Affinity Labeling of Bovine Carboxypeptidase Aγ^{Leu} by *N*-Bromoacetyl-*N*-methyl-L-phenylalanine. I. Kinetics of Inactivation*

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ABSTRACT: Bovine carboxypeptidase A$_\gamma^{Leu}$ is rapidly inactivated upon incubation with the substrate analog *N*-bromoacetyl-*N*-methyl-L-phenylalanine. A parallel loss of esterase and peptidase activities is observed. The inactivation is first order with respect to remaining active enzyme. A kinetic analysis of the inactivation process suggests the following. (1) A reversible enzyme–inhibitor complex is formed prior to inactivation, as might be expected of an active-site-directed reagent. (2) Phenylacetate, a competitive inhibitor of carboxypeptidase A, and *N*-bromoacetyl-*N*-methyl-L-phenylalanine compete for the same site. (3) The apparent first-order rate constant extrapolated to infinite inhibitor concentration increases sharply between pH 6.0 and 7.5 and levels off at 0.200 min^{-1} at pH 7.5 and 25.0° while the concentration of inhibitor which produces the half-maximal rate of inactivation increases (1–5 mM) between pH 6.0 and 8.5. Experiments in which ^{14}C-labeled inhibitor is used to correlate the extent of modification and loss of enzymic activity indicate that a maximum of 2.0 inhibitor molecules may be incorporated per carboxypeptidase molecule. If the reaction is performed in the presence of 0.05 M D-phenylalanine, one inhibitor molecule is incorporated without loss of activity. Removal of D-phenylalanine and further incubation with reagent result in a parallel loss in activity and incorporation of an additional molecule of reagent. These data suggest that the loss of activity of carboxypeptidase A upon treatment with *N*-bromoacetyl-*N*-methyl-L-phenylalanine is caused by modification of a single amino acid residue which is very likely a part of the active site.

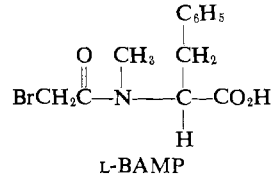
Carboxypeptidase A[1] is a zinc metalloenzyme of molecular weight 34,500 which catalyzes the hydrolysis of esters and peptides containing a free α-carboxylate group. Experiments employing group specific reagents for tyrosine, the dicarboxylic acids, and arginine have implicated these types of residues as critical for normal catalytic function toward peptide substrates (Vallee and Riordan, 1968).

Based in part upon the X-ray structure of a complex between CPA[2] and glycyl-L-tyrosine, Lipscomb *et al.* (1968) proposed a mechanism of catalysis which involves Arg-145 as binding site for the α-carboxyl group, Tyr-248 as proton donor, and Glu-270 as nucleophile. Roholt and Pressman (1967) have isolated a peptide containing Tyr-248 labeled with ^{131}I after iodination of CPA, and Pétra (1971) and Pétra and Neurath (1971) have shown that modification of Glu-270 with Woodward reagent K causes a parallel loss of esterase and peptidase activities.

In order to obtain additional information regarding the functional residues of CPA, we have undertaken a study of the affinity labeling of this enzyme. The method involves reaction of the enzyme with a compound which both resembles a substrate and possesses a group which will form covalent linkage with amino acid side chains (Singer, 1967).

Initial attempts to attach an affinity label to CPA were made with bromoacetyl-L-phenylalanine, which possesses the desired components (*i.e.*, a free α-carboxylate group, a hydrophobic side chain, and a reactive group). Although this compound does rapidly and irreversibly inhibit CPA, it is also a substrate which is hydrolyzed during the reaction.

Since compounds in which the amide hydrogen is replaced by a methyl group are not hydrolyzed by carboxypeptidase (Stahmann *et al.*, 1946), L-BAMP was synthesized and tested.



L-BAMP

This report presents the kinetics of inactivation of CPA$_\gamma^{Leu}$ by L-BAMP.

Experimental Section

Materials

Carboxypeptidase A$_\gamma^{Leu}$, prepared as described earlier (Pétra and Neurath, 1969) by DEAE-cellulose chromatography of carboxypeptidase A (Worthington Biochemical Corp.), was the generous gift of Dr. P. H. Pétra. Enzyme stock solutions were prepared by washing the crystals with cold distilled water and dissolving them in cold 5 M NaCl. Enzyme thus prepared exhibited minimal specific activities, in terms of first-order constants, of 18.0 and 300 sec^{-1} toward carbobenzoxyglycyl-L-phenylalanine (CGP) and hippuryl-DL-phenyllactic acid (HPLA), respectively.

Carbobenzoxyglycyl-L-phenylalanine and sodium hippuryl-

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[1] For an explanation of the nomenclature, see Pétra and Neurath (1969).

[2] The following abbreviations are used: CPA, carboxypeptidase A; BAMP, *N*-bromoacetyl-*N*-methyl-L-phenylalanine; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-phenyllactic acid; CM, carboxymethyl; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

DL-phenyllactate were purchased from Fox Chemical Co.; *trans*-cinnamic acid, indoleacetic acid, 4-phenylbutyric acid, and bromoacetyl bromide from Aldrich Chemical Co.; glycyl-L-tyrosine from Cyclo; D-phenylalanine and L-phenylalanine from Fluka; Sephadex G-25 from Pharmacia Fine Chemicals; Triton X-100, 2,5-diphenyloxazole (PPO), and 1,4-bis[2-(5-phenyloxazolyl)]benzene ((POPOP) from Packard; and *N*-α-acetyl-L-cysteine was from Calbiochem.

β-Phenylpropionic acid (hydrocinnamic acid) was purchased from Eastman Chemical Co. and recrystallized as described by Pétra and Neurath (1969) prior to use.

Bromoacetic acid (practical grade) was purchased from J. T. Baker Chemical Co. and recrystallized from pentane. [1-^{14}C]Bromoacetic acid (lot 465-073) was obtained from New England Nuclear and used without further purification.

Methods

Analytical Procedures. The pH of solutions was measured with an Orion Digital pH Motor (Model 801) equipped with a glass electrode.

Optical density measurements and spectrophotometric assays were performed with a Gilford Model 2000 spectrophotometer. The temperature of the cell housing was maintained at 25.0 ± 0.1°.

Radioactivity measurements were made with a Packard Tri-Carb liquid scintillation spectrometer, Model 3003. An efficiency of 56% was observed when 2 ml of aqueous sample were counted in 15 ml of toluene–Triton X-100 (2:1, v/v) which contained 2.67 g/l. of PPO and 0.067 g/l. of POPOP.

Enzyme Assays. Peptidase activity was determined by following the decrease in absorption at 223.7 nm when CGP is hydrolyzed by CPA (Whitaker *et al.*, 1966). The assay solution contained 1.18 mM CGP, 0.45 M KCl, and 0.05 M Tris-chloride (pH 7.5).

Esterase activity was determined spectrophotometrically as described by McClure *et al.* (1964). The assays were performed by adding a suitable aliquot of CPA to 3 ml of a 1 mM solution of HPLA in 0.45 M KCl–0.05 M Tris-chloride (pH 7.5), and monitoring the increase in absorbance at 254 nm.

Protein concentration was obtained from the absorption at 278 nm after corrections for light scattering and assuming $A_{278}^{0.1\%} = 1.88$ (Bargetzi *et al.*, 1963).

Synthesis of N-Bromoacetyl-N-methyl-L-phenylalanine. *N*-Bromoacetyl-*N*-methyl-L-phenylalanine (L-BAMP) was prepared by acylating *N*-methyl-L-phenylalanine (Fischer and Lipschitz, 1915) with bromoacetyl bromide in alkaline solution (Fischer, 1905). The product crystallized from ethanol–water. After one recrystallization the product appeared pure as judged by thin-layer chromatography in chloroform–glacial acetic acid (95:5; v/v, R_F 0.28) and by its sharp melting point of 109°. *Anal.* (by Alfred Bernhardt, Mikroanalytisches Laboratorium, West Germany). Calcd for $C_{12}H_{14}BrNO_3$: C, 48.04; H, 4.70; N, 4.67. Found: C, 47.94; H, 4.66; N, 4.61.

Synthesis of N-Bromoacetyl-N-methyl-D-phenylalanine. *N*-Bromoacetyl-*N*-methyl-D-phenylalanine was synthesized as described above for the L isomer, mp 113°.

Synthesis of N-([1-^{14}C]Bromoacetyl)-N-methyl-L-phenylalanine. ^{14}C-labeled L-BAMP was synthesized by acylating *N*-methyl-L-phenylalanine with [1-^{14}C]bromoacetyl chloride as described above for the acid bromide. The acid chloride was prepared as follows. [1-^{14}C]Bromoacetic acid (18 mg; 2.3 mCi/mmole) and unlabeled bromoacetic acid (675 mg) were dissolved in 20 ml of anhydrous ether and 1.0 g of phosphorus pentachloride added. After all PCl_5 had dissolved (1 hr), the solution was evaporated on a rotary evaporator at 30°.

The specific activity of L-[^{14}C]BAMP was determined by drying the crystalline product over phosphorus pentoxide, preparing a neutralized stock solution, and counting suitable aliquots. The value obtained (7.6×10^{10} cpm/mole) was confirmed by incubating 1 ml of a 4 mM solution of inhibitor with 1 ml of 4 mM *N*-acetylcysteine at pH 8.9 for 2 hr. An aliquot was removed and hydrolyzed in 6 N HCl as described by Moore and Stein (1963). One portion of the hydrolysate was subjected to amino acid analysis and the amount of CM-cysteine formed was determined. An equal amount was chromatographed on the Spinco Model 120B amino acid analyzer (Spackman *et al.*, 1958) and the eluate collected in a fraction collector. The fractions corresponding to CM-cysteine were analyzed for radioactivity. A value of 7.8×10^{10} cpm/mole was determined by this method for the specific activity of L-[^{14}C]BAMP.

Kinetics of Inactivation. Carboxypeptidase A_γ^{Leu} solutions were prepared at approximately 0.2 mg/ml in a solution containing 2 N NaCl–0.10 M Tris-acetate at the desired pH, and suitable concentrations of a competitive inhibitor. The enzyme solution was incubated at 25.0 ± 0.1° and an equal volume of a neutralized solution of L-BAMP was added to initiate the reaction. Aliquots were removed during the course of inactivation to monitor esterase or peptidase activities. Values for k, the pseudo-first-order rate constant, were calculated from eq 1 where E_1 and E_2 are the observed activities

$$\ln\left(\frac{E_1}{E_2}\right) = k(T_2 - T_1) \qquad (1)$$

at times T_1 and T_2.

τ, the half-time for inactivation, was calculated from k according to eq 2.

$$\tau = \frac{\ln 2}{k} \qquad (2)$$

Incorporation of Radioactivity. A solution of carboxypeptidase A_γ^{Leu} (2 mg/ml in 2 N NaCl–0.2 M Tris-chloride, pH 7.5, containing where required competitive inhibitor) was mixed at 25.0° with an equal volume of 4 mM L-[^{14}C]BAMP and the solution incubated at 25° in the dark. At suitable intervals 2-ml samples were removed and the protein and small molecules separated on a 1.7 × 15 cm column of Sephadex G-25 equilibrated with 0.25 M NaCl–0.05 M Tris-chloride (pH 7.5). The protein concentration, activities toward HPLA and CGP, and radioactivity incorporated were determined as described earlier.

Results

The time course of inactivation of CPA_γ^{Leu} by 1.00 mM L-BAMP at pH 7.5 and 25° is presented in Figure 1. The reaction is first order with respect to active enzyme remaining, with a half-time of 25 min. Incubation for longer periods of time or with greater concentrations of inhibitor results in complete inactivation. The parallel loss of esterase and peptidase activities observed in this experiment and under a variety of conditions suggests that modification alters one or more groups whose integrity is required for both activities.

The inactivation of CPA_γ^{Leu} by D-BAMP (10 mM) is also shown in Figure 1. As in the case of the L isomer, the reaction is first order with respect to remaining active enzyme and both

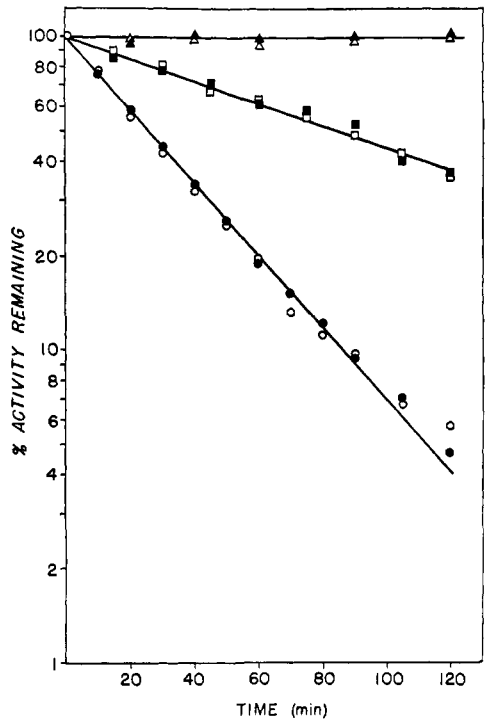

FIGURE 1: Loss of peptidase and esterase activities of CPAγ^{Leu} as a function of time during incubation with L-BAMP, D-BAMP, and bromoacetate. CPAγ^{Leu} (0.1 mg/ml) was treated with 1 mM L-BAMP, 10 mM D-BAMP, or 10 mM bromoacetate in a solution containing 1 N NaCl–0.05 M Tris-chloride (pH 7.5.) The esterase (L-BAMP, ●; D-BAMP, ■; bromoacetate, ▲) and peptidase (L-BAMP, ○; D-BAMP, □; bromoacetate, Δ) activities of the enzyme were monitored as a function of time as described in the Experimental Section.

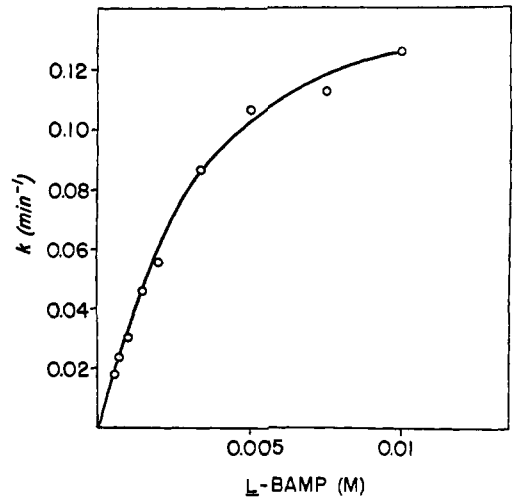

FIGURE 2: Pseudo-first-order rate constant, k, for inactivation as a function of BAMP concentration at pH 7.5. Incubation mixtures contained ~0.1 mg/ml of CPAγ^{Leu} in 1 N NaCl–0.05 M Tris-chloride (pH 7.5), including different amounts of BAMP. Esterase activity was monitored as a function of time at each concentration of BAMP, and pseudo-first-order rate constants were calculated as described in the Experimental Section.

esterase and peptidase activities are lost simultaneously. At an inhibitor concentration 10 times that of the L isomer, a somewhat greater half-time (~90 min) is observed. Bromoacetate at a concentration of 10 mM has no effect on enzyme activity.

To characterize further the reaction of L-BAMP and CPA$_\gamma^{Leu}$, the rate of enzyme inactivation as a function of reagent concentration was investigated. The variation of k, the pseudo-first-order rate constant for the inactivation process, with L-BAMP at pH 7.5 is shown in Figure 2. The hyperbolic relationship suggests that enzyme and inhibitor form a reversible complex prior to inactivation according to eq 3. Equation 4 was derived by Meloche (1967) to relate the half-time for inactivation to the inhibitor concentration in such a system,

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}I \xrightarrow{k_2} E_{inact} \quad (3)$$

$$\tau = \frac{K_{inact}T}{[I]} + T \quad (4)$$

where τ is the half-time for inactivation as determined from first-order rate plots and $K_{inact} = (k_{-1} + k_2)/k_1$. [I] is the inhibitor concentration, and T is the half-time predicted at infinite inhibitor concentration. Values for K_{inact} and T can be obtained from plots of τ against the reciprocal of the inhibitor concentration.

More nearly accurate estimates of T and K_{inact} should be obtained from linear plots of τ[I] against [I] according to the following rearrangement of eq 4.

$$\tau[I] = TK_{inact} + T[I] \quad (5)$$

When the data presented in Figure 2 are replotted according to eq 5, a straight line is obtained whose y-axis intercept is TK_{inact} and the slope equals T. Least-squares analysis yields $T = 3.61 \pm 0.37$ min, $TK_{inact} = 0.0175 \pm 0.0017$ min M^{-1} at pH 7.5, and thus $K_{inact} = 4.8$ mM at this pH.

To characterize further the reaction between L-BAMP and CPA$_\gamma^{Leu}$, the effects of pH on T and on K_{inact} were investigated. Plots of product of half-time and concentration of L-BAMP against the concentration of L-BAMP were obtained in the pH range of 5.8 and 8.4, and T and K_{inact} determined by least-squares analysis. T was converted into k_2, the apparent first-order rate constant for the reaction

$$E\text{-}I \xrightarrow{k_2} E_{inact} \quad (6)$$

by eq 7

$$k_2 = \frac{\ln 2}{T} \quad (7)$$

The relationship between log k_2 and pH presented in Figure 3 suggests the following mechanism

$$\begin{array}{ccc} E + I & \rightleftharpoons & E\text{-}I \xrightarrow{k_2^0} E_{inact} \\ \uparrow\downarrow K_{a,E} & & \uparrow\downarrow K_{a,EI} \\ EH & & EHI \end{array} \quad (8)$$

where the unprotonated form of a residue at the active site is required for inactivation. If this mechanism is correct, the equation relating k_2^0, the pH-independent first-order rate constant, and k_2, the observed first-order rate constant at a given pH is

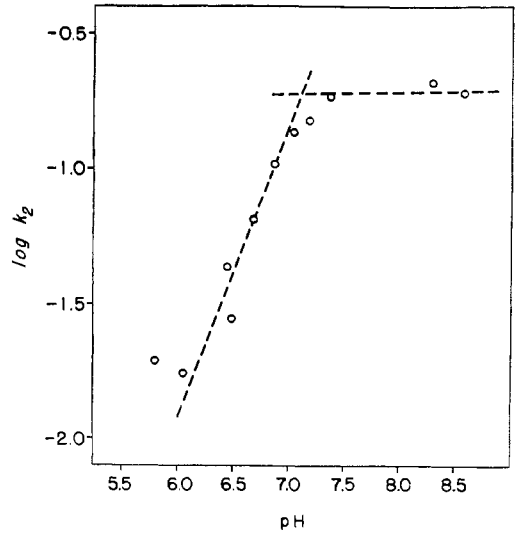

FIGURE 3: Effect of pH on log k_2. k_2, the apparent first-order rate constant for the reaction EI → E_{inact}, was determined as described in the text.

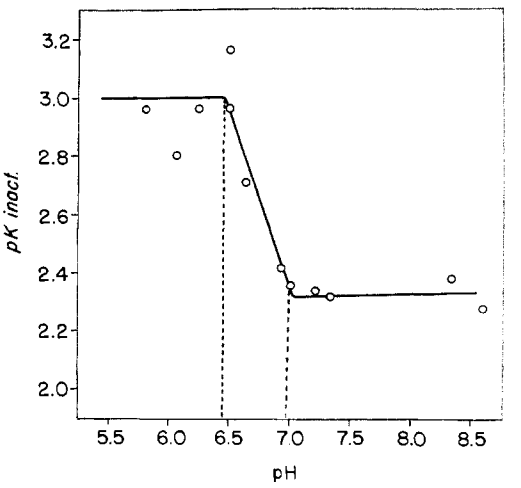

FIGURE 4: Effect of pH on pK_{inact}. K_{inact} was determined by least-squares analysis of plots of τ[I] against [I] as described in the text.

$$k_2^0 = k_2 \times \left(1 + \frac{[H]}{K_{a,EI}}\right) \quad (9)$$

Plots of log k_2 against pH for such a system should consist of a flat portion at pH values significantly greater than p$K_{a,EI}$ joined by a curved region to a line of unit slope at p$K_{a,EI}$ > pH. This situation is identical with that for enzyme-catalyzed reactions where the rate of catalysis is affected by an ionizable group (Dixon and Webb, 1967). A pK_a of approximately 7.0 is obtained at the intersection of the extrapolated linear portions of Figure 3. The pH-independent first-order rate constant, k_2^0, is approximately 0.2 min^{-1}.

In the case of enzyme-catalyzed reactions, plots of pK_m against pH exhibit inflection points at pK_a values of groups important either for binding or catalysis. Plots of pK_m against pH consist of linear portions joined by curved segments. An increase in slope is observed with increasing pH at the pK_a of a group in the E–I complex while a decrease in slope is observed with increasing pH at the pK_a of a group in the free enzyme (Dixon and Webb, 1967). K_{inact} varies with pH as follows

$$K_{inact} = K^0_{inact} \times \frac{\left(1 + \frac{[H]}{K_{a,E}}\right)}{\left(1 + \frac{[H]}{K_{a,EI}}\right)} \quad (10)$$

where K_{inact} and K^0_{inact} are the observed and pH-independent constants, respectively.

Figure 4 represents the variation of pK_{inact} with pH. The downward inflection at pH 6.5 (*i.e.*, from 0 to −1 slope) presumably occurs at p$K_{a,E}$ and the upward inflection at pH 7.0 (*i.e.*, from −1 to 0 slope) at the p$K_{a,EI}$ observed in the plot of log k_2 against pH. However, because the change in K_{inact} is small (1–5 mM) in this pH range and because the error incurred in the estimation of K_{inact} is relatively great, values of p$K_{a,E}$ and p$K_{a,EI}$ obtained from Figure 4 are only approximate.

Evidence for active-site modification is provided by the greater ability of L-BAMP to inhibit CPA_γ^{Leu} relative to D-BAMP or bromoacetate, by the formation of a reversible complex between L-BAMP and enzyme prior to inactivation (*vide supra*), and by the reduced rates of inactivation in the presence of competitive inhibitors of CPA. Table I presents a comparison of the efficacy of several competitive inhibitors of carboxypeptidase as protecting agents against inactivation by L-BAMP, in terms of K_i values. Although a negative correlation exists between the half-time for inactivation in the presence of β-phenylpropionate, indoleacetate, phenylacetate, 4-phenylbutyrate, and glycyl-L-tyrosine and their respective K_i's, D-phenylalanine is more effective in protecting from enzyme inactivation than would be predicted from its reported K_i.

Meloche (1967) has shown that the equation relating τ and affinity-label concentration [I] in the presence of a competitive inhibitor is

$$\tau = \frac{K_{inact}T}{[I]} \times \left(1 + \frac{[X]}{K_i}\right) + T \quad (11)$$

where [X] is the concentration of competitive inhibitor and K_i the dissociation constant of the E–X complex. If the affinity label and competitive inhibitor bind at the same site, plots of τ against 1/[I] should be linear with a slope of $k_{inact}T(1 + [X]/K_i)$ and a y-axis intercept of T. The relationship between τ and [L-BAMP] in the absence of competitive inhibitor and in the presence of 3 and 6 mM phenylacetate is presented in Figure 5. The values for the dissociation constant of the enzyme–phenylacetate complex are 0.9 and 1.4 mM in the presence of 3 and 6 mM, respectively. These are in reasonable agreement with the reported value of approximately 0.4 mM (Elkins-Kaufman and Neurath, 1949).

The extent of modification of carboxypeptidase as a function of time of incubation with L-[^{14}C]BAMP was measured by incorporation of radioactivity (Figure 6). Two residues of L-BAMP are introduced per enzyme molecule in the absence of competitive inhibitor. An average of one molecule of L-BAMP per molecule enzyme is incorporated after 0.5-hr incubation while it takes nearly 12 hr to add the second residue of L-BAMP, suggesting that the presumed two sites of modification have different reactivities.

The competitive inhibitor D-phenylalanine in concentra-

TABLE I: Comparison of the Half-Times for Inactivation of CPA$_\gamma^{Leu}$ by L-BAMP in the Presence of Competitive Inhibitors and Their K_i Values.

Inhibitor	τ^a (min)	Esterase		Peptidase	
		K_i (M)	Ref	K_i (M)	Ref
None	25				
β-Phenylpropionate	174	1.52×10^{-4}	*b*	6.2×10^{-5}	*c*
Indoleacetate	156			7.8×10^{-5}	*d*
D-Phenylalanine	275			2.0×10^{-3}	*c*
Phenylacetate	47	4.1×10^{-4}	*b*	3.9×10^{-4}	*c*
4-Phenylbutyrate	39	1.37×10^{-3}	*b*	1.13×10^{-3}	*c*
Glycyl-L-tyrosine	27			2.0×10^{-3}	*e*

[a] Half-times were determined as described in the Experimental Section for the inactivation of CPA$_\gamma^{Leu}$ upon incubation with 1 mM L-BAMP and 1 mM competitive inhibitor. [b] Kaiser and Carson (1965). [c] Elkins-Kaufman and Neurath (1949). [d] Smith *et al.* (1951). [e] Yanari and Mitz (1957).

tions of 0.01 and 0.05 M reduces the number of residues incorporated per enzyme molecule to approximately one in a 24-hr period. Although there was negligible loss of activity in the sample protected by 0.05 M D-phenylalanine, approximately 20% loss was observed after 24 hr in 0.01 M D-phenylalanine.

Modification of CPA$_\gamma^{Leu}$ (preincubated with unlabeled L-BAMP for 24 hr in the presence of 0.05 M D-phenylalanine) with radioactive L-BAMP results in the rapid introduction of approximately one residue of L-BAMP. The simplest interpretation consistent with these data is to assume that L-BAMP modifies two sites in the CPA$_\gamma^{Leu}$ and that D-phenylalanine protects the more reactive site but has little if any effect on the rate of modification of the less reactive site.

β-Phenylpropionate, unlike D-phenylalanine, protects both sites from modification by L-BAMP. (This is not at all surprising since a similar compound, β-(*p*-iodophenyl)propionate, binds to carboxypeptidase A at several loci (Steitz *et al.*, 1967).) There is no loss in activity during the 24-hr incubation with L-BAMP in the presence of 0.05 M β-phenylpropionate, demonstrating that modification under these conditions does not occur at a functional residue.

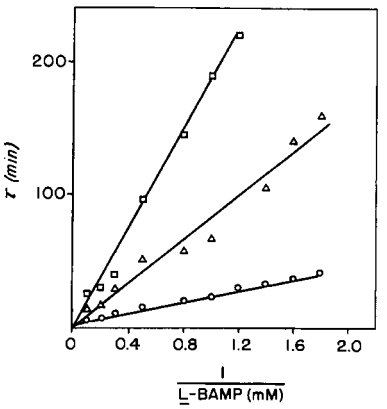

FIGURE 5: Half-time (τ) for the inactivation of CPA$_\gamma^{Leu}$ as a function of the reciprocal of [BAMP] in the presence and absence of phenylacetate, a competitive inhibitor. Data were obtained and analyzed as described in the text. The solutions contained no phenylacetate (○), 3 mM phenylacetate (△), and 6 mM phenylacetate (□).

Figure 7 depicts the correlation between extent of modification and loss of enzymatic activity for CPA$_\gamma^{Leu}$ and for "modified" CPA$_\gamma^{Leu}$ (*i.e.*, CPA$_\gamma^{Leu}$ which had been preincubated for 24 hr with unlabeled L-BAMP in the presence of 0.05 M D-phenylalanine). Nearly stoichiometric relations between L-BAMP incorporation and enzyme inactivation are observed over the entire course of modification of pretreated CPA$_\gamma^{Leu}$. In this case approximately 1.1 residues/molecule are incorporated when inactivation is complete (~5 hr). Incubation for an additional 19 hr produces an average increase of only 0.1 residue/molecule.

CPA$_\gamma^{Leu}$ is also stoichiometrically inactivated by L-BAMP within the range of 0–30% inactivation; however, additional amounts of L-BAMP are incorporated as inactivation proceeds. Approximately 1.8 residues/molecule are present when inactivation is complete and further incubation increases this ratio to 2:1.

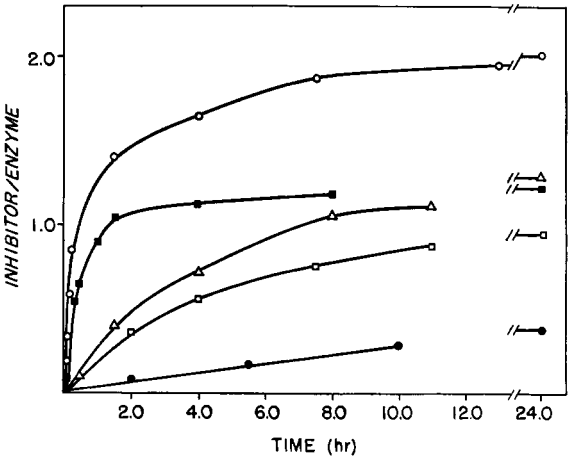

FIGURE 6: Incorporation of ^{14}C-labeled inhibitor as a function of time. Incubation mixtures contained ~2 mg/ml of CPA$_\gamma^{Leu}$ in 1 N NaCl, 0.002 M [1-^{14}C]-*N*-bromoacetyl-*N*-methyl-L-phenylalanine, and 0.1 M Tris-chloride (pH 7.5). The solvent contained no competitive inhibitor (○), 0.01 M D-phenylalanine (△), 0.05 M D-phenylalanine (□), or 0.05 M β-phenylpropionate (●). One sample (■) was prepared by modifying CPA$_\gamma^{Leu}$ which had been previously treated with unlabeled BAMP as described in the text.

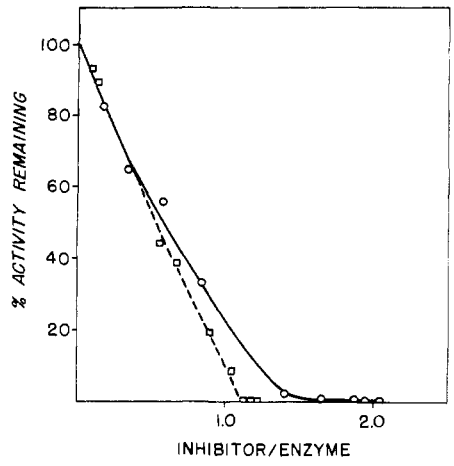

FIGURE 7: Per cent esterase activity remaining as a function of the extent of modification. Procedures for the preparation of samples and determination of extent of modification and esterase activity are described in the Experimental Section. Reactions of $CPA\gamma^{Leu}$ (○) and "modified" $CPA\gamma^{Leu}$ (□) (*i.e.*, enzyme which had been treated with unlabeled BAMP for 24 hr in the presence of 0.05 M D-phenyl alanine) with [^{14}C]BAMP are depicted.

Thus incorporation of one residue of L-BAMP per molecule of enzyme is sufficient to produce inactivation. Additional modification occurs at a slower rate but without affecting enzymatic activity.

Discussion

The present investigation of the affinity labeling of CPA was undertaken for two reasons: (1) to confirm and extend the current views of the active site of bovine CPA, and (2) to provide a reagent which may be of some utility in comparing bovine CPA and other enzymes of similar specificity.

In order for an affinity label such as *N*-bromoacetyl-*N*-methyl-L-phenylalanine to be of use in achieving either goal it should possess the following characteristics. (1) It should be capable of reacting with the various amino acid side chains that might participate in catalytic function, and (2) it should react at a minimal number of alternate sites.

The reagent L-BAMP is an α-bromo acid amide and is thus capable of reacting with lysine, histidine, aspartic acid, glutamic acid, cysteine, methionine, and with the α-amino group. The products formed by condensation of L-BAMP with amino acid side chains are converted into CM-amino acids by acid hydrolysis.

The second requirement, that of active-site direction, derives from the relation of L-BAMP to chloroacetyl-L-phenylalanine, one of the earliest known synthetic substrates for carboxypeptidase A (Putnam and Neurath, 1946). In keeping with the behavior of substrates, the L isomer of BAMP is much more effective than D-BAMP or bromoacetate. Further kinetic evidence for active-site modification of CPA by L-BAMP includes the predicted formation of an enzyme–inhibitor complex prior to inactivation, the correlation between strength of binding of competitive inhibitors of CPA and their ability to protect it from inactivation by L-BAMP, and the demonstration that phenylacetate and L-BAMP compete for the same site.

Incorporation of radioactive L-BAMP by CPA indicates that under appropriate conditions, the reaction is specific and that multiple labeling should not be a problem in identifying sites of modification. A maximum of two residues can be incorporated but one shows preferential reactivity. Modification of the more reactive site causes loss of enzymatic activity which can be prevented by D-phenylalanine without affecting the rate of reaction at the second site.

Having confirmed that L-BAMP satisfies the criteria of broad reactivity, reaction at the active site, and minimal side reactions, all being mandatory for the effective employment of an affinity label, the original goals may now be addressed. The identification of Glu_{270} as the critical site of modification of CPA by L-BAMP and some implications of the kinetics of inactivation (*vide supra*) are presented in the following paper (Hass and Neurath, 1971).

The reactions of L-BAMP with bovine carboxypeptidase B and bovine procarboxypeptidase A-S5 are currently being investigated in this laboratory in an attempt to extend the utility of this reagent in demonstrating the homologies of carboxypeptidases A and B and in probing the reactivity of the corresponding amino acid residues in the zymogen.

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