AFFINITY LABELLING OF CARBOXYPEPTIDASE B: MODIFICATION OF A METHIONYL RESIDUE.

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Modification of porcine carboxypeptidase B with [^{14}C]-bromoacetyl-p-aminobenzylsuccinic acid results in a marked decrease of the activity toward both peptide and ester substrates. The inactivation correlates with the incorporation of 1 equivalent of inhibitor molecule per carboxypeptidase molecule. Evidence is presented that inactivation is due to alkylation of a single, presumably methionyl residue in the active center of the enzyme.

In order to further define the functional residues in the active center of carboxypeptidase B we extend studies of the affinity labeling of this enzyme (1-4). A fundamental feature of affinity labeling reagents (5) is their intrinsic capability of reacting with a wide variety of amino acid side chains. Selectivity arises from their specific binding to a single locus on the enzyme surface. It is very important for carboxypeptidase B that the reagent should react at a minimal number of alternate sites due to the various modes of binding of substrates and inhibitors (4)(6)(7). Recently Byers and Wolfenden (8) investigated a variety of carboxylic and dicarboxylic acids as inhibitors of carboxypeptidase A. Among these, benzyl-L-succinic acid was unusually potent. Due to the strong similarities between CPA and CPB we expected that this inhibitor might be a strong inhibitor of CPB as well. If so it will serve as a basis for a suitable affinity label particularly if it could posess a group able to form a covalent linkage with the enzyme. This report shows that benzylsuccinic acid

Abbreviations: CP, carboxypeptidase; Pla, Phenyllactate; BAABS, bromoacetyl-p-amino-DL-benzylsuccinic acid; HA, hippuryl-L-arginine; HAA, hippuryl-L-argininic acid; HPLA, hippuryl-L-phenyllactate.

is indeed a very potent competitive inhibitor of CPB and that substitution at the para position with a bromacetylamino residue yields a very useful reagent for the modification of carboxypeptidases.

EXPERIMENTAL

Carboxypeptidase B was purchased from the Worthington Biochemical Corp; the substrates hippuryl-L-arginine, hippuryl-L-argininic acid from Cyclo Corp; and t-Boc(alanyl)$_2$-phenyllactat; t-Boc(alanyl)$_2$-phenylalanine from Miles-Yeda, Rehovot; L-and DL-benzylsuccinic acid were prepared as previously described (8). All other chemicals were of the best grade available. Buffers were extracted with 0.1% dithizone in carbon tetrachloride to avoid contamination by adventitious metal ions. [^{14}C]-Bromoacetyl-p-aminobenzylsuccinic acid was prepared as follows: 1.5 mmole of [^{14}C]-bromoacetic acid and 1.5 mmole N-hydroxysuccinimide dissolved in 6 ml dioxane were coupled with 1.5 mmole dicyclohexylcarbodiimide for 2 hours at 0°. The urea derivative was removed by filtration and the active ester in the dioxane solution was then coupled with 1.2 mmole p-aminobenzylsuccinic acid dissolved in 4 ml 80% dioxane. The reaction mixture was kept at room temperature for 6 hours and then evaporated to dryness. The oily residue was dissolved in ethylacetate and water. The organic phase was washed twice with H_2O, dried over Na_2SO_4 and evaporated to dryness. The compound was crystallized from ethylacetate-petroleum ether; m.p. 126°, Neutral equiv 358.

Amino Acid Analyses were performed on a Beckman-Unichrom amino acid analyzer. Analysis of tryptophan by MCD was kindly carried out by Dr. J.F. Rirodan. Zn^{++} concentrations were determined on a Varian-Techtron atomic adsorption spectrometer, model AA5.

Protein Concentrations were measured by the absorbance at 278 nm using a molar absorptivity of 7.34×10^4 M^{-1} cm^{-1}.

Activity Measurements All assays were performed in 0.1M NaCl-0.05M Tris

buffer pH 7.5 in a thermostatted (25°) cell compartment of a Cary 16 K spectrophotometer. The initial velocity (<6%) of substrate hydrolysis was followed by measuring either the increase of absorbance at 254 nm in the case of hippuryl derivatives or the decrease of absorbance at 225nm for other substrates, as described previously (9).

Modifications were performed at 25° by incubation of a solution of 5 mg/ml CPB in 0.5M Tris-0.1M NaCl buffer pH 7.0, containing various concentrations of reagent. The modified enzyme was separated from excess reagent by passing it through a short Bio-gel P-4 column.

RESULTS

Both the racemic and the L-isomer of benzylsuccinate were found to produce linear inhibition, competitive, against all four substrates tested: hippurylarginine, hippurylargininic acid, t-Boc(Ala)$_2$Pla and t-Boc(Ala)$_2$ Phe. The Ki value calculated from double recpirocal plots are given in Table I. Inhibition could be reversed by dilution or dialysis. These are the most effective reversible inhibitors of CPB as was also observed by Byers and Wolfenden (8), who introduced them for CPA. The unusual potency of benzyl succinate suggests that the incorporation of an active group might produce an effective affinity label.

Incubation of CPB with 9mM bromoacetylaminobenzylsuccinate (BAABS) at pH 7.0, 25° produces a slow loss of peptide activity, which reaches 20% of the control value within 24hr. As shown in Fig 1. the rate of peptidase inactivation is greater that that of esterase inactivation. Inactivation is faster at pH 6 than at pH 9 (Fig 2). Due to the intrinsic instability of CPB, modification could not be carried out at pH values below 6. All further modifications were therefore carried out at pH 7.0.

Incorporation of the reagent into carboxypeptidase was measured directly by means of radioactivity. Loss of activity and incorporation of ^{14}C-reagent follow similar time course. As shown in Fig 3 the enzyme

TABLE I

DISSOCIATION CONSTANTS FOR COMPETITIVE INHIBITION OF CPB ACTIVITY BY BENZYLSUCCINIC ACID[a].

Inhibitor \ Substrate	Hippuryl-L-Arginine[a]	Hippuryl-L-Argininic acid[a]	t-Boc-Ala-Ala-Phe(L_3)[b]	t-Boc-Ala-Ala-PLA(L_3)[c]
Benzylsuccinic acid (DL)	1.2×10^{-6}M	1.2×10^{-6}M	1.3×10^{-6}M	8.0×10^{-7}M
Benzylsuccinic acid (L)	6.7×10^{-7}M	-	-	4.2×10^{-7}M

[a] pH 7.5, 25°C. The parameters were calculated from a least-squares treatment of Lineweaver-Burk plots. [b] In 0,05M Tris 0.1M NaCl buffer. [c] In 0,01M Tris 0.1M NaCl buffer.

is stoichiometrically inactivated with the incorporation of about 1 mole of reagent/mole of enzyme. Such selective labeling indicates modification via a specific process. As shown in Fig 4. the time course of inactivation depends upon the initial concentration of BAABS. However, with increasing concentration of the reagent the rate of inactivation becomes maximal, indicative of saturation kinetics, and implying the formation of a reagent-enzyme complex prior to inactivation. Evidence that inactivation is due to modification of an active-site residue comes from the protection afforded by various inhibitors or poor substrates (Table II).

Zinc and amino acid analysis including tryptophan of the native and alkylated protein were similar except that the alkylated enzyme had about one less methionyl residue. The reagent, and α-bromo acid amide, is capable of reacting with various amino acid side chains. No new ninhydrin or radioactivity peak was seen in the amino acid analysis in the region of carboxymethylated tyrosine, histidine lysine or cysteine. Measurement of ^{14}C

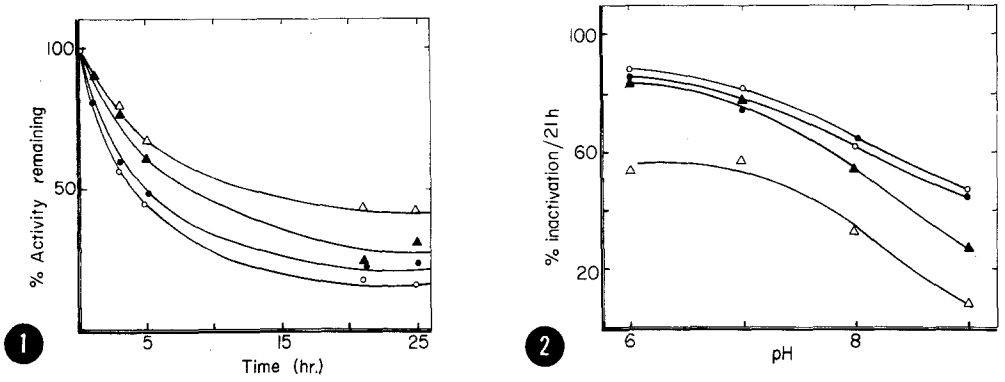

Figure 1 Rate of inactivation of CPB by BAABS, at pH 7.0 25°. CPB (5mg/ml) was treated with 9mM BAABS in 0.5M Tris - 0.1M NaCl buffer. Activity was monitored as a function of time. (●) HA (Δ) HAA (o) t-BOC(Ala)$_2$Phe (▲) HPLA.

Figure 2 Activity remaining after reaction of CPB with BAABS for 21 hours as a function of pH. 0.5M Tris - 0.1M NaCl buffer, 9mM reagent. (o) t-Boc(Ala)$_2$Phe (●) HA (▲) HPLA (Δ) HAA

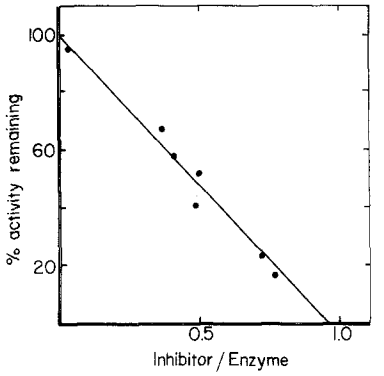

Figure 3 Incorporation of BAABS as a function of peptidase activity remaining. (pH 7.0, 25°)

indicated a single peak which appeared at the breakthrough fraction in the amino acid analyzer. This might be due to a fraction of the sulfonium salt of methionine not destroyed during acid hydrolysis.

Finally, modified CPB was treated under conditions which might be expected to cleave an ester bond (Table III). Incubation with 2M hydroxylamine or 0.01N alkali produced little if any loss of label. These data

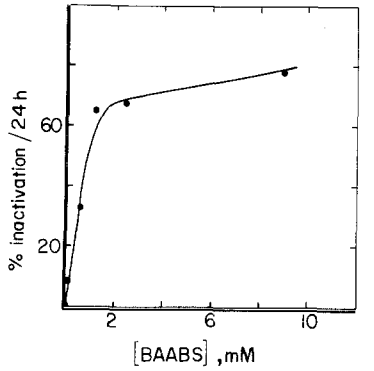

Figure 4 Rate of inactivation of carboxypeptidase B by BAABS as a function of reagent concentration. (pH 7.0 25°).

TABLE II

INACTIVATION OF CARBOXYPEPTIDASE B BY BAABS IN THE PRESENCE OF INHIBITORS[a].

Inhibitor	% inactivation[b]	mole BAABS/ mole enzyme[c]
none	69	0.70
Argininic acid (10mM)	32	0.35
Acetyl-L-Arginine (10mM)	56	0.54
L-Phenyllactic acid (50mM)	50	0.49
β-phenylpropionic acid (25mM)	2	0.03

[a] Carboxypeptidase B (5 mg/ml), was reacted with 7.5mM BAABS at pH 7.0 25° for 20 hrs. [b] Activity toward hippurylarginine. [c] By radioactivity.

are in contrast to the relatively rapid ester cleavage previously seen in CPB modified with bromoacetyl-D-arginine (3)(4), indicating that BAABS and bromoacetyl-D-arginine modify different residues. The label could be removed by treatment with SH reagents (Table III).

TABLE III

RELEASE OF LABEL FROM CPB INACTIVATED WITH [^{14}C] BAABS

Treatment	Residues/ enzyme molecule	Activity %
none	0.72	24
pH 9.0, 24h, 25°	0.72	22
pH 9.0, 2M NH_2OH, 24h, 25°	0.70	15
pH 12, NaOH, 60 min, 25°	0.65	34
pH 12, NaOH, 15 min, 37°	0.62	36
pH 8.0, 75mM cystein 30 min 37°	0.36	20
pH 7.5, 73mM mercaptethanol 60 min 25°	0.45	50

DISCUSSION

The evidence presented in this report indicates that alkylation of carboxypeptidase B with BAABS results in the incorporation of 1 mole of reagent into the active center of the enzyme with a concomitant loss of four of its enzymic activities. The alkylation reaction exhibits saturation kinetics (Fig 4) indicative of the formation of a reversible complex prior to covalent bond formation. Thus BAABS exhibits the characteristics of a specific active site directed, irreversible inhibitor.

Amino acid analysis of this alkylated enzyme showed that only one methionyl residue was modified and no other alkylated amino acid derivatives were formed. Tryptophan, unstable to the conditions of acid hydrolysis, was quantitatively determined by MCD which gave similar values for both the

native or the alkylated enzyme. The label is not labile to 2M hydroxylamine treatment nor to exposure to alkali. It is most unlikely, therefore, that the alkylation involves the formation of an ester i.e. with glutamic or aspartic acid. On the other hand, the label could be removed by treatment with cysteine and mercaptoethanol. This reversal of alkylation has been shown recently (10) (11) to be due to the decomposition of the alkylsulfonium salt formed by alkylation of a methionyl residue.

The high degree of specificity exhibited by BAABS for the modification at the active center of CPB might result from (a) an enhanced reactivity of this residue towards alkylating agents in general or (b) specific binding of the reagent. The fact that BAASB inactivates CPB at a much slower rate when compared either to other affinity reagents, BrAc-D-Arg (3) (4) or BrAc-N-methyl-Phe (2), or to alkylation of methionyl residues in other proteins (12) indicates that the methionyl residue does not posses an enhanced reactivity and that the specific binding of the reagent is the main reason for the specificity exhibited by BAASB. Furthermore, the saturation kinetics (Fig 4) are entirely consistent with this suggestion. Residues modified so far with other similar reagents (2-4) are not methionyl residues i.e. Tyr-248 and Glu-270. In those cases the alkylating moiety was located in close proximity to the scissile bond of a normal substrate. Therefore it, would most likely interact with a residue that presumably functions in the catalytic step.

The present chemical modification differentiates to a certain degree the two types of activity that this enzyme possesses. Thus, the peptidase activity decreases when assayed at standard conditions, to a greater extent than the esterase activity. Although additional investigation is required to locate the precise site of alkylation and the possible differential role of the methionyl residue, the hydrophobic nature of the reagent employed in this study and the fact that the reactive side chain is carried on that part of the molecule, tempts to suggest that the methionyl residue modified

is part of the substrate recognition site in the hydrophobic pocket of CPB (13)(4). This possibility is reminiscent of the role of methionine-192 in Chymototrypsin (14) where it appears to function as a flexible hydrophobic lid on the substrate binding pocket.

Since benzylsuccinate (8) and its derivatives are also very potent inhibitors of carboxypeptidase A it is expected that the reagent BAASB could be of use in the investigation of carboxypeptidase A and should provide an additional means of comparing the active center of carboxypeptidases A and B.

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