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Role of the *leu-3* Cistron in the Regulation of the Synthesis of Isoleucine and Valine Biosynthetic Enzymes of *Neurospora*

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The production by *Neurospora* of the enzymes of isoleucine and valine synthesis in response to specific end product-derived signals depends upon the presence of an effective *leu-3* regulatory product and its effector α-isopropylmalate (α-IPM). In *leu-3*$^+$ strains, threonine deaminase production is repressed as a function of available isoleucine, acetohydroxy acid synthetase as a function of valine, and the isomeroreductase and dihydroxy acid dehydratase as a function of isoleucine and leucine. In the absence of an effective *leu-3* regulatory product, α-isopropylmalate, or both, the production of isoleucine and valine biosynthetic enzymes is fixed at or near fully repressed levels even under conditions of severe end product limitation. Thus, in addition to its involvement in the regulation of expression of the three structural genes of leucine synthesis, the *leu-3* α-IPM regulatory product is necessary for full expression of at least four genes specifying the structure of the enzymes of isoleucine and valine synthesis. It is suggested that the *leu-3* α-IPM regulatory element may facilitate transcription of the genetically dispersed cistrons either by imposing specificity on ribonucleic acid polymerase for structurally similar promoters adjacent to each of the cistrons or by "opening" promoters after interaction with nearly identical stretches of deoxyribonucleic acid near each of the structural genes.

The product of the *leu-3* cistron as well as the positive effector α-isopropylmalate (α-IPM) are required for expression of the *leu-1* and *leu-2* cistrons of *Neurospora* in the synthesis of β-isopropylmalate dehydrogenase (2-hydroxy-4-methyl-3-carboxy-valerate: NAD$^+$ oxidoreductase EC 1.1.1.85) and isopropylmalate isomerase (2-hydroxy-4-methyl-3-carboxy-valerate hydro-lyase EC 4.2.1.33) (11, 26). In addition, the *leu-3* product is required to attain maximum derepression of the first enzyme of the leucine pathway, α-IPM synthetase [3-hydroxy-4-methyl-3-carboxyvalerate 2-oxo-3-methylbutyrate-lyase (CoA-acetylating) EC 4.1.3.12] (specified by the *leu-4* cistron). The involvement of the *leu-3* product in the regulation of expression of each of the *leu* cistrons suggested that it might play a coordinating regulatory role governing leucine biosynthesis from its earliest specific precursors. Since α-ketoisovalerate is both the immediate precursor of valine and of α-IPM, the first intermediate of the leucine pathway, the analysis of the regulatory function of the *leu-3* product was extended to a study of its role in the synthesis of the enzymes of the isoleucine and valine pathway.

There are four enzymes in *Neurospora* involved specifically in isoleucine and valine synthesis. Three of these catalyze reactions common to the synthesis of both amino acids and the fourth, threonine deaminase (L-threonine hydro-lyase EC 4.2.1.16) generates α-ketobutyrate, the first precursor of isoleucine from threonine. The gene specifying the structure of threonine deaminase has not yet been identified but the structural genes of the three enzymes of the common pathway have been mapped. The structural gene for acetohydroxy acid synthetase (acetolactate pyruvate-lyase EC 4.1.3.18), the first enzyme of the common pathway, *ilv-3* (by common agreement, *iv*, the previous designation for the genes of the isoleucine and valine pathway of *Neurospora* has been changed to *ilv* to conform with general microbial genetic notation), is on linkage group IV and *ilv-2* and *ilv-1* which specify the isomeroreductase (2,3-dihydroxyisovalerate: NADP$^+$ oxidoreductase EC 1.1.1.89) and dihydroxy acid dehydratase (2,3-dihydroxy acid hydrolyase EC 4.2.1.9), the second and third enzymes of the pathway, are within five units of each other on linkage group V (29). These three enzymes, though specified by nuclear genes and presumably synthesized on cytoplasmic ribosomes, are found localized in mitochondria (18). This system, then, may eventually afford

the opportunity to study the relationship of localization to regulation and catalysis.

In this paper we describe some properties of the regulatory system governing formation of the isoleucine and valine biosynthetic enzymes, its relation to regulation of the enzymes of the leucine pathway, and the requirement of the *leu-3* product and its positive effector α-IPM for maximum isoleucine valine enzyme production.

MATERIALS AND METHODS

Strains. The isoleucine- and valine-requiring (*ilv*) mutants used in these studies were obtained from the Fungal Genetics Stock Center, Arcata, Calif. Before use they were made reasonably isogenic to the leucine-requiring strains by several successive crosses to *leu* mutants until heterokaryotic compatibility was obtained.

Preparation of extracts. Mycelia were usually obtained after growth at 34 C for 20 to 24 h in Fernbach flasks containing 1 liter of synthetic medium. Growth on nonlimiting medium was initiated by inoculation with 1 ml of a conidial suspension (optical density at 550 nm [OD_{550}] = 1.0). If the growth rate was to be reduced by limitation of an auxotrophic requirement, 2 to 4 ml of the conidial suspension was used as the inoculum to assure equivalent mycelial yields after 24 h of incubation.

Reproducible procedures for obtaining high yields of structurally intact mitochondria were difficult to apply to the large number of repetitive preparations required for these studies. Indeed, it seemed preferable, in those cases where the assay procedure permitted, to measure the totality of enzyme produced and avoid, for the present, the difficult question of the efficiency of enzyme localization at different levels of enzyme production. However, comparisons of the specific activities of the isomeroreductase and dihydroxy acid dehydratase in mitochondria and whole mycelial extracts from which they were obtained indicated reasonable concordance between the two over a wide range of enzyme production levels. The specific activity of threonine deaminase in mitochondrial preparations, on the other hand, was consistently found to be so much lower than that of whole mycelial extracts as to indicate that most, if not all, of the enzyme is in the cytosol. Threonine deaminase, isomeroreductase, and dihydroxy acid dehydratase, therefore, were measured after ammonium sulfate precipitation from mycelial extracts obtained by homogenization and sonic oscillation according to the method of Gross (11). The extraction buffers used were 0.05 M Tris buffer [tris(hydroxymethyl)aminomethane-hydrochloride], pH 8.2, containing 0.01 M $MgSO_4$ for threonine deaminase and dihydroxy acid dehydratase, and 0.05 M Tris, pH 7.5, containing 0.01 M $MgSO_4$ and 5×10^{-4} dithiothreitol for the isomeroreductase (15, 16). The specific activity of threonine deaminase was determined in the protein that precipitated between 0 and 80% ammonium sulfate, and the isomeroreductase and dihydroxy acid dehydratase were measured in the fraction that precipitated between 60 and 80%.

The efficiency of extraction of each of the enzymes by the sonic disruption procedure was determined by extracting with 0.3% sodium deoxycholate samples of supernatant and sedimented material after 27,000 × *g* centrifugation of disrupted STD8A mycelia. One-hundred percent of threonine deaminase activity, 80% of the dihydroxy acid dehydratase, and only 40% of the isomeroreductase were found solubilized by sonic disruption. However, use of detergent in the extraction of the isomeroreductase was rejected because two-thirds of the activity was lost and the specific activity halved after sodium deoxycholate treatment.

The specific activity of acetohydroxy acid synthetase in whole mycelial extracts is generally low and excessive nonspecific acetoin production limits the accuracy of the enzyme assay. Mitochondrial preparations, however, are relatively free of interfering acetoin synthesis and apparently contain most of the cell's acetohydroxy acid synthetase. The production of this enzyme, then, was measured in mitochondria prepared by a procedure derived from Caroline et al. (6). Mycelia were washed with 0.1 M Tris buffer, pH 7.6, in 0.1 M sucrose, then ground in a cold mortar with twice the wet weight of both acid-washed sand and 0.15% bovine serum albumin in 0.25 M sucrose. The suspension was centrifuged twice at 1,500 × *g* for 15 min to remove sand and cell debris. The supernatant was centrifuged at 12,000 × *g* for 30 min and the reddish-brown mitochondrial pellet was extracted for 10 min with 0.015 M Tris buffer, pH 8.5, containing 0.225% sodium deoxycholate, 25% (vol/vol) glycerol, and 1 mM Cleland's reagent. Centrifugation of this suspension at 37,000 × *g* for 15 min produced the supernatant extract used to assay acetohydroxy acid synthetase activity. Sodium deoxycholate extraction of mitochondria was found to be more efficient than sonic disruption in solubilizing protein and yielding acetohydroxy acid synthetase preparations of higher specific activity.

Isomeroreductase and dihydroxy acid dehydratase are stable when stored as ammonium sulfate precipitates at −20 C. Threonine deaminase is unstable and, consequently, was assayed immediately after preparation. Acetohydroxy acid synthetase prepared from detergent-extracted mitochondria was also assayed immediately.

Enzyme assays. Threonine deaminase activity was measured in 0 to 80% ammonium sulfate precipitates dissolved in 0.05 M Tris, pH 8.3, containing 10% ammonium sulfate. Activity was measured in a 2-ml reaction mixture containing 80 μmol of L-threonine, 0.04 μmol of pyridoxal-5′ monophosphate, and 9.1 ml of enzyme extract in 0.05 M Tris buffer, pH 9.0. The reaction was terminated by the addition of 3 ml of 0.025% 2,4-dinitrophenylhydrazine in 0.3 N HCl. The α-ketobutyrate produced was measured as the 2,4-dinitrophenylhydrazine at 445 nm according to the method of Friedemann and Haugen (9). The formation of 1 nmol of α-ketobutyrate under these assay conditions yields a ΔOD_{445} of 0.00377.

Acetohydroxy acid synthetase activity was measured in a 1-ml reaction mixture containing 160 μg of thiamine pyrophosphate, 5 μmol of $MgSO_4$, 20 μmol

of pyruvate, and 0.1 ml of enzyme extract in 0.1 M phosphate buffer, pH 7.6. Reactions were terminated by the addition of 0.1 ml of 6 N H_2SO_4 and were then heated at 60 C for 15 min to convert the acetolactate formed to acetoin (21). Acetoin was determined according to the procedure of Westerfield (31). Where required, acetoin produced by α-acetolactate decarboxylase was determined by stopping reactions with 0.1 ml of 2.5 N NaOH. The formation of 1 μmol of acetoin under these assay conditions yields a ΔOD_{530} of 0.00468.

It should be pointed out that in addition to the pH 7.5 biosynthetic acetohydroxy acid synthetase there is a second, non-biosynthetic, pH 6.0 α-acetolactate synthesizing activity present in the cytosol fraction. This pH 6.0 α-acetolactate synthesizing activity is associated with the pyruvate dehydrogenase complex (12, 17). Mutants lacking the pH 7.5 biosynthetic activity require isoleucine and valine although the pH 6.0 activity is still present (6).

Isomeroreductase activity was measured by the procedure of Kiritani et al. (16) in 60 to 80% ammonium sulfate precipitates dissolved in 0.05 M Tris buffer, pH 7.5, containing 0.01 M $MgSO_4$. Dihydroxy acid dehydratase activity was measured in 60 to 80% ammonium sulfate precipitates dissolved in 0.05 M Tris buffer, pH 8.2, containing 0.01 M $MgSO_4$. The 1.0-ml reaction mixture employed contained 10 nmol of α,β-dihydroxyisovalerate and 0.1 ml of enzyme extract in 0.05 M Tris buffer, pH 7.9 (3). α-Ketoisovalerate formed was measured by the method of Friedemann and Haugen (9).

There is some question as to whether the dihydroxy acid dehydratase activity measured is that of a single molecular species. The possibility that there might be two species of dihydroxy acid dehydratase in *Neurospora* has been suspected because 35 to 40% of the dihydroxy acid dehydratase activity is consistently found in the cytosol no matter how much care is taken to prevent mitochondrial disruption and all known *ilv-1* mutants produce some active enzyme (2, 3). However, a comparison of the enzymes purified from mitochondrial and cytosol fractions by Altmiller and Wagner (2) showed that both are indistinguishable with regard to molecular weight (135,000 to 155,000), acrylamide gel electrophoretic mobility and antigenicity. The enzymes from both sources have identical substrate binding constants. More importantly, however, mutations in the *ilv-1* cistron alters equivalently the enzymes of mitochondrial and cytosol origin with respect to thermal sensitivity, pH dependence and kinetics (1) and, as pointed out above, the mitochondrial and cytosol enzyme activities vary concordantly over a wide range of production levels. It seems reasonable to conclude, then, that at the very least the cytosol and mitochondrial enzymes have a common polypeptide specified by *ilv-1*.

Specific activity units for all of the enzymes are expressed as nanomoles of product formed per milligram of protein per minute. Each determination of the specific activity of threonine deaminase was obtained from at least three different assays of the formation of α-ketobutyrate as a function of protein concentration. The specific activities for the other enzymes were determined by at least two independent trials.

Assays of threonine deaminase, dihydroxy acid dehydratase, and isomeroreductase were performed at 34 C, and the assay of acetohydroxy acid synthetase was performed at 30 C. Protein was determined either by the method of Warburg and Christian (30) or that of Lowry et al. (20).

α,β-Dihydroxyisovalerate, α-acetolactate (as the acetylated ethyl ester), 5',5',5'-trifluoroleucine, and thiaisoleucine-hydrochloride were products of Reef Laboratory. All other chemicals were from standard commercial sources.

RESULTS

Isoleucine and valine biosynthetic enzymes production by the wild type. As indicated in Table 1 production of all of the isoleucine and valine biosynthetic enzymes is noncoordinately repressed (the term repressed is used here merely as a synonym for reduced without connotation of mechanism) when an excess of the three relevant amino acids, isoleucine, valine,

TABLE 1. *Effect of growth in presence of branched-chain amino acids on levels of isoleucine and valine biosynthetic enzymes production by wild-type STD8A*

Supplement to minimal medium	Sp act			
	Threonine deaminase	Acetohydroxy acid synthetase	Isomeroreductase	Dihydroxy acid dehydratase
None	262 ± 16 (7)[a]	16 ± 0.8 (4)	34 ± 1 (10)	114 ± 10 (13)
2.3 mM Leu + Ile + Val[b]	82 ± 14 (8)	3 ± 0.9 (3)	17 ± 3 (11)	71 ± 8 (10)
2.3 mM Ile + Val	86	4	33	95
2.3 mM Leu + Val	191		30	83
2.3 mM Ile + Leu	61		16	70
2.3 mM Val	183	5	45	113
2.3 mM Leu	196	17	32	86
2.3 mM Ile	72	31 ± 4 (4)	36	88

[a] Except those for which standard deviations and number of independent determinations (in parentheses) are provided all specific activities expressed are the average of at least two independent determinations.

[b] Leu, Leucine; Ile, isoleucine; Val, valine.

and leucine are supplied during growth of wild-type *Neurospora* (STD8A). Production of each of the enzymes, however, responds differentially to specific amino acid supplementation. Isoleucine, the feedback inhibitor of threonine deaminase (see below), is very effective in repressing threonine deaminase production, whereas leucine or valine is relatively ineffective. On the other hand, acetohydroxy acid synthetase production is repressed by valine, the feedback inhibitor of this enzyme. Isoleucine, surprisingly, stimulates acetohydroxy acid synthetase production, an effect which appears to be completely reversed specifically by valine.

Isomeroreductase and dihydroxy acid dehydratase production are repressed more effectively by combined leucine and isoleucine supplementation. The dehydratase, however, appears somewhat more resistant to repression than the other enzymes. Threonine has been found to be ineffective in repression of threonine deaminase or any of the other isoleucine and valine biosynthetic enzymes.

It appears then, that the production of threonine deaminase in *Neurospora* is not under multivalent control as it is in *Salmonella* and *Escherichia coli* (8). Instead, its production, as well as that of acetohydroxy acid synthetase, is regulated, at least in part, by the specific feedback regulators of enzyme activity.

Threonine deaminase and acetohydroxy acid synthetase, catalysis, and feedback inhibition. Since there appears to be no published description of the properties of the *Neurospora* threonine deaminase, we record here some of the properties of the enzyme relevant to these investigations. The *Neurospora* threonine deaminase is very unstable even after precipitation by ammonium sulfate (30% of its activity is lost per day at −20 C). The enzyme is not stabilized by extraction in the presence of 10^{-4} M L-isoleucine but is stabilized somewhat by ammonium sulfate (40 mM), a property which might be related to the reported activation by ammonium of the enzyme from *Saccharomyces cerevisiae* (13). The specific activity of the enzyme is strongly dependent on protein concentration, falling precipitously at concentrations below 0.1 mg per ml. Consequently, the specific activity of the enzyme must be determined from the slope of activity as a function of protein concentration significantly greater than 0.1 mg per ml. The enzyme has a broad pH optimum between pH 9.0 and 10.0 and is equally sensitive to inhibition (97%) by isoleucine (2.5 mM) over the entire pH range tested (pH 7.1 to 10.6). Valine (2.5 mM) has no significant effect on enzyme activity as a function of pH.

Lineweaver-Burke plots show a marked curvature upwards at low substrate concentrations indicating substrate cooperativity. This curvature is reduced in the presence of 5 mM L-valine. K_m estimates from the linear portions of reciprocal plots, i.e., at high threonine concentrations, yield values of approximately 4 mM either in the absence or presence of the effector L-valine. The Hill coefficient of L-threonine is 1.4 (1.3 in the presence of 4 mM L-valine) which indicates the probable involvement of interacting binding sites on the enzyme (7). The concentration of L-isoleucine necessary for half-maximal inhibition of the enzyme is 1.1×10^{-4} M. The Hill coefficient for L-isoleucine, in the presence of a saturating concentration of L-threonine, is 3.9. Of the compounds tried so far only isoleucine and thiaisoleucine (17% inhibition at 2×10^{-3}M) inhibit the enzyme significantly.

Acetohydroxy acid synthetase, as described by Glatzer et al. (10), is inhibited most effectively by valine. However, inhibition is not complete even at high valine concentrations. As indicated in Table 2, about 30% of the enzyme activity is resistant to inhibition whether produced at the level characteristic of wild type grown in minimal medium, at the somewhat higher level obtained when grown in the presence of isoleucine, or at the very high production level characteristic of the *leu-1* mutant grown on limiting leucine. The valine-resistant fraction, however, appears to be considerably larger at very low levels of enzyme production by the wild type or *leu-3* grown in the presence of excess branched-chain amino acids. In addition to valine, which inhibits maximally at less than 0.1 mM, about 60% of acetohydroxy acid synthetase activity is inhibited by α-aminobutyrate, isoleucine and phenylalanine at a concentration of 2 mM; at the same concentration, about 20 to 30% of the activity is inhibited by leucine, threonine, and alanine. The same enzyme species seems sensitive to all of the compounds, for various combinations of valine with isoleucine, leucine, and phenylalanine do not inhibit more effectively than valine alone.

The valine-sensitive acetohydroxy acid synthetase activity is lost within 2 to 4 min of incubation in the absence of substrate at 35 C, a loss coincident with inactivation of 70% of catalytic activity. The consistent detection of a heterogeneous response to valine inhibition as well as the differential thermolability suggests that *Neurospora* may produce two species of acetohydroxy acid synthetase, one sensitive and the other insensitive to valine as does *Salmo-*

nella typhimurium and some strains of *E. coli* (5, 23). That valine resistance is not an artifact introduced by extraction is suggested by the fact that the growth of *Neurospora* is not inhibited by valine as is *E. coli* K-12 which produces only a valine-sensitive enzyme (5).

The detection of valine-sensitive and valine-insensitive acetohydroxy acid synthetase activities raises the question whether two different genes and two different regulatory signals are involved in their synthesis. As noted above, however, the data of Table 2 indicate that the fraction of enzyme inhibitable by valine remains constant over a very wide range of production levels and only at very low levels of enzyme production does the inhibitable fraction fall significantly below 0.7. If there are two enzymes, then, their regulation must be coordinate over a fairly wide range of synthesis. In addition, both feedback-sensitive as well as feedback-insensitive activities are virtually eliminated by mutation of the *ilv-3* cistron (6). The simplest conclusion, then, is that either the two forms of acetohydroxy acid synthetase share a common subunit or the enzyme exists normally in two conformational states.

The properties of the solubilized acetohydroxy acid synthetase were found to be virtually the same as those of the mitochondrial-bound enzyme of Glatzer et al. (10) with the following exceptions. Acetohydroxy acid synthetase, immediately after solubilization, is fully as sensitive to valine as when associated with mitochondria. However, valine sensitivity is very labile after solubilization of the enzyme (about 50% of the sensitivity is lost per minute at 35 C in the absence of valine); the Ki for valine is 1.0×10^{-5} M for the solubilized enzyme as compared to 5.1×10^{-4} M reported for the enzyme measured in intact mitochondria; the solubilized enzyme activity is not stimulated by flavine adenine dinucleotide at 10^{-5} M (the acetohydroxy acid synthetase of *S. cerevisiae* also appears to be insensitive to flavine adenine dinucleotide [21]).

Enzyme production by leucine auxotrophs. *Leu-1* (β-IPM dehydrogenase deficient) and *leu-2* (IPM isomerase deficient) mutants accumulate α-IPM which has been implicated as a positive effector in the regulation of IPM isomerase and β-IPM dehydrogenase (11). These two mutants not only produce high levels of the leucine biosynthetic enzymes when grown under conditions of leucine limitation (11, 14, 26), but also produce elevated levels of three out of four of the isoleucine and valine biosynthetic enzymes (Table 3). Acetohydroxy acid synthetase, isomeroreductase, and dihydroxy acid dehydratase production by the mutants are, respectively, 4 times, 1.5 to 3 times, and 1.5 to 2 times higher than that of wild type grown on minimal medium. The synthesis of acetohydroxy acid synthetase varies most dramatically, there being a 20-fold difference between the enzyme level of *leu-1* or *leu-2* grown on limiting leucine and wild type grown on excess branched-chain amino acids (Table 1). Threonine deaminase production, however, is not elevated above the wild-type level. In fact, for reasons not understood, the level of threonine deaminase production by leucine or isoleucine and valine auxotrophs never quite reaches that of the wild type grown on minimal medium (Tables 1, 3, and 4).

Production of threonine deaminase and isomeroreductase is not repressed when either

TABLE 2. *Feedback inhibition of acetohydroxy acid synthetase activity in wild type and various mutants*

Mutant gene (strain)	Supplement to minimal medium	Sp act in absence of inhibitor(s)	Inhibitors (mM)	Inhibition (%)
leu-1 (D221-1-27A)	0.23 mM Leu	81	Val (2)	72
Wild type (STD8A)	2.3 mM Ile	28	Val (2)	77
	None	18	Val (2)	72
			Val (4)	67
			Val(2) + Leu(2)	69
			Val(2) + Ile(2)	68
			Val(2) + Phe(2)[a]	65
			Val(2) + Leu(4)	69
			Val(2) + Ile(4)	73
			Val(2) + Leu(4) + Ile(4)	67
	2.3 mM Leu + Ile + Val	4	Val (2)	29
leu-3 (R156-P1-20A)	0.23 mM Leu	16	Val (2)	68
	2.3 mM Leu + Ile + Val	3	Val (2)	45

[a] Phe, Phenylalanine.

TABLE 3. *Levels of isoleucine and valine biosynthetic enzymes in leucine auxotrophs*

Mutant gene (strain)	Supplement to minimal medium	Sp act			
		Threonine deaminase	Acetohydroxy acid synthetase	Isomeroreductase	Dihydroxy acid dehydratase
leu-1 (D221-1-27A)	0.15 mM Leu[a]	198	70[b]	48	172
	2.3 mM Leu	218	17	46	124
	2.3 mM Leu + Ile + Val	195	12	51	121
leu-2 (R86-18-40A)	0.15 mM Leu	219	68	77	221
	2.3 mM Leu	221	16	98	171
	2.3 mM Leu + Ile + Val	210	11	94	173
leu-3 (R156-P1-20A)	0.15 mM Leu	92	16	17	82
	2.3 mM Leu	98	13	20	75
	2.3 mM Leu + Ile + Val	87	3	16	58
leu-4 (R108-9-5A)	0.15 mM Leu	81	13	15	64
	2.3 mM Leu	96	10	19	78
	2.3 mM Leu + Ile + Val	75	3	20	62
leu-1 leu-2 (R86-20-7A)	0.15 mM Leu	191	87	57	231
	2.3 mM Leu + Ile + Val	197	13	80	135
leu-1 leu-3 (R156-8-1A)	0.15 mM Leu	92	17	16	69
	2.3 mM Leu + Ile + Val		5		
leu-2 leu-3 (R156-6-43A)	0.15 mM Leu	83	14	15	71
	2.3 mM Leu + Ile + Val		2		
leu-3 leu-4 (R203-4-194a)	0.15 mM Leu	177	17	15	68
	2.3 mM Leu + Ile + Val	93	4	18	57
leu-1 leu-4 (R108-10-34a)	0.15 mM Leu[c]	102	15	12	68
	2.3 mM Leu + Ile + Val[c]	95	5	14	63

[a] Conditions of leucine limitation.
[b] Instead of 0.15 mM, the medium was supplemented routinely with 0.23 mM leucine to increase the yield of mycelia used in the isolation of mitochondria for acetohydroxy acid synthetase determinations.
[c] Calcium pantothenate, 50 μg/ml, was added to the growth medium because this strain also contains a *pan-1* mutation.

leu-1 or *leu-2* are grown on excess leucine (2.3 mM) or all of the branched-chain amino acids and the production of dihydroxy acid dehydratase is reduced, at most, to levels slightly higher than that of the wild type grown on minimal medium. Although production of acetohydroxy acid synthetase is most responsive to repression, the minimum level of enzyme observed in *leu-1* and *leu-2* mutants is four times higher than that of the fully repressed wild type.

Leu-4 (α-IPM synthetase deficient) and *leu-3* mutants (R156) produce threonine deaminase, isomeroreductase, and dihydroxy acid dehydratase at levels equivalent to that of fully repressed wild type even when grown under conditions of leucine limitation. Although acetohydroxy acid synthetase production by *leu-3* and *leu-4* when grown under leucine limitation is above the fully repressed level, the amount produced is no higher than that produced by the wild type grown in minimal medium.

A comparison of enzyme production by a *leu-1 leu-2* double mutant with that of the *leu-3* single mutant demonstrates the importance of the *leu-3*$^+$ allele in regulating isoleucine and valine biosynthetic enzyme synthesis. The *leu-1 leu-2* double mutant is physiologically equivalent to the *leu-3* mutant in that it produces little or no IPM isomerase or β-IPM dehydrogenase and accumulates α-IPM (11). However, the *leu-1 leu-2* double mutant, which can produce a normal *leu-3* gene product, produces the isoleucine and valine biosynthetic enzymes at levels equivalent to those of the *leu-1* and *leu-2* single mutants. On the other hand, *leu-1 leu-3*, *leu-2 leu-3*, *leu-1 leu-4*, or *leu-3 leu-4* double mutants,

TABLE 4. *Levels of isoleucine and valine biosynthetic enzymes in trifluoroleucine-resistant mutants*

Mutant gene (strain)	Supplement to minimal medium	Sp act			
		Threonine deaminase	Acetohydroxy acid synthetase	Isomeroreductase	Dihydroxy acid dehydratase
leu-4^{FLR92} (FLR92-2-260A)	None	298	33	85	119
	2.3 mM Leu + Ile + Val	261	17	96	117
leu-4^{FLR92} *leu-1* (FLR92-1-314A) ..	0.15 mM Leu	221	40[a]	93	272
	2.3 mM Leu + Ile + Val	228	23	109	168
leu-4^{FLR92} *leu-3* (R156-10-84A) ...	0.15 mM Leu	71	12	17	77
	2.3 mM Leu + Ile + Val		2		

[a] See footnotes of Table 3.

like the *leu-3* and *leu-4* single mutants, produce the isoleucine-valine at the low levels characteristic of *leu-3* and *leu-4* mutant strains.

The *leu-4*^{FLR92} mutant differs from wild type only in producing feedback-insensitive α-IPM synthetase, and, as a consequence, overproduces leucine and its intermediates (among them the presumed inducer α-IPM) during growth (11). When *leu-4*^{FLR92} is grown on minimal medium the production of acetohydroxy acid synthetase and isomeroreductase is elevated more than twofold over that of wild type grown under the same conditions, whereas the production of threonine deaminase and dihydroxy acid dehydratase is at the wild type level (Table 4). Threonine deaminase, isomeroreductase, and dihydroxy acid dehydratase production is not repressed during growth of the feedback-insensitive strain on excess branched-chain amino acids. Acetohydroxy acid synthetase production is repressible, but only to a level four times higher than that of fully repressed wild type.

A comparison of *leu-4*^{FLR92}, *leu-1*, and *leu-4*^{FLR92} *leu-3* double mutants grown on limiting leucine again demonstrates the requirement of a functional *leu-3* product for high production levels of the isoleucine and valine biosynthetic enzymes. Both double mutants overaccumulate α-IPM because of the feedback insensitivity of their α-IPM synthetase and subsequent blocks in the leucine pathway. However, even under growth conditions promoting the greatest endogenous accumulation of α-IPM (leucine limiting conditions), the production of threonine deaminase, isomeroreductase, and dihydroxy acid dehydratase by *leu-4*^{FLR92} *leu-3* is no higher than that of fully repressed wild type, and the production of acetohydroxy acid synthetase by *leu-4*^{FLR92} *leu-3* like the *leu-3* single mutant is no higher than production of the enzyme by wild type grown on minimal medium.

Enzyme production by ilv mutants. The behavior of leucine auxotrophic and feedback-negative strains, then, suggests that maximum production of the enzymes of the isoleucine and valine pathway, like production of the leucine biosynthetic enzymes, is in some way dependent on the effector α-IPM and the *leu-3*$^+$ product. To study further the relation between isoleucine- and valine-mediated regulation and the function of the *leu-3* product, measurements were made of enzyme production by representative *ilv* single and *ilv leu* double mutants. Growth of *ilv* mutants and *ilv leu* double mutants is severely inhibited when the concentration of any of the required branched-chain amino acids supplied is present in significantly lower concentration than the others. This inhibition, presumably due to competition for a common permease (24, 27, 32) precluded limiting growth by restricting the supply of each of the required amino acids singly. This problem has been encountered and overcome in *S. cerevisiae* by using glycyl dipeptides of isoleucine and valine in place of the free amino acids to limit growth (22). Presumably, the dipeptides are taken up in yeast by a mechanism different from that by which the amino acids are taken up. Unfortunately, the *ilv* mutants of *Neurospora* cannot utilize the dipeptides and, as a consequence, for the purpose of these investigations, advantage was taken of the fact that growth inhibition is minimal when the exogenous supply of each of the amino acids is approximately equimolar.

The data in Table 5 reveal that the level of acetohydroxy acid synthetase production by *ilv-2* (isomeroreductase deficient) and *ilv-1* (a leaky dihydroxy acid dehydratase mutant) when grown on limiting amounts of isoleucine and valine is about twice that of wild type grown on minimal medium. Under similar growth conditions isomeroreductase production

TABLE 5. *Levels of isoleucine and valine biosynthetic enzymes in Ilv and Ilv Leu double mutants*

Mutant gene (strain)	Supplement to minimal medium	Sp act			
		Threonine deaminase	Acetohydroxy acid synthetase	Isomeroreductase	Dihydroxy acid dehydratase
ilv-3 (T344-D-1A)	0.15 mM Ile + Val[a]	199	Trace	41	142
	2.3 mM Leu + Ile + Val	101	Trace	22	75
ilv-3 leu-1 (T344-3-17A)	0.15 mM Leu + Ile + Val	202	Trace	50	179
	2.3 mM Leu + Ile + Val	208	Trace	42	116
ilv-3 leu-3 (T344-3-27A)	0.15 mM Leu + Ile + Val	83	Trace	21	88
	2.3 mM Leu + Ile + Val	76	Trace	23	73
ilv-2 (T322-D-12A)	0.15 mM Ile + Val	174	33[b]	Trace	123
	2.3 mM Leu + Ile + Val	77	4	Trace	69
ilv-2 leu-1 (T322-2-38A)	0.15 mM Leu + Ile + Val	181	41	Trace	200
	2.3 mM Leu + Ile + Val	238	12	Trace	115
ilv-2 leu-3 (T322-3-3A)	0.15 mM Leu + Ile + Val	76	14	Trace	73
	2.3 mM Leu + Ile + Val	69	3	Trace	68
ilv-1 (T304-D-75A)	0.15 mM Ile + Val	185	29	47	14
	2.3 mM Leu + Ile + Val	95	3	25	10
ilv-1 leu-1 (T304-3-50A)	0.15 mM Leu + Ile + Val	178	37	63	21
	2.3 mM Leu + Ile + Val	247	9	49	17
ilv-1 leu-3 (T304-2-9A)	0.15 mM Leu + Ile + Val	88	12	16	11
	2.3 mM Leu + Ile + Val	91	3	17	10

[a] Conditions of isoleucine and valine limitation.
[b] See footnotes of Table 3.

by *ilv-1* and *ilv-3* mutants is somewhat higher than that of wild type grown on minimal medium and the effect, if any, on dihydroxy acid dehydratase production by *ilv-2* and *ilv-3* is also small. As previously noted for the *leu* mutants, the maximum levels of threonine deaminase production by the *ilv* mutants are consistently lower than that of the wild type. However, unlike *leu-1* and *leu-2* mutants, the production of each of the isoleucine and valine enzymes can be repressed fully by growth in the presence of excess branched-chain amino acids.

The regulatory phenotype of the *ilv leu* double mutants is primarily that of the *leu* gene involved. This is reflected by the *ilv leu-1* double mutants grown on limiting branched-chain amino acids in higher production levels of acetohydroxy acid synthetase, isomeroreductase, and dihydroxy acid dehydratase, and in resistance of the production of all four enzymes to repression by excess amino acid supplementation. Similarly, the *leu-3* phenotype is expressed predominantly in *ilv leu-3* double mutants in that all of the enzymes are produced at the low levels characteristic of *leu-3* irrespective of the mode of growth.

Isoleucine and valine biosynthetic enzyme production by a conditionally constitutive leu-3 mutant and the "leaky" leu-3 mutant from which it was derived. The *leu-3* mutant R229 produces very low but measurable amounts of the leucine biosynthetic enzymes IPM isomerase and β-IPM dehydrogenase. This mutant differs from the relatively tight mutant, R156, in yielding prototrophic conditionally constitutive (for leucine enzyme production) revertants (26). IPM isomerase and β-IPM dehydrogenase production by conditionally constitutive revertants of strain R229 (*leu-3*^{cc}) is independent of the positive effector α-IPM but enzyme production is enhanced greatly if leucine or isoleucine and valine are supplied during growth.

A comparison of the data in Table 6 with those of Table 3 indicates that the leaky *leu-3* mutant R229 produces about twice as much threonine deaminase and, perhaps, slightly more dihydroxy acid dehydratase than strain R156 when grown on leucine-limiting medium but, in all other respects the two mutants are

TABLE 6. *Levels of isoleucine and valine biosynthetic enzymes in leu-3 and leu-3^cc single and multiple mutants*

Mutant gene (strain)	Supplement medium	Sp act			
		Threonine deaminase	Acetohydroxy acid synthetase	Isomeroreductase	Dihydroxy acid dehydratase
leu-3 (R229-1-2a)[a]	0.15 mM Leu	182	19[b]	16	109
	2.3 mM Leu + Ile + Val	90	4	19	52
leu-3[cc] (R229,r21-1-11a)[a]	0.15 mM Leu	196	9 ± 2(3)	36	80
	2.3 mM Leu + Ile + Val	142	5	30	76
leu-3[cc] *leu-4* (R229,r21-8-98a)	0.15 mM Leu	203	22	37	90
	2.3 mM Leu + Ile + Val	163	5	33	83
leu-3[cc] *ilv-2* (R229,r21-6-23a)	0.15 mM Ile + Val	174	28	Trace	123
	2.3 mM Leu + Ile + Val	143	5	Trace	74
leu-3[cc] *leu-2* (R229,r21-3-7a)	0.15 mM Leu	205	59	52	95
	2.3 mM Leu + Ile + Val	139	10	42	75

[a] These strains also bear the *inos* mutation 89601. Growth media were supplemented with 25 μg of i-inositol per ml.

[b] See footnotes of Table 3.

equivalent. The *leu-3*[cc] mutant (R229r21) differs from the strain from which it was derived (R229) by producing twice the level of isomeroreductase, significantly less acetohydroxy acid synthetase, and by displaying resistance to repression of threonine deaminase and isomeroreductase production. The relative production levels of the dihydroxy acid dehydratase and the isomeroreductase by *leu-3*[cc] indicates clearly that the synthesis of these two enzymes does not respond coordinately to the *leu-3*[cc] product even though they are specified by the only known pair of linked structural genes in the branched-chain amino acid pathway. The data show instead that the isomeroreductase is produced by *leu-3*[cc] and *leu-3*[cc] *leu-2* at levels two to three times higher than the original *leu-3* mutant, whereas the production of dihydroxy acid dehydratase remains near the relatively low level characteristic of *leu-3*.

The increased production of acetohydroxy acid synthetase and the isomeroreductase by the *leu-3*[cc] *leu-2* double mutant might suggest some responsiveness to α-IPM on the part of the *leu-3*[cc] product in its role in regulating the production of these two enzymes. But, in view of the fact that threonine deaminase as well as the acetohydroxy acid synthetase and isomeroreductase are produced by the *leu-3*[cc] *leu-4* double mutant at levels higher than expected of a strain unable to synthesize α-IPM (see Table 3), it appears that the effect of the *leu-3*[cc] product is at least partially independent of α-IPM in the regulation of the three enzymes.

The low yield of acetohydroxy acid synthetase characteristic of the *leu-3*[cc] mutant strain used in these experiments is not a general property of *leu-3*[cc] mutants. Another independently derived *leu-3*[cc] mutant, R203r219, which is quite similar to strain R229r21 in all other respects, produces the acetohydroxy acid synthetase at levels more nearly characteristic of *leu-3* auxotrophic mutants. Until a more detailed genetic analysis is done the significance of this discrepancy cannot be assessed.

DISCUSSION

The intervention of the *leu-3* product and its effector α-IPM in the regulation of synthesis of the four enzymes of the isoleucine and valine biosynthetic pathway seems established by the data reported here. When coupled with the previously reported demonstration of the requirement for an active *leu-3* product in the production of IPM isomerase and β-IPM dehydrogenase and for the relaxation of restraints on the synthesis of α-IPM synthetase (11, 26), it becomes apparent that the *leu-3* product is involved in the regulation of at least seven structural genes of branched-chain amino acid biosynthesis in *Neurospora*. In addition, there is some evidence that the *leu-3* product is involved in regulating the production of a branched-chain amino acid permease (A. Olshan, unpublished observations; 26).

The salient feature of *leu-3* α-IPM mediated regulation of isoleucine and valine biosynthetic enzyme production is the requirement of a

normal *leu-3* product and α-IPM for the relaxation of restraints on synthesis of the enzymes in response to end product related signals. This is manifested in *leu-3*$^-$ and *leu-4*$^-$ mutants by the synthesis of the isoleucine and valine biosynthetic enzymes at, or only slightly higher than basal levels even when they are subjected during growth to severe end product deprivation. Thus, as has already been deduced for the regulation of synthesis of enzymes of the leucine pathway, at least two kinds of interrelated pathway-specific regulatory signals are involved, a set of signals which exert control over specific *ilv* cistrons as a function of end product availability and a "positive" signal derived from *leu-3* and α-IPM.

Several mechanisms can be suggested for function of the *leu-3* α-IPM regulatory product in multisite regulation of *ilv* and *leu* cistrons if it is assumed, for simplicity, that both positive and negative regulation are exerted at the level of transcription and that end product regulation in *Neurospora*, as in the enteric bacteria, is mediated by site specific repressors. Accordingly, the *leu-3* product might be required for the release of repressors from their respective operators. More likely, however, in view of the requirement of the *leu-3* α-IPM product for enzyme synthesis at low end product concentration (i.e., when the effective concentration of repressor is low) and its involvement in regulation of all of the unlinked *ilv* and *leu* cistrons, the *leu-3* α-IPM product might facilitate the attachment of ribonucleic acid (RNA) polymerase to promoters of similar structure adjacent to each of the structural genes. This could be accomplished if the *leu-3* α-IPM regulatory product interacted with RNA polymerase in a manner analogous to that proposed for the T7 sigma factor (28), increasing its binding site specificity for a unique set of *ilv* and *leu* cistron promoters. Alternatively, the *leu-3* α-IPM regulatory product by interaction with specific, nearly identical stretches of deoxyribonucleic acid adjacent to or within the promoters of each of the cistrons, like the cyclic adenosine monophosphate binding protein and cyclic adenosine-5′-monophosphate (4), might open promoters that were relatively unavailable to RNA polymerase. In this vein, it seems appropriate to point out that the level of production of three of the four *ilv* enzymes when maximally repressed is reasonably substantial and about the same as that obtained in the absence of an effective *leu-3* α-IPM regulatory product. In view of this it seems reasonable to assume the involvement of a relatively high degree of unregulated transcription which suggests the possible participation of secondary promoters near each of the cistrons responsible for maintaining the basal transcription rate.

Enzyme localization and regulation. The fact that three of the four isoleucine and valine biosynthetic enzymes are localized in the mitochondrion (18) (virtually all of the threonine deaminase is found in the cytosol) raises the question of the relevance of enzyme localization to regulation and catalysis. It was, therefore, of considerable interest to find that the synthesis of acetohydroxy acid synthetase and isomeroreductase is not only discoordinate but that regulation of synthesis of the two enzymes involves different negative effectors, valine for acetohydroxy acid synthetase, and isoleucine and leucine for the isomeroreductase. Synthesis of the isomeroreductase and dihydroxy acid dehydratase also appears discoordinate despite the fact that the same effectors are involved. In addition, the *leu-3*^{cc} product differentially affects the production of the two enzymes.

Since the specific activities of the isomeroreductase and dihydroxy acid dehydratase were measured in extracts of whole mycelia, whereas only mitochondrial-bound acetohydroxy acid synthetase activity was measured, it might be argued that discoordinate synthesis might not affect the proportionality of the three enzymes in mitochrondria but instead the totality of enzyme synthesized, much of which, at high levels of synthesis, would be found in the cytosol. However, it is the production level of acetohydroxy acid synthetase that varies the greatest. As a consequence, if the acetohydroxy acid synthetase as well as the isomeroreductase and biosynthetic dihydroxy acid dehydratase are, under normal circumstances, principally localized in mitochondria, as claimed by Leiter et al. (18), the concentration of the three enzymes in mitochondria can be proportional only over a limited range of enzyme production levels or, for some reason, localization has little to do with organization of the enzymes in an array of fixed molar composition.

Despite some uncertainty as to whether there are two acetohydroxy acid synthetases, one sensitive to feedback inhibition by valine and another minor valine insensitive species, as well as a second species of dihydroxy acid dehydratase, it appears that the *leu-3* product plays an ubiquitous role in the regulation of synthesis of all of the enzymes of branched-chain amino acid synthesis. If regulation indeed involves similar *leu-3* product recognition sites near each of the seven or eight structural cistrons, something like the repetitive "address" loci suggested by Paul (25) might play an integral part

in regulating the synthesis of groups of enzymes without regard to linkage. In any case, with the involvement of the *leu-3* product in the regulation of isoleucine and valine biosynthesis, the coordination of the synthesis of enzymes of the leucine pathway with the synthesis of the enzymes leading to its immediate precursor has been achieved in *Neurospora* by a mechanism quite different from the multivalent repression mechanism of the enteric bacteria (8, 19).

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