THE SOLUTION CONFORMATION OF BOVINE CARBOXYPEPTIDASE A: REACTION WITH 2-HYDROXY-5-NITROBENZYL BROMIDE AND *N*-METHYLNICOTINAMIDE CHLORIDE *

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Received 26 January 1970

1. Introduction

The recent completion of the amino acid sequence [1] and of the high resolution electron density map [2] of crystalline bovine carboxypeptidase A lead to a study of the position of various amino acid side chains of the enzyme in an aqueous environment. For this reason, the availability of the tryptophanyl residues of this enzyme have been examined by two methods: chemical modification by 2-hydroxy-5-nitrobenzyl bromide (HNBB) [3, 4] and complex formation with *N*-methylnicotinamide chloride [15]. These experiments revealed that the seven tryptophanyl residues present in the enzyme [1] are unavailable. However, in contrast to earlier reports, [3, 4] modification of the protein by 2-hydroxy-5-nitrobenzyl bromide at pH 7.0 occurs rapidly and apparently specifically at the α-amino group, suggesting that under certain conditions, the specificity of 2-hydroxy-5-nitrobenzyl bromide is wider than previously thought.

* Supported by research grants from the National Institutes of Health (GM 04617 and GM 15731), the American Cancer Society (P-79K), the Office of Naval Research (NONR 477-35), and the National Science Foundation (GB 4990).

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2. Experimental procedure

2.1. *Materials*

Crystalline carboxypeptidase A (Anson) and twice-crystallized pepsin were purchased from Worthington Biochemical Corporation. 2-Hydroxy-5-nitrobenzyl bromide was purchased from Cyclo Chemical Corporation and recrystallized from benzene before use. *N*-Methylnicotinamide chloride was prepared from nicotinamide and methyl iodide, followed by treatment with silver chloride, as described by Karrer et al. [16].

2.2. *Modification of carboxypeptidase A with 2-hydroxy-5-nitrobenzyl bromide at neutral pH*

50 μl aliquots of an HNBB solution (85 μmoles in 0.2 ml of dry acetone) were added with stirring at 5 min intervals to a neutral solution (8.5 ml) containing 0.5 μmoles of carboxypeptidase A in 0.1 M NaCl-0.2 M sodium acetate was at 0° in an ice bath. After the final addition, the sample was centrifuged and the clear supernatant containing the modified enzyme was dialyzed at 5°C against neutral 1.0 M NaCl-0.2 M sodium acetate. Protein concentration, esterase activity and the concentration of HNB groups were determined on aliquots withdrawn from the above stock solution of modified enzyme. Experiments were also carried out in the presence of 0.02 M β-phenylpropionate (β-PP) under the above conditions and, separately, in a buffer of 0.05 M sodium phosphate, pH 6.5, containing 1 M NaCl but no β-phenylpropionate. In some experiments, excess reagent was removed by gel filtration.

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2.3. *Isolation of the labelled peptides from modified carboxypeptidase*

Modified carboxypeptidase A was digested sequentially by thermolysin and pepsin as described previously [7, 8]. Spectrophotometric analysis indicated that over 90% of the material absorbing at 410 nm had been solubilized.

Fractionation was carried out on a 0.9 × 50 cm column of SE-Sepahdex (G-25) at 55°C. The column was developed with a double linear gradient of pyridine-acetate [9] and monitored by ninhydrin analysis after alkaline hydrolysis [10]. In addition, 50 μl aliquots from alternate fractions were withdrawn and treated with 1.0 N NaOH to locate the yellow peptides. The fractions containing the modified peptides were further purified on Sephadex G-25 and G-50 equilibrated with 0.1 M ammonium acetate (pH 9.0).

2.4. *Titration of carboxypeptidase A with N-methylnicotinamide chloride*

The titration was performed in the manner described by Deranleau et al. [5] except that a buffer of 0.05 M sodium phosphate-1.0 M NaCl (pH 6.5) was employed. Light scattering measurements were performed in a similar manner on a Sophica light scattering photometer.

3. Results

3.1. *Modification of carboxypeptidase A with 2-hydroxy-5-nitrobenzyl bromide at neutral pH*

The modification of carboxypeptidase A with HNBB under several conditions is summarized in table 1. As shown in experiment no. 1, about 75% of the activity toward hippuryl-β-phenyllactate (HPLA) was retained when one HNB group was incorporated per enzyme molecule at pH 7.0. However, when the modification was carried out in the presence of the competitive inhibitor, β-phenylpropionic acid (experiment no. 2), more than 90% of the esterase activity was retained in the modified enzyme when less than one HNB group was incorporated. Modification at pH 6.5 led to a slightly greater degree of incorporation of HNB groups (1.3) with a loss of about 30% of the esterase activity (experiment no. 3). Further treatment of the sample isolated from experiment no. 3 with reagent did not lead to greater modification, although an additional 30% of the original esterase activity was lost (experiment no. 4). On the basis of these experiments, we concluded that the loss of activity during the modification reaction is due mainly to denaturation and not directly to the modification of groups essential for function. The apparent protection afforded by β-phenylpropionic acid against loss in activity probably results from stabilization of the modified enzyme rather than steric protection of essential groups.

In order to identify the site of substitution in the molecule, the modified enzyme was subjected to digestion by thermolysin, followed by pepsin. Separation of the soluble peptic peptides on SE-Sephadex yielded only two yellow peaks, Pp I and Pp II. Pp I contained only one major peptide. Pp II had to be purified further on Sephadex G-25 and yielded three yellow fractions, designated Pp II-1, Pp II-2, and Pp II-3. Fractions Pp II-1 and Pp II-2 were contaminated with several ninhydrin positive components and were present in insufficient quantities to enable further purification. Both Pp I and Pp II-3 were finally purified by high voltage electrophoresis at pH 3.75.

Data on the amino acid composition of purified peptide Pp I and peptide Pp II-3, before and after purification by preparative high voltage electrophoresis, are summarized in table 2. Peptides Pp I and Pp II-3 had the same amino acid composition and were assigned unambiguously to residues 9–12 in the complete amino acid sequence of carboxypeptidase Aα [1]. These residues correspond to residues 2–5 in carboxypeptidase Aγ, the predominant form in the enzyme preparation used in this investigation [11]. Since no tryptophanyl residues are located in this segment and these peptides account for 81% of the total 410 nm absorption, we concluded that both peptides Pp I and Pp II-3 are derived from the amino terminal segment of the protein and that specific modification of the α-amino group of the *N*-terminal asparagine residue (*vide infra*) prevents the appearance of one residue of aspartic acid in the acid hydrolysate.

3.2. *Amino terminal analysis of modified carboxypeptidase A*

In support of this conclusion, *N*-terminal analyses were performed by the cyanate method of Stark and Smyth [12] as modified by Pétra and Neurath [11]. The results of these experiments are summarized in table 3. Although the amount of alanine (the *N*-terminal residue of the α form) is somewhat higher than

Table 1
The modification of carboxypeptidase A with 2-hydroxy-5-nitrobenzyl bromide at 0°C.

Experiment	Buffer	pH	β-PP	Number of HNB groups	% Esterase activity with HPLA
1	1.0 M NaCl 0.2 M Na Acetate	7.0	–	1.13	75
2	1.0 M NaCl 0.2 M Na Acetate	7.0	+	0.87	94
3	1.0 M NaCl 0.04 M Na Phosphate	6.5	–	1.34	67
4[a]	1.0 M NaCl 0.04 M Na Phosphate	6.5	–	1.34	35
5[b]	1.0 M NaCl 0.20 M Na Acetate	7.0	+	1.00	90[c]

[a]This sample was prepared by treating the preparation in experiment no. 3 again.
[b]This experiment was performed on a preparative scale with 10 μmoles of enzyme (350 mg).
[c]The percentage of peptidase activity for this material, as measured by the method of Whitaker et al. [14], was 103%.

Table 2
Amino acid composition of fractions Pp I and Pp II-3[a].

Amino Acid	Peptide Pp II-3		Peptide Pp I
	Before Electrophoretic Purification	After Electrophoretic Purification	After Purification by Electrophoresis
Threonine	1.28	1.00 (1)	0.99 (1)
Serine	0.11	–	–
Glutamic Acid	0.37	0.20	–
Glycine	0.32	0.16	–
Alanine	1.00	1.00 (1)	1.0 (1)
Valine	0.17	–	–
Isoleucine	0.31	0.09	–
Tyrosine	1.80	1.61 (2)	2.29 (2)

[a]The values are expressed as residues per molecule, assuming the presence of alanine in both peptides. Numbers is parentheses refer to the presumed integral values.

usually seen in such preparations, both alanine and serine (the *N*-terminal residue of the β form) are usually found [11]. Following reaction with the HNBB reagent, a marked decrease in all *N*-terminal residues was observed, consistent with the conclusion that the α-amino group is the main site of modification.

3.3. *Titration by N-methylnicotinamide*

The above results suggest that none of the tryptophanyl residues is available for reaction with the HNBB reagent at neutral pH. To test this conclusion further, solutions of the enzyme were titrated under similar conditions with *N*-methylnicotinamide, which forms a yellow (charge transfer) complex with tryptophan residues available to the solvent [5, 13]. Although some absorption was observed during successive additions of *N*-methylnicotinamide chloride, no yellow color was obtained. Analysis of the spectra after cor-

Table 3
N-terminal analyses of native and HNB-carboxypeptidase A*.

Residue	Native carboxypeptidase A	Modified carboxypeptidase A
Aspartic acid	0.72	0.23
Serine	0.15	0.10
Alanine	0.22	0.08
Total	1.09	0.41

* Determined by the method of Stark and Smyth [12].

rection for light scattering showed a complete lack of a complex with the visible absorbance characteristics of tryptophan-*N*-methylnicotinamide complexes.

4. Discussion

The reaction of carboxypeptidase A with HNBB at neutral pH in aqueous buffers seems to be confined to the amino terminal residue and little or no reaction with any tryptophan residue is observed. It should be noted that although the extent of reaction appears to correspond to the incorporation of one mole of HNB per mole of protein (table 1), this number was calculated from the extinction coefficient of the HNB-tryptophan derivative. Since the site of modification is an amino group, the exact number of HNB groups incorporated is somewhat more difficult to determine. Moreover, the effect of double substitutions, i.e., 2 moles of HNB per amino group, may also contribute to inaccuracy in estimating the total incorporation [3]. The incomplete protection of the α-amino group (table 3) and the recovery of the same peptide in two fractions from SE-Sephadex columns suggests that some double labelling does occur.

The modified peptides in fractions Pp II-1 and Pp II-2, (amounting to 19% of the total material absorbing at 410 nm) which were not identified are most probably derived from the amino terminal portion of the α and β forms of carboxypeptidase A present in the commercial preparations [11].

The preservation of the esterase or peptidase activity of the modified enzyme as measured with small synthetic substrates would seem to rule out any direct catalytic role for the α-amino group. The absence of any change in the optical rotatory dispersion spectra mitigates against a major structural change, and the ready availability of the α-amino group to modification argues against any type of internal binding of this group in the native enzyme in solution.

The results of the HNBB modifications and the *N*-methylnicotinamide titrations together indicate that none of the tryptophanyl side chains is freely available in a manner detectable by either of these methods. On this basis, it may be concluded that the tryptophanyl residues of carboxypeptidase A are located in the interior of the molecule in solution and presumably play a role in stabilizing its three-dimensional structure.

The identification of the α-amino group as a site of modification by HNBB was not detected by Horton and Koshland [3]. It is noteworthy that this site is unlikely to react in acidic solutions because of the pK_a usually assigned to this group (∼7.5–8.0) and, hence, should not interfere with total tryptophan determinations with HNBB [4]. However, application of this reagent to studies with other proteins in neutral solutions should be made with appropriate caution in the light of these findings.

Acknowledgements

The authors would like to thank Dr. Philip H. Pétra for help in the petidase assays of carboxypeptidase A and Mr. R.R.Granberg and Mr. Thomas A. Oas for amino acid analyses of the samples. Mr. C.V. Shih is also thanked for skillful assistance in the *N*-methylnicotinamide chloride titrations of carbocypeptidase A.

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