CONFORMATIONAL AND ACTIVITY CHANGES ON SUBTILISIN CLEAVAGE OF CARBOXYPEPTIDASE A*

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Carboxypeptidase A digestion with subtilisin Carlsberg yields, carboxypeptidase S, with 4-5 fold the esterase and 1/2 the peptidase activity of the native enzyme. An environmentally sensitive probe suggests that these changes are due to conformational alterations in the active center region of the protein.

Limited proteolytic cleavage is well-known as a biological mechanism for the activation of zymogens, hormonogens and kininogens, for the clotting of blood and milk, the conversion of ovalbumin into plakalbumin, the formation of ribonuclease S, and a number of other transformations (1). In addition, selective proteolysis has been employed to examine conformational changes in proteins (2) and to study the relationship of enzyme structure with function.

Native carboxypeptidase A of bovine pancreas is highly resistant to mammalian proteases but can be sensitive to a number of bacterial enzymes. We have found that under appropriate conditions, cleavage of carboxypeptidase by subtilisin Carlsberg is limited to two peptide bonds. The formation of the modified derivative is recognizable by virtue of its altered enzymatic characteristics. This product, termed carboxypeptidase S, has 4-5 fold the esterase and half the peptidase activity of the native enzyme. The hypothesis that these new activities are the result of conformational changes has been

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examined by incorporation of an environmentally sensitive azochromophore (3) into the native protein and following its circular dichroic spectrum during limited proteolysis.

EXPERIMENTAL: Carboxypeptidase (Worthington) 10 mg/ml, in 0.02 M Veronal - 2 M NaCl, pH 7.5, digested at 1° or 9° with a protease concentration of 0.1 mg/ml. Esterase and peptidase activities were determined using hippuryl-DL-phenyllactate and carbobenzoxyglycyl-L-phenylalanine as substrates, respectively, as previously described (4). The degree of digestion was monitored with ninhydrin and the appearance of amino groups is expressed in terms of phenylalanine equivalents per mole. N-terminal analyses were performed with 1-fluoro-2,4-dinitrobenzene (5). Coupling of carboxypeptidase with diazotized p-arsanilic acid (DAA) was carried out with crystalline enzyme. The detailed characteristics of the product, DAA-carboxypeptidase, will be reported elsewhere (6,7). Circular dichroic measurements were performed at 25° using a Cary 60 spectropolarimeter with a model 6001 attachment as previously described (3).

RESULTS AND DISCUSSION: Initial experiments, monitored by measuring both the appearance of amino groups with ninhydrin and the changes in enzymatic activity, showed that native carboxypeptidase can be digested by Nagarse (Biddle Sawyer Corp.), the extracellular alkaline protease from Bacillus amyloliquefaciens, Table I. Over a period of five days, approximately 19 peptide bonds are cleaved and peptidase activity decreases by 20% relative to the control. However, esterase activity actually increases, giving first indications that an altered but active form of carboxypeptidase is being generated during the course of digestion.

The presence of 0.1 M β-phenylpropionate, an inhibitor of carboxypeptidase, protects the enzyme against proteolysis and the concomitant changes in activity (Table I). Diisopropylphosphofluoridate inactivates Nagarse (8) and completely abolishes its capacity to alter the activities of carboxypeptidase (Table I). Since digestion of carboxypeptidase with Nargarse is rather extensive, it seemed

TABLE I

NAGARSE HYDROLYSIS OF CARBOXYPEPTIDASE

	Activity[a]		Bonds
	Esterase	Peptidase	Hydrolyzed[b]
Carboxypeptidase	100	100	--
" + Nagarse	153	82	19.3
" + " + 0.1 M βφP	101	102	0.1
" + (" + 5mM DFP)	100	104	1.3

[a]Expressed as a per cent of the control.

[b]Expressed as the increase in amino groups as determined with ninhydrin. βφP = β-phenylpropionate; DFP = diisopropylphosphofluoridate. Digestion was carried out at 1° for 5 days.

desirable to search for other proteolytic enzymes which might alter activities by more specific and limited cleavage. Of the proteases studied, subtilisin

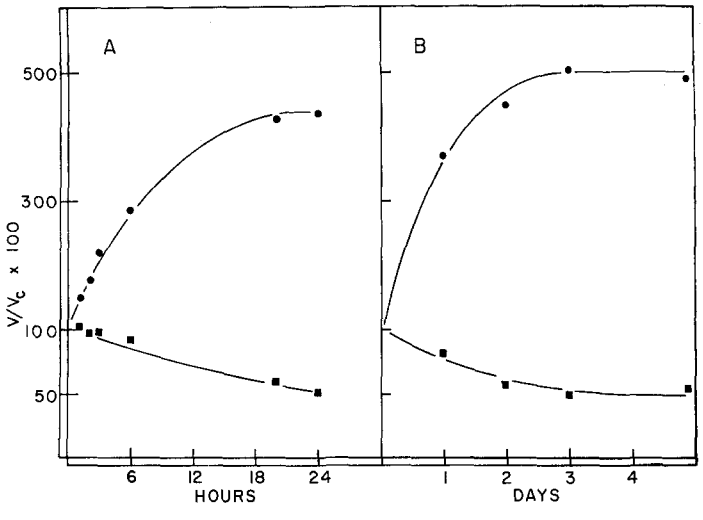

Figure 1. Changes in esterase (●) and peptidase (■) activities on digestion of carboxypeptidase (10 mg/ml) with subtilisin Carlsberg (0.1 mg/ml) in 0.02 M Veronal-2 M NaCl, pH 7.5 buffer at 9° (A) and at 1° (B).

TABLE II

PROTEOLYSIS OF CARBOXYPEPTIDASE

Protease	Activity		Bonds
	Esterase	Peptidase	Hydrolyzed
None, control	100	100	--
Elastase, Worthington	104	98	0
Thermolysin[a]	101	82	0
A. oryzae Proteinase, Henley Corp.	107	96	0.1
Pronase (purified)[a]	123	78	4.3
Pronase, Calbiochem	139	80	15.3
Nagarse, Biddle Sawyer	153	82	19.3
Subtilisin Novo, Biddle Sawyer	260	67	35.5
Subtilisin Carlsberg[b]	550	61	30.9

Conditions as in Table I

[a]Kindly provided by Dr. K. Fuwa

[b]Kindly provided by Dr. M. Ottesen

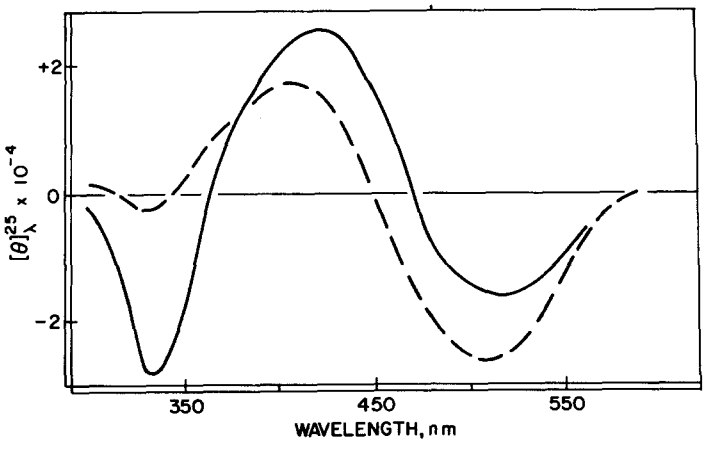

Figure 2. Circular dichroic spectrum of DAA-carboxypeptidase (———) and DAA-carboxypeptidase S (------). Spectra were run in 0.05 M Tris-HCl-1 M NaCl at pH 7.5 as previously described (3).

Carlsberg was by far the most effective (Table II). The time course of digestion of carboxypeptidase by subtilisin Carlsberg was followed at 9° (Fig. 1A) and at 1° (Fib. 1B). At both temperatures peptidase activity decreases to about 50% while esterase activity increases to more than 400% of the control. The yield of modified product varies from 80-90% under these conditions. At higher temperatures the amount of non-specific proteolysis increases markedly and the yield of product is reduced. Carboxypeptidase modified by subtilisin Carlsberg, will be referred to as carboxypeptidase S, the S signifying subtilisin.

Carboxypeptidase S can be crystallized from the digestion mixture by dialysis against distilled water. N-terminal analysis indicates the presence of two new residues, serine and glycine, in addition to aspartic acid present in the native enzyme, Table III. Little or no differences are apparent between the amino acid composition of carboxypeptidase S and that of the native enzyme.

An alteration in protein conformation would seem to be the most probable explanation for the selective, proteolytic effects of subtilisin on the enzymatic function of carboxypeptidase. In order to test this hypothesis we have examined the subtilisin digestion of carboxypeptidase which had first been coupled with diazotized arsanilic acid. It has been shown that this derivative, DAA-carboxypeptidase, contains a single arsanilazo group located on tyrosine 248, at the active center of the enzyme (9). The azochromophore is optically active giving rise to the circular dichroic spectrum shown in Fig. 2.

The esterase and peptidase activities of DAA-carboxypeptidase are about 55% and 60%, respectively, of those of the native enzyme according to the standard assay. On digestion of this enzyme with subtilisin Carlsberg, at 9° for 24 hours, esterase activity increases almost 4-fold while peptidase activity falls by about 30%. These changes in activities are relatively quite analogous to those seen on digestion of native carboxypeptidase. Apparently coupling with DAA does not affect its susceptibility to subtilisin digestion.

TABLE III

CHANGES IN N-TERMINAL RESIDUES ON DIGESTION OF CARBOXYPEPTIDASE WITH SUBTILISIN

	DNP - Asp	DNP - Ser	DNP - Gly
CPD	1.0	<0.1	0
CPD-S	1.0	0.24	0.37

Digestion at 9° in pH 7.5, 0.02 M Veronal - 2 M NaCl buffer, 24 hours. The reaction was stopped by 5mM DFP. The samples were dialyzed to remove contaminating peptides prior to reaction with fluorodinitrobenzene. The results are expressed as moles of DNP-amino acid found per mole of DNP-Asp. The recoveries of DNP-amino acids determined with models were 67%, 51% and 27% for DNP-Asp, -Ser and -Gly, respectively.

During the course of proteolysis the circular dichroic spectrum of DAA-carboxypeptidase undergoes marked changes, Fig. 2. The change at 335 nm is particularly dramatic, decreasing by 24,000°, and correlates directly with the alteration in esterase activity. Since the azochromophore is located on tyrosine 248, these circular dichroic changes would seem to reflect alterations in the environment of the active center brought about by subtilisin cleavage. The close similarity of the enzymatic consequences of limited proteolysis of native and DAA-carboxypeptidase would suggest that similar changes in active site topology occur for both enzymes and that these local conformational changes account for the increased esterase and decreased peptidase activities observed in carboxypeptidase S.

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