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Aminoacetone synthase from goat liver

Involvement of arginine residue at the active site and on the stability of the enzyme

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The arginine-specific reagents phenylglyoxal and butane-2,3-dione inactivated goat liver aminoacetone synthase with pseudo-first-order kinetics, with the rate dependent on modifier concentration. Phenylglyoxal and butane-2,3-dione appeared to react with one arginine residue per enzyme molecule. The inactivated enzyme could be re-activated by Tris, suggesting additional evidence of modification of the arginine residue. Acetyl-CoA, one of the substrates, completely protected the enzyme from inactivation. Glycine gave partial protection. Protection by substrates against inactivation by phenylglyoxal and butane-2,3-dione suggested the presence of an essential arginine residue at the substrate-binding region. Experiments with [7-^{14}C]phenylglyoxal in the presence of acetyl-CoA showed that only the arginine residue at the active site could be modified by phenylglyoxal. The stability of the enzyme is dependent on the presence of both EDTA and Mg^{2+}.

INTRODUCTION

Aminoacetone synthase is the enzyme which acts on glycine and acetyl-CoA to form aminoacetone. This enzyme, first reported to be present in guinea-pig liver (Urata & Granick, 1963), has subsequently been isolated and purified from a number of sources, including bovine liver (Fubara *et al.*, 1986) and *Escherichia coli* (Mukherjee & Dekker, 1987). The biological significance of this enzyme is not yet clear. It has been suggested that the true enzyme product is the unstable 2-amino-3-oxobutyrate, which is spontaneously decarboxylated to the detectable product aminoacetone. Hence the names 2-amino-3-oxobutyrate CoA ligase or 2-amino-3-oxobutanoate glycine lyase have also been suggested for this enzyme (EC 2.3.1.29).

2-Amino-3-oxobutyrate is also an enzymic product of L-threonine dehydrogenase, which utilizes L-threonine and NAD^+ as substrates (Boylan & Dekker, 1981). L-Threonine is converted into glycine and acetyl-CoA by the combined action of the enzymes L-threonine dehydrogenase and aminoacetone synthase (Aoyama & Motokawa, 1981). However, aminoacetone is oxidized to methylglyoxal by the enzyme aminoacetone oxidase, which is present in goat liver (Ray & Ray, 1987) and plasma (Ray & Ray, 1983).

Both bovine liver and *E. coli* enzymes contain bound pyridoxal phosphate as coenzyme. The amino acid composition of the enzyme has been determined. However, the amino acid residue(s) present at the active site of the enzyme has not yet been determined. It has been shown that many negatively charged molecules bind to proteins via electrostatic interactions with arginyl residues (Riordan *et al.*, 1977). Since one of the substrates of aminoacetone synthase is negatively charged acetyl-CoA, we have attempted to explore the possible involvement of arginine residue(s) in the function of this enzyme by using phenylglyoxal and butane-2,3-dione as modifying reagents.

The results described herein strongly suggest the presence of an essential arginine residue at the substrate-binding region of aminoacetone synthase. Some other important properties of the enzyme have also been described.

MATERIALS AND METHODS

Materials

All the biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-100 was the product of Pharmacia Fine Chemicals, Uppsala, Sweden. [7-^{14}C]Phenylglyoxal was purchased from Amersham International, Amersham, Bucks., U.K. Aminoacetone hydrochloride was prepared from glycine and acetic anhydride by the method of Hepworth as described previously (Ray & Ray, 1983). Calcium phosphate gel was prepared as described by Colowick (1955). Other chemicals were of analytical grade and obtained from local manufacturers.

Enzyme assay

The enzyme aminoacetone synthase was assayed by either of the following procedures.

Colorimetric. The standard reaction mixture contained 50 mM-Tris/HCl buffer, pH 8.3, 25 mM-glycine, 0.3 mM-acetyl-CoA and the requisite amount of the enzyme in a total volume of 0.5 ml. The reaction was terminated by adding 50 μl of 0.1 M-sodium acetate/acetic acid buffer, pH 4.6, and 50 μl of acetylacetone and heated in a 100 °C water bath for 10 min. After removal of the precipitated protein by centrifugation (3000 *g* for 10 min), the supernatant was treated with 1.0 ml of Ehrlich–Hg reagent (Urata & Granick, 1963), and the reading was taken after 10 min at 552 nm to determine the aminoacetone formed ($\epsilon_{552} = 5.33 \times 10^4\ M^{-1} \cdot cm^{-1}$). A unit of enzyme activity is defined as the amount of the enzyme which forms 1 μmol of aminoacetone/min.

Spectrophotometric. The standard reaction mixture contained 50 mM-Tris/HCl buffer, pH 8.3, 25 mM-glycine, 0.3 mM-acetyl-CoA, 0.25 mM-5,5′-dithiobis-(2-nitrobenzoate) and the requisite amount of the enzyme in a total volume of 0.5 ml. The increase in A_{412} was monitored. Assuming $\epsilon_{412} = 1.36 \times 10^4\ M^{-1} \cdot cm^{-1}$, a unit of activity is defined as the formation of 1 μmol of CoA/min. The deviation between two types of assay was not more than 10–15%.

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The specific activity of the enzyme is defined as units of enzyme activity per mg of protein. With BSA as standard, protein was determined either by the method of Lowry *et al.* or by that of Warburg & Christian as outlined by Layne (1957). Polyacrylamide-gel electrophoresis (Davis, 1964) and SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) were performed by the methods described in the references.

Enzyme-modification experiments

Modification experiments using butane-2,3-dione were performed by incubating the enzyme in 50 mM-sodium phosphate buffer, pH 8.0, containing 20 mM-sodium borate. Borate ions at this concentration do not react with the enzyme. Modification with phenylglyoxal was carried out in 20 mM-sodium phosphate buffer, pH 8.0. Phenylglyoxal solution was made in dimethyl sulphoxide. Dimethyl sulphoxide has no effect on the enzyme activity. In all cases control experiments were performed without modifying reagents. The temperature of the incubation was 28 °C. At the indicated time, samples were removed for measuring the enzyme activity. In the substrate-protection experiments, substrates were added to the incubation medium before addition of the modifier. The amount of acetyl-CoA or glycine transferred with the samples to the assay mixture of the enzyme has no effect on the rate of enzyme activity, as was revealed by addition of the transferred amount of acetyl-CoA or glycine to the assay mixture of the native or inactivated enzyme.

Incorporation of [7-^{14}C]phenylglyoxal into aminoacetone synthase in the absence and presence of acetyl-CoA

In two tubes, aminoacetone synthase (2.8 nmol) in 0.2 ml of 50 mM-sodium phosphate buffer, pH 8.0, was incubated with [7-^{14}C]phenylglyoxal (7×10^6 c.p.m.) at 27 °C for 45 min. The concentration of phenylglyoxal was 5 mM. In one of the tubes acetyl-CoA (1.5 mM) was added before addition of phenylglyoxal. After ensuring almost complete inactivation, the contents of both tubes were passed separately through a Sephadex G-50 centrifuge column (Maniatis *et al.*, 1982) to remove excess reagents. The column was equilibrated with 50 mM-sodium phosphate buffer, pH 8.0. The eluate was analysed for radioactivity incorporated into the enzyme.

Re-activation of butane-2,3-dione- and phenylglyoxal-inactivated enzyme

For re-activation experiments with butane-2,3-dione-inactivated enzyme, 20 μg of the enzyme was incubated for 45 min at 28 °C in a total volume of 100 μl containing 20 mM-sodium phosphate buffer, pH 8.0, 20 mM-sodium borate and 3.5 mM-butane-2,3-dione. Then 50 μl of the inactivated enzyme was transferred to 50 μl of 100 mM-Tris/HCl buffer, pH 8.0, and incubated for 1 h at 28 °C, and samples were assayed for enzyme activity.

The re-activation experiment with phenylglyoxal (6 mM)-inactivated enzyme was performed in a similar manner, but without addition of borate.

Purification of the enzyme

Preparation of mitochondria. All operations were carried out at 0–4 °C unless otherwise mentioned. A 100 g portion of goat liver obtained from the local slaughterhouse was homogenized in a Waring Blendor in 500 ml of 0.25 M-sucrose. The homogenate was centrifuged for 10 min at 1000 *g* and the pellet was discarded. The supernatant was centrifuged at 12000 *g* for 25 min. After the supernatant was discarded, the pellet was suspended in 200 ml of buffer (hereafter termed SEMP buffer) containing 0.25 M-sucrose, 25 mM-sodium phosphate buffer, pH 7.4, 20 mM-$MgCl_2$ and 10 mM-EDTA, and centrifuged at 12000 *g* for 15 min. After discarding the supernatant, the pellet, designated as mitochondria, was processed for further purification.

Preparation of mitochondrial supernatant. Mitochondria prepared as above from 100 g of goat liver were suspended in SEMP buffer. The total volume of the suspension was 100 ml. The suspension was freeze–thawed four times and centrifuged at 12000 *g* for 15 min, and the supernatant was retained. The pellet was suspended in SEMP buffer to a volume of 50 ml and stirred for 40 min. After a similar centrifugation, the pellet was discarded and the supernatant was pooled with the above supernatant and used for further purification.

Calcium phosphate gel treatment. All centrifugation at this step was for 4 min at 1000 *g*, and the incubation of calcium phosphate gel with the enzyme preparation was for 15 min. Calcium phosphate gel (65 ml; dry wt. 17 mg/ml) was centrifuged and the supernatant discarded. To the pellet 90 ml of the above pooled enzyme (protein 6.5 mg/ml) suspension was added with stirring. The gel was centrifuged and the supernatant was discarded. The pellet was treated with 60 ml of SEMP buffer and again centrifuged and the supernatant discarded. To the pellet, 60 ml of SEMP buffer containing 0.25 M-NaCl was added with stirring. After centrifugation the supernatant was retained. After a similar treatment of the pellet with 30 ml of SEMP buffer containing 0.25 M-NaCl, both supernatants were pooled. The above pooled enzyme fraction was precipitated by adding solid $(NH_4)_2SO_4$ (to 80% saturation) and centrifuged at 12000 *g* for 15 min. The supernatant was discarded and the pellet was dissolved in a minimum volume of SEMP buffer.

The above enzyme fraction was heated to 58 °C for 1 min in a water bath and rapidly cooled. The denatured protein was removed by centrifugation, and the enzyme was dialysed against SEMP buffer. Calcium phosphate gel (24 ml; dry wt. 17 mg/ml) was centrifuged and the supernatant discarded. To the pellet, 6 ml of the dialysed enzyme containing 20 mg of protein was added with stirring. The enzyme was adsorbed in the gel. The gel was successively washed with 6 ml each of SEMP buffer containing no NaCl, 50 mM- and 100 mM-NaCl. The washings were discarded. The gel was then washed with 2×6 ml of SEMP buffer containing 250 mM-NaCl. These two washings containing the enzyme activity were pooled and treated with solid $(NH_4)_2SO_4$ (to 80% saturation). After centrifugation at 15000 *g* for 15 min, the supernatant was discarded and the pellet was dissolved in SEMP buffer and subjected to gel filtration.

Gel filtration on a Sephadex column. The above enzyme fraction was filtered on a Sephadex G-100 column (75 cm × 1.9 cm). The most active fractions were pooled. The volume of the pooled enzyme fraction was concentrated against sucrose and again passed through a Sephadex G-100 column (75 cm × 1.9 cm). Fractions containing highest specific activity and showing a major band on polyacrylamide-gel electrophoresis were pooled (2–3 tubes). SDS/polyacrylamide-gel electrophoresis of this enzyme fraction revealed a major band (about 90–95% of the total protein) and one minor band (result not shown).

The specific activity (μmol of aminoacetone or acetyl-CoA formed/min per mg of protein) of the enzyme at this stage is 12.7, which is quite comparable with the specific activity of aminoacetone synthase obtained from bovine liver (Fubara *et al.*, 1986). The specific activities of bovine liver enzyme (Fubara *et al.*, 1986) and *E. coli* enzyme (Mukherjee & Dekker, 1987) were reported to be 16.4 and 2.7 respectively.

RESULTS AND DISCUSSION

Inactivation of aminoacetone synthase with butane-2,3-dione

Incubation of the enzyme with butane-2,3-dione resulted in a progressive loss of enzyme activity. The detailed time course of inactivation is shown in Fig. 1. The inactivation followed pseudo-first-order kinetics. The enzyme was inactivated almost completely when incubated for 40 min with 3.5 mM-butane-2,3-dione. A plot of pseudo-first-order rate constant (k) versus log of butane-2,3-dione concentration was a straight line with a slope of 1.04, indicating that 1 mol of arginine/mol of the enzyme is involved in the activity.

Inactivation by phenylglyoxal

Further evidence that the aminoacetone synthase inactivation was due to modification of an arginyl residue was obtained with phenylglyoxal, another chemical modification reagent specific for the arginyl residue (Riordan, 1973; Takahashi, 1968, 1977). The enzyme was inactivated on treatment with phenylglyoxal, and this inactivation also followed pseudo-first-order kinetics and was dependent on phenylglyoxal concentration (Fig. 2). The slope of a plot of log k versus log of phenylglyoxal concentration was determined to be 1.02 for the enzyme.

Re-activation of the phenylglyoxal- and butane-2,3-dione-inactivated aminoacetone synthase

Enzymes inactivated by diones have been found to be re-activated on incubation with a nucleophilic buffer such as Tris/HCl (Patthy & Smith, 1975). Aminoacetone synthase after inactivation with phenylglyoxal was re-activated almost completely on incubation with 50 mM-Tris/HCl buffer, pH 8.3, for 1 h, suggesting additional evidence in favour of modification of arginyl residue(s). The enzyme inactivated by butane-2,3-dione was also re-activated on incubation with 50 mM-Tris/HCl buffer (Table 1).

Protection of the enzyme by substrates against inactivation by phenylglyoxal and butane-2,3-dione (Fig. 3)

The substrates acetyl-CoA and glycine were found to protect the enzyme activity against inactivation by phenylglyoxal and butane-2,3-dione. The K_m values of acetyl-CoA and glycine for the enzyme were found to be 0.07 mM and 10 mM respectively. By measuring the rate of the enzyme activity at various concentrations of one substrate, keeping the other at saturating concentration (for glycine 25 mM, and for acetyl-CoA 0.3 mM), the K_m values were estimated from Lineweaver–Burk (1934) double-reciprocal plots. At concentrations of 0.2 mM (about 3 times the K_m value) and 1 mM, acetyl-CoA could protect the enzyme activity to the extent of 45% (almost half-maximal protection) and 100% respectively. Glycine at a concentration of 10 mM (K_m value for glycine) could give half-maximal protection. At 50 mM, it could protect the enzyme activity by about 50%. Increasing the concentration of glycine could not afford further protection. The protective effect of glycine probably is not due to simple reversal of the modification reaction by this nucleophile, because, unlike the nucleophilic buffer Tris/HCl, glycine could not re-activate the enzyme that had been inactivated by butane-2,3-dione or phenylglyoxal.

Several investigators have evaluated a variety of aldehyde compounds for their ability to react with various amino acids and protein moieties. Included in the studies were phenylglyoxal and butane-2,3-dione, with the finding that phenylglyoxal is the most arginine-specific reagent (Takahashi, 1968, 1977; Yankeelov, 1970; Riordan, 1973).

The complete inactivation of aminoacetone synthase by

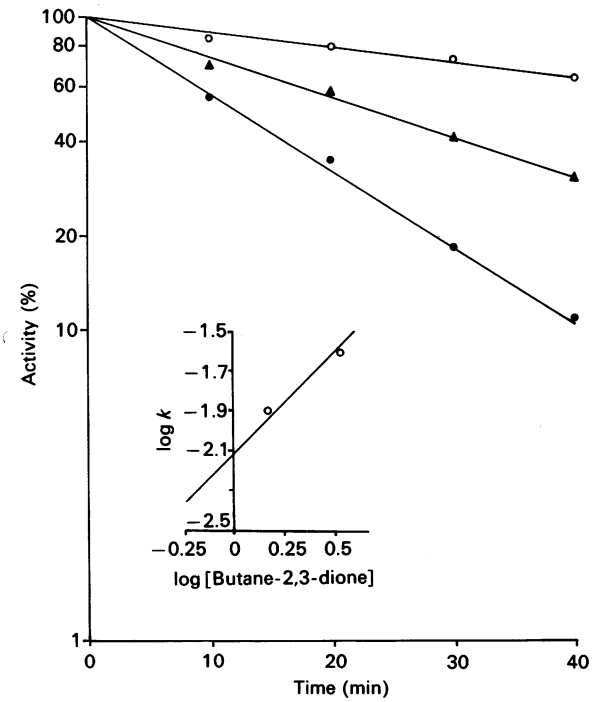

Fig. 1. Inactivation of aminoacetone synthase by butane-2,3-dione

The enzyme was incubated at 28 °C in different tubes with various concentrations of butane-2,3-dione in 50 mM-sodium phosphate buffer, pH 8.0, containing 20 mM-borate. In the control, the enzyme was incubated under identical condition but without butane-2,3-dione. At the indicated time intervals, samples were removed for measuring the enzyme activity. The butane-2,3-dione concentrations were 0.7 mM (○), 1.5 mM (▲) and 3.5 mM (●). Inset: plot of log of pseudo-first-order rate constant (k) of butane-2,3-dione inactivation versus log of butane-2,3-dione concentration (mM).

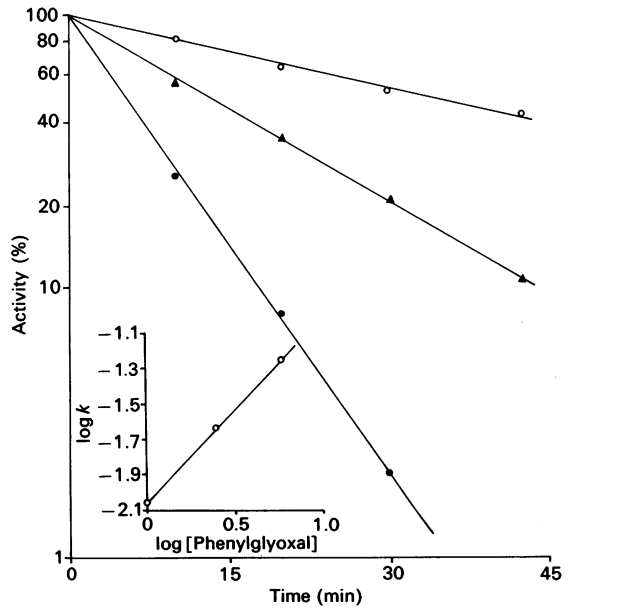

Fig. 2. Inactivation of aminoacetone synthase by phenylglyoxal

The enzyme was incubated at 28 °C in different tubes in 20 mM-sodium phosphate buffer, pH 8.0, and with various concentrations of phenylglyoxal. A control tube with only dimethyl sulphoxide was run under identical condition. The phenylglyoxal concentrations were 1.0 mM (○), 2.5 mM (▲) and 6 mM (●). Inset: plot of log of pseudo-first-order rate constant (k) of phenylglyoxal inactivation versus log of phenylglyoxal concentration (mM).

Table 1. Re-activation of phenylglyoxal- and butane-2,3-dione-inactivated aminoacetone synthase

The details of the inactivation and re-activation procedures of the enzyme are described in the Materials and methods section.

Modifying reagent	Incubation with 50 mM-Tris/HCl buffer	Activity (%)
None	−	100
Phenylglyoxal (1.5 mM)	−	30
Phenylglyoxal (1.5 mM)	+	100
Phenylglyoxal (3.5 mM)	−	8
Phenylglyoxal (3.5 mM)	+	93
Butane-2,3-dione (2.5 mM)	−	22
Butane-2,3-dione (2.5 mM)	+	88
Butane-2,3-dione (6.5 mM)	−	2
Butane-2,3-dione (6.5 mM)	+	76

phenylglyoxal and butane-2,3-dione in the presence of borate strongly suggests that an essential arginine residue may be critically involved at the catalytic site of the enzyme.

Evidence that the modified arginyl residue of aminoacetone synthase acted as an anionic recognition site was further obtained from a protection experiment with the negatively charged acetyl-CoA. The observed complete regeneration of the enzyme activity by a nucleophilic buffer such as Tris is also consistent with the modification of an arginine residue on the enzyme surface.

Experiments with [7-^{14}C]phenylglyoxal

The complete protection by acetyl-CoA of the enzyme against inactivation by phenylglyoxal provided us with a method to label specifically the arginine residue at the active site of the enzyme, and also to determine the total number of arginine residue(s) modified by phenylglyoxal. To determine the latter, we used [7-^{14}C]phenylglyoxal and studied its incorporation into the enzyme. We found that when the enzyme was completely inactivated, only 1.2 nmol of [7-^{14}C]phenylglyoxal was incorporated per nmol of the enzyme. When the enzyme was protected with acetyl-CoA, very little radioactivity (0.28 nmol) was incorporated, indicating that only the arginyl residue at the acetyl-CoA-binding region reacted with phenylglyoxal.

Dependence of the enzyme on added EDTA and Mg^{2+} for stability

Aminoacetone synthase from goat liver was found to be strongly dependent on added EDTA and Mg^{2+} for stability. During the course of purification of this enzyme, it lost its activity rapidly when extracted in Tris/HCl buffer containing only 0.25 M-sucrose. Elution of the enzyme from a Sephadex column with eluting buffer containing either 20 mM-sodium phosphate and 0.25 M-sucrose or 20 mM-Tris/HCl and 0.25 M-sucrose resulted in a total loss of activity.

A systematic study revealed that the enzyme could be stabilized by addition of EDTA and Mg^{2+}. Typical stabilizing agents such as dithiothreitol, 2-mercaptoethanol, glycerol or EDTA alone or in combination failed to protect the enzyme. The total dependence of the enzyme for stability on the presence of added EDTA and Mg^{2+} could be demonstrated clearly when partially purified enzyme fractions (purified in the presence of EDTA and Mg^{2+}) were passed through a series of Sephadex G-50 columns, each equilibrated and eluted with or without EDTA and Mg^{2+} (Table 2).

Table 2 shows that in the absence of EDTA and Mg^{2+} the activity of the enzyme is rapidly lost, whereas it is effectively

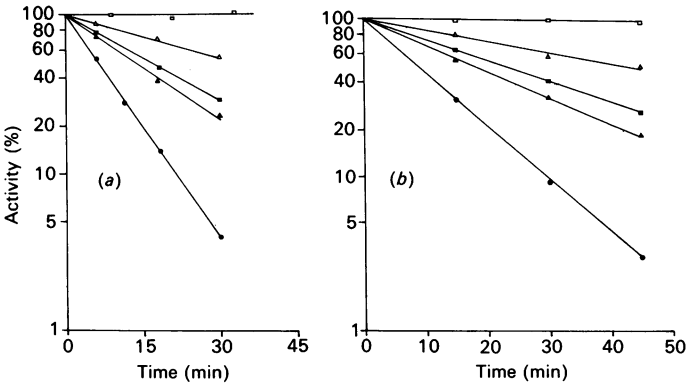

Fig. 3. Substrate protection of aminoacetone synthase from inactivation by phenylglyoxal and butane-2,3-dione

(*a*) Substrate protection against phenylglyoxal inactivation. The enzyme was incubated at 28 °C in different tubes each containing 6 mM-phenylglyoxal and various concentrations of acetyl-CoA or glycine. Substrates were added to the incubation medium before addition of phenylglyoxal. In three separate control tubes, enzyme was incubated under identical conditions without phenylglyoxal, but containing, in tube 1 no substrate, in tube 2 1.0 mM-acetyl-CoA and in tube 3 50 mM-glycine. (*b*) Substrate protection against butane-2,3-dione inactivation. The enzyme was incubated at 28 °C in different tubes with 4 mM-butane-2,3-dione in 50 mM-sodium phosphate buffer, pH 8.0, containing 20 mM-borate and various concentrations of acetyl-CoA or glycine. Control tubes were maintained as for phenylglyoxal inactivation. For both (*a*) and (*b*), at the indicated time intervals samples were removed for measuring the enzyme activity: ●, in absence of any substrate; ■, in presence of 0.1 mM-acetyl-CoA; □, in presence of 1.0 mM-acetyl-CoA; ▲, in presence of 10 mM-glycine; △, in presence of 50 mM-glycine. All the control tubes retained 100% enzyme activity.

Table 2. Recovery of activity of the enzyme from a Sephadex G-50 column equilibrated with different metal ions and chelators

Abbreviation: CDTA, *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid.

Metal ion	Chelator	Activity (%)
None	None	0
None	EDTA (10 mM)	0
Mg^{2+} (20 mM)	EDTA (10 mM)	100
Mg^{2+} (10 mM)	EDTA (10 mM)	98
Mg^{2+} (5 mM)	EDTA (5 mM)	102
Mg^{2+} (2 mM)	EDTA (2 mM)	106
Mg^{2+} (1 mM)	EDTA (1 mM)	115
Mg^{2+} (0.5 mM)	EDTA (0.5 mM)	109
Mg^{2+} (20 mM)	None	0
Mg^{2+} (20 mM)	EGTA (10 mM)	0
Mg^{2+} (20 mM)	CDTA (10 mM)	0
Mg^{2+} (20 mM)	Citrate (10 mM)	0
Mg^{2+} (20 mM)	ATP (5 mM)	0
Mn^{2+} (20 mM)	EDTA (10 mM)	0
Zn^{2+} (20 mM)	EDTA (10 mM)	0
Fe^{3+} (20 mM)	EDTA (10 mM)	0
Ca^{2+} (20 mM)	EDTA (10 mM)	58
Ca^{2+} (20 mM)	EGTA (10 mM)	0
Ca^{2+} (20 mM)	CDTA (10 mM)	0
Li^{+} (20 mM)	EDTA (10 mM)	46
Li^{+} (20 mM)	EGTA (10 mM)	0
Li^{+} (20 mM)	CDTA (10 mM)	0

protected in the presence of EDTA (10 mM) and Mg^{2+} (20 mM). We have studied a range of Mg^{2+} and EDTA concentrations, and found that Mg^{2+} at a concentration as low as 0.5 mM and EDTA at 0.5 mM could completely protect the enzyme (Table 2). EDTA

or Mg^{2+} alone could not afford any protection. Other bivalent-metal chelators such as EGTA, *trans*-1,2-diaminocyclohexane-*NNN'N'*-tetra-acetic acid and ATP were found to be completely ineffective in protecting the enzyme. However, we could not explain the exact nature of the ligand(s) which brings about this protective effect. Probably the Mg^{2+}–EDTA complex is effective in protecting the enzyme.

A number of metal ions have been tested for the stability of the enzyme. Of the metal ions tested, only Ca^{2+} and Li^{+} could partially replace Mg^{2+} (Table 2). Addition of substrates alone had no protective effect. The substrate glycine in combination with EDTA (1 mM) could afford partial protection. Glycine at a concentration of 1 mM could protect the enzyme activity to the extent of 50% in the presence of EDTA. Increasing the concentration of glycine had no further protective action.

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