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α-Isopropylmalate Synthase from *Alcaligenes eutrophus H 16*

II. Substrate Specificity and Kinetics*

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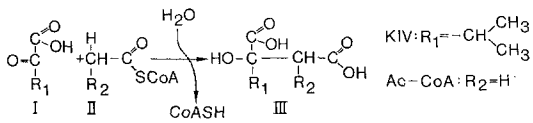
Abstract. The purified isopropylmalate synthase of *Alcaligenes eutrophus H 16* reacted with the following α-keto acids and acyl-coenzyme A derivatives (in the sequence of decreasing affinities): α-ketoisovalerate, α-keto-n-valerate, α-ketobutyrate and pyruvate; acetyl-CoA, propionyl-CoA, butyryl-CoA, malonyl-CoA, valeryl-CoA, and crotonyl-CoA. α-Ketoisocaproate, however, is a strong inhibitor of the enzyme. All reactions catalyzed by isopropylmalate synthase were inhibited to the same extent by the endproduct L-leucine. The substrate saturation curves of α-ketoisovalerate or other α-keto acids and of acetyl-coenzyme A or other acyl-CoA derivatives had intermediary plateau regions; the Hill coefficient alternated between n_H-values higher and lower than 1.0, indicating changes from positive to negative and from negative to positive cooperativity for the substrates. The products, isopropylmalate and free coenzyme A, showed competitive inhibition patterns against both substrates (α-ketoisovalerate and acetyl-CoA). Free coenzyme A (1 μM) inactivated the enzyme irreversibly. The 3′-phosphate of coenzyme A and the free carboxyl group of α-ketoisovalerate were involved in optimal binding of these substrates, but 3′-dephospho-acetyl-coenzyme A and the methylester of α-ketoisovalerate were also converted by this enzyme. A CH_3-CH_2-grouping of the α-keto acids seemed to be necessary for binding this substrate.

Key words: Hydrogen bacteria – *Alcaligenes eutrophus H 16* – Leucine biosynthesis – α-Isopropylmalate synthase – Cooperativity changes – Product inhibition – Substrate specificity.

The first enzyme in the pathway specific for biosynthesis of L-leucine, α-isopropylmalate (IPM) synthase (E.C. 4.1.3.12), catalyzes the addition of acetyl-coenzyme A (Ac-CoA) to α-ketoisovalerate (KIV) with the formation of α-isopropylmalate (IPM) and coenzyme A (CoA-SH):



Instead of α-ketoisovaleric acid other α-keto acids are converted by the enzyme, too. This unspecificity has been detected when studying the IPM synthases from *Neurospora crassa* (Webster and Gross, 1965), *Saccharomyces* spec. (Ulm et al., 1972) and *Salmonella typhimurium* (Kohlhaw et al., 1969).

However, no study has been made on the specificity of the enzyme for its second substrate acetyl-coenzyme A. Positive cooperativity was described for the bacterial enzyme in respect to substrate acetyl-coenzyme A. The saturation curves for KIV and for both substrates of the enzyme from fungi (Ulm et al., 1972; Webster and Gross, 1965) were hyperbolic. The enzyme reaction with various coenzyme A derivatives as well as the kinetics of substrate saturation and product inhibition are the focal topics of this paper.

MATERIALS AND METHODS

Organisms and Culture Conditions. The organism and the experimental conditions are identical to those described in our previous paper (Wiegel and Schlegel, 1977).

Enzyme Assay. The fluorimetric assay, the optical assay at 232 nm and the DTNB-assay were as described previously (Wiegel and Schlegel, 1977). The concentration of acetyl-coenzyme A was determined using citrate synthase (Srere et al., 1963) but the concentrations of other thioesters of coenzyme A and its analogues were determined by a modified nitroprussid assay (Bartsch, 1956).

Abbreviations Used. CoA = Coenzyme A; Tris = Tris(hydroxymethyl)aminomethane hydrochloride; DTNB = 5,5′-dithiobis-(2-nitrobenzoic acid); IPM = α-Isopropylmalate; KIV = α-Ketoisovalerate

* Prepared from doctoral thesis of the University of Göttingen 1973

which was calibrated with acetyl-CoA and N-acetyl-S-acetoacetyl-cysteamine.

Chemicals. KIV and pantethine were obtained from Sigma Chemical Company, St. Louis, U.S.A. Keto acids except KIV, were obtained from Fluka AG, Buchs, Switzerland. Malonyl-CoA, palmityl-CoA were purchased from Serva Entwicklungslabor, Heidelberg, Germany. Coenzyme A and 3'-dephospho-CoA were from C. F. Boehringer, Mannheim, Germany. The acyl-coenzyme A derivatives were prepared by the method of Simon and Shemin (1953) from coenzyme A and the corresponding anhydrides; β-hydroxybutyryl-coenzyme A was a gift from Dr. P. Hillmer. Pantetheine was a gift from Dr. Madan. Free coenzyme A present in the preparation of the acyl derivatives was oxidized with iodine-saturated ethanol (0.1 ml per 10 ml acyl-CoA solution), and the iodine was removed by a fourfold extraction with a tenfold volume of diethylether. KIV-methylester was prepared with diazomethane and its purity was determined by gas and thin layer chromatography. Other chemicals were of the highest chemical purity available.

RESULTS

Saturation with Substrates

Substrate saturation curves for acetyl-coenzyme A and α-ketoisovaleric acid (KIV) as variable substrates were measured at different pH-values, at varied temperatures and with Tris-HCl or potassium phosphate buffers (Figs. 1 – 3, not all different conditions shown).

Curves with intermediary plateau regions were obtained in all cases. The saturation curve with substrate acetyl-coenzyme A (Fig. 3C) using purified enzyme after preparative plate-gel electrophoresis (Wiegel and Schlegel, 1977) showed the significance of the intermediary plateau. The experimental error of the individual values was less than $\pm$ 0.3 nmoles coenzyme A formed per minute; the deviation from a hyperbolic curve was significant. Such curves have been described for CTP synthase and other enzymes by Teipel and Koshland (1969). These curves were considered indicative for both positive and negative cooperativity. Half-maximal activities were obtained with 60 µM KIV at 30° C and 120 µM KIV at 40° C and 7.5 µM acetyl-CoA at 30° C. At high concentrations and at pH 8 both substrates exerted inhibitory effects; these effects were especially pronounced in Tris-HCl buffer at pH 7.6 (Fig. 3B) with acetyl coenzyme A as substrate. From the Hill-plot Hill coefficients n_H were derived which changed with in-

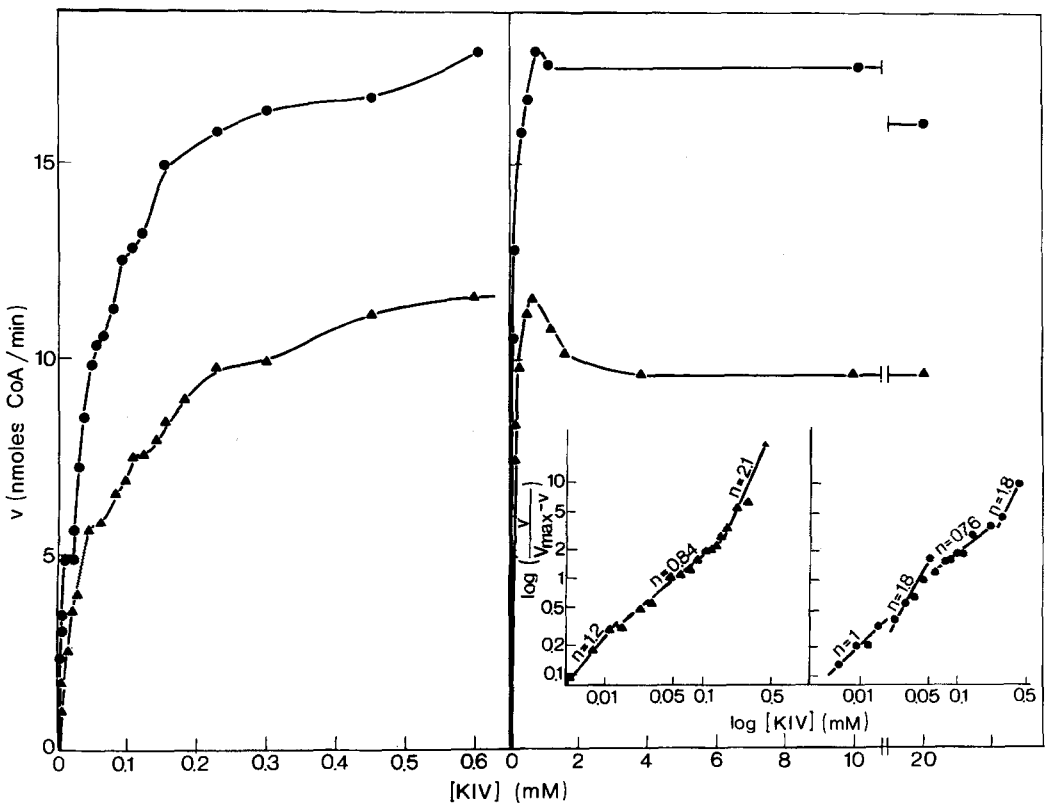


Fig. 1. Substrate (α-ketoisovalerate) saturation curves of IPM synthase at pH 8.1 and 30° C. The concentration of acetyl-CoA was 0.0875 mM (●) and 0.26 mM (▲). The buffer used was 250 mM potassium phosphate. Initial velocities (v) were determined with DTNB assay which was started with 110-fold purified enzyme. Hill-plots of the data are inserted

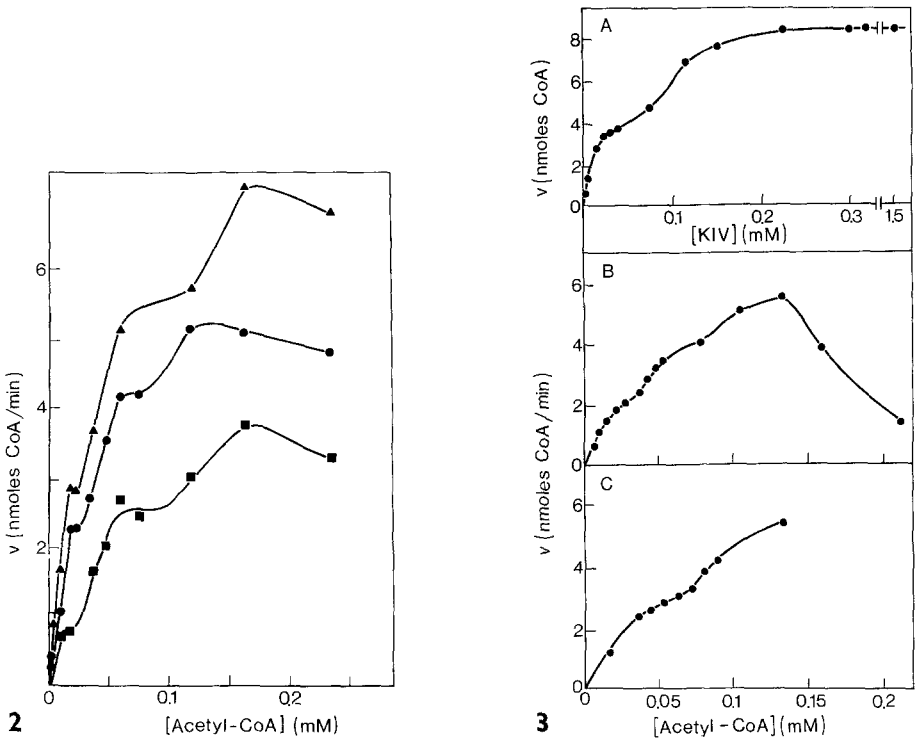


Fig. 2. Substrate (acetyl-CoA) saturation curves of IPM synthase at pH 8.0 and 30° C at different fixed concentrations of substrate KIV: 1.5 mM (▲), 0.125 mM (●) and 0.025 mM (■). The buffer used was 250 mM Tris-HCl containing 250 mM potassium chloride. Initial velocities (v) were determined with DTNB assay which was started with 110-fold purified enzyme

Fig. 3 A–C. Substrate (α-ketoisovalerate and acetyl-CoA) saturation curves of IPM synthase with substrates at different conditions. Initial velocities (v) were determined using the optical assay at 232 nm (A and B) and using the DTNB assay at 412 nm (C) at 30° C. The buffer used was 250 mM Tris-HCl pH 7.65 (A) pH 7.6 (B) and pH 8.1 (C). The fixed substrate concentration was 0.15 mM acetyl-CoA (A), 0.75 mM KIV (B) and 1.625 mM KIV (C), respectively, the other substrate was varied as indicated. The assay was started with purified enzyme (A and B: 109-fold; C: 112-fold, including preparative gel electrophoresis after Sephadex G-200 step; for details see "Materials and Methods")

creasing substrate concentrations to n_H higher than 1, lower than 1 and again higher than 1 (Fig. 1).

At higher acetyl-CoA concentrations (higher than 3 mM) the highly purified (preparative gel electrophoresis) IPM synthase exhibited a minor thioesterase activity (pH 8.0). This thioesterase activity could be inhibited by L-leucine in a similar manner as IPM synthase activity.

Substrate Specificity

The purified IPM synthase from *Alcaligenes eutrophus H 16*-A 81 was able to use several α-keto acids as substrates with equal or with shorter chain lengths than KIV. Glyoxylic acid, α-ketocaproic and α-keto acids with more than 5 carbon atoms were not used as substrates. Glyoxylic acid was not inhibitory up to a concentration of 100 mM while α-ketoisocaproic acid (an analogue of both substrate KIV and of end-product leucine) inhibited the enzyme completely at 0.5 mM concentration. Furthermore, the enzyme was inhibited by α-keto acids with more than 5 carbon atoms and even by the corresponding fatty acids (Table 1): Isocaproate, the corresponding fatty acid of leucine, was the most effective fatty acid.

In addition to acetyl-coenzyme A, higher homologous and analogous acyl-coenzyme A compounds also served as substrates (Table 2). If α-ketobutyrate or pyruvate was used instead of KIV, the rates with all coenzyme A derivatives were higher than with the natural substrate KIV. With β-hydroxybutyryl-CoA or acetoacetyl-CoA the rates were very low, even with α-ketobutyrate. As in the case of the natural substrate conversion, L-leucine also inhibited the reaction of these acetyl-CoA compounds (60–70% at 2 mM L-leucine and pH 8 or 85–92% at 0.5 mM L-leucine and pH 7.5).

Also with these substrates all saturation curves were bumpy with plateaus. Therefore, the apparent K_m-values (Table 2) are only approximate. No marked changes of the saturation concentration for the α-keto acids were observed, when different acyl-CoA esters

Table 1. Effect of α-keto acids and homologous fatty acids on the activity of IPM synthase

Number of C-atoms	Keto acids	% Activity			Fatty acids, K-salt	% Activity	
		0.5 mM	2 mM	10 mM		10 mM	50 mM
1					Formate	100	100
2	Glyoxylate	n.d.	100	100	Acetate	100	100
3	Pyruvate[a]	n.d.	108	n.d.	Propionate	100	98
4	2-Ketobutyrate[a]	n.d.	204	n.d.	Butyrate	98	74
5	2-Ketoglutarate	n.d.	100	100	–		
5	4-Ketovalerate[b]	n.d.	90	94	n-Valerate	82	78
6	2-Ketocaproate	n.d.	77	n.d.	n-Caproate	82	53
6	5-Ketocaproate[b]	n.d.	82	100			
6	2-Ketoisocaproate	0.1	< 0.1	< 0.1	iso-Caproate	45	0.1
6	2-Keto, 3-methylvalerate	29	2	0.1	–		
6	2-Ketogluconate	n.d.	94	98	–		
6	2-Ketoadipate	n.d.	94	74	–		
8	2-Ketooctanate	n.d.	75	43	n-Octanate	n.d.	63
9	2-Ketononanate	n.d.	83	71	n-Nonanate	n.d.	65

Enzyme preparation after chromatography on Sephadex G-200 was used. The enzyme was assayed using the DTNB-assay at 412 nm. (KIV = 0.09 mM; acetyl-CoA = 0.47 mM; Tris-HCl = 250 mM; pH = 8.0.) The activity in the assay without additions of analogues was taken as 100%

[a] The degree of substrate saturation of the enzyme with those α-keto acids which served as substrates was higher than with KIV as substrate alone. Thus, in the presence of both KIV and those α-keto acids, the reaction rates were greater than only with KIV = 100%

[b] In the absence of substrate KIV, the aldol addition reaction of the enzyme with these keto acids up to 100 mM was very low

n.d. = not determined

Table 2. Specificity for substrates of the IPM synthase

Acyl-CoA compound	App. K_m[a] (mM)	Rel. v_{max}[b]				
		KIV app. K_m = 0.06 mM	KnV app. K_m = 0.4 mM	KB app. K_m = 1.8 mM	Pyruvate app. K_m = 10 mM	KIV-OCH_3 app. K_m = 5 mM
Acetyl-CoA	0.075	1	0.6	4	1	0.9
Propionyl-CoA	0.1	0.25	0.01	1	6	n.d.
Butyryl-CoA	0.4	1.5	< 0.001	5	1.6	n.d.
Valeryl-CoA	0.9	< 0.01	< 0.001	0.1	0.2	n.d.
Malonyl-CoA	0.5	0.6	0.1	2	1.2	n.d.
Crotonyl-CoA	0.9	0.02	< 0.001	1	0.5	n.d.
Acetoacetyl-CoA	–	< 0.001	< 0.001	0.06	0.075	n.d.
3'-De-P-Acetyl-CoA	0.125	1	n.d.	n.d.	n.d.	n.d.

[a] App. K_m values = concentration at half maximum velocity determined with acyl-CoA and KIV as the substrates and the app. K_m values for α-keto acids were determined with acetyl-CoA as the second substrate obtained from Lineweaver-Burk diagrams without considering the deviations

[b] Relative maximal velocity = v_{max} measured with the analogous coenzyme A derivatives and α-keto acids compared to v_{max} measured with acetyl-CoA and KIV

KnV = α-keto-n-valerate; KB = α-ketobutyrate; KIV–OCH_3 = methylester of α-ketoisovaleric acid; n.d. = not determined. Enzyme preparations after chromatography on Sephadex G-200 and after preparative gel electrophoresis

were used; similarly no alterations of the saturation concentration for the acyl-CoA esters were detected when different α-keto acids were used.

Product Inhibition

The reaction of IPM synthase was competitively inhibited by the product α-isopropylmalate (IPM), with respect to both substrates KIV (Fig. 4B) and acetyl-CoA (Fig. 4A). The apparent K_i-values of IPM with respect to the enzyme substrate KIV were calculated to be 0.5 mM at a substrate concentration of 0.07 mM acetyl-CoA, and 0.7 mM at 0.175 mM acetyl-CoA. The K_i-values of IPM against acetyl-CoA are 0.9 mM at 0.375 mM KIV concentration and 4 mM at 0.75 mM KIV, respectively.

The addition of the product IPM did not alter the multiphasic course of the substrate saturation

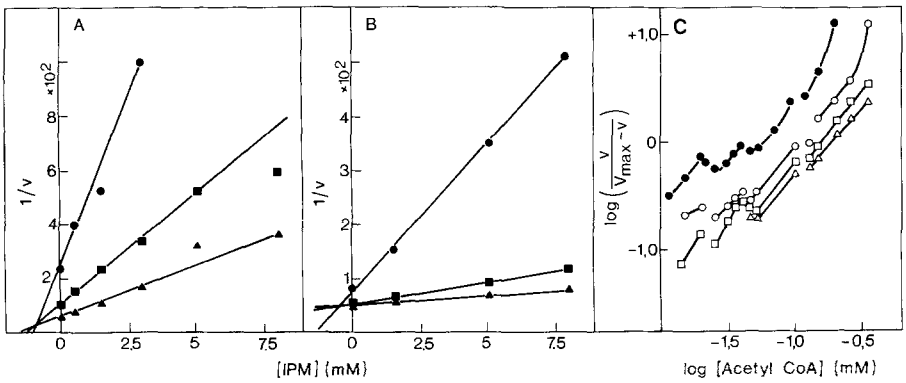


Fig. 4A–C. Inhibition of IPM synthase by its product isopropylmalate (*IPM*). The 110-fold purified enzyme was assayed in 250 mM Tris-HCl (pH 8.0 containing 100 mM KCl) at 30° C using the optical assay with DTNB. Velocity (v) is expressed as ΔOD_{412}/min. (A) (Dixon plot) Varying the concentration of IPM at different acetyl-CoA concentrations 0.4 mM (▲), 0.16 mM (■) and 0.04 mM (●) and at a fixed concentration of 0.375 mM KIV. (B) (Dixon plot) As (A) except substrate KIV was 1.5 mM (▲), 0.75 mM (■), and 0.075 mM (●) and the acetyl-CoA concentration employed was 0.175 mM. (C) (Hill plot) Varying the concentration of acetyl-CoA at different concentrations of product IPM 0 mM (●), 1.5 mM (○), 5 mM (△), 8 mM (□). KIV was used at a concentration of 0.75 mM. The slopes were calculated with the aid of slide-rule calculator SR-51A

curve significantly, as shown in Figure 4C, for substrate acetyl-coenzyme A.

The second product of the enzyme reaction, coenzyme A, inhibited the enzyme at low coenzyme A concentrations (Fig. 5) with respect to the enzyme substrates KIV and acetyl-CoA competitively, too. However, the interpretation of the kinetics of inhibition by coenzyme A suffered under a concommitant time dependent inactivation by coenzyme A (Fig. 6). By very high substrate concentrations (40 mM KIV; 0.17 mM acetyl-CoA) the inhibition exerted by the simultaneous addition of both products (0.12 mM coenzyme A; 2.5 mM IPM) was alleviated.

The time dependent decrease of the activity of IPM synthase by coenzyme A was highly dependent on the pH-value; in the presence of 2 mM KIV the enzyme activity decreased at pH 7.5 but not at pH 8.2. L-valine exerted a modest stabilizing effect on the enzyme activity at pH 7.5, however, not at pH 8.2. Furthermore, the inactivation by coenzyme A could be reversed neither by the incubation nor by dialysis of the enzyme in the presence of metal ions, SH-reagents, L-leucine and/or L-valine either in the presence or in the absence of one of the substrates (Fig. 6).

In contrast to coenzyme A, the other reaction product IPM (10 mM), did not influence the stability of the enzyme either at pH 7.2 or at pH 8.2, even at incubation periods up to 8 h.

The experiments on the inhibition of IPM synthase by coenzyme A were extended to some coenzyme A analogues and their derivatives (Table 3). 3′-Dephospho-coenzyme A exerted an inhibitory effect slightly less than coenzyme A; pantetheine had only a weak inhibitory effect. The oxidized compounds CoA-S-S-CoA and pantethine (S-S) as well as AMP, ADP and

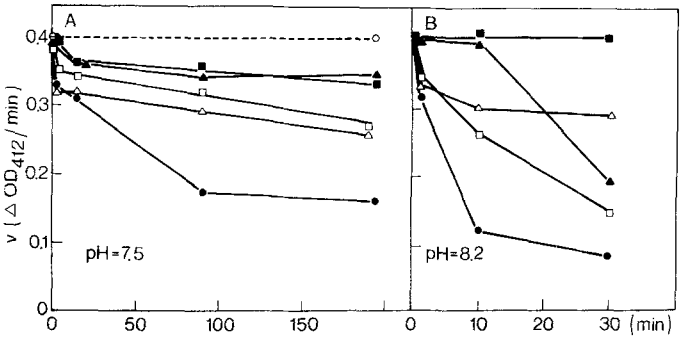


Fig. 5. (A) Decrease in enzyme activity caused by the effect of the product coenzyme A using different substrate (*KIV*) concentrations at pH 7.5 and 30° C (Dixon plot). The initial velocities (v) were determined employing the optical assay at 232 nm using 0.5 cm quartz cuvettes (1 ml volume) and the 110-fold purified enzyme. The substrate concentrations were 0.075 mM acetyl-CoA and 0.075 mM (■), 0.09 mM (●) and 0.75 mM (▲) KIV

ATP (each at 5 mM concentration) had no affect on the enzyme. However, contrary to the 5′-nucleoside phosphates the 3′-nucleoside phosphates and cyclic AMP (each 5 mM) at half maximal substrate saturation exerted an inhibition of 62% or 73%, respectively. At very low concentration (10^{-5} M) palmityl-CoA exerted a very strong inhibition.

DISCUSSION

The α-isopropylmalate (IPM) synthase from *Alcaligenes eutrophus H 16* has a rather low specificity; this is in contrast to other synthase enzymes such as malate synthase, homocitrate synthase and citrate synthase which catalyze the condensation of acetyl-

CoA and glyoxylate, α-ketoglutarate and oxaloacetate, respectively, with high substrate specificity. The low specificity for the α-keto acid substrates is shared with the IPM synthases from other organisms (Kohlhaw et al., 1969; Sai et al., 1969; Tracy and Kohlhaw, 1975; Webster and Gross, 1965). The low specificity of IPM synthase towards the coenzyme A derivatives as substrates had not been observed before. The highest reaction rates were obtained when propionyl-CoA and pyruvate or butyryl-CoA and α-ketobutyrate were the substrates. Besides the purity of the enzyme there are two arguments in favour of the conclusion that the observed reactions were really catalyzed by the same IPM synthase: The shape of the substrate saturation curve was similar to that obtained with the natural substrates KIV and acetyl-CoA; the inhibition of the enzyme activity by L-leucine was similar when either substrate analogues or KIV and acetyl-CoA were used. Experimental support for low substrate specificity of IPM synthase was also obtained by in vivo experiments (Hill and Schlegel, 1969). In the living cell the synthesis of L-leucine is apparently guaranteed by the high affinity of the enzyme to its "natural" substrates acetyl-CoA and KIV. Only under special conditions the low specificity of IPM synthase confers a special advantage to the cell; e.g. α-ketobutyrate was synthesized from acetyl-CoA and pyruvate in a mutant of *A. eutrophus H 16* lacking threonine deaminase activity (Reh, 1970).

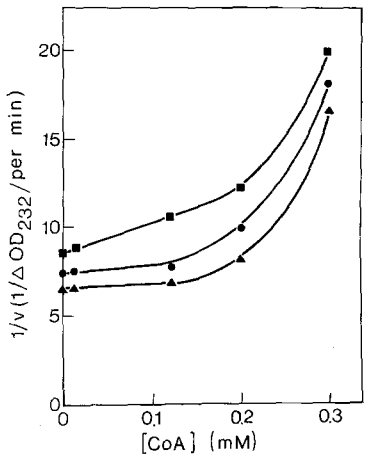


Fig. 6. Inactivation of IPM synthase by product coenzyme A. The preincubation was carried out at 23° C in 50 mM potassium phosphate pH 8.2 (A) and at pH 7.5 (B) containing 100 mM KCl. Supplements present during the preincubation were: none (○), 1 μM CoA (▲), 10 μM CoA (●), 10 μM CoA plus 2 mM KIV (■) or 1 mM L-valine (□), or 1 mM L-leucine (△); samples were withdrawn at the indicated time and assayed for residual IPM synthase activity. The initial velocities were determined using the DTNB assay, which contained 250 mM Tris-HCl, pH 8.2 and high substrate concentration (0.94 mM acetyl CoA; 1.5 mM KIV). The assay was started with 25 μl of the preincubation mixture. Coenzyme A and leucine transferred from the incubation mixture with the enzyme do not cause any detectable inhibition under the conditions used

The binding of α-keto acids at the catalytic site of the enzyme is not only due to the α-ketofunction of the substrate but is apparently influenced by the CH_3-CH_2-grouping, too. This conclusion is drawn from the experiments with substrate analogues; glyoxylate, formate and acetate had no inhibitory effect on the reaction, but propionate and higher homologues caused inhibition (Tables 1 and 2). Hill and Schlegel (1969) working with crude extracts indicated that glyoxylate may be a substrate for IPM synthase, the present work with the purified enzyme, does not support this hypothesis.

The apparent K_m-value for α-ketoisovaleric methylester was about two orders of magnitude higher than

Table 3. The effect of coenzyme A analogues and derivatives on the IPM synthase activity

Supplement	(M)[a]	% Activity	
		KIV = 1.5 mM acetyl-CoA = 0.94 mM	KIV = 0.375 mM acetyl-CoA = 0.094 mM
		100	100
Coenzyme A	4×10^{-5}	91	75
	8×10^{-5}	64	54
3'-Dephospho-CoA	4.4×10^{-5}	101	80
	9×10^{-5}	79	54
Pantetheine (−SH)	4.5×10^{-5}	96	94
	9×10^{-5}	91.5	77
CoAS-SCoA[b]	2×10^{-3}	99	100
Pantethine (-S-S-)[b]	2×10^{-3}	100	99
N'-Acetyl-S-acetoacetyl-Cysteamine[b]	1×10^{-3}	–	100
	2×10^{-3}	100	100
S-Acetyl-pantetheine	6×10^{-5}	79	70
Palmityl-CoA[c]	9×10^{-6}	8	0

[a] Determined by DTNB-assay
[b] Determined by weight
[c] Determined by nitroprussid assay.
The enzyme activity was determined in Tris-HCl (250 mM) pH = 8.0 using DTNB-assay. The enzyme preparation after DEAE-cellulose chromatography was used

that for KIV. One can conclude, therefore, that for optimal binding a free carboxyl-group is necessary. As has been shown in the case of the biosynthesis of malate (Eggerer and Klette, 1967) and of citrate (Eggerer, 1965) in the catalytic mechanism of the aldol addition reaction the carboxylate anion of the α-keto acid is involved. According to Eggerer and Klette (1967) the mechanism is the same in all analogous enzyme reactions, the IPM synthase reaction included. On the basis of this mechanism the methylester of an α-keto acid would not react or only very slowly. However, the IPM synthase of strain *H 16* reacted with the α-ketoisovaleric acid methylester at almost equal rate as KIV. The methylester caused an increase of the K_m, however, did scarcely affect the reaction velocity. A modified reaction mechanism for the isopropylmalate synthase from *A. eutrophus H 16* has to be assumed, therefore.

The binding of acetyl-CoA at the active site of the enzyme apparently involves the panthenyl and the adenosyl group, not, however, the cysteamine residue; N,S-acylcysteamine compounds, are apparently no appropriate analogues for the IPM synthase from *H 16* (Table 3).

The inhibition (95%) of IPM synthase by 9 μM palmityl-CoA appears to be an in vitro reaction without physiological significance. Similar inhibitory effects have been described for citrate synthase (Srere, 1965); the inhibition may be due to a non-specific adsorption and to structural changes of the enzyme (Vagelos, 1971). The inactivation of IPM synthase by 1–10 μM coenzyme A at pH 7.5 especially at low concentration of the second substrate KIV is apparently of physiological significance. Our results are in accordance with those of Tracy and Kohlhaw (1975), who studied the yeast enzyme. This was inactivated when the concentration of free coenzyme A was increased. The inactivation may represent a protection mechanism which serves to maintain a certain level of acetyl-coenzyme A; free coenzyme A serves as an indicator and directly affects IPM synthase provoking its inactivation.

Conformational changes may explain the observed deviations from normal Michaelis-Menten kinetics as well as the anomalous temperature dependence of the enzyme (Wiegel and Schlegel, 1977). Substrate saturation curves with an intermediary plateau have been described and discussed for other enzymes by Teipel and Koshland (1969) and by Levitzki and Koshland (1969). The described curves may indicate that IPM synthase from strain *H 16* undergoes conformational changes when binding KIV which facilitate the binding of a second substrate molecule (positive cooperativity, $n_H > 1$); this renders difficult the binding of a third substrate molecule (negative cooperativity, $n_H < 1$), while the binding of the fourth molecule is facilitated again ($n_H > 1$). The substrate (acetylcoenzyme A) saturation curves have to be considered in an analogous manner. The maximum values of the Hill coefficients obtained are indicative for at least three binding sites for KIV and four sites for acetyl-coenzyme A. With respect to the intermediary plateaus (Levitzki and Koshland, 1969; Teipel and Koshland, 1969), four binding sites were the minimum if the saturation curve had one bump, but six binding sites if two intermediary plateaus were considered.

There are two findings which may suggest that a "Rapid Equilibrium Random Bi Bi" mechanism (Cleland, 1963) is responsible for the observed kinetics of this enzyme: (i) the IPM synthase was competitively inhibited by the products α-isopropylmalate (IPM) and coenzyme A, with respect to both substrates acetyl-coenzyme A and KIV, (ii) the half saturation concentration of one substrate was constant at different concentrations of the second substrate. However, since the saturation curves were typical "bumpy curves", further detailed binding studies are necessary to confirm the mechanism. Binding studies are also necessary to prove the presence of and the influence of time dependent, slow transients (Ainslie et al., 1972), induced by the substrates.

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