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Pathway for Oxidative Dissimilation of Glycerol in *Bacillus subtilis*

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It has been suggested that the main pathway for oxidative glycerol dissimilation in *Bacillus subtilis* involves a nicotinamide adenine dinucleotide (NAD)-dependent glycerol dehydrogenase (8). In this paper, evidence is presented that supports the notion that glycerol dissimilation in this organism proceeds primarily via an NAD-independent glycerol phosphate dehydrogenase. In strains 168 and W23 of *B. subtilis*, activity corresponding to the dihydroxyacetone kinase and NAD-dependent glycerol dehydrogenase reported by Wiame et al. (8) was found; however, glycerol kinase and an inducible NAD-independent glycerophosphate dehydrogenase of higher specific activity were also found in the same strains. It was also observed that approximately 10% of mutants unable to grow on glycerol as the major source of carbon and energy are lacking the NAD-independent glycerophosphate dehydrogenase. The mutations were induced with ethyl methane sulfonate, and the mutants were isolated by replica plating after penicillin selection in minimal medium supplemented with tryptophan, histidine, methionine, proline, threonine, serine, and valine at 10 μg/ml (containing 0.3% glycerol as the carbon source). The mutant character is transformable at a frequency characteristic of single site mutations. The mutants grow on all carbon sources, except glycerol, that support the growth of the parent strain, e.g., glucose, mannitol, succinate, fumarate, galactose, and amino acids. These mutants have unchanged activity for glycerol dehydrogenase, indicating that it is the NAD-independent enzyme that is involved in the dissimilation of glycerol. The NAD-dependent glycerol dehydrogenase apparently is the means by which the cell synthesizes glycerol from dihydroxyacetone or glyceraldehyde and 2,3-butanediol from acetoin (1). Although the cells contain the enzymes (glycerol dehydrogenase and dihydroxyacetone kinase) that might lead to dihydroxyacetone phosphate through an NAD-linked system, they probably do not use this pathway for the dissimilation of glycerol.

The NAD-independent glycerophosphate dehydrogenase activity is induced by growth on glycerol and is repressed by growth on glucose (Table 1). A mutant of strain 168, designated B24, which is unable to grow on glycerol as a carbon source, does not form the NAD-independent enzyme. Glycerol gives no reaction in the assay system. The reaction with α-glycerophosphate does not require and is not stimulated by either NAD or nicotinamide adenine dinucleotide phosphate.

Neither the oxidation of reduced nicotinamide adenine dinucleotide (NADH) nor the reduction of NAD is stimulated by dihydroxyacetone phosphate or α-glycerophosphate. Although the activities are measured in fairly crude extracts and may, therefore, be somewhat inaccurate, it seems clear that the NAD-independent enzyme has a much greater activity than the glycerol dehydrogenase. Extracts of cells of strain 168 grown in minimal medium show an activity of 1.2×10^{-2} μmoles per min per mg of protein when assayed by the reduction of NAD in the presence of glycerol. This may be compared to the activity of 6.2×10^{-2} μmoles per min per mg of protein shown by extracts of the same strain grown in the same medium when assayed for NAD-independent α-glycerophosphate dehydrogenase activity.

The activity of the NAD-dependent glycerol dehydrogenase, although variable in extracts of cells grown on different media, does not respond to changes in the glycerol concentration of the growth medium. The finding that the NAD-independent enzyme activity is inducible adds considerable support to the notion that it is on the pathway of oxidative glycerol dissimilation. Activities for glycerol kinase and dihydroxyacetone kinase were found to be 0.04 and 0.08 μmoles per min per mg of protein, respectively, when measured by NAD reduction or NADH oxidation coupled with α-glycerophosphate dehydrogenase. The reaction mixture for glycerokinase activity at 25 C contained 0.3 ml of 0.1 M glycerol, 0.3 ml of 3 N hydrazine, 0.5 ml of 1 M carbonate, *p*H 9.5, 0.6 ml of 0.1 M adenosine

TABLE 1. *Variation of NAD-independent L-α-glycerophosphate dehydrogenase activity with growth conditions*[a]

Strain	Carbon source	Enzyme activity (μmoles per min per mg of protein)
168	Vitamin Free Casamino Acids (2 mg/ml)	4.7×10^{-3}
168	Vitamin Free Casamino Acids (2 mg/ml) plus glycerol (2 mg/ml)	6.2×10^{-2}
168	Glucose (2 mg/ml) plus glycerol (2 mg/ml)	8.9×10^{-3}
B24	Vitamin Free Casamino Acids (2 mg/ml) plus glycerol (2 mg/ml)	8.4×10^{-5}

[a] NAD-dependent glycerol dehydrogenase activity of strain 168 in Vitamin Free Casamino Acids (2 mg/ml) plus glycerol (2 mg/ml) was 1.2×10^{-2} μmole per min per mg of protein. This enzymatic activity was determined at 25 C from the initial rate of reduction of NAD in 0.1 M pyrophosphate buffer at *p*H 8.7.

[b] Enzymatic activities were determined at 25 C according to the procedure of E. C. C. Lin et al. (4), using lysozyme lysates of cells growing exponentially in minimal medium (5), supplemented with 0.5 mg of Vitamin Free Casamino Acids per ml, plus the carbon sources listed above. The assay involves the reduction of 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide in the presence of phenazine methosulfate and α-glycerophosphate.

triphosphate (ATP), 0.6 ml of 0.1 M $MgCl_2$, 0.2 ml of 0.02 M NAD, 0.3 mg of L-α-glycerophosphate dehydrogenase, plus extract and water to make 3 ml. The mixture for dihydroxyacetone kinase activity contained 0.3 ml of 0.1 M dihydroxyacetone, 2.5 ml of 0.05 M phosphate buffer, *p*H 7.5, 0.6 ml of 0.1 M ATP, 0.6 ml of 0.1 M $MgCl_2$, 0.03 ml of 0.014 M NADH, 0.3 mg of L-α-glycerophosphate dehydrogenase, plus 0.1 ml of extract. These activities may be properties of the same enzyme, since purified glycerol kinases from other sources are able to phosphorylate dihydroxyacetone, often at greater rates than glycerol (2). The activity of glycerol kinase is not inducible by growth on glycerol.

The finding of both glycerol dehydrogenase and glycerophosphate dehydrogenase in the same strain has been made previously in other organisms. Several pathways have been described for glycerol dissimilation in bacteria. One involves an L-α-glycerophosphate dehydrogenase that is independent of NAD (7), whereas another involves an NAD-dependent glycerol dehydrogenase (3). Some strains of enteric bacteria have one of these enzymes, others have both (6). One or both of these enzymes can exist in an inducible form.

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