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Biosynthesis of Branched-Chain Amino Acids in Yeast: Regulation of Leucine Biosynthesis in Prototrophic and Leucine Auxotrophic Strains

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The first enzyme in the biosynthesis of leucine in yeast, α-isopropylmalate synthetase, is inhibited by L-leucine. In a mutant resistant to the analogue 5',5',5'-trifluoroleucine, the enzyme is markedly resistant to inhibition by L-leucine. Growth ing the presence of exogenous L-leucine results in repression of the second and third enzymes of the pathway. The first enzyme is not repressed unless both L-leucine and L-threonine are supplied in the medium. Comparison of levels of the remaining two enzymes in leucine auxotrophs grown under conditions of leucine excess and leucine limitation reveals deviations from the wild-type derepression pattern in some mutants. In some, repression of the synthetase by leucine alone was observed. In others, the repressibility of the dehydrogenase was lost. It is unlikely that these deviations were due to the same primary mutational event that caused leucine auxotrophy. No mutants were found in which an altered gene was recognized to be clearly responsible for the level of the leucine-forming enzymes.

In the previous report, the biochemical block in a representative strain from each of 10 classes of leucine-requiring yeast mutants was reported (8). It was noted that the mutant strains differed from the wild type in that one or more enzyme activities were missing and, in some cases, in the amount of the remaining enzyme or enzymes. To determine whether these "secondary" differences between the mutants and the wild type were due to the genetic lesions in the *le* loci affected or to other undefined genetic differences, a survey of additional strains was undertaken. At the same time, a comparison was made of the effects of excess and limiting leucine on the enzyme levels in the wild type and in various leucine auxotrophs in order to determine whether the pattern observed was related to the *le* locus affected.

MATERIALS AND METHODS

Organisms, media, and growth conditions. The media employed were those described in the previous paper (8). The wild-type organism, a *Saccharomyces* species derived from several different species, strain 60615, and the leucine auxotrophs were from the Lindegren yeast collection. Some had additional growth requirements. The strain numbers and characteristics of the strains are given in relation to specific experiments.

The techniques used in preparing the genetic crosses and isolating the progeny have been described elsewhere (6).

When the effect of excess leucine was examined, batch cultures in minimal medium supplemented with 2×10^{-3} M L-leucine (8) were employed. The effect of limiting leucine was examined in two ways. (i) Batch cultures, in medium supplemented with 2×10^{-4} M L-leucine and an excess of any additional growth factors, were grown until the leucine was exhausted. The density of the cultures was followed at the later stages by withdrawing 3-ml samples from the flasks aseptically at appropriate intervals and measuring the turbidity in a Klett-Summerson colorimeter with a blue no. 42 filter. (ii) In a modified chemostat (1), fresh medium containing 2×10^{-4} M L-leucine was pumped into an aerated culture that was kept at a constant volume of 500 ml. The replacement time was about 15 hr. The culture in the growth vessel was used for the preparation of the extract.

Preparation of the extracts and enzyme assays. The extracts were prepared and the enzyme activities were assayed as before (8), except that in some cases an alternative method for the determination of α-isopropylmalate (α-IPM) isomerase activity was employed. Rather than measuring the increase in optical

density at 235 nm due to the formation of dimethylcitraconate (DMC) from β-isopropylmalate (β-IPM), dimethylcitraconate itself was used as substrate (at a concentration of 2×10^{-4} M) and the decrease in optical density observed (3). DMC was obtained from the Reef Laboratory, Lafayette, Ind. The assay employing β-IPM yielded specific activity values about 3.5 times as high as the assay employing DMC.

RESULTS

Effect of leucine on the synthesis of α-IPM. In *Salmonella* (C. Jungwirth and H. E. Umbarger, Federation Proc., p. 10, 1962) and in *Neurospora* (4), α-IPM synthetase is inhibited by L-leucine. L-Leucine, at a concentration of 10^{-2} M, resulted in almost complete inhibition of α-IPM synthetase activity in an extract of the wild strain (Fig. 1). In contrast, the α-IPM synthetase activity in a mutant, strain 60615/fl-1, selected for growth on 5×10^{-3} M 5′,5′,5′-trifluoroleucine, was inhibited only about 30% by 10^{-2} M L-leucine.

Effect of L-leucine on the level of the leucine-forming enzymes. It was anticipated that the three enzymes in the pathway to leucine would be repressed in cells grown with excess L-leucine. Both the isomerase and the dehydrogenase were repressed, although the magnitude of the repression was not great (Table 1). The synthetase, however, was not repressed but rather was consistently higher in cells grown with L-leucine. The same observations were made on strain 60615/fl-1, in which the α-IPM synthetase was partially resistant to leucine. (Even though this strain showed an elevated level of the isomerase, the extent of repression by leucine was similar.)

When the cells were grown with a complex amino acid mixture, such as acid-hydrolyzed casein (e.g., Difco Casamino Acids) or a pancreatic digest of casein (e.g., Difco tryptone), both the normal and "feedback-resistant" α-IPM synthetase were repressed; in the wild type, the isomerase and dehydrogenase were repressed further than they were by L-leucine alone. In the mutant, however, the repression caused by L-leucine alone was counteracted by tryptone, especially in the case of the dehydrogenase. Adding additional L-leucine to the tryptone had no effect.

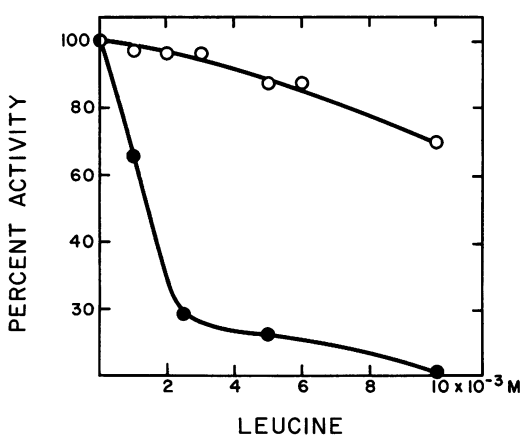

FIG. 1. *Effect of L-leucine on α-isopropyl malate synthetase activity of a crude extract of yeast. Symbols: ●, strain 60615 extract; ○, strain 60615/fl-1.*

Effect of threonine on the level of α-IPM synthetase. In an attempt to determine which of the components in tryptone were required for the repression of α-IPM synthetase described above, combinations of selected amino acids were tested with leucine (Table 2). Supplementing the medium with a mixture of the three branched-chain amino acids resulted in a slight repression; however, a mixture of the three plus L-threonine mimicked exactly the effect of tryptone.

Although the effect of L-threonine cannot yet be explained, the results shown in Table 3 may be useful. The data are given in relative specific activities because of the ease in comparison with the level of synthetase in minimal medium-grown cells, and also because of an unexplained variation in specific activity that was encountered during the performance of these experiments. The values representing repeat experiments show that the relative effects were in remarkably good agreement. The specific activities of the minimal medium cultures, however, varied during this period from 0.0081 to 0.030 μmole of product per mg of protein per min. Further evidence that the values represented day-to-day variation and not flask-to-flask variation was obtained by comparing the results of duplicate experiments performed on the same day in several cases.

The effect of L-leucine in elevating the synthetase is not specific, since L-isoleucine and, to a lesser extent, L-valine do the same (Table 3). L-Threonine alone represses, but this effect is antagonized by L-valine and by L-isoleucine. The effect of threonine, however, is not antagonized by L-leucine; rather, the repression is almost as great as it was with tryptone (Table 2). When leucine and either valine or isoleucine were used to supplement the medium, the level of synthetase was only slightly, though probably not significantly, higher than the minimal medium level.

In attempting to analyze the effect of threonine, several compounds that might have been derived by degradation of threonine (α-aminobutyrate, glycine, and serine) were tested in the presence of leucine. Cells grown in the presence of leucine

TABLE 1. *Effect of* L-*leucine on the level of leucine-forming enzymes*

Additions to minimal medium	Specific activity					
	Wild strain 60615			Trifluoroleucine-resistant strain 60615/fl-1		
	Synthetase	Isomerase	Dehydrogenase	Synthetase	Isomerase	Dehydrogenase
None	0.018	0.084	0.040	0.031	0.114	0.043
	0.021	0.097	0.038			
L-Leucine, 2×10^{-3} M	0.061	0.038	0.026	0.042	0.048	0.031
	0.059	0.044	0.024	0.050	0.035	0.027
Tryptone, 0.5%	0.012	0.008	0.008	0.010	0.078	0.039
Tryptone plus L-Leucine				0.013	0.064	0.038

TABLE 2. *Replacement of tryptone in repression of α-IPM synthetase activity in wild-type yeast*

Additions to minimal medium	Relative specific activity[a]
None	100
L-Valine, L-Isoleucine, L-Leucine, 2×10^{-3} M each	87
As above plus L-threonine, 2×10^{-3} M	35
Tryptone, 0.5%	36

[a] Percentage of the specific activity of the extract of cells grown in minimal medium, which was 0.026 μmoles of coenzyme A released per mg of protein per min.

and either glycine or serine overcame the leucine effect, the cells exhibiting minimal medium levels of the synthetase. The addition of α-aminobutyrate did not appear to alter the leucine effect. Aspartic acid, a precursor of threonine, added with leucine partially overcame the leucine effect (relative specific activity of 152).

Levels of α-IPM isomerase and β-IPM dehydrogenase activities in strains lacking α-IPM synthetase activity. In the previous paper, the only strain reported to lack α-IPM synthetase was strain 70458, with a lesion in the *le*-6 locus. Interestingly, this strain exhibited no detectable α-IPM isomerase and very little β-IPM dehydrogenase when the strain was cultivated in a medium containing excess leucine. To determine whether the second and third enzymes might be elevated under conditions of leucine limitation, extracts were prepared from strain 70458 grown in batch cultures with excess and limiting leucine and in a chemostat with leucine limiting. It was possible to obtain cells with a higher level of dehydrogenase when a chemostat was employed (Table 4). However, the use of a chemostat did not result in extracts in which unequivocal isomerase activity could be detected. Similar results were obtained with a second *le*-6 mutant, strain 70480. In this strain, the dehydrogenase activity was even lower. However, when a batch culture in medium containing limiting L-leucine (2×10^{-4} M) was allowed to grow until leucine was exhausted, a small amount of isomerase was detected. This was the only instance in which both isomerase and dehydrogenase were detected in an *le*-6 strain, and is the basis for concluding that the *le*-6 locus affects primarily the synthetase.

Effect of limiting and excess leucine on enzyme levels in isomeraseless mutants. In the previous report (8), it was shown that the leucine auxotrophs that lacked α-IPM isomerase activity varied widely in the levels of synthetase and dehydrogenase activities; these levels were in extracts prepared from cells grown with excess L-leucine. The questions that arise immediately are whether the secondary differences from the wild type are locus-specific and whether the various auxotrophs respond to leucine in the growth medium in the same way as does the wild type (i.e., an elevation of the synthetase and a repression of the dehydrogenase when grown with excess L-leucine). To determine the response to exogenous leucine, extracts prepared from cells grown with excess L-leucine were compared with those prepared from cells grown with limiting L-leucine in a chemostat. In addition, three different *le*-1 strains were examined (Table 5).

The pattern observed in the prototrophic strain 60615 was found in only one of the auxotrophs, strain Q-121, an *le*-5 mutant. In some strains, the synthetase was actually derepressed by limiting L-leucine, a pattern that is seen in some bacterial systems. In other strains it was unaffected. The dehydrogenase was derepressed on limiting L-leucine in some strains but, in others, the enzyme levels were essentially the same in excess and limiting L-leucine. Furthermore, the repressibility of the two enzymes seemed to be independently controlled and varied among the three *le*-1 strains. Thus, in strain 75315 (*le*-10), containing a highly repressible synthetase, the dehydrogenase was about that of the wild type

TABLE 3. *Effect of various amino acid supplements on the level of α-IPM synthetase activity in wild-type yeast*

Other additions	Amino acids added to medium[a]							
	None		Leucine		Valine		Isoleucine	
Threonine	67	70	41	40	106		104	
	73		44	42				
Isoleucine	230	235	113		218[b]	223		
Valine	169		116					
Leucine	180	213						
	(0.008)[c]	(0.022)						
	233	212						
	(0.024)	(0.020)						

[a] All amino acids added at a concentration of 2×10^{-3} M. All values are expressed in per cent of the specific activity of an extract prepared from cells grown in minimal medium on the same day from the same inoculum. Figures in the right side of each column represent values obtained with 2% glucose in the medium.

[b] L-Valine added at a concentration of 4×10^{-3} M.

[c] Actual specific activities shown in parentheses.

TABLE 4. *Effect of leucine limitation on enzyme levels in le-6 (synthetaseless) mutants*

Strain	Growth condition	Specific activity	
		Isomerase	Dehydrogenase
70458	Batch, 2×10^{-3} M L-leucine	ND[a]	0.008
70458	Chemostat, 2×10^{-4} M L-leucine	ND	0.014
70480	Batch, 2×10^{-3} M L-leucine	ND	Trace
70480	Chemostat, 2×10^{-4} M L-leucine	ND	0.008
70480	Batch, 2×10^{-4} M L-leucine	0.006	0.005

[a] Not detected.

TABLE 5. *Effect of leucine on the enzyme levels in isomeraseless mutants*

Strain	Locus affected	Growth conditions	Specific activity	
			Synthetase	Dehydrogenase
Q-1118	*le*-1	Batch[a]	0.067	0.044
		Chemostat[b]	0.021	0.038
Q-171	*le*-1	Batch	0.042	0.086
		Chemostat	0.085	0.126
Q-178	*le*-1	Batch	0.020	0.024
		Chemostat	0.020	0.073
65279	*le*-4	Batch	0.015	0.011
		Chemostat	0.053	0.015
Q-121	*le*-5	Batch	0.013	0.010
		Chemostat	0.005	0.043
74394	*le*-7	Batch	0.017	0.018
		Chemostat	0.016	0.053
75290	*le*-8	Batch	0.031	0.002
		Chemostat	0.027	0.004
75315	*le*-10	Batch	0.003	0.024
		Chemostat	0.003	0.023

[a] L-Leucine, 2×10^{-3} M.

[b] L-Leucine, 2×10^{-4} M.

in excess L-leucine and was not derepressed. In contrast, strain 74394 (*le*-7) exhibited a derepressible dehydrogenase, but the level of its synthetase was unaffected by exogenous L-leucine.

Effect of L-leucine on enzyme levels in le-2 (dehydrogenaseless) mutants. The response to exogenous L-leucine varied from one *le*-1 mutant to another just as it did with the three *le*-2 mutants that were tested (Table 6). In all three mutants, the synthetase levels were essentially unaffected by exogenous L-leucine, although, in strain Q-174, the synthetase was near the (derepressed) level found in prototrophic cells grown in the presence of exogenous L-leucine. In the other two strains, it was at the (repressed) level found in cells grown without L-leucine. The isomerase pattern was again not constant in the three strains, with one strain exhibiting a slight, but probably insignificant, repression and two strains exhibiting derepression, when grown with limiting L-leucine. The results obtained with the three different *le*-1 and three different *le*-2 mutants revealed that the regulation pattern of the synthetase was not a consequence of lesions in the *le*-1 or *le*-2 loci.

Examination of synthetase levels in the progeny of a cross between le-1 and le-2. To determine whether the response of the synthetase was a consequence of the specific kind of lesion in these

TABLE 6. *Effect of* L-*leucine on enzyme levels in le-2 (dehydrogenaseless) mutants*

Strain	Locus affected	Growth condition	Specific activity	
			Synthetase	Isomerase
Q-110	*le*-2	Batch	0.033	0.081
		Chemostat	0.024	0.064
Q-174	*le*-2	Batch	0.044	0.061
		Chemostat	0.047	0.118
83295	*le*-2	Batch	0.028	0.056
		Chemostat	0.028	0.134

loci (which primarily affected the isomerase or dehydrogenase, respectively), a cross between *le*-1 and *le*-2 mutants of opposite mating type was made. The tetrads of these asci were dissected and maintained as separate clones. One parent, strain 83026 of mating type α, carried an *le*-2 marker and an *hi*-4 (histidine deficiency) marker, lesions which have been located on the same arm of chromosome III. The other parent, strain 85039 of mating type α, carried an *le*-1 marker and a *tr*-5 (tryptophan deficiency) marker, lesions which have been located on the same arm of chromosome VII. It was thus possible to recognize evidence in some of the progeny of crossovers occurring between the *le* and *tr* or *hi* markers.

The results of the examination of these progeny are shown in Table 7. In neither parent was the synthetase repressed or derepressed by growth in the presence of excess L-leucine. When both L-leucine and L-threonine were in excess, repression did occur. Furthermore, the two parent strains differ not only in the primary lesions affecting leucine biosynthesis, but also in the levels of the synthetase.

The few progeny strains tested were sufficient to demonstrate that the level of synthetase is controlled by factors independent of either the *le*-1 or the *le*-2 gene. It is also clear that additional factors that affect the response to exogenous L-leucine, and were not apparent in either parent, have segregated in 7 of the 12 progeny classes. These factors are of two kinds: one allowing repression and the other allowing a "derepression" when L-leucine was in excess. Of special interest was strain 89173, in which the synthetase was repressed by L-leucine to nearly as low a level as found in any extract thus far examined, and in which the synthetase was the highest found in any extract when L-leucine was limiting. It would be of interest to define these factors and to study their genetic control; however, at the present time there is no known way to screen for their presence except by examination of extracts.

The results of tests for isomerase and dehydrogenase in the progeny were, as expected, completely in accord with the view that the *le*-1 and *le*-2 lesions affect the isomerase and the dehydrogenase, respectively (Table 7). (Owing to the scarcity of the substrate β-isopropylmalate required for the assay of the dehydrogenase, that enzyme activity was examined only in cases in which its presence or absence could not be logically deduced.)

DISCUSSION

In considering the regulation of the leucine biosynthetic pathway, it appears that end product inhibition plays a significant role. The loss of end product sensitivity was found to accompany the acquisition of resistance to trifluoroleucine, an analogue of leucine that inhibits growth of the wild strain. The mutant, 60615/fl-1, was found to excrete leucine into the medium, whereas the wild-type parent did not. In a separate study, 14 of 30 trifluoroleucine-resistant isolates were found to have α-IPM synthetases with altered sensitivity to L-leucine (H. Bussey, *personal communication*).

The regulation of enzyme levels through repression, derepression, or induction is less clear. The addition of leucine to the medium does lead to levels of the isomerase and dehydrogenase that are lower than the levels in minimal medium-grown cells. It seems likely that leucine itself plays a direct role (perhaps via some derivative which would be a corepressor). The alternative that the subsequent two enzymes are induced by α-IPM, a mechanism which seems to operate in *N. crassa* (2), should be considered in this system. However, strain 60615/fl-1, in which the leucine sensitivity of α-IPM synthetase in drastically reduced (but not absent), still exhibits a reduction in isomerase and dehydrogenase levels when grown in the presence of excess L-leucine. If the repression of these two enzymes occurred only via the quenching of α-IPM formation by leucine, no repression would be expected, since, in addition to the lack of inhibition of α-IPM synthetase, the enzyme was actually higher than the minimal medium level. Although not understood at this time, it may be significant that tryptone, which brought about in the wild type an even greater repression of the isomerase and dehydrogenase than did L-leucine alone, actually reduced the repression of the isomerase and abolished that of the dehydrogenase in strain 60615/fl-1.

TABLE 7. *Analysis of the progeny of a cross between an le-1 and an le-2 mutant*

Parental type		α-IPM synthetase activity[a]				Isomerase	Dehydrogenase			Other features[b]
Strain	Genotype	Minimal leucine	Limiting leucine	Excess leucine	Excess leucine plus threonine					
83026 (a)	*le*-2, *hi*-4		0.017	0.021	0.008	+	−			LSL, 0
85039 (α)	*le*-1, *tr*-5		0.068	0.068	0.034	−	+			HSL, 0
Progeny from cross								Apparent crossover on chromosomes III and VII		
Strain	Ascus	Phenotype								
89170	1	HI, LE, TR	0.051		0.043		(+)[c]	(+)	−	−	HSL, 0
89171	1	HI, le, TR		0.053	0.093		+	(−)	+	−	HSL, D
89172	1	hi, le, tr		0.033	0.016		−	(+)	+	−	LSL, R
89173	1	hi, le, tr		0.138	0.009		−	−	−	−	LSL, R
89210	2	HI, le, tr		0.074	0.074	0.036	−	(+)	−	−	HSL, 0
89211	2	HI, le, tr		0.070	0.029		−	(+)	−	−	LSL, R
89212	2	hi, le, TR		0.067	0.043		(+)	(−)	−	−	HSL, R
89213	2	hi, le, TR		0.047	0.048	0.029	+	(−)	−	−	HSL, 0
89222	3	hi, le, TR		0.016	0.025		−	(−)	−	+	LSL, D
89223	3	HI, LE, TR	0.049		0.058		(+)	(+)	−	−	HSL, D
89224	3	hi, LE, tr	0.020		0.022		(+)	(+)	+	+	LSL, 0
89225	3	HI, le, tr		0.079	0.071		−	(−)	+	−	HSL, 0

[a] Expressed as micromoles per milligram of protein per minute.
[b] HSL, (LSL) = high (low) synthetase on excess leucine; D, R, 0 = derepression, repression, or no significant effect due to excess leucine.
[c] Designations in parentheses are inferred results.

The effect of L-threonine on the synthesis of α-IPM synthetase is also unclear. It is the only amino acid tested that, added singly to the medium, led to repression in the wild type. Of interest is the fact that, whereas leucine enhances the threonine effect, L-valine and L-isoleucine abolish it. That the leucine effect on the level of the synthetase may not be a direct one is indicated by the observation that L-isoleucine is as effective and L-valine almost as effective in derepressing that enzyme as is L-leucine. Studies in progress are concerned with the effect of exogenous amino acids on the composition of the free amino acid pool and the degree of charging of the various amino acid acceptor ribonucleic acids. Perhaps the composition of these pools will be more readily correlated with the state of repression of the enzymes than is the composition of the medium.

Although the pattern of repression of α-IPM synthetase similar to that in the prototrophic strain 60615 was found in some of the auxotrophic strains examined, differences in level and in repressibility or derepressibility between one auxotroph and another were found. (At the present time, it appears that the factors determining the level of the synthetase are probably independent of the lesion affecting the other two specific enzymes in the pathway to leucine.) Whether any of the effects observed on either the isomerase or dehydrogenase level are directly related to a lesion in a structural gene affecting the other enzyme cannot be determined. In the case of the *le*-1 and *le*-2 lesions, the strain differences in enzyme levels of the remaining enzymes appear to be unrelated to the primary lesions. In some of the mutants employed in this study, the mutagenesis that led to the deficiencies in the leucine biosynthetic enzymes was perhaps accompanied by minor lesions in other genes that were not sufficient to lead to recognizable biochemical deficiencies when the simple criterion of growth or no growth is employed but which, nevertheless, markedly altered the cytoplasm from that of the wild type. It may be that further study of regulation of these enzymes should be confined to those mutants that appear to have the wild type pattern of response to leucine. Such a procedure

seems preferable to the use of prototrophic revertants of the various mutants as control strains for each mutant, a procedure employed by de Robichon-Szulmajster and Magee (7) to "normalize" the different cytoplasmic compositions of different yeast mutants.

Although the pattern of enzyme regulation presently appears complex, it may eventually be possible to define that pattern more simply than now appears possible. However, the observations that have been made on this system and the factors that have been considered during this study may be useful in guiding other workers interested in the regulation of biosynthetic enzymes in yeast. It is unfortunate that, at the present time, there are no clearly defined regulatory mutants. Therefore, it is not possible to provide evidence for a mechanism of control analogous to that postulated by Jacob and Monod (5) for bacteria, nor is it possible to postulate any alternative mechanism for the regulation of this system.

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