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ACTIVATION AND INHIBITION OF CARBOXYPEPTIDASE G_1 BY DIVALENT CATIONS

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SUMMARY

The metal requirements of carboxypeptidase G_1, an exopeptidase derived from *Pseudomonas stutzeri* and active against glutamyl- and aspartyl-terminal peptides, have been investigated. When the enzyme is rendered metal-free by incubation with the chelating resin, Chelex 100, the resulting inactive enzyme is re-activated only by Zn^{2+}, which produces a progressive increase in hydrolytic activity over the concentration range 10^{-7}–$5 \cdot 10^{-5}$ M. Prior addition of heavy metals such as Co^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, or Mn^{2+} to the metal-free enzyme inhibits reconstitution of the active Zn^{2+} complex. Addition of these inhibitory cations to the fully constituted Zn^{2+}–enzyme complex produces no inhibition. Atomic absorption analysis of the active enzyme indicates the binding of four atoms of Zn^{2+} per enzyme molecule, or two atoms to each of the two enzyme subunits.

There was a partial irreversible loss of carboxypeptidase G_1 activity following brief exposure to either a metal binding resin or low pH. In both instances, bovine serum albumin (10 mg/ml) substantially protected the enzyme from inactivation.

INTRODUCTION

Numerous bacterial and mammalian enzymes have been described which require Zn^{2+} for activity[1]. In particular, the studies of bovine carboxypeptidase A (peptidyl-L-amino acid hydrolase, EC 3.4.2.1), a single chain protein of 34 000 molecular weight which binds one atom Zn^{2+} per enzyme molecule, have elucidated the importance of the metal ion in determining enzyme activity and substrate specificity[2]. This enzyme possesses C-terminal exopeptidase activity *versus* a broad range of substrates with particular affinity for aromatic C-terminal peptides.

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More recently, a Zn^{2+}-activated bacterial exopeptidase, carboxypeptidase G, has been described which specifically cleaves C-terminal glutamyl residues from peptide linkage[3]. The loosely bound Zn^{2+} cofactor of this enzyme was readily removed by Sephadex G-100 gel filtration, with loss of enzyme activity.

We have recently isolated a carboxypeptidase, named carboxypeptidase G_1, (ref. 4) which hydrolyzes C-terminal glutamyl and aspartyl residues, and which displays a high affinity for folic acid and its derivatives. This enzyme, which possesses antitumor activity *in vitro* and *in vivo*[5], differs from carboxypeptidase G in its ability to cleave aspartyl-terminal peptides, its greater affinity for folates, and its failure to bind to DEAE-cellulose in dilute buffer at pH 7.3. (ref. 4) Carboxypeptidase G_1, a dimeric protein of 92 000 molecular weight, is also activated by Zn^{2+}. This paper describes the stoichiometry and affinity of Zn^{2+} binding to carboxypeptidase G_1, and defines the effect of substitution of various other heavy metals on enzyme activity. In addition, evidence is presented for the stabilization of metal-free carboxypeptidase G_1 by bovine serum albumin. The implications of these findings for the use of this enzyme as an antineoplastic agent are discussed.

METHODS

Reagents

Chelex 100 resin (200–400 mesh) was purchased from BioRad. Dithizone (diphenylthiocarbazide) and 1,10-phenanthroline were purchased from Pfaltz-Bauer. Tris-Base "Ultra-Pure" grade, and crystalline bovine serum albumin, were from Schwartz-Mann. Divalent metals, including Zn^{2+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Mg^{2+}, and Ca^{2+} were purchased from Fisher as the chloride salts, "certified" grade, and contained less than 0.006% heavy metal contamination, with the following exceptions: Co^{2+} (0.03% Ni^{2+}); Ni^{2+} (0.01% Pb^{2+}); Mn^{2+} (0.02% Zn^{2+}); and Cu^{2+} (0.015% Fe^{2+}). Other sources of materials were previously described[4].

Preparation of glassware

Polyethylene containers were used where ever possible. Plastic pipettes were used throughout. All glassware was acid washed[6]. Cuvettes were soaked in a cleaning solution of equal parts of 3 M HCl and absolute ethanol for 18 h prior to rinsing with 0.001% dithizone in carbon tetrachloride, and finally with double distilled water.

Preparation of buffers and metal solutions

Double distilled water was used throughout. Buffers were extracted with 0.001% dithizone in carbon tetrachloride, and the solutions clarified by further extractions with carbon tetrachloride. Metal solutions were prepared and stored in 20-ml plastic vials; the salts were dissolved in 0.1 ml of 0.1 M HCl and appropriate dilutions made to give final concentrations in the range of 10^{-1} to 10^{-4} M.

Preparation of metal-free carboxypeptidase G_1

In a typical experiment, 1.0 ml carboxypeptidase G_1, specific activity 200–600 units/mg of protein and activity 25–100 units/ml, was added to a 2-ml suspension containing Chelex 100 resin, 1 g; Tris–HCl (pH 7.3), 1.5 mmoles; and where indicated bovine serum albumin, 30 mg. The suspension was then swirled gently in a metabolic

shaker at 25 °C for 2 h. The suspension was centrifuged for 10 min at 5000 rev./min and the supernatant saved for experimental use.

Carboxypeptidase G_1 enzyme activity was assayed as previously described[4]. Briefly, 1–10 μl of an enzyme solution was added to an assay mixture containing methotrexate, 60 nmoles; Tris–HCl (pH 7.3), 0.05 mmole; and $ZnCl_2$, 0.1 μmole, in a total volume of 1 ml at 37 °C. For studies of enzyme activation and inhibition by metal ions, Zn^{2+} was not included in the basic assay solution, and specific metal addition was made as described in the individual experiments. The rate of hydrolysis of methotrexate was determined by following the decrease in absorbance at 320 nm. At this wavelength, a ΔA of 8.3 absorbance units·mmole^{-1}·l was determined for the hydrolysis of methotrexate[3].

Atomic absorption analysis

The molar Zn^{2+} content of carboxypeptidase G_1 was determined by the single spike height method[7]. $1.03 \cdot 10^{-5}$ M carboxypeptidase G_1 of specific activity 600 units/mg of protein, yielding one major active band of protein on disc electrophoresis[4], was dialyzed for 48 h *versus* three changes of a 1000-fold volume of metal-free 0.01 M Tris–HCl (pH 7.3) prior to analysis by atomic absorption.

Enzyme acidification

The activity of a solution containing 5–15 units/ml carboxypeptidase G_1 (specific activity 250 units/mg), 0.01 M Tris–HCl (pH 7.3) and 10^{-4} M $ZnCl_2$, was determined by the standard assay. 2 M HCl was then added slowly with stirring to lower pH to the appropriate level. A 5–10-μl aliquot of the acidified carboxypeptidase G_1 solution was then assayed in the standard pH 7.3 solution for activity. After 15 min, the pH of the enzyme solution was returned to 7.3 by addition of 2 M NaOH, and an aliquot of the solution was again assayed for residual enzyme activity. In specified experiments, bovine serum albumin (10 mg/ml) was included in the enzyme solution.

RESULTS

During enzyme purification it was observed that a dialyzable factor was required for enzyme activity and stability. Activity was gradually lost when carboxypeptidase G_1 was dialyzed against metal-free 0.01 M Tris–HCl buffer, pH 7.3 (Table I).

TABLE I

STABILITY OF CARBOXYPEPTIDASE G_1 DURING DIALYSIS

3 ml of carboxypeptidase G_1 (4 units/ml) was dialyzed at 4 °C for 24 h against three changes of 100 ml of 0.01 M Tris–HCl (pH 7.3).

Additions to dialysate	*Activity remaining after 24 h (% of initial)*
None	66
1,10-Phenanthroline, 10^{-5} M	43
1,10-Phenanthroline, 10^{-4} M	3
Zn^{2+}, 10^{-5} M	100
Zn^{2+}, 10^{-4} M	100

The addition of the chelating agent, 1,10-phenanthroline, to the dialysate led to a greater loss of activity. As the assay of enzyme activity was performed in the presence of 10^{-4} M Zn^{2+}, it was apparent that the loss of activity could not be reversed by re-exposure to Zn^{2+}. However, when carboxypeptidase G_1 was dialyzed against a Tris buffer solution containing 10^{-5} M Zn^{2+}, no loss of activity was observed.

Metal-free carboxypeptidase G_1 was prepared by incubation of the enzyme with Chelex 100 resin. As previously reported, during incubation with Chelex the enzyme gradually lost all activity over a 2-h period, as judged by assay in the absence of Zn^{2+}, but full activity was restored by adding Zn^{2+} to the assay solution[4]. The reconstitution of active enzyme, presumably as a carboxypeptidase G_1–Zn^{2+} complex, was concentration dependent, with 50% of activity restored at $2 \cdot 10^{-6}$ M (Fig. 1).

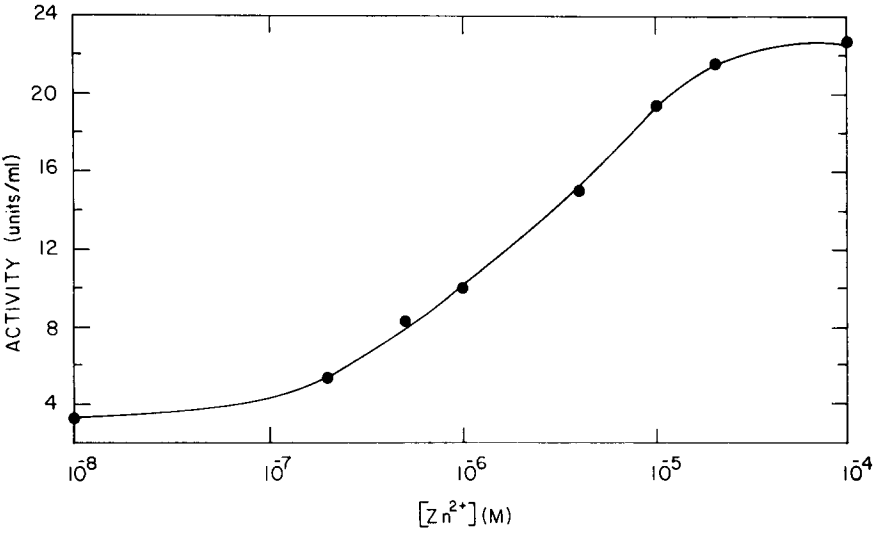

Fig. 1. Re-activation of metal-free carboxypeptidase G_1 by Zn^{2+}. 72 units of enzyme were added to 3 g Chelex resin which had been equilibrated with 2 ml of 0.05 M Tris–HCl (pH 7.3), and 0.83 M NaCl, and the volume was brought to 3 ml with water. The suspension was incubated at 25 °C for 2 h. The supernatant was removed as in Fig. 1, and a 2-μl aliquot was then assayed for hydrolytic activity in the presence of increasing concentrations of Zn^{2+}, as described in Methods.

Attempts to demonstrate activation of metal-free carboxypeptidase G_1 by prolonged incubation with stoichiometric quantities of Zn^{2+}, 10^{-9}–10^{-11} M, were uniformly unsuccessful.

The order of addition of Zn^{2+} and substrate to metal-free enzyme had no effect on activity. Prior addition of substrate to the enzyme did not prevent subsequent full activation of the metal-free enzyme by Zn^{2+}.

Enzyme content of Zn^{2+} was determined by atomic absorption analysis following a 48-h dialysis against metal-free buffer. Zn^{2+} content was 3.80 gatoms per mole carboxypeptidase G_1, a ratio consistent with the binding of two atoms of metal ion for each of the two enzyme subunits[4]. However, since Zn^{2+} was included in all buffers during the process of enzyme purification, it can not be stated with certainty that Zn^{2+} is the metal found in the "native" enzyme.

Addition of bovine serum albumin to the Chelex incubation suspension was

TABLE II

STABILIZATION OF METAL FREE CARBOXYPEPTIDASE G_1 BY ALBUMIN

Carboxypeptidase G_1 (1.3 units/ml) was incubated with Chelex 100 (see Methods) in duplicate tubes, one of which contained bovine serum albumin (10 mg/ml). After 2 h incubation the metal-free supernatants were recovered by centrifugation, stored at 4 °C, and assayed at the time intervals indicated in the standard assay solution.

Time (h)	*Activity (units/ml)*	
	Carboxypeptidase G_1 alone	*Carboxypeptidase G_1 with albumin*
0	0.21	0.66
2	0.15	0.66
5	0.09	0.69
17	0.06	0.54

found to stabilize dilute solutions of carboxypeptidase G_1 (Table II), and facilitated the study of the inhibitory effect of divalent cations on enzyme activation by Zn^{2+}. Although serum albumin is known to have a weak metal chelating capability[8], bovine serum albumin at a concentration of $1.5 \cdot 10^{-4}$ M had no effect on the Zn^{2+} activation of carboxypeptidase G_1. A series of divalent cations were tested for ability to activate metal-free carboxypeptidase G_1. None of the metal ions listed in Table III

TABLE III

Zn^{2+} ACTIVATION OF METAL-FREE CARBOXYPEPTIDASE G_1: INHIBITION BY PRIOR ADDITION OF DIVALENT METALS

Metal-free carboxypeptidase G_1 (0.05 unit) was added to 1 ml of Zn^{2+}-free assay mixture containing 10^{-4} M of the designated cation. After 1 min incubation, 1 μl of 0.01 M $ZnCl_2$ was then added to the cuvette and the rate of hydrolysis determined. The control rate was determined by addition of enzyme to an assay solution lacking the inhibitory cation.

Cation	*Activity (% control)*
Hg^{2+}	60
Co^{2+}	14
Cu^{2+}	19
Ni^{2+}	27
Mn^{2+}	44
Ca^{2+}	100
Mg^{2+}	100

activated the enzyme when added to the assay solution over the concentration range, 10^{-7} to 10^{-3} M. However, Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, and Hg^{2+}, but not Ca^{2+} or Mg^{2+}, markedly inhibited subsequent activation of the enzyme by Zn^{2+} when added to the assay solution prior to Zn^{2+}. When Zn^{2+} was added to the metal-free enzyme prior to the addition of the other metal ions, no inhibition was seen.

Since other Zn^{2+} activated proteins are reversibly inactivated by lowering pH, due to the competition of H^+ for metal binding sites on the protein[1], the effect of H^+ on activity and stability of carboxypeptidase G_1 was examined. When the pH of an enzyme solution was lowered to 5 or less, the enzyme showed a progressive loss of

TABLE IV

ALBUMIN PROTECTION OF CARBOXYPEPTIDASE G_1 DURING ACIDIFICATION

Activity expressed as % control. See Methods for details.

pH during acidification	*Activity at low pH*		*Activity when pH restored to 7.3*	
	+Albumin	*−Albumin*	*+Albumin*	*−Albumin*
2.0	7	0	15	3
2.5	13	8	37	16
3.0	29	21	70	26
4.0	83	50	83	73
5.0	100	100	100	100

activity which was only partially reversed by returning the pH of the solution to 7.3 (Table IV). When albumin (10 mg/ml) was present, however, protection was afforded the enzyme from irreversible inactivation at low pH.

DISCUSSION

The preceding studies have characterized carboxypeptidase G_1 as a Zn^{2+} activated enzyme. This activation proceeds over the concentration range of approx. 10^{-7}–$5 \cdot 10^{-5}$ M Zn^{2+}, and in this respect resembles carboxypeptidase G, the glutamate specific C-terminal exopeptidase described by Goldman and Levy[3], which was activated by Zn^{2+} in the concentration range 10^{-6} to 10^{-5} M. Other divalent cations failed to activate metal-free carboxypeptidase G_1, although several metals, including Mn^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+} substantially inhibited the reconstitution of the active complex of Zn^{2+}–carboxypeptidase G_1, presumably by occupying the enzyme's metal binding site(s) and preventing Zn^{2+} attachment. Consistent with this interpretation is the failure of these cations to inhibit the enzyme when added after the addition of Zn^{2+}.

Failure of Co^{2+} to activate the metal-free carboxypeptidase G_1 represents a departure from the substantial stimulatory effect of this ion on the apoenzyme form of the three most carefully studied Zn^{2+} metalloenzymes, carboxypeptidase A (ref. 2), carbonic anhydrase[9], and alkaline phosphatase from *Escherichia coli*[10]. In addition the presence of substrate does not prevent metal activation of carboxypeptidase G_1 as was described in the case of carboxypeptidase A (ref. 11).

In addition to the requirement of Zn^{2+} for enzyme activity, the metal cation appears to stabilize carboxypeptidase G_1. Thus, dialysis against metal-free buffer or the chelating agent 1,10-phenanthroline, or removal of metal ions with the chelating resin Chelex 100 leads to a gradual irreversible loss of enzyme activity. Metal removal has been shown to promote depolymerization of two other Zn^{2+}-containing enzymes, alkaline phosphatase[12] and yeast alcohol dehydrogenase[13]; alkaline phosphatase readily re-aggregates upon addition of Zn^{2+}, but the dehydrogenase is irreversibly inactivated. Albumin, which stabilizes carboxypeptidase G_1 in the metal-free state and during exposure to low pH, has been frequently used to stabilize other enzymes, although the mechanism of this stabilization is uncertain.

Because of the antitumor activity of carboxypeptidase G_1 (ref. 5), its metal re-

quirements and stability are of considerable importance. The Zn^{2+} concentration in human serum is approx. 10^{-5} M, but varies with sex and age[12]. This level would satisfy the activation requirements of carboxypeptidase G_1 and would enhance its stability.

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