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Leucine Biosynthesis in Yeast

Identification of Two Genes (LEU4, LEU5) that Affect α-Isopropylmalate Synthase Activity and Evidence that LEU1 and LEU2 Gene Expression is controlled by α-Isopropylmalate and the Product of a Regulatory Gene[1]

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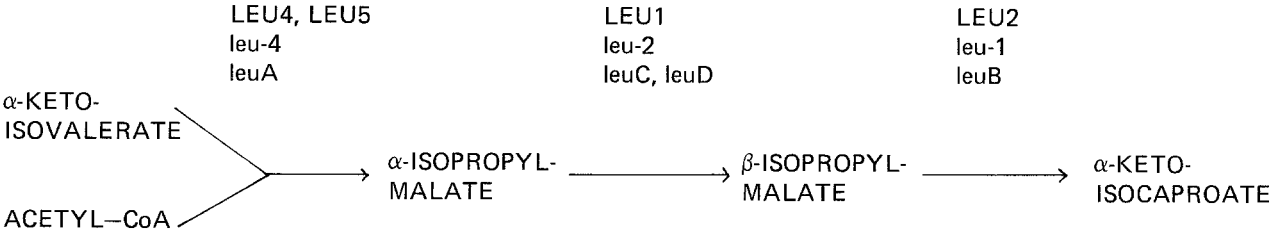
Summary. Tetrad analysis indicates that α-isopropylmalate synthase activity of yeast is determined by two separate genes, designated *LEU4* and *LEU5*. *LEU4* is identified as a structural gene. *LEU5* either encodes another α-isopropylmalate synthase activity by itself or provides some function needed for the expression of a second structural gene. The properties of mutants affecting the biosynthesis of leucine and its regulation suggest that the expression of *LEU1* and *LEU2* (structural genes encoding isopropylmalate isomerase and β-isopropylmalate dehydrogenase, respectively) is controlled by a complex of α-isopropylmalate and a regulatory element (the *LEU3* gene product). Similarities and differences between yeast and *Neurospora crassa* with respect to leucine biosynthesis are discussed.

Key words: *Saccharomyces cerevisiae* – Isoenzymes – Induction

Introduction

The biosynthesis of leucine via the isopropylmalate (IPM) pathway occurs in three specific steps, catalyzed by α-IPM synthase (EC 4.1.3.12), IPM isomerase (dehydratase) (EC 4.2.1.33), and β-IPM dehydrogenase (EC 1.1.1.85), respectively (Scheme 1). The regulation of this pathway has been studied most extensively in enteric bacteria (Soper et al. 1976; Calvo 1983) and in fungi (Gross 1969; Kohlhaw 1983). In *Salmonella typhimurium* and *Escherichia coli*, the three leucine pathway-specific enzymes are encoded by four contiguous genes. These genes constitute an operon which is controlled mainly by attenuation (Calvo 1983). Leucine regulation in fungi differs in two important ways from the pattern observed in bacteria. First, the genes do not constitute an operon. In fact, most of the genes that have been localized (*leu-1, leu-2, leu-3*, and *leu-4* of *N. crassa* and *LEU1* and *LEU2*



Scheme 1. Biosynthesis of the keto acid precursor of leucine. The designation of genes is for *Saccharomyces cerevisiae, Neurospora crassa*, and *Escherichia coli*, respectively (top to bottom)

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[2] At the time these experiments were performed, no strong evidence for isoenzymes of α-IPM synthase existed. Therefore, no effort was made to find out whether the observed effects were due to changes in both isoenzymes or in only one of them

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of yeast), lie within different linkage groups (Gross 1969; Mortimer and Schild 1980). Although *leu-3* and *leu-4* of *N. crassa* belong to the same linkage group, they are not tightly linked (Gross 1969). Second, leucine regulation in *Neurospora* and yeast involves intricate combinations of the modulation of enzyme activity, repression and induction.

In *N. crassa*, the intracellular level of α-IPM regulates leucine biosynthesis by facilitating the expression of the *leu-2* and *leu-1* genes in concert with a putative regulatory protein (the *leu-3* gene product) (Gross 1965; Kashmiri and Gross 1970; Polacco and Gross 1973; Reichenbecher et al. 1978). Control of *leu-2* and *leu-1* expression by leucine is therefore an indirect result of feedback inhibition and/or repression of α-IPM synthase.

The overall meachnism of regulation of leucine biosynthesis in yeast appears to be similar to that of *N. crassa*, but there are significant differences. One important point of difference is α-IPM synthase whose properties, regulation, and localization are distinct in yeast. Thus, the subunit molecular weight of the yeast synthase is 1.5 times that of its *N. crassa* counterpart (Kohlhaw 1983). Furthermore, the yeast enzyme is not only inhibited by leucine (Satyanarayana et al. 1968; Ulm et al. 1972), but is also inactivated by coenzyme A, a product of the reaction (Tracy and Kohlhaw 1975, 1977; Hampsey and Kohlhaw 1981). Coenzyme A inactivation, regarded to be part of a system which regulates the distribution of acetyl-CoA among anabolic and catabolic pathways, has not been reported for *N. crassa* α-IPM synthase. A further difference between the two enzymes is their intracellular localization. Most of the α-IPM synthase of yeast is located in the mitochondrial matrix (Hampsey et al. 1983), whereas the *N. crassa* enzyme appears to be cytosolic (S. R. Gross, personal communication). Differences also exist in the way these organisms regulate the synthesis of α-IPM synthase. *N. crassa* utilizes both repression control by leucine and regulation by the putative α-IPM-regulatory protein complex (Gross 1969). In yeast, excess leucine in the growth medium consistently causes a significant *increase* in the level of α-IPM synthase (Brown et al. 1975; this paper), possibly because the enzyme is subject to the cross-pathway regulation known as "general control" of amino acid biosynthesis (Hsu et al. 1982; see Discussion). Cross-pathway regulation involving α-IPM synthase has not been described for *N. crassa*.

The *LEU2* gene of yeast and its flanking regions have recently been sequenced (Andreadis et al. 1982). Other *leu* genes of yeast are now being isolated and characterized. It therefore seemed timely to gather more basic knowledge on the biosynthesis of leucine in this organism. We show in this paper that the α-IPM synthase activity of yeast is in all likelihood determined by at least two genes. Judging by the properties of mutant strains of yeast, we conclude that α-IPM induces the synthesis of IPM isomerase and β-IPM dehydrogenase, in conjunction with the product of a regulatory gene, much like in *N. crassa*.

Materials and Methods

Strains. The strains of *S. cerevisiae* used in this study are listed in Table 1.

Growth Conditions. Cells were grown aerobically at 30 °C. After at least 7–8 generations, cells were harvested in late log phase ($O.D._{580}$ ca. 0.85 on Spectronic 20 photometer). Cells used for determination of enzyme activities were grown in medium M-N supplemented with 0.1% ammonium sulfate and 2% glucose (Fink 1970). Complete (YEPD) and minimal media used for all other purpose were those described by Fink (1970).

Genetic Techniques. Genetic crosses, tetrad analysis, random spore plating, and complementation tests were performed as described by Fink (1970) and Sherman et al. (1979).

Cell-Free Extracts used for assaying IPM isomerase and β-IPM dehydrogenase were prepared from cells that had been frozen for at least 15 h at −20 °C. Typically, 1 g of cells (wet weight) was suspended in 1.5 ml of 0.1 M potassium phosphate buffer, pH 6.9, containing 1.25 M ammonium sulfate, 20% (v/v) glycerol, and 0.03% sodium azide. The suspension was passed twice through a French pressure cell at 138 megapascals. Extracts were clarified by centrifugation at 39,000 x g for 20 min.

Permeabilization of Yeast Cells for the purpose of assaying α-IPM synthase was performed as described by Miozzari et al. (1978).

Enzyme Assays. α-Isopropylmalate synthase activity was determined as described by Tracy and Kohlhaw (1977). Specific activity is expressed as nanomoles of product formed per min per mg of cells (wet weight). Wet weights were standardized by determining whole cell protein by a modified biuret method (Herbert et al. 1971). The "in situ" assay was chosen because of its greater reproducibility compared to assays performed with cellfree extracts.

Isopropylmalate isomerase was assayed by the method of Cho-Chung and Umbarger (1970), using disodium dimethylcitraconate as substrate. Specific activity is expressed as nanomoles of substrate utilized per min per mg of protein. β-Isopropylmalate dehydrogenase was assayed by the colorimetric method described previously (Hsu and Kohlhaw 1980). Specific activity is expressed as nanomoles of product formed per min per mg of protein. All assays were performed at 30 °C.

Determination of Amino Acid Pools. Cells from 0.3 l of culture were harvested in late log phase on a Reeve Angel glass fiber filter, washed three times with ice-cold physiological saline solution, weighed, resuspendended in 10 ml ice-cold 5% trichloroacetic acid solution, and stirred on ice for 20 min. Cells were then collected on a fresh filter and washed three times with 5% trichloroacetic acid solution. The filtrate was extracted two times with anhydrous ether, and the aqueous phases pooled and lyophilized. The residue was then redissolved in 50 mM citrate buffer, pH 2.2, and subjected to amino acid analysis on a Durrum D-500 analyzer. One micromole of norleucine was carried through the entire procedure as an internal standard.

Table 1. List of strains

Strain	Description	Source or reference
S288cα	Wild type	YGSC[a)]
HB190	*a leu4 leu5*; leucine auxotroph derived from S288cα by EMS mutagenesis; see text for further details	H. E. Umbarger, H. Brown and M. Hayt
SK103	α *LEU4*^{fbr}; spontaneous trifluoroleucine-resistant mutant derived from S288cα; contains feedback-resistant α-IPM synthase; see text for further details	This work
STX26-2C-7C	*a leu1 his1 trp2 gal2*	YGSC
STX26-2C-7D	α *leu1 his1 trp2 gal2*	YGSC
S2072A	*a leu1 arg4 trp1 gal2*	YGSC
AH22	*a leu2-3 leu2-112 his4-519 can1 gal2*	G. R. Fink
DBY746	α *leu2-3 leu2-112 his3-Δ1 ura3-52 trp1-289*	YGSC
DBY747	*a leu2-3 leu2-112 his3-Δ1 ura3-52 trp1-289*	YGSC
XK5-1	α *leu1 LEU4*^{fbr}; recovered from cross between STX26-2C-7C and SK103	This work
XK6-14	*a leu2-3 leu2-112 LEU4*^{fbr}; recovered from cross between DBY747 and SK103	This work
6668-6B	*a leu3-781 inos*$^{-}$; spontaneous mutant derived from S288cα; leaky	G. R. Fink
6668-6C	α *leu3-781 inos*$^{-}$; as above	G. R. Fink
RH657	*a leu3*; spontaneous mutant derived from wild type X2180; leaky	Niederberger et al. (1981)
XK10-35	α *leu3 ura3-52 his3-Δ1*; recovered from cross between RH657 and DBY746	This work
XK1-36	*a leu2-3 leu2-112 leu3*; recovered from cross between DBY747 and 6668-6C	This work
CG219	α *ura3-52*	C. Giroux and T. D. Petes
N349-3B	*a cdc6 his4 ura1 MAL2 gal1 SUC*	YGSC
CG237	*a his4*C-712 *ura3*-52 *can*^{R}	C. Giroux and T. D. Petes
XK14-13C	*a LEU4*^{fbr} *his4*; trifluoroleucine-resistant strain recovered from a cross between SK103 and N349-3B	This work
XK12-11B	α *leu4 leu5 ura3*-52; leucine auxotroph recovered from a cross between HB190 and CG219	This work
XK12-27A	α *leu4 leu5*; leucine auxtroph recovered from a cross between HB190 and CG219	This work
XK12-8A	α *leu5 ura3*-52; see text	This work
XK12-8B	α *leu4 leu5*; see text	This work
XK12-8C	*a leu4 ura3*-52; see text	This work
XK21-14A	*a leu4 ura3*-52; trifluoroleucine-sensitive leucine bradytroph recovered from a cross between XK12-11B and XK14-13C	This work
XK21-30C	α *leu4 his4*; similar to XK21-14A (see text)	This work
XK21-32D	*a leu4 his4*; similar to XK21-14A (see text)	This work
XK22-5A	α *leu4 leu5*; leucine auxotroph recovered from a cross between XK21-14A and XK12-27A	This work

[a] YGSC, Yeast Genetic Stock Center

α-IPM Excretion. The concentration of α-IPM in the growth medium at the time of harvest was determined by the procedure of Calvo and Gross (1970).

Special Chemicals. 5′5′5′-Trifluoroleucine was a gift from H. S. Anker and D. F. Steiner, University of Chicago. β-IPM, synthesized by Reef Laboratories and containing 45% biologically active isomer (*threo*-D$_S$-β-isopropylmalate) and dimethyl citraconate were gifts from H. E. Umbarger, Purdue University.

Results

Complementation Data

Table 2 shows that genetic complementation occurred between *leu1* and *leu2* mutants. Two independently isolated classes of leucine bradytrophs (represented by

Table 2. Complementation tests[a]

a \ α	STX26-2C-7D (*leu1*)	DBY746 (*leu2*)	6668-6C (*leu3*)	XK10-35 (*leu3*)
AH22 (*leu2*)	+	N.T.[b]	+	+
6668-6B (*leu3*)	+	+	–	–
RH657 (*leu3*)	+	+	–	N.T.
HB190 (*leu4 leu5*)	+	+	+	+

[a] Plus signs indicate complementation, minus signs, noncomplementation. The relevant genotypes are shown in parentheses
[b] N.T., not tested

Table 3. Properties of a trifluoroleucine-resistant mutant

	Strain	
	S288cα	SK103
Spec. activity of α-IPM synthase[a]	0.34	0.81
Doubling time in minimal medium (h)	1.9	2.2
$K_{i,app}$ for leucine (mM)[b]	0.2	15.0
Leucine pool (μmol/g cells [wet weight])[c]	0.39	6.0

[a] Strains were grown on minimal medium with no additions. See Materials and Methods for assay details
[b] Concentration of leucine required for 50% inhibition
[c] The pools of 15 other amino acids differed by less than 2-fold

6668-6B and 6668-6C on the one hand and RH657 and XK10-35 on the other hand) complemented all other classes of mutants, but did not complement one another. On this basis, they are assigned to the same complementation group (i.e. *leu3*). Strain HB190, isolated as a leucine auxotroph after EMS-mutagenesis of S288c (H. E. Umbarger, personal communication), and designated *leu4 leu5* (see below) was found to complement *leu1*, *leu2*, and *leu3* mutants.

Characterization of Mutants Affecting α-IPM Synthase

Among the mutant strains that proved to be especially useful for the present study were two strains with lesions affecting α-IPM synthase, *viz.* SK103 and HB190.

Strain SK103 is a spontaneous trifluoroleucine-resistant mutant of S288c. It contained about $2^1/_2$ times more α-IPM synthase than its wild type parent (Table 3). The mutant α-IPM synthase was about 75 times less sensitive to leucine than enzyme from the parent strain. The free leucine pool was elevated 15-fold in the mutant. Similar results had been obtained with another trifluoroleucine-resistant strain, SK305 (Hampsey 1982). Both strains excreted leucine or its ketoacid precursor (Hampsey 1982; Kohlhaw 1983). When SK103 was crossed to a strain that contained intact *leu* genes (N349-3B), seventeen of eighteen tetrads analyzed segregated 2:2 with respect to the trifluoroleucine-resistance marker (the eighteenth tetrad segregated 3:1, $TFL^S:TFL^R$). In ten trifluoroleucine-resistant spores (obtained from five tetrads) the α-IPM synthase was feedback-resistant.These results indicate that trifluoroleucine-resistance in strain SK103 is due to a mutation in a single gene, which we have designated *LEU4*, and that the trifluoroleucine-resistance phenotype and the feedback-resistance character of α-IPM synthase cosegregate.

Strain HB190 had been shown, in preliminary experiments, to be devoid of not only α-IPM synthase, but also IPM isomerase and β-IPM dehydrogenase activities (Bigelis 1974). However, the complementation data shown above (Table 2) and the results of cloning experiments (unpublished results, see Discussion) indicate that the *leu*$^-$ phenotype of HB190 is in fact an α-IPM synthase-negative phenotype. To analyze strain HB190 genetically, it was crossed to CG219 (wild type with respect to leucine). The tetrad analysis of this cross is shown in Table 4 (cross XK12). The tetrads do not show the 2:2 segregation of *leu*$^+$ and *leu*$^-$ expected for a single gene difference between the two parents. Instead, the segregation pattern (7:2:20, PD:NPD:T) is consistent with segregation of two mutant genes, both of which must be mutant in order to show the *leu*$^-$ phenotype. We therefore attribute the lack of α-IPM synthase in HB190 to lesions in two genes, either of which is adequate (but not necessarily optimal) for synthase function. The two genes are designated *LEU4* and *LEU5*.

When strain XK12-11B (derived from and having the same *leu*$^-$ phenotype as HB190) was crossed to strain XK14-13C (derived from and having the same trifluoroleucine-resistance phenotype as SK103), tetrad analysis showed the following (see Table 4, cross XK21): (i) with respect to the *leu*$^-$ phenotype, the ratio of parental ditypes:nonparental ditypes:tetratypes was 9:0:36. This ratio is similar to the one seen with cross XK12.

Table 4. Tetrad analysis

Cross designation	Cross[a]	Tetrads dissected	Segregation of *leu*$^{-}$[b]		
			$2^+:2^-$	$4^+:0^-$	$3^+:1^-$
XK12	HB190 (*leu4 leu5*) x CG219 (*leu*$^+$)	29	7	2	20
XK21	XK14-13C (*LEU4*^{fbr}) x XK12-11B (*leu4 leu5*)	45[c]	9	0	36
XK22	XK21-14A (*leu4*) x XK12-27A (*leu4 leu5*)	8	8	0	0
XK30	CG237 (*leu*$^+$) x 6668-6C (*leu3*)	16	15	0	1
XK31	CG237 (*leu*$^+$) x XK12-8B (*leu4 leu5*)	11	1	2	8
XK36	XK21-32D (*leu4*) x XK12-8A (*leu5*)	26	2	4	20
XK37	XK12-8C (*leu4*) x XK21-30C (*leu4*)	4	0	4	0
XK43	XK12-8C (*leu4*) x XK22-5A (*leu4 leu5*)	15	15	0	0

[a] Relevant genotype shown in parentheses
[b] All other markers tested (mating type, ura$^-$, and his$^-$) segregated 2:2
[c] All 45 tetrads segregated 2:2 with respect to trifluoroleucine-resistance

(ii) With respect to the trifluoroleucine-resistance phenotype, segregation was entirely 2:2. This implies that the trifluoroleucine-resistance allele of the *LEU4* locus derived from strain SK103 must be functional in leucine biosynthesis. It also implies that the locus of the resistance mutation must correspond to one of the loci which is defective in strain HB190. Any strain derived from cross XK21 which is phenotypically *leu*$^+$ and trifluoroleucine-sensitive (examples: XK21-14A, -30C, and -32D) must carry a defective allele at the *LEU4* locus and a functional allele at the second locus defective in strain HB190. These strains are therefore designated *leu4 LEU5*.

The *LEU4* locus most likely corresponds to a structural gene for α-IPM synthase, since the trifluoroleucine-resistant allele produces an altered, feedback-resistant enzyme. The fact that *leu4 LEU5* strains are not auxotrophic for leucine implies that an additional synthase activity is present in these strains. The *LEU5* gene could either encode the other α-IPM synthase or provide a function required for the expression of the second synthase gene.

In another experiment (Table 4, cross XK22), a *leu4 LEU5* strain (XK21-14A, see Table 1) was crossed to a *leu4 leu5* strain (XK12-27A), in order to construct a diploid homozygous for *leu4* and heterozygous for *leu5*. The *leu*$^-$ phenotype of this cross segregated 2:2, implying that the *leu5* mutation segregates as a single marker.

To identify a *LEU4 leu5* strain, a tetratype tetrad (XK12-8) obtained from the cross between HB190 and CG219 (see above) was used for further analysis. When strain XK12-8A was crossed to XK21-32D (Table 4, cross XK36) the *leu*$^-$ phenotype segregated 4:2:20, PD:NPD:T. Since both parents are prototrophic for leucine and XK21-32D is *leu4 LEU5* (see above), XK12-8A must be *LEU4 leu5*. The *leu*$^-$ spores obtained in this cross are then a result of the combination of the *leu4* allele from XK21-32D and the *leu5* allele from XK12-8A. The cross of XK12-8B (which is the only leucine auxotroph in tetrad XK12-8) to CG237 (a wild type with respect to leucine) gave a *leu*$^-$ phenotype segregation of 1:2:8, PD:NPD:T (Table 4, cross XK31). This segregation pattern is very similar to that seen in cross XK12 (HB190 x CG219, Table 4). Hence, XK12-8B is designated *leu4 leu5*. Crossing XK12-8C to XK22-5A (derived from and having the same *leu*$^-$ phenotype as HB190) resulted in a *leu*$^-$ segregation of 15:0:0, PD:NPD:T (Table 4, cross XK43), which is identical to the segregation pattern seen in cross XK22. This implies that there is a single gene difference between XK12-8C and XK22-5A, and that XK12-8C must be *leu4 LEU5* (since XK12-8A is already *LEU4 leu5*). This notion was confirmed in the cross XK37 (Table 4) where XK12-8C was crossed to a defined *leu4 LEU5* strain (XK21-30C). In this case all four tetrads examined segregated $4^+:0^-$ with respect to the leucine requirement. In addition, 24 random

Table 5. Enzyme activities in cells derived from selcted spores

Spore	α-IPM Synthase-related Genotype[a]	Phenotype	Specific Activities[b]		Doubling time h
			α-IPM Synthase	IPM Isomerase	
XK12-8A	*LEU4 leu5*	*leu*$^+$	0.19	32	2.3
XK12-8B	*leu4 leu5*	*leu*$^-$	Not detectable	Not detectable	–
XK12-8C	*leu4 LEU5*	*leu*$^+$	Trace	22	3.2
XK12-8D	*LEU4 LEU5*	*leu*$^+$	0.39	36	2.2

[a] See text for further details

[b] See Materials and Methods for assay details. The synthase data were kindly provided by L. F. Chang of this laboratory. Specific activities for the wild type parent (CG219) were 0.35 (α-IPM synthase) and 29 (IPM isomerase) for cells grown in the absence of leucine. The values listed in the Table represent the average of four determinations. Cells were grown in M-N medium supplemented with 0.1% ammonium sulfate and 2% glucose (Fink 1970). The growth medium for strain XK12-8B contained in addition 0.2 mM leucine

spores obtained from this cross were all *leu*$^+$. By elimination, the remaining spore in the tetrad XK12-8 (i.e., XK12-8D) must be *LEU4 LEU5*.

Enzyme Activities and Growth Rates of Strains Obtained from a Tetratype Tetrad of Cross XK12

α-IPM synthase and IPM isomerase activities of one representative tetrad of the tetratype class obtained from cross XK12 are shown in Table 5. The enzyme levels of XK12-8D and XK12-8B resembled those of the parents (near [above] wild type levels and no detectable activity, respectively). The α-IPM synthase level in XK12-8C was near the limit of detection, while the level in XK12-8A was approximately 60% of the wild type level. The synthase in XK12-8A was inhibited by leucine in an apparently normal fashion (data not shown). The isomerase levels in XK12-8C and XK12-8A were 75% and 110% of normal, respectively. In the absence of leucine, XK12-8C grew more slowly than XK12-8A. The growth rate of XK12-8D was nearly identical to that of wild type.

Enzyme Activities in Mutants Affecting the Biosynthesis of Leucine and its Regulation

The Lindegren wild type (strain 60615) responds to the presence of leucine in minimal medium by only a small decrease in the specific activities of IPM isomerase and β-IPM dehydrogenase, while actually showing an increase in the specific activity of α-IPM synthase (Satyanarayana et al. 1968; Brown et al. 1975). In media with leucine plus threonine or in tryptone media, strong repression of isomerase and dehydrogenase was observed. Using a different wild type (S288c), we found that leucine alone (2 mM) repressed both isomerase and dehydrogenase below detectable limits (Table 6). The α-IPM synthase level concomitantly increased approximately two-fold. This response of the synthase is typical (Table 6). In all instances (with the exception of the synthase-less strain HB190), the level of the synthase rose 1.4- to 3.4-fold above that characteristic of minimally grown wild type cells *either* when leucine was present in excess *or* when leucine limitation was imposed. A possible reason for this response is discussed below.

We confirmed the results of Bigelis (1974) who reported that strain HB190 is devoid of all three leucine pathway-specific activities. Even when the cells were grown with limiting amounts of leucine, no enzyme activities were found.

The α-IPM synthase levels of strain SK103, a feedback-resistant mutant (see above), were increased 2.4-fold over wild type levels even when no leucine was added to the medium. Recall that this strain accumulates leucine and has a free leucine pool 15 times larger than that of strain S288c (see Table 3). Growth of SK103 in leucine medium caused a small additional increase in the synthase level. Under both conditions, i.e., with or without externally added leucine, the levels of IPM isomerase and β-IPM dehydrogenase in SK103 were not only not repressed, but were strongly elevated, compared to wild type (Table 6). It is noteworthy in this context that SK103 excreted small amounts of α-IPM.

The level of β-IPM dehydrogenase in isomerase-less mutants (*leu1* strains STX26-2C-7C and S2072A, Table 6) and the level of IPM isomerase in a dehydrogenase-less mutant (*leu2* strain AH22, Table 6) were repressed by excess leucine, but repression was less severe than in wild type cells. When leucine was limiting, the level of

Table 6. Enzyme activities in a wild type and various mutant strains of *S. cerevisiae*

Strain	Relevant Genotype	Additions to Medium	α-IPM Synthase	Activities[a]		α-IPM[b] Excretion
				IPM Isomerase	β-IPM Dase	
S288c	Wild type	None	1.0	1.0	1.0	–
		2 mM leu	2.2	<0.1	<0.1	N.T.[c]
HB190	*leu4 leu5*	0.2 mM leu	<0.1	<0.1	<0.1	–
		2 mM leu	<0.1	<0.1	<0.1	N.T.
SK103	*LEU4*^{fbr}	None	2.4	3.5	5.2	±
		2 mM leu	3.4	3.0	4.1	N.T.
STX26-2C-7C	*leu1*	0.2 mM leu	2.1	<0.1	2.5	+
		2 mM leu	1.7	N.T.	0.7	N.T.
S2072A	*leu1*	0.2 mM leu	N.T.	<0.1	2.3	N.T.
		2 mM leu	N.T.	N.T.	0.9	N.T.
AH22	*leu2*	0.2 mM leu	2.3	7.0	<0.1	+
		2 mM leu	2.9	0.5	N.T.	N.T.
XK5-1	*LEU4*^{fbr} *leu1*	0.2 mM leu	3.4	<0.1	12.8	+
		2 mM leu	2.6	N.T.	18.5	+
XK6-14	*LEU4*^{fbr} *leu2*	0.2 mM leu	1.8	8.8	<0.1	+
		2 mM leu	1.8	11.3	N.T.	+
RH657	*leu3*	None	1.6	0.1	<0.1	N.T.
		0.2 mM leu	1.4	<0.1	<0.1	+
		2 mM leu	1.8	<0.1	<0.1	N.T.
6668-6B	*leu3*	0.2 mM leu	3.3	<0.1	<0.1	+
		2 mM leu	2.3	<0.1	<0.1	N.T.
XK1-36	*leu2 leu3*	0.2 mM leu	1.4	<0.1	<0.1	+
		2 mM leu	1.9	<0.1	N.T.	N.T.

[a] All strains were grown at least two times in batch cultures. Enzyme activities are expressed relative to those found in wild type cells with no additions to the medium. Actual specific activities in wild type cells (no additions) were: α-IPM synthase, 0.34; IPM isomerase, 28.3; β-IPM dehydrogenase, 10.1. See Materials and Methods for definition of units. Each batch of cells was assayed at least four times. The overall error is ± 20% of the mean

[b] –: no excretion; ±: 1–10 μg/ml of medium; +: > 10 μg ml of medium (at time of harvest)

[c] N.T.: not tested

the dehydrogenase in the *leu1* strains rose about 2.4 fold over minimally grown wild type, and the level of the isomerase in the *leu2* strain rose 7-fold. Both *leu1* and *leu2* strains excreted α-IPM into the growth medium. *LEU4*^{fbr} *leu1* and *LEU4*^{fbr} *leu2* double mutants (strains XK5-1 and XK6-14, Table 6) had very high levels of the dehydrogenase and the isomerase, respectively, under conditions of either excess or limiting leucine. Both strains again excreted α-IPM.

The two *leu3* mutants listed in Table 6 exhibited "normal" behavior as far as their synthase levels were concerned. They, too, excreted α-IPM. The overproduction of α-IPM and their very slow growth in the absence of leucine apparently stems from the virtual absence of IPM isomerase and β-IPM dehydrogenase. The levels of these enzymes were at the limit of detection even when cells were grown with limiting leucine or without leucine. With respect to the levels of α-IPM synthase and IPM isomerase as well as the excretion of α-IPM, a *leu2 leu3* double mutant did not differ significantly from the *leu3* mutants.

Discussion

The evidence we have presented for the existence of two α-IPM synthase activities must be compared to other more circumstantial observations suggesting that there are α-IPM synthase isoenzymes. First, Brown et al. (1975) found that the synthase levels of glucose-grown cells were 2–3 times lower than those of acetate-grown cells. The synthase from glucose-grown cells appeared to be more stable in vivo and more sensitive to leucine than enzyme from acetate-grown cells. Second, it is apparent-

ly difficult to isolate spontaneous synthase-negative mutants. For example, among 500 independently isolated spontaneous *leu* mutants, none was α-IPM synthase deficient (G. R. Fink, personal communication, and our own observations). Finally, Hampsey et al. (1983) observed that antibody prepared against highly purified α-IPM synthase precipitated two major polypeptides when added either to extracts or to reaction mixtures obtained after in vitro translation of total yeast RNA. One polypeptide corresponded in size to the subunit of the previously purified α-IPM synthase (i.e., ca. 65,000), while the other was about 2,000 daltons smaller. There was no obvious precursor-product relationship between the two polypeptides. Only the larger polypeptide was incorporated into intact mitochondria. It was also present in much greater amounts than the smaller one when total cellular protein labeled in vivo was used for immunoprecipitation. However, no definite connection has as yet been established between the two polypeptides studied by Hampsey et al. and *LEU4* and *LEU5*.

Judged by the results presented in Table 5, the strain that we have designated *LEU4 leu5* contained much more α-IPM synthase than the *leu4 LEU5* strain. However, these data must be interpreted with caution. For example, it is not clear how the very low synthase activity in the *leu4 LEU5* strain is to be reconciled with an almost normal isomerase level in cells grown with limiting or no leucine (assuming induction of the isomerase by α-IPM, see below), and with relatively good growth in the absence of leucine. It is possible that the synthase activity produced in the *leu4 LEU5* strain is labile and therefore grossly underestimated. (In vitro lability of the second synthase would also explain why so little biochemical evidence for α-IPM synthase isoenzymes has been forthcoming.) In order to resolve these questions, attempts are now underway to clone the *LEU4* and *LEU5* genes by functional complementation of the synthase-less mutant HB190. One of the genes has recently been isolated on a 6.5 kilo-basepair fragment (L. F. Chang, T. S. Cunningham, and G. B. Kohlhaw [1983] Fed. Proc. 42, Abs., p. 1969). Transformation of strain HB190 with a plasmid containing this fragment not only resulted in an α-IPM synthase level that was 20 times higher than wild type, but also restored IPM isomerase and β-IPM dehydrogenase activities.

α-IPM synthase activity was consistently 2- to 3-fold higher than the basal wild type level when *either* excess leucine was added to the growth medium *or* leucine limitation was imposed (see Table 6). This phenomenon can be best explained by the following two facts: (a) α-IPM synthase is under the "general control" of amino acid biosynthesis (Hsu et al. 1982)[2]; and (b) *both* limiting leucine *and* excess leucine elicit derepression of enzymes known to be under the "general control" (Niederberger et al. 1981). Excess leucine (i.e., an initial medium concentration of at least 2 mM) probably does so by causing an amino acid imbalance. Because α-IPM synthase is under the "general control", it is difficult to judge whether the enzyme is also specifically repressed by leucine, as it is in *N. crassa*.

As pointed out in the Introduction, there are numerous differences between the α-IPM synthases of yeast and *N. crassa*. The likely existence of isoenzymes in yeast may now be included among these differences. By contrast, the regulation of IPM isomerase and β-IPM dehydrogenase in yeast resembles the pattern that has been reported for *N. crassa* (Gross 1969). Thus, the level of the isomerase and the dehydrogenase in yeast cells appears to be largely a function of the in vivo activity of the synthase (as opposed to the synthase activity measured in vitro), i.e., the isomerase and dehydrogenase levels respond to the endogenously produced α-IPM, which probably acts as an inducer. For example, even though a medium concentration of 2 mM leucine causes an increase in the activity of the synthase in wild type cells, it is also likely to cause inhibition of the enzyme and thus to limit or eliminate α-IPM production. If α-IPM were an inducer of *LEU1* and *LEU2*, then very little isomerase and dehydrogenase should be synthesized. It then also follows that: (i) An α-IPM synthase-negative mutant should synthesize little or no isomerase and dehydrogenase. (ii) A strain containing feedback-resistant α-IPM synthase should continue to make isomerase and dehydrogenase even in the presence of leucine. (iii) *Leu1* or *leu2* mutants, which are expected to accumulate α-IPM, should show increased amounts of dehydrogenase or isomerase. The extent of the increase should be a function of the amount of leucine present. The increase should be especially strong (and should no longer be reduced by excess leucine) in double mutants in which a lesion in *LEU1* or *LEU2* is combined with a mutation causing feedback resistance of the synthase. All of these predictions were indeed fulfilled.

In a further analogy to *N. crassa*, mutants were found (designated *leu3*) that showed very low expression of *LEU1* and *LEU2* but were able to complement *leu1* and *leu2* mutants. The leucine-requiring phenotype of the *leu3* mutants segregated in Mendelian fashion when a *leu3* strain was crossed to a LEU^+ strain (Table 4, cross XK30), indicating that the observed phenotype was due to a lesion in a single gene. The fact that the *leu3* mutants contain near normal levels of α-IPM synthase and excrete α-IPM indicates that the *leu3* mutation does not impede α-IPM production (as is the case with HB190), but rather affects an event (or events) subsequent to the production of α-IPM that normally facilitate(s) the expression of *LEU1* and *LEU2*. We postulate that the *LEU3* gene of yeast encodes a regulatory factor capable of interacting with α-IPM and that formation of a *LEU3* gene product-α-IPM complex is required for the expression of *LEU1*

and *LEU2*. An analysis of transformants carrying the *LEU3* gene, now in progress, is expected to shed more light on the mechanism of action of *LEU3*, including the level at which this regulation occurs.

On the basis of some structural features of the 5′-noncoding region adjacent to the *LEU2* gene, a case has been made for a more direct participation of leucine in the regulation of *LEU2* (Andreadis et al. 1982), in addition to its indirect action as inhibitor of α-IPM synthase. However, the results reported here, especially the properties of the feedback-resistant strain, suggest that any regulatory action of leucine that goes beyond modulation of α-IPM synthase activity is probably of minor importance.

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Note Added in Proof

It has been observed that a *leu4 LEU5* strain (XK21-14A) shows no detectable IPM isomerase activity when grown in the presence of 2 mM leucine. This suggests that the α-IPM synthase activity in this strain is regulated by leucine