

# Biosynthesis of Isoleucine and Valine in *Rhodopseudomonas spheroides*: Regulation of Threonine Deaminase Activity

G. J. BARRITT<sup>1</sup>

Biochemistry Department, John Curtin School of Medical Research, The Institute of Advanced Studies,  
Australian National University, Canberra, A.C.T., Australia

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The activities of threonine deaminase, acetoxy acid synthetase, acetoxy acid reductoisomerase, dihydroxy acid dehydrase, and transaminase B were detected in cell-free extracts of *Rhodopseudomonas spheroides*. No significant repression or derepression of threonine deaminase activity was observed.

In *Escherichia coli* and *Salmonella typhimurium*, multivalent repression of four of the five enzymes of the isoleucine-valine biosynthetic pathway, as well as feedback inhibition of threonine deaminase (EC 4.2.1.16) and acetoxy acid synthetase activities, both contribute to the regulation of the biosynthesis of isoleucine and valine (5, 15). The pathways which lead to the biosynthesis of isoleucine and valine in *Rhodopseudomonas spheroides* have not been elucidated, although threonine deaminase activity has been detected in cell-free extracts (2). Moreover, Datta (2) has shown that the activity of this enzyme is inhibited by isoleucine and activated by valine, and the mechanism of the feedback inhibition and activation has been investigated (2, 3; G. Barritt and J. F. Morrison, unpublished data). Since little was known about the regulation of the level of threonine deaminase in *R. spheroides*, it was of interest to determine whether repression of the synthesis of threonine deaminase is also an important factor in the regulation of isoleucine biosynthesis in *R. spheroides*. This report describes the use of auxotrophs of *R. spheroides* in a study of the regulation of threonine deaminase activity in this organism.

## MATERIALS AND METHODS

A wild-type strain of *R. spheroides* was obtained from C. A. Appleby, originally from the stock of R. Y. Stanier. Mutations were induced in this strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1), the mutants were concentrated by penicillin selection (4), and auxotrophs with a requirement for both isoleucine and valine

or for threonine were isolated by replica plating (10). A culture of the *E. coli* K-12 auxotroph, JP58 (*ilvC*<sup>-</sup>), was kindly provided by J. Pittard. The *R. spheroides* cells were grown in a modification of the medium described by Lascelles (8). One liter of minimal medium contained: DL-malic acid, 2.1 g; glucose, 2.0 g; K<sub>2</sub>HPO<sub>4</sub>, 11.8 g; NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 6.8 g; (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 2.2 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g; CaCl<sub>2</sub>, 0.04 g; nicotinic acid, 3 mg; thiamine-hydrochloride, 3 mg; biotin, 30 μg; D-pantothenate, 8 mg; pyridoxal-hydrochloride, 8 mg; ferric citrate, 3 mg; and trace element solution (13), 1.0 ml. The pH of the medium was adjusted to 6.8. Threonine deaminase activity was measured by using essentially the method of Datta (2); one unit of activity is defined as the amount of enzyme which catalyzes the formation of 1 μmole of α-ketobutyrate per hr. Acetoxy acid synthetase activity was assayed by the method of Magee and de Robichon-Szulmajster (11), acetoxy acid reductoisomerase and dihydroxy acid dehydrase (EC 4.2.1.9) activities were measured by the method of Szentirmai et al. (14), and transaminase B (EC 2.6.1.15) activity was estimated qualitatively (7). Protein was estimated by the biuret method (9).

## RESULTS AND DISCUSSION

The activities of threonine deaminase (2) and the four enzymes which in *E. coli* are involved in the biosynthesis of isoleucine and valine from α-ketobutyrate and pyruvate, respectively (15), were detected in cell-free extracts of wild-type *R. spheroides*. When the activities of three of these enzymes were measured in cell-free extracts of nine isoleucine-valine auxotrophs, it was found that, in mutant F6 and in seven other auxotrophs, including F9 and F21, acetoxy acid synthetase activity was very low or absent (Table 1). In the other isoleucine-valine mutant which was

<sup>1</sup> Present address: Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106.

TABLE 1. Activities of acetohydroxy acid synthetase, acetohydroxy acid reductoisomerase, and dihydroxy acid dehydrase in cell-free extracts of *R. spheroides* auxotrophs which require isoleucine and valine<sup>a</sup>

Strain of <i>R. spheroides</i>	Specific enzyme activity ( $\mu$ moles per hr per mg of protein)		
	Acetohydroxy acid synthetase	Acetohydroxy acid reductoisomerase	Dihydroxy acid dehydrase
Wild-type	0.36	0.12	0.76
F6	0.01	0.17	1.24
F31	0.24	0.14	0.04

<sup>a</sup> Strains of *R. spheroides* were grown under photosynthetic conditions (8) in a medium which contained: tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g;  $\text{KH}_2\text{PO}_4$ , 1.0 g;  $\text{K}_2\text{HPO}_4$ , 1.0 g; and DL-isoleucine, DL-valine, and DL-leucine, 0.1 g each, in a volume of 1 liter, adjusted to pH 6.8. The cells were harvested in early stationary phase, cell-free extracts were prepared in 0.05 M Bicine-NaOH (pH 8.0), which contained 0.05 M potassium phosphate, 0.2 mM L-isoleucine, 4 mM DL-allothreonine, 20  $\mu$ M pyridoxal phosphate, 0.5 mM ethylenediaminetetraacetic acid, and 1 mM dithiothreitol, by using an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., London, England), and the resulting suspension was centrifuged ( $36,000 \times g$  for 10 min).

tested (F31), dihydroxy acid dehydrase activity was found to be very low (Table 1). The isoleucine-valine auxotrophs grew in minimal medium which was supplemented with both isoleucine and  $\alpha$ -ketoisovalerate, but no growth was observed in the presence of both  $\alpha$ -ketobutyrate and valine, or in the presence of either valine or isoleucine alone. These results indicate that in *R. spheroides*, the pathways for the synthesis of isoleucine and valine from threonine and pyruvate, respectively, are similar to those found in *E. coli* (15).

When the isoleucine-valine auxotroph, F9, was grown aerobically in minimal medium supplemented with L-valine, L-leucine (slow growth was observed in the absence of leucine), and growth-limiting concentrations of glycyl-L-isoleucine (16), no marked increase in threonine deaminase activity was observed from the time at which limitation of growth began until 20 hr after rapid growth had ceased (Fig. 1A). The threonine deaminase activity observed under these conditions differed little from the activity (0.8  $\mu$ mole per hr per mg of protein) present when the same auxotroph was grown in the presence of excess concentrations of L-isoleucine. Furthermore, these enzyme levels were similar to the level of activity present in wild-type *R. spheroides* cells (Fig. 1A, Table 2). Similar results were obtained with two different isoleucine-valine auxotrophs (F6 and F21). The activity of threonine deaminase in the cells of a slow-growing revertant of

mutant F9 (F9/25) was also measured, first, when the cells were grown in minimal medium, and second, when the cells were grown in minimal medium supplemented with excess L-isoleucine, L-valine, and L-leucine. It was found that there was no significant difference between the activity of threonine deaminase in the cells grown in minimal medium and the activity of the enzyme in the cells grown in the presence of excess isoleucine, valine, and leucine (Table 2). By contrast, the activity of threonine deaminase in cells of the *E. coli* auxotroph, JP58 (*ilvC*<sup>-</sup>), grown in the presence of growth-limiting concentrations of glycyl-L-isoleucine, was 25 times higher than that observed when this auxotroph was grown in the presence of excess L-isoleucine, L-valine, and L-leucine (Table 2).

When the *R. spheroides* mutant T2 (Thr<sup>-</sup>) was grown in the presence of growth-limiting concentrations of L-threonine, again, no increase in threonine deaminase activity was observed (Fig. 1B), and the enzyme activity was similar to the level (1.0  $\mu$ mole per hr per mg of protein) observed in cells of the same auxotroph which were grown in the presence of excess concentrations of L-threonine and L-isoleucine. Similar results were obtained with two other threonine auxotrophs. The activity of threonine deaminase present in wild-type *R. spheroides* cells was also measured, and the results indicate that the enzyme activity in cells grown in minimal medium is comparable with that observed in cells grown in minimal medium supplemented with excess L-isoleucine, L-valine, L-leucine, and D-pantothenate (Table 2).

It was considered possible that repression or derepression of threonine deaminase might only be observed when *R. spheroides* cells were grown under photosynthetic conditions. However, no derepression of threonine deaminase was observed when the auxotroph F9 (*Ilv*<sup>-</sup>) was grown photosynthetically (8) in minimal medium which was supplemented with excess L-valine (50  $\mu$ g/ml), L-leucine (50  $\mu$ g/ml), and growth-limiting concentrations of glycyl-L-isoleucine (25  $\mu$ g/ml).

The results indicate that in *R. spheroides* the level of threonine deaminase is not subject to marked fluctuations as a result of repression or derepression. This finding contrasts with the multivalent repression of threonine deaminase and other enzymes of the isoleucine-valine biosynthetic pathway, which has been observed in *E. coli* and *S. typhimurium* (5). Although there appears to be no repression or derepression of threonine deaminase in *R. spheroides*, the activity of this enzyme is inhibited by isoleucine, and valine can reverse this inhibition (2). Moreover, it was found that the activity of acetohydroxy acid syn-

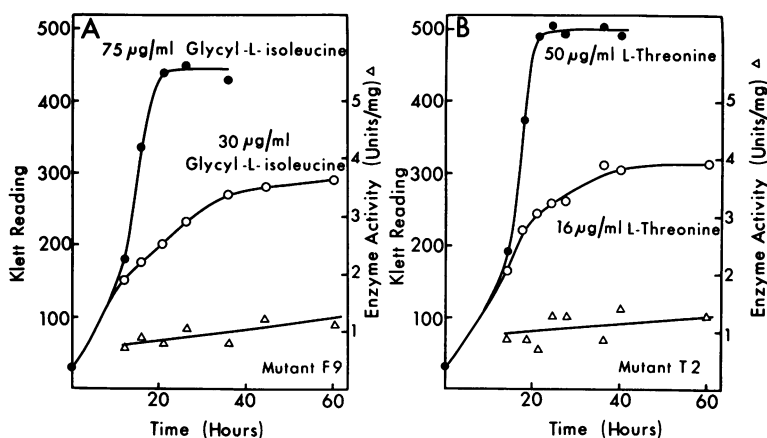


FIG. 1. Threonine deaminase activity. (A) Cell growth (O) and threonine deaminase activity present in cell-free extracts ( $\Delta$ ) of the *R. spheroides* auxotroph F9, which lacks acetohydroxy acid synthetase activity (*Ilv*<sup>-</sup>). The cells were grown aerobically in minimal medium supplemented with L-leucine (50  $\mu$ g/ml), L-valine (50  $\mu$ g/ml), and a limiting amount of glycyl-L-isoleucine (30  $\mu$ g/ml). Cell growth in the presence of excess glycyl-L-isoleucine (75  $\mu$ g/ml) is also shown ( $\bullet$ ). (B) Cell growth (O) and threonine deaminase activity present in cell-free extracts ( $\Delta$ ) of the *R. spheroides* auxotroph T2, in which either homoserine kinase (EC 2.7.1.39) or threonine synthetase (EC 4.2.99.2) activity is absent (*Thr*<sup>-</sup>). The cells were grown aerobically in minimal medium supplemented with L-threonine (16  $\mu$ g/ml). Cell growth in the presence of excess L-threonine (50  $\mu$ g/ml) is also shown ( $\bullet$ ). The cells were grown in 250-ml volumes of medium in 1-liter flasks which were shaken at 37 C. Growth was followed turbidimetrically by using a Klett-Summerson photoelectric colorimeter (filter no. 42), and a Klett reading of 100 units corresponds to 140  $\mu$ g (dry weight) of cells per ml. Samples (30 ml) were withdrawn aseptically, and cell-free extracts were prepared as described in the footnote of Table 1. (It was shown that the isoleucine present in the extraction buffer does not inhibit the activity of threonine deaminase under the assay conditions used.)

TABLE 2. Level of threonine deaminase activity in strains of *R. spheroides* and *E. coli* K-12 grown under aerobic conditions

Bacterial strain	Growth medium <sup>a</sup>	Threonine deaminase specific activity ( $\mu$ moles per hr per mg of protein)
Wild-type <i>R. spheroides</i>	Excess isoleucine <sup>b</sup>	0.9
	Minimal medium	0.9
F9/25 ( <i>Ilv</i> <sup>-</sup> ) <sup>c</sup>	Excess isoleucine <sup>b</sup>	0.8
	Minimal medium	1.0
<i>E. coli</i> JP58 ( <i>ilvC</i> <sup>-</sup> )	Excess isoleucine	0.7
	Limiting isoleucine	16.6

<sup>a</sup> Strains of *R. spheroides* were grown aerobically in minimal medium which was supplemented with 2 g of glutamic acid per liter; the *E. coli* strain was grown in medium 56 (12) which also contained thiamine-hydrochloride, 0.1 mg; L-arginine, 0.15 g; and glucose, 2 g per liter of medium. The minimal medium was supplemented with L-isoleucine, L-valine, and L-leucine, at 50  $\mu$ g/ml (excess isoleucine); or with glycyl-L-isoleucine, at 30  $\mu$ g/ml and L-valine and L-leucine at 50  $\mu$ g/ml (limiting isoleucine). The cells were grown as described in the legend of Fig. 1 and were harvested at late logarithmic phase. Cell-free extracts were prepared as described in footnote a, Table 1.

<sup>b</sup> Supplemented with 8 mg of D-pantothenate per liter.

<sup>c</sup> Mutant F9/25, which was isolated by the method of Gerhart and Holoubek (6), exhibits a partial requirement for both isoleucine and valine.

TABLE 3. Inhibition by valine of acetohydroxy acid synthetase activity in cell-free extracts of *R. spheroides*<sup>a</sup>

Concn of valine (mM)	Specific enzyme activity ( $\mu$ moles per hr per mg of protein)
0	0.50
0.1	0.35
1.0	0.21
10.0	0.12

<sup>a</sup> Wild-type *R. spheroides* cells were grown in minimal medium under photosynthetic conditions (8), and cell-free extracts were prepared as described in footnote a, Table 1. Acetohydroxy acid synthetase activity was measured by the method of Magee and de Robichon-Szulmajster (11), by using a concentration of pyruvate of 5.0 mM.

thetase is inhibited by valine (Table 3). Thus, it appears that in *R. spheroides* the biosynthesis of isoleucine and valine is regulated by a mechanism of feedback inhibition which is similar to that observed in *E. coli* (15).

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