Role of Vitamin B₆ Biosynthetic Rate in the Study of Vitamin B₆ Synthesis in Escherichia coli

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Nutritional auxotrophs of Escherichia coli synthesize vitamin B₆ compounds at a rate of $1 \times 10^{-10}$ to $2 \times 10^{-10}$ moles per hr per mg (dry weight) of cells when they are suspended in minimal medium lacking their required nutrients. A few auxotrophs have been found to stop or reduce vitamin B₆ synthesis during such an experiment. These include thiamineless, citrate synthaseless, and pyridoxineless mutants as well as mutants which require four carbon compounds for growth. Glycolaldehyde was found to restore vitamin B₆ synthesis in the last named of these mutants without restoring normal growth. A class of pyridoxineless mutants which responded with normal growth to 0.4 mM glycolaldehyde or $0.15 \times 10^{-3}$ mM pyridoxol was also found. The results suggest that a thiamine pyrophosphate-requiring step as well as glycolaldehyde may be involved in pyridoxal phosphate biosynthesis.

The sequence of reactions leading to the biosynthesis of pyridoxal phosphate in bacteria is largely unknown. Although several groups have recently presented preliminary or general data on this subject (6, 10, 16), no overall picture of the reactions involved has emerged, nor has any one, unique biosynthetic reaction been identified.

A previously unused approach to solving this synthetic pathway lies in the identification of those compounds (if any) in Escherichia coli that are required for continued synthesis of vitamin B₆. These compounds can be identified as necessary for vitamin B₆ biosynthesis if mutants lacking the capability to synthesize them are simultaneously unable to synthesize vitamin B₆. This type of measurement can be made by analyzing the vitamin B₆ content of cultures of various mutants during starvation for their required nutrients. Final identification of particular nutrients as necessary or direct precursors of vitamin B₆ as opposed to indirect or metabolically "coupled" compounds would be made by some other test, e.g., showing that the specific activity of vitamin B₆ was the same as that of the appropriately labeled radioactive test compound when tested in a mutant unable to make that test compound.

The purpose of this report is to communicate the results of one set of such tests made with E. coli B mutants in an attempt to identify metabolites required for vitamin B₆ synthesis. During these tests three unexpected findings were made which appear to bear directly upon vitamin B₆ biosynthesis.

MATERIALS AND METHODS

Bacterial strains. The several strains used in this study are listed in Table 1. Strains WGI, WG15, and WG139 have been described previously (4, 5, 8). WG1032 was identified as AroA phenotype by the nutritional test procedure of Pittard and Wallace (14). WG1390 was identified as citrate synthaseless (1) by assay of extracts of the organisms by the method of Srere et al. (15). All other strains were identified primarily by nutritional requirements.

Media. In all cases the basic medium used was the glucose minimal medium previously described (5). For growth of each different mutant this medium was supplemented as necessary with nutrients at the following final concentrations: pyridoxol, $6 \times 10^{-4}$ M; thiamine, $3 \times 10^{-3}$ M; L-glutamate, $3.4 \times 10^{-3}$ M; L-aspartate, $3.7 \times 10^{-3}$ M; L-lysine, $6.8 \times 10^{-4}$ M; L-leucine, $1.9 \times 10^{-4}$ M; uracil, $1.8 \times 10^{-4}$ M; and L-arginine, $5.8 \times 10^{-4}$ M. For strain WG1032 the following compounds were present during growth: L-tryptophan, $2.4 \times 10^{-4}$ M; L-phenylalanine, $3.0 \times 10^{-4}$ M; L-tyrosine, $2.8 \times 10^{-4}$ M; p-aminobenzoic acid, $1.5 \times 10^{-4}$ M, and p-hydroxybenzoic acid, $3.6 \times 10^{-4}$ M.

Starvation procedure. A 50-ml culture of the mutants in glucose minimal medium plus the required nutrients was inoculated with the appropriate mutants and grown overnight at 37°C, with vigorous shaking. In the morning, a portion of these cells containing 100 mg (dry weight) of cells was centrifuged, washed with saline, and used to inoculate 1 liter of identical medium. Shaking at 37°C was resumed until the mass at least doubled. Then the culture was centrifuged and washed.

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with saline, and a portion was used to inoculate unsupplemented glucose minimal medium to the cell densities indicated in each experiment. Duplicate 5-ml samples were withdrawn immediately and mixed at once with 5 ml of 0.11 \( \times \) \( \text{H}_2\text{SO}_4 \). Sampling was repeated every half hour for at least 3 hr. The cell mass at each sampling time was determined from a standard curve relating apparent absorbancy at 420 or 650 nm to dry mass of cells. Samples were stored overnight at 2 C and hydrolyzed for 5 hr at 121 C, and portions then were assayed for total pyridoxine as described previously (3). In every case the entire experiment was repeated at least once with essentially the same results.

**RESULTS**

The general procedure used in this study was based upon that of Wilson and Pardee (18). Cultures of mutants growing exponentially in a glucose minimal medium supplemented with whatever nutrients the particular mutants required were centrifuged, washed, and suspended in unsupplemented glucose minimal medium. Samples were withdrawn at different times, hydrolyzed with acid, and then assayed for total vitamin \( B_6 \) by measuring the growth response of *Saccharomyces carlsbergensis* to portions of the hydrolysate. The slope of the curve obtained by plotting the total vitamin \( B_6 \) content of the culture per milligram of cells at each time against time was equal to the rate of vitamin \( B_6 \) synthesis (18). The initial hypothesis upon which this work was based was that the vitamin \( B_6 \) biosynthetic rates would fall to a low, zero, or negative value if the particular nutrient required by the mutant was necessary for vitamin \( B_6 \) biosynthesis but would remain near some average value if there was no direct connection between vitamin \( B_6 \) synthesis and the mutation at hand.

Before assuming that such variations in this vitamin \( B_6 \) biosynthetic rate could be interpreted as being related to vitamin \( B_6 \) biosynthesis, it was necessary to show that pyridoxineless mutants at least gave zero rates of vitamin \( B_6 \) synthesis. Figure 1 shows such data obtained when two pyridoxineless mutants were used in this experimental procedure. Similar data for other pyridoxineless genotypes has appeared (7). One can see that cell mass continued to increase during starvation, a behavior characteristic of vitamin mutants (18). In these particular experiments the medium was capable of supporting growth to 1.5 mg of dry cell mass per ml, so that the cells could be presumed to have finally stopped growth when they did because of their starvation for vitamin \( B_6 \). More importantly, the total vitamin \( B_6 \) content of the culture actually decreased while the cell mass increased. The result was a curve with negative slope or negative rate of \( B_6 \) synthesis. It was reasonable, therefore, to expect that either low or negative rates of \( B_6 \) synthesis might be found when mutants with alterations in pathways related to vitamin \( B_6 \) biosynthesis were tested in this manner.

Some of the mutants tested in this present series behaved in a manner similar to that previously reported for other mutants (3), that is, they continued to synthesize vitamin \( B_6 \) during starvation. Figure 2 shows results of four of these experiments. The data in this figure are essentially identical to those shown here. No additional AroA mutants were found.

Of more immediate interest however was the finding of a few mutant strains which were not

**FIG. 1. Effect of vitamin \( B_6 \) starvation upon two \( \text{pdx} \) mutants. \( \text{WG15 (pdxD15)} \) and \( \text{WG139 (pdxC139)} \) were shifted at time 0 to vitamin \( B_6 \)-free glucose minimal medium. Samples were taken at various times to measure cell mass in mg of dry weight per ml (○), total vitamin \( B_6 \) per ml (□), and total vitamin \( B_6 \) per mg of cells (●).**

**TABLE 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Requirements</th>
<th>Mutagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>WG1</td>
<td>wild type</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>WG15</td>
<td>( \text{pdxD15} )</td>
<td>Pyridoxine</td>
<td>UV*</td>
</tr>
<tr>
<td>WG139</td>
<td>( \text{pdxC139} )</td>
<td>Pyridoxine</td>
<td>UV</td>
</tr>
<tr>
<td>WG662</td>
<td>( \text{pyrA} )</td>
<td>Arginine and uracil</td>
<td>NTG*</td>
</tr>
<tr>
<td>WG1002</td>
<td>( \text{leu-2} )</td>
<td>Leucine</td>
<td>NTG</td>
</tr>
<tr>
<td>WG1032</td>
<td>( \text{aroA10} )</td>
<td>Tyrosine and phenylalanine</td>
<td>NTG</td>
</tr>
<tr>
<td>WG1139</td>
<td>( \text{lvs-5} )</td>
<td>Lysine</td>
<td>NTG</td>
</tr>
<tr>
<td>WG1225</td>
<td>( \text{thi-22} )</td>
<td>Thiamine</td>
<td>NTG</td>
</tr>
<tr>
<td>WG1256</td>
<td>( \text{ppc-3} )</td>
<td>4-Carbon acid</td>
<td>NTG</td>
</tr>
<tr>
<td>WG1368</td>
<td>( \text{ppc-4} )</td>
<td>4-Carbon acid</td>
<td>UV</td>
</tr>
<tr>
<td>WG1390</td>
<td>( \text{gli-1} )</td>
<td>Glutamate or ( \alpha )-keto-glutarate</td>
<td>UV</td>
</tr>
</tbody>
</table>

* UV, ultraviolet light.
* NTG, \( N \)-methyl-\( N' \)-nitro-\( N \)-nitroguanidine.
Samples were nutrients required for growth. The data of this type isolated in this laboratory, namely, WG1390 and WG1427, were found to have no citrate synthase activity when assayed by the method of Srere et al. (15). The results of a typical experiment with a citrate synthaseless mutant are shown in Fig. 4, which shows no growth during starvation for glutamate and some loss of total vitamin B₆ to give a net negative slope to the curve for vitamin B₆ per milligram of cells.

Figure 3 shows that the vitamin B₆ biosynthetic rate was negative throughout the starvation of WG1225 for thiamine. This result suggests that vitamin B₆ may be required for vitamin B₆ synthesis. The data in Fig. 3 show that the cell mass of WG1225 increased sixfold during the period that vitamin B₆ increased barely twofold. Vitamin B₆ biosynthesis was found to resume in thiamine-starved WG1225 immediately upon the addition of thiamine to a final concentration of 100 μg/liter.

Niacinless and pantothenateless mutants of other strains of E. coli were also tested but were found to make vitamin B₆ at normal rates throughout vitamin starvation. Nonleaky mutants for these and other vitamins were not found in E. coli B even after an extensive search using several types of mutagen.

The second mutant type to exhibit a negative vitamin B₆ biosynthetic rate was that group of mutants which have an absolute requirement for either glutamate or α-ketoglutarate. Mutants of this type have been shown by Ashworth et al. to lack citrate synthase (1). Two of the mutants of this type were WG1225, a mutant which showed negative or very low vitamin B₆ biosynthetic rates in these tests. The first of these was WG1225, a mutant which required thiamine for growth.

Phenotypically pyridoxineless but which showed negative or very low vitamin B₆ biosynthetic rates in these tests. The first of these was WG1225, a mutant which required thiamine for growth.
WG1390, a mutant of this type, made normal amounts of vitamin B₆ in media containing α-ketoglutarate or glutamate but failed to incorporate any significant amount of radioactivity into pyridoxal phosphate when grown in glucose minimal medium with uniformly labeled 14C-glutamate (Dempsey, in preparation) as tested by the method described previously (6). This suggested that the carbon skeleton of glutamate itself was not necessary for vitamin B₆ synthesis and that, instead, a less direct role of glutamate or α-ketoglutarate was involved.

The third mutant type to show a vitamin B₆ biosynthetic rate markedly lowered from the average value of 1.1 × 10^{-10} moles per hr per mg of cells (3) was the type of mutant designated Ppc by geneticists (17). Mutants of this phenotype usually lack phosphoenolpyruvate carboxylase and require a compound such as succinate, fumarate, aspartate, glutamate, or α-ketoglutarate for normal growth.

Results of experiments with two of these mutants are shown in Fig. 5. The rate of synthesis of vitamin B₆ in these mutants was 0.4 × 10^{-10} mole hr per mg or less than half the average rate of 1.1 × 10^{-10} moles per hr per mg (3). A large number of compounds was then tested for ability to restore vitamin B₆ biosynthesis to these mutants. The earlier finding of Morris and Woods that glycolaldehyde evidently played a role in vitamin B₆ biosynthesis (13) led to the inclusion of this compound in this test. Fortuitously, the result was that only glycolaldehyde had a positive effect. As can be seen in Fig. 5, addition of glycolaldehyde to a final concentration of 2 × 10^{-4} M appeared to restore vitamin B₆ biosynthesis without affecting cell growth.

This experiment was repeated, but instead of adding glycolaldehyde after starvation, different 2-carbon compounds, each at 5 × 10^{-4} M, were tested by having them present from the time starvation was initiated. Figure 6 shows that glycolaldehyde was the most effective of the compounds in restoring vitamin B₆ synthesis (middle panel) and that glyoxylate was most effective in restoring growth to the culture (left panel); rate of synthesis (right panel) was most effectively restored by glycolaldehyde. Glycolate was also tried in this series with results identical to those shown for acetaldehyde.

This finding suggested the interpretation that citrate synthaseless mutants might have failed to synthesize vitamin B₆ because they lacked isocitrate and, consequently, a source of 2-carbon units. If this were the case, then citrate synthaseless mutants suspended in media containing 2-carbon compounds ought to synthesize vitamin B₆. Table 2 shows that vitamin B₆ synthesis was not restored to normal in these mutants simply by the presence of 2-carbon compounds. In similar tests it was found that the presence of glycolaldehyde at 2 × 10^{-4} M had no effect upon vitamin B₆ synthesis by WG1225 during starvation for thiamine.

The possibility that glycolaldehyde was a precursor of vitamin B₆ was first suggested by Morris (11). The above evidence added support to the argument that this 2-carbon compound was involved in vitamin B₆ biosynthesis. When glycolaldehyde was then tested by the replica plate method as a replacement for the vitamin B₆ requirement of over 100 Pdx mutants of all five Pdx groups, the finding was that 10^{-4} M glycolaldehyde could satisfy the growth requirement of some group I Pdx mutants as measured at 37 C after 24 hr. This group had previously been divided into three feeding classes or phenotypes on the basis of cross-feeding properties (8). A member of each phenotype was then tested to determine whether glycolaldehyde would support normal growth in terms of growth rate as well as growth extent. The growth rates of aerobic cultures of one mutant of each of PdxB, PdxC, and PdxD classes were tested. Figure 7 shows that 4.2 × 10^{-4} M glycolaldehyde was equivalent in growth-supporting ability to 1.5 × 10^{-3} M pyridoxol for two of the three classes. Samples of these cultures grown on glycolaldehyde had normal contents of vitamin B₆.

Figure 1 shows that PdxC and PdxD mutants do not synthesize vitamin B₆ when suspended in glucose minimal medium. These data for PdxB have already been published (7). Based on this type of test, these mutants clearly are true pyridoxineless mutants. The finding that they reach
full growth in media containing no more vitamin B₆ than E. coli normally makes also indicates that they must be considered pyridoxineless mutants. Therefore, group I Pdx mutants are composed of pyridoxineless mutants which respond only to vitamin B₆ and pyridoxineless mutants which respond to either vitamin B₆ or glycolaldehyde.

DISCUSSION

Mutants which lack citrate synthase do not grow or make vitamin B₆ in the absence of α-ketoglutarate or glutamate, but these mutants do grow normally and make normal amounts of vitamin B₆ when α-ketoglutarate or glutamate are present. The failure of these mutants to synthesize vitamin B₆ during starvation for glutamate might be due to the diminished adenosine triphosphate supply in the presence of a defective Krebs cycle, but this seems likely to be at most only a partial explanation because some adenosine triphosphate should be available for vitamin B₆ synthesis from fermentation of glucose via the Embden Meyerhof route. An equally likely explanation might derive from the fact that glutamate is a cosubstrate of 3-phosphohydroxypropionate: glutamate aminotransferase, an enzyme which has been shown to be essential for vitamin B₆ biosynthesis (7); and in an absence of glutamate this essential reaction could not proceed.

Mutants which require thiamine for growth show greatly reduced vitamin B₆ synthesis during thiamine starvation. A relationship between the biosynthesis of these two compounds in yeast has been evident for some time (12), but it remains to be determined whether that relationship is as intimate as the sharing of a common precursor or as remote as the presence in the pyridoxal phosphate biosynthetic scheme of an enzyme which uses thiamine pyrophosphate as a coenzyme and vice versa. On the basis of numerous baffling observations with a large number of pyridoxineless mutants over the last several years, I favor the latter interpretation, but hard data in its support are not yet available. The existence of a class of pyridoxineless mutants which require either pyridoxine or glycolaldehyde for growth suggests that another type of pyridoxal phosphate "Km" mutant has been found. [This name applies to certain mutants of pyridoxal phosphate holoenzymes which respond with growth either to vitamin B₆ or to the product of the respective biosynthetic pathway such as iso-
leucine (9), lysine (2), or serine (7).] But in this case, in contrast to the others, the mutants are unable to make any vitamin B₆ without glycolaldehyde being present. This finding implicates glycolaldehyde or a derivative directly in vitamin B₆ biosynthesis. In addition, the two phenotypes PdxC and PdxB which respond to glycolaldehyde or vitamin B₆ not only are very closely linked to each other genetically (4, 5), but also are linked to a nonglycolaldehyde-responsive pyridoxineless mutant phenotype PdxD to form a group of three separate pdx genes (5) supporting the interpretation that these are true pyridoxineless mutants. The data may presently be interpreted as suggesting that PdxB and PdxC mutants have blocks in enzyme(s) before the enzyme block in PdxD. This may be represented by: X \xrightarrow{PdxB} Y \xrightarrow{PdxC} Z \rightarrow \text{vitamin B}_6. \text{In this scheme metabolites X and Y are unknown and metabolite Z either is glycolaldehyde or is readily derived from it.}

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