Site-Directed Mutagenesis on (Serine) Carboxypeptidase Y. A Hydrogen Bond Network Stabilizes the Transition State by Interaction with the C-Terminal Carboxylate Group of the Substrate†

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ABSTRACT: The three-dimensional structure of (serine) carboxypeptidase Y suggests that the side chains of Trp49, Asn51, Glu65, and Glu145 could be involved in the recognition of the C-terminal carboxylate group of peptide substrates. The mutations Trp49 → Phe; Asn51 → Ala, Asp, Glu, Gln, Ser, or Thr; Glu65 → Ala; and Glu145 → Ala, Asp, Asn, Gln, or Ser have been performed. Enzymes with Ala at these positions were also produced as double and triple mutations. These mutations have only little effect on the esterase activity of the enzyme, consistent with the absence of a hydrogen bond acceptor in the P_1' position of such substrates. On the other hand, removal of the hydrogen-bonding capacity by incorporation of Ala at any of these four positions results in reduced peptidase activity, in particular when Asn51 and Glu145 are replaced. The results are consistent with Trp49 and Glu65 orienting Asn51 and Glu145 by hydrogen bonds, such that these can function as hydrogen bond donors (Glu145 only in its protonated carboxylic acid form) with the C-terminal α-carboxylate group of the peptide substrate as acceptor. However, it appears that strong interactions are formed only in the transition state since the combined removal of Asn51 and Glu145 reduces k_{cat} about 100-fold and leaves K_M practically unchanged. The results obtained with enzymes in which Asn51 or Glu145 has been replaced with other residues possessing the capacity to donate a hydrogen bond demonstrate that there is no flexibility with respect to the nature of the hydrogen bond donor at position 145, whereas enzymes with Gln, Ser, or Thr at position 51 exhibit much higher activity than N51A, although none of them reaches the wild-type level. With carboxypeptidase Y as well as other serine carboxypeptidases the binding of peptide substrates in the ground state (K_M) is adversely affected by an increase in pH. It is shown that deprotonation of a single ionizable group with a pK_a of 4.3 on the enzyme is responsible for this pH effect. The results show that the group involved is either Glu65 or Glu145, the latter being the more probable. The effect of this ionization on K_M is explained by charge repulsion between the carboxylate group of the substrate and that of Glu145, hence preventing substrate from binding.

Carboxypeptidase Y (CPD-Y)[1] is an exopeptidase located in the vacuole of *Saccharomyces cerevisiae* (Breddam, 1986). The enzyme belongs to the group of serine carboxypeptidases, and studies using chemical modifications (Hayashi et al., 1973) and site-directed mutagenesis (Bech & Breddam, 1989) have identified the catalytically essential serine and histidine residues. The gene (*PRC1*) coding for CPD-Y has been cloned and sequenced (Stevens et al., 1986; Valls et al., 1987). The three-dimensional structure of CPD-Y was recently determined (J. Endrizzi, K. Breddam, and S. J. Remington, in preparation), and the structure of the homologous carboxypeptidase II from wheat (CPD-WII) is also known (Liao & Remington, 1990; Liao et al., 1992). Examination of the topology of the tertiary structures of both enzymes has classified them as belonging to a group of enzymes having a newly identified enzyme fold, the α/β hydrolase fold, which also includes acetylcholinesterase from *Torpedo californica*, dienelactone hydrolase from *Pseudomonas* sp. B13, haloalkane dehalogenase from *Xanthobacter autotrophicus*, and lipase from *Geotrichum candidum* (Ollis et al., 1992).

A prominent feature of a carboxypeptidase must be the ability to recognize and bind the carboxylate terminus of a peptide substrate (Riordan, 1973). In (metallo)carboxypeptidase A (CPD-A), binding of the C-terminal carboxylate group of peptide substrates involves the formation of a salt bridge with an arginine residue situated in a dead-end pocket. Additionally, the carboxylate group accepts two hydrogen bonds from the side chains of a tyrosine residue and an asparagine residue (Christianson & Lipscomb, 1987; Gardell et al., 1987). In the serine carboxypeptidases the mechanism of carboxylate recognition has not been identified. The influence of pH on the binding of peptides suggested that a histidine residue had a function analogous to that of the arginine residue in CPD-A (Breddam, 1986). However, examination of the two three-dimensional structures now available revealed no positively charged amino acid residues in or close to the regions predicted to interact with the C-terminus of peptide substrates. Thus, it would appear that only hydrogen bonds are involved and that the pH dependence of peptide binding needs a different explanation.

The structure of CPD-WII complexed with arginine (a reaction product) shows an interaction between the carboxylate group of the free amino acid and the side chains of Asn51 and Glu145 as well as the backbone amide of Gly52. Furthermore, these amino acid residues are situated in regions which are conserved among serine carboxypeptidases (Sørensen et al., 1986, 1987; Breddam et al., 1987), and thus, they are good candidates for the hydrogen bond donors involved in the

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[1] Abbreviations: CPD, carboxypeptidase; FA, furylacryloyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine.

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binding of the C-terminus of peptide substrates. Asn51 and Glu145 are part of a hydrogen-bonding network also including Trp49 and Glu65. We have investigated the enzymatic consequences of replacing Trp49, Asn51, Glu65, and Glu145 by site-directed mutagenesis.

MATERIALS AND METHODS

Materials. The plasmid pUC19 (Yanisch-Perron et al., 1985), the shuttle vector pRA21 (Olesen & Kielland-Brandt, 1993) harboring a GAL4 promoter controlled structural gene for CPD-Y, the pRA21-derived plasmid pRA21Δ*Bam* (R. Andersen, unpublished) containing a deletion of a 1112-bp *Bam*HI–*Bam*HI fragment, the yeast strain W2579 (Nielsen et al., 1990), and the *Escherichia coli* strain $DH_5\alpha$ (Hanahan 1985) were from in-house collections. The synthetic oligonucleotides were synthesized on an Applied Biosystems 380 DNA synthesizer. Restriction endonucleases and T4 ligase were from Boehringer Mannheim, Germany. *Taq* DNA polymerase was from Perkin-Elmer, Norwalk, CT. Bicine, Mes, and Hepes were from Sigma, St. Louis, MO. Sephacryl-S300 was from Pharmacia-LKB Biotechnology, Sweden, and DEAE-Fractogel 650 was from Merck, Germany. GYBS-Sepharose was prepared as described by Johansen et al. (1976). FA-Phe-Ala-OH, FA-Phe-Gly-OH, FA-Phe-Leu-OH, FA-Phe-Val-OH, and FA-Ala-OBzl were synthesized as previously described (Breddam, 1984). FA-Phe-OMe was from Bachem, Switzerland.

Site-Directed Mutagenesis. A plasmid, pUC-α30, was constructed by inserting a 1112-bp *Bam*HI fragment of the *PRC1* gene containing the coding region for all amino acid residues involved in the formation of the active site into the unique *Bam*HI site in the polylinker of pUC19. The orientation of the fragment was opposite that of the *lacZ* gene. pUC-α30 contains unique *Bst*XI, *Eco*RI, *Nae*I, and *Sma*I restriction sites which can be used in the cloning and mutagenesis procedures described below. The mutations W49F and N51X were made by using the polymerase chain reaction (PCR) (Innis et al., 1990) in a Perkin-Elmer Cetus DNA thermal cycler using a Gene Amp kit (Perkin-Elmer Cetus) on pUC-α30 with GTTTCTGTCCTTGTGAGACAAAATTTCAGA (oligo wt1) and with GGATCCGGTCATCCTTTTCTTGAACGGG (oligo W49F), GCAAAGGATCCGGTCATCCTTTGGTTGGCAGGGGGCCA (oligo N51A), GCAAAGGATCCGGTCATCCTTTGGTTGGACGGGGGCCA (oligo N51D), GGATCCGGTCATCCTTTGGTTGGAAGGGGGCCA (oligo N51E), GGATCCGGTCATCCTTTGGTTGCAAGGGGGT (oligo N51Q), GGATCCGGTCATCCTTTGGTTGAGTGGGGGT (oligo N51S), or GGATCCGGTCATCCTTTGGTTGACTGGGGGT (oligo N51T). Underlined nucleotides are different from wild type. Cleavage with *Bst*XI allowed insertion of the PCR fragment into a *Sma*I–*Bst*XI vector fragment of pUC-α30. The mutations E145X were made by PCR with GCAAGGCGATTAAGTTGGGT(oligo pUC19 sq1) and GGCGTAGGAAGCCCCAGCGAT (oligo E145A), GGCGTAGGAATCCCCAGCGAT (oligo E145D), GGCGTAGGAATTCCCAGCGAT (oligo E145D), GGCGTAGGATTGCCCAGCGAT (oligo E145Q), or GGCGTAGGATGACCCAGCGAT (oligo E145S) on pUC-α30. Cleavage of the PCR fragment with *Eco*RI allowed introduction into a *Nae*I–*Eco*RI vector fragment of pUC-α30. The mutations E65A and N51A+E65A were produced by fusion of two overlapping PCR fragments using either pUC-α30 or pUC-α30-N51A as template. Fragment 1 was generated with CTGTTCTTTGCATTAGGACCC (oligo E65A) and oligo wt1, and fragment 2 was generated with oligo pUC19 sq1 and oligo E145A. An additional PCR reaction was performed on the fused fragment with oligo pUC19 sq1 and oligo wt1. The resultant fragment was cut with *Eco*RI and *Bst*XI, thus removing the unwanted mutation at position 145, and ligated into a pUC-α30 vector fragment cut with the same restriction enzymes. N51A+E145A, E65A+E145A, and N51A+E65A+E145A were made by properly combining the above listed mutations, exploiting the *Eco*RI site in the polylinker and exploiting that *Bst*XI cleaves between position 65 and 145. The mutated sequence was introduced into the *PRC1* gene by transferring the mutated 1112-bp *Bam*HI fragment into the vector pRA21Δ*Bam*. The fragment inserted into pUC-α30 was controlled for the absence of any nonsilent secondary mutation by sequencing using the *Taq* Dye-Dideoxy terminator cycle sequencing kit and the Model 373A DNA-sequencing system from Applied Biosystems, Foster City, CA.

Purification of Mutant CPD-Y. Mutant enzymes were purified from a 1-L culture grown under the conditions previously described (Nielsen et al., 1990). Growth media containing secreted mutant enzyme were adjusted to pH 4.4 with concentrated acetic acid and then directly applied to the GYBS-Sepharose affinity column according to the procedure of Johansen et al. (1976). N51A, which did not bind effectively to GYBS-Sepharose, was subjected to diafiltration against 10 mM NaH_2PO_4, pH 7.0, using a Pellicon system (Millipore) and then purified by ion-exchange chromatography on a DEAE-Fractogel 650 column (2.6 × 6 cm). The column was washed until A_{280} was below 0.01, and elution was accomplished with a linear salt gradient from 0 to 0.5 M NaCl in 10 mM NaH_2PO_4, pH 7.0. The eluate was concentrated using equipment from Amicon and applied to a Sephacryl-S300 column (1 × 100 cm) equilibrated with 50 mM NaH_2PO_4 and 0.1 M NaCl, pH 7.0. Fractions with constant specific activity were pooled, concentrated, and dialyzed against water. All enzyme preparations were stored frozen in water at −18 °C.

Characterization of CPD-Y and Mutant CPD-Y. The purity of the mutant enzymes was ascertained by SDS–PAGE on 12.5% homogeneous gels using the PhastSystem from Pharmacia. The concentration of CPD-Y mutants was determined spectrophotometrically using A_{280} (1 mg/mL = 1.48) (Johansen et al., 1976). Activity toward peptide substrates was determined in 50 mM Mes and 1 mM EDTA, pH 6.5, and activity toward ester substrates was determined in 50 mM Hepes, 1 mM EDTA, and 2.5% (v/v) CH_3OH, pH 7.5 (Breddam, 1984). The hydrolysis of FA-Phe-Gly-OH with wild-type enzyme, N51Q, N51S, N51T, and E145A was also measured at pH 5.5 in 50 mM Mes and 1 mM EDTA. The influence of ionic strength on the catalytic parameters was determined in 20 mM Mes and 1 mM EDTA, pH 6.5, in the absence of NaCl and in the presence of 0.5 M NaCl. For the pH dependencies of the kinetic constants, activity measurements were performed in 20 mM buffer, 0.1 M NaCl, and 1 mM EDTA using the following buffers: formic acid, pH 3.00–4.25; acetic acid, pH 4.25–5.50; Mes, pH 5.25–6.50; Hepes, pH 6.50–8.00; and Bicine, pH 8.00–8.30. The kinetic parameters for hydrolysis of substrates as well as the pK_a values describing the pH profiles of the catalytic parameters were determined using the nonlinear regression data analysis program Grafit 3.01 (Erithacus Software). Hydrolysis rates were determined spectrophotometrically at 329–353 nm, depending on the concentration range of the substrate, and 25 °C using a Perkin-Elmer λ9 spectrophotometer. Precau-

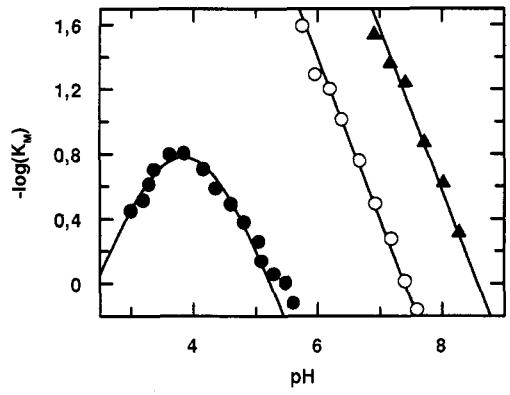


FIGURE 1: pH dependence of $-\log K_M$ for wild-type CPD-Y for the hydrolysis of FA-Phe-Gly-OH (●), FA-Phe-Ala-OH (○), and FA-Phe-Leu-OH (▲). The $-\log K_M$ values obtained for hydrolysis of FA-Phe-Ala-OH and FA-Phe-Leu-OH were fitted to $-\log K_{M,obs} = -pH + b$, where b is a constant, and the values obtained for hydrolysis of FA-Phe-Gly-OH were fitted to $-\log K_{M,obs} = \log((K^{-1}_{M,max} \exp_{10}(pH - 3.44)/(\exp_{10}(pH - 3.44) + 1)) - (K^{-1}_{M,max} \exp_{10}(pH - pK_{a2})/(\exp_{10}(pH - pK_{a2}) + 1)))$; 3.44 represents the pK_a of the acidic leg and pK_{a2} represents the pK_a for the basic leg of the bell curve.

tions were taken to ensure that the substrate solutions were not subjected to light (Kanstrup & Buchardt, 1991).

The pK_a of FA-Phe-Gly-OH was determined by proton NMR of 0.2 mM FA-Phe-Gly-OH and 100 mM NaCl at 25 °C with pH values in the range 2.0–5.5 using a 500-MHz Bruker spectrometer. The spectral width was 7462.69 Hz. Trace amounts of acetone were used as an internal standard.

RESULTS

Ionic Interactions Are Not Important for Substrate Binding. Crystallographic and solution studies of complexes of CPD-A with inhibitors or products have revealed that the C-terminal carboxylate group forms a salt linkage with the side chain of Arg145 (Riordan, 1973; Christianson & Libscomb, 1987), and as a consequence addition of salt adversely affects the rate of hydrolysis (Williams & Auld, 1986). To examine the possibility that substrate binding in CPD-Y could also be dependent on ionic interactions, the kinetic parameters for the hydrolysis of the peptide substrate FA-Phe-Ala-OH in the presence and absence of 0.5 M NaCl were determined. The following values in the absence (presence) of salt were found: $k_{cat} = 8700 \pm 130$ min^{-1} (8000 ± 110 min^{-1}), and $K_M = 0.15 \pm 0.006$ mM (0.13 ± 0.005 mM). Thus, addition of salt has very little influence on the kinetic parameters for the CPD-Y-catalyzed hydrolysis of peptide substrates, and ionic interactions therefore contribute very little to substrate binding.

CPD-Y Recognizes the Carboxylate Form of the Peptide Substrate. Serine carboxypeptidases bind peptide substrates most efficiently at low pH values, and most of them are very active at pH values as low as 3.0 (Breddam, 1986). This raises the question of which form of the C-terminus of the peptide substrates binds to the enzyme, the carboxylic acid form or the carboxylate form. In serine carboxypeptidases $K_M \sim K_s$ (Breddam, 1986), and consequently, the influence of pH on K_M was determined. To secure reliable determinations in the acidic pH range, where peptides bind very efficiently to CPD-Y, the substrate FA-Phe-Gly-OH, characterized by fairly inefficient binding to the enzyme at neutral pH, was chosen. The results were plotted as $-\log K_M$ versus pH (Figure 1), and they fitted a bell shape, consistent with pK_a values of 3.58 ± 0.14 for the acidic leg and 4.10 ± 0.12 for the basic leg. The pK_a values describe ionization of groups either in the free enzyme or in the free substrate according to Dixon (1953). Thus, one of these values could correspond

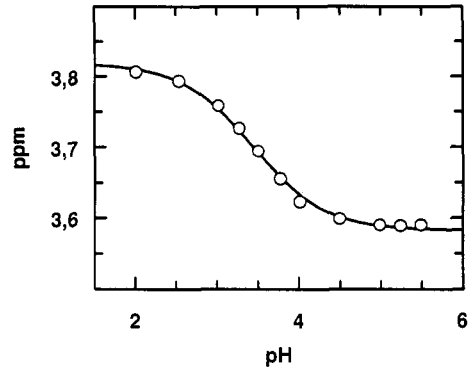


FIGURE 2: Dependence of the chemical shift of the α-carbon protons on the degree of ionization of the C-terminal carboxylate group of FA-Phe-Gly-OH. The curve is the best fit to ppm(obs) = $(\alpha + \beta \exp_{10}(pH - pK_a))/(\exp_{10}(pH - pK_a) + 1)$, where α and β are the limits in chemical shifts at low and high pH, respectively.

to the dissociation of the substrate, and consequently, the pK_a of FA-Phe-Gly-OH was determined by proton NMR, exploiting the dependence of the chemical shift of the C-terminal α-carbon protons on the degree of ionization of the C-terminus. The chemical shifts could be fitted to a pK_a of 3.44 ± 0.01 (Figure 2). This value corresponds closely to that of the acidic leg of the $-\log K_M$ curve, and we therefore conclude that this part of the curve is determined by the state of ionization of the substrate and that CPD-Y preferentially binds the charged form of the substrate. Setting the pK_a of the acidic leg of the bell shape to the pK_a of the substrate, a new fit yields a pK_a of 4.24 ± 0.04 for the basic leg of the curve (Figure 1), suggesting that deprotonation of an acidic side chain on the enzyme adversely affects peptide binding.

The pH dependence of K_M in CPD-Y-catalyzed peptide hydrolysis was further investigated using the substrates FA-Phe-Ala-OH and FA-Phe-Leu-OH, and plots of $-\log K_M$ against pH yielded straight lines with slopes of -0.93 ± 0.03 and -0.90 ± 0.05, respectively. These slopes are close to -1, which indicates that K_M is dependent on a single ionizable group (Dixon, 1953), and values for K_M were therefore fitted to such a theoretical curve (Figure 1). With FA-Phe-Ala-OH and FA-Phe-Leu-OH no points below pH 5.7 and 6.5, respectively, could be obtained due to very low values of K_M, and consequently, the maximum value of $-\log K_M$ could not be determined.

Production and Purification of CPD-Y Mutants at Positions 49, 51, 65, and 145. The three-dimensional structures of CPD-Y and CPD-WII (Liao et al., 1992) have shown that the side chains of Trp49, Asn51, Glu65, and Glu145, all situated in the S_1' [2] binding subsite of the enzyme, are linked by a chain of hydrogen bonds. To test the role of these residues in the binding of the C-terminal carboxylate of the substrate, we constructed the following series of mutants: W49F, N51A, N51D, N51E, N51Q, N51S, N51T, E65A, E145A, E145D, E145N, E145Q, E145S, N51A+E65A, N51A+E145A, E65A+E145A, and N51A+E65A+E145A. All mutations resulted in active enzymes which were secreted into the media due to a mutation in the vacuolar protein sorting pathway in the strain of *S. cerevisiae* chosen as expression system (Rothman et al., 1986; Robinson et al., 1988; Nielsen et al., 1990). The secreted enzymes were produced in amounts of 20–100 mg/L, except W49F, which was only produced in amounts of 2 mg/L. Enzymes that did not contain the

[2] The binding site notation is that of Schechter and Berger (1967). Accordingly, P_n denotes a substrate position, and S_n denotes the corresponding enzyme binding subsite.

Table 1: Influence of Mutational Alterations at Positions 49, 51, 65, and 145 of CPD-Y on Its Esterase Activity

	FA-Phe-OMe			FA-Ala-OBzl		
	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})
wild type[a]	1100	0.39	28 000	9100	0.054	170 000
W49F	3900	0.31	13 000	5100	0.062[b]	83 000
N51A	4200	0.15	28 000	3700	<0.014	>250 000
N51D	4500	0.16	28 000	3600	<0.015	>240 000
N51E	2200	0.92[b]	2200	3900	0.023[b]	170 000
N51Q	3300[b]	1.1[b]	3000	5100	0.046[b]	110 000
N51S	6900	0.17	41 000	7700	0.022[b]	350 000[b]
N51T	2600	0.51[b]	5100	8300	0.035[b]	240 000[b]
E65A	8900	0.73[b]	12 000	14 000[b]	0.16[b]	87 000
E145A	5300	0.33	16 000	4400	0.022	200 000
E145D	4000	0.37	11 000	2400	<0.015	>160 000
E145N	2000	0.29	7100	1700	<0.015	>110 000
E145Q	710	0.89	800	32	0.029[b]	1100
E145S	2400	0.10	24 000	2200	<0.015	>150 000
E51A+E65A	11 000	0.39[b]	28 000	6200	0.032	200 000
E51A+E145A	3800	0.15	26 000	4400	<0.015	>290 000
E65A+E145A	200	0.16[b]	1200	1800	0.016	110 000
E51A+E65A+E145A	69	0.44[b]	160	950	0.18	5300

[a] Data obtained with the wild-type enzyme are from Breddam (1984). [b] Standard deviation ≤ ±20%; all other kinetic parameters were determined with a standard deviation ≤ ±10%.

mutation N51A could be purified by an affinity chromatographic procedure. N51A did not bind to the affinity resin, and consequently, N51A, N51A+E65A, N51A+E145A, and N51A+E65A+E145A were purified by ion-exchange chromatography and gel filtration chromatography. All enzymes were homogeneous as determined by SDS–PAGE.

Influence of the Mutational Replacements on Ester Hydrolysis. The mutant enzymes with replacements at positions 49, 51, 65, and 145 were investigated for their ability to catalyze the hydrolysis of the ester substrates FA-Phe-OMe and FA-Ala-OBzl (Table 1). The hydrolysis of FA-Phe-OMe and FA-Ala-OBzl was predicted not to be drastically affected by the mutations since the alcohol moiety of ester substrates has no capacity to form a hydrogen bond. However, a mutation resulting in a small conformational change in the enzyme could affect the neighboring catalytic apparatus, leading to reduced activity. With most enzymes k_{cat}/K_M values for the hydrolysis of FA-Phe-OMe and FA-Ala-OBzl were similar to the values obtained with the wild-type enzyme. With N51E, N51Q, and N51T, k_{cat}/K_M values for the hydrolysis of FA-Phe-OMe were reduced by a factor 13, 9 and 5, respectively, whereas k_{cat}/K_M for the hydrolysis of FA-Ala-OBzl was similar to the value obtained with the wild-type enzyme. The adverse effect on the hydrolysis of FA-Phe-OMe could be due to loss of hydrophobic interactions between the enzyme and the -OMe group, an effect which may be covered by the much tighter binding of the -OBzl group. k_{cat}/K_M for the hydrolysis of both FA-Phe-OMe and FA-Ala-OBzl was only significantly reduced with E145Q and the triple mutant N51A+E65A+E145A, indicating that in these enzymes a conformational change accompanying these mutations had impaired the catalytic apparatus.

Function of Hydrogen Bonds in Peptide Hydrolysis. The function of Trp49, Asn51, Glu65, and Glu145 in the binding of the C-terminal carboxylate group of peptide substrates was investigated by characterization of the mutants with Phe at position 49 and Ala at the other positions, hence eliminating the hydrogen-bonding capacity. The kinetic constants k_{cat} and K_M for the hydrolysis of a series of FA-Phe-X-OH substrates (X = Gly, Ala, Val, and Leu) were determined (Table 2).

Wild-type CPD-Y exhibits a preference for hydrophobic amino acid residues in the P_1' position, i.e., Leu > Val > Ala > Gly. All the mutants listed in Table 2 hydrolyzed all four peptide substrates with reduced k_{cat}/K_M values. For each mutant the reductions were only slightly dependent on the substrate, and as a consequence the P_1' preference was maintained in all the mutants described. However, the extent of the reduction was dependent on the nature of the mutation performed. The k_{cat}/K_M for the hydrolysis of FA-Phe-Gly-OH was drastically reduced by incorporation of Ala at position 51 or 145: with N51A and E145A the k_{cat}/K_M values were 1% and 0.4%, respectively, of the values obtained with the wild-type enzyme, compared with 16% with E65A and 8% with W49F. Similar differences were obtained with the other FA-Phe-X-OH substrates with X = Ala, Val, and Leu. Thus, the binding of the C-terminal carboxylate group of peptide substrates to the enzyme is primarily due to Asn51 and Glu145 and to a much lesser extent Glu65 and Trp49. Compared to the values obtained with N51A and E145A, the k_{cat}/K_M values with the double mutants N51A+E65A, N51A+E145A, and E65A+E145A were only slightly lower.

A more complicated picture was seen when the individual kinetic parameters k_{cat} and K_M were investigated. With the double mutants E65A+E145A and N51A+E145A the reductions in k_{cat}/K_M were almost exclusively due to a more than 100-fold reduction in k_{cat}. With W49F the reductions were primarily due to an increase in K_M. With the other mutants, i.e., N51A, E65A, E145A, and N51A+E65A, the reductions in k_{cat}/K_M were due to effects on both k_{cat} and K_M. With N51A atypical values were obtained for the hydrolysis of FA-Phe-Gly-OH: a 500-fold reduced k_{cat} and a 6-fold reduced K_M were observed. Unfortunately, K_M was too high to be determined with E145A for the hydrolysis of FA-Phe-Gly-OH. However, exploiting that this enzyme (see below) binds peptide substrates more tightly at low pH allowed the determination of k_{cat} and K_M at pH 5.5. Thus, it could be established that with E145A, as was the case with N51A, the reduction in k_{cat}/K_M for the hydrolysis of FA-Phe-Gly-OH is mainly due to a large reduction in k_{cat}.

The pH Dependence of Peptide Binding Is Determined by Either Glu65 or Glu145. To test the hypothesis that the deprotonation of either Glu65 or Glu145 could be responsible for the increase in K_M at pH > 4 (Liao et al., 1992), the mutant enzymes E65A and E145A were studied. With E145A the influence of pH on K_M for the hydrolysis of the substrates

Table 2: Removal of the Hydrogen-Bonding Capacity in the Carboxylate Binding Site: Kinetic Parameters for Hydrolysis of Peptide Substrates with Wild-Type CPD-Y, W49F, N51A, E65A, E145A, N51A+E65A, N51A+E145A, and E65A+E145A

enzyme	substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
wild type[a]	FA-Phe-Leu-OH	4900	0.021	230 000
	FA-Phe-Val-OH	6500	0.047	140 000
	FA-Phe-Ala-OH	6700	0.18	38 000
	FA-Phe-Gly-OH	5800	5.4	1100
	FA-Phe-Gly-OH[b]	3700	0.41	9000
W49F	FA-Phe-Leu-OH	2300	0.065	35 000
	FA-Phe-Val-OH	4500	0.24	19 000
	FA-Phe-Ala-OH	4300	1.1	4000
	FA-Phe-Gly-OH	nd[c]	nd	93
N51A	FA-Phe-Leu-OH	1100	0.14[d]	7900
	FA-Phe-Val-OH	2500	0.52	4900
	FA-Phe-Ala-OH	1200	1.7[d]	670
	FA-Phe-Gly-OH	10	0.84[d]	12[d]
E65A	FA-Phe-Leu-OH	940	0.032	29 000
	FA-Phe-Val-OH	720	0.070	10 000
	FA-Phe-Ala-OH	7600	0.61	12 000
	FA-Phe-Gly-OH	nd	nd	180
E145A	FA-Phe-Leu-OH	830	0.052	16 000
	FA-Phe-Val-OH	1400	0.19	7800
	FA-Phe-Ala-OH	1100	1.2	910
	FA-Phe-Gly-OH	nd	nd	4.0
	FA-Phe-Gly-OH[b]	20	1.1	18
N51A+E65A	FA-Phe-Leu-OH	900	0.44	2000
	FA-Phe-Val-OH	1900	1.7	1100
	FA-Phe-Ala-OH	nd	nd	140
	FA-Phe-Gly-OH	nd	nd	0.61
N51A+E145A	FA-Phe-Leu-OH	39	0.062	640
	FA-Phe-Val-OH	41	0.21	200
	FA-Phe-Ala-OH	21	0.30	71
	FA-Phe-Gly-OH	14	3.0[d]	5.0
E65A+E145A	FA-Phe-Leu-OH	25	0.035	710
	FA-Phe-Val-OH	24	0.12	220
	FA-Phe-Ala-OH	30	0.32	92
	FA-Phe-Gly-OH	4.8	2.2[d]	2.2

[a] Data obtained with the wild-type enzyme, except for the hydrolysis of FA-Phe-Gly-OH[b], are from Breddam (1984). [b] Data obtained at pH 5.5, all other kinetic parameters were determined at pH 6.5. [c] nd, not determined due to too high K_M values. [d] Standard deviation $\leq \pm 20\%$; all other kinetic parameters were determined with a standard deviation $\leq \pm 10\%$.

FA-Phe-Ala-OH and FA-Phe-Leu-OH was determined and plotted as $-\log K_M$ (Figure 3, panel A). The tight binding of FA-Phe-Leu-OH to the enzyme prevented determination of the K_M values at pH values below 6.5. Within the studied pH range the results fitted a straight line with a slope of −0.87 ± 0.04, and the curve represents the best fit to one with a slope of −1. FA-Phe-Ala-OH binds less tightly to the enzyme, and therefore the minimum value could be determined. The results were fitted to a titration curve with a maximum at low pH and a minimum approaching 0 at high pH and plotted as $-\log K_M$ versus pH (Figure 3, panel A). The plateau at low pH allows estimation of the pK_a of the free enzyme as 5.23 ± 0.08, which is 1 pH unit higher than the corresponding pK_a of the wild-type enzyme. At high pH the curve for $-\log K_M$ approaches a straight line with a slope of −1, parallel to the curve for FA-Phe-Leu-OH. Thus, with both the wild-type enzyme and E145A the plots of $-\log K_M$ versus pH yield straight lines with slopes close to −1, and accordingly, K_M increases with pH under the influence of the deprotonation of a single ionizable group. pH profiles were also determined for E65A, and the plots of $-\log K_M$ are shown in Figure 3, panel B. The pH profile for $-\log K_M$ for the substrate FA-Phe-Leu-OH is likewise described by a straight line with a slope of −0.80 ± 0.047, which is fairly close to −1, and therefore, K_M for the hydrolysis of FA-Phe-Leu-OH with E65A also depends on one ionizable residue. For the substrate FA-Phe-Ala-OH a plateau is reached at low pH indicating a pK_a of 4.92 ± 0.08 for the free enzyme.

The observation that increasing pH has an adverse effect on the binding of peptide substrates to E65A and E145A, similar to that observed with the wild-type enzyme, seems to contradict a model involving Glu65 or Glu145 as the residue responsible for the pH dependence of K_M. However, the possibility existed that in E145A a negative charge could develop on Glu65 with an adverse effect on binding as a consequence, and likewise on Glu145 in E65A. To test this possibility, the pH profile of K_M for the hydrolysis of FA-Phe-Ala-OH was determined with the double mutant E65A+E145A. With this enzyme K_M only increased 3-fold in the pH range 3.7–7.9, and accordingly, the plot of $-\log K_M$ in Figure 3, panel C, is almost flat. The results fit a simple titration curve with $K_M(max) = 0.35 \pm 0.01$ and $K_M(min) = 0.11 \pm 0.02$. The pK_a for the ES complex (K_M) is 5.40 ± 0.16, and the pK_a for the free enzyme ($1/K_M$) is 4.70 ± 0.14. These values reflect ionization of a group on the enzyme that influences the binding of peptide substrates but is not essential. The identity of this group has not been further investigated. Thus, either Glu65 or Glu145 is responsible for the adverse effects on substrate binding at pH > 4.

The influence of the Asn51 → Ala mutation on the pK_a of the glutamic acid responsible for the pH dependence of K_M was investigated using N51A and N51A+E145A and the substrate FA-Phe-Ala-OH. As expected, the profiles dis-

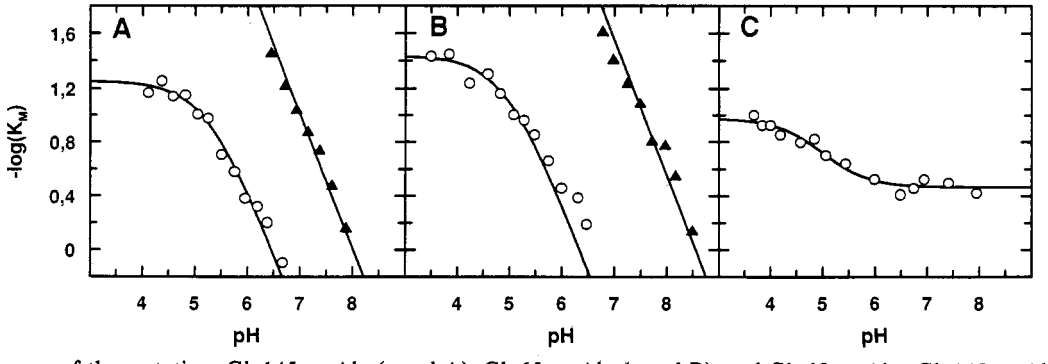


FIGURE 3: Influence of the mutations Glu145 → Ala (panel A), Glu65 → Ala (panel B), and Glu65 → Ala+Glu145 → Ala (panel C) on the pH dependence of $-\log K_M$ for the hydrolysis of FA-Phe-Ala-OH (○) and FA-Phe-Leu-OH (▲). The data for hydrolysis of FA-Phe-Ala-OH with E145A and E65A were fitted to $-\log K_{M,obs} = \log(K^{-1}_{M,max} - K^{-1}_{M,max} \exp_{10}(pH - pK_a)/(\exp_{10}(pH - pK_a) + 1))$ (panels A and B) and with E65A+E145A to $-\log K_{M,obs} = \log(K^{-1}_{M,max} + K^{-1}_{M,min} \exp_{10}(pH - pK_a)/(\exp_{10}(pH - pK_a) + 1)$ (panel C). The data for hydrolysis of FA-Phe-Leu-OH were fitted to $-\log K_{M,obs} = -pH + b$, where b is a constant (panels A and B).

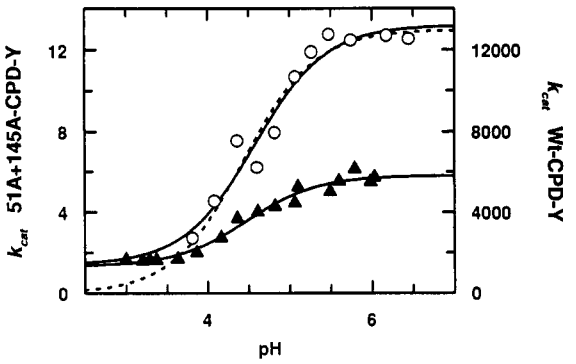


FIGURE 4: pH dependence of k_{cat} (min^{-1}) for wild-type CPD-Y (▲) and N51A+E145A (○) catalyzed hydrolysis of FA-Phe-Gly-OH. Solid lines represent best fits to $k_{cat,obs} = (k_{cat,min} + k_{cat,max} \exp_{10}(pH - pK_a))/(\exp_{10}(pH - pK_a) + 1)$ (wild-type CPD-Y and N51A+E145A), and the dashed line represents the best fit to $k_{cat,obs} = k_{cat,max} \exp_{10}(pH - pK_a)/(\exp_{10}(pH - pK_a) + 1)$ (N51A+E145A only).

played the same shape as that of the wild-type enzyme (see below). The pK_a values for the free forms of these enzymes were 4.93 ± 0.08 and 5.28 ± 0.07, respectively. Thus, removal of Asn51 does not eliminate the influence of pH on K_M.

Binding of the C-Terminal Carboxylate Group Does Not Perturb the pH Dependence of k_{cat}. The influence of pH on k_{cat} for the hydrolysis of FA-Phe-Gly-OH with the wild-type enzyme was investigated. The results could be fitted to a simple titration curve with a pK_a of 4.47 ± 0.08, a minimum of 1300 min^{-1} at low pH, and a maximum of 5800 min^{-1} at high pH (Figure 4). Thus, k_{cat} only increased by a factor of 4.5 within the pH range 3.0–6.5. Similar curves were obtained with the substrates FA-Phe-Ala-OH and FA-Phe-Leu-OH (data not shown). The influence of the carboxylate binding site on this pH profile was investigated with N51A+E145A since the groups primarily involved in the hydrogen-bonding to the carboxylate group have been eliminated in this double mutant (Figure 4). Unfortunately, no k_{cat} values could be determined below pH 3.75 due to increasing K_M values and reduced substrate solubility. The available data therefore do not reveal whether k_{cat} is approaching 0 or a value > 0 (wild-type situation) at low pH. Consequently, the results (open symbols) have been fitted to two curves, one with $k_{cat} = 0$ (dashed curve) and one with $k_{cat} > 0$ (solid curve) as the lower limit. The corresponding pK_a values were estimated as 4.42 ± 0.08 (dashed line) and 4.56 ± 0.17 (solid line), respectively. Both values are similar to that obtained with the wild-type enzyme. The pH profiles for k_{cat} were also determined with the single mutants N51A and E145A using FA-Phe-Ala-OH as substrate and the shape of the curves were almost identical to those obtained with the wild-type enzyme (data not shown). Thus, it appears that the binding site for the C-terminal carboxylate group does not contribute to the low pK_a of the pH profile of k_{cat}. It is noteworthy, however, that the difference in k_{cat} between the acidic form and the basic form of the enzymes is larger for N51A+E145A compared to the wild-type enzyme.

Flexibility of the Hydrogen Bond Donors at Positions 51 and 145. The results presented above suggest that both Asn51 and Glu145 (in its protonated state) function in the recognition of the C-terminal carboxylate group of peptide substrates. We have investigated the tolerance to other hydrogen bond donors by producing a series of mutants with other amino acids with this capacity, i.e., Glu, Asp, Ser, Thr, Asn, or Gln, introduced at these positions. The kinetic parameters for the hydrolysis of the peptide substrates FA-Phe-X-OH (X = Gly, Ala, Val, or Leu) were determined (Table 3). With N51Q, N51S, and N51T the k_{cat}/K_M values obtained at pH 6.5 were consistently higher than the corresponding values obtained with N51A regardless of the size of the side chain of the leaving group. In contrast, N51D hydrolyzed these substrates with k_{cat}/K_M values similar to, and in the case of N51E even lower than, the values obtained with N51A. All the N51X mutants hydrolyzed the peptide substrates investigated with k_{cat}/K_M values lower than the corresponding wild-type values. However, with N51D, N51E, N51S, and N51T the reductions in k_{cat}/K_M were most pronounced for substrates with a small side chain, whereas with N51Q the reduction in k_{cat}/K_M was almost independent of the size of the side chain. As a consequence, the highest k_{cat}/K_M for the hydrolysis of FA-Phe-Gly-OH is obtained with N51Q, whereas for the hydrolysis of FA-Phe-Leu-OH it is obtained with N51S. With N51D and N51E, k_{cat} decreased and K_M increased relative to the wild-type values but were close to those obtained with N51A. With N51Q, N51S, and N51T, K_M values were similar to the wild-type value for the hydrolysis of FA-Phe-Leu-OH, but with decreasing volume of the side chain of the leaving group

Table 3: Flexibility of the Hydrogen Bond Donor at Positions 51 and 145: Kinetic Parameters for Hydrolysis of Peptide Substrates with N51D, N51E, N51Q, N51S, N51T, E145D, E145N, E145Q, and E145S

enzyme	substrate	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
N51D	FA-Phe-Leu-OH	760	0.076	10 000
	FA-Phe-Val-OH	2900	0.58[a]	5000
	FA-Phe-Ala-OH	nd	nd	840
	FA-Phe-Gly-OH	nd	nd	8.5
N51E	FA-Phe-Leu-OH	580	0.16[a]	3700
	FA-Phe-Val-OH	930	0.27	3400
	FA-Phe-Ala-OH	310[a]	1.5[a]	210
	FA-Phe-Gly-OH	nd[b]	nd	3.6
N51Q	FA-Phe-Leu-OH	910	0.028	32 000
	FA-Phe-Val-OH	1500	0.085	17 000
	FA-Phe-Ala-OH	6300	0.64[a]	9800
	FA-Phe-Gly-OH	nd	nd	230
	FA-Phe-Gly-OH[c]	2800	3.6[a]	780
N51S	FA-Phe-Leu-OH	4200	0.036	120 000
	FA-Phe-Val-OH	8100	0.17[a]	48 000
	FA-Phe-Ala-OH	5900	0.60[a]	9800
	FA-Phe-Gly-OH	nd	nd	160
	FA-Phe-Gly-OH[c]	1800	2.5	720
N51T	FA-Phe-Leu-OH	2000	0.031	64 000
	FA-Phe-Val-OH	5300	0.16	34 000
	FA-Phe-Ala-OH	7500	1.1[a]	7000
	FA-Phe-Gly-OH	nd	nd	69
	FA-Phe-Gly-OH[c]	2400	4.5[a]	530
E145D	FA-Phe-Leu-OH	150	0.17	870
	FA-Phe-Val-OH	200	0.68	290
	FA-Phe-Ala-OH	nd	nd	77
	FA-Phe-Gly-OH	nd	nd	0.28
E145N	FA-Phe-Leu-OH	56	0.081	700
	FA-Phe-Val-OH	49	0.068[a]	720
	FA-Phe-Ala-OH	56	1.1[a]	52
	FA-Phe-Gly-OH	nd	nd	0.42
E145Q	FA-Phe-Leu-OH	10	0.017[a]	590
	FA-Phe-Val-OH	12	0.036	340
	FA-Phe-Ala-OH	120	0.49	240
	FA-Phe-Gly-OH	nd	nd	26
E145S	FA-Phe-Leu-OH	410	0.062	6600
	FA-Phe-Val-OH	390	0.089	4400
	FA-Phe-Ala-OH	170	0.59	280
	FA-Phe-Gly-OH	nd	nd	0.88

[a] Indicates a standard deviation ≤ ±20%. Otherwise the kinetic parameters were determined with a standard deviation ≤ ±10%. [b] nd, not determined due to too high K_M values. [c] Data obtained at pH 5.5; all other kinetic parameters were determined at pH 6.5.

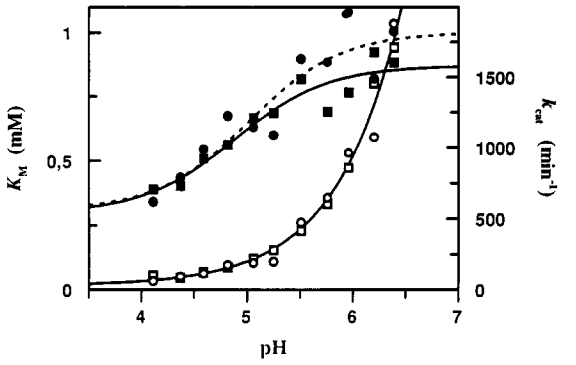


FIGURE 5: pH dependence of k_{cat} and K_M with N51A (■, □) and N51D (●, ○) for the hydrolysis of FA-Phe-Ala-OH. For k_{cat} (closed symbols) the solid line (N51A) and the dashed line (N51D) represent best fits to $k_{cat,obs} = (k_{cat,min} + k_{cat,max}(\exp_{10}(pH - pK_a)))/(\exp_{10}(pH - pK_a) + 1)$. For K_M (open symbols) the solid line represents best fit to $K_{M,obs} = K_{M,min}(\exp_{10}(pH - pK_a) + 1)$, where pK_a is the pK_a of the free form of N51A obtained by analyzing the inverse plot.

increased K_M values were observed. With N51Q, k_{cat} for the hydrolysis of FA-Phe-Leu-OH was reduced to 20%, but with this enzyme hydrolysis of substrates with smaller volumes of the side chain on the leaving group is accompanied by an increase in k_{cat}, approaching the wild-type level. With N51S and N51T all peptide substrates were hydrolyzed with k_{cat} values close to those obtained with the wild-type enzyme.

The observation that the kinetic parameters obtained with N51D were almost identical to those obtained with N51A, despite the widely different structures of the incorporated amino acids, was further investigated. The pH dependency of k_{cat} and K_M for the hydrolysis of FA-Phe-Ala-OH with N51D was determined. The pH profiles were compared to the corresponding pH profiles obtained with N51A, and the curves obtained for the two enzymes are nearly indistinguishable in the pH range 4.0–6.5 (Figure 5).

The catalytic parameters for the E145D-, E145N-, E145Q-, and E145S-catalyzed hydrolysis of FA-Phe-X-OH (X = Gly, Ala, Val, or Leu) were determined (Table 3). These results demonstrate that introduction of other hydrogen bond donors at position 145 causes reduction of k_{cat}/K_M to a level comparable to or lower than those obtained with E145A. The large reductions in k_{cat}/K_M were found mainly to be due to reductions in k_{cat}.

DISCUSSION

The three-dimensional structure of CPD-Y identifies a hydrogen-bonding network consisting of Trp49, Asn51, Glu65, and Glu145 within the region of the enzyme where the C-terminal carboxylate group of peptide substrates binds (Figure 6). These amino acid residues are also found in CPD-WII (Liao et al., 1992), and in the complex of this enzyme with arginine the carboxylate group interacts with Asn51 and Glu145, suggesting that these two amino acid residues together could function in binding of the C-terminal carboxylate group of substrates as well. However, the presence of two glutamic acid residues within the binding position for the C-terminus of a peptide substrate demanded that the state of protonation of the different acidic groups be established in order to fully understand these interactions. To address this question, the influence of pH on –log K_M for the hydrolysis of peptide substrates was determined, and the resulting pH profile could be fitted to a bell-shaped curve with the acidic leg ($pK_a = 3.4$) matching the pK_a of the C-terminal carboxylate group of the substrate and the basic leg ($pK_a = 4.2$) reflecting ionization of a group in the enzyme. Thus, CPD-Y, like the metalloenzyme CPD-A (Riordan, 1973; Christianson & Lipscomb, 1987), binds the carboxylate form of a peptide substrate. However, in contrast to CPD-A, ionic interactions are not responsible for the recognition of the C-terminal carboxylate group, as high ionic strength had no influence on the catalytic parameters. Glu145 has been suggested as the residue responsible for the adverse effect on binding above pH 4 (Liao et al., 1992), i.e., the residue determining the basic leg of the bell curve. Investigation of the role of Glu145 in determining the pH profile of K_M for peptide hydrolysis was complicated by the presence of another acidic amino acid residue (Glu65) hydrogen bonded to Glu145. However, the fact that with the mutant enzyme E65A+E145A the influence of pH on K_M for peptide hydrolysis was essentially eliminated confirmed that deprotonation of one or two of the acidic groups (Glu65/Glu145) was responsible for the pH dependence of K_M.

The next question was whether increasing pH from 3 to 8.3 causes accumulation of one or two charges (when only the two glutamic acids are considered). Two observations strongly suggest that only a single charge is developed. First, the basic leg of the pH profile for –log K_M obtained with the wild-type enzyme can be assigned to the deprotonation of a single residue, and second, the two single mutants E65A and E145A exhibited pH profiles for –log K_M shaped like that of the wild type, although the pK_a had been shifted 0.7–1 pH unit upwards. Thus, the presence of a single glutamic acid within this region of the enzyme is sufficient to create an adverse effect on peptide binding.

It was considered whether the acidic form has a charge of 0 or –1 and the basic form correspondingly has a charge of

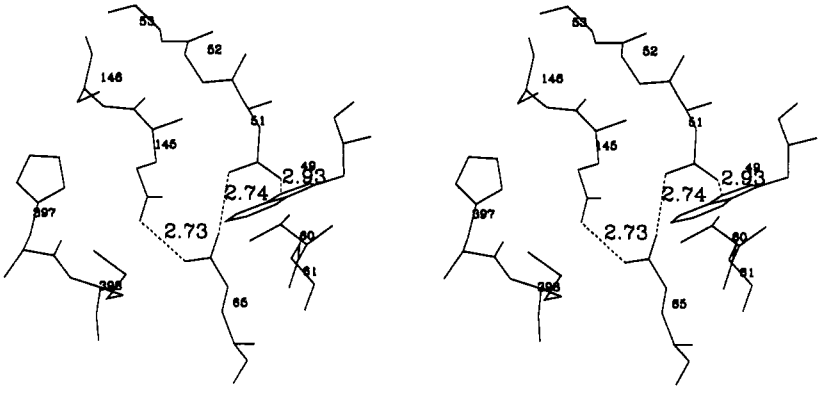


FIGURE 6: Stereoview of the active site of CPD-Y (J. Endrizzi, K. Breddam, and S. J. Remington, in preparation). The hydrogen bond network between Trp49, Asn51, Glu65, and Glu145 is indicated by dashed lines. Large numerals represent the distances between atoms involved in hydrogen bonding, and small numerals refer to the number of a given residue. The catalytically essential Ser146 and His397 are positioned in close proximity to the hydrogen bond network.

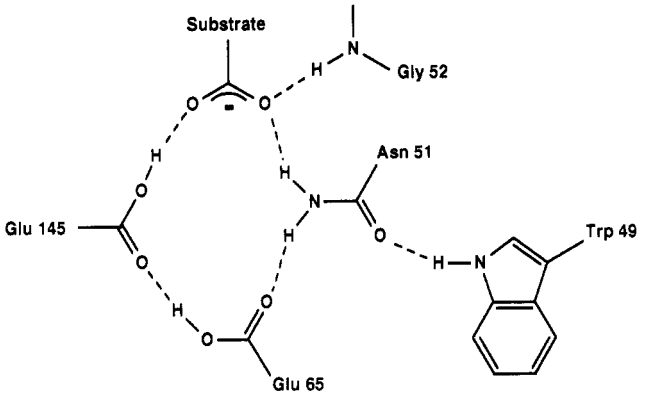


FIGURE 7: A schematic representation of the hydrogen bond network in the complex between CPD-Y and the substrate in the transition state. The model is based on the structure of the complex between arginine and CPD-WII (Liao et al., 1992) and the results presented in this paper.

−1 or −2, respectively, when only a single charge is developed on the glutamic acid pair in the pH range investigated. We expected that the presence of two adjacent negative charges in the wild-type enzyme, directed against each other and in close proximity of the essential Ser146, would cause sufficient conformational change to influence the hydrolytic activity. However, no dramatic effect on k_{cat} for peptide hydrolysis is observed in the pH range around the pK_a of the deprotonating Glu. Similarly k_{cat} for the hydrolysis of FA-Ala-OBzl remains constant in the pH range 7–9.5 (Breddam, 1983). We therefore believe that only a single negative charge is formed and that the substrate binding species is the double-protonated one. The deprotonated form binds peptide substrates very poorly, if at all, due to charge repulsion. The pH profiles do not tell which of the two glutamic acids dissociate. However, the three-dimensional structure of CPD-WII in complex with arginine (Liao et al., 1992) points to Glu145 as the one being involved in peptide binding, and the fact that the Glu145 → Ala mutation is much more detrimental to peptide hydrolysis compared to the Glu65 → Ala replacement provides evidence for this.

The role of the amino acid residues involved in the hydrogen bond network in catalysis was evaluated by replacing them with residues without the capacity to form a hydrogen bond. With ester substrates the effects on the catalytic parameters were generally minor, confirming that the leaving groups of these substrates do not interact with Trp49, Asn51, Glu65, and Glu145. These results also show that the mutations, with a few exceptions, leave the enzyme fully functional. With peptide substrates k_{cat}/K_M was reduced with all mutants. However, the effect was much larger with N51A and E145A than with W49F and E65A, suggesting that Asn51 and Glu145 interact directly with the C-terminus of the substrate, whereas the role of Trp49 and Glu65 probably is to orient the other two residues in the network. The fact that the reaction product arginine in complex with CPD-WII interacts with Asn51 and Glu145 (Liao et al., 1992) provides further evidence for this.

Thus, with the double-protonated species as the substrate binding form and Asn51 and Glu145 as the residues directly interacting with the C-terminus of the substrate the following model is proposed (Figure 7): A hydrogen bond between the indole NH of Trp49 and the γO of Asn51 and one between the γN of Asn51 and the δO of Glu65 leaves one proton of Asn51 free for the formation of a hydrogen bond with one of the partially negatively charged carboxylate oxygens of the substrate. This oxygen atom also accepts a hydrogen bond from the backbone amide of Gly52. Glu65 forms a hydrogen bond with Glu145, and as Glu65 accepts a hydrogen bond from Asn51, it must donate one to Glu145, leaving one -OH group free on Glu145 to interact with the carboxylate group of the substrate.

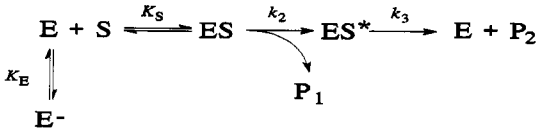
The contribution of Asn51 and Glu145 to peptide hydrolysis could be estimated. The Asn51 → Ala and Glu145 → Ala mutations reduced k_{cat}/K_M for the hydrolysis of FA-Phe-Gly-OH by factors of 100 and 300, respectively, corresponding to $\Delta\Delta G_T^{\ddagger}$ values[3] of 11.2 kJ/mol and 13.9 kJ/mol, respectively. However, k_{cat}/K_M for the hydrolysis of FA-Phe-Gly-OH with the double mutant N51A+E145A was only reduced by a factor 220 ($\Delta\Delta G_T^{\ddagger}$ = 13.4 kJ/mol). Accordingly, the effects of these two mutations are not additive, suggesting that both interactions are required for transition-state stabilization. In this comparison it is necessary to take into consideration the different pK_a values of the K_M and k_{cat} dependencies. The k_{cat} dependence is not altered by the mutations (see below), but the pK_a value for the K_M dependence was shifted upwards by the mutations, i.e., pK_a values of 4.3 with the wild-type enzyme and 4.9–5.3 with the three mutants. This prevents a direct comparison of the maximum effect of these mutations, but the K_M values with the mutants are underestimated by approximately a factor of 10. Thus, the true differences in k_{cat}/K_M between wild-type enzyme and mutants are much larger, and if this is taken into consideration, the $\Delta\Delta G_T^{\ddagger}$ values listed above should be increased by 5.7 kJ/mol. The energy of a hydrogen bond donated to a charged acceptor has been determined to be in the range of 12–25 kJ/mol (Fersht et al., 1985), and the measured effects of the Asn51 → Ala and Glu145 → Ala mutations, corrected as described, are in fair agreement with this; thus, it is probable that both amino acid residues act as hydrogen bond donors to the C-terminal carboxylate group of peptide substrates.

Among serine endopeptidases k_{cat} increases from 0 at acidic pH, dependent on the deprotonation of the essential His with a pK_a value around 7 (Fersht, 1985). We must assume that His397 functions in a similar way in CPD-Y considering that this enzyme has the same catalytic triad as the serine endopeptidases (J. Endrizzi, K. Breddam, and S. J. Remington, in preparation) and that mutational removal of His397 leads to inactivation of the enzyme (Bech & Breddam, 1989). With CPD-Y, k_{cat} for the hydrolysis of peptides does increase with pH but is dependent on an unusually low pK_a value (4.47). Furthermore, k_{cat} does not approach 0 at acidic pH with the result that it only increases by a factor of 4.5 over the entire pH range investigated. Two possibilities exist: hydrolysis at low pH may take place by a mechanism different from that of the serine endopeptidases, or alternatively, the pK_a of His397 is below 3.0 and is not the 4.47 value on which k_{cat} is dependent in the pH range investigated in this study. The pH profile of k_{cat} for the hydrolysis of the ester substrate Bz-Tyr-OEt (Christensen et al., 1992) is similar to that reported here for a peptide substrate, and consequently, it is unlikely that the unusual shape of the pH profile is determined by the carboxylate group of the peptide substrate. With the double mutant N51A+E145A the pH profile for k_{cat} yields a pK_a identical to the value obtained with the wild-type enzyme, demonstrating that the ionization of the residue influencing k_{cat} is not perturbed by the residues involved in binding of the C-terminal carboxylate group. Thus, the high k_{cat} values observed at low pH need a different explanation.

The characterization of the double mutant N51A+E145A in which both possibilities for hydrogen bonds to the car-

[3] $\Delta\Delta G_T^{\ddagger}$(observed(A → B)) = $-RT\ln((k_{cat}/K_M(B))/(k_{cat}/K_M(A)))$.

Scheme 1



$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad K_M = \frac{K_S k_3 (1 + K_E/[H])}{k_2 + k_3}$$

boxylate group of the substrate had been eliminated provided an interesting piece of information. This enzyme hydrolyzes FA-Phe-Gly-OH with k_{cat} and K_M values that are 0.24% and 50% of the values obtained with the wild-type enzyme. Similar results were obtained with FA-Phe-Ala-OH, FA-Phe-Val-OH, and FA-Phe-Leu-OH, which bind more strongly to the enzyme due to hydrophobic interactions. Thus, the two hydrogen bonds in question do not appear to stabilize the ground state (Michaelis complex) very much; instead, by lowering the activation energy (k_{cat}), they appear to facilitate the formation of the transition-state intermediate where the full strength of the hydrogen bond seems to be realized. Upon formation of the tetrahedral intermediate the scissile C–N bond is transformed from its partial double bond character to single-bond character, and consequently, the bond length is slightly increased and rotational freedom is simultaneously obtained. We believe that the hydrogen bonds provide strain on the peptide bond, hence reducing part of the delocalization energy. In this context, it would be interesting if the interaction between Arg145 in CPD-A and the α-carboxylate group of a substrate serves a similar purpose. With the single mutant N51A the k_{cat} value for the hydrolysis of FA-Phe-Gly-OH was as low as with the double mutant N51A+E145A, signifying that two hydrogen bonds are required to facilitate the formation of the transition state. However, for the hydrolysis of FA-Phe-Ala-OH, FA-Phe-Val-OH, and FA-Phe-Leu-OH the k_{cat} values were 30–60 times higher with the single mutant compared with the double mutant, suggesting that the combination of a hydrogen bond from Glu145 and hydrophobic interactions with the P_1' side chain provides significant transition-state stabilization. Interestingly, this stabilization takes place at the expense of the binding in the ground state, suggesting that binding energy has been converted into transition-state stabilization. With the other single mutant, E145A, the large decrease in k_{cat}/K_M for the hydrolysis of FA-Phe-Gly-OH was likewise a consequence of a large reduction in k_{cat} and not a significant effect on K_M. Since the other peptide substrates were hydrolyzed with kinetic parameters following the pattern obtained with N51A, it may be concluded that a hydrogen bond from Asn51 together with hydrophobic interactions also secures stabilization of the transition-state intermediate. The fact that the removal of the hydrogen-bonding capacity at position 145 obtained by the Glu145 → Ala mutation primarily affects k_{cat} for the hydrolysis of FA-Phe-Gly-OH would seem to contradict that elimination of this capacity by deprotonation of Glu145 only affects K_M. However, the effect of pH is not due to the loss of the hydrogen bond but to the charge repulsion between the negatively charged carboxylate group on the substrate and the deprotonated form of Glu145 which probably prevents formation of the Michaelis complex (see Scheme 1). It is apparent from the equations for k_{cat} and K_M that only the latter is dependent on pH.

The involvement of two hydrogen bond donors in the C-terminal recognition of peptide substrates raised the question of whether other hydrogen bond donors could function in this capacity, in particular since carboxypeptidase III from germinated barley, as the only known serine carboxypeptidase, contains a Thr at this position (Sørensen et al., 1989). The results presented here demonstrate that at position 51 several hydrogen bond donors (Gln, Ser, and Thr) facilitate peptide bond hydrolysis in the sense that these mutant enzymes exhibit significantly higher k_{cat}/K_M values than a mutant enzyme without this capacity (N51A). However, none of the mutants reached the wild-type activity. Interestingly, an enzyme with a Ser at position 51 hydrolyzed all the investigated peptide substrates with higher k_{cat}/K_M values than an enzyme with a Thr at this position. Thus, it seems likely that Thr51 in CPD-MIII is organized slightly differently compared to a Thr introduced at this position in CPD-Y.

The damaging influence of the hydrogen bond being too short (N51Q) or too long (N51S) was dependent on the size of the leaving group. With small leaving groups (-Gly-OH and -Ala-OH) the same reduction in k_{cat}/K_M was observed regardless of the direction of the change in the length of the hydrogen bond. With large leaving groups (-Val-OH and -Leu-OH) a long hydrogen bond is superior to a short one, suggesting that with a short hydrogen bond and a large side chain on the leaving group the substrate may be anchored in a rigid binding mode leaving little flexibility in the transition state to obtain the optimal interaction with the Gln at position 51. It is noteworthy that in contrast to N51A an effective transition state stabilizing interaction can be obtained with N51Q and N51S without the requirement of a side chain being present on the leaving group of the substrate.

Introduction of Asp at position 51 constituted a special case since this amino acid residue may ionize. With N51D the pH dependence of K_M for the hydrolysis of FA-Phe-Ala-OH was consistent with the ionization of a single amino acid residue, and therefore ionization of Asp51 does not seem to contribute. There are two explanations of this: (a) Asp51 has been shifted away to a position where its ionization does not influence the pH dependence of substrate binding, or (b) it does not ionize due to its participation in the hydrogen bond network in analogy to that of Asn51 in the wild-type enzyme. The first possibility would require that the hydrophilic Asp51 shift away from the water-accessible surface, and we consider that less likely than option b. If interpretation b is correct, the low activity of N51D (similar to that of N51A) and the identical N51D and N51A pH dependencies of k_{cat} and K_M for peptide hydrolysis may be explained by the absence of an available hydrogen bond donor for the α-carboxylate group of the substrate. This model also accounts for the results obtained with N51E, which hydrolyzes the investigated peptide substrates with slightly lower k_{cat}/K_M values than those obtained with N51D.

With a similar series of mutants at position 145 it was observed that all the enzymes hydrolyzed peptide substrates with lower k_{cat}/K_M values than those obtained with E145A, which is unable to donate a hydrogen bond. Thus, at position 145 no flexibility exists with respect to the nature of the hydrogen bond donor. Considering the results obtained with the position 51 mutants, we suspect that a rearrangement of the groups involved in the hydrogen bond network takes place in response to the substitutions at position 145.

Hydrogen bonds are involved in numerous enzyme–substrate interactions, and their contribution to substrate binding in the ground state (Bonneau et al., 1991) as well as in the transition state (Leatherbarrow et al., 1985; Bryan et al., 1986; Wells et al., 1986) has been studied by site-directed mutagenesis. The results presented here demonstrate some

latitude concerning the length of the hydrogen bond between the α-carboxylate group of the substrate and the hydrogen bond donor at position 51. It is further suggested that part of this flexibility is due to a similar latitude in the interaction between the C-terminal side chain and the S_1' binding pocket. Such latitude for hydrogen bonds involved in transition-state stabilization has not previously been observed with other enzymes. In subtilisin BPN', Asn155, which interacts with the oxyanion of the carbonyl group of the substrate, could not be changed to Ser or Gln without drastically reducing activity. In fact, the latter mutant enzymes hydrolyze a peptide substrate with k_{cat} values comparable to those of a mutant with an Ala at this position (Braxton & Wells, 1991). The fact that the hydrogen bond is situated close to the scissile bond in the subtilisin case while it is further removed in the CPD-Y case may be the reason for this difference.

The described involvement of hydrogen bonds in the binding of the C-terminal carboxylate group of peptide substrates represents a new mechanism of biological recognition of carboxylate groups. A comparison of the primary structures of other serine carboxypeptidases reveals that Asn51 and Glu145 are situated in very conserved regions despite the fact that the enzymes exhibit only 25% identity. Thus, the type of recognition described here will probably exist in other serine carboxypeptidases, some of which are involved in polypeptide processing (Dmochowska et al., 1987; Latchinian-Sadek & Thomas, 1993) and in other reactions of physiological importance (Jackman et al., 1990, 1992). Interestingly, the same sequences can also be identified in the primary structures of lipase from *Geotrichum candidum* (Shimada et al., 1990) and acetylcholinesterase from *Torpedo californica* (Schumacher et al., 1986). In those enzymes, however, the asparagine is changed to the larger and much more hydrophobic tyrosine in accordance with these enzymes having no requirement for a carboxylate binding site, but the glutamic acid corresponding to Glu145 is conserved. Since the esterase/peptidase ratio characteristic of CPD-Y can be increased 1000-fold by the removal of the hydrogen-bonding capacity, one can imagine that a carboxypeptidase activity might be engineered into these esterases by employing the results presented in this paper.

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