MALT CARBOXYPEPTIDASE CATALYZED AMINOLYSIS REACTIONS

by

KLAUS BREDDAM and MARTIN OTTESEN

Department of Chemistry, Carlsberg Laboratory,
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

Keywords: Phenylguanidine, carboxypeptidase, barley, malt, enzymatic synthesis, peptide synthesis.

Malt carboxypeptidase catalyzes the formation of peptide bonds using N-benzoyl-arginine esters as acyl components and amino acid amides or amino acid methyl esters as nucleophiles. With seven different nucleophiles yields of 50–95% were obtained while no aminolysis was observed with H-Pro-NH₂ and H-Glu- α -NH₂. The enzyme is easily saturated with nucleophile indicating the formation of a complex between nucleophile and acyl-enzyme intermediate prior to deacylation. The nature of the side-chain of the amino acid esters used as nucleophiles strongly influences the apparent dissociation constant of this complex ($K_{N(\text{app})}$) while less dependence on the side-chain is observed with amino acid amides. $K_{N(\text{app})}$ is drastically increased by the presence of phenylguanidine in the reaction medium suggesting a competition between this substance and the nucleophile for the same binding site. This site has previously been identified as the S'_i binding site of malt carboxypeptidase (Carlsberg Res. Commun. 48, 573–582 (1983)). The influence of the nucleophile H-Val-NH₂ on the kinetic parameters for the cleavage of N_n-CBZ-Lys-p-nitrophenylester indicates that the acylation step is rate-limiting, and that H-Val-NH₂ has two different modes of binding to the enzyme.

1. INTRODUCTION

It is well known that serine and sulfhydryl proteases in addition to hydrolysis of peptide bonds also hydrolyse N-blocked amino acid esters and peptide esters. These reactions proceed via an acyl-enzyme intermediate, and when other nucleophiles are added to the reaction mixture, e.g. amino compounds, the acyl-

enzyme intermediate will react both with water to give a hydrolysis product and with the amine to give an aminolysis product. BENDER et al. (1) and FASTREZ and FERSHT (9) found that when methanol, ethanol, glycine amide or alanine amide were added as nucleophiles to chymotrypsin it reacted with ester substrates according to the simple reaction model shown in Scheme

Abbreviations: Bu = butyl; Bz = N-benzoyl; CBZ = Carbobenzoxyl; EDTA = ethylene diamine tetraacetic acid, sodium salt; HPLC = high performance liquid chromatography; Mes = 2-(N-morpholino)ethane sulfonic acid; Np = p-nitrophenyl; PhGu = phenylguanidine hydrogencarbonate. Amino acids abbreviated in upper case refer to the L-enantiomer and those in lower case refer to the D-enantiomer. 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 (11). Accordingly, the binding site for the C-terminal amino acid residue of the substrate is denoted S'_i and those for the amino acid residues in the amino-terminal direction away from the scissile bond are denoted S₁, S₂, ..., S_i.

$$dP_2/dt = k_3[W][ES']$$

$$dP_3/dt = k_4[N][ES']$$

$$\frac{P_3}{P_2} = \frac{k_4[N]}{k_3[W]}$$

Scheme 1. Competition between water (W) and other nucleophile (N) in the serine protease catalyzed hydrolysis of ester substrates as proposed by BENDER et al. (1). The enzyme (E) and substrate (S) initially forms a complex (ES) with the dissociation constant K_s . k_2 is the rate constant for the essentially irreversible conversion of ES complex to the acyl-enzyme intermediate (ES'). k_3 and k_4 are the rate-constants for the deacylation reactions with W and N as nucleophiles, respectively. P_1 is the alcohol leaving group of the ester substrate. P_2 and P_3 are the hydrolysis and aminolysis products, respectively. This model assumes that neither water nor added nucleophile bind to the enzyme prior to the reaction with the acyl-enzyme intermediate.

I, in which it is assumed that neither water nor the added nucleophile bind to the enzyme prior to the reaction with the acyl-enzyme intermediate. Hence, the ratio of the two reaction products being formed, $[P_3]/[P_2]$, is a linear function of the ratio $[N]/[W]$ between the concentrations of added nucleophile and water. FINK and BENDER (10) showed that the sulfhydryl protease papain catalyzed hydrolysis of esters with methanol and ethanol as added nucleophiles according to Scheme 1. However, when higher alcohols were added as nucleophiles deviations were observed and to explain this they had to introduce a more complex reaction scheme which took into account specific binding of the nucleophiles to the enzyme and the enzyme-substrate complex (10).

The serine exopeptidase, carboxypeptidase Y from baker's yeast, functions similar to the serine and sulfhydryl endopeptidases: it hydrolyzes both peptide and ester bonds, and in the presence of nucleophiles it catalyzes both aminolysis and alcoholysis reactions, a property

which has been demonstrated to be useful in enzymatic peptide synthesis (2-6, 13-15). Another serine carboxypeptidase from malt has recently been isolated in high purity in this laboratory and characterized with respect to hydrolysis of peptide and ester substrates (7, 8). Phenylguanidine has a specific function as a modifier of this enzyme by binding to the S'_1 binding site, i.e. the binding site of the leaving group of the substrate. In the present paper this malt carboxypeptidase is characterized with respect to aminolysis reactions and the effect of addition of phenylguanidine is used to demonstrate that amino acid amides and amino acid methyl esters bind to the S'_1 binding site prior to their attack on the acyl-enzyme intermediate.

2. MATERIALS AND METHODS

2.1. Materials

Malt carboxypeptidase and Bz-Arg-OBu were prepared as previously described (7, 8). Mes, Bz-Arg-OMe and H-Ser-NH₂·HCl were obtained from Sigma, USA. Phenylguanidine hydrogencarbonate was obtained from EGA Chemie, W. Germany. All other derivatives of amino acids were purchased from Bachem, Switzerland. Other reagents and solvents were of analytical purity and obtained from Merck, W. Germany.

2.2. Methods

The reactions were performed in a pH stat (12). The reaction volumes were 2 ml and Bz-Arg-OMe and Bz-Arg-OBu were used as substrates in the presence of 5 mM-EDTA. Most experiments were performed at pH 7.5 because the enzyme is unstable at higher pH values. 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 C-18 reverse phase column. The following eluant system was used: 50 mM-triethyl ammonium phosphate, pH 3.0 (A-buffer) and 50 mM-triethyl ammonium phosphate, pH 3.0 in 50% CH₃CN (B-buffer) employing various linear and concave

Figure 1. Malt carboxypeptidase catalyzed aminolysis of Bz-Arg-OBu using H-Val-NH₂ as nucleophile at different concentrations. Reaction conditions: 2 mM-Bz-Arg-OBu, 0.01–0.3 M-H-Val-NH₂, 5 mM-EDTA, 0.3 μM-enzyme, pH 7.5. Reactions were quenched at the indicated times and analyzed by HPLC (see section 2.2). —○—○—, Bz-Arg-OBu; —●—●—, Bz-Arg-OH; —■—■—, Bz-Arg-Val-NH₂; —x—x—, fraction of aminolysis, i.e. Bz-Arg-Val-NH₂/(Bz-Arg-OH + Bz-Arg-Val-NH₂).

gradients. All separations were carried out at room temperature and monitored at 254 nm. The per cent composition of the reaction mixtures could be calculated directly from the integrated peak areas since all components had the benzoyl group as a common chromophore. Aminolysis and hydrolysis products were initially collected and identified by amino acid analysis. The fraction of aminolysis was expressed 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. Influence of added nucleophiles

Previously it was observed that malt carboxypeptidase had high activity towards ester substrates with a positively charged side-chain (8), and for this reason, Bz-Arg-OBu and Bz-Arg-OMe were selected as substrates in the aminolysis reactions. The action of malt carboxypeptidase on Bz-Arg-OBu with the amino acid amide H-Val-NH₂ as added nucleophile resulted in the formation of both Bz-Arg-OH and Bz-Arg-Val-NH₂ due to the partitioning of the acyl-enzyme

intermediate between the hydrolysis reaction and the aminolysis reaction. The fraction undergoing aminolysis reaction increased with increasing concentrations of H-Val-NH₂ but it did not exceed approximately 0.90 (Figure 1). It is seen that 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-Val-NH₂ indicating an inhibitor function of this nucleophile. The correlation between fraction of aminolysis and concentration of H-Val-NH₂ shown in Figure 2 was unaffected by the addition of 2 M-NaCl or by replacing Bz-Arg-OBu with Bz-Arg-OMe as substrate. Using Bz-Arg-OBu as substrate and the amino acid ester H-Val-OMe as nucleophile resulted in formation of the aminolysis product Bz-Arg-Val-OMe. Also in this case the fraction of aminolysis reached a constant value but it was at a lower level than was observed with H-Val-NH₂ (Figure 2).

The observation that malt carboxypeptidase becomes saturated with nucleophiles such that the fraction of aminolysis does not exceed 0.90

Figure 2. The influence of the concentration of nucleophile on the fraction of aminolysis using Bz-Arg-OBu as substrate and H-Val-NH₂ (—○—○—) or H-Val-OMe (—●—●—) as nucleophile. The reaction conditions were the following: 2 mM-Bz-Arg-OBu, 5 mM-EDTA, 0.3 – 0.6 μ M-malt carboxypeptidase, pH 7.5. The concentration of nucleophile is indicated. Reaction time: 2 – 20 min. 80 – 95% of the substrate was consumed in the reaction.

with H-Val-NH₂ and 0.50 with H-Val-OMe is incompatible with a proportionality with the nucleophile concentration as predicted from

Scheme 1. These results indicate that the nucleophiles bind to the acyl-enzyme complex prior to the deacylation reaction and the capability of the enzyme to bind nucleophiles may conveniently be estimated from the concentration of nucleophile required to produce a fraction of aminolysis which is half the maximal aminolysis fraction. This concentration is designated $K_{N(\text{app})}$ since it is related to the dissociation constant of the complex between acyl-enzyme and nucleophile. Table I lists the $K_{N(\text{app})}$ values for a series of amino acid amides and amino acid methyl esters and presents in addition the fractions of aminolysis observed at saturation with the nucleophiles. It is seen that $K_{N(\text{app})}$ for the amino acid esters varies much more with the nature of the amino acid side-chain than $K_{N(\text{app})}$ for the corresponding amino acid amides. Furthermore, the fraction of aminolysis with amino acid amides is consistently higher than with amino acid methyl esters. The amino acid amides H-Pro-NH₂ and H-Glu- α -NH₂, the D-enantiomers H-val-NH₂ and H-ala-OMe, and glycine N-methyl amide gave no aminolysis products.

The influence of pH on malt carboxypeptidase catalyzed aminolysis reactions was studied with Bz-Arg-OBu as substrate and H-Val-NH₂ as nucleophile (Figure 3). The enzyme reached saturation with H-Val-NH₂ at all pH values

Table I.

Malt carboxypeptidase catalyzed synthesis of peptide bonds using Bz-Arg-OBu as substrate and amino acid amides and esters as nucleophiles

Substrate	Nucleophile	Aminolysis product	Fraction of aminolysis at saturation	$K_{N(\text{app})}$	$K_{N(\text{app})}$ with PhGu
Bz-Arg-OMe	H-Gly-NH ₂	Bz-Arg-Gly-NH ₂	0.90	0.03	0.4
Bz-Arg-OMe	H-Ser-NH ₂	Bz-Arg-Ser-NH ₂	0.90	0.03	
Bz-Arg-OMe	H-Leu-NH ₂	Bz-Arg-Leu-NH ₂	0.90	0.03	0.4
Bz-Arg-OBu	H-Val-NH ₂	Bz-Arg-Val-NH ₂	0.90	0.015 ^{a)}	> 0.3
Bz-Arg-OBu	H-Gly-OMe	Bz-Arg-Gly-OMe	0.65	> 3.0 ^{b)}	> 3.0 ^{b)}
Bz-Arg-OBu	H-Ala-OMe	Bz-Arg-Ala-OMe	0.50	0.05	1.0
Bz-Arg-OBu	H-Val-OMe	Bz-Arg-Val-OMe	0.50	0.1	0.6

$K_{N(\text{app})}$ was determined from plots of yield of aminolysis versus concentration of amine component (see Figure 2) in the absence and in the presence of 10 mM-PhGu. Reaction conditions: 2 mM-substrate, 5 mM-EDTA, 0.3 – 3 μ M-malt carboxypeptidase, 0.002 – 4.0 M-amine component, pH 7.5, 25 °C. Reaction time: 2 – 20 minutes. Conditions were selected such that 80 – 95% of the acyl component was consumed in the reactions. a) the same value of $K_{N(\text{app})}$ was obtained with Bz-Ala-OMe as substrate, b) saturation was not obtained with H-Gly-OMe as amine component and PhGu was found not to influence the yield of aminolysis at concentrations of H-Gly-OMe up to 4.0 M.

Figure 3. Influence of the concentration of H-Val-NH₂ on the fraction of aminolysis at different pH values. Reaction conditions: 2 mM-Bz-Arg-OBu, 5 mM-EDTA, 0.3 – 3 μ M-malt carboxypeptidase. Reaction time 2 – 20 minutes. pH 8.0, $\circ$ – $\circ$ –; pH 7.5, $\bullet$ – $\bullet$ –; pH 6.5, $\blacksquare$ – $\blacksquare$ –; pH 6.0, $\times$ – $\times$ –; pH 5.5, ∇ – ∇ –; pH 5.0, $\blacktriangle$ – $\blacktriangle$ –.

except pH 5.0 and the maximal fraction of aminolysis varied only slightly with pH. However, the concentration of nucleophile needed to saturate the enzyme increased drastically with decreasing pH with $K_{N(\text{app})}$ reflecting a group with a pK_a below 5.5 (Figure 4).

The cleavage of peptide bonds by malt carboxypeptidase proceed like the cleavage of ester bonds via an acyl-enzyme intermediate. Consequently, it is to be expected that peptides also can function as substrates for aminolysis reactions. To demonstrate this, Bz-Lys-Ala-OH was used as substrate and H-Val-NH₂ as nucleophile, and the reaction was studied in the pH range 3.5 to 7.0. It was observed that the fraction of aminolysis was maximal at pH 4.25 where it reached approximately 0.4 (Figure 5) while at pH 7.0 only a small amount of Bz-Lys-Val-NH₂ was formed. However, due to the relatively low yield of this reaction no further studies were performed.

Figure 4. The influence of pH on $K_{N(\text{app})}$ derived from the curves shown in Figure 3. It is assumed that the maximal yield at pH 5.0 is 85%.

Figure 5. The influence of pH on the fraction of aminolysis in malt carboxypeptidase catalyzed transpeptidation reaction. Reaction conditions: 5 mM-Bz-Lys-Ala-OH, 1.5 M-H-Val-NH₂, 5 mM-EDTA, 0.3 μM-malt carboxypeptidase. Reaction time: 5–15 min.

3.2. The influence of phenylguanidine (PhGu) on aminolysis reactions

The results described in section 3.1. demonstrate that the acyl-enzyme complex has a binding site for the added nucleophiles and it is likely that this binding site is identical with the S_i' binding site for the leaving group in the acylation reaction. Hence, it would be expected that addition of PhGu, which previously has been shown to bind to the S_i' binding site in malt carboxypeptidase (8), would have an effect on the aminolysis reactions. This was investigated by addition of PhGu to the malt carboxypeptidase catalyzed aminolysis of Bz-Arg-OBu using H-Val-NH₂ and H-Val-OMe as nucleophiles. Although the limited solubility of the nucleophiles did not allow sufficiently high concentrations to be used to obtain complete saturation it appeared (Figure 6) that the the same maximal fractions of aminolysis was being approached as was observed without PhGu (see Figure 2). However, as seen from a comparison between Figure 6 and Figure 2 much higher concentrations of nucleophile were needed to saturate the enzyme in the presence of PhGu than in the absence of this substance, i.e. PhGu caused an increase in

Figure 6. Influence of PhGu on malt carboxypeptidase catalyzed aminolysis reactions using Bz-Arg-OBu as acyl component and H-Val-NH₂ (—○—○—) or H-Val-OMe (—●—●—) as amine components. The reaction conditions were the following: 2 mM-Bz-Arg-OBu, 5 mM-EDTA, 10 mM-PhGu, 0.3 μM–0.6 μM-malt carboxypeptidase, pH 7.5. Reaction time: 2–20 minutes. The concentration of nucleophile is indicated. 80–95% of the acyl component was consumed in the reactions.

$K_{N(app)}$. As seen from Table I similar effects of PhGu were observed with other nucleophiles, with the exception of H-Gly-OMe, suggesting that PhGu competes with these nucleophiles for the S_i' binding site.

3.3. Kinetic studies of malt carboxypeptidase catalyzed aminolysis reactions

The influence of H-Val-NH₂ on the cleavage of Bz-Arg-OBu by malt carboxypeptidase (section 3.1.) was studied at 2 mM-substrate concentration which was approximately 100 fold higher than K_m (8). Hence, the rate of substrate disappearance reflected k_{cat} , which was seen to decrease by the addition of H-Val-NH₂ (Figure 1). However, nitrophenylesters are more convenient substrates for kinetic studies of this type since it is possible spectrophotometrically to follow the rate of release of the leaving group, i.e. nitrophenol. N_α-CBZ-Lys-ONp is an excellent substrate of malt carboxypeptidase and it was used as substrate with H-Val-NH₂ as nucleophile. When the fraction of aminolysis was studied by HPLC

Figure 7. Influence of the concentration of H-Val-NH₂ on the fraction of aminolysis using N_α-CBZ-Lys-ONp as substrate. Reaction conditions: 2 mM-N_α-CBZ-Lys-ONp, 0.1 M-Mes, 1 mM-EDTA, 0.7 μM-malt carboxypeptidase, pH 5.5. The concentration of H-Val-NH₂ varied as described in the Figure. 80 – 95% of the substrate was converted in the reaction which lasted 5 – 15 minutes.

determination of the amounts of N_α-CBZ-Lys-OH and N_α-CBZ-Lys-Val-NH₂ being formed the shape of the curve (Figure 7) again indicated a saturation with nucleophile with a maximal fraction of aminolysis of approximately 0.80 and a $K_{N(\text{app})}$ of approximately 0.4 M. When the rate of disappearance of N_α-CBZ-Lys-ONp was determined spectrophotometrically as a function of the concentration of H-Val-NH₂ the kinetic parameters indicated that k_{cat} rapidly decreased to approximately 50% and then remained constant, suggesting that H-Val-NH₂ exhibited partial non-competitive inhibition with an approximate K_i of 0.03 M. The constant value of k_{cat} at increasing nucleophile concentrations above 0.2 M suggested that the rate-limiting step in malt carboxypeptidase catalyzed action on ester substrates lies ahead of the deacylation step, i.e. acylation is presumably rate-limiting (12). In contrast to this, K_m increased linearly over the entire concentration range of H-Val-NH₂ (0 – 1.35 M), indicating that the nucleophile also exhibited competitive inhibition. The K_i of this inhibition is 0.50 M which is much larger than the K_i of 0.03 M for the non-competitive inhibition and this suggests that H-Val-NH₂ binds to the enzyme at two different positions.

Figure 8. Influence of the concentration of H-Val-NH₂ on the kinetic parameters for the disappearance of N_α-CBZ-Lys-ONp. The reactions were performed in 0.05 M-Mes, 1 mM-EDTA, pH 5.5. The concentration of Z-Lys-ONp varied from 0.1 – 1.0 mM, and 7×10^{-9} M-malt carboxypeptidase was used. The kinetic parameters were determined from linear Lineweaver-Burk plots at different concentrations at H-Val-NH₂ included in the assay mixture.

4. DISCUSSION

The results presented in section 3 for the action of malt carboxypeptidase on ester substrates in the presence of added amine nucleophiles showed saturation phenomena which deviated from the simple model in Scheme 1 which predicted a proportional partitioning of the acyl-enzyme complex between hydrolysis and aminolysis. FINK and BENDER (10) have previously observed similar binding effects when larger n-alkyl alcohols were added to papain acting on p-nitrophenyl substrates. They found that their results could only be explained by adopting the reaction scheme presented in Scheme 2 in which the added nucleophile binds to the enzyme at two different positions. ES is the noncovalent enzyme-substrate complex, ES' is the acyl-enzyme, P₁ is the substrate leaving group, P₂ is the hydrolysis product and P₃ is the aminolysis product formed when the nucleophile N is present. K_N is the dissociation constant for nucleophile bound to the same site as the leaving group, i.e. the S'_i binding site of the enzyme. K_i is the dissociation constant for nu-

Scheme 2. Model proposed by FINK and BENDER (10) to account for the binding of nucleophiles to papain. The added nucleophile binds at two different positions of the enzyme with the dissociation constants K_N and K_i , respectively. Water is assumed not to bind to the enzyme prior to its participation in the deacylation reaction. α and β are factors which account for the possible influence of bound nucleophile on the magnitudes of k_2 and k_3 , respectively. Other abbreviations used are defined in Scheme 1.

cleophile bound at a different site. α and β are factors which account for the possible influence of nucleophile bound at this position on the magnitudes of k_2 and k_3 , respectively. In our studies, the influence of H-Val-NH₂ on the steady-state parameters k_{cat} and K_m for the action of malt carboxypeptidase on N_α-CBZ-Lys-ONp similarly demonstrated the existence of two binding modes for the added nucleophile, one causing competitive inhibition with a K_i of 0.50 M, the other causing non-competitive inhibition with an approximate K_i of 0.03 M. These results are compatible with the mechanism presented in Scheme 2 when it is assumed that $K_N = 0.50$ M and $K_i = 0.03$ M for H-Val-NH₂ at pH 5.5. The fact that $K_{N(app)}$ was determined to approximately 0.4 M from the correlation of fraction of aminolysis, $P_3/(P_2 + P_3)$, versus nucleophile concentration, $[N]$, and that this is in agreement with the kinetically determined K_i value of 0.50 M supports that Scheme 2 is a good description of the action of malt carboxypeptidase on ester substrates and that $K_{N(app)}$ is a reasonably good measure for K_N .

PhGu has previously been demonstrated to bind to the S'_i binding site of malt carboxypepti-

dase since it weakened the binding of substrates with bulky leaving groups (8). The present results demonstrated that addition of PhGu to malt carboxypeptidase catalyzed aminolysis reactions drastically increased $K_{N(app)}$ for all the tested nucleophiles (except H-Gly-OMe), but it had no effect on the maximal level of aminolysis. This is consistent with a competition between PhGu and the binding of nucleophiles at the S'_i position. The fact that PhGu had no effect on the aminolysis reactions with H-Gly-OMe but strongly affected $K_{N(app)}$ for H-Ala-OMe and H-Val-OMe might suggest that it is the side-chains of amino acid methyl esters which are bound to the S'_i binding site in such a manner that they occupy or overlap with the binding site for PhGu. No such differences were observed with amino acid amides since PhGu affected $K_{N(app)}$ for all amino acid amides tested including H-Gly-NH₂. This suggests that these two types of nucleophiles exhibit slightly different binding modes within the S'_i binding site of the enzyme. These different modes of binding may explain why the fraction of aminolysis at saturation with nucleophile is much higher with amino acid amides than with amino acid methyl esters.

However, the binding of both types of nucleophiles appear to be stereospecific since the D-enantiomers H-val-NH₂ and H-ala-OMe gave no aminolysis products.

The nucleophiles used in the present study exist in solution both in an unprotonated (amine) form and a protonated (ammonium) form. However, only the amine form participates as nucleophile in the deacylation reaction and consequently, $K_{N(app)}$ refer to the dissociation constant of the complex between the amine form of the nucleophile and the acyl-enzyme intermediate. Thus, in the case of H-Val-NH₂ it would be expected that $K_{N(app)}$ would reflect the deprotonation of a group with a pK_a of 8.0 which is the pK_a of H-Val-NH₂. However, the aminolysis was dependent on a group with a pK_a below 5.5 which suggested that H-Val-NH₂ when bound to the enzyme exhibited a pK_a significantly different from that observed in solution. The deacylation reaction with H-Val-NH₂ as nucleophile represents the reverse of an alkylation reaction with H-Val-NH₂ as leaving group. Thus, after cleavage of a peptide bond it is conceivable that the leaving group remains unprotonated while still bound to this particular binding site even at pH values far below its pK_a in solution, and the dissociation of this group from the enzyme will be dependent on the dissociation constant. It remains to be established whether this feature is functionally significant but one might imagine that the protonation and subsequent ejection of the leaving group could be linked to a conformational change of the active site region of the enzyme.

REFERENCES

1. BENDER, M.L., G.E. CLEMENT, C.R. GUNTER & F.J. KEZDY: The kinetics of α -chymotrypsin reactions in the presence of added nucleophiles. *J. Am. Chem. Soc.* 86, 3697-3703 (1964)
2. BREDDAM, K., F. WIDMER & J.T. JOHANSEN: Carboxypeptidase Y catalyzed transpeptidations and enzymatic peptide synthesis. *Carlsberg Res. Commun.* 45, 237-247 (1980)
3. BREDDAM, K., F. WIDMER & J.T. JOHANSEN: Influence of the substrate structure on carboxypeptidase Y catalyzed peptide bond formation. *Carlsberg Res. Commun.* 45, 361-367 (1980)
4. BREDDAM, K., F. WIDMER & J.T. JOHANSEN: Carboxypeptidase Y catalyzed C-terminal modifications of peptides. *Carlsberg Res. Commun.* 46, 121-128 (1981)
5. BREDDAM, K., F. WIDMER & J.T. JOHANSEN: Carboxypeptidase Y catalyzed C-terminal modification in the B-chain of porcine insulin. *Carlsberg Res. Commun.* 46 361-371 (1981)
6. BREDDAM, K., F. WIDMER & J.T. JOHANSEN: Amino acid methyl esters as amine components in CPD-Y catalyzed peptide synthesis: Control of side reactions. *Carlsberg Res. Commun.* 48, 231-237 (1983)
7. BREDDAM, K., S. SØRENSEN & M. OTTESEN: Isolation of a carboxypeptidase from malted barley by affinity chromatography. *Carlsberg Res. Commun.* 48, 217-230 (1983)
8. BREDDAM, K. & M. OTTESEN: Influence of guanidine derivatives on the specificity of malt carboxypeptidase. *Carlsberg Res. Commun.* 48, 573-582 (1983)
9. FASTREZ, J. & A.R. FERSHT: Demonstration of the acyl-enzyme mechanism for the hydrolysis of peptides and anilides by chymotrypsin. *Biochemistry* 12, 2025-2034 (1973)
10. FINK, A.L. & M.L. BENDER: Binding sites for substrate leaving groups and added nucleophiles in papain-catalyzed hydrolyses. *Biochemistry* 8, 5109-5118 (1969)
11. SCHECHTER, I. & B. BERGER: On the size of the active site of proteases. I. Papain. *Biochem. Biophys. Res. Commun.* 27, 157-162 (1967)
12. SEYDOUX, F. & J. YON: Compétition nucléophile dans les réactions d'hydrolyse enzymatique. Analyse cinétique et application à l'hydrolyse trypsique de quelques esters. *Eur. J. Biochem.* 3, 42-56 (1967)
13. WIDMER, F. & J.T. JOHANSEN: Enzymatic peptide synthesis. Carboxypeptidase Y catalyzed formation of peptide bonds. *Carlsberg Res. Commun.* 44, 37-46 (1979)
14. WIDMER, F., K. BREDDAM & J.T. JOHANSEN: Carboxypeptidase Y catalyzed peptide synthesis using amino acid alkyl esters as amine components. *Carlsberg Res. Commun.* 45, 453-463 (1980)
15. WIDMER, F., K. BREDDAM & J.T. JOHANSEN: Influence of the structure of amine components in carboxypeptidase Y catalyzed amide bond formation. *Carlsberg Res. Commun.* 46, 97-106 (1981)