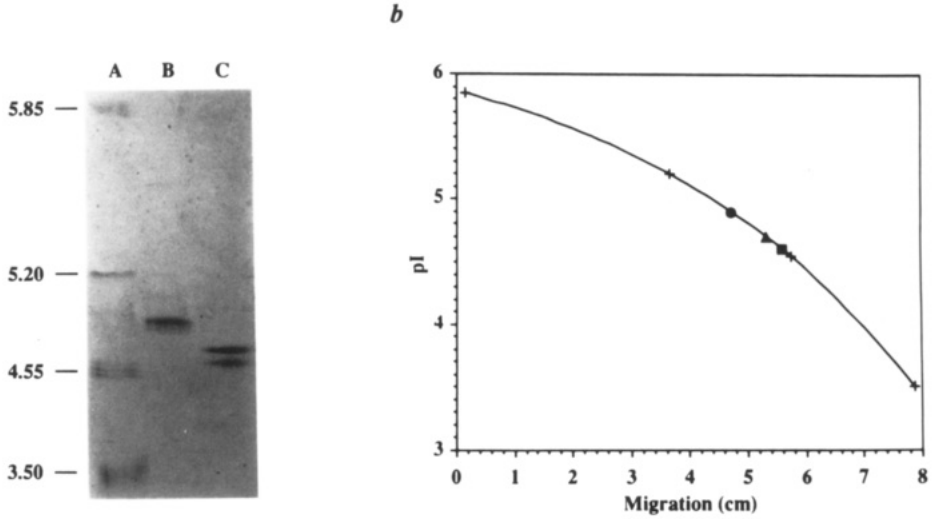



FIGURE 2: Mutation of Ser-214 to Lys-214 introduces an additional positive charge into rat anionic trypsin. (a) Coomassie-stained isoelectric focusing gel of (A) standards, (B) S214K rat anionic trypsin, and (C) wild-type rat anionic trypsin. Standards are bovine carbonic anhydrase (p*I* 5.85), β-lactoglobulin (p*I* 5.20), soybean trypsin inhibitor (p*I* 4.55), and amyloglucosidase (p*I* 3.50). (b) Interpolation of the isoelectric point of (●) S214K (4.90) and (▲, ■) wild-type (4.70 and 4.60) rat anionic trypsins. The doublet in lane C of panel a is probably due to autolysis of the sample. Methods: Approximately 10 μg total of standard proteins and 5 μg per lane of each trypsin sample were electrophoretically focused under nitrogen at 25 W, constant power, at 10 °C for 50 min from a position 5 cm from the anode on a 14-cm LKB Ampholine PAG plate, pH 4.0–6.5. The potential varied from 650 to 2370 V and the current from 35 to 9.5 mA. The gel was fixed and stained according to the manufacturer's instructions.

well tolerated in the polar cave of trypsin.

The only other change in the structure of trypsins S214K and S214E is at the catalytic Ser195. In trypsin-like serine proteases, Nε2 of His57 and Ser195-Oγ of the catalytic triad form a weak and highly distorted hydrogen bond (approximately 3.0 Å) that is characterized by an angle (Ser915Cβ–Ser195Oγ–His57Nε2) of about 90° (Bode & Huber, 1986; James et al., 1980; Smith et al., 1989). Structural adjustments induced by substrate binding are believed to promote the geometry necessary for catalysis (Kraut, 1977; Kossiakoff, 1987), and therefore the ground state structure is not a good predictor of catalytic potential. This is borne out by the evidence that although the Ser195-Oγ to His57-Nε2 distance in the less active mutant trypsin S214K is 3.7 Å (Table II) (and the bond angle 63°) (Marquart et al., 1983), the distance is 3.9 Å in trypsin S214E, which is a very active enzyme. These distances are long though not unprecedented; a refined structure of chymotrypsin has a 3.7-Å distance between His57-Nε2 and Ser195-Oγ (Cohen, 1981).

*Charge State of Lys214 and Glu214.* Buried, unpaired lysine side chains are unusual in proteins. A survey of lysines in 36 protein structures in the Brookhaven Protein Data Bank indicated that only 5 lysines had solvent-inaccessible side chains (Rashin & Honig, 1984). We examined the protein context of the 555 lysines in the 36 structures, and glycogen phosphorylase, and analyzed the environments of the 15 (2.7%) lysines with ostensibly buried Nζ atoms. Fourteen were eliminated because they were in unrefined structures (three), are charge compensated (eight), or lie mostly on the protein surface (three). The only examples of truly buried (no solvent accessibility) and uncompensated lysine side chains were found in glycogen phosphorylase and very recently in a staphylococcal nuclease mutant (Stites et al., 1991) and a T4 lysozyme mutant (Dao-pin et al., 1991). Thus, replacement of Ser214 in trypsin with lysine provides a rare example of a buried, formal charge.

This raises two questions concerning Lys214. Is its p$K_a$ lowered 3 or 4 units so that it is present in neutral form in the polar microenvironment of the protein (described above) or, if it is ionized, what balance of forces stabilizes the charged form of an amino acid removed from solvent?

Analytical isoelectrofocusing results show that trypsin, trypsin S214E, and trypsin S214A have essentially the same p*I* (data not shown). Thus, it appears that Glu214 in trypsin S214E is uncharged while trypsin S214K has a higher isoelectric point by 0.2 pH unit (Figure 2), consistent with an additional positive charge. Although the simplest explanation is that Lys214 is charged, another formal possibility is that Asp102 becomes neutral upon the loss of the hydrogen bond to Ser214. This is unlikely since previous calculations found that the p$K_a$ of Asp102 is as low as 0 (Warshel & Russell, 1986; A. S. Yang and B. Honig, unpublished results), making it improbable that loss of one hydrogen bond (from Ser214) in trypsin S214K could result in a neutral Asp102.

Electrostatic calculations using the program DelPhi (Gilson & Honig, 1988; Nicholls & Honig, 1991) provide strong evidence that Lys214 is charged. To determine whether our results were dependent on the variables used to describe the molecular charge distribution, all calculations were carried out with the DISCOVER (Hagler et al., 1979), AMBER (Weiner & Kollman, 1981; Weiner et al., 1984), and CHARMM (Brooks et al., 1983) parameter sets (which differ in the assignments of partial charges to the polar atoms). The p$K_a$ of an amino acid in a protein relative to its value in solution is given by the difference in electrostatic potential at the site of protonation in the two environments (Bashford & Karplus, 1990; Honig & Hubbell, 1984; Warshel & Russell, 1986). Using methods described previously (Sharp & Honig, 1990), we find that the energetic cost of burying a lysine in the position observed in trypsin S214K is 15 ± 1.5 kcal/mol in the absence of all other interactions (the range of numbers is determined by results obtained with different parameter sets). This is compensated by a stabilization energy of −10.1 ± 1.3 kcal/mol arising from the L99-O, the T229-Oγ, and a buried water molecule. A further −7 kcal/mol is provided by the negative charge on Asp102, 4.8 Å away. Thus, the change in electrostatic energy of Lys214 in the protein relative to water is about −2 kcal/mol. This translates into a p$K_a$ *increase* of about 1.4 units. Calculations accounting for interactions with the entire protein predict a p$K_a$ of Lys214 of 12.6, 12.7, and 12.3, respectively,

Table III: Kinetic Constants for Hydrolysis of Succinyl-Ala-Phe-Lys-AMC by Trypsin and Trypsin S214(K,E,A) in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 20 mM $CaCl_2$ at 25 °C[a]

| enzyme | $k_{cat}$ ($min^{-1}$) | $K_m$ ($\mu M$) | $k_{cat}/K_m$ ($min^{-1}$ $\mu M^{-1}$) | normalized[b] (%) |
|---|---|---|---|---|
| trypsin | 490 ± 19 | 192 ± 12 | 2.56 ± 0.06 | 100 |
| trypsin S214K | 4.2 ± 0.8 | 6500 ± 1400 | 0.00064 ± 0.00006 | 0.86 |
| trypsin S214E | 220 ± 70 | 6700 ± 2000 | 0.033 ± 0.001 | 45 |
| trypsin S214A | 900 ± 10 | 260 ± 10 | 3.4 ± 0.1 | 184 |

[a] The values are the means of two determinations ± the standard deviations for each pair of determinations. Each determination included the observed hydrolysis rates for reactions at five concentrations of substrate. [b] $= k_{cat}$(trypsin)/$k_{cat}$(mutant trypsin).

for the three different charge sets. Thus, independent of parameter set, Lys214 is expected to be charged and more stable in the observed position than it would be solvated.

The neutral state of the Glu214 side chain in trypsin S214E which was predicted by its proximity to charged Asp102, and seen by isoelectric focusing, is corroborated by DelPhi calculations. A negative charge on Glu214 is indeed not favorable in the vicinity of Asp102: the electrostatic interaction energies for the two positions of the Glu214 side chain are +7.5 and +6.0 kcal/mol. Again, it is possible that Asp102 is protonated and Glu214 is charged, but calculations show that arrangement to be unstable by +7.0 kcal/mol (L. Jin and R. J. Fletterick, unpublished results).

*Functional Consequences of the Buried Charge in Trypsin S214K.* Kinetic analysis of trypsin S214K, trypsin S214A, and trypsin S214E utilized the electrostatically neutral tripeptide substrate Suc-AFK-AMC at pH 8.0. The data for trypsin S214A clearly show that Ser214 can be replaced without mitigating enzyme activity (Table III). This mutant has 130% of the activity of trypsin as judged by $k_{cat}/K_m$. The cause of the increase in activity compared to the wild-type enzyme is unknown but could result from increased polarizability at the active site since the serine hydroxyl is most likely replaced by a water molecule in trypsin S214A.

For trypsin S214K and S214E, $K_m$ has increased 34-fold relative to trypsin (Table III). Most of the decrease in binding affinity is likely a consequence of Trp215 blocking access of the substrate to the main-chain atoms. The large increase in $K_m$ skews the kinetic parameter $k_{cat}/K_m$ such that the efficiency of the enzyme appears to be greatly reduced compared to the catalytic rate. The three-dimensional structures of the mutant enzymes suggest a basis for these changes in $K_m$ in that the free energies of the ground states of the variant enzymes are perturbed by the substitution at 214. The side chain of Trp215 must assume a more native conformation on formation of the E·S complex. Consequently, the free energies of the E·S complex and transition state are perturbed.

The loss of catalytic power, with respect to the transition state, is then measured by changes in $k_{cat}$ not $k_{cat}/K_m$, but we cannot fractionate the experimental change in $k_{cat}$ due to structural adjustments that may alter the free energy of E·S, or E·S‡, versus the effects of the substitutions on the chemistry of the transition state complex. The kinetic constant $k_{cat}$ will reflect the free energy difference between E·S and the transition state complex E·S‡. Thus, the decrease in $k_{cat}$ to 1% of that found for trypsin S214K for this substrate could be due either to structural changes or to the electrostatic effects of the Lys214 positive charge. We can make only a crude estimate of the effect of disordering the indole of Trp215. Trypsin S214E has a $k_{cat}$ which is approximately half that measured for trypsin, or 50-fold higher than that of trypsin S214K. This suggests that disordering the Trp215 side chain does not necessarily compromise the enzyme's activity. Regarding the change in the electrostatics at the active site, we can make a semiquantitative estimate of the expected effect from the charge added at position 214.

The extent to which the positive charge of Lys214 may undermine the electrostatic role of Asp102 in the catalytic mechanism can be estimated by calculating its electrostatic interaction with the charge distribution of the assumed transition state. FDPB calculations of these interactions were carried out as previously described in a study of cow and rat trypsins (Soman et al., 1989). To account for the exclusion of water molecules by substrate, molecular graphics was used to build amino acids 14 and 15 of bovine pancreatic trypsin inhibitor (BPTI) into the active site of trypsin S214K where they occupy positions found in the trypsin–BPTI complex (Marquart et al., 1983). We have used three representations of the transition state to determine the sensitivity of our calculations to the uncertainty in its detailed atomic structure and charge set. The three models are (a) a positive charge on the Nε2 of His57 and a negative charge in the oxyanion hole, (b) half a charge on the Nε2 and Nδ1 of His57 and a negative charge in the oxyanion hole, and (c) the model for the transition state suggested by Warshel and co-workers in which the charge is delocalized over His57, Ser195, and the oxyanion hole (Warshel et al., 1989a). The interaction of Lys214 with these three models for the transition state was calculated using DelPhi. The polar groups that change as a consequence of the mutation were found to have a negligible effect. (This calculation for trypsin S214E yielded the same conclusion.)

Lys214 was calculated to destabilize the transition state by 1.3, 2.2, and 1.8 kcal/mol in models a, b, and c, respectively. The corresponding reductions in $k_{cat}$ are factors of 9, 41, and 22. The experimentally observed effect on catalysis is a 2.8 kcal/mol increase in the activation energy. Our calculations suggest that between 46% and 79% of the loss in $k_{cat}$ for trypsin S214K could be due to electrostatic effects.

## Conclusions

Ser214 could be called the fourth member of the catalytic triad on the basis of its constancy throughout the serine protease family. Although purported to stabilize Asp102 by hydrogen bonding, removal of the hydroxyl group in trypsin S214A does not reduce enzyme activity on a tripeptide amide substrate. The two buried water molecules which form a hydrogen-bonding network with Ser214 in trypsin are a fortuitous structural feature which allows us to replace Ser with Lys or Glu. However, the buried side chains displace Trp215, resulting in inefficient recognition of peptide substrates as reflected in $K_m$. This complicates quantification of the electrostatic role of Asp102. Nonetheless, comparisons between trypsins S214E and S214K show similar structural perturbations, yet trypsin S214K is only 2–3% as active as trypsin S214E. It is intriguing that this difference in $k_{cat}$ between the two mutants is predicted by electrostatic calculations as largely arising from alteration of the electric field at the catalytic triad.

**Registry No.** Asp, 56-84-8; Ser, 56-45-1; Ala, 56-41-7; Glu, 56-86-0; Lys, 56-87-1; trypsin, 9002-07-7; succinyl-Ala-Phe-Lys-AMC, 73207-91-7.

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