Affinity Labeling of Bovine Carboxypeptidase Aγ^{Leu} by *N*-Bromoacetyl-*N*-methyl-L-phenylalanine. II. Sites of Modification*

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ABSTRACT: Reaction of bovine carboxypeptidase A_γ^{Leu} with the affinity-label *N*-bromoacetyl-*N*-methyl-L-phenylalanine (L-BAMP) results in enzyme inactivation by incorporation of the label into the carboxyl group of the active site. Comparison of the chromatographic elution profiles of the cyanogen bromide fragments of the enzyme labeled in the presence and absence of the competitive inhibitor D-phenylalanine demonstrates that inactivation is accompanied by modification of a residue in fragment F_I (residues 104–301). On the basis of the isolation of a single radioactive peptide (glutamic acid, phenylalanine) from a thermolytic digest of F_I and the known amino acid sequence of carboxypeptidase A_α, this residue was identified as Glu_{270}. In addition, 0.6–0.8 residue of L-BAMP was incorporated into the amino-terminal cyanogen bromide fragment, F_N, when carboxypeptidase A_γ^{Leu} was reacted in the presence or absence of D-phenylalanine with 2 mM reagent at pH 7.4 for 24 hr. The amino groups of the amino-terminal asparagine (0.5 residue/molecule) and His_{13} (~0.2 residue/molecule) were identified as additional sites of modification. Significantly, these residues were not modified in carboxypeptidase A_β^{Leu}.

In the preceding paper (Hass and Neurath, 1971) we presented kinetic evidence that *N*-bromoacetyl-*N*-methyl-L-phenylalanine (L-BAMP)[1] inactivates carboxypeptidase A_γ^{Leu} by reaction at the active site. Two molecules of L-BAMP per molecule of enzyme were maximally introduced when CPA_γ^{Leu} was incubated with 2 mM L-BAMP at pH 7.5 for 24 hr.

Two amino acid residues or classes of residues reactive toward L-BAMP could be distinguished. One is more rapidly modified by L-BAMP than the other, is protected by both D-phenylalanine and β-phenylpropionate, and is modified at a rate equal to that of inactivation. The other less reactive class of residues is protected by β-phenylpropionate but not by D-phenylalanine and is modified without affecting the catalytic activity of the enzyme.

In order to identify the principal sites of reaction of carboxypeptidase A with L-BAMP, the modified enzyme was degraded with cyanogen bromide. The modified amino acid residues were identified by peptide analysis and by alignment with the known sequence of carboxypeptidase A (Bradshaw *et al.*, 1969).

Experimental Section

Materials

Carboxypeptidase A_γ^{Leu} labeled with *N*-([1-^{14}C]bromoacetyl)-*N*-methyl-L-phenylalanine in the presence and absence of 0.05 M D-phenylalanine or 0.05 M β-phenylpropionate was prepared and characterized with regard to enzymic activity and extent of modification as described previously (Hass and Neurath, 1971).

CPA_β^{Leu} was prepared from activated pancreatic juice by affinity chromatography and subsequent crystallization (Reeck *et al.*, 1971). Separation from CPA_β^{Val} was achieved by ion-exchange chromatography (Pétra and Neurath, 1969).

N-([1-^{14}C]Bromoacetyl)-*N*-methyl-L-phenylalanine (specific activity 1.36×10^{11} dpm/mole) was prepared as previously described (Hass and Neurath, 1971). *N*-Carboxymethyl-L-aspartic acid, *O*-carboxymethyl-L-tyrosine, and a mixture of ε-carboxymethyl-L-lysine and ε-dicarboxymethyl-L-lysine were synthesized as described by Korman and Clarke (1956). A mixture of 1-carboxymethyl-, 3-carboxymethyl-, and 1,3-dicarboxymethyl-L-histidine was prepared according to Crestfield *et al.* (1963).

Thermolysin was purchased from Daiwa Kasei, Osaka, Japan; Sephadex G-75 and SE-Sephadex C-25 from Pharmacia Fine Chemicals; Dowex 1-X2 and Dowex 50-X4 from Bio-Rad; and phenyl isothiocyanate from Eastman.

Pyridine was redistilled from solid ninhydrin (Hill and Delaney, 1967) prior to use in chromatographic systems.

All other chemicals were of reagent grade and were used without further purification.

Methods

Analytical Procedures. Radioactivity measurements, esterase and peptidase assays, optical density determinations, and pH measurements were performed as described previously (Hass and Neurath, 1971).

Glycolic acid was determined by the colorimetric method of Takahashi *et al.* (1967). The value of 0.218 obtained for the optical density of a 10 nM standard was in good agreement with that of 0.226 reported earlier (Takahashi *et al.*, 1967).

Amino acid analyses were performed on the Spinco Model 120B amino acid analyzer (Spackman *et al.*, 1958) using an accelerated system to be described.[2] Samples containing

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[1] The following abbreviations are used: CPA, carboxypeptidase A; L-BAMP, *N*-bromoacetyl-*N*-methyl-L-phenylalanine; CM, carboxymethyl; CGP, carbobenzoxyglycyl-L-phenylalanine.

[2] R. R. Granberg, unpublished observations.

norleucine as internal standard (Walsh and Brown, 1962) were hydrolyzed with 6 N HCl at 110° *in vacuo* for 24 hr prior to analysis (Moore and Stein, 1963). Radioactive amino acid derivatives were identified and quantitated as follows. The dried hydrolysates were dissolved in 0.01 M HCl containing cysteic acid which was included as a marker. Suitable aliquots were subjected to amino acid analyses, counted to determine recoveries, and applied to the 50 cm column of the amino acid analyzer. The eluate was collected in a fraction collector (68 ml/hr, 1.6 min/tube). Cysteic acid (14 ml) was located with ninhydrin (Moore and Stein, 1963) and 1 ml of each tube was counted. The position of glycolic acid (25–26 ml) was determined as described by Takahashi *et al.* (1967). Recovery of radioactivity was 95 ± 3%.

Radioactive amino acid derivatives were characterized by their electrophoretic mobility at pH 6.5, 3.75, and 2.1 (2 kV, 45 min) and descending paper chromatography in pyridine–1-butanol–acetic acid–H_2O (10:15:3:12, v/v) and then compared to standard amino acids and their corresponding derivatives. The dried chromatograms were stained with ninhydrin (Bennett, 1967) to locate the standards and then cut into 0.5-in. sections which were counted in 10 ml of scintillation fluid.

Subtractive Edman degradations were performed by the method of Konigsberg and Hill (1962) as modified by Shearer *et al.* (1967).

Cleavage with Cyanogen Bromide and Separation of the Fragments. Carboxypeptidase A labeled with radioactive L-BAMP was cleaved by treatment with cyanogen bromide in 70% formic acid (Nomoto *et al.*, 1969). The resulting fragments, F_I (residues 104–301),[3] F_{III} (residues 23–103), and a mixture of F_N (residues 8–22 in CPA_γ^{Leu}, 3–22 in CPA_β^{Leu}) and F_C (residues 302–307), were separated on a 3 × 100 cm column of Sephadex G-75 equilibrated with 0.1 M propionic acid (Nomoto *et al.*, 1969). The optical density was monitored at 280 or 290 and the radioactivity determined by counting aliquots of alternate tubes.

Fragments F_N and F_C were separated on a 1.4 × 80 cm column of Sephadex G-25 equilibrated with 50% acetic acid (Nomoto *et al.*, 1969). The separation was monitored by ninhydrin reaction after alkaline hydrolysis (Hirs *et al.*, 1956) and the radioactivity determined in alternate tubes.

Thermolytic Digestion of F_I. Lyophilized F_I was dispersed in 0.01 M Tris-Cl–0.002 M $CaCl_2$ (pH 7.0) and digested at 37° with thermolysin. Thermolysin (1% by weight) was added to initiate digestion and an additional 1% introduced after 2 hr. Digestion was terminated after another 2 hr by adjusting the pH to 2.4. The amount of radioactivity solubilized during each digestion was estimated by counting 25 μl before and after centrifugation. The core was then washed with water (pH 2.4) and again digested as described. After four treatments, more than 96% of the total counts had been solubilized. The supernatants were combined and lyophilized.

Purification of the Radioactive Thermolytic Peptides from F_I. The lyophilized thermolytic digest of F_I was dissolved in 0.05 M pyridine–acetic acid (pH 2.4), an aliquot counted, and the remainder applied to a 1.5 × 80 cm column of SE-Sephadex C-25 equilibrated with 0.05 M pyridine–acetic acid (pH 2.4) at 55°. The peptides were eluted at a flow rate of 36 ml/hr (3.3-ml fractions) using a double linear gradient composed of 200 ml of 0.05 M pyridine–acetic acid (pH 2.4) and 200 ml of 0.5 M pyridine–acetic acid (pH 3.75) followed by 200 ml of 0.5 M pyridine–acetic acid (pH 3.75) and 200 ml of 2.0 M pyridine–acetic acid (pH 5.0) (Pétra and Neurath, 1971). The fractions were monitored by ninhydrin color after alkaline hydrolysis (Hill and Delaney, 1967) and by radioactivity. The radioactive fractions were divided into two pools, SE-1 and SE-2 (see Figure 3). Each pool was evaporated to dryness at 40° and dissolved in 3% pyridine.

Pool SE-1 was purified by chromatography at 37° on a 0.9 × 15 cm column of Dowex 1-X2 using a linear gradient of 200 ml of 3% pyridine (pH 8.8) and 200 ml of 2.0 M pyridine–acetic acid (pH 5.0). Fractions of 2 ml were collected at a flow rate of 30 ml/hr and monitored by radioactivity and ninhydrin color after alkaline hydrolysis. The radioactive fractions were pooled and evaporated. The purified pool, SE-1-DX, was dissolved in 50% acetic acid and its radioactivity and amino acid content determined after acid hydrolysis.

Pool SE-2 was purified by chromatography on Dowex 1-X2 as described for the chromatography of SE-1 (*vide supra*) and subjected to preparative paper electrophoresis at pH 6.5. The radioactive pool, SE-2-DX-1, obtained by chromatography on Dowex 1-X2, was evaporated, dissolved in 1.0 ml of H_2O, and applied in a 3-in. strip to Whatmann No. 3MM paper. After electrophoresis at pH 6.5 for 2 hr at 2 kV, the paper was dried at room temperature. A 0.25-in. strip was cut lengthwise from the chromatogram, stained with ninhydrin, and cut into 0.5-in. sections for radioactivity determinations. The radioactive zone was eluted with 50% acetic acid at room temperature. This pool, SE-2-DX-1-P, was subjected to radioactivity measurements, to amino acid and glycolic acid analyses after acid hydrolysis, and to subtractive Edman degradation (*vide supra*).

Stability of Incorporated Label in Base. Enzymatically inactive carboxypeptidase A_γ^{Leu} containing an average of 1.65 residues of radioactive L-BAMP was crystallized by dialysis at 4° against 0.01 M sodium phosphate (pH 7.5). The crystals were dissolved in cold 0.1 N NaOH (~1 mg of protein/ml) at 0°. Aliquots were removed at various times and mixed with an equal volume of cold glacial acetic acid. A zero time control was prepared by dissolving the enzyme in 50% acetic acid. The protein was separated from low molecular weight material on a 1.2 × 15 cm column of Sephadex G-25 equilibrated with 50% acetic acid and monitored by optical density at 280 nm. The protein was then analyzed for radioactivity and the protein concentration was determined by amino acid analysis.

Results

The general procedure for the identification of the modified residues involved degradation of the labeled enzyme with cyanogen bromide and analysis of the fragments containing the label.

Cleavage of Modified CPA_γ^{Leu} with Cyanogen Bromide. CPA_γ^{Leu} which had been modified by treatment with 2 mM L-[^{14}C]BAMP for 24 hr in the absence of competitive inhibitor contained an average of 1.7–1.9 labeled residues/molecule and was catalytically inactive. The fragments obtained by cyanogen bromide cleavage were separated on Sephadex G-75 as shown in Figure 1A. Significant amounts of radioactivity were associated with fragment F_I (residues 104–301), containing an average of 1.1 residues/molecule, and with the [F_N + F_C] pool. Fragment F_{III} contained only 0.04 residue of inhibitor/molecule following a second chromatography on Sephadex G-75.

The separation of F_N (residues 8–22) and F_C (residues 302–

[3] Amino acids are numbered from 1 to 307 where 1 is the amino-terminal residue of CPA_α (see Bradshaw *et al.*, 1969).

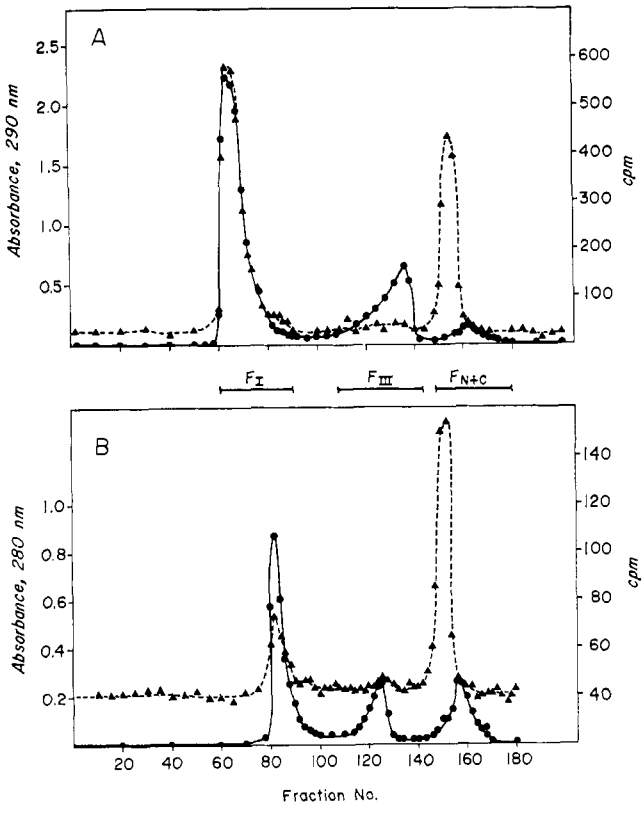


FIGURE 1: Elution pattern for the cyanogen bromide fragments of modified CPAγ^{Leu} on a 3 × 100 cm column of Sephadex G-75 developed with 0.1 M propionic acid at 36 ml/hr at 23°. Fractions of 3.6 ml were collected and their absorbance was determined at 280 or 290 nm (●) and radioactivity measured by counting 0.050-ml aliquots (▲). The fragments were derived from 150 mg of CPAγ^{Leu} modified in the absence of D-phenylalanine (A) and 70 mg of CPAγ^{Leu} modified in the presence of D-phenylalanine (B) as described in the text.

307) on Sephadex G-25 is shown in Figure 2. F_N contained an average of 0.7 residue/molecule, whereas F_C was devoid of label.

Incubation of CPA$_\gamma^{Leu}$ with L-[^{14}C]BAMP in the presence of 0.05 M D-phenylalanine resulted in the incorporation of an average of 0.96 residue of inhibitor/molecule with less than 10% loss of enzymatic activity. The elution profile of the cyanogen bromide fragments of this derivative is presented in Figure 1B. In this instance, relatively little radioactivity (<0.1 residue/molecule) was incorporated into F_I, while F_N was modified to approximately the same extent as the corresponding fragment obtained from enzyme labeled in the absence of D-phenylalanine. These data indicate that inactivation occurs by the alkylation of a residue in F_I and that modification of F_N has no effect on enzymatic activity.

Identification of Radioactive Amino Acid Derivatives. Amino acid analysis of acid hydrolysates of CPA$_\gamma^{Leu}$ labeled with L-[^{14}C]BAMP yielded three peaks of radioactivity. The first peak fraction, tentatively identified as N-CM-Asp (*vide infra*), eluted with cysteic acid in the breakthrough volume (14 ml); the second peak fraction eluted with glycolic acid (Takahashi *et al.*, 1967) at 25–26 ml using an accelerated system;[2] and the final one with 3-CM-His (88 ml).

The amounts of each radioactive product found in hydrolysates of CPA$_\gamma^{Leu}$ labeled with L-[^{14}C]BAMP in the presence and absence of competitive inhibitors are presented in Table

TABLE I: Distribution of Radioactive Amino Acid Derivatives.[a]

Sample	Residues Inhibitor/Molecule		
	N-CM-Asp	Glycolate	3-CM-His
Unprotected[b]	0.55	0.92	0.30
+D-Phenylalanine[c]	0.53	0.04	0.16
+β-Phenylpropionate[d]	0.26	0.06	0.08
F_I[e]	0.04	0.81	0.03
F_N[e]	0.42	0.02	0.20
OH^-[f]	0.46		0.12
CPA$_\beta^{Leu}$[g]		0.94	

[a] Determined as described in the text. [b] CPA$_\gamma^{Leu}$ incubated with 2 mM L-[^{14}C]BAMP for 24 hr at pH 7.5. [c] Same as in *b* except incubation mixture contained 0.05 M D-phenylalanine. [d] Same as in *b* except incubation mixture contained 0.05 M β-phenylpropionate. [e] Cyanogen bromide fragments of CPA$_\gamma^{Leu}$ labeled as in *b*. [f] CPA$_\gamma^{Leu}$ labeled as in *b* after incubation with 0.1 N NaOH at 0° for 1 hr. [g] CPA$_\beta^{Leu}$ prepared as in *b*.

I. CPA$_\gamma^{Leu}$ labeled in the presence and absence of D-phenylalanine contained *N*-CM-Asp (~0.5 residue/molecule) and 3-CM-His (~0.2 residue/molecule). These derivatives occur in the amino-terminal fragment F_N. The enzyme modified in the absence of D-phenylalanine yielded in addition approximately 1 residue/molecule of glycolic acid. This product is found in hydrolysates of F_I but not F_N (Table I).

Unlike D-phenylalanine, β-phenylpropionate protected both sites in F_I and in F_N against alkylation by L-BAMP. Since the ratio of N-CM-Asp to 3-CM-His was approximately the same for CPA$_\gamma^{Leu}$ modified with L-BAMP in the presence and absence of this inhibitor, it is likely that the binding of β-phenylpropionate at a single site prevents alkylation of the α-amino group of Asn_8 and of the imidazole group of His_{13}.

Thermolytic Digestion of F_I and Peptide Isolation. The thermolytic digest of fragment F_I of the L-[^{14}C]BAMP-labeled

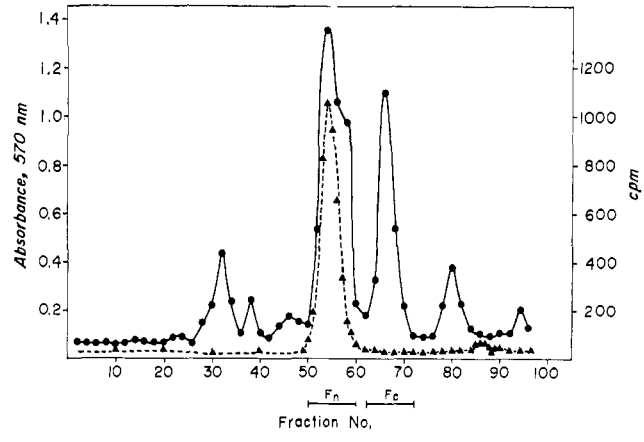


FIGURE 2: Elution profile of cyanogen bromide fragments F_N and F_C on a 1.4 × 80 cm column of Sephadex G-25 eluted at 23° with 50% acetic acid at 12 ml/hr. Fractions of 1.8 ml were collected. Protein was determined on aliquots by ninhydrin analyses after basic hydrolysis (●) and radioactivity measured on 0.025 ml of alternate tubes (▲). The sample was the [F_N + F_C] pool described in Figure 1A.

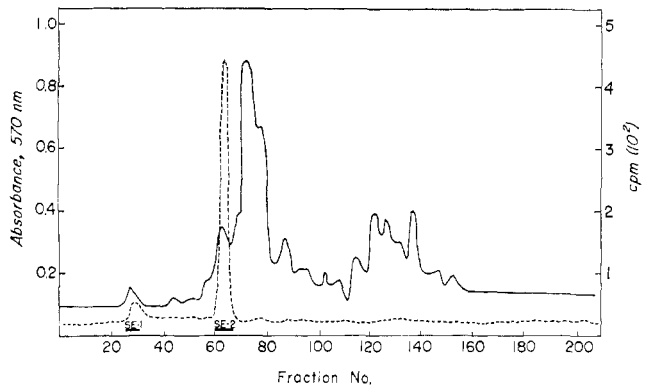


FIGURE 3: Elution pattern on a 1.5 × 80 cm column of SE-Sephadex C-25 at 35° of the thermolytic peptides of cyanogen bromide fragment F_I derived from CPA_γ^{Leu} labeled with L-[^{14}C]BAMP. Elution was carried out at 36 ml/hr using a double linear gradient of pyridine–acetic acid buffers as described in the text. The column was monitored by ninhydrin analysis after alkaline hydrolysis (———). Fractions of 3.3 ml were collected. Radioactivity (- - - -) was monitored by counting 0.05-ml aliquots.

enzyme was chromatographed on SE-Sephedex as shown in Figure 3. The two radioactive peaks, SE-1 and SE-2, were present in the ratio 1:15 based upon radioactivity, but greater amounts of SE-1 were found when thermolytic digestion of F_I was performed at pH 8.0 rather than pH 7.0. In all experiments SE-1 and SE-2 accounted for over 85% of the radioactivity applied to the column.

The radioactive component in SE-1 chromatographed as a broad peak (SE-1-DX) on Dowex 1-X2 (see Experimental Section). Amino acid analysis of this material revealed less than stoichiometric amounts of any amino acid relative to radioactivity. Attempts to purify SE-1 or SE-1-DX by paper electrophoresis resulted in a loss of radioactivity, presumably because the radioactive component was soluble in toluene, the medium used for electrophoresis. Based on these data, the radioactive component of SE-1 was tentatively identified as *N*-glycolyl-*N*-methyl-L-phenylalanine; this compound probably was the result of spontaneous hydrolysis of the ester formed during alkylation of Glu_{270} (*vide infra*).

The radioactive component in SE-2 chromatographed as a single peak (SE-2-DX-1) on Dowex 1-X2 (Figure 4). The

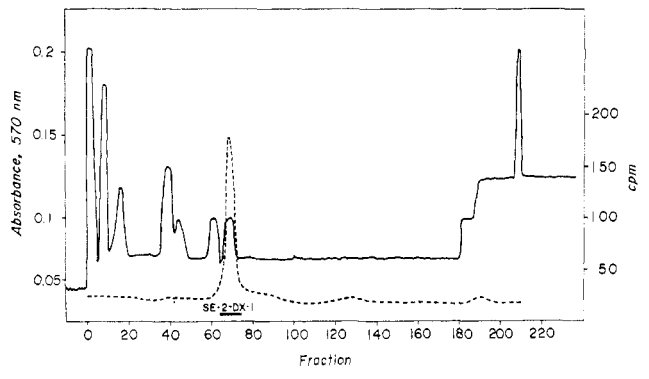
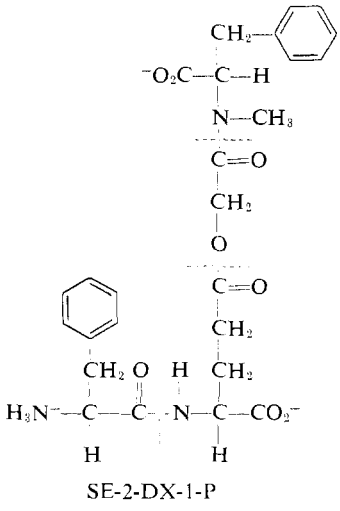


FIGURE 4: Elution profile of SE-2 chromatographed on a 0.9 × 15 cm column of Dowex 1-X2 at 37° using a linear gradient of pyridine–acetic acid buffers as described in the text. The column was developed at 30 ml/hr and fractions of 2 ml were collected. Ninhydrin analysis (———) after alkaline hydrolysis was performed and radioactivity (- - - -) monitored by counting 0.05-ml aliquots.

radioactivity in SE-2-DX-1 accounted for 95% of that applied to the column. This component was separated from contaminants by preparative paper electrophoresis at pH 6.5.

Amino acid analysis indicated that based on radioactivity this peptide contained glutamic acid (1.1) and phenylalanine (0.9). After one step of the Edman degradation the phenylalanine content was reduced to 0.05 residue/glutamic acid residue. In addition to these amino acids, 0.7 residue of glycolic acid was detected in the peptide by the spectrophotometric method of Takahashi *et al.* (1967). Since there is a single Phe-Glu sequence in CPA (Bradshaw *et al.*, 1969), modification of CPA_γ^{Leu} in the F_I region must have occurred by the alkylation of the γ-carboxyl of Glu_{270} forming an ester. Thermolysin cleaved the peptide bonds between Thr_{268} and Phe_{269} and between Glu_{270} and Leu_{271} to give the modified peptide SE-2-DX-1-P. Subsequent acid hydrolysis (- - - -)



SE-2-DX-1-P

released phenylalanine, glutamic acid, glycolic acid, and *N*-methylphenylalanine. The latter was difficult to quantitate because of its low color value with ninhydrin.

Substitutions in F_N. Radioactive derivatives obtained after acid hydrolysis of labeled F_N eluted with the breakthrough volume and with 3-CM-His during amino acid analysis (Table I). Chromatography in pyridine–butanol–acetic acid–H_2O (10:15:3:12, v/v) and electrophoresis at pH 2.1, 3.75, and 6.5 all indicated the presence of two radioactive components. Since one of these (the less radioactive one) migrated with 3-CM-His in all systems tested and since F_N contained only one histidine, His_{18}, this residue must have been one of the sites of partial modification.

The radioactive product which resulted from acid hydrolysis of F_N modified with L-BAMP at the α-amino group of the amino terminal asparagine was *N*-CM-aspartic acid. This compound was eluted with the breakthrough volume during amino acid analysis and migrated with the major radioactive component of acid hydrolysates of F_N. Further evidence that the α-amino group of CPA_γ^{Leu} is the major site of modification in the F_N region is the low value of aspartic acid during amino acid analysis (Table II). A reduced aspartic acid value would result from modification of the α-amino group of Asn_8 but not from alkylation of the β-carboxylate groups of Asp_{16} or Asp_{20}. F_N which is modified at the α-amino group might be expected to be resistant to Edman degradation and, in fact, relatively little change in specific activity was observed after two runs of the degradation (Table II). Aspartic acid was reduced from 2.3 to 2.0 residues in turn 1 and tyrosine from

TABLE II: Edman Degradation of F_N Labeled with L-[^{14}C]-BAMP.[a]

Amino Acid[b]	Composition	Step 1	Step 2
Aspartic acid	2.30 (3)[c]	**2.01**	2.02
Threonine	1.71 (2)	1.78	1.82
Glutamic acid	0.92 (1)	0.96	1.04
Alanine	1.00 (1)	1.00	1.00
3-CM-Histidine	0.18	0.16	0.17
Isoleucine	0.95 (1)	0.93	0.94
Leucine	1.04 (1)	0.96	0.96
Tyrosine	2.27 (3)[c]	2.47	**1.69**
Phenylalanine	0.98 (1)	1.03	1.01
Histidine	0.65 (1)	0.61	0.66
Homoserine and lactone	0.58 (1)	0.48	0.46
^{14}C	0.65	0.62	0.61

[a] Values are expressed in terms of molar ratios relative to alanine. Theoretical values are in parentheses and boldface entries indicate amino acids removed at each step. [b] All other amino acids were present at less than 0.2 residue/alanine. [c] The observed values for aspartic acid and tyrosine in F_N from unmodified CPA$_\gamma^{Leu}$ are 2.8 and 2.6 residues, respectively (Pétra and Neurath, 1969).

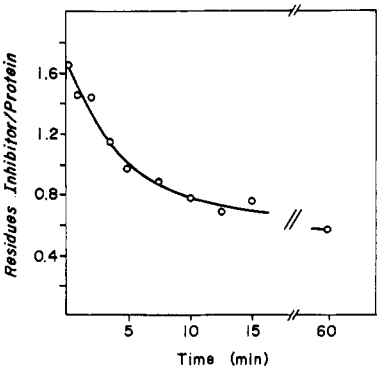


FIGURE 5: The stability of incorporated L-[^{14}C]BAMP in 0.1 N NaOH at 0°. CPAγ^{Leu} which had been modified with L-[^{14}C]BAMP was incubated with 0.1 N NaOH at 0°. At the times noted, aliquots were desalted and the specific radioactivity of the protein peak was determined. The protein concentration was determined by amino acid analysis.

2.5 to 1.7 in turn 2. Thus approximately 50% of the α-amino groups of F_N were modified.

Carboxypeptidase A_β contains an additional five amino acid residues at the amino terminus not present in the γ form. When CPA$_\beta^{Leu}$ was incubated with 2 mM L-[^{14}C]BAMP, one residue of inhibitor was rapidly incorporated and no further incorporation occurred during 24 hr. The only radioactive derivative (Table I) found after acid hydrolysis was glycolic acid, suggesting that in contrast to CPA$_\gamma$, neither modification of the α-amino group nor of the imidazole group of His_{13} occurred.

Stability of Incorporated Label to Base. When CPA$_\gamma^{Leu}$ containing an average of 1.7 residues of L-[^{14}C]BAMP/molecule was incubated at 0° in 0.1 N NaOH, approximately 1 residue of inhibitor/molecule was maximally released within 15 min (Figure 5). No enzymatic activity was regained under these conditions.

A portion of the sample exposed to 0.1 N NaOH for 1 hr was subjected to acid hydrolysis and analyzed for radioactive amino acids. Although the amounts of *N*-CM-L-aspartic acid and 3-CM-L-histidine were unchanged, glycolic acid was completely lost (Table I).

Discussion

Modification of Glu_{270}. Comparison of the elution profiles of the cyanogen bromide fragments of CPA$_\gamma^{Leu}$ labeled with L-[^{14}C]BAMP in the presence and absence of D-phenylalanine demonstrates that inactivation results from alkylation of a residue in fragment F_1. The isolation of a single radioactive peptide containing after acid hydrolysis phenylalanine, glutamic acid, glycolic acid, and *N*-methyl-L-phenylalanine identifies the critical site of modification as Glu_{270}. The rapid removal of 1 residue of inhibitor/molecule upon treatment of the labeled enzyme with base is consistent with the formation of an ester during the modification of CPA$_\gamma^{Leu}$ with L-BAMP.

Carboxylates are relatively poor nucleophiles and the reaction of L-BAMP with CPA is one of the relatively few examples of modification of these groups by α-halo acids or α-halo acid amides (for a review, see Wilcox, 1971). A similar type of reactivity of a carboxylate group of the active site with halo acids has been reported for ribonuclease T_1 (Takahashi *et al.*, 1967). If it is assumed that L-BAMP and glycyl-L-tyrosine (Lipscomb *et al.*, 1968) bind similarly to CPA, the high reactivity can be in part explained by the proximity of Glu_{270} and the bound reagent. However, the apparent first-order rate constant for the model reaction of bromoacetate with acetate estimated from the observed second-order rate constant of 5×10^{-5} min^{-1} l. mole^{-1} (Smith, 1943) employing the proximity corrections as described by Storm and Koshland (1970) is 2.7×10^{-3} min^{-1}, as compared to 0.2 min^{-1} for the apparent first-order rate constant for inactivation of CPA$_\gamma^{Leu}$ by L-BAMP (Hass and Neurath, 1971). The considerably higher rate of enzyme inactivation may indicate that CPA catalyzes reaction between Glu_{270} and L-BAMP at the active center.

The basic form of a group with a p$K_{a,EI}$ of 7.0 participates in the inactivation of CPA$_\gamma^{Leu}$ by L-BAMP (Hass and Neurath, 1971). Since the ascending limbs of pH profiles for the CPA-catalyzed hydrolysis of CGP (Neurath and Schwert, 1950), of *N*-acyl tripeptides (Auld and Vallee, 1970), and of *O*-acetylmandelate (Carson and Kaiser, 1966) also exhibit inflections at approximately pH 7.0, it is likely that the same group participates in enzymatic catalysis and in the inactivation of CPA by L-BAMP. Although it seems reasonable to identify this group as Glu_{270} as proposed originally by Lipscomb *et al.* (1968), the possibility that Glu_{270} has a "normal" p$K_{a,EI}$ (~4.5) and that the basic form of another group (p$K_{a,EI}$ = 7.0) is required for the enzymatic catalysis of either hydrolysis of substrates or inactivation by L-BAMP has not been eliminated. However, the following evidence strongly supports the view that this residue is Glu_{270}: (1) the modification by L-BAMP of Glu_{270} would require the basic form of this nucleophile; the observed effect of pH on the rate of inactivation is consistent with a p$K_{a,EI}$ of 7.0 for Glu_{270}; (2) the rate of modification by Glu_{270} by Woodward's reagent K (Pétra and Neurath, 1971), a reaction also requiring an ionized carboxylate, increases with pH and also exhibits an inflection at pH 7.0; and (3) the rate of inactivation of a critical carboxyl (presumably Glu_{270}) by 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-

imide metho-*p*-toluenesulfonate (Riordan and Hayashida, 1970) decreases with pH with an inflection at pH 7. Unlike α-halo acid amides (*e.g.*, L-BAMP) and isoxazolium salts (*e.g.*, Woodward's reagent K), carbodiimides react with the protonated form of carboxylic acids.

Modification of F_N. In addition to the alkylation of Glu_{270} which results in inactivation, L-BAMP modifies the α-amino group of Asn_8 and the imidazole group of His_{13} without affecting the enzymatic activity of CPA. Although Korman and Clarke (1956) demonstrated that α-amino groups of peptides and amino acids may be alkylated by bromoacetate, this is to our knowledge the first reported evidence of this type of reaction occurring in proteins.

Several experimental observations suggest that there is a hydrophobic binding site for L-BAMP in the amino-terminal region of CPA_γ^{Leu} and that the bound inhibitor reacts with either the α-amino group or with His_{13}. Evidence for the existence of this binding site includes: (1) unique reactivity of these two residues toward L-BAMP in CPA_γ^{Leu}; (2) parallel protection of modification of these residues by β-phenylpropionate; and (3) reaction of the α-amino group of CPA_γ with 2-hydroxy-5-nitrobenzyl bromide (Radhakrishnan *et al.*, 1970).

The reactivity of the α-amino group and of His_{13} of CPA_γ^{Leu} toward L-BAMP must be ascribed to a unique conformation in the amino-terminal region not present in CPA_β^{Leu} which does not react at the corresponding sites. The particular reactivity of these two groups toward L-BAMP does not result from such intrinsic properties as abnormal pK_a values or accessibility to solvent since the reaction of these residues with 0.2 M bromoacetate is not observed upon incubation at pH 7.0 for 3 days.[4]

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[4] P. H. Pétra, personal communication.