Studies on the Hydrolytic Properties of (Serine) Carboxypeptidase Y

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Received November 20, 1995; Revised Manuscript Received March 21, 1996[⊗]

ABSTRACT: The activity of serine carboxypeptidases is dependent on a catalytic triad, an oxyanion hole, and a binding site equivalent to those found in the serine endopeptidases. The action of carboxypeptidase Y on substrates containing amino acids, alcohols, and amines as leaving groups is described. It is demonstrated that the features common to serine endopeptidases and carboxypeptidases are sufficient for hydrolysis of ester bonds. However, rapid hydrolysis of amide bonds is dependent on interactions between the C-terminal carboxylate group of the substrate and the C-terminal recognition site of the enzyme. Furthermore, on the basis of the pH dependencies of wild-type and mutant enzyme, combined with the ability of the enzyme to utilize binding energy to promote catalysis, alternative models for the high activity of carboxypeptidase Y at low pH are discussed. They describe how the catalytically essential histidine is maintained in its active deprotonated state through perturbation of its pK_a value in the enzyme−substrate complex.

Carboxypeptidase Y (CPD-Y)[1] is a 64 kDa serine carboxypeptidase isolated from *Saccharomyces cerevisiae* [for reviews see Breddam (1986) and Remington and Breddam (1994)]. In contrast to the metallocarboxypeptidases, e.g., carboxypeptidase A, it has been found to catalyze hydrolysis reactions with a large variety of leaving groups, e.g., amino acids, *p*-nitroaniline, and various alcohols. The serine carboxypeptidases have been classified as α/β hydrolases (Ollis *et al*., 1992; Endrizzi *et al*., 1994), which compose a diverse family of structurally related hydrolases containing a catalytic triad of the nucleophile−histidine−acid type. The α/β hydrolase family contains enzymes capable of hydrolyzing dienelactones, acetylcholine, haloalkanes, lipids, and peptides (Ollis *et al*., 1992). However, despite the diversity of this structural family, it so far does not comprise cysteine or serine endopeptidases. The three-dimensional structures of CPD-Y and carboxypeptidase WII from wheat (Endrizzi *et al*., 1994; Liao & Remington, 1990; Liao *et al*., 1992; Bullock *et al*., 1994) combined with chemical modification studies (Hayashi *et al*., 1975) have shown that the catalytic triad consists of Ser146, Asp338, and His397 (CPD-Y numbering). The three-dimensional arrangement of the functional groups of these three residues together with the observations that Ser146 may be irreversibly acylated with DFP and that an acyl intermediate may be detected in the hydrolysis of indole acryloyl imidazole (Martin *et al*., 1980) suggests that the serine carboxypeptidases employ a catalytic mechanism similar, if not identical, to that of the well-characterized serine endopeptidases (Bender *et al*., 1962; Fersht, 1985). The serine endopeptidases hydrolyze peptide and ester substrates in a two-step reaction during which the catalytically essential serine is acylated by the substrate. A key feature of this mechanism is the ability of the histidine to abstract a proton from the serine prior to or simultaneously with the nucleophilic attack on the scissile bond. This can take place only when the histidine is in the deprotonated form. As a consequence, the pH profile of k_{cat} is determined by the pK_a of the histidine rendering these enzymes virtually inactive at low pH. Nevertheless, important differences do exist. In contrast to the serine endopeptidases, the serine carboxypeptidases are highly active at low pH, and from pH 3 to 9, k_{cat} only increases by a factor of 3−4 (Breddam, 1986). Furthermore, k_{cat} titrates with a pK_a value about 1.5 units below the value typically found for the essential histidine in the serine endopeptidases (Bender *et al*., 1962; Fersht, 1985). To explain this, recent models suggest that Glu145 or the C-terminal carboxylate of the substrate functions as an acceptor of the inhibitory proton from the positively charged (inactive) catalytic His397 (Bullock *et al*., 1994; Christensen, 1994). However, we have previously shown that the serine carboxypeptidase catalyzed hydrolysis of peptide bonds is highly dependent on interaction between the C-terminal carboxylate group of the substrate and the hydrogen bond donors Asn51 and Glu145 (in its protonated form) (Mortensen *et al*., 1994). In the present communication, this model and the significance of Glu145 in C-terminal recognition are investigated and the question of whether Glu145 may have more than a single function within the enzyme is addressed. Alternative models describing how the catalytically essential histidine is maintained in its deprotonated state through perturbation of its pK_a value in the enzyme−substrate complex is discussed.

MATERIALS AND METHODS

Materials. MES and HEPES were from Sigma, U.S.A. FA-Phe-OMe and FA-Phe-OGly-OH were from Bachem, Switzerland. FA-Phe-OEt, FA-Phe-NHEt, FA-Phe-Gly-OH,

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[⊗] Abstract published in *Advance ACS Abstracts*, May 15, 1996.

[1] Abbreviations: CPD-Y, carboxypeptidase Y from yeast; DFP, diisopropyl phosphofluoridate; FA, furylacryloyl; Phg, phenylglycine; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); -OGly-OH, glycolic acid; Ph, phenyl; PMSF, phenylmethylsulfonyl fluoride; PRC1, structural gene for CPD-Y; EDTA, ethylenedinitrilotetraacetic acid.

S0006-2960(95)02758-9 CCC: $12.00

FA-Ala-OCH$_2$Ph, FA-Ala-NHCH$_2$Ph, FA-Ala-Phg-OH, FA-Ala-NH(CH$_2$)$_2$Ph, and FA-Ala-Phe-OH were synthesized as previously described (Breddam, 1984). The substrates were crystallized from water:ethanol. Wild-type CPD-Y, FA-Phe-Ala-OH, and FA-Phe-Leu-OH were from Carlbiotech, Copenhagen, Denmark. [^{3}H]DFP was from Amersham, U.S.A. PMSF was from Boehringer Mannheim, Germany. pUC19 and M13mp19 were from in-house collections (Yanisch-Perron *et al*., 1985). Coordinates for the three-dimensional structure of wild-type and E65A+E145A were kindly provided by Dr. S. James Remington. The water accessible surface area of His397 in the wild-type and the mutant enzyme was determined using Naccess (Hubbard & Thornton, 1993).

Preparation of Mutant Enzymes. S146A was constructed as described previously for E145A (Mortensen *et al*., 1994) using oligo S146A: GGCGTAGGCTTCCCCAGCGAT. Nucleotides underlined are different from wild-type. E65A+E145A was prepared as previously described by Mortensen *et al*. (1994). H397A and D338A was constructed using the Oligonucleotide-Directed *in Vitro* Mutagenesis System Version 2 kit from Amersham, 5′-GGCGATAAAGCTTTCATCTGT-3′ (oligo D338A), 5′-CGCCATGGTTCCATTTGACGTCCCTGAA-3′ (oligo H397A), and a M13mp19 subclone of the structural gene for CPD-Y as previously described by Bech and Breddam (1989). H397G was constructed using the polymerase chain reaction on a pUC19 derivative containing a 1112 bp *Bam*HI-*Bam*HI fragment of *PRC1* with the oligo: 5′-CGGCATGGTTCCATTTGACGTCCCTGAA-3′ (oligo H397G) and a 24-mer reverse sequencing primer for M13mp19 equivalent to primer #1233 from New England Biolabs, MA. The resulting PCR product was subcloned as a *Msc*I-*Bam*HI fragment into the pRA21 expression vector, which contains the *PRC1* gene under control of the *GAL1* promotor. Mutant enzymes were produced by introduction of pRA21 into the yeast strain W2579, which is deficient in intracellular sorting (*vps1*) and deleted in the *PRC1* gene (Δ*prc1*), as previously described by Mortensen *et al*. (1994) and purified by affinity chromatography using the method of Johansen *et al*. (1976). Reaction of S146A, H397A and H397G with PMSF was performed using a 10-fold excess of PMSF in 100 mM NaH$_2$PO$_4$ buffer, pH 7.0. The PMSF-treated mutant enzymes were repurified by affinity chromatography, and this treatment was repeated until no additional loss of activity was observed. All mutant enzyme preparations were homogenous as determined by SDS−PAGE.

Determination of Kinetic Parameters. The k_{cat} and K_M values for the hydrolysis of FA-Phe-OMe and FA-Phe-Leu-OH were determined at 25 °C in the buffers 50 mM HEPES, 2.5% (v/v) CH$_3$OH, 1 mM EDTA, pH 7.5, and 50 mM MES, 1 mM EDTA, pH 6.5, respectively. The k_{cat} and K_M values for the hydrolysis of FA-Ala-OCH$_2$Ph, FA-Ala-NHCH$_2$Ph, FA-Ala-Phg-OH, FA-Ala-NH(CH$_2$)$_2$Ph, and FA-Ala-Phe-OH were determined at 25 °C in the buffer 50 mM MES, 2.5% (v/v) CH$_3$OH, 1 mM EDTA, pH 6.5. The k_{cat} and K_M values for the hydrolysis of FA-Phe-OEt, FA-Phe-OGly-OH, and FA-Phe-Gly-OH were determined at 25 °C in the buffer 50 mM HAc, 2.5% (v/v) CH$_3$OH, 1 mM EDTA, pH 5.5. Hydrolysis rates were measured spectrophotometrically at 337−346 nm using a Perkin Elmer λ7 spectrophotometer thermostated to 25 °C. The standard deviations on the catalytic parameters were below 10%. The influence of pH on the catalytic parameters for the hydrolysis of the substrates FA-Ala-OBzl, FA-Phe-OEt, FA-Phe-OGly-OH, FA-Phe-Leu-OH, and FA-Phe-Ala-OH was determined in 20 mM buffer, 0.1 M NaCl, 1 mM EDTA and 2.5% CH$_3$OH using the following buffers: acetic acid, pH 4.0−5.5; MES, pH 5.5−6.5; HEPES, pH 6.5−8.5. No apparent change in the catalytic parameters associated with changes in buffer type was observed. Hydrolysis rates were determined spectrophotometrically at 329−346 nm and 25 °C using a Perkin Elmer λ7 spectrophotometer. The kinetic parameters as well as the pK_a values describing their pH profiles were determined using the nonlinear regression analysis program Grafit 3.01 (Leatherbarrow, 1993). For the wild-type enzyme the equations used for the determination of the pK values were based on the reaction schemes outlined in Figure 1, schemes A and B, valid with uncharged and charged substrates, respectively (Christensen, 1994). For uncharged substrates the following equations were used:

$$k_{cat}(\mathrm{pH}) = [(k_{cat,1}\exp_{10}(\mathrm{pH} - \mathrm{p}K_{ES}) + k_{cat,2})/(1 + \exp_{10}(\mathrm{pH}-\mathrm{p}K_{ES}))]$$

$$K_M(\mathrm{pH}) = [K_{S,2}(1 + \exp_{10}(\mathrm{pH} - \mathrm{p}K_E) + \exp_{10}(\mathrm{p}K_{EH} - \mathrm{pH}))/(1 + \exp_{10}(\mathrm{pH} - \mathrm{p}K_{ES}))]$$

$$k_{cat}/K_M(\mathrm{pH}) = [((k_{cat,1}/K_{S,1})\exp_{10}(\mathrm{pH} - \mathrm{p}K_E)) + (k_{cat,2}/K_{S,2}))/(1 + \exp_{10}(\mathrm{pH} - \mathrm{p}K_E) + \exp_{10}(\mathrm{p}K_{EH} - \mathrm{pH}))]$$

For the charged substrates the following equations were used:

$$k_{cat}(\mathrm{pH}) = [(k_{cat,1} + k_{cat,2}\exp_{10}(\mathrm{p}K_{EHS} - \mathrm{pH}))/(1 + \exp_{10}(\mathrm{p}K_{EHS} - \mathrm{pH}))]$$

$$K_M(\mathrm{pH}) = [(K_{S,1}(1 + \exp_{10}(\mathrm{p}K_S - \mathrm{pH}))(1 + \exp_{10}(\mathrm{pH} - \mathrm{p}K_E) + \exp_{10}(\mathrm{p}K_{EH} - \mathrm{pH})))/(1 + \exp_{10}(\mathrm{p}K_{EHS} - \mathrm{pH}))]$$

$$k_{cat}/K_M(\mathrm{pH}) = [((k_{cat,1}/K_{S,1}) + (k_{cat,2}/K_{S,2})\exp_{10}(\mathrm{p}K_{EH} - \mathrm{pH}))/((1 + \exp_{10}(\mathrm{pH} - \mathrm{p}K_E) + \exp_{10}(\mathrm{p}K_{EH} - \mathrm{pH}))(1 + \exp_{10}(\mathrm{p}K_S - \mathrm{pH})))]$$

It should be noted that titration of the substrate was taken into account for the charged substrates with the term $(1 + \exp_{10}(\mathrm{p}K_S) - \mathrm{pH})$. For E65A+E145A schemes C and D (Figure 1) were used for uncharged and charged substrates, respectively. For the charged as well as uncharged ester substrates the following equations were used:

$$k_{cat}(\mathrm{pH}) = [k_{cat}/(1 + \exp_{10}(\mathrm{p}K_{ES} - \mathrm{pH}))$$

$$K_M(\mathrm{pH}) = [(K_{S,1}(1 + \exp_{10}(\mathrm{p}K_E - \mathrm{pH})))/(1 + \exp_{10}(\mathrm{p}K_{ES} - \mathrm{pH}))]$$

$$K_{cat}/K_M(\mathrm{pH}) = [(k_{cat}/K_{S,1})/(1 + \exp_{10}(\mathrm{p}K_E - \mathrm{pH}))]$$

For the charged peptide substrates the following equations were used:

$$k_{cat}(pH) = [(k_{cat,1}\ exp_{10}(pH - pK_{ES}) + k_{cat,2})/ (1 + exp_{10}(pH - pK_{ES}) + exp_{10}(pK_{EHS} - pH))]$$

$$K_M(pH) = [(K_{S,2}(1 + exp_{10}(pH - pK_E) + exp_{10}(pK_{EH} - pH)))/ (1 + exp_{10}(pH - pK_{ES}) + exp_{10}(pK_{EHS} - pH))]$$

$$K_{cat}/K_M(pH) = [((k_{cat,1}/K_{S,1})exp_{10}(pH - pK_E) + (k_{cat,2}/K_{S,2}))/ (1 + exp_{10}(pH - pK_E) + exp_{10}(pK_{EH} - pH))]$$

The pH dependence of k_{cat} for the wild-type CPD-Y-catalyzed hydrolysis of FA-Phe-Leu-OH was described by the equation $k_{cat}(pH) = [(k_{cat,1} + k_{cat,2}\ exp_{10}(pK_{EHS} - pH))/ (1 + exp_{10}(pH - pK_{ES}) + exp_{10}(pK_{EHS} - pH))]$, whereas the pH dependence of K_M was described by the same equation as with the other charged substrates.

For two enzymes or substrates, A and B, the difference in transition state stabilization energy was calculated from the k_{cat}/K_M values: $\Delta\Delta G_T^{\ddagger} = -RT \ln[(k_{cat}/K_M(B))/(k_{cat}/K_M(A))]$ (Fersht, 1985). The $\Delta\Delta G_T^{\ddagger}$ values obtained when comparing substrates with and without a C-terminal carboxylate group were compensated for the presence of enzyme unable to bind substrate due to deprotonation of Glu145 by multiplying the observed k_{cat}/K_M value for the charged substrate by $(1 + exp_{10}(pH - pK_{Glu145}))$. This allows assessment of the transition state activation energy contributed by the interactions between the enzyme and the C-terminal carboxylate group.

[^{3}H]DFP Inactivation of Wild-Type and Mutant CPD-Y. Wild-type enzyme as well as mutant enzymes (16.5 μM) were treated with a 5-fold molar excess of [^{3}H]DFP in 100 mM NaH_2PO_4 at pH 7.0 for 2 h at room temperature. Upon completion of the reaction, the sample was diluted 4-fold and the protein was precipitated using deoxycholate and trichloroacetic acid (Peterson, 1977). The precipitate was washed twice with ethanol:ether (1:1), dried, and resuspended in 100 μL of 10 mM Tris, 1 mM EDTA, pH 8.0. An aliquot of the reaction mixture was added to 5 mL of Packard Gold scintillation fluid cocktail and counted in a Beckman LS6000IC scintillation counter for 5 min.

Determination of Deacylation Rates. The reaction was carried out in 50 mM MES, 1 mM EDTA, pH 6.5, containing 12.5 μM indole acryloyl imidazole and a 1.1-fold excess of CPD-Y or a 2-fold excess of E65A+E145A at 25 °C. Spectra were recorded at 60-s intervals on a Perkin Elmer λ9 spectrophotometer. The rate of deacylation was determined according to Martin *et al*. (1980).

Reactivation of His397 Mutant Enzymes. Reconstitution of activity toward FA-Phe-OMe through addition of imidazole was carried out at 25 °C in 50 mM HEPES, 1 mM EDTA, 2.5% CH_3OH, pH 7.5. The ionic strength of the reaction mixture was kept constant through adjustment with NaCl. The concentration of FA-Phe-OMe in the assay was 0.2 mM, and the hydrolysis reaction was followed spectrophotometrically at 337 nm on a Perkin Elmer λ9 spectrophotometer. The observed reaction rates were normalized to 1, equal to the rate in the absence imidazole.

RESULTS

Does the High Activity of CPD-Y at Low pH Depend on Glu145? It has been proposed that transferral of a proton from the protonated form of the catalytic histidine to either the carboxylate group of Glu145 or the C-terminal carboxylate of the substrate may account for the high activity of serine carboxypeptidases at low pH (Bullock *et al*., 1994: Christensen, 1994). This model can be tested by analyzing a mutant enzyme in which Glu145 has been changed to a residue without the capacity to accept a proton, e.g., E145A. This mutant enzyme should reflect the state of ionization of His397 and therefore exhibit a pH profile equivalent to that of the serine endopeptidases. However, this experiment is complicated by the observation that the carboxylate group of Glu145 forms a hydrogen bond with the buried carboxylate group of Glu65, thus forming a glutamic acid bridge (Liao *et al*., 1990; Endrizzi *et al*., 1994; Mortensen *et al*., 1994; Mortensen & Breddam, 1994). To eliminate the possibility that in E145A a "liberated" Glu65 could functionally substitute for Glu145, the double mutant was constructed. The resulting enzyme, i.e., E65A+E145A, has no acidic groups within the active site apart from Asp338 belonging to the catalytic triad. The three-dimensional structure of E65A+E145A shows that the structural changes within the active site are limited to movement of the flexible side chain of Met398 (Sørensen *et al*., 1995). Furthermore, CPD-Y and E65A+E145A were reacted with indole acryloyl imidazole to establish whether the rate of deacylation was affected by the mutation. Values of 9×10^{-4} s^{-1} and 4×10^{-4} s^{-1} were obtained for CPD-Y and E65A+E145A, respectively. Thus, the rate of deacylation as well as the three-dimensional structure is only slightly affected by the mutation, and consequently, the catalytic apparatus is essentially unaffected.

The pH dependence of the catalytic parameters for the hydrolysis of a substrate without an α-terminal carboxylate group, FA-Ala-OBzl, was determined (Figure 2). The pH dependence of wild-type CPD-Y showed that the enzyme exhibits a high k_{cat} even at low pH, i.e., 3000 min^{-1} at pH 4.5, and that k_{cat} increased to around 5500 min^{-1} as pH was increased to 8.5. K_M decreased from 0.34 to 0.04 mM within the same pH interval and k_{cat}/K_M increased from 11 000 to 150 000. The data for the pH dependence of the determined parameters were fitted to the equations derived from scheme A, describing a system in which the uncharged substrate does not bind to the enzyme when both His397 and Glu145 are protonated (Christensen, 1994). The following pK values were determined: $pK_{ES} = 6.1$, $pK_E = 6.8$, and $pK_{EH} = 5.3$. The pH dependence for E65A+E145A showed an overall decrease of both k_{cat} and K_M. Importantly, below pH 6, k_{cat}/K_M values for the mutant enzyme actually exceed those for the wild-type enzyme, indicating that Glu145 is not important for the activation of His397 at low pH. The data for the pH dependence of E65A+E145A cannot be described by the equations derived from scheme A since in this enzyme Glu145 has been replaced. A simpler scheme without the upper line, i.e., pK_E, pK_{ES}, and $K_{S,1}$, was not relevant either, since it would imply a pH independent k_{cat}. Consequently, the data were fitted to the equations derived from scheme C, although it isn't possible to determine whether k_{cat} approaches zero or a lower limit. The following pK values were determined: $pK_{ES} = 4.3$ and $pK_E = 4.9$.

To investigate whether the change in the pK values were a general feature or one related to the uncharged ester substrate, the pH dependencies of k_{cat} for the hydrolysis of a charged ester substrate, FA-Phe-OGly-OH, and a peptide

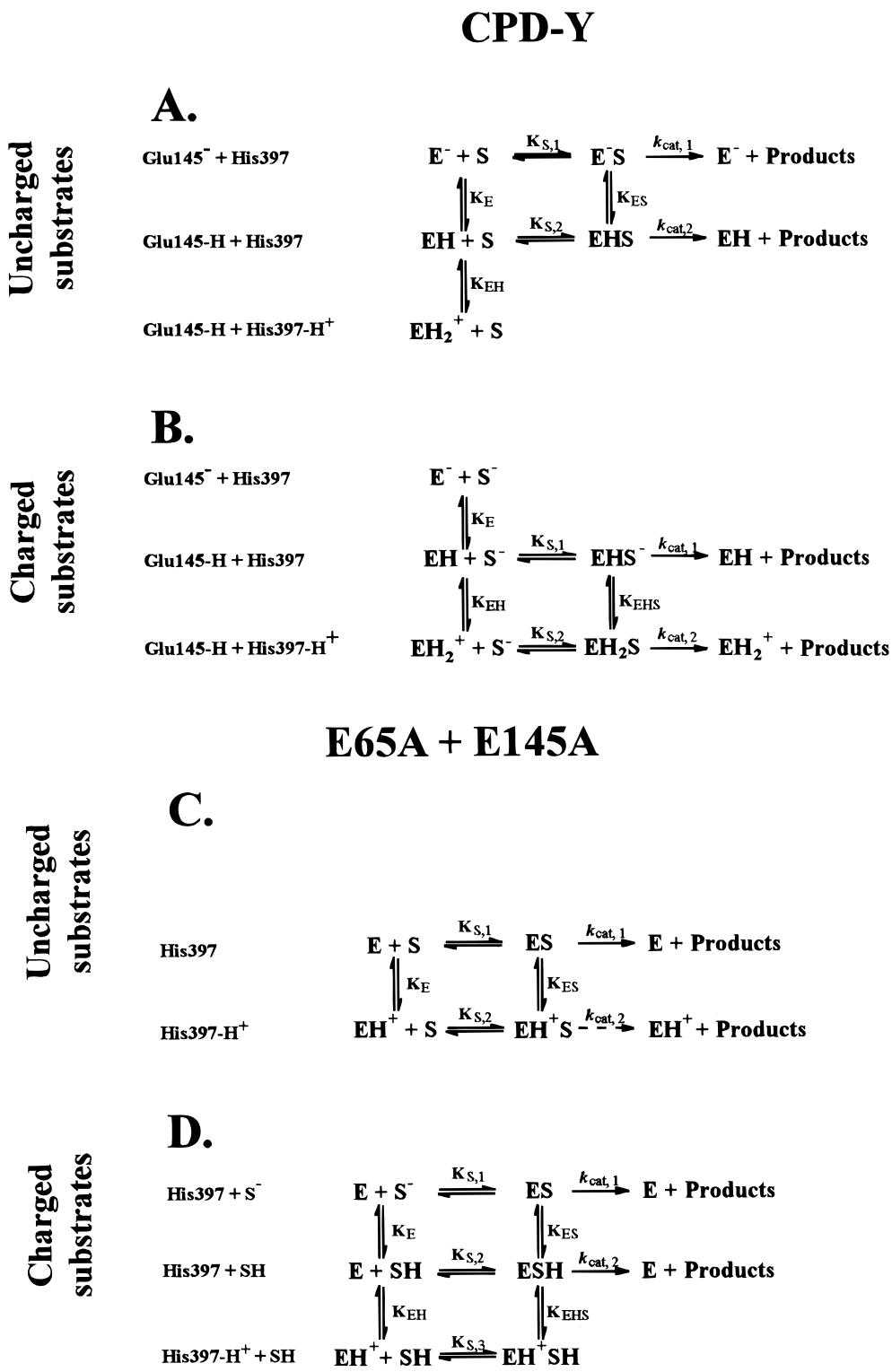

FIGURE 1: Schemes for the pH dependence of wild-type CPD-Y with an uncharged and a charged substrate, schemes A and B, respectively, and for the mutant enzyme with an ester and a peptide substrate, schemes C and D, respectively. To the left of each scheme are indicated the species that are believed to be present in the reaction of the corresponding line in the reaction scheme.

substrate, FA-Phe-Ala-OH, were determined (Figure 3). With FA-Phe-OGly-OH and wild-type CPD-Y the pH dependence of the catalytic parameters is consistent with scheme B, which is valid for substrates containing a C-terminal carboxylate, since it accounts for the inability of the enzyme to accommodate the substrate when both the C-terminal carboxylate and Glu145 are deprotonated. With E65A+E145A the hydrolysis of FA-Phe-OGly-OH is better described by scheme C, which describes the hydrolysis of an ester substrate without a C-terminal carboxylate (FA-Ala-OBzl), consistent with the fact that in this enzyme the C-terminal recognition site has been removed. The pK_{ES} values for wild type and E65A+E145A were found to be 5.9 and 4.8, respectively.

With FA-Phe-Ala-OH and wild-type CPD-Y scheme B also applied. However, with E65A+E145A the pH dependence of k_{cat} for the hydrolysis of FA-Phe-Ala-OH is bell-shaped, and this may be described according to scheme D. The acidic part of the bell-shape is characterized by a pK_{EHS} = 3.9, whereas the basic part of the bell-shape is character-

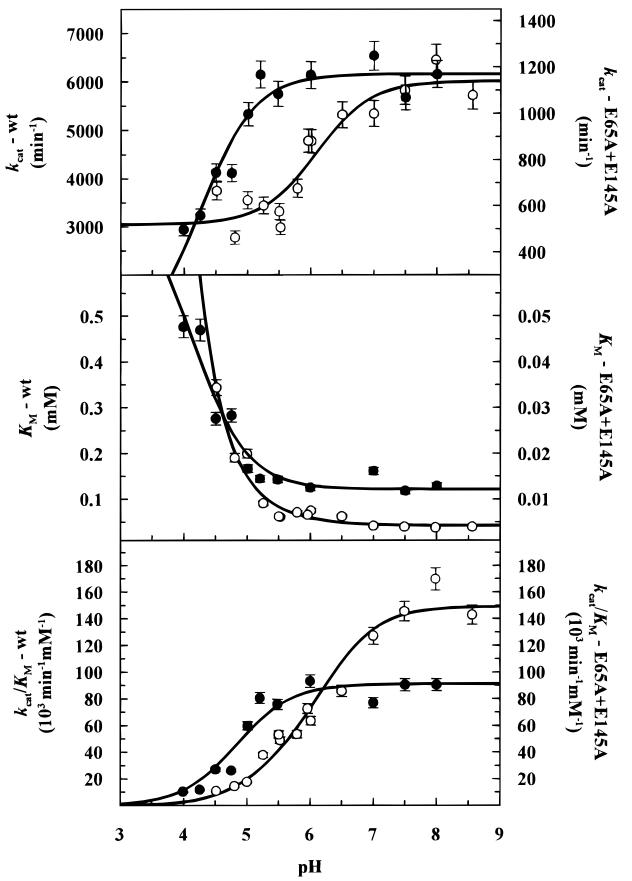

FIGURE 2: pH dependence of the catalytic parameters for the hydrolysis of FA-Ala-OBzl catalyzed by wild-type CPD-Y (○) and E65A+E145A (●). For CPD-Y the fitted parameters for the curve drawn for k_{cat} are pK_{ES} = 6.1 ± 0.2, $k_{cat,1}$ = 6000 ± 250 min^{-1}, and $k_{cat,2}$ = 3000 ± 250 min^{-1}; those for K_M are pK_{ES} = 6.1 ± 0.2, pK_{EH} = 5.2 ± 0.1, $K_{S,1}$ = 0.04 ± 0.01 mM, and $K_{S,2}$ = 0.06 ± 0.01 mM; and those for k_{cat}/K_M are pK_E = 6.3 ± 0.3, pK_{EH} = 5.2 ± 0.3, $k_{cat,1}/K_{S,1}$ = 150 000 ± 7 000 min^{-1} mM^{-1} and $k_{cat,2}/K_{S,2}$ = 42 000 ± 7 000 min^{-1} mM^{-1}. For E65A+E145A the fitted parameters for the curve drawn for k_{cat} are pK_{ES} = 4.3 ± 0.1, k_{cat} = 1170 ± 50; those for K_M are pK_E = 4.8 ± 0.2, pK_{ES} = 4.1 ± 0.2, $K_{S,1}$ = 0.01 ± 0.002 mM; and those for k_{cat}/K_M are pK_E = 4.8 ± 0.1 and $k_{cat,1}/K_{S,1}$ = 91 000 ± 5 000 min^{-1} mM^{-1}.

ized by pK_{ES} = 6.5. The reason for the drop in k_{cat} in the basic pH range is currently unknown.

A control experiment ensured that the shape of the pH dependence did not originate from the addition of exogenous acetate to the reaction mixture, i.e., the catalytic parameters determined in 20 mM MES, 100 mM NaCl, 1 mM EDTA, pH 5.5, and in 20 mM HAc, 100 mM NaCl, 1 mM EDTA, pH 5.5, were identical (data not shown).

Evaluation of the Members of the Catalytic Triad. The observed kinetic differences relative to the serine endopeptidases warranted an investigation of the importance of the individual members of the catalytic triad for catalysis. The mutant enzymes S146A, D338A, H397A, and H397G were prepared. With these mutant enzymes, which are expected to be virtually inactive, even small contaminating amounts of wild-type enzyme or an endogenous protease may contribute significantly to the activity. The Ser146→Ala substitution can only be accomplished by changing a single base pair. Accordingly, the possibility existed that the mutant enzyme S146A was contaminated with extremely small amounts of wild-type activity as the result of translational errors (Schimmel, 1989). The possibility of the presence of

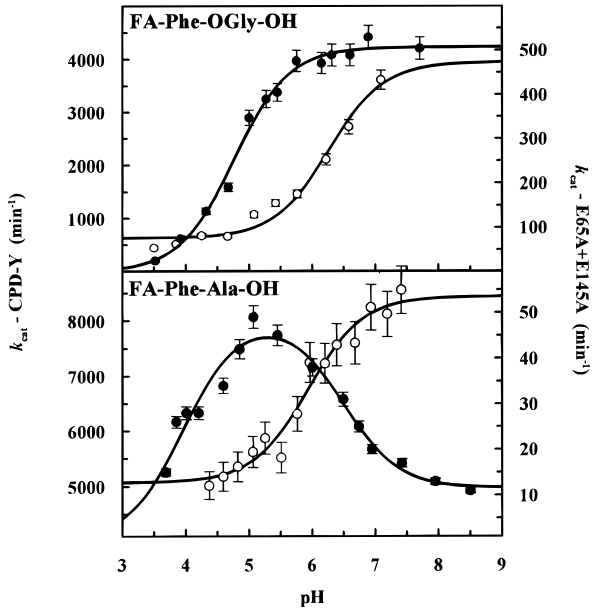

FIGURE 3: pH dependence of k_{cat} for the hydrolysis of FA-Phe-OGly-OH and FA-Phe-Ala-OH by wild-type CPD-Y (○) and E65A+E145A (●). For FA-Phe-OGly-OH the fitted parameters for the curve drawn for k_{cat} of CPD-Y are pK_{EHS} = 6.3 ± 0.1, $k_{cat,1}$ = 4000 ± 250 min^{-1}, and $k_{cat,2}$ = 630 ± 80 min^{-1}; those for (E65A+E145A) are pK_{ES} = 4.8 ± 0.04 and k_{cat} = 510 ± 10 min^{-1}. For FA-Phe-Ala-OH the fitted parameters for the curve drawn for k_{cat} of CPD-Y are pK_{EHS} = 5.9 ± 0.1, $k_{cat,1}$ = 8450 ± 150 min^{-1}, and $k_{cat,2}$ = 5100 ± 140 min^{-1}; those for (E65A+E145A) are pK_{ES} = 6.5 ± 0.1, pK_{EHS} = 4.0 ± 0.1, $k_{cat,1}$ = 12 ± 2 min^{-1}, and $k_{cat,2}$ = 49 ± 2 min^{-1}.

Table 1: Reaction of CPD-Y and Enzymes Altered in the Catalytic Triad with [^{3}H]DFP at pH 7.0

enzyme	cpm/μg of protein (average of three counts)	% reacted with [^{3}H]DFP
CPD-Y	363	100
S146A	19	5
H397A	21	6
H397G	13	4
bovine serum albumin	19	5

contamination with a serine type carboxypeptidase was investigated by treating S146A, as purified by affinity chromatography, with PMSF. This inhibitor acts by irreversibly acylating the catalytic serine, and therefore the activity of only the contaminating wild-type enzyme or other serine proteases, and not S146A, is expected to be inhibited by PMSF. Incubation with PMSF reduced the activity to 3% of that of the original preparation, and the residual activity was found to be resistant to any further treatment with PMSF. Excess reagent and PMSF-modified enzyme were subsequently removed by affinity chromatography, exploiting the fact that this derivative does not bind to the affinity resin (data not shown). The mutant enzymes H397A and H397G were prepared in the same way, but for D338A this question of contamination was not addressed due to the comparatively high activity of this enzyme, i.e., less than 1000-fold reduction in k_{cat}/K_M compared to the wild-type enzyme. Reaction of the S146A, H397A, and H397G mutant enzymes (not previously treated with PMSF) with [^{3}H]DFP caused very little incorporation of radioactivity, indicating that activity inhibited by the PMSF treatment does not originate from the mutant enzymes (Table 1).

Table 2: Catalytic Parameters for the Hydrolysis of FA-Phe-Leu-OH and FA-Phe-OMe at pH 6.5 and 7.5, Respectively, with Wild-Type CPD-Y, S146A, D338A, H397A, and H397G

	FA-Phe-Leu-OH				FA-Phe-OMe	
enzyme	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat}/K_M (mut)/ k_{cat}/K_M (wt)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat}/K_M (mut)/ k_{cat}/K_M (wt)
wild-type	4.9×10^3	0.021	2.3×10^5	1	2.8×10^4	1
S146A	7.5×10^{-4}	0.15	4.9×10^{-3}	2.1×10^{-8}	1.7×10^{-2}	6.0×10^{-7}
D338A	6.2	0.014	4.4×10^2	1.9×10^{-3}	1.1×10^2	3.9×10^{-3}
H397A	3.3×10^{-2}	0.052	0.63	2.7×10^{-6}	0.40	1.4×10^{-5}
H397G	1.3×10^{-2}	0.075	0.18	7.8×10^{-7}	0.26	9.2×10^{-6}

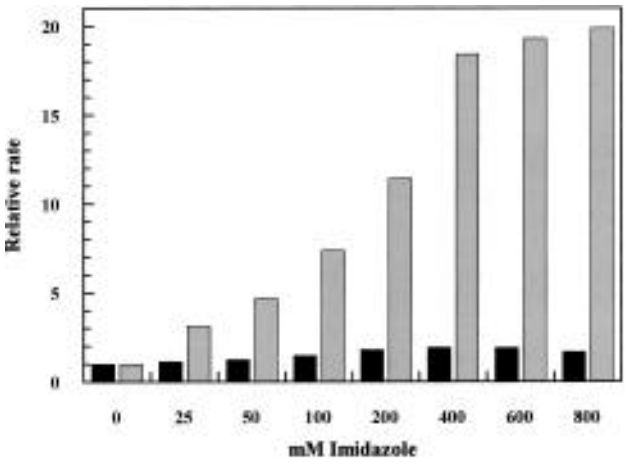

FIGURE 4: Reactivation of H397A (solid bars) and H397G (hatched bars) through addition of exogenous imidazole at pH 7.5.

The mutant enzymes were kinetically characterized using a peptide as well as an ester substrate. The results demonstrate that with S146A, H397A, and H397G the k_{cat} values for both ester and peptide hydrolysis were reduced 10^5-10^7-fold, whereas the K_M values were only slightly affected, confirming the importance of these residues in catalysis (Table 2). With D338A k_{cat} was only reduced by 10^3-fold.

Previously, Carter *et al*. (1991) and Perona *et al*. (1994) have described how lost functions in subtilisin and trypsin can be reconstituted through addition of exogenous compounds. Accordingly, it was tested whether the activity of H397A and H397G toward an ester substrate (FA-Phe-OMe) could be reconstituted by addition of imidazole (Figure 4). A 20-fold increase in activity could be obtained with the H397G enzyme, but the increase in activity was limited by inhibition of the enzyme at high imidazole concentrations. In contrast, a less than 2-fold activation is observed with H397A. The fact that the subtilisin BPN′ mutant H57A regains significant activity upon addition of imidazole (Carter *et al*., 1991) suggests a slightly different arrangement of the catalytic triad in CPD-Y.

Significance of C-Terminal Interaction in Catalysis. Previous studies have demonstrated that the interaction between the C-terminal carboxylate group of peptide substrates and the carboxylate recognition site of the enzyme is a condition for high k_{cat} values (Mortensen *et al*., 1994). In contrast, the hydrolysis of alkyl ester substrates is not dependent on such an interaction. This could be due either to the absence of a carboxylate group on such substrates or to the more labile ester not requiring such additional interaction for hydrolysis. To investigate this, substrates were synthesized which combine the two types of scissile bonds (ester/amide) with different leaving groups (presence/absence of a C-terminal carboxylate group).

Table 3: Kinetic Parameters for the CPD-Y-Catalyzed Hydrolysis of FA-Ala-X Substrates Containing Modified Leaving Group Moieties at pH 6.5

substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
FA-Ala-O-CH_2Ph	6900	0.07	102 000
FA-Ala-NH-CH_2-Ph	nd[a]	nd	2
FA-Ala-Phg-OH	6100	0.53	11 400
FA-Ala-NH-$(CH_2)_2$Ph	0.20	0.21	0.9
FA-Ala-Phe-OH	16 500	0.60	27 500

[a] Not determined due to low solubility of the substrate, i.e., $[S]_{max} < K_M$.

Scheme 1

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E{\bullet}P_2 + P_1 \xrightarrow{k_3} E + P_2$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad K_M = \frac{k_3 K_S}{k_2 + k_3} \quad \frac{k_{cat}}{K_M} = \frac{k_2}{K_S}$$

The CPD-Y-catalyzed hydrolysis of various substrates may be described by a simple ping-pong mechanism (Scheme 1) (Douglas *et al*., 1976), and consequently, k_{cat}/K_M equals k_2/K_S. Thus, k_{cat}/K_M is independent of whether acylation or deacylation is the rate-determining step of the reaction and, therefore, is a suitable parameter for comparing substrates containing different scissile bonds.

The catalytic parameters for the hydrolysis of these substrates were determined at pH 6.5, since at this pH CPD-Y exhibits maximum activity toward both charged and uncharged substrates (Table 3). FA-Ala- represents the acyl component in all substrates. With benzyl alcohol as leaving group the k_{cat}/K_M was 50 000-fold higher than with benzylamine, confirming the higher susceptibility of the ester bond as compared with the amide bond. Addition of an α-carboxylate group to the leaving group of the amide substrate (L-phenylglycine) increases k_{cat}/K_M 5700-fold, the beneficial effect being due to an interaction with the carboxylate recognition site on the enzyme. This is also illustrated by the 30 000-fold difference in k_{cat}/K_M values obtained with the H_2N-CH_2-CH_2-Ph/phenylalanine pair of leaving groups, the difference being due to an effect on k_{cat} exclusively (Table 3).

A similar experiment was performed with a series of substrates containing FA-Phe- as the acyl component and ethanol, ethylamine, glycolic acid, and glycine as leaving groups (Table 4). As expected, a large difference in k_{cat}/K_M was observed with the leaving group pair ethylamine/ethanol, i.e., k_{cat}/K_M for FA-Phe-NHEt was 0.3 min^{-1} mM^{-1} as compared with 54 900 min^{-1} mM^{-1} for FA-Phe-OEt. In

Table 4: Kinetic Parameters for Hydrolysis of FA-Phe-X Substrates (X = -NH-CH_2-COO^-, -O-CH_2-COO^-, -O-CH_2-CH_3, and -NH-CH_2-CH_3) Catalyzed by Wild-Type CPD-Y and E65A+E145A at pH 5.5

	CPD-Y			E65A+E145A		
substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
FA-Phe-Gly-OH	3700	0.410	9000	4.8	2.2	2.2
FA-Phe-NHEt	nd[a]	nd	0.3	nd	nd	nd
FA-Phe-OGly-OH	1450	0.160	8700	285	0.067	4500
FA-Phe-OEt	4100	0.075	54 900	290	0.028	10 200

[a] Not determined due to low solubility of the substrate, i.e., $[S]_{max} < K_M$.

contrast, with a substrate pair containing a C-terminal carboxylate the k_{cat}/K_M values are almost identical, i.e., 9000 min^{-1} mM^{-1} for FA-Phe-Gly-OH as compared with 8700 min^{-1} mM^{-1} for FA-Phe-OGly-OH. With the same substrate pair and E65A+E145A the following values were found, i.e., 2.2 min^{-1} mM^{-1} for FA-Phe-Gly-OH as compared with 4500 min^{-1} mM^{-1} for FA-Phe-OGly-OH. To further examine the influence of a C-terminal carboxylate on ester hydrolysis, the pH dependencies of the catalytic parameters for the CPD-Y-catalyzed hydrolysis of FA-Phe-OEt and FA-Phe-OGly-OH were determined (Figure 5). The k_{cat} values were found to be 3-fold higher with ethanol (HOEt) as leaving group than with glycolic acid (HOGly-OH), containing the additional α-carboxylate group, throughout the pH range investigated. The pH dependence of K_M for FA-Phe-OEt resembles that for FA-Ala-OBzl (scheme A) although the increase in K_M at low pH is less pronounced. In contrast, the pH dependence of K_M for FA-Phe-OGly-OH is consistent with the pK values, pK_E = 5.7 and pK_{EH} = 5.0, which correspond to the pK values found for the hydrolysis of peptide substrates (scheme B), e.g., for FA-Phe-Ala-OH the pK values are pK_E = 5.9 and pK_{EH} = 5.0 (data not shown). Another significant feature is that the K_M values for FA-Phe-OGly-OH are smaller than those for FA-Phe-OEt at low pH, presumably due to a beneficial effect of the C-terminal carboxylate on substrate binding at low pH, in a manner similar to the peptide substrates. As a result, the k_{cat}/K_M values for the hydrolysis of FA-Phe-OGly-OH exceeds those for the hydrolysis of FA-Phe-OEt at pH values below pH 4.5.

Previous investigations on the pH dependence of k_{cat} for the hydrolysis of peptides substrates have shown k_{cat} to decrease somewhat at high pH with a pK value of 7−8 (Fukuda & Kunugi, 1985; Breddam, 1985). In the present investigation the pH dependence was determined for FA-Phe-Leu-OH, which allows determination of the catalytic parameters at high pH. With FA-Phe-Leu-OH it was observed that k_{cat} decreases with a pK value of 8.4 (see Figure 6) and K_M increases in agreement with the observations made with other peptide substrates. The pK value for the titration of Glu145, which is responsible for this increase in K_M, was found to be 5.3 ± 0.2. Similar values were found for the substrates FA-Phe-Ala-OH and FA-Phe-Gly-OH (data not shown).

The C-terminal binding site of CPD-Y is composed of Asn51, Glu65, and Glu145. An alignment of CPD-Y with other serine carboxypeptidases shows that Glu65 and Glu145 are conserved throughout the family of serine carboxypeptidases whereas Asn51 may be replaced by residues having a similar hydrogen bonding potential, i.e., Thr and Gln

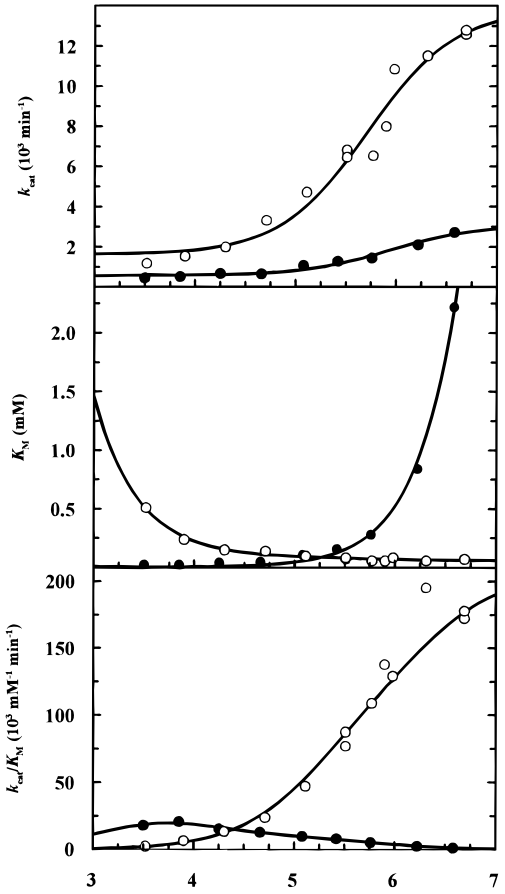

FIGURE 5: pH dependence of the catalytic parameters for the hydrolysis of FA-Phe-OEt (○) and FA-Phe-OGly-OH (●) catalyzed by wild-type CPD-Y. For FA-Phe-OEt the fitted parameters for the curve drawn for k_{cat} are pK_{ES} = 5.7 ± 0.1, $k_{cat,1}$ = 14 000 ± 750 min^{-1}, and $k_{cat,2}$ = 1600 ± 450 min^{-1}; those for K_M are pK_E = 5.9 ± 0.1, pK_{EH} = 4.2 ± 0.1, pK_{ES} = 5.7 ± 0.1, and $K_{S,2}$ = 0.09 ± 0.01 mM; and those for k_{cat}/K_M are pK_E = 6.3 ± 0.2, pK_{EH} = 5.2 ± 0.1, $k_{cat,1}/K_{S,1}$ = 110 000 ± 5 000 min^{-1} mM^{-1}, and $k_{cat,2}/K_{S,2}$ = 210 000 ± 7 000 min^{-1} mM^{-1}. For FA-Phe-OGly-OH the fitted parameters for the curve drawn for k_{cat} are pK_{EHS} = 5.9 ± 0.2, $k_{cat,1}$ = 3100 ± 250 min^{-1}, and $k_{cat,2}$ = 570 ± 75 min^{-1}; those for K_M are pK_E = 5.6 ± 0.1, pK_{EH} = 5.2 ± 0.2, and $K_{S,1}$ = 0.24 ± 0.06; and those for k_{cat}/K_M are pK_E = 5.9 ± 0.5, pK_{EH} = 4.5 ± 0.6, $k_{cat,1}/K_{S,1}$ = 8600 ± 5800 min^{-1} mM^{-1}, and $k_{cat,2}/K_{S,2}$ = 20 500 ± 2 000 min^{-1} mM^{-1}.

(Olesen & Breddam, 1995; Mortensen *et al*., 1994). Asn51 and Glu145 are of importance for the hydrolysis of peptide substrates whereas the main function of Glu65 apparently is to maintain the correct orientaiton of Asn51 and Glu145 (Mortensen *et al*., 1994). With FA-Ala-Phe-OH as substrate, k_{cat}/K_M is reduced from 27 500 to 216 min^{-1} mM^{-1} for E65A+E145A. This reduction in catalytic efficiency originates from a large decrease in k_{cat}, from 16 500 to 67 min^{-1}, whereas K_M is reduced from 0.60 to 0.31 mM. This is in

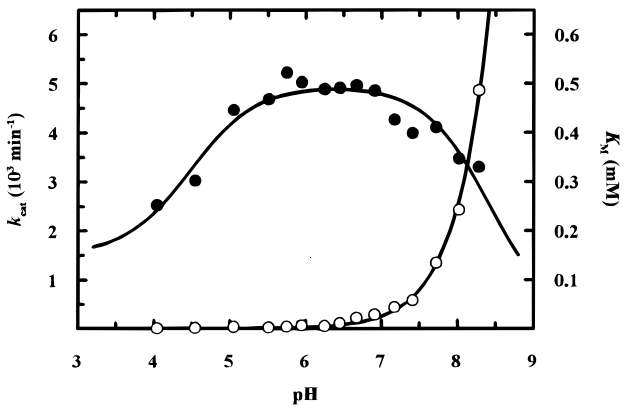

FIGURE 6: pH dependence of k_{cat} (●) and K_M (○) for the wild-type CPD-Y-catalyzed hydrolysis of FA-Phe-Leu-OH. For k_{cat} the parameters describing the pH-dependence are $pK_{EHS} = 4.5 \pm 0.3$, $pK_{ES} = 8.4 \pm 0.1$, $k_{cat,1} = 5000 \pm 150$ min^{-1}, and $k_{cat,2} = 1500 \pm 550$ min^{-1}; those for K_M are $pK_{EHS} = 4.5 \pm 0.3$, $pK_E = 5.3 \pm 0.1$, $pK_{EH} = 3.9 \pm 0.2$, $pK_S = 3.44$, and $K_{S,1} = 0.6 \pm 0.05$ μM.

agreement with C-terminal interactions only being necessary for transition state stabilization in the hydrolysis of peptides.

DISCUSSION

The roles of the individual members of the catalytic triad in serine proteases have previously been thoroughly investigated with subtilisin BPN′ (Carter & Wells, 1988). With CPD-Y the reduction in k_{cat} when the catalytic residues are substituted for alanines is similar to what was observed with subtilisin BPN′. Furthermore, the effect on k_{cat} was larger with the peptide substrate than with the ester substrate in agreement with the ester bond being more labile than the peptide bond, which also corresponds to the findings made with subtilisin. Thus, based on these observations, combined with the fact that the S146A and H397A enzymes do not react with DFP, it is concluded that the serine carboxypeptidases share the basic catalytic features with the serine endopeptidases. However, the results with addition of exogenous imidazole to the His397 mutants suggest some steric differences in the arrangement of the catalytic triad between subtilisin BPN′ and CPD-Y.

Serine proteases usually hydrolyze ester bonds at higher rates than corresponding peptide bonds, reflecting the high energy associated with the breakdown of the partially double-bonded nature of the peptide bond (Fersht, 1971), and CPD-Y is not an exception. With CPD-Y the substitution of an ester bond for a peptide bond is associated with a significant increase in the transition state activation energy, i.e., $\Delta\Delta G_T^{\ddagger}$(FA-Ala-OCH$_2$Ph→FA-Ala-NHCH$_2$Ph) = 26.9 kJ mol^{-1}. Addition of a carboxylate group to a peptide substrate greatly enhances the rate of peptide bond hydrolysis, i.e., $\Delta\Delta G_T^{\ddagger}$(FA-Ala-NHCH$_2$Ph→FA-Ala-Phg-OH) = −21.4 kJ, $\Delta\Delta G_T^{\ddagger}$(FA-Ala-OCH$_2$Ph→FA-Ala-Phg-OH) = 5.4 kJ, and $\Delta\Delta G_T^{\ddagger}$(FA-Ala-NH(CH$_2$)$_2$Ph→FA-Ala-Phe-OH) = −25.6 kJ mol^{-1}. If these values are compensated for the fact Glu145, at pH 6.5, is partially deprotonated and thus, unable to interact with the carboxylate of the substrate the following $\Delta\Delta G_T^{\ddagger}$ values are found: $\Delta\Delta G_T^{\ddagger}$(FA-Ala-NHCH$_2$Ph→FA-Ala-Phg-OH) = −28 kJ mol^{-1}, $\Delta G_T^{\ddagger}$(FA-Ala-OCH$_2$Ph→FA-Ala-Phg-OH) = −2 kJ mol^{-1}, and $\Delta\Delta G_T^{\ddagger}$(FA-Ala-NH(CH$_2$)$_2$-Ph→FA-Ala-Phe-OH) = −33 kJ mol^{-1}. Since $\Delta\Delta G_T^{\ddagger}$(FA-Ala-OCH$_2$Ph→FA-Ala-Phg-OH) is −2 kJ mol^{-1} as compared with 26.9 kJ mol^{-1} for $\Delta\Delta G_T^{\ddagger}$(FA-Ala-OCH$_2$Ph→FA-Ala-NHCH$_2$Ph), it can be concluded that the addition of a carboxylate group to the amine leaving group overcomes the major part of the energy barrier for peptide bond hydrolysis. The $\Delta\Delta G_T^{\ddagger}$ values resulting from removal of the α-carboxylate group from the phenylalanine or phenylglycine leaving groups correspond to the energy of formation of two strong hydrogen bonds between a charged and an uncharged hydrogen bonding pair (Fersht *et al*., 1985; Bone & Agard, 1991).

Comparison of k_{cat}/K_M values for the substrate pairs FA-Phe-OGly-OH/FA-Phe-Gly-OH and FA-Phe-OEt/FA-Phe-NHEt, provides an additional piece of information. With the substrate pair containing a C-terminal carboxylate the $\Delta\Delta G_T^{\ddagger}$(FA-Phe-Gly-OH→FA-Phe-OGly-OH) = −0.084 kJ mol^{-1}, whereas for the pair without this moiety, $\Delta\Delta G_T^{\ddagger}$(FA-Phe-NHEt→FA-Phe-OEt) = −31.0 kJ mol^{-1}. Thus, there is no significant difference in the energy required to hydrolyze an ester and a peptide bond when both substrates possess a C-terminal carboxylate group.

The C-terminal interaction may also be interrupted by altering the enzyme. In E65A+E145A the hydrogen bonding potential at position 145 has been removed, and with FA-Ala-Phe-OH as substrate $\Delta\Delta G_T^{\ddagger}$(wt→E65A+E145A) = 12 kJ mol^{-1}. With E65A+E145A and the substrate pair containing a C-terminal carboxylate group, $\Delta\Delta G_T^{\ddagger}$(FA-Phe-Gly-OH→FA-Phe-OGly-OH) = −18.9 kJ mol^{-1} as compared with the −0.084 kJ mol^{-1} for the wild-type enzyme. Thus, the C-terminal interactions are much more important for hydrolysis of a peptide substrate than for an ester substrate. Furthermore, the loss of transition state activation energy due to substitution of Glu145 for Ala is approximately half of that found for complete loss of C-terminal interaction. This is consistent with the fact that Glu145 is not the only hydrogen bond donor but functions together with Asn51 (Mortensen *et al*., 1994).

Bullock *et al*. (1994) and Christensen (1994) have explained the high activity of CPD-Y at low pH by Glu145 or the α-carboxylate group of the substrate serving as "proton sink" for the essential His397 belonging to the catalytic triad. Previously, it has been demonstrated that, in the ground state, Glu145 is protonated and the α-carboxylate group of the substrate is deprotonated (Mortensen *et al*., 1994). If another proton is delivered to this system in the transition state, e.g., from His397, it would no longer be possible to form the predicted strong hydrogen bond between a charged and an uncharged partner (see above) but only a weaker one between two uncharged partners, resulting in a loss of binding energy equal to 8−9 kJ mol^{-1} (Fersht *et al*., 1985; Bone & Agard, 1991). Therefore, such a scheme for deprotonation of His397 seems unlikely. Furthermore, if Glu145 should serve as a "proton sink" it would be expected that the substitution of Glu145 for an alalnine would adversely affect the ability of the enzyme to catalyze hydrolysis reactions at acidic pH. However, the mutant enzyme E65A+E145A, with no acidic groups within the active site apart from Asp338 belonging to the catalytic triad, hydrolyzes FA-Ala-OBzl with higher k_{cat}/K_M than the wild-type enzyme at pH values below 6 (Figure 2). Furthermore, the observation that scheme C (Figure 1) accounts for E65A+E145A-catalyzed hydrolysis of both the tested ester substrates, combined with the high similarity of the pH dependencies observed with wild-type enzyme and E65A+E145A, indicates that neither the

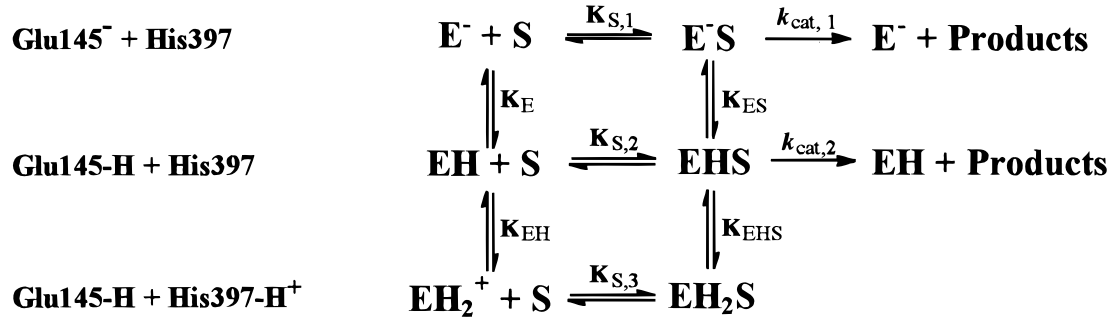

FIGURE 7: Scheme for the pH dependence containing two additional reaction pathways, symbolized with the parameters $K_{S,3}$ and pK_{EHS}, which cannot be validated on the basis of the data presented in the present manuscript but are necessary to explain a system in which His397 is highly perturbed in the ES complex.

presence of a C-terminal carboxylate on the substrate nor Glu145 can serve to explain the high k_{cat} values observed at low pH.

For the uncharged substrate, k_{cat}/K_M approaches zero at low pH but k_{cat} remains high. This unusual pH dependence of k_{cat} may be explained by perturbation of the pK_a of the catalytic histidine, in the enzyme substrate complex. This may be achieved either by amino acid residues within the enzyme or by moieties within the substrate affecting the pK_a value of His397 in the enzyme substrate complex.

His397 may be perturbed within the enzyme substrate complex by amino acid residues within the active site, reducing its pK_a value below the pH range investigated, i.e., below pH 3.5. The scheme outlined in Figure 7 describes the situation in which the titration of His397 in the enzyme substrate complex is characterized by pK_{EHS} rather than pK_{ES} and the substrate is capable of binding to the enzyme when both Glu145 and His397 are protonated, in contrast to the model described in scheme A (Figure 1) (Christensen, 1994). Such a scheme is consistent with scheme C describing the pH dependence for E65A+E145A, in which the uncharged substrate is capable of binding to the enzyme when His397 is protonated.

Accordingly, the increase in k_{cat} with pH must be due to something other than the catalytic His residue. Thus, the value of pK_{EHS} may reflect the pK value of His397 when Glu145 is protonated. As pH is increased Glu145 becomes deprotonated (pK_a = 5.3) stabilizing the protonated form of His397. Thus, the charged form of Glu145 counteracts the deprotonation of His397 by increasing its pK_a in the ES complex from below 3.5 to around 6. If this is the case, the argument of Bullock *et al*. (1994) against a highly perturbed catalytic His might not reflect the situation in which the pK value of His397 is highly perturbed, since it represents k_{cat} at a pH value above pK_{ES}.

Glu145, in its protonated state, is essential in transition state activation of peptide substrates through interaction with the C-terminal carboxylate group. The model presented (Figure 7) predicts that for such substrates k_{cat} will decrease with the pK_a value of Glu145 (5.3), since deprotonation prior to acylation will prevent formation of the acyl-enzyme. A decrease in k_{cat} in the basic pH range is observed with the substrate FA-Phe-Leu-OH, but with a pK_a value of 8.4 (Figure 6). Similar findings has previously been reported for CPD-Y and other serine carboxylpeptidases with pK_a values of 7−8 (Fukuda & Kunugi, 1985; Breddam, 1985). The unexpectedly high pK value is probably due to perturbation of the pK_a value of Glu145 due to the hydrogen bond with the C-terminal carboxylate of the substrate in the Michaelis complex. Thus, the pK_{ES} value must represent the titration of the catalytic His in the acyl-enzyme, which would explain the similarity in the pH profiles for k_{cat} observed with charged and uncharged substrates.

Atypically low pK_a values of His residues have previously been observed, e.g., in triose phosphate isomerase His95 has a pK_a of 4.5 due to interactions with an α-helix microdipole (Knowles, 1991) and in a derivative of papain resembling the acyl-enzyme the catalytic histidine (His159) has a pK value of around 3.5 (Johnson *et al*., 1981). In CPD-Y, no positively charged residues which could destabilize the protonated His397 are found (Endrizzi *et al*., 1994), and thus the reason for a low value could be the small accessible surface of His397 (7.6 Å^2) as compared to that of the corresponding histidines in the serine endopeptidases. This is supported by the observation that the increase in the accessible surface area of His397 from 7.6 Å^2 in the wild-type enzyme to 21 Å^2 in E65A+E145A is associated with an increase of the pK value for the His residue in the ES complex, i.e., from below 3.5 to 4.3.

Bullock *et al*. (1994) have argued against such a scheme, stating that the rate of proton transfer from the catalytic serine to the histidine constitute an upper limit for k_{cat}. The free energy barrier of this transfer is determined by the difference in the pK_a values of the catalytic residues, i.e., $\Delta G^{\ddagger} = -RT\,\Delta\ln(K_a)$. A p$K_a$ value of 13−15.7 for the β-OH of serine has been estimated (Bullock *et al*., 1994; Bender *et al*., 1975; Golubev *et al*., 1994). With CPD-Y, the highest k_{cat} values thus far observed are around 15 000 min^{-1}. Using the Arrhenius equation ($k = A\exp(-\Delta G^{\ddagger}/RT)$) ($A$ is a pre-exponential factor equal to 10^{13}) and a pK_a of 13−15.7 for Ser146 (see above) leads to minimum values of 2.4−5.1 as the pK_a of His397. On the basis of these considerations a low pK_a value of His397 cannot be excluded. Thus, the high activity of CPD-Y and other serine carboxypeptidases at acidic pH may be explained by an unusually acidic pK_a value of His397, which, apart from Glu65, Glu145, and Asp338, is the only ionizable residue within the active site of CPD-Y.

Perturbation of His397 may also be accomplished by moieties within the substrate, capable of accepting a proton from the catalytic histidine during catalysis. According to the conventional mechanism, a proton is transferred from the essential serine to the histidine and the scissile peptide bond becomes polarized concomitantly with the nucleophilic attack. As a consequence, the pK_a value of the amide nitrogen increases such that it subsequently can receive this

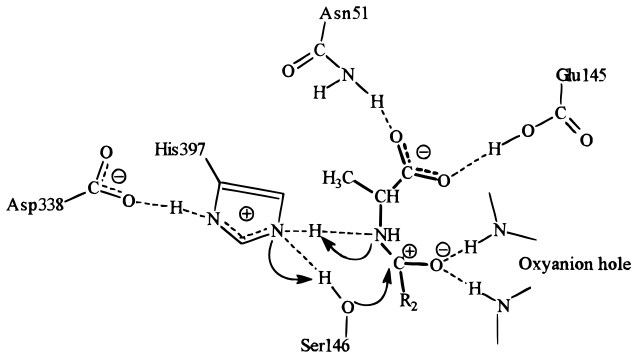

FIGURE 8: Possible mechanism for acylation of CPD-Y with a peptide substrate at low pH.

proton. If the peptide bond of the substrate can be polarized prior to the nucleophilic attack by the catalytic serine it is conceivable that it may be able to activate a positively charged histidine by accepting a proton, hence perturbing the pK_a of the essential histidine. If so, the enzyme may function at pH values significantly below the pK_a of the essential histidine. The serine endopeptidases do not possess a mechanism for polarizing the scissile peptide bond, but the serine carboxypeptidases interact with the α-carboxylate group of the substrate by means of two hydrogen bond donors (Asn51 and Glu145). This interaction facilitates the formation of the transition state, possibly by distortion of the peptide bond leading to the required polarization, hence increasing the pK_a of the amide nitrogen (Figure 8). The required distortion of the peptide bond may be obtained by stabilization of a specific tautomeric form of the substrate. A number of enzyme−substrate interactions may contribute to this, e.g., the oxyanion $-O^-$ with the oxyanion hole (approximately 40 kJ mol^{-1}) (Fersht *et al*., 1985; Wells *et al*., 1986; Daggett *et al*., 1991), the C-terminal carboxylate with Asn51 and Glu145 (30−35 kJ mol^{-1}) as well as a hydrogen bond between the protonated form of the catalytic histidine, and the amide nitrogen of the substrate (approximately 5−8 kJ mol^{-1}) (Fersht *et al*., 1985; Bone & Agard, 1991). Combined, these interactions may stabilize one tautomeric form by 75−85 kJ mol^{-1}, enough to compensate for most of the resonance energy [approximately 74 kJ mol^{-1} according to Fersht (1971)] lost upon stabilization of one tautomer. In addition to this, the interactions between the positively charged carbon atom of the amide bond and the partially negatively charged γ-O of Ser146 become stronger and favor the transfer of the proton from the Ser146 to His397 as well as formation of the tetrahedral intermediate. The three-dimensional structure of carboxypeptidase WII co-crystallized with arginine shows a hydrogen bond between the catalytic histidine and the α-amino group of an arginine occupying the position of the leaving group (Liao *et al*., 1992), supporting the assumption that the nitrogen of the leaving group could have such a function in catalysis. In addition to this, recent computational studies of water-assisted amide hydrolysis have suggested that the protonation of the amide nitrogen is an important step in amide hydrolysis (Antonczak *et al*., 1994). With the ester substrates the mechanism might resemble that outlined for the peptide substrates. However, the energy required to distort the ester bond is significantly lower eliminating the need for additional C-terminal interactions, i.e., the binding energy from hydrophobic interactions and interactions with the tetrahedral intermediate are sufficient to bring about the reaction. Furthermore, the hydrogen bonding network suggested in the mechanism at low pH (Figure 8) is similar to that suggested by Lang (1964) describing the acid-catalyzed ester hydrolysis.

In summary, the high activity of CPD-Y at low pH may be due to a highly perturbed catalytic histidine in the enzyme substrate complex. Two different models for the mechanism of perturbation have been discussed. One describes how the enzyme may function at low pH by direct perturbation of the catalytic histidine by an apolar environment and how the pK_a value of this residue may be increased by the deprotonation of Glu145. The other describes how serine carboxypeptidases may transfer a proton directly from the essential His to the scissile nitrogen during acylation at low pH (Figure 8). On the basis of the current data it is not possible to discriminate between the two models.

ACKNOWLEDGMENT

We thank Dr. S. James Remington, University of Oregon, for helpful and critical discussion.

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BI952758E