Multivalent Repression of Isoleucine-Valine Biosynthesis in *Saccharomyces cerevisiae*

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Regulation of the biosynthesis of four of the five enzymes of the isoleucine-valine pathway was studied in *Saccharomyces cerevisiae*. A method is described for limiting the growth of a leucine auxotroph by using valine as a competitor for the permease. Limitation for isoleucine and valine was accomplished by the use of peptides containing these amino acids conjugated with glycine as nutritional supplements for auxotrophs. The enzymes were repressed on synthetic medium containing isoleucine, valine, and leucine, as well as on broth supplemented with these amino acids. Limitation for any of the three branched-chain amino acids led to derepression of the isoleucine-valine biosynthetic pathway. Maximal derepression ranged from 3-fold for threonine deaminase to approximately 10-fold for acetohydroxyacid synthase. (Two of the enzymes, acetohydroxyacid synthase and dihydroxyacid dehydratase, may be controlled by a mechanism different from that regulating threonine deaminase.) Possible molecular mechanisms for multivalent repression are discussed.

The discovery, by Freundlich, Burns, and Umbarger (6), of multivalent repression in isoleucine-valine biosynthesis in *Escherichia coli* revealed a mechanism which regulates the levels of the enzymes of biosynthetic pathways leading to several products. This form of regulation, in which a deficiency of any one of the several products leads to derepression of the enzymes for the pathway, has also been shown to operate in the threonine-methionine pathway in *E. coli* (2, 4). It seems to be an alternative to the mode of regulation in which several enzymes, each repressed by one of the end products, catalyze a single step (13). So far, multivalent repression has been found only in these two pathways, and only in bacteria.

The regulation of the isoleucine-valine biosynthetic pathway in *E. coli* K-12 is complex. There are two, or possibly three, operons involved (2, 6, 14), and only one, that containing the enzymes catalyzing steps 1, 4, and 5 in Fig. 1, is repressed multivalently. If the appropriate nutritional mutant is limited for the exogenous supply of isoleucine, valine, or leucine, these enzymes are coordinately derepressed 10- to 20-fold. Enzyme 2 on the other hand, is only derepressed by limitation for valine.

Because multivalent repression is a relative rarity even in bacterial systems, one might expect isoleucine-valine biosynthesis to be controlled differently in eucaryotes, whose regulatory mechanisms are in general less efficient. That differences

between the two groups of organisms may occur is shown by the work of Gross (7), who demonstrated that leucine biosynthesis in *Neurospora crassa* is regulated by sequential induction, the product of the first enzyme of the pathway being the inducer for the next two enzymes. The two enzymes which were induced may have been coordinately induced but did not map adjacent one to the other.

In earlier reports (1, 10, 11), we examined regulation of the activity of the first two enzymes concerned with isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*. In this paper, we show that at least four of the five enzymes in the pathway are repressed multivalently by isoleucine, valine, and leucine, and that two of them may be depressed coordinately, at least over part of the range which we have been able to examine.

MATERIALS AND METHODS

Strains. M2 (*trp iso-2*), M6 (*trp iso-1*), M7 (*trp iso-3*), and M12 (*trp iso-5*) were isolated by S. N. Kakar (8) and were obtained from D. Hawthorne. MD 13 (*trp leu*) is a leucine auxotroph isolated by mutagenesis with *N*-methyl-*N*-nitro-*N*,_i-nitrosoguanidine from a segregant of a cross between M2 and NR 211-1a. The latter strain was obtained from H. Hefner.

Media. Cells were grown on one of five media. Medium Y contained 10 g of Difco yeast extract, 10 g of Difco peptone, and 30 g of glucose per liter, and was supplemented with L-isoleucine, L-valine, and L-leucine, each at a concentration of 5×10^{-3} M. Minimal medium (M) was the same as medium G

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

described previously (1) except that the pH was adjusted to 6.2 by the addition of 40 ml of 1 M K_2HPO_4 per liter; 1 ml of 0.5% L-tryptophan, 20 ml of 0.1 M L-valine, and 5 ml of 0.1 M L-isoleucine were added per liter. Repressing medium (RM) was the same as medium M except that 50 ml of 0.1 M valine, 50 ml of 0.1 M isoleucine, and 100 ml of 0.05 M L-leucine were added per liter. Isoleucylglycine medium (IG) was the same as medium M, except that the valine was increased to 50 ml/liter, 270 mg of L-isoleucylglycine per liter was added, and isoleucine was omitted. Glycylvaline medium (GV) was medium M with 2.5 ml of valine per liter, 1 g of glycyl-DL-valine per liter, and 5 ml of 0.1 M L-isoleucine per liter. The latter two media were filter-sterilized.

Preparation of cells. For assay of the enzymes, the cells were shaken overnight in medium Y at 30°C and then were inoculated into 100 ml of the appropriate medium in a 1-liter Erlenmeyer flask. The latter cultures were shaken overnight at 30°C so that at least two divisions took place before harvesting. The inocula were adjusted so that the cell density at harvest would be between 10^7 and 5×10^7 /ml. (Stationary phase is reached at about 2×10^8 /ml in any medium except Y, where cells grow to a density of about 10^9 /ml.)

The cultures 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, and 0.01 M dithiothreitol. They were made permeable by the addition of 0.5 ml of toluene followed by rapid mixing on a Vortex mixer for 30 sec and incubation for 90 sec at 30°C. The cells were then centrifuged at $27,000 \times g$ and were resuspended to give between 2 and 10 mg of protein per ml, measured as described below. The wash after toluene treatment reduces the concentration of allosteric effectors which might affect the assays.

Enzyme assays. The assays for aceto-hydroxyacid synthase (AHA synthase) and threonine deaminase have been described previously (10, 11). AHA synthase was routinely assayed at pH 8.0 to minimize contributions of the acetoin-forming system (10).

Dihydroxyacid dehydrase (DHA dehydrase) was assayed by a modification of the Friedmann-Haugen method. Cells which had been made permeable were incubated with 0.05 ml of 0.05 M $MgSO_4$ for 10 min at 30°C. The reaction was started by the addition of 0.05 ml of a 10 mg/ml solution of DL-dihydroxyiso-

valerate, sodium salt (prepared by Cyclo Chemical Co.), in 0.5 M Tris-hydrochloride (pH 7.7). After 20 min, the reaction was stopped by the addition of 0.25 ml of 0.3% dinitrophenylhydrazine in 2 N HCl, and 10 min later 1.5 ml of 1.5 N NaOH was added. After another 10 min, 1.5 ml of absolute ethyl alcohol was added. The tubes were mixed vigorously on a vortex mixer and centrifuged. The optical density (OD) of the reaction mixture was read at 520 nm on a Bausch & Lomb Spectronic-20 colorimeter, and the quantity of ketoacid in each was determined from a standard curve prepared with pyruvate. A zero-time blank was always subtracted because the cells often contained some dinitrophenylhydrazine-positive material.

Reductoisomerase was assayed on a Gilford 2400 spectrophotometer in a reaction mixture containing (per ml) 0.02 ml of 0.05 M MgSO_4 , 0.01 ml of reduced nicotinamide adenine dinucleotide phosphate (NADPH; 10 mg/ml), 0.03 ml of 0.25 M acetohydroxybutyrate (Reef Laboratories, Lafayette, Ind.), and 0.9 ml of 0.5 M Tris-hydrochloride (pH 7.7). The reaction was followed for 8 min, and the Δ OD/minute at 340 nm was calculated for the last 6 min. The rate of oxidation of NADPH in a reaction mixture lacking substrate was subtracted from this value to give the true enzymatic rate. Assays of all four enzymes were always conducted in duplicate.

Protein assays. Samples of the cell preparations, diluted 1:10 or 1:20 in 1 M NaOH, were boiled for 5 min and then centrifuged. The supernatant fluid was assayed by the method of Lowry et al. (9), with bovine serum albumin as a standard. Suitably diluted and boiled samples of the Tris-glycerol-DTT buffer were added to the standard curve, since DTT reacts to some extent in the Lowry assay.

RESULTS

Limitation of growth. Probably because of competition of the two amino acids for the same permease, it is very difficult to grow isoleucine-valine auxotrophs in a chemostat in a medium limiting for one or the other of these amino acids. Therefore, the cells were grown in batch culture, and peptides were used in place of the free amino acids to limit growth. For isoleucine, 0.27 mg of L-isoleucylglycine per ml was used. For valine, 1 mg of DL-glycylvaline per ml served. Figure 2 shows that the generation times for a typical auxotroph were 12 hr on isoleucylglycine and 7.5 hr on glycylvaline. Medium M gave a generation time of 2.75 hr. To show that the cells are in fact limited by these peptides, one can restore the appropriate amino acid and decrease the generation time to the normal level. Thus, auxotrophs grown on these peptides as nutritional supplements could be expected to be derepressed for the biosynthetic enzymes.

Neither glycylleucine nor leucylglycine would serve as a source of leucine for a leucine auxotroph, so advantage was taken of the competition

FIG. 2. Growth of M12 on isoleucylglycine and glycylvaline. The cells were grown overnight on M medium and then transferred to the appropriate medium. Growth was monitored by the increase in turbidity at 650 nm. Symbols: Δ , M medium; $\blacksquare$, GV medium; $\circ$, GV medium + 5×10^{-3} M L-valine; $\square$, IG medium; $\bullet$, IG medium + 2.5×10^{-3} M L-isoleucine.

of leucine and valine for the same permease. Figure 3 shows that the growth of MD13, a leucine auxotroph, was strongly inhibited by valine at concentrations of 1.5×10^{-2} (generation time = 7 hr) and 2×10^{-3} M (generation time = 9.25 hr) when leucine was present at 5×10^{-4} M. Increasing the leucine to 10^{-2} M restored normal growth (generation time = 3.25 hr). Both concentrations of valine were used to derepress this strain.

Derepression of isoleucine-valine biosynthetic enzymes. Table 1 shows the activities of the isoleucine-valine biosynthetic enzymes in various mutants grown on various media. Each value is the average of at least two experiments. It can be seen that derepression of four- to fivefold is possible for each of the enzymes except threonine deaminase. The latter increases at most threefold under conditions of isoleucine limitation and shows very little increase when valine is the limiting nutrient. Although all of these strains are presumably derived from the same parent, differing by a single mutation, they show rather different basal levels of the various enzymes. Furthermore, M7 shows the highest specific activity of all of the enzymes on medium M, rather than on IG or GV medium. RM medium is less effective than Y medium in repressing the enzymes in this strain, whereas the converse is true in the other strains.

FIG. 3. Effect of added valine on the growth of MD13. The cells were grown overnight on minimal medium + 5×10^{-4} ML-leucine and then transferred to medium containing 5×10^{-4} ML-leucine (○), 5×10^{-4} ML-leucine + 1.5×10^{-2} ML-valine (●), 5×10^{-4} ML-leucine + 2×10^{-2} ML-valine (□), or 5×10^{-3} ML-leucine + 2×10^{-2} ML-valine (△).

Table 1 also shows the results of limitation for leucine in MD13. The levels of the AHA synthase in this strain are routinely lower than in any of the others, and the ratio of derepressed to repressed levels is greater (ninefold versus fivefold in M7). It can be seen that the enzymes involved in valine biosynthesis are in fact derepressed by limitation for leucine as well as for isoleucine and valine. Threonine deaminase shows about a two-fold increase in the culture limited on leucine.

Relative levels of the various enzymes. Kakar and Wagner (8) showed that the genes *iso-1*, *iso-2*, *iso-3*, and *iso-5* are not linked in *S. cerevisiae*. One would, therefore, expect that the derepression of the enzymes would not be coordinate. Figure 4, a plot of the specific activity under various growth conditions of threonine deaminase versus DHA dehydrase, shows that those enzymes are not coordinately controlled. Although a reasonably straight-line relationship can be drawn, the line does not extrapolate through the origin. Similar relationships can be shown for threonine deaminase versus AHA synthase and for reductoisomerase against any of the other three enzymes. In MD13, AHA synthase and DHA dehydrase, however, show an almost linear correlation (Fig. 5A). Figure 5B demonstrates that the enzymes are not coordinately controlled over their entire range in M12; there is a region in which dehydrase increases without a concomitant increase in synthase. The fact that both curves extrapolate to a point close to the origin (0.07 units of dehydrase for MD13 and 0.013 units of synthase for M12) indicates that the synthesis of these enzymes may

TABLE 1. Levels of isoleucine and valine biosynthetic enzymes under various conditions^a

Conditions	Specific activity ^b			
	TD	AHA synthase	RI	DHA dehydrase
M2 (AHA synthase ⁻)				
Y (nutrient + iso, val + leu)	1.38	—	.115	.117
RM (minimal + optimal iso, val + leu)	1.87	—	.116	.074
M (minimal + iso, val)	2.09	—	.156	.223
IG (limiting iso)	4.70	—	.492	.595
GV (limiting val)	3.66	—	.236	.192
M6 (TD ⁻)				
Y	—	.085	.115	.084
RM	—	0.59	.138	.032
M	—	.160	.354	.165
IG	—	.249	.463	.172
M12 (RI ⁻)				
Y	1.78	.154	—	.183
RM	1.64	0.59	—	.056
M	3.35	.190	—	.365
IG	3.34	.280	—	.333
GV	2.57	.232	—	.266
M7 (DHA dehydrase ⁻)				
Y	1.93	.085	.274	—
RM	2.38	.136	.315	—
M	3.10	.446	.650	—
IG	4.28	2.49	.419	—
GV	3.54	.187	.330	—
MD13 (leu ⁻)				
Y	1.48	.015	.198	.167
RM	1.51	.032	.193	.102
M	2.24	0.46	.359	.134
M + 0.15 M val (limiting leu)	2.99	.142	.448	.520
M + 0.020 M val (limiting leu)	2.39	.134	.361	.430

^a Abbreviations: TD, threonine deaminase; AHA synthase, acetohydroxyacid synthase; RI, reductoisomerase; DHA dehydrase, dihydroxyacid dehydrase; iso, isoleucine; val, valine; leu, leucine; Y, RM, M, IG, and GV are media for which the compositions are given in Materials and Methods.

^b Expressed as micromoles of product per 20 min per milligram of protein. All values are the average of two or more experiments.

be regulated by a mechanism which differs in some way from that for threonine deaminase. That this difference exists is indicated by the isolation of mutants derepressed for synthase and

FIG. 4. Ratio of threonine deaminase to dihydroxyacid dehydrase (DH dehydrase) under various growth conditions in M2 and M12. The line is plotted by the least-square method. Intercept = 1.05 ± 0.2 units of threonine deaminase activity; correlation coefficient = 0.79.

dehydrase but not for threonine deaminase (P. T. Magee, *in preparation*).

DISCUSSION

Multivalent repression, not heretofore shown in eucaryotes, regulates the biosynthesis of isoleucine and valine in *S. cerevisiae*. Although the role of the vitamin pantothenic acid was not investigated in this study, it is clear that isoleucine, leucine, and valine are all necessary to repress the biosynthetic enzymes, and that limitation for any of these causes a derepression of these enzymes. This derepression may vary from 3-fold for threonine deaminase to 5- to 10-fold for AHA synthase. In bacteria, derepression for these enzymes may be as much as 50-fold (2). Eucaryotes in general show a much smaller range of repressed to derepressed enzyme levels than bacteria.

The variation in the repression patterns of the various strains is worthy of comment. M7 seems totally derepressed on minimal medium, the values for AHA synthase and reductoisomerase being higher there than on any medium for any other strain. Furthermore, growth on limiting medium (IG or GV) gives lower specific activities for these two enzymes. RM medium also fails to depress these activities to a low level, although medium Y is somewhat more effective. Since M7 is an original isolate and not a segregant of the original isolate, it is quite possible that it carries a secondary mutation responsible for the elevated specific activities. It seems unlikely that this mutation is associated with any nutritional leakiness

FIG. 5. Ratio of acetohydroxyacid synthase (AHA synthase) to dihydroxyacid dehydrase (DH dehydrase) under various growth conditions in various strains. Lines plotted by least-square method. (A) MD13. Intercept = 0.073 ± 0.072 units of dehydrase activity. Correlation coefficient = 0.69. (B) M12. The line is fitted by eye. Intercept is 0.0125 units of AHA synthase.

for another co-repressor, since M7 grows normally on M medium. The possibility that one of the products which accumulates in M7 (e.g., dihydroxyisovaleric acid) is an inducer, in analogy with the leucine pathway in *N. crassa* (7), seems to be eliminated by the fact that strains in which no dihydroxyacids can occur are normally derepressed on limiting medium.

IG medium seems in all cases to be more effective

tive than GV medium in derepressing the enzymes, but the generation time on IG medium is longer. The activity of AHA synthase is strictly dependent upon the cells being in log phase; the enzyme is destroyed in stationary-phase cells (Magee and de Robichon-Szulmajster, *unpublished data*).

The lack of coordination in the regulation of two of these four biosynthetic enzymes is to be expected, given the fact that they are not linked genetically. The question of whether there is a single regulatory mechanism acting upon all four, as is the case with the unlinked arginine genes in *E. coli* (7a), will remain open until regulatory mutants have been thoroughly studied. The fact that the plot of threonine deaminase versus synthase or dehydrase extrapolates to a basal level of about one rather than zero may indicate that the regulation of this enzyme, although physiologically similar, involves a molecular mechanism different from that for the other two.

Evidence to be reported elsewhere (Hartwell, McLaughlin, and Magee, *in preparation*) indicates that the isoleucyl-transfer ribonucleic acid (tRNA) synthase plays a part in multivalent repression in yeast, since strains with a temperature-sensitive isoleucyl-tRNA synthase show derepression for at least two of the enzymes in the pathway. Very similar results have been reported for strains of *E. coli* with a temperature-sensitive valyl-tRNA synthase. There is at present no evidence as to the role of leucyl-tRNA synthase in regulation of the isoleucine-valine pathway, although Printz and Gross (12) have shown that mutants of *N. crassa* with a lesion in the leucine-activating enzyme are not derepressed for the enzymes specific for the biosynthesis of leucine.

How the lack of any one of two or possibly three very complex molecules functions to turn on specific protein synthesis is presently far from clear. One possibility is that the system is analogous to the *lac* operon, with a single molecule serving as repressor. This molecule would have sites for each of the co-repressors; a lack of any one of the co-repressors would cause the molecule to lose its affinity for the operator and allow transcription of the appropriate genetic material. A second, closely related possibility is that the repressor has a single site for an inducer; this inducer would be any of the three types of uncharged tRNA. The site on the repressor would presumably have an affinity for some common sequence on the RNA molecules. Many other possibilities, some involving regulation at the translational level, can also be envisioned. To

distinguish among these possibilities and to elucidate the molecular mechanism will require analysis of many sorts of mutants, among them derepressed strains and strains with lesions of various sorts in protein synthesis.

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