REACTION OF 2-HYDROXY-5-NITROBENZYLATED CARBOXYPEPTIDASE A WITH ^{14}C-1-FLUORO-2,4-DINITROBENZENE[1]

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SUMMARY: Carboxypeptidase A was treated with dimethyl(2-hydroxy-5-nitrobenzyl) sulfonium chloride, in 20 times its molar concentration, at pH 7.5. Hydroxy-nitrobenzylated enzyme was isolated from the reaction mixture and examined for modification of amino acid residues. Modification of tryptophan was evidenced both by diminished recoveries from a variety of acid hydrolysates, and from quenching of indole fluorescence of the modified protein in denaturing as well as non-denaturing solvents. N-Terminal amino group alkylation did not appear to have occurred, on the basis of essentially full recoveries of α-N-DNP-amino acids from acid hydrolysates following reaction of the enzyme with ^{14}C-fluoro-dinitrobenzene.

2-Hydroxy-5-nitrobenzyl bromide (HNB-Br)[2] has received wide application since its introduction as a tryptophan-selective protein reagent and reporter group (1, 2). Not only has HNB-Br been used to implicate the involvement of tryptophyl residues in enzymatic activities (3, 4), in toxin toxicity (5), and in the response of muscle tissue to insulin (6), but it has also provided the basis for a colorimetric assay for tryptophan in proteins (7).

Recently, however, it was reported that treatment of bovine pancreatic carboxypeptidase A with HNB-Br at pH 7 appeared to result in specific alkylation of α-amino groups afforded by the population of N-terminal residues, aspartate (asparagine), serine, and alanine (8), rather than alkylation of tryptophan (9). It was noted that problems of partial denaturation of the enzyme had been encountered in this study, as had also been observed in our laboratory (10).

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[2]The abbreviations used are: HNB, 2-hydroxy-5-nitrobenzyl; DNP, 2,4-dinitrophenyl.

In order to circumvent problems of denaturation of proteins and rearrangements of HNB-labeled tryptophan species which can arise from the use of acetone (11), we have utilized dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium salts to introduce the HNB reporter group into the structure of enzymes in aqueous solutions (10). Treatment of L-tryptophan ethyl ester with dimethyl-HNB-sulfonium halide was found to result in nearly quantitative formation of two isomeric monoalkylated products in which the HNB group was attached to the β-carbon atom of the indole ring (12). The same isomeric species had been observed as the major products of reaction with HNB-Br (13), though in smaller yields (12). Hydroxynitrobenzylation of the free amino group had not been observed with tryptophan ethyl ester, but the possibility for such alkylation of α-amino groups in proteins appeared to warrant further study.

Accordingly, experiments were undertaken to quantitate the free α-amino groups in HNB-labeled carboxypeptidase A, through reaction with ^{14}C-labeled 1-fluoro-2,4-dinitrobenzene (14).

MATERIALS AND METHODS

Materials. Carboxypeptidase A (Anson), twice crystallized, was purchased from Worthington Biochemical Corporation. Dimethyl(2-hydroxy-5-nitrobenzyl)-sulfonium chloride was prepared as previously described (10). 1-Fluoro-2,4-dinitrobenzene-^{14}C(U), specific activity 31.5 mCi/mmole, was obtained from Amersham/Searle Corporation, and checked for radiochemical purity by paper and thin-layer chromatography (98-100% in four systems). Cold fluorodinitrobenzene, DNP-L-alanine, DNP-L-serine, ε-DNP-L-lysine·HCl, and O-DNP-L-tyrosine were purchased from Sigma Chemical Company; DNP-L-aspartate was prepared by treating 50 mg of L-aspartic acid (Mann) with a 2-fold molar excess of fluorodinitrobenzene in 5% $NaHCO_3$ (1% ethanol).

Preparation of HNB-Labeled Carboxypeptidase A. To 50-80 mg of carboxypeptidase A in 0.2 M Tris-chloride, pH 7.5, containing 3 M NaCl, was added solid dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride, to a final molar concentration of 20 times that of the enzyme, at room temperature with

continuous stirring. The pH was maintained at 7.5 with 0.2 N NaOH delivered from a pH-stat, over a period of two hours. The mixture was then dialyzed exhaustively in the cold, first against 0.005 M Tris-chloride, pH 8.0, containing 0.04 M LiCl, and then against 0.05 M Tris-0.04 M LiCl, pH 7.5, containing 0.05 M β-phenylpropionate.

Isolation of HNB-Labeled Carboxypeptidase. The dialyzed enzyme preparation was passed through a Sephadex G-25 column (2.5 x 30-cm) equilibrated with 0.05 M Tris-0.04 M LiCl, 0.05 M β-phenylpropionate, pH 7.5, and then fractionated on a 2.5 x 30-cm column of triethylaminoethyl-cellulose (Bio-Rad Cellex-T) using a stepwise elution procedure as shown in Figure 1. The HNB-labeled enzyme, identified by UV-visible spectra and by enzymatic activity (with hippuryl-β-phenyllactate and with carbobenzoxyglycyl-L-phenylalanine) emerged in two well resolved peaks, 1 and 4, which together represented a recovery of approximately 85% of the protein. The fractions comprising each peak were combined, concentrated with Aquacide I (Calbiochem, Lot 900873), and dialyzed against 0.05 M Tris-1.0 M NaCl, pH 7.5, to remove β-phenylpropionate prior to activity assays and enzyme characterization. The degree of alkylation was ascertained from UV-visible spectra in alkaline solution (2, 7), using a Cary 15 spectrophotometer. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer.

Dinitrophenylation. Unmodified carboxypeptidase A and HNB-labeled enzyme were each treated with ^{14}C-labeled fluorodinitrobenzene (prepared to specific activity 0.15 mCi/mmole) by incubating approximately 0.03 μmole enzyme with 6.7 μmoles fluorodinitrobenzene and 1.4 mmoles $NaHCO_3$, in a total volume of 2.0 ml, for 24 hours at 30° in the dark with continuous shaking. The insoluble DNP-protein was collected and washed successively with water, ethanol, and ether, then dried under nitrogen, and hydrolyzed in 2 ml of 6 N HCl (containing norleucine as internal standard) in evacuated, sealed Pyrex tubes at 110° for 16 hours. The acid hydrolysates were each extracted twice with 2-ml portions of ether. The aqueous acidic fractions and the ether extracts were each eva-

porated to dryness. The dried, aqueous portions were dissolved in citrate buffer and subjected to analysis on a Beckman 120C Amino Acid Analyzer to determine protein content. The ether extracts, containing α-DNP-amino acids, were dissolved in ethanol, and aliquots were subjected to quantitative thin-layer chromatography on 20 x 5-cm sheets of Baker-flex cellulose buffered with 0.1 M potassium phthalate, pH 6.0, and developed with tert-amyl alcohol saturated with the same buffer. DNP-Amino acid standards and dinitrophenol were included in each chromatogram.

Following chromatography, spots corresponding to DNP-aspartate (R_f 0.04), DNP-serine (0.24), and DNP-alanine (0.46) were cut out and placed in vials with 10 ml of Omnifluor solution (4 g per liter toluene) for scintillation counting in a Packard Tri-Carb Model 314 EX system.

RESULTS AND DISCUSSION

Dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride in a mole ratio of 20:1 reacts with carboxypeptidase A at pH 7.5 to form products in which the predominant species (Peak 1, Figure 1) contains 1.0 mole of HNB group per mole enzyme, on the basis of spectral measurement (Figure 2). Analysis of acid

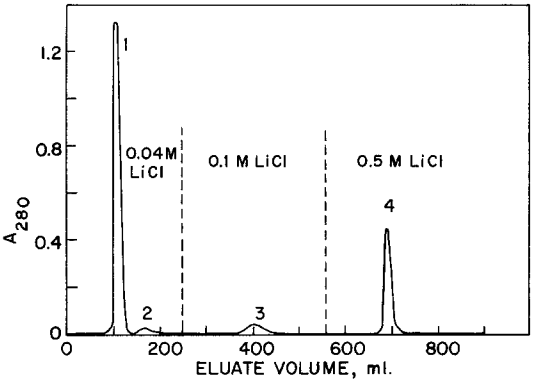

Fig. 1. TEAE-Cellulose Chromatography of HNB-Labeled Carboxypeptidase A. Load: ~53 mg in 12 ml; eluant: 0.05 M Tris-chloride, pH 7.5, containing 0.05 M β-phenylpropionate and 0.04 M, 0.1 M, and 0.5 M LiCl, as indicated; other conditions as described in text. Eluate monitored continuously at 280 mμ (Buchler Uviscan III). Peak 1, HNB-enzyme (1:1); peaks 2 and 3, solvolysis products of reagent; peak 4, HNB-enzyme (2:1).

hydrolysates revealed no detectable differences in amino acid composition of native and of HNB-labeled enzyme preparations, save a lowered tryptophan recovery. When acid hydrolysis was conducted in the presence of 4% thioglycolic acid, a complete disappearance of tryptophan from the labeled enzyme was observed, but 90-98% recovery of tryptophan was obtained from native enzyme. The presence of 2% thioglycolic acid resulted in the recovery of 15-30% less tryptophan from the HNB-enzyme than from native carboxypeptidase. Analysis of acid hydrolysates of dinitrophenylated HNB-carboxypeptidase revealed 5.8 residues of tryptophan

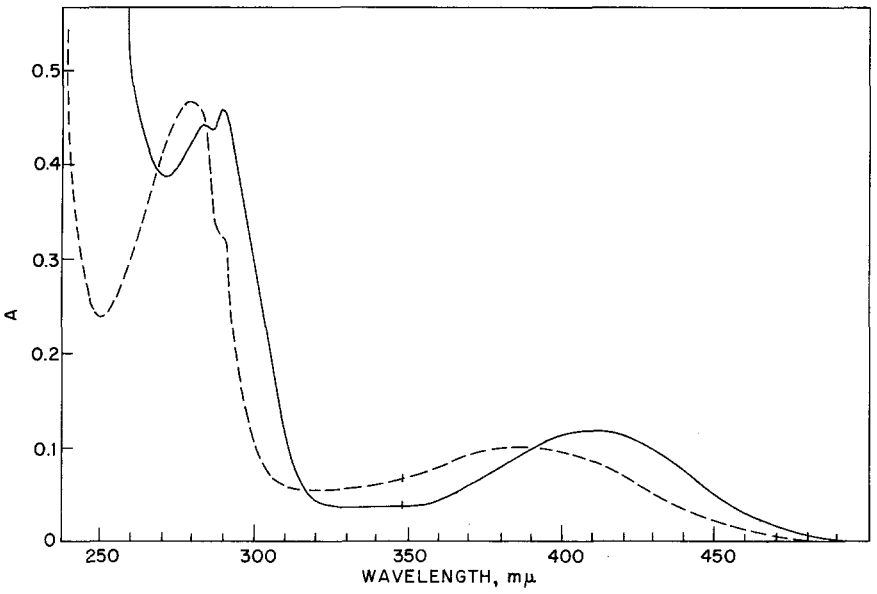

Fig. 2. UV-Visible Absorption Spectra of HNB-Carboxypeptidase A (Peak 1). Broken line: pH 7.5; continuous line: pH > 12. Degree of label, 1.0 mole of reporter group/mole enzyme.

as compared to 6.9 from the dinitrophenylated native enzyme. The recoveries of amino acids from three separate preparations of HNB-carboxypeptidase as compared to the control enzyme were: aspartate, 100-102%; alanine, 99.6-100.5%; and serine, 97-101%. Spectral titrations (15) revealed a lowered reactivity of HNB-carboxypeptidase with N-bromosuccinimide as compared to that of native enzyme. Furthermore, the incorporation of 1.0 mole of HNB led to a 50% quenching of fluorescence of carboxypeptidase excited at 280 mμ in Tris-NaCl, pH 7.

TABLE I

Dinitrophenylation of Native and HNB-labeled Carboxypeptidase A

	Native enzyme			HNB-enzyme		
	1	2	3	1	2	3
Based on radioactivity:						
μmoles DNP-aspartic acid	0.0145	0.0192	0.0057	0.0045	0.0038	0.0049
μmoles DNP-serine	0.0066	0.0056	0.0024	0.0008	0.0009	0.0026
μmoles DNP-alanine	0.0043	0.0047	0.0020	0.0007	0.0001	0.0016
Total μmoles N-terminal DNP residues recovered	0.0254	0.0295	0.0101	0.0060	0.0048	0.0091
Based on amino acid analyses:						
Total μmoles protein recovered	0.0336	0.0385	0.0135	0.0067	0.0050	0.0125
$\frac{\text{μmoles N-term. DNP residues recovered}}{\text{μmole protein recovered}}$	0.76	0.77	0.75	0.89	0.96	0.73

Following complete denaturation in 6 M guanidinium chloride, the relative quantum yield for HNB-carboxypeptidase was found to be 10.5 as compared to 14.5 for the unlabeled enzyme. Thus it appears that the incorporation of HNB groups into carboxypeptidase A at pH 7.5 results in modification of tryptophan.

Table I presents the results of dinitrophenylation of native and HNB-labeled carboxypeptidase A. In view of the recoveries of ^{14}C-DNP-aspartate, -alanine, and -serine from acid hydrolysates of the dinitrophenylated proteins, it may be concluded that hydroxynitrobenzylation of the enzyme does not diminish the reactivity of its α-amino groups with fluorodinitrobenzene. Thus, in the case of reaction of carboxypeptidase A with the HNB-sulfonium salt, at pH 7.5, in the absence of added acetone or other organic solvent, denaturation is negligible and modification appears to be limited to tryptophyl residues, without significant alkylation of α-amino groups.

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