Mechanistic Study on Carboxypeptidase Y-Catalyzed Transacylation Reactions. Mutationally Altered Enzymes for Peptide Synthesis

Uffe H. Mortensen, Henning R. Stennicke, Mette Raaschou-Nielsen, and Klaus Breddam*

Contribution from Carlsberg Laboratory, Department of Chemistry, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark

Received August 18, 1993®

Abstract: In (serine) carboxypeptidase Y the recognition of the C-terminal carboxylate group of peptide substrates is due to the side chains of Asn51 and Glu145 functioning as hydrogen bond donors. Carboxypeptidase Y mutants, where these amino acid residues have been substituted for other residues, have been investigated for their applicability in transacylation reactions. It is shown that Glu145 is not important for the binding of amino acid nucleophiles, consistent with the fact that at basic pH, where synthesis reactions are carried out, Glu145 cannot act as a hydrogen bond donor when deprotonated. In fact, its substitution for Ala is beneficial for the yield of synthesis, an effect which is probably due to complete or partial elimination of the charge repulsion between the α-carboxylate group of the amino acid nucleophiles and Glu145 creating more favorable binding modes for the nucleophiles. Replacement of Asn51 with Ala eliminates the capacity of the enzyme to accept amino acids as nucleophiles, suggesting that a hydrogen bond donor at position 51 is required. Incorporation of other hydrogen bond donors at this position, i.e. Ser and Gln, shows that the distance of the bound nucleophile to the acylated Ser146 is important for synthesis yields. With Ser (long distance), Asn (wild-type), and Gln (short distance) the yields using H-Val-OH as nucleophile were 5%, 32%, and 97%, respectively. On the other hand, a mutant enzyme with a Gln at position 51 results in a lower k_{cat} for the hydrolysis of peptide substrates as compared to an Asn (wild-type) or a Ser at this position. Thus, short distance is favorable for synthesis and unfavorable for hydrolysis. A novel mechanism for carboxypeptidase Y-catalyzed transacylation reactions, describing the fraction of aminolysis (fa) by the parameters fa_{max} and $K_{N,app}$, is suggested. This mechanism contains the new feature that hydrolysis of the acyl–enzyme is possible with the leaving group/nucleophile bound within the S_1' binding site. It is further shown that the low yields often encountered in transpeptidation reactions are due to attack by water on the acyl–enzyme intermediate, while the leaving group remains bound to the enzyme. This reaction may be suppressed by mutational alterations of the enzyme, hence increasing its synthetic capability, in particular in amidation reactions.

Introduction

Serine carboxypeptidases catalyze the hydrolysis of C-terminal peptide bonds of peptides and proteins with an acidic pH optimum.[1] In a recent study the binding site in carboxypeptidase Y (CPD-Y) for the negatively charged C-terminal carboxylate group of peptide substrates was identified.[2] It was shown that the carboxylate group binds to the side chains of Asn51 and Glu145 in the S_1' binding pocket.[3] Both side chains act as hydrogen bond donors, but to fulfill this function, Glu145 must be in its protonated form, and this explains the pH dependence of peptide binding. When Glu145 is deprotonated, the enzyme does not bind the carboxylate group of the substrate; in fact it appears that charge repulsion between enzyme and substrate completely prevents the substrate from binding. The side chains of Asn51 and Glu145 appear to be oriented by hydrogen bonds with Glu65 and Trp49, which therefore, have an indirect function.

Serine carboxypeptidases also catalyze the hydrolysis of peptide esters, but this activity increases with pH and remains constant in the pH range 7–9.5. Thus, at basic pH the esterase activity is high and the peptidase activity is low.[1] These unique properties combined with an ability to catalyze transacylation reactions with amino acids or amino acid derivatives as nucleophiles render serine carboxypeptidases useful in peptide synthesis.[4-7] Within this group of enzymes CPD-Y is the most commonly used. Using CPD-Y, a peptide ester as substrate, and amino acid amides as nucleophiles, the yields are generally high (>85%), but requirement for subsequent deamidation of the product complicates this method of peptide elongation. With amino acid methyl esters, medium yields (40–80%) are obtained, but this method is complicated by the risk of oligomerization. With amino acids as nucleophiles yields exceeding 60% are obtained in a few cases, but yields of 10–40% are more common and H-Pro-OH, H-Glu-OH, and H-Asp-OH are not accepted as nucleophiles. Such unsatisfactory yields are unfortunate since this route of peptide elongation is the simplest of the three listed. The low yields with amino acids as nucleophiles are not due to degradation of the product since the reaction is carried out at basic pH where the peptidase activity is very low (see above), thus securing accumulation of the peptide product in the reaction mixture.

High yield of the aminolysis reaction is dependent on tight binding of the nucleophile, preferably in a mode which secures efficient attack on the acyl–enzyme intermediate.[8] The inefficiency of amino acids as nucleophiles when compared to amino acid amides could be due to their α-carboxylate group adversely affecting binding to the enzyme. Consistent with previous observations with another serine carboxypeptidase[8] we may assume that the amino acid nucleophile when bound to CPD-Y occupies a position equivalent to that occupied by the C-terminal amino acid residue of peptide substrates. Thus, the α-carboxylate

* Abstract published in *Advance ACS Abstracts*, December 15, 1993.

(1) Breddam, K. *Carlsberg Res. Commun.* **1986**, *51*, 83–128.
(2) Mortensen, U. H.; Remington, S. J.; Breddam, K. *Biochemistry*, in press.
(3) The binding site notation is that of Schechter and Berger: Schechter, I.; Berger, B. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157–162.
(4) Widmer, F.; Breddam, K.; Johansen, J. T. *Carlsberg Res. Commun.* **1980**, *45*, 453–463.
(5) Breddam, K.; Widmer, F.; Johansen, J. T. *Carlsberg Res. Commun.* **1980**, *45*, 361–367.
(6) Widmer, F.; Breddam, K.; Johansen, J. T. *Carlsberg Res. Commun.* **1981**, *46*, 97–106.
(7) Breddam, K.; Widmer, F.; Johansen, J. T. *Carlsberg Res. Commun.* **1983**, *48*, 231–237.
(8) Breddam, K.; Ottesen, M. *Carlsberg Res. Commun.* **1984**, *49*, 473–481.

0002-7863/94/1516-0034$04.50/0

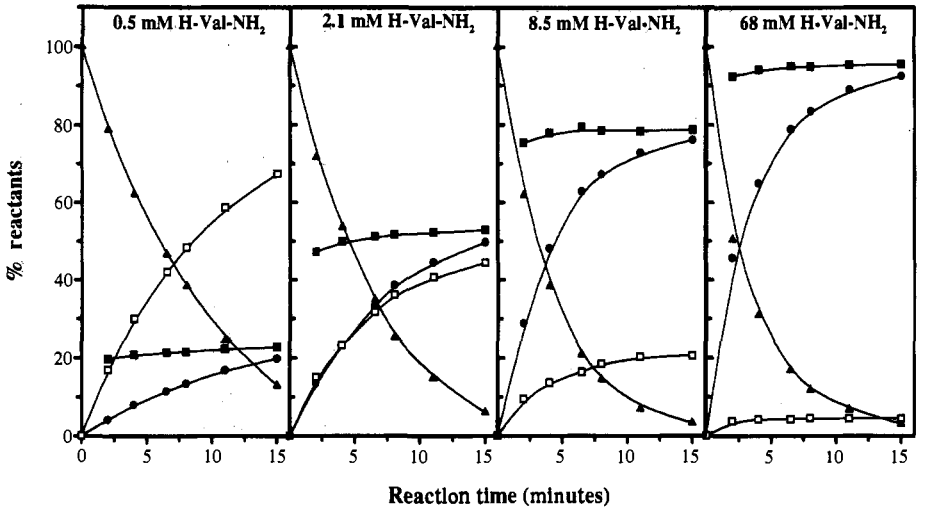


Figure 1. Dependence of time and concentration of nucleophile on the composition of reactants in the aminolysis reaction when using FA-Ala-OBzl as substrate and H-Val-NH_2 as nucleophile: (-▲-) % substrate (FA-Ala-OBzl); (-□-) % hydrolysis product (FA-Ala-OH), (-●-) % aminolysis product (FA-Ala-Val-NH_2) of the total amount of reactants in the reaction mixture; and (-■-) fraction of aminolysis (fa).

group of amino acid nucleophiles would be predicted to interact with the enzyme in a manner similar to that of the C-terminal carboxylate group of peptide substrates. These interactions are obviously not optimal for aminolysis, but it was recently shown that mutational replacements in CPD-Y of the amino acid residues involved in the binding of the C-terminal carboxylate group of peptide substrates had dramatic effects on the peptidase activity of the enzyme, while the esterase activity was left essentially unchanged.[2] Such alterations will also influence the binding of amino acid nucleophiles prior to their attack on the acyl–enzyme intermediate, and this is used here to obtain mechanistic insight into CPD-Y catalyzed transacylation reactions.

Experimental Section

Carboxypeptidase Y, FA-Ala-Gln-OH, FA-Ala-Ala-OH, and FA-Ala-Leu-OH were obtained from Carlbiotech, Copenhagen, Denmark. All amino acids/amino acid derivatives as well as the substrates FA-Ala-Arg-OH,[10] FA-Ala-Lys-OH, Bz-Gly-Phe-OH, and Bz-Gly-OPhe-OH[9] were purchased from Bachem, Switzerland. The mutants E65A, E65Q, E145A, E145Q, E65A+E145A, E65Q+E145Q, N51S, N51Q, N51A, and N51A+N145A were prepared as previously described.[2] The preparation of FA-Ala-Val-OH, FA-Ala-Phe-OH, and FA-Ala-OBzl was carried out as previously described.[11]

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. Five microliters of substrate (8 mM FA-Ala-OBzl or FA-Ala-Xaa-OH in methanol) was added to 190 μL of nucleophile solution followed by 5 μL of enzyme diluted to the appropriate concentration in the buffer described above, 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 reverse-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.[12] 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) and by cochromatography of authentic standards. The fraction of aminolysis (fa) was expressed as the ratio between the formed aminolysis product and the sum of all products being formed; that is, unconsumed substrate was disregarded in the calculations. $K_{N(app)}$ and fa_{max} (the highest possible fa obtained at saturation with nucleophile) were determined by fitting the obtained fa values to the equation $fa = fa_{max} / (1 + K_{N(app)}/N)$ using the Grafit program.[13]

The enzymatic hydrolysis of FA-Ala-OBzl and the peptide substrates was followed on a Perkin-Elmer λ9 spectrophotometer. The substrates were dissolved in methanol at a concentration of 0.5–8 mM. A total of 25 μL of substrate solution was added to 955 μL of 50 mM HEPES and 5 mM EDTA, pH 7.5, to give initial substrate concentrations in the cuvette, S_0, of 12.5–200 μM. The cleavage of the substrate was followed with time by monitoring the decrease in absorbance at 337 nm, and from this the initial rates of hydrolysis, v_0, was determined. For FA-Ala-OBzl the k_{cat}/K_M value was determined by fitting to the following form of the Michaelis–Menten equation: $v_0 = e_0(k_{cat}/K_M)/(1/K_M + 1/S_0)$. For the peptide substrates the k_{cat}/K_M values were determined by fitting to the equation $v_0 = (k_{cat}/K_M)e_0 S_0$, which is valid when $S_0 \ll K_M$. All fits of the parameter k_{cat}/K_M were performed using the Grafit program.[13]

Results and Discussion

Transacylation Reactions with Wild-Type CPD-Y. The ability of CPD-Y to catalyze transacylation reactions with amino acids or amino acid derivatives acting as nucleophiles in competition with water is most conveniently studied with an ester substrate. Transacylation reactions should preferably be performed at slightly basic pH to maximize the esterase activity and minimize the peptidase activity.[1] Thus, when amino acids are used as nucleophiles, the product, a peptide, is very slowly degraded by the enzyme and as a consequence, it accumulates in the reaction mixture.

FA-Ala-OBzl is hydrolyzed at very high k_{cat}/K_M by CPD-Y,[11] and the prepared mutant enzymes also exhibit high esterase activity,[2] allowing the use of low concentrations of enzyme (8 μM). The fact that the peptide products are hydrolyzed at much lower k_{cat}/K_M prevents degradation of the aminolysis product. Initially, such reactions were performed with H-Val-NH_2 and H-Val-OH as nucleophiles, and in these cases the hydrolysis reaction competes with the following aminolysis reaction: FA-Ala-OBzl + H-Val-X → FA-Ala-Val-X + HOBzl (X = NH_2 or OH). The fraction of aminolysis (fa) increased with increasing concentrations of H-Val-X (Figures 1 and 2). The correlation between the fraction of aminolysis and the concentration of nucleophile is shown in Figure 3, and it is apparent that the fraction of aminolysis did not exceed a value characteristic of the nucleophile. The correlation between the fraction of aminolysis and concentration of H-Val-OH was identical with 0.5 M NaCl

(9) Bz = *N*-benzoyl; Bz-Gly-OPhe-OH = hippuryl-L-β-phenylactic acid.
(10) FA = furylacryloyl.
(11) Breddam, K. *Carlsberg Res. Commun.* **1984**, *49*, 535–554.
(12) Breddam, K.; Widmer, F.; Johansen, J. T. *Carlsberg Res. Commun.* **1980**, *45*, 237–247.
(13) Leatherbarrow, R. J. *Grafit*, version 3.01; Erithacus Software Ltd.: Staines, UK, 1990.

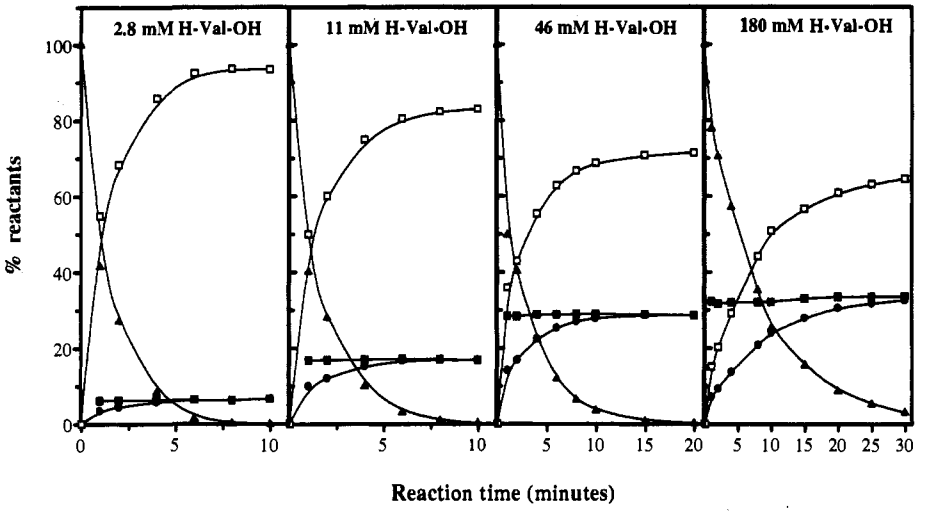


Figure 2. Dependence of time and concentration of nucleophile on the composition of reactants in the aminolysis reaction when using FA-Ala-OBzl as substrate and H-Val-OH as nucleophile: (-▲-) % substrate (FA-Ala-OBzl); (-●-) % aminolysis product (FA-Ala-Val-OH); (-□-) % hydrolysis product (FA-Ala-OH) of the total amount of reactants in the reaction mixture; and (-■-) fraction of aminolysis (fa).

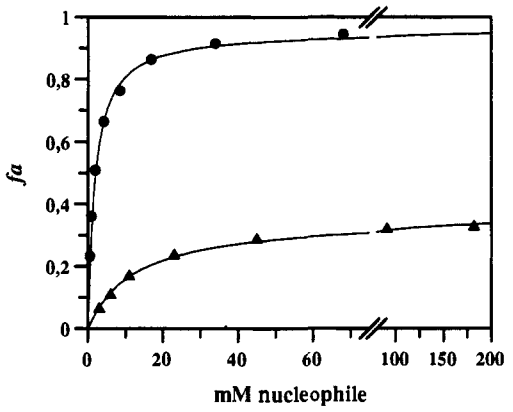


Figure 3. Saturation curves achieved with FA-Ala-OBzl as substrate and H-Val-OH and H-Val-NH_2 as nucleophiles: (-▲-) fa with H-Val-OH and (-●-) fa with H-Val-NH_2.

included in the reaction medium. Hence, the ratio of rates of the hydrolysis and aminolysis reaction is not affected by ionic strength (data not shown).

These results are consistent with the formation of a complex between nucleophile and the acyl–enzyme intermediate prior to deacylation as previously suggested.[8,14,15] The saturation curves can be described by eq 1, where $P_{2,t}$ and $P_{3,t}$ are the concentrations of hydrolysis and aminolysis products, respectively, in an aminolysis reaction where the concentration of nucleophile (N) may be considered constant as a function of time, i.e. $N \gg P_{2,t} + P_{3,t}$, and the product (P_3) is not degraded within the time of the experiment. The upper limit of fa, termed fa_{max}, is reached

$$fa = \frac{P_{3,t}}{P_{2,t} + P_{3,t}} = \frac{fa_{max}}{1 + \frac{K_{N(app)}}{N}} \quad (1)$$

when $N \gg K_{N(app)}$. The maximum value of fa_{max} is 1. The concentration at which $fa_{max}/2$ is reached, termed $K_{N(app)}$, describes the affinity of the nucleophile for the enzyme and the dissociation of the aminolysis product.

The results obtained with H-Val-OH and H-Val-NH_2 as nucleophiles show that fa_{max} and $K_{N(app)}$ is increased and decreased, respectively, by blocking the carboxylate group with NH_2. Thus,

(14) Drøhse, H.; Breddam, K.; Christensen, U. *Biocatalysis* **1991**, *5*, 109–120.

(15) Christensen, U.; Drøhse, H.; Mølgaard L. *Eur. J. Biochem.* **1992**, *210*, 467–476.

Table 1. CPD-Y-Catalyzed Aminolysis of an Ester Substrate Using Amino Acids as Nucleophiles: The Influence of Amino Acid Structure on the Parameters for the Aminolysis Reaction[a]

nucleophile	fa_{sat}	fa_{max}	$K_{N(app)}$
H-Gly-OH	0.60	0.92 ± 0.01	1500 ± 50
H-Ser-OH	0.78	0.87 ± 0.01	310 ± 20
H-Arg-OH	0.87	0.87 ± 0.01	23 ± 2
H-Lys-OH	0.79	0.81 ± 0.03	190 ± 30
H-His-OH	0.20	0.27 ± 0.01	87 ± 9
H-Gln-OH	0.64	0.90 ± 0.02	150 ± 8
H-Glu-OH	0	nd	nd
H-Ala-OH	0.85	0.91 ± 0.02	83 ± 7
H-Val-OH	0.30	0.32 ± 0.01	13 ± 1
H-Leu-OH	0.32	0.35 ± 0.01	10 ± 1
H-Phe-OH	0.14	0.15 ± 0.01	9.5 ± 1
H-Pro-OH	0	nd	nd

[a] All reactions were performed at pH 7.5, as described in the Experimental Section. The values for $K_{N(app)}$ are in mM. The concentrations at which fa_{sat} was obtained were H-Gly-OH = 2.9 M, H-Ser-OH = 3.2 M, H-Lys-OH = 2.7 M, H-His-OH = 0.25 M, H-Gln-OH = 0.37 M, H-Glu-OH = 1.1 M, H-Ala-OH = 1.4 M, H-Val-OH = 0.48 M, H-Leu-OH = 0.14 M, H-Phe-OH = 0.14 M, H-Pro-OH = 1.0 M, and H-Arg-OH = 2.0 M.

the amino acid amide is a much better nucleophile than the corresponding amino acid.

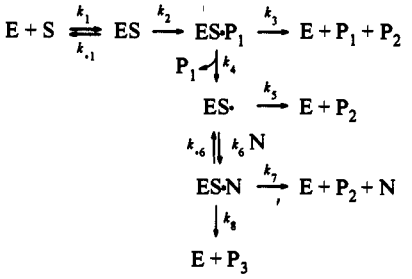
The influence of the side chain of the amino acid nucleophile on fa_{max} and $K_{N(app)}$ was investigated. $K_{N(app)}$ decreased in the following order: H-Gly-OH > H-Ser-OH > H-Lys-OH > H-Gln-OH > H-His-OH > H-Ala-OH > H-Val-OH > H-Leu-OH > H-Phe-OH (Table 1). No aminolysis product was obtained with H-Pro-OH and H-Glu-OH, consistent with previous observations.[6] Thus, hydrophilic amino acids bind much less efficiently than hydrophobic ones, and this correlates with the preference of CPD-Y with respect to the P_1' position of peptide substrates.[1] The highest fa_{max} values were obtained with the amino acids that bound least efficiently: with H-Gly-OH, H-Ala-OH, H-Ser-OH, and H-Arg-OH the fa_{max} values exceeded 0.8, whereas with the hydrophobic and efficiently binding H-Val-OH, H-Leu-OH, and H-Phe-OH values below 0.4 were obtained.

With amino acid amides as nucleophiles, fa_{max} consistently exceeds 0.85, essentially independent of the hydrophobicity of the side chain. Therefore, it is probable that the low values observed with some amino acids are due to the interaction between the enzyme and the α-carboxylate group of the amino acid when combined with tight binding of its side chain to the enzyme. This combination evidently results in a binding mode which is unfavorable for the aminolysis reaction.

Table 2. k_{cat}/K_M Values for *N*-Blocked Ester and Peptide Substrates

substrate	k_{cat}/K_M (s^{-1} M^{-1})
FA-Ala-OBzl	$2.5 \times 10^6 \pm 2.5 \times 10^5$
FA-Ala-Gln-OH	$1.6 \times 10^3 \pm 1.2 \times 10^2$
FA-Ala-Lys-OH	$2.6 \times 10^3 \pm 2.0 \times 10^2$
FA-Ala-Arg-OH	$1.1 \times 10^4 \pm 1.2 \times 10^3$
FA-Ala-Ala-OH	$1.6 \times 10^4 \pm 1.7 \times 10^3$
FA-Ala-Val-OH	$3.8 \times 10^4 \pm 2.3 \times 10^3$
FA-Ala-Leu-OH	$6.3 \times 10^4 \pm 6.7 \times 10^3$
FA-Ala-Phe-OH	$8.0 \times 10^4 \pm 2.5 \times 10^3$

Scheme I. Reaction Scheme Utilized in the Hydrolysis of Peptide and Ester Substrates and Aminolysis Reactions by CPD-Y[a]



[a] k_1 and k_{-1} represent the rate of association/disassociation of enzyme and substrate. k_2 represents the rate of formation of the acyl–enzyme + leaving group complex (acylation). k_3, k_5, and k_7 represent the rate of hydrolysis of the acyl–enzyme. k_4 represents the rate of dissociation of the leaving group and the acyl–enzyme. k_6 (N) and k_{-6} represent the rate of association/disassociation of acyl–enzyme and nucleophile. k_8 represents the rate of formation of the aminolysis product and the subsequent dissociating of the enzyme–product complex.

It has previously been suggested that low yields in aminolysis reactions were due to degradation of the products,[14,15] a theory based on the fact that any product of a CPD-Y-catalyzed aminolysis reaction will be a substrate for CPD-Y. In the aminolysis reaction FA-Ala-OBzl + H-Xaa-OH → FA-Ala-Xaa-OH + HOBzl, this would require that k_{cat}/K_M for the hydrolysis of FA-Ala-Xaa-OH would be of similar magnitude as that for FA-Ala-OBzl. However, as seen in Table 2, the values obtained with a series of FA-Ala-Xaa-OH substrates are 50–100-fold lower than that for the hydrolysis of FA-Ala-OBzl. This is also illustrated by the reactions shown in Figures 1 and 2. In both cases the ratio of aminolysis to hydrolysis remained constant during the reaction, independent of the amount of substrate remaining in the reaction mixture.

Revised Reaction Model. The results listed in Table 1 show that fa_{max} consistently is below 1, and since this cannot be ascribed to degradation of products, it is required that previous models for transacylation reactions[8,14,15] are modified. A fa_{max} value below 1 suggests that water may attack the acyl–enzyme intermediate when an amino acid nucleophile is bound to the S_1' binding site of the enzyme. As a consequence, it is reasonable to assume that water also may attack the acyl–enzyme intermediate, while the leaving group remains bound to the acyl–enzyme. The simplest model accounting for these observations is outlined in Scheme 1. The enzyme (E) attacks the peptide or ester bond of the substrate (S) forming an acyl–enzyme intermediate in complex with the leaving group (ES·P_1). Subsequently, the leaving group diffuses out of the active site (ES·) and the added nucleophile (N) enters the vacant position forming ES·N. Ultimately, the added nucleophile attacks ES· and deacylation takes place forming P_3 (aminolysis), which is the desired product. All ES· species may be attacked by water leading to hydrolysis and subsequent formation of P_2 (hydrolysis).

Assuming that no conversion of the aminolysis product occurs, the expressions for fa_{max} and $K_{N(app)}$ corresponding to Scheme 1 may be deduced as follows:

In the steady state the rates by which P_2 and P_3 are produced can be expressed as

$$\frac{dP_2}{dt} = k_3(ES\cdot P_1) + k_5(ES\cdot) + k_7(ES\cdot N) \quad (2)$$

$$\frac{dP_3}{dt} = k_8(ES\cdot N) \quad (3)$$

From the rate equations the amount of P_2 and P_3 produced at a certain time (t) will be

$$P_2 = \int_0^t k_3(ES\cdot P_1) + k_5(ES\cdot) + k_7(ES\cdot N)\, dt \quad (4)$$

$$P_3 = \int_0^t k_8(ES\cdot N)\, dt \quad (5)$$

The expressions for P_2 and P_3 with the expression for the fraction of aminolysis (fa) can be combined into the following equation:

$$fa = \frac{P_3}{P_2 + P_3} = \frac{\int_0^t k_8(ES\cdot N)\, dt}{\int_0^t k_3(ES\cdot P_1) + k_5(ES\cdot) + k_7(ES\cdot N) + k_8(ES\cdot N)\, dt} \quad (6)$$

From steady-state considerations based on Scheme 1 the following expressions for the fractions ES·/ES·N and ES·P_1/ES·N can be obtained:

$$\frac{ES\cdot}{ES\cdot N} = \frac{k_{-6} + k_7 + k_8}{k_6(N)} = \frac{K_N}{N} \quad (7)$$

$$\frac{ES\cdot P_1}{ES\cdot N} = \frac{(k_5 + k_6(N))ES\cdot}{k_4 ES\cdot N} - \frac{k_{-6}}{k_4} \quad (8)$$

Rearrangement of eq 6 and subsequent substitution of eqs 7 and 8 into eq 6 leads to the following expression for fa as a function of the nucleophile concentration:

$$fa = \frac{\int_0^t k_8(ES\cdot N)\, dt}{\int_0^t k_8(ES\cdot N)(1 + k_3/k_4)(1 + (k_7/k_8) + (k_5 K_N/k_8 N))\, dt} \quad (9)$$

When the concentration of the nucleophile can be considered constant, i.e. [N] ≫ [S], eq 9 can be reduced to eq 10:

$$fa = \frac{1}{(1 + k_3/k_4)(1 + k_7/k_8)} \Big/ \left(1 + \frac{(k_5/k_8)K_N}{(1 + k_7/k_8)N}\right) \quad (10)$$

At this point it is possible to extract two important parameters from the expression for fa in eq 10: fa_{max} and $K_{N(app)}$ (eqs 11 and 12).

$$fa_{max} = \frac{1}{(1 + k_3/k_4)(1 + k_7/k_8)} \quad (11)$$

$$K_{N(app)} = \frac{(k_5/k_8)K_N}{1 + k_7/k_8} = \frac{K_N'}{1 + k_7/k_8} \quad (12)$$

K_N' describes the affinity for the nucleophile, the reaction of the acyl–enzyme with the nucleophile, and the hydrolytic degradation

Table 3. Use of CPD-Y Mutants for Aminolysis of Ester Substrates Using Amino Acids as Nucleophiles[a]

	H-Val-OH		H-Leu-OH	
mutant	fa_{max}	$K_{N(app)}$	fa_{max}	$K_{N(app)}$
wild-type	0.32 ± 0.01	13 ± 1	0.35 ± 0.01	10 ± 0.8
N51A + E145A	0[b]	nd	0[b]	nd
N51A	0.02[b]	nd	0[b]	nd
N51S	0.05[b]	nd	0.03[b]	nd
N51Q	0.97 ± 0.02	61 ± 3	0.96 ± 0.01	42 ± 1
E145A	0.48 ± 0.01	79 ± 6	0.30 ± 0.01	40 ± 2
E145Q	0.68 ± 0.02	41 ± 4	0.66 ± 0.06	29 ± 8
E65A	0.91 ± 0.01	69 ± 2	0.96 ± 0.01	42 ± 1
E65Q	0.92 ± 0.02	75 ± 4	0.97 ± 0.01	56 ± 1
E65A + E145A	>0.8	>500	>0.8	>80
E65Q + E145Q	1.00 ± 0.03	240 ± 15	>0.8	>80

[a] All reactions were performed at pH 7.5, as described in the Experimental Section. The values for $K_{N(app)}$ are in mM. [b] The low values prevented the demonstration of saturation of the enzyme, and consequently they must be regarded as fa_{sat} values.

Table 4. N51Q-Catalyzed Aminolysis of an Ester Substrate Using Amino Acids as Nucleophiles: The Influence of Amino Acid Structure on the Parameters for the Aminolysis Reaction[a]

nucleophile	fa_{sat}	fa_{max}	$K_{N(app)}$
H-Gly-OH	0.28	>0.8	>20 000
H-Ser-OH	0.66	0.87 ± 0.01	1100 ± 40
H-Lys-OH	0.73	0.97 ± 0.06	960 ± 140
H-His-OH	0.18	0.53 ± 0.06	520 ± 80
H-Gln-OH	0.42	1.00 ± 0.03	540 ± 30
H-Glu-OH	0	nd	nd
H-Ala-OH	0.84	0.99 ± 0.01	250 ± 10
H-Val-OH	0.87	0.97 ± 0.02	61 ± 3
H-Leu-OH	0.74	0.96 ± 0.01	42 ± 1
H-Phe-OH	0.74	0.89 ± 0.03	29 ± 1
H-Pro-OH	0	nd	nd

[a] All reactions were performed at pH 7.5, as described in the Experimental Section. The values for $K_{N(app)}$ are in mM. The concentrations of nucleophile at which fa_{sat} was obtained are described in Table 1.

of the acyl–enzyme. Thus, the magnitude of K_N' depends on the acyl component as well as the chosen nucleophile, but it is independent of the leaving group. This is in agreement with the observation that $K_{N(app)}$ values obtained with H-Val-NH_2 as nucleophile and different amino acid leaving groups are identical (Table 5).

With ester substrates it is known that there is no dependence on the nature of the alcohol leaving group[5] on the fa, i.e. $k_3 \ll k_4$. If the hydrolysis due to k_3, i.e. breakdown of the ES·P_1 complex into free enzyme and hydrolysis product, is much slower than the conversion of ES·P_1 into ES by release of P_1 from the complex, the expression for $K_{N,app}$ is unchanged, while fa_{max} and $fa_{max}/K_{N(app)}$ are reduced to

$$fa_{max} = \frac{1}{1 + k_7/k_8} \tag{13}$$

$$\frac{fa_{max}}{K_{N(app)}} = \frac{1}{K_N'} \tag{14}$$

Accordingly, the fraction of aminolysis will be unaffected by the choice of leaving group and will depend only upon the choice of nucleophile. This situation is found when using amino acid nucleophiles together with ester substrates. Under conditions where the nucleophile concentration is much smaller than $K_{N(app)}$, fa/[N] becomes equal to $fa_{max}/K_{N(app)}$ (see eq 1) and the initial slope of the saturation curve is determined solely by K_N' when $k_3 \ll k_4$. Consequently, some information about the interactions between the acyl–enzyme and the nucleophile can be obtained even for nucleophiles that only interact weakly with the acyl–enzyme, i.e. high $K_{N(app)}$ values, or exhibit low solubility in aqueous

Table 5. Influence of the Amino Acid Nucleophile/Leaving Group on the fa_{max} Values Obtained with Wild-Type CPD-Y in the Reactions FA-Ala-Xaa-OH + H-Yaa-NH_2 → FA-Ala-Yaa-NH_2 + H-Xaa-OH (A) and FA-Ala-OBzl + H-Xaa-OH → FA-Ala-Xaa-OH + HOBzl (B)

H-Xaa-OH	fa_{max} with amino acid acting as leaving group (A)	fa_{max} with amino acid acting as nucleophile[c] (B)
H-Gln-OH	0.93 ± 0.01[a]	0.90
H-Lys-OH	0.99 ± 0.01[a]	0.81
H-Arg-OH	1.00 ± 0.02[a]	0.87
H-Ala-OH	0.94 ± 0.01[b]	0.91
H-Val-OH	0.22 ± 0.01[b]	0.32
H-Leu-OH	0.16 ± 0.01[b]	0.35
H-Phe-OH	0.06 ± 0.01[b]	0.15

[a] H-Gly-NH_2 was used as nucleophile. [b] H-Val-NH_2 was used as nucleophile. Using FA-Ala-OBzl as substrate, the observed fa_{max} was 1.00 ± 0.01 with H-Gly-NH_2 and 0.98 ± 0.01 with H-Val-NH_2. [c] The results are from Table 1.

solutions. In this context the special case where $k_7 \ll k_8$ can be considered. This is the situation when essentially no hydrolysis takes place at ES·P_1 or ES·N. Hence, it is possible to achieve fa_{max} values approximately equal to 1 and a $K_{N(app)}$ equal to K_N'. This situation is typically found when ester substrates are used together with amino acid amide nucleophiles,[6] but it is also found with peptide substrates with hydrophilic amino acid leaving groups, e.g. Arg and Lys.

Alternatively, if hydrolysis occurs at the ES·P_1, i.e. k_3 comparable to k_4, while the hydrolysis due to k_7, i.e. breakdown of the ES·N complex to free enzyme and products (P_1 and P_2), can be considered to be insignificant compared to the conversion of ES·N into P_3, the expression for fa_{max} and $K_{N(app)}$ can be reduced to

$$fa_{max} = \frac{1}{1 + k_3/k_4} \tag{15}$$

$$K_{N(app)} = K_N' \tag{16}$$

This situation is found in the reactions where amino acid amide nucleophiles, e.g. H-Val-NH_2 and H-Gly-NH_2, are used together with peptide substrates, as described later. Consequently, in such reactions it is possible to evaluate the ratio k_3/k_4 for various amino acid leaving groups on the basis of the obtained fa_{max} values.

Transacylation Reactions with CPD-Y Mutants. Amino acid nucleophiles must by necessity occupy a position similar to that of the P_1' amino acid residue of peptide substrates. The binding of the C-terminal carboxylate group of peptide substrates is dependent on hydrogen bonds from the side chain of Asn51 and Glu145, the latter with the carboxylic acid group in its protonated form, and the optimal position of these two side chains is secured by hydrogen bonds from Glu65 and Trp49.[2] However, this is the situation at low pH, where the enzyme efficiently binds peptide substrates; at pH 7.5, where the aminolysis reactions were carried out, Glu145 is in its deprotonated negatively charged form, which cannot participate in the binding of the α-carboxylate group. In fact, this negative charge may substantially influence the binding mode of the amino acid nucleophile. Hence, it was deemed probable that the transacylation reactions would be influenced by mutational replacements of Asn51 and Glu145 and possibly also, due to indirect effects, by replacement of Glu65.

CPD-Y mutants with replacements at positions 51, 65, and 145 were investigated for their ability to catalyze transacylation reactions using H-Val-OH and H-Leu-OH as nucleophiles (Table 3). The complete removal of the hydrogen bond donating capacity of the side chains at positions 51 and 145 by incorporation of Ala at both positions, i.e. N51A + E145A, abolished the capacity of the enzyme to accept amino acids as nucleophiles. The same result was obtained with the single mutant N51A, suggesting that Asn51 is important for the binding of amino acid nucleophiles

prior to the attack on the acyl–enzyme intermediate. With the other single mutant E145A, fa_{max} was slightly elevated or remained essentially unchanged, while $K_{N(app)}$ was elevated 4–5 times higher, and this is consistent with the notion that Glu145 at pH 7.5 plays a minor role in the binding of amino acid nucleophiles.

It is common to all the position 65 and 145 mutants, i.e. E65A, E65Q, E145A, E145Q, E65A+E145A, and E65Q+E145Q, that $K_{N(app)}$ with H-Val-OH and H-Leu-OH increased by a factor of 5–10. Simultaneously, an increase in fa_{max} was observed. With E65A and E65Q the fa_{max} values exceeded 0.90, and as a result, it is possible with these enzymes to obtain much higher yields when hydrophobic amino acids are used as nucleophiles. Hence, although Glu65 and Glu145 do not appear to be directly involved in the binding of the α-carboxylate group of amino acid nucleophiles at the pH where the reaction is carried out, they do exert an indirect influence such that their replacement affects the binding mode as indicated by the elevated fa_{max} values.

The results obtained with N51A suggested that a hydrogen bond donor at position 51 is required for the binding of amino acid nucleophiles. Substitution of Asn51 for Ser or Gln affected fa_{max} in different directions. With N51S and H-Val-OH and H-Leu-OH as nucleophiles, fa_{sat} was 0.05 and 0.03, respectively (Table 3). With N51Q the fa_{max} values were 0.97 and 0.96, respectively, and with the wild-type enzyme fa_{max} was 0.35 with H-Leu-OH and 0.32 with H-Val-OH. These values correlate with the volume of the group at position 51, and they probably signify a dependence on the distance between the acylated Ser146 and the nucleophilic amino group of the amino acid interacting through a hydrogen bond with the side chain at position 51. If this interpretation is correct, the smaller the distance, the smaller the probability for nucleophilic attack by water. $K_{N(app)}$ also correlates with the volume of the group at position 51: with N51Q $K_{N(app)}$ values of 61 mM (H-Val-OH) and 42 mM (H-Leu-OH) were obtained, as compared with 15 and 10 mM, respectively, with the wild-type enzyme. It was not possible to determine fa_{max} and $K_{N(app)}$ with N51S due to the low level of aminolysis. Thus, the smaller the distance between the nucleophilic amino group and the acylated Ser146, the less tight the binding.

With FA-Phe-OMe as substrate and H-Val-OH as nucleophile, the same influence of the group at position 51 on fa_{max} was observed (data not shown). In this synthesis an intermediate with the FA-Phe moiety acylated on Ser146 in combination with bound H-Val-OH must exist. This is also true during the hydrolysis of the peptide substrate FA-Phe-Val-OH, and the kinetic parameters for the hydrolysis of this substrate by these mutants provide some insight into the cause of this effect. The values have previously been published:[2] k_{cat} = 1500 min^{-1}, K_M = 0.085 mM, k_{cat}/K_M = 18000 min^{-1} mM^{-1} (N51Q); k_{cat} = 8100 min^{-1}, K_M = 0.17 mM, k_{cat}/K_M = 48000 min^{-1} mM^{-1} (N51S), as compared with k_{cat} = 6500 min^{-1}, K_M = 0.047 mM, k_{cat}/K_M = 140000 min^{-1} mM^{-1} (wild-type). The dependence of k_{cat}/K_M on the nature of the hydrogen bond donor at position 51 may be attributed to the effects of a too short (N51Q) or a too long hydrogen bond (N51S) for optimal binding of the transition state to the enzyme.[2] Normally, in serine protease catalyzed peptide bond hydrolysis, the acylation step is rate-limiting. However, this is not the case in CPD-Y catalyzed hydrolysis of FA-Phe-X-OH substrates (X = amino acid residue) since the k_{cat} values show only little dependence on the structure of X.[11] Thus, the rate of the deacylation step exerts an influence on k_{cat}. The k_{cat} values for the hydrolysis of FA-Phe-Val-OH increase in the order N51Q < wild-type < N51S, and this correlates inversely with the fa_{max} values with H-Val-OH as nucleophile, which decrease in the order N51Q > wild type > N51S.

To understand this, the results obtained with N51Q may be considered. With N51Q and H-Val-OH as nucleophile, fa_{max} is very high (0.97), suggesting that the space between Gln51 and the acylated Ser146 is sufficient for productive binding of the nucleophile. The high fa_{max} also implies that the probability for nucleophilic attack by water is low, and since water acts as nucleophile in the hydrolysis of FA-Phe-Val-OH, this might explain the low k_{cat} values. If so, the correlation described above is related to the fact that an acyl–enzyme intermediate in complex with H-Val-OH is common and important for both the hydrolysis and the aminolysis reactions. Thus, during peptide hydrolysis water attacks the acylated Ser146 while the leaving group interacts with the side chain at position 51, and the rate of this step is adversely influenced by the reduction in the distance between the acylated Ser146 and the bound leaving group due to the Asn51 → Gln mutation. Consistent with this, increasing the distance by the Asn51 → Ser mutation results in a reduced fa_{max} and an increased k_{cat}. These results do not warrant the conclusion that all deacylation takes place with the leaving group bound; it is highly probable that part of it takes place after the leaving group has diffused away. With the wild-type enzyme and N51S the former route may dominate, hence accounting for the high k_{cat} values. In N51Q, on the other hand, it may exist at a very low frequency due to the reduced space between the acylated Ser146 and Gln51, preventing the access of water to the acylated Ser146. As a consequence, k_{cat} is reduced. The existence of two routes of deacylation, although not recognized, probably exists among other serine proteases as well.

The results in Table 3 show that among the mutants tested the highest fa_{max} values were obtained with N51Q, and as a consequence, this enzyme was deemed the most suitable supplement to the wild-type enzyme as a catalyst in this particular type of peptide synthesis. This was further investigated by determination of the $K_{N(app)}$ and fa_{max} values for a number of different amino acid nucleophiles using 51Q (Table 4), and the results may be compared to those obtained with the wild-type enzyme (Table 1). For H-His-OH and the hydrophobic amino acids the fa_{max} values were significantly higher with N51Q as compared with those obtained with the wild-type enzyme. However, with some amino acids the Asn51 → Gln substitution had no or only a small effect on fa_{max}, i.e. those where fa_{max} with the wild-type enzyme was 0 or exceeded 0.9. With all nucleophiles $K_{N(app)}$ was significantly higher with N51Q as compared with the wild-type enzyme. The value of fa obtained at the highest possible concentration of nucleophile, i.e. fa_{sat}, has been listed in Table 3. A comparison of the fa_{sat} values obtained with the wild-type enzyme leads to the conclusion that N51Q is superior to the wild-type enzyme with hydrophobic amino acids as nucleophiles.

Transpeptidation Reactions with Wild-Type CPD-Y. A number of peptides produced by recombinant DNA methods requires subsequent modifications such as incorporation of C-terminal amide groups. Serine carboxypeptidases are particularly useful in this context due to their strict specificity for the C-terminus of peptides, eliminating the risk for cleavage of internal peptide bonds,[1] as is often seen with endopeptidases.[16] Modifications of this type are performed by a transpeptidation reaction at neutral pH, i.e. without prior esterification or other chemical modification of the C-terminal amino acid. In such reactions the C-terminal amino acid residue, acting as leaving group, is replaced with an amino acid derivative, functioning as nucleophile in the reaction in competition with water. By this method, C-terminal amidation of GRF(1–29)[17] and calcitonin[18,19] has been accomplished in high yield. However, with CPD-Y, the most commonly employed

(16) Bongers, J.; Offord, R. E.; Felix, A. M.; Campbell, R. M.; Heimer, E. P. *Int. J. Pept. Protein Res.* **1992**, *40*, 268–273.
(17) Breddam, K.; Widmer, F.; Meldal, M. *Int. J. Pept. Protein Res.* **1991**, *37*, 153–160.
(18) Henriksen, D. B.; Breddam, K.; Møller, J.; Buchardt, O. *J. Am. Chem. Soc.* **1992**, *114*, 1876–1877.
(19) Henriksen, D. B.; Rolland, M.; Jakobsen, M. H.; Buchardt, O.; Breddam, K. *Pept. Res.* **1992**, *5*, 321–324.

serine carboxypeptidase, high yield of transpeptidation is only achieved when the leaving group is a small or hydrophilic amino acid.[20,21]

Although satisfactory yield may be obtained when the C-terminal amino acid residue can be chosen freely, it would be beneficial, due to higher rate and specificity of the reaction, if a leaving group matching the preference of the enzyme was permissible. This would also allow modification of peptides derived from peptide block polymers,[22] where the choice of leaving group is restricted, due to the limited number of endopeptidases available for specific conversion of peptide polymers into peptide monomers. Altered leaving group preferences would also permit modification of a wider range of peptides and proteins as isolated from natural sources. The low yields caused by the described leaving group dependence have prevented further development of methods for incorporation of labeled amino acid residues into peptides and proteins with the purpose of facilitating amino acid sequence determinations.[23]

The situation where a peptide functions as substrate was investigated with an amino acid amide as nucleophile and FA-Ala-Xaa-OH (Xaa= H-Arg-OH, H-Lys-OH, H-Gln-OH, H-Ala-OH, H-Val-OH, H-Leu-OH, and H-Phe-OH) as substrates. The observed fa_{max} values were 1.00, 0.99, 0.93, 0.94, 0.22, 0.16, and 0.06, respectively (Table 5). Hence, fa_{max} is inversely related to the hydrophobicity of the amino acid leaving group. This difference relative to ester substrates may be due to alcohol leaving groups lacking the α-carboxylate and α-amino groups characteristic of amino acid leaving groups. To further investigate this, aminolysis reactions with H-Gly-NH_2 as nucleophile and the substrates Bz-Gly-Phe-OH and Bz-Gly-OPhe-OH, which are distinguished only by the nature of the scissile bond (peptide/ester bond), were carried out. With the peptide substrate (ester substrate) the fa_{max} values were 0.33 ± 0.01 (0.96 ± 0.01) and the $K_{N(app)}$ values were 0.63 ± 0.06 M (0.47 ± 0.05 M). The difference in the fa_{max} values obtained with these substrates suggests that the low fa_{max} values found with some amino acids are caused primarily by the presence of the α-amino group.

A comparison of the fa_{max} values obtained when identical amino acids act as nucleophile or leaving group shows that the values are similar (see Table 5). This may be due to the intermediate FA-Ala-E·H-Xaa-OH being common to both reactions. The reaction model in Scheme 1 assumes that water may attack the acylated enzyme in the FA-Ala-E·H-Xaa-OH intermediate whether H-Xaa-OH originates from the substrate (FA-Ala-Xaa-OH) or is added as nucleophile. Accordingly, we can assume that k_3 and k_7 are identical, since they represent the rate of hydrolysis within identical intermediates. This is in agreement with the observation that amino acids which result in low fa_{max} values when used as nucleophiles also result in low fa_{max} values when acting as leaving groups (see Table 5). Consequently, differences in the fa_{max} values with the same amino acid as nucleophile or leaving group must be due to differences in k_4 and k_8. However, the results presented here suggest that the magnitudes of k_4 and k_8 are rather similar, although they describe very different reactions (see Scheme 1). From a synthetic point of view the magnitudes of the individual parameters are of minor interest, since it is the ratios k_3/k_4 and k_7/k_8 that determine the yield of the aminolysis reaction, as outlined in the special cases listed in eqs 13 and 15.

Transpeptidation Reactions with Mutant CPD-Y. The different fa_{max} values obtained with the various amino acids acting as leaving groups (nucleophiles) may originate from interactions within the S_1' binding site, affecting the positioning of the α-amino group and, hence, regulating the access of water to the acyl component covalently attached to the essential serine residue (see above). Thus, it might be possible to achieve increased fa_{max} values in transpeptidation reactions if the binding mode of a specific amino acid within the S_1' binding site is altered to protect the acyl–enzyme from nucleophilic attack by water. As a result, the significance of the ES·N and ES·P_1 intermediates in the reaction course would be reduced, in accordance with eqs 13 and 15.

Table 6. Influence of the Amino Acid Leaving Group on the fa_{max} and $K_{N(app)}$ Values Obtained with N51S, N51Q, and Wild-Type CPD-Y in the Reactions FA-Ala-Xaa-OH + H·Val-NH_2 → FA-Ala-Val-NH_2 + H·Xaa-OH (A) and FA-Ala-OBzl + H-Xaa-OH → FA-Ala-Xaa-OH + HOBzl (B)

enzyme	H-Xaa·OH	fa_{max} (A)	$K_{N(app)}$ (mM) (A)	fa_{max} (B)
wild-type	H-Ala-OH	0.94 ± 0.01	1.8 ± 0.1	0.91[a]
	H-Val-OH	0.22 ± 0.01	1.5 ± 0.1	0.32[a]
	H-Leu-OH	0.16 ± 0.01	1.6 ± 0.1	0.35[a]
	H-Phe-OH	0.06 ± 0.01	1.1 ± 0.1	0.15[a]
N51S	H-Ala-OH	0.88 ± 0.01	1.9 ± 0.1	0.56 ± 0.05[b]
	H·Val-OH	0.13 ± 0.01	1.8 ± 0.1	nd[c]
	H-Leu·OH	nd	nd	nd
	H-Phe-OH	nd	nd	nd
N51Q	H·Ala-OH	0.98 ± 0.01	3.1 ± 0.1	0.99[d]
	H·Val-OH	0.91 ± 0.01	2.4 ± 0.1	0.97[d]
	H-Leu-OH	0.80 ± 0.01	2.4 ± 0.1	0.96[d]
	H·Phe-OH	0.77 ± 0.01	3.1 ± 0.1	0.89[d]

[a] Results taken from Table 1. [b] The $K_{N(app)}$ found with H-Ala-OH in reaction B was 140 ± 4 mM. [c] nd = no aminolysis products are detected, suggesting a fa_{max} value below 0.01. [d] Results from Table 3.

The fact that mutational alteration of Asn51, Glu65, and Glu145 influences fa_{max} in reactions with ester substrates (Table 3) suggests modified k_7/k_8 ratios (see eq 13). Changes in fa_{max} values due to alteration of the k_7 values should result in similar changes in the fa_{max} values for the transpeptidation reaction, because k_3 and k_7 are believed to be identical (see Scheme 1). On the other hand, changes due to altered reactivities of the amino acid nucleophiles in reactions with ester substrates, i.e. modified k_8 values, would not necessarily result in identical changes in fa_{max} for the corresponding transpeptidation reactions, i.e. the same amino acid acting as leaving group. This is due to k_4 and k_8 representing the rates of different reactions (see Scheme 1).

To investigate the origin of the changes in fa_{max}, the reaction FA-Ala-Xaa-OH + H-Val-NH_2 → FA-Ala-Val-NH_2 + H-Xaa-OH was studied with the mutants N51S and N51Q (Table 6) since this reaction allows evaluation of the k_3/k_4 ratio (see eq 15) and subsequent comparison with the changes in k_7/k_8. With the mutant N51Q the fa_{max} values were 0.98, 0.91, 0.80, and 0.77 with Xaa = Ala, Val, Leu, and Phe, respectively. The fa_{max} values with Xaa = Val, Leu, and Phe were 4–5-fold higher than those obtained with the wild-type enzyme and, furthermore, quite similar to those obtained in the reaction FA-Ala-OBzl + H-Xaa-OH (Xaa = Ala, Val, Leu, and Phe) → FA-Ala-Xaa-OH + HOBzl (see Table 6). Thus, also in transpeptidation reactions the mutation N51 → Q appears to protect the acyl–enzyme toward nucleophilic attack by water (reduction in k_3 and k_7). In contrast, with a Ser at position 51 the fa_{max} values obtained in the reaction FA-Ala-Xaa-OH + H-Val-NH_2 → FA-Ala-Val-NH_2 + H-Xaa-OH were significantly lower than those obtained with the wild-type enzyme (Table 6). Hence, fa_{max} was reduced to 0.13 with Xaa = Val, while no products were found with Xaa = Leu or Phe. This is consistent with the fact that no products were observed with FA-Ala-OBzl as substrate and H-Val-OH, H-Leu-OH, or H-Phe-OH as nucleophile (Table 6). Thus, with N51S the decreased fa_{max} values observed with FA-Ala-OBzl were also found with FA-Ala-Xaa-OH as substrate, hence repeating the pattern found with N51Q: changes in fa_{max} with an amino acid acting as leaving group are also found when it acts as nucleophile.

(20) Breddam, K.; Widmer, F.; Johansen, J. T. *Carlsberg Res. Commun.* **1980**, *45*, 237–247.
(21) Breddam, K.; Widmer, F.; Johansen, J. T. *Carlsberg Res. Commun.* **1981**, *46*, 121–128.
(22) Schellenberger, V.; Pompejus, M.; Fritz, H.-J. *Int. J. Pept. Protein Res.* **1992**, *41*, 326–332.
(23) Carles, C.; Gueguen, P.; Ribadeau-Dumas, B. *FEBS Lett.* **1987**, *212*, 163–167.

The significantly lower fa_{max} values obtained in N51S-catalyzed transpeptidation reactions are due to hydrolysis within the ES·N and ES·P_1 intermediates being favored relative to the aminolysis reaction. As stated in the previous section, the distance between the acyl–enzyme and the α-amino group of the nucleophile/leaving group appears to be decisive.

From these results it is evident that the binding mode of an amino acid within S_1' of the acyl–enzyme may be influenced by mutational replacements in the vicinity, and as a result, they can favor either hydrolysis or aminolysis. Finally, the fact that fa_{max}, with the same amino acid acting as nucleophile and leaving group, is influenced in an identical manner by a single amino acid substitution in CPD-Y provides further support for the proposed reaction model.

Conclusions

A novel mechanism for CPD-Y catalyzed transacylation reactions has been proposed. It suggests that CPD-Y utilizes a mixed-ordered random bi–bi mechanism in addition to the traditional Ping-Pong mechanism that is believed to be the exclusive mechanism of the serine endopeptidases.[24] Since the carboxypeptidases from germinating barley and *Penicillium janthinellum*[25,26] exhibit similar properties, this mechanism may be valid for all the serine carboxypeptidases. Furthermore, the fact that the glutamic acid specific protease from *Bacillus licheniformis* exhibits saturation with nucleophile in aminolysis reactions[27] suggests that hydrolysis of the acyl–enzyme may also take place with the leaving group/nucleophile bound within the active site of serine endopeptidases. Thus, this model may be more widely applicable and valid for serine endopeptidases as well.

CPD-Y-catalyzed aminolysis reactions are best performed at basic pH, where esterase activity is maximal and peptidase activity minimal. Under these conditions many amino acid nucleophiles are bound in a less than optimal mode. However, by mutational replacements of Asn51 and Glu145 the mode of binding may be changed such that it more effectively competes with water in the deacylation process. The higher fa_{max} values obtained with some mutants suggest a beneficial effect of shifting the bound nucleophile closer to the acylated Ser146, possibly due to a simultaneous exclusion of water. The produced mutants of CPD-Y are useful alternatives to wild-type CPD-Y and other serine carboxypeptidases in peptide synthesis as well as in transpeptidation reactions.

(24) Fersht, A. R. In *Enzyme structure and mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985.
(25) Breddam, K. *Carlsberg Res. Commun.* **1985**, *50*, 309–323.
(26) Breddam, K. *Carlsberg Res. Commun.* **1988**, *53*, 309–320.
(27) Rolland-Fulcrand, V.; Breddam, K. *Biocatalysis* **1993**, *7*, 75–82.