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ALPHA-ISOPROPYLMALATE SYNTHASE FROM YEAST

A ZINC METALLOENZYME

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Summary

Highly purified α-isopropylmalate synthase (3-hydroxy-4-methyl-3-carboxyvalerate 2-oxo-3-methylbutyrate-lysase (CoA-acetylating), EC 4.1.3.12) from *Saccharomyces cerevisiae* is inactivated by various chelating agents. Atomic absorption spectrometry indicates that the enzyme contains approx. four gatoms of zinc per dimer of molecular weight of 130 000. Dialysis against ethylenediaminetetraacetic acid at an initial concentration of 0.1 mM reduces the zinc content to about two gatoms of zinc per dimer. While such enzyme remains active, it has altered kinetic properties and is stimulated by Mn^{2+}, in contrast to untreated enzyme. Dialysis against ethylenediaminetetraacetic acid at an initial concentration of 50 mM reduces the zinc content by more than 80% and causes almost complete loss of enzymatic activity. Activity can be restored by the addition of Zn^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, or Cd^{2+}.

Introduction

α-Isopropylmalate synthase (3-hydroxy-4-methyl-3-carboxyvalerate 2-oxo-3-methylbutyrate-lyase (CoA-acetylating), EC 4.1.3.12) catalyzes the condensation of acetyl-CoA and α-ketoisovalerate, the first specific reaction in the biosynthesis of leucine. The enzyme from yeast is stimulated by monovalent cations such as K^+, NH_4^+, or Na^+, but not by divalent metals, when these are added to the assay mixture [1]. Like α-isopropylmalate synthases from other sources [2,3], it is, however, inhibited by EDTA. These observations and the fact that some enzymes catalyzing aldol condensations depend on divalent metals for activity while others do not [4—12] prompted us to examine the possible role of divalent metals in yeast α-isopropylmalate synthase. We report

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Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid.

here that the enzyme contains tightly bound zinc which is necessary for catalytic activity.

Materials and Methods

Organism. Saccharomyces cerevisiae strain SK101, isolated as a 5′,5′,5′-trifluoroleucine-resistant derivative of S288cα, was employed as a source for α-isopropylmalate synthase because of its 2—3-fold elevated level of the enzyme when compared with wild type. Cells grown aerobically on a yeast extract/salts medium (containing 2% glucose) [13] were harvested in late logarithmic phase.

Special chemicals. $(NH_4)_2SO_4$ ('Ultra Pure') and Tris base ('Ultra Pure') were from Schwarz/Mann. 1,10-Phenanthroline and 1,7-phenanthroline were from K and K Laboratories. Acetyl-CoA was prepared from CoA and acetic anhydride following the procedure of Simon and Shemin [14].

Purification and assay of α-isopropylmalate synthase. The enzyme was purified to near homogeneity (greater than 95% purity) essentially as described by Tracy and Kohlhaw [15]. Activity was determined by measuring the amount of CoA produced within a timed incubation period with 5,5′-dithiobis(2-nitrobenzoate) [16].

Treatment of buffers and dialysis tubing. Buffers prepared with doubly glass-distilled water were extracted with dithizone to render them metal free [17]. They were stored in new poly(ethylene) containers rinsed with hot doubly distilled water. Dialysis tubing was boiled for 10 min in a solution containing 50 mM EDTA and 5% Na_2CO_3 and then rinsed repeatedly with doubly glass-distilled water.

Atomic absorption spectrometry. Prior to analysis, enzyme samples were dialyzed against metal-free 10 mM Tris-HCl buffer, pH 7.5. The dialysates served as blanks. A Perkin-Elmer Model 460 atomic absorption spectrophotometer equipped with an HGA-2200 graphite furnace was used for the metal determinations. Standard curves were prepared from solutions of the chloride salts of the metals tested.

Results

Effect of chelators on α-isopropylmalate synthase activity

Incubation of highly purified α-isopropylmalate synthase with three different chelators (1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonate, and EDTA) led to a time-dependent loss of activity (Fig. 1A). The effect of 1,7-phenanthroline, which is not a chelating agent, was negligible compared to that of 1,10-phenanthroline, indicating that the inactivation by the latter compound was due to interaction with metal. EDTA was ineffective at a concentration of 1 mM, but had a strong inactivating effect at concentrations above 10 mM. In all cases, activity could be recovered by adding Zn^{2+} or other divalent cations at a concentration slightly exceeding the concentration of chelating agent. Inactivation by 8-hydroxyquinoline-5-sulfonate was completely prevented by the presence of α-ketoisovalerate and slowed down by the pres-

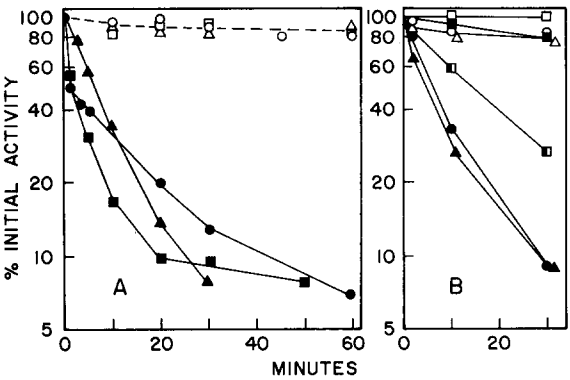

Fig. 1. (A) Effect of chelating agents on the activity of α-isopropylmalate synthase. Purified enzyme (concentration 1.4 μM) was incubated at 23°C in 50 mM Mops/KOH buffer, pH 7.2, containing 1.2 M $(NH_4)_2SO_4$, 10% (v/v) glycerol, and the following additions: ○, none; △, 1 mM 1,7-phenanthroline; □, 1 mM EDTA; ■, 50 mM EDTA; ▲, 1 mM 1,10-phenanthroline; ●, 1 mM 8-hydroxyquinoline-5-sulfonate. At the indicated times, aliquots were withdrawn, diluted 12.5-fold into the assay mixture, and assayed for activity. (B) Effect of ligands on inactivation of α-isopropylmalate synthase by 8-hydroxyquinoline-5-sulfonate (8-HQS). Experimental conditions were as above. ○, control, no addition; □, 0.5 mM α-keto-isovalerate or 1 mM acetyl-CoA; △, 2 mM L-leucine; ●, 1 mM 8-HQS; ■, 1 mM 8-HQS plus 0.5 mM α-keto-isovalerate; ◧, 1 mM 8-HQS plus 1 mM acetyl-CoA; ▲, 1 mM 8-HQS plus 2 mM leucine.

ence of acetyl-CoA. The feedback inhibitor leucine, on the other hand, even when present at ten times $K_{i,app}$, had no protective effect (Fig. 1B).

Identification and stoichiometry of bound metal

Atomic absorption spectrometry indicated that purified α-isopropylmalate synthase contained approx. 4 gatoms of zinc/mol of enzyme (Table I), calculated on the basis of a molecular weight of 130 000 for enzyme dimer, the form in which native enzyme is likely to exist [15]. By contrast, there were at most 0.47 gatoms of manganese present/mol of enzyme and only traces of cobalt.

Stepwise removal of zinc from α-isopropylmalate synthase and properties of partially and completely zinc-depleted enzyme

When native enzyme ('holoenzyme') was dialyzed against 0.1 mM EDTA, enzyme was obtained which had lost about half of its original zinc. Table II

TABLE I

METAL CONTENT OF α-ISOPROPYLMALATE SYNTHASE

The metal content was determined by atomic absorption spectrometry. A—C refer to three different preparations of highly purified enzyme with specific activities between 145 and 165. Enzyme concentration varied between $1.0 \cdot 10^{-8}$ M and $4.7 \cdot 10^{-8}$ M.

Element	gatoms/130 000 daltons		
	A	B	C
Zn	4.12	4.00	3.19
Mn	0.47	0.05	
Co	<0.01	<0.01	0.08

TABLE II

PROPERTIES OF α-ISOPROPYLMALATE SYNTHASE WITH DIFFERENT ZINC CONTENT

For the holoenzyme highly purified enzyme was dialyzed for 36 h at 4°C against several changes of 50 mM Mops/KOH buffer, pH 7.2, containing 1.2 M $(NH_4)_2SO_4$ and 10% (v/v) glycerol (buffer A). Loss of activity during dialysis was negligible. For the two-Zn-enzyme highly purified enzyme was dialyzed for 20 h at 4°C against several changes of buffer A containing 0.1 mM EDTA. Dailysis was then continued for 16 h against two changes of buffer A without EDTA. For the apoenzyme highly purified enzyme was dialyzed for 20 h at 4°C against four changes of buffer A containing 50, 10, 1 and 0.1 mM EDTA, respectively. Dialysis was then continued for 16 h against two changes of buffer A without EDTA.

	Holoenzyme	Two-Zn-enzyme	Apoenzyme
gatom Zn/mol	4.1	1.9	0.6
Specific activity *			
No metal added	149	191	<5
5 μM Zn^{2+} added	133	178	161
20 μM Mn^{2+} added	187	535	482
$K_{m,app}$ (acetyl-CoA) (μM) **			
No metal added	43	357	—
5 μM Zn^{2+} added	38	43	41
20 μM Mn^{2+} added	50	333	385
$K_{m,app}$ (α-ketoisovalerate) (μM) **			
No metal added	48	38	—
5 μM Zn^{2+} added	46	48	40
20 μM Mn^{2+} added	56	130	181
$K_{i,app}$ (L-leucine) (μM) ***			
No metal added	220	230	—
5 μM Zn^{2+} added	220	230	230
20 μM Mn^{2+} added	230	110	110

* μmol of CoA formed/h per mg of protein. The final concentrations of the substrates in the assay mixture were: acetyl-CoA, 800 μM; α-ketoisovalerate, 400 μM.

** When acetyl-CoA was the variable substrate, the α-ketoisovalerate concentration was 400 μM; when α-ketoisovalerate was the variable substrate, the acetyl-CoA concentration was 800 μM.

*** $K_{i,app}$ refers to the apparent inhibitor constant for leucine, i.e. the concentration of leucine causing 50% inhibition under the conditions of the assay.

shows that this 'two-Zn-enzyme' was fully active, but that its apparent K_m value for acetyl-CoA had increased more than 8-fold. The K_m value returned to normal when Zn^{2+} was added to the assay mixture, but remained high when Mn^{2+} was added. The addition of Mn^{2+} to this enzyme also resulted in an elevated K_m value for α-ketoisovalerate and in an almost 3-fold increase in specific activity. When holoenzyme was dialyzed first against 50 mM EDTA and subsequently against decreasing concentrations of the chelator, greater than 80% of the zinc was removed, and almost all of the catalytic activity was lost. Activity could be restored with Zn^{2+} or Mn^{2+}, with Mn^{2+} again leading to a significant increase above the original specific activity. In addition to Zn^{2+} and Mn^{2+}, Fe^{2+}, Co^{2+}, and Cd^{2+} also restored activity. However, only Zn^{2+}-regenerated enzyme exhibited native K_m values. Apoenzyme whose activity had been restored with Zn^{2+} could not be further activated by Mn^{2+}. Dialysis of such enzyme overnight against metal-free buffer led to a loss of activity of only 20%, indicating that the Zn^{2+} was again tightly bound to the enzyme.

Interestingly, Mn^{2+}-stimulated two-Zn-enzyme or apoenzyme was about twice as sensitive to leucine inhibition than the other enzyme preparations (Table II).

Discussion

The conclusion that α-isopropylmalate synthase is a zinc metalloenzyme is based primarily on the atomic absorption spectrometry data and on the fact that zinc remains bound to the enzyme during extensive purification and cannot be removed by dialysis unless chelators are present. (No Zn^{2+} was added during the purification.) On the basis of their interaction with EDTA, two types of zinc binding sites can be distinguished. Type I sites lose their zinc upon dialysis against 0.1 mM EDTA. Since this does not cause any loss of activity, the question may be asked whether type I zinc is adventitious. This is unlikely, however, since the kinetic properties of two-Zn-enzyme are distinctly different from those of holoenzyme, suggesting some relationship between type I zinc and catalytic activity. Type II zinc is apparently more tightly bound than type I zinc, since it can only be removed by higher concentrations of EDTA. Removal of this zinc is accompanied by essentially complete loss of activity.

Is there a logical role for a divalent metal ion in the reaction catalyzed by α-isopropylmalate synthase? An examination of enzymes catalyzing aldol condensations shows that there is no obligatory requirement for divalent metals. Among these enzymes, most of which are either aldolases or oligocarboxylic acid synthases, examples may be found for no metal requirement, for metal activation, and for metalloenzymes. Indeed, the aldolases are commonly divided into two classes on the basis of whether or not divalent metal participates in catalysis. Class I aldolases operate by forming a Schiff base between dihydroxyacetone phosphate and a lysine residue, and have no metal requirements. The prototype of these enzymes is rabbit muscle aldolase [4]. By contrast, class II aldolases do not form Schiff bases, but are sensitive to chelating agents and typically contain divalent metal. For example, D-fructose-1,6-bisphosphate aldolase from yeast [5] as well as L-rhamnulose-1-phosphate aldolase from *Escherichia coli* [6] contain tightly bound zinc whose removal leads to loss of activity. Activity can be restored by Zn^{2+} and by other ions of the first transition period. From magnetic resonance studies with Mn^{2+}-substituted yeast aldolase, Mildvan et al. concluded that the metal has the dual role of polarizing the carbonyl group of dihydroxyacetone phosphate and of correctly orienting the substrate [7]. With the oligocarboxylic acid synthases, the situation is similar, albeit not as clearly defined. On the one hand, citrate (*si*)-synthase (the common citrate synthase) apparently does not require divalent metal and is not inhibited by chelators. Also, Schiff base formation has been ruled out as part of the mechanism [8]. Instead, a mechanism has been proposed by which the enolization of acetyl-CoA is brought about with the aid of the carboxylate anion of oxaloacetate [9,10]. Citrate (*re*)-synthase, on the other hand, an anabolic enzyme found in *Clostridium* sp., does require Mn^{2+}, Co^{2+}, or Zn^{2+} for optimal activity [11]. Similarly, malate synthase requires Mg^{2+} for activity [12]. Eggerer and Klette have shown that yeast malate synthase acts as an acetyl-CoA enolase and that this function depends on added Mg^{2+} which can be replaced by Co^{2+}, Mn^{2+}, or Zn^{2+} [10]. Yeast α-isopropylmalate synthase now joins this group of condensing enzymes as the first example for a metalloenzyme. It is possible that the zinc in this enzyme, too,

serves as electrophile in the enolization of acetyl-CoA and/or promotes the correct alignment of the substrates. However, with the information presently available, we cannot clearly distinguish between a structural or mechanistic role. Indirect evidence for essential metal being in the proximity of the active center comes from the observation that both acetyl-CoA and α-ketoisovalerate protect the enzyme against inactivation by 8-hydroxyquinoline-5-sulfonate, whereas leucine has no such effect. Also, since the 'two-Zn-enzyme' has a much higher K_m for acetyl-CoA than holoenzyme, some of the zinc may be involved in substrate binding.

Addition of Mn^{2+} to α-isopropylmalate synthase from which zinc had been either partially or fully removed resulted in enzyme with about three times the specific activity of the original holoenzyme. At the same time, the apparent K_m values for both acetyl-CoA and α-ketoisovalerate increased significantly. The molecular mechanism for this change in catalytic properties is not clear. However, the coupling of decreased substrate affinity with increased turnover is reminiscent of a similar effect observed when α-ketoisovalerate was replaced with the 'non-physiological' substrate α-ketobutyrate [1].

Since added Mn^{2+} had no significant effect on holoenzyme or on Zn^{2+}-treated apoenzyme, manganese probably cannot replace zinc once the latter has bound to the enzyme. Which of the two metals combines with α-isopropylmalate synthase would obviously depend on their affinity for the enzyme as well as on their relative abundance within the cell. A partially purified preparation of α-isopropylmalate synthase (with a purity of about 20%) exhibited low K_m values and was not stimulated by Mn^{2+} [1], a behavior similar to that of highly purified enzyme. It is therefore likely that the form in which the enzyme exists in the cell under the growth conditions employed is the four-Zn-holoenzyme.

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