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ENZYMATIC PROPERTIES OF MALT CARBOXYPEPTIDASE II IN HYDROLYSIS AND AMINOLYSIS REACTIONS

by

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Carboxypeptidase II from malted barley is a serine carboxypeptidase which exhibits peptidase activity with a pH optimum below 4.0 and amidase, esterase, and peptidyl amino acid amide hydrolase activities with a pH optimum above 7.5. These different pH-optima reflect variations of the pH dependence of K_m. The enzyme hydrolyses effectively substrates containing basic and hydrophobic amino acid residues on the P_1 and/or P_1' positions. Addition of salt or phenylguanidine inhibits the activity towards substrates with basic amino acid residues in either of these positions and enhances the activity towards peptide substrates with hydrophobic amino acid residues in both positions and towards ester substrates with hydrophobic amino acid residues in the P_1 position. A binding site model to account for the observations is proposed.

Malt carboxypeptidase II catalyses the formation of peptide bonds using an N-blocked amino acid ester as acyl component and amino acids, amino acid methyl esters or amino acid amides as nucleophiles. Peptides may also be used as acyl components with the result that the nucleophile is incorporated in place of the C-terminal amino acid residue, i.e. a transpeptidation takes place.

The enzyme is easily saturated with amino acid amides indicating the formation of a complex between nucleophile and acyl-enzyme intermediate prior to deacylation. The apparent dissociation constant of this complex ($K_{N(app)}$) is approximately 1 mM with H-Lys-NH_2 and H-Arg-NH_2 while it is 13 mM with amino acid amides with uncharged side-chains. At saturation with these nucleophiles the fraction of aminolysis are 0.80 – 0.95. However, saturation cannot be achieved with amino acids and amino acid esters as nucleophiles and the fractions of aminolysis are lower than with amino acid amides. The specificity of carboxypeptidase II renders it suitable for synthesis of peptide bonds where the acyl and/or the imine portion is donated by a basic amino acid residue.

Abbreviations: Bicine = N,N-bis(2-hydroxyethyl)glycine; Bu = butyl; Bz = N-benzoyl; EDTA = ethylene diamine tetraacetic acid; FA = furylacryloyl; Hepes = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC = high performance liquid chromatography; Mes = 2-(N-morpholino)ethane sulfonic acid; PhGu = phenyl guanidine hydrogencarbonate; Z = carbobenzoxy. Other abbreviations of amino acids, amino acid derivatives and peptides are according to the guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature. The binding site notations for the enzyme is that of SCHECHTER and BERGER (16). Accordingly, the binding site for the C-terminal amino acid residue of the substrate is denoted S_1' and those for the amino acid residues in the amino-terminal direction away from the scissile bond are denoted S_1, S_2,...., S_i. The substrate positions all denoted P_1', P_1, P_2, ... P_i in correspondence with the binding sites.

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1. INTRODUCTION

The serine carboxypeptidase from yeast, carboxypeptidase Y, is an effective tool in enzymatic peptide synthesis because it catalyzes aminolysis of peptide esters with formation of new peptide bonds (1, 18, 19, 20) and transpeptidation reactions which exchange the C-terminal amino acid residues of existing peptides (10, 11). However, the yield of synthesis is variable, reflecting the side-chain specificity of the enzyme and consequently, the availability of other serine carboxypeptidases with different specificities is desirable.

Ion exchange chromatography of extracts from malted barley has indicated the presence of five different carboxypeptidases, termed carboxypeptidases I to V (14). Carboxypeptidases I and II have been purified (3, 9) and they are both inhibited by diisopropylfluorophosphate and hence, serine carboxypeptidases. It appears that these two enzymes are similar in terms of physical-chemical properties but different with respect to their specificities towards peptide substrates (3, 9). Carboxypeptidase I has been shown to catalyze the hydrolysis and aminolysis of peptide esters (4, 7) but it suffers from lack of stability at basic pH values (3) where peptide synthesis preferentially should be performed (7, 18) and in addition, it is available only in rather small amounts (3). Carboxypeptidase II, on the other hand, is available in large amounts, it exhibits a better stability at basic pH values and a specificity which differs from that of carboxypeptidase Y (9). In the present paper carboxypeptidase II has been studied with respect to its ability to synthesize peptide bonds.

2. MATERIALS AND METHODS

2.1. Materials

The following materials were prepared as previously described: malt carboxypeptidase II, FA-Phe-Phe-OH, and FA-Phe-Ala-OH (9); FA-Phe-Gly-OH, FA-Phe-NH_2, and FA-Phe-Gly-NH_2 (3); FA-Phe-Leu-OH, FA-Ala-OEt, FA-Phe-OEt, FA-Phe-Val-NH_2, FA-Ala-OBzl, and FA-Ala-OMe (8); Bz-Arg-OBu and Bz-Pro-OMe (4); Bz-Gly-OMe and Bz-Phe-OMe (1); Bz-Ile-OMe (2), Bz-Lys-Ala-OH (5). FA-Arg-OMe was synthesized in the following manner: 3 mmol FA-N-hydroxysuccinimide ester was dissolved in 7 ml dioxane and 7 ml water containing 6 mmol $NaHCO_3$ and 3 mmol H-Arg-OMe · 2HCl was added. After 2 hours reaction the dioxane was evaporated and pH lowered to 4.0 by addition of 1 M-HCl. After extraction with ethylacetate the aqueous phase, containing 90% FA-Arg-OMe and 10% FA-Arg-OH as judged by HPLC analysis, was lyophilized. This preparation was utilized without further purification since attempts to crystallize the substrate failed.

H-Thr-NH_2 was obtained from Carlsberg Biotechnology, Denmark; H-Arg-NH_2 · 2 acetate from Senn Chemicals, Switzerland; H-Gly-OH and solvents from Merck, W. Germany; Bz-Val-OMe, Bz-His-OMe and Bz-Met-OMe from Vega-Fox, USA; H-Val-OH, H-Gly-NH_2 · HCl, H-Arg-OH · HCl, H-Ser-OH, H-Ser-NH_2, Hepes, Mes, Bicine, Bz-Arg-OMe and Bz-Arg-OEt from Sigma, USA. All other amino acids, amino acid derivatives, peptide and peptide ester substrates were from Bachem, Switzerland. Phenyl guanidine bicarbonate was obtained from EGA Chemie, W. Germany.

2.2. Methods

All enzymatic activities were determined spectrophotometrically at 254 nm for Bz-substrates and at 329 – 350 nm for FA-substrates using a Cary 219 spectrophotometer thermostated at 25 °C. k_{cat} and K_m values were graphically determined from Lineweaver-Burk plots. The pH profiles for the action of malt carboxypeptidase II was carried out in 25 mM buffer, 1 mM-EDTA. The following buffers were used: acetic acid, pH 3 – 5.8; Mes, pH 5.5 – 7; Hepes, pH 7 – 8; Bicine, pH 8 – 9.

Aminolysis reactions (1 ml) were carried out in a pH stat (18) in the presence of 5 mM-EDTA. pH did not exceed 8.5 since malt carboxypeptidase II is unstable at pH values above 8.7. Unless specifically stated, the reactions were allowed to proceed until 80 – 95% of the substrate had been consumed. At this stage aliquots were removed for determination of the reactant composition by HPLC, using equipment from Waters Associates, USA, and a 10 μ Radial-Pak C-18 reverse phase column. The following eluant system was

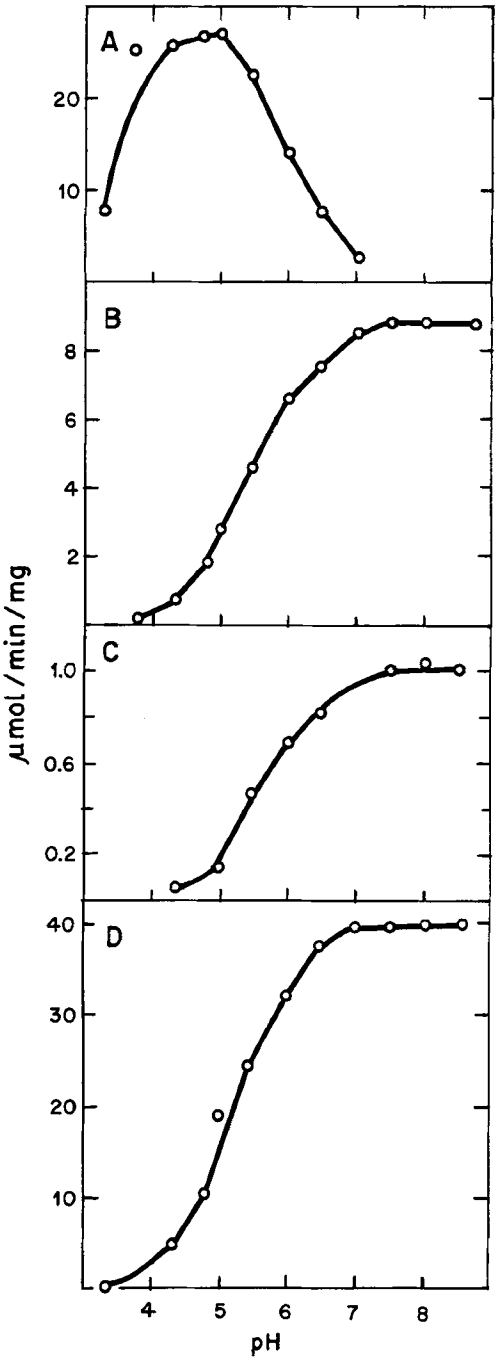


Figure 1. pH profiles for the malt carboxypeptidase II catalyzed hydrolysis of FA-Phe-Gly-OH (A), FA-Phe-Gly-NH_2 (B), FA-Phe-NH_2 (C) and FA-Phe-OMe (D). The assays were performed at 0.2 mM substrate in 25 mM buffer, 1 mM-EDTA, 1.0 M-NaCl, 2.5% CH_3OH. The buffers employed are listed in section 2.2.

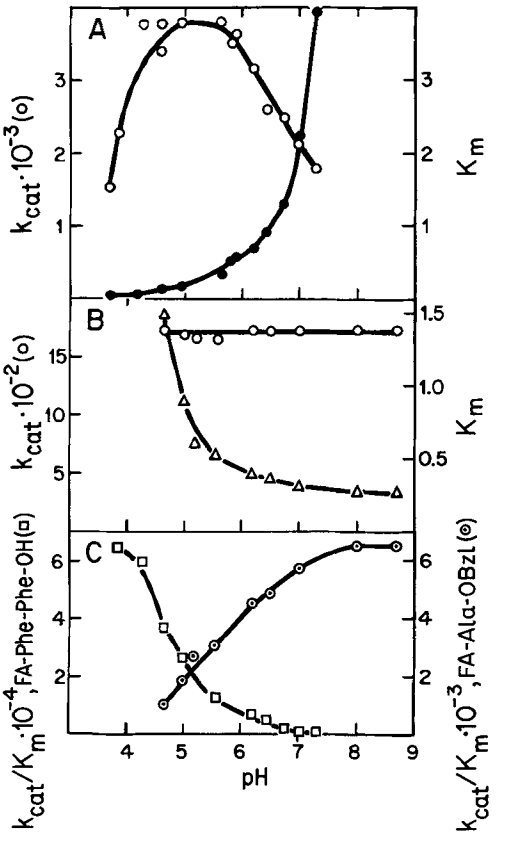


Figure 2. The influence of pH on the kinetic parameters for the carboxypeptidase II catalyzed hydrolysis of FA-Phe-Phe-OH (A) and FA-Ala-OBzl (B). Panel C shows the pH dependence of k_{cat}/K_m for the two substrates. The assays were performed in 25 mM buffer, 1 mM-EDTA. With FA-Ala-OBzl the assay mixture contained 2.5% CH_3OH. The buffers employed are listed in section 2.2.

used: 50 mM-triethyl ammonium phosphate, pH 3.0 (A-buffer) and 50 mM-triethyl ammonium phosphate, pH 3.0 in 50% CH_3CN (B-buffer) employing various linear and concave gradients. All separations were carried out at room temperature and monitored at 254 nm. The per cent composition of the reaction mixture was estimated directly from the integrated peak areas since all components had the Bz- or Z-group as dominant chromophore. Aminolysis and hydrolysis products were collected and identified by amino acid analysis. The fraction of

Table I. Hydrolysis of Bz-X-OMe substrates (X= amino acid residue) with malt carboxypeptidases I and II

Substrate	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	
	Carboxypeptidase II	Carboxypeptidase I
Bz-Gly-OMe	< 5	< 5
Bz-Ala-OMe	19	370
Bz-Val-OMe	7.1	22
Bz-Ile-OMe	7.5	17
Bz-Leu-OMe	310	420
Bz-Met-OMe	160	1200
Bz-Phe-OMe	5700	14600
Bz-Pro-OMe	< 5	< 5
Bz-Asp-OMe	< 5	< 5
Bz-Lys-OMe	2200	18300
Bz-Arg-OMe	950	25300
Bz-His-OMe	76	5600
Bz-Thr-OMe	< 5	69

Assay conditions: 0.5 mM substrate, 2.5% CH_3OH, 25 mM-Hepes, 1 mM-EDTA pH 8.0, 25 °C. With Bz-Lys-OMe, Bz-Arg-OMe and Bz-His-OMe CH_3OH was omitted. The rate of hydrolysis was measured spectrophotometrically at 254 nm and followed to more than 95% completion. From the progression curves k_{cat}/K_m values were determined utilizing the integrated form of the Michaelis-Menten equation (17). The values were based on the molecular weights of the monomer (60,000). The values for malt carboxypeptidase I were taken from ref. 4.

aminolysis was expresssed as the ratio between the formed aminolysis product and the sum of all products being formed, i.e. unconsumed substrate was disregarded in the calculations.

3. RESULTS

3.1. Enzymatic properties of malt carboxypeptidase II

The pH profiles for the peptidase, esterase, amidase and peptidyl amino acid amide hydrolase activities of malt carboxypeptidase II towards FA-Phe$\downarrow$Gly-OH, FA-Phe$\downarrow$OMe, FA-Phe$\downarrow$$NH_2$ and FA-Phe$\downarrow$Gly-NH_2, respectively, are shown in Figure 1. The stability of the enzyme (9) allowed the enzymatic activity to be studied in the pH range 3.4 – 8.7. The pH profiles reflect k_{cat}/K_m since the hydrolysis reactions were carried out at a substrate concentration below K_m. The hydrolysis of FA-Phe$\downarrow$OMe, FA-Phe$\downarrow$$NH_2$ and FA-Phe$\downarrow$Gly-NH_2 is optimal at pH > 7, and apparently dependent on deprotonation of a group with a pK_a of 5.2 – 5.6. In contrast, the hydrolysis of the peptide substrate FA-Phe$\downarrow$Gly-OH is optimal at pH 4.7. The difference in pH

Table II. The influence of the size of the alcohol leaving group on the kinetic parameters of malt carboxypeptidase II catalyzed ester hydrolysis.

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
Bz-Arg-OMe	0.30	350	1200
Bz-Arg-OEt	0.13	110	860
Bz-Arg-OBu	0.037	770	21000

The substrates were assayed in 25 mM-Hepes, 1 mM-EDTA, pH 8.0 at 25 °C.

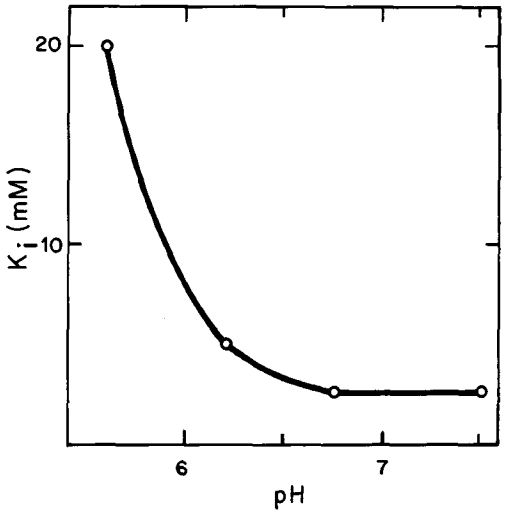


Figure 3. The competitive inhibition of PhGu on the hydrolysis of FA-Ala↓Lys-OH. The influence of pH on K_i. The inhibition of PhGu was studied in 25 mM buffer, 1 mM-EDTA. The buffers employed are listed in section 2.2.

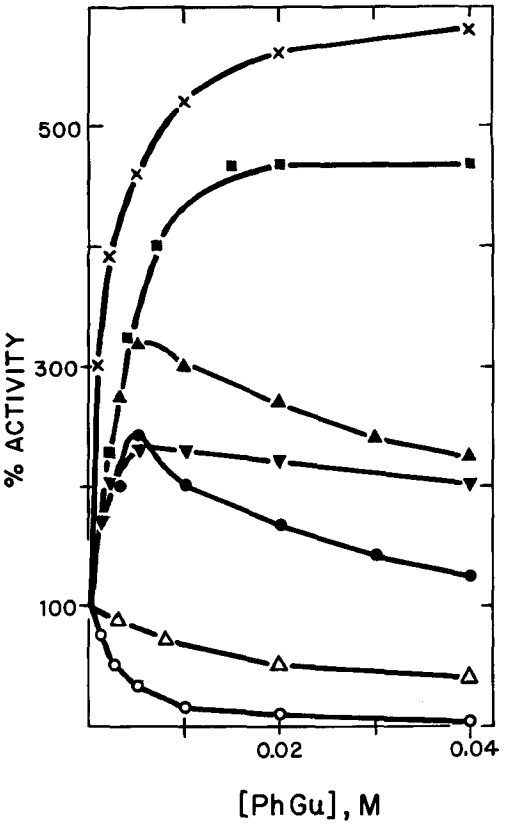


Figure 4. The influence of PhGu on the activities of malt carboxypeptidase. The assays with FA-Arg↓OMe (△), FA-Phe↓OMe (X), FA-Phe↓NH_2 (■) and FA-Phe↓Gly-NH_2 (▼) were performed in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5 and those with FA-Ala↓Lys-OH (○), FA-Phe↓Gly-OH (▲) and FA-Phe↓Phe-OH (●) were performed in 0.05 mM-Mes, 1 mM-EDTA, pH 6.75. A substrate concentration of 0.2 mM was used and the assay mixture contained 2.5% CH_3OH except with FA-Ala↓Lys-OH and FA-Arg↓OMe. The indicated concentrations of PhGu were included in the assays.

profile between ester and peptide hydrolysis was investigated in further detail with the substrates FA-Ala↓OBzl and FA-Phe↓Phe-OH. k_{cat} for the hydrolysis of FA-Phe↓Phe-OH (Figure 2A) increases with pH and reaches a maximum at pH 4.6 and again declines above pH 6. For FA-Ala↓OBzl k_{cat} is constant in the pH range 4.6 – 8.7. On the other hand, K_m increases with pH for the hydrolysis of FA-Phe↓Phe-OH (Figure 2A) while it decreases with pH for the hydrolysis of FA-Ala↓OBzl (Figure 2B). These different effects of pH on K_m explain why k_{cat}/K_m for the hydrolysis of FA-Phe↓Phe-OH is optimal below pH 4 while for FA-Ala↓OBzl it is optimal above pH 7.5 (Figure 2C). High K_m values precluded determinations at pH values above 7.3 for FA-Phe↓Phe-OH and below 4.6 for FA-Ala↓OBzl.

The specificity of carboxypeptidase II with respect to the P_1 position of peptide substrates has previously been determined (9) and the specificity with respect to the P_1 position of ester substrates was investigated at pH 8.0 using a series of substrates with the general formula Bz-X↓OMe (Table I). The highest k_{cat}/K_m is obtained for Bz-Phe↓OMe and this value is 15 - 30 times higher than the value obtained with substrates with an aliphatic side-chain with an unbranched β carbon, i.e. Leu and Met. The substrates with a branched β carbon, i.e. Val and Ile, are hydrolysed with very low rates, and those with Pro, Gly, Thr and Asp are hydrolysed with negligible rates. The substrates where the X-position is occupied by an amino acid residue with a positively charged side-chain, i.e. Arg and

Table III. The effects of PhGu and NaCl on the kinetic parameters for the malt carboxypeptidase II catalyzed hydrolysis reactions.

Substrate		Effector		
		0	20 mM-PhGu	1 M-NaCl
FA-Phe-OMe[a)]	k_{cat}	5600	9500	5700
	K_m	0.80	0.12	0.24
	k_{cat}/K_m	7000	79000	24000
	% k_{cat}/K_m		1100	340
FA-Phe-OEt[a)]	k_{cat}	5200	8200	5400
	K_m	0.75	0.18	0.26
	k_{cat}/K_m	6900	46000	21000
	% k_{cat}/K_m		670	300
FA-Ala-OEt[a)]	k_{cat}	140	2200	220
	K_m	4.3	4.3	2.1
	k_{cat}/K_m	33	510	105
	% k_{cat}/K_m		1500	320
FA-Ala-OBzl[a)]	k_{cat}	1600	2800	1600
	K_m	0.36	0.13	0.13
	k_{cat}/K_m	4400	22000	12000
	% k_{cat}/K_m		500	270
FA-Arg-OMe[a)]	k_{cat}	180	230	430
	K_m	0.19	0.77	2.7
	k_{cat}/K_m	950	300	160
	% k_{cat}/K_m		32	17
FA-Phe-Phe-OH[b)]	k_{cat}	3500	1100	2000
	K_m	2.8	0.56	0.83
	k_{cat}/K_m	1300	2000	2400
	% k_{cat}/K_m		150	180
FA-Ala-Lys-OH[b,c)]	k_{cat}	17100	17100[c)]	9600
	K_m	0.53	1.4[c)]	1.6
	k_{cat}/K_m	32000	12000[c)]	6000
	% k_{cat}/K_m		38	19

k_{cat} values are in min^{-1}, K_m values in mM and k_{cat}/K_m in $min^{-1} \cdot mM^{-1}$.
a) The substrates were assayed in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5.
b) The substrates were assayed in 0.05 M-Mes, 1 mM-EDTA, pH 6.75.
c) The assays were carried out in only 5 mM-PhGu.

Lys, are hydrolysed with relatively high rates. The concentrations of Bz-Arg$\downarrow$OMe, Bz-Lys$\downarrow$OMe and Bz-Phe$\downarrow$OMe could be varied sufficiently to allow determination of k_{cat} and K_m from linear Lineweaver-Burk plots. The values were for Bz-Phe-OMe: $k_{cat} = 5600\ min^{-1}$, $K_m = 1.0$ mM; for Bz-Arg$\downarrow$OMe: $k_{cat} = 350\ min^{-1}$, $K_m = 0.30$ mM; and for Bz-Lys$\downarrow$OMe: $k_{cat} = 850\ min^{-1}$, $K_m = 0.30$ mM.

The influence of the size of the alcohol leaving group of ester substrates was investigated using three Bz-Arg$\downarrow$OX substrates (X = Me, Et, Bu) (Table II). It is seen that K_m decreases with increasing size/hydrophobicity of the leaving

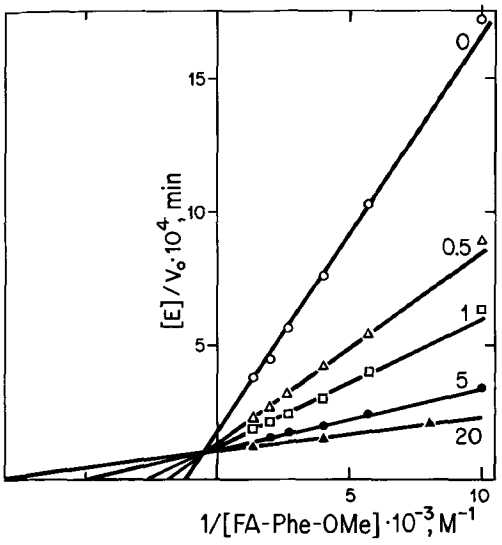


Figure 5. Lineweaver-Burk plots of the hydrolysis of FA-Phe↓OMe in 0.05 M-Hepes, 1 mM-EDTA, 2.5% CH_3OH, pH 7.5 containing variable concentrations of PhGu. The numbers indicate the concentrations of PhGu in mM.

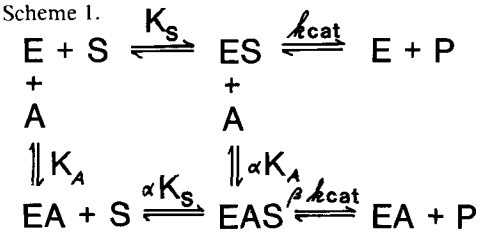
group. k_{cat} also varies with the size of the leaving group in the following manner: Bz-Arg↓OBu > Bz-Arg↓OMe > Bz-Arg↓OEt.

3.2. Influence of PhGu on malt carboxypeptidase II

PhGu has previously been shown to have a pronounced influence on the enzymatic properties of malt carboxypeptidase I (4) and hence, it was tested whether malt carboxypeptidase II also is affected by this substance. Lineweaver-Burk plots at different concentrations of PhGu indicates that it acts as a competitive inhibitor with respect to the hydrolysis of FA-Ala↓Lys-OH and K_i decreases with pH with the lowest values being observed in the pH range 6.75 to 7.5 (see Figure 3). For this reason the influence of PhGu on the ability of carboxypeptidase II to hydrolyse peptide substrates was studied at pH 6.75 in spite of the much more acidic pH optimum of its peptidase activity while the influence of PhGu on the hydrolysis of ester and amide substrates was studied at pH 7.5, i.e. the optimum of these activities. PhGu decreases rates of hydrolysis of an ester substrate containing a basic amino acid residue in the P_1 position, i.e. FA-Arg↓OMe, and a peptide substrate with a basic amino acid residue in the P_1' position i.e. FA-Ala↓Lys-OH (Figure 4, open symbols). In contrast, PhGu increases the rates of hydrolysis of the uncharged substrates FA-Phe↓OMe and FA-Phe↓NH_2 until the activities reach constant levels indicating that the enzyme becomes saturated with this substance (Figure 4, closed symbols). The rates of hydrolysis of peptide and peptide amide substrates where both the P_1 and P_1' positions are occupied by uncharged amino acid residues are also increased by the addition of PhGu but at concentrations above 5 mM the rates decrease (Figure 4).

The kinetic parameters for the hydrolysis of a series of substrates in the absence and in the presence of 20 mM-PhGu (Table III) confirm that the influence of PhGu is dependent on the substrate: with FA-Phe↓OMe, FA-Phe↓OEt, and FA-Ala↓OBzl K_m is reduced and k_{cat} is increased; with FA-Ala↓OEt k_{cat} is drastically increased while K_m is unchanged; with FA-Arg↓OMe both k_{cat} and K_m increase and with FA-Phe↓Phe-OH both k_{cat} and K_m decrease. These effects of PhGu were studied in further detail by determination of the kinetic parameters for these substrates at different concentrations of PhGu. As an example, the Lineweaver-Burk plots obtained for FA-Phe↓OMe are shown in Figure 5. The effects of PhGu can be described in terms of a nonessential activator/mixed inhibitor mechanism, shown in its general form in Scheme 1, as described by SEGEL (17). α and β are the factors by which K_s and k_{cat} change when A occupies the enzyme and K_A is the dissociation constant of the EA complex. If rapid equilibrium assumptions are applied to Scheme 1 values for α, β and K_A can be obtained from a plot of $K_m^\circ/\Delta K_m$ and $k_{cat}^\circ/\Delta k_{cat}$ versus 1/[PhGu] as described by SEGEL (17) and modified by ROHRBACH et al. (15). The

Scheme 1.



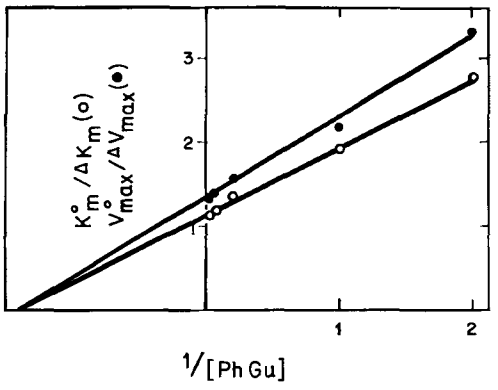


Figure 6. Replot of $V^{\circ}_{max}/\Delta V_{max}$ and $K^{\circ}_m/\Delta K_m$ versus 1/[FA-Phe-OMe] as described in ref. 15. The intersection of both lines with the X-axis is equal to $-1/\alpha K_A$ while the intersection of $V^{\circ}_{max}/\Delta V_{max}$ with the Y-axis equals $1/(\beta-1)$ and the intersection of $K^{\circ}_m/\Delta K_m$ equals $1/(1-\alpha)$.

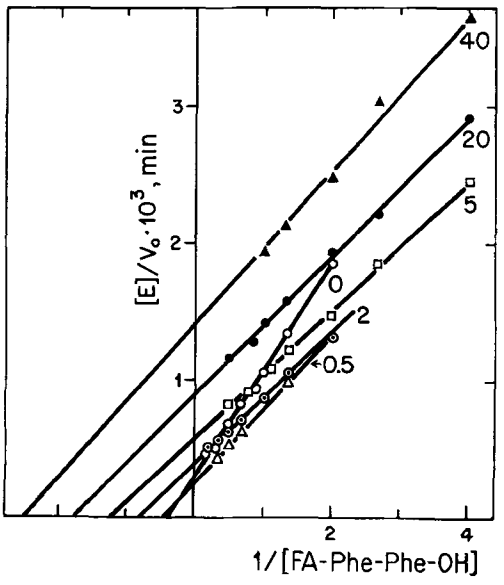


Figure 7. Lineweaver-Burk plots of the hydrolysis of FA-Phe↓Phe-OH in 0.05 M-Mes, 1 mM-EDTA, 2.5% CH_3OH, pH 6.5 containing variable concentrations of PhGu. The numbers indicate the concentrations of PhGu in mM.

plot obtained for FA-Phe↓OMe is shown in Figure 6. The values for α, β and K_A obtained in this manner for the substrates used in Table III are listed in Table IV. It is seen that K_A varies between 6 and 8 mM when determined by assays of esterase activity and that β with these substrates is higher than unity, and highest for FA-Ala↓OEt with a value of 26. The α values are below 1 for FA-Phe-OMe, FA-Phe↓OEt and FA-Ala↓OBzl, 1.0 for FA-Ala↓OEt and importantly, significantly higher than 1 for the charged ester substrate FA-Arg↓OMe. For FA-Ala↓Lys-OH the competitive nature of the inhibition of PhGu dictates that α is infinity while β is 1. With FA-Phe↓Phe-OH as substrate the influence of PhGu is rather complex. Up to 0.5 mM-PhGu the Lineweaver-Burk plots pivot clockwise (Figure 7) corresponding to an increase in k_{cat} and a decrease in K_m similar to the observations with FA-Phe↓OMe. However, further increases in the concentration of PhGu produce a series of parallel Lineweaver-Burk plots corresponding to reductions in both k_{cat} and K_m indicating that PhGu at high concentrations exhibits uncompetitive inhibition. For this mode of inhibition a K_i of approximately 20 mM was determined from a plot of $1/K_m$ and $1/k_{cat}$ versus [PhGu] as described by SEGEL (17) (plot not shown).

Table IV. Binding of PhGu to malt carboxypeptidase II: the influence of the substrate structure on the α, β and K_A values.

Substrate	α	β	K_A or K_i (mM)
FA-Phe-OMe[a]	0.12	1.8	5.8
FA-Phe-OEt[a]	0.17	1.8	6.3
FA-Ala-OEt[a]	1.0	26	8
FA-Ala-OBzl[a]	0.31	1.9	7.0
FA-Arg-OMe[a]	9	4	8
FA-Ala-Lys-OH[b]	∞	1.0	3
FA-Phe-Phe-OH[b]	complex, see text		

The α, β and K_A values (see Scheme 1) were calculated on the basis of k_{cat} and K_m values, determined at a minimum of five different concentrations of PhGu. a) The hydrolysis was carried out in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5. b) hydrolysis carried out in 0.05 M-Mes, 1 mM-EDTA, pH 6.75.

3.3. The influence of added salt on malt carboxypeptidase II catalyzed hydrolysis

The malt carboxypeptidase II catalysed hydrolysis of ester and peptide substrates contain-

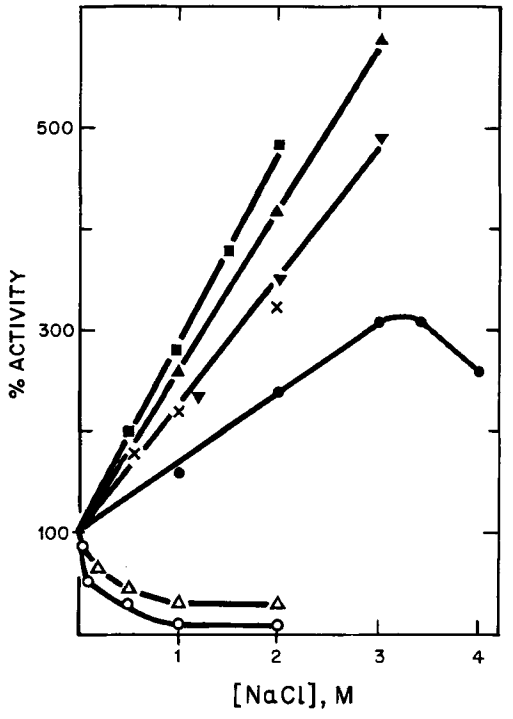


Figure 8. The influence of NaCl on the activities of malt carboxypeptidase. The activities were assayed as described in Figure 4.

ing basic amino acid residues on the P_1 or P_1' position, i.e. FA-Arg↓OMe and FA-Ala↓Lys-OH, are adversely affected by the addition of NaCl to the assay medium (Figure 8, open symbols). With substrates containing uncharged amino acid residues on these positions NaCl has the opposite effect: the rates of hydrolysis of FA-Phe↓OMe, FA-Phe↓NH_2, FA-Phe↓Gly-NH_2, and FA-Phe↓Gly-OH increase with the concentration of NaCl (Figure 8, closed symbols). This is also observed with FA-Phe↓Phe-OH as substrate up to 3 M-NaCl; at higher concentrations a reduction in the rate of hydrolysis is observed (Figure 8, closed symbols). For hydrophobic substrates the solubility decreases with ionic strength. However, at 1 M-NaCl the influence of NaCl on the kinetic parameters for the hydrolysis of a series of substrates could be compared (Table III). For the hydrophobic substrates, i.e. FA-Phe↓OMe and FA-Phe↓OEt, FA-Ala↓OEt, FA-Ala↓OBzl and FA-Phe↓Phe-OH K_m decreases while it increases for FA-Ala↓Lys-OH and FA-Arg↓OMe, containing positively charged groups. k_{cat} remains constant or is slightly increased for all ester substrates while it is decreased for the peptide substrates.

These effects of NaCl were studied by determination of the kinetic parameters for each substrate at different concentrations of NaCl and analyzing the results in terms of the general scheme for non-essential activation and mixed type inhibition as described in section 3.2. With the hydrophobic ester substrates, i.e. FA-Phe↓OMe, FA-Phe↓OEt, FA-Ala↓OBzl and FA-Ala↓OEt, the values for α are around 0.05 and β varies between 1.0 and 2.5 (Table V). With FA-Arg↓OMe and FA-Ala↓Lys-OH the values for α are higher than unity, i.e. 50 and 2, respectively, and the values for β are 4 and 0.5, respectively. Thus, the influence of NaCl on the hydrolysis of substrates containing positively charged amino acid residues differs from that on those containing uncharged amino acid residues. This is also apparent from the widely different K_A values observed with these two groups of substrates: 0.05 M and 0.09 M with FA-Arg↓OMe and FA-Ala↓Lys-OH, respectively, and values ranging from 1.3 M to one exceeding 20 M with the hydrophobic substrates. The influence of Na_2SO_4 on the hydrolysis of FA-Phe↓OMe has also been studied. The values for α and β are identical to those obtained with NaCl, i.e. 0.06

Table V. The effect of NaCl on malt carboxypeptidase II: influence of the substrate structure on the α, β and K_A values for NaCl.

Substrate	α	β	K_A or Ki (M)
FA-Phe-OMe[a,c]	0.06	1.0	6
FA-Phe-OEt[a]	0.06	1.0	3
FA-Ala-OEt[a]	< 0.04	2.5	> 20
FA-Ala-OBzl[a]	0.19	1.0	1.3
FA-Arg-OMe[a]	50	4	0.05
FA-Ala-Lys-OH[b]	1.9	0.5	0.09

The α, β and K_A values (see Scheme 1) were calculated on the basis of k_{cat} and K_m values, determined at a minimum of five different concentrations of NaCl. a) The hydrolysis was carried out in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5. b) the hydrolysis carried out in 0.05 M-Mes, 1 mM-EDTA, pH 6.75. c) with Na_2SO_4 the values for α, β and K_A are 0.06, 1.0 and 3 M, respectively.

and 1.0, respectively, but the value for K_A is 3 M as compared with 6 M for NaCl.

3.4. Malt carboxypeptidase II catalyzed peptide synthesis

The ability of carboxypeptidase II to catalyze the aminolysis of N-blocked amino acid esters has been investigated at pH 8.5 where the enzyme remains stable for at least 4 hours. At this pH the enzyme exhibits maximal esterase and amidase activity and minimal peptidase activity (see Figure 1). The reaction was initially studied with Bz-Arg↓OBu as substrate and H-Gly-NH_2 as nucleophile. Two products were formed: Bz-Arg-OH and Bz-Arg-Gly-NH_2 due to the partitioning of the acyl-enzyme intermediate between the hydrolysis reaction and the aminolysis reaction. The fraction undergoing aminolysis increased with increasing concentrations of H-Gly-NH_2 but did not exceed 0.95 (data not shown). The fraction of aminolysis was constant at each concentration of nucleophile and thus essentially independent of the concentration of substrate remaining in the reaction mixture. However, the rate of substrate disappearance decreased significantly with increasing concentrations of H-Gly-NH_2 indicating an inhibitor function of the nucleophile. The observation that the enzyme becomes saturated with nucleophile such that the fraction of aminolysis does not exceed 0.95 is compatible with the nucleophile binding to the acyl enzyme intermediate prior to the deacylation reaction. The capability of the enzyme to bind nucleophiles in this fashion may conveniently be estimated from the concentration of nucleophile required to produce a fraction of aminolysis which is half the maximal aminolysis fraction as previously described for carboxypeptidase I (7). This concentration is designated $K_{N(app)}$ since it is related to the dissociation constant of the complex between acyl-enzyme and nucleophile. Table VI lists the $K_{N(app)}$ values for a series of amino acid amides. It is observed that $K_{N(app)}$ is identical (13 mM) for H-Gly-NH_2, H-Ser-NH_2 and H-Val-NH_2 while it is only approximately 1 mM for H-Lys-NH_2 and H-Arg-NH_2. The action of carboxypeptidase II on Bz-Arg-OBu in the presence of amino acids or amino acid methyl esters also yields aminolysis products but with these nucleophiles a maximum of aminolysis could not be reached. The values obtained at the highest concentrations of nucleophile

Table VI. Malt carboxypeptidase II catalyzed aminolysis of Bz-Arg-OBu using amino acids, amino acid amides and amino acid methyl esters as nucleophiles.

Nucleophile	Maximum fraction of aminolysis	$K_{N(app)}$ (M)
H-Gly-NH_2	0.95	0.013
H-Ser-NH_2	0.85	0.013
H-Val-NH_2	0.90	0.013
H-Arg-NH_2	0.75	~ 0.001
H-Lys-NH_2	0.85	~ 0.001
H-Gly-OH[a)]	0.65 (3.0 M)	
H-Ser-OH[a)]	0.50 (3.0 M)	
H-Val-OH[a)]	0.30 (0.5 M)	
H-Gly-OMe[a)]	0.40 (3.0 M)	
H-Ser-OMe[a)]	0.25 (3.0 M)	
H-Val-OMe[a)]	0.55 (1.0 M)	

Conditions: 2 mM-Bz-Arg-OBu, 5 mM-EDTA, 0.4 – 4 μM-carboxypeptidase II. Reaction time: 5 – 50 min.
[a)] Saturation with these nucleophiles could not be obtained.

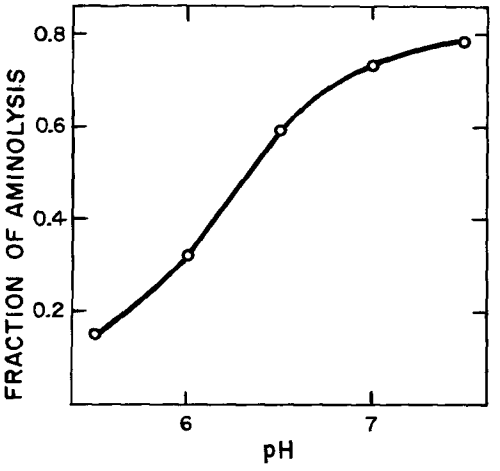


Figure 9. The influence of pH on the fraction of aminolysis in carboxypeptidase II catalyzed transpeptidation reactions. Reaction conditions: 2 mM-Bz-Lys↓Ala-OH, 5 mM-EDTA, 1 M-H-Gly-NH_2, containing 0.12 μM (pH 5.5), 0.20 μM (pH 6.0), 0.24 μM (pH 6.5), 0.55 μM (pH 7.0) or 0.80 μM (pH 7.5) carboxypeptidase II. Reaction time: 25 – 80 min.

tested are somewhat lower (0.25 – 0.65) than those obtained with amino acid amides (Table VI).

The ability of malt carboxypeptidase II to catalyze transpeptidation reactions was initially studied with the excellent substrate Bz-Lys↓Ala-OH (k_{cat} = 2000 min^{-1}, K_m = 0.12 mM) and with H-Gly-NH_2 (1 M) as nucleophile. Two products were formed: Bz-Lys-OH and Bz-Lys-Gly-NH_2 and at pH 5.5 the fraction of aminolysis was only 0.15 but this value increased with pH to 0.80 at pH 7.5 (Figure 9). It was not feasible to increase pH further due to the decreased peptidase activity at basic pH values. An increase in the concentration of H-Gly-NH_2 to 2 M did not increase the fraction of aminolysis at pH 7.5 but at acidic pH it was increased significantly suggesting that the enzyme was not saturated with nucleophile in this pH range. With H-Val-NH_2, H-Thr-NH_2 or H-Ser-NH_2 as nucleophiles and Bz-Lys↓Ala-OH as substrate the fractions of aminolysis at pH 7.5 were 0.70, 0.85 and 0.80, respectively.

The influence of the nature of the C-terminal amino acid residues of the peptide substrate on the fraction of aminolysis was studied with a series of Z-Ala-X-OH substrates (X = amino acid residue) using H-Gly-NH_2 as nucleophile (Table VII). It is seen that the fractions of aminolysis are high (0.80 – 0.90) when X = Gly, Ala or Ser while they are low (0.15 – 0.35) for all the other substrates.

4. DISCUSSION

It is characteristic for the serine carboxypeptidases thus far investigated that they hydrolyse peptides with an acidic pH optimum while they act on substrates with blocked C-terminus, i.e. peptide esters and amides, with a basic pH optimum (13). With malt carboxypeptidase II k_{cat} for peptide hydrolysis correlates with the deprotonation of a group with a pK_a around 4 and the protonation of a group with a pK_a around 7. The fact that k_{cat} for ester hydrolysis is constant in the pH range 4.6 to 8.7 precludes the involvement of a group with a pK_a around 7 in ester hydrolysis but the significance of a group with a pK_a around 4 cannot be established since the kinetic constants could not be determined below pH 4.6. However, it is probable that a common group determines both activities as in the case of carboxypeptidase Y (2, 8) but with the important difference that it exhibits a pK_a around 4 in carboxypeptidase II as compared with 5.5 in carboxypeptidase Y. The

Table VII. The influence of the substrate structure on the fraction of aminolysis in malt carboxypeptidase II catalyzed aminolysis reactions using H-Gly-NH_2 as nucleophile.

Substrate	enzyme conc. (μM)	reaction time (min)	fraction of aminolysis (Z-Ala-Gly-NH_2)
Z-Ala-Gly-OH	7.5	500	0.80
Z-Ala-Ala-OH	1.5	90	0.90
Z-Ala-Ser-OH	1.5	270	0.90
Z-Ala-Arg-OH	3.7	10	0.35
Z-Ala-Lys-OH	3.7	12	0.15
Z-Ala-Pro-OH	28	120	0.20
Z-Ala-Asn-OH	1.5	35	0.35
Z-Ala-His-OH	1.5	80	0.35
Z-Ala-Val-OH	3.7	30	0.35
Z-Ala-Met-OH	14	15	0.20
Z-Ala-Phe-OH	14	80	0.20
Z-Ala-Asp-OH	28	300	–

Conditions: 20 mM substrate, 1 M-H-Gly-NH_2, 5 mM-EDTA, pH 7.5.
– indicates that no reaction has taken place.

inactivation of serine carboxypeptidases with diisopropylfluorophosphate suggests mechanistic similarities with the serine endopeptidases and consequently, these groups should be histidyl residues (13). However, alternative mechanisms are possible, in particular since a pK_a of 4 as observed for carboxypeptidase II is unusually low for a histidyl residue.

K_m for peptide hydrolysis appears to be dependent on the protonation of a group with $pK_a > 7$ as observed in carboxypeptidase Y and it is probable that it at low pH functions as a positively charged binding site for the negatively charged carboxylate group of peptide substrates. Consistent with this, K_m for hydrolysis of ester substrates, containing blocked carboxyl groups, is essentially constant above pH 6 and the different pH optima of k_{cat}/K_m for hydrolysis of esters (pH > 7.5) and peptides (pH < 4) are consequently due to different pH dependencies of K_m. Since similar differences were observed with carboxypeptidase Y (2, 8), this is possibly general for serine carboxypeptidases.

The influence on k_{cat} of the alcohol leaving group of ester series Bz-Arg↓OMe, -OEt, -OBu, indicates that the deacylation step is not rate-limiting in ester hydrolysis in accordance with the observations for malt carboxypeptidase I (4) and carboxypeptidase Y (2).

The malt carboxypeptidase II catalyzed hydrolysis of ester substrates with the general formula Bz-X↓OMe indicates a wide specificity with preference for substrates where X = Phe, Arg, Lys over those where the X-position is occupied by an amino acid residue with an uncharged aliphatic or acidic side-chain. The high k_{cat}/K_m value for the hydrolysis of Bz-Phe↓OMe is mainly due to a high k_{cat} value while for Bz-Arg↓OMe and Bz-Lys-OMe it is mainly due to a low K_m value. The previous study with peptide substrates of the general formula Z-Ala↓X-OH indicated a wide specificity with preference for substrates where X = Arg, Lys or hydrophobic amino acid residues (9). Thus, malt carboxypeptidase II possesses the ability to hydrolyse substrates containing either hydrophobic or positively charged amino acid residues on both the P_1 and the P_1' positions. This is hardly consistent with the general understanding that a proteolytic enzyme contains only a single

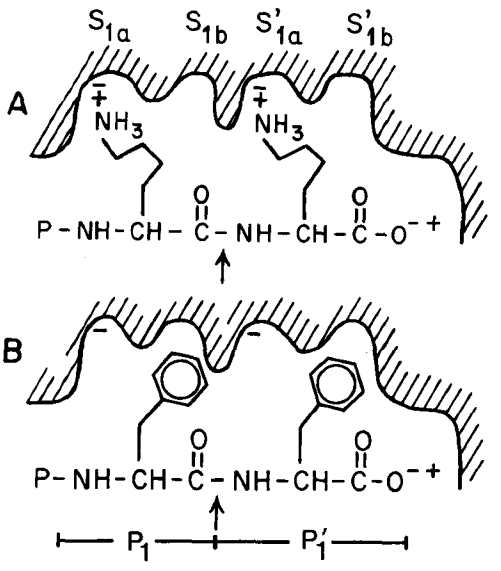


Figure 10. Schematic representation of the binding regions in malt carboxypeptidase II. The binding notation is that of SCHECHTER and BERGER (see abbreviations), and the dead-end structure is assumed to account for the exo-peptidase activity of the enzyme. (A) shows a proposed binding mode of a peptide with lysyl residues in the P_1 and P_1' positions. It is assumed that the positively charged side-chains of Lys interact with negatively charged groups located in the S_{1a} and S'_{1a} portions of the S_1 and S_1' binding sites, respectively. (B) shows a proposed binding mode of an analogous peptide with phenylalanyl residues in these positions. Here it is assumed that the hydrophobic side-chains interact with the hydrophobic S_{1b} and S'_{1b} regions of the S_1 and S_1' binding sites, respectively.

binding site for each position of the substrate. It is more likely that within the S_1 and the S_1' binding sites separate areas exist to secure the interaction with these side-chains of different nature. A postulated binding region of the active site of malt carboxypeptidase II, incorporating this hypothesis, is shown in Figure 10. It is assumed that the S_{1a} and S'_{1a} portion of the S_1 and S_1' binding sites, respectively, contain negatively charged groups which interact with the positively charged side-chains of e.g. FA-Arg↓OMe and FA-Ala↓Lys-OH. The S_{1b} and S'_{1b} portions, on the other hand, are assumed to be hydrophobic regions which provide the inter-

action with hydrophobic side-chains of the substrate. Since the K_m values for FA-Phe↓OMe and FA-Ala↓Phe-OH are significantly higher than for FA-Arg↓OMe and FA-Ala↓Lys-OH, respectively, it would appear that the ionic interactions between substrate and enzyme are stronger than the hydrophobic interactions.

The observation that NaCl and PhGu inhibit the malt carboxypeptidase II catalyzed hydrolysis of substrates containing positively charged groups in the P_1 or P_1' positions and activate the hydrolysis of substrates containing hydrophobic groups in both positions support the hypothesis that each of the S_1 and S_1' binding sites are divided into two regions with affinities for basic (S_{1a} and S_{1a}') and hydrophobic (S_{1b} and S_{1b}') amino acid residues, respectively. The hydrolysis of FA-Ala-Lys-OH is competitively inhibited by PhGu, indicating that this substance completely abolishes this activity by preventing the interaction between the lysyl residue of the substrate and the S_{1a}' binding site. The hydrolysis of FA-Arg↓OMe is only partially inhibited by PhGu. At saturation with this substance K_m for the hydrolysis of FA-Arg↓OMe is increased drastically suggesting an adverse effect of PhGu on the binding of this substrate to the enzyme. It is conceivable that the ionic interaction between enzyme and substrate, i.e. binding of the arginyl residue to the S_{1a} binding site, is completely abolished at saturating concentrations of PhGu. Nevertheless, the enzyme retains an ability to hydrolyse FA-Arg↓OMe and interestingly, with four fold higher k_{cat}.

Binding of PhGu to malt carboxypeptidase II strongly activates its hydrolysis of substrates with hydrophobic groups on the P_1 and P_1' positions. With the ester substrates the results are consistent with a K_A of 6-8 mM and since this value is in fair agreement with the value for K_i obtained for FA-Ala↓Lys-OH (3 mM) and FA-Arg-OMe (8 mM) it is probable that the activation observed with the uncharged ester substrates is due to binding of PhGu to the binding sites for charged amino acid residues, i.e. to the S_{1a} and S_{1a}' binding sites. The ionic nature of the binding of PhGu to the enzyme is supported by the fact that in the presence of 1 M-NaCl, saturation of the enzyme with PhGu cannot be obtained, i.e. the activating effect of PhGu on the hydrolysis of FA-Phe↓OMe is negligible. It appears that the enzyme saturated with PhGu hydrolyses substrates containing bulky/hydrophobic groups in either the P_1 (Phe) or P_1' (– OBzl) positions with significantly reduced K_m. This suggests that such groups when bound to the S_{1b} or S_{1b}' sites interact with the phenyl group of the PhGu bound at the S_{1a} or S_{1a}' sites in such a manner that the binding of these substrates is increased. When non-bulky groups are bound to both the S_{1b} and S_{1b}' binding sites, viz. FA-Ala↓OEt, the interaction with the PhGu appears to increase k_{cat} rather than to increase binding of the substrate.

It would be expected that binding of PhGu to the S_{1a} and S_{1a}' sites influenced the hydrolysis of the peptide substrate FA-Phe↓Phe-OH, containing hydrophobic amino acid residues on both the P_1 and the P_1' positions, in a manner similar to that observed for the ester substrates containing bulky/hydrophobic groups. However, a similar decrease in K_m and increase in k_{cat} is observed only at low concentrations of PhGu; at higher concentrations an apparent third binding mode of PhGu reduces the activity of the enzyme towards this substrate. This inhibition ($K_i \sim 20$ mM) is uncompetitive, i.e. PhGu binds in this mode only to the enzyme-substrate complex. The activities of malt carboxypeptidase II towards other peptide and peptide amide substrates, i.e. FA-Ala↓Phe-OH, FA-Phe↓Gly-OH, FA-Phe↓Ala-OH, FA-Phe↓Val-OH, FA-Phe↓Gly-NH_2 and FA-Phe↓Val-NH_2, are similarly activated at low concentrations and inhibited at high concentrations of PhGu. The hydrolysis of FA-Phe↓NH_2 is also activated at low concentrations of PhGu but it is not inhibited at high concentrations. Thus, the uncompetitive mode of inhibition is observed only when the P_1' position is occupied by an amino acid or an amino acid amide.

It appears that the S_{1a} and S_{1a}' binding sites also may be saturated with NaCl with the consequence that the activities towards FA-Arg↓OMe and FA-Ala↓Lys-OH are partially inhibited. The K_i values are 50 mM and 90 mM for FA-Arg↓OMe and FA-Ala↓Lys-OH, respectively, and NaCl concentrations of that magnitude have very little influence on the activities towards substrates with hydrophobic amino

acid residues on the P_1 and P_1' positions. However, at much higher concentrations of NaCl the hydrolysis of uncharged ester substrates is activated, mainly due to a decrease in K_m. This activation is consistent with NaCl functioning as a non-essential activator for the hydrolysis of uncharged substrates by binding to the enzyme according to Scheme 1. However, since K_A is dependent on the nature of the substrate this interpretation is probably not correct. It is more probable that the increased ionic strength of the assay medium amplifies the hydrophobic interaction between enzyme and substrate and the K_A is then only a measure for the concentration of NaCl at which this effect becomes significant. This is consistent with the magnitude of K_A being related to the solubility of the substrate with the lowest values being observed with the least soluble substrate, i.e. FA-Ala$\downarrow$OBzl. For Na_2SO_4 the value of K_A is only 3 M with FA-Phe$\downarrow$OMe as substrate as compared with 6 M for NaCl consistent with the decreased solubility of the substrate in Na_2SO_4.

The action of malt carboxypeptidase II on peptide esters in the presence of amino acids or amino acid derivatives indicate that these substances compete with water as nucleophiles in the deacylation of the acyl-enzyme intermediate. The saturation phenomena observed with amino acid amides further indicate that these are bound to the acyl-enzyme intermediate prior to their participation in the deacylation reaction in analogy with previous observations with malt carboxypeptidase I (7). It is probable that the aminolysis reaction with amino acids and amino acid methyl esters involve a similar binding step but the high dissociation constant of these complexes ($K_{N(app)}$) make the demonstration of this impossible. Assuming that amino acid amides are bound to the same site as the leaving group in the acylation reaction, i.e. the S_1' binding site, the results with different amino acid amides support a division of this binding site into two regions with the one, presumably the S_{1a}' binding site, providing tight ionic binding of H-Lys-NH_2 and H-Arg-NH_2. The other region of the S_1' binding site provides a much less tight binding of amino acid amides containing uncharged side-chains. However, this region cannot be assigned to the S_{1b}' site since the hydrophobic properties of this site would have predicted better binding of H-Val-NH_2 than of H-Ser-NH_2 and H-Gly-NH_2 while in fact, all these uncharged nucleophiles exhibit identical $K_{N(app)}$. A similar lack of dependence on the side-chain of amino acid amides has previously been observed in malt carboxypeptidase I catalyzed aminolysis reactions (7).

The specificities of malt carboxypeptidases I (4) and II thus indicate that both enzymes contain S_1 binding sites with separate binding regions for positively charged and uncharged amino acid side-chains. The S_1' binding site appears only to be divided in carboxypeptidase II since carboxypeptidase I essentially is unable to hydrolyse peptides containing C-terminal Arg or Lys (3).

The specificity of carboxypeptidase Y towards the C-terminus of peptide chains and its ability to catalyze aminolysis of peptide esters using amino acids or amino acid derivatives as amine components has rendered this enzyme a useful tool in the step-wise synthesis of polypeptides (1, 18, 19, 20). In addition, when the enzyme acts on polypeptides in the presence of nucleophiles it catalyzes transpeptidation reactions, i.e. the incorporation of the nucleophile in place of the C-terminal amino acid residue. This reaction can be utilized to alter the C-terminal amino acid residue, e.g. the conversion of porcine insulin to human insulin (12), to convert peptides, e.g. those obtained by genetic engineering, to peptide amides, or to incorporate various probes into peptides and proteins (10, 11). However, carboxypeptidase Y with a preference for hydrophic amino acid residues on the P_1 and P_1' position (2, 3) is not suitable for the synthesis and semisynthesis of all peptide bonds. Although chemically modified derivatives of this enzyme with altered specificities may be useful for many reactions and among these the semisynthesis of human insulin (6) a demand exists for serine carboxypeptidases with specificities which can supplement that of carboxypeptidase Y. One such alternative is malt carboxypeptidase II since it is available in large quantities (9). The results presented in this paper indicate that this enzyme is particularly suited in the synthesis of peptide bonds where a basic amino acid residue donates the acyl portion and/or the

imine portion. The aminolysis reactions of Bz-Arg↓OBu using a number of different nucleophiles including H-Arg-NH_2 and H-Lys-NH_2 are examples of how the enzyme can be employed in the step-wise synthesis of such peptides, and the transpeptidation reactions with Bz-Lys-Ala-OH using a series of amino acid amides as nucleophiles are examples of how it can be employed in the semisynthesis of peptides and proteins.

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