The α-Isopropylmalate Synthetase of *Neurospora*. I. The Kinetics and End Product Control of α-Isopropylmalate Synthetase Function*

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ABSTRACT: α-Isopropylmalate synthetase which catalyzes the first step in leucine biosynthesis, the formation of α-isopropylmalate from acetyl coenzyme A and α-ketoisovalerate, has been isolated from *Neurospora*. The enzyme has a broad pH optimum between pH 7 and 8 and requires potassium ions for activity. The K_m value of the enzyme at pH 7.5 is 2.45×10^{-5} M for acetyl coenzyme A and 1.0×10^{-5} M for α-ketoisovalerate.

Pyruvate and α-ketobutyrate can substitute for α-ketoisovalerate as substrates but display K_m values of 5.8×10^{-3} and 2.1×10^{-3} M, respectively. Leucine and its structural analog 5′,5′,5′-trifluoro-DL-leucine are effective inhibitors of synthetase function. The inhibition is reversed by the structurally unrelated substrate acetyl coenzyme A. The inhibition kinetics change as a function of pH from competitive at pH 7.5 to mixed at lower pH values. The pH dependence of inhibition differs significantly from the pH dependence of catalysis. A relation between bound inhibitor and increased thermal stability was established by the observation of a positive correlation between inhibition efficiency and protection from thermal inactivation as a function of pH. In addition, a leucine-resistant enzyme obtained from a fluoroleucine-resistant strain was found to have the same thermal sensitivity in the presence of leucine as did the normal enzyme in the absence of leucine. The results are interpreted in terms of an allosteric conformational transition induced by bound inhibitor.

Interest in the structure and function of the *Neurospora* α-isopropylmalate synthetase (referred to hereafter as the synthetase)[1] stems from the fact that the enzyme exhibits several biologically important features. The synthetase catalyzes the first reaction in the biosynthetic sequence leading to the formation of leucine and is regulated by the end product of the reaction sequence (Umbarger, 1961). In addition, the synthesis of the synthetase is controlled by a leucine-concentration-dependent repression mechanism (S. R. Gross, in preparation). Furthermore, active synthetase can be formed by complementation interactions between differently defective products of *leu*-4 alleles.

This paper contains the results of a study of the catalytic properties of the normal synthetase—the reaction catalyzed, the kinetics of catalysis and of end product inhibition, as well as a description of the purification procedure employed in obtaining the enzyme for physical analysis. The accompanying paper contains a more detailed structural analysis of the synthetase and its relation to complementation interactions.

Experimental Methods and Procedures

Biological. The synthetase was obtained from extracts of a *leu*-1 mutant, D3:23A, grown under conditions of derepression on limiting leucine (Gross *et al.*, 1963). Routinely, 20 ml of a dense conidial suspension (OD_{550} 0.5) was added to 12 l. of synthetic medium containing 1% sucrose and 15 mg/l. of DL-leucine. After 24-hr incubation with vigorous aeration at 34°, the growth medium was supplemented with 120 mg of DL-leucine and incubation was continued for an additional 36 hr. Mycelia obtained from 36 l. of medium, about 180 g wet weight, were used as the source of enzyme in the purification procedures described.

FLR_{92}-1-216A, a trifluoroleucine-resistant *leu*-1 (D221) double mutant (S. R. Gross, in preparation) grown under conditions of derepression, was the source of leucine-resistant synthetase. The synthetase produced by this mutant is about 500 times less sensitive to leucine inhibition than the normal enzyme.

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[1] The biosynthetic precursors of leucine have been referred to variously as derivatives of isocaproic, malic, and succinic acids. The investigators now working in this area of metabolism have agreed with Strassman and Ceci (1963) to refer to the product of the condensation of α-ketoisovalerate and acetyl coenzyme A (CoA) as α-isopropylmalic acid [referred to previously as β-hydroxy-β-carboxyisocaproic acid (Jungwirth *et al.*, 1961) and 2-isopropylmalic acid (Calvo *et al.*, 1962)]. Similarly, the product of isomerization will be referred to henceforward as β-isopropylmalic acid.

Chemical. *N*-Ethylmaleimide (NEM)[2] and a highly purified lithium salt of acetyl coenzyme A (CoA) were obtained from Mann Research Laboratories. Acetyl-^{14}C-CoA with a specific activity of 40 mc/mmole was obtained from New England Nuclear Corp. 1-Aminocyclopentane-1-carboxylic acid (cycloleucine) and phosphotransacetylase were purchased from California Corp. for Biochemical Research. 5′,5′,5′-Trifluoro-DL-leucine (fluoroleucine) was a gift from Dr. H. S. Anker, Department of Biochemistry, University of Chicago, Chicago, Ill. DL-Azaleucine was provided by Dr. T. J. McCord, Department of Chemistry, Abilene Christian College, Abilene, Texas. 3-Carboxy-4-nitrophenyl disulfide was purchased from the Aldrich Chemical Co. and recrystallized twice from glacial acetic acid before use. DEAE Sephadex A-50, Sephadex G-50, and G-200 were obtained from Pharmacia. Hydroxylapatite was prepared by the method of Tiselius *et al.* (1956).

The amount of acetyl CoA present in all preparations was determined by the phosphotransacetylase method of Stadtman (1957). Free CoA contamination of the preparations of acetyl CoA used was determined to be negligible by thin layer chromatography on cellulose plates with ethanol–0.1 M sodium acetate buffer, pH 4.5 (60:40 v/v), as the solvent.

Assays of Synthetase Activity. Synthetase activity was measured by a spectrophotometric procedure that takes advantage of the fact that the thiol of CoA reacts quantitatively with NEM between pH 6.5 and 7.5 (Roberts and Rouser, 1958; Alexander, 1958). In this procedure, hereafter referred to as the NEM assay, the decrease in absorption due to the loss of the double bond plus the loss of optical density at 232 mμ resulting from the spectral shift in the conversion of acetyl CoA to CoA (Stadtman, 1957) yields a ΔOD_{232} of $-1.08/0.1$ μmole of CoA formed. The reaction mixture usually contained 0.1 μmole of acetyl CoA, 0.5 μmole of α-ketoisovalerate (α-KIV), 0.1 μmole of NEM, and 0.1 mmole of potassium phosphate buffer, pH 7.5, plus 0.01 ml of enzyme in a total volume of 1 ml. The change in optical density was followed as a function of time at 30° against a control mixture without acetyl CoA in a Beckman-Gilford recording spectrophotometer. The reaction kinetics were linear over a wide range of enzyme and substrate concentrations. A unit of enzyme activity catalyzes the formation of 0.1 μmole of CoA per minute at 30°.

The production of free thiol groups was measured also by SH exchange with 3-carboxy-4-nitrophenyl disulfide (Ellman, 1959). This assay is referred to hereafter as the CNPD assay. Unlike NEM which, surprisingly, in view of the thiol content of the synthetase (see below) does not inhibit catalysis, CNPD is a strong inhibitor of synthetase activity. Except for the absence of NEM and the presence of 50 μmoles of phosphate, pH 7.5, buffer instead of 0.1 mmole, the CNPD reaction mixture was the same as described for the NEM assay. The reaction was terminated by the addition of 1.5 ml of acetone, then 1 ml of a 10^{-3} M solution of CNDP was added, the yellow color was allowed to develop for 10 min, then measured in 13-mm test tubes with a Bausch and Lomb Spectronic 20 spectrophotometer (ΔOD_{410} 0.415/0.1 μmole of CoA).

In addition to the above, synthetase activity was measured by the rate of disappearance of α-KIV from a reaction mixture containing 0.1 μmole of α-KIV, 0.12 μmole of acetyl CoA, 0.1 mmole of phosphate buffer, pH 7.5, and 0.1 ml of enzyme in a total volume of 1.0 ml. The reaction was terminated by the addition of 1.5 ml of 7.5% trichloroacetic acid, 1 ml of a 0.1% solution of 2,4-dinitrophenylhydrazine was then added, and the solution was incubated for 10 min at 25°. The 2,4-dinitrophenylhydrazone formed was measured by the method of Friedemann and Haugen (1943) after extraction with toluene.

Thermal Inactivation. The synthetase used in studies of thermal inactivation was freed of leucine by passing a 1-ml sample through a Sephadex G-50 column (21 × 1.3 cm) equilibrated with 0.1 M phosphate buffer. The leucine-free enzyme solution was then diluted tenfold into prewarmed 0.1 M phosphate buffer and at intervals the heating was terminated by pipetting 0.1-ml aliquots into tubes precooled at 0°.

Miscellaneous Procedures. Protein was determined by the method of Lowry *et al.* (1951) and the spectrophotometric procedure of Warburg and Christian (1941). ^{14}C was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Sedimentation velocity patterns were obtained using a Beckman/Spinco Model E ultracentrifuge operated at 59,780 rpm at 25° with a 12-mm single-sectored cell. Sucrose density gradient centrifugation was performed in a Spinco Model L ultracentrifuge as described by Martin and Ames (1961).

The protein elution patterns for all columns were monitored with a LKB 4701 A Uvicord ultraviolet absorptiometer. Protein solutions were concentrated without significant change in salt concentration by using a LKB 6300 A Ultrafilter. Before use the dialysis tubing (Visking) was always placed on the frame and equilibrated under negative pressure for 12 hr against 0.1 M phosphate buffer, pH 6.5, containing 2×10^{-4} M L-leucine.

Results

Purification of α-Isopropylmalate Synthetase. Mycelia from three 12-l. cultures were collected by filtration, washed copiously with, and suspended in 500 ml of 0.1 M phosphate buffer, pH 6.5, containing 2×10^{-4} M L-leucine (hereafter referred to as phosphate–leucine buffer). The presence of L-leucine was necessary throughout the purification for stabilization of the synthetase. The mycelia were homogenized in a Lourdes blender until an homogeneous free-flowing slurry was obtained. Care was taken in this, as in all subsequent operations, to maintain the temperature between 0 and

[2] Abbreviations used throughout the text: NEM, *N*-ethylmaleimide; CoA, coenzyme A; α-KIV, α-ketoisovalerate; PCMB, *p*-mercuribenzoate; CNPD, 3-carboxy-4-nitrophenyl disulfide.

TABLE I: Enzyme Purification.

Fraction	Total Protein (mg)	Activity (units)	Specific Activity (units/mg of protein)	Purification Factor
Crude extract	39,800	36,000	0.9	—
Ammonium sulfate I	11,400	28,000	2.5	2.8
Ammonium sulfate II	2,880	25,900	9.0	10.0
DEAE Sephadex I	575	18,400	32.0	35.6
DEAE Sephadex II	302	12,400	41.0	45.6
Hydroxylapatite	74.5	5,300	71.0	79.0
G-200 Sephadex	14.5	2,100	145.0	161.0

5°. The homogenate was then sonicated intermittently (about 2-min exposures) with a 75 w, 20 kc Branson Sonifier until disruption was complete (about 13–15-min total exposure).

After removal of debris by low-speed centrifugation (all low-speed centrifugations were carried out at 8000*g* for 30 min), 20 mg of protamine sulfate/100 mg of protein was added and the resulting precipitate was removed by centrifugation. Solid ammonium sulfate (43 g/100 ml) was added slowly and the precipitate formed, ammonium sulfate fraction I, was collected by centrifugation. Ammonium sulfate precipitates were stored routinely at −20°.

Two samples of ammonium sulfate fraction I were pooled and suspended in 100 ml of phosphate–leucine buffer, dialyzed overnight against 4 l. of the same buffer, then centrifuged for 90 min at 100,000*g*. The concentration of protein in the supernatant solution was adjusted to 15 mg/ml and 60 ml of saturated ammonium sulfate in phosphate–leucine buffer was added/100 ml of solution. The precipitate formed was discarded after centrifugation and an additional 20 ml of saturated ammonium sulfate was added/100 ml of supernatant solution. The precipitate, ammonium sulfate fraction II, was collected by centrifugation.

Four samples of ammonium sulfate fraction II were pooled and dissolved in approximately 80 ml of phosphate–leucine buffer and dialyzed overnight against the same buffer. The phosphate concentration of the dialyzed protein solution was adjusted to 0.035 M by the addition of a 2×10^{-4} M solution of leucine, and the solution was placed on a DEAE A-50 Sephadex column (30 × 4 cm) equilibrated with 0.035 M phosphate buffer, pH 6.5, containing 2×10^{-4} M leucine. A 1200-ml linear gradient of 0.035–0.35 M phosphate buffer, pH 6.5, containing 2×10^{-4} M leucine was applied to the column, and the protein elution pattern was monitored. The synthetase was eluted from the column at about 0.12 M as part of a complex protein peak. All fractions with a specific activity of 20 or more were pooled and concentrated to approximately 30 ml by ultrafiltration. The enzyme solution was then diluted with 60 ml of a 2×10^{-4} M solution of leucine and placed on a second DEAE A-50 Sephadex column (28 × 2.5 cm) prepared as before. The enzyme was eluted with a 900-ml linear gradient of 0.035–0.35 M phosphate buffer, pH 6.5, containing 2×10^{-4} M leucine. All fractions with a specific activity of 40 or more were pooled and concentrated to 20 ml by ultrafiltration.

The concentrated solution from the second DEAE Sephadex column was diluted with 46 ml of a 2×10^{-4} M solution of leucine and placed on an hydroxylapatite column (15 × 2 cm) which had been equilibrated with 0.03 M phosphate buffer, pH 6.5, containing 2×10^{-4} M leucine. After the unadsorbed protein had passed through, the synthetase was eluted with a 950-ml linear gradient of 0.03–0.2 M phosphate buffer at pH 6.5 containing 2×10^{-4} M leucine. The synthetase emerged as a definite protein peak at about 0.085 M phosphate buffer. All fractions with a specific activity of 70 or more were pooled, concentrated to 15 ml by ultrafiltration, and lyophilized.

The lyophilized protein was dissolved in 3 ml of phosphate–leucine buffer, then placed on and run through a Sephadex G-200 column (80 × 2.5 cm) which had been equilibrated against phosphate–leucine buffer. The synthetase was not excluded by the gel and emerged as a distinct peak after a column void volume equivalent had passed through. Preparations obtained after gel filtration had a specific activity of 140–145 and were the subject of the chemical and physical studies described below. Purified synthetase could be stored frozen in phosphate–leucine buffer at −20° with little loss of activity. The results of a typical purification procedure are presented in Table I.

Sedimentation analysis indicated that the synthetase obtained by the purification procedure described was within the acceptable limits of purity required for physical and chemical analyses. The slight asymmetry of the leading edge of the sedimentation velocity pattern in Figure 1 suggested no more than a 5–8% contamination of the preparation with extraneous protein. Only a single band of protein containing all of the detectable enzymatic activity was found after electrophoresis on cellulose acetate at pH 6.5 and 7.5. Electrophoresis at pH 9.5 on acrylamide gels revealed the presence of two

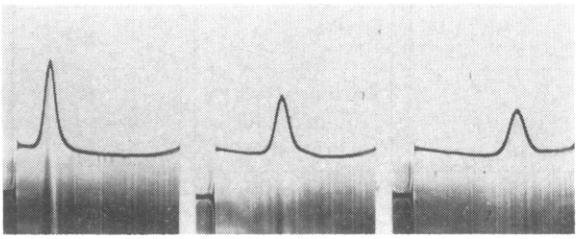

FIGURE 1: Sedimentation velocity of α-isopropylmalate synthetase. Schlieren patterns of a 9 mg/ml solution of the synthetase photographed at 24-min intervals during centrifugation at 59,780 rpm in phosphate–leucine buffer at 25°.

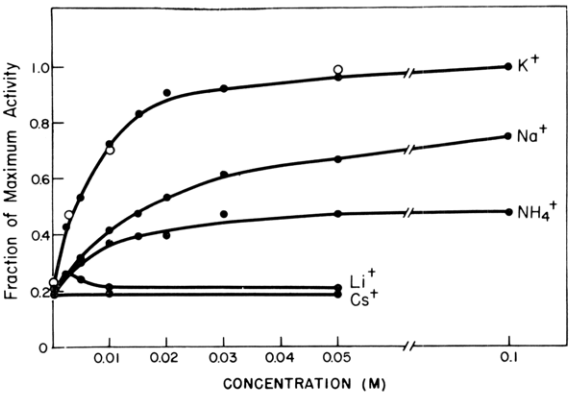

FIGURE 2: The dependence of α-isopropylmalate synthetase activity on monovalent cation concentration. Spectrophotometric assays were carried out in 0.05 M Tris–HCl, pH 7.5, buffer in the presence (●) and absence (○) of NEM.

minor bands in addition to a single primary band containing 90–95% of the protein applied.

Some Properties of the Condensation Reaction and α-Isopropylmalate Synthetase. α-Isopropylmalate synthetase catalyzes the condensation of α-KIV and acetyl CoA yielding α-isopropylmalate and CoA (Jungwirth *et al.*, 1963; Strassman and Ceci, 1963). The stoichiometry of the reaction is reported in Table II. As is indicated, for every mole of α-KIV and acetyl CoA consumed, a mole of CoA-SH appeared. Of importance however is the demonstration that NEM has no discernible effect on the reaction kinetics of the synthetase.

TABLE II: Stoichiometry of the α-Isopropylmalate Synthetase Reaction.

Measurement	Amount Enzyme/ μg protein	mμmoles[a] Minutes 1	2	3	60
Acetyl CoA disappearance (NEM assay)	0.65	6.4	12.8	19.0	
	1.30	12.8	24.6	37.0	
CoA appearance (CNPD assay)	0.65	6.5	13.3	20.1	
	1.30	13.3	26.8	39.0	114
α-KIV disappearance	0.65	5.5	13.5	18.0	
	1.30	11.0	24.0	36.0	102
α-KIV disappearance in presence of NEM	0.65	5.0	11.0	18.0	
	1.30	13.0	25.0	39.0	

[a] Each value presented is an average of two separate determinations of two separate reaction mixtures. The reaction mixtures incubated for 1, 2, and 3 min contained 100 mμmoles of α-KIV, 110 mμmoles of acetyl CoA, and 50 μmoles of phosphate buffer, pH 7.5, in a total volume of 1 ml. Those reaction mixtures incubated for 60 min contained 500 mμmoles of α-KIV when complete conversion of acetyl CoA to CoA was measured, and those reaction mixtures in which the complete disappearance of α-KIV was determined contained 200 mμmoles of acetyl CoA.

The amount of α-isopropylmalate formed at equilibrium was measured in a reaction mixture containing 0.1 μmole of α-KIV and 0.1 μmole of acetyl-1-^{14}C CoA. The acetyl-1-^{14}C CoA preparation contained 1.04×10^6 cpm of which 0.95×10^6 cpm was actually acetyl CoA as determined by thin layer chromatography and by the phosphotransacetylase assay. All of the counts, 0.96×10^6 cpm, were recovered in the α-isopropylmalate formed. The reaction therefore very heavily favors synthesis of α-isopropylmalate.

The activity of the synthetase is strongly dependent on the presence of certain monovalent cations. In this respect it resembles another enzyme involved in leucine biosynthesis, β-isopropylmalate dehydrogenase (Burns *et al.*, 1963). As indicated in Figure 2, K^+ is most stimulatory, Na^+ and NH_4^+ are less effective, Li^+ is stimulatory only at low concentrations, and Cs^+ is ineffective.

No divalent cations are required for activity. Ba^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+} have no effect when tested in concentrations as high as 10^{-3} M in the presence of 0.1 M KCl in 0.5 M Tris–HCl buffer, pH 7.5. However, Zn^{2+} even in concentrations as low as 10^{-4} M destroys activity. Cl^-, PO_4^{3-}, Br^-, and F^- in concentrations below 0.02 M have little effect on K^+ stimulation but at concentrations above 0.02 M Br^- and especially F^- are inhibitory. PO_4^{3-} seems required in some way for maintaining constant reaction kinetics. The initial velocity of reaction performed in either 0.1 M phosphate buffer or 0.05 M Tris–HCl buffer containing 0.1 M KCl at pH 7.5 is usually the same, but the reaction carried out in the Tris–HCl–KCl buffer departs from linearity over long reaction periods. For this reason, 0.1 M phosphate buffer was used for assaying the synthetase whenever possible.

Potassium phosphate (0.03–0.5 M) seems to have some specific stabilizing effect on the enzyme, for the activity of the enzyme is lost even at 0° when left in buffers of lower ionic strength. Other annoying problems such as the tendency of the enzyme to irreversibly

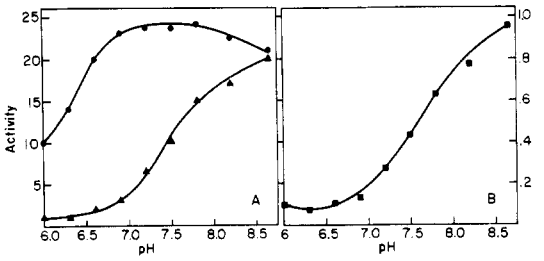

FIGURE 3: The pH dependence of catalysis and leucine inhibition of α-isopropylmalate synthetase. Activity is expressed as the disappearance of α-KIV, in mμmoles, per 5-min incubation at 30°. The pH dependence of synthetase activity in the presence (▲) and absence (●) of 2×10^{-4} M L-leucine are presented in A (left). B (right) is a plot of the fraction of enzyme activity in the presence of leucine as a function of pH.

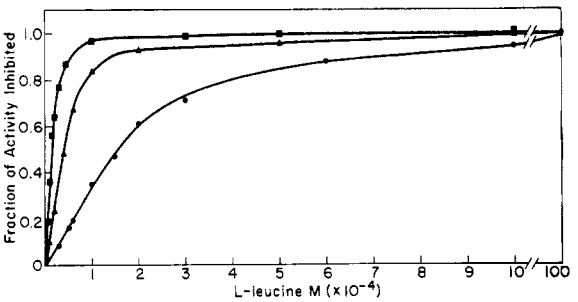

FIGURE 4: The inhibition of α-isopropylmalate synthetase as a function of leucine concentration. The fraction of enzyme activity inhibited is plotted as a function of leucine concentration at pH 6.5 (■), pH 7.0 (▲), and pH 7.5 (●).

adsorb to Sephadex, dialysis tubing, etc., also arise as a consequence of low salt concentrations. Consequently, the salt concentration was kept between 0.03 and 0.5 M phosphate.

As illustrated in Figure 3A, the pH optimum for the synthetase reaction is broad, ranging from pH 7.0 to 8.0. The activity falls rapidly below pH 6.8 and only gradually between pH 8.0 and 8.7. A pH of 7.5 was chosen for routine assays of the enzyme.

The binding specificity of the *Neurospora* synthetase is similar to that of the synthetase from *Salmonella* (Kohlhaw and Umbarger, 1965). Pyruvate and α-ketobutyrate could be substituted for α-KIV in reactions catalyzed by purified synthetase, but oxalacetate and α-ketoisocaproate could not. The K_m of the enzyme for α-KIV at pH 7.5 is 1.0×10^{-5} M while the K_m values obtained for pyruvate and α-ketobutyrate are 5.8×10^{-3} M and 2.1×10^{-3} M, respectively. However, the V_{max} of the enzyme was much higher with α-ketobutyrate as the substrate than with α-KIV as substrate. In this case the specificity of the enzyme seems to be more intimately related to substrate-binding specificity rather than catalytic specificity.

End Product Inhibition of the Synthetase. Like many other enzymes that catalyze the first reaction in a biosynthetic sequence, the synthetase is subject to inhibition by the end product of the reaction sequence, L-leucine. As indicated in Table III, of all the compounds tested, only L-leucine and DL-fluoroleucine were inhibitory at pH 7.5. At pH 6.5, DL-azaleucine, L-isoleucine, L-phenylalanine, and L-valine exhibited some inhibition at high concentration (10^{-2} M) which was reversible by high substrate concentration.

Leucine inhibition and the catalytic activity of the synthetase vary independently of each other as a function of pH as illustrated in Figure 3A. Leucine inhibition varied from 80% at pH 7.0 to 25% at pH 8.0, a range of pH values in which little variation in catalytic activity was observed. Neither the carboxyl nor the amino groups of leucine are significantly titratable in this range (the pK values are 2.36 and 9.6). Therefore, the dependence of inhibition on pH is due probably to titration of some ionizable group or groups on the enzyme. Figure 3B is a plot of the fraction of enzyme activity in the presence of 2×10^{-4} M leucine as a function of pH. The data indicate a pK of about 7.5 for inhibition.

TABLE III: The Effect of Leucine-Like Compounds on α-Isopropylmalate Synthetase Activity.

Compound Added (10^{-2} M)	mμmoles of α-KIV Disappearance in 3.5 min, pH 7.5[a]	5.5 min, pH 6.5[a]
None	38	43
L-Leucine	0	0
DL-Fluoroleucine	3	0
DL-Azaleucine	36	13
L-Isoleucine	36	16
L-Phenylalanine	37	32
L-Valine	36	35
Cycloleucine	42	43
L-Threonine	38	43
L-Histidine	40	43

[a] The values presented are averages of duplicate determinations.

The fraction of synthetase activity inhibited as a function of leucine concentration is plotted in Figure 4. The concentration dependence of inhibition increases with pH and is complete at high concentrations of leucine. No significant deviations from a direct concentration dependence were noted at low concentrations of leucine.

Figure 5 contains Lineweaver-Burk plots of the reaction velocity as a function of substrate concentration at several inhibitor concentrations. The kinetics of leucine inhibition as a function of acetyl CoA concentration, at pH 7.5, were competitive (in the sense that

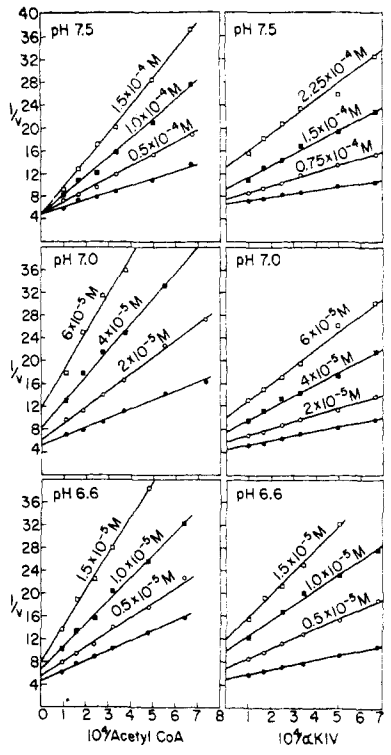

FIGURE 5: Lineweaver–Burk plots of the reaction velocity of α-isopropylmalate synthetase as a function of substrate concentration at several concentrations of L-leucine. The concentration of L-leucine employed for each set of determinations is presented above the corresponding line. The α-KIV concentration employed was 0.5 μmole/reaction mixture for those determinations involving varying acetyl CoA concentrations, and 0.125 μmole of acetyl CoA was used when α-KIV concentrations were varied.

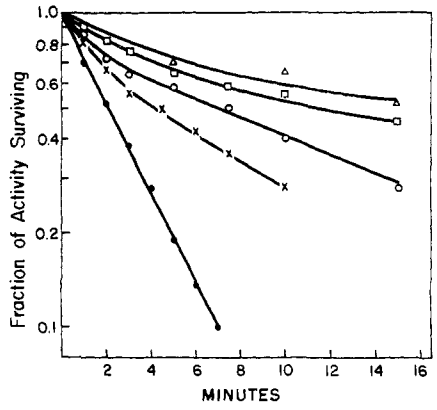

FIGURE 6: The effect of L-leucine on the thermal stability of α-isopropylmalate synthetase at 48° at pH 6.5. The fraction of activity surviving is presented as a function of time in the absence of leucine (●), and in the presence of 5×10^{-5} M (×), 1×10^{-4} M (○), 2×10^{-4} M (□), and 1×10^{-3} M (△) L-leucine.

V_{max} was unaffected by inhibitor concentration). Below pH 7.5, mixed inhibition kinetics (Dixon and Webb, 1964) were obtained for varying concentrations of acetyl CoA. Mixed inhibition kinetics were obtained for leucine as a function of varying α-KIV concentrations at all pH values studied. The properties of leucine inhibition kinetics were the same regardless of the state of purity of the enzyme.

The complexity of the inhibition of the synthetase by leucine is indicated also by the fact that the values of K_i calculated either from the slopes or the intercepts of the inhibition patterns of Figure 5 decrease with increasing inhibitor concentration. This suggests the involvement of two or more cooperatively interacting inhibitor binding sites per molecule of enzyme, as has been suggested for the inhibition of threonine deaminase by isoleucine (Changeux, 1963; Monod *et al.*, 1963).

In order to determine whether more than one molecule of leucine was bound per molecule of enzyme, the data in Figure 4 were replotted according to Hill's empirical equation as described in Monod *et al.* (1963). Straight lines, each with a slope of 1.51, were obtained at the three pH values studied. Ideally, the Hill plot should yield lines the slopes of which are related to the degree of interaction of inhibitor binding sites. Complete interaction of binding sites should yield lines with slopes equal to the number of sites involved. Unfortunately, the slope obtained permits only the conclusion that leucine inhibition involves the participation of more than one binding site, but the relation between bound inhibitor and functional enzyme is not straightforward. It is important to note that a similar analysis of substrate binding yields lines with a slope of 1.0. This suggests that only one kind of binding site is involved for each of the substrates, with little or no interaction between them.

The kinetic data in Figure 5 illustrate yet another property of the synthetase and its relation to end product inhibition. The K_m for acetyl CoA is 3.50×10^{-5} M at pH 6.6, 2.80×10^{-5} M at pH 7.0, and 2.45×10^{-5} M at pH 7.5. The change in K_m values as a function of pH indicates that the binding of acetyl CoA is more favorable for catalysis under conditions in which sensitivity to leucine inhibition diminishes. The observation that the leucine-resistant synthetase obtained from FLR_{92} appears to bind acetyl CoA somewhat better than the normal enzyme (the K_m at pH 7.5 is 2.0×10^{-5} M) also is indicative of an inverse relationship between the affinity of the enzyme for acetyl CoA and inhibition by leucine. The K_m values for α-KIV of the leucine-resistant enzyme and the normal enzyme are the same, 1.0×10^{-5} M at pH 7.5.

Heat Inactivation. The interaction of inhibitor and synthetase is characterized further by an increased resistance of the enzyme to thermal inactivation. As illustrated in Figure 6, the thermal stability of the enzyme at pH 6.5 increases as a function of leucine concentration. As indicated in Figures 6 and 7, the kinetics of thermal inactivation of the enzyme in the absence of leucine are first order when measured at 48° or higher.

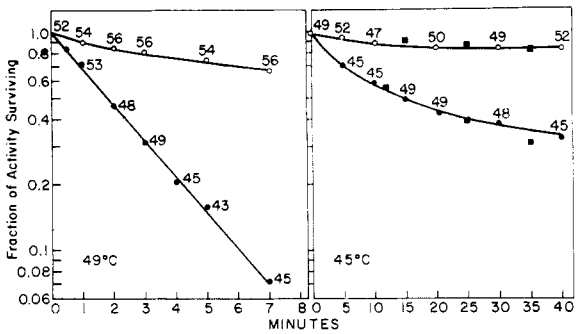

FIGURE 7: Thermal inactivation of α-isopropylmalate synthetase at 49 and 45° at pH 6.5 in the presence and absence of L-leucine. The fraction of activity surviving is presented as a function of time in the absence of leucine (●) and in the presence of 2×10^{-4} M L-leucine (○) with rapid cooling, and as measured at 30° immediately after heating (■). The numbers refer to the per cent inhibition by 2×10^{-4} M L-leucine displayed by the surviving enzyme at each interval.

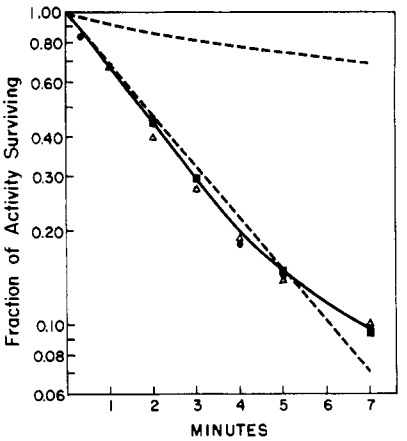

FIGURE 8: The kinetics of thermal inactivation of the leucine-insensitive α-isopropylmalate synthetase at 49°, pH 6.5. The solid line represents the kinetics of thermal inactivation of the leucine-insensitive enzyme in the absence (●) of leucine and in the presence of 2×10^{-4} M (△) and 8×10^{-3} M (■) leucine. The interrupted lines represent the kinetics of thermal inactivation of the normal enzyme in the absence of L-leucine (lower line) and in the presence of 2×10^{-4} M L-leucine (the data are from Figure 7). The protein concentration of both the normal and leucine-insensitive enzyme was about 1.2 mg/ml.

Inactivation of the enzyme in the presence of leucine, or at 48° or less in the absence of leucine, yields more complex curves, suggestive of reversible transitional states. However, little or no difference in survival of enzyme activity can be observed between samples assayed immediately after heating and samples cooled to 0° before assay. It seems improbable then that renaturation of the enzyme upon cooling after subcritical heating could have led to the unusual kinetics observed. As indicated in Figure 7, at each interval the enzyme that survives heating in either the presence or absence of leucine is as sensitive as the original preparation to leucine inhibition. It also should be noted that the leucine sensitivity of the normal enzyme has never been observed to disappear without concomitant loss of enzymatic activity. Extensive purification, freezing and thawing, exposure to high salt concentrations, etc., have failed to separate the two properties.

That increased thermal stability is imparted by leucine bound to the inhibitor site or sites is suggested further by the observation that the leucine-insensitive enzyme obtained from the fluoroleucine-resistant strain FLR_{92} is also insensitive to thermal stabilization by leucine. A plot of the results of a typical thermal inactivation experiment is shown in Figure 8. Little protection can be observed at concentrations up to 8×10^{-3} M L-leucine. The inactivation curve obtained is complex at low survival of activity which again suggests the possible involvement of some transitional conformation during heat inactivation.

As indicated previously, the efficiency of inhibition of the synthetase by leucine decreases as a function of increased pH. In a strikingly similar way leucine protection of the synthetase from thermal inactivation also decreases as a function of increased pH. Figure 9A contains the results of thermal inactivation experiments carried out at pH 6.5, 7.5, and 8.3 in the presence and absence of 2×10^{-4} M L-leucine. The protection afforded by leucine is greatest at pH 6.5, less at pH 7.5, and entirely lost at pH 8.3. Inactivation was carried out at 45° at pH 8.3 instead of 48° because the inactivation rate at 48° was too rapid to be measured accurately.

Some aspects of the specificity of the protection of the synthetase from heat inactivation at pH 6.5 are illustrated in Figure 9B by the behavior of several leucine-like compounds. At 1×10^{-2} M, a concentration ten times higher than required for maximal protection by leucine, only L-isoleucine and L-phenylalanine were reasonably effective and DL-azaleucine only slightly effective. DL-Fluoroleucine was found to be as effective as leucine in protecting the enzyme. Cycloleucine, L-threonine, and L-histidine had no stabilizing effect. Therefore, only those compounds in Table III which inhibited the enzyme at pH 6.5 were able to protect the synthetase from thermal inactivation at comparable concentrations. Valine, however, behaved exceptionally, for it had no demonstrable effect on the thermal stability of the enzyme at this pH, but inhibited activity slightly at 1×10^{-2} M. It should be noted that the leucine-insensitive synthetase from strain FLR_{92} is neither inhibited nor protected from heat inactivation at pH 6.5 by 1×10^{-2} M concentrations of any compound in Table III.

Sucrose density gradient centrifugation of the synthetase in the presence and absence of leucine was performed in order to determine whether aggregation or

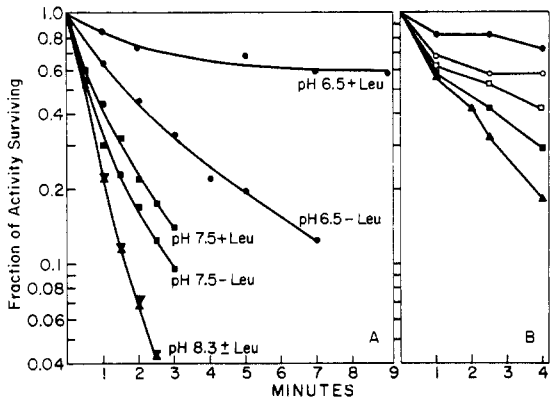

FIGURE 9: The pH dependence of leucine protection of α-isopropylmalate synthetase from thermal inactivation. In Figure 9A the synthetase activity surviving is plotted as a function of time at 48° at pH 6.5 and 7.5 and at 45° at pH 8.3 in the presence and absence of 2×10^{-4} M L-leucine. Figure 9B is a plot of the synthetase activity surviving as a function of time at pH 6.5 in the presence of the following compounds at 10^{-2} M: leucine (●), isoleucine (○), phenylalanine (□), DL-4-azaleucine (■), and in the absence of any addition (▲). Because of the ultraviolet absorption of phenylalanine, the production of free CoA was measured after 4-min incubations using the CNPD assay procedure.

dissociation of protein subunits was involved in the leucine inhibition and thermal stabilization interactions. Samples of the normal synthetase isolated and purified in either the presence of phosphate–leucine buffer or in phosphate buffer only were freed completely of leucine by passage through Sephadex G-50. The sedimentation velocity of such leucine-free preparations of the synthetase was compared with that of enzyme prepared in the usual manner by centrifugation in sucrose density gradients with and without leucine present. The sedimentation velocity of the leucine-resistant enzyme was also determined in sucrose density gradients with and without leucine. The $s_{20,w}$ values presented in Table IV were calculated according to the method of Martin and Ames (1961) using bovine hemoglobin with an $s_{20,w}$ of 4.45 (Tanford and Kirshner, 1964) as an internal standard in all runs. No significant deviations from the calculated $s_{20,w}$ of 6.68 ± 0.50 of the normal synthetase in the presence of leucine were observed under the various conditions tried. The sedimentation velocity of the normal enzyme with and without leucine present was also measured by analytical ultracentrifugation and found to be the same. Furthermore, the enzyme that survived thermal inactivation at 45° for 50 min (40% survival) had the same sedimentation coefficient as the normal enzyme. Hence neither leucine inhibition nor the complex nature of thermal inactivation of the synthetase seems to involve subunit aggregation or dissociation.

TABLE IV: Sucrose Density Gradient Centrifugation Determinations of Sedimentation Coefficients of Normal and Leucine-Insensitive Synthetase in the Presence and Absence of Leucine.[a]

	Average $s_{20,w}$ (S)	No. of Determn
Normal synthetase plus leucine	6.68 ± 0.50	10
Normal synthetase without leucine	6.42 ± 0.26	4
Heated normal synthetase without leucine	6.40 ± 0.11	2
FLR_{92} synthetase without leucine	6.40 ± 0.24	2
FLR_{92} synthetase with leucine	6.55 ± 0.50	2

[a] Centrifugation was carried out in sucrose gradients (5–20%) made up in 0.1 M phosphate buffer, pH 6.5, with 2×10^{-4} M leucine where indicated. The sample of heated synthetase was obtained after 50 min at 45° in the absence of leucine (40% of the initial activity survived).

Discussion

α-Isopropylmalate synthetase is the key enzyme in the regulation of leucine biosynthesis in *Neurospora*. As has been shown here, the catalytic function of the synthetase is controlled by leucine. Thus in this pathway as in most others studied in a variety of organisms (Umbarger, 1961), the end product of a biosynthetic sequence controls its own synthesis by regulating the rate of the first reaction unique to that pathway. In addition to this, however, the catalytic activity of the synthetase in *Neurospora* has a regulatory function on the rate of synthesis of the enzymes subsequent to it in the pathway (S. R. Gross, in preparation). The structures of the three leucine biosynthetic enzymes are determined by four cistrons widely distributed throughout the *Neurospora* genome (Gross, 1962). Although the rate of synthesis of each of the enzymes can be shown to be dependent upon leucine concentration, only the synthesis of the synthetase is controlled directly by a leucine-concentration-dependent repression mechanism. The product of the synthetase reaction, α-isopropylmalate, however, is an inducer of synthesis of the two enzymes converting it to α-ketoisocaproate, the keto acid analog of leucine (S. R. Gross, in preparation). Thus, the sensitivity of the *Neurospora* synthetase to leucine inhibition has three important related physiological roles, the regulation of leucine biosynthesis by end product inhibition, the regulation of the synthesis of other enzymes of the pathway as a function of α-isopropylmalate synthesis, and the control of its own rate of synthesis by the regulation of the amount of a repressor substance produced.

Like the condensing enzyme of the citric acid cycle (Stern *et al.*, 1951), α-isopropylmalate synthetase catalyzes the condensation of an α-keto acid with acetyl

CoA. In this case the physiological products are α-isopropylmalate and CoA. The specificity of the enzyme for α-KIV is not absolute. Pyruvate and α-ketobutyrate can be substituted for α-KIV, but have K_m values that are from 200 to 600 times greater than that of the K_m of α-KIV. The observation that the V_{max} of the reaction involving α-ketoisobutyrate is actually higher than the V_{max} of the same enzyme preparation for α-KIV suggests strongly that the major contributing factor in substrate specificity is substrate binding site specificity rather than catalytic specificity.

The synthetase performs its enzymatic function in a straightforward manner, in the sense that the steady-state kinetics of the reaction catalyzed in the absence of inhibitor do not suggest the involvement of binding-site and/or catalytic-site interactions of the sort that would be manifest in changes of V_{max} or K_m as a function of substrate concentration. The kinetics of enzyme inhibition by leucine, however, are unusual in several respects. First, the kinetics of inhibition apparently undergo a drastic change in form as a function of pH. At pH 7.5, leucine seems only to affect the K_m of acetyl CoA because the inhibition of the reaction by leucine as a function of acetyl CoA concentration is of the competitive type. At lower pH values the inhibition is of the mixed variety suggestive of an effect on the catalytic properties of the enzyme in addition to an effect on substrate binding.

Second, acetyl CoA, the substrate with little or no structural resemblance to leucine, is the effective one in reversing the inhibition. α-KIV reverses leucine inhibition only slightly, if at all.

Third, it was shown that the pH dependence of leucine inhibition is different from the pH dependence of the catalytic activity of the enzyme. The titration of leucine inhibition as a function of pH indicated the involvement in inhibition of an ionizable group or groups on the protein with a pK of about 7.5. The pK of this group differed by at least 0.5 pH unit from the apparent pK of the catalytic rate-determining ionizable groups of the protein. From this, as well as considerations of the structural differences between substrate and inhibitor in addition to the kinetics of inhibition, it appears that leucine may act not by altering the structure of the substrate binding site *per se* but instead by determining whether or not acetyl CoA can reversibly get to its binding site on the protein.

All of the data presented thus far are most easily interpreted to suggest that the enzyme has different nonoverlapping binding sites for substrate and inhibitor. This interpretation is supported by the observation that the substrate binding constants of the leucine-resistant synthetase obtained from FLR_{92} are virtually the same as that of the normal enzyme (if anything the K_m of the mutant enzyme for acetyl CoA is slightly more favorable for catalysis). One might have expected a large effect on substrate binding of an amino acid substitution that leads to resistance to inhibition if the inhibition observed were due to the involvement of identical or extensively overlapping substrate and inhibitor binding sites. Thus, the synthetase seems to fall into the general class of allosteric (Monod *et al.*, 1963) proteins which have sterically different but catalytically interrelated substrate and inhibitor binding sites which can undergo reversible conformational alterations in response to binding of substrate and/or inhibitor.

Because the synthetase is a rather large protein with an $s_{20,w}$ of 6.9, has a molecular weight of about 143,000, and is a multimer consisting probably of three identical polypeptide subunits (Webster *et al.*, 1965) and because of the analogous behavior of other enzymes which undergo allosteric transitions (Datta and Gest, 1964; Tomkins *et al.*, 1963), one might expect dissociation or association interactions between subunits to account for some of the unusual kinetic properties of leucine inhibition. However, no such interactions were observed, as determined by either sucrose density gradient centrifugation or sedimentation velocity in the presence or absence of inhibitor.

However, evidence suggestive of a reversible conformational alteration upon occupation of the inhibitor binding site was obtained when it was observed that leucine appreciably protected the enzyme from thermal inactivation. Leucine thermal protection of the protein is effective under conditions most favorable for inhibition. In addition, the specificity of thermal protection is the same as the specificity of inhibition (only those compounds protect that inhibit activity). The correlation between protection and the ability to occupy inhibitor binding sites is supported also by the observation that the leucine-resistant enzyme obtained from FLR_{92} is not protected appreciably in the presence of leucine or any other leucine-like compound which inhibited the normal enzyme and is as sensitive to thermal inactivation in the absence of leucine as is the normal enzyme.

A demonstration of the involvement of a conformational transition upon occupancy of the inhibitor binding site will have to await direct physical analysis. However, an estimate of the extent of the conformational alteration involved may be obtained from a calculation of the difference in activation energy of thermal inactivation of the synthetase in the presence and absence of leucine. The activation energy of inactivation calculated from the data presented is about 65,000 kcal/mole in the absence of inhibitor and 102,000 kcal/mole in the presence of leucine. Thus, the enzyme seems to be held together much more tightly in the presence of inhibitor than in its absence. Whether a few highly energetic bonds are involved rather than a relatively large number of hydrophobic and/or hydrogen bonds cannot be determined.

Although the involvement of an "allosteric transition" seems common in end product inhibition, several features of the synthetase are worth special emphasis. Perhaps the most important of these is the fact that the reaction catalyzed by the synthetase is relatively uncomplicated and describable in terms of straightforward Michaelis–Menten kinetics. Furthermore, although the enzyme is probably made up of three identical sub-

units, the allosteric interactions observed do not seem to involve multimer–monomer transitions.

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