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Involvement of Threonine Deaminase in Multivalent Repression of the Isoleucine–Valine Pathway in *Saccharomyces cerevisiae*

(leucine/feedback/nonsense/missense)

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ABSTRACT A strain (MAR33) of *Saccharomyces cerevisiae* containing a threonine deaminase [L-threonine hydrolyase (deaminating) EC 4.2.1.16] with decreased feedback sensitivity has been shown to have a specific activity of acetohydroxy acid synthetase higher than that of the parent strain (MD11) when both are grown on minimal medium. When strain MAR33 is grown on minimal medium supplemented only with isoleucine, the specific activity of the synthetase is reduced to that found in the parent strain. Another strain, D106-1A, contains a nonsense mutation in the middle of the gene for threonine deaminase. When this strain is grown on minimal medium containing appropriate supplements (which include a nonrepressing concentration of isoleucine), or on minimal medium supplemented with isoleucylglycine (which acts as a limiting source of isoleucine), acetohydroxy acid synthetase remains repressed. Leucine limitation causes partial derepression. With the reversion of the nonsense mutation, either intragenically or via a suppressor for the mutation, partial derepression of the synthetase returns. When D106-1A is diploidized with either M15, a mutant lacking the synthetase, or MD9, a strain containing the enzyme, normal, partially derepressed, values for this enzyme are found. This indicates that threonine deaminase is necessary for derepression, and that it possibly acts as an inducer.

The involvement of a biosynthetic enzyme of a specific amino-acid pathway in repression of other enzymes of the same pathway has been considered by several investigators (1–7). Our possession of several mutants affected in the allosteric properties of threonine deaminase permitted us to explore the involvement of this enzyme in the repression of acetohydroxy acid synthetase in *Saccharomyces cerevisiae*. Threonine deaminase and acetohydroxy acid synthetase are the first two enzymes in the isoleucine–valine biosynthetic pathway. The properties of these two enzymes in *S. cerevisiae* have been examined (8–10). Regulation of the isoleucine–valine pathway has been examined in *S. cerevisiae* (11, 12), in *Escherichia coli* (13–15), and in *Salmonella typhimurium* (16). All of these organisms show multivalent repression of at least some of the enzymes of the pathway. Full repression occurs only in the presence of all three amino acids: isoleucine, valine, and leucine. Starvation for one of these compounds causes derepression of the enzymes of the pathway, even in the presence of an excess of the other two (11).

METHODS AND RESULTS

Threonine deaminase mutated in its allosteric properties and the repression of acetohydroxy acid synthetase

MAR33, a haploid strain of *S. cerevisiae*, contains a single mutation that renders threonine deaminase 100-fold less sensitive to isoleucine inhibition than the enzyme of MD11 (the parent strain) (17). Parent and mutant strains were grown in minimal and repressing medium, which is minimal medium supplemented with isoleucine, valine, and leucine, each at 5 mM. The cultures were made permeable with toluene, and the acetohydroxy acid synthetase ("synthetase"), which catalyzes the first common step in the synthesis of isoleucine and valine, was assayed (11). As shown in Table 1, when MAR33 is grown in minimal medium, the increase in the specific activity of synthetase is 5.7-fold higher than its repressed level, whereas that of MD11 is only 2.4-fold higher than its repressed level. According to an analysis by Student's t test, this difference between the means of the MAR33 and MD11 values is significant at a 99% confidence level. When the strains are grown in minimal medium supplemented with (only) isoleucine at 5 mM, which is the concentration used in repressing medium, the specific activity of the synthetase is the same for both MAR33 and MD11 (Table 1).

To test whether the effect segregated with the gene conferring feedback resistance, strain MAR33 was crossed with M14, a strain lacking dihydroxy acid dehydrase, the enzyme after the synthetase in the isoleucine–valine pathway (18), and the resulting diploid was allowed to sporulate. Ten random spores of this cross were analyzed, two of which had inherited the normal threonine deaminase. The increased specific activity of synthetase in minimal medium and the mutation affecting the isoleucine inhibition of threonine deaminase of MAR33 segregated together in all cases examined. Thus, it appears that the altered regulatory behavior is a result of the threonine deaminase mutation or is closely linked to it. No difference was observed between the spores auxotrophic for isoleucine and valine and the prototrophs, indicating that the presence of a mutation in the third step of the biosynthetic pathway did not affect the regulatory behavior of the synthetase, thereby reducing the possibility that accumulated intermediates of the pathway act as inducers of the enzyme.

A nonsense mutation in the threonine deaminase gene affects the levels of acetohydroxy acid synthetase

Strain D106-1A, which has a nonsense mutation in the middle of *ilv1*, the gene for threonine deaminase (29), and is in addition a leucine auxotroph, was grown in the following media: (*a*) repressing medium, (*b*) minimal medium containing nonrepressing concentrations of L-leucine and L-isoleucine, and (*c*) medium containing a nonrepressing concentration of leucine and a limiting supply of isoleucine, which was fur-

nished either by supplementing the culture with 1.5 mM isoleucylglycine or by inhibition by valine of isoleucine uptake (11). M21, a missense mutant of *ilv1*, was grown under the same conditions. Synthetase was assayed as before. As can be seen in Table 1, D106-1A gives neither partial derepression of synthetase when grown on minimal medium containing the growth requirements, nor complete derepression when grown on limiting isoleucine. The lack of derepression by D106-1A under these conditions contrasts with the derepression of synthetase obtained when M21 is grown under the same conditions. Strain M6, whose threonine deaminase shows a greatly reduced affinity for threonine, leading to isoleucine auxotrophy (30), has been shown to behave like M21 (11). Leucine may be limited in an auxotroph of *S. cerevisiae* by the addition of an excess of valine to leucine-containing medium. The valine competes for the permease and slows leucine uptake. When D106-1A is grown in medium that is limiting for leucine (20 mM valine + 0.15 mM leucine), but contains adequate isoleucine for growth, then the synthetase is partially derepressed.

TABLE 1. *Specific activity of acetohydroxy acid synthetase in* ilv1 *mutants of* Saccharomyces cerevisiae

Strain	Conditions	Acetohydroxy acid synthetase: Specific activity	Acetohydroxy acid synthetase: Standard deviation
Haploid strains			
MAR 33	M	0.315	±0.099
	R	0.055	±0.004
	M + Ile (5 mM)	0.159	±0.042
MD 11	M	0.142	±0.035
	R	0.060	±0.004
	M + Ile (5 mM)	0.143	±0.036
D106-1A	M	0.063	±0.008
	R	0.066	±0.009
	M + Isoleucylglycine	0.070	±0.010
	M + 0.5 mM Ile, 20 mM Val	0.052	±0.005
	M + 0.5 mM Leu, 20 mM Val	0.150	±0.020
M21	M	0.162	
	R	0.053	
	M + Isoleucylglycine	0.305	
	M + 0.5 mM Ile, 20 mM Val	0.330	
APB 1 to 6 (*ilv1*$^+$)	M	0.120	±0.020
	R	0.045	±0.015
APB 7 (*ilv1 SUP*)	M	0.125	
	R	0.065	
Diploid strains			
D106-1A × M15	M	0.083	
	R	0.032	
D106-1A × MD9	M	0.123	
	R	0.059	
M15 × MD13	M	0.076	
	R	0.040	
M2 × YM33	M	0.074	
	R	0.035	

MAR33 contains a mutation in the *ilv1* gene that renders the threonine deaminase 100-fold less sensitive to isoleucine inhibition. MD11 is the parent strain of MAR33. D106-1A contains a nonsense (29) and M21 a missense mutation (18) in *ilv1*. APB 1 to 6 are six *ilv1*$^+$ revertants of D106-1A, and APB 7 contains a suppressor for the D106-1A nonsense mutation. M15 is *ilv1*$^+$ *ilv2*$^-$, MD13 is *ilv1*$^+$ *ilv2*$^+$ *leu1*$^-$, and YM33 has a missense mutation in *ilv1*. M and R stand for minimal and repressed conditions, respectively. The preparation of cultures and the acetohydroxy acid synthetase assay have been described (11).

To determine whether the unusual regulatory behavior of synthetase found in D106-1A was in fact due to the nonsense mutation in the threonine deaminase gene, revertants of the mutation were obtained and examined for derepression. Six revertants were assayed for acetohydroxy acid synthetase and all six gave specific activities similar to the wild type when grown on minimal medium. An average value for all six revertants is given in Table 1, line 5. The strain containing the nonsense mutation also shows normal partial derepression on minimal medium when a suppressor for the mutation is present. This suppressor was found in MAR33, which was crossed with D106-1A. *Ilv*$^+$ progeny were tested and some were found to give *ilv*$^-$ in 25% yield when back-crossed with an *ilv*$^+$ strain, MD9. The level of enzyme in such a suppressed mutant is shown in Table 1, line 6.

Since the nonsense mutation in the threonine deaminase seemed responsible for the lack of derepression of synthetase, the question arose as to whether the effect was dominant or recessive. To answer this question, two diploids were constructed, one of D106-1A and M15, and one of D106-1A and MD9. M15 is an *ilv2* mutant that lacks synthetase activity, but has a normal threonine deaminase; MD9 has a normal complement of isoleucine–valine biosynthetic enzymes. The synthetase was assayed in cultures grown under minimal and repressed conditions. The synthetase of both diploids grown in minimal medium gave partially derepressed values similar to those obtained with the wild-type haploid strain and with the control diploids M15 × MD13 and M2 × YM33, indicating that the presence of the normal threonine deaminase, as supplied by M15 or MD9, permitted the derepression of the acetohydroxy acid synthetase from D106-1A (Table 1, Section 2). The nonsense mutation is thus recessive in its effect upon regulation. MD13 is a *leu*$^-$ auxotroph, M2 is another *ilv2* mutant, and YM33 is an *ilv1* mutant.

The values obtained in Table 1 were found in cultures narvested in mid-exponential phase. It seemed possible that the effect of the threonine deaminase mutations might be to alter the physiological state of the cells, with a consequent effect on the specific activity of synthetase. We therefore determined the differential rate of synthetase synthesis in cultures of MAR33, D106-1A, and M21 after dilution from repressing to minimal medium. 150-ml cultures were grown overnight under repressed conditions to about 2×10^7 cells/ml and were then centrifuged, washed, and diluted 10-fold into 1000 ml of minimal medium. 200-ml samples were taken at 0, 1.5, 3, 4, 7, and 10 hr, the cells were made permeable, and the acetohydroxy acid synthetase was assayed. As can be seen in Fig. 1, D106-1A shows a brief period of rapid enzyme synthesis, then slows to a very low differential rate, giving about 0.02 umits of enzyme per 10^6 cells. M21, after 4 hr, gives a differential rate about 3-fold higher, 0.06 units/10^6 cells. MAR33, under these conditions, makes about 0.22 units/10^6 cells, a differential rate 10-fold higher than that of

D106-1A. The data further indicate that the initial rate of synthesis in M21 is a rapid as in MAR33, whereas in D106-1A the initial rate is considerably slower.

In summary, a strain carrying a nonsense mutation in the gene coding for threonine deaminase cannot be derepressed for synthetase by starvation for isoleucine. A mutant with threonine deaminase genetically desensitized to isoleucine makes synthetase at an accelerated rate, unless excess isoleucine is present.

DISCUSSION

Since the concept of repression was first introduced (19), several systems have been examined. The lactose operon of *E. coli* has been thoroughly explored and its negative-control mechanism has been largely elucidated (20, 21). In the case of repression of biosynthetic enzymes, the situation is not as well understood, although there has been evidence that control might be exerted at the level of both transcription and translation (22), with strong evidence for translational control (23). There is additional evidence that repression might involve tRNAs (24, 25), aminoacyl-tRNA synthetases (26-28), and completed biosynthetic enzymes (1-7). The best evidence that a completed enzyme might be involved in the repression of other enzymes of the same pathway has been obtained in the histidine pathway of *S. typhimurium*, where it has been shown that the binding site for feedback inhibition of the first enzyme of the histidine pathway is also involved in repression (3). This is similar to the finding with MAR33. This strain is not as repressed as is its parent, MD11, when both are grown in minimal medium, but it can be partially repressed by the addition of 5 mM isoleucine. The fact that MAR33 has a mutation affecting the isoleucine feedback-inhibition site suggests that the binding of isoleucine to the inhibitory site of threonine deaminase is required for repression, and thus that threonine deaminase itself is involved.

The simplest test for the involvement of a gene product in regulation is to use a strain with either a deletion or a nonsense mutation in that gene. Unfortunately, any test of the effect of a nonsense mutation in the first enzyme of the histidine pathway on the repression of the subsequent histidine enzymes is complicated by a polarity effect due to the polycistronic nature of the message. In the case of the isoleucine–valine pathway in *S. cerevisiae* this is not a problem, since the genes are not juxtaposed. We were, thus, able to examine the repression pattern in D106-1A, a nonsense mutant in *ilv1*.

When only a fragment of the threonine deaminase polypeptide is made, which is the case in D106-1A, the synthesis of acetohydroxy acid synthetase remains repressed. On the other hand, strains M21 and M6, which have missense mutations in *ilv1*, give a normal response when grown on minimal medium, providing evidence that neither accumulation of threonine nor lack of α-ketobutyrate is involved in the unusual regulatory behavior of D106-1A. It has been shown that the effect of the incomplete threonine deaminase donated by D106-1A in the diploid D106-1A × MD9 is recessive, the synthetase being partially derepressed on minimal medium. Further evidence that intact threonine deaminase is necessary for derepression can be seen from the regulatory behavior in the D106-1A × M15 diploid. This diploid contains one functional acetohydroxy acid synthetase as supplied by D106-1A, and one complete and functional threonine deaminase as supplied by M15. The acetohydroxy acid synthetase in this strain shows a normal repression to derepression ratio, implying that the presence of complete threonine deaminase is sufficient for derepression. (The somewhat lower specific activities found in diploids containing only one functional gene for acetohydroxy acid synthetase is believed to reflect gene dosage. The amounts of enzyme in the two control diploids, M2 × YM33 and M15 × MD13, each of which contains only one copy of *ilv2*, are consistent with the results obtained with the diploid D106-1A × M15.)

The experiments presented in Fig. 1 show that the three strains, missense mutant, nonsense mutant, and desensitized mutant, all make acetohydroxy acid synthetase at different rates, as would be predicted by their specific activities. The rates vary over a 10-fold range, giving firm evidence of the role of threonine deaminase in regulation of the second enzyme.

Therefore, we conclude that intact threonine deaminase appears to be necessary for derepression by limitation for isoleucine of at least one enzyme of the pathway. A number of mechanisms can be envisaged for this effect. The least interesting would be stabilization of synthetase by its substrate, α-ketobutyrate. This possibility is rather unlikely in view of the fact that at least two missense mutants that lack functional threonine deaminase can be derepressed by starvation for isoleucine; therefore the nonderepressibility seems specific for the nonsense mutation and is probably due to the missing polypeptide piece, rather than to the loss of function. An analogous mechanism might be stabilization of synthetase by

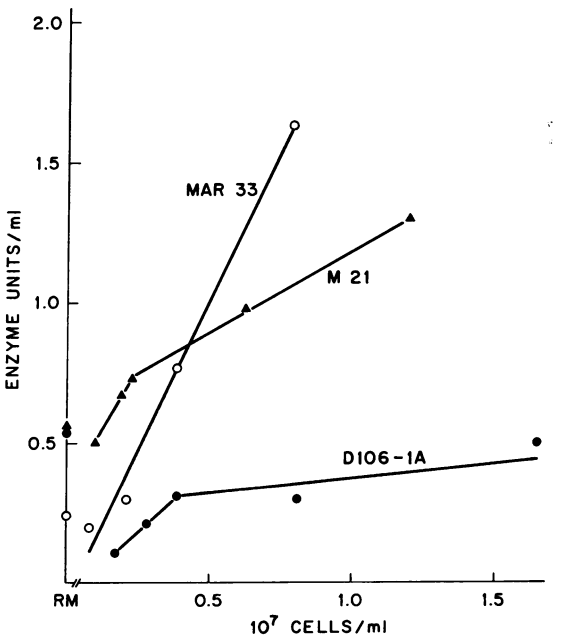

FIG. 1. The following 150-ml cultures were grown under repressed conditions to about 2 × 10^7 cells/ml: MAR33, which contains a threonine deaminase 100-fold less sensitive to isoleucine as inhibitor than the wild type; M21, which contains a missense mutation in *ilv1*, the gene for threonine deaminase; and D106-1A, which contains a nonsense mutation in *ilv1*. The cultures were centifuged, washed, and inoculated into 1000 ml of minimal medium at 2× 10^6 cells/ml. 200-ml samples were taken at 0, 1.5, 3, 4, 7, and 10 hr. The cultures were made permeable and the acetohydroxy acid synthetase was assayed. Enzyme units are in μmol/20 min per mg protein. O, MAR33; ▲, M21; ●, D106-10A.

the *ilv1* gene product itself. This would require the *ad hoc* assumption that MAR33 makes a better stabilizer than does the wild type. This possibility is very difficult either to rule out or to prove, but there are two pieces of evidence that render it unappealing as an explanation. The first is that D106-1A *can* be derepressed, at least partially, by starvation for leucine. If the low specific activity of synthetase were due strictly to the lack of a stabilizing factor in this strain, there should be no derepression, no matter what is limiting. A second argument against stabilization by the *ilv1* gene product is the lack of proportionality between the specific activities of threonine deaminase and the synthetase in mutants deficient in one or another of the biosynthetic enzymes (11). One might expect at least some relationship between these values, but none is apparent.

The most interesting possible mechanism for the data reported here is the involvement of the *ilv1* gene product in regulation of the synthesis of the synthetase, at either the translational or the transcriptional level. Such involvement might be mediated *via* formation of a complex between the deaminase and nascent synthetase, facilitating translation of the synthetase message. Alternatively, theomine deaminase might function as a repressor, binding directly to the synthetase gene or its messenger. In either case, isoleucine would need to bind to its inhibitory site for repression to take place. Both these models are subject to the criticism that they do not account for derepression by leucine limitation in D106-1A.

It is possible to account for all the results we report here if threonine deaminase has a role in regulating the concentration of a leucyl-tRNA species that is itself the key intermediate in multivalent repression, binding the charged tRNA in the absence of isoleucine and valine [both of which appear to have sites on the enzyme (17)], and releasing it in their presence. Leucine starvation would then have a primary effect, depletion of leucyl-tRNA, and would not require intact deaminase for derepression. This model is made more plausible by the report that purified threonine deaminase from *S. typhimurium* binds leucyl-tRNA (6). We are presently investigating the binding of leucyl-tRNA to purified threonine deaminase from *S. cerevisiae*.

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