POSITIVE COOPERATIVITY IN THE PORCINE CARBOXYPEPTIDASE B-CATALYZED HYDROLYSIS OF NEUTRAL PEPTIDE SUBSTRATES*

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Summary

Sigmoid saturation curves indicative of positive cooperativity have been observed for the porcine carboxypeptidase B-catalyzed hydrolysis of neutral N-acyl dipeptides. At 100 mM substrate, benzoylglycylphenylalanine was hydrolyzed at the same rate as benzoylglycyllysine. Slopes of 1.6-2.0 for the linear Hill plots indicated at least two ligand binding sites.

The pancreatic peptidase CPB*** (peptidyl-L-lysine hydrolase, EC 3.4.2.2) hydrolyses peptides and esters of the type RCO-NHCHR$_1$COOH and RCO-OCHR$_1$COOH where RCO is a protected amino acid or peptide and R$_1$ is a basic side-chain (1). Neutral molecules where R$_1$ is benzyl are also substrates for the bovine (2) and dogfish (3) enzymes, but they have been considered not to be substrates for the porcine enzyme (1,4,5). We report here studies on the hydrolysis of several neutral N-protected peptides by porcine CPB. Other very recent papers have also reported the

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*** Abbreviations: CPB, carboxypeptidase B; CPA, carboxypeptidase A; BGL, α-N-(benzoylglycyl)-L-lysine; BGP, benzoylglycyl-L-phenylalanine; BGN, benzoylglycyl-L-norleucine; BGL(Ac), α-N-(benzoylglycyl)-ε-N-acetyl-L-lysine; BAP, benzoyl-L-alanyl-L-phenylalanine; ZGP, benzyloxycarbonylglycyl-L-phenylalanine.

hydrolysis of neutral esters (6,7) and peptides (7) by porcine CPB.

Materials

BGL (from water-dioxane, m.p. 235-237°, lit. m.p. 236-238° (8)) was prepared from benzoylglycine and ε-N-benzyloxycarbonyl-L-lysine methyl ester (9) using dicyclohexylcarbodiimide (10) followed by saponification and hydrogenolysis. BGL(Ac) (m.p. 107-110° dec, $[\alpha]_D^{24}$ +8.9° (1, HOAc)) was obtained by leaving BGL in methanol containing 2 equivalents of acetic anhydride (11) for 30 min and adding ether. The product could be quantitatively (amino acid analyzer) converted to BGL by boiling in N HCl for 1 min. BGN (m.p. 138-139°), BGP (m.p. 146-147°), ZGP (m.p. 127.5-128.5°, lit. m.p. 125-126° (8)) and BAP (m.p. 143-144°, $[\alpha]_D^{23}$ +27.1° (1, EtOH)) were prepared from the N-protected amino acid and the amino acid ethyl ester using N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (12) followed by saponification, and crystallized from ethyl acetate-ligroin. The BAP is considered to have been optically pure on the basis of the demonstration by us using the amino acid analyzer that the EEDQ-mediated coupling of benzoyl-L-alanine and ε-N-benzyloxycarbonyl-L-lysing methyl ester followed by saponification and hydrogenolysis gave a benzoylated L,L-dipeptide containing <1% of the diastereomer. Peptides were subjected to t.l.c. on silica gel GF-254 (Merck) using n-butanol-acetic acid-water (4:1:1) as solvent. All peptides gave only one spot when the plates were examined for u.v. fluorescence quenching or stained with ninhydrin or chlorine-starch/iodide (13). They also gave the expected amino acid ratios after acid hydrolysis.

Methods

The enzyme, CPB-COBC from Worthington, was dissolved in

0.1 M NaCl and stored at -20°. Enzyme activity was determined by measuring the amino acid released, using the short column of a Beckman model 120B amino acid analyzer. Incubation was carried out in 0.1 M N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)-0.2 M NaCl, pH 8.0, at 27°C. Enzyme was added at zero time and aliquots were removed from the reaction mixture at fixed time intervals and pipetted into 20% sulfosalicylic acid to terminate the reaction. Enzyme concentration was determined by measuring the absorbance at 278 nm using a molar extinction coefficient of 7.4×10^4 (4) and assuming a molecular weight of 34,300 (4).

Results

Fig. 1 shows the effect of substrate concentration on the rates of hydrolysis of the three neutral peptides of benzoylglycine

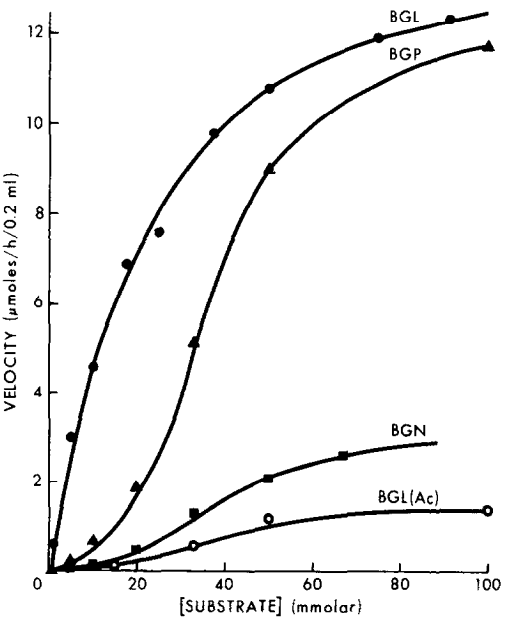

Fig. 1. Effect of substrate concentration on the initial rates of hydrolysis of dipeptides of benzoylglycine by bovine CPB at 27°C. [E] = 7.47×10^{-8} M for BGL, BGP and BGN; 7.47×10^{-6} for BGL(Ac).

Also included for comparison purposes are data for the hydrolysis of the 'normal' basic substrate BGL. It is seen that in contrast to the curve for BGL, which is the usual one observed for substrates exhibiting Michaelis-Menten kinetics, the curves for the neutral substrates are all S-shaped, similar to those found for allosteric enzymes displaying positive cooperative behaviour (14). Moreover, for BGP, the activation phenomenon was so pronounced that at 100 mM substrate, the rate of cleavage was nearly the same as the rate for the hydrolysis of BGL. At 1 mM substrate, the ratio of rates BGP/BGL was 1/40. In the presence of an equal concentration of BGL, the hydrolysis of BGP was almost totally inhibited. It is also to be noted that despite V_{max} values varying by as much as three orders of magnitude, $X_{0.5}$ (14), the ligand concentration at $V_{max}/2$, was about the same, 40 mM for the three neutral substrates.

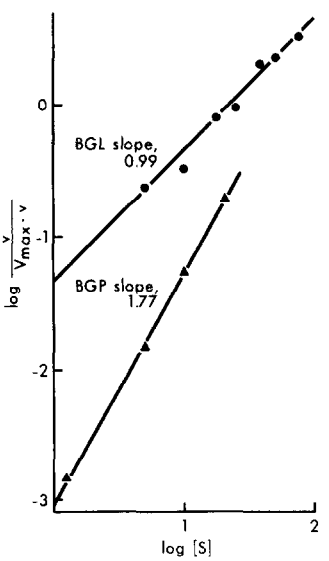

Fig. 2. Hill plots of some of the data in Fig. 1, for [S] <40 mM. Slope was 1.57 (intercept at -2.84) for BGN, and 1.99 (intercept at -3.27) for BGL(Ac). V_{max} values (μmol/h/0.2 ml) used in the calculations: BGL, 15.6, obtained from a plot of v/[S] versus [S]; BGP, 12.0; BGN, 3.0; BGL(Ac), 1.5.

When Hill plots were constructed from the data in Fig. 1 using V_{max} values estimated from the curves, the neutral substrates gave lines with slopes between 1.5 and 2.0 in contrast to the basic substrate which gave a line of slope 0.99 (Fig. 2). The slopes in excess of one are indications of the participation of at least two substrate binding sites (14).

Similar sigmoid velocity-substrate curves were obtained ($E = 6.19 \times 10^{-8}$ M) for ZGP ($X_{0.5} \simeq 40$ mM) exhibiting substrate inhibition above 50 mM, and BAP ($X_{0.5} \simeq 7$ mM; $V_{max} = 5.5$ µmol/h/0.2 ml observed over the range 20-50 mM). Up to 50 mM substrate, ZGP was hydrolyzed at about 1/3 the rate for BGP. The slopes of the Hill plots for ZGP ([S] = 2-20 mM; estimated $V_{max} = 4$ µmol/h/0.2 ml) and BAP ([S] = 0.5-5.0 mM) were 1.74 and 1.60 respectively.

Discussion

The results demonstrate unequivocally that neutral N-acylated peptides are substrates for CPB. The hydrolysis of BGP observed in this work could not have been due to contamination of the enzyme by CPA because over the same substrate concentration range, the hydrolysis of BGP by CPA follows a different kinetic pattern, The Lineweaver-Burk plot for the latter curved downwards (15) while that obtained from the data presented here curved upwards (16). Moreover, the hydrolysis of BGP by CPB was prevented by BGL. Failure to observe hydrolysis of BGP by porcine CPB in earlier experiments (4) could have been due to the much lower substrate concentration used, since the activation becomes pronounced only at [S] > 20 mM.

This is the first report of substrate activation for CPB. Sigmoid curves have been observed for the hydrolysis of an ester by trypsin (17) and CPA (18). BGP (15) and ZGP (19) act as activators of their own hydrolysis by CPA, the latter also

acting as an inhibitor (20). The activation of CPB by the substrates reported here seems to be a general property for neutral N-acyl dipeptides, since it was displayed by benzoylglycyl, benzoylalanyl and benzyloxycarbonylglycyl dipeptides. The lower $X_{0.5}$ value of 7 for the alanyl peptide compared to the similar $X_{0.5}$ values of 40 for the four glycyl peptides would indicated a participating role for the side-chain methyl group in the binding of the former to the enzyme.

On the basis of affinity chromatography, two mutually interacting binding sites, one for neutral substrates and one for basic substrates, have been suggested for CPB (21). The activation site responsible for the phenomena described here could be adjacent to or form part of the catalytic site, or it could be topologically distant from it as is the case for allosteric enzymes. The allosteric enzymes so far studied have displayed cooperative behaviour because of the presence of quaternary structure. There is, however, no evidence for the existence of sub-units in either CPB (1) or CPA (22). The term "autosteric" has been proposed by Koshland (23) for cases where adjacent or overlapping binding modes give rise to cooperative behaviour. Autosteric effects may be responsible for the kinetics observed here. Multiple modes of non-productive binding have previously been proposed in order to explain the anomolous kinetic behaviour of CPA (24). X-ray diffraction studies and model-building experiments of the enzyme surface involved with substrate recognition (25) have identified a secondary binding site down the cleft of the active site which could accommodate modifier molecules. On the basis of the proposed structural homology of CPA and CPB (26), it is tempting to propose a binding site for activator molecules

in CPB similar to that in CPA to explain the kinetic anomalies common to both enzymes. The effect of modifiers on the kinetic behaviour of CPB towards neutral and basic peptide substrates (16) will be reported elsewhere.

References

1. J.E. Folk, in The Enzymes (P.D. Boyer, Ed.), Academic Press, New York, 1971. Vol. III, p. 57.
2. E. Wintersberger, D.J. Cox and H. Neurath, Biochemistry, 1, 1069 (1962).
3. J.W. Prahl and H. Neurath, Biochemistry, 5, 4137 (1966).
4. J.E. Folk, K.A. Piez, W.R. Carroll and J.A. Gladner, J. Biol. Chem., 235, 2272 (1960).
5. E.C. Wolff, E.W. Schirmer and J.E. Folk, J. Biol. Chem., 237, 3094 (1962).
6. O.A. Roholt and D. Pressman, Eur. J. Biochem., 18, 79 (1971).
7. M. Sokolovsky and N. Zisapel, Biochem. Biophys. Acta, 250, 203 (1971).
8. K. Hofmann and M. Bergmann, J. Biol. Chem., 134, 225 (1940).
9. J.R. Coggins, R. Demayo and N.L. Benoiton, Can. J. Chem., 48, 385 (1970).
10. J.C. Sheehan and G.P. Hess, J. Amer. Chem. Soc., 77, 1067 (1955).
11. D.W. Thomas, B.C. Das, S.D. Gero and E. Lederer, Biochem. Biophys. Res. Commun., 32, 519 (1968).
12. B. Belleau and G. Malek, J. Amer. Chem. Soc., 90, 1651 (1968).
13. H.N. Rydon and P.W.G. Smith, Nature, 169, 922 (1952).
14. D.E. Koshland, in The Enzymes (P.D. Boyer, Ed.), Academic Press, New York, 1970. Vol. I, p. 342.
15. D.S. Auld and B.L. Vallee, Biochemistry, 9, 602 (1970).
16. G.J. Moore, Ph.D. Thesis, University of Ottawa, April, 1972.
17. C.G. Trowbridge, A. Krehbiel and M. Laskowski, Biochemistry, 2, 843 (1963).
18. E.T. Kaiser, S. Awazu and F.W. Carson, Biochem. Biophys. Res. Commun., 21, 444 (1965).
19. J.R. Whitaker, F. Menger and M.L. Bender, Biochemistry, 5, 386 (1966).
20. R. Lumry, E.L. Smith and R.R. Glantz, J. Amer. Chem. Soc., 73, 4330 (1951).
21. H. Akanuma, A. Kasuga, T. Akanuma and M. Yamasaki, Biochem. Biophys. Res. Commun., 45, 27 (1971).
22. I. Schecter and E. Zazepizki, Eur. J. Biochem., 18, 469 (1971).
23. D.E. Koshland and K.E. Neet, Ann. Rev. Biochem., 37, 359 (1968).
24. B.L. Vallee, J.F. Riordan, J.L. Bethune, T.L. Coombs, D.S. Auld and M. Sokolovsky, Biochemistry, 7, 3547 (1968).
25. J.A. Hartsuck and W.N. Lipscomb, in The Enzymes (P.D. Boyer, Ed.), Academic Press, New York, 1971. Vol. III, p. 1.
26. G. Reeck, K.A. Walsh, M.A. Hermodson and H. Neurath, Proc. Natl. Acad. Sci. USA, 68, 1226 (1971).