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Mechanism of Feedback Inhibition by Leucine. Binding of Leucine to Wild-Type and Feedback-Resistant α-Isopropylmalate Synthases and Its Structural Consequences†

Elizabeth Teng-Leary and Gunter B. Kohlhaw*

ABSTRACT: Binding of the feedback inhibitor, leucine, to a wild-type and a feedback-resistant form of α-isopropylmalate synthase from *Salmonella typhimurium* has been studied by equilibrium dialysis and ultraviolet difference spectroscopy. Binding of leucine to wild-type enzyme is positively cooperative, and the affinity for leucine increases with decreasing enzyme concentration. At a protein concentration of 1 mg/ml, the apparent binding constants for leucine are 10.5 μM for wild-type enzyme and about 1.5 mM for the feedback-resistant enzyme. This difference is similar to that observed in leucine inhibition kinetics. No more than two leucine sites per tetramer can be saturated. Binding of leucine has also been examined in the presence of either substrate. Acetyl-CoA has an antagonistic effect on leucine binding, yielding a biphasic Hill plot with mixed positive and negative cooperativity. α-Ketoisovalerate, the other substrate, increases the affinity for leucine, thereby lowering the positive cooperativity of leucine binding. A mixed effect is observed when leucine binding is studied in the presence of acetyl-CoA and α-ketoisocaproate, a very slowly reacting analog of the natural substrate α-ketoisovalerate. Ultraviolet absorption difference spectroscopy suggests that similar conformational changes occur upon binding of leucine to either the wild-type or the feedback-resistant enzyme. The concentrations of leucine required for a half-maximal effect in difference spectroscopy correlate closely with the apparent binding constants obtained in equilibrium dialysis experiments. The difference spectra observed upon binding of α-ketoisovalerate or acetyl-CoA are similar to each other and dissimilar to that caused by leucine. In contrast to wild-type α-isopropylmalate synthase, the feedback-resistant enzyme is not dissociated by inhibitory concentrations of leucine. The feedback resistance of the mutated enzyme is discussed in terms of strongly impaired leucine binding which is accompanied by the loss of preferential binding of the inhibitor to enzyme dimers.

For the past several years, this laboratory has been studying α-IPM[1] synthase, the first enzyme specific to leucine biosynthesis. The enzyme from *Salmonella typhimurium* is of particular interest because it is subject to an association–dissociation equilibrium which is affected by various ligands (Leary and Kohlhaw, 1970, 1972). Physical and chemical studies have indicated that the highest aggregate assumed by the native enzyme is a tetramer composed of very similar, if not identical polypeptide chains with a molecular weight of about 50,000 (Kohlhaw and Boatman, 1971; Bartholomew and Calvo, 1971; Leary and Kohlhaw, 1972). Kinetic studies have shown little or no cooperativity between substrate sites, but pronounced positive cooperativity between leucine sites, with

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[1] Abbreviation used is: α-IPM, α-isopropylmalate.

Hill coefficients, however, not exceeding the value of 2.0 (Kohlhaw *et al.*, 1969; also, this paper). In the present study, both equilibrium dialysis and ultraviolet difference spectroscopy have been used to investigate in a more direct way the interaction of wild-type α-IPM synthase and of a feedback-resistant enzyme form with the feedback inhibitor, leucine. The results reported here together with information obtained previously suggest that both isomerization and polymerization equilibria are important for the regulation of enzymatic activity. The results also suggest that the feedback resistance of the mutated enzyme is due to impaired binding of leucine and not to an inability to transmit the allosteric signal to the active site.

Experimental Section

Materials

Uniformly labeled L-[^{14}C]leucine (specific radioactivity 200–312 Ci/mol) was obtained from ICN Chemical and Radioisotope Division, from Schwarz-Mann, or from New England Nuclear. Bovine serum albumin was from Pentex Inc. Coenzyme A (lithium salt, dihydrate) was from P-L Biochemicals, Inc. α-Ketoisovaleric acid (sodium salt) and 5,5′-dithiobis(2-nitrobenzoic acid) were from Sigma Chemical Co. The equilibrium dialysis membrane was size $^{20}/_{32}$ from Union Carbide, Chicago. All other chemicals used were of the best available grade.

Methods

Enzyme Purification and Determination of Enzyme Purity. α-IPM synthase from *S. typhimurium* strain CV-19 was isolated essentially as previously described (Kohlhaw *et al.*, 1969; Kohlhaw, 1970; Leary and Kohlhaw, 1972). The enzyme from this strain is considered to have wild-type properties. The feedback-resistant enzyme from strain CV-241 (Calvo and Calvo, 1967) was isolated following a modification of the above-mentioned procedure (Soper *et al.*, 1973). The purity of the enzyme from each preparation was determined by means of sodium dodecyl sulfate polyacrylamide gel electrophoresis following the procedure of Fairbanks *et al.* (1971), since enzyme which appears to be homogeneous in normal polyacrylamide gel electrophoresis was found to contain minor impurities upon sodium dodecyl sulfate gel electrophoresis. The gels were scanned at 550 nm with a Gilford linear transport (Model 2410-S) connected to a Gilford spectrophotometer (Model 240). The intensity of the protein bands stained with Coomassie Blue was kept below a ΔOD of 1.5 where Beer's Law is obeyed. All enzyme preparations used in this study were between 84 and 95% pure by the above-outlined analysis. Only experiments performed with enzyme from a single preparation were compared quantitatively. Enzyme molarities in each individual case were calculated taking into account the purity indicated by densitometry scans of sodium dodecyl sulfate gels.

Protein Determination. Protein concentration was determined by the Biuret method with bovine serum albumin as a standard (Gornall *et al.*, 1949). Nitrogen determination of dried bovine serum albumin was performed to give the true protein content before a standard curve was made. The interference by glycerol (10–40%, v/v), used for storage of α-IPM synthase, was corrected for in the biuret assay through appropriate standard curves. Dry weight analysis of the purified enzyme corresponded closely to the biuret protein determination.

Measurement of Enzyme Activity. α-IPM synthase activity was assayed with the end point assay using 5,5′-dithiobis-(2-nitrobenzoate) to measure CoA formed during a timed incubation of enzyme with acetyl-CoA and α-ketoisovalerate at pH 8.5 and 37° (Kohlhaw *et al.*, 1969). The final volume was 0.125 ml. Lower pH values than 8.5 were used for kinetic studies in order to compare more directly with equilibrium dialysis. Specific activity is defined as micromoles of CoA formed per hour per milligram of protein.

Equilibrium Dialysis. Acrylic multimicro equilibrium dialysis cells (200 μl) from Interscience Scientific Products were used for all equilibrium dialysis experiments. The membranes were treated and stored according to Englund *et al.* (1969). Parafilm gaskets were used to sandwich the membrane to prevent leakage. A glass bead (diameter 1 mm) was placed in each chamber to aid in stirring. Samples of 50 μl were used in each chamber of the cell. The assembled dialysis cells were left on a rotating wheel at 4° for 3–4 hr. Under these conditions equilibrium in the presence of protein was reached in 2 and 3 hr, respectively, depending on whether ligand was included in both or only one chamber at the start of the dialysis experiment. Thereafter, triplicates of 10-μl samples were removed from each chamber with a Hamilton syringe. Samples were counted in 10 ml of a toluene-based scintillation fluid with a Beckman CPM 100 liquid scintillation counter at room temperature. Enzyme concentrations in equilibrium dialysis experiments were in the range of 0.4–8.0 mg/ml (8–160 μM in terms of monomers), and leucine concentrations were in the range of 1.5–1000 μM. The buffer used unless otherwise stated was 50 mM potassium phosphate (pH 6.8), with 10% (v/v) glycerol. Calculations from the amino acid composition of α-IPM synthase (Bartholomew and Calvo, 1971) indicated that, under the conditions employed, the Donnan effect should be negligible. At the conclusion of each experiment, at least 80% of the enzymatic activity was recovered. No corrections were made for the small amount of enzyme inactivated during an experiment as this was later shown to be unnecessary (*cf.* Results section).

Ultraviolet Difference Spectra. Difference spectra were measured in a Cary 15 spectrophotometer. The cell compartments were thermostated at 22°. The buffer used was 50 mM potassium phosphate (pH 6.8). Further details are given in the respective legends.

Results

Leucine Inhibition of Wild-Type and Feedback-Resistant α-IPM Synthases at pH 6.8. In order to quantitate leucine sensitivity at the pH at which equilibrium dialysis studies were to be performed, the activity of both the wild-type and the feedback-resistant enzymes was measured as a function of leucine concentration at pH 6.8. The results are shown in Figure 1. At saturating substrate concentrations (0.8 mM acetyl-CoA and 4 mM α-ketoisovalerate) the apparent K_i values, *i.e.*, the concentrations of leucine required for 50% inhibition, were 22 μM and 20 mM, respectively, for the wild-type and the feedback-resistant enzyme. With both enzymes, positive cooperativity was observed, as indicated by the values of the Hill coefficient. The cooperativity was more pronounced with wild-type enzyme. The apparent K_i values were a function of substrate concentration. Thus, when the substrate concentrations were reduced to 0.4 mM acetyl-CoA and 0.1 mM α-ketoisovalerate, the apparent K_i value of the wild-type enzyme was lowered approximately 2.5-fold to 8 μM, whereas that of the feedback-resistant enzyme was lowered about 7-fold to 3 mM. The Hill coefficients were practically unaffected.

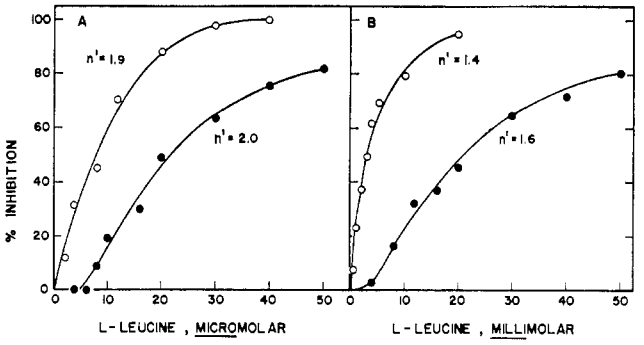


FIGURE 1: Leucine inhibition kinetics of wild-type (A) and feedback-resistant (B) α-IPM synthase. All assays were performed in 80 mM potassium phosphate buffer (pH 6.8) at 37°. The substrate concentrations were as follows: 0.8 mM acetyl-CoA and 4 mM α-ketoisovalerate (closed symbols), and 0.4 mM acetyl-CoA and 0.1 mM α-ketoisovalerate (open symbols). n' designates Hill coefficients, derived from plots of $[(v_0 - v_i)/v_i]$ *vs.* log [leucine], where v_0 is the reaction velocity in the absence and v_i is the reaction velocity in the presence of the inhibitor.

Binding of Leucine to Wild-Type and Feedback-Resistant α-IPM Synthases. A difference of the same order of magnitude as seen with the kinetic studies was observed when the two enzymes were saturated with leucine in equilibrium dialysis experiments (Figure 2). The $I_{0.5}$ values (the concentrations of leucine required for half-maximal saturation) were 10.5 μM and 1.5 mM, respectively, for the wild-type and feedback-resistant enzymes. In all binding experiments performed to date with the wild-type enzyme, which also include gel filtration (Hummel and Dreyer, 1962) and rate of dialysis measurements (Colowick and Womack, 1969), the maximum number of leucine molecules bound per monomer ($\bar{\nu}_t$) has never significantly risen above 0.5. For the feedback-resistant enzyme, which is also composed of monomers of about 50,000 molecular weight, $\bar{\nu}_t$ could not be accurately determined because the high leucine concentrations required for saturation would greatly reduce the specific radioactivity of leucine resulting in unacceptably large errors. However, the value for $I_{0.5}$ listed above, calculated with the assumption of $\bar{\nu}_t = 0.5$, is supported by the results of ultraviolet difference spectroscopy presented below (Figure 8).

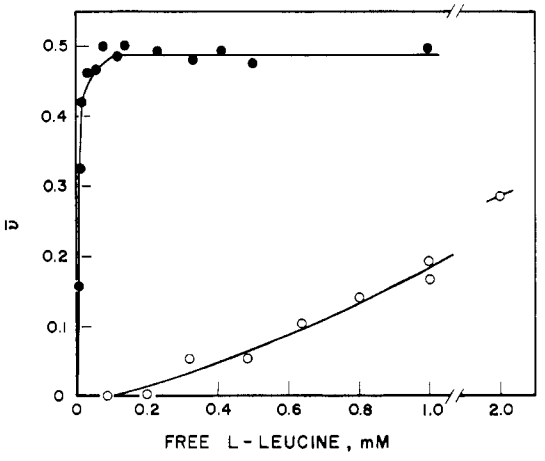


FIGURE 2: Saturation curves comparing the binding of leucine to wild-type (●) and feedback-resistant (○) α-IPM synthase. Protein concentration was 1 mg/ml. $\bar{\nu}$ is the number of moles of leucine bound per mole of enzyme monomer. Because of the low affinity of the feedback-resistant enzyme for leucine, full saturation of the enzyme was not feasible. The data of the feedback-resistant enzyme represent an average of three experiments with repeated sample counting.

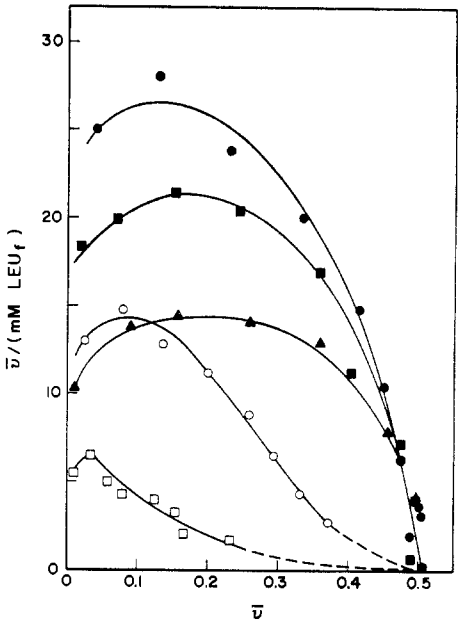


FIGURE 3: Scatchard plots of leucine binding to wild-type enzyme at varying protein concentrations (closed symbols) and in the presence of acetyl-CoA (open symbols). Protein concentrations were 1 mg/ml (●), 3 mg/ml (■), and 8 mg/ml (▲). The results represented by the open symbols were obtained at 1 mg/ml of protein and the acetyl-CoA concentrations were as follows: (○) 1.2 μM; (□) 5.1 μM. The plots are based on the mass law binding equation of Scatchard (1949), which for the present purpose may be written $\bar{\nu}/[I] = (\bar{\nu}_t - \bar{\nu})/K$, where $\bar{\nu}$ is the number of moles of inhibitor bound per mole of enzyme monomer, [I] is the concentration of free leucine, and $\bar{\nu}_t$ is the total number of binding sites per monomer.

Inactivation of wild-type enzyme to one-third of its original specific activity by repeated freezing and thawing did not affect the total number of leucine sites saturable, although the value of $I_{0.5}$ did increase slightly.

Figure 3 displays Scatchard plots of leucine binding to wild-type enzyme. Curves with closed symbols represent results obtained at enzyme concentrations between 1 and 8 mg per ml. Expansion of this range was not possible without sacrificing accuracy and reproducibility of the results. The $I_{0.5}$ values decreased from 17.5 μM at 8 mg/ml of enzyme to 10.5 μM at 1 mg/ml, reflecting an increase in the affinity of the enzyme for leucine with decreasing enzyme concentration. The convex curvature seen is indicative of positive cooperativity between leucine sites; straight lines would be expected for equivalent, independent sites. The Hill coefficients (calculated as described in the legend of Figure 4) remained at about 1.4 at all enzyme concentrations tested. With some enzyme preparations, a higher value of the Hill coefficient was observed. However, it never exceeded 1.9. The apparent total number of binding sites, *i.e.*, 0.5/monomer, did not change under the various conditions.

Binding of leucine to wild-type α-IPM synthase was also studied in the presence of substrates. The presence of acetyl-CoA led to very complex Scatchard plots, as shown in Figure 3 (open symbols). Increasing the acetyl-CoA concentration resulted in a greatly diminished ability of the enzyme to bind leucine. The total number of available binding sites again did not exceed 0.5/monomer. Because of the complex nature of leucine saturation in the presence of acetyl-CoA, the corres-

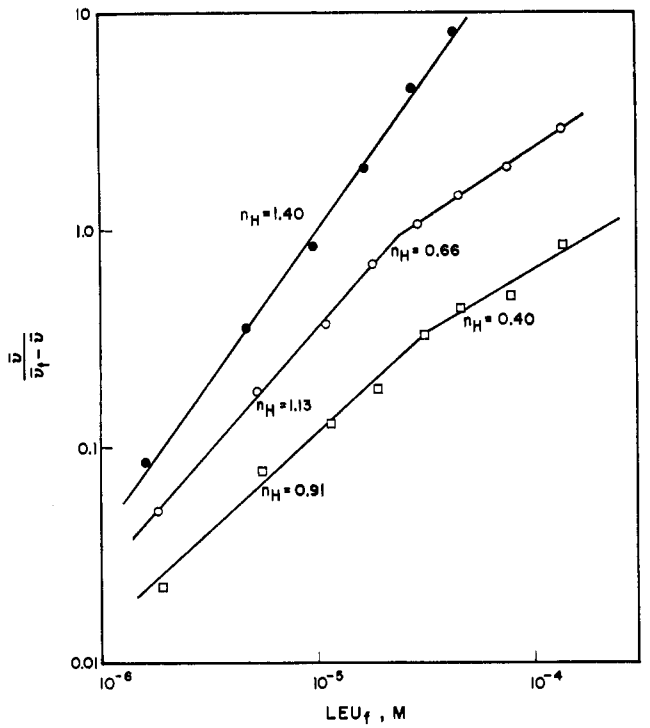


FIGURE 4: Hill plot of leucine binding to wild-type α-IPM synthase in the presence of acetyl-CoA. Replot of part of the data presented in Figure 3. The same symbols are used to designate various acetyl-CoA concentrations. The equation on which the plot is based is: $\log [\bar{\nu}/(\bar{\nu}_t - \bar{\nu})] = n_H \log [I] - \log K$, where $\bar{\nu}$, $\bar{\nu}_t$, and [I] have the same meaning as in Figure 3, and n_H is the Hill coefficient.

ponding Hill plots are also shown (Figure 4). It is evident from Figures 3 and 4 that the presence of acetyl-CoA also causes a decrease in the positive cooperativity between leucine sites. In fact, two phases can be distinguished in the Hill plots, and the second phase clearly exhibits negative cooperativity with a Hill coefficient of 0.66 in the presence of 1.2 μM acetyl-CoA and 0.40 in the presence of 5.1 μM acetyl-CoA. The concentration of acetyl-CoA necessary for a half-maximal antagonistic effect is in the micromolar range, approximately 100-fold lower than the apparent K_m value for acetyl-CoA obtained in kinetic studies.

Results much different from those seen with acetyl-CoA were obtained when the effect of the other substrate, α-ketoisovalerate, on leucine binding was examined. In the presence of 1 mM α-ketoisovalerate, the ability of the enzyme to bind leucine was increased, while at the same time cooperativity was greatly decreased (Figure 5). However, the effect of acetyl-CoA on leucine binding is relatively much more pronounced than the effect of α-ketoisovalerate. This conclusion was drawn from an experiment in which enzyme was saturated with leucine in the presence of acetyl-CoA and α-ketoisocaproate. α-Ketoisocaproate is a very sluggishly reacting homolog of α-ketoisovalerate (the maximum velocity reached with α-ketoisocaproate is less than 2% of that reached with α-ketoisovalerate) and a competitive inhibitor with respect to α-ketoisovalerate (Kohlhaw *et al.*, 1969). When 1.2 μM acetyl-CoA and 1 mM α-ketoisocaproate were incubated under the conditions of the equilibrium dialysis experiments, less than 1% of the substrates was turned over in 3 hr. The effect of α-ketoisocaproate alone on leucine binding was very similar to that of α-ketoisovalerate (Figure 5). It is evident from Figure 5 that acetyl-CoA at a concentration of 1.2 μM

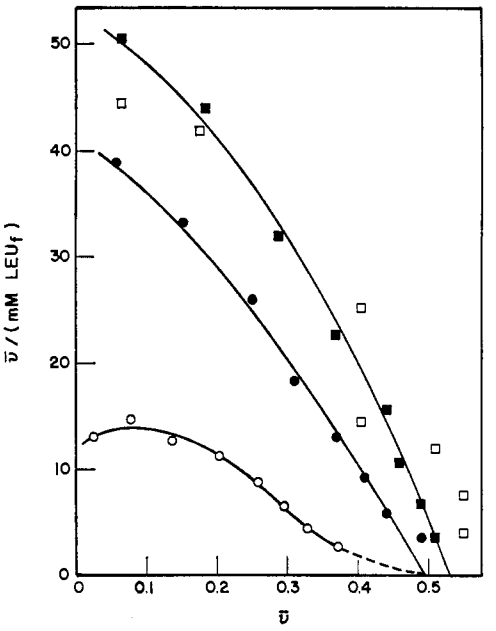


FIGURE 5: Scatchard plot of leucine binding to wild-type enzyme in the presence of substrates. Protein concentration was 1 mg/ml. Leucine concentrations were from 1.8 to 140 μM. Substrate concentrations were as follows: (■) 1 mM α-ketoisovalerate; (□) 1 mM α-ketoisocaproate; (●) 1 mM α-ketoisocaproate plus 1.2 μM acetyl-CoA. For comparison, the experiment with 1.2 μM acetyl-CoA alone is again included (○).

was capable of partially reversing the effect of 1 mM α-ketoisocaproate.

Figure 6 illustrates leucine binding to wild-type enzyme at different pH values. Although the affinity of the enzyme for leucine decreased with increasing pH, the total number of leucine binding sites again remained unchanged. Furthermore, a leucine binding study performed at room temperature (22°) indicated higher binding affinity for leucine without any effect on the number of binding sites.

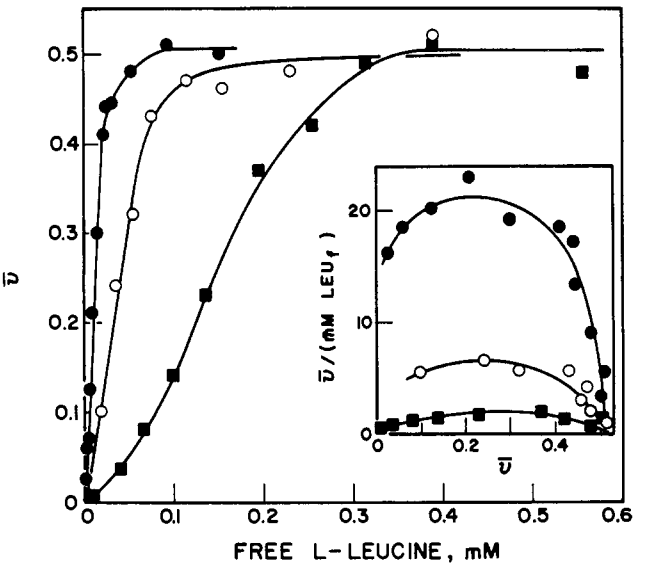


FIGURE 6: Leucine binding to wild-type enzyme at varying pH values. Protein concentration was 1 mg/ml. Leucine concentrations were in the range of 1.2–600 μM. (●) 50 mM potassium phosphate buffer (pH 6.8) with 10% glycerol; (○) 50 mM potassium phosphate buffer (pH 7.8) with 10% glycerol; (■) 50 mM Tris phosphate buffer (pH 8.5) with 75 mM KCl and 10% glycerol.

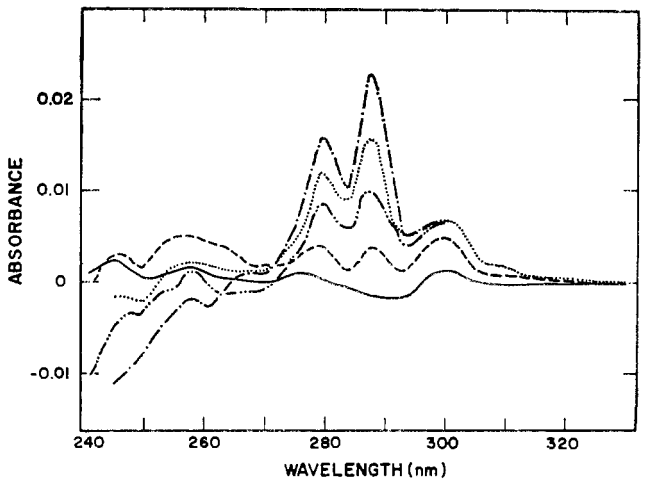


FIGURE 7: Ultraviolet difference spectra of wild-type α-IPM synthase upon addition of leucine. Identical 1-ml quartz cuvets with 1-cm light path were used. Protein concentration was 1.5 mg/ml ($OD_{280} \approx 0.8$). Base line (solid line) was set with protein against protein. Final leucine concentrations were: ---, 4 μM; ·-·-·-·, 10 μM; · · · ·, 20 μM; -·-·, 100 μM. Increasing the leucine concentration to 1 and 10 mM had no additional effect.

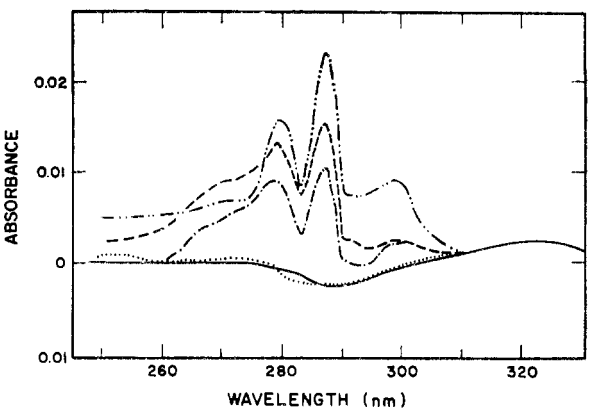


FIGURE 8: Ultraviolet difference spectra of the feedback-resistant enzyme upon addition of leucine. Quartz cuvets (1 ml) with a light path of 1 cm were used. Protein concentration was 1.5 mg/ml. To avoid significant protein concentration changes upon leucine addition, all leucine solutions were made to 1.5 mg/ml of α-IPM synthase. For every addition of leucine–enzyme solution to the sample cuvet, an identical aliquot of enzyme solution with the same protein concentration was added to the reference cuvet. Base line (solid line) was set with protein against protein. L-Leucine concentrations were as follows: · · · · 0.1 mM; -·-, 1 mM; ---, 2 mM; -·-·-, 10 mM. No additional change was observed at 20 mM leucine. All curves were corrected for leucine absorption. However, light scattering caused by a slight protein precipitation during the course of the experiment resulted in a slight shift of the base line. No attempts were made to correct for the scattering.

Ultraviolet Difference Spectra of Enzyme and Enzyme–Ligand Complexes. Previous findings that leucine causes an appreciable decrease in the reactivity of sulfhydryl groups of α-IPM synthase (Kohlhaw, 1970) suggested a conformation change upon leucine binding. We further investigated the question of conformational changes by means of ultraviolet difference spectra which, in contrast to the above-mentioned SH titrations, could be obtained under conditions resembling those of the majority of the binding studies. Figure 7 represents difference spectra obtained with wild-type enzyme and varying concentrations of leucine. Two major positive difference peaks can be identified at 287 and 279 nm, respectively, corresponding to a net red shift. A minor positive peak appears at about 300 nm. Although this experiment does not yield information on the behavior of individual amino acid residues, it may be interpreted as reflecting a mean transition of aromatic amino acid side chains from a more exposed to a less exposed environment (Laskowski, 1970). The ratio of the two major peaks did not significantly vary with different leucine concentrations; $\Delta A_{287}/\Delta A_{279}$ was 1.41 at 4 μM, 1.40 at 10 μM, 1.45 at 20 μM, and 1.55 at 100 μM leucine. The spectral change observed with 100 μM leucine represents the maximal change, since increasing the leucine concentration to as high as 10 mM had no further effect. The leucine concentration required for half-maximal change (at 287 nm) was about 10 μM, a value which is very close to the total concentration of leucine required to half-saturate the wild-type enzyme in binding studies (13 μM).

Figure 8 shows that the feedback-resistant enzyme, upon interaction with inhibitory concentrations of leucine, appears to undergo a conformational change very similar to that of the wild-type enzyme. No difference spectrum was observed at a leucine concentration sufficient to induce maximum change in wild-type enzyme. Owing to the high concentrations of leucine employed in this experiment and the resulting increase in ionic strength and refractive index, good difference spectra were more difficult to obtain. It is clear, nevertheless, that major positive peaks were again apparent at 287 and 279 nm, with a minor peak at 300 nm. The $\Delta A_{287}/\Delta A_{279}$ ratio was 1.30 at 1 mM leucine, 1.36 at 2 mM, and 1.51 at 10 mM leucine. Again, the leucine concentration required for half-maximal change at 287 nm, about 0.9 mM, agrees quite closely with the half-saturation value obtained in equilibrium dialysis studies where a total of 0.5 leucine site/monomer was assumed for the feedback-resistant enzyme.

When wild-type enzyme was incubated with 1 mM α-ketoisovalerate, the difference spectrum (Figure 9A) was very different from that given by leucine. A broad negative peak was observed, corresponding to a net blue shift. Upon addition of 0.125 mM leucine to the enzyme–α-ketoisovalerate complex, the spectrum typical for the enzyme–leucine complex appeared.

Addition of 7.5 μM acetyl-CoA to wild-type enzyme gave rise to a difference spectrum similar to, but not identical with, the one observed in the presence of α-ketoisovalerate. Again, addition of leucine caused the appearance of the difference spectrum typical for the enzyme–leucine complex (Figure 9B).

None of the difference spectral changes observed in this study were recognizably time dependent. The first scan was recorded a few seconds after mixing the samples.

Comparison of Gel Filtration and Leucine Inhibition Data of the Feedback-Resistant Enzyme. Wild-type α-IPM synthase is known to be dissociated by leucine at concentrations which also cause inhibition (Kohlhaw *et al.*, 1969; Leary and Kohlhaw, 1970). A crude preparation of the feedback-resistant enzyme, on the other hand, did not appear to be subject to dissociation by leucine (Kohlhaw *et al.*, 1969). In an effort to quantitatively compare the lack of dissociability and the residual inhibition by leucine of the feedback-resistant enzyme, both gel filtration and inhibition experiments were carried out under similar conditions (*i.e.*, relatively high enzyme concentration and low temperature). When chromatographed on a Sephadex G-200 column (0.9 × 60 cm) which was equilibrated at 5° with 50 mM potassium phosphate buffer (pH 6.8) either with or without 50 mM leucine, the feedback-resistant enzyme, applied at a concentration of 0.2 mg/ml, was found to have an apparent average molecular weight of

162,000 both in the absence and in the presence of leucine. Leucine inhibition kinetics were performed in 50 mM potassium phosphate buffer (pH 6.8) at 5°, and at an enzyme concentration of 0.175 mg/ml. Substrate concentrations were 0.8 mM for acetyl-CoA and 4 mM for α-ketoisovalerate. Under these conditions, the mutant enzyme was found to have an apparent K_i value of 16 mM. It is evident, therefore, that the residual inhibition of the feedback-resistant enzyme occurs without an alteration of the quaternary structure.

Discussion

We have consistently observed saturation of wild-type α-IPM synthase with leucine to be apparently complete with only 0.5 site/monomer (*i.e.*, one site per dimer) occupied. No one satisfactory explanation can be given at the present time, although several alternatives are being considered: (1) incomplete saturation of leucine binding sites; (2) the existence of "functional dimers"; (3) nonidentical subunits. These will be discussed in turn.

(1) The first explanation seems unlikely in view of the fact that the saturation curve clearly leveled off after 0.5 site/monomer had been saturated and that no further increase was observed even though the leucine concentration was increased to about 100 times $I_{0.5}$. Furthermore, maximum change of the difference spectrum was observed at a leucine concentration (0.1 mM) just saturating 0.5 site/monomer in the equilibrium dialysis studies. A leucine concentration as high as 10 mM did not give rise to additional change (Figure 7). It is nevertheless possible that optimal conditions for the binding of more leucine have not yet been found.

(2) Results of sedimentation equilibrium experiments conducted under conditions similar to those employed in the binding experiments may be interpreted as reflecting a predominance of dimeric species in the presence of leucine, although the existence of monomers is also indicated (Leary and Kohlhaw, 1970). While it cannot be ruled out that the formation of a dimer is required before leucine binding can occur, the more attractive possibility is that the dimer has the highest affinity for leucine of all molecular weight forms present (the protein concentration dependence of leucine binding, established in Figure 3, only suggest that species *smaller than the tetramer* bind leucine preferentially). Extreme negative cooperativity between subunits or asymmetric association of monomers would then have to be invoked to account for the observation that only one leucine site per dimer can be saturated. Further support for the predominance of dimers in the presence of leucine comes from cross-linking experiments conducted previously (Kohlhaw and Boatman, 1971). Enzymes cross-linked at pH 8.5 after prior incubation with leucine consisted mainly of dimers and to a lesser extent of monomers. In the absence of leucine, a monomer–dimer–tetramer pattern was obtained with tetramers being predominant.

(3) As far as the identity of polypeptide chains is concerned, both genetic (Margolin, 1963; Calvo and Worden, 1970) and physical–chemical evidence (Leary and Kohlhaw, 1970, 1972; Bartholomew and Calvo, 1971) is consistent with only one type of subunit. It is, of course, possible that minor chemical differences (introduced after the biosynthesis of the polypeptide chains) have escaped detection and that the enzyme actually consist of two sets of nonidentical subunits. Further experimentation is needed to clarify these points.

It was of special interest to us to learn whether the binding studies reported here would help explain the leucine-resistance of α-IPM synthase from strain CV-241. It is clear from the data obtained that leucine binding to the resistant enzyme is impaired, the binding constant being over 100-fold higher than that of the wild-type enzyme. Once leucine binds, however, it causes both inhibition and the appearance of a difference spectrum which is practically indistinguishable from that obtained with wild-type enzyme upon leucine binding. It appears, therefore, that transfer of the feedback inhibition signal can still occur with the feedback-resistant enzyme and is accompanied by conformational changes resembling those seen with the normal enzyme. The available evidence is consistent with the notion that, with the wild-type enzyme, species smaller than the tetramer, probably dimers, have a higher affinity for leucine and that therefore the equilibrium is shifted in their favor when leucine is added to the enzyme. While a monomer–dimer–tetramer equilibrium is still likely to exist with the feedback-resistant enzyme (because of the pattern seen after cross-linking (Soper *et al.*, 1973) and the observed average molecular weight of 162,000), there is no indication that any one of these species is stabilized by leucine (*cf.* the gel filtration experiments). In other words, the mutation appears to have abolished the ability of dimers to bind leucine preferentially; with the feedback-resistant enzyme all molecular weight forms exhibit the same low affinity toward leucine. To what extent the observed increase in subunit inter-

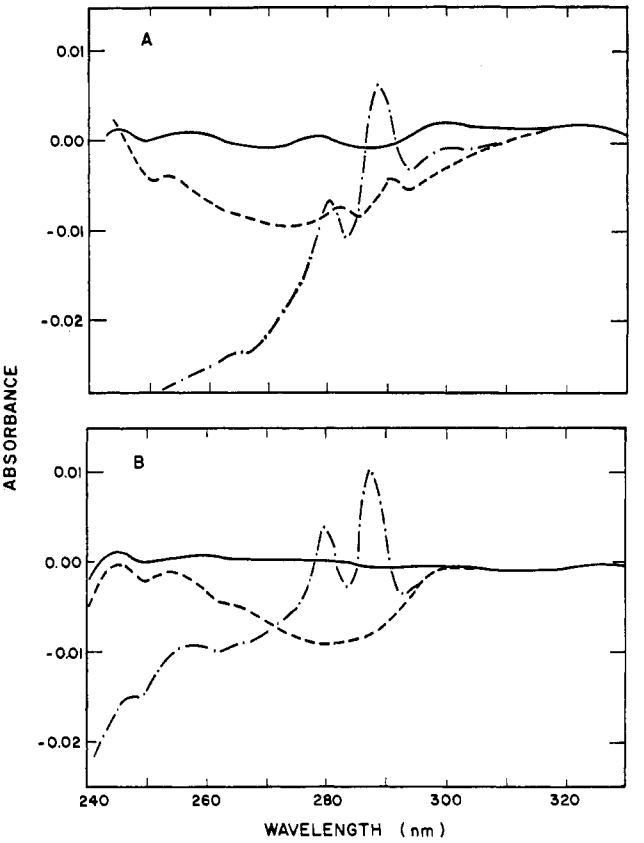
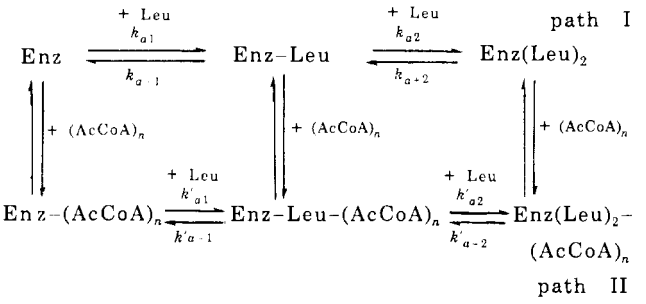


FIGURE 9: Uv difference spectra of wild-type α-IPM synthase in the presence of α-ketoisovalerate (A) and acetyl-CoA (B) followed by addition of leucine. Identical 3-ml tandem quartz cuvets with a total light path of 0.874 cm were used. Base line (solid line) was set with protein in one compartment and ligand in the other compartment of both the sample and reference cuvets. The sample cuvet was tipped to mix the protein and ligand solution to produce the enzyme–ligand complex. Protein concentration was 1.5 mg/ml: (A) ----, 1 mM α-ketoisovalerate; ---, 1 mM α-ketoisovalerate + 0.125 mM leucine; 0.25 mM leucine did not give rise to additional difference spectrum; (B) ---, 7.5 μM acetyl-CoA; ---, 7.5 μM acetyl-CoA + 2 mM leucine; 4 mM leucine had no additional effect.

SCHEME I



actions contributes to the leucine resistance, is not known at present.

It should be mentioned at this point that attempts to show an enzyme concentration dependence of leucine *inhibition* have so far not met with success, probably because it has not been possible to vary the enzyme concentration over a wide enough range. Stopped-flow experiments may have to be employed in these studies.

It is of interest that, although the feedback-resistant enzyme cannot be further dissociated by leucine, its binding of and inhibition by leucine are still positively cooperative. Apparently the cooperativity of α-IPM synthase derives mainly from the conformational change of the enzyme upon leucine binding and only to a lesser extent from the polymerization–depolymerization of the subunits.

All three ligands tested (*i.e.*, the inhibitor, leucine, and the two substrates, α-ketoisovalerate and acetyl-CoA) have been shown to cause distinct conformational alterations of α-IPM synthase. However, while the SH reactivity of the enzyme is decreased by both α-ketoisovalerate and leucine (Kohlhaw, 1970), the ultraviolet difference spectra obtained upon binding of either of these two ligands differ greatly; the difference spectrum observed upon leucine binding indicates a net burial of aromatic amino acid residues, that obtained upon interaction of α-ketoisovalerate indicates a net exposure of residues. Binding of acetyl-CoA, too, appears to cause a net exposure of aromatic residues. On the other hand, α-ketoisovalerate enhances leucine binding, while acetyl-CoA acts in an antagonistic fashion. These results confirm once again that care must be taken in interpreting such difference spectra beyond the simple statement that they indicate some conformational change.

It is intriguing that a substrate (α-ketoisovalerate) should give rise to a conformation favoring the binding of the inhibitor. For the catalytic reaction, however, the effect of α-ketoisovalerate alone is irrelevant, and our results indicate that, if both substrates are present, the effect of acetyl-CoA, which is a strong antogonist of leucine, would predominate.

In the presence of acetyl-CoA, leucine binding exhibits a complex pattern which involves both positive and negative cooperativity (Figures 3 and 4). The formation of a ternary complex, as shown in the following scheme (Scheme I), is suggested as one possible way to explain how such a binding pattern can arise, assuming a total of two leucine sites per tetramer. Scheme I postulates two possible paths for leucine binding. Path I shows leucine binding in the absence of acetyl-CoA where $k_{a1} \gg k_{a-1}$, $k_{a2} \gg k_{a-2}$, and $k_{a2} > k_{a1}$, and positive cooperativity of the leucine sites would result. Path II represents the binding of leucine to an enzyme–acetyl-CoA complex forming an enzyme–leucine–acetyl-CoA ternary complex, where $k'_{a1} \ll k'_{a-1}$, $k'_{a2} \ll k'_{a-2}$, $k'_{a2} < k'_{a1}$, and the binding of the second leucine molecule to this ternary complex would occur with negative cooperativity. Negative cooperativity can also arise from a combination of paths I and II where $k_{a1} > k'_{a2}$. The fraction of leucine undergoing each route would depend on the concentration of acetyl-CoA present.

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