Role of Serine 214 and Tyrosine 171, Components of the S_2 Subsite of α-Lytic Protease, in Catalysis†

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ABSTRACT: The function of a hydrogen bond network, comprised of the hydroxyl groups of Tyr 171 and Ser 214, in the hydrophobic S_2 subsite of α-lytic protease, was investigated by mutagenesis and the kinetics of a substrate analog series. To study the catalytic role of the Tyr 171 and Ser 214 hydroxyl groups, Tyr 171 was converted to phenylalanine (Y171F) and Ser 214 to alanine (S214A). The double mutant (Y171F:S214A) also was generated. The single S214A and double Y171F:S214A mutations cause differential effects on catalysis and proenzyme processing. For S214A, k_{cat}/K_m is (4.9×10^3)-fold lower than that of wild type and proenzyme processing is blocked. For the double mutant (Y171F:S214A), k_{cat}/K_m is 82-fold lower than that of wild type and proenzyme processing occurs. In Y171F, k_{cat}/K_m is 34-fold lower than that of wild type, and the proenzyme is processed. The data indicate that Ser 214, although conserved among serine proteases and hydrogen bonded to the catalytic triad [Brayer, G. D., Delbaere, L. T. J., & James, M. N. G. (1979) *J. Mol. Biol. 131*, 743], is not essential for catalytic function in α-lytic protease. A substrate series (in which peptide length is varied) established that the mutations (Y171F and Y171F:S214A) do not alter enzyme–substrate interactions in subsites other than S_2. The pH dependence of k_{cat}/K_m for Y171F and Y171F:S214A has changed less than 0.5 unit from that of wild type; this suggests the catalytic triad is unperturbed. In wild type, hydrophobic interactions at S_2 increase k_{cat}/K_m by up to (1.2×10^3)-fold with no effect on K_m. The consequence of the S_2 hydroxyl group mutations is to destabilize hydrophobic binding in the S_2 subsite. In Y171F and Y171F:S214A, the k_{cat}/K_m values for good dipeptide substrates (containing hydrophobic P_2 side chains) decrease 25–45-fold compared to wild type, while k_{cat}/K_m for poor substrates is unaffected. We conclude the Tyr 171 and Ser 214 hydroxyls do not interact directly with the substrate, but facilitate catalysis by maintaining S_2 in an optimal and exact structure.

α-Lytic protease[1] is a serine hydrolase that can bind peptides through six subsites.[2] The extended binding subsites function to increase k_{cat}/K_m by a factor of 1.6×10^6. Of the six subsites, S_2 is of particular importance since binding interactions there increase k_{cat}/K_m or k_{cat} several hundred-fold (Bauer et al., 1981). Structural features of S_2 which are indicated from crystallographic (Fujinaga et al., 1985) and molecular modeling (Epstein, 1992) data are summarized in Figure 1, part A, and the schematic diagram in part B. One edge of the pocket is composed of the imidazole ring of His 57, which is part of the catalytic triad. Residues Phe 94 and Tyr 171 form sides of the pocket in aromatic–aromatic interactions such as described for other proteins (Burley & Petsko, 1985, 1986). Of interest is the hydrogen bond network formed by the hydroxyl groups of Tyr 171 and Ser 214 and the carboxylate of Asp 102 ($O_\delta 1$) (Brayer et al., 1978, 1979; Fujinaga et al., 1985). S_2 is hydrophobic. Binding contacts between these two hydroxyl groups and the substrate are not apparent (Epstein, 1992). The P_2 side chain makes strong hydrophobic contacts with His 57, Tyr 171, and Phe 94 (Bone et al., 1987, 1989).

The purpose of this study is to delineate the components of S_2 involved in catalysis. We focused on the hydroxyl groups of Tyr 171 and Ser 214 and prepared α-lytic protease mutants Y171F, S214A, and Y171F:S214A. Ser 214 is conserved in both primary and topological structures among the serine proteases (James et al., 1978; Brayer et al., 1978; McGrath et al., 1992), yet the function of the Ser 214 carbinol side chain is unknown. Hydrogen bond donation from Ser 214 was postulated to stabilize the Asp 102–ImH^+ ion pair that forms in the tetrahedral transition state (Brayer et al., 1978, 1979; Warshel & Russell, 1986; Warshel et al., 1989). The function of the hydroxyl group of Tyr 171 is unknown; this amino acid residue is present in the S_2 subsites of other bacterial serine proteases (Brayer et al., 1978; Read et al., 1983).

To facilitate structure–function studies, we and others have cloned α-lytic protease (Silen et al., 1988; Epstein & Wensink, 1988, 1989). The nucleotide sequence revealed that the 198-residue serine protease resides at the CO_2-terminus of a 397-residue preproenzyme molecule. The prepeptide directs secretion, and the prodomain is believed to fold the protease

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[1] The following nomenclature is used throughout: α-Lytic protease is the mature protease encoded from Ala 1 to Gly 198. The preproenzyme refers to the 397-residue prepro-α-lytic protease protein; the prodomain is the 166-residue protein from Ala −166 to Thr −1, and the prepeptide is the 33-residue signal sequence from Met −199 to Ala −167. In the bimolecular folding experiments the preprodomain includes residues Met −199 to Asp 8, and the prodomain refers to residues Ala −166 to Asp 8. DNA fragments are denoted by the restriction endonuclease site used for mapping or cloning. For example ptz18[*Sac*II$_{884}$–*Sac*II$_{1020}$]:Y171F refers to the *Sac*II fragment from nucleotides 884 to 1020 cloned into the *Sac*II sites of modified ptz18 and carrying the Y171F mutation. Y171F denotes the mutation of Tyr 171 to phenylalanine, and S214A denotes the mutation of Ser 214 to alanine. Chymotrypsin numbering system is used. In the text, peptide substrates and inhibitors are denoted by bold numbers as well as by their respective amino acid sequences.

[2] The scissile bond is between substrate residues P_1 and P'_1. Peptide residues on the substrate are denoted as P_1, P_2, etc., (NH_2-terminal to the scissile bond), and P'_1) P'_2, etc., (P'_n, refers to CO_2-terminal residues). The corresponding binding pockets (subsites) of the enzyme are similarly denoted as S_n and S'_n according to the nomenclature of Schecter and Berger (1967).

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domain into its active conformation (Silen et al., 1989; Silen & Agard, 1989; Epstein & Wensink, 1988). Proteolytic removal of the prodomain from activated α-lytic protease is thought be autocatalytic.

EXPERIMENTAL PROCEDURES

Reagents. Enzymes used in DNA manipulations were purchased from New England Biolabs, IBI, U.S. Biochemical Corp., and Amersham. Radiolabeled compounds were from New England Nuclear. Peptide synthesis reagents were purchased from Aldrich Chemical Co. Blocked amino acids and peptides were products of Bachem or Peptides International. *N*-MeOSuc-AlaAlaProAla-CH_2Cl was generously provided by Enzyme Systems Products.

Synthesis. Peptides *N*-Suc-ValAla-pNA (**6**), *N*-Suc-LeuAla-pNA (**7**), *N*-Suc-ProAla-pNA (**8**), and *N*-Suc-GlyAla-pNA (**10**) were synthesized according to the mixed-anhydride procedure (Anderson et al., 1967). The purity of each compound was checked by thin-layer chromatography on Merck precoated silica gel in at least two solvent systems: system I, butanol–acetic acid–water 4:1:1; system II, chloroform–methanol 9:1 system III, chloroform; system IV, chloroform–methanol–acetic acid 8:1.7:0.3. The identity of each peptide was confirmed by ^1H NMR and ^{13}C NMR. Synthetic procedures and NMR data are available in the supplementary material.

Expression. Plasmids ptac11 (Amann et al., 1983) or pACYC184 (Chang & Cohen, 1978) were used for expression in *Escherichia coli*; ptz18/19 (U.S. Biochemical Corp.) modified with *Msc*I and *Sac*II sites was used for mutagenesis and sequencing. DNA encoding prepro-α-lytic protease domains was isolated from pαLP1 and purified by electrophoresis from 1% agarose gels onto DEAE-type NA-45 paper (Epstein & Wensink, 1988). An *Eco*RI site was inserted at $SnaBI_{250}$ in pαLP1 (Figure 2). The *Sna*BI–*Eco*RI linker sequence is 5′-GTACATGAATTCATGTAC-3′ and leaves the wild-type fMet initiation codon unchanged (Silen et al., 1988; Epstein & Wensink, 1988). The 1.5-kbp *Eco*RI fragment [$EcoRI_1(\Delta SnaBI_{10})$–$EcoRI_{1499}$] was cloned into ptac11 to yield the expression plasmid pDE200. α-Lytic protease was expressed in *E. coli* DH21 cells (Hanahan, 1983). Cells harboring pDE200 were grown to midlog stage at 37 °C in LB media supplemented with 50 μg/mL ampicillin. The cell culture was cooled to 22 °C and α-lytic protease expression begun by addition of IPTG[3] to 0.1 mM. Induction continued at 22 °C for 8–14 h.

Mutagenesis. Mutations were prepared by standard methods (Sayers et al., 1988). Oligodeoxynucleotides had the following sequences (mutant codon in italics): Y171F noncoding strand, 5′-CACCGCACCTTCGGC*GAA*GTTGGCGGTGAC-3′; S214A/G coding strand, 5′-GCGCAGGGCGTGATG*G(C/G)*GGGCGGCAACGTGCAG-3′; A/G214S coding strand, 5′-GCGCAGGGCGTGATG*TCG*GGCGGCAACGTGCAG-3′. The Y171F mutation was prepared on the [$SacII_{884}$–$SacII_{1020}$] DNA fragment (αLP codon 123) (Figure 2B). The Ser 214 mutations (codon 159) were prepared on the [$MscI_{839}$–$EcoRI_{1499}$] fragment. Fragments containing the mutations were cloned back into wild-type αLP DNA. For example, Y171F:S214A was prepared in two steps. (1) The [$SacII_{884}$–$SacII_{1020}$]:Y171F fragment was cloned into

[3] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; CMK, chloromethyl ketone; DFP, diisopropyl fluorophosphate; IPTG, isopropyl β-D-thiogalactopyranoside; OMTK, turkey ovomucoid third domain: TFMK, peptidyl trifluoromethyl ketone.

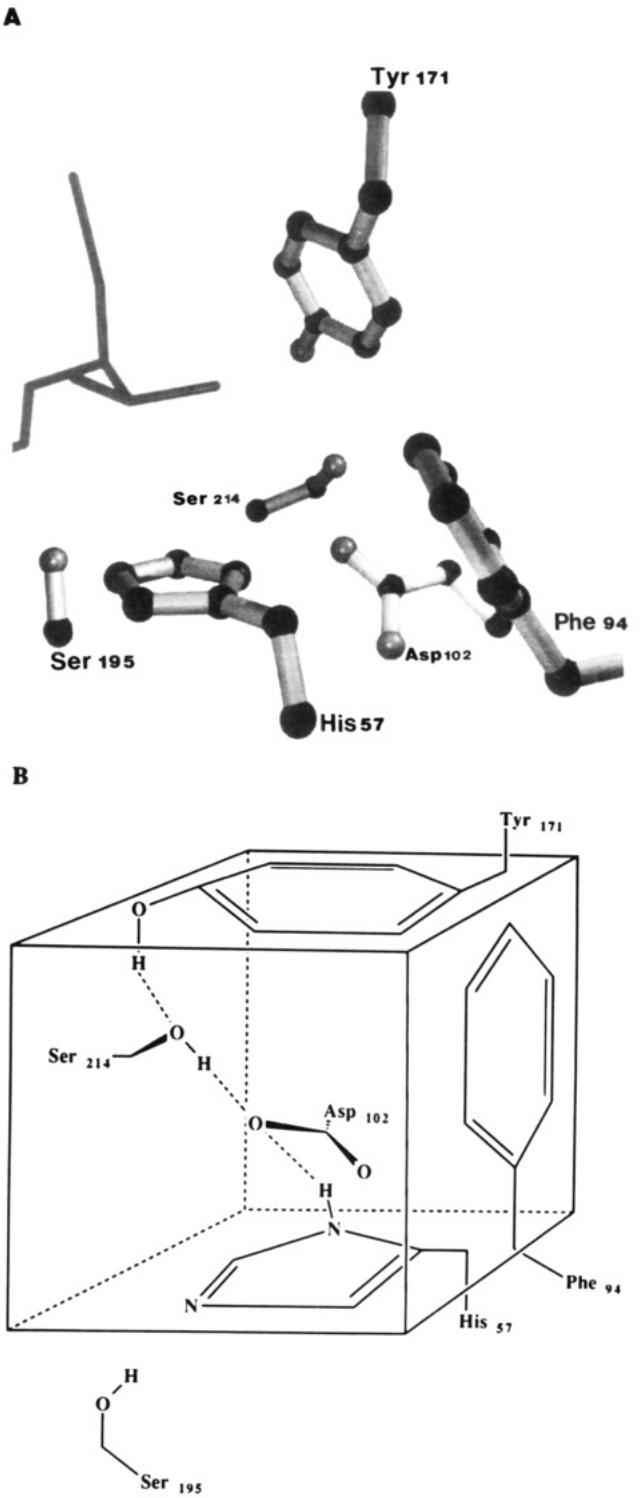

FIGURE 1: Structure of the S_2 subsite of α-lytic protease. (A) Rayshade-generated image of the crystallographic structure of the S_2 subsite of α-lytic protease into which a P_2 side chain is modeled. The side chains of residues composing the S_2 subsite and catalytic triad of α-lytic protease are shown in ball and stick format. Clockwise from the top are Tyr 171, Phe 94, Asp 102, Ser 214, His 57, and Ser 195. In solid black (unlabeled) is a P_2 threonine side chain modeled from the turkey ovomucoid third domain inhibitor (OMTK). The crystallographic data for α-lytic protease (1.7-Å resolution) are from Fujinaga et al. (1985) and that for the OMTK inhibitor (1.8-Å resolution) are from Read et al. (1983). The active site region of α-lytic protease was modeled using the computer program Hydra. The average displacement for active site residues His 57, Asp 102, and Ser 195 is 0.220 Å. The maximum displacement is 0.535 Å. (B) Schematic model of the unliganded S_2 subsite of α-lytic protease illustrating the interresidue hydrogen bonds and aromatic–aromatic interactions between S_2 side chains and the catalytic triad.

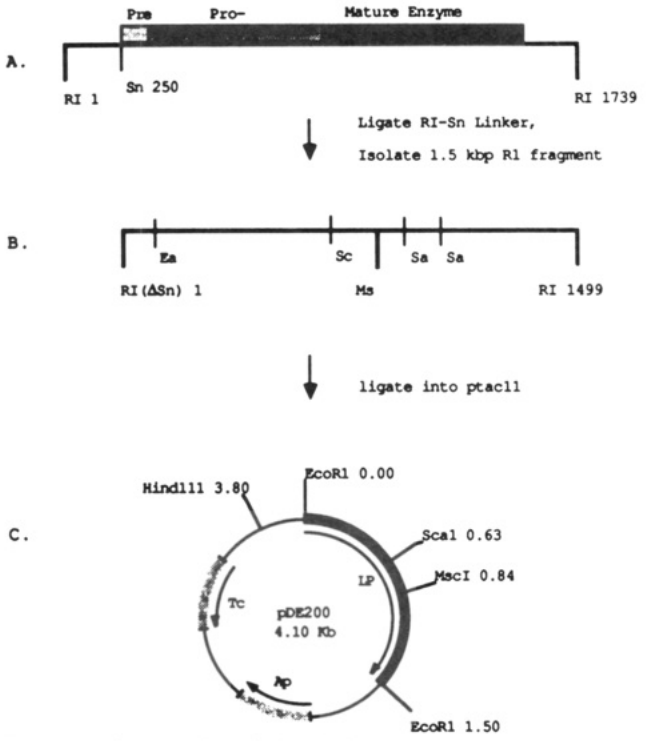

FIGURE 2: Construction of the α-lytic protease expression plasmid. (A) Restriction map and coding region structure for the 1.75-kbp insert in plasmid pαLP1. (B) Generation of the 1.5-kbp *Eco*RI fragment by *Eco*RI–*Sna*bI linker insertion into the *Sna*bI$_{250}$ site of pαLP1. The wild-type fMet initiation codon and coding sequence are unchanged by this linker insertion. Restriction nuclease sites from left to right are RI, *Eco*RI$_1$; ΔSn, *Sna*bI$_{10}$; Ea; *Eag*I$_{104}$; Sc, *Sca*I$_{626}$; Ms, *Msc*I$_{839}$; Sa, *Sac*II$_{884}$; Sa, *Sac*II$_{1020}$; and RI, *Eco*RI$_{1499}$. (C) Ligation of the 1.5-kbp *Eco*RI fragment into ptac11 to generate pDE200, which encodes the 397-residue preproenzyme of α-lytic protease.

ptz18[*Msc*I$_{839}$–*Eco*RI$_{1499}$]:S214A. (2) The [*Msc*I$_{839}$–*Eco*RI$_{1499}$]:Y171F:S214A and wild-type [*Eco*RI$_1$(Δ*Sna*bI$_{10}$)–*Msc*I$_{839}$] fragments were ligated into ptac11.

Nucleotide sequence was determined using dideoxynucleotides (Sanger et al., 1979). The mutated DNA fragments were reisolated from the expression plasmids and cloned into ptz18 or -19. Sequence of the mutated fragments (including cloning sites) was determined for both strands. All nucleotides are observed except S214A nucleotides 897–9, 908–9, and 1062–7 of the coding strand and 883–5 of the noncoding strand; Y171F nucleotides 987 of the coding strand and 883–5 and 896–7 of the noncoding strand; Y171F:S214A nucleotides 875–6 and 1062-7 of the coding strand and 883–4, 894–5, 941–2, and 1052–3 of the noncoding strand (the nucleotide sequence for the complementary strand at these compressions was determined and was wild type).

Reversion Mutagenesis. To test whether in vitro mutagenesis had generated undetected mutations elsewhere in the coding sequence, we mutated both S214A and S214G back to wild type. Site-directed mutagenesis was carried out on fragments [*Msc*I$_{839}$–*Eco*RI$_{1499}$]:S214A and -:S214G subcloned from the respective expression plasmids. The consequences of mutation on processing were eliminated by reversion to wild-type sequence, and wild-type catalytic activity was reobtained.

pFold and pEnz Expression Plasmids. Procedures for preparing the plasmids pFold, pEnz, and pEnz.S214A, the α-lytic protease prodomain and mature enzyme expression vectors, respectively, are described in the supplementary material. The plasmids pFold and pEnz are compatible in *E. coli* and were cotransformed into DH21 selected with Ap and Cm. Conditions for coexpression are identical to those described for the intact preproenzyme, adding 30 μg/mL chloramphenicol.

α-Lytic Protease Purification. All steps were performed at 4 °C except FPLC Mono-S chromatography. Cell paste (approximately 100 g) was suspended in 0.03 M sodium citrate (pH 5.75), 10% glycerol, 0.01 M KCl (1 mL of buffer/g of cells). The suspension was sonicated and centrifuged and the supernatant liquid applied to a carboxymethyl Amberlite CG-50 (Sigma) column (20 cm × 2.5 cm) in buffer A [0.033 M sodium citrate (pH 5.75) (1 g of resin/mL of supernatant liquid)]. Ten column volumes of buffer A were applied, after which α-lytic protease was eluted with 0.5 M sodium citrate (pH 6.2). Combined fractions were concentrated and chromatographed over a 200-mL G75 (Pharmacia) column (50 cm x 2.5 cm) equilibrated in 0.1 M KCl, 0.05 M Tris–HCl (pH 7.5). α-Lytic protease elutes at 90–120 mL of eluant, and combined fractions were dialyzed against buffer B [0.01 M KCl, 0.01 M sodium citrate (pH 6.3)] and then applied to a 5-mL CM-Sepharose (Pharmacia) column (5 cm x 0.75 cm). The column was eluted in three volumes of buffer B, after which a linear gradient to 0.5 M NaCl was developed over five column volumes. α-Lytic protease elutes at 0.3 M NaCl and was concentrated, dialyzed against 0.01 M Tris–HCl (pH 7.0), and passed over 0.25-mL DEAE-Sephacel (Pharmacia) in an 11-mL polypropylene Econo-Column (Bio-Rad). The eluant was dialyzed against 0.01 M KCl, 0.01 M sodium citrate (pH 6.2) and applied to an FPLC Mono-S (1 mL) column (Pharmacia) over 10 min in 0.01 M sodium citrate (pH 6.2). The Mono-S column was eluted for an additional 5 min in 0.01 M sodium citrate (pH 6.2) (1 mL/min), after which a linear gradient was developed over 60 min to 0.125 M NaCl. Mutant and wild-type α-lytic proteases elute as broad peaks at 52.5–55.8 min. To activate the protease (Westler, 1980), the enzymes were dialyzed against 0.5 M NaCl for 24 h and dialysis was continued against 0.1 M KCl until the enzyme was fully active, usually 24 h. Mutant and wild-type α-lytic proteases were homogeneous when visualized by Coomassie blue stained SDS–PAGE.

SDS–PAGE and Immunoblot Analysis of Expressed Protein. Cells from liquid culture (1.5 mL) were collected by centrifugation, resuspended in 90 μL of 0.01 M Tris–HCl (pH 7.5), boiled 5 min in sample buffer, and electrophoresed on SDS–PAGE (1% SDS, 15% polyacrylamide) (Laemmli, 1970). To prevent autolysis, purified α-lytic proteases were inhibited with *N*-Z-AlaAlaProAla-CF_3 (a gift from C. P. Govardhan) prior to SDS–PAGE Proteins were transferred to nitrocellulose according to Towbin et al. (1979). Rabbit anti-α-lytic protease antibodies were raised against *Lysobacter* enzyme, a gift from W. W. Bachovchin. Immune sera were partially purified by passing a 10-fold dilution of the sera over *E. coli* lysate immobilized onto nitrocellulose [dilution buffer: 1% gelatin, 0.02 M Tris–HCl (pH 7.5), 0.5 M NaCl]. Immunoblot (Burnette, 1981) reagents and prestained low molecular weight standards were from Bio-Rad.

Protein Sequencing. NH_2-terminal sequence analysis was obtained from α-lytic protease bound to Immobilon membrane (Matsudaira, 1987). The first seven residues of the NH_2-terminal sequence of *E. coli* derived wild-type, Y171F, and Y171F:S214A and wild-type α-lytic protease from pEnz were ANIVGGI.

α-Lytic Protease Enzyme Assay. The reaction progress of α-lytic protease catalyzed hydrolysis of peptidyl *p*-nitroanilides was measured spectrophotometrically at 410 nm by the release

of *p*-nitroaniline. Typically, a solution of 0.05 M Tris–HCl (pH 8.75) and substrate in Me_2SO was incubated at 25 °C. Reaction was initiated by addition of enzyme (final Me_2SO concentration ≤2% v/v). The molar extinction coefficient for *p*-nitroaniline at 410 nm was determined to be 8100 M^{-1} cm^{-1}. Peptidyl *p*-nitroanilide concentration was determined from the molar extinction coefficient after incubation of peptide in 0.1 N NaOH for 24 h at 22 °C. The cloned wild-type protease active site concentration was determined by two methods: (1) comparison of V_{max} values of cloned and *Lysobacter* α-lytic proteases and (2) active site labeling with [^{3}H]DFP (8.6 Ci/mmol) (Jansen et al., 1950; Whitaker, 1970). The concentrations of Y171F, S214A, and Y171F:S214A were determined by active site labeling with [^{3}H]DFP. [^{3}H]DFP-labeled enzyme was recovered from a 50-mL G25 (Pharmacia) column (30 cm x 1.25 cm) eluted in 0.05 M Tris–HCl (pH 7.5), 0.1 M KCl, 22 °C. Labeled protein eluted at 16–21 mL; unincorporated [^{3}H]DFP eluted at 26–40 mL. The specific activity of the labeled enzyme was determined by liquid scintillation counting. Based on protein concentration, [^{3}H]-DFP incorporation was stoichiometric. A standard curve, using triplicate samples, was generated with known amounts of *Lysobacter* α-lytic protease; $E^{1\%}_{280} = 10$ (Whitaker, 1970). The standard error for Y171F, Y171F:S214A, and S214A molarity was 15%, 10%, and 5%, respectively. Typically, a 5–10-fold molar excess of [^{3}H]DFP to enzyme was incubated in 0.05 M Tris–HCl (pH 7.5) for 8 h, at 22 °C.

Kinetic constants k_{cat}, K_m, and k_{cat}/K_m were determined from nonlinear least squares fit of the data to the Michaelis–Menten equation using the computer program HYPERO (Cleland, 1979). Each experiment consisted of determining initial velocities in duplicate or triplicate and at four to eight substrate concentrations. Enzyme concentrations ranged from 5.05 nM to 2.5 μM.

pH Dependence of k_{cat}/K_m. In the pH range 5.93–8.10, α-lytic protease was assayed at 25 °C in 0.05 M potassium phosphate buffer at an ionic strength, *I*, of 0.15, adjusted with KCl. In the range 7.03–8.75, activity was determined in 0.05 M Tris–HCl at $I = 0.15$. In all cases, the pH of the buffer solution was determined at 25 °C after adjusting the ionic strength. The pH-dependent values for k_{cat}/K_m were measured in duplicate or triplicate and were calculated from the slope of *v* versus [S] curves at two to five substrate concentrations, where $[S] \ll K_m$. In all cases the slope was linear. Enzyme concentrations were 50 nM for wild type, 110 nM for Y171F, and 96 nM for Y171F:S214A. The values for k_{cat}/K_m as a function of pH were fit to the equation $\log Y = \log (C/[1 + K/H])$ using the computer program HABELL (Cleland, 1979).

Chloromethyl Ketone Inactivation Kinetic Assay. The kinetics of inactivation by *N*-MeOSuc-AlaAlaProAla-CH_2-Cl were determined in the presence of substrate by the continuous assay method (Tian & Tsou, 1982; Waley, 1985) under standard α-lytic protease assay conditions. The change in absorbance as a function of time was nonlinear and fit eq 1, where P is the product concentration, v_o is the uninhibited

$$P = v_s t + (v_o - v_s)(1 - \exp(-k_{obs} t))/k_{obs}$$

velocity, v_s is the steady-state velocity, and k_{obsd} is the observed first-order rate constant for the approach to steady state. A replot of k_{obs} values versus [I] yielded a hyperbolic line with an intercept at 0. Inactivation kinetics were determined at five to six inhibitor concentrations. The inactivation kinetics were also determined by incubation of enzyme and inhibitor in the absence of substrate, followed by dilution of an aliquot of incubated enzyme into a standard assay solution.

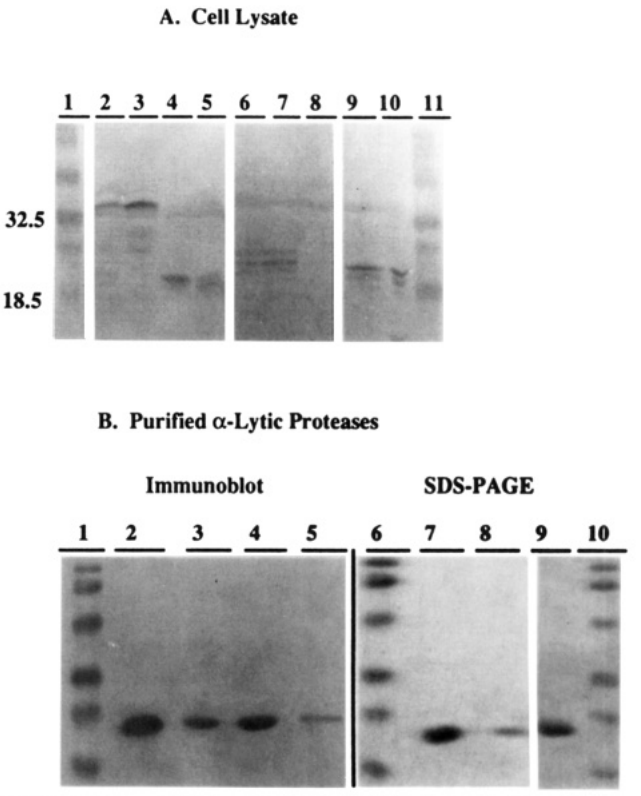

FIGURE 3: Immunoblot analysis of *E. coli* derived α-lytic proteases. (A) Immunoblot analysis of *E. coli* whole-cell extracts containing expressed wild-type and mutant α-lytic proteases: lane 1, prestained molecular weight standards (top to bottom 85, 49.5, 32.5, 27.5, and 18.5 kDa, respectively); lane 2, S214A extract; lane 3, S214G extract; lane 4, A214S extract; lane 5, G214S extract; lane 6, Y171F extract; lane 7, Y171F:S214A extract; lane 8, *E. coli* DH21 extract; lane 9, wild-type pDE200 extract; lane 10, *bona fide* α-lytic protease (*Lysobacter enzymogenes*); lane 11, prestained low molecular weight SDS–PAGE standards. (B) Purified α-lytic proteases: immunoblot and Coomassie blue stained SDS–PAGE. Immunoblot: lane 1, prestained molecular weight standards (top to bottom 130, 75, 50, 39, 27, and 17 kDa, respectively); lane 2, wild type (*L. enzymogenes*); lane 3, Y171F; lane 4, Y171F:S214A; lane 5, S214A. SDS–PAGE: lane 6, prestained molecular weight standards; lane 7, wild type (*L. enzymogenes*); lane 8, Y171F; lane 9, Y171F:S214A; lane 10, prestained molecular weight standards.

Amino acid analysis was performed on acid hydrolysates of 5 nmol of CMK-inactivated and untreated wild-type α-lytic protease. Samples were derivatized with *o*-phthalaldehyde, (OPA) (Chang et al., 1983) and chromatographed on an OPA-HS C-8 HPLC column (Alltech) equilibrated in 0.05 M NaOAc (pH 5), 4% THF, 10% methanol. A linear gradient to 60% methanol was developed over 25 min at a flow rate of 0.5 mL/min.

RESULTS

Expression of Wild-Type and Mutant α-Lytic Proteases. We expressed wild-type prepro-α-lytic protease in *E. coli*. The data (Figure 3A, lane 9) show that wild-type α-lytic protease and likely precursor molecules, in the 20-kDa size range, comigrate on SDS–PAGE with *Lysobacter* protein (lane 10). A protein corresponding to a 38–kDa proenzyme species (Silen et al., 1988; Epstein & Wensink, 1988) is not detected. Purified enzyme is homogeneous and the NH_2-terminal sequence is correct (Experimental Procedures). The data in Table I show that the cloned and *Lysobacter* enzymes have similar kinetic parameters and substrate specificity. Anal-

Table I. Kinetic Parameters for Wild-Type Cloned and *Lysobacter*-Derived α-Lytic Proteases

	E. coli[a]			*E. coli*[b]			*L. enzymogenes*		
substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
acAAPApNA (1)	40 (±10)	4.3 (±1)	1.0×10^4	40 (±1)	5.2 (±0.4)	8.0×10^3	41 (±4)	3.4 (±0.5)	1.2×10^4
acAAPVpNA (2)	2 (±0.1)	5.2 (±0.6)	3.8×10^2	2 (±0.1)	5.5 (±0.6)	3.8×10^2	2.6 (±0.2)	5.7 (±1)	4.5×10^2

[a] Wild-type cloned α-lytic protease derived the intact preproenzyme expressed from pDE200. [b] Wild-type α-lytic protease activated by the bimolecular folding reaction between the proenzyme and protease domains.

Table II. Kinetic Parameters of Mutant and Wild-Type (*L. enzymogenes*) α-Lytic Protease

	wild type			Y171F			Y171F:S214A			S214A		
substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
acAAPApNA (1)	41 (±4)	3.4 (±0.5)	12000	2 (±0.2)	5.0 (±1)	400	2 (±0.4)	13 (±2)	154	nd[a]	nd	2.65
acAAPVpNA (2)	2.6 (±0.2)	5.7 (±1)	456	0.43 (±0.2)	24 (±16)	18	0.7 (±0.5)	40 (±28)	17	nd	nd	0.14
sucAAVApNA (3)	48 (±4.4)	0.5 (±0.1)	97960	12 (±4)	4 (±2)	3000	25 (±6)	13 (±4)	1900	nd	nd	nd
acAPApNA (4)	12 (±0.08)	7 (±1)	1714	1 (±0.80)	20 (±2)	50	1 (±0.06)	47 (±3)	21	0.01 (±0.002)	28 (±15)	0.35
acPApNA (5)	0.8 (±0.02)	27 (±12)	30	nd	nd	0.6	nd	nd	0.8			
sucVApNA (6)	1.25 (±0.08)	5.0 (±2)	250	0.14 (±0.01)	14 (±2)	10	0.06 (±0.003)	11 (±1)	5.5			
sucLApNA (7)	1.6 (±1)	24 (±20)	67	0.1 (±0.02)	13 (±4)	8	0.08 (±0.08)	7 (±1)	11			
sucPApNA (8)	0.4 (±0.06)	8 (±2)	50	nd	nd	1	nd	nd	2			
sucAApNA (9)	0.12 (±0.01)	7 (±2)	17	0.02 (±3× 10^{-3})	9 (±3)	2	0.01 (±0.004)	4 (±0.4)	2.5			
sucGApNA (10)	nd	nd	0.2	nd	nd	0.1	nd	nd	0.2			
sucApNA (11)	2.5×10^{-3} ($\pm 3 \times 10^{-4}$)	12 (±2)	0.2	4×10^{-4} ($\pm 6 \times 10^{-5}$)	13 (±3)	0.035	1×10^{-3} ($\pm 4 \times 10^{-4}$)	14 (±2)	0.07			

[a] nd, not determined.

ogous results were obtained using the alkaline phosphatase promoter and signal sequence as an α-lytic protease expression system (Silen et al., 1989).

Prepro-Y171F and Y171F:S214A mutants process to the mature enzyme. As with wild type, protein in the 20-kDa size range is the predominant expression product with Y171F and Y171F:S214A. In the region of the SDS–PAGE gel where 38-kDa proenzyme is expected to migrate, immunologically cross-reacting material is not observed (Figure 3A, lanes 6 and 7). Purified Y171F and Y171F:S214A are homogeneous and the correct size as determined by SDS–PAGE, immunoblotting (Figure 3B), and NH_2-terminal sequencing (Experimental Procedures). On average, 1–2 mg of each enzyme is purified from approximately 100 g of cells.

Impairment of Processing by Mutation S214A. In contrast to wild type, immunoblot analysis of prepro-S214A expressed in *E. coli* (Figure 3A, lane 2) shows that a protein of about 38 kDa is the predominant species in vivo and that the 19.8-kDa mature enzyme is not apparent. An identical result is obtained for S214G (Figure 3A, lane 3). The known size of the α-lytic protease gene product and the specificity of the anti-α-lytic protease antibody allow us to conclude that the newly identified 38-kDa proteins are the respective proenzymes of S214A and S214G. We estimate that about 95% of the expressed steady-state S214A protein remains in the proenzyme form. To test whether in vitro mutagenesis had generated additional nucleotide changes outside the codon of interest, S214A and S214G were mutated back to wild type (Figure 3A, lanes 4 and 5). For both Ser 214 mutants, the effects of the primary mutation on processing were eliminated, and wild-type activity was reobtained.

S214A Purification. We purified on average 30 μg of active serine protease from approximately 100 g of recombinant *E. coli* harboring the prepro-S214A expression plasmid. By the following criteria we conclude that this is the mature 19.8-kDa S214A enzyme. During the purification procedure, which involves five column chromatographic steps, S214A comigrates with wild-type α-lytic protease (Experimental Procedures). Purified S214A cross-reacts with anti-α-lytic protease antisera (Figure 3B, lane 5). The activity is not due to low-level contamination of wild-type α-lytic protease since it is only weakly inhibited by high concentrations (187 μM) of a peptidyl trifluoromethyl ketone inhibitor, *N*-Boc-AlaProAla-CF_3, which fully inhibit wild-type enzyme (wild type K_i = 3 μM). The enzyme activity is present only in DH21 cells harboring the S214A expression plasmid. Finally, the enzyme is inactivated by DFP, and by a peptidyl chloromethyl ketone, *N*-MeOSuc-AlaAlaProAla-CH_2Cl peptide (**12**) (data not shown).

Prodomain Activation. Prepro-α-lytic protease activation likely involves unimolecular folding and cleavage reactions, but a bimolecular folding reaction has been reported (Silen & Agard, 1989). We obtained similar results using a different coexpression system. The protease and prodomains were subcloned onto separate plasmids, and the two domains were coexpressed (Experimental Procedures). The wild-type enzyme produced by coexpression of both domains was purified (yielding 50 μg of α-lytic protease from approximately 100 g of cells) and its kinetic parameters, substrate specificity (Table I), and NH_2-terminal sequence are identical to enzyme from the intact gene. We employed the bimolecular folding reaction to test whether S214A processing is blocked because the propeptide cannot fold the mutant protease domain. Coexpression of secreted proenzyme and (S214A) enzyme domains did not yield active, folded S214A, even though the wild-type and S214A protease domains are expressed at similar levels (data not shown). These results suggest that S214A mutation alters the structure of the enzyme so that proper folding cannot take place.

Effect of Mutation on Proximal Subsites. Kinetic parameters for wild-type α-lytic protease and three mutant enzymes are listed in Table II. It is important to establish whether structural changes other than at S_2 have occurred. Interactions at S_1 and the extended binding subsites are of concern. The ability of S_1 to function in the mutant enzymes is assessed from the kinetic parameters for *N*-Suc-Ala-pNA peptide (**11**). This peptide interacts primarily at S_1. The data show that the k_{cat}/K_m value of **11** fo Y171F is 5-fold

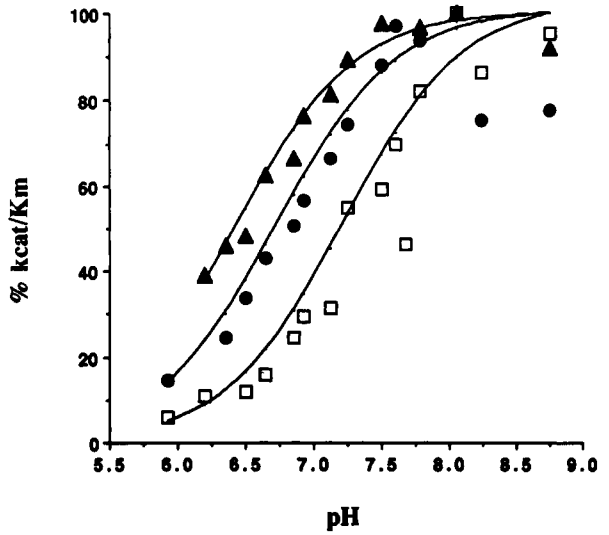

FIGURE 4: pH dependence of k_{cat}/K_m for mutant and wild-type α-lytic proteases: ▲, Y171F; ●, wild-type α-lytic protease (*L. enzymogenes*); □, Y171F:S214A. The k_{cat}/K_m values for each enzyme were normalized to 100% activity obtained at the pH maxima.

lower than its value for wild type, and similarly, the k_{cat}/K_m value of peptide **11** for Y171F:S214A is 3 times lower. No change occurs in the K_m value of this small substrate as a result of mutation. These results lead us to conclude that interactions at the S_1 subsite are affected slightly, if at all. The effect of mutation on interactions at S_3 was evaluated by comparing the k_{cat}/K_m values of *N*-Ac-Pro-Ala-pNA (**5**) and *N*-Ac-AlaProAla-pNA (**4**). The ratio of K_{cat}/K_m for the two peptides for wild-type enzyme is 50. The corresponding ratio for Y171F and Y171F:S214A is 80 and 25, respectively. The data indicate that interactions at the S_3 subsite are not significantly altered. Finally, the ratio of k_{cat}/K_m values for peptides **1** and **4** with wild type, Y171F, S214A, and Y171F:S214A mutant enzymes is the same (about 8-fold). Thus, we conclude that interactions at S_4 remain intact.

His 57 is a component of both S_2 and the catalytic triad; the basicity of His 57 can be assessed by examining the pH dependence of k_{cat}/K_m (Robillard & Schulman, 1974; Bachovchin & Roberts, 1978). Peptide *4*, *N*-Ac-AlaProAla-pNA, was used in each pK_a determination. Figure 4 shows that the pH dependence of k_{cat}/K_m for Y171F yields a single ionization constant with a $pK_a = 6.4 \pm 0.04$; the observed pK_a of Y171F is slightly lower than the wild-type pK_a of 6.7 ± 0.04. The wild-type pK_a value determined here is identical with previous measurement (Kaplan & Whitaker, 1969). The pH dependence of k_{cat}/K_m for Y171F:S214A yields a single ionization constant with pK_a of 7.2 ± 0.08. Similar results ($pK_a = 7.3 \pm 0.15$) are obtained with **1** and Y171F:S214A (data not shown). Taken together, these data indicate that changes in the active site ionization state as result of mutation are slight.

Effect of Tyr 171 and Ser 214 Mutations at S_2. The data in Table II show that the Tyr 171 and Ser 214 mutations primarily affect k_{cat}/K_m or k_{cat}. K_m is not affected significantly by mutation; in general, K_m increases less than 7-fold compared to wild type. The largest effect on k_{cat}/K_m is observed with the S214A mutant. For S214A, with the tripeptide substrate **4**, $k_{cat}/K_m = 0.35\ M^{-1}\ s^{-1}$, which is (4.9×10^3)-fold lower than wild type. Surprisingly, introduction of a second-site mutation (Y171F) increases k_{cat}/K_m 60-fold, so that for the double mutant (Y171F:S214A) $k_{cat}/K_m = 21\ M^{-1}\ s^{-1}$. Therefore, the significant loss in catalytic efficiency (and mature enzyme processing; see above) of the S214A single mutant is partly offset by the Y171F:S214A double mutation. Y171F:S214A is a pseudorevertant. The k_{cat}/K_m values of Y171F and Y171F:S214A are 34- and 82-fold lower than that of wild type, respectively (cf. **4**).

Table III. Inactivation of Mutant and Wild-Type α-Lytic Proteases by a Peptidyl Chloromethyl Ketone

enzyme	k_i (s^{-1})	K_I (mM)	k_i/K_I ($M^{-1}\ s^{-1}$)
wild type[a]	0.004	0.11	36
	(0.002)	(0.09)	(22)
Y171F	0.0140	12	1.2
	(0.005)	(9.8)	(0.5)
Y171F:S214A	0.005	0.33	15
	(0.003)	(0.27)	(11)

[a] α-Lytic protease derived from *L. enzymogenes*. The inactivation kinetics for *N*-MeOSuc-AlaAlaProAla-CH_2Cl (**12**) were measured in the presence (no parentheses) and absence (parentheses) of substrate (Experimental Procedures).

Binding interactions in the S_2 subsite in mutant and wild-type enzyme are evaluated from kinetic analysis of dipeptide substrates (Table II, **5–10**) for which the size/hydrophobicity of P_2 is varied. For wild-type S_2, the decreasing order of discrimination for P_2 side chains, determined by k_{cat}/K_m is

$$Val > Leu \geq Pro > Ala > Gly \quad (1250:335:255:85:1)$$

The S_2 specificity of Y171F and Y171F:S214A is qualitatively similar to wild type, although the magnitude of discrimination, in both mutants, is lower than that for wild type. For Y171F, the decreasng order of discrimination is

$$Val \geq Leu > Ala \geq Pro > Gly \quad (100:80:20:10:1)$$

and for the double mutant it is

$$Leu > Val > Ala \geq Pro > Gly \quad (55:27:12:10:1)$$

In the mutants, k_{cat}/K_m for good dipeptide substrates is reduced, whereas for poor substrates, k_{cat}/K_m is not changed (Table II). For example, in Y171F:S214A, the k_{cat}/K_m value of the best dipeptide substrate, **6**, with valine at P_2, is 45-fold lower than that of wild type. With a less specific substrate, **9**, containing alanine at P_2, the k_{cat}/K_m value with Y171F:S214A in only 7-fold lower than that of wild type. Finally, with the worst dipeptide substrate, **10**, with glycine at P_2, the k_{cat}/K_m values of Y171F:S214A and wild type are equivalent. Similar observations are made with Y171F when compared to wild type. Thus, hydrophobic interactions which occur in the transition state are altered by mutation.

Enzyme Inactivation. We used two inactivators to examine the effect of mutation on the catalytic triad. Wild-type, Y171F, S214A, and Y171F:S214A enzymes are completely inactivated by diisopropyl fluorophosphate (DFP) under identical conditions. Thus, the nucleophilicity of Ser 195 has been maintained. Peptidyl chloromethyl ketones (CMK) are inactivators of serine proteases that alkylate His 57 (Schoellmann & Shaw, 1963). We find that wild-type α-lytic protease is inactivated by a peptidyl chloromethyl ketone, *N*-MeOSuc-AlaAlaProAla-CH_2Cl (**12**), with an apparent second-order rate constant equal to $37\ M^{-1}\ s^{-1}$ (Table III). Amino acid analysis of wild-type CMK-inactivated enzyme indicates that the single histidine residue in α-lytic protease is altered upon treatment with **12** (data not shown). Mutants Y171F and Y171F:S214A also are inactivated by **12**, and the kinetic parameters are reported in Table III. For all three enzymes, inactivation is not reversed upon dialysis.

Effect of Nucleophiles on Reaction Rate. In this study we employed peptidyl *p*-nitroanilides (pNA) as amide substrate analogs. In serine protease catalyzed reactions, the rate-

limiting step for amide hydrolysis is acylation; for ester hydrolysis, it is deacylation (Bender & Kezdy, 1965). To evaluate whether deacylation had become rate limiting in the Y171F and Y171F:S214A enzymes, the effect of added nucleophiles on the rate of reaction was determined (Stein et al., 1984, 1987). Increasing concentrations of added nucleophiles (from 0.01 to 0.1 M hydroxylamine, valine methyl ester, alanine amide, or glycine amide) have no effect on the rate of *N*-Ac-AlaProAla-pNA, (**4**; 0.02 M) hydrolysis for wild-type, Y171F, or Y171F:S214A enzymes (data not shown).

DISCUSSION

A hydrogen bond network in the hydrophobic S_2 subsite of α-lytic protease was examined by mutagenesis. Mutants (S214A, Y171F, Y171F:S214A) were prepared in order to investigate the role of the Ser 214 and Tyr 171 hydroxyl groups in the catalytic process. Of particular interest is Ser 214, which is part of a hydrogen-bonding network that includes Asp 102 of the catalytic triad. Meaningful results are obtained only if one can define the subsites affected by mutation. We have shown (Results) that mutations Y171F and Y171F:S214A primarily alter binding interactions in the S_2 pocket and have little, if any, effect on the S_1 (which contains Ser 195 and the oxy anion binding site), S_3, or S_4 subsite or on the catalytic triad. Low yield and low enzymatic activity prevented us from defining the range of interactions altered by the single S214A mutation.

The pH dependence of k_{cat}/K_m is altered slightly by the Y171F and Y171F:S214A mutations. It has been proposed that Ser 214 functions to stabilize charge buildup in the Asp–COO^-–ImH^+ ion pair during catalysis (James et al., 1978; Brayer et al., 1978, 1979; Warshel & Russell, 1986; Warshel et al., 1989). The pK_a observed for Y171F:S214A is 0.5 unit greater than that for wild type and is expected to increase in the double mutant since a hydrogen bond that is donated to the Asp 102 carboxylate is removed as a consequence of mutation. The observed pK_a for Y171F is 0.3 unit less than for wild type and may be due to repositioning Asp 102. Since the changes in pK_a are slight, the data suggest that Ser 214 functions in catalysis other than by modulating the ionization state of the catalytic triad.

The data in Table II show that mutation primarily affects k_{cat}/K_m or k_{cat}. The largest effect on k_{cat}/K_m is obtained with S214A. For **1** and **4** k_{cat}/K_m is reduced approximately (5×10^3)-fold while K_m is increased only 4-fold. The mutations affect transition-state interactions, but have little effect on ground-state interactions. Therefore, a conformational change (of the enzyme or the substrate) must occur, in converting from the ground-state complex to the transition state complex. A comparison of X-ray structural data of native α-lytic protease to that for the enzyme complexed with peptidyl-boronic acid tetrahedral intermediate analogs does not reveal significant structural difference between the ground and tetrahedral-intermediate states of the enzyme (Fujinaga et al., 1985; Bone et al., 1987, 1989). Analogous results have been obtained for chymotrypsin complexed with TFMK inhibitors (Brady et al., 1990). Structural changes which occur in moving from the ground state to the transition-state complex may be very subtle and thus may not be detected by X-ray crystallography.

It is important to consider what the maximum effect an S_2 mutation, one which abolishes all P_2–S_2 transition-state interactions, can have on k_{cat}/K_m. For proline in the P_2 position, the effect can be estimated by comparing **8** and **11**. Elimination of all S_2 subsite interactions would result in a 250-fold decrease in k_{cat}/K_m. The data in Table II show that for peptides with proline in P_2, k_{cat}/K_m is reduced less than 250-fold for mutants Y171F and Y171F:S214A. Therefore, transition-state interactions at S_2 are partially lost in Y171F and Y171F:S214A. In contrast for S214A, a greater than 10^3-fold decrease in k_{cat}/K_m is observed. It is, therefore, probable that extensive structural changes, likely within the extended binding region, have taken place in S214A.

From the S214A data alone, one might infer Ser 214 is essential for catalytic function. However, the results of the Y171F:S214A second-site mutation suggest that a different interpretation is required. Since the effects of the S214A mutation are partially offset in the double mutant, it is likely Ser 214 is not essential for catalytic function in α-lytic protease. This suggests the S214A mutation results in a complete loss of structure in the S_2 subsite or catalytic triad, rather than loss of specific interactions between the substrate and the Ser 214 hydroxyl. It seems unlikely that the loss of a specific interaction between the Ser 214 hydroxyl group and the substrate could be compensated for by removal of the Tyr 171 hydroxyl group. Molecular modeling (Epstein, 1992) and crystal structure data (Bone et al., 1987) indicate the Try 171 and Ser 214 hydroxyl groups do not interact with substrate. A loss of binding site structure, necessary for precise catalytic interactions, is consistent with the failure to process the proenzyme. In addition, the S214A enzyme domain also may be a poor substrate in the prodomain stabilized folding reaction (Results). The mechanism through which substitution of phenylalanine for tyrosine compensates for the S214A mutation is not known. We speculate that removal of the Ser 214 OH group leaves an "unsatisfied" Tyr 171 hydroxyl group hydrogen-bonding partner, which then interacts with another group on the enzyme, possibly the Asp 102 carboxylate, or remains solvated by bulk solution. Alternatively, in S214A, the Tyr 171 hydroxyl group simply may participate in repulsive interactiions with the hydrophobic P_2 side chain.

The data in Table II extend the previous observation (Bauer et al., 1981) that hydrophobic binding interactions in S_2 are used selectively to increase k_{cat}. The data (Table II) show that for wild type, k_{cat}/K_m for **6** is 1250 times larger than that for **10**. **6** has valine in P_2 whereas **10** contains glycine in P_2. Thus, significant transition-state interactions occur in the S_2 subsite. For Y171F, k_{cat}/K_m of **6** is 100-fold larger than k_{cat}/K_m of **10**. Thus, the magnitude of difference in k_{cat}/K_m values for the two peptides has decreased as a consequence of the Y171F mutation. For Y171F:S214A, k_{cat}/K_m for **6** is only 27-fold larger than that for **10**, a further decrease in the discrimination of P_2 hydrophobic substituents. It is very likely that the increase in k_{cat}/K_m (observed for mutant and wild-type enzymes) is due to specific and precise hydrophobic interactions between the S_2 subsite and the P_2 side chain of the substrate. Therefore, as a result of mutation a significant portion, up to 98%, of the hydrophobic interaction between substrate and subsite is lost. The data suggest that the precise hydrophobic binding interactions which are required for stabilization of the transition state, and optimal catalytic efficiency, cannot occur in the mutants.

The data for inactivation by the peptidyl chloromethyl ketone **12** are summarized in Table III. K_I is the dissociation constant of a reversible complex that is formed prior to alkylation of His 57. If this complex is stabilized by transition-state interactions available to the substrate during the hydrolytic process, one would expect that K_I wild type < Y171F:S214A ≤ Y171F. Clearly different interactions stabilize the reversible complex among the three enzymes,

some of which may involve transition-state interactions utilized for acyl transfer. The magnitude of the rate constant for the alkylation (k_i) probably is determined primarily by the position of the chloromethyl group relative to His 57. For wild-type and mutant α-lytic proteases, k_i is not greatly different. This indicates that, prior to inactivation, the orientation of the chloromethyl group and His 57 side chain are very similar in the wild-type and mutant enzyme complexes.

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SUPPLEMENTARY MATERIAL AVAILABLE

Synthetic methods and NMR spectral data for compounds in this paper and the method for preparing the expression plasmids pFold, pEnz, and pEnz.S214A (6 pages). Ordering information is given on any current masthead page. Canadian residents should add 7% GST.

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