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Involvement of Threonine Deaminase in Repression of the Isoleucine-Valine and Leucine Pathways in *Saccharomyces cerevisiae*

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L-Threonine deaminase (L-threonine dehydratase [deaminating], EC 4.2.2.16) has been shown to be involved in the regulation of three of the enzymes of isoleucine-valine biosynthesis in yeast. Mutations affecting the affinity of the enzyme for isoleucine also affected the repression of acetohydroxyacid synthase, dihydroxyacid dehydrase, and reductoisomerase. The data indicate that isoleucine must be bound for effective repression of these enzymes to take place. In a strain with a nonsense mutation midway in *ilv 1*, the gene for threonine deaminase, starvation for isoleucine or valine did not lead to derepression of the three enzymes; starvation for leucine did. The effect of the nonsense mutation is recessive; it is tentatively concluded, therefore, that intact threonine deaminase is required for derepression by two of the effectors for multivalent repression, but not by the third. A model is presented which proposes that a regulatory species of leu $tRNA^{leu}$ is the key intermediate for repression and that threonine deaminase is a positive element, regulating the available pool of charged leu tRNA by binding it.

Regulation of amino acid biosynthesis has proven to be considerably more complex than regulation of carbohydrate catabolism. The multiplicity of genetic loci which affect repression of the enzymes of the histidine pathway provides an example (24). Regulation of branched amino acid pathways is even more complicated, and complete elucidation of the mechanism of regulation of the pathways has not yet been achieved.

The isoleucine-valine pathway is shown in Fig. 1. Its regulation has been studied in *Saccharomyces cerevisiae* (4, 6, 8, 19, 20), *Escherichia coli* (10, 12, 21-23), and *Salmonella typhimurium* (13). In each of these organisms, multivalent repression (12) has been shown to exist. Full repression occurs only in the presence of all three amino acids: isoluecine, valine, and leucine. Derepression of the enzymes of the pathway is achieved by starvation for any one of the end products, even in the presence of an excess of the other two (12).

We have previously presented evidence that threonine deaminase is involved in the regulation of synthesis of acetohydroxyacid synthase (AHA synthase) in *S. cerevisiae* (4). A role for biosynthetic enzymes in the regulation of the synthesis of their pathways has been suggested by several investigators (1, 3, 4, 7, 9, 13, 15-17, 26). We present in this report evidence that threonine deaminase is also involved in regulation of the dihydroxyacid dehydrase (DHA dehydrase) and reductoisomerase. Evidence is presented that threonine deaminase may be necessary for derepression of these enzymes.

MATERIALS AND METHODS

Strains. The strains used are listed in Table 1.

Media. Cells were grown on one of three media. Minimal medium was the M medium described previously (20). Repressing medium was the same as M medium except that L-isoleucine, L-valine, and L-leucine, each at 5×10^{-3} M, were present (20). Y medium contained 10 g of Difco yeast extract, 10 g of Difco peptone, and 30 g of glucose per liter (20). Supplements were added, when the strains required them for growth, at the following levels: L-leucine, 2 mM; L-isoleucine, 4 mM; DL-valine, 4 mM; L-histidine, 1 mM; L-tryptophan, 0.5 mM; adenine and uracil, each at 4 mM.

Preparation of cells. The preparation of cells for enzyme assays has been described previously (19).

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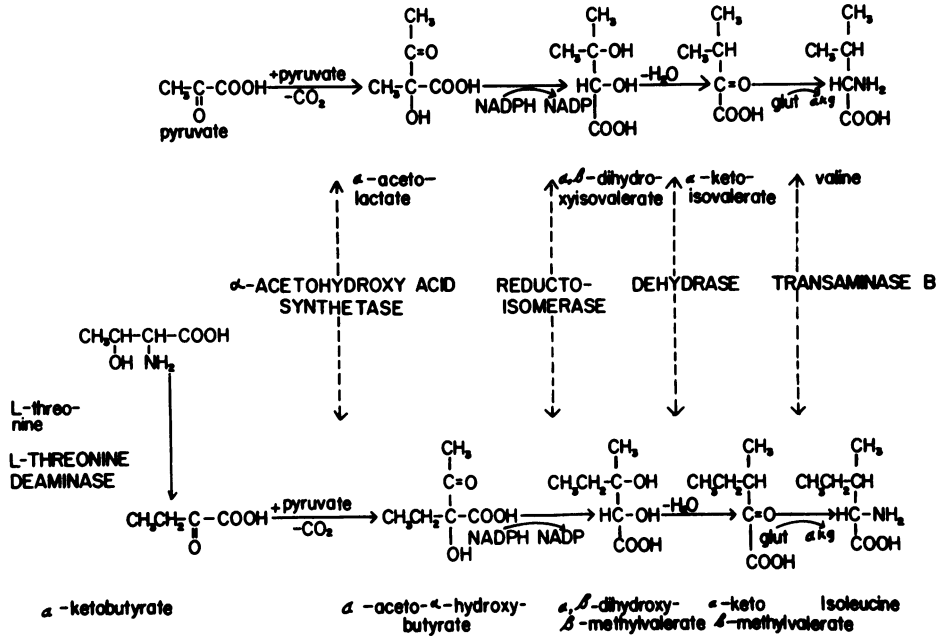

FIG. 1. *Biosynthetic pathway for isoleucine and valine in Saccharomyces cerevisiae.*

TABLE 1. *Yeast strains used*

No.	Description	Origin
MD9	α, *his1*	Isolated by Betz et al. (2)
MD11	a, *trp 2*	Isolated by Betz et al. (2)
MD13	a, *trp 2, leu 1*	Isolated by Kakar and Wagner (14)
M21	α, *his, ilv 1*	Isolated by Kakar and Wagner (14)
MD15	α, *his1, ilv 2*	Isolated by Kakar and Wagner (14)
M14	α, *his1, ilv 3*	Isolated by Kakar and Wagner (14)
1875	α, *his, ura, ade, ilv 4*	
M6	a, *trp 2, ilv 1* (threonine deaminase sixfold more sensitive to isoleucine inhibition [5])	Isolated by Kakar and Wagner (14)
MAR33	a, *trp 2, ilv 1^R* (threonine deaminase desensitized 100-fold to isoleucine inhibition [18])	Derived from Strain MD11 (2)
TIR-9	a, *his 1, ilv 1^R* (threonine deaminase desensitized 10-fold to isoleucine inhibition [18])	Derived from strain MD 9 (2)
D106-1a	a, *ade, ura, leu, ilv 1* (nonsense mutation midway in *ilv 1* [27])	Obtained from Zimmerman (27)
APB1	a, *ade, ura, leu, ilv 1 SUP*)contains suppressor for the D106-1a nonsense mutation)	Isolated by Bollon (4)
APB7	a, *ade, ura, leu* (revertant of D106-1a)	Isolated by Bollon (4)
D106-1a5	a, *ilv 1 nonsense, ilv 3*	Segregate isolated from diploid D1061a × M14
D106-1a6	a, *ilv 1 nonsense, leu 1*	Segregate isolated from diploid D106-1a × MD13

Cells were shaken overnight in medium Y at 30 C, inoculated into 100 ml of the appropriate medium in a 1-liter Erlenmeyer flask, and again shaken overnight at 30 C.

When the cultures reached a density between 10^7 and 5×10^7 cells/ml, the cells were harvested by centrifugation and washed once with 40 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.7). The cells were then suspended in 5 ml of buffer containing 25% glycerol, 0.5 M tris-hydrochloride (pH 7.7), 0.001 M ethylenediaminetetraacetate (EDTA), and 0.01 M dithiothreitol.

The cells were made permeable by the addition of 0.5 ml of toluene followed by rapid mixing on a Vortex mixer for 30 s and incubation for 90 s at 30 C. The cells were then centrifuged at 27,000 × *g* and were resuspended to give between 2 and 10 mg of protein per ml, measured as described below.

Cells were prepared for the threonine deaminase kinetic experiments as described previously (2). Cells were grown, centrifuged, and resuspended in the Tris-glycerol buffer as just described. Instead of the toluene treatment, cells were broken by shaking for 90 s with an equal volume of 0.5-μm glass beads on a Bronwill shaker cooled with CO_2. The extracts were centrifuged at 20,000 × *g* at −5 C for 15 min, and the supernatant fluid was passed through a Bio-Gel P-10 column (0.9 by 25 cm) equilibrated with Tris-glycerol-EDTA-dithiothreitol buffer. The eluate was used for the kinetic experiments.

Extracts for the α-isopropylmalate synthetase (α-IPM synthase) assay were prepared by a slight modification of the procedure of Satyanarayana, Umbarger, and Lindegren (25). Cells were grown and centrifuged as previously described but were washed with 0.01 M potassium phosphate buffer (pH 6.5) containing 2×10^{-3} M leucine. The washed cells were resuspended in 0.01 of the original volume of the same buffer and centrifuged at 15,000 rpm for 30 min. A 1-g amount of cells was usually ground to a fine paste with 2 g of alumina in a precooled mortar and pestle. After addition of 2 g more, grinding was continued for 5 min longer. The paste was extracted with 0.05 M potassium phosphate buffer containing 2×10^{-3} M leucine. The paste was centrifuged at 15,000 rpm for 30 min. Dialyzed crude extracts were prepared by dialyzing 5 ml of the extract against 500 ml of 0.01 M potassium phosphate buffer (pH 6.5) containing 2×10^{-3} M leucine for 1 h at 4 C with stirring.

Materials. Actidine (cycloheximide) was obtained from Calbiochem. Dihydroxyisovalerate was prepared by CycloChemical Co. Ethyl-α-acetyl-α-acetoxy-hydroxy-butyrate was obtained from Reef Chemical Co.

Enzymes assays. The assays for AHA synthase, DHA dehydrase, reductoisomerase, and protein have been described (20). The αIPM synthase activity was assayed according to Satyanarayana, Umbarger, and Lindegren (25).

RESULTS

Effect of mutations in ilv 1 on repression of the enzymes of the isoleucine-valine pathway. Strain MAR33, which is a strain containing threonine deaminase 100-fold less sensitive to feedback inhibition by isoleucine, was grown in minimal medium and in repressing medium. Also grown under the same conditions was strain MD11, which is the parent of strain MAR33 and contains a normal threonine deaminase. AHA synthase, reductoisomerase, and DHA dehydrase were assayed as described in Materials and Methods. Table 2 shows that the two strains gave equivalent values for the three enzymes on repressing medium. On minimal medium, however, strain MAR33 gave twofold higher values for all three enzymes. Thus, a sixfold derepression of the enzymes was obtained on minimal medium for strain MAR33, compared to threefold for strain MD11 on minimal medium.

We have previously shown (4) that isoleucine

TABLE 2. *Enzyme activities in ilv 1 mutant MAR33*

Medium	Strain	Specific activities[a]		
		Acetohydroxy-acid synthase	Dihydroxy-acid dehydrase	Reductoisomerase
Minimal	MD11	0.142 ± 0.025	0.371 ± 0.043	0.440 ± 0.058
	MAR33	0.315 ± 0.041	0.730 ± 0.064	0.931 ± 0.085
	TIR9	0.153 ± 0.021	0.351 ± 0.039	0.475 ± 0.061
	M6	0.171 ± 0.023	0.379 ± 0.041	0.483 ± 0.063
	MAR seg 1-10	0.329 ± 0.039	0.754 ± 0.069	0.954 ± 0.089
Repressing	MD11	0.060 ± 0.011	0.179 ± 0.029	0.251 ± 0.038
	MAR33	0.057 ± 0.009	0.183 ± 0.027	0.231 ± 0.035
	TIR9	0.059 ± 0.013	0.163 ± 0.027	0.233 ± 0.039
	M6	0.072 ± 0.018	0.183 ± 0.025	0.239 ± 0.041
	MAR seg 1-10	0.053 ± 0.012	0.178 ± 0.031	0.241 ± 0.040
Minimal plus 5 mM isoleucine	MD11	0.143 ± 0.021	0.378 ± 0.041	0.473 ± 0.059
	MAR33	0.147 ± 0.023	0.359 ± 0.037	0.436 ± 0.053
	MAR seg 1-10	0.141 ± 0.020	0.361 ± 0.039	0.461 ± 0.060

[a] Specific activities ± standard deviations expressed as micromoles of product per 20 min per milligram of protein. Average of five determinations for all strains except the segregants of MAR33 (MAR seg 1-10), for which the results from 10 segregants are averaged.

alone at 5 mM in minimal medium reduces AHA synthase activity in strain MAR33 to the parental minimal level. The same effect was seen for the other two enzymes, reductoisomerase and DNA dehydrase. Table 2 shows that the specific activities of all three enzymes from strain MAR33 grown in excess isoleucine were identical to the levels of strain MD11, regardless of whether the parent was grown on minimal medium plus or minus 5 mM isoleucine.

To eliminate the possibility that some accumulated intermediates may account for the increased depression, strain MAR33 was crossed with strain MD15 which lacks AHA synthase activity because of a mutation in *ilv2*, an *ilv 2 ilv* 1^R recombinant was isolated, and the enzymes were assayed. Regulation of the enzymes in the recombinant was similar to that in the parent, strain MAR33. As we have reported previously, the increased derepression of AHA synthase in strain MAR33 segregated with the mutation affecting isoleucine inhibition (4); in 10 cases examined, the other two enzymes showed parallel behavior (Table 2).

Two other strains were available containing threonine deaminases altered in kinetic properties: strain TIR 9 which contains a threonine deaminase 20-fold less sensitive to isoleucine inhibition, and strain M6 which contains a threonine deaminase 6-fold more sensitive to isoleucine inhibition (5). They were grown on minimal and repressing medium, and the three enzymes were assayed. For both strains, the minimal and repressed values for the three enzymes were comparable to wild type (Table 2).

Strain D106-la, which contains a nonsense mutation in *ilv 1*, and strain M21, which contains a missense mutation in *ilv 1*, were also grown on minimal and repressing medium. As can be seen in Table 3, the AHA synthase, DHA dehydrase, and reductoisomerase of strain D106-la appeared repressed after growth on minimal medium; the ratio of specific activity on minimal medium to that on repressing medium for all three enzymes was close to one. This agrees with our earlier finding for AHA synthase alone (4). Strain M21, on the other hand, gave the normal minimal to repressed ratios found in strain MD11.

When strain APB1, revertant of the strain bearing the *ilv 1* nonsense mutation, was grown on minimal and repressing medium and the three enzymes were assayed, normal minimal values were obtained. The same result was obtained when a suppressor for the nonsense mutation was present, strain APB7 (Table 3). As can be seen in Table 7, the specific activities of threonine deaminase from the suppressed strain APB7 and an *ilv* 1^+ strain, MD9, were similar.

Differential rates of synthesis of AHA synthase, DHA dehydrase, and reductisomerase in strains D106-1A, MAR33, M21, and M6. Although the regulatory response of all three enzymes in strain M6 appeared to be normal under our standard conditions for determination of specific activity, a more sensitive test was sought. We decided to look at the differential rates of enzymes synthesis under conditions of escape from repression. Strain M6 was grown in 150 ml of repressing medium to about 2×10^7 cells/ml, and the 150-ml culture was centrifuged, washed, and diluted 10-fold

TABLE 3. *Effect of a nonsense mutation in ilv 1 on the isoleucine-valine biosynthetic enzymes*

Medium	Strain	Specific activities[a]		
		Acetohydroxy-acid synthase	Dihydroxy-acid dehydrase	Reductoisomerase
Minimal	D106-1a	0.052 ± 0.011	0.120 ± 0.018	0.280 ± 0.031
	M21	0.130 ± 0.021	0.340 ± 0.037	0.490 ± 0.061
	APB7	0.125 ± 0.019	0.340 ± 0.034	0.480 ± 0.057
	APB1	0.120 ± 0.021	0.330 ± 0.038	0.451 ± 0.062
	D106 seg 1-6	0.057 ± 0.009	0.131 ± 0.021	0.271 ± 0.034
Repressing	D106-1a	0.062 ± 0.010	0.150 ± 0.028	0.230 ± 0.032
	M21	0.067 ± 0.013	0.162 ± 0.025	0.254 ± 0.034
	APB7	0.054 ± 0.012	0.182 ± 0.029	0.241 ± 0.031
	APB1	0.059 ± 0.010	0.140 ± 0.022	0.204 ± 0.031
	D106 seg 1-6	0.061 ± 0.005	0.149 ± 0.025	0.231 ± 0.037

[a] Specific acitivities ± standard deviations expressed as micromoles of product per 20 min per milligram of protein. Average of five determinations for all strains except the segregants of D106 seg 1-6), for which the results from six segregants are averaged.

into 1,000 ml of minimal medium containing 20 mM valine plus 2.5 mM isoleucine. Under these conditions, isoleucine is limiting because of competition with valine, probably at the level of permeation (20). Samples of 200 ml were taken at 0, 2, 4, 6, 8, and 10 h. The cells were made permeable, and the AHA synthase, DHA dehydrase, and reductoisomerase were assayed as described in Materials and Methods. It can be seen from Fig. 2 that the differential rate of synthesis of AHA synthase from strain M6 was biphasic with a low early differential rate of 0.05 unit/10^6 cells which, after slightly more than one generation, accelerated to 0.20 unit/10^6 cells. Strain M21 gave a constant differential rate of 0.20, fourfold higher than the early phase of strain M6. The doubling time for both strains was 7 h. Similar results were obtained when isoleucylglycine (1.5×10^{-3} M) was used as a source of limiting isoleucine instead of the isoleucine-valine combination. We interpret this lag as being due to the time required to reduce the isoleucine pools below the level required to saturate the threonine deaminase inhibition site. The maximal differential rate found here under derepressing conditions is very close to the rate found in strain MAR33 in minimal medium (4).

Differential rates of DHA dehydrase and reductoisomerase were determined for strains MAR33, M21, and D106-1a. The same procedure as described above was used with the exception that the cells upon coming from 150 ml of repressing medium were diluted into minimal medium containing required supplements for growth rather than into limiting isoleucine medium.

As can be seen in Fig. 3 and 4, the differential rates of synthesis of the two enzymes differed for the three strains tested. The reductoisomerase from strain MAR33 had a differential rate of formation of 1.76 units/10^6 cells, compared to 0.22 for strain D106-1a and 0.70 for strain M21 (Fig. 3). The three strains also differed in their differential rates of formation of DHA dehydrase as shown in Fig. 4: strain MAR33 gave a differential rate of 1.02 units/10^6, 17-fold higher than strain D106-1a and 5-fold higher than strain M21. These results are similar to those obtained for the differential rates of AHA synthase synthesis in the same three strains under the same conditions. Strain MAR33 gave a differential rate of 0.22 unit/10^6 cells, 10-fold higher than that of strain D106-1a, which was 0.02 unit/10^6 cells, and 3.7-fold higher than the 0.06 unit/10^6 cells for strain M21 (4).

To test whether the differential rates for all three enzymes actually represented synthesis and not activation of the enzymes, 10 μg of

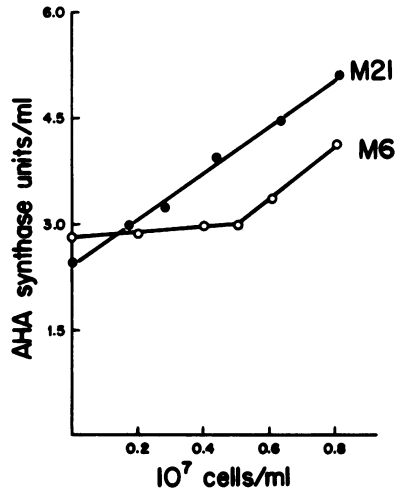

FIG. 2. *Differential rate of acetohydroxyacid synthetase synthesis.* (○) *Strain M6;* (●) *strain M21.*

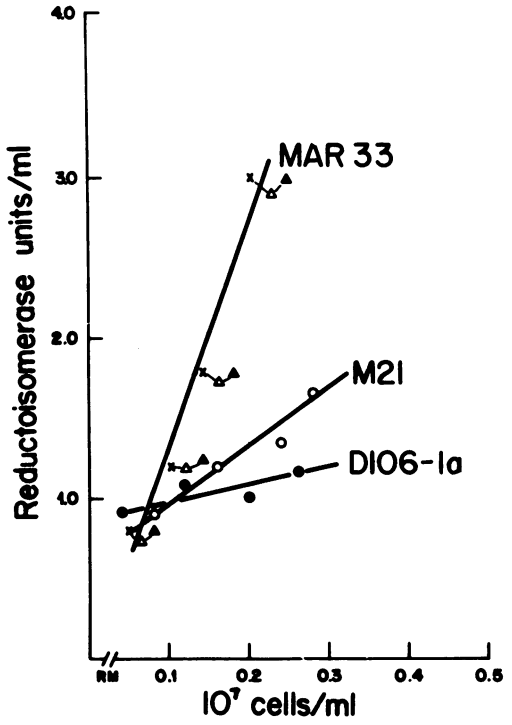

FIG. 3. *Differential rate of reductoisomerase synthesis in minimal medium after shifting from repressing medium (RM). Symbols:* ●, *strain D106-1a;* ○, *strain M21;* ×, *strain MAR33;* △ *and* ▲, *reductoisomerase synthesis 2 and 4 h, respectively, after the addition of 10 μg of cycloheximide/ml to the strain MAR33 cultures.*

cycloheximide/ml was added to the cultures at different times after the shift into minimal medium; 100-ml samples were removed at 2- and 4-h intervals, and the enzymes were assayed. As can be seen in Fig. 3 and 4, there was no increase in enzyme activity after the cycloheximide has been added, implying that protein synthesis is required for the increase in enzyme activity. The possible involvement of an activator that requires protein synthesis cannot be ruled out.

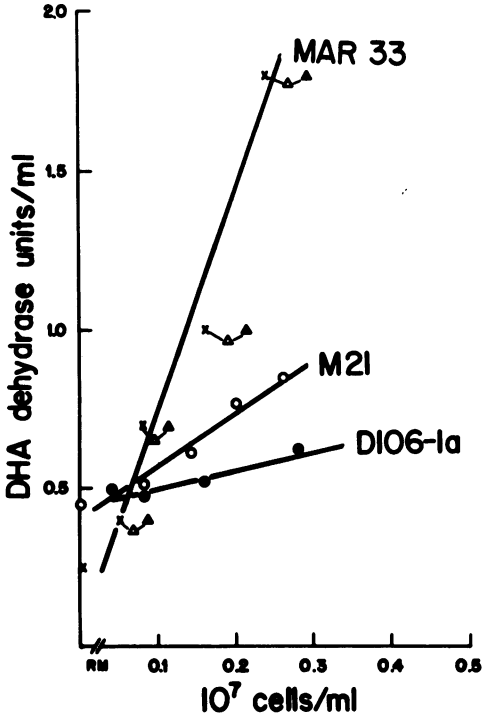

FIG. 4. *Differential rate of dihydroxyacid (DHA) dehydrase synthesis in minimal medium after shifting from repressing medium (RM). Symbols: ●, strain D106-1a; ○, strain M21; ×, strain MAR33; Δ and ▲, DNA dehydrase synthesis 2 and 4 h, respectively, after the addition of 10 μg of cycloheximide/ml to the strain MAR33 cultures.*

Differential involvement of leucine in multivalent repression. Since AHA synthase, reductoisomerase, and DHA dehydrase from strain D106-1a remained repressed when the strain was grown on minimal medium, we looked at derepression via limitation for any one of the effectors of multivalent repression. Limitation for isoleucine was achieved by growing strain D106-1a, which requires isoleucine for growth, on medium containing 0.5 mM isoleucine, 20 mM valine, and other supplements needed for growth. Isoleucine limitation was achieved by inhibition of isoleucine uptake by valine (20). Limitation for leucine was achieved in a similar fashion, strain D106-1a being grown on medium containing 0.5 mM leucine plus 20 mM valine. Limitation for valine was achieved by growing strain D106-1a5, which contains the nonsense lesion in *ilv 1* and a lesion in *ilv 3*, on medium containing 0.5 mM valine plus 25 mM isoleucine (20). Strain M21 (*ilv 1*) was also grown under limiting isoleucine conditions, and strain M14 (*ilv 3*) and strain M13 (*leu 1*) were grown under limiting valine and leucine conditions, respectively. The three enzymes were assayed as described in Materials and Methods. As can be seen in Table 4, none of the three enzymes was derepressed in D106-1a under conditions limiting for isoleucine or valine, but all were partially derepressed under limiting leucine conditions. This agrees with the earlier finding that AHA synthase from strain D106-1a is not dere-

TABLE 4. *Derepression of D106-1a on limiting leucine*

Medium	Strain	Specific activities[a]		
		Acetohydroxy-acid synthase	Dihydroxy-acid dehydrase	Reductoisomerase
Isoleucine limiting	M21 (*ilv 1*)	0.320 ± 0.037	0.710 ± 0.063	0.920 ± 0.084
	D106-1a	0.060 ± 0.011	0.133 ± 0.035	0.221 ± 0.034
Valine limiting	M14 (*ilv 3*)	0.320 ± 0.041	–	0.859 ± 0.079
	D106-1a5 (*ilv 1* nonsense, *ilv 3*)	0.052 ± 0.012	–	0.234 ± 0.031
Leucine limiting	MD13 (*leu 1*)	0.142 ± 0.021	0.448 ± 0.051	0.520 ± 0.061
	D106-1a6 (*ilv 1* nonsense, *leu 1*)	0.120 ± 0.021	0.420 ± 0.049	0.498 ± 0.053

[a] Specific activities ± standard deviations expressed as micromoles of product per 20 min per milligram of protein. Average of five determinations.

pressed under limiting isoleucine or valine conditions, but is partially derepressed under conditions limiting for leucine (4).

Since only limiting leucine gave partial derepression of the three enzymes of strain D106-1a, the first enzyme of the leucine biosynthetic pathway, αIPM synthase, was examined in strains MAR33, MD11, and D106-1a. One-liter cultures of strains MAR33 and D106-1a were prepared from overnight Y medium cultures. The cells were grown in minimal medium, minimal plus 5 mM L-leucine and 5 mM L-threonine, and minimal plus 5 mM L-leucine, 5 mM L-isoleucine, and 5 mM L-threonine. The cultures were centrifuged, the cells were broken, and the extract was dialyzed according to the procedure of Satyanarayana and Umbarger (25). As can be seen in Table 5, the specific activity of αIPM synthase from strain MAR33 grown under minimal conditions was approximately twice that of the enzyme from strain MD11 grown under the same conditions. The repressed levels were the same for both strains. In a medium containing threonine and leucine, slightly higher values of the enzymes were obtained for strain MAR33 than for strain MD11. As can be seen, the addition of 5 mM isoleucine to the threonine- and leucine-containing medium resulted in the reduction of the specific activity to that obtained with strain MD11 under the same conditions. The one *ilv* 1^R segregant tested gave results identical to strain MAR33. The enzyme from strain D106-1a grown under minimal conditions appeared repressed. The minimal value was obtained in a strain D106-1a revertant also grown under minimal conditions. These results are similar to the findings with the isoleucine-valine enzyme.

Requirement of ilv 1 gene product (threonine deaminase) for derepression of the isoleucine-valine enzymes. Since strain D106-1a gave repressed enzyme values on minimal medium, it was desirable to examine whether the absence of completed *ilv 1* gene product accounted for the lack of derepression. Several diploids were constructed. Strain D106-1a was mated with three different *ilv* 1^+ strains, each auxotrophic for one of the three isoleucine-valine enzymes studied here. As previously reported, when strain D106-1a was mated with strain MD15 which is *ilv* 1^+, *ilv 2*, and the diploid was grown on minimal and repressing medium, the AHA synthase gave a normal minimal to repressed ratio. This can be seen in Table 6. When strain D106-1a was mated with strain M14 which is *ilv* 1^+, *ilv 3*, or with strain 1875 which is *ilv* 1^+, *ilv 4*, the same kind of result was obtained. Partial derepression of DHA dehydrase was obtained when strain D106-1a × M14 was grown on minimal medium, and partial derepression of reductoisomerase was obtained when strain D106-1a × 1875 was grown on minimal medium. It therefore seems that the presence of complete threonine deaminase as furnished by any one of the *ilv* 1^+ strains mentioned above is needed for the derepression of the enzymes examined. Taken with the results on the revertant and the suppressor strains, this result provides firm evidence that the enzymes examined in D106-1a can be normally derepressed in the presence of complete threonine deaminase.

Since Zimmerman et al. (28, 29) have shown intragenic complementation in diploids containing *ilv* 1^- and *ilv* 1^+ genes, it seemed possible that such complementation might obscure the dominance-recessivity tests just described. If complementation occurred between the strains D106-1a and MD9 *ilv 1* products in the diploid of the two strains, a hybrid enzyme would be made containing the incomplete fragments from the strain D106-1a *ilv 1* gene. In such a case, the hybrid threonine deaminase might be active, giving a heterogeneous population of threonine deaminase in the diploid. To test such a possibility, extracts were prepared as described in Materials and Methods from strain MD9 and the diploid strain D106-1a × MD9. Samples containing 100 μg of

TABLE 5. *α-Isopropyl malate synthetase specific activity in ilv 1 and ilv* 1^+ *strains*[a]

Medium[b]	Strains				
	MD11	MAR33	D106-1a18	MAR33 seg	APB-7
Minimal	0.182 ± 0.20	0.374 ± 0.044	0.111 ± 0.010	0.358 ± 0.038	0.202 ± 0.018
Repressing + thr	0.102 ± 0.010	0.056 ± 0.010	0.122 ± 0.010	0.102 ± 0.012	0.098 ± 0.010
Thr + leu	0.126 ± 0.010	0.182 ± 0.016	—	0.190 ± 0.016	0.134 ± 0.014
Thr + leu + iso	0.134 ± 0.014	0.106 ± 0.008	—	0.118 ± 0.012	0.122 ± 0.014

[a] Enzyme specific activity is expressed as micromoles of product per 20 min per milligram of protein ± standard deviation. Average of five determinations.

[b] Thr, L-threonine; leu, L-leucine; iso, L-isoleucine. Each amino acid concentration is 5×10^{-3} M.

protein were put on polyacrylamide gels and subjected to electrophoresis at 3 A/tube for 8 h. The gels were then stained for threonine deaminase activity according to the method of Datta (11). As can be seen in Fig. 5, only one band of activity was observed for each strain. To substantiate the conclusion that no active hybrid enzyme is formed, 100-ml cultures of the haploid and diploid strains were grown in minimal medium to 5×10^8 cells/ml and the extracts were prepared as described previously (2). As can be seen in Fig. 6, the isoleucine inhibition curves for both haploid and diploid strains were similar. The Hill numbers for isoleucine for the enzymes of both strains are quite similar, being between 2 and 3 for strain MD9 and for strain D106-1a × MD9. Differences in the kinetics of isoleucine inhibition of threonine deaminases from wild-type strains and diploid strains of wild type and *ilv 1* auxotrophs have been observed (28). Although no active hybrid threonine deaminase is formed in the diploid, the tests just reported do not eliminate the possibility of inactive hybrid enzyme.

To test for inactive hybrid enzyme, the specific activity for threonine deaminase was measured for the haploid strain MD9 and the diploid strains D106-1a × MD9, MD11 × M21, and MD11 × MD9. As can be seen in Table 7, the diploid strain D106-1a × MD9 showed a specific activity for threonine deaminase of 1.53, not very different from 1.65 for strain MD9 and 1.78 for strain MD11 × MD9. The specific activity observed in the MD11 × M21 strain was 1.43. If there is an inactive hybrid enzyme formed, it does not substantially reduce the specific activity of the D106-1a × MD9 diploid extract; however, it must be born in mind that the diploid containing two intact *ilv 1* genes does not show a specific activity significantly greater than the diploid containing one intact *ilv 1* gene.

DISCUSSION

This report presents further evidence supporting our observation that threonine deaminase is involved in multivalent repression (4). We have shown that strain MAR33, a strain containing a mutation in *ilv 1* which renders threonine deaminase 100-fold less sensitive to isoleucine inhibition, gives derepressed values for AHA synthase under minimal conditions. Strain D106-1a, a strain containing a nonsense mutation in the middle of the *ilv 1* gene, gives repressed values for AHA synthase under minimal conditions. These observations are further substantiated by the data presented in this report, namely, that in strain MAR33 and D106-1a the regulatory responses of DHA dehydrase and reductoisomerase show behavior parallel to the AHA synthase under the same conditions. The altered regulation of synthesis of the three enzymes from strain MAR33 and D106-1a can best be seen in Table 8, which gives a summary of the differences among the three strains containing different *ilv 1* alleles. As can be seen in Tables 2 and 3, the altered regulatory behavior of synthesis of the three enzymes appears to segregate with the *ilv 1* mutation in the respective strains.

It can be seen in Tables 2 and 4 that the three enzyme levels obtained from MAR33 cells grown in minimal medium are similar to enzyme levels from cells grown under conditions limiting for any of the three end products: isoleucine, leucine, or valine. Since the enzyme levels obtained under limiting conditions are

TABLE 6. *Recessivity of ilv 1 nonsense effect on repression*

Medium	Diploid strain	Specific activities[a]		
		Acetohydroxy-acid synthase	Dihydroxy-acid dehydrase	Reductoisomerase
Minimal	D106-1a (*ilv 1* nonsense)			
	× MD9 (*ilv 1*$^+$)	0.123 ± 0.021	0.370 ± 0.041	0.420 ± 0.063
	× MD15 (*ilv 2*)	0.115 ± 0.013	—	—
	× M14 (*ilv 3*)	—	0.330 ± 0.039	—
	× 1875 (*ilv 4*)	—	—	0.660 ± 0.074
Repressing	× MD9	0.059 ± 0.009	0.133 ± 0.018	0.250 ± 0.029
	× MD15	0.045 ± 0.007	—	—
	× M14	—	0.0114 ± 0.015	—
	× 1875	—	—	0.275 ± 0.031

[a] Specific activities are expressed as micromoles of product per 20 min per milligram of protein ± standard deviation. Average of five determinations.

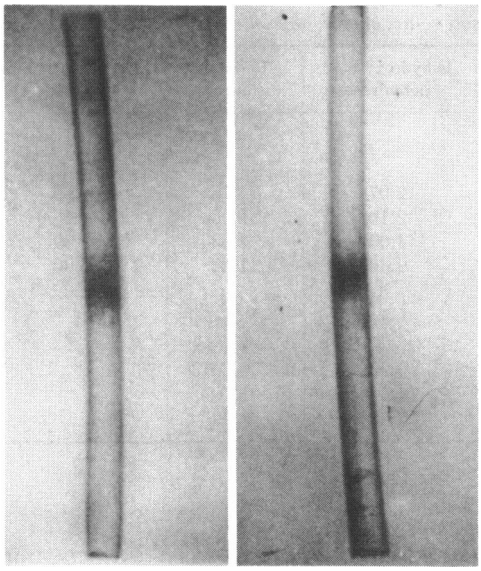

FIG. 5. *Acrylamide gels of threonine deaminase activity from haploid strain MD9 (left) and diploid D106-1a × MD9 (right).*

maximal and therefore represent the maximal degree of derepression obtained (20), strain MAR33 grown in minimal medium appears to be maximally derepressed for the three enzymes tested.

The absence of increased levels of activity of the three enzymes from strain MAR33 grown in minimal medium containing 10 μg of cycloheximide/ml indicates that protein synthesis is necessary for the observed increase in activity, implying that enzyme synthesis is being measured. It is possible that the increased activity is due to activation of the enzymes by some factor which itself requires protein synthesis, though there is at present no evidence for such a factor in yeast.

Although the nature of the involvement of threonine deaminase in multivalent repression remains obscure, the isoleucine inhibition site is probably involved. This conclusion follows from the nature of the *ilv 1* mutation in strain MAR33, which seems to affect only those kinetic properties relating to isoleucine inhibition, and from the fact that the specific activities of all three enzymes from strain MAR33 are reduced to the wild-type minimal values when 5 mM isoleucine is present. Although strain M6 shows altered regulation of the AHA synthase (Fig. 2), the complex alteration of kinetic properties of the strain M6 threonine deaminase prevents the drawing of further conclusions about site involvement in multivalent repression. It does appear that threonine deaminase does not have to be catalytically active to participate in multivalent repression since, in several cases examined, strain M21 and other *ilv 1* auxotrophs showed normal regulation for the isoleucine-valine enzymes.

Our previous suggestion that leucine involvement in multivalent repression is different from that of isoleucine and valine is made more likely by the fact that only limiting leucine conditions result in derepression of all three enzymes in strain D106-1a (Table 4). It is also interesting that the regulation of the biosynthesis of the first leucine enzyme αIPM synthase, is altered in strains MAR33 and D106-1a. The increased activity of the αIPM synthase from strain MAR33 grown on minimal medium seems to be a significant effect. The slight decrease in activity when isoleucine is added to minimal medium containing leucine and threonine is not pronounced enough to be considered significant, although the effect is in the expected direction. Since it has been shown

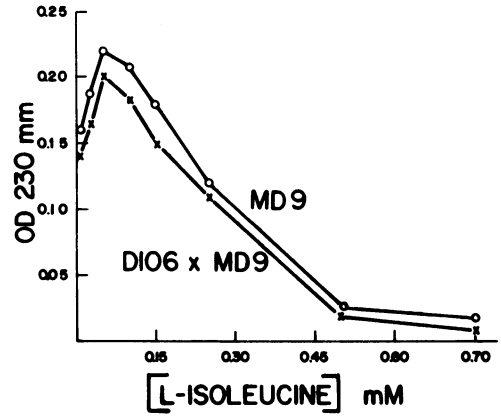

FIG. 6. *Effect of L-isoleucine on the activity of threonine deaminase from strain MD9* (○) *and strain D106-1a × MD9* (×).

TABLE 7. *Threonine deaminase specific activity in different yeast strains*[a]

Strain	Specific activity[b]
MD9	3.30 ± 0.24
D106-1a × MD9	3.06 ± 0.30
MD11 × M21	2.86 ± 0.26
MD11 × MD9	3.56 ± 0.30
APB7	3.20 ± 0.20

[a] Strains were grown in minimal medium.

[b] Specific activity is expressed as micromoles of product per 20 min per milligram of protein ± standard deviations. Average of five determinations.

TABLE 8. *Summary of the effects of ilv 1 mutation on the isoleucine, valine, and leucine biosynthetic enzymes*

Strain and genotype	Acetohydroxy-acid synthase	Dihydroxy-acid dehydrase	Reducto-isomerase	Isopropyl malate synthase
Ratio of specific activity[a] on minimal medium to specific activity on repressed				
MD11 (*ilv 1*$^+$)	2.37	2.07	1.75	1.79
M21 (*ilv 1*$^-$)	1.94	2.10	1.93	—
MAR33 (*ilv 1*^{R})	5.52	4.00	4.03	3.89
D106-1a (*ilv 1* ochre)	0.84	0.80	1.22	0.90
Differential rates of synthesis[b] in minimal medium				
M21	0.06	0.20	0.70	
MAR33	0.22	1.02	1.76	
D106-1a	0.02	0.06	0.22	

[a] Micromoles of product per 20 min per milligram of protein.
[b] Micromoles of product per 20 min per 10^6 cells.

previously (25) as well as in this report that leucine and threonine but not isoleucine and valine are required for repression of the leucine enzymes, the effect upon the αIPM synthase of strain MAR33 and strain D106-1a is surprising. One possible conclusion is that multivalent repression of the isoleucine-valine enzymes and repression of the leucine enzyme are mediated by a common factor. A possible factor that we previously proposed (4) was a regulatory species of leucine transfer ribonucleic acid ($tRNA^{leu}$). Evidence for such a species has been obtained in this laboratory (Magee, P. T., submitted to J. Bacteriol. 1972).

To test whether the regulatory effect of the *ilv 1* mutation in strain D106-1a was dominant or recessive, diploids were made between strain D106-1a and several strains containing a normal *ilv 1* locus. As is shown in Table 5, the altered regulation in strain D106-1a appears to be recessive, implying that the threonine deaminase, only a fragment of which is synthesized in D106-1a owing to the nonsense mutation in the middle of the *ilv 1* gene, may be required for derepression. One difficulty with the interpretation of the diploid data is the possibility of intragenic complementation. It is conceivable that strain D106-1a threonine deaminase is a repressor with isoleucine and valine binding sites missing, but with a site for leucyl-tRNA remaining. Such a model explains the strain D106-1a effect; the derepressed state in the diploid could be explained by dilution of the super repressor fragment by complementation. Zimmerman and colleagues (28, 29) have shown that, in diploids containing *ilv 1* and *ilv 1*$^+$ alleles, intragenic complementation occurs, resulting in hybrid threonine deaminase. As reported in this paper, when extracts from strains MD9 and MD9 × D106-1a were subjected to polyacrylamide electrophoresis, only one band of threonine deaminase activity was obtained. All of the enzyme activity in such extracts was normally sensitive to isoleucine, whereas Zimmerman et al. were sometimes able to show the existence of hybrid enzyme by altered isoleucine sensitivity. It thus seems unlikely that active hybrid enzyme is formed in diploids of strain D106-1a × MD9. However, the same workers showed that strain D106-1a could complement (negatively) certain temperature-sensitive alleles. Because of the lack of correspondence between gene dosage and specific activity (Table 7), one cannot rule out the possibility that inactive hybrid enzyme may be formed. Another approach will be to examine the three enzymes in strains containing proximal nonsense mutations, that is, close to the N-terminal end, so that little or no threonine deaminase is made.

Two models might explain threonine deaminase involvement in derepression. One possibility would be that threonine deaminase induces the synthesis of the enzymes either at the transcriptional or the translational level. In the presence of repressing concentrations of isoleucine and valine (both of which appear to have sites on threonine deaminase) and leucine (which may bind to threonine deaminase via $leucyl\text{-}tRNA^{leu}$), the inducer would be inactivated. This model has the shortcoming that the ability of strain D106-1a to be derepressed via leucine can only be explained by attributing special properties to the fragment provided.

Another model, proposed by A. Bollon and presented previously (4), is based on the data

for strains MAR33 and D106-1a presented in this paper and the finding of Hatfield and Burns concerning the binding of leucyl-tRNA to an immature form of threonine deaminase in *Salmonella typhimurium* (13).

This model proposes that threonine deaminase regulates repression of the pathway secondarily, by binding a regulatory species of leucyl-tRNA^{leu} which is itself necessary for repression. Since all three end products, isoleucine, valine, and leucine, are needed for multivalent repression, leucine would be necessary to charge fully the regulatory species of leucyl-tRNA^{leu}, and isoleucine and valine would prevent the binding of the leucyl-tRNA^{leu} to threonine deaminase, making the tRNA available for repression. In the absence of either isoleucine or valine, both of which have sites on yeast threonine deaminase, the enzyme would bind the regulatory species of leucyl-tRNA^{leu} and derepression would occur. According to this model, leucyl-tRNA would act as a negative control element, and threonine deaminase would function as a positive element by regulating the available pool of the charged leucyl-tRNA.

Consistent with the last model are the results of the αIPM synthase in strains MAR33 and D106-1a (Table 5), the derepression of the enzymes of strain D106-1a by only leucine limitation (Table 4), and the recent finding in this laboratory of a leucyl-tRNA^{leu} species involved in multivalent repression (Magee, submitted to J. Bacteriol). It might be possible to distinguish between the two models discussed here since the binding of leucyl-tRNA^{leu} to threonine deaminase should occur less efficiently in the presence of isoleucine and valine.

Although none of the mentioned models explains the involvement of isoleucyl tRNA synthetase in multivalent repression (18), it is possible that the involvement of the synthetase is indirect via the regulation of threonine deaminase. In yeast, threonine deaminase does not appear to be under multivalent control but appears to be repressed only by isoleucine and threonine (6, 20).

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