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α-Isopropylmalate Synthase from *Alcaligenes eutrophus* H 16

III. Endproduct Inhibition and Its Relief by Valine and Isoleucine

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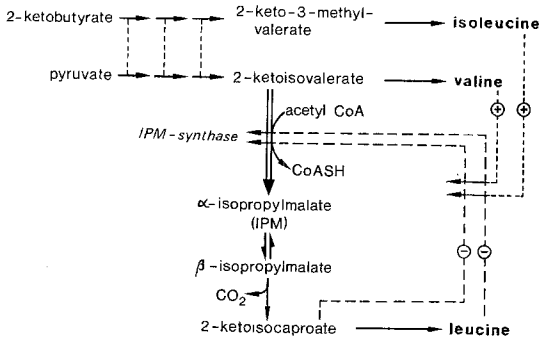
Abstract. The α-isopropylmalate synthase (EC 4.1.3.12) from *Alcaligenes eutrophus* H 16 was inhibited by L-leucine and α-ketoisocaproate. The extent of inhibition was influenced by substrate- and inhibitor concentrations as well as by the pH. Intermediary plateaus, which always appeared in the inhibition curves, suggested cooperative effects. The maximal Hill coefficient was found to be two. At low concentrations of leucine the inhibition mechanism was of the competitive type with respect to substrate acetyl coenzyme A and of the noncompetitive type with respect to substrate α-ketoisovalerate. The inhibition was specifically relieved by the addition of valine or isoleucine. The anomalous effect of temperature on enzyme activity was diminished by leucine. The Arrhenius energy of the reaction increased from about 11 kcal/mole in the absence of leucine to about 18 kcal/mole in the presence of leucine. The further addition of valine reversed this effect. The physiological relevance of the α-ketoisocaproate-mediated inhibition is discussed.

Key words: Hydrogen bacteria – *Alcaligenes eutrophus* H 16 – Leucine biosynthesis – α-isopropylmalate synthase – Regulation – Feedback inhibition – Relief of inhibition by valine and isoleucine – Inhibition by α-ketoisocaproate – Temperature anomaly.

The α-isopropylmalate route of leucine biosynthesis (Scheme 1) is, so far known, common to aerobic, microaerobic and phototrophic bacteria and even to plants (Stieglitz and Calvo, 1974; Wiegel and Schlegel, 1977c; Miflin and Cave, 1972; Scheme 1). In various organisms, however, the regulation of this pathway is differently realized on the level of enzyme synthesis and of enzyme activity.

In all organisms studied in this respect, the first enzyme of this pathway, the α-isopropylmalate (IPM) synthase, is inhibited by the endproduct L-leucine. Stieglitz and Calvo (1974) reported that IPM synthases from different organisms exhibit hyperbolic or sigmoidal inhibition curves. Under standardized conditions, maximal inhibition occurred on different levels. The structure-function relationships of IPM synthases from various organisms are unknown except for the enzyme from *Salmonella typhimurium* (Kohlhaw et al., 1969). Here the association-dissociation equilibrium was influenced by the concentration of protein and leucine. The level of inhibition correlated with this equilibrium; leucine favoured the dissociation into the catalytical inactive monomers (Kohlhaw and Boatman, 1971). Such an association-dissociation equilibrium was not observed for the enzyme from *A. eutrophus* H 16 but cannot generally be excluded at this time (Wiegel and Schlegel, 1977a).

Unlike the enzyme from *S. typhimurium* the leucine-mediated inhibition of the IPM synthase from *A. eutrophus* was reversed by the addition of valine or isoleucine, both of which are endproducts of the branched chain amino acid biosynthesis (Hill and



Scheme 1

Abbreviations. IPM = α-isopropylmalate; KIC = α-ketoisocaproate; KIV = α-ketoisovalerate; DTNB = 5:5 Dithiobis-(2-nitrobenzoe acid)

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Schlegel, 1969a). This was found to be a common effect on IPM synthases from many bacteria (e.g. several species of *Rhodospirillum*, *Rhodopseudomonas*, *Bacillus*, *Pseudomonas* and all strains of *A. eutrophus* and *Corynebacterium autotrophicum*) however, not on the enzyme from species of the Enterobacteriaceae (Wiegel and Schlegel, 1977c). We therefore reported the antagonism between leucine and valine in detail.

The general properties of the synthase (EC 4.1.3.12) from *A. eutrophus* have been described in the preceding papers (Wiegel and Schlegel, 1977a, b) including specificity of the enzyme to its substrate, cooperative effect of the substrates, product inhibition and inactivation by product coenzyme A. This paper describes the regulation of the IPM synthase activity by the endproduct inhibitor L-leucine, by α-ketoisocaproate and by the antagonistic effectors, valine and isoleucine.

MATERIALS AND METHODS

Cultivation of *Alcaligenes eutrophus* H 16 (ATCC 17699) and its valine and isoleucine autotrophic mutant A 81 (Reh and Schlegel, 1969), the preparation of crude extract, the purification of α-isopropylmalate synthase and the different assays used, were described previously (Wiegel and Schlegel, 1976a, b). If not otherwise indicated, the enzyme preparation resulting from the DEAE-cellulose step (11.5–11.7 U/mg protein) was used. For the calculation of lines in reciprocal plots, the calculator SR-51 A from Texas Instruments was used.

Assay with Toluene-Treated Cells. The cell suspension (5 ml of the wildtype or 1 ml of the mutant; OD_{436} about 10 to 18) was poured into 5 ml ice and centrifugated at 5000 × g and at 0° C for 5 min. The cells were resuspended in 1 ml 250 mM potassium phosphate pH 7.5. After adding 0.01 ml toluene, the suspension was shaken intensively and incubated for 10 min at 30° C. The standardized fluorimetric assay (see above) was started with 0.05–0.2 ml of this suspension. Determining the reversal of the leucine-mediated inhibition, the cell suspension was incubated first with leucine for 1 min.

RESULTS

Effect of Amino Acids on Activity and Inhibition of IPM-Synthase

Hill and Schlegel (1969a), using crude extract, showed that the pH-optimum of feedback inhibition (pH 7.2) differed from that of the catalytical activity (above pH 8.0). Therefore, the influences of L- and D-amino acids on the purified enzyme as well as on gel-filtrated crude extract, were tested at pH 7.5 and pH 8.0. No amino acid (10 mM at pH 8.1 and 1 mM at pH 7.5) caused an activation of IPM-synthase. Besides the feedback inhibitor L-leucine only D-leucine as well as L- and D-histidine caused a significant inhibition (more than 10%). At pH 8.1 and 7.5 the inhibition by histidine was 11% and 12% respectively, however, L- and D-histidine inactivated the enzyme, too. In the presence of 1 mM histidine (pH 7.5) less than 5% activity remained after 1 h.

Table 1. The effect of added amino acids on the leucine-mediated inhibition of IPM-synthase from *Alcaligenes eutrophus* H 16

Added amino acid	Remaining activity (%)	
	at pH 7.4[a]	at pH 8.1[b]
L-Leucine	14	63
L-Leucine + L-threonine	18	49.5
L-Leucine + L-alanine	17	74
L-Leucine + L-methionine	27	71
L-Leucine + L-histidine	9	37
L-Leucine + D-histidine	9	–
L-Leucine + L-isoleucine	50	71
L-Leucine + D-isoleucine	24	60
L-Leucine + L-valine	65.5	80
L-Leucine + D-valine	16	63
L-Leucine + L-phenylalanine	27	65
L-Leucine + 2-amino-4-methylpentanoic amide (L-leucine amid)	14.3 (100)[c]	
L-Leucine + l-amino cyclopentane-l-carboxylic acid (Cycle-leucine)	12.9 (63)	
L-Leucine + DL-2-aminobutanoic acid	50 (100)	
L-Leucine + DL-4-aminobutanoic acid	41 (100)	
L-Leucine + DL-2-aminopentanoic acid (Norvaline)	46.5 (91)	
L-Leucine + DL-2-amino-3-methyl-hexanoic acid (Allo-isoleucine)	16.4 (93)	
L-Leucine + DL-2-amino-3-methyl-pentanoic methyl ester (Isoleucine methyl ester)	26 (100)	

The addition of the following amino acids (D- and L-form) had no significant effect: glycine, serine, proline, tryptophane, tyrosine, arginine, lysine, asparagine, aspartic acid, glytamine and homoserine (only D-form tested)

[a] Activity was determined using the optical assay at 232 nm at 25° C. 1 ml of the assay mixture contained 250 µmole Tris-HCl pH 7.4, 0.75 µmole KIV, 0.094 µmole acetyl coenzyme A 0.05 µmole leucine and 1 µmole of added amino acid as indicated. The assay was started by the addition of purified enzyme (11.7 U/mg of protein). 100% activity corresponded to ΔOD_{232} per min = 0.28

[b] Activity at pH 8.1, determined using the DTNB-assay. 1 ml of the assay mixture contained 250 µmole Tris-HCl pH 8.1, 0.75 µmole KIV 0.15 µmole acetyl coenzyme A, 5 µmole leucine and 10 µmole of the other amino acid as indicated. 100% activity corresponded to ΔOD_{412} per min = 0.27

[c] The values in brackets are the activity at pH 7.4, determined as under ([a]), however, without added leucine

In the presence of L-leucine some of the amino acids acted as activators (Table 1). The two branched chain amino acids L-valine and L-isoleucine were most effective. This effect was observed in experiments with a highly purified enzyme (12 U/mg of protein; after preparative gel electrophoresis) as well as with toluene-treated cells (approaching in vivo conditions). These

findings supported the results obtained with crude extracts reported previously (Hill and Schlegel, 1969a). The analogous compounds α-aminovalerate, α-aminobutyrate, γ-aminobutyrate, norleucine, norvaline, cycloleucine and glycylvaline were also antagonists of leucine, however, these amino acids were not regarded as physiological compounds. L-Valine and L-isoleucine were accepted as physiological important antagonists. D-Valine and D-isoleucine did not cause any relief.

Catalytic Activity, Leucine Inhibition and Its Reversal as a Function of pH

The catalytic activity of the purified enzyme and the extent of the leucine-mediated inhibition varied independent of each other as a function of pH (Fig. 1). At the pH-optimum of the catalytic activity (pH 8.5) 0.05 mM leucine caused an inhibition of about 20%, however, at pH 7.8 of about 85%. Contrary to this pH-dependent difference of activity (65%) in the presence of leucine, in the absence of leucine or in the presence of an excess of valine, the enzyme activity at pH 7.8 was only about 15% less than at pH 8.5. Only a slight effect on the extent of inhibition was observed when Tris-HCl was replaced by triethanolamin-HCl or potassium phosphate.

The reversal of the leucine-mediated inhibition by valine or isoleucine was affected by the pH, too. In the presence of valine, the pH for maximal inhibition by leucine shifted from pH 7.4 to 7.0, due to the pH optimum for the relief of the inhibition (pH 7.5; Fig. 1C). At pH 9.0 and 9.3 valine (2.5 mM) reacted as an inhibitor (10% and 35% inhibition, respectively), however, in the presence of 1.0 mM leucine valine (2.5 mM) caused no inhibition at these pH-values. Isoleucine reacted similarly to valine.

Kinetics of Leucine-Mediated Inhibition

In view of the remarkable dependence on pH, the kinetics of leucine-mediated inhibition were studied at three different pH-values and with different substrate concentrations. Using the v versus $[I]$ plot the inhibition curves at pH 8.1 (Fig. 2A), at pH 7.6 and at pH 7.35 (Fig. 2B) seemed to be more hyperbolic than sigmoidal. However, using the Dixon plot ($1/v$ versus I) the curves were not linear (Figs. 2C and 3A). They obviously contained intermediary plateaus, e.g. leucine exhibited cooperative effects. Whether these effects were homotropic or due to the interference with the cooperative effect of the substrate, has not yet been decided on the basis of initial rate experiments. The inhibition did not reach 100% even at 50 mM leucine. This indicated a negative cooperativity in accordance with a Hill coefficient lower than one (Fig. 4). However, the cooperativity depended on the pH and on the leucine and substrate concentrations employed. This is shown by the different Hill coefficients (n_H) obtained for leucine (Fig. 4): the values varied between 0.6 (e.g. negative cooperativity) and 2 (e.g. positive cooperativity). Values higher than one were obtained at pH 7.6 and leucine concentrations

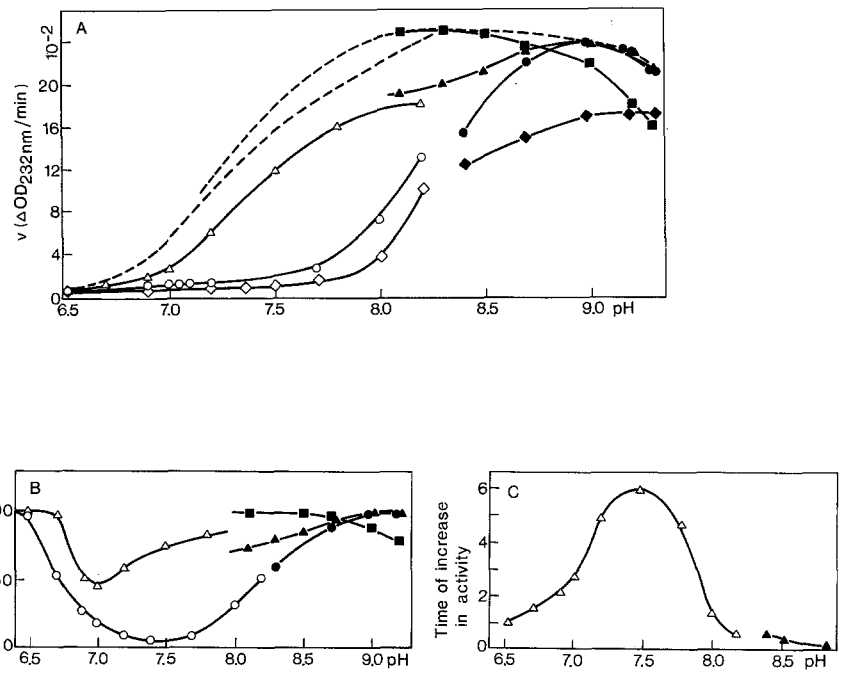


Fig. 1A–C
pH Dependence of IPM-synthase activity, of inhibition by leucine and its relief by valine. (A) Activity was determined by using the optical assay at 232 nm at 30° C. One ml of the assay mixture contained 250 μmole Tris-HCl (*filled symbols*) or potassium phosphate (*open symbols*) 0.75 μmole KIV 0.188 μmole acetyl coenzyme A and the amino acids as followed 0.05 μmole (◆), 0.2 μmole (●) leucine; 2.5 μmole valine (■); 0.05 μmole leucine plus 2.5 μmole valine (▲), no additions (*dotted line*). (B) The pH-optimum of inhibition by leucine. The data from (A) were replotted; the activities in the absence of leucine were taken as 100% and the activity (%) obtained in the presence of added amino acids were plotted versus the pH-value. (C) The pH-optimum of the relief of leucine-mediated inhibition by valine. Times of increase in activity by the addition of 2.5 mM valine (in the presence of 0.05 mM leucine) is plotted versus pH. The graph is based on the data of (A)

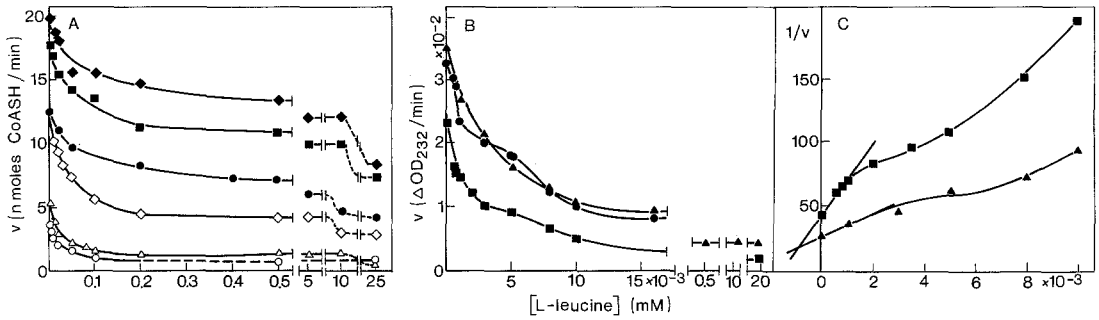


Fig. 2A–C. Reaction velocity of the IPM-synthase as a function of leucine concentration at different levels of substrate KIV and acetyl coenzyme A at pH 8.1 (A) and 7.35 (B). The activity was determined by using the DTNB assay with 250 mM Tris-HCl pH 8.1 (A) and the optical assay at 232 nm 250 mM Tris-HCl pH 7.35 (B). The substrate concentrations used under (A) were 1.5 mM KIV, 0.705 mM acetyl coenzyme A (◆); 0.375 mM KIV, 0.705 mM acetyl coenzyme A (■); 0.0375 mM KIV, 0.705 mM acetyl coenzyme A (●); 0.0375 mM KIV, 0.141 mM acetyl coenzyme A (△); 1.5 mM KIV, 0.141 mM acetyl coenzyme A (◇); 1.5 mM KIV, 0.047 mM acetyl coenzyme A (○). The substrate concentrations used under (B) were: 0.75 mM KIV, 0.141 mM acetyl coenzyme A (▲); 0.15 mM KIV, 0.141 mM acetyl coenzyme A (●); 0.75 mM KIV, 0.188 mM acetyl coenzyme A (■). For recognizing the type of inhibition exerted by leucine with respect to acetyl coenzyme A at pH 7.35 and at low inhibitor concentration, the Dixon-plot was drawn (C)

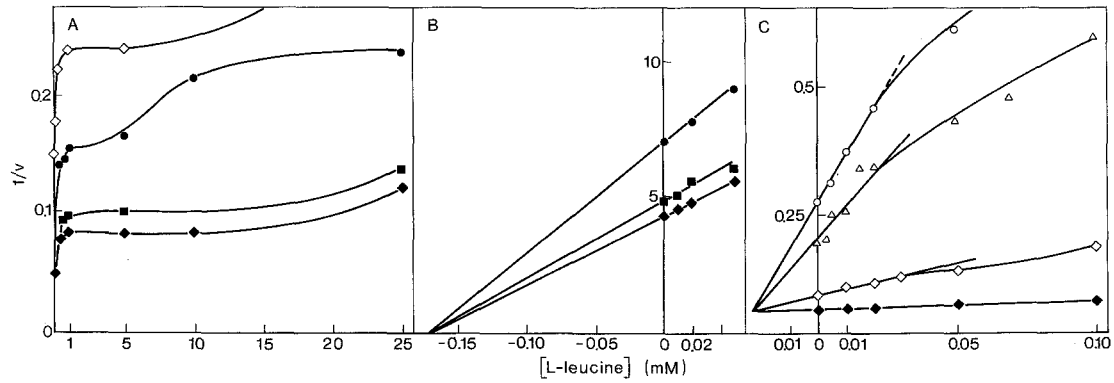


Fig. 3A–C. Reciprocal plot of reaction velocity as a function of leucine concentration at different substrate concentrations (A). For low leucine concentration the inhibition patterns are demonstrated in (B) different concentrations of KIV) and (C) (different concentrations of acetyl coenzyme A). Conditions and symbols used are as described under Figure 2

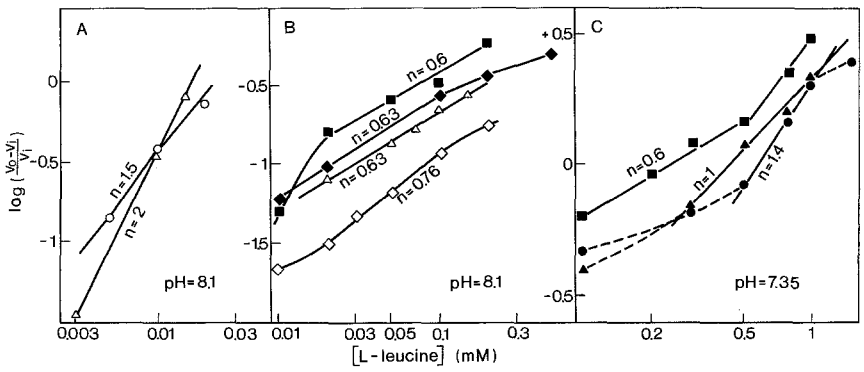


Fig. 4A–C
Modified Hill plot of the reaction velocity as a function of leucine concentration. The data of Figure 2A were used for (A) and (B) and of Figure 2B for (C). The values for n represent the slopes of the corresponding lines [log $([V_0 - V_i]/V_i) = n$ log [I] + log C; V_0 = reaction velocity in the absence of the inhibitor I; V_i = reaction velocity in the presence of inhibitor I]

at less than 0.03 mM as well as at pH 8.1 and low substrate concentrations ($< S_{05}$). At pH 8.1 and higher substrate concentrations the Hill coefficients for leucine were lower than one.

The values for half of the maximal inhibition ($I_{0.5}$) taken from the v versus I plot depended on the substrate concentration and they were in the range of 0.025 and 0.25 mM leucine at pH 8.1 and in the range

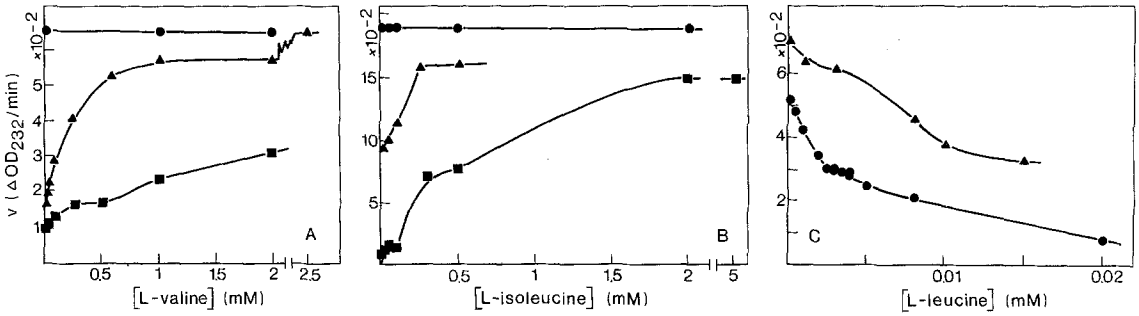


Fig. 5A–C. Antagonism between valine or isoleucine and leucine at the leucine-mediated inhibition of the IPM synthase. The antagonistic effects are shown as a function of the concentration of valine at 0.1 (▲), 1.0 mM (■) and without leucine (●) (A), of the concentration of isoleucine at 0.01 (▲), 0.05 mM (■) and without leucine (●) (B), and of the concentration of leucine at 0.1 (▲) and 0.04 (●) mM valine (C). Initial velocities were determined by monitoring the change of 232 nm. The substrate concentrations and the pH of the Tris-HCl buffer (250 mM) used were: (A) 0.75 mM KIV, 0.094 mM acetyl coenzyme A and pH 7.65; (B) and (C) 0.375 mM KIV, 0.094 mM acetyl coenzyme A and pH 7.4

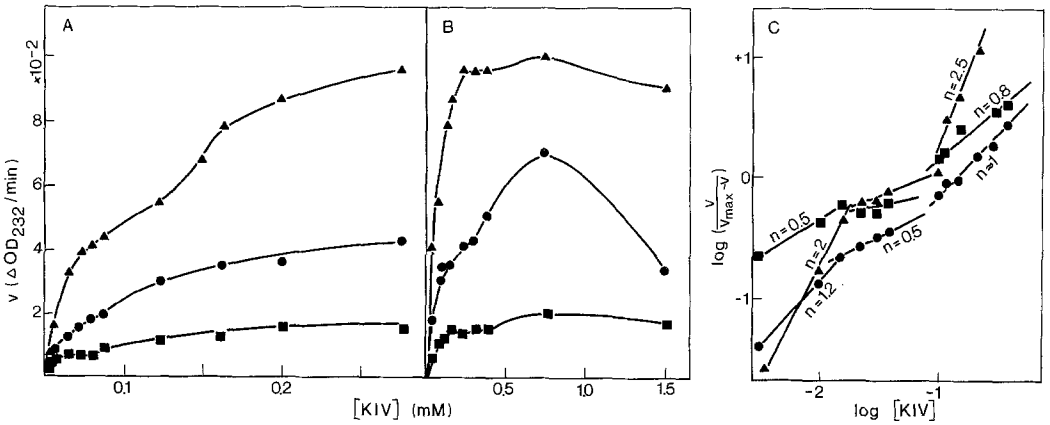


Fig. 6A–C. Substrate (KIV)-saturation curves in the presence and absence of leucine and leucine plus valine (A, B). The continuous optical assay at 232 nm was used. One ml of the assay mixture contained 250 μmole Tris-HCl, pH 7.6; 0.15 μmole acetyl coenzyme A, KIV as indicated and amino acids as followed: (▲) none; (■) 0.1 μmole leucine; (●) 0.1 μmole leucine plus 0.25 μmole valine. Highly purified enzyme (12.05 U/mg of protein) was used. (C) Hill plot of the data from (A) and (B)

of 0.003 and 0.006 mM leucine at pH 7.35. At low leucine concentrations, the cooperative effects were not significant. For this range it is suggested from the Dixon plot (Figs. 2C and 3B, C), that the inhibition mechanism is most likely to be of the noncompetitive type with respect to substrate KIV and of the competitive type with respect to substrate acetyl coenzyme A. The K_i-values for leucine were 0.0025 (pH 8.1), 0.002 (pH 7.6) and 0.001 mM leucine (pH 7.35) if acetyl coenzyme A was varied and 0.2 mM (pH 8.1) if substrate KIV was varied. At pH 7.6 a K_i of 0.1 mM leucine and a competitive inhibition was obtained for concentrations between 0.06 and 0.4 mM leucine.

Antagonism of Leucine Inhibition by Valine or Isoleucine

The leucine-mediated inhibition was relieved with increasing concentration of L-valine or L-isoleucine (Fig. 5). In the presence of 1 mM (0.1 mM) leucine, the addition of valine caused an increase in enzyme activity from 15–34% (from 25–45%). The effect of isoleucine was less pronounced. The inhibition was totally reversed at a 100-fold concentration of valine over leucine. The intermediary plateaus of the inhibition curves were more pronounced in the presence of valine (Fig. 5C) or isoleucine. Owing to the cooperative effects on the basis of the kinetics no conclusions could be drawn on the mechanism of this antagonism.

Substrate Saturation in the Presence of Amino Acids

In the presence of leucine or valine or both amino acids, the saturation curves for the substrates were still polyphasic (Fig. 6A, B). In the absence of leucine the Hill coefficient depended on the substrate saturation and altered from values higher than one to lower than one and again to higher than one, e.g. a change from positive to negative and back to positive cooperativity (Fig. 6C). In the presence of leucine the

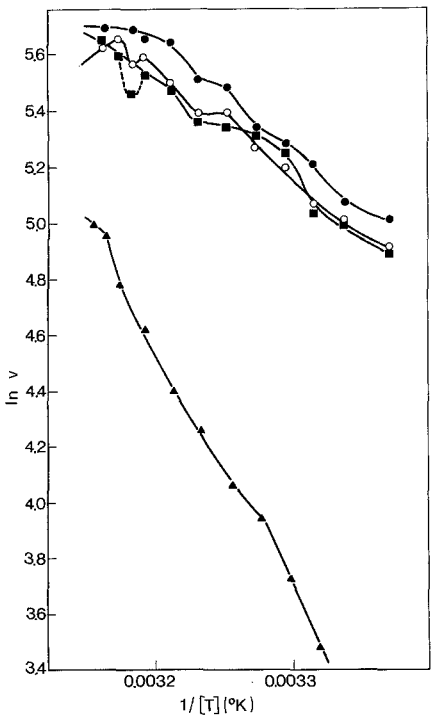
Hill coefficients still changed in the same range of substrate concentration, however, they were always lower than one, e.g. in the presence of leucine, the substrate KIV exhibited only negative cooperativity. If the leucine-mediated inhibition was reversed, positive cooperativity was observed again and the Hill coefficient increased with the extent of relief. No alteration of the substrate (KIV) saturation curves was observed in the presence of valine only, or of an excess of valine with respect to leucine.

The Influence of the Amino Acids on the Anomalous Temperature Dependence of Enzyme Activity

The temperature optimum of the enzyme activity (at pH 7.5) increased from 44° to 45° and to 48° C in the presence of 2 mM valine and 0.1 mM leucine, respectively. The presence of leucine resulted in a broad temperature optimum. In the presence of leucine (pH 7.5) the Arrhenius energy increased from about 11 kcal per mol (Wiegel and Schlegel, 1977a) to about 18 kcal per mol (Fig. 7). If the leucine-mediated inhibition was totally relieved by the addition of an excess of valine the activation energy changed again to 11 kcal per mol. A remarkable effect of leucine was that it diminished the temperature anomaly (Wiegel and Schlegel, 1977a). Contrary to leucine, valine intensified the expression of the intermediary plateaus (Fig. 7).

Inhibition by α-Ketoisocaproate

α-Ketoisocaproate (KIC), the immediate precursor of leucine, reacted as a ketoanalogue of the feedback inhibitor leucine as well as a higher homologue of substrate α-ketoisovalerate (KIV). This conclusion was drawn by the following results: IPM-synthase from *A. eutrophus* excerted no activity neither with KIC nor with α-ketocaproate as substrate, however, 5 mM KIC inhibited the enzyme activity totally at pH 8.0. The inhibition exerted by 0.1 mM KIC and 1 mM α-ketooctanoate at pH 7.7 was partially relieved by 10 mM valine; 70% and 12% relief, respectively. Contrary to this, the inhibition excerted by 0.1 mM leucine was relieved totally. In accordance with these findings were the results obtained with a leucine insensitive IPM-synthase from a 5′,5′,5′-tri-



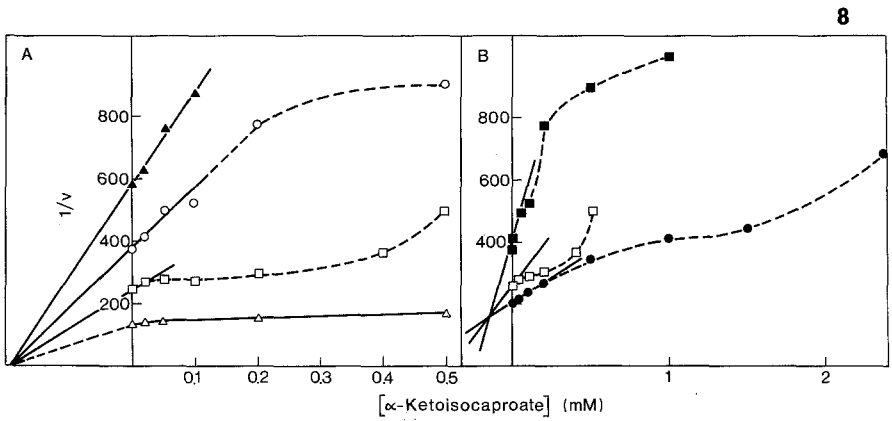


Fig. 7. The effect of temperature on IPM-synthase activity in the absence and presence of amino acids. The fluorimetric assay and 70-fold purified enzyme was used. 1 ml of the assay mixture contained 250 μmole potassium phosphate pH 7.5, 20 μmole KIV, 20 μmole acetyl phosphate, 0.2 mg coenzyme A (grade II), 0.01 mg phosphotransacetylase and following supplements: (○) none; (■) 5 μmole valine; (▲) 0.05 μmole leucine; (●) 0.05 μmole leucine plus 5 μmole valine. The assay mixture was preincubated for 5 min without the enzyme and for further 20 min after adding the enzyme at the temperature indicated. To avoid cross-overs the *upper curve* (●) in the figure was shifted on the ordinate to higher values; the original value for $T^{-1} = 0.00332$ was identical with that of the curve without additions (○). The values are the means of two parallel experiments

Fig. 8A and B. Reaction velocity of IPM-synthase as a function of the α-ketoisocaproate (KIC) concentration at different saturation levels of substrate α-ketoisovalerate (KIV) (A) and acetyl coenzyme A (B). Initial velocity (v) was determined using the DTNB assay, which was started with purified enzyme (11 U/mg of protein). 250 mM Tris-HCl buffer was used. The substrate concentrations were: (A) 0.24 mM acetyl coenzyme A; 0.0225 (▲), 0.045 (○), 0.15 (□) and 2.25 mM (△) KIV; (B) 0.15 mM KIV; 0.05 (■), 0.24 (□), and 0.7 (●) mM acetyl coenzyme A

fluoroleucine resistant mutant (H16- leu 101) using the fluorimetric assay and gel filtrated (Sephadex G 25) crude extract. At pH 7.5 0.1 mM KIC and 0.1 mM, leucine caused an inhibition of 28% and of 16%, respectively, whereas in the corresponding extract from the wildtype an inhibition of 60% and 78%, respectively, was obtained.

The inhibition curves (Fig. 8, Dixon-plot) of KIC contained intermediary plateaus, however, it was not recognizable whether the cooperative effects were due to the analogy of leucine or of KIV. At low inhibitor concentrations the cooperative effects were not significant. For this range the inhibition was competitive (pH 8.0; $K_i = 0.1$ mM) with respect to substrate acetyl coenzyme A and noncompetitive (pH 8.0; $K_i = 0.2$ mM) with respect to substrate KIV. These effects were comparable to the effects of leucine. In opposition to these effectors, the inhibition pattern of the substrate KIV analogue α-ketooctanoate were competitive to KIV ($K_i = 8$ mM at 0.23 mM acetyl coenzyme A) at low saturation concentrations. The $K_{0.5}$ for KIV increased from about 0.06 mM to 0.1 and to 0.2 mM in the presence of 1 mM and 10 mM ketooctanoate, respectively. However, at high substrate KIV concentrations (2.25 mM KIV and 0.24 mM acetyl coenzyme A) 1 mM KIC excerted only about 3% inhibition, this effect was not comparable to that of leucine.

DISCUSSION

Leucine inhibition and different pH optima for feedback inhibition and for the catalytic activity were common to all IPM synthases so far studied (Webster and Gross, 1965; Kohlhaw et al., 1969; Ulm et al., 1972; Stieglitz and Calvo, 1974; Wiegel and Schlegel, 1977a,c and unpublished results). On the basis of the pH effect, Kohlhaw et al. (1969) suggested, that histidine might be involved in the binding sites of leucine. This assumption was also made for the enzyme from *Alcaligenes eutrophus*. The differences in the pH optima of IPM synthases from different organisms in the presence of leucine may be due to the different neighbourhood of the histidine; similar effects were reported for histidine-residues of the active centre of pancreatic ribonuclease (Rüterjans and Witzel, 1969). The structure of the binding sites of leucine or of the active centre of the IPM synthase from *A. eutrophus* is not known; however, it may be speculated that the L- and D-histidine with that of the protein, resulting in the change of enzyme stability or structure. The histidine-mediated inactivation of IPM synthase was also observed in crude extracts from other organisms (unpublished results).

The feedback inhibition of the enzyme from *A. eutrophus* was specific; only L-leucine, its immediate precursor α-ketoisocaproate (KIC) and some physiologically unimportant analogues caused a significant inhibition. The reported K_i- and $I_{0.5}$-values for leucine were in the same magnitude as those reported for other IPM-synthases (Lit. see above). However, the inhibition kinetics of this enzyme were more complex: inhibition curves with intermediary plateaus had not been reported for IPM synthases before. Only at low leucine concentrations could the type of inhibition be detected, which was similar to that reported for the enzyme from *Salmonella typhimurium* (Kohlhaw et al., 1969). The assumption that leucine is an allosteric inhibitor of the IPM synthase is supported by the fact that the substrate KIV reacted in a noncompetitive manner, the substrate acetyl coenzyme A in a competitive manner with respect to the leucine inhibition (even though the structure of KIV is much more similar to leucine than that of acetyl coenzyme A), and by the cooperative effects of leucine. The maximal Hill coefficient for leucine was two, suggesting two or more bindings sites. Two binding sites (based on the Hill coefficients) were also reported for the enzyme from *S. typhimurium* (Kohlhaw et al., 1969) and *Saccharomyces* spec. (Ulm et al., 1972). However, the intermediary plateaus obtained with the enzyme from *A. eutrophus* suggested four binding sites for leucine. This conclusion was drawn on the basis of the assumption made by Teipel and Koshland (1969). More than two binding sites for leucine need not be postulated, if the intermediary plateaus are due to heterotrophic effects of the substrates on leucine inhibition.

Low concentrations of α-ketoisocaproate (KIC) exerted a noncompetitive inhibition with respect to substrate KIV. The inhibition of the IPM synthase at low physiologically relevant concentrations of KIC suggested that this is a further regulation mechanism of branched chain amino acid synthesis. This is in contrast to the effects observed with the enzyme from *S. typhimurium*. In *A. eutrophus*, especially at a low transaminase activity, the inhibition of IPM synthase by KIC would prevent its accumulation. This consideration is in accordance with the findings that the amount of leucine excreted by 5′,5′,5′-trifluoroleucine resistant mutants of *A. eutrophus* was always very low, especially in comparison to the excretion of valine and isoleucine (Hill and Schlegel, 1969b; Reh and Schlegel, 1969; Wiegel, unpublished results) and that the excretion of KIV under N-limiting growth conditions was very low compared with that of *S. typhimurium* or of *Saccharomyces* spec. (Vollbrecht, pers. comm.).

The relief of the leucine-mediated inhibition of IPM synthase by valine or isoleucine has to be con-

sidered as a positive regulation mechanism for leucine biosynthesis. This effect of valine is widely distributed among IPM synthases from different genera of bacteria (Wiegel and Schlegel, 1977c) and is comparable to the well known relief of the isoleucine-mediated inhibition of the threonine deaminase (EC 4.2.1.16) by valine as described for *S. typhimurium* (Freundlich and Umbarger, 1963), for *Escherichia coli* (Changeaux, 1963), for *A. eutrophus* (Reh and Schlegel, 1969), for *Bacillus subtilis* (Hatfield and Umbarger, 1970). In both examples the antagonistic relationships between the amino acids favour the maintenance of a balanced ratio of the branched chain amino acids in the cells. In contrast to the effect caused by valine on the threonine deaminase from *S. typhimurium* and *E. coli*, valine was not an activator of the IPM synthase from *A. eutrophus* and did not cause a change in the substrate saturation curves; intermediary plateaus were obtained in the presence and absence of valine with or without leucine. Owing to these cooperative effects, we failed to decide whether valine and leucine bind at a common modifier site, but cause different effects, or whether they have different binding sites. Some of our results suggested that valine and isoleucine bind not at the inhibitor site as leucine does: e.g. only in the presence of valine was the anomalous temperature dependence strongly pronounced, however, the presence of leucine minimized the anomaly; valine was inhibitory at pH 9.0, but not in the presence of leucine; and valine intensified the intermediary plateau of the leucine inhibition curves. The assumption that only one binding site exists for all three amino acids was supported by the similarity in the structure of valine, isoleucine and leucine, by the similar effect of valine and leucine on the inactivation by coenzyme A (Wiegel and Schlegel, 1977b) and by EDTA (unpublished results). This problem is similar to that reported by Hatfield and Umbarger (1970) for the valine-isoleucine antagonism observed with the threonine deaminase from *B. subtilis*.

The regulation of the leucine pool size in bacteria seems to be of central importance. Leucine plays a role in the regulation of other biosynthetic and metabolic pathways, too ("metabolic interlock", e.g. Rebello and Jensen, 1970; Quay and Oxender, 1976). In *E. coli* limitation for leucine was both necessary and sufficient for derepression of branched chain amino acid transport. These results and other observations supported the idea of a hierarchy of regulation of transport and biosynthesis of amino acids (Quay and Oxender, 1976). Although these phenomena have not yet been examined in *A. eutrophus*, these considerations emphasize the importance of the reported relief of leucine-mediated inhibition by valine or isoleucine for the overall regulation of the amino acid pool size in this and other bacteria (Wiegel and Schlegel, 1977c).

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