METALLOCARBOXYPEPTIDASES: A CADMIUM - CARBOXYPEPTIDASE B WITH PEPTIDASE ACTIVITY

Nava Zisapel and Mordechai Sokolovsky

Department of Biochemistry

The George S. Wise Center for Life Sciences

Tel-Aviv University, Tel-Aviv, Israel.

Received June 12, 1973

The replacement of zinc by a series of other metal ions (Co, Cd, Mn) resulted in enzymatically active carboxypeptidases both as peptidases and esterases. The effect of the metal replacement on the kinetic parameters varies for the various substrates. Cd-CPB, previously known for its lack of peptidase activity, shows enhenced activity as long as the substrate interact with four subsites (including the N-terminal blocking group). Kinetic measurements and chemical modification revealed differences in the nature of the residues necessary for the proper alignment along the active site between the Zn and Cd carboxypeptidases. A simple explanation of the data observed is based on the assumption that replacement of the metal affects the microenvironment of the active site.

Peptidase activity of carboxypeptidase B when assayed with hippuryl-L-arginine and hippuryl-L-lysine, is virtually lost by replacing Zn^{2+} with Cd^{2+} (1). Both metallocarboxypeptidases are active however towards the ester substrate hippurylargininic acid (1). The experiments to be reported were designed to discern whether the loss of peptidase activity by metal replacement is dependent on the substrate investigated (size and C-terminal amino acid) (2) (3) or whether the species of metal ion at the active site determines the magnitude of the specific activity as well as the specificity of the enzyme.

EXPERIMENTAL

Carboxypeptidase B (Code: COBC) was purchased from Worthington Biochemical Corp.; hippuryl-L-arginine, hippuryl-L-phenyllactate and

---

Abbreviations: CP, Carboxypeptidase; HA, Hippuryl-L-arginine; HAA, Hippuryl-L-argininic acid; HPLA, Hippuryl-L-phenyllactate; Z, Carbobenzoxy; Bz, Benzoyl; Pla, phenyllactate.

hippuryl-L-argininic acid from Cyclo Corp.; Z-Ala_3 from Miles-Yeda, Rehovot; Z-Ala_2-Phe, Z-Ala-Arg, Z-Ala_2-Arg were prepared as described previously (3). Z-Gly-Phe peptides were a gift from Dr. S. Blumberg and Dr. M. Fridkin. The synthesis of esters of phenyllactic acid will be described in a forthcoming communication.

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.

Apocarboxypeptidase. Apoenzyme was prepared by dialysis of 2×10^{-4}M carboxypeptidase against four changes of a 200-fold volume excess of 4×10^{-3}M 1,10-phenanthroline in 0.01M acetate buffer (pH 5.5) followed by dialysis vs four changes of acetate buffer (pH 5.5) and 2 changes of 0.1M NaCl-0.05M Tris buffer (pH 7.5).

Metallocarboxypeptidases. Apoenzyme samples (10^{-4}M) were dialyzed at 4° against two changes of a 100 fold volume excess of 10^{-3}M metal ion in 0.1M NaCl-0.05M Tris (pH 7.5). The amount of zinc present in the apoenzyme and in all of the metalloenzymes, was less than 0.06 g-atom of zinc per mole of enzyme, as determined by atomic absorption spectrometry. The amount of metal present in the reconstituted metalloenzymes, determined by atomic absorption spectrometry (Varian Techtron AA-5) was always in the range of 0.96 to 1.1 g-atom of metal per mole of enzyme.

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

A stock solution of enzyme (1×10^{-4}M) was diluted before each set of runs and kept at 25°. These solutions were used within 1 hr. Rate measurements were carried out spectrophotometrically at 25° in a Cary Model 16K recording spectrophotometer, with 3 ml of substrate solution in 1 cm cuvets in a thermostated cell compartment. The hydrolyses of hippuryl-L-argininic acid and hippuryl-L-phenyllactate were followed at 254 nm (4) (5). The concentration of CPB in these assay mixtures

TABLE I

STABILITY CONSTANTS[a] OF METALLOCARBOXYPEPTIDASE B.

Metal ion	K dis	pK dis
Mn^{2+}	1.0×10^{-4}	4.0
Co^{2+}	1.25×10^{-5}	4.9
Zn^{2+}	1.5×10^{-6}	5.8
Cd^{2+}	7.7×10^{-7}	6.1

[a]Determined by equilibrium dialysis in 0.1M NaCl-0.05M Tris, pH 7.5, 25°.

were 2.5×10^{-9}M and 2×10^{-8}M, respectively. The hydrolyses of all other peptides were followed at 225 nm, using 0.7 ml of substrate solution in 0.2 cm light path cuvets. All substrates were dissolved in 0.05M Tris-0.1M NaCl buffers. The hydrolysis of all other esters was followed in the pH-stat in 0.001M Tris-0.09M NaCl. Metalloenzymes were assayed in the presence of 1 mM metal ion. The values of kcat and Km were calculated from Lineweaver-Burk plots. The substrate concentration was varied approximately 5-7 fold both below and above the value of Km.

RESULTS

The apparent stability constants at 25°, for the complexes of CPB with Mn^{2+}, Co^{2+}, Zn^{2+} and Cd^{2+} determined by equilibrium dialysis at pH 7.5 are shown in Table I. The stability constant for the Cd-CPB was determined by competing the metal ion for Zn^{2+} and Co^{2+}. The stability constant was calculated from the ratio of the two types of metallocarboxypeptidase present at equilibrium. The same order of stability

constants was observed previously for its counterpart CPA (6). The evidence for the participation of a zinc ion in catalysis has been amply documented (7). The replacement of zinc by a series of other metal ions resulted in enzymatically active carboxypeptidases towards both peptide and ester substrates and is shown in Table II. Surprisingly, the cadmium enzyme which lacks activity towards hippuryl-L-arginine is highly active when assayed with Z-Ala_2-Phe. Hence, the hydrolysis of several N-blocked peptides and esters that differ in size and C-terminal amino acid were examined (Table III). The Cd enzyme is active as a peptidase towards tripeptides as does the Zn enzyme and possesses enhenced activity towards esters. The apparent pKa's calculated from the pH dependence of Km for the hydrolysis of hippurylargininic acid were 8.1 and 7.55 for the Zn and Cd-CPB respectively. Similarly, pKa's of 7.9 and 7.4 were obtained from the pH dependence of Km for the hydrolysis of Boc-Ala_2-Pla by Zn and Cd-CPB. A representative plot of the pH dependence of Km and kcat is shown in Fig. 1.

DISCUSSION

The replacement of zinc ion by Cd, Co and Mn ions resulted in enzymatically active carboxypeptidases towards peptide and ester substrates. The effect of metal replacement on the values of the kinetic parameters varies for the various substrates (Table II). While the Km values of the Zn, Co and Mn enzymes are essentially identical for the hydrolysis of hippurylarginine, the kcat values differ considerably e.g. kcat of the Co enzyme as was also reported earlier (1) is about 70 fold and 8 fold larger relative to the Mn-CPB and Zn-CPB respectively. The values of Km and kcat for the hydrolysis of the corresponding ester, hippurylargininic acid are changed in the same direction in the sequence: Cd > Mn > Zn > Co; the cobalt enzyme shows the lowest values of Km and kcat. On the other hand replacement of the metal is almost without any effect on the kinetic

TABLE II

KINETIC CONSTANTS FOR PEPTIDES AND ESTERS HYDROLYSIS BY METALLOCARBOXYPEPTIDASE[a]

Metallo-Carboxy-peptidase	Bz-Gly-Arg (HA)		Hippurylargininic acid (HAA)		$Z\text{-}Ala_2\text{-}Phe$		Hippurylphenyllactate (HPLA)	
	Km(M)	kcat (min^{-1})	Km(M)	kcat (min^{-1})	Km(M)	kcat (min^{-1})	Km(M)	kcat (min^{-1})
Mn^{+2}	2.6×10^{-4}	480	1×10^{-4}	1.9×10^{4}	6.4×10^{-4}	5.1×10^{3}	8.1×10^{-3}	3.3×10^{4}
Co^{+2}	2.6×10^{-4}	3×10^{4}	8.5×10^{-6}	9.1×10^{3}	6.4×10^{-4}	3.9×10^{3}	1.4×10^{-2}	1.2×10^{5}
Cd^{+2}	not hydrolysed		2.2×10^{-4}	4.1×10^{4}	8.2×10^{-4}	4.4×10^{3}	irregular	
Zn^{+2}	2.6×10^{-4}	5.3×10^{3}	3×10^{-5}	8.2×10^{3}	3×10^{-3}	6×10^{3}	7.5×10^{-3}	2.7×10^{4}

[a]Assays performed in 0.05M Tris - 0.1M NaCl (pH 7.5, 25°), containing 1×10^{-3}M metal ion.

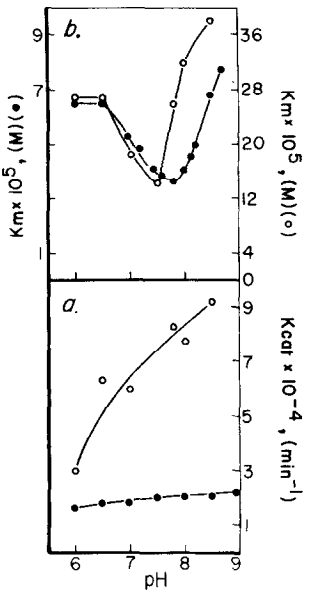

Figure 1 The pH dependence of kcat (a) and Km (b) for hippurylargininic acid hydrolysis by Zn-CPB (•) and Cd-CPB (o).

parameters for the hydrolysis of Z-Ala_2-Phe. Such data imply significant and probably varying participation of metal in the hydrolysis of peptide and ester substrates.

The data of Table III clearly demonstrates that the replacement of zinc by cadmium does not change the "specificity" of the enzyme, but does change the structural requirements for the substrate. Thus, Cd-CPB is active as a peptidase towards tripeptides as does the Zn-enzyme, but will not hydrolyze dipeptides. Significantly, the presence of glycyl residues in the peptides markedly alters the peptidase activity (Table III). Comparison of the alanyl and glycyl peptides indicates a possible role for the methyl side chain group for the proper alignment of substrate along the active site of the enzyme (3).

A simple explanation for the observed behavior of the cadmium enzyme could be based on the assumption that the presence of a bulky blocking group at the third position in the substrate prevents its binding at the productive binding site (3) and imposes non-productive binding.

TABLE III

HYDROLYSIS OF PEPTIDES AND ESTERS BY Cd-CPB

Substrate	Relative Rate % of rate of Zn-CPB
Bz-Gly-Arg[a]	0
Z-Ala-Arg[a]	0
Z-Ala-Ala-Arg[a]	130
Z-Gly-Phe[b]	0
Z-Ala-Phe[b]	0
Z-Ala-Ala-Ala[c]	120
Z-Ala-Ala-Phe[b]	140
Z-Gly-Gly-Phe[a]	<5
Bz-Gly-Phenyllactate[a]	40
Boc-Ala-Ala-Phenyllactate[b]	410
Bz-Gly-Argininic acid[a]	340

Assays performed in 0.05M Tris - 0.1M NaCl (pH 7.5, 25°) with: [a] 10^{-3}M substrate; [b] 5×10^{-3}M substrate; [c] 1×10^{-2}M substrate.

This hypothesis is supported by the observation that hippuryl-L-arginine which is not a substrate for Cd-CPB will inhibit competitively the hydrolysis of other peptide and ester substrates by the cadmium enzyme. A similar observation has been reported by Folk et al. (1). The effect of the blocking group could be related to its size, hydrophobicity or both. Internal chain extension by one alanyl residue, moving the blocking group to the fourth position, allows the proper alignment of the substrate at the active site and probably imposes more contraints on its binding so that some of the nonproductive binding forms are obviated and catalysis is observed. The changes in the structural requirements effected by the

replacement of zinc by cadmium are also manifasted in the shifts observed in the pKa's. Thus, additional contacts between the metal ion and the active site region could result in changes in the pKa's associated with binding, catalysis or both. The assumption that due to their different geometries the various metals might affect differently the environmental conditions of the active site (8), could satisfactorilly account for the above observation. Comparison of the nitration (9) of Zn and Cd-CPB at pH 8.0 was then carried out. Nitration occurred in both enzymes, but was twice as rapid for the Cd-CPB. This might indicate that the micro-environment of the tyrosyl residue at the active site, recently shown to interact with the metal (10), has been changed so that nitration is facilitated. It is not clear at this stage if the same tyrosyl residue is nitrated in both cases; structural and kinetic studies now in progress, should help to clarify the problem.

REFERENCES

1. Folk, J.E., Wolff, E.C. and Schirmer, E.W. J. Biol. Chem. 237, 3100-3104 (1962).
2. Zisapel, N. and Sokolovsky, M. Biochem. Biophys. Res. Commun. 46, 357-361 (1972).
3. Zisapel, N., Kurn-Abramowitz, N. and Sokolovsky, M. Eur. J. Biochem. (1973) in press.
4. Wolff, E.C., Schirmer, E.W. and Folk, J.E., J. Biol. Chem., 237, 3094-3099 (1962).
5. McClure, W.O., Neurath, H. and Walsh, K.A. Biochemistry 3, 1897-1901 (1964).
6. Coleman, J.E. and Vallee, B.L., J. Biol. Chem. 236, 2244-2249 (1961).
7. Folk, J.E. in The Enzymes (P.D. Boyer, ed. 3rd edition) Academic Press 3, 57-77 (1971).
8. Vallee, B.L. and Williams, R.J.P. Proc. Natl.Acad. Sci. U.S.A. 59, 498-502 (1968).
9. Sokolovsky, M., Eur. J. Biochem. 25, 267-273 (1972).
10. Shaklai, N., Zisapel, N. and Sokolovsky, M. Proc. Natl. Acad. Sci. U.S.A. (1973) in press.