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SERINE CARBOXYPEPTIDASES. A REVIEW.

by

KLAUS BREDDAM

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

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Carboxypeptidases are proteolytic enzymes which only cleave the C-terminal peptide bond in polypeptides. Those characterized until now can, dependent on their catalytic mechanism, be classified as either metallo carboxypeptidases or as serine carboxypeptidases. Enzymes from the latter group are found in the vacuoles of higher plants and fungi and in the lysosomes of animal cells. Many fungi, in addition, excrete serine carboxypeptidases. Apparently, bacteria do not employ this group of enzymes.

Most serine carboxypeptidases presumably participate in the intracellular turnover of proteins and some of them, in addition, release amino acids from extracellular proteins and peptides. However, prolyl carboxypeptidase which cleaves the C-terminal peptide bond of angiotensin II and III is a serine carboxypeptidase with a more specific function.

Serine carboxypeptidases are usually glycoproteins with subunit molecular weights of 40,000 – 75,000. Those isolated from fungi apparently contain only a single peptide chain while those isolated from higher plants and animals in most cases contain two peptide chains linked by disulfide bridges. However, a number of the enzymes aggregate forming dimers and oligomers.

It is probable that the well-known catalytic mechanism of the serine endopeptidases is also employed by the serine carboxypeptidases but presumably with the difference that the pK_a of the catalytically essential histidyl residue is somewhat lower in the carboxypeptidases than in the endopeptidases. However, the leaving group specificity of these two groups of enzymes differ since the carboxypeptidases only release C-terminal amino acids from peptides (peptidase activity) and not longer peptide fragments. In addition, they release C-terminal amino acid amides (peptidyl amino acid amide hydrolase activity) or ammonia (amidase activity) from peptide amides and alcohols from peptide esters (esterase activity) and this property they share with the serine endopeptidases. Like other proteolytic enzymes the serine carboxypeptidases contain binding sites which secure the interaction between enzyme and substrate. In this laboratory, the properties of these have been studied for three serine carboxypeptidases, i.e. carboxypeptidase Y from yeast and malt carboxypeptidases I and II, by means of kinetic studies, chemical modifications of amino acid side-chains located at these binding sites and exchange of such amino acid residues by site-directed mutagenesis.

Serine carboxypeptidases, such as carboxypeptidase Y and malt carboxypeptidase II which are available in large quantities, can be applied for several purposes. Their broad specificity and ability to release amino acids from the C-terminus of a peptide chain can be employed in determination of amino acid sequences, and their ability to catalyze transpeptidation reactions and aminolysis of peptide esters can be employed to exchange C-terminal amino acid residues in peptides and in step-wise synthesis of polypeptides, respectively. The type of reactions catalyzed by these enzymes is limited by their specificities but, fortunately, some of the derivatives of carboxypeptidase Y with changed specificity due to chemical modifications and genetic substitutions of amino acid side-chains located at binding sites can be employed with advantage. These modified enzymes are examples on how the different activities of an enzyme can be perturbed by "protein engineering", hence rendering the enzyme particularly suitable for certain processes.

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Abbreviations: Bu = butyl; Br-Hg-CPD-Y = $^{+}$Hg-CPD-Y reactivated by bromide; Bz = N-benzoyl; Bzl = benzyl; CPD-Y = carboxypeptidase Y; DFP = diisopropylphosphorofluoridate; FA = furylacryloyl; Hepes = N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; $^{+}$Hg-CPD-Y = CPD-Y inactivated by mercuric ions; HPLC = high performance liquid chromatography; I-Hg-CPD-Y = $^{+}$Hg-CPD-Y reactivated by iodide; Mes = 2-(N-morpholino)ethane sulfonic acid; PAB-CPD-Y = CPD-Y modified with phenacylbromide; Ph-Hg^{+} = phenylmercuric ions; Ph-Hg-CPD-Y = CPD-Y modified with phenylmercuric ions; p-HMB = parahydroxymercuribenzoate; Pr = propyl; Z = N-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 site notations for the enzyme is that of SCHECHTER and BERGER (163). 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. Amino acid residues in the substrate are referred to as P_1, P_2,..., P_i, and P_1' in correspondence with the binding site. A vertical arrow indicates the bond in the substrate being cleaved, henceforth termed the scissile bond.

CONTENTS

1. INTRODUCTION

It was suggested in 1960 by B.S. HARTLEY (62) that proteases could be divided into four groups based on the catalytic mechanism employed: 1) Acid or aspartyl proteases, 2) sulfhydryl proteases, 3) serine proteases and 4) metallo proteases. This classification primarily comprised endopeptidases because the catalytic mechanisms of exopeptidases were less understood at the time. However, more recent studies have demonstrated that the bacterial metallo endopeptidase thermolysin and the mammalian metallo exopeptidase carboxypeptidase A have similar constellations of essential amino acid residues in their active sites (104) and, as shown in the present paper, another group of carboxypeptidases exhibit mechanistic similarities with the serine endopeptidases. Hence, it is reasonable to include these two groups of exopeptidases in the original classification of proteolytic enzymes and it is possible that each of the four groups in the original classification of proteolytic enzymes may in the future be subdivided into endopeptidases and exopeptidases.

Exopeptidases of widely different specificities have been isolated: carboxypeptidases and dipeptidyl carboxypeptidases release C-terminal amino acid residues and C-terminal dipeptides, respectively, and analogously aminopeptidases and dipeptidyl aminopeptidases release N-terminal amino acid residues and N-terminal dipeptides, respectively. In addition, a variety of di- and tripeptidases have been described. It is characteristic for all of these enzymes that they only cleave peptide bonds at a specific location relative to the terminus of polypeptide chains in contrast to endopeptidases which, provided the side-chain specificity requirements are met, have the capacity to cleave a peptide bond regardless of its location in the peptide chain and even in the proximity of the terminals. Thus, an endopeptidase may also function as an exopeptidase although normally of low efficiency as demonstrated for trypsin (192). A comparison of the three-dimensional structures of the active sites of carboxypeptidase A and thermolysin illustrates the nature of the restrictions imposed upon an exopeptidase. The specificity of (metallo) carboxypeptidase A towards the C-terminus of a peptide chain can be attributed to the positively charged Arg-145 which binds the C-terminal carboxylate group of the peptide substrate (157), and together with Tyr-248 forms a dead-end pocket, incapable of accomodating more than a single amino acid residue on the C-terminal side of the scissile bond (153). In contrast, the metallo endopeptidase thermolysin exhibits no restrictions with regard to the length of the peptide chain on either side of the scissile bond (104). In terms of the nomenclature of SCHECHTER and BERGER (163) carboxypeptidase A possesses only a single leaving group binding site, i.e. S_1', as opposed to several in thermolysin, i.e. S_1', S_2' ...., S_i'. Three dimensional structures of other exopeptidases are not known, but it is highly conceivable that similar dead-end pockets as observed in carboxypeptidase A account for the specificities of all these enzymes.

All known carboxypeptidases may be classified either as metallo carboxypeptidases or as enzymes belonging to a group which originally by ZUBER and MATILE (204) was termed "acid carboxypeptidases" because the optimum for their hydrolysis of peptide bonds was in the acidic pH range as opposed to the metallo carboxypeptidases which hydrolyze peptides with the highest rates at basic pH values. However, this group of enzymes was renamed by HAYASHI et al. (72) because the original name suggested that they utilized the mechanism of the acid proteases and no evidence has been presented for this. On the contrary, all enzymes in this group are inhibited by DFP, a specific inhibitor of the serine proteases (151). The term "serine carboxypeptidases" was therefore adopted (EC 3.4.16).

While the metallo carboxypeptidases are well-characterized both with respect to phylogenetic relations and catalytic mechanism, much less is known about the serine carboxypeptidases and only for one of the enzymes, carboxypeptidase Y from yeast, is the primary structure known (36). However, the interest for these enzymes has increased in recent years as evidenced by the number of publications concerning their cellular location, physiological function, structure, mechanism of action, and applications in sequence determination and enzymatic peptide synthesis.

2. DISTRIBUTION AND FUNCTION OF SERINE CARBOXYPEPTIDASES

A literature survey suggests that all carboxypeptidases found in bacteria are metallo enzymes (e.g. 22) since the D,D-carboxypeptidases from various strains of bacteria which contain an essential serine residue (193, 201) incorrectly have been classified as carboxypeptidases: in addition to cleaving off a C-terminal amino acid residue from a peptide these enzymes also catalyze transpeptidation reactions with nucleophiles containing several amino acid residues, e.g. pentapeptides (164), and this is in conflict with the nucleophile specificity of the serine carboxypeptidases which is limited to single amino acid residues (see section 6.3). Hence, the D,D-carboxypeptidases possess several binding sites for the leaving group, i.e. S'_1, S'_2,..., S'_1, and cannot be classified as serine carboxypeptidases.

As seen from Tables I, II and III the serine carboxypeptidases are widely distributed in fungi and higher plants, and the observation that lysosomal cathepsin A is a serine carboxypeptidase (47) suggests that these enzymes also are common in animal tissues. In all organisms where they appear they presumably serve one of the following purposes: as intracellular enzymes, which are wide-spread in fungi as well as in higher plants and animals, they participate in the general turnover of proteins and in providing free amino acids from storage proteins; as extracellular enzymes, which primarily are found among fungi, they presumably function to cleave off amino acids needed for nutrition from external peptides and proteins.

2.1. Serine carboxypeptidases from fungi

As listed in Table I several strains of penicillum and aspergillus excrete extracellular serine carboxypeptidases (87, 202). Aspergillus saitoi in addition contains two intracellular serine carboxypeptidases with immunological properties similar to those of the excreted enzyme, but at present it cannot be established whether these enzymes are related or identical (88).

The yeasts Saccharomyces cerevisiae, i.e. baker's yeast, and Rhodotorula glutinis have both been shown to contain an intracellular

Table I. Serine carboxypeptidases from fungi

Source	Molecular weight			pI	carbo hydrate %	refe-rence
	non-dissociating conditions	dissociating, not reducing	dissociating, reducing			
Bakers yeast	64000	n.d.	61000	3.6	15-22	72, 98
Pycnoporus sanguinus	50000	n.d.	54000	4.8	n.d.	89
Aspergillus saitoi	155000	51000	n.d.	n.d.	n.d.	86
Aspergillus oryzae						
Type I	120000	n.d.	n.d.	n.d.	n.d	140
Type II	105000	n.d.	n.d.	n.d.	n.d.	141
Type III	61000	n.d.	n.d.	n.d.	n.d.	142
Type IV	43000	n.d.	n.d.	n.d.	n.d.	143
Type 0-1	63000	n.d.	n.d.	4.1	n.d.	180, 181
Type 0-2	63000	n.d.	n.d.	4.2	n.d.	180, 181
Aspergillus niger	136000	61000	73000	4.1	22	114
Rhodotorula glutinis	80000	n.d.	n.d.	n.d.	n.d.	78
Penicillum janthinellum						
Type S-1	48000	n.d.	48000	3.7	3	79
Type S-2	135000	65000	n.d.	4.7	7	79

n.d. = not determined

serine carboxypeptidase (68, 78) while extracellular counterparts have not been identified for either organism. Carboxypeptidase Y from baker's yeast resides primarily in the vacuoles like proteases A and B and aminopeptidase I (116). Specific inhibitors of each of these enzymes are located in the cytoplasm surrounding the vacuoles (16, 39, 118, 119, 125, 146, 161) presumably regulating proteolysis (39).

Carboxypeptidase Y is synthesized as a 59,000 molecular weight precursor (139) which, due to glycosylation and incorporation of phosphate, attains a molecular weight of 67,000 in the endoplasmatic reticulum and subsequently, one of 69,000 in the Golgi body (175). This inactive precursor is converted to the active enzyme by cleavage of a peptide bond near the N-terminus of the zymogen thereby releasing a peptide with a molecular weight of 6,000 – 9,000 (45, 65, 75). However, the proteolytic enzyme involved in this activation and its location in the cell is unknown, but it is probable that this process takes place subsequent to transferral of the precursor from the Golgi body to the vacuole (175). Other enzymes from yeast vacuoles (134) and from the lysosome (66, 67, 170), the organelle in animal cells with equivalent function (126), are similarly synthesized as precursors. This principle of protein synthesis may serve several purposes to cells: it may help the correct folding and crosslinking of peptide chains during protein synthesis, e.g. proinsulin (172), or in cases where the precursor is inactive it may protect the cell against unwanted enzymatic action, e.g. trypsinogen (144). Alternatively, an additional N-terminal peptide segment may function as a signal peptide guiding the enzyme to the correct location in the cell (75) in accordance with the hypothesis of BLOBEL and coworkers (18, 40). Studies by T. STEVENS et al. (176) have established that a signal sequence of 15 amino acid residues near the N-terminus is responsible for the translocation of pro-carboxypeptidase Y across the endoplasmatic reticulum and that another short sequence is required for efficient delivery of the protein to the vacuole. The carbohydrate portion of carboxypeptidase Y (see section 3) serves no such recognition functions since the carbohydrate-free enzyme synthesized in the presence of tunicamycin was seggregated into the vacuoles to the same extent as the intact glycoprotein (166).

2.2. Serine carboxypeptidases in higher plants

Serine carboxypeptidases have been found in various fruits and seeds (Table II) (49, 50, 109, 110, 111, 127, 128, 165, 188, 205) and sometimes they are induced during germination (49, 52, 91, 92, 152). Plant stems and leaves have also been shown to contain serine carboxypeptidases (41, 49, 171, 191) and in one of these cases wounding of a single leaf caused a three fold increase of carboxypeptidase activity in other unwounded leaves (191).

ZUBER and MATILE demonstrated in 1968 (204) that the serine carboxypeptidases in higher plants were intracellular enzymes localized in vacuoles and they assumed that they functioned in intracellular protein turnover. This observation has been confirmed in the more recent literature, but the picture is complex since higher plants are composed of many organs and tissues. Thus, in rice plants three apparently different serine carboxypeptidases have been found (49, 50). One was found in the leaves and it also appeared in the seeds during germination. Another enzyme was primarily present in resting seeds and it decreased during germination, and a third enzyme was transiently present in young roots and shoots. These results suggest that different tissues of plants have different carboxypeptidases in amounts which depend on the developmental stage of each tissue. However, it has not been established whether these carboxypeptidases represent isoenzymes. The carboxypeptidases isolated from germinated barley grains exhibit a similar complexity (11, 28, 37, 135, 154, 190, 200).

The serine carboxypeptidase induced during germination of cotton seeds functions together with other proteolytic enzymes to release amino acids from storage proteins and thus support the growth of the emerging tissues of the seedling (91, 92). The synthesis of this carboxypeptidase stops when the rate of disappearance of the storage protein is maximal. The carboxypeptidases from germinated barley have similar functions, rendering these enzymes important in the production of beer (52). In this process carboxy-

Table II. Serine carboxypeptidases from higher plants

Source	Molecular weight			pI	carbo hydrate %	refe-rence
	non-dissociating conditions	dissociating, not reducing	dissociating, reducing			
Barley (malt)						
Carboxypeptidase I	100000	51000	19000 32000	5.7	8	28
Carboxypeptidase II	120000	61000	27000 34000	n.d.	15	37
Rice bran	110000	n.d.	21000 35000	7.8	n.d.	50
Wheat bran	118000	58000	25000 35000	6.0	12	188
Leaves of wounded tomato plants	105000	n.d.	18000 37000	5.2	n.d	191
Germinating cotton seedings	85000	n.d.	24000 31000 33000	n.d.	1	91
Germinated wheat						
Type A	63000	n.d.	60000	5.7.	n.d.	152
Type B	54000	n.d.	56000	n.d.	n.d.	152
Rice leaves	120000	n.d.	n.d.	6.0	n.d.	49
Watermelon						
Type F-I	89000	n.d.	n.d.	4.4	n.d	127
Type F-II	110000	n.d.	n.d.	5.0	n.d	127
Citrus Natsudaidai	93000	n.d.	n.d.	n.d	10	109
Citrus orange	120-150000	n.d.	n.d.	n.d.	n.d.	205
Orange leaves	175000	n.d.	n.d.	4.5	n.d.	171
Mandarin orange						
Type C_{Ua}	96000	n.d.	n.d.	4.8	7	57
Type C_{Ub}	112000	n.d.	n.d.	4.7	6	57

n.d. = not determined

peptidases are responsible for the degradation of polypeptides released from the kernels by endopeptidases. After mashing, 10-15% of the grain-protein has been converted to free amino acids (52). In this context it has also been suggested that a chelator-insensitive carboxypeptidase during malting solubilizes β-glucans released from the barley, presumably by releasing amino acids from the glucan (9).

2.3. Mammalian serine carboxypeptidases

Among the intracellular lysosomal proteolytic enzymes, cathepsin A (47, 48, 100, 101, 102, 130) has been positively identified as a serine carboxypeptidase. The catalytic properties of the enzyme named cathepsin B2 suggest that it also is a carboxypeptidase (1) but inhibition data have so far not been presented. DOI (47) has argued that catheptic carboxypeptidase and ca-

Table III. Serine carboxypeptidases from mammalians

Source	Molecular weight			pI	carbo hydrate %	refe-rence
	non-dissociating conditions	dissociating, not reducing	dissociating, reducing			
Rat liver cathepsin						
Type A_I	100000	n.d.	several	4.7	n.d.	130
Type A_{II}	200000	n.d.	several	4.8	n.d.	130
Type A_{III}	420000	n.d.	several	4.9	n.d.	130
Pig kidney cathepsin						
Type A,L	500000	n.d.	20000 25000 50000	5.8	n.d.	100, 102
Type A,S	100000	n.d.	20000 25000 50000	5.0	n.d.	101, 102
Human kidney prolyl carboxypeptidase	115000	66000 45000	n.d.	n.d.	n.d.	147

n.d. = not determined

thepsin A is really the same enzyme, but both enzymes are incompletely characterized with respect to physico-chemical and immunological properties. The function of the lysosomal enzymes in mammals is presumably similar to the function of the enzymes localized in the vacuoles of yeast cells.

The enzymes in various human organs, in urine and in leucocytes which cleave the C-terminal Pro-Phe peptide bond in angiotensins II and III – thereby inactivating their hypertensive activity – have also been demonstrated to be serine carboxypeptidases (147, 148). The enzyme isolated from the kidney is presumably located in the lysosome, but its importance in regulation of the level of angiotensin and hence blood pressure is still unknown (147).

3. PHYSICO-CHEMICAL PROPERTIES OF SERINE CARBOXYPEPTIDASES

Tables I, II, and III list the molecular weights, isoelectric points and contents of carbohydrate of serine carboxypeptidases isolated from fungi, higher plants and animals, respectively. It is observed that all investigated serine carboxypeptidases, with the exception of the one from rice leaves, are acidic proteins which, apart from the one from cotton seeds, contain covalently linked carbohydrate. The molecular weights range from 43,000 to approximately 500,000 under non-dissociating conditions, i.e. as determined by gelfiltration or ultracentrifugation in the absence of urea, SDS etc. Where measurements were performed under dissociating conditions but in the absence of reducing agents, e.g. mercaptoethanol, molecular weights of 45,000 to 65,000 were obtained, suggesting that the higher molecular weights are due to association of monomers into dimers or oligomers. ICHISHIMA (86) has demonstrated for the carboxypeptidase from Aspergillus saitoi that the association is influenced by the salt concentration: in the presence of NaCl the molecular weight is around 140,000, and in the absence of NaCl the enzyme exists in two forms of molecular weights 51,000 and 140,000, respectively. However, similar studies of the influence of ionic strength on the association properties have not been performed with other serine carboxypeptidases.

With respect to the number of peptide chains in the molecule important differences are apparent between enzymes isolated from fungi (Table I) and those isolated from higher plants (Table II): serine carboxypeptidases from fungi contain only a single peptide chain with a molecular weight of 48,000 – 73,000 (including attached carbohydrate) while those isolated from tomato plants, rice bran, wheat bran and germinated barley all contain two peptide chains of molecular weights 32,000 – 37,000 for the largest chain and 19,000 – 27,000 for the smallest chain. Since these enzymes under non-dissociating conditions are characterized by molecular weights of 100,000 – 120,000 it may be assumed that they normally are dimers and that the monomers of molecular weights 50,000 – 60,000 are composed of two different peptide chains linked by disulfide bridges. This structure may well be shared by other serine carboxypeptidases isolated from higher plants which have not been investigated in similar detail. The enzyme from germinating cotton seedlings was shown to contain three peptide chains of molecular weights 33,000, 31,000 and 24,000, but it is conceivable, as pointed out by the authors, that the peptide chain of molecular weight 31,000 is derived from the one of molecular weight 33,000, such that this enzyme in reality is composed of two peptide chains.

The limited data available on the subunit structure of serine carboxypeptidases from animals (Table III) suggest similarities with the structure of the serine carboxypeptidases from higher plants. The cathepsins A,S and A,L from porcine kidney have molecular weights of 100,000 and 500,000, respectively. However, both enzymes are composed of peptide chains of molecular weights 20,000, 25,000 and 55,000 (102), which might indicate that the two enzymes contain identical subunits but different degrees of association.

The structural differences between serine carboxypeptidases from fungi and those from higher plants and animals could be due to different mechanisms of converting the zymogens to active enzymes. Several zymogens, e.g. chymotrypsinogen, have been shown to be activated by a proteolytic cleavage of internal peptide bonds located between two disulfide bridges, such that the active species comprises two peptide chains linked by disulfide bridges (145) and this could similarly account for the existence of two peptide chains in the serine carboxypeptidases from plants. The existence of only a single peptide chain in the active enzymes from fungi suggests that potential zymogens of these enzymes might be activated by cleavage of an internal peptide bond located on the N-terminal side of the disulfide bridges. This mechanism of activation has been demonstrated for the zymogen of carboxypeptidase Y (see section 2.1).

The amino acid composition of several serine carboxypeptidases has been determined (28, 37, 79, 91, 98, 110, 111, 114, 188, 191) and it is characteristic that the enzymes isolated from fungi have much higher contents of the amino acids Asx and Glx and lower contents of the basic amino acids, i.e. Lys and Arg, than those from higher plants. All the enzymes contain disulfide bridges, and the enzymes from yeast (6, 27) penicillum (79) and carboxypeptidase I from barley (28) have been shown in addition to contain a single buried sulfhydryl group (further details are given in section 6). Approximately 98% of the amino acid sequence of carboxypeptidase Y has been identified by SVENDSEN and coworkers (124, 179) utilizing traditional Edman degradation, and DNA sequencing has recently completed the primary structure (T. STEVENS, personal communications) (36, 176). At present, approximately 80% of the amino acid sequences of the A-chains of malt carboxypeptidases I and II are known and they indicate 35% homology with each other and approximately 30% homology with the N-terminal portion of yeast carboxypeptidase (S. SØRENSEN, K. BREDDAM, I. SVENDSEN and M. OTTESEN, unpublished observations) suggesting that the serine carboxypeptidases from higher plants and those from fungi are related. Very little homology with sequences of other known proteolytic enzymes has been observed, suggesting that serine carboxypeptidases are not or only distantly related to other serine proteases.

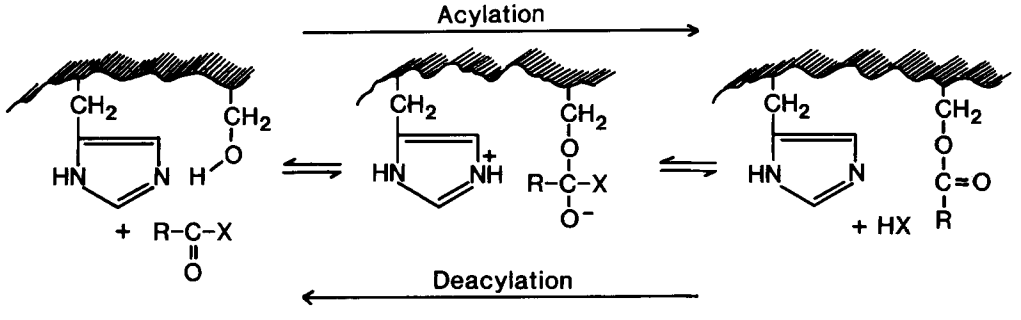
Apart from the enzyme from cotton seedlings (91) all the serine carboxypeptidases, which have been analyzed for carbohydrate content, have turned out to be glycoproteins (Table I) contain-

ing neutral sugar (galactose and/or mannose) as well as glucosamine. Carboxypeptidase Y contains four carbohydrate moieties which are attached to the protein through asparagine side chains (65, 183) at positions tentatively identified by amino acid sequencing (36). All four positions are characterized by an Asn-X-Thr/Ser sequence which is typical in glycoproteins with N-linked carbohydrates (85). Like in other enzymes from the vacuoles of yeast and from the lysosomes of mammalian cells (64, 66, 67, 184) each carbohydrate moiety in carboxypeptidase Y is composed of two N-acetylglucosamine residues and a branched mannose moiety (63, 184). Three of these carbohydrate moieties may be cleaved off by endo-β-N-acetylglucosaminidase H and each of these contains from 11 to 18 mannose residues and 1 or 2 phosphate residues. The fourth moiety which is only released from denatured carboxypeptidase (183) contains 8 – 12 mannose residues and no phosphate (184). This moiety is presumable attached before final folding of the protein with the consequence that it becomes inaccessible rendering further attachment of mannose residues impossible as opposed to the three accessible carbohydrate moieties (184).

The heterogeneity of the carbohydrate portion of carboxypeptidase Y has resulted in enzyme preparations of widely different carbohydrate content (120) and enzyme with four phosphate groups has been separated from enzyme with five phosphate groups by ion exchange chromatography (63). This probably explains why an apparently homogeneous preparation of yeast carboxypeptidase (98) with no evidence for the existence of isoenzymes as evaluated from sequence studies (179) has been shown to contain at least two species with different isoelectric points and slightly different activities (98). It has not been clarified whether the phosphate and/or carbohydrate attached to the enzyme influence enzymatic activity. Enzymatic removal of the three accessible carbohydrate moieties did not alter the peptidase activity of carboxypeptidase Y (183), but since denaturation of the enzyme was needed to remove the fourth moiety, this could not be fully answered (43, 183). More direct evidence might be obtained by kinetic characterization of the carbohydrate-free carboxypeptidase Y produced by yeast grown in the presence of tunicamycin (65) but such results have hitherto not been described. It may be noted that malt carboxypeptidase I also exists in two species with identical N-terminal sequences but different isoelectric points. The reasons for this heterogeneity may be the same as for yeast carboxypeptidase.

4. THE CATALYTIC MECHANISM OF THE SERINE ENDOPEPTIDASES

The results of chemical modifications and kinetic studies during the 1950ies and 60ies suggested that a seryl and a histidyl residue were involved in the catalytic mechanism of the serine



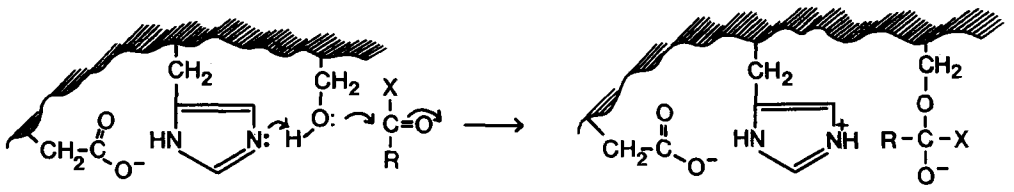
Scheme 1. The reaction mechanism of the serine proteases as proposed by BENDER and KEZDY (12). Im = imidazole, X = OR or NHR in the acylation and OH in the deacylation reaction.

endopeptidases, and based on these observations BENDER and KEZDY (12) proposed the reaction mechanism shown in Scheme 1. It is seen that the nucleophilic attack by the hydroxyl group of the reactive seryl residue on the carbonyl carbon atom of the substrate is catalyzed by a histidyl residue functioning as a general base. This leads to the formation of a tetrahedral intermediate and an imidazolium ion. The intermediate breaks down by general acid catalysis to an acyl-enzyme, an imidazole base, and an alcohol or amine depending on the nature of the substrate. The acyl-enzyme is hydrolysed through the reverse reaction pathway with water functioning as nucleophile instead of the hydroxyl group of the seryl residue. The first X-ray crystallographic studies of chymotrypsin confirmed that a histidyl residue is located in the proximity of the active site seryl residue (131) and later crystallographic studies have provided support for the existence of both tetrahedral (83) and acyl-enzyme intermediates (76, 77) in the reaction sequences of these enzymes.

X-ray crystallography has furthermore revealed that the histidyl side-chain also interacts with a buried aspartyl side-chain (19) and it is generally accepted that all three amino acid residues, i.e. Ser, His, and Asp, are functionally essential because they are present in all serine endopeptidases which so far have been investigated by X-ray crystallography. BLOW et al. (19) proposed that the side-chains of Asp, His, and Ser form a triad linked by hydrogen bonds thereby constituting a "charge relay system" which functions by a partial transfer of the hydrogen atom of the hydroxyl group of the seryl residue to the aspartyl carboxylate group, hence forming a strongly nucleophilic alkoxide group. However, KRAUT and coworkers (108, 133) have shown for several serine endopeptidases that such a hydrogen bond does not exist between the seryl and the histidyl residues and consequently, the seryl side-chain could hardly be strongly nucleophilic. They proposed that the seryl residue reacts with the substrate only because it is in the optimum position to attack the substrate's tetrahedrally distorted carbon atom which has been induced by its binding to the enzyme. Thus, the only function of the His-Asp couple would be to transfer a proton from the seryl -OH to the leaving group in the acylation reaction. After years of dispute over the state of ionization of the His-Asp couple ROBERTS and coworkers (4, 5) have now convincingly demonstrated, by means of ^{15}N and ^{13}C NMR spectroscopy that the pK_a of the imidazolium cation of the histidyl residue of α-lytic protease is approximately 7, and that the enzyme is in its active form, i.e. pH > 7, when the histidyl side-chain is protonated at N-3 and unprotonated at N-1. They consequently suggested that the tetrahedral adduct is formed by the mechanism shown in Scheme 2 which deviates from the mechanisms proposed by other investigators (19, 84) by the aspartyl side-chain remaining unprotonated during the reaction. It was suggested that this negatively charged group instead functions in one of the following ways: a) to favour the correct imidazole tautomer, b) to orient the imidazole ring, or c) to stabilize the imidazolium cation. All these studies were performed with "resting" enzyme, i.e. in the absence of substrate, but crystallographic studies on the complex between trypsin and pancreatic trypsin inhibitor have suggested that a hydrogen bond between the seryl and histidyl residues does exist (83) and NMR studies of this enzyme-inhibitor complex imply partial formation of an alkoxide anion on the seryl residue (121). These results are in conflict with those obtained by KRAUT and coworkers (108, 132), suggesting that important differences exist between "resting" and "working" enzyme (174), such that observations with resting enzyme are insufficient to explain important aspects of its function.

X-ray crystallography studies on several serine endopeptidases suggest that the binding sites responsible for the proper interaction between enzyme and substrate are of three categories: (a) binding sites for the backbone of polypeptide substrates, (b) binding sites for the amino acid side-chains, and (c) the oxyanion binding site.

The interaction between enzyme and the backbone of the acyl portion of polypeptide substrates was first observed in chymotrypsin (167) and in subtilisin (107, 158). However, similar interactions with the backbone of the imine portion of peptide substrates have not



Scheme 2. Mechanism of formation of the tetrahedral intermediate as proposed by BACHOVCHIN and ROBERTS (4).

been demonstrated. In subtilisin X-ray crystallography has indicated the existence of four binding sites for the side-chains at the P_1, P_2, P_3, and P_4 positions of the substrate (158, 159), and for trypsin, chymotrypsin, and elastase similar results have been obtained (169, 173, 177). A crevice in chymotrypsin for the side-chain in the P_1 position is hydrophobic and shaped such that aromatic side-chains fit much better than other hydrophobic and hydrophilic side-chains (173). A corresponding cleft in trypsin is similar in geometry, but it contains a negatively charged aspartyl side-chain which renders the enzyme specific for substrates with a positively charged side-chain, i.e. Lys and Arg, in the P_1 position (177). In elastase two bulky side-chains protrude into the hydrophobic crevice for the amino acid side-chain at the P_1 position hence, reducing its size and consequently, elastase allows the binding of the methyl side-chain of an alanyl residue in the substrate much better than more bulky side-chains (169). Subtilisin is characterized by a much wider specificity than chymotrypsin, trypsin, and elastase and consistent with this it contains a binding site for the side-chain at the P_1 position which is sterically much less restrictive than in the other enzymes. X-ray studies of the complexes between trypsin and pancreatic trypsin inhibitor (83) have indicated that the side chains of the amino acid residues in the P_1' and P_2' positions of both inhibitors interact with the enzyme but in a less specific manner than observed on the other side of the scissile bond. However, kinetic studies of several serine endopeptidases have provided evidence for the importance of interactions between enzyme and the side-chains on both sides of the scissile bond as reviewed by SVENDSEN (178).

The binding sites of proteolytic enzymes determine their specificity but they probably also function to stabilize the transition state configuration. ROBERTUS et al. (159) have suggested that this is partially achieved by an increase in the binding strength between enzyme and substrate at the P_1 position as the Michaelis complex is converted into the tetrahedral intermediate and HENDERSON (76, 77) has proposed the existence of a specific binding site for the oxyanion in the serine endopeptidases. He suggested that hydrogen bonds were formed between the substrate carbonyl oxygen of the acyl-enzyme intermediate and two amide groups in the backbone of chymotrypsin, and that these bonds might be stronger in a tetrahedral transition state. A true tetrahedral transition state with a covalent bond between the serine at the active site of the enzyme and the substrate has not yet been observed, but X-ray crystallography of the complexes between trypsin and pancreatic trypsin inhibitor indicate that the reactive carbonyl group in the inhibitor is tetrahedrally distorted with the oxygen atom hydrogen bonded to two amide groups of the backbone of the enzyme located in the oxyanion binding site (83). However, it appears that no covalent bond has been formed between the substrate and the seryl residue in the active site since an identical complex is formed with trypsin where the active site serine has been converted to dehydroalanine (83). Hence, the intermediate may be considered a pretransition state Michaelis complex as opposed to a true transition state, and its formation is apparently dependent on the existence of a complementary surface on the enzyme, i.e. the oxyanion binding site and the binding sites for the side-chains and backbone of the substrate. However, the results are controversial since NMR studies have failed to provide confirma-

tion of the tetrahedrally skewed carbonyl group (155).

Additional evidence for the existence of an oxyanion binding site has been obtained by studying the three dimensional structures of complexes between serine endopeptidases and transition state analogues, i.e. substances which with the reactive seryl residue form stable adducts with tetrahedral structures resembling the proposed transition state shown in Scheme 1. Thus, in trypsin treated with DFP an oxygen atom of the diisopropylphosphoryl group accepts hydrogen bonds from the two amide groups located in the proposed oxyanion binding site (177). Phenylboronic acid, 2-phenylethylboronic acid (105, 117, 132), phenylarsonic acid (17, 59), and various aromatic sulfonyl flourides (76) have been shown to form similar tetrahedral adducts to the active site serine hydroxyl group in serine endopeptidases.

5. THE CATALYTIC MECHANISM OF SERINE CARBOXYPEPTIDASES

As discussed in section 4 two different features are responsible for the catalysis of serine endopeptidases: a) a catalytic triad, consisting of Ser, His, and Asp, and b) multiple binding sites for the substrate. Similar features are probably responsible for the catalytic activity of serine carboxypeptidases, but short of X-ray studies an evaluation of their catalytic properties must be based on the results of kinetic studies, chemical modifications, and site-directed mutagenesis.

5.1. Modification of catalytically essential amino acid residues

DFP has been shown to specifically modify the seryl residue in the active site of numerous serine endopeptidases causing inactivation of these enzymes (e.g. 151). In fact, inactivation of a proteolytic enzyme with this reagent has become presumptive evidence for the involvement of a seryl residue in the catalytic mechanism. All activities of the carboxypeptidases listed in Tables I to III are inhibited by DFP demonstrating that a seryl residue is essential for their catalytic activity, hence justifying their classification as serine carboxypeptidases. The site of modification has been identified as a seryl residue in three of these enzymes, i.e. carboxypeptidase Y (71) and carboxypeptidases C_{Ua} and C_{Ub} from mandarin orange (57). The amino acid sequence around the reactive seryl residue is compared with those of subtilisin and mammalian serine endopeptidases in Scheme 3. It is observed that similarities exist between the carboxypeptidases from mandarin orange and mammalian endopeptidases while the sequence around the seryl residue in carboxypeptidase Y exhibits only slight analogy with other enzymes. It should be noted that all the listed enzymes contain a glycyl residue in position 3 in the N-terminal direction of the active site seryl residue suggesting it to have arisen as a result of a convergent evolution.

Enzymes	Amino acid sequences
	*
Carboxypeptidase C_{Ua}	-Glu-Gly-Asp-Ser-Gly-Gly-Glu-Leu-
Carboxypeptidase C_{Ub}	-Glu-Gly-Asp-Ser-Gly-Gly-Glu-Leu-
Carboxypeptidase Y	-Ala-Gly-Glu-Ser-Tyr-Ala-His-Gly-
Trypsin(bovine)	-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Val-
Chymotrypsin(bovine)	-Met-Gly-Asp-Ser-Gly-Gly-Pro-Leu-
Elastase(pig)	-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-
Plasmin(human)	-Gln-Gly-Asp-Ser-Gly-Gly-Pro-Leu-
Thrombin(bovine)	-Glu-Gly-Asp-Ser-Gly-Gly-Pro-Phe-
Subtilisin BPN	-Asn-Gly-Thr-Ser-Met-Ala-Ser-Pro-

Scheme 3. Amino acid sequences around the reactive seryl residue (marked with an asterisk) of typical serine proteases. Data from ref. 57.

Various chloromethylketones have been demonstrated to specifically modify the essential histidyl residue of the serine endopeptidases (150, 168), and HAYASHI et al. (69) have demonstrated that a reagent of this type also modifies a single histidyl residue in carboxypeptidase Y abolishing both esterase and peptidase activities. Unfortunately, the position of the modified residue in the sequence has not yet been identified. The involvement of a histidyl residue in the catalytic mechanism of other serine carboxypeptidases has similarly been implicated: the carboxypeptidase from Aspergillus saitoi is also inhibited by a chloromethylketone (86), and the carboxypeptidases from Penicillum janthinellum (79) and mandarin orange (112) are inhi-

bited by photooxidation. The probable importance of a seryl and a histidyl residue suggest that serine carboxypeptidases employ a mechanism similar to the one employed by the serine endopeptidases. This is supported by the fact that carboxypeptidase Y, as shown in this laboratory, is inhibited by p-aminophenylarsonic acid and phenylboronic acid (98), two compounds which are considered to be transition state analogues for the serine endopeptidases (see section 4). However, as yet no evidence has been presented for the involvement of an aspartyl residue in the catalytic mechanism of the serine carboxypeptidases.

5.2. Kinetic evidence for the serine mechanism

In the reaction sequence of carboxypeptidase Y catalyzed hydrolysis reactions only indirect evidence points to the existence of an acyl-enzyme intermediate (122). DOUGLAS et al. (51) have by means of stopped-flow spectrophotometry observed burst kinetics in the carboxypeptidase Y catalyzed hydrolysis of 4-nitrophenyl trimethylacetate and shown this to be in agreement with the existence of a trimethylacetyl-enzyme in the reaction course. However, for a number of the serine carboxypeptidases listed in Tables I, II and III it is observed that esterase activity is optimal approximately two pH units higher than peptidase activity and this differs from the serine endopeptidases for which the hydrolysis of all substrates is dependent on the deprotonation of a histidyl residue (4) with a pK_a around 7.0 (12). This brings up the question whether the influence of pH on the various activities of the serine carboxypeptidases is compatible with the proposed mechanism of the serine endopeptidases.

Among the serine carboxypeptidases the influence of pH on the kinetic parameters of ester and peptide hydrolysis have only been studied for carboxypeptidase Y and malt carboxypeptidase II. With the former enzyme the present author has shown (27) that k_{cat} for the hydrolysis of Bz-Ala↓OBzl is dependent on the deprotonation of an ionizable group with a pK_a of 5.2 – 5.5 and it remains constant in the pH range 7.5 – 9.5 (Figure 1A). K_m for the hydrolysis of Bz-Ala↓OBzl is dependent on the deprotonation of an

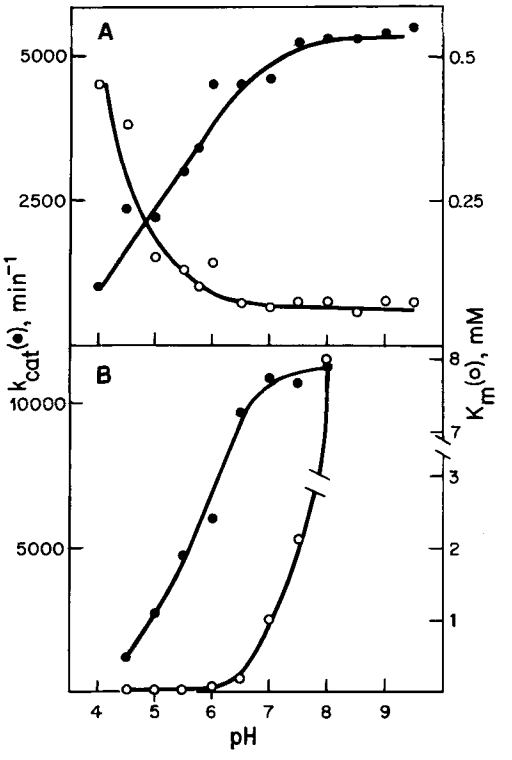


Figure 1. The influence of pH on k_{cat} and K_m for carboxypeptidase Y catalyzed hydrolysis of the ester Bz-Ala↓OBzl (Panel A) (27) and the peptide FA-Phe↓Ala-OH (panel B) (34).

ionizable group with a pK_a below 5 and remains constant in the pH range 6 – 9.5. Similar results have been reported by HAYASHI using Ac-Phe↓OEt as substrate (70). For the peptide substrates Z-Phe↓Leu-OH (70) and FA-Phe↓Ala-OH (34) k_{cat} is dependent on the deprotonation of a group with a pK_a of 5.4 while K_m increases with protonation of a group with a pK_a around 7 (Figure 1B). Thus, the different pH optima for hydrolysis of ester and peptide substrates are primarily due to the influence of pH on K_m for the hydrolysis of peptide substrates. For amide substrates the pH dependence of k_{cat} and K_m have not been determined because these substrates cannot be dissolved in sufficiently high concentrations, but the pH profiles for hydrolysis rates suggest similarities with ester substrates (8, 28), presumably due to the fact that ester and amide substrates both contain a blocked C-terminal

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\quad P_1$$

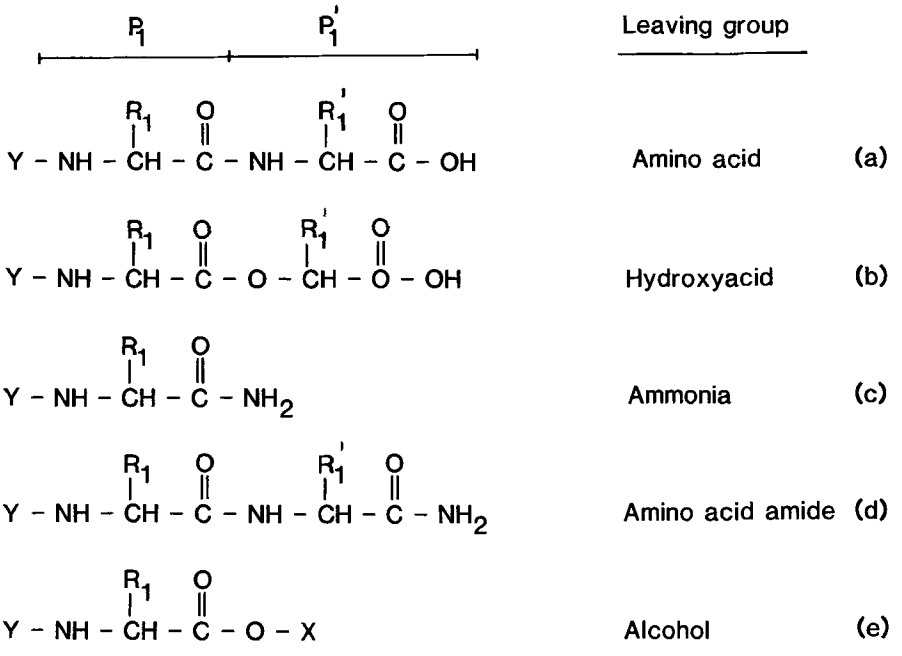
$$k_{cat} = \frac{k_2 \cdot k_3}{k_2 + k_3} \qquad K_m = K_s \cdot \frac{k_3}{k_2 + k_3}$$

Scheme 4. Reaction mechanism for the serine proteases. E = enzyme; S = substrate; ES = Michaelis complex; K_s = dissociation constant of the ES complex; ES' = the acyl-enzyme intermediate; P_1 = leaving group; P_2 = hydrolysis product; k_2 and k_3 are the rate constants for the acylation and deacylation reactions, respectively.

carboxylic acid group. Similar observations have been made with malt carboxypeptidase II but with the difference that k_{cat} for the hydrolysis of ester and peptide substrates, i.e. FA-Ala↓OBzl and FA-Phe↓Phe-OH, respectively, is dependent on the deprotonation of a group with a pK_a around 4.0 (38).

The fact that k_{cat} for the hydrolysis of both ester and peptide substrates depend on ionizable groups with pK_a values around 5.4 and 4.0 for carboxypeptidase Y and malt carboxypeptidase II, respectively, suggest that the ionization of a single residue in both enzymes determine their activities towards all substrates regardless of whether the carboxylate group on the substrate is blocked or not. This residue presumably corresponds to the essential histidyl residue in the serine endopeptidases, and as mentioned in section 5.1 a histidyl residue is indeed essential to the action of carboxypeptidase Y. Accordingly, the high activity of the serine carboxypeptidases in the acidic pH ranges relative to the corresponding endopeptidases can be explained by unusually acidic essential histidyl residues. A number of serine carboxypeptidases have been reported to exhibit optima towards peptides significantly below that of malt carboxypeptidase II (e.g. 115, 141, 142, 143). However, the substrates used in these studies contain side-chains which ionize in this pH range, e.g. Z-Glu↓Tyr-OH, and accordingly the observed pH profiles do not necessarily reflect ionizations on the enzyme.

Assuming the reaction sequence of the serine endopeptidases (Scheme 4) to be valid for the serine carboxypeptidases as well, it is possible to estimate the relative magnitudes of k_2 and k_3 for some of these enzymes. For carboxypeptidase Y (70), watermelon carboxypeptidase (129) and cathepsin A (103) it has been demonstrated that k_{cat} for the hydrolysis of N-blocked dipeptides is strongly dependent on the nature of the leaving group, i.e. the C-terminal amino acid residue. A similar dependence of k_{cat} on the alcohol leaving group of ester substrates has been demonstrated for carboxypeptidase Y (8, 27), malt carboxypeptidases I and II (30, 38). With the serine endopeptidases the leaving group influences k_{cat} for the hydrolysis of amide bonds while for the hydrolysis of ester substrates it is independent of the nature of the leaving group. This is consistent with $k_2 \ll k_3$ for the hydrolysis of amide bonds and $k_2 \gg k_3$ for the hydrolysis of ester bonds (12), and consequently, with the serine carboxypeptidases $k_2 \ll k_3$ for the hydrolysis of both amide and ester bonds. However, the limited data must be evaluated with caution since the situation where $k_2 \ll k_3$ cannot be distinguished from $k_2 \sim k_3$ as long as only the steady-state kinetics of hydrolysis reactions have been studied. Determination of kinetic parameters in the presence of added nucleophiles is more informative and such studies have been performed with malt carboxypeptidase I in this laboratory using Z-Lys↓ONp as substrate and H-Val-NH_2 as added nucleophile (33). Under these conditions the acyl-enzyme intermediate is partitioned between hydrolysis and aminolysis products, i.e. Z-Lys-OH and Z-Lys-Val-NH_2, respectively (see section 6.3). In case the deacylation reaction were rate-limiting, k_{cat} for the disappearance of substrate should increase by the addition of H-Val-NH_2. However, no variation of k_{cat} was observed, suggesting that the rate-limiting step lies ahead of the deacylation step, i.e. acylation is rate-limiting ($k_2 \ll k_3$). The acylation is assumed to be rate-limiting for all N-blocked amino acid esters with the exception of substrates characterized by an unusually low k_3, e.g. trimethylacetyl nitrophenylester (51). Since the rate of acylation for ester substrates is much faster than for amide substrates the acylation step is also assumed to be rate-limiting for the



Scheme 5. Substrates of the serine carboxypeptidases. Y = peptidyl or N-blocking group; R_1 and R_1' = amino acid side-chain; X = Me, Et or Bzl.

hydrolysis of N-blocked dipeptides, and consequently $k_{cat} \sim k_2$ and $K_m \sim K_s$ (see Scheme 4).

6. BINDING SITES IN SERINE CARBOXYPEPTIDASES

6.1. The influence of the substrate structure

The influence of systematic alterations in the structure of the substrate on the kinetic parameters for its hydrolysis is a commonly used method to obtain information about the nature of the binding sites (reviewed by SVENDSEN (178)). The degree of complementation between enzyme and substrate is perhaps best reflected by k_{cat}/K_m since proteolytic enzymes as discussed by FRUTON (56) and FERSHT (54) appear to use part of the binding energy to increase k_{cat} and consequently the value for K_s (see Scheme 4) is not necessarily a true measure for the affinity of the enzyme for different substrates.

Carboxypeptidases are characterized by their inability to hydrolyse any other peptide bond than the C-terminal in a polypeptide chain. Such hydrolysis reactions may formally be considered as transacylation reactions with a peptide as acyl-donor, water as acyl-acceptor, and an amino acid as leaving group. However, like the serine endopeptidases, the serine carboxypeptidases also catalyze reactions with other acyl-donors and acyl-acceptors, e.g. various alcohols and amines. Thus, two types of specificity must be considered: the specificity in the acylation reaction towards the acyl-donor which is the substrate, and the specificity in the deacylation reaction towards the acyl-acceptor, i.e. the nucleophile. The present section describes only hydrolysis reactions and hence only the specificity towards the acyl-donor.

Carboxypeptidase Y and malt carboxypeptidases I and II have in the present laboratory been shown to catalyze the hydrolysis of substrates of the types listed in Scheme 5 (23, 28, 37, 98), and it is probable that other serine carboxypeptidases which are less extensively characterized have similar specificities. It is apparent that serine carboxypeptidases do not exhibit an absolute requirement for a carboxylate ion on the leaving

Table IV. Influence of the leaving group on the kinetic parameters for carboxypeptidase Y catalyzed hydrolysis reactions.

Substrate (Scissile bond)	pH	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	ref.
Bz-Gly↓Phe-OH	6.5	230	7.7	30	70,73
Bz-Gly-OGly-OH	6.5	1800	56	32	[a]
Bz-Gly-OPhe-OH	6.5	1100	0.05	22000	98
Bz-Gly-OMe	7.5	150	48	3.1	[a]
Bz-Gly-OEt	7.5	150	21	7.1	[a]
Bz-Gly-O^{i}Pr	7.5	260	20	13	[a]
Bz-Gly-OBzl	7.5	1100	1.7	650	[a]
Bz-Ala-OGly-OH	6.5	3500	14	250	[a]
Bz-Ala-OMe	7.5	3100	6.3	490	27
Bz-Ala-OBzl	7.5	7400	0.07	106000	27
Bz-Phe-Gly-OH	6.5	690	1.1	630	[a]
Bz-Phe-OGly-OH	6.5	2100	2.4	880	[a]
Bz-Phe-OMe	7.5	9100	0.18	51000	27
Z-Phe-Gly-OH	6.5	8400	4.0	2100	70, 73
Z-Phe-NH_2	8.0	320	10	32	8
Z-Phe-NH-CH_3	7.5		not hydrolysed		70, 73
FA-Phe-Gly-OH	6.5	5800	5.4	1100	34
FA-Phe-Ala-OH	6.5	9700	0.14	61000	34
FA-Phe-Val-OH	6.5	6500	0.047	140000	34
FA-Phe-Leu-OH	6.5	4900	0.021	230000	34
FA-Phe-NH_2	7.5	–	–	89	34
FA-Phe-Gly-NH_2	7.5	–	–	160	34
FA-Phe-Val-NH_2	7.5	–	–	6200	34
FA-Phe-OMe	7.5	11000	0.39	28000	34

[a] K.BREDDAM. unpublished results

group since they also, like the serine endopeptidases, release alcohols and amines without a negative charge. This is in contrast to the metallo carboxypeptidases which are specific for peptides and depsipeptides. Thus, although serine carboxypeptidases do not cleave internal peptide bonds they combine elements from the specificity of the serine endopeptidases with the specificity requirements of the metallo carboxypeptidases. Nevertheless, carboxypeptidase Y contains only a single active site since modification of either of the essential seryl or histidyl residues abolishes the activities towards both peptides and alkyl esters (see section 5.1).

In order to analyze the specificity of serine carboxypeptidases in further detail it is necessary to analyze how the specificity constant k_{cat}/K_m is influenced by variations in each of the structural features which characterize the substrate (see Scheme 5): a) the nature of the scissile bond, b) the importance of the negatively charged carboxylate ion of the leaving group, c) the nature of the N-blocking group Y, and d) the nature of the amino acid side-chains R_1 and R'_1. The latter aspect is normally referred to as side-chain specificity, and for the sake of convenience the nature of X in alkyl esters is included in this concept.

Table IV lists the influence of the leaving group on the kinetic parameters for carboxypep-

tidase Y catalyzed hydrolysis of amide and ester bonds. Comparing substrates which only differ in the nature of the scissile bond, one being an amide bond the other an ester bond reveals that the ester bond is hydrolysed with much higher rates than the amide bond. Thus, k_{cat}/K_m for the hydrolysis of Bz-Gly↓OPhe-OH, an ester substrate with a carboxylate group on the leaving group, is 2-3 orders of magnitude higher than for Bz-Gly↓Phe-OH, the corresponding peptide substrate. For substrates without a carboxylate group on the leaving group similar differences are observed: k_{cat}/K_m for the hydrolysis of FA-Phe↓OMe is approximately 200 times higher than for the hydrolysis of FA-Phe↓NH_2 and FA-Phe↓Gly-NH_2. Similar observations have also been made with the malt carboxypeptidases and several serine endopeptidases. This influence of the scissile bond can be attributed to the higher reactivity of an ester bond as compared with an amide bond.

The inherent strength of the amide bond where ammonia functions as leaving group is similar to the strength of one where the leaving group is an amino acid. Nevertheless, as shown in this laboratory, k_{cat}/K_m for the carboxypeptidase Y catalyzed hydrolysis of FA-Phe↓Gly-OH is 7-12 times higher than for FA-Phe↓NH_2 and FA-Phe↓Gly-NH_2, and for FA-Phe↓Val-OH it is 23 times higher than for FA-Phe↓Val-NH_2 (Table IV). This implicates a specific influence of the carboxylate group and one might expect that ester substrates would be similarly influenced by a carboxylate group such that k_{cat}/K_m for the hydrolysis of depsipeptides would be much higher than for corresponding alkyl esters. However, such an effect of a carboxylate group was not observed in all cases: Bz-Gly↓OGly-OH and Bz-Gly↓OPhe-OH are hydrolysed by carboxypeptidase Y with higher k_{cat}/K_m than the corresponding alkyl ester, i.e. Bz-Gly↓OMe and Bz-Gly↓OBzl, respectively, but Bz-Phe↓OGly-OH and Bz-Ala↓OGly-OH are hydrolysed with lower k_{cat}/K_m than the corresponding alkyl esters, i.e. Bz-Phe↓OMe and Bz-Ala↓OMe, respectively (Table IV).

The observed effects of a C-terminal carboxylate group in peptide substrates suggest the existence of a positively charged binding site on the enzyme for this group. In metallo carboxypeptidase A K_m for hydrolysis of peptides is constant in the pH range 5 – 9 (3) consistent with the arginyl residue ($pK_a \sim 12$) being the binding site for the C-terminal carboxylate group of the substrate (153, 157). Contrary to this, studies in this laboratory indicate that K_m for the carboxypeptidase Y (34) and malt carboxypeptidase II (38) catalyzed hydrolysis of N-blocked dipeptides increases sharply above pH 5.5 and provided that K_m is a measure for K_s (see section 5.2) this pH dependence presumably eliminates an arginyl residue as binding site for C-terminal carboxylate groups. It is more likely that a positively charged histidyl side-chain functions as anion binding site at acidic pH values. However, the state of ionization of the proposed

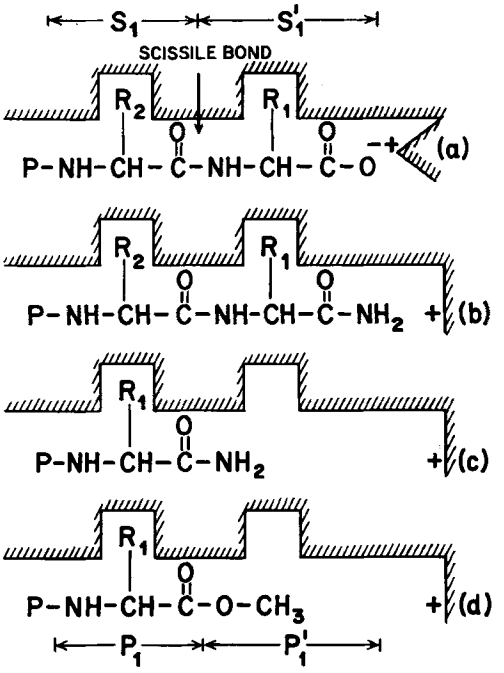


Figure 2. Schematic presentation of the binding regions in the active site of carboxypeptidase Y. The binding notation is that of SCHECHTER and BERGER (163) (see abbreviations), and the dead-end structure of the active site is assumed to account for the exo-peptidase specificity of carboxypeptidase Y. The figure demonstrates conceivable binding modes of peptides, peptide amides, and peptide esters, accounting for the peptidase (a), peptidyl amino acid amide hydrolase (b), amidase (c), and esterase (d) activities of the enzyme.

anion binding site has apparently no influence on the binding of ester substrates to these enzymes since K_m for the hydrolysis of esters is essentially constant in the pH range 6-9. A possible explanation could be that this binding site is not in the proximity of the alcohol leaving group of ester substrates but only "moves into position" as a consequence of binding of a substrate with a charged C-terminal as is suggested in Figure 2 which also illustrates how the various types of substrates might bind productively to the enzyme.

Kinetic studies with N-blocked dipeptides and N-blocked amino acid esters have for some serine carboxypeptidases provided information about the side-chain specificities, i.e. the influence of R_1, R_1' and X on k_{cat}/K_m (Table V). Carboxypeptidase Y hydrolyses both ester and peptide substrates with aromatic and other hydrophobic amino acid residues in the P_1 position much faster than those with hydrophilic amino acid residues in this position. Thus, k_{cat}/K_m for the hydrolysis of Bz-Phe↓OMe is 4,000 times higher than for Bz-Arg↓OMe, and similarly with peptide substrates k_{cat}/K_m for the hydrolysis of Z-Phe↓Ala-OH is 20,000 times larger than for Bz-Lys↓Ala-OH (27). These results indicate that the binding site for the side-chain at the P_1 position is hydrophobic like that of chymotrypsin (see section 4). In contrast, the serine carboxypeptidases from watermelon, malt and porcine kidney rapidly hydrolyse ester substrates both with hydrophobic and positively charged residues, i.e. Arg, Lys, or His, in the P_1 position (Table V) (30, 38, 103, 129). Available data for peptide hydrolysis by these enzymes suggest a preference for hydrophobic amino acid residues in the P_1 position, but substrates with positively charged residues have not been included in systematic studies (103, 129). However, it is conceivable that they all hydrolyse such substrates with high rates as it has been demonstrated for malt carboxypeptidases I and II (K. BREDDAM, unpublished results). Thus, the binding sites for the side-chain at the P_1 position in the enzymes from malt, watermelon and porcine kidney are presumably more complex than that of carboxypeptidase Y since they seem to accomodate an aromatic and a positively charged side-chain equally well. Work in this laboratory with malt carboxypeptidases I and II has demonstrated that the high k_{cat}/K_m 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 (30, 38). Detailed kinetic studies with malt carboxypeptidase II indicate that addition of NaCl has an adverse effect on the binding of FA-Arg↓OMe while the binding of FA-Phe↓OMe appears tighter at higher ionic strength suggesting ionic forces to be important for the interaction between binding site, and the positively charged side-chain of Arg, while hydrophobic forces are responsible for the interaction with the uncharged side-chain of Phe (38). These observations are consistent with a negatively charged residue on the enzyme being important for the binding of positively charged side-chains without having any particular influence on binding of uncharged side-chains (see Figure 3). The results with malt carboxypeptidase I are qualitatively identical (30). The subtilisins have a similar wide specificity with respect to the P_1 position, and it has been suggested that this originates from different modes of productive binding for substrates with an aromatic and a basic residue in the P_1 position (60).

Other serine carboxypeptidases have not been studied in similar detail, but their substrate preferences suggest that they exhibit specificities with respect to the P_1 position corresponding to that of either carboxypeptidase Y or to those of the carboxypeptidases from watermelon, malt, and human kidney. Human prolyl carboxypeptidase is an exception since it, due to its special function (see section 2), only cleaves substrates where the P_1 position is occupied by a prolyl residue.

With the exception of malt carboxypeptidase II all the enzymes listed in Table V exhibit preferences for substrates with hydrophobic R_1' (see Scheme 5) suggesting that they contain hydrophobic binding sites for this side-chain. However, it should be noted that aliphatic hydrophobic amino acids, i.e. Val, Ile, Leu, Met, and Ala, are released faster than aromatic residues, i.e. Phe and Tyr. The preference of malt carboxypeptidase II for basic R_1', i.e. Arg and Lys, renders this enzyme unique among serine carboxypeptidases. Since it also releases hydrophobic amino acid residues its binding site for

Table V. Specificity of serine carboxypeptidases

Source of carboxypeptidase	Substrates	pH	P_1 position	Scissile bond	P'_1 position
Carboxypeptidase Y from yeast	Peptides	6.5	Phe > Leu > Ala > > His > Glu > Gly > > Lys		Met,Ile,Leu > Phe > Ala > Arg > Ser > Pro > Lys > Asn > Gly > Asp
(8,27,34,70)	Esters	7. 5	Phe > > Leu > Met > Ala > Ile, Val > Thr, His, Arg > Gly > Lys > Asp,Pro		Bzl > Et > Me
Watermelon (129)	Peptides	5.2	Phe > Ala, Leu > Glu > Gly > > Pro		Ala,Leu > Val > Phe > Tyr > Pro > Gly > Glu
	Esters	7.0	Arg > Tyr,Phe		
Malt Carboxypeptidase I (28,30)	Peptides	4.7	Phe,Lys > Ala[a)]		Val > Ala,Ile,Met > Phe > Pro > Ser > Gly,Asp > His,Lys,Arg
	Esters	7.5	Arg > Lys > Phe > His > Ala > Thr > Val, Ile > Pro, Asp, Gly		Bu > Pr > Et > Me
Malt Carboxypeptidase II (37,38)	Peptides	4.7			Lys > Arg > > Ile,Met > Phe,Val > His > Ala > Ser > Asp,Asn > Gly > Pro
	Esters	8.0	Phe > Lys > Arg > Leu > Met > His > Ala > Val,Ile > Gly,Thr,Pro,Asp		Bzl > OMe > OEt
Cathepsin A from pig kidney	Peptides	5.2	Phe > Ala > Leu,Glu > Gly > > Pro		Ala > Val > Leu > Phe > Pro > Gly > Arg
(103)	Esters	8.0	Arg > Phe,Tyr		

The preference of each enzyme is listed in order of decreasing k_{cat}/K_m values. In cases where k_{cat}/K_m values deviate less than 20% a comma is used instead of the larger than symbol. The peptide substrates used in these studies are N-blocked dipeptides, and the ester substrates are N-blocked amino acid esters. [a)]K. BREDDAM, unpublished results.

the side-chain at the P_1' position presumably contains separate regions to secure the interaction with these groups of different nature. This is supported by the fact that addition of NaCl has an adverse effect on the binding of FA-Ala↓Lys-OH, whereas the binding of FA-Phe↓Phe-OH is enhanced (38). This binding site therefore appears similar to that for the R_1 group in both carboxypeptidases I and II. The influence of the nature of X, i.e. the alcohol leaving group of ester substrates, on k_{cat} has only been investigated for carboxypeptidase Y and malt carboxypeptidases I and II, and for these enzymes an increase in k_{cat}/K_m is observed with increasing size (hydrophobicity) of the leaving group, suggesting that the alcohol leaving group binds to hydrophobic regions of the S_1' binding site.

It is known that carboxypeptidase Y hydrolyses the substrates listed in Scheme 5 only when the amino acid residues in the P_1 and P_1' positions are in the L-form (7, 70) and when the amino group of the amino acid in the P_1 position is blocked, i.e. no hydrolysis is observed when Y = H. k_{cat}/K_m for carboxypeptidase Y catalyzed hydrolysis of peptide, amide, and ester substrates is strongly affected by the nature of the N-blocking group occupying the P_2 position and decrease in the following order: Z, Bz, FA, and Ac (8, 27, 34, 70). It is unknown how the length of the peptide chain influences k_{cat}/K_m for the hydrolysis of the C-terminal ester or amide bond.

6.2. Studies with modifiers

The effects on catalysis of substances which bind specifically to the binding sites, i.e. modifiers, have previously been utilized to study the nature of binding sites in proteolytic enzymes as illustrated by the work of INAGAMI and MURACHI on trypsin (93). This enzyme hydrolyses substrates with positively charged side-chains, i.e. Lys and Arg, in the P_1 position with high rates but hydrolyses only very slowly substrates with other amino acid residues in this position. However, in the presence of positively charged substances like methyl ammonium, ethyl ammonium, or 1-propylammonium ions k_{cat} for the hydrolysis of Ac-Gly↓OEt is drastically increased while K_m remains unchanged. They concluded that the combination of an unspecific substrate and an ammonium ion simulates the behaviour of the specific substrate with a positively charged residue in the P_1 position. Later work has demonstrated similar effects of numerous other amine and guanidine compounds (162, 182, 189).

Among the serine carboxypeptidases three cases have been reported where addition of a substance produce activity changes which can be related to its binding to particular substrate binding sites. 3-phenyl-l-propanol increases k_{cat} and decreases K_m for the carboxypeptidase Y catalyzed hydrolysis of Z-Gly↓Phe-OH (6). Since the hydrolysis of Z-Phe↓Leu-OH, which has a bulky amino acid residue in the P_1 position, is inhibited by phenylpropanol this substance presumably binds to the binding site for the side-chain at the P_1 position of the substrate. The hydrophobic nature of 3-phenyl-l-propanol combined with the fact that the negatively charged 3-phenylpropionic acid does not bind at this binding site is consistent with it being hydrophobic.

The ability of malt carboxypeptidase II to hydrolyse substrates containing either hydrophobic or positively charged amino acid residues on both the P_1 and P_1' positions combined with the effects of added NaCl already suggested that within the S_1 and S_1' binding sites separate areas exist to secure the interaction with these side-chains of different nature (see section 6.1). A postulated binding region of the active site of malt carboxypeptidase II, incorporating this hypothesis, is shown in Figure 3. 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 interaction with hydrophobic side-chains of the substrate. It appears that phenylguanidine binds to the S_{1a} and S_{1a}' binding sites of malt carboxypeptidase II with an attenuation of the binding of FA-Arg↓OMe and FA-Ala↓Lys-OH as a result (38). In contrast, the binding of uncharged substrates containing bulky groups in either the P_1 or P_1' positions, e.g. FA-Phe↓OMe, is enhanced in the

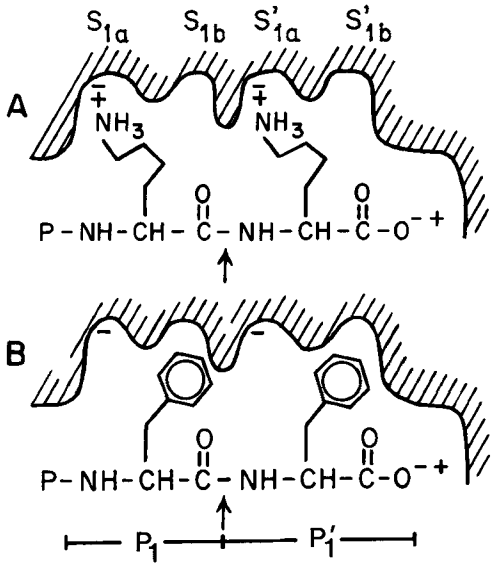
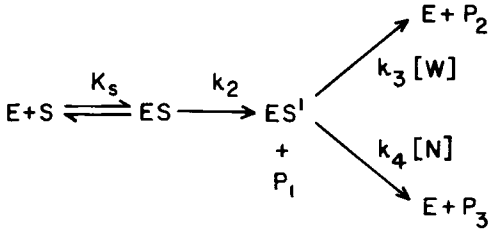


Figure 3. Schematic representation of the binding regions in malt carboxypeptidase II. The binding notation is that of SCHECHTER and BERGER (see abbreviations), and the dead-end structure is assumed to account for the exo-peptidase activity of the enzyme. (A) shows a proposed binding mode of a peptide 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.

presence of phenylguanidine. This suggests that such groups when bound to the S_{1b} or S_{1b}' sites interact favourably with the phenyl group of the phenylguanidine bound at the S_{1a} or S_{1a}' sites. When non-bulky groups are bound to both the S_{1b} and S_{1b}' binding sites, viz. FA-Ala↓OEt, the interaction with the phenylguanidine appears to increase k_{cat} rather than to increase binding of the substrate. These results with phenylguanidine support the model of the binding region of malt carboxypeptidase II shown in Figure 3.

The specificity of malt carboxypeptidase I (see Table V) suggests that its S_1 binding site comprises two distinct regions as indicated for malt carboxypeptidase II in Figure 3, but its inability to hydrolyse substrates with basic residues in the P_1' position suggests the existence of only a single region in S_1' binding site. However, it appears that phenylguanidine does not bind to the S_{1a} binding site of malt carboxypeptidase I since it enhances the rate of the hydrolysis of Bz-Arg↓OMe. Thus, in spite of apparent similarities in the S_1 binding sites of the two malt carboxypeptidases, as judged by their specificities, certain structural differences must exist to account for their different behaviour towards phenylguanidine. The apparent absence of an S_{1a}' binding site in carboxypeptidase I would predict that phenylguanidine should not bind to its S_1' binding site. However, in the presence of this substance the activities towards FA-Phe↓Ala-OH and FA-Phe↓Gly-NH_2 are decreased to less than 3% of the control whereas the activities towards FA-Phe↓OMe and FA-Phe↓NH_2 are increased to 380% and 220% of the control, respectively. These results indicate that when the leaving group of the substrate is large, i.e. an amino acid or an amino acid amide, it competes with phenylguanidine for the same site. The activation observed with FA-Phe↓OMe and FA-Phe↓NH_2 suggests that the binding of phenylguanidine affects the catalytic properties of the enzyme without adversely affecting its ability to bind substrates with small leaving groups, i.e. the enzyme may conceivably bind both groups simultaneously. A kinetic study of the effects of phenylguanidine on the hydrolysis of ester substrates with leaving groups of different size confirmed this interpretation (30). While k_{cat} for the hydrolysis of all the substrates, i.e. Bz-Arg↓OMe, -OEt, -OPr and -OBu, is increased by the presence of this substance, K_m is affected in a manner which depends on the size of the leaving group: it decreases for Bz-Arg↓OMe and increases for Bz-Arg↓OEt, -OPr and -OBu. Thus, it appears that phenylguanidine binds to the binding site for the leaving group of ester substrates, i.e. the S_1' binding site, and that this position in the enzyme functions as the binding site for the leaving groups of all types of substrates. The effects of chemical modifications of a methionyl residue located at the S_1' binding site of carboxypeptidase Y on its specificity (see section 6.4) indicates that this conclusion is valid for carboxypeptidase Y as well.



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

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

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

Scheme 6. Competition between water (W) and other nucleophile (N) in the serine protease catalyzed hydrolysis of ester substrates as proposed by BENDER et al. (12). The enzyme (E) and substrate (S) initially form 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 intermedidate (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.

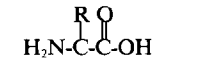
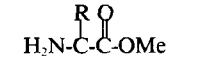
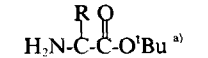
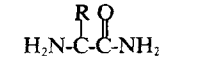
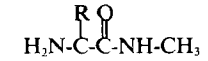
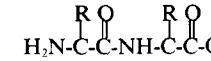
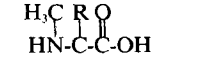
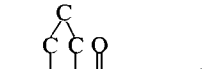
6.3. Binding sites for nucleophiles

In the serine protease catalyzed hydrolysis of amide and ester bonds water functions as nucleophile. When such reactions are allowed to proceed in aqueous solution with another nucleophile added, e.g. an amine compound, a partitioning of the acyl-enzyme may take place to yield a hydrolysis product and an aminolysis product. BENDER et al. (13) and FASTREZ and FERSHT (53) 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 6, 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.

Carboxypeptidase Y and the malt carboxypeptidases have similarly in this laboratory been demonstrated to catalyze transacylation reactions to other nucleophiles than water, e.g. to alcohols, amino acids and amino acid derivatives (24, 33, 38, 195, 196, 197). For carboxypeptidase Y it has been shown, using N-blocked amino acid esters as substrates, that the fraction of aminolysis, $P_3/(P_2+P_3)$, is highly dependent on the nature of the amine nucleophile added. With H-Asp-OH, H-Asp-α-OMe, H-Asp-α-NH_2, H-Glu-OH, H-Glu-α-OMe, H-Glu-α-NH_2, H-Pro-OH, H-Pro-OMe, and H-Pro-NH_2 no aminolysis is observed. However, with the other L-amino acids, amino acid methyl esters, and amino acid amides aminolysis is observed with the fraction of aminolysis varying with the nature of the side-chain as seen from Table VI. D-amino acids, D-amino acid methyl esters, and D-amino acid amides do not react with the acyl-enzyme, suggesting that the reaction with the nucleophile is stereospecific. No aminolysis takes place with secondary amines, e.g. proline and sarcosine, and more bulky substituents on the carbonyl group than -OMe and -NH_2, e.g. -OEt, -O^{t}Bu, and -NH-CH_3 result in decreased fractions of aminolysis with the exception of H-Gly-OEt and H-Gly-O^{t}Bu.

The variations in the efficiency of the nucleophiles listed in Table VI cannot be attributed to different nucleophilicities. This combined with the stereospecificity of the enzyme towards the nucleophile would suggest that binding of the added nucleophile to the acyl-enzyme intermediate, presumably at the S_1' binding site, preceeds its participation in the deacylation reaction. In fact, the results listed in Table VI suggest that the nucleophiles which react with the acyl-enzyme intermediate exhibit structural resemblance with the leaving groups (P_1') of the substrates described in Scheme 5. However, the fact that proline is released from the C-terminus of peptides by carboxypeptidase Y and nevertheless cannot function as nucleophile in the deacylation reaction suggest that certain differences between the structures of substances which can function as leaving groups and those

Table VI. Carboxypeptidase Y catalyzed aminolysis reactions.

Nucleophile	Fraction of aminolysis
	0.10 – 0.65
Substitutions on carbonyl group:	
	0.25 – 0.90
	0 – 0.10
	0.70 – 0.90
	0.10
	0
Substitutions on amino group:	
	0
	0

All reactions are performed with Bz-Ala-OMe as substrate at pH 9.5 using various concentrations of nucleophile. R = amino acid side-chain with the exception of the side chains of Asp and Glu. Fraction of aminolysis is defined as $P_3/(P_2+P_3)$ with P_2 = hydrolysis product and P_3 = aminolysis product (see Scheme 5). [a] H-Gly-O^{t}Bu is an exception since the fraction of aminolysis with this nucleophile is as high as with the methyl ester. Results from ref. 196 & 197.

which can function as nucleophiles do exist. Although the release of an amino acid ester from a peptide ester would be difficult to demonstrate due to the much faster release of the alcohol group it should be noted that this reaction has not been demonstrated and yet, amino acid methyl esters can function as nucleophiles.

The studies of carboxypeptidase Y catalyzed aminolysis reactions were originally aimed at the synthetic aspects of such reactions rather than investigating in detail the causes for the different fractions of aminolysis with nucleophiles of different structures. Consequently, the aminolysis reactions were often performed at arbitrary concentrations of nucleophiles, and from such results it is not possible to decide whether a high fraction of aminolysis with a given nucleophile is due to a tight binding of the nucleophile to the enzyme or alternatively, due to an effective attack of the nucleophile on the acyl-enzyme intermediate. Malt carboxypeptidase I similarly catalyzes aminolysis reactions of ester and peptide substrates when various amino acid amides and methyl esters are used as nucleophiles, and these reactions have now been investigated in further detail (33) than the corresponding reactions with carboxypeptidase Y. The influence of the concentration of nucleophile on the fraction of aminolysis demonstrated that the enzyme was easily saturated with nucleophile indicating the formation of a complex between nucleophile and acyl-enzyme intermediate prior to deacylation. This observation is consistent with the fact that phenylguanidine, which has been shown to bind to the S_1' binding site of malt carboxypeptidase I (see section 6.2), when added to aminolysis reactions causes a drastic increase in $K_{N(app)}$, the apparent dissociation constant of the complex between acyl-enzyme and the tested nucleophiles. This indicates a competition between phenylguanidine and the nucleophiles for the S_1' binding site of the enzyme. Phenylguanidine has no effect on the aminolysis reactions with H-Gly-OMe but strongly affects $K_{N(app)}$ for H-Ala-OMe and H-Val-OMe suggesting that it is the side-chains of amino acid methyl esters which are bound to the S_1' binding site in such a manner that they occupy or overlap with the binding site for phenylguanidine. No such differences were observed with amino acid amides: phenylguanidine affected $K_{N(app)}$ for all amino acid amides tested including H-Gly-NH_2. This indicates that ester and amide nucleophiles exhibit slightly different binding modes within the S_1' binding site of the enzyme. This could explain why amino acid amides are more effective as nucleophiles than amino acid methyl esters, viz. the fraction of aminolysis at saturation with nucleophile is much higher with amino acid amides (0.90) than with amino acid methyl esters (0.50). It may also explain why for amino acid amides $K_{N(app)}$ is lower and less dependent on the nature of the

amino acid side-chain than for amino acid methyl esters. However, the binding of both types of nucleophiles appears to be stereospecific since with the D-enantiomers H-val-NH_2 and H-ala-OMe no aminolysis products are obtained.

FINK and BENDER (55) have previously observed similar binding effects when larger n-alkyl alcohols were added to papain acting on p-nitrophenyl ester substrates. They found that their results could only be explained by adopting a reaction scheme in which the added nucleophile binds to the enzyme at two different positions, one of which is identical to the binding site for the leaving group, i.e. the S_1' binding site. By studying the influence of H-Val-NH_2 on the steady-state parameters, k_{cat} and K_m, for the action of malt carboxypeptidase on Nα-CBZ-Lys-ONp it was demonstrated that this enzyme also has two binding modes for the added nucleophile, of which only one causes aminolysis of the acyl-enzyme intermediate (33). More recently RIECHMANN and KASCHE have found that nucleophiles similarly bind to chymotrypsin prior to their participation in the deacylation reaction (156), suggesting that such binding is general for proteolytic enzymes. The results with malt carboxypeptidase I (33) demonstrate that studies of the competition between water and added nucleophiles in the deacylation reaction may provide information about the binding sites of proteolytic enzymes which cannot be obtained from the study of hydrolysis reactions.

6.4. Modifications of binding sites

Chemical modifications of amino acid side-chains in enzymes have been used in numerous cases to correlate loss of a certain amino acid residue with changes in activity in order to establish which side-chains are functionally essential. In such studies it is important to distinguish between two types of side-chains: a) those which are essential to catalysis (see section 5.1) and b) those which alone or in combination with others function in the binding of substrate, e.g. the side-chains in chymotrypsin which form the hydrophobic crevice for the side-chain in the P_1 position of the substrate. While chemical modifications of essential amino acid residues usually abolish the catalytic activity, modifications of side-chains at binding sites may have different effects on activity, depending on the nature of the reagent and the importance of the particular side-chain as previously demonstrated with some proteolytic enzymes (44, 80, 178). Recently, it has become possible by site-directed mutagenesis to specifically exchange amino acid residues in proteins (206). Modifications of this type are in many ways less drastic than the chemical modifications provided that the mutated enzyme is able to attain its proper tertiary structure. Both chemically modified and mutated enzymes can provide insight into the nature of the binding sites beyond what is obtained by kinetic studies and in addition, these may have an altered specificity which is of potential interest in processes where proteolytic enzymes are used (see section 7).

6.4.1. Modifications at the S_1 binding site

It has long been known that carboxypeptidase Y from baker's yeast contains a cysteinyl residue (71) and this has recently, in this laboratory, been identified in the amino acid sequence as Cys-341 (36). This residue is inaccessible to iodoacetate and Ellman's reagent unless the enzyme is denatured. However, without denaturation of the enzyme it reacts rather slowly with p-HMB ($k = 0.45\ mM^{-1} \cdot sec^{-1}$) producing the spectral change expected for a cysteine and it reacts quickly with Hg^{++} ($k = 1000\ mM^{-1} \cdot sec^{-1}$) (6). Only stoichiometric amounts of p-HMB or Hg^{++} are bound, and Hg^{++} apparently binds tighter to the sulfhydryl group than the mercuribenzoate group since addition of 1 equivalent Hg^{++} to carboxypeptidase Y previously modified with p-HMB results in an instantaneous displacement of the mercuribenzoate group from the cysteinyl residue. The different reactivities of mercurials and iodoacetate may be due to different abilities to penetrate the enzyme molecule before reaction with the sulfhydryl group or alternatively, due to different abilities to produce a conformational change rendering the buried cysteinyl residue accessible.

Modification of the cysteinyl residue with organomercurials causes significant inhibition

Table VII. Relative specificity of carboxypeptidase Y and carboxypeptidase Y modified with I-Hg$^+$ and Ph-Hg$^+$.

Substrate	CPD-Y	I-Hg-CPD-Y	Ph-Hg-CPD-Y
Bz-Gly-OMe	1	1	1
Bz-Ala-OMe	160	89	43
Bz-Val-OMe	58	111	4
Bz-Ile-OMe	84	360	2
Bz-Leu-OMe	1800	1700	68
Bz-Met-OMe	900	6100	30
Bz-Phe-OMe	16000	17000	790
Bz-Lys-OMe	0.18	46	0
Bz-Arg-OMe	3.9	30	0
Bz-His-OMe	4.2	21	0
Bz-Thr-OMe	5.8	13	0.30

The numbers which represent relative k_{cat}/K_m values can only be compared within the same column. The results are from ref. 27.

of the enzyme, but this residue is nevertheless not essential for catalysis since substantial activity is retained towards some substrates (6, 27), and the pH dependence of k_{cat} for ester hydrolysis remains unchanged (27). This is consistent with recent experiments where Cys-341 has been exchanged by site-directed mutagenesis with a seryl residue or a glycyl residue. These derivatives of carboxypeptidase Y are active, but the activity towards peptide substrates is only around 10% (J. WINTHER, M.C. KIELLAND-BRANDT and K. BREDDAM, unpublished results).

Studies in this laboratory have shown that Hg^{++}, in the absence of halides, i.e. Cl^-, Br^-, SCN^-, and CN^-, causes complete inactivation of carboxypeptidase Y regardless of the nature of the substrate (27). This total lack of activity of the modified enzyme is possibly due to the mercuric ion forming a -S-Hg-X-bridge between the sulfhydryl group and some other amino acid side-chain X capable of forming a rather tight complex with Hg^{++}, e.g. X = His, Asp, or Glu (194). This explanation is supported by the observation that addition of halides which form tight complexes with Hg^{++} (194), partially reactivates the enzyme without removing the metal from the enzyme (27). It is conceivable that this is due to the halide displacing X from the mercuric ion bound to the sulfhydryl group thus forming an active enzyme species, e.g. -S-Hg-Cl when Cl^- is added.

BAI and HAYASHI (6) found that the inhibition of carboxypeptidase Y by organomercurials is most pronounced when the enzyme is assayed towards substrates with bulky side-chains in the P_1 position, indicating that the sulfhydryl group is located at the binding site for this side-chain. This information warranted an extensive study by the present author of the influence of modification with mercurials on the specificity of carboxypeptidase Y with respect to the P_1 position (27). The kinetic constants for a series of ester substrates with the general formula Bz-X-OMe, where X = amino acid residue, have been determined for unmodified carboxypeptidase Y, carboxypeptidase Y modified with I-Hg$^+$ (Hg^{++} modified enzyme assayed in the presence of I^-), and carboxypeptidase Y reacted with phenylmercuric chloride. Modification with both mercurials causes a decrease in k_{cat} and an increase in K_m for the hydrolysis of all substrates tested with the exception of Bz-Lys-OMe where modification of I-Hg$^+$ caused a 40 times increase in k_{cat}. A comparative assessment of the specificity of these three enzymes has been made by calculating relative k_{cat}/K_m values with Bz-Gly-OMe serving as reference for each enzyme (Table VII). As previously mentioned carboxypeptidase Y exhibits a strong preference for substrates with hydrophobic residues in the P_1 position, i.e. Phe, Leu, and Met, and only slowly hydrolyses substrates with hydrophilic residues, i.e. Arg, Lys, Thr, and Gly. The two modified

enzymes retain the highest k_{cat}/K_m for substrates with hydrophobic residues although their relative preference for the different substrates deviate substantially and differ from the preferences of unmodified enzyme. With carboxypeptidase Y modified with Ph-Hg^+, the relative k_{cat}/K_m is reduced drastically for all substrates except Bz-Ala$\downarrow$OMe. This is equivalent to an increased preference for Bz-Gly$\downarrow$OMe and Bz-Ala$\downarrow$OMe, and can be explained by the Ph-Hg^+ group introducing a steric hindrance into the binding site for the side-chain at the P_1 position with the consequence that the hydrolysis of substrates characterized by a bulky side-chain in the P_1 position, e.g. Bz-Phe$\downarrow$OMe, is much more affected by the Ph-Hg^+ modification than the hydrolysis of substrates with non-bulky side-chains in this position, i.e. Bz-Gly$\downarrow$OMe and Bz-Ala$\downarrow$OMe. Introduction of the I-Hg^+ group has not similar steric effects since the hydrolysis of Bz-Gly$\downarrow$OMe and Bz-Phe$\downarrow$OMe are affected to the same extent, i.e. the relative k_{cat}/K_m for Bz-Phe$\downarrow$OMe remains unchanged. However, the drastic increase in the relative k_{cat}/K_m for the hydrolysis of Bz-Lys$\downarrow$OMe and the slight increase for substrates with Val, Ile, Met, Arg, His, and Thr in the P_1 position indicate that the properties of the binding site of the modified enzyme is different from that of the unmodified enzyme. The exact nature of this change is difficult to evaluate; possibly the effects of the I-Hg^+ group on Bz-Lys$\downarrow$OMe hydrolysis are due to attenuation of an unfavourable interaction between enzyme and substrate.

Malt carboxypeptidase II contains no sulfhydryl group but it is still inactivated by Hg^{++} (37). However, while the carboxypeptidase Y-Hg^+ complex exhibits a dissociation constant far below 10^{-8} M in the pH range 4 – 10 (K. BREDDAM, unpublished results) the formation of a malt carboxypeptidase II-Hg^+ complex is dependent on deprotonation of a group on the enzyme with an apparent pK_a of 6.4 and the dissociation constant is rater high ($1.3 \cdot 10^{-6}$ M at pH 7.5). The comparatively poor binding of Hg^{++} to carboxypeptidase II is also apparent from the lack of inhibition in the presence of iodide since this indicates that Hg^{++} binds less tightly to the enzyme than to iodide. The site of reaction in malt carboxypeptidase II is unknown, but the essential histidyl residue is a possibility since the histidyl side-chain is known to form complexes with Hg^{++} (194). In this context it should be noted that CHAMBERS et al. (42) by X-ray crystallography have demonstrated that Ag^+ binds to a position between the aspartyl and histidyl side-chains of the catalytic triad in trypsin, and Hg^{++} might be expected to have a similar affinity for this position.

The influence of Hg^{++} on malt carboxypeptidase I which does contain a sulfhydryl group is essentially identical to that on malt carboxypeptidase II (28) and thus, it is probable that the inactivation is not due to reaction with the sulfhydryl group. This is consistent with this group being inaccessible to p-HMB as opposed to the sulfhydryl group in carboxypeptidase Y (27). Dilution of malt carboxypeptidase I inactivated by Hg^{++} apparently causes a dissociation of the enzyme-Hg^{++} complex. However, the activity of the enzyme was only partially recovered and with increasing reaction period a decreasing proportion of the enzyme could be reactivated. These results indicate that the initial enzyme-Hg^{++} complex undergoes an irreversible time dependent inactivation presumably due to denaturation of the enzyme. The time-course of this denaturation suggests a complex reaction scheme which might involve conformational changes as well as a secondary reaction of Hg^{++} with the sulfhydryl group.

p-HMB inhibits a number of the serine carboxypeptidases from fungi (Table I) and the various types of cathepsin A (Table III) and hence, it is probable that these enzymes contain a sulfhydryl group of reactivity as the one in carboxypeptidase Y. The carboxypeptidases from higher plants (Table II) and human prolyl carboxypeptidase, on the other hand, are not inhibited by p-HMB. An inhibitory effect of Hg^{++} is often seen with these enzymes but as shown with the malt carboxypeptidases this does not necessarily indicate the presence of a sulfhydryl group.

The serine endopeptidases protease B from yeast (106) and thermitase from Thermoactinomyces vulgaris (10) also contain a single sulfhydryl group. Both enzymes are inhibited by p-HMB and Hg^{++} but not by other reagents known to modify thiols indicating that their sulfhydryl group is buried and of similar accessibility as the

one in carboxypeptidase Y. A peptide containing the cysteinyl residue of thermitase has been shown to exhibit homology with a stretch of the amino acid sequence of subtilisin. The position of the cysteinyl residue in thermitase corresponds in subtilisin to Val-68 which in the three-dimensional structure is located behind the histidyl residue of the catalytic triad. The fact that so many genetically unrelated but functionally similar enzymes contain a sulfhydryl group suggests that it has a function although it is not strictly required in catalysis as indicated by the studies with carboxypeptidase Y (6, 27). Possibly, it could have a function in stabilizing the protein structure since carboxypeptidase Y is destabilized by chemical modification of the sulfhydryl group (27) and by replacement of the cysteinyl residue with a glycyl or seryl residue by site-directed mutagenesis (J. WINTHER, M.C. KIELLAND-BRANDT, K. BREDDAM, unpublished results).

6.4.2. Modification at the S_1' binding site

The S_1' binding site in serine carboxypeptidases presumably consists of binding sites for both the side-chain and the carboxylate group of the C-terminal amino acid residue of the substrate (see Figure 2). KUHN et al. (113) have, based on chemical modifications with phenylglyoxal, suggested that an arginyl side-chain functions as the carboxylate binding site in carboxypeptidase Y, but results obtained in the present laboratory on chemical modifications of carboxypeptidase Y with phenylglyoxal and butanedione have not provided evidence for this conclusion (21). Malt carboxypeptidase I has also been treated with these reagents and no inactivation of this enzyme was observed (K. BREDDAM, unpublished results). In addition, kinetic studies with both carboxypeptidase Y and malt carboxyptidase II have suggested that the binding of peptides is dependent on the deprotonation of an ionizable group with a pK_a around neutral (see section 6.1) which is far below the pK_a of an arginyl side-chain, but instead suggests the involvement of a histidyl residue or, alternatively, the N-terminal α-amino group in the binding of peptide carboxylate groups.

KUHN et al. (113) have described that alkylation of a methionyl residue in carboxypeptiase Y with iodoacetamide causes a reduction in the peptidase activity of the enzyme without significantly altering the esterase activity. In this laboratory, this methionyl residue has been identified as Met-398 in the amino acid sequence (36) and in addition it has been alkylated with phenacylbromide, m-nitrophenacylbromide and p-nitrophenacylbromide (34) and oxidized with H_2O_2 (35). All these derivatives of carboxypeptidase Y have been separated from residual unmodified enzyme by affinity chromatography and characterized kinetically (34, 35). Modification of carboxypeptidase Y with phenacylbromide influences k_{cat}/K_m for the hydrolysis of ester and amide substrates in a manner which is dependent on the size of the leaving group of the substrate (Table VIII). With substrates containing a non-bulky leaving group, i.e. -OMe and $-NH_2$, k_{cat}/K_m is increased while it is decreased for substrates with larger leaving groups, i.e. -OEt and -Gly-NH_2, and in particular for those with very bulky leaving groups, i.e. -OBzl and -Val-NH_2. These results suggest that Met-398 is located in the S_1' binding site, and presumably in the binding pocket for the side-chain of the C-terminal amino acid residue since the hydrolysis of FA-Phe$\downarrow$Val-NH_2 is much more affected by the modification than the hydrolysis of FA-Phe$\downarrow$Gly-NH_2. This suggests that bulky leaving groups of ester substrates bind to this position as well. Similar activities were obtained with carboxypeptidase Y alkylated with m-nitrophenacylbromide and p-nitrophenacylbromide. The enzymes in which the same methionyl residue has been alkylated with iodoacetamide to form a non-bulky methionyl sulfonium derivative (34) or oxidized with H_2O_2 to form a methionyl sulfoxide derivative (35) hydrolyse all ester and amide substrates, including those containing small leaving groups, at reduced rates relative to unmodified carboxypeptidase Y. Thus, it appears that the modified enzyme hydrolyses substrates with small leaving groups at higher rates than unmodified CPD-Y only when a bulky hydrophobic group, e.g. the phenacyl group, is introduced into the S_1' binding site.

Alkylation of Met-398 with phenacylbromide

Table VIII. Hydrolysis of ester, amide and peptide substrates by carboxypeptidase Y and phenacylbromide modified carboxypeptidase Y.

Substrate	Enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
FA-Phe↓OMe	CPD-Y	11000	0.39	28000
	PAB-CPD-Y	6400	0.078	82000
FA-Leu↓OMe	CPD-Y	4000	0.36	11100
	PAB-CPD-Y	2300	0.041	56000
FA-Phe↓OEt	CPD-Y	11000	0.059	190000
	PAB-CPD-Y	4500	0.11	41000
FA-Ala↓OEt	CPD-Y	4600	1.0	4600
	PAB-CPD-Y	30000	14	2100
FA-Ala↓OBzl	CPD-Y	9100	0.054	170000
	PAB-CPD-Y	680	0.43	1600
FA-Val↓NH_2	CPD-Y			0.95
	PAB-CPD-Y			1.7
FA-Leu↓NH_2	CPD-Y			52
	PAB-CPD-Y			160
FA-Phe↓NH_2	CPD-Y			89
	PAB-CPD-Y			220
FA-Phe↓Gly-NH_2	CPD-Y			160
	PAB-CPD-Y			78
FA-Phe↓Val-NH_2	CPD-Y			6200
	PAB-CPD-Y			71
FA-Phe↓Gly-OH	CPD-Y	5800	5.4	1100
	PAB-CPD-Y	180	1.6	110
FA-Phe↓Val-OH	CPD-Y	9700	0.14	61000
	PAB-CPD-Y	260	0.42	620
FA-Phe↓Leu-OH	CPD-Y	4900	0.021	230000
	PAB-CPD-Y	41	0.033	1200

The results are from ref. 34.

reduces k_{cat} for the hydrolysis of all peptides to approximately 2% of the values obtained with unmodified CPD-Y while K_m is only slightly increased (Table VIII). The small influence of the alkylation on K_m suggests that the postulated positively charged binding site which is expected to function when the enzyme forms tight complexes with peptide substrates (see section 6.1) is unaffected by the alkylation of the methionyl residue. This is consistent with the pH dependence of K_m for peptide hydrolysis remaining unchanged by the modification with phenacylbromide relative to unmodified carboxypeptidase Y (34). The drastic reduction in k_{cat} is unlikely to be due to the loss of Met-398 since oxidation of this residue only reduces k_{cat} for peptide hydrolysis to 35 - 50% of the values obtained with unmodified CPD-Y (35). Furthermore, the derivative alkylated with iodoacetamide hydrolyses peptides with kinetic parameters similar to those of the derivative alkylated with phenacylbromide indicating that the bulkiness of the group introduced only has little influence. It is more likely that the sulfonium ion introduced into all the alkylated derivatives is the cause of the reduction in k_{cat}, and it may be speculated that this group prevents a conformational change which might be essential for the high k_{cat} values characteristic for hydrolysis of peptides. The fact that hydrolysis of ester and amide substrates is adversely affected only when the leaving group of the substrate is bulky would then suggest that the postulated conformational change is not essential for the hydrolysis of substrates with blocked C-terminus.

To further study the role of Met-398 this amino acid residue has been substituted by a leucyl residue or an arginyl residue by means of site-directed mutagenesis (199). Compared with carboxypeptidase Y, Leu-398-CPD-Y generally exhibits unchanged or moderately reduced k_{cat} values for the hydrolysis of peptide and ester substrates, whereas K_m is affected in a way which is dependent on the group occupying the P_1' position. For the substrates FA-Phe$\downarrow$Gly-OH, FA-Phe$\downarrow$Ala-OH, and FA-Ala$\downarrow$OEt, which all are characterized by relatively small groups at the P_1' position, K_m is increased, while for FA-Phe$\downarrow$Leu-OH, FA-Phe$\downarrow$Phe-OH, and FA-Ala$\downarrow$OBzl, all characterized by bulky and strongly hydrophobic leaving groups, K_m is decreased. Correspondingly, Leu-398-CPD-Y exhibits decreased k_{cat}/K_m values towards substrates with small leaving groups, and increased values for the substrates with large leaving groups. The apparent tighter binding to Leu-398-CPD-Y of substrates with bulky and hydrophobic P_1' groups is in accordance with the leucyl side-chain being slightly smaller and more hydrophobic than the methionyl side-chain. The increased preference for Phe in the P_1' position is essentially independent of the amino acid residue occupying the P_1 position (199) and thus, it appears that only the specificity with respect to the P_1' position is affected by the Met to Leu mutation which is consistent with Met-398 being located in the S_1' binding site. Arg-398-CPD-Y exhibits kinetic parameters similar to those obtained with the enzyme in which Met-398 has been modified with iodoacetamide and this is consistent with the structural resemblance between an arginyl residue and this particular methionyl sulfonium derivative (L. BECH, J. WINTHER, M.C. KIELLAND-BRANDT, K. BREDDAM, unpublished results).

Treatment of carboxypeptidase Y with H_2O_2 in acetate buffer rather than in phosphate buffer converted both Met-398 and Met-313 to the corresponding sulfoxide derivatives (35, 36). The influence of the buffer on the oxidation reaction is presumably due to acetic acid in the presence of H_2O_2 being converted to peracetic acid, a much more potent oxidizing agent than H_2O_2 itself. The enzyme with both Met-313 and Met-398 oxidized is, relative to the enzyme with only Met-398 oxidized, characterized by 2-4 fold higher activity towards all substrates. It has not yet been possible to establish whether this increase in activity observed upon modification of Met-313 is due to this residue being located in a particular binding site. However, it should be noted that while oxidation of Met-398 has very little influence on the thermal stability of carboxypeptidase Y, the additional oxidation of Met-313 causes a drastic decrease in the stability (35).

Treatment of carboxypeptidase Y with fluorodinitrobenzene resulted in the specific modification of a tyrosyl residue, forming a DNP-O-Tyr derivative (35). The specificity of this modified enzyme suggests that this particular tyrosyl resi-

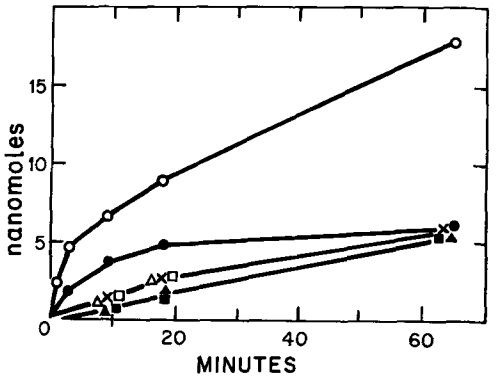


Figure 4. Digestion of oxidized ribonuclease with carboxypeptidase Y. Reaction conditions: 0.5 mM oxidized ribonuclease, 0.1 M-Mes, pH 7.0, 0.27 μM-CPD-Y. For the amino acids released from the C-terminus the following symbols were used: Val,○; Ser,●; Ala,×; Asp,□; Phe,△; His,▲; Pro,■.

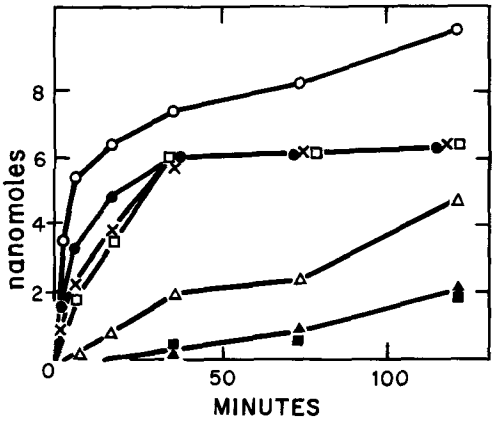


Figure 5. Digestion of oxidized ribonuclease with carboxypeptidase Y. Reaction conditions: 0.5 mM oxidized ribonuclease, 0.1 M-Mes, pH 5.0, 0.8 μM-carboxypeptidase Y. The symbols are listed in Figure 4.

due is located in the S_1' binding site. However, its position in the amino acid sequence has not yet been identified.

7. APPLICATIONS OF SERINE CARBOXYPEPTIDASES

7.1. Determination of C-terminal sequences

The ability of carboxypeptidases to sequentially release amino acids from the C-terminus of a peptide chain has frequently been used in the elucidation of primary structures of peptides and proteins. Originally metallo carboxypeptidases A and B which are optimally active at basic pH values were used (2) but recently serine carboxypeptidases, and in particular carboxypeptidase Y, have gained wide-spread use because these enzymes release all C-terminal amino acids from peptides including Pro (74, 185) which is not released by the metallo carboxypeptidases (2). The ability of serine carboxypeptidases to act on peptide amides (see section 6.1) allows C-terminal sequence determinations on these substances although the C-terminal amino acid residue in cases where the amino acid amide is released faster than ammonia (23, 34) has to be identified as the released amino acid amide. Carboxypeptidase Y functions in denaturing agents such as 0.5% SDS (123) and 6 M-urea (72), and this allows it to be used to digest peptides which are otherwise insoluble. However, in 6 M-urea carboxypeptidase Y is inactivated with time, e.g. at pH 7.0: $t_{1/2} = 2$ hours, and for this reason the reaction should preferably be completed within a short time. No loss of activity is registered in 3 M-urea (K. BREDDAM, unpublished results).

The rates of hydrolysis with serine carboxypeptidases are strongly affected by the nature of the amino acid residues in the P_1 and P_1' positions of peptide substrates (see Table V), and consequently the individual steps in the sequential release of amino acids from peptides are characterized by widely different rates. In cases where a slow step is followed by one or several fast steps the results are ambiguous and this is often encountered when the non-specific carboxypeptidase Y is used for the digestions. An example is shown in Figure 4, where oxidized ribonuclease is digested with carboxypeptidase Y at pH 7.0. The sequence of the C-terminal segment of this peptide is: -Pro-Val-His-Phe-Asp-Ala-Ser-Val-OH, but from the results in Figure 4 it is only possible to deduce the correct positions of the two amino acids released first. This is because carboxypeptidase Y catalyzed peptide hydrolysis is very slow at pH 7.0 for substrates with an Asp residue in the P_1 position (70). Thus, in the case of oxidized ribonuclease, the release of Ala

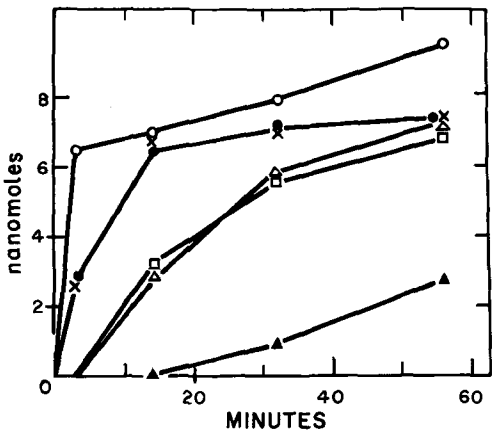


Figure 6. Digestion of oxidized ribonuclease with malt carboxypeptidase I. Reaction conditions: 0.5 mM oxidized ribonuclease, 0.1 M-Mes, pH 5.0, 0.25 μM-malt carboxypeptidase. The symbols are listed in Figure 4.

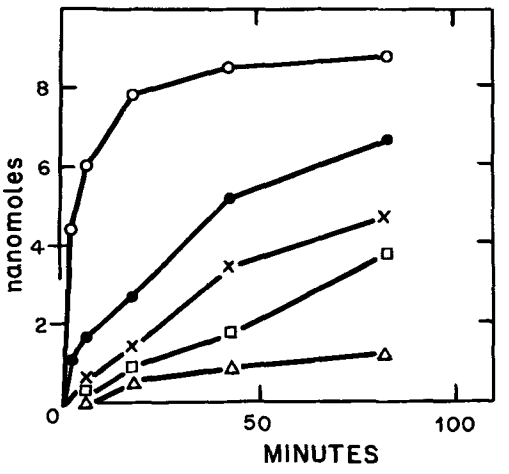


Figure 7. Digestion of oxidized ribonuclease with Ph-Hg-CPD-Y. Reaction conditions: 0.5 mM oxidized ribonuclease, 0.1 M-Mes, pH 5.0, 2.2 μM-Ph-Hg-CPD-Y. The symbols are listed in Figure 4.

is very slow, while the following amino acids are relased with comparatively high rates. Problems of this nature may in some cases be met by increasing the rate of the slow step or by decreasing the rate(s) of the following fast step(s). This may be accomplished by alterations in the reaction conditions, e.g. pH, ionic strength, and concentration of substrate. Thus, substrates which at the P_1 position contain an Asp or Glu are hydrolyzed much faster at pH 5.0 than at pH 7.0 while for those containing His at this position the opposite relation holds. These effects of pH are due to carboxypeptidase Y hydrolysing substrates with charged side-chains in the P_1 position much slower than substrates with an uncharged side-chain (27, 70). By lowering the pH from 7.0 to 5.0 in the digestion of oxidized ribonuclease Ala is released almost as fast as Ser, while the His-Phe bond is cleaved with a lower relative rate than at pH 7.0, allowing the position of Phe in the sequence to be decided (Figure 5).

In cases where alterations in the reaction conditions are not sufficient to obtain an interpretable sequential release of amino acids from the C-terminus of peptides additional information may be obtained using an enzyme with a different specificity. Figure 6 shows the time course for the action of malt carboxypeptidase I on oxidized ribonuclease. It is apparent that the Pro-Val bond is cleaved slower than with carboxypeptidase Y and that Pro is not released at all.

Although a combination of the results obtained with carboxypeptidase Y and malt carboxypeptidase I may indicate substantial parts of the sequence, a single satisfactory time course has not been produced with either enzyme. However, when carboxypeptidase Y modified with Ph-Hg^+ is used a reaction course is obtained which easily can be interpreted (Figure 7). This is because modification of carboxypeptidase Y with Ph-Hg^+ results in a drastic decrease in k_{cat}/K_m for the hydrolysis of substrates with Phe in the P_1 position while the hydrolysis of substrates with less bulky side-chains in the P_1 position is less affected (27). Consequently, this modification causes a much more pronounced decrease in the rate of cleavage of the Phe-Asp bond in ribonuclease than the other peptide bonds towards the C-terminus. This experiment demonstrates that in cases where an optimization of the conditions of the digestion experiment is insufficient to produce a time course which can be interpreted, modified enzymes with altered specificities may be employed with advantage.

The extent of the digestion is also dependent on the specificity of the enzyme used. This is

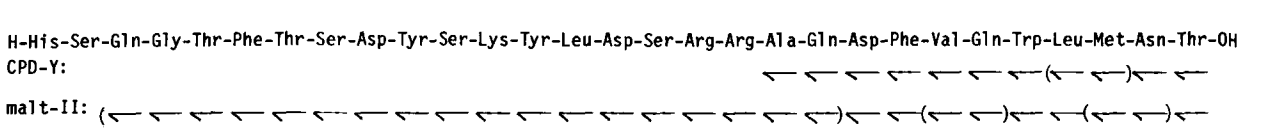


Figure 8. Digestion of porcine glucagon with serine carboxypeptidases. Conditions: 285 μM-glucagon, 2.2 μM malt carboxypeptidase II or 1.9 μM-carboxypeptidase Y, 0.05 M-sodium acetate, pH 4.0. The final aliquot was taken at 3 h. The arrows indicate the interpretable sequential release of amino acids from the peptide. The arrows placed in parenthesis indicate that the release of these amino acids can be measured, but their positions within this peptide segment cannot be distinguished.

demonstrated by digestion of glucagon with carboxypeptidase Y and malt carboxypeptidase II. The former of these enzymes will only very slowly release amino acids from peptides with Arg or Lys in the penultimate position and consequently, the Arg-Arg sequence in the middle of the glucagon peptide will represent an essentially unsurmountable obstacle for this enzyme (Figure 8). However, malt carboxypeptidase II exhibits a preference for this type of sequence (37, 38) and digests glucagon down to the N-terminal dipeptide, i.e. H-His-Ser-OH. Thus, the C-terminal sequence can be determined with carboxypeptidase Y, and after thermal denaturation of this enzyme, malt carboxypeptide II can be added to provide additional sequence information. Recent experiments have shown that malt carboxypeptidase II is particularly suited for the determination of C-terminal sequences of tryptic peptides since it rapidly releases the C-terminal Lys or Arg of such peptides and the subsequent amino acid residues with lower rates (K. BREDDAM and M. OTTESEN, to be published).

7.2. Debittering of bitter peptides

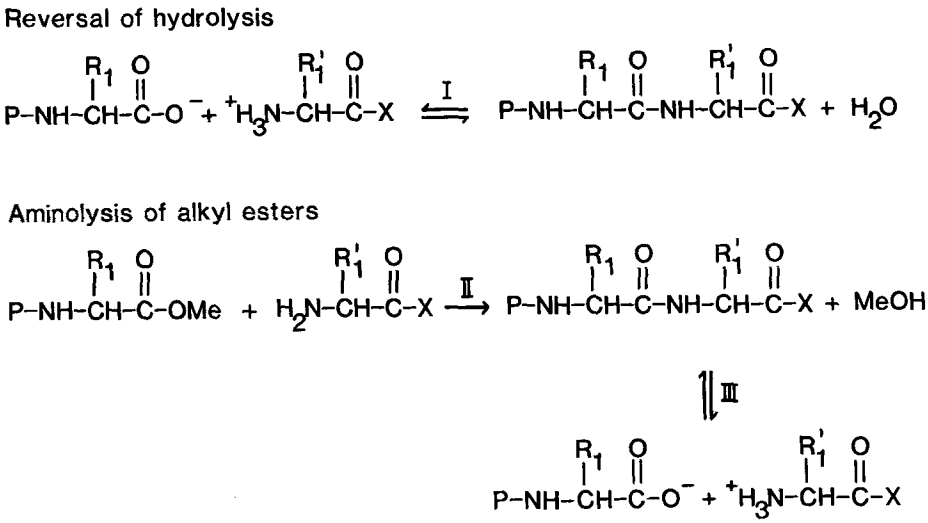
Enzymatic hydrolysates of various proteins frequently contain peptides with bitter taste which limit their use in food. The serine carboxypeptidase from wheat bran has been demonstrated to release hydrophobic amino acids from such peptides obtained by pepsin hydrolysis of casein with the result that the bitterness decreases (187). This suggests that carboxypeptidases and other exopeptidases might have applications in food chemistry.

7.3. Use of serine carboxypeptidases in peptide synthesis

BERGMAN, FRUTON and FRAENKEL-CONRAT demonstrated in 1937 (14, 15) that chymotrypsin and papain catalyzed the condensation of an acylamino acid and an amino acid amide thus forming a peptide bond according to reaction I in Scheme 7. Reactions of this type have since been shown to exhibit equilibrium constants K_{syn} of 0.3 – 0.5 M^{-1} at neutral pH values (46, 81, 82) and are consequently displaced in favour of hydrolysis. Significant product yields can, however, be obtained (a) if the product continuously is removed from the equilibrium, e.g. by precipitation (95, 96, 97), or (b) by using large concentrations of the reactants thereby displacing the equilibrium in favour of synthesis (186), or (c) by changing the polarity of the reaction medium through the addition of organic solvents (81).

Protease catalyzed aminolysis reactions of peptide esters (reaction II, Scheme 7) are energetically much more favourable than condensation reactions (reaction I) (reviewed by J. FRUTON (56)). The ester substrate rapidly forms an acyl-enzyme intermediate which further reacts, in competition with water, with the amine nucleophile to form a new peptide bond. By terminating the reaction before final equilibrium can be established through reactions where the aminolysis product functions as substrate, e.g. via reaction III, it is achieved that the yield of aminolysis is not based on an equilibrium constant as in reaction I but rather on the relative rates of aminolysis and hydrolysis of ester substrates (see section 6.3).

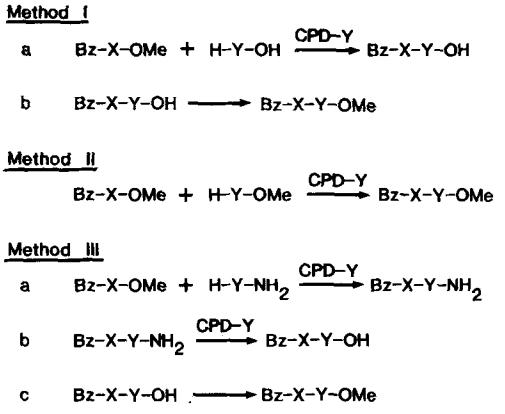
While all proteolytic enzymes may catalyze reaction I, reaction II is catalyzed only by those



Scheme 7. Two different principles of enzymatic peptide synthesis.

enzymes which hydrolyse alkyl esters, i.e. serine and sulfhydryl proteases. The structures of the acyl and amine components which may participate in the reaction are determined by the specificity of the enzyme used, and studies in this laboratory have revealed that serine carboxypeptidases, e.g. carboxypeptidase Y and the malt carboxypeptidases, as expected, catalyze only aminolysis reactions with amino acids and amino acid derivatives as amine components (33, 38, 196, 197) as opposed to endopeptidases that in addition to amino acid derivatives also accept polypeptides as nucleophiles (136, 149). Consequently, when a carboxypeptidase is used a peptide chain will only be elongated by a single amino acid residue in each aminolysis reaction. This may be achieved in three different ways using amino acids, amino acid amides, or amino acid methyl esters as amine components according to the reactions listed in Scheme 8.

When amino acids are used as nucleophiles (method I, reaction a) it is important to perform the reaction at a pH where the enzyme exhibits high esterase activity and almost no peptidase activity such that the product formed in the reaction is not degraded by the enzyme. In the case of carboxypeptidase Y this is achieved at pH > 9 (see section 5.2) where the enzyme remains stable (27). Unfortunately, the yield of the aminolysis reaction depends strongly on the particular amino acid and it is rarely higher than 50% (197). Malt carboxypeptidase I is unstable at pH values above 7.5, and at this pH the yield of



Scheme 8. Three different ways of performing one elongation step in carboxypeptidase Y catalyzed peptide synthesis. Bz-X-OMe represents the peptide ester functioning as acyl component in the reaction, and H-Y-OH, H-Y-OMe, and H-Y-NH_2 represent the amino acid, amino acid methyl ester, and amino acid amide, respectively, functioning as amine components in the reactions. Bz-X-Y-OMe represents the acyl-component in the subsequent elongation reaction. The esterification reactions Ib and IIIc are performed chemically.

aminolysis using amino acids as nucleophiles was below 10% conceivably due to an unfavourable ratio of esterase to peptidase activity (K. BREDDAM, unpublished results). However, malt carboxypeptidase II is more stable at basic pH values and at pH 8.5 the yields of aminolysis appear to be of the same magnitude as obtained with carboxypeptidase Y (38).

The use of amino acid esters as amine components (Scheme 8, method II) is complicated by the fact that the aminolysis product, like the acyl component used in the reaction, is a peptide ester and hence, it may react further in one or more additional steps resulting in lower yields of the desired products (196). However, with carboxypeptidase Y it has been demonstrated that the yield of the initial aminolysis product can be drastically improved by utilizing that N-blocked amino acid benzyl esters are much better substrates of carboxypeptidase Y than the corresponding methyl esters (29). Thus, by using N-blocked amino acid benzyl esters as acyl-components and amino acid methyl esters as amine components, peptide methyl esters are formed as the major product because they are turned over by the enzyme with a lower rate than the initial benzyl ester acyl component. Similarly, it has been shown that high yields of the initial aminolysis product can be obtained in cases where the side-chain specificity of the enzyme is such that the peptide ester formed as aminolysis product is a poor substrate of carboxypeptidase Y relative to the substrate used as acyl component in the reaction (29). Thus, when e.g. Bz-Ala$\downarrow$OMe functions as acyl component and H-Gly-OMe as amine component, the aminolysis product, Bz-Ala-Gly$\downarrow$OMe is a poor substrate of carboxypeptidase Y relative to Bz-Ala$\downarrow$OMe, and consequently, Bz-Ala-Gly$\downarrow$OMe reacts no further at the concentrations of enzyme and time needed to consume all Bz-Ala$\downarrow$OMe. This is due to the ability of carboxypeptidase Y to hydrolyse ester substrates with Ala in the P_1 position much faster than those with Gly in this position (27). Conversely, when H-Phe-OMe is used as the nucleophile the product, Bz-Ala-Phe$\downarrow$OMe, is a better substrate than Bz-Ala$\downarrow$OMe, because Phe is preferred to Ala in the P_1 position (27), and Bz-Ala-Phe$\downarrow$OMe reacts further so that it is obtained in a yield of 2% only (29). These results demonstrate that the side-chain specificity of the enzyme is very important for the yield. Consequently, it would be advantageous to have several serine carboxypeptidases with different specificities available. This is illustrated by Bz-Arg-Ala-OMe being produced in 50% yield from Bz-Arg$\downarrow$OBu and H-Ala-OMe using malt carboxypeptidase I compared to 2% with carboxypeptidase Y which hydrolyses Bz-Arg$\downarrow$OBu very slowly (K. BREDDAM, unpublished results).

Since carboxypeptidase Y and malt carboxypeptidase II are the only serine carboxypeptidases which are available in such quantities that they can be commercially utilized in enzymatic synthesis, it is fortunate that the specificity of carboxypeptidase Y with respect to the P_1 position of the ester substrate may be altered by modification of its sulfhydryl group with mercurials (see section *6.4.1*). Thus, work in this laboratory has shown that carboxypeptidase Y modified by Ph-Hg^+ is particularly suitable for the synthesis of peptide esters Bz-X-Y-OMe where X is a nonbulky amino acid residue and Y is a bulky amino acid residue. This is because the preference of this enzyme towards ester substrates with bulky amino acid residues in the P_1 position is lower than that observed with the unmodified enzyme such that the reactivity of Bz-X-Y$\downarrow$OMe relative to Bz-X$\downarrow$OMe (-OBzl) is reduced when compared with the unmodified enzyme. The HPLC chromatograms in Figure 9 compare the reactant composition of a carboxypeptidase Y catalyzed synthesis of Bz-Ala-Leu-OMe from Bz-Ala$\downarrow$OBzl and H-Leu-OMe (panel A) with the corresponding synthesis with carboxypeptidase Y modified with Ph-Hg^+ (panel B). It is apparent that the initial coupling product, Bz-Ala-Leu-OMe, is accumulated in much higher yield with the modified enzyme than with the unmodified enzyme.

When amino acid amides are used as nucleophiles (Scheme 8, method III, reaction a) it is not critical to perform the reaction at high pH since the esterase activities of carboxypeptidase Y and the malt carboxypeptidases are far higher than their amidase activities (28, 37) regardless of pH such that the product, a peptide amide, usually is not degraded by the enzyme. The yield of aminolysis is 85 – 95% for most amino acid

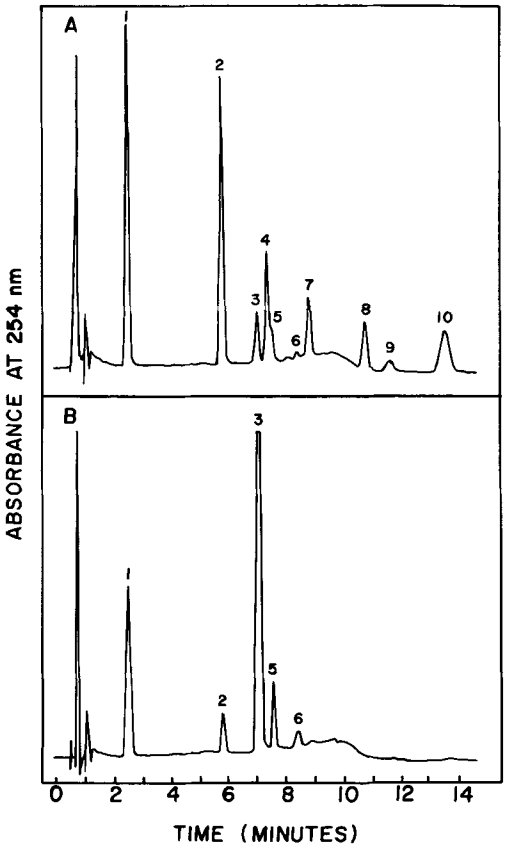
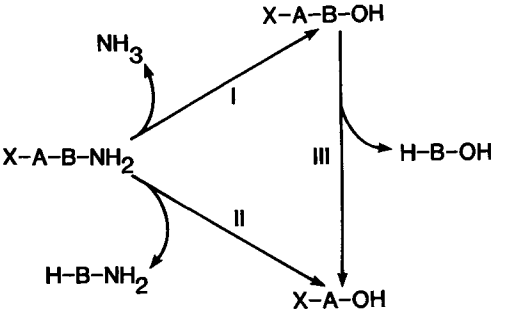


Figure 9. HPLC chromatograms of peptides formed by CPD-Y (Panel A) and Ph-Hg-CPD-Y (Panel B) catalyzed reaction of Bz-Ala-OBzl in the presence of H-Leu-OMe. The following peaks were assigned: 1: Bz-Ala-OH (hydrolysis product), 2: Bz-Ala-Leu-OH, 3: Bz-Ala-Leu-OMe (aminolysis product), 5: Bz-Ala-OBzl (substrate), 4, 6, 7, 8, 9, 10 are oligomerization products containing more than one leucyl residue. The results are from ref. 29.



Scheme 9. Carboxypeptidase Y catalyzed deamidation reactions. Reactions I, II, and III represent amidase, peptidyl amino acid amide hydrolase, and peptidase activities, respectively. X = N-blocking group; A and B = amino acid residues.

amides using either the malt carboxypeptidases or carboxypeptidase Y, but further elongation of the peptide amide produced in reaction IIIa requires that it is deamidated (reaction IIIb) prior to esterification (reaction IIIc). Chemical methods are not suitable for the deamidation reaction and no native enzyme has been described which specifically releases NH_3 from peptide amides. Serine and sulfhydryl proteases do exhibit amidase activity, but in addition they cleave internal peptide bonds, and are consequently of limited value in the deamidation of polypeptide amides. The serine carboxypeptidases also exhibit amidase activity (Scheme 9, reaction I) and in the case of carboxypeptidase Y the formed peptide is not degraded via the peptidase activity of the enzyme (Scheme 9, reaction III) provided that the reaction is performed at pH 10 (23). However, carboxypeptidase Y in addition exhibits peptidyl amino acid amide hydrolase activity and consequently also releases the C-terminal amino acid amide from peptide amides (Scheme 9, reaction II). The relative rates of reactions I and II are strongly dependent on the C-terminal sequence of the peptide amide (Table IX) but usually the yield of the deamidated product formed via reaction I is lower than 70% (23, 34). Thus, deamidation with carboxypeptidase Y can generally not be performed in a satisfactory yield but, fortunately, it has recently been demonstrated in this laboratory that most peptide amides can be deamidated in significantly higher yields (80-100%) using carboxypeptidase Y modified with phenacylbromide (Table IX). This difference between the two enzymes is due to alkylation of a methionyl residue in the S_1' binding site of carboxypeptidase Y enhancing the amidase activity, i.e. reaction I in Scheme 9, while the peptidyl amino acid amide hydrolase activity, i.e. reaction II in Scheme 9, is decreased (see section *6.4.2*). The amidase and peptidyl amino acid amide hydrolase activities of malt carboxy-

peptidase I are influenced in a similar manner by addition of phenylguanidine to the reaction medium (see section 6.2) and when used in malt carboxypeptidase I catalyzed deamidation reactions this substance increases the yields of deamidation substantially (30). Although the unique side-chain specificity of malt carboxypeptidase I (30) potentially could be useful in deamidation reactions, viz. deamidation of peptide amides with C-terminal lysyl, arginyl or histidyl residues, malt carboxypeptidase I is available only in small quantities and consequently, deamidation of most peptide amides is best performed with carboxypeptidase Y modified with phenacylbromide.

It is thus apparent that peptide chains may be elongated by reactions catalyzed by serine carboxypeptidases, provided the peptide ester acyl-component is a substrate of the enzyme. Unfortunately, Pro cannot be incorporated by either of the methods I, II, and III in Scheme 8, and Asp and Glu only when the carboxylic acid group in the side-chain is blocked, e.g. by esterification (196, 197). However, replacements of specific amino acid residues in the S_1' binding site by site-directed mutagenesis can conceivably be employed to enhance the binding of such problematic nucleophiles. The carboxypeptidase Y containing Leu at position 398 in place of Met exhibits an increased preference for hydrophobic residues in the P_1' position and this derivative would presumably bind hydrophobic nucleophiles better than the unmodified enzyme. In analogy, replacements with hydrophilic amino acid residues might favour binding of hydrophilic nucleophiles. No general rule can be formulated to decide which of the methods in Scheme 8 is the most suitable for incorporation of a specific amino acid, but the recent development which allows the deamidation of peptide amides in high yields suggest that method III in spite of the extra reaction step (reaction IIIb) will gain more wide-spread use, especially when it is taken into consideration that the yields obtained by methods I and II rarely exceed 65% which is significantly less than the expected overall yield of method III (80 – 90%).

Serine and sulfhydryl endopeptidases do not catalyze reaction I in Scheme 8 but they do catalyze reactions II and IIIa (136, 149) and may therefore be used in step-wise synthesis in combination with serine carboxypeptidases. However, presumably they will primarily be used to catalyze reactions in which peptides function as amine components. In general, enzyme assisted peptide synthesis is characterized by somewhat lower yields than those characteristic of the chemical methods (61). Nevertheless, enzymatic peptide synthesis have advantages in many cases since it is stereospecific and requires minimal protection of functional groups in the side-chains (198).

In conventional peptide synthesis ester groups can be used to protect carboxylic acid groups but this requires that the ester group subsequently is removed without otherwise affecting the elongated peptide. ROYER (160) has previously demonstrated that the high esterase and low peptidase activity of carboxypeptidase Y at pH > 8 renders this enzyme a useful tool in such reactions. However, observations in this laboratory suggest that carboxypeptidase Y even under these conditions exhibits significant peptidase activity, in particular towards peptides with hydrophobic amino acid residues in the P_1 and P_1' positions. Thus, after removal of the ester group

Table IX. Deamidation of peptide amides by carboxypeptidase Y and carboxypeptidase Y modified with phenacylbromide.

Peptide amide	% Yield	
	CPD-Y	PAB-CPD-Y
Z-Ala-Gly-NH_2	2	10
Z-Val-Gly-NH_2	46	49
Z-Ala-Ser-NH_2	0	35
Bz-Phe-Gln-NH_2	11	81
Bz-Ala-Ala-NH_2	31	100
Z-Gly-Ala-NH_2	100	
Z-Val-Ala-NH_2	94	
Bz-Ala-Thr-NH_2	9	85
Bz-Ala-Val-NH_2	10	96
Bz-Ala-Ile-NH_2	10	92
Bz-Ala-Leu-NH_2	60	98
Bz-Ala-Phe-NH_2	66	96
Bz-Phe-His-NH_2	28	92
Z-Ala-Met-NH_2	36	100

The results are from ref. 34.

I. Conversion of peptides to peptide esters

(a) Bz-X-Y-OH + MeOH ⟶ Bz-X-OMe + H-Y-OH

(b) Bz-X-Y-OH + H-Z-OMe ⟶ Bz-X-Z-OMe + H-Y-OH

II. Conversion of peptides to peptide amides

(a) Bz-X-Y-OH + NH_3 ⟶ Bz-X-NH_2 + H-Y-OH

(b) Bz-X-Y-OH + H-Z-NH_2 ⟶ Bz-X-Z-NH_2 + H-Y-OH

III. Conversion of peptides to other peptides

Bz-X-Y-OH + H-Z-OH ⟶ Bz-X-Z-OH + H-Y-OH

Scheme 10. Carboxypeptidase Y catalyzed exchange of C-terminal residues in peptides. X, Y, and Z represent amino acid residues.

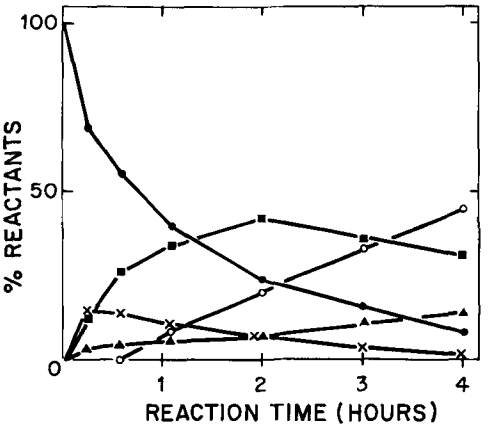


Figure 10. The action of carboxypeptidase Y on Bz-Lys-Ala-OH in the presence of H-Thr-NH_2. Bz-Lys-Ala, -●-●-; Bz-Lys-OH, -▲-▲-; Bz-Lys-Thr-NH_2, -■-■-; Bz-Lys-Ala-Thr-NH_2, -×-×-; Bz-Lys-Thr-Thr-NH_2 + Bz-Lys-Thr-Thr-OH, -○-○-. The results are from ref. 31.

from the peptide ester carboxypeptidase Y may degrade the unblocked peptide. Carboxypeptidase Y modified with phenacylbromide has been shown to exhibit negligible peptidase activity (see section *6.4.2*) and compared with unmodified carboxypeptidase Y this modified enzyme removes ester groups from peptide esters more specifically and can consequently with advantage be applied in such reactions (34).

7.4. Exchange of C-terminal amino acid residues in peptides

All hydrolytic reactions catalyzed by carboxypeptidase Y proceed via an acyl-enzyme intermediate, and studies in this laboratory have shown that all substrates of the enzyme in principle can be used as acyl-components in transacylation reactions to other nucleophiles than water. It is of particular interest that unblocked peptides may function as acyl-donors such that the C-terminal amino acid residue can be exchanged for various other groups as shown in Scheme 10 (23, 25).

Carboxypeptidase Y catalyzed transpeptidation reactions using N-blocked dipeptides as acyl components and amino acid amides as amine components normally result in formation of only two products, a transpeptidation product where the C-terminal amino acid residue has been replaced and a hydrolysis product. However, a carboxypeptidase Y catalyzed reaction with Bz-Lys↓Ala-OH as acyl component and H-Thr-NH_2 as amine component demonstrated that other products could be formed as well in cases where the acyl component is a poor substrate of the enzyme (30). The product composition (Figure 10) indicates that Bz-Lys↓Ala-OH functions as acyl component in three reactions forming Bz-Lys-Thr-NH_2, Bz-Lys-Ala-Thr-NH_2, and Bz-Lys-OH, and that Bz-Lys-Thr↓NH_2 reacts further to yield Bz-Lys-Thr-OH and Bz-Lys-Thr-Thr-NH_2 (Scheme 11). The formation of these additional products is due to Bz-Lys↓Ala-OH being a very poor substrate of carboxypeptidase Y (27) such that the rates of the otherwise fast reactions I and III are reduced to approximately the rates of reactions II, IV and V. The lysyl residue in the P_1 position of the substrate is unquestionably the cause for this atypical reaction course. Consistent with this the reaction course was drastically altered when carboxypeptidase Y modified by Hg^{++} was used, hence increasing the preference for Lys in the P_1 position (26) (Figure 11): only Bz-Lys-Thr-NH_2 (95%) and Bz-Lys-OH (5%) were formed. With malt carboxypeptidase II which exhibits high activity towards Bz-Lys-Ala-OH a yield of 85% was obtained using only 0.5 μM enzyme as compared with 12 μM modified carboxypeptidase Y. These results demonstrate that the speci-

I. Reactions with Bz-Lys-Ala-OH:

(a) Bz-Lys-Ala-OH + H-Thr-NH_2 ⟶ Bz-Lys-Thr-NH_2 + H-Ala-OH (transpeptidation)

(b) Bz-Lys-Ala-OH + H-Thr-NH_2 ⟶ Bz-Lys-Ala-Thr-NH_2 + H_2O (condensation)

(c) Bz-Lys-Ala-OH + H_2O ⟶ Bz-Lys-OH + H-Ala-OH (hydrolysis)

II. Reactions with Bz-Lys-Thr-NH_2

(a) Bz-Lys-Thr-NH_2 + H_2O ⟶ Bz-Lys-Thr-OH + NH_3 (hydrolysis)

(b) Bz-Lys-Thr-NH_2 + H-Thr-NH_2 ⟶ Bz-Lys-Thr-Thr-NH_2 + NH_3 (transpeptidation)

Scheme 11. Reaction courses for the action of carboxypeptidase Y on Bz-Lys-Ala-OH in the presence of a nucleophile (H-Thr-NH_2).

specificity of the enzyme employed imposes severe restrictions on the type of reactions which are feasible and in addition, they demonstrate how chemically modified derivatives of enzymes may be useful.

Although the yields of carboxypeptidase Y catalyzed transpeptidation reactions are strongly dependent on the structures of both the acyl and amine components used in the reaction (23, 24) they represent attractive possibilities in protein semisynthesis. Exchange of C-terminal amino acid residues in peptides, incorporation of labelled amino acids as well as reporter groups and conversion of peptides to peptide esters are possibilities to be considered.

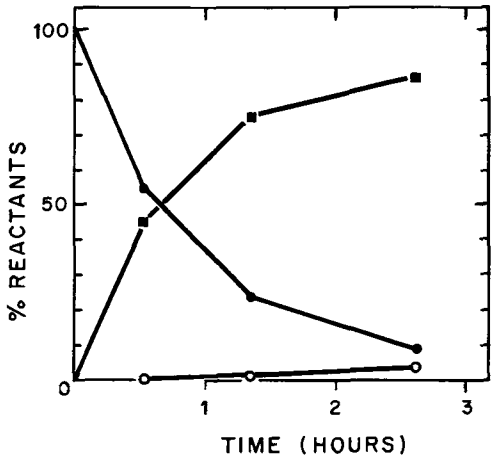


Fig. 11. The action of Cl-Hg-CPD-Y on Bz-Lys-Ala-OH in the presence of H-Thr-NH_2. Bz-Lys-Ala-OH, -●-●-; Bz-Lys-OH, -○-○-; Bz-Lys-Thr-NH_2, -■-■-. The results are from ref. 31.

Porcine and human insulins only differ at the C-terminal position of the B-chain, porcine insulin containing an alanyl residue and human insulin a threonyl residue. The semisynthetic conversion of porcine insulin to human insulin has been solved in various ways (26, 32, 58, 94, 99, 137, 138). In the procedure originating from this laboratory (26) the C-terminal amino acid residue of porcine insulin is exchanged by a carboxypeptidase Y catalyzed transpeptidation reaction using porcine insulin as acyl component and H-Thr-NH_2 as amine component forming human insulin amide, des$(Ala)^{B30}$(Thr-NH_2)^{B30}insulin. However, like Bz-Lys↓Ala-OH porcine insulin is a poor substrate of carboxypeptidase Y because it too contains a lysyl residue at the P_1 position. The reaction course of the transpeptidation reactions with porcine insulin and Bz-Lys-Ala-OH are consequently very reminiscent in terms of products formed and the rather low yields of the transpeptidation products. Fortunately, however, the yield of human insulin amide could be increased from approximately 25% to approximately 65% by modification of carboxypeptidase Y with Hg^{++} in analogy with the observations with Bz-Lys↓Ala-OH, and human insulin could subsequently be obtained from human insulin amide by a carboxypeptidase Y catalyzed deamidation reaction in essentially quantitative yield (32).

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