THE EFFECTIVE USE OF AFFINITY CHROMATOGRAPHY FOR THE STUDY OF COMPLEX FORMATION OF BOVINE CARBOXYPEPTIDASE B WITH BASIC AND AROMATIC AMINO ACID ANALOGUES

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SUMMARY: The affinity chromatography method was applied to the study of the binding of carboxypeptidase B to basic and aromatic amino acid analogues. This method revealed that these two kinds of ligands occupy different sites on the enzyme and the affinity of its binding site(s) for aromatic substrates or inhibitors was profoundly influenced by the occupation of the other site(s) by ε-aminocaproic acid.

Bovine carboxypeptidase B (CPB) has been shown to bear considerable catalytic activity on the hydrolysis of typical substrates for carboxypeptidase A (CPA): hippuryl-*d*,*l*-phenyllactic acid and carbobenzoxyglycyl-L-phenylalanine (Z-Gly-Phe-OH) (1). In the course of kinetic investigation of the CPA-like function of CPB, it has been suggested that the inhibition of the CPB-catalyzed hydrolysis of Z-Gly-Phe-OH by ε-aminocaproic acid, a lysine analogue, is of uncompetitive type, indicating the apparent cooparative formation of the inactive ternary complex of CPB, with the aromatic substrate and ε-aminocaproic acid (2).

The present paper deals with the effective use of affinity chromatography to prove directly the formation of such ternary complexes.

Fig. 1 illustrates the possible molecular species of a given enzyme (E) within the bed of the matrix to which a specific ligand of the enzyme (S) is covalently fixed. As is obvious from the illustration, the possible effect of the presence of an unfixed ligand (I) on the migration of the enzyme through the column of the matrix should be one of the following three types,

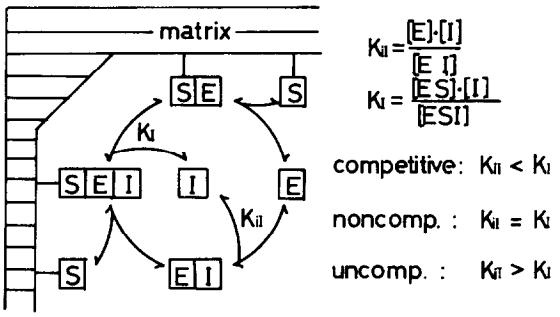

Fig. 1 Possible molecular species of a given enzyme within the affinity matrix. S: covalently fixed ligand to matrix, I: unfixed ligand.

1) COMPETITIVE TYPE EFFECT (Where (ESI) complex is less stable than (EI) complex.): In this case, the increase of the concentration of (I) induces the increase of the fraction of the enzyme in the mobile phase ([E] + [EI]) and the retardation of the enzyme on the column is reduced by the presence of (I).

2) NONCOMPETITIVE TYPE EFFECT (Where the stability of (ESI) complex is the same as that of (EI) complex, or the binding of (I) to the enzyme does not affect the enzyme affinity for (S).): In this case, the retardation of the enzyme is not affected by the presence of (I), because the fraction of the enzyme in the mobile phase is not affected by (I).

3) UNCOMPETITIVE TYPE EFFECT (Where (ESI) complex is more stable than (EI) complex.): In this case the increase of the concentration of (I) causes the decrease of the fraction of the enzyme in the mobile phase, and thus, the retardation of the enzyme is enhanced by (I).

MATERIALS AND METHODS

Bovine pancreatic CPB and CPA were prepared according to the methods of Wintersberger *et al.* (1) and Cox *et al.* (3), respectively.

Two kinds of substrate analogue-coupled matrices were prepared by coupling the azide of CM-Sephadex (C 50, capacity: 4.5 ± 0.5 meq/gm) with

ε-aminocaproyl-D-arginine or ε-aminocaproyl-D-phenylalanine (4). By amino acid analysis, 0.49 mmole of arginine and 0.65 mmole of phenylalanine were found per gram of each dry matrix, respectively.

Protein concentration was determined by measuring the optical density at 280 mμ (1) or by the Folin-Lowry's method (5).

RESULTS AND DISCUSSION

As shown in Fig. 2, CPB was adsorbed to the column of the arginine-coupled matrix and was dissociated from the column with ε-aminocaproic acid, a competitive inhibitor of the CPB-catalyzed hydrolysis of benzoylglycyl-L-arginine (Bz-Gly-Arg-OH) (2,6). The following three control experiments indicated that the nonspecific interactions such as ionic ones play an insignificant role as far as the present "affinity chromatography" is concerned:

1) CPB itself was not markedly retarded by the original CM-Sephadex under the same conditions as those shown in the legend of Fig. 2.

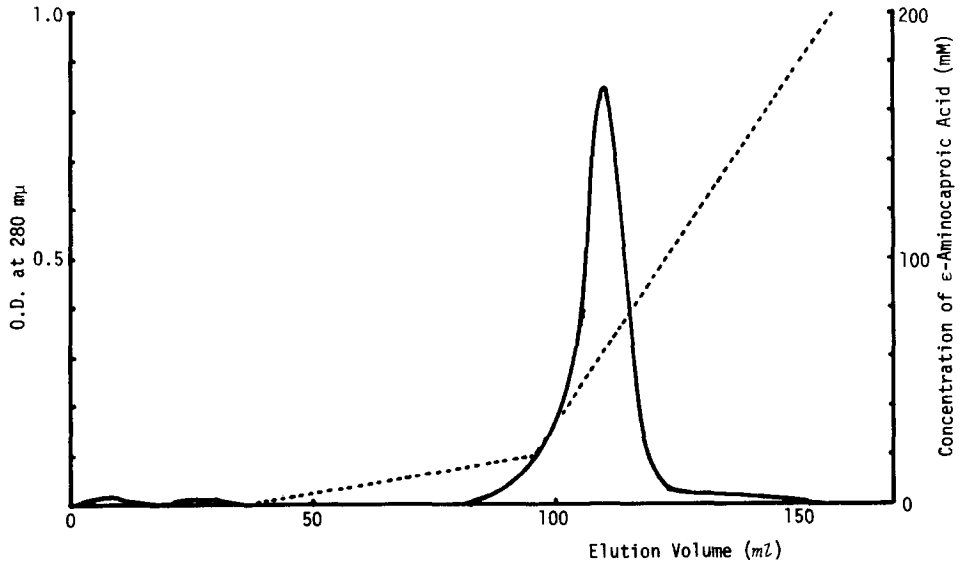

Fig. 2 Elution of CPB from ε-aminocaproyl-D-arginine-coupled CM-Sephadex column by ε-aminocaproic acid. Amount of enzyme applied: 10 mg, column size: 1.0 cm x 11.2 cm, solid line: optical density at 280 mμ, eluant: 10 mM sodium phosphate buffer containing 100 mM NaCl, a gradient from 0 to 200 mM ε-aminocaproic acid was applied as indicated by the dotted line, pH 7.05 throughout.

2) CPB was retained on the column of the arginine-coupled matrix even in the presence of 1M NaCl.

3) CPB was not eluted from the arginine-coupled matrix with 100 mM γ-aminobutyric acid, which is a homologue of ε-aminocaproic acid with poor specific interaction with CPB (7), under the same conditions as those shown in the legend of Fig. 2.

The dissociation of CPB from the column shown in Fig. 2 is therefore interpreted in terms of the competition of ε-aminocaproic acid with the acyl-D-arginine group of the matrix in the formation of binary complex with CPB.

CPB was not markedly retained on the column of the phenylalanine-coupled matrix even at low ionic strength in a pH range of 7-9. But by the addition of such low concentration of ε-aminocaproic acid as 20 μM to the eluant, however, the enzyme was tightly adsorbed to the phenylalanine-coupled matrix.

Fig. 3 shows the results of chromatography of CPB on the column of the matrix, where the concentration of ε-aminocaproic acid in the eluant was decreased exponentially. The enzyme was first eluted from the column when the

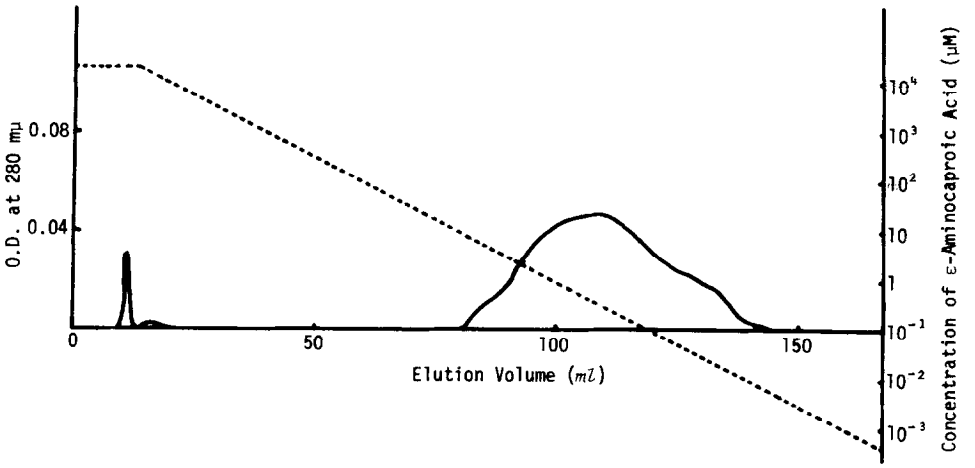

Fig. 3 Elution of CPB from ε-aminocaproyl-D-phenylalanine-coupled CM-Sephadex column with the effect of decreasing concentration of ε-aminocaproic acid. Amount of enzyme applied: 5.0 mg, column size: 1.0 cm x 6.8 cm, solid line: optical density at 280 mμ, eluant: 10 mM sodiun phosphate buffer containing 100 mM NaCl, an exponential gradient from 20 to +0 mM ε-aminocaproic acid was applied as indicated by the dotted line, pH 7.05 throughout.

concentration of the lysine analogue was decreased approximately to 10^{-1} μM. The order of this concentration is in reasonable agreement with the kinetic inhibition constant of ε-aminocaproic acid towards the CPB-catalyzed hydrolysis of Z-Gly-Phe-OH (5×10^{-1} μM obtained under the similar conditions to those of the chromatography). The results lead to one of the following two possible interpretaions:

1) The complex of CPB and the lysine analogue bears a strong affinity for the matrix coupled with the aromatic substrate analogue.

2) The adsorption of the enzyme to the aromatic column is stabilized by ε-aminocaproic acid.

Is either case, the cooperative formation of a ternary complex of CPB with the aromatic substrate analogue and the basic amino acid analogue was verified by these results. The distinct difference in the inhibition constants of ε-aminocaproic acid towards CPB-catalyzed hydrolysis: 5×10^{-1}μM for Z-Gly-Phe-OH and 4.1×10^{-1} mM for Bz-Gly-Arg-OH (2), can also be explained by the difference in the types of inhibition in these two cases, namely, competitive type for the basic substrate and uncompetitive type for the aromatic substrate.

The competition of β-phenylpropionic acid, a phenylalanine analogue, with the aromatic substrate analogue fixed to the matrix was also examined. As shown in Fig. 4, CPB, cooperatively adsorbed to the column of phenylalanine-coupled matrix in the presence of ε-aminocaproic acid, was eluted with β-phenylpropionic acid. The result suggests the formation of ternary complex of CPB with ε-aminocaproic acid and β-phenylpropionic acid in the mobile phase.

The formation of the ternary complexes mentioned above justifies to postulate that CPB possesses at least two binding subsites : one for basic amino acid residues and another for aromatic or hydrophobic ones, and that the subsites mutually interact, probably through conformational changes, as exemplified by the cooperative binding of the ligands to the enzyme. The kinetic results elucidating the role of these subsites in the catalytic mechanism will be published elswhere.

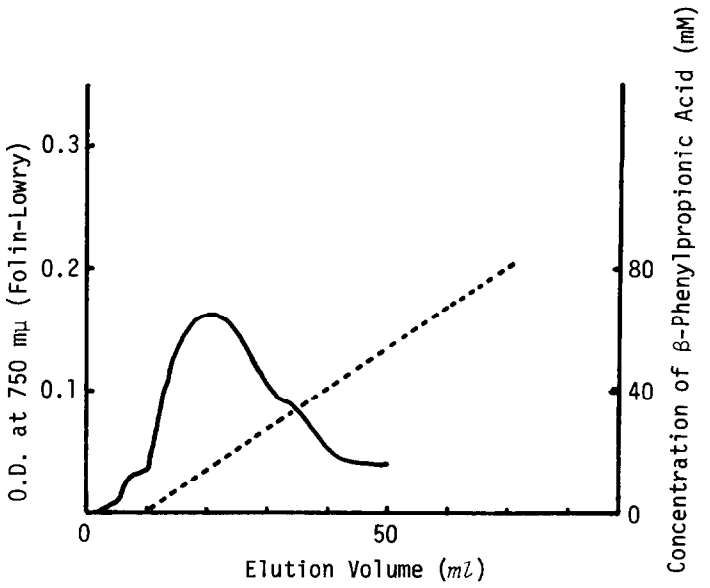

Fig. 4 Elution of CPB from ε-aminocaproyl-D-phenylalanine-coupled CM-Sephadex column with the effect of increasing concentration of β-phenylpropionic acid. Amount of enzyme applied: 5.0 mg, column size: 1.0 cm x 6.8 cm, solid line: optical density at 750 mμ (Folin–Lowry), eluant: 10 mM sodium phosphate buffer containing 100 mM NaCl and 20 mM ε-aminocaproic acid was used as the starting buffer and a linear gradient from 0 to 80 mM of Na β-phenylpropionate was applied as indicated by the dotted line, while the concentration of NaCl as decreased from 100 to 20 mM to maintain the constant Na^+ concentration in the eluant, pH 7.05 throughout.

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