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Evidence for Two Distinct CoA Binding Sites on Yeast α-Isopropylmalate Synthase*

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α-Isopropylmalate synthase (EC 4.1.3.12) from *Saccharomyces cerevisiae* was purified to a purity of about 95%. The molecular weight of the enzyme is approximately 127,000, as determined by sedimentation equilibrium centrifugation and by intersubunit cross-linking. Under denaturing conditions, one major species (95%) with molecular weight of about 65,000 is obtained. The dimeric structure of the enzyme is apparently unaffected by the presence of various ligands, including substrates, the feedback inhibitor leucine, and the inactivating combination of CoA plus Zn^{2+}.

Our previous observation that CoA, a product of the reaction, causes a very specific, Zn^{2+}-dependent, reversible inactivation of yeast α-isopropylmalate synthase (Tracy, J., and Kohlhaw, G. B. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 1802–1806) was further analyzed. Evidence is presented for the existence of two distinct CoA sites on each enzyme subunit. The first site (product site) interacts with CoA and desulfo-CoA, both of which are competitive inhibitors with respect to acetyl-CoA, with apparent K_i values of 70 and 90 μM, respectively. Equilibrium dialysis experiments show that up to one product site per subunit can be saturated with CoA, and that the binding capacity changes in parallel with the specific activity of the enzyme. The dissociation constant for CoA binding at the product site is approximately 65 μM. The second site ("regulatory site") appears to be absolutely specific for CoA. Binding of CoA to this site occurs only when Zn^{2+} is present, is independent of the specific activity of the enzyme, and does not eliminate CoA binding at the product site. Thus, when Zn^{2+} is present, the number of CoA sites saturable per subunit increases by 1.0. A dissociation constant of about 35 μM can be calculated for the Zn^{2+}-dependent binding of CoA.

The evolutionary establishment of a second CoA binding site is interpreted as further corroborating the idea that the CoA-mediated inactivation of α-isopropylmalate synthase has regulatory significance.

Yeast α-isopropylmalate synthase (EC 4.1.3.12), which catalyzes the first committed step in the biosynthesis of leucine, *i.e.* the reaction between acetyl-CoA and α-ketoisovalerate to give α-isopropylmalate and CoA, is subject to feedback inhibition by leucine (1, 2). In addition, the enzyme has been shown to undergo a highly specific, time-dependent reversible inactivation by CoA (2, 3). That this latter effect is not simple product inhibition was suggested by the following observations: CoA-mediated inactivation cannot be prevented by acetyl-CoA nor can it be reversed by subsequent addition of excess acetyl-CoA and α-ketoisovalerate (reversal is possible, however, by removal of CoA); also, CoA inactivation requires the addition of divalent metal ions, preferably Zn^{2+}, while no such requirement exists for catalytic activity. Recent work has established that at least two more biosynthetic enzymes in acetyl-CoA utilizing pathways in yeast are specifically and reversibly inactivated by CoA. These are homocitrate synthase (EC 4.1.3.21) (3), the first committed enzyme in lysine biosynthesis, and hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) (4), a key enzyme in sterol biosynthesis. Taking into account the additional observations that the CoA-sensitive enzymes are all associated with the mitochondria and that citrate synthase (EC 4.1.3.7) is completley resistant to CoA-mediated inactivation, we have proposed a regulatory mechanism according to which a decrease in the intramitochondrial acetyl-CoA concentration is accompanied by an increase in the concentration of free CoA which in turn inactivates biosynthetic, acetyl-CoA utilizing reactions (3). The result would be a channeling of acetyl-CoA into the citrate cycle whenever a critically low concentration of this key metabolite is reached.

The possible regulatory role of CoA prompted us to study in greater detail the interaction between CoA and α-isopropylmalate synthase. We present evidence in this report for the existence of a distinct, "regulatory" CoA site on the surface of each α-isopropylmalate synthase subunit.

EXPERIMENTAL PROCEDURES

Reagents—CoA (90% pure), oxidized CoA, 3′-dephospho-CoA, and (1-N^6-etheno)-CoA were purchased from P-L Biochemicals, Inc. The purity of CoA as supplied was routinely checked by a stoichiometric assay using phosphotransacetylase from *Clostridium kluyveri* (5) and by titration with DTNB.[1] [*G*-^{3}H]CoA (specific radioactivity, 800 mCi/mmol) was obtained from New England Nuclear. The radiochemical purity (95%) was confirmed by thin layer chromatography in isobutyric acid:ammonium hydroxide (28%):water (57:4:39). Dimethylsuberimidate was obtained from Pierce Chemicals. Guani-

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[1] The abbreviations used are: DTNB, 5,5′-dithiobis-(2-nitrobenzoate); SDS, sodium dodecyl sulfate.

dine HCl (ultra-pure) and enzyme grade ammonium sulfate were bought from Schwarz/Mann. Desulfo-CoA, an analogue in which the sulfhydryl group is replaced by hydrogen, was prepared by treatment of CoA with Raney nickel catalyst as described by Chase *et al.* (6). The compound was then purified by chromatography on acid-washed charcoal and on Dowex 1-X2 (7). It was identified as desulfo-CoA by several criteria: (*a*) the absorption spectrum at pH 7.5 showed a maximum at 258 nm and a minimum at 235 nm; (*b*) the compound gave a single ultraviolet absorbing spot on thin layer plates that did not correspond to CoA, oxidized CoA, or 3′ dephospho-CoA; (*c*) it failed to react with DTNB; and (*d*) it was a competitive inhibitor of phosphotransacetylase with respect to CoA. Acetyl-CoA was prepared by acetylation with acetic anhydride after the method of Simon and Shemin (8). All other chemicals were of the best grade available.

Organism – Commercially obtained bakers' yeast (*Saccharomyces cerevisiae* grown by Anheuser-Busch Inc., St. Louis, Mo.) was used throughout these studies.

Assay of α-Isopropylmalate Synthase Activity – The activity was measured by determining with DTNB the amount of CoA liberated within a timed incubation period. The general procedure has been described (9). The standard assay mixture contained 25 μmol of Tris/HCl buffer, pH 8.5, 10 μmol of KCl, 0.1 μmol of acetyl-CoA, 0.5 μmol of α-ketoisovalerate, and enzyme in a total volume of 0.125 ml. When assays were performed at pH 7.2, 4-morpholinopropanesulfonic acid-KOH buffer was substituted for Tris/HCl buffer. One unit of α-isopropylmalate synthase activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of CoA/h under the standard assay conditions. Specific activity is expressed in units/mg of protein.

Protein Determination – Protein concentration was measured either by the biuret method (10), using lyophilized bovine serum albumin as a standard, or by the method of Warburg and Christian (11).

Purification of α-Isopropylmalate Synthase – The procedure employed here (see Table I) was a modification of a previous method which had yielded partially purified enzyme (2). Essentially, the polyethylene glycol fractionation of the previous method was replaced with an ammonium sulfate fractionation (0 to 50% and 50 to 70% saturation), and the hydroxyapatite chromatography was replaced with combined hydrophobic and affinity chromatography on L-leucine-Sepharose, the principle of which has been described (9). The purified enzyme was stored at 4° as a suspension in 3.6 M ammonium sulfate in the presence of 5 mM dithiothreitol.

Disc Gel Electrophoresis – Proteins were electrophoresed in 7.5% polyacrylamide disc gels following the procedure outlined by Davis (12), except that stacking and sample gels were omitted. Samples containing 10% (v/v) glycerol were applied directly to the surface of the gels. If enzyme activity was to be determined in gels, the gels were pre-electrophoresed for 30 min to remove traces of the ammonium persulfate catalyst.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis – SDS polyacrylamide gel electrophoresis was done according to the general procedure of Weber and Osborn (13). Carboxymethylation of α-isopropylmalate synthase was performed with iodoacetate (14) after denaturation with 6 M guanidine HCl and reduction.

Cross-linking of α-Isopropylmalate Synthase – Purified α-isopropylmalate synthase (0.5 mg/ml), dissolved in 0.2 M triethanolamine/HCl buffer, pH 8.5, was treated for 30 min at 23° with 2 mg/ml of dimethylsuberimidate. This was followed by SDS-polyacrylamide gel electrophoresis (13).

Sedimentation Equilibrium Analysis – The Spinco model E analytical ultracentrifuge used was equippped with electronic speed control and an RTIC temperature control unit. A 12-mm aluminum-filled epon 2.5° double-sector synthetic boundary centerpiece was used for long column meniscus depletion experiments (15), whereas a 12-mm Kel-F 2.5° double-sector centerpiece was used for short column work (16). No fluid base was used (17). Molecular weights were calculated in the usual way from plots of the squared distance from the center of rotation *versus* the logarithm of fringe displacement (16). The partial specific volume was assumed to be 0.728 ml/g at 20°, a value previously determined for α-isopropylmalate synthase from *Salmonella typhimurium* (18). A very similar value had been calculated from the amino acid composition of the enzyme from *Neurospora crassa* (19).

Equilibrium Dialysis – Since CoA traverses cellophane membranes only slowly, the porosity of the membranes was increased by treatment with $ZnCl_2$ solution (20). After treatment, the membranes were soaked overnight in 50 mM Na_2EDTA, exhaustively washed, and stored at 4° in 50% ethanol. The treated membrane allowed equilibration of CoA within 2 to 3 h. It also allowed diffusion of cytochrome *c*, but not of ovalbumin or α-isopropylmalate synthase. Prior to each experiment, the enzyme was dialyzed for 16 h at 4° against 1000 volumes of 50 mM 4-morpholinopropanesulfonic acid KOH buffer, pH 7.5, containing 10% glycerol and, when required, 50 μM $ZnCl_2$. The dialysate was used to prepare the ligand solutions. Dialysis was carried out on a rotating wheel at 4° for 6 h. Duplicate 15-μl samples were removed from each chamber of the dialysis cells and discharged into 100 μl of water. Radioactivity was measured after adding 10 ml of scintillation cocktail.

Radioactivity Measurements – A toluene-based scintillation cocktail was used. It contained 7.0 g of 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole, 80 g of naphthalene, 400 ml of ethylene glycol monomethyl ether, and 600 ml of scintillation grade toluene. The counting efficiency of [^{3}H]CoA in 10 ml of this cocktail containing 1% (v/v) water was 20% using a Beckman CPM-100 scintillation counter.

RESULTS

Purification of Yeast α-Isopropylmalate Synthase – Table I summarizes the procedure which was used to isolate α-isopropylmalate synthase from a cell-free yeast extract. SDS-polyacrylamide gel electrophoresis of the purified material re-

TABLE I

Purification of yeast α-isopropylmalate synthase

Assays were performed at pH 8.5.

Step	Total activity	Total protein	Specific activity	Purification	Recovery
	10^{-3} × units	*g*	*units/mg*	*–fold*	*%*
1. Cell-free extract[a]	61.2	63	0.97	1.0	100
2. Streptomycin sulfate treatment (1.5%, w/v)	64.8	51	1.27	1.3	106
3. Ammonium sulfate precipitation					
(0–50% supernatant)	49.4	17.6	2.8	2.9	81
(50–70% pellet)	46.4	5.2	8.9	9.2	76
4. Leucine-Sepharose chromatography[b]					
4a	32.3	0.303	107	110	53
4b	22.4	0.057	393	405	37
4c	10.8	0.025	432	445	18
5. Ammonium sulfate precipitation (75% saturated)	9.9	0.023	430	444	16

[a] The cell-free extract was prepared by suspending 1 part of yeast cells (washed wet cake) in 1 part of 50 mM potassium phosphate buffer, pH 7.5, which contained 1.5 mM phenylmethylsulfonylfluoride, and passing the suspension through an Aminco French pressure cell. This was followed by centrifugation at 18,000 × *g*.

[b] Steps 4a, b, and c refer to three successive adsorptions to and elutions from L-leucine-Sepharose columns with intermittent pooling of the most active fractions. Adsorptions and elutions were performed following the principle of combined hydrophobic and affinity chromatography (9).

vealed one major protein band and two small bands of lower molecular weight. From a densitometric scan (Fig. 1) it was estimated that the major species constituted 95% of the total protein, with the two contaminants equaling approximately 2% and 3% of the total. The major component was identified as α-isopropylmalate synthase after gel electrophoresis in the absence of SDS. When a gel was incubated for 30 min in standard assay medium and subsequently immersed in 1 mM DTNB, a single yellow band appeared which coincided with the major protein species.

Molecular Weight Determinations of Native and Denatured Enzyme—When highly purified, fully active α-isopropylmalate synthase was analyzed by sedimentation equilibrium centrifugation, the data yielded a straight line when plotted according to Yphantis (16). From the slope of the line, the apparent molecular weight was calculated to be 127,000. A similar experiment was performed with enzyme which had been denatured with 6 M guanidine HCl. The data again yielded a straight line in an Yphantis plot, corresponding to a molecular weight of 64,500. The size of the α-isopropylmalate synthase subunit was also determined by SDS-polyacrylamide gel electrophoresis after rapid denaturation of the enzyme with SDS and 2-mercaptoethanol. As shown in Fig. 2, a value of about 67,000 was found for yeast α-isopropylmalate synthase. The same value was obtained with reduced, carboxymethylated enzyme and with gels containing 5% and 7.5% acrylamide, respectively. Also shown in Fig. 2 is the position of the subunit of α-isopropylmalate synthase from *Salmonella typhimurium*, confirming its well established molecular weight of about 50,000 (27, 28).

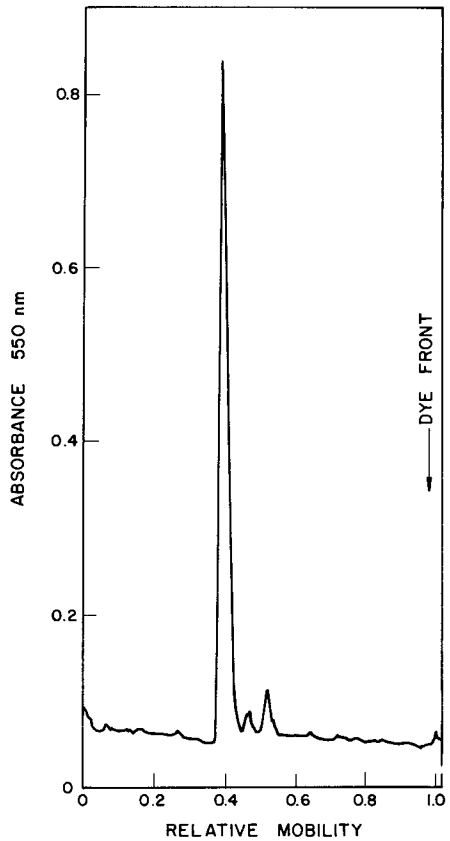

FIG. 1. Densitometric scan of purified α-isopropylmalate synthase obtained after SDS-polyacrylamide gel electrophoresis. The gel, stained for protein with Coomassie brilliant blue R250, was scanned at 550 nm using a Gilford model 240 spectrophotometer equipped with a Gilford model 2410-S linear transport and connected to a Sargent model SRLG recorder. The absorbance was proportional to protein concentration up to a value of about 1.2. From the relative peak areas, this preparation was estimated to be 95% pure.

More evidence for a dimeric structure of the yeast enzyme was obtained when the enzyme was cross-linked with dimethylsuberimidate in the presence and absence of various ligands. In parallel experiments, the enzyme was incubated for 15 min at 23° and subsequently cross-linked in the presence of (*a*) 10 mM L-leucine; (*b*) 0.8 mM acetyl-CoA plus 4 mM α-ketoisocaproate (an inhibitory homologue of α-ketoisovalerate), (*c*) 1 mM CoA plus 50 μM $ZnCl_2$, or (*d*) in the absence of ligands. After cross-linking, the enzyme was denatured with SDS and electrophoresed on SDS-polyacrylamide gels using cross-linked rabbit muscle aldolase as a standard. Strikingly, only one species, corresponding to a molecular weight of 127,500, was observed whether or not ligands were present. The concentration of leucine present before and during cross-linking was sufficient to completely inhibit the enzyme. Therefore, in contrast to what was seen with α-isopropylmalate synthase from *S. typhimurium* (27), feedback inhibition of the yeast enzyme apparently does not involve dissociation, which is supported by gel filtration data (2). A similar argument can be put forward with respect to the combined effect of CoA and Zn^{2+}. While the enzyme was completely inactivated under the conditions specified above, there was apparently no change in its quaternary structure.

The molecular weight determinations, taken together, indicate that yeast α-isopropylmalate synthase is a relatively stable dimeric protein composed of subunits with a molecular weight of about 65,000.

Involvement of CoA Sulfhydryl Group in CoA-mediated Inactivation—The previously observed failure of closely related analogues of CoA, such as 3′-dephospho-CoA or 1-N^6-etheno-CoA, to inactivate α-isopropylmalate synthase emphasized the specificity of this effect (3). The ineffectiveness of oxidized CoA (3) hinted at a possible involvement of the sulfhydryl group. Further evidence for such involvement was obtained when desulfo-CoA, an analogue in which the sulfhy-

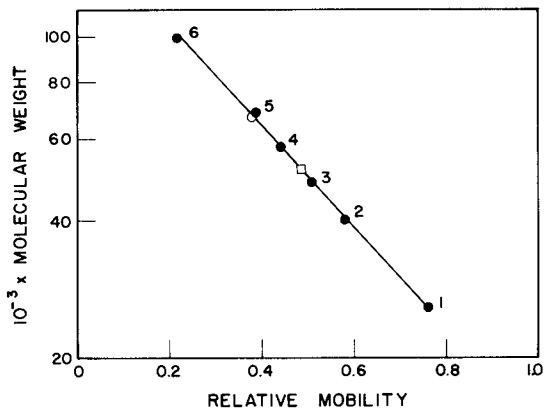

FIG. 2. Subunit molecular weight of α-isopropylmalate synthase as determined by SDS-polyacrylamide gel electrophoresis. Approximately 3-μg samples of α-isopropylmalate synthase from yeast (○), α-isopropylmalate synthase from *Salmonella typhimurium* strain CV-19 (□ see Ref. 9), and selected standards (●), were electrophoresed in separate 6% gels. The standards used were: *1*, α-chymotrypsinogen, M_r = 25,700 (21); *2*, rabbit muscle aldolase, M_r = 40,000 (22); *3*, pig heart fumarase, M_r = 48,500 (23); *4*, bovine liver catalase, M_r = 57,500 (24); *5*, bovine serum albumin, M_r = 68,000 (25); and *6*, rabbit muscle phosphorylase, M_r = 921,500 (26). The *straight line* is a least squares fit of the standards.

TABLE II

CoA sulfhydryl group requirement for inactivation of α-isopropylmalate synthase

Purified α-isopropylmalate synthase (25 μg/ml), dissolved in 50 mM 4-morpholinopropane sulfonic acid-KOH buffer, pH 7.5, containing 10% (v/v) glycerol, was incubated at 30° with the indicated additions. After 5 min, 10-μl samples were withdrawn and diluted 12.5-fold into the standard assay medium. Relative activity is expressed as percentage of activity at zero time.

Additions	Relative activity remaining after 5 min
	%
None	95
11 μM CoA, 25 μM $ZnCl_2$	9
100 μM desulfo-CoA, 25 μM $ZnCl_2$	94
1 mM desulfo-CoA, 25 μM $ZnCl_2$	90
11 μM CoA	95
25 μM $ZnCl_2$	93

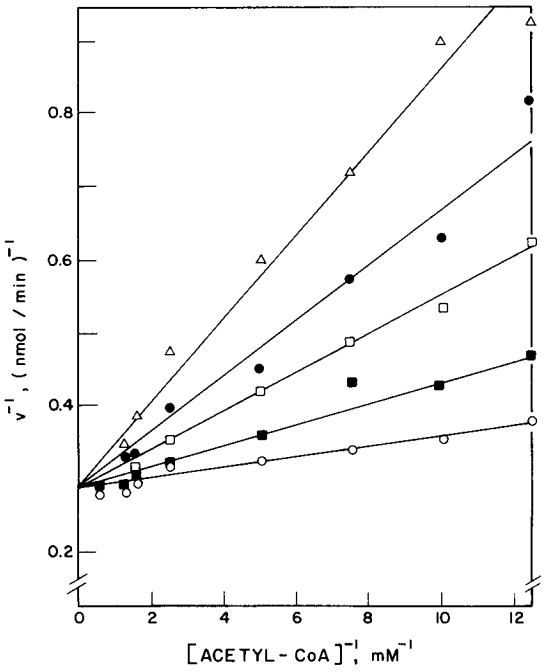

FIG. 3. Double reciprocal plot of initial velocity as a function of the acetyl-CoA concentration at several CoA concentrations. Assays were performed at pH 7.2. The protein concentration was 8 μg/ml. The α-ketoisovalerate concentration was maintained at 4 mM. CoA concentrations were: ○, no CoA; ■, 60 μM; □, 100 μM, ●, 170 μM; △, 340 μM.

dryl group has been replaced by hydrogen, was tested for its ability to inactivate (Table II). Although α-isopropylmalate synthase was sensitive to 11 μM CoA, concentrations of desulfo-CoA up to 1 mM had no significant inactivating effect. It may be concluded that the sulfhydryl group of CoA is indispensable for inactivation. The implications of this conclusion will be discussed below.

Product Inhibition by CoA and CoA Analogues – There was no inactivation of α-isopropylmalate synthase by CoA when divalent metals were absent (3) (Table II). It was therefore possible to study "normal" CoA product inhibition without the complications of CoA-mediated inactivation by simply omitting Zn^{2+} and other divalent metals from the assay medium. Fig. 3 shows that CoA was a competitive inhibitor with respect to acetyl-CoA when α-ketoisovalerate was saturating. A secondary plot of slopes *versus* CoA concentration was linear and

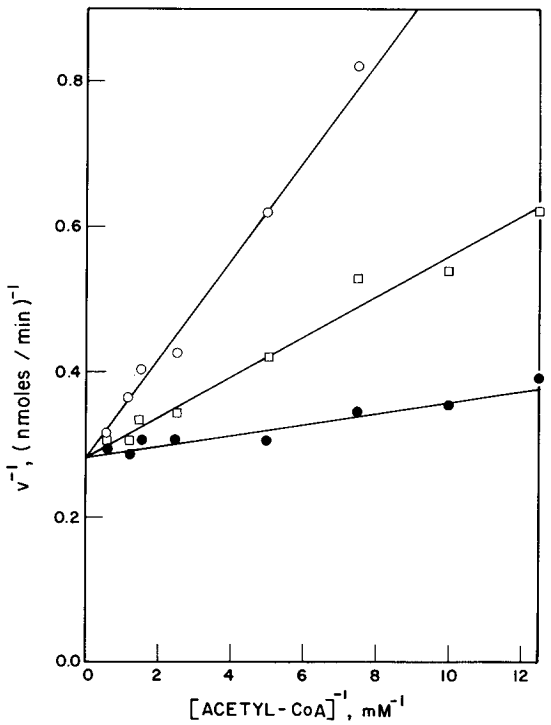

FIG. 4. Double reciprocal plot of initial velocity as a function of the acetyl-CoA concentration at various desulfo-CoA concentrations. Assays were performed as described in the legend to Fig. 3. The desulfo-CoA concentrations were: ●, no desulfo-CoA; □, 250 μM; ○, 500 μM.

yielded an apparent K_i value of 70 μM. When acetyl-CoA was saturating and α-ketoisovalerate was the variable substrate, CoA concentrations up to 340 μM had no effect on activity. Product inhibition was also studied with analogues of CoA. 3′-Dephospho-CoA and 1-N^6-etheno-CoA did not inhibit the enzyme even at low acetyl-CoA concentrations. However, competitive inhibition (with respect to acetyl-CoA) by desulfo-CoA was comparable to that observed with CoA itself (Fig. 4). An apparent K_i value of about 90 μM was obtained from a secondary plot. As with CoA, no inhibition was seen with desulfo-CoA at concentrations up to 500 μM when the α-ketoisovalerate concentration was varied and the acetyl-CoA concentration kept saturating.

Binding of CoA to α-Isopropylmalate Synthase – CoA binding was first studied in the absence of Zn^{2+}, *i.e.* in the absence of CoA-mediated inactivation. In the experiment shown in Fig. 5, three enzyme preparations of different specific activity were used. The two preparations with lower specific activity were generated from the preparation with the highest specific activity by storage for different lengths of time in the absence of dithiothreitol. As is evident from Fig. 5*B*, a linear dependence of the CoA-binding capacity, r_t, on enzyme specific activity was observed. This suggests that the CoA product site was destroyed to the extent that specific activity was lost during storage. The value of r_t approached 1.0 when enzyme with the highest specific activity was used. An approximate dissociation constant of 65 μM for CoA binding in the absence of Zn^{2+} was obtained from a Scatchard plot of the data of Fig. 5*A* (*upper curve, cf.* Fig. 7).

The two preparations with specific activity of about 44 and 225 units/mg, respectively, were also employed to study CoA binding in the presence of 50 μM Zn^{2+}. The results are shown in Fig. 6. Enzyme with approximately half-maximal specific

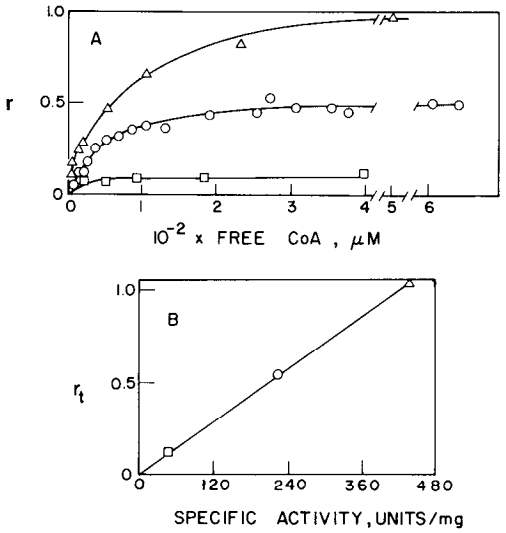

FIG. 5. Binding of CoA to α-isopropylmalate synthase as a function of enzyme specific activity. *A*, equilibrium dialysis was performed with enzyme (2 mg/ml) of the following specific activities (units/mg): □, 47; ○, 225; △, 430. The binding ratio, *r*, is the number of moles of CoA bound/mol of enzyme monomer. No Zn^{2+} was present during these experiments, and no enzyme activity was lost. *B*, plot of r_t, the total number of CoA binding sites/enzyme monomer saturable under a given set of conditions, *versus* enzyme specific activity. Three different plots were employed to estimate the values of r_t: (*a*) *r versus* $[CoA]_{free}$, shown in *A*; (*b*) $r/[CoA]_{free}$ *versus r* (Scatchard plot); and (*c*) *r versus* log $[CoA]_{free}$ (29, 30). The Scatchard plots (data from the *upper two curves* of *A* only) were linear. The r_t value for the preparation with the lowest specific activity is only approximate.

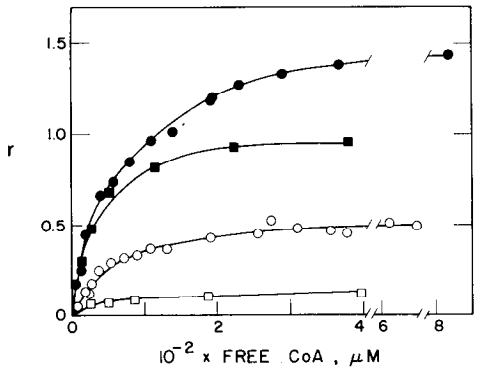

FIG. 6. Binding of CoA to α-isopropylmalate synthase in the absence and in the presence of Zn^{2+}. Enzyme concentration was 2 mg/ml. The symbol *r* is used as defined in the legend to Fig. 5. □, enzyme with specific activity of 47, no Zn^{2+} (replot of data in Fig. 5*A*); ■, enzyme with initial specific activity of 41, 50 μM Zn^{2+} present; ○, enzyme with specific activity of 225, no Zn^{2+} (replot of data in Fig. 5*A*); ●, enzyme with initial specific activity of 225, 50 μM Zn^{2+} present. When Zn^{2+} was present, no measurable enzyme activity remained after equilibrium dialysis.

activity (225 units/mg), which bound a total of about 0.5 mol of CoA/mol of monomer in the absence of metal, was found to bind about 1.6 mol of CoA/mol of monomer when Zn^{2+} was present (Scatchard plot extrapolation). Likewise, enzyme with approximately one-tenth of the maximal specific activity, which bound a total of about 0.1 mol of CoA/mol of monomer in the absence of Zn^{2+}, was able to bind slightly more than 1.0 mol of CoA/mol of monomer in the presence of Zn^{2+} (Scatchard plot extrapolation, *cf.* Fig. 7). In other words, the presence of Zn^{2+} increased the CoA binding capacity of α-isopropylmalate synthase by almost exactly one site per monomer. Interestingly, the intactness of this Zn^{2+}-dependent site did not appear to be affected by the specific activity of the enzyme. From the experiment with enzyme of low specific activity where the contribution of CoA binding to the product site should be minimal (*filled squares* in Figs. 6 and 7), a dissociation constant of 35 μM for CoA binding to the Zn^{2+}-dependent site was calculated.

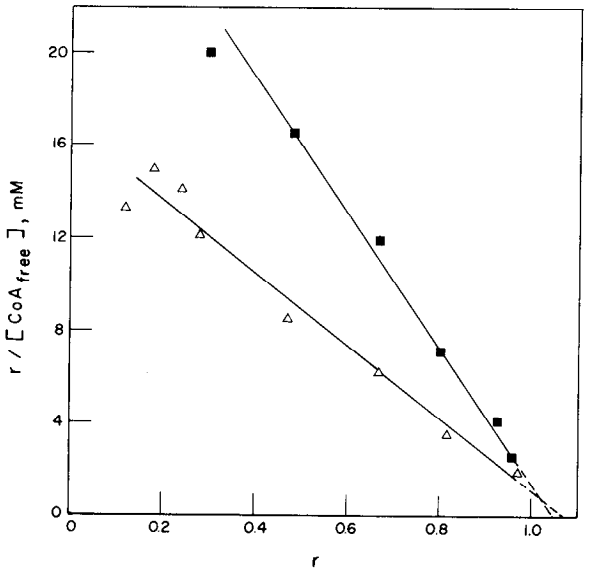

FIG. 7. Scatchard plots of data shown in Figs. 5 and 6. △, CoA binding to enzyme of high specific activity (430) in the absence of Zn^{2+}; ■, CoA binding to enzyme of low specific activity (41), but in the presence of 50 μM Zn^{2+}. The symbol *r* has the same meaning as in Figs. 5 and 6. The *straight lines* were fitted to the data by the method of least squares.

DISCUSSION

Our data indicate that CoA plays a dual role in its interaction with yeast α-isopropylmalate synthase. It interacts with the enzyme both as a reaction product and as an inactivating agent. The fact that inactivation requires Zn^{2+} made it possible to dissect the two functions. Thus, in the absence of Zn^{2+} (and other divalent metal ions) CoA is a product inhibitor which is strictly competitive with the substrate acetyl-CoA. The fact that desulfo-CoA also is a competitive inhibitor with respect to acetyl-CoA, while analogues with modifications in the adenosine portion of CoA have no effect, suggests that binding at the product site requires an intact nucleotide moiety, but does not require the sulfhydryl group of CoA. From product inhibition data, an apparent K_i value of 70 μM was calculated for CoA, which is in close agreement with the dissociation constant of about 65 μM obtained from direct binding studies.

In the presence of Zn^{2+}, CoA assumes the role of inactivator. As such, it is very specific, with none of the above mentioned CoA analogues, including desulfo-CoA, having a significant effect. It is noteworthy that acetyl-CoA, at concentrations up to several hundred-fold above that of CoA, is unable to prevent or to reverse the CoA-mediated inactivation; it also has no inactivating effect by itself. These observations clearly point to a participation of the CoA sulfhydryl group in the inactivation process. At least four possibilities for the involvement of the sulfhydryl group may be considered: (*a*) formation of a mixed disulfide between CoA and an enzyme sulfhydryl in a metal-catalyzed oxidation; (*b*) formation of a mixed disulfide between CoA and an enzyme disulfide bridge in a disulfide

exchange reaction; (*c*) formation of a thioester bond with an enzyme carboxyl group; and (*d*) participation of the CoA sulfhydryl group in a coordination complex with Zn^{2+}. The first of these possibilities was ruled out when it was found that CoA inactivation proceeded equally well in the complete absence of molecular oxygen as it did in its presence (3). Another argument against this mechanism is the fact that inactivated enzyme can be reactivated by dialysis (3). The second possibility, a disulfide exchange reaction, cannot be ruled out at present. A decision on this mechanism must wait until information becomes available concerning the existence of disulfide bridges in the enzyme. The third mechanism, thioester formation, would required the input of energy. A CoA-enzyme intermediate of this type has in fact been generated only from acyl-CoA substrates (with CoA transferases, Refs. 31 to 33). In an experimental approach with respect to the fourth possibility, we have studied Zn^{2+} binding to CoA by gel filtration and have observed that a $Zn^{2+} \cdot$CoA complex exists (7). The failure of desulfo-CoA to react with Zn^{2+} under the same conditions indicated the participation of the CoA sulfhydryl group in the formation of this complex. While these results do not prove that a $Zn^{2+} \cdot$CoA complex is the inactivating agent, they do mean that such a mechanism must be given serious consideration.

The differences in the behavior of CoA as reaction product on the one hand and as inactivator on the other led to the assumption that there might be two distinct sites for CoA on the enzyme surface. Equilibrium binding studies provided direct evidence for this assumption and at the same time revealed the complex nature of the CoA-mediated inactivation. The data of Fig. 6 show that c_z, the binding capacity for CoA (*i.e.* the total number of CoA sites saturable) in the presence of Zn^{2+}, can be described by the expression:

$$c_z = c_p + 1$$

where c_p signifies the CoA binding capacity in the absence of Zn^{2+} (*i.e.* at the product site only). This means that in the presence of Zn^{2+}, a new CoA site is opened up. At the same time, the binding capacity for CoA at the product site remains unchanged even though the enzyme is inactivated. It appears, therefore, that CoA-mediated inactivation and inactivation by aging proceed by different mechanisms. In the case of enzyme aging, the binding capacity for CoA at the product site does change in parallel with the loss of activity (*cf.* Fig. 5). Presumably, the CoA-mediated inactivation is directed primarily toward the α-ketoisovalerate site. The observation that high concentrations of α-ketoisovalerate can protect against CoA-mediated inactivation, whereas acetyl-CoA cannot, is consistent with this view.

A possible arrangement of the various binding sites proposed to exist on a subunit of yeast α-isopropylmalate synthase is shown schematically in Fig. 8. A separate binding site for leucine has tentatively been included in this model although there is no direct evidence for this at present. There are, however, at least two facts which are consistent with there being a separate site for leucine: (*a*) the existence of α-isopropylmalate synthase mutants with strongly reduced sensitivity to leucine (1, 34), and (*b*) the fact that leucine inhibition is noncompetitive with respect to both substrates (2).

In all likelihood, the second CoA site was established and retained during evolution because it provided the yeast cell with a selective advantage. It is conceivable that this advantage consisted of the acquisition of a sensor for changes in the mitochondrial CoA concentration, a sensor which differed

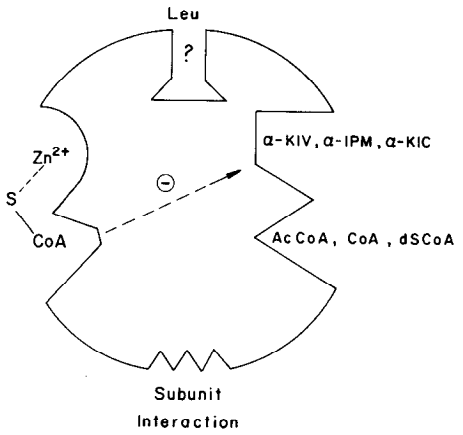

FIG. 8. Proposed binding sites on yeast α-isopropylmalate synthase subunit. The interaction between CoA and Zn^{2+} is only one of two plausible alternatives for the involvement of the CoA sulfhydryl group in the Zn^{2+}-dependent inactivation; the other is interaction between CoA and an enzyme disulfide bridge exposed by the prior binding of Zn^{2+}. See text for further details. *AcCoA*, acetyl-CoA; *dSCoA*, desulfo-CoA; *α-KIV*, α-ketoisovalerate; *α-IPM*, α-isopropylmalate; *α-KIC*, α-ketoisocaproate.

from the CoA product site in two important ways. It was more sensitive, and it was not subject to competition by acetyl-CoA. In other words, CoA-mediated inactivation, in contrast to CoA product inhibition, was independent of the absolute concentration of acetyl-CoA, which made it possible for the cell to regulate acetyl-CoA utilization at any desirable level of acetyl-CoA. We think that the regulatory mechanism which becomes manifest as CoA-mediated inactivation is triggered by a decrease in the (mitochondrial) acetyl-CoA concentration to some critical value. This decrease in the acetyl-CoA concentration would be coupled to an increase in the concentration of free CoA, which in turn would lead to the inactivation of α-isopropylmalate synthase and at least two other biosynthetic, acetyl-CoA utilizing enzymes.[2] For reasons discussed earlier (see introduction), the likely result of the selective inactivation by CoA would be a channeling of acetyl-CoA into the citrate cycle.

For the CoA-mediated inactivation to be an effective control mechanism, it should be reversible. We have obtained reactivation of CoA-inactivated enzymes by dialysis (3). It has also been observed that the addition of an excess of oxidized glutathione can lead to reactivation *in vitro* (7), probably by means of a disulfide exchange reaction. *In vivo* reactivation might require the participation of CoA recycling enzymes such as acetyl-CoA synthetase or pyruvate oxidase. Further work is required to establish this point.

It is of interest to note that the molecular weight of the yeast α-isopropylmalate synthase subunit (about 65,000) is significantly higher than that found for α-isopropylmalate synthase from both *S. typhimurium* (approximately 50,000; Refs. 27 and 28; also Fig. 2) and *Neurospora crassa* (43,000 to 50,000; Refs. 19 and 35). It is not known whether the enzyme from *N. crassa* is sensitive to CoA. We have observed, however, that the bacterial enzyme is not subject to specific, reversible inactiva-

[2] We have recently observed that the following acetyl-CoA consuming enzymes are not inactivated by CoA: acetyl-CoA carboxylase (EC 6.4.1.2), glucosaminephosphate acetyltransferase (EC 2.3.1.4), D-amino-acid acetyltransferase (EC 2.3.1.36), and malate synthase (EC 4.1.3.2); also, all of these enzymes appear to be extramitochondrial (A. Tan-Wilson and G. Kohlhaw, unpublished experiments).

tion by CoA.[3] The question may therefore be asked whether the increase in the size of the yeast α-isopropylmalate synthase subunit is related to the CoA sensitivity of this enzyme. It is tempting to speculate that partial sequence duplication, possibly of the active site region, followed by extensive modification during evolution, led to the structure proposed in Fig. 8. Alternatively, gene fusion between the gene coding for α-isopropylmalate synthase and a gene coding for another protein with a CoA binding site may have provided the evolutionary basis for the second CoA site.

[3] J. Tracy, E. Teng-Leary, and G. Kohlhaw, unpublished observations.

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