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Kinetic characterization of carboxypeptidase-Y-catalyzed peptide semisynthesis Prediction of yields

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Summary. Carboxypeptidase-Y-catalyzed peptide semisynthesis has been characterized at pH 7.5, 25°C from initial rate steady state kinetic and progress reaction studies of hydrolysis and aminolysis of α-N-benzoyl-L-tyrosine 4-nitroanilide using the natural L-amino acids and their amides as nucleophiles. The reaction mechanism previously shown to account for carboxypeptidase-Y-catalyzed aminolysis reactions (Christensen et al., 1992) was found also to account for all of the reactions studied here. It involves in addition to the classical serine proteinase mechanism: i) complex formation between the free enzyme and the nucleophile, an interaction characterized by the competitive inhibition constant, K_i, and ii) reaction of the nucleophile with the acylated enzyme forming a complex of enzyme and aminolysis product, characterized by the aminolysis kinetic parameter, K'_N.

A competitive inhibitory effect showing binding to the free enzyme is seen mainly with large hydrophobic amino acids and their amides i.e. the same residues as those preferred on either side of the scissile bond in carboxypeptidase-Y substrates. The stoichiometry of the inhibition is 1:1 and the actual binding position most likely is that of the leaving group of substrates, S'_1.

Aminolysis effects are obtained with a wide range of amino acids and amino acid amides, exceptions are Pro and, probably due to their low solubility, Tyr, Trp, Asp and Glu. The K'_N-values show relatively little dependence on the chemical nature of the side groups, but a marked difference between the amino acid and its amide. The amides interact more strongly. The kinetic parameter, k_c/K_m, of the hydrolysis of the aminolysis products is another important factor in peptide semisynthesis. The k_c/K_m-values obtained of the amidated aminolysis products are much less than those of the products formed with free amino acids. All in all this leads to rather efficient aminolysis with the L-amino acid amides and poor aminolysis with the L-amino acids.

Keywords: Amino acids – Carboxypeptidase-Y – Peptide semisynthesis – Kinetics – Yields

Abbreviations: $BzTyrNHPhNO_2$: α-N-benzoyl-L-tyrosinyl 4-nitro-aniline, Xaa: L-amino acids, Xaaa: L-amino acid amides, Z-Phe: Carbobenzoxy-L-phenylalanine, Z-Met: Carbobenzoxy-L-methionine, BzTyr: α-N-benzoyl-L-tyrosine, AlaVal: L-alanyl-L-valine, ValAla: L-valyl-L-alanine.

Introduction

Carboxypeptidase-Y from baker's yeast belongs to the group of serine carboxypeptidases. These enzymes are functionally similar, but structurally unrelated to the serine endopeptidases (Breddam, 1986; Liao and Remmington, 1990). Carboxypeptidase-Y catalyzes the hydrolysis of peptide and ester bonds and exhibits a rather broad substrate specificity (Bai et al., 1975; Hayashi et al., 1975; Breddam, 1986). Aminolytic reaction catalyzed by the enzyme has been successfully used in enzymic peptide semisynthesis (Widmer and Johansen, 1979; Breddam et al., 1981; Hellio et al., 1988). In order to better understand the rate and yield controlling factors of such processes, we are currently studying the mechanism of carboxypeptidase-Y-catalyzed aminolysis reactions. In previous papers (Christensen et al., 1992, Christensen, 1993) we suggested that they are adequately described by the reaction in Fig. 1, and further indicated how to obtain the kinetic parameters that characterize the aminolysis reactions. Here we have studied the carboxypeptidase-Y-catalyzed hydrolysis and aminolysis reactions of $BzTyrNHPhNO_2$ using the 20 natural L-amino acids, their amides and some N-substituted-L-amino acids as nucleophiles and report the values of the kinetic parameters that characterize these reactions.

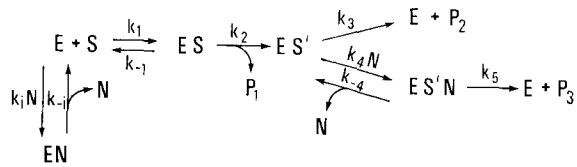

Fig. 1. Reaction scheme illustrating carboxypeptidase-Y-catalyzed aminolysis in initial rate conditions: E is the free enzyme. ES, ES', $ES'N$ are enzyme substrate complexes. EN is the enzyme-nucleophile inhibitory complex. S is the substrate, P_1, P_2, and P_3 are products formed during the reaction. N is the nucleophile. k_1. k_{-1}, k_2, k_3, k_4, k_5, k_i and k_{-i} are individual rate constants. $K_i = k_{-i}k_i$; $K'_N = k_3(k_{-4} + k_5)/k_4k_5$; k_c/K_M of the substrate: $(k_c/K_M)_S = k_1k_2/(k_{-1} + k_2)$; $k_c = k_2k_3/(k_2 + k_3)$. The reaction, $E + P_3 \rightarrow ES'N$, with the rate $k_{-5}EP_3$, has to be taken into consideration in time course experiments, since only at zero time, $P_3 = 0$. k_c/K_M of hydrolysis of P_3: $(k_c/K_M)_{P_3} = k_{-5}k_{-4}/(k_5 + k_{-4})$

Materials and methods

Enzyme

Aqueous stock solutions of carboxypeptidase-Y from baker's yeast, stable for several months when kept at −20°C, were kindly provided by Dr. K. Breddam (Carlsberg Laboratory,

Copenhagen, Denmark). The active site concentration of the enzyme (≈ 300 μM) was determined by titration using pivalic acid 4-nitrophenol as described earlier (Drøhse et al., 1991).

Chemicals

$BzTyrNHPhNO_2$, amino acids, (Xaa), amino acid amides, (Xaaa), BzTyr, Z-Phe, Z-Met, AlaVal and ValAla were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland), Merck (Darmstadt, Germany) or from Sigma (St. Louis, MO). Buffer was 10 mM $H_2PO_4^-/HPO_4^{2-}$, 0.1 M NaCl, pH 7.5.

Steady state kinetic experiments

All experiments reported here were performed at pH 7.5, 25°C. The reactions of carboxypeptidase-Y-catalyzed hydrolysis and aminolysis of $BzTyrNHPhNO_2$ in the presence and absence of nucleophiles were studied spectrophotometrically as previously described (Christensen et al., 1992). The natural L-amino acids (Xaa), and their amides (Xaaa) and some N-substituted L-amino acid, including a number of dipeptides, were used as nucleophiles. Series of initial rate experiments were performed in which the formation of BzTyr (P_2) was measured at 256 nm ($\Delta\varepsilon = 980$ M^{-1} cm^{-1}) and the formation of 4-nitroaniline (P_1) was measured at 410 nm ($\Delta\varepsilon = 8500$ M^{-1} cm^{-1}) using 100–300 nM concentrations of the enzyme, 20–60 μM concentrations of $BzTyrNHPhNO_2$ and various appropriate concentrations of the nucleophiles. Also series of full time course curves were measured at 256 nm as well as at 410 nm.

Data analysis

Initial rates

In the presence of a nucleophile that gives rise to aminolysis the steady state initial rate equations of the production of the products, P_1 and P_2 according to Fig. 1 are always different, thus for a substrate of which acylation is rate determining (Christensen et al., 1992, Christensen, 1993):

$$v_{P_1} = dP_1/dt = k_c E_0/(1 + K_m(1 + N/K_i)/S), \tag{1}$$

and

$$v_{P_2} = dP_2/dt = k_c E_0/(1 + N/K'_N)(1 + K_m(1 + N/K_i)/S) \tag{2}$$

where k_c and K_m are the regular steady state kinetic parameters obtained in the absence of the nucleophile, N. The ratio of the initial rate of P_1 production to that of P_2 production thus equals:

$$v_{P_1}/v_{P_2} = 1 + N/K'_N \tag{3}$$

when the two initial rates are obtained at the same concentrations of substrate, S, as well as of total enzyme, E_0. K'_N-values were obtained from the concentration dependencies on N of the ratio of measured initial rates of P_1 and of P_2 at identical experimental conditions (Eqn. 3). K'_N determines the initial production (yield) of the aminolysis product, P_3. At zero time, $t \approx 0$:

$$\frac{dP_3/dt}{dP_2/dt + dP_3dt} = \frac{1}{K'_N/N + 1} \tag{4}$$

K_i-values were obtained from measured initial rates of P_1-production in the presence of series of concentrations of the nucleophile, N, at conditions $S \ll K_m$. The dependence on the concentration of N of the reciprocal of such rates were analyzed. According to Eqn. 1: this leads to a linear dependence on N.

$$v_{P_1}^{-1}(S \ll K_m) = (K_m/k_c S E_0)(1 + N/K_i) \tag{5}$$

To obtain values of the kinetic parameters the appropriate equations were fitted to the data using a non-linear regression analysis program.

Time courses

(k_c/K_m)-values of the hydrolysis of the aminolysis products were obtained from time course measurements. Here the initial rate condition $P_3 = 0$ is not valid and the net production of P_3 stops at the time, t, when (Christensen et al., 1992):

$$\frac{P_3(t)}{S(t)} = \frac{N(k_c/K_m)_S}{K'_N(k_c/K_m)_{P_3}} \tag{6}$$

When the concentration of the nucleophile, N, K'_N and $(k_c/K_m)_S$ of the substrate are known $(k_c/K_m)_{P_3}$ of the aminolysis product may be obtained, if $P_3(t)$ and $S(t)$ are measured. In the time course experiments, the law of mass action requires that at any time:

$$P_3 = P_1 - P_2 \qquad \text{and} \qquad S = S_0 - P_1 \tag{7}$$

P_3 and S were obtained as functions of time by subtractions according to Eqn. 7 of the relevant normalized (division by $\Delta\varepsilon$) time courses measured at identical experimental conditions. The maximum value of P_3 i.e. $P_3(t)$, and the corresponding value of S, $S(t)$, were identified and the k_c/K_m-value of the aminolysis product, P_3 was calculated using Eqn. 6, the known concentration of the nucleophile, N, the K'_N-value determined and the known $(k_c/K_M)_S$-value. The results given are the mean values of 4 determinations at various concentrations of the nucleophile.

Results

Aminolysis reactions of carboxypeptidase-Y has been investigated using the natural L-amino acids and their amides as nucleophiles. The results of kinetic initial rate measurements were analyzed according to the reaction scheme shown in Fig. 1, which in addition to a normal serine proteinase mechanism takes into account two kinds of interaction with nucleophiles: i) with the free enzyme, giving rise to competitive inhibition of substrate hydrolysis and ii) with the acylated enzyme to yield an aminolysis product, a process first of all characterized by the value of K'_N (Christensen et al., 1992). Fig. 2 shows some typical competitive inhibition results. The actual experimental results shown are those of studies of the effects of Met and Met-amide on the ratios of reciprocal initial rates of 4-nitroaniline, P_1, release in BzTyrNHPhNO$_2$-hydrolysis in the presence and absence of nucleophile. The corresponding K_i-values were obtained according to Eqn. 5. Similar experiments were performed in the presence of series of concentrations of all of the amino acids and their amides and the K_i-values obtained are given in Table 1. It is seen that the L-amino acids falls into four groups with respect to inhibition of carboxypeptidase-Y. Phe, Tyr, Trp and Leu each are rather strong inhibitors ($K_i \sim 10$ mM). Ile, Val and Met each interact significantly, but more weakly, whereas His, Arg and Cys are very weak inhibitors and the rest do not inhibit at all. (Some of these of course are only slightly soluble.) The L-amino acid amides, in general, are inhibiting significantly less than the corresponding L-amino acids, only Trp-amide, which is a rather strong inhibitor and the amides of the very weakly inhibiting L-amino acids show

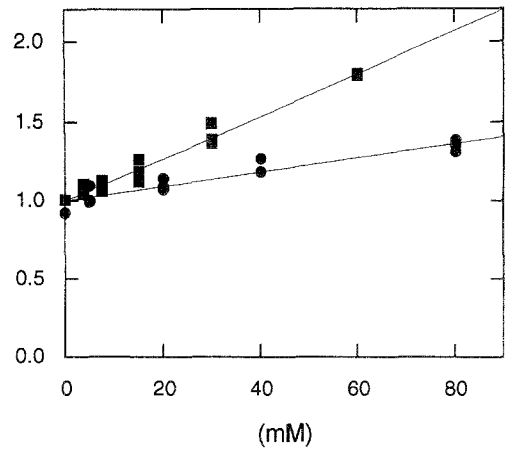

Fig. 2. Typical examples of competitive inhibition effects on carboxypeptidase-Y-catalyzed production of 4-nitroaniline, P_1, from $BzTyrNHPhNO_2$ in the presence of nucleophiles. The effects of Met (■) and of Met-amide (●) are illustrated. Ordinate: Ratio of initial rates of P_1-production obtained in the absence and in the presence of the nucleophile, from Eqn. 5: $v_{P1,0}/v_{P1,N} = 1 + N/K_i$. Abscissa: Millimolar concentration of nucleophiles. Experimental conditions: pH 7.5, 25°C

Table 1. Inhibition constants of L-amino acids (Xaa) and their amides (Xaaa) characterizing their competitive inhibitory effects on carboxypeptidase-Y, pH 7.5, 25°C

L-amino acid (Xaa)	Activity of saturated solution* (Xaa) (M)	K_i (Xaa) (mM)	K_i (Xaaa) (mM)
Phenylalanine	0.5	8.2	25
Tyrosine	0.003	~16	50
Tryptophan	0.05	11	10
Leucine	0.17	10	360
Isoleucine	0.27	27	180
Valine	0.45	33	320
Alanine	1.5	M	~3500
Glycine	2.4	M	M
Methionine	0.4	75	220
Histidine	~0.3	430	120
Arginine	~1	350	490
Lysine	0.7	M	M
Serine	2.4	M	M
Threonine	1.5	M	M
Cysteine	—	240	ND
Asparagine	0.2	M	M
Aspartic acid	0.04	M	M
Glutamine	0.3	M	M
Glutamic acid	0.06	M	M
Proline	~5	M	500

* Calculated from activity coefficients and solubilities from Sober (1968).

M Measurements performed, but no effect observed (<5%) at saturating conditions. Also Z-Phe, Z-Met, BzTyr, AlaVal and ValAla showed no effects.

ND Not determined.

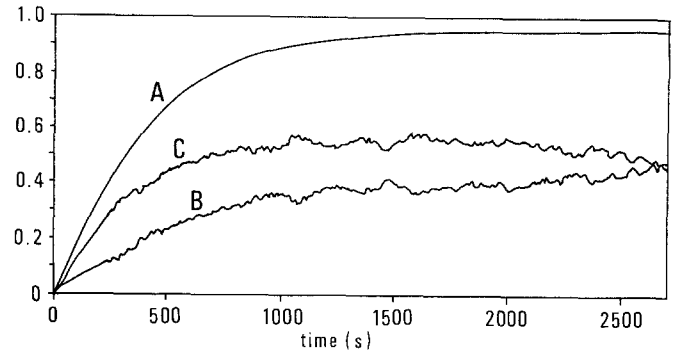

Fig. 3. Typical example of time courses of hydrolysis (*A*) and aminolysis (*C*) reactions in carboxypeptidase-Y-catalyzed processing of BzTyrNHPhNO$_2$ in the presence of nucleophiles. In this example N = Ser(0.34 M). Curve *A*: P_1/S_0 measured at 410 nm. Curve *B*: P_2/S_0 measured at 256 nm. Curve *C*: Their difference, P_3/S_0. Experimental conditions: pH 7.5, 25°C

effects similar to those of the corresponding L-amino acids. Interactions of the carboxylate function seems important of Met, Val, Ile and in particular Leu inhibition, but less important of Phe, Tyr and in particular Trp-interaction, whereas His, Arg and Cys probably interact mainly through their side chains. N-substituted L-amino acids such as Z-Phe and Z-Met showed no inhibition, nor did dipeptides such as AlaVal and ValAla (Results not shown).

Fig. 3 shows a typical result of a time course experiment. The one shown is with L-Ser as the nucleophile. Curve A shows the time course of production of 4-nitroaniline, P_1, obtained at 410 nm, curve B shows that of BzTyr, P_2, obtained at 256 nm, curve C their difference, which represents the time course of the aminolysis product, here BzTyrSer, P_3 (Eqn. 7), from which the time, t, at which the product attains its maximal value, $P_3(t)$, is obtained. The ratio $P_3(t)/S(t)$ is used to determined $(k_c/K_m)_{P_3}$ according to Eqn. 6, when K'_N is known. The K'_N-value is obtained from the ratio of initial rates (Eqn. 3) of series of such experiments. Fig. 4 shows an example and illustrates the expected linear dependence on N, which was obtained in all cases. The resultant K'_N- and $(k_c/K_m)_{P_3}$-values obtained on each of the amino acids and their amides are given in Tables 2 and 3. The $(k_c/K_M)_{P_3}$-values show the expected leaving group specificity of the enzyme as do the inhibition pattern (Table 1). In contrast to this the K'_N-values, which reflect the interaction of the nucleophile with the acylated enzyme, show little, if any specificity. Of the L-amino acids (Table 2) those with strong hydrophillic side chains show very weak interactions as seen from the values of K'_N (> 1 M) of Glu, Gln, Asp, Asn, His, Arg and Lys. The acceptance of a charged side chain or one that prefers two hydrogen bonds thus is low, whereas hydrophobic and slightly hydrophillic side chains are better accepted ($K'_N = 0.2 \pm 0.1$ M). The interactions of the acylated enzyme with L-amino acid amides (Table 3) are significantly stronger with K'_N-values approximately one tenth those of the L-amino acids. Again the loss of specificity in the acylated enzyme is apparent.

Yields of aminolysis products are governed by K'_N and $(k_c/K_M)_{P_3}$ of product hydrolysis, but of course also by the concentration of the nucleophile (Eqns. 4 and 6) (Christensen et al., 1992). Since the values of either kinetic parameter is

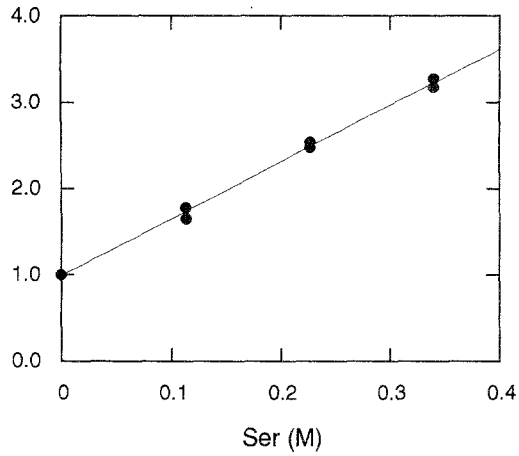

Fig. 4. A typical example of data used to determine K'_N-value. The effect of Ser on the ratio of carboxypeptidase-Y-catalyzed initial rates of production of 4-nitroaniline, P_1, and of BzTyr, P_2 (Eqn. 3) is illustrated. Ordinate: v_{P1}/v_{P2}. Abscissa: Molar concentration of nucleophile, here Ser. Experimental conditions: pH 7.5, 25°C

Table 2. Characteristic kinetic parameters of carboxypeptidase Y catalyzed aminolysis of BzTyr-substrates obtained with L-amino acids as nucleophiles, pH 7.5, 25°C

L-amino acid (Xaa)	Used konc. range (M)	K'_N (M)	k_c/K_M of BzTyrXaa (M^{-1} s^{-1})	Theoretical maximum yield* (%)
Phenylalanine	$0-7 \times 10^{-3}$	>0.2	ND	—
Tyrosine	—	ND	ND	—
Tryptophan	—	ND	ND	—
Leucine	0–0.15	0.26	90000	40**
Isoleucine	0–0.15	0.24	90000	38**
Valine	0–0.5	0.32	10000	70
Alanine	0–1	0.15	8000	90
Glycine	0–2	0.25	400	92
Methionine	0–0.4	0.11	200000	80**
Histidine	0–0.3	1.5	2000	17
Arginine	0–1	1.8	500	35
Lysine	0–1	1.3	1000	60
Serine	0–2	0.15	2000	94
Threonine	0–1	0.15	2000	91
Cysteine		ND	ND	—
Asparagine	0–0.2	1.5	400	12
Aspartic acid	0–0.04	>1	ND	<5
Glutamine	0–0.3	2.0	500	16
Glutamic acid	0–0.05	>1	ND	<5
Proline	0–5	≫10	ND	<5

* Calculated from Eqn. 4 using the activity of the saturated L-amino acid solution as N.
** The product is hydrolysed rather fast ((k_c/K_M) is large) and not even from an ester substrate would one actually get these yields.
ND Not determined.

Table 3. Characteristic kinetic parameters of carboxypeptidase Y catalyzed aminolysis of BzTyr-substrates obtained with L-amino acid amides as nucleophiles, pH 7.5, 25°C

Amide of L-amino acid (Xaaa)	K'_N (mM)	k_c/K_M of BzTyrXaaa (M^{-1} s^{-1})
Phenylalanine amide	≈42	≥20000
Tyrosine amide	ND	ND
Tryptophan amide	ND	ND
Leucine amide	36	21000
Isoleucine amide	45	18000
Valine amide	36	6000
Alanine amide	58	3000
Glycine amide	109	800
Methionine amide	11	7500
Histidine amide	43	700
Arginine amide	75	500
Lysine amide	105	800
Serine amide	28	500
Threonine amide	42	500
Cysteine amide	68	500
Asparagine amide	85	≤200
Aspartic acid amide	ND	ND
Glutamine amide	72	≤200
Glutamic acid amide	ND	ND
Proline amide	ND	ND

ND Not determined. Concentration ranges used: 0–150 mM, except of phenylalanine amide 0–7 mM.

low of the L-amino acid amides large yields are obtained of their aminolysis products. The L-amino acids, however, are not as efficient as nucleophiles for aminolysis reactions and here their solubilities or rather the activities of saturated solutions also become important parameters for an evaluation of obtainable yields. Relevant data are given in Tables 1 and 3.

Discussion

In this study methods to obtain values of kinetic parameters determining aminolysis reactions catalyzed by enzymes that follow the reaction mechanism of Fig. 1 is developed and used to characterize aminolysis reactions catalyzed by carboxypeptidase-Y. One of these has recently been thoroughly investigated and shown to be in accordance with Fig. 1 (Christensen et al., 1992). Here is investigated the aminolysis reactions of the substrate, $BzTyrNHPhNO_2$ with all the natural L-amino acids, their amides, and a number of amino acid derivatives. The results are in accordance with Fig. 1 and thus confirm the validity of the reaction scheme (Fig. 1) as a model of carboxypeptidase-Y-catalyzed aminolysis reactions.

As is illustrated in Fig. 2 some of the amino acids and -amides behave as competitive inhibitors of the enzymic process corresponding with the formation

of a nucleophile-enzyme-complex, *EN*, to which substrates cannot bind. From the inhibition constants, K_i, obtained (Table 1) a specificity pattern similar to that on either side of the scissile bond of carboxypeptidase-Y substrates (Breddam, 1986) is apparent. The linear concentration dependencies (Fig. 2 shows an example) correspond with a 1:1-stoichiometry of the inhibitory enzyme-nucleophile complex and strongly indicate that binding occurs in only one binding position. Further, as is also seen from Table 1, the complexes are generally weakened in the absence of a free carboxylate ion of the nucleophile ($K_{i,\mathrm{xaa}} < K_{i,\mathrm{xaaa}}$) a phenomenon that parallels the leaving group specificity of the enzyme. It is unlikely that the amino acids amides bind productively as substrates. Dipeptides, in spite of their better leaving groups, -Xaa versus $-NH_2$, are not forming inhibitory complexes (Table 1) and are not processed by the enzyme (Breddam, 1986). This actually indicates that a free charged α-amino function of an amino acid residue is not accepted in the S_1-binding position of carboxypeptidase-Y. Moreover, one of the characteristics of carboxypeptidase catalysis is to reject the product, P_2 from the S_1-binding position after hydrolysis when the free carboxylic group is formed, and another is to bind substrates with leaving groups containing free carboxylic groups in the S'_1-position. In accordance with the first of these, BzTyr, Z-Phe, and Z-Met were found not to inhibit the enzyme. Indeed, binding of inhibitory L-amino acids and -amides at the leaving group site, S'_1, of carboxypeptidase-Y is indicated. The K_i-values given in Table 1 thus represent the dissociation constants of complexes of carboxypeptidase-Y and L-amino acids or -amides formed by interactions at the leaving group site of the free enzyme.

In the aminolysis reactions the nucleophiles also interact at the leaving group site, but of the acylated enzyme. The K'_N-values obtained (Tables 2 and 3) characterize the reactions of α-N-benzoyl-L-tyrosinylated carboxypeptidase-Y with L-amino acids and -amides. Again there is a marked difference between the interactions of L-amino acids and those of their amides, the acylated enzyme interacts more strongly with the amides, $K'_{N,\mathrm{xaa}} > K'_{N,\mathrm{xaaa}}$. These interactions show little side chain specificity, but two groups of L-amino acids can be discerned: i) the highly hydrophillic L-amino acids, which show very weak interactions, $K'_N > 1$ M and ii) the rest, including even glycine, which show just weak interactions, $K'_N \approx 0.2 \pm 0.1$ M. Although the L-amino acid amides interact more strongly, they also show this unspecific pattern: i) highly hydrophillic L-amino acid amides, but also glycine amide, $K'_N \approx 90 \pm 20$ mM, and ii) the rest, $K'_N \approx 40 \pm 20$ mM.

The leaving group specificity of carboxypeptidase-Y substrates has been studied previously (Hayashi et al., 1975; Bech and Breddam, 1988) and the k_c/K_M-values of BzTyrXaa (Table 2) and of BzTyrXaaa (Table 3) carboxypeptidase-Y substrates are in general agreement with the known specificity pattern. It is advantageous to obtain such values from aminolysis studies, (Fig. 3, Eqns. 6 and 7), since the determination during the reaction saves costly synthesis and purification of substrates. Moreover Tables 2 and 3 contain data on a more complete list of leaving groups than previous studies. Our main interest in obtaining the k_c/K_M-values, however, is due to their importance as kinetic parameters determining the yields in carboxypeptidase-Y-catalyzed aminolysis

reactions (Eqn. 6). As discussed previously the theoretical maximal yield (Table 2, Eqn. 4) is obtained only if the substrate that undergoes the aminolysis is a better i.e. more specific, substrate than the aminolysis product, so that $(k_c/K_M)_S \gg (k_c/K_M)_{P_3}$ (Christensen et al., 1992). As seen from the results presented here carboxypeptidase-Y efficiently catalyzes aminolysis with the L-amino acid amides, but more poorly with the L-amino acids as nucleophiles.

Carboxypeptidase-Y is an exopeptidase and prefers, but do not require a free carboxylate group on the leaving group of its substrates, not only L-amino acids, but also L-amino acid amides (and alcohols of esters) are released. In contrast to trypsin-like enzymes, it shows considerable leaving group specificity, which is due to binding at the active site of specific leaving groups leading to low K_M with k_c maintained (Breddam, 1986). From the results in this study, it seems particulary noteworthy that the leaving group site specificity of the enzyme is lost in the presence of an acylating moiety, indicating that important structural changes in that part of the active site of the enzyme takes place during catalysis.

Acknowledgements

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