Modification of Bovine Carboxypeptidase B with *N*-Bromoacetyl-*N*-methyl-L-phenylalanine†

G. Michael Hass, Mary A. Govier, Donald T. Grahn, and Hans Neurath*

ABSTRACT: Bovine carboxypeptidase B is irreversibly inactivated by *N*-bromoacetyl-*N*-methyl-L-phenylalanine (L-BAMP). The reaction is first-order with respect to active enzyme remaining and is accompanied by a parallel loss of esterase and peptidase activities. There is no evidence for the initial formation of a reversible enzyme–reagent complex since the pseudo-first-order rate constant of inactivation is proportional to reagent concentration (0–20 mM). The competitive inhibitor ε-aminocaproate protects against inactivation by L-BAMP. Digestion with thermolysin of the enzyme labeled with radioactive (^{14}C) reagent yields a single radioactive dipeptide apparently identical with that obtained from similarly labeled and digested bovine carboxypeptidase A (Hass, G. M., and Neurath, H. (1971), *Biochemistry 10*, 3541). The modified residue is believed to correspond to Glu_{270} of the active site (numbering of the sequence of carboxypeptidase A).

Bovine carboxypeptidases A (EC 3.4.2.1) and B (EC 3.4.-2.2) are pancreatic exopeptidases. Carboxypetidase A preferentially hydrolyzes terminal peptide bonds in which the amino donor is a hydrophobic amino acid whereas the specificity of carboxypeptidase B is analogously directed toward basic amino acids. Much similarity has been found between the amino acid sequence of carboxypeptidase A and partial sequences of carboxypeptidase B, suggesting that they are of common evolutionary origin (Reeck *et al.*, 1971). The two enzymes respond similarly to the removal of zinc by chelating agents (Wintersberger *et al.*, 1965) and to modification of a tyrosine residue (Plummer, 1969) or a carboxylate group (Plummer, 1971). Since the participation of these types of groups in the catalytic mechanism of carboxypeptidase A is well documented (Vallee and Riordan, 1969), the evolutionary divergence of these proteins appears manifest primarily as alteration of binding site structures.

The reagent L-BAMP[1] was originally designed as an affinity label of carboxypeptidase A and was found to inactivate the enzyme by modification of Glu_{270} of the active site. Since bovine carboxypeptidase B also hydrolyzes substrates of carboxypeptidase A, it was expected that the same reagent could be of use in the investigation of carboxypeptidase B and other metalloexopeptidases. This report describes the modification of bovine carboxypeptidase B with L-BAMP and the identification of the principal site of alkylation.

Experimental Procedure

Materials

Bovine carboxypeptidase B (fraction II) was prepared from activated pancreatic juice as described by Reeck *et al.* (1971). The specific activity of enzyme preparations in terms of first-order rate constants was 2.7×10^3 min^{-1} when assayed with hippuryl-L-arginine as described below.

N-Bromoacetyl-*N*-methyl-L-phenylalanine and *N*-([1-^{14}C]-bromoacetyl)-*N*-methyl-L-phenylalanine (specific activity 16.6×10^{10} dpm/mole) were prepared and characterized as described earlier (Hass and Neurath, 1971a).

Thermolysin was purchased from Daiwa Kasei; sodium hippuryl-DL-phenyllactate and hippuryl-L-arginine·$2H_2O$ were from Fox Chemical Co.; Sephadex G-25 and SE-Sephadex C-25 from Pharmacia Fine Chemicals; Dowex 1-X2 from Bio-Rad; and Triton X-100, 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene were from Packard.

Methods

Analytical Procedures. Optical density measurements, spectrophotometric assays, radioactivity measurements, amino acid analyses, and identification and quantitation of radioactive amino acids were performed as described previously (Hass and Neurath, 1971a,b).

Glycolic acid was determined by the colorimetric method of Takahashi *et al.* (1967).

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

Enzyme Assays. Carboxypeptidase B activity was monitored by the increase in absorbance at 254 nm observed during hydrolysis of the peptide substrate hippuryl-L-arginine. A suitable aliquot of enzyme was added to 3 ml of a 1 mM solution of substrate in 0.1 M NaCl–0.025 M Tris-HCl (pH 7.5) (Wintersberger *et al.*, 1962).

Esterase activity was determined at 25° using an automatic titrator to monitor the release of protons during the hydrolysis of hippuryl-DL-phenyllactate. The assay solution contained 10 mM substrate, 45 mM NaCl, and 5 mM Veronal (pH 7.5) (Wintersberger *et al.*, 1962).

Rates of hydrolysis were converted to specific activities using the protein concentration as determined either by absorbance at 280 nm, assuming $A_{280}^{0.1\%} = 2.1$ (Cox *et al.*, 1962), or by amino acid analysis, assuming 22 alanine residues/molecule (Cox *et al.*, 1962).

Kinetics of Inactivation and Incorporation of Radioactivity. The rate of inactivation of carboxypeptidase B by L-BAMP and the extent of modification of the enzyme as a function of per cent activity remaining were measured as described earlier (Hass and Neurath, 1971a).

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received May 15, 1972.* This work has been supported by research grants from the National Institutes of Health (GM 15731) and the American Cancer Society (NP-18N).

[1] Abbreviations used are: L-BAMP, *N*-bromoacetyl-*N*-methyl-L-phenylalanine; AC, ε-aminocaproate; BrAc-D-Arg, *N*-bromoacetyl-D-arginine; CPA, carboxypeptidase A; CPB, carboxypeptidase B.

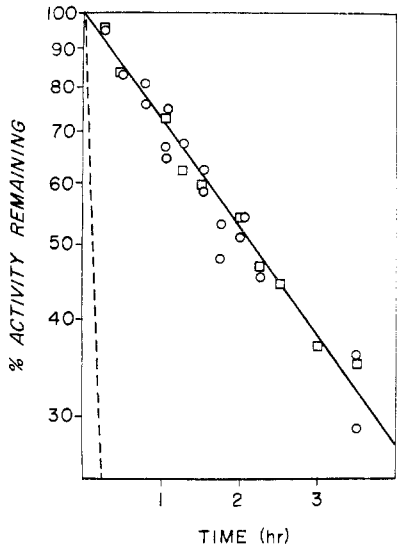


FIGURE 1: Rate of inactivation of carboxypeptidase B by L-BAMP at pH 7.5, 25°. Carboxypeptidase B (~0.2 mg/ml) was treated with 10 mM L-BAMP in 1 N NaCl–0.1 M Tris-acetate. The esterase (○) and peptidase (□) activities were monitored as a function of time. The broken line represents the rate of inactivation of CPAγ^{Leu} under these conditions (Hass and Neurath, 1971a).

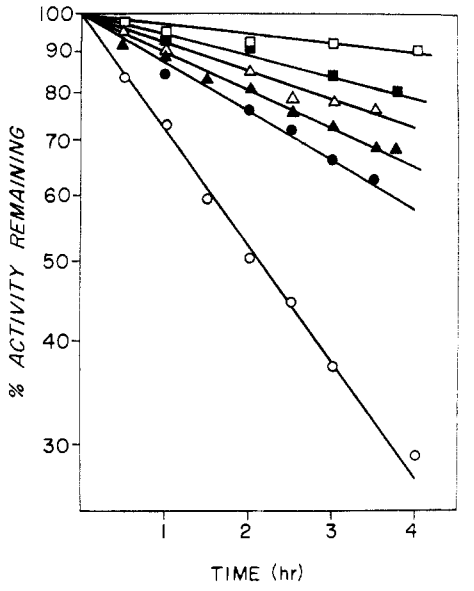


FIGURE 2: Inactivation of carboxypeptidase B by 10 mM L-BAMP in the presence of various concentrations of ε-aminocaproate. Incubation mixtures contained carboxypeptidase B (~0.2 mg/ml), L-BAMP (10 mM), NaCl (1 N), and Tris-acetate (0.1 M) (pH 7.5), and the following concentrations of ε-aminocaproate: none (○), 2.5×10^{-4} M (●), 5.0×10^{-4} M (▲), 7.5×10^{-4} M (△), 1×10^{-3} M (■), and 2.5×10^{-3} M (□). The peptidase activities were monitored as a function of time.

Isolation of Radioactive Peptide Derivative. For the preparation of carboxypeptidase B labeled with radioactive L-BAMP for peptide analysis the same reaction conditions were employed except that protein concentrations were 3.5–7.0 mg/ml. The final concentration of L-[^{14}C]BAMP was 5 mM. When inactivation reached 80%, the solution was dialyzed at 4° against 0.45 M KCl–0.05 M Tris-HCl (pH 7.5), and then against several changes of water. The solution was lyophilized and redissolved in 5% formic acid. Aliquots were removed for the estimation of protein concentration and radioactivity and the solution was then diluted with 10 volumes of distilled water and again lyophilized.

Carboxypeptidase B labeled with L-[^{14}C]BAMP was digested with thermolysin (Hass and Neurath, 1971b). The lyophilized peptides were dissolved in 4 ml of 0.05 M pyridine–acetic acid (pH 2.4) and fractionated on a 1.5 × 80 cm column of SE-Sephadex C-25 as described earlier (Hass and Neurath, 1971b).

The single radioactive pool, SE-1, obtained by chromatography on SE-Sephadex was further purified by chromatography at 37° on a 0.9 × 15 cm column of Dowex 1-X2 at a flow rate of 19 ml/hr. The peptides were eluted with a continuous gradient of 100 ml each of 3% pyridine, 0.5 M pyridine–acetic acid (pH 6.0), 1.0 M pyridine–acetic acid (pH 6.0), and 2.0 M pyridine–acetic acid (pH 5.0) (Bradshaw *et al.*, 1969). The fraction volume was 2 ml.

The elution of peptides was monitored by automated ninhydrin analysis after alkaline hydrolysis (Hill and Delaney, 1967). Radioactive peptide derivatives were detected by counting 0.1-ml aliquots from alternate fractions.

The single radioactive pool (SE-1-DX-1), eluted from Dowex 1-X2, was purified to homogeneity by preparative paper electrophoresis at pH 6.5 (Hass and Neurath, 1971b). A control was also eluted and analyzed to correct for impurities in the paper.

Peptide purification was also monitored at each step by electrophoresis of aliquots at pH 6.5 and 3.75 for 1.25 hr. The dried chromatograms were stained with ninhydrin and cut into 0.5-in. sections. Radioactive peptides were located by counting the sections in 10 ml of scintillation fluid.

Stability of Incorporated Radioactivity. Carboxypeptidase B containing approximately 5% residual activity after extended incubation with L-[^{14}C]BAMP was dialyzed exhaustively at 4° against distilled water and lyophilized. Aliquots of 0.5 mg were dissolved in 1 ml of the following buffer solutions and incubated at 25° for 24 hr: (1) 0.1 M NaCl–0.2 M Tris-HCl (pH 7.0), (2) 0.1 M NaCl–0.2 M Tris-HCl (pH 9.0), (3) 2.0 M NH_2OH–0.2 M Tris-HCl (pH 7.0), and (4) 2.0 M NH_2OH–0.2 M Tris-HCl (pH 9.0). The protein was separated from small molecules on a 0.6 × 20 cm column of Sephadex G-25 equilibrated with 0.45 M KCl–0.05 M Tris-HCl (pH 7.5). The fractions containing protein were pooled and the protein concentration, enzymatic activity, and radioactivity were determined.

Results

Incubation of carboxypeptidase B with 10 mM L-BAMP produces a slow inactivation which is first-order with respect to remaining active enzyme (Figure 1). A parallel loss of peptidase and esterase activity is observed.

The relationship between k, the pseudo-first-order rate constant for inactivation of carboxypeptidase B, and the concentration of L-BAMP (2.5–20 mM) is linear. The slope of this line (0.6 l. mole^{-1} min^{-1}) is the apparent second-order rate constant for the reaction of carboxypeptidase B and L-BAMP.

Evidence for inactivation by modification of an active-site residue is the protection afforded by the competitive inhibitor ε-aminocaproate. The effect of various concentrations of ε-aminocaproate (AC) on the inactivation of carboxypeptidase B (CPB) by 10 mM L-BAMP is shown in Figure 2. Scrutton and Utter (1965) have derived an equation (eq 4) to describe a system such as that represented by eq 1–3. It is assumed

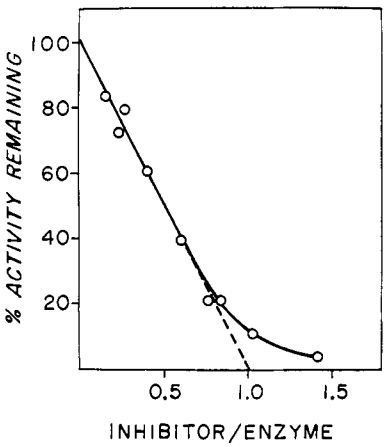


FIGURE 3: Incorporation of L-[^{14}C]BAMP as a function of peptidase activity remaining. Procedures for the preparation and analysis of samples are described in the Experimental Section. The solid line represents incorporation of reagent during the entire inactivation process and the broken line is an extrapolation of the extent of modification in the range 0–80% inactivation to zero activity.

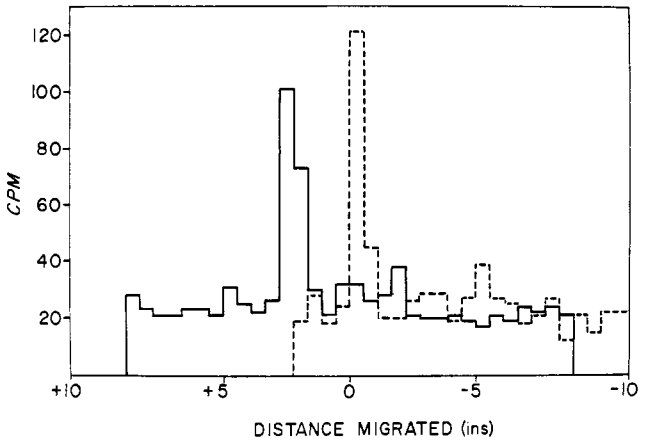


FIGURE 4: Radioactive derivatives observed after electrophoresis of a thermolytic digest of carboxypeptidase B which had been inactivated 80% with L-[^{14}C]BAMP. Electrophoresis was performed at pH 3.75 (- - - -) and 6.5 (———) for 1 hr at 2 kV. Radioactivity was determined as described in the Experimental Procedure section.

that the concentrations of L-BAMP and ε-aminocaproate are considerably greater than that of carboxypeptidase B and that the rate of reversible dissociation of the ε-aminocaproate–carboxypeptidase B complex is greater than that of inactivation.

$$\text{CPB} + \text{L-BAMP} \xrightarrow{k_1} \text{E}_{\text{inact}} \quad (1)$$

$$\text{CPB–AC} + \text{L-BAMP} \xrightarrow{k_2} \text{E}_{\text{inact}} \quad (2)$$

$$\text{CPB} + \text{AC} \underset{}{\overset{K_i}{\rightleftharpoons}} \text{CPB–AC} \quad (3)$$

$$\frac{V_i}{V_0} = \frac{k_2}{k_1} + K_i\left(\frac{1 - \frac{V_i}{V_0}}{[\text{AC}]}\right) \quad (4)$$

In eq 4 V_0 and V_i are the pseudo-first-order rate constants for inactivation in the absence and presence of ε-aminocaproate, respectively, and K_i is the dissociation constant of the carboxypeptidase B–ε-aminocaproate complex.

A plot of the data of Figure 2 according to this equation indicates that the relationship between V_i/V_0 and $[1 - (V_i/V_0)]/[\text{AC}]$ is linear. The ordinate intercept is approximately zero, indicating that the reaction represented by eq 2 does not contribute significantly to the inactivation process. The value for K_i (2.3×10^{-4} M), calculated from the slope of this plot, is somewhat less than that of 1×10^{-3} M determined earlier for porcine carboxypeptidase B (Wolff *et al.*, 1962).

The extent of modification of carboxypeptidase B by L-BAMP as a function of per cent activity remaining is presented in Figure 3. The enzyme is stoichiometrically inactivated up to approximately 80% with incorporation of more than 1 mole of reagent/mole of enzyme as inactivation proceeds. Extrapolation of the linear portion of this plot to zero activity demonstrates that inactivation results from the incorporation of a single residue of L-BAMP.

Further evidence for the specificity of modification is the identification of radioactive amino acid derivatives after acid hydrolysis of carboxypeptidase B which had been inactivated 80% with L-[^{14}C]BAMP. Three radioactive peaks are eluted from the 50-cm column of the amino acid analyzer. The first peak emerges with the breakthrough (24 ml) and accounts for 0.03 residue/molecule; the second peak elutes together with glycolic acid (36 ml) and represents 0.79 residue/molecule; and the third peak coincides with ε-carboxymethyllysine (0.06 residue/molecule). It is concluded from these data that radioactive glycolic acid results from the hydrolysis of the alkylated residue which is critical for activity.

Trypsin, chymotrypsin, and pepsin were employed unsuccessfully in initial attempts to identify the critical site of modification. Digestion of carboxypeptidase B with trypsin proceeds at an unacceptably slow rate (Elzinga and Hirs, 1968) and treatment with pepsin or chymotrypsin apparently results in loss of the label as judged by the absence of radioactive peptide derivatives during paper electrophoresis at pH 2.1, 3.75, and 6.5.

Thermolysin produces satisfactory digestion patterns provided that the modified carboxypeptidase B is first denatured by brief exposure to 5% formic acid. The initial digestion solubilizes about 80% of the radioactivity and the remainder becomes soluble during further digestion of the core. Electrophoresis at pH 6.5 and 3.75 of the thermolytic peptides (Figure 4) reveals one major radioactive zone, suggesting that a single site is modified by L-BAMP and that digestion with thermolysin produces a homogeneous radioactive peptide.

The first step in the purification of the labeled thermolytic peptide was chromatography on SE-Sephadex C-25 using a double linear gradient of pyridine–acetic acid buffers (Figure 5). Over 90% of the radioactivity applied to the column was found in pool SE-1 (fractions 57–62) which eluted immediately preceding the neutral peptides.

Chromatography of pool SE-1 on a column of Dowex 1-X2 (Figure 6) yielded a single radioactive zone, SE-1-DX-1, which accounted for approximately 95% of the radioactivity applied.

Final purification of the radioactive peptide was achieved by preparative paper electrophoresis at pH 6.5. This step was necessary despite the relatively low yields (30–50%) since amino acid analysis and paper electrophoresis indicated that pool SE-1-DX-1 was heterogeneous. The purified peptide, SE-1-DX-1-P, contained 0.98 ± 0.1 residue of glutamic acid and 0.90 ± 0.02 residue of phenylalanine (based on radioactivity present). In addition, an average of 0.7 residue of

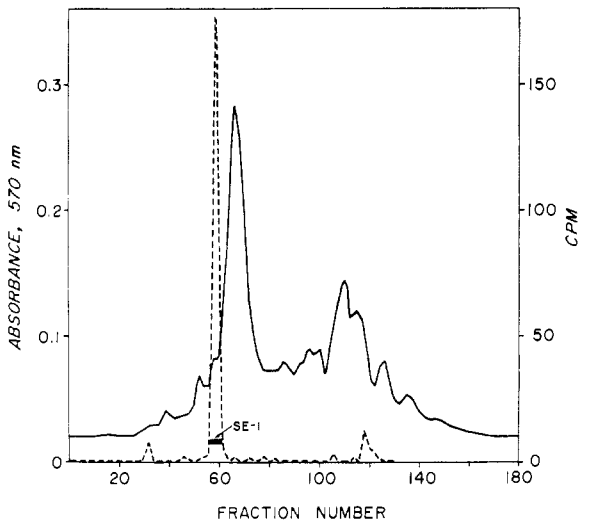


FIGURE 5: Chromatography on a 1.5 × 80 cm column of SE-Sephadex C-25 at 55° of the thermolytic peptides derived from carboxypeptidase B which had been 80% inactivated with L-[^{14}C]BAMP. Peptides were eluted at 36 ml/hr using a double linear gradient of pyridine–acetic acid buffers. Fractions of 3.3 ml were collected. The column was monitored by automatic ninhydrin analysis after alkaline hydrolysis (———) of 8% of the eluate. Radioactivity (– – – –) was determined by counting 0.1-ml aliquots of alternate tubes.

glycolic acid (presumably resulting from the acid hydrolysis of the modified dipeptide as shown below) was found using the colorimetric method of Takahashi *et al.* (1967). After a single turn of Edman degradation, phenylalanine was found at only 15% of the amount of glutamic acid.

These data suggest that inactivation of carboxypeptidase B by L-BAMP results from the specific alkylation of the γ-carboxylate of a glutamic acid residue occurring in the sequence -X-Phe-Glu-Y-, where Y is a hydrophobic amino acid for which thermolysin exhibits specificity (*i.e.*, Phe, Leu, Tyr) (Matsubara *et al.*, 1966).

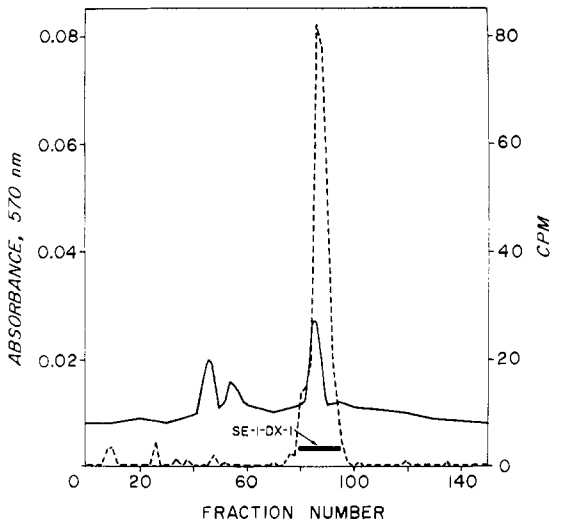


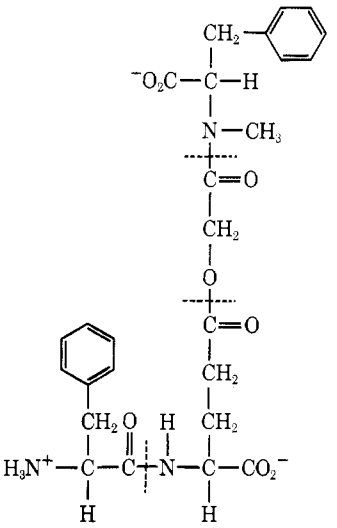
FIGURE 6: Chromatography of SE-1 of a 0.9 × 15 cm column of Dowex 1-X2 using the continuous gradient of pyridine–acetic acid buffers described in the text. The flow rate was 19 ml/hr and the fraction size 2.0 ml. The column was monitored by automatic ninhydrin analysis after alkaline hydrolysis (———) of 17% of the eluate. Radioactivity (– – – –) was determined by counting 0.1-ml aliquots of alternate fractions.

TABLE I: Release of Label from Carboxypeptidase B Inactivated with L-[^{14}C]BAMP.

Treatment	L-BAMP[a] Residues/ Molecule	L-BAMP[a] Reactivation (%)	BrAc-D-arginine[b] Residues/ Molecule	BrAc-D-arginine[b] Reactivation (%)
None	1.5 ± 0.1	0	1.09	0
pH 7.0	1.5 ± 0.1	0	1.05	2
pH 7.0, 2 M NH_2OH	1.3 ± 0.1	0	0.98	5.7
pH 9.0	1.4 ± 0.1	0	1.05	2.5
pH 9.0, 2 M NH_2OH	1.2 ± 0.1	0	0.09	26.2

[a] Values determined as described in the text. [b] From Plummer (1971).

The structure of the modified dipeptide, SE-1-DX-1-P (*vide infra*), is presumably identical with that isolated from thermolysin digests of carboxypeptidase A which had been modified with L-BAMP (Hass and Neurath, 1971b).



Finally, carboxypeptidase B which had been inactivated >95% by L-[^{14}C]BAMP was treated under conditions which might be expected to cleave ester bonds (Table I). Incubation for 24 hr at 25° at pH 7.0 and 9.0 produced little if any loss of label. Hydroxylamine (2 M) also had little effect, resulting in an average loss of only 0.2 and 0.3 residue per molecule at pH 7.0 and 9.0, respectively. These data are in contrast to the relatively rapid release ($\tau_{1/2} \sim 7$ hr) of glycolyl-D-arginine by 2 M hydroxylamine (pH 9.0) from carboxypeptidase B which had been modified with bromoacetyl-D-arginine (Plummer, 1971), suggesting that L-BAMP and bromoacetyl-D-arginine modify different residues.

Discussion

The affinity labeling of bovine carboxypeptidase A by the reagent L-BAMP was recently described (Hass and Neurath, 1971a,b). The present report of the modification of carboxy-

peptidase B by L-BAMP extends the use of this reagent as an affinity label and provides an additional means of comparing the active sites of carboxypeptidases A and B.

The most notable difference in the modification of these enzymes is that carboxypeptidase B is inactivated at a much slower rate at pH 7.5 than is carboxypeptidase A. A partial explanation, at least, for this slower rate is the lower affinity of L-BAMP for carboxypeptidase B. In the case of carboxypeptidase A (Hass and Neurath, 1971a) the pseudo-first-order rate constant for inactivation by L-BAMP is a hyperbolic function of the reagent concentration, suggesting the formation of a reversible L-BAMP–carboxypeptidase A complex prior to inactivation (eq 5). The dissociation constant

$$\text{CPA} + \text{L-BAMP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{CPA–L-BAMP} \xrightarrow{k_2} \text{CPA}_{\text{inact}} \quad (5)$$

for this carboxypeptidase A–L-BAMP complex is approximately 5 mM at pH 7.5 (Hass and Neurath, 1971a). In contrast, no evidence for saturation of carboxypeptidase B with L-BAMP is observed up to 20 mM reagent. This absence of a saturation effect raises the question as to whether L-BAMP behaves as an affinity label for carboxypeptidase B. The high degree of specificity exhibited by L-BAMP for the glutamic acid residue at the active site of carboxypeptidase B could result from (1) an enhanced reactivity of this residue toward alkylating agents in general, or (2) specific binding of L-BAMP at the active site. The following considerations strongly mitigate against the first possibility. (1) The observed second-order rate constant (0.6 l. mole^{-1} min^{-1}) for the reaction of L-BAMP with the γ-carboxylate of Glu_{270} of the active site is considerably greater than those ($\sim 5 \times 10^{-5}$ l. mole^{-1} min^{-1}) observed for model reactions of carboxylates and alkylating agents (Smith, 1943). (2) Iodoacetate, an alkylating agent of similar chemical reactivity as L-BAMP, does not inactivate carboxypeptidase B (Wintersberger *et al.*, 1962). Thus the most likely explanation for the specificity of the modification of carboxypeptidase B is that L-BAMP exhibits preferential (although weak) binding at the active site of the enzyme.

The stronger binding of L-BAMP to carboxypeptidase A than to carboxypeptidase B is consistent with the substrate specificities of the enzyme since carboxypeptidase A preferentially hydrolyzes substrates with hydrophobic amino acids in the carboxyl position while carboxypeptidase B exhibits greater (but not exclusive) specificity for basic amino acids. Unfortunately the apparent first-order rate constants (k_2) for inactivation observed at saturating levels of L-BAMP cannot be compared due to the weak binding of L-BAMP to carboxypeptidase B.

Although inactivation of carboxypeptidase B by L-BAMP occurs at a slower rate than that of carboxypeptidase A, several similarities are found in the kinetic properties of the inactivation processes. These include the following. (1) Inactivation is first-order with respect to active enzyme. (2) A parallel loss of esterase and peptidase activities is observed. (3) The observed rate of inactivation is enhanced relative to that observed with a compound of similar chemical reactivity lacking active-site direction (iodoacetate) (Wintersberger *et al.*, 1965). (4) Competitive inhibitors afford protection against inactivation by L-BAMP.

The most obvious similarity between the modification of carboxypeptidases A and B is that in both instances the γ-carboxylate of a critical glutamic acid residue is alkylated and that the preceding amino acid in the sequence is phenylalanine. Since there is a single Phe-Glu sequence in carboxypeptidase A, this information was sufficient to identify the critical site of modification as Glu_{270} (Hass and Neurath, 1971b). The amino acid sequence of carboxypeptidase B has not been completed and there may be more than one Phe-Glu sequence; therefore, substantial effort was directed toward the isolation of longer radioactive peptide derivatives using peptic and chymotryptic digests. Treatment with these enzymes was unsuccessful, however, presumably due to loss of label (*vide supra*). A possible mechanism consistent with the specificity of pepsin and chymotrypsin would be enzymatic cleavage of the Phe–Glu bond followed by spontaneous cyclization of the γ ester of glutamate to give pyroglutamate with loss of label.

Although additional investigations may be required to locate the precise site of alkylation, the identification of a critical glutamic acid residue at the active site of carboxypeptidase B provides additional evidence that carboxypeptidases A and B are not only homologous but also promote catalysis by analogous mechanisms. Significant differences between the enzymes may only occur at the binding site whereas the catalytic groups tyrosine, zinc, and glutamic acid are identical.

Finally, it is of importance to note that Plummer (1971) has identified a carboxylate group at the active site of carboxypeptidase B by modification with bromoacetyl-D-arginine. He concluded that the site of modification by this reagent may be at the binding rather than the catalytic site since treatment with 2 M hydroxylamine at pH 9.0 produced a rapid loss of label with significant ($\sim$30%) recovery of activity. This tentative conclusion is in agreement with our observation that hydroxylamine is without effect on carboxypeptidase B modified at the catalytic site with L-BAMP.

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Subunit Interactions in Aspartate Transcarbamylase†

Robert A. Cook

ABSTRACT: Binding studies of cytidine triphosphate (CTP) to native *Escherichia coli* asparate transcarbamylase reveal both positive and negative cooperative-like behavior. Equilibrium dialysis studies at 4 and 23° are consistent with six binding sites for CTP on the transcarbamylase. Complete saturation of the CTP binding sites is not attained at 23°. Complex Hill plots are obtained with at least two interaction coefficients values (η_H), with one break in the Hill plot occurring at approximately 35% saturation. The results are explained by a ligand-induced sequential model with subunit interactions which lead to a mixture of positive and negative cooperativity. A model involving dissimilar binding sites is also discussed.

Aspartate transcarbamylase of *Escherichia coli* has been the subject of numerous physicochemical and kinetic studies due to the availability of large quantities of homogeneous enzyme and the central role this enzyme has played in the development of allosteric theory. The enzyme is now generally accepted to be hexameric in structure, consisting of six regulatory subunits (mol wt 17,000) and six catalytic subunits (mol wt 33,500) (Weber, 1968; Meighen *et al.*, 1970; Rosenbusch and Weber, 1971). The enzyme can be dissociated by a variety of physical techniques to yield two types of subunits, a catalytic trimer (mol wt 100,000) and a regulatory dimer (mol wt 33,000) (Gerhart and Schachman, 1965). Based on the unusual association pattern of subunits following dissociation of the native molecule and the crystallographic data of Wiley and Lipscomb (1968), several models for the transcarbamylase molecule have been proposed (Gerhart, 1970; Rosenbusch and Weber, 1971; Markus *et al.*, 1971).

Support for the hexameric structure has come primarily from binding studies on native aspartate transcarbamylase and the isolated subunits. By the method of continuous variation, Hammes *et al.* (1970) have determined a total of six binding sites for BrCTP[1] on the native enzyme and three binding sites for carbamyl phosphate on the isolated catalytic trimer. Three binding sites for succinate, an aspartate analog, have also been indicated on the isolated catalytic trimer (Rosenbusch and Weber, 1971).

The determination of the allosteric mechanism involved in the regulation of the transcarbamylase requires more detailed binding and kinetic studies with the native, untreated enzyme. The binding of CTP to native aspartate transcarbamylase has been examined by the use of spin-label probes (Buckman, 1970) and by equilibrium dialysis (Changeux *et al.*, 1968; Winlund and Chamberlin, 1970). The results of these authors have not been in agreement. The original binding experiments of Changeux *et al.* (1968) revealed four independent binding sites for CTP, in agreement with the original tetrameric model for the enzyme. The binding experiments of Winlund and Chamberlin (1970) revealed six binding sites total, and these authors have suggested two different classes of CTP binding sites, including three independent "tight" sites and three independent "weak" binding sites. The CTP binding results of Buckman (1970) have been explained in terms of three independent "tight" binding sites but six independent "weak" binding sites. The binding of succinate to native transcarbamylase has been examined by the technique of equilibrium dialysis (Changeux *et al.*, 1968). A total of four binding sites for succinate were obtained, a value well below the expected value of six based on the number of catalytic subunits.

The discrepancy between the binding data for ligands and the proposed hexameric structure of the transcarbamylase prompted a detailed examination of the binding characteristics of the inhibitor, CTP, and the substrate analog, succinate. In this paper, the binding of CTP has been examined by equilibrium dialysis utilizing [^{14}C]CTP in an effort to understand the mechanism of enzyme inhibition.

The similarity of the present results to the results obtained in an examination of yeast glyceraldehyde-3-phosphate dehydrogenase (Cook and Koshland, 1970) has prompted an explanation of the results on the basis of "sequential" changes (Conway and Koshland, 1968; Koshland *et al.*, 1966; Kirtley and Koshland, 1967) of subunit conformation induced by the binding of CTP. A combination of both positive and negative cooperative effects is concluded.

Experimental Section

Materials. Aspartate transcarbamylase was purified from a strain of *Escherichia coli* kindly provided by Dr. J. Gerhart. The enzyme was purified essentially as described by Gerhart and Holoubek (1967). The only modification of the original procedure was an additional DEAE-cellulose column step added at the end of the purification to remove a minor contaminant band visible in cellogel electrophoresis. The enzyme then appeared to be homogeneous when tested by cellogel electrophoresis in Tris–borate buffer (pH 8.6) (Boyer *et al.*,

† From the Department of Biochemistry, University of Western Ontario, London, Ontario, Canada. *Received September 8, 1971.* This work was supported by a research grant from the Medical Research Council (MA-3848).

[1] Abbreviations used are: BrCTP, 5-bromocytidine triphosphate; CTP, cytidine triphosphate; n_H, Hill coefficient; n_M, maximum number of binding sites; S_f, free substrate; S_b, bound substrate; E_t, total enzyme; K_i', binding constant.