A ROLE FOR A PYRIDOXINE DERIVATIVE IN THE MULTIVALENT REPRESSION OF THE ISOLEUCINE AND VALINE BIOSYNTHETIC ENZYMES*

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Received January 8,1973

SUMMARY: Starvation of a *pdx* mutant of *Escherichia coli* strain B in the presence of repressing levels of isoleucine, valine and leucine leads to a derepression of the normally repressible *ilv* genes. The derepression of the *ilvA* gene under these conditions results in the accumulation of apothreonine deaminase. Addition of pyridoxine leads to a sudden increase in threonine deaminase activity, and to restoration of repression. The pyridoxine component needed for the repression signal is probably not threonine deaminase but, more likely, some transient ("immature") form of the enzyme.

Hatfield and Burns (1) have demonstrated that threonine deaminase reconstituted *in vitro* by mixing pyridoxal-5'-phosphate and the apoenzyme must undergo a "maturation" process before it is catalytically active. They further demonstrated that isoleucine and valine impeded the maturation of the "immature" holoenzyme and that leucyl tRNA was bound specifically to the immature form. These observations led to the idea that immature threonine deaminase might be the aporepressor needed for multivalent repression of the isoleucine and valine forming enzymes. That threonine deaminase is related to repression in some way was further indicated by a pleiotropic mutation that affected induction of the isomeroreductase (enzyme 3 in the isoleucine pathway), and both the repressibility and endproduct inhibition of threonine deaminase (2).

It was recently observed that an isoleucine deficiency occurs very soon after pyridoxine-deficient (*pdx*) mutants are starved for the vitamin (3). If this deficiency be due to a cessation of threonine deaminase formation, it allows a test of one consequence of the Hatfield and Burns (1) model. If the multivalent repression signal can be generated during the pyridoxine starvation

*Supported by U.S. Public Health Service Grants GM12522 and AM14157.
†Recipient of a U.S. Public Health Service Predoctoral Fellowship, GM46084.

period, the postulated role of immature threonine deaminase would be disproven. A role for the apoenzyme in repression, however, would still be a possibility. Experiments directed to this question are described in this report.

MATERIALS AND METHODS

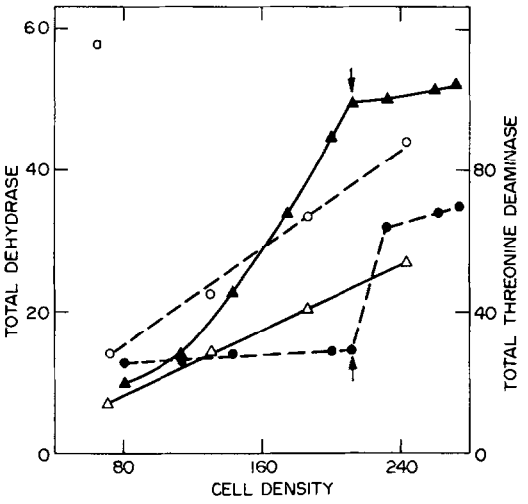
The minimal medium used was that of Davis and Mingioli (4). The following supplements were employed where indicated: isoleucine and leucine, 0.4 mM; valine, 1 mM; pyridoxine, 8×10^{-7} M. Growth of organisms, preparation of cell extracts and assays for enzyme activity were described previously (5,6,7).

When cells were to be transferred to fresh medium, they were harvested by centrifugation at room temperature. The cells were washed twice with warm 50 mM potassium phosphate buffer, pH 7.5, and resuspended at about the same density in pre-warmed (37°) minimal medium.

Organism. Strain WG1473 (*pdx-204*, phage resistant) was derived from the wild type *E. coli* strain B by nitrosoguanidine mutagenesis. Measurement of transduction frequency as described previously (8) revealed that the *pdx-204* allele lies at a position on the *E. coli* chromosome represented by minute 1 on the map of Taylor (9). Thus, it belongs to linkage group II (8). The phage resistance is to an unidentified virulent phage.

RESULTS

Effect of vitamin B_6 compounds (B_6) on the maintenance of multivalent repression. Growth of strain WG1473 with excess branched-chain amino acids and pyridoxine (its required nutrilite) results in repression of the isoleucine and valine forming enzymes (about 40 percent that in a non-supplemented pyridoxine medium). Such cells, washed and transferred to a similar medium, maintained that repression. When transferred to a medium containing the repressing amino acids but no pyridoxine, there was no increase in threonine deaminase in the culture and the differential rate of dehydrase formation was constant for only 20 to 30 minutes. Thereafter, growth decelerated and the dehydrase became derepressed. Fig. 1 shows that adding pyridoxine after 110 min of starvation resulted in a rapid appearance of threonine deaminase and restora-



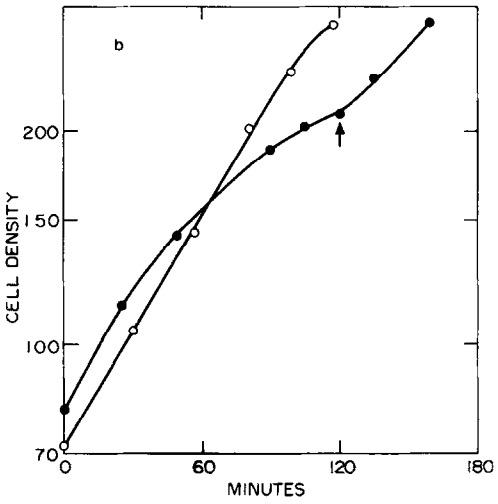


Figure 1a. Strain WG1473 was grown in minimal medium with pyridoxine and the three branched-chain amino acids. The cells were harvested, divided into two parts and transferred to medium with the branched-chain amino acids, with (○, △) or without (●, ▲) pyridoxine. Pyridoxine was added to the pyridoxine-starved culture at the point indicated by the arrows. At the time of transfer and various times thereafter, 25 ml samples were assayed for threonine deaminase (○, ●) and dehydrase (△, ▲). Total enzyme activity is expressed as specific activity (μmoles product/min/mg protein) times the Klett reading of the culture when the sample was removed.

Figure 1b. Growth of strain WG1473 in medium containing the branched-chain amino acids, with (○) or without (●) pyridoxine. Cell density is expressed in Klett units (#42 filter) (80 Klett units approximately 3 x 10^8 cells/ml).

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tion of repression. These results demonstrate that, in addition to the three branched-chain amino acids, multivalent repression requires some pyridoxine derivative.

The stability of apothreonine deaminase during pyridoxine starvation. That the activity increase that occurred after adding pyridoxine was due to the conversion of apo- to holoenzyme was shown by the fact that the increase was chloramphenicol insensitive. When the addition of pyridoxine was delayed after adding chloramphenicol, it was found that the B_6-stimulated threonine deaminase activity (apoenzyme) appeared to decay at an exponential rate with a half life of about 1/2 hour. This decay rate allows us to calculate that the amount of apoenzyme formed during B_6 starvation was greater than the repressed level of active enzyme that would have been formed had B_6 been present. Other

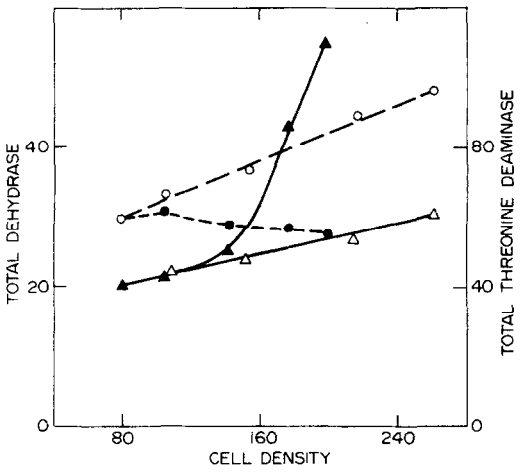

Fig. 2.

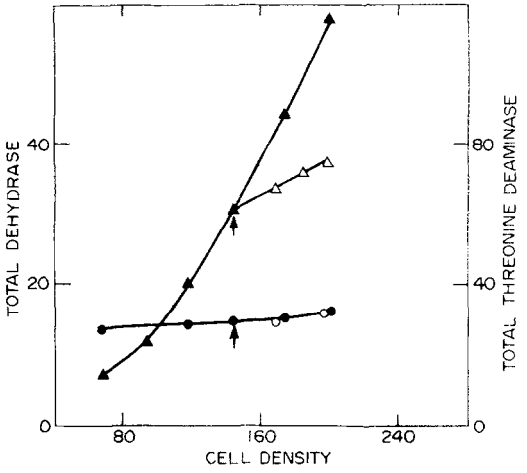

Fig. 3.

Figure 2. Strain WG1473 was grown in medium supplemented only with pyridoxine. The cells were transferred, as indicated in Fig. 1a, into medium containing the branched-chain amino acids with (O, Δ) or without (●, ▲) pyridoxine. Samples were assayed for threonine deaminase (O, ●) and dehydrase (Δ, ▲).

Figure 3. Cells were grown as described in Fig. 1a, then transferred to medium with the branched-chain amino acids, lacking pyridoxine. At the time indicated by the arrows, one-half of the culture received 5 x 10^{-5} M 4-deoxypyridoxine (O, Δ), the other half received no addition (●, ▲). Threonine deaminase (O, ●) and dehydrase (Δ, ▲) activities were determined as indicated in Fig. 1a.

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experiments showed that the decay was probably not due to decay in enzyme activities converting pyridoxine to pyridoxal phosphate. Thus, the *ilvA* gene as well as the *ilvD* gene (corresponding to the dehydrase) was derepressed during B_6 starvation.

Is threonine deaminase the vitamin B_6 derivative needed for coordinate repression? That the derepression during pyridoxine starvation was not due to a reduction in the specific activity of threonine deaminase was indicated by examining the onset of derepression of dehydrase in cells in which threonine deaminase activity was twice that of the cells represented in Fig. 1. The higher level of threonine deaminase activity was achieved by growing strain WG1473 without the amino acid supplement. As Fig. 2 shows, the onset of derepression of the dehydrase, during pyridoxine starvation in the presence of

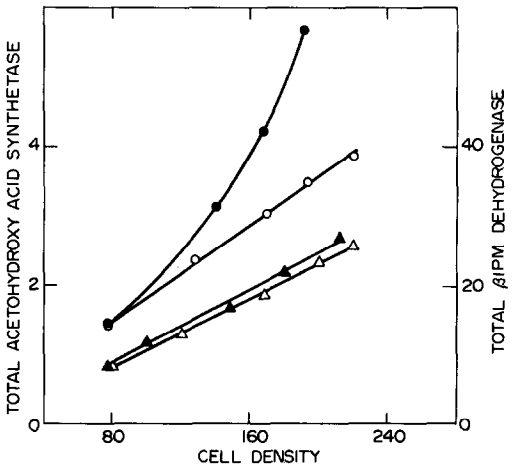

Figure 4. Cells were grown as described in Fig. 1a, then transferred to medium containing the branched-chain amino acids, with (O, Δ) or without (●, ▲) pyridoxine. Samples were assayed for acetohydroxy acid synthetase (O, ●) and β-isopropylmalate dehydrogenase (Δ, ▲) activities.

repressing amounts of the branched-chain amino acids, was essentially the same as shown in Fig. 1. Throughout the experiment, the derepression of the dehydrase was equal to or greater than that in the experiment (Fig. 1) in which the cells actually contained less threonine deaminase activity. We conclude, therefore, that the vitamin B_6 derivative needed for the generation of the multivalent repression signal is not threonine deaminase itself. It could be, however, a derivative of apothreonine deaminase to which pyridoxal phosphate had been added [quite possibly the immature enzyme of Hatfield and Burns (1)].

The effect of 4-deoxypyridoxine on multivalent repression. An attempt to restore repression of the *ilvADE* operon without converting the apoenzyme to active threonine deaminase was made by adding 4-deoxypyridoxine to a pyridoxine-starved culture. As can be seen in Fig. 3, 4-deoxypyridoxine, added after 70 min of starvation, mimicked the effect of pyridoxine in allowing repression but was inactive in the conversion of apo- to holothreonine deaminase.

The effect of pyridoxine starvation on repression of other branched-chain amino acid biosynthetic enzymes. As Fig. 4 shows, acetohydroxy acid synthetase was also derepressed during pyridoxine starvation. In this experiment, the

increased activity was at least as valine-sensitive as that in the presence of pyridoxine. Thus, we presume that the *ilvB* gene itself is also derepressed. In contrast, a leucine forming enzyme, β-isopropylmalate dehydrogenase, was not derepressed by pyridoxine starvation. Thus, a vitamin B_6 derivative, possibly a product of the *ilvA* gene, is involved along with leucine in the repression of the *ilv* genes, but the repression of the *leu* operon is apparently independent of any vitamin B_6 derivatives.

DISCUSSION

Two observations have implicated some role for threonine deaminase in the repression of the isoleucine and valine biosynthetic enzymes. One is the observation that a mutation in the *ilvY* region resulted in the simultaneous loss of inducibility of the isomeroreductase (the product of the *ilvC* gene), in an alteration of several properties of threonine deaminase, including loss of feedback inhibition by isoleucine and in an altered repression of the *ilvADE* operon (2). The second observation is the binding of leucyl tRNA by immature threonine deaminase that led to the model of Hatfield and Burns (1). An implicit feature of this model is that pyridoxal phosphate is required for multivalent repression, since it is required to form the immature tetramer.

The experiments described in this paper were designed to show that a vitamin B_6-containing component was not required for multivalent repression. The results, on the contrary, are most readily interpreted by the Hatfield and Burns model but, of course, do not prove it. The results further indicate that if some product of the *ilvA* gene is involved in repression, it is probably not active threonine deaminase itself.

The above results are compatible with a model in which some, perhaps unstable, product of the *ilvA* gene, intermediate between the apoenzyme and active holoenzyme, is essential for multivalent repression of the *ilvADE* operon. The results obtained in experiments (to be reported later) measuring inducibility of the isomeroreductase during pyridoxine starvation are consistent with the idea that some product of the *ilvA* gene may also be "upsilon,"

the product of the *Y* region of the *ilv* cluster which, in turn, may be part of the *ilvA* gene (10). Furthermore, the results obtained using 4-deoxypyridoxine indicate that the function of the *ilvA* gene product active in repression is different from that involved in the induction of the *ilvC* gene. The experiments in which induction has occurred are, however, more complex and are being further studied.

Pyridoxine starvation also results in formation of progressively increasing amounts of both apotransaminase B and other pyridoxal phosphate-dependent apoenzymes as well (results not shown). While any of these might be the component needed for multivalent repression, there is no supporting evidence to implicate any of them in such a role.

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