CHEMICAL EVIDENCE FOR A FUNCTIONAL ARGININE RESIDUE IN CARBOXYPEPTIDASE B

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Modification of porcine carboxypeptidase B with phenylglyoxal at pH 7.9 results in a marked decrease of the activity toward the peptides hippurylarginine and $Z(Ala)_3$ and toward the ester hippurylphenyllactate. Analysis of the kinetics of the modified enzyme revealed that only the k_{cat} values have been changed while the Km values are essentially unchanged. On the other hand, the activity of the modified enzyme toward the basic ester, hippurylargininic acid is slightly increased, due to an increase in both k_{cat} and Km values. The inactivation correlates with the modification of about one arginyl residue. The possible role of this arginyl residue in the mechanism of action of carboxypeptidase B is discussed.

Chemical modifications have proved useful in showing the participation of a tyrosyl residue (which corresponds to tyrosine-248 in carboxypeptidase A) in the mechanism of action of porcine carboxypeptidase B (CP-B) (1)(2)(3). It was therefore hoped that other functional residues could be identified by the same approach. The specificity requirement of CP-B for a free carboxylate ion in the C-terminus of the substrate might be due to an ionic interaction with a positively charged lysyl or arginyl residue of the enzyme. In previous studies reagents which react with lysyl residues have failed to alter the activities of CP-B (3). A specific reagent for the modification of arginyl residues in proteins, phenylglyoxal (4), was therefore used in an attempt to identify this functional positive residue. The present report describes the effect of the modification of a arginyl residue of CP-B on the various activities of the enzyme.

Abbreviations: CP, Carboxypeptidase; HA, Hippuryl-L-arginine; HAA, Hippuryl-L-argininic acid; $Z(Ala)_3$, Carbobenzoxy-L-Ala-L-Ala-L-Ala; HPLA, Hippuryl-L-phenyllactic acid.

EXPERIMENTAL

Carboxypeptidase B (Code: COBC) was purchased from the Worthington Biochemical Corp.; the substrates HA and HPLA from Cyclo Corp.; HAA was either as described previously (1) or from Cyclo Corp.; $Z(Ala)_3$ from Miles-Yeda, Rehovot; Phenylglyoxal hydrate was purchased from K & K Lab. The ^{14}C-labeled phenylglyoxal was prepared from acetophenonecarbonyl-^{14}C (4), purchased from New England Nuclear. All other chemicals were of the best grade available. Buffers were extracted with 0.1% dithizone in CCl_4, to avoid contamination by adventitious metal ions. Water was glass redistilled.

Modifications were performed at 37° by a 1 hour incubation of a 0.15% solution of CP-B in 0.3M borate buffer pH 7.9, containing various concentrations of phenylglyoxal. The modified enzyme was separated from excess reagent by passing it through a short Biogel P-4 column, equilibrated with 0.05M borate buffer pH 7.9.

Kinetic experiments were performed at 25° $\pm$ 0.1, by using a Cary 16K recording spectrophotometer, and 3 ml of substrate solution in 1 cm cuvettes (HA and HAA) or 0.75 ml substrate solution in 0.1 or 0.2 cm light path cuvettes (HPLA and $Z(Ala)_3$) in a thermostated cell compartment. The hydrolyses of the substrates were followed as previously described (5) at pH 7.9 in 0.05M Tris - 0.1M NaCl buffers. k_{cat} and Km values were calculated from a least-square treatment of Lineweaver-Burk plots.

Amino acid analyses were performed on a Beckman-Unichrom amino acid analyzer. Zn^{++} concentrations were determined on a Varian-Techtron atomic absorption spectrophotometer, model AA5.

RESULTS

The effect of variation of the molar excess of phenylglyoxal on the esterase and peptidase activities of CP-B is shown in Fig. 1. With a 185-fold molar excess of reagent, the esterase activity towards HAA slightly increases, while the other 3 activities fall simultaneously to about 15% of the control

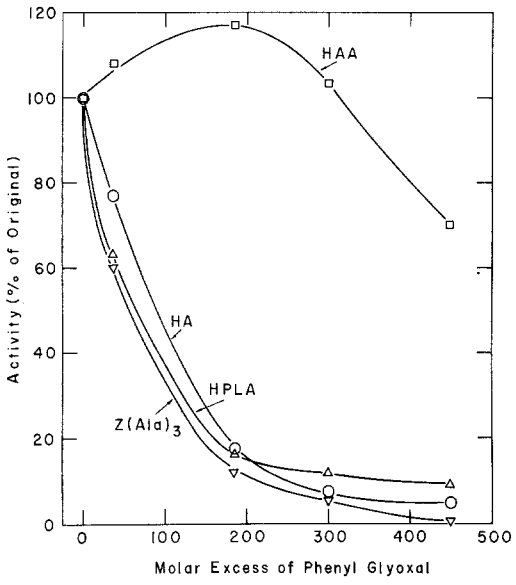

Fig. 1.

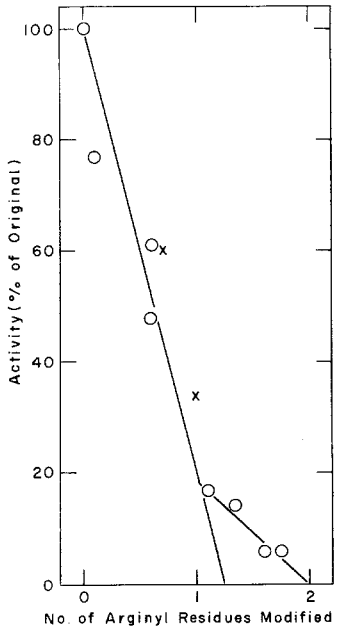

Fig. 2.

Figure 1. Effect of variation of the molar excess of phenylglyoxal on peptidase and esterase activities of CP-B. O——O HA; □——□ HAA; Δ——Δ HPLA; ∇——∇ $Z(Ala)_3$. CP-B (1.5 mg/ml) was incubated for 1 hour with the reagent at 37° in 0.3M borate buffer, pH 7.9.

Figure 2. Correlation of the activity of phenylglyoxal-modified CP-B toward HA with the number of arginyl residues modified. O, Determined by amino acid analysis; x, Determined by incorporation of ^{14}C-phenylglyoxal (10^5 cpm/μmole).

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and thereafter gradually decrease with increasing amounts of reagent. The modified CP-B elutes from Sephadex G-75 as a single symmetrical peak in the same position as that of the native enzyme.

Loss of activity correlates with the modification of about one arginyl residue as determined both by amino acid analysis and by incorporation of ^{14}C-phenylglyoxal (Fig. 2). No other differences were found between the amino acid compositions of the native and modified enzyme, which both contain 0.98g-atom of zinc.

The modification of the arginyl residue(s) of CP-B was found to be stable with time. Thus, modified CP-B which had 17% of the activity of native

TABLE I

KINETIC PARAMETERS FOR CP-B AND PHENYLGLYOXAL-MODIFIED CP-B[a].

Substrate	Native CP-B		Modified CP-B[b]		Modified CP-B[c]	
	Km mM	k_{cat} sec^{-1}	Km mM	k_{cat} sec^{-1}	Km mM	k_{cat} sec^{-1}
HA	0.26	158	0.31	112	0.32	27.6
HAA	0.037	395	0.068	370	0.109	595
$Z(Ala)_3$	8.5	27.8	8.1	16.0	7.8	4.4

a. pH 7.9, 0.05 M Tris - 0.1 M NaCl, 25°. The parameters were calculated from a least-squares treatment of Lineweaver-Burk plots.

b. Modified with a 30-fold molar excess of phenylglyoxal.

c. Modified with a 180-fold molar excess of phenylglyoxal.

CP-B toward HA, retained the same degree of inhibition when stored for 16 days at 4° in 50 mM borate buffer, pH 7.9.

Addition of the inhibitor 3-phenylpropionate prior to the modification could not prevent the activity changes even at concentrations as high as 0.3M. The non-basic substrate analog Z-L-Ala-D-Ala-L-Ala (up to 0.5M) had also little protective effect against the modification. Other known inhibitors of CP-B, e.g. argininic acid and ε-aminocaproic acid, could not be used due to their reaction with the modifying reagent.

The difference in response to modification between the activity toward HAA and the other substrates expressed itself in the kinetic properties of the modified CP-B (Table I). While the Km values for the three substrates, i.e. HA,

HPLA and $Z(Ala)_3$, remained essentially unchanged, the Km for HAA is increased three-fold. The k_{cat} values for the three substrates is reduced to a fraction of the k_{cat} of the native enzyme, whereas the k_{cat} value for HAA is increased by about 50%.

DISCUSSION

Modification of CP-B with a 140-fold molar excess of phenylglyoxal at pH 7.9, alters the activities of the enzyme. The changes in activity correlate with the loss of about one arginyl residue. Similar experiments using diacetyl as the modifying reagent, have implicated the participation of an arginyl residue in the peptidase activity of CP-A (6). The modification of CP-B with phenylglyoxal differentiates between the various types of activities of CP-B. Thus, toward the basic peptide, HA, and the non-basic ester and peptide substrates, HPLA and $Z(Ala)_3$, no change in Km was observed and the change in activity was only due to a parallel decrease in k_{cat}. On the other hand, a completely different behavior was observed for the basic ester substrate HAA: the Km is increased three-fold and more, while the k_{cat} is increased up to 50%. Hence, it would seem that modification of one arginyl residue in CP-B affects the binding (assuming that Km is a measure of binding) of the various types of substrates in different ways. A possible explanation is that productive binding of the three substrates, HA, HPLA and $Z(Ala)_3$ to the modified enzyme is abolished, the reduced k_{cat} reflecting the fraction of the unmodified enzyme and thus leaving the measured Km unchanged. However, a productive mode of binding of the basic ester, HAA, is preserved in the modified enzyme. This does not necessarily imply identical modes of binding of this ester to the native and modified CP-B. This explanation is in agreement with the suggested multiple substrate binding modes of CP-B (5) and of the homologous enzyme - CP-A (7).

The fact that 3-phenylpropionate did not prevent the loss of activity, suggests that the inhibitor binds to the enzyme in a way which does not interfere with modification of the arginyl residue essential for catalytic activity. This

means that this arginyl residue is not located in the "hydrophobic pocket" of the enzyme, which in CP-A (8) is known to be the binding site of the side chain of the C-terminal amino acid of the substrate and that of inhibitors, such as 3-phenylpropionate (8). One should therefore consider the possibility that this residue is located outside of the active site and that its modification leads to a conformational change which hampers enzymic activity. This, however, seems very unlikely because such a gross conformational change would be expected to cause a parallel decrease in all four activities of the enzyme, which was not observed. Furthermore, no change in the intrinsic fluorescence properties of the enzyme were observed upon modification.

An attractive hypothesis is that the arginyl residue modified is the one involved in "anchoring" the C-terminus portion of the substrate into the position needed for the cleavage. Examination of a space-filling model of the active site of CP-A indicated that the guanido group of Arg-145, the residue that is involved in binding the carboxylate of the peptide substrate (8), sticks outside of the hydrophobic pocket. Considering the possibility that CP-B and CP-A are homologous both in amino acid sequence and in the three-dimensional configuration (9)(10), one can offer an explanation for the lack of protection of 3-phenylpropionate against modification of arginyl residues in contrast to the protection that is seen on modification of the tyrosyl residue (1)(2). Apparently, the inhibitor binds at the "hydrophobic site" and will prevent reaction with residues located in that area, i.e. tyrosyl-248. On the other hand, since the guanido group of Arg-145 is not part of the protected area, the inhibitor can not prevent its modification. If this was the case, then one would expect to see similar behavior in CP-A. Indeed, 3-phenylpropionate protects against modifications of the tyrosyl-248 in CP-A and has only slight effect on the modification of an arginyl residue in CP-A upon treatment with diacetyl (6).

Experiments designed to verify the hypothesis concerning the binding modes of substrates as well as to identify the essential arginyl residue of CP-B are now under way.

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