Site-Directed Mutagenesis on (Serine) Carboxypeptidase Y from Yeast. The Significance of Thr60 and Met398 in Hydrolysis and Aminolysis Reactions

Steen Bech Sørensen,[†] Mette Raaschou-Nielsen,[†] Uffe H. Mortensen,[†] S. James Remington,[‡] and Klaus Breddam*,[†]

Contribution from the Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark, and Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

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Abstract: In (serine) carboxypeptidase Y, the flexible side chain of Met398 forms one side of the S_1' binding pocket and the β- and γ-carbon atoms of Thr60 form the opposite side. Met398 has been substituted with the residues Gly, Ala, Val, Ile, Leu, Phe, and Tyr while Thr60 has been substituted with the residues Ala, Val, Leu, Met, Phe, and Tyr by site-directed mutagenesis, and the resulting enzymes have been characterized with respect to their P_1' substrate preferences using the substrate series FA-Phe-Xaa-OH (Xaa = Gly, Ala, Val, or Leu) and FA-Ala-Yaa-OH (Yaa = Leu, Gln, Glu, Lys, or Arg). The results show that Met398 is much more important for transition state stabilization than Thr60 although it appears that the selected nonbulky amino acid residue (Thr) at position 60 is important for high k_{cat} values. The results further suggest that bulky amino acid side chains at position 398 are able to adjust the size of the S_1' pocket such that favorable interactions with the substrate can be obtained with even small P_1' side chains, e.g., Gly. Accordingly, the hydrolysis of substrates with bulky/hydrophobic P_1' side chains is less dependent on the nature of the amino acid residue at position 398 than that of a substrate with a nonbulky P_1' side chain. The three-dimensional structure of the mutant enzyme E65A + E145A has been determined, and it provides support for the high mobility of the Met398 side chain. In transpeptidation reactions the substitutions at position 398 also influence the interactions between the binding pocket and the amino acid leaving group as well as the added nucleophile competing with water in the deacylation reaction. Much higher aminolysis was obtained with some of the mutant enzymes, presumably due to a changed accessibility of water to the acyl−enzyme intermediate while the nucleophile/leaving group is bound at the S_1' binding site.

Introduction

The serine carboxypeptidase from *Saccharomyces cerevisiae*, carboxypeptidase Y (CPD-Y), releases all accessible amino acids from the C-terminus of peptides and proteins including the charged amino acids and proline.[1−3] The enzyme may be used for C-terminal modifications of peptides and proteins due to its ability to catalyze transacylation reactions with amino acids or amino acid derivatives as nucleophiles.[4,5] CPD-Y exhibits a P_1' preference[6] for hydrophobic amino acid residues, but the fact that arginine is released at significant rates shows that the S_1' binding site accommodates amino acid side chains of widely different properties. The three-dimensional structure of carboxypeptidase Y recently became available, and it shows that the S_1' binding pocket is comprised of Thr60, Phe64, Tyr256, Tyr269, Leu272, and Met398[7] (see Figure 1). The wide substrate preference may be due to the fact that the S_1' binding pocket is sufficiently large to allow multiple binding modes but the documented high flexibility of Met398[7] could also play a role. Previously, such flexibility of substrate and structural elements of other enzymes has been shown to be involved in the definition of substrate preference.[8−11]

In CPD-Y-catalyzed C-terminal modification of peptides the S_1' binding site interacts with the P_1' amino acid residue of the substrate, the released P_1' amino acid (leaving group), and finally the added nucleophile taking the position of the leaving group. In these complexes, with the exception of the former, water may attack the acyl−enzyme intermediate, leading to hydrolysis. Previously, it has been shown that structural alterations within the region of the enzyme where the C-terminal carboxylate group of the substrate binds may drastically change the rate of such undesired hydrolytic activity.[12] The implication is that the position of the leaving group or the added nucleophile relative to the acylated Ser146 is decisive for the accessibility of water. If so, it would be expected that this relationship could also be affected by altering the S_1'−P_1' interactions.

The side chains of Met398 and Thr60 each constitute part of the side wall within the S_1' binding pocket, the former being

* To whom correspondence should be addressed.
† Carlsberg Laboratory.
‡ University of Oregon.
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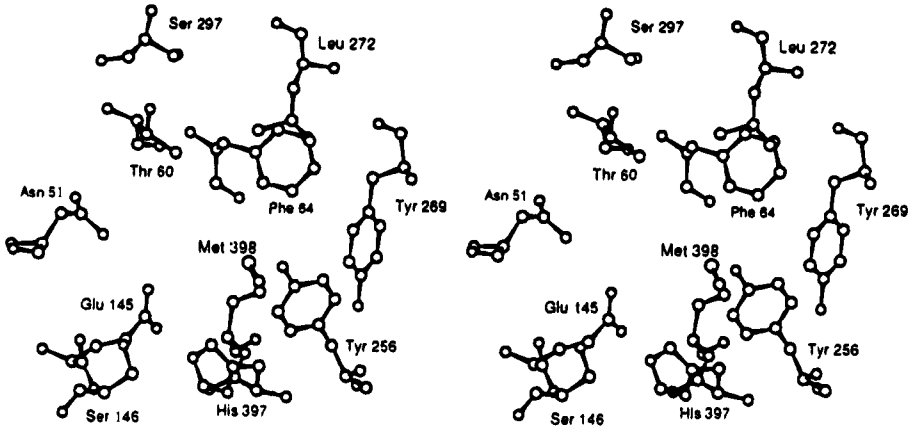

Figure 1. Stereo drawing of the active site of carboxypeptidase Y comprising the residues in the S_1' binding pocket as well as the catalytic residues Ser146 and His397 and the residues Asn51 and Glu145 involved in the recognition of the C-terminal carboxylate group.

rather mobile.[7] Predictably, these amino acid side chains will be decisive for the possible binding modes of P_1' amino acid residues and nucleophiles. In the present study, mutations at these positions have been performed and 14 mutant enzymes including one enzyme with replacements at both positions have been isolated in order to investigate the contribution of Met398 and Thr60 to the enzymatic properties of the enzyme. The production of mutant enzymes with improved properties for C-terminal modifications has been an additional aim of this work.

Experimental Section

CPD-Y was obtained from Peptech, Hilleroed, Denmark. The mutations T60X or M398X in the structural gene for CPD-Y and isolation of the resulting enzymes were carried out as previously described.[12] The purity of the enzymes was ascertained by sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis.

The double mutant enzyme E65A + E145A was prepared, purified, deglycosylated, and crystallized as described previously.[7,12] Fragments of crystals of wild-type enzyme were used as seeds, which resulted in isomorphous crystals of the mutant enzyme suitable for data collection. Triply redundant diffraction data were collected from a single crystal using a Xuong−Hamlin multiwire detector and reduced using the supplied software[13] and the PROTEIN program by W. Steigemann.[14] $F_o - F_c$ and $2F_o - F_c$ electron density maps were calculated using the model of the wild-type enzyme[7] for phasing purposes, after replacement of the side chains of Glu65 and Glu145 with alanine. The model and electron density maps were inspected, and adjustments to the model were made using the FRODO program.[15] The atomic model was refined using the "conjugate direction" option in the TNT program suite.[16]

The synthesis of the various FA substrates[17] has previously been described.[18,19] All enzymatic activities toward FA-substrates were determined spectrophotometrically at 329−343 nm using a Perkin-Elmer Lambda 7 or Lambda 9 spectrophotometer thermostated to 25 °C. The hydrolysis was carried out in 0.05 M Mes and 1 mM EDTA, pH 6.5. k_{cat} and K_M values were determined using the Grafit program.[20] Precautions were taken to ensure that the substrate solutions were not subjected to light.[21]

Aminolysis reactions were carried out in the following way: the nucleophile was dissolved in 50 mM Hepes and 5 mM EDTA, and the pH was adjusted to 7.5. Substrate (5 μL) (8 mM FA-Ala-OBzl or FA-Ala-Xaa-OH (Xaa = Ala, Leu, Phe) dissolved in methanol) was added to 190 μL of nucleophile solution followed by 5 μL of enzyme, resulting in a substrate concentration of 0.2 mM. During the reaction, 20 μL aliquots were removed from the reaction mixture and added to 50 μL of 1% trifluoroacetic acid to quench the reaction. The reactant composition was determined by HPLC using a Waters HPLC equipped with a C-18 Waters Novapac 4 μm reversed phase column and various gradients of acetonitrile in 0.1% trifluoroacetic acid. The separation was monitored at 302 nm, allowing the direct quantification of the products from the integrated peak areas.[22] The composition of the reaction mixture was determined at least twice during the reaction, the first time when 20−50% (preferably 35%) of the ester substrate had been consumed in the reaction and the second time when 50−90% (preferably 80%) of the substrate had been consumed. The products were collected and identified by amino acid analysis after acid hydrolysis (Pharmacia Alpha Plus analyzer). The fraction of aminolysis (*fa*) was expressed as the ratio between the aminolysis product and the sum of all FA-containing products being formed; i.e., unconsumed substrate was disregarded in the calculations.

The $K_{N(app)}$, representing the nucleophile concentration at which *fa* is half the maximal value (a measure for the dissociation constant of the nucleophile from the acyl−enzyme complex), and fa_{max} (the highest possible *fa* obtained at saturation of the enzyme with nucleophile) were determined by fitting the values of *fa* obtained at a minimum of seven concentrations of nucleophile to a saturation curve[23,24] using the Grafit program.[20]

With all mutant enzymes a single *fa* value was determined for the reaction FA-Ala-Leu-OH + H-Gly-NH_2 → FA-Ala-Gly-NH_2 + H-Leu-OH. The concentration of H-Gly-NH_2 was 2 M which normally is much higher than $K_{N(app)}$, and thus the value of *fa* is a measure of fa_{max}. Otherwise the procedure was as described above.

Results

The S_1' binding pocket of CPD-Y has two sides,[7] henceforth referred to as the right side and the left side seen from the catalytic triad (see Figure 1). Met398 is situated on the right side with the whole side chain facing toward the cavity of the pocket and directed away from the backbone of the substrate. Thr60 is situated on the left side with the β- and γ-carbons facing toward the cavity. Thus, both side chains are predicted

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Table 1. Influence of the Nature of the Amino Acid Residues at Positions 60 and 398 of CPD-Y on k_{cat}/K_M (min^{-1} mM^{-1}) for the Hydrolysis of Peptide Substrates at pH 6.5[a]

enzyme	FA-Ala-Leu-OH	FA-Ala-Gln-OH	FA-Ala-Glu-OH	FA-Ala-Lys-OH	FA-Ala-Arg-OH
wild type	48 000	630	46	710	3 000
M398G	1 500	26	2	44	99
M398A	9 700	28	2	26	160
M398V	3 700	12	2	15	120
M398I	6 600	32	4	34	220
M398L	16 000	330	16	510	2 300
M398F	4 400	390	30	170	660
M398Y	440	60	5	86	480
T60A	17 000	260	48	320	1 700
T60V	17 000	250	44	150	320
T60L	19 000	220	11	350	600
T60M	36 000	140	13	160	470
T60F	11 000	290	39	230	610
T60Y	10 000	180	14	360	830
T60F + M398F	530	47	7	40	100

[a] The assays were performed as described in the Experimental Section. All kinetic parameters were determined with a standard deviation $\leq \pm 10\%$.

Table 2. Influence of the Nature of the Amino Acid Residue at Position 398 of CPD-Y on the Catalytic Parameters of CPD-Y-Catalyzed Peptide Hydrolysis[a]

	enzyme							
	wt	M398G	M398A	M398V	M398I	M398L	M398F	M398Y
FA-Phe-Gly-OH								
k_{cat} (min^{-1})	5800	nd	nd	nd	nd	nd	3900	9900
K_M (mM)	5.4	nd	nd	nd	nd	nd	4.6	7.0
k_{cat}/K_M (min^{-1} mM^{-1})	1100	24	18	23	37	670	850	1400
FA-Phe-Ala-OH								
k_{cat} (min^{-1})	6700	1400	780	1900	1500	7500	3200	2800
K_M (mM)	0.18	1.3	0.33	0.30	0.42	0.14	0.20	0.67
k_{cat}/K_M (min^{-1} mM^{-1})	38 000	1100	2400	6100	3600	53 000	16 000	4100
FA-Phe-Val-OH								
k_{cat} (min^{-1})	6500	830	2000	2000	1800	2000	210	460
K_M (mM)	0.047	0.053	0.097	0.13	0.024	0.051	0.026	0.22
k_{cat}/K_M ($min^{-1}mM^{-1}$)	140 000	16 000	20 000	15 000	75 000	38 000	8300	2100
FA-Phe-Leu-OH								
k_{cat} (min^{-1})	4900	520	1900	1200	1600	1200	280	260
K_M (mM)	0.021	0.026	0.036	0.026	0.035	0.016	0.012	0.090
k_{cat}/K_M ($min^{-1}mM^{-1}$)	230 000	20 000	54 000	46 000	46 000	72 000	23 000	2900

[a] The assays were performed at pH 6.5 as described in the Experimental Section. All kinetic parameters were determined with a standard deviation $\leq \pm 10\%$. wt = wild-type enzyme. nd = not determined, due to K_M values exceeding the applicable substrate concentration range.

to contribute hydrophobic interactions to the binding of the P_1' side chain. In the present study, the significance of such interactions is investigated by incorporation of other hydrophobic amino acid residues at positions 60 and 398. Thr60 has been replaced with Ala, Val, Leu, Met, Phe, and Tyr, and Met398 has been substituted with Gly, Ala, Val, Ile, Leu, Phe, or Tyr. A double mutant where both residues have been exchanged for Phe has also been produced.

Role of Thr60 and Met398 in Hydrolysis Reactions. The significance of the amino acid residues at positions 60 and 398 for the wide substrate preference of CPD-Y was investigated. The influence of the different substitutions on k_{cat}/K_M for the hydrolysis of the substrate series FA-Ala-Xaa-OH was determined. With Xaa = Leu, Gln, Glu, Lys, or Arg this series comprises substrates of widely different properties. Using FA-Ala-Leu-OH as substrate, k_{cat}/K_M was found to be strongly dependent on the nature of the amino acid side chain at position 398 but only slightly dependent on that of the amino acid residue at position 60 (Table 1). A similar trend was observed with FA-Ala-Gln-OH, FA-Ala-Glu-OH, and FA-Ala-Lys-OH. The results obtained with FA-Ala-Arg-OH deviated somewhat due to the rather low values obtained with bulky substituents on position 60. With the double mutant enzyme T60F + M398F all k_{cat}/K_M values determined for the FA-Ala-Xaa-OH substrates were further decreased by a factor of 4−8 relative to M398F.

The effects of replacements of Thr60 and Met398 on the enzymatic properties were further investigated by determination of k_{cat} and K_M values using the hydrophobic substrate series FA-Phe-Xaa-OH (Xaa = Gly, Ala, Val, or Leu). With the mutant enzymes M398G, M398A, M398V, and M398I the k_{cat}/K_M values for the hydrolysis of FA-Phe-Gly-OH were reduced to 2−3% of the wild-type value. The corresponding figures for the mutant enzymes M398L, M398F, and M398Y were 60−130% of the wild-type value, increasing from M398L through M398F to M398Y (Table 2). A similar pattern was found with FA-Phe-Ala-OH, although less pronounced. However, in this case the k_{cat}/K_M values obtained with M398L, M398F, and M398Y decreased from M398L through M398F to M398Y. This may be ascribed to steric repulsion between the side chain of Ala in the substrate and bulky position 398 substituents like Phe and Tyr. This effect was even more pronounced with the more bulky P_1' amino acid side chains of FA-Phe-Val-OH and FA-Phe-Leu-OH. As a result, the k_{cat}/K_M values obtained with M398Y are quite similar among the FA-Phe-Xaa-OH (Xaa = Ala, Val, Leu) substrates. Thus, the mutant enzyme M398Y is unspecific within this series. Furthermore, it should be noted that the variation in k_{cat}, K_M, and k_{cat}/K_M for the hydrolysis of FA-Phe-Val-OH or FA-Phe-Leu-OH with the mutant enzymes M398G, M398A, M398V, M398I, and M398L is rather small. Thus, surprisingly, the hydrolysis of substrates with a bulky/hydrophobic P_1' side chain is less dependent on the size of

Table 3. Influence of the Nature of the Amino Acid Residue at Position 60 on the Catalytic Parameters of CPD-Y-Catalyzed Peptide Hydrolysis[a]

	enzyme							
	wt	T60A	T60V	T60L	T60M	T60F	T60Y	T60F+M398F
FA-Phe-Gly-OH								
k_{cat} (min^{-1})	5800	2400	8000	nd	6400	3400	nd	1700
K_M (mM)	5.4	1.8	2.3	nd	1.8	1.5	nd	3.1
k_{cat}/K_M (min^{-1} mM^{-1})	1100	1400	3500	3600	3600	2200	1300	540
FA-Phe-Ala-OH								
k_{cat} (min^{-1})	6700	7800	5100	4000	3300	7700	7500	670
K_M (mM)	0.18	0.18	0.16	0.22	0.12	0.19	0.19	0.21
k_{cat}/K_M (min^{-1} mM^{-1})	38 000	42 000	33 000	18 500	28 000	41 000	40 000	3200
FA-Phe-Val-OH								
k_{cat} (min^{-1})	6500	4700	1200	1300	7600	500	520	14
K_M (mM)	0.047	0.059	0.020	0.017	0.039	<0.015	<0.015	<0.015
k_{cat}/K_M (min^{-1} mM^{-1})	140 000	81 000	57 000	77 000	195 000	>33 000	>35 000	>930
FA-Phe-Leu-OH								
k_{cat} (min^{-1})	4900	2900	890	1400	6300	650	415	30
K_M (mM)	0.021	0.027	<0.015	0.012	0.029	<0.015	<0.015	0.017
k_{cat}/K_M (min^{-1} mM^{-1})	230 000	110 000	>59 000	117 000	219 000	>43 000	>28 000	1800

[a] The assays were performed at pH 6.5 as described in the Experimental Section. All kinetic parameters were determined with a standard deviation $\leq \pm 10\%$. wt = wild-type enzyme. nd = not determined, due to K_M values exceeding the applicable substrate concentration range.

hydrophobic amino acid residues at position 398 than that of a substrate with a nonbulky P_1' side chain.

With the mutant enzymes T60X a general increase in k_{cat}/K_M for the hydrolysis of FA-Phe-Gly-OH to 120−330% of the wild-type value due to decreased K_M values was observed (Table 3). A general decrease in k_{cat}/K_M, in most cases due to decreased k_{cat} values, was observed with the substrates in which the P_1' amino acid residue was Ala, Val, or Leu. The only exception is T60M hydrolyzing FA-Phe-Val-OH with a k_{cat}/K_M that is significantly higher than the wild-type value. For the two substrates FA-Phe-Val-OH and FA-Phe-Leu-OH a 10-fold reduction in k_{cat} was observed with the mutant enzymes T60F and T60Y. This k_{cat} effect was even more pronounced with the double mutant T60F + M398F characterized by a reduction in k_{cat} of more than 100-fold. Otherwise only small variations in k_{cat} were observed (Table 3).

Crystallographic Studies. Previously,[7] binding of the inhibitor benzylsuccinic acid to wild-type carboxypeptidase Y has been shown to cause a shift in the side chain of Met398 possibly due to the more hydrophobic environment within the complex. This might also be achieved by the removal of hydrophilic residues in the proximity of the otherwise hydrophobic S_1' binding pocket. The δ-carbons of Glu65 and Glu145, which function in C-terminal recognition, are situated 5.2 and 4.5 Å from the α-carbon of Met398. Thus, the potential for hydrophobicity-induced structural changes within the S_1' binding pocket was investigated by crystallization of the mutant enzyme E65A + E145A.

The double mutant enzyme E65A + E145A crystallized isomorphously to the wild-type enzyme in the cubic space group $P2_13$ with cell parameter a = 112.0 Å and one molecule in the asymmetric unit. Data collection, which resulted in a data set 92% complete to 2.6 Å resolution, and statistics for the refined atomic model of the double mutant enzyme are presented in Table 4. After manual adjustment of the model, automated refinement converged after 56 cycles and the refined atomic model has an R-factor of 0.18 for all 14 110 reflections observed in the 20.0−2.6 Å range. Thirty-eight solvent (water) molecules and three carbohydrate residues are included in the model. The model has been deposited with the Protein Data Bank (access code 1CPY).

An initial electron density map proved to be straightforward to interpret and is shown in Figure 2. Two matched positive and negative density features at the 7σ level indicated a change in two of the side chain conformational angles of Met398 (X1 and X3) so that the side chain now occupies the space vacated by removal of the side chains of Glu65 and Glu145 (Figure 3; open bonds are wild-type coordinates superimposed on the refined mutant enzyme structure). Smaller difference density features, not associated with the atomic model, are suggestive of solvent structure such as ordered water molecules, but these have not been modeled. In addition, the aromatic groups of Phe64 and His397 shift slightly toward the space vacated by the conformational change at Met398. Given the rather limited 2.6 Å resolution of the diffraction pattern, it is not clear that the minor differences in the backbone conformation of residues 64−65, 145−146, and 397−398 shown in Figure 3 are significant. No other features appear elsewhere in the electron density maps, suggesting that the consequences of the mutation are strictly local.

Table 4. Data Collection and Atomic Model Statistics for the CPD-Y Mutant Enzyme E65A + E145A

Data Collection	
space group	$P2_13$
unit cell parameter	112.0 Å
number of crystals	1
number of observations	48 636
resolution	20−2.6 Å
unique reflections	14 110
completeness[a]	92%
R merge[b]	0.072
Atomic Model Statistics	
initial R-factor[c]	0.241
final R-factor (all data)	0.180
number of protein atoms	3287
number of solvent atoms	38
Rms Deviations	
ideal bond lengths	0.023 Å
ideal bond angles	3.3°
restrained thermal parameters	3.1 Å^2

[a] Completeness is the ratio of observed reflections to those theoretically possible. [b] R merge = $\Sigma(I_{hkl} - \langle I \rangle)/\Sigma I_{hkl}$ where $\langle I \rangle$ is the average of the individual observations of I_{hkl}. [c] R-factor is the standard crystallographic residual $\Sigma(|F_o| - |F_c|)/\Sigma|F_o|$.

The results of the crystallographic study confirm the high flexibility of Met398 and predict an ability of the S_1' binding site of the enzyme to adapt to the size and properties of the residue at the P_1' site of the substrate. Furthermore, the lack of conformational changes associated with the main chain at locations 145 and 65 supports the suggestion that these side

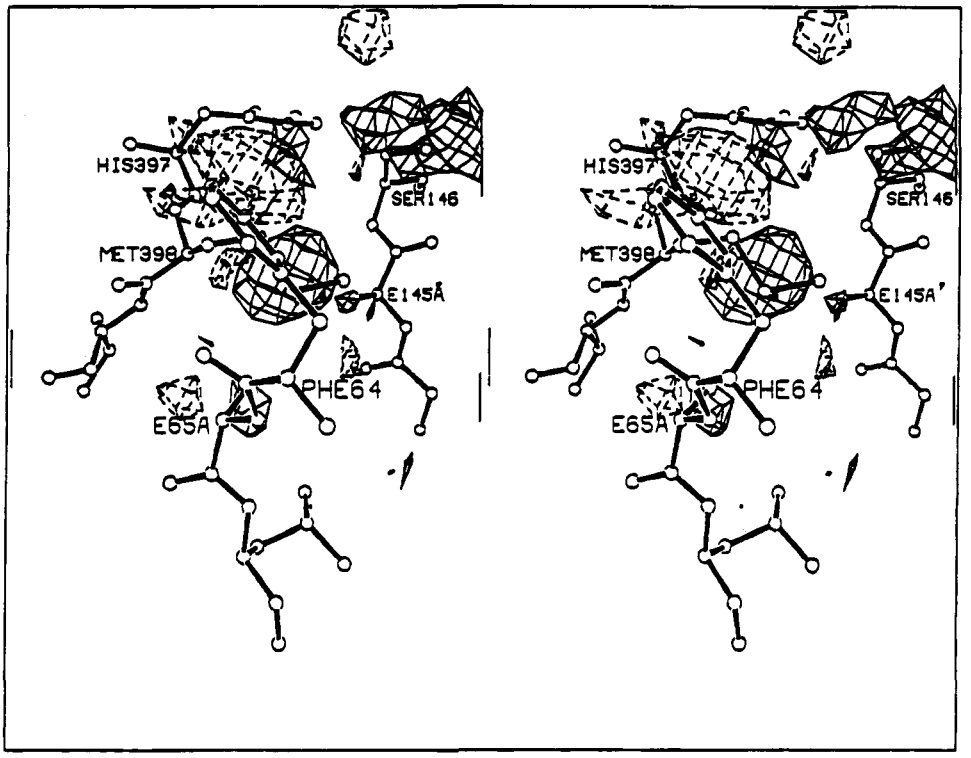

Figure 2. Stereo view of the original $F_o - F_c$ electron density map, calculated after truncation of the side chains of Glu65 and Glu145 to alanine. The final refined model is superimposed. Contour levels are -3σ (dashed lines) and $+3\sigma$ (solid lines).

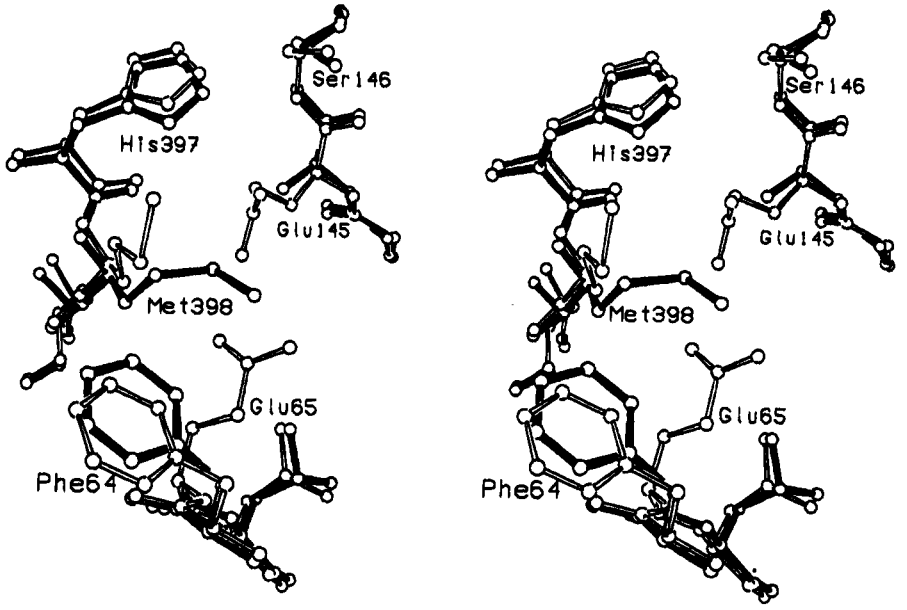

Figure 3. Stereo drawing of the final model of the double mutant enzyme E65A + E145A (solid bonds) superimposed on the wild type (open bonds) in the vicinity of the mutations.

chains are important for interaction with the carboxy terminus of a peptide substrate. Otherwise they are not involved in establishing or maintaining the structure of the active site, as catalytic activity toward ester substrates is not impaired by the mutation.[12]

Role of Positions 60 and 398 in Binding of Nucleophiles. Nucleophiles other than water, e.g., amino acids, may function in the deacylation reaction. The transition state in the hydrolysis of a peptide substrate must by necessity be identical to the corresponding transition state in its synthesis, and this offers an alternative way to study $P_1'-S_1'$ interactions. Two parameters were determined: $K_{N(app)}$ which is a measure of the affinity of the nucleophile to the acylated enzyme, and fa_{max} which is the fraction of aminolysis obtained when the enzyme is saturated with nucleophile.[24] All reactions studied in this section were designed such that the rate of degradation of the product was negligible compared to the rate of conversion of the substrate. As a consequence *fa* was constant with time (see Experimental Section) and reflects the ratio of the rates of the competing aminolysis and hydrolysis reactions.[24]

Wild-type carboxypeptidase Y catalyzes the transpeptidation reaction FA-Ala-Leu-OH + H-Gly-NH_2 → FA-Ala-Gly-NH_2 + H-Leu-OH with a fa_{max} value of only 0.15. In an initial screening to investigate the applicability of the mutant enzymes in transpeptidation reactions, an approximate value for fa_{max} was obtained by determination of *fa* at 2 M H-Gly-NH_2. With the T60X mutant enzymes *fa* values in the range 0.03−0.66 were obtained while with the M398X mutant enzymes the *fa* values were 0.89−1.0 (data not shown). Accordingly, the ability of the mutant enzymes M398A, M398V, M398I, M398L, and

Table 5. Transpeptidation Reactions with CPD-Y Mutant Enzymes, Mutated at Position 398 Using FA-Ala-Xaa-OH (Xaa =Ala, Leu, Phe) as Substrate and H-Gly-NH_2 as Nucleophile[a]

enzyme		substrate		
		FA-Ala-Ala-OH	FA-Ala-Leu-OH	FA-Ala-Phe-OH
CPD-Y (wt)	$K_{N(app)}$	128 ± 6	118 ± 18	105 ± 20
	fa_{max}	0.96 ± 0.01	0.15 ± 0.01	0.04 ± 0.002
M398A	$K_{N(app)}$	93 ± 4	109 ± 6	113 ± 5
	fa_{max}	0.98 ± 0.01	0.89 ± 0.01	0.90 ± 0.01
M398V	$K_{N(app)}$	153 ± 8	149 ± 6	148 ± 6
	fa_{max}	1.0 ± 0.01	0.97 ± 0.01	0.91 ± 0.01
M398I	$K_{N(app)}$	132 ± 10	142 ± 16	137 ± 12
	fa_{max}	1.0 ± 0.02	1.0 ± 0.04	0.82 ± 0.02
M398L	$K_{N(app)}$	72 ± 3	66 ± 5	57 ± 4
	fa_{max}	1.0 ± 0.01	0.90 ± 0.02	0.29 ± 0.01
M398F	$K_{N(app)}$	421 ± 14	336 ± 53	512 ± 28
	fa_{max}	1.0 ± 0.01	0.98 ± 0.05	1.0 ± 0.02

[a] All reactions were performed at pH 7.5 as described in the Experimental Section. The values for $K_{N(app)}$ are in mM. wt = wild type.

Table 6. Use of CPD-Y Mutant Enzymes Altered at Position 398 for Aminolysis of the Ester Substrate FA-Ala-OBzl Using Free Amino Acids as Nucleophiles[a]

enzyme		nucleophile		
		H-Ala-OH	H-Leu-OH	H-Phe-OH
CPD-Y (wt)	$K_{N,(app)}$	83 ± 7	10 ± 1	10 ± 1
	fa_{max}	0.91 ± 0.02	0.35 ± 0.01	0.15 ± 0.01
M398A	$K_{N(app)}$	245 ± 66	7 ± 0.3	4 ± 0.5
	fa_{max}	0.67 ± 0.06	0.49 ± 0.01	0.86 ± 0.02
M398V	$K_{N(app)}$	269 ± 10	4 ± 0.1	2 ± 0.2
	fa_{max}	0.76 ± 0.01	0.88 ± 0.01	0.94 ± 0.01
M398I	$K_{N(app)}$	198 ± 11	3 ± 0.2	1 ± 0.07
	fa_{max}	0.92 ± 0.02	0.96 ± 0.01	0.93 ± 0.01
M398L	$K_{N(app)}$	62 ± 4	7 ± 1	4 ± 0.3
	fa_{max}	0.94 ± 0.01	1.0 ± 0.02	0.63 ± 0.01
M398F	$K_{N,(app)}$	266 ± 9	62 ± 2	40 ± 5
	fa_{max}	0.96 ± 0.01	0.87 ± 0.02	0.78 ± 0.04

[a] All reactions were performed at pH 7.5 as described in the Experimental Section. The values for $K_{N(app)}$ are in mM. wt = wild type.

M398F to accept amino acid amides or amino acids as nucleophiles was investigated in further detail.

The fa_{max} and $K_{N(app)}$ values in the transpeptidation reaction FA-Ala-Xaa-OH + H-Gly-NH_2 → FA-Ala-Gly-NH_2 + H-Xaa-OH (Xaa = Ala, Leu, Phe) were determined using the five 398 mutant enzymes mentioned above (Table 5). For each mutant enzyme fa_{max} was dependent on the substrate whereas $K_{N(app)}$ was essentially independent, consistent with earlier observations.[24] With Xaa = Ala, fa_{max} approached the maximum value of 1.0 with the wild-type enzyme, and this level was maintained with all the investigated M398X mutant enzymes. With Xaa = Leu or Phe, fa_{max} was low with the wild-type enzyme and increased dramatically with all the mutant enzymes, in some cases to 1.0.

According to a previous model for CPD-Y-catalyzed transacylation reactions[24] the observed dependence of fa_{max} on the position 398 substituent in the reaction FA-Ala-Xaa-OH + H-Gly-NH_2 → FA-Ala-Gly-NH_2 + H-Xaa-OH would also be expected in the reaction FA-Ala-OBzl + H-Xaa-OH → FA-Ala-Xaa-OH + HOBzl where H-Xaa-OH acts as the nucleophile (Xaa = Ala, Leu, or Phe). With the wild-type enzyme, $K_{N(app)}$ decreased with increasing hydrophobicity of the nucleophile although the values for H-Leu-OH and H-Phe-OH were identical (Table 6). These results are consistent with the hydrophobic P_1' preference of the enzyme (Table 2). None of the replacements investigated altered this relation, signifying that the S_1' pocket remains hydrophobic. Relative to the wild-type enzyme, M398F showed poorer binding of all the nucleophiles, but with the other mutant enzymes the effects were in both directions depending on the nucleophile. With H-Ala-OH as nucleophile, $K_{N(app)}$ is significantly increased with M398A, M398V, M398I, and M398F whereas it is decreased with M398L (Table 6). Thus, the nature of the side chain at position 398 influences the binding of the H-Ala-OH nucleophile to the enzyme. A similar dependence is seen when Ala functions as the leaving group in hydrolysis reactions (see the previous section). With M398A, M398V, M398I, and M398L the values of $K_{N(app)}$ obtained with H-Leu-OH or H-Phe-OH as nucleophile were either similar to or lower than those observed with the wild-type enzyme.

Using H-Leu-OH or H-Phe-OH as nucleophile rather low fa_{max} values were obtained with the wild-type enzyme, but these values increased with all the mutants, and in some cases dramatic increases were observed (Table 6). This is consistent with the expectations since the same dependence of fa_{max} on the position 398 substituent was found when H-Leu-OH and H-Phe-OH acted as leaving groups in a transpeptidation reaction with FA-Ala-Xaa-OH as substrate (see above). With H-Ala-OH as nucleophile the effects on fa_{max} were in both directions dependent on the substituent at position 398. However, it should be considered that the fa_{max} value obtained with the wild-type enzyme is 0.91, close to the maximum value of 1.0.

Discussion

One aspect of the substrate preference of CPD-Y is its ability to accommodate hydrophobic as well as hydrophilic (charged/uncharged) bulky P_1' side chains. The k_{cat}/K_M values for the hydrolysis of such substrates were only slightly dependent on the size of hydrophobic amino acid residues at position 60. Thus, Thr60 does not seem to provide significant interactions which stabilize the transition state. However, the decrease in K_M for the hydrolysis of FA-Phe-Val-OH and FA-Phe-Leu-OH observed with T60F and T60Y shows that a bulky and hydrophobic side chain at position 60 stabilizes the ground state. However, this is achieved at the expense of k_{cat}, i.e., the activation energy for reaching the subsequent transition state. Thus, it appears that the nonbulky side chain at position 60 of the wild-type enzyme is selected to maximize k_{cat}. In contrast, the size of hydrophobic amino acid residues at position 398 has a pronounced influence on k_{cat}/K_M for the hydrolysis of such substrates. Thus, it may be concluded that bulky, hydrophobic P_1' side chains interact with Met398. This is also the case with the hydrophobic portion of hydrophilic side chains. Their distal hydrophilic portion is probably exposed to the solvent upward. To achieve this, the conformation of the hydrophobic part may differ among the hydrophilic side chains, and this may be why the results suggest some interaction between Thr60 and the side chain of Arg in FA-Ala-Arg-OH.

Another aspect of the substrate preference of CPD-Y is its ability to release nonbulky amino acids, e.g., Gly and Ala, at fairly high rates. The three-dimensional structure of carboxypeptidase Y shows that the side chain of Met398 forms the right side of the S_1' binding pocket and points away from the backbone of the substrate (see Figure 1). Thus, substrates with a C-terminal Gly (no side chain) would not be predicted to have contacts with any of the position 398 mutants. This is difficult to reconcile with the fact that k_{cat}/K_M with such a substrate is strongly dependent on the nature of the amino acid residue at position 398. However, the structure of the E65A + E145A mutant enzyme shows that the Met398 side chain is sufficiently flexible to reach the backbone of the substrate. If the bulky side chains of Leu, Phe, or Tyr at position 398 are mobile to a similar extent, they may swing down and fill out the S_1' subsite, hence providing contacts to the glycyl residue of FA-Phe-Gly-OH. Thus, masking of the hydrophilic Glu145 by the C-

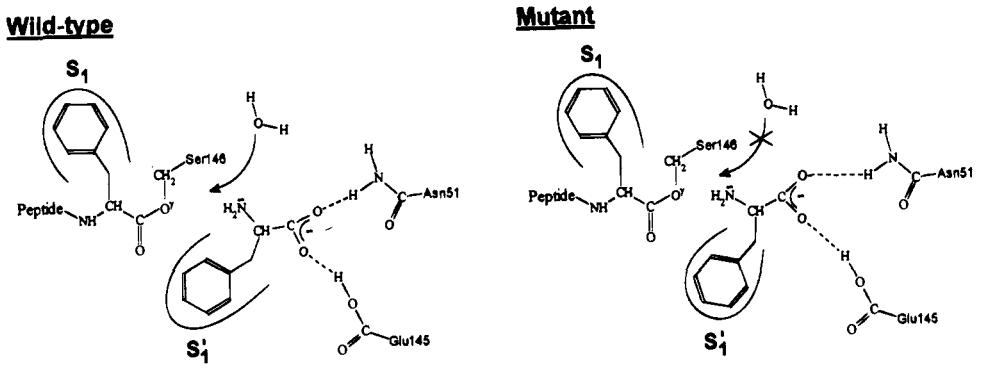

Figure 4. A model for how the changed S_1' binding pocket in the mutant enzymes through its interaction with the side chain of the leaving group/nucleophile may influence the access of water to the acyl–enzyme intermediate.

terminus of the substrate provides the basis for the movement of the side chain at position 398. Favorable interactions appear to be prevented in the mutants M398A, M398V, and M398I, possibly due to an adverse effect of the branched β-carbon atom in M398V and M398I and the short side chain in M398A.

Similar structural changes may also take place during binding of FA-Phe-Ala-OH, but with the most bulky position 398 substituents, i.e., Phe and Tyr, steric repulsion appears to play a role due to the presence of a C-terminal methyl side chain on the substrate. With FA-Phe-Val-OH and FA-Phe-Leu-OH less mobility would be anticipated. However, it is surprising that the k_{cat}/K_M values obtained with M398A exceed many of the values obtained with more hydrophobic amino acid residues at position 398. Thus, the interaction is not purely hydrophobic but seems rather to be complicated by steric repulsion.

The three-dimensional structure of CPD-Y suggested that the β-carbon group of the P_1' side chains in substrates binds close to Thr60.[7] However, a general increase in k_{cat}/K_M values was observed with the mutant enzymes substituted at position 60 hydrolyzing FA-Phe-Gly-OH which do not possess such a group. This is an indication that the amino acid residue at position 60 in the mutant enzymes is capable of establishing more favorable interactions than Thr in the wild-type enzyme with the glycine in the P_1' position of substrates.

Previously, Met398 has been alkylated with phenacyl bromide, hence forming a bulky sulphonium derivative.[18] As a result, the phenacyl moiety must protrude into the pocket, preventing optimal interactions with bulky P_1' side chains. This explains the low activity with such substrates. However, this modification leads to increased activity toward substrates with nonbulky P_1' substituents, e.g., $-OMe$ or $-NH_2$,[18] possibly due to the modified side chain of Met398 also being very flexible with the ability to interact with such leaving groups.

If the replacements of Thr60 and Met398 were independent of each other, the change in interaction energy $\Delta\Delta G_T$ of the transition state complex for the double mutant T60F + M398F would be the sum of the corresponding terms for the single mutant enzymes T60F and M398F.[9,25] However, a comparison of the $\Delta\Delta G_T$ values calculated on the basis of the k_{cat}/K_M values for hydrolysis of FA-Phe-Ala-OH with the three mutant enzymes in question revealed that this is not the case. For the double mutant, $\Delta\Delta G_T = 6.1$ kJ/mol which does not equal the sum (1.9 kJ/mol) of -0.2 kJ/mol for T60F and 2.1 kJ/mol for M398F. Thus, the replacements of the amino acid residues at positions 60 and 398 are mutually dependent on each other and presumably accompanied by structural changes in the enzyme/substrate complex in accordance with the mobility of Met398 observed by Endrizzi et al.[7]

It appears that Met398 through its mobility, as demonstrated in the three-dimensional structure of the double mutant enzyme E65A + E145A (Figure 3), contributes to the wide P_1' substrate and nucleophile preference of CPD-Y by securing interactions with amino acids which otherwise would fit only poorly into the S_1' binding pocket, e.g., Gly, Ala, Lys, and Arg. A more restricted substrate preference may be engineered by limiting this mobility. Thus, the Leu over Gly preference characteristic of the wild-type enzyme is increased 15-fold in M398A.

In transacylation reactions with free amino acids acting as leaving groups (transpeptidation) or as nucleophiles, substitutions at position 398 influence the interactions between the binding pocket and the free amino acid. With some of the mutant enzymes much higher fa_{max} values were obtained. It has previously been shown[24] that low fa_{max} values are mainly due to hydrolysis within the complex between the acyl–enzyme intermediate and the amino acid nucleophile; i.e., water and the bound nucleophile compete for the attack on the acyl–enzyme intermediate. This complex also exists when the substrate is a peptide, in which case the C-terminal amino acid acts as the leaving group. In this case, hydrolysis within the complex also reduces fa_{max} since it is a prerequisite that the leaving group has departed from the active site before the nucleophile can enter and bind within the S_1' binding site prior to nucleophilic attack. This provides an explanation for the fact that the fa_{max} values in reactions where a particular amino acid (Ala, Leu, or Phe) acts as the leaving group, i.e., transpeptidation reactions, correlate with those obtained when the same amino acid acts as the nucleophile. These effects on fa_{max} are probably due to a subtle influence on the accessibility of water to attack the acyl–enzyme intermediate while the nucleophile/leaving group is bound at the S_1' binding site. Hence, the interactions between the binding pocket and side chain of the leaving group/nucleophile may be transmitted to the region of the acylated Ser146 as illustrated in Figure 4. This effect may be exploited, possibly in combination with other structural alterations, to produce an enzyme that, at high concentrations of nucleophile, essentially excludes the action of water and thus specifically catalyzes the exchange of the C-terminal amino acid in peptides for the nucleophile, i.e., "a peptide ligase".[26] Such enzymes could improve the applicability of CPD-Y in C-terminal modifications of peptides and proteins. In this regard the ability of CPD-Y to catalyze amidation of peptides produced by fermentation with genetically manipulated microorganisms is particularly topical since good methods to carry out this posttranslational modification found in numerous peptide hormones of pharmaceutical interest are lacking.

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