Isolation and Structure of an Active-Center Peptide of Bovine Carboxypeptidase B Containing the Zinc-binding Sulfhydryl Group*

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ABSTRACT: A peptide containing the thiol group involved in the binding of zinc in bovine carboxypeptidase B was isolated and its amino acid sequence was determined.

The reactive sulfhydryl group was labeled with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, and the yellow pepitide was isolated from a chymotryptic digest of the labeled protein. Composition, end-group analysis, and degradation by Nagarse and by acid suggest the following amino acid sequence for this active-center peptide: Val.Leu.Pro.Glu.Ser.Gln,-Ile.Gln.Pro.Thr.CySH.Glu.Glu.Thr.

The investigation described in the preceding paper (Wintersberger *et al.*, 1965) has shown that bovine pancreatic carboxypeptidase B contains a single thiol group which is involved in the binding of zinc to the apoenzyme. The additional six half-cystine residues are disulfide bonded and hence do not react with sulfhydryl reagents in the native or denatured holo- or apoenzyme. As a result of these observations, it has become possible to label the zinc-binding thiol group with alkylating agents and to isolate from enzymatic digests of the labeled protein a tetradecapeptide containing the cysteine residue normally involved in the binding of zinc. The yellow maleimide derivative *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, commonly referred to as DDPM[1] (Witter and Tuppy, 1960), was found to be particularly suited for this purpose. The isolation and amino acid sequence of this peptide are described in the present report.

Materials and Methods

Carboxypeptidase B was prepared from aqueous extracts of acetone powders of beef pancreas glands as described by Wintersberger *et al.* (1962). The material used was the same as that described in the preceding report (Wintersberger *et al.*, 1965).

α-Chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corp. *Nagarse* was the lyophilized crystalline bacterial protease from Nagarse and Co., Ltd., Osaka, Japan. *N-(4-Dimethylamino-3,5-dinitrophenyl)maleimide* was obtained from Aldrich Chemical Corp.

Protein concentration was estimated from the absorbance at 280 mμ, using $E^{1\%}_{280m\mu}$ = 21 (Cox *et al.*, 1962). *Amino acid analyses* and analyses for amino acid derivatives were performed with a Beckman/Spinco Model 120 automatic amino acid analyzer according to the method of Spackman *et al.* (1958). Dinitrophenylations were carried out in solutions containing 1% triethylamine, as described by Sanger and Thompson (1953).

Digestions with carboxypeptidase A were carried out in 0.1 M ammonium acetate, *p*H 8.0, using molar ratios of peptide to enzyme of approximately 50:1. The liberated amino acids were separated by the method of Richmond and Hartley (1959) and quantitated according to Tigane *et al.* (1961).

Results

As previously described, the reaction of carboxypeptidase B with DDPM proceeds at *p*H 4.6 essentially stoichiometrically, 0.9 residue of the alkylating agent becoming substituted per protein molecule (Wintersberger *et al.*, 1965). For the purpose of labeling and subsequent isolation of a labeled peptide, in a typical case, 100 mg of carboxypeptidase B was dissolved in 2 ml of 4 M lithium chloride (reagent grade) and diluted with 18 ml of 0.1 M sodium acetate buffer, *p*H 4.6. DDPM (20 mg; a 20-fold molar excess) was dissolved in 1 ml acetone and added to the protein solution. The mixture was incubated overnight at 37°. The yellow DDPS protein precipitated during incubation and was centrifuged off and washed twice with 20 ml water and then five times with acetone to remove excess reagent. After two additional washings with water, the protein was suspended in 10 ml water, and the suspension was adjusted to *p*H 8 with 0.2 N NaOH and digested with 6 mg of chymotrypsin at 40° (molar substrate-to-enzyme ratio about 10:1). During digestion, the *p*H was main-

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[1] Abbreviations used in this work: DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DDPS, *N*-(4-dimethylamino-3,5-dinitrophenyl)succinimido; *S*-SC, *S*-succino-.

tained at *p*H 8 in a *p*H-stat (Radiometer TTT-1 in conjunction with an Ole Dich recorder), under nitrogen, using 0.2 N NaOH for titration. After 6 hours, the solution was completely clear and no further base uptake occurred. The solution was adjusted to *p*H 5.5 with 1 N HCl and filtered through a 1.8- × 10-cm column of washed talc. The yellow peptide became adsorbed and, after the column was washed with about 10 ml water, the peptide was eluted with 50 ml ethanol containing 0.2% glacial acetic acid (Witter and Tuppy, 1960). The eluate was dried in a desiccator and the DDPS peptide was purified further by high-voltage electrophoresis in pyridine acetate buffer, *p*H 6.5. The total amount of material was applied to Whatman No. 3 paper in a band 10 cm wide and subjected to electrophoresis at 2000 v for 70 minutes. The yellow peptide moved rapidly toward the anode faster than any other peptide of the chymotryptic digest (mobility approximately 0.5 with respect to cysteic acid). The yellow band was cut out, stitched onto another sheet of filter paper (Richmond and Hartley, 1959), and again subjected to high-voltage electrophoresis at *p*H 6.5, 2000 v, for 75 minutes. This resulted in the removal of the remaining slower-moving impurities. Final purification of the peptide was achieved by chromatography in butanol–acetic acid–water (3:1:1) in which system the yellow material moved quite fast (R_F approximately 0.55). After elution and drying, the yield of the peptide with regard to carboxypeptidase B was approximately 30%.

Composition and Structure of DDPS-Peptide

The amino acid composition was determined after 24 hours of hydrolysis in 6 N HCl at 105°, and is given in Table I. The composition corresponds to Val,*S*-SC-Cys,Leu,Ile,Pro_2,Ser,Thr_2,Glu_5. Dinitrophenylation of the intact tetradecapeptide yielded DNP-valine. Digestion with carboxypeptidase A at *p*H 8 and 25° for 1, 4, 7, and 24 hours released threonine and glutamic acid, with threonine coming off first (Table II). Hence, valine appears to be the N-terminal and threonine the C-terminal residue.

TABLE I: Amino Acid Composition of DDPS-Peptide.[a]

Amino Acid	μMoles	Residues	
S-SC-cysteine[b]		0.93	1
Threonine	0.522	1.94	2
Serine	0.241	0.90	1
Glutamic acid	1.488	5.4	5
Proline	0.530	1.97	2
Valine	0.283	1.05	1
Isoleucine	0.275	1.02	1
Leucine	0.273	1.01	1

[a] Also traces (less than 0.01 μmole) of glycine, alanine, tyrosine, and aspartic acid. [b] The color value of *S*-SC-cysteine was estimated to be 68% of that of aspartic acid.

TABLE II: Digestion of DDPS-Peptide with Carboxypeptidase A.

Digestion Time (hr)	μMoles Amino Acid Released	
	Threonine	Glutamic Acid
1	0.016	0.006
4	0.035	0.009
7	0.028	0.017
24	0.035	0.031

Further degradation of the peptide was carried out with Nagarse. In a typical case, to 0.9 μmole of peptide dissolved in 1 ml of 0.1 M ammonium acetate buffer, *p*H 8, 10 μl of a 1% Nagarse solution was added and the mixture was incubated for 16 hours at 25°. High-voltage electrophoresis of the digest at *p*H 6.5 for 60 minutes yielded four main fractions, two neutral (N_2 and N_3) and two acidic ones (N_1 and N_4), and a small amount of another acidic peptide (N_5). Only peptide N_4 was yellow. The neutral peptides were further purified by high-voltage electrophoresis at *p*H 2.1 followed by chromatography in butanol–acetic acid–water (4:1:5).

Peptide N_1 was acidic, and after acid hydrolysis yielded equal amounts of glutamic acid, proline, leucine, and valine (Table III). Dinitrophenylation yielded DNP-valine, whereas digestion with carboxypeptidase A failed to release detectable amounts of any amino acids, suggesting that proline is either the terminal or penultimate amino acid residue. Partial acid hydrolysis of the tetrapeptide (6 N HCl, 105°, 10 minutes) and separation of the reaction products by high-voltage electrophoresis at *p*H 6.5 and 2.1, each for 60 minutes, at 2000 v, gave the following products: (Val,Leu),(Val,Leu,Pro), glutamic acid, and unhydrolyzed peptide. The sequence of peptide N_1 is thus Val.Leu.Pro.Glu.

Peptide N_2 proved to be a neutral dipeptide which, on acid hydrolysis, yielded serine and glutamic acid (Table III). Dinitrophenylation yielded DNP-serine and the structure of this dipeptide is, therefore, Ser.Gln.

Peptide N_3 was neutral also and after acid hydrolysis gave the following composition: Ile,Glu,Pro,Thr (Table III). Dinitrophenylation yielded DNP-isoleucine, whereas carboxypeptidase A failed to release any amino acids, suggesting that, as in the case of peptide N_1, proline was the terminal or penultimate amino acid residue. Partial acid hydrolysis (6 N HCl, 105°, 10 minutes) and separation of the products by high-voltage electrophoresis gave the following products: (Ile,Glu); (Ile,Glu,Pro); large amounts of threonine and smaller quantities of proline. Since the peptide is neutral, glutamic acid exists in the form of glutamine

TABLE III: Amino Acid Compositions of Peptides Obtained from DDPS Carboxypeptidase B.

		Amino Acids (residues/mole)							
		S-SC-Cys	Thr	Ser	Glu	Pro	Val	Ile	Leu
Chymotryptic peptide		0.93	1.94	0.90	5.4	1.97	1.05	1.02	1.01
Nagarse peptides	N_1				1.2	0.9	1.07		1.00
	N_2			0.95	1.05				
	N_3		1.01		1.07	0.94		0.97	
	N_4	0.8	0.91		2.1				

and the sequence of the peptide is, therefore, Ile.Gln.-Pro.Thr.

Peptide N_4 was highly acidic with an electrophoretic mobility at *p*H 6.5 of 0.74 with respect to cysteic acid. This peptide was yellow and thus contained the DDPS group. After acid hydrolysis, the following amino acid composition was obtained (Table III): *S*-SC-cysteine, Glu_2, Thr. Dinitrophenylation followed by acid hydrolysis yielded DNP-*S*-SC-cysteine, as determined by comparison in chromatographic behavior with an authentic sample. Since DNP-*S*-SC-cysteine does not separate well from DNP-glutamic acid in the two-dimensional system used for the separation of DNP-amino acids (*t*-amyl alcohol–ammonia in the first dimension, 1.5 M phosphate buffer, *p*H 6, in the second dimension), chromatography was carried out in isoamyl alcohol saturated with 1 N acetic acid. In this solvent, DNP-glutamic acid moved with an R_F of 0.81, as compared to 0.64 for DNP-*S*-SC-cysteine, and 0.63 for the unknown. When electrophoresis was carried out in pyridine acetate buffer, *p*H 3.5, DNP-*S*-SC-cysteine moved faster than DNP-glutamic acid, and the unknown again had the same mobility as the DNP-*S*-SC-cysteine standard. Digestion of peptide N_4 with carboxypeptidase A removed threonine and glutamic acid, threonine being released first. The remaining dipeptide was still acidic on electrophoresis, indicating that in the sequence DDPS-Cys.Glu the glutamic acid residue was not amidated. The C-terminal sequence Glu.Thr, suggested by digestion with carboxypeptidase A and obtained in peptide N_4 as well as in the entire tetradecapeptide, alassifies N_4 as the C-terminal peptide and establishes the sequence of peptide N_4 as *S*-SC-Cys.Glu.Glu.Thr.

Peptide N_5, which was obtained in small yields from the Nagarse digest of the tetradecapeptide, had the somposition Val,Glu_2,Leu,Ser,Pro. Carboxypeptidase A released first glutamine and then serine. Dinitrophenylation followed by acid hydrolysis yielded DNP-valine. Composition as well as the C-terminal sequence of this peptide provide evidence that the dipeptide N_2 is adjacent to peptide N_1 and hence that the sequence of peptides obtained from Nagarse digests of the tetradecapeptide is N_1-N_2-N_3-N_4. The amino acid sequence of the peptide is therefore Val.Leu.Pro.Glu.-Ser.Gln.Ile.Gln.Pro.Thr.CySH.Glu.Glu.Thr. (Table IV).

TABLE IV: Amino Acid Sequence of Cysteinyl Peptide of the Active Center of Carboxypeptidase B.

Chymotryptic peptide: H.Val.(Leu,Ile,Ser,*S*-SC-Cys, Thr,Pro_2,Glu_4)Glu.Thr.OH

Nagarse peptides:
N_1: H.Val(Leu,Pro,Glu)
N_2: H.Ser.Gln.OH
N_3: H.Ile(Gln,Pro,Thr)
N_4: *S*-SC-Cys.Glu.Glu.Thr.OH
N_5: H.Val(Leu,Pro,Glu)Ser.Gln.OH

Products of partial acid hydrolysis:
of N_1: (Val,Leu,Pro); (Val,Leu); Glu
of N_3: (Ile,Glu,Pro); (Ile,Glu); Pro; Thr

Total sequence: H.Val.Leu.Pro.Glu.Ser.Gln.Ile.Gln. Pro.Thr.Cys.Glu.Glu.Thr.OH

Discussion

The present experiments have provided a tentative structure for a tetradecapeptide containing the single cysteine residue which, in native bovine pancreatic carboxypeptidase B, is involved in the binding of zinc to the enzyme protein. The evidence for the involvement of this group in metal binding has been presented in the preceding paper and need not be detailed again (Wintersberger *et al.*, 1965). The labeling of this thiol with the yellow maleimide derivative of Witter and Tuppy (1960), DDPM, has provided a marker for the identification of the peptide bearing this group, which has been isolated from a chymotryptic digest of the protein. The splitting of a peptide bond adjacent to a threonyl residue is unusual for chymotryptic action but not without precedent. Dus *et al.* (1962) found in a chymotryptic digest of the heme peptide.$CySO_3H$.His.Thr.-Phe.Asp. free phenylalanine and some free threonine,

indicating cleavage before and after threonine in addition to the usual cleavage after phenylalanine. Although the present peptide could be prepared in good yield, the limited amount of enzyme available when this investigation was conducted restricted the number and kinds of procedures that could be applied to the elucidation of the amino acid sequence. For this reason, the present formulation of the structure of this thiol peptide must be considered to be tentative.

Because of the previously noted similarities in composition and mechanism of action of carboxypeptidases A and B (Cox *et al.*, 1962; Wintersberger *et al.*, 1965), it is of obvious interest to compare the structure of the peptide described herein with the analogous peptide isolated from carboxypeptidase A. A preliminary report by Sampath Kumar *et al.* (1963) gives the structure of the latter peptide as Gly.Lys.Ala.Gly.(Ala,-Ser).Ser.(Pro,Ser,CySH).Ser.Glu.Thr.Tyr., which is obviously quite different from that proposed in the present work for the corresponding peptide of carboxypeptidase B. The lack of correspondence in structure of these two peptides was unexpected in the light of analogous observations on other enzymes that are supposed to operate by a common mechanism. Thus, in the case of chymotrypsin, trypsin, and other "serine-histidine" enzymes, striking similarities in peptide sequences containing the active-center serine and histidines were found (Walsh and Neurath, 1964; Smillie and Hartley, 1964). Analogous findings were obtained by comparing the peptides containing the active-center thiol of papain (Light *et al.*, 1964), and ficin (Wong and Liener, 1964). The lack of homology between the cysteinyl peptides of carboxypeptidase A and B may suggest that these two enzymes have evolved independently, as reflected also by the considerable difference in sulfur-containing amino acids (Cox *et al.*, 1962). Alternatively, it might be suggested that the difference in amino acid sequence in the region of the zinc-binding thiol is of no moment as this group merely serves as a support for the crucial metal atom but, itself, is not a part of the active site. A clarification of the possible homologies between carboxypeptidases A and B must await the elucidation of the entire amino acid sequence of both of these enzymes.

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References

Cox, D. J., Wintersberger, E., and Neurath, H. (1962), *Biochemistry 1*, 1078.

Dus, K., Bartsch, R. G., and Kamen, M. D. (1962), *J. Biol. Chem. 237*, 3083.

Light, A., Frater, R., Kimmel, J. R., and Smith, E. L. (1964), *Proc. Natl. Acad. Sci. U.S. 52*, 1276.

Richmond, V., and Hartley, B. S. (1959), *Nature 184*, 1869.

Sampath Kumar, K. S. V., Walsh, K. A., Bargetzi, J.-P., and Neurath, H. (1963), *Aspects Protein Struct. Proc. Symp. Madras, 1963*, 319.

Sanger, F., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 353.

Smillie, L. B., and Hartley, B. S. (1964), *J. Mol. Biol. 10*, 183.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Tigane, E., Wade, E. H. M., Wong, J. T., and Hanes, C. S. (1961), *Can. J. Biochem. Physiol. 39*, 427.

Walsh, K. A., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U.S. 52*, 884.

Wintersberger, E., Cox, D. J., and Neurath, H. (1962), *Biochemistry 1*, 1069.

Wintersberger, E., Neurath, H., Coombs, T. L., and Vallee, B. L. (1965), *Biochemistry 4*, 1526 (this issue; preceding paper).

Witter, A., and Tuppy, H. (1960), *Biochim. Biophys. Acta 45*, 429.

Wong, R. C., and Liener, I. E. (1964), *Biochem. Biophys. Res. Commun. 17*, 470.