INFLUENCE OF GUANIDINE DERIVATIVES ON THE SPECIFICITY OF MALT CARBOXYPEPTIDASE

by

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Malt carboxypeptidase (Carlsberg Res. Commun. 48, 217-230, 1983) catalyzes hydrolysis of ester substrates with the general formula Bz-X-OMe with preference for substrates where X = Phe, Arg or Lys over those where the X-position is occupied by an amino acid residue with a neutral or acid aliphatic side chain. The rapid hydrolysis of Bz-Phe-OMe is mainly due to a high k_{cat} value while for Bz-Arg-OMe it is mainly due to a low K_m value. Addition of salts result in decreased rates of hydrolysis of substrates where X = Lys or Arg (positively charged) and increased rates of hydrolysis of substrates where X = Phe or Leu (hydrophobic).

Guanido compounds influence the enzymatic properties of malt carboxypeptidase. Kinetic data for the hydrolysis of various substrates indicate that phenylguanidine binds to the S_1' binding site of the enzyme in such a manner that the rate of cleavage of ester or amide substrates with large groups in the P_1' position, i.e. -OEt, -OPr, -OBu, -Ala-OH, -Gly-NH₂, is reduced while substrates with small groups in this position i.e. -OMe, -NH₂ are hydrolyzed with increased rates. These observations have applications in deamidation reactions since the presence of phenylguanidine in the reaction medium results in a significantly increased yield of the deamidated product.

Abbreviations: Ac = acetyl; BiGu = 2-guanidobenzimidazol; Bu = butyl; Bz = benzoyl; DFP = diisopropylphosphorofluoridate; EDTA = ethylene diamine tetraacetic acid sodium salt; FA = furylacryloyl; Gu = guanidine hydrochloride; Hepes = N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; HPLC = high performance liquid chromatography; MeGu = methyl guanidine hydrochloride; Mes = 2-(N-morpholino)ethane sulfonic acid; PhGu = phenylguanidine hydrogencarbonate; PpGu = 1-(3-phenylpropylamino) guanidine hydrochloride; Pr = propyl; TEAP = triethyl ammonium phosphate; Tris = tris(hydroxy methyl) aminomethane; Z = carbobenzoxy. Abbreviations of amino acids, amino acid derivatives and peptides are according to the guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature. The binding notation for the enzyme and the substrates is that of SCHECHTER and BERGER. Accordingly, amino acid residues in the substrate are referred to as $P_1, P_2, \dots, P_i$ in the amino-terminal direction away from the scissible bond and P_1' for the carboxy-terminal residue that is hydrolyzed off. Enzyme subsites are denoted $S_1, S_2, \dots, S_i$ and S_1' in correspondence with the substrate.

1. INTRODUCTION

In a recent paper from this laboratory the isolation of a "serine carboxypeptidase" (E.C. 3.4.16) from malted barley was described (5) and it was demonstrated that this enzyme exhibited activities similar to carboxypeptidase Y from baker's yeast, the most extensively characterized enzyme in this class (9, 13). However, the carboxypeptidases from malt and yeast differ substantially with respect to molecular weight and other physical-chemical characteristics.

The binding sites of proteolytic enzymes determine their specificity and chemical modifications of amino acid residues in these sites have provided information about enzyme-substrate interactions for such enzymes as subtilisin (20), thermolysin (1) and carboxypeptidase Y (4). In the latter case with interesting applications as a consequence (5, 6). The work by INAGAMI and MURACHI demonstrated that information about binding sites could be obtained by addition of various effectors (11). Hence, it was of interest to determine the side group specificity of malt carboxypeptidase and to investigate whether effectors would influence its enzymatic properties. In the present paper, it is demonstrated that malt carboxypeptidase exhibits a side group specificity with respect to the P₁ position of the substrate which is reminiscent of the specificity of subtilisin, and that phenylguanidine drastically change the enzymatic properties of the enzyme.

2. MATERIALS AND METHODS

2.1. Materials

Malt carboxypeptidase was isolated by affinity chromatography as previously described (5). All synthesized substrates were checked by HPLC as described in reference 22, and it was ascertained that the purity was higher than 98%. Bz-Arg-OPr and Bz-Arg-OBu were prepared by esterification of Bz-Arg-OH with propanol/HCl and butanol/HCl, respectively. The reactions went to completion as judged by HPLC analysis. After evaporation of the alcohol, the oil containing the esters was washed extensively with ether to remove residual alcohol, resulting in a white solid. Melting points: 143-144 °C (Bz-Arg-OPr), 138-140 °C (Bz-Arg-OBu). Bz-Phe-OEt was synthesized from Bz-Phe-OH with ethanol/HCl and crystallized

from ethanol/water. Melting point: 102-103 °C. Bz-Pro-OMe was synthesized from Bz-Pro-OH with methanol/HCl and recrystallized from ethanol/water. Melting point: 88-89.5 °C. Bz-Phe-His-NH₂ was made by enzymatic synthesis in the following way: 1.4 g histidine amide · 2 HCl was dissolved in 25 ml 1 mM-EDTA and pH was adjusted to 9.5 by addition of NaOH. 5 mg carboxypeptidase Y was added followed by dropwise addition of 600 mg Bz-Phe-OEt dissolved in 3 ml methanol. A precipitate was formed and after 25 minutes the reaction was quenched by addition of 200 ml CH₃CN. HPLC identification indicated that the reaction mixture consisted of 65% Bz-Phe-His-NH₂, 10% Bz-Phe-OH and 25% Bz-Phe-OEt. The reaction mixture was evaporated to dryness. Bz-Phe-His-NH₂ was dissolved in water by addition of HCl until pH 2.6 and subsequent recrystallization from ethanol/water yielded the pure product. Melting point: 189-192 °C. The following compounds were synthesized as previously described: Bz-Gly-OMe (3), Bz-Phe-OMe (3), Bz-Ile-OMe (4), FA-Phe-NH₂ (5) and FA-Phe-Gly-NH₂ (5). FA-Phe-Ala-OH was a gift from Dr. S.B. SØRENSEN.

Bz-Ile-OH, Bz-Pro-OH, Bz-Val-OMe, Bz-His-OMe · HCl and Bz-Met-OMe were from Vega-Fox Chemicals, USA. Methyl guanidine hydrochloride, Hepes, Trisma base, Mes, Bz-Arg-OMe and Bz-Arg-OEt were obtained from Sigma, USA. Phenylguanidine hydrogencarbonate, 1-(3-phenylpropylamino) guanidine hydrochloride and 2-guanidino benzimidazol were obtained from EGA Chemie, W. Germany. All other peptides were purchased from Bachem, Switzerland. Other reagents and solvents were of analytical purity and obtained from Merck, W. Germany.

2.2. Methods

Esterase activity towards N-benzoylated amino acid esters was determined spectrophotometrically at 254 nm using a Cary 219 spectrophotometer thermostated at 25 °C. The specificity study was performed at pH 7.5 (the pH optimum for malt carboxypeptidase catalyzed ester hydrolysis) using 0.3 mM-substrate, 0.05 M-Hepes, 1 mM-EDTA. The hydrolysis was followed to completion and k_{cat}/K_m values were determined from the integrated form of the

Michaelis-Menten equation as described in reference 19. k_{cat} and K_m values for the malt carboxypeptidase catalyzed hydrolysis of Bz-Arg-OMe, Bz-Arg-OEt, Bz-Arg-OPr and Bz-Arg-OBu in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5 were graphically determined from Lineweaver-Burk plots, using substrate concentrations from 0.02 to 1.5 mM.

Esterase, amidase, peptidyl amino acid amide hydrolase and peptidase activities towards FA-Phe¹OMe, FA-Phe¹NH₂, FA-Phe¹Gly-NH₂ and FA-Phe¹Ala-OH, respectively, were measured spectrophotometrically at 337 nm and 25 °C. Esterase activity towards Ac-Arg-OMe was determined by the pH-stat method in 0.1 M-KCl, 1 mM-EDTA, pH 7.5 (12).

Apparent dissociation constants ($K_{\text{d(app)}}$) for the binding of BiGu, PpGu, PhGu and MeGu to the enzyme were determined according to the following equation (based on the assumption that the compounds at saturation bind to the enzyme in a ratio of 1:1):

$$K_{\text{d(app)}} = \frac{[\text{X}][\text{E}]}{[\text{XE}]} = \frac{[\text{X}](V_{\text{sat}} - v)}{v - v_0}$$

where [X], [XE] and [E] are the concentrations of free effector, effector bound to enzyme and free enzyme, respectively; V_{sat} and v represent rates at saturating and given concentrations of effector. v_0 represents the rate in the absence of effector. [X] was assumed to be equal to the concentration of added X. This holds since $K_{\text{d(app)}}$ is much larger than the total concentration of enzyme. The equation was used in its logarithmic form:

$$\log[\text{X}] - \log K_{\text{d(app)}} = \log \frac{v - v_0}{V_{\text{sat}} - v}$$

Deamidation reactions of Bz-Ala-Phe-NH₂ and Bz-Phe-His-NH₂ were followed by HPLC using the Waters liquid chromatography system (23). The following eluant system was used: A-buffer: 50 mM-TEAP, pH 3.0, B-buffer: 50% CH₃CN + 50% A. All separations were carried out at room temperature and monitored at 254 nm. The per cent composition could be calculated directly from the integrated peak areas since all products had the benzoyl group as a common chromophore.

3. RESULTS

3.1. Specificity of malt carboxypeptidase

The specificity of malt carboxypeptidase with respect of the P₁ position of ester substrates was investigated for the hydrolysis of a series of substrates with the general formula Bz-X-OMe (Table I). Substrates where the X position is occupied by an amino acid with a positively charged side chain, i.e. Lys, Arg and His, are hydrolyzed with high rates. Among the other substrates tested, high rates are only obtained with Bz-Phe-OMe whereas medium rates are observed for substrates with an aliphatic side chain with an unbranched β -carbon, i.e. Leu, Met, Ala. The substrates with a branched β -carbon, i.e. Val, Ile and Thr are hydrolyzed with very low rates. Bz-Gly-OMe, Bz-Pro-OMe and Bz-Asp-OMe are not hydrolyzed at all. The concentrations of Bz-Arg-OMe and Bz-Phe-OMe could be varied sufficiently to allow calculation of k_{cat} and K_m values from linear Lineweaver-Burk plots. The values were for Bz-Phe-OMe: $k_{\text{cat}} = 29000 \text{ min}^{-1}$, $K_m = 2.0 \text{ mM}$ and for Bz-Arg-OMe: $k_{\text{cat}} = 8400 \text{ min}^{-1}$, $K_m = 0.39 \text{ mM}$.

Table I.

The specificity of malt carboxypeptidase with respect to the X-position of Bz-X-OMe substrates (X = amino acid residue).

Substrate	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
Bz-Gly-OMe	0
Bz-Ala-OMe	370
Bz-Val-OMe	22
Bz-Ile-OMe	17
Bz-Leu-OMe	420
Bz-Met-OMe	1200
Bz-Phe-OMe	14600
Bz-Lys-OMe	18300
Bz-Arg-OMe	25200
Bz-His-OMe	5600
Bz-Thr-OMe	69
Bz-Pro-OMe	0
Bz-Asp-OMe	0

k_{cat}/K_m values were determined from full hydrolysis experiments using the following assay conditions: 0.3 mM-substrate, 0.05 M-Hepes, 1 mM-EDTA, 2.5% methanol, pH 7.5. Methanol was omitted with Bz-Arg-OMe, Bz-Lys-OMe, Bz-His-OMe and Bz-Asp-OMe.

The hydrolysis of N-blocked amino acid methyl esters is influenced by addition of salts as seen in Figure 1. Both NaCl and Na₂SO₄ inhibit the hydrolysis of substrates where the X position is occupied by a positively charged amino acid residue, i.e. Arg or Lys, while activation is ob-

Figure 1. The effect of salts on the activity of malt carboxypeptidase towards Bz-Arg-OMe (●-●-), Bz-Lys-OMe (▲-▲-), Bz-Leu-OMe (▼-▼-) and Bz-Phe-OMe (■-■-). The abscissa is ionic strength, closed symbols are with NaCl and open symbols with Na₂SO₄. The assays were performed at 0.5 mM-substrate in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5. The assays with Bz-Phe-OMe and Bz-Leu-OMe contained 2.5% (v/v) methanol. Addition of 2.5% (v/v) methanol to assays with Bz-Arg-OMe had no influence on activity neither in the absence nor in the presence of NaCl.

served for substrates where X is an uncharged amino acid residue, i.e. Phe or Leu. The inhibition of Bz-Arg-OMe and Bz-Lys-OMe hydrolysis is only partial, since the activity of the enzyme reaches constant levels at high concentrations of salt. The kinetic constants for the hydrolysis of Bz-Arg-OMe in the presence of 1 M-NaCl are: $k_{\text{cat}} = 8000 \text{ min}^{-1}$ and $K_m = 2.0 \text{ mM}$. In the absence of NaCl the values are: $k_{\text{cat}} = 8400 \text{ min}^{-1}$ and $K_m = 0.39 \text{ mM}$. Since only K_m is affected, the inhibition is competitive.

3.2. Influence of guanidine derivatives on the specificity of malt carboxypeptidase

An investigation of the effects of substrate analogues indicated guanidine derivatives to have a strong influence on the enzymatic proper-

Figure 2. Effect of various guanidine derivatives on the activity of malt carboxypeptidase towards FA-Phe-OMe. The assays were performed at 0.3 mM-substrate in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5. Panel A: Activation of malt carboxypeptidase by PhGu (▼-▼-) and BiGu (-x-x-). Panel B: Inhibition of malt carboxypeptidase by MeGu (-▲-▲-), Gu (-■-■-) and PpGu (-○-○-).

ties of malt carboxypeptidase. As demonstrated in Figure 2A the rate of hydrolysis of FA-Phe-OMe is increased by PhGu and BiGu about 4 and 1.7 times, respectively. In contrast, Gu, MeGu and PpGu inhibit the enzyme partially (Figure 2B). The results in Figure 2 may be used for calculation of apparent dissociation constants ($K_{d(app)}$) for the enzyme-effector complexes as shown in Figure 3 (see section 2.2). The values were: PhGu: $1.0 \cdot 10^{-4}$ M; BiGu: $3.1 \cdot 10^{-5}$ M; MeGu: $3.7 \cdot 10^{-4}$ M; Gu: $1.5 \cdot 10^{-4}$ M; PpGu: $1.6 \cdot 10^{-4}$ M.

The activation of the malt carboxypeptidase catalyzed hydrolysis of FA-Phe-OMe by PhGu has been studied further. Specific buffer effects are not involved since the same activation by PhGu is observed when 0.05 M-Tris or 1 M-NaCl is present. Neither 10 mM-benzylamine nor 10 mM-aniline had any activating effect, suggesting that in addition to an aromatic residue the guanidine group is essential for the activation. However, $K_{d(app)}$ for PhGu is slightly influenced by ionic strength: In the presence of 0.3 M-NaCl and 1.0 M-NaCl $K_{d(app)}$ is $1.4 \cdot 10^{-4}$ M and $2.5 \cdot 10^{-4}$ M, respectively, compared to $1.0 \cdot 10^{-4}$ M in the absence of NaCl. It was investigated whether MeGu and PhGu are bound to the same position on the enzyme by comparing the curve for PhGu activation (Figure 2A) with a similar curve obtained in the presence of 10 mM-MeGu. In the event that MeGu and PhGu competed for the same position on the enzyme, high concentrations of PhGu would displace MeGu, and consequently the activity of the enzyme at saturation should not be influenced by the presence of MeGu. However, the presence of 10 mM-MeGu reduces the activity of the enzyme-PhGu complex (at saturation with PhGu) to 65%, suggesting the formation of a ternary enzyme-PhGu-MeGu complex where PhGu and MeGu are bound to the enzyme at different positions. Although MeGu and PhGu apparently bind to the enzyme at different positions the binding of MeGu reduces the affinity for PhGu since $K_{d(app)}$ for PhGu increases nine fold to $8.9 \cdot 10^{-4}$ M.

As shown in Figure 4, $K_{d(app)}$ for the binding of PhGu to malt carboxypeptidase depends strongly on pH and it apparently correlates with the deprotonation of a group with a pK_a below 6.5. The degree of activation is also dependent on

Figure 3. Determination of $K_{d(app)}$ for the compounds shown in Figure 2 using the method described in section 2.2. The symbols correspond to those used in Figure 2. The following $K_{d(app)}$ were determined: PhGu: $1.0 \cdot 10^{-4}$ M; BiGu: $3.1 \cdot 10^{-5}$ M; MeGu: $3.7 \cdot 10^{-4}$ M; Gu: $1.5 \cdot 10^{-4}$ M; PpGu: $1.6 \cdot 10^{-4}$ M.

Figure 4. Influence of pH on $K_{d(app)}$ of the enzyme-PhGu complex ($\blacktriangle$ - $\blacktriangle$). K_d was estimated from the influence of the concentration of phenylguanidine on the activity of malt carboxypeptidase towards FA-Phe-OMe according to the procedure described in section 2.2. Assay conditions: 0.05 M-Hepes or 0.05 M-Mes, 1 mM-EDTA, 0.3 mM-FA-Phe-OMe, 2.5% methanol, $4.7 \cdot 10^{-9}$ M enzyme. The percentage activation (V_{max}/V_c) at each pH value is also indicated ($\circ$ - $\circ$). V_{max} is the rate of hydrolysis at saturating concentrations of PhGu: 5 mM at pH 7 to 8 and 50 mM at pH 6 and 6.5. V_c is the rate of hydrolysis in the absence of PhGu at the indicated pH value.

Table II.

Influence of MeGu, PhGu and BiGu on the malt carboxypeptidase catalyzed hydrolysis of different ester substrates.

Substrate	(v/v ₀) × 100 in the presence of		
	MeGu	PhGu	BiGu
Bz-Ala-OMe	55	570	280
Bz-Leu-OMe	45	900	290
Bz-Phe-OMe	45	310	170
FA-Phe-OMe	55	380	170
Bz-Arg-OMe	50	340	220
Ac-Arg-OMe		280	
Bz-Phe-OEt	55	50	35
Bz-Arg-OEt	50	80	60

The assays were performed at a substrate concentration of 0.5 mM in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5. All assays except those with Ac-Arg-OMe, Bz-Arg-OMe and Bz-Arg-OEt contained 2.5% (v/v) methanol. The following concentrations of effector was used: 10 mM-MeGu, 0.75 mM-PhGu and 0.1 mM-BiGu.

pH, with the highest activation about pH 8. At pH 5.0 where saturation of the enzyme with PhGu cannot be obtained due to the limited solubility of PhGu, the enzyme-PhGu complex is still more active than the enzyme alone: at 50 mM-PhGu v/v₀ is 1.8.

Table II summarizes the effects of MeGu, PhGu and BiGu on the activity of malt carboxypeptidase towards different ester substrates. MeGu inhibits all of the substrates regardless of the amino acid residue in the P₁ position and the alcohol residue in the P_i position of the substrate. In contrast, PhGu and BiGu increase the rate of hydrolysis of the N-blocked methyl esters while the rates of hydrolysis of the ethyl esters Bz-Arg-OEt and Bz-Phe-OEt are reduced, thus demonstrating a specific influence of the leaving group of the substrate.

The effects of MeGu and PhGu on the enzymatic properties of malt carboxypeptidase were further investigated by determination of the kinetic constants for a series of ester substrates Bz-Arg-*OX* with varying leaving group (X = Me, Et, Pr, Bu) (Table III). With all of these substrates MeGu decrease *k*_{cat} and increase *K*_m, and there is no apparent correlation with the size of the alcohol leaving group of the substrate. The effects of PhGu on the kinetic parameters for the hydrolysis of this series of substrates is more interesting. With all of the substrates an increase in *k*_{cat} is seen, the highest with Bz-Arg-OMe which increases approximately 3 fold. In contrast to this, *K*_m varies in both directions: it decreases from 0.39 mM to 0.16 mM with Bz-Arg-OMe and increases approximately 4 fold with Bz-Arg-OEt,

Table III.

The influence of MeGu and PhGu on malt carboxypeptidase catalyzed hydrolysis of ester substrates with different alcohol leaving group.

		Substrate			
		Bz-Arg-OMe	Bz-Arg-OEt	Bz-Arg-OPr	Bz-Arg-OBu
No effector added	<i>k</i> _{cat}	8400	11400	11000	11600
	<i>K</i> _m	0.39	0.11	0.034	0.022
	<i>k</i> _{cat} / <i>K</i> _m	21500	104000	324000	527000
+ MeGu	<i>k</i> _{cat}	6300	6700	6300	6000
	<i>K</i> _m	1.15	0.17	0.065	0.042
	<i>k</i> _{cat} / <i>K</i> _m	5500	39000	97000	140000
+ PhGu	<i>k</i> _{cat}	23600	20000	16400	19900
	<i>K</i> _m	0.16	0.43	0.12	0.095
	<i>k</i> _{cat} / <i>K</i> _m	150000	47000	140000	210000

The rate of hydrolysis was determined spectrophotometrically in 0.05 M-Hepes, 1 mM-EDTA, pH 7.5, using substrate concentrations of 0.03 - 1.3 mM. *k*_{cat} values are in min⁻¹ and *K*_m values in mM. MeGu and PhGu were used at saturating concentrations, 5 mM and 0.75 mM, respectively.

Figure 5. Effect of PhGu on the activities of malt carboxypeptidase. All assays were performed in 0.05 M-Hepes, 1 mM-EDTA, 2.5% methanol, pH 7.5 at a substrate concentration of 0.3 mM. The indicated concentrations of phenylguanidine were included in the assays. FA-Phe-OMe, $\circ-\circ-$; FA-Phe-Ala-OH, $\blacksquare-\blacksquare-$; FA-Phe-Gly-NH₂, $\blacktriangle-\blacktriangle-$; FA-Phe-NH₂, $\nabla-\nabla-$.

-OPr and -OBu. These results indicate that PhGu interacts with the S_i position of the enzyme to which the leaving group of ester substrates is bound and it does this in a manner which renders methyl esters better substrates of the enzyme and ethyl, propyl and butyl esters worse substrates. HPLC analysis of the reaction mixtures has confirmed that in the presence of PhGu Bz-Arg-OH is the only product formed from Bz-Arg-OMe suggesting that PhGu does not function as a nucleophile in the reaction.

Since PhGu interacts with the binding of the leaving group of ester substrates to malt carboxypeptidase, it was investigated whether it interacted similarly with the leaving group of peptide and peptide amide substrates. The substrates: FA-Phe¹OMe, FA-Phe¹Ala-OH, FA-Phe¹NH₂ and FA-Phe¹Gly-NH₂ were used to measure the esterase, peptidase, amidase and peptidyl amino acid amide hydrolase activities, respectively. As seen from Figure 5, binding of PhGu to the enzyme reduces its activity towards FA-Phe¹Ala-OH

Figure 6. The influence of PhGu on malt carboxypeptidase catalyzed deamidation reactions. The figures list the appearance of Bz-X-OH and Bz-X-Y-OH versus the degree of conversion of substrate (Bz-X-Y-NH₂). Panel A: Deamidation of Bz-Ala-Phe-NH₂. Triangles = Bz-Ala-OH, circles = Bz-Ala-Phe-OH; closed symbols: reaction performed in the presence of 10 mM-PhGu, open symbols: reaction performed in the absence of PhGu. Reaction conditions: 2.5 mM-Bz-Ala-Phe-NH₂, 10% (v/v) DMF, 1 mM-EDTA, 5.0 μ M-enzyme, pH 7.5. The last point is at 60 minutes. Panel B: Deamidation of Bz-Phe-His-NH₂. Triangles = Bz-Phe-OH, circles = Bz-Phe-His-OH; closed symbols: reaction performed in the presence of 10 mM-PhGu, open symbols: reaction performed in the absence of PhGu. Reaction conditions: 2.5 mM-Bz-Phe-His-NH₂, 10% (v/v) DMF, 1 mM-EDTA, 5.0 μ M-enzyme, pH 7.5. The last point is at 40 minutes.

and FA-Phe¹Gly-NH₂ to less than 3% of the control. Contrary to this, the rate of hydrolysis of FA-Phe¹OMe and FA-Phe¹NH₂ is increased to 380% and 220% of the control, respectively. These results indicate that the hydrolysis of substrates with large groups in the P₁ position, i.e. -Ala-OH and -Gly-NH₂, is inhibited while the hydrolysis of those with small groups in this position is activated, i.e., -OMe and -NH₂.

The effect of PhGu on the various activities of malt carboxypeptidase is reflected similarly in the deamidation of peptide amides. Reaction of Bz-Ala-Phe-NH₂ with malt carboxypeptidase forms two products: Bz-Ala-Phe-OH by release of NH₃ (amidase activity) and Bz-Ala-OH. The latter arise either by direct release of H-Phe-NH₂ (peptidyl amino acid amide hydrolase activity) or by secondary release of H-Phe-OH from Bz-Ala-Phe-OH (peptidase activity). The results in Figure 6A demonstrate that binding of PhGu to the enzyme exerts a pronounced effect on the yield of the deamidation product Bz-Ala-Phe-OH. In the absence of PhGu, the yield was less than 45% while in the presence of PhGu, the yield was 95-100%. Figure 6B demonstrates a similar effect of PhGu on the deamidation of Bz-Phe-His-NH₂, where the yield of deamidation product Bz-Phe-His-OH is increased from 3-15% in the absence of PhGu to 55-70% in the presence of PhGu. Thus, the reactions competing with the deamidation reaction are clearly suppressed in both cases.

4. DISCUSSION

The X-ray structures of two enzymes which employ similar catalytic mechanisms, the metallo enzymes thermolysin and carboxypeptidase A, have indicated the explanation for the inability of a carboxypeptidase to cleave any other peptide bonds than the C-terminal as opposed to a protease which cleaves peptide bonds in the interior of polypeptide chains. Thermolysin possesses multiple binding sites for the leaving group, S₁, S₂...S_n, located in an extended cleft of the enzyme while carboxypeptidase A possesses only a single binding site for the leaving group i.e. S₁, which is located in a dead-end pocket in the enzyme (14, 16, 17). The active sites of the serine proteases are similarly located in clefts (15, 18), and in analogy with carboxypeptidase A we might expect dead-end structures in the serine

carboxypeptidases. In spite of such conceivable differences between serine proteases and serine carboxypeptidases they share the capability to release alcohols from peptide esters and ammonia and/or the C-terminal amino acid amide from peptide amides (5) because both groups of enzymes possess the required binding sites for these activities: S₁ in the C-terminal direction, and S₁, S₂..., S_n in the N-terminal direction of the scissile bond. Thus, it is reasonable to compare the side chain specificity of malt carboxypeptidase catalyzed ester hydrolysis with the specificities of proteases as well as other serine carboxypeptidases.

The malt carboxypeptidase catalyzed hydrolysis of ester substrates with the general formula Bz-X-OMe indicate 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 a neutral or acidic aliphatic 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 it is mainly due to a low K_m value. The competitive inhibition by NaCl of malt carboxypeptidase catalyzed hydrolysis of Bz-Arg-OMe indicates the presence of a negatively charged residue on the enzyme which participates in the binding of substrates with positively charged side chains in the P₁ position. Apparently this residue does not influence the binding of substrates with a neutral side chain in the P₁ position. The subtilisins have a similar wide specificity and it has been suggested that this is due to different modes of productive binding for substrates with an aromatic and a basic residue in the P₁ position (8). A similar situation could explain the wide specificity of malt carboxypeptidase. The specificity of malt carboxypeptidase catalyzed hydrolysis of ester substrates has some resemblance with Penicillocarboxypeptidase S-2 (10), but it is very different from that of carboxypeptidase Y from yeast, which with respect to the P₁ position of ester substrates has a specificity similar to that of chymotrypsin, i.e. it hydrolyses Bz-Phe-OMe very fast but almost completely lack the capability to hydrolyse Bz-Arg-OMe and Bz-Lys-OMe (4).

The acylation step appears to be rate-limiting in the malt carboxypeptidase catalyzed hydro-

lysis of the ester substrate Z-Lys-ONp (to be published). Provided this also applies to the ester substrates Bz-Arg-OX ($X = \text{Me, Et, Pr, Bu}$), K_m will be approximately equal to K_s and the decreasing K_m values in the sequence -OMe, -OEt, -OPr, -OBu then suggest that the interaction between enzyme and alcohol leaving group is mainly due to hydrophobic interactions. This may explain why MeGu apparently does not interact with the S_i position whereas the more hydrophobic PhGu interacts in a manner which results in a decrease in K_m for Bz-Arg-OMe and an increase in K_m for Bz-Arg-OEt (-OPr, -OBu). Thus, binding of PhGu presumably changes the properties of the S_i site such that the binding of Bz-Arg-OMe becomes tighter. The increased k_{cat} for all the substrates Bz-Arg-OX is then attributed to an increased rate of the rate-limiting step, i.e. acylation.

The different effects of PhGu on the activities of malt carboxypeptidase towards FA-Phe-OMe, FA-Phe-NH₂, FA-Phe-Ala-OH and FA-Phe-Gly-NH₂ indicate that when the leaving group of the substrate is large, i.e. -Ala-OH and -Gly-NH₂, it competes with PhGu for the same site. However, when the leaving group of the substrate is small, i.e. -NH₂ and -OMe, PhGu exerts an activating effect, indicating that the S_i binding site may accommodate PhGu and the small leaving group simultaneously.

At present, no enzyme has been described which catalyzes the specific release of NH₃ from the C-terminal of peptide amides. Although serine and sulphhydryl proteases exhibit amidase activity, they cleave in addition internal peptide bonds and are thus of limited use in deamidation reactions. The serine carboxypeptidases have similar limitations, since these enzymes release both ammonia and the C-terminal amino acid amide (2). The present observation that PhGu increases the amidase activity of malt carboxypeptidase while it decreases the peptidase and peptidyl amino acid amide hydrolase activities is of interest since the yields of deamidation reactions may be increased as demonstrated with Bz-Phe-His-NH₂ and Bz-Ala-Phe-NH₂. Such deamidation reactions are essential in stepwise enzymatic peptide synthesis, where they function as deblocking reactions (2, 6, 23).

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