Vol. 82, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 14, 1978 Pages 907-912

Mn^{++}-SPECIFIC REACTIVATION OF EDTA INACTIVATED α-ISOPROPYLMALATE SYNTHASE FROM ALCALIGENES EUTROPHUS H 16

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Received April 18,1978

SUMMARY

The α-isopropylmalate synthase (EC 4.1.3.12) from Alcaligenes eutrophus H 16 was inactivated by EDTA in a time-dependent reaction. Only the addition of Mn^{++} plus dithiothreitol could restore the activity. The substrate, α-ketoisovalerate, prevented the inactivation; the feedback inhibitor, leucine, and it's antagonist, valine, increased the rate of inactivation. Except for α,α'-bipyridyl, chelating reagents, other than EDTA had no effect on the enzyme stability. It is suggested that the α-isopropylmalate synthase is a metallo enzyme - the evidence points to Mn^{++} as the metal ion - and that this enzyme uses a mechanism of catalysis which differs from that of the analogous malate synthase (EC 4.1.3.2) and citrate synthase (EC 4.1.3.4).

INTRODUCTION

The α-isopropylmalate (IPM) synthase is the first enzyme of the branched-chain amino acid pathway which specifically leads to the synthesis of leucine. This enzyme catalyzes the formation of α-isopropylmalate (IPM) from α-keto-isovalerate (KIV) and acetyl coenzyme A. The metal content of IPM synthases has not been studied so far. The experiments presented in this paper provide evidence that the IPM synthase from A. eutrophus H 16 is a metalloprotein which may contain Mn^{++} as the functional metal ion.

MATERIALS AND METHODS

The purification, some physical properties, and the kinetics of this enzyme have been previously described [1,2,3]. The purified enzyme used in this investigation had an activity of 11.7 U (μmoles of IPM per min and mg of protein at 30°C). The assays for IPM synthase activity have been previously described [1,2]. Inactivated enzyme (0.18 mg protein/0.3 ml) was prepared by dialysis against 100 mM Tris-HCl buffer, pH 8.5, containing 2 mM EDTA (2 x 500 ml), for 60 h, then dialysis against several changes of EDTA free buffer to remove all metal ions and EDTA. The resulting enzyme had no detectable IPM synthase activity.

0006-291X/78/0823-0907$01.00/0

RESULTS

The IPM synthase is inactivated when incubated with 1 mM EDTA. The degree of inactivation depends on time and on the pH of the incubation solution (Fig. 1). At the pH optimum (pH 8.2), the IPM synthase is stable for more than 24 h in the absence of EDTA; however, in the presence of EDTA, about 20 and 0% activity remained after 20 min and 5 h, respectively. The kinetics of inactivation are not of first or second order. EDTA affected the activity of IPM synthase in the crude extract to the same extent as the highly purified enzyme. At pH values higher than 8.3, and lower than 7.2, a pH-dependent inactivation was observed; however, this inactivation was always significantly lower than that caused by EDTA. The inactivation, caused by incubation with EDTA in the absence of substrates, was not relieved by dialysis against various phosphate buffers (50 or 250 mM, pH 7.5 or 8.2) alone or containing 1 mM α-ketoisovalerate, 50 mM valine, 1-10 mM mercaptoethanol, Mn^{++} or other metal ions or of any combinations of these supplements. However, if dithiothreitol (0.1 mM final concentration) was added in the presence of 1 mM Mn^{++}, a completely reactivated enzyme was obtained. No differences in the allosteric properties [3,5] of either the untreated or the reactivated enzyme could be detected with respect to their inhibition by leucine (K_i; non linear kinetics) or to the reaction with the leucine antagonist, valine. Both the metal and the SH-reagent were required for reactivation (Table 1). The excess of metal and SH reagent were removed after 10 seconds incubation by passing through a Sephadex G-25 column (1.5 x 10 cm, 100 mM potassium phosphate, pH 7.5). This was necessary because prolonged incubation resulted in lower initial activities and in a lability of IPM synthase activity; e.g., an incubation time of 2 min resulted in 95, 57 and 5% activity after 15, 120, and 500 min, respectively. This lability may be due to the reduction of a critical disulfide bond of the protein by dithiothreitol; the presence of disulfide bonds had been determined previously [2]. The substrate, α-ketoisovalerate (2 mM), protected the IPM synthase from inactivation by EDTA

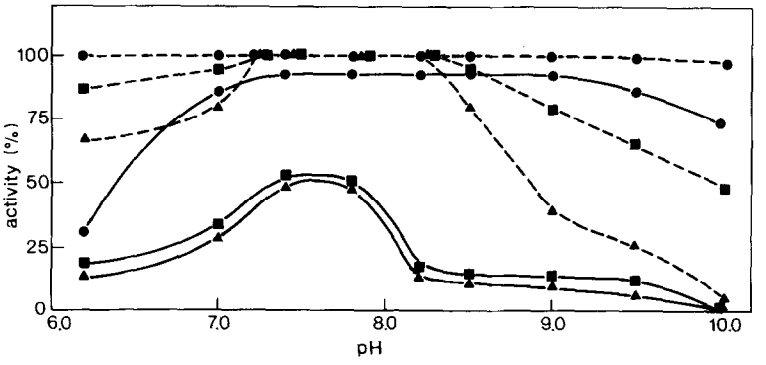


Fig. 1. The effect of pH on the EDTA-mediated inactivation of the α-isopropylmalate synthase.

Enzyme (0.02 mg), having a spec. activity of 11.7, was incubated at 25°, in 0.25 ml of 125 mM Tris-HCl or potassium phosphate buffer, at pH values indicated without EDTA (dashed line) and with 1 mM EDTA (solid line). Aliquots (0.025 ml) were taken at 1 (-o-), 20 (-□-), and 60 min (-▲-), and were assayed at 30° for IPM-synthase activity using the DTNB method. The assay (1 ml) contained 250 µmole Tris-HCl, 20 µmole KIV, and 1 µmole acetyl coenzyme A; after adding the enzyme solution (0.025 ml) to the assay, the EDTA concentration was 0.04 mM which exhibits no inactivation in the presence of KIV.

Table 1: Reactivation of IPM Synthase by Mn^{++} and Dithiothreitol

Enzyme Treatment[1)]	Additions	Spec. activity
None	None	11.7
None	Dithiothreitol (0.1 mM)	0.2[2)]
EDTA	None	0
EDTA	Dithiothreitol (0.1 mM)	0
EDTA	$MnCl_2$ (1 mM)[3)]	0
EDTA	$MnCl_2$ plus dithiothreitol[3)]	11.7[4)]

1) The enzyme was purified to a spec. activity of 11.7 U/mg of protein. The treatment with EDTA includes dialysis as described under "Materials and Methods."

2) Measured after 30 min of incubation.

3) With the exception of Zn^{++} (5% reactivation), the addition of metals other than Mn^{++} did not result in reactivation; we tried 1 mM of $Fe^{++}/^{+++}$, Ba^{++}, Mg^{++}, Ca^{++}, Cd^{++}, Ni^{++}, Cu^{++}, Hg^{++}, Pb^{++}, WO_4^{3-} and MoO_4^{4-}. Also CH_3SH (0.01-10 mM) instead of dithiothreitol caused no significant reactivation.

4) After 10 sec incubation, see text.

at all pH values tested (pH 6.2, 7.4 and 8.5). Consequently, EDTA has only a minor effect on the enzyme activity in the assay mixture; the low inhibition (10-20%) which occurred under those conditions was relieved by removing the EDTA by dialysis, or by reducing the free EDTA concentration by adding divalent metal ions. Such a low and reversible inactivation by EDTA is also reported for the enzyme from Salmonella typhimurium [4]. The feedback inhibitor, leucine, and its antagonist, valine [3,5], increased the rate of inactivation by EDTA at pH 6.2, 7.4 (optimum for feedback inhibition) and pH 8.5, although, the enzyme is not sensitive to feedback inhibition at pH 8.5 [3].

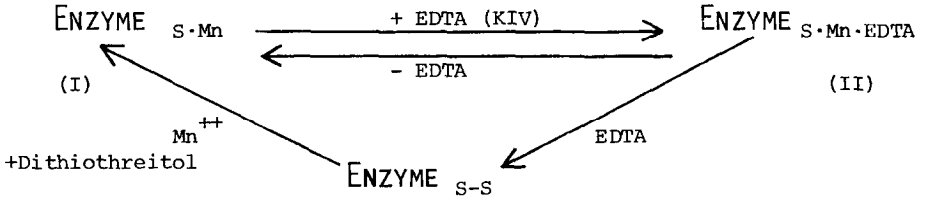
Incubation with various metal-EDTA (1:1) complexes resulted in different degrees of inactivation of the enzyme, which indicates [6] that the chelating properties of EDTA are primarily responsible for the observed effects. The extent of inactivation correlated with the dissociation constant of the metal-EDTA complex, except for Hg^{++} and Mn^{++}. The Hg-EDTA complex has a low dissociation constant, but a strong inactivation (82% after 45 min) occurred; in contrast to the Mn-EDTA complex, which has a much higher dissociation constant, but no inactivation occurred.

α,α'-Bipyridyl (10 mM) caused inactivation at pH 8.3; 20 and 5% activity remained after 20 and 60 min, respectively. This inactivation was also not relieved by dialysis against EDTA free buffer. Titan yellow (2,2'[(diazo-amino)-di-p-phenylene]bis[6-methyl-7-benzothiazole sulfonic acid]disodium salt; 1 mM) caused slight inactivation at pH 6.2 and 8.2; the remaining activity was 65 and 70%, respectively, after 1 h. However, this inactivation was relieved in the DTNB assay mixture during monitoring of activity; after 10 min, 100% activity was again obtained. Aeration with CO for 10 min, and the addition of 1 mM Na_2S, 1 mM KCN, 100 mM NaN_3, Dowex Chelating Resin A 1, 1-10 mM p-nitrobenzolazo-α-naphthol (Magneson) or 1-10 mM 8-hydroxychinoline-5-sulfonate to the enzyme had no effect on the activity nor on the stability of the enzyme up to 90 min at pH 6.2, 7.5, and 8.5.

DISCUSSION

From the inactivation with EDTA, and the specific reactivation with Mn^{++} (see Table 1), it is suggested that the IPM synthase from Alcaligenes eutrophus H 16 is a metallo enzyme; the evidence points to Mn^{++} as the metal ion. Furthermore, there is evidence that a sulfhydryl group(s) of the protein is involved in the binding of the metal ion: the reactivation takes place only in the presence of dithiothreitol; the Hg-EDTA (1:1) complex inactivated the enzyme, although no free available SH-group was detectable with DTNB [1]; and in the absence of EDTA, the incubation with mercuribenzoate up to 60 min has no effect on the enzyme. This suggestion would also explain the strong inactivation of this enzyme by the product coenzyme A [2]: the free SH group of coenzyme A attacks the binding of the putative metal ion, which presumably is at the catalytic center (see below).

The observations enable us to draw the following working hypothesis:



(III)

By removing EDTA, form II, the slightly inhibited enzyme, can be converted to the active form I. Form III, the inactive enzyme is formed from II in a time and pH dependent reaction. To convert III into I the presence of Mn^{++}-ions and dithiothreitol is necessary.

Eggerer and his coworkers studied the reaction mechanism of malate synthase (EC 4.1.3.2) and citrate synthase (EC 4.1.3.4) [7,8] which catalyze a similar aldol reaction as the IPM synthase. They showed that the catalysis of the first reaction step in the enzymatic aldol reaction, the enolisation of acetyl coenzyme A, is the rate determining step which needs the free carboxylate anion of the α-ketoacid. In addition, the malate synthase from Saccharomyces spec. and Pseudomonas ovalis requires additional Mg^{++}-ions.

Contrary to these synthases, the IPM synthase from A. eutrophus H 16 does not require either added divalent ions in the assay [1] or the free carboxylate anion of α-ketoisovalerate. The latter conclusion is drawn from the fact that this enzyme uses the methylester of α-ketoisovalerate with the same V_{max} as the free acid [2]. This suggests another mechanism of catalysis [7]. Eggerer speculated that the catalytic effect of the carboxylate anion can be substituted by the effect of a metal ion in the protein [7]. The suggestion that a metal ion(s) is located in the catalytic reaction center of this IPM synthase, and that it is involved in the catalytic reaction, supports this speculation.

ACKNOWLEDGEMENT

I am grateful to J. Klemme, H. Fischer and J. Brewer for their stimulating discussions. The author is very indebted to Professor H. G. Schlegel's support of this work. The chelating agents were a generous gift from M. Ziegler.

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