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6-Phospho-D-Gluconate:NAD+ 2-Oxidoreductase (Decarboxylating) from Slow-Growing Rhizobia

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6-Phospho-D-gluconate:NAD+ 2-oxidoreductase (decarboxylating) (NAD+-6PGD) was detected in several slow-growing strains of rhizobia, and no activity involving NADP+ was found in the same extracts. By contrast, fast-growing strains of rhizobia had NADP+-6PGD activity; most of them also had NAD+-6PGD activity. NAD+-6PGD was partially purified from the slow-growing strain *Rhizobium japonicum* 5006. The reaction was shown to be an oxidative decarboxylation.

6-Phosphogluconate is an important substrate in the intermediary metabolism of D-glucose in bacteria. This compound is a common metabolite for both the Entner-Doudoroff pathway and the hexose monophosphate shunt. There is some evidence (7-9) for the presence of the Entner-Doudoroff pathway in most strains of rhizobia studied, including slow-growing ones. By contrast, the oxidative portion of the hexose monophosphate shunt does not seem to be present in all the rhizobia strains (8, 9); the key enzyme, 6-phosphogluconate dehydrogenase (6PGD), specific for oxidized nicotinamide adenine dinucleotide phosphate (NADP+), was found in fast-growing (7, 9), but not in slow-growing, strains (8, 9).

However, both fast- and slow-growing rhizobia have been reported to contain NAD+-dependent 6PGD (G. Martínez-Drets and A. Arias, Actas V Reun. Lat. Am. de Rhizobium, Rio, Brazil, p. 15, 1970; K. Mulongoy and G. H. Elkan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K169, p. 164). We now report partial purification of this enzyme from the slow-growing strain *Rhizobium japonicum* 5006 and the nature of the reaction catalyzed.

MATERIALS AND METHODS

Bacterial strains. *Rhizobium* strains were obtained from different sources (Table 1). They were kept in 2% agar stabs of mannitol yeast medium (10).

Culture of organisms. Cells were grown in the medium of Keele et al. (8) with 2 g of yeast extract per liter and incubated at 30°C on an oscillatory shaker (100 rpm). Culture media were inoculated with 1% of a 72-h subculture of bacteria, and the cells were collected after 96 h. Cells were centrifuged at 11,600 × *g*, washed in 0.005 M sodium phosphate buffer (pH 7.0), and kept frozen at −20°C. The yield of wet cells was about 8 g per liter of medium.

Cell-free extract preparation. Frozen cell paste, 16 g, was suspended in 25 ml of 0.005 M sodium phosphate buffer (pH 7.0), disrupted by sonic exposure (eight 1-min treatments with a Branson sonifier) and centrifuged at 14,000 × *g* for 30 min. This cell-free extract was employed for the measurement of the enzyme activities.

Enzyme studies. 6PGD was determined by measuring the reduction of NAD+ or NADP+ at 340 nm in a spectrophotometer (Beckman model DU). The incubation mixture contained (in a total volume of 1.0 ml): glycylglycine buffer (pH 8.0), 70 μmol; $MgSO_4$, 10 μmol (Table 1 only); NAD+ or NADP+, 0.5 μmol; sodium 6-phosphogluconate, 2.5 μmol; and sufficient diluted enzyme solution to produce an absorbance change of about 0.030 per min. Corrections were made for the small amount of NADH or NADPH formed in controls without substrate or with boiled extract. Specific activities were expressed in micromoles of NADH or NADPH formed per minute and per milligram of protein at 30°C. Gluconate dehydrogenase (EC 1.1.99.3) activity was determined by following NAD+ or NADP+ reduction at 340 nm. The incubation mixture contained: glycine-sodium hydroxide buffer (pH 10.0), 20 μmol; $MgSO_4$, 2 μmol; NAD+ or NADP+, 0.3 μmol; sodium gluconate, 15 μmol; and enzyme solution (0.3 mg of protein), in a total volume of 1.0 ml. NADH oxidase was measured by following the disappearance of NADH at 340 nm in an incubation mixture containing: NADH, 0.1 μmol; glycylglycine buffer (pH 8.0), 70 μmol; and cell-free extract, in a total volume of 1.0 ml.

The presence of the Entner-Doudoroff pathway was detected by measuring the conversion of 6-phosphogluconate to pyruvate by the method of Keele et al. (8).

Chemical and analytical methods. Sodium 6-phospho[1-^{14}C]gluconate was prepared enzymatically from D-[1-^{14}C]glucose-6-phosphate, disodium salt (New England Nuclear Corp.), and NADP+

TABLE 1. *Rhizobia strains employed and 6PGD activity*

Strain	Source[a]	Sp act (×10³)	
		NAD⁺	NADP⁺
Rhizobium japonicum E2	INTA	47	<0.5
R. japonicum 5006	IPEACS	66	<0.5
R. lupini SV623	UUS	16	<0.5
R. lupini D48	IPVP	68	<0.5
Rhizobium sp. (for cowpea) 316U6	USDA	17	<0.5
Rhizobium sp. (for cowpea) 9931	ATCC	21	1.8
Rhizobium sp. (for cowpea) CB33	CSIRO	34	<0.5
Rhizobium sp. CB627	CSIRO	19	<0.5
Rhizobium meliloti U210	PA	<0.5	51
R. meliloti U211	PA	<0.5	24
R. trifolii TA1	DATA	55	167[b]
R. trifolii WU290	WAU	15	51
R. trifolii NZ29	DANZ	50	237[b]
R. leguminosarum 10004	ATCC	25	91
Rhizobium sp. (for *Lotus corniculatus*) B816	DMKA	6	100[b]
Rhizobium sp. (for *Lotus corniculatus*) U24	PA	4	28

[a] INTA, Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina; IPEACS, Instituto de Pesquises e Experimentaçao Agropecuaria, Rio de Janeiro, Brasil; UUS, Department of Bacteriology, University of Uppsala, Uppsala, Sweden; IPVP, Institut de Recherche et Production Végétale, Prague, Czechoslovakia; USDA, U.S. Department of Agriculture, Washington, D.C.; ATCC, American Type Culture Collection, Rockville, Md.; CSIRO, Commonwealth Science and Industrial Research Organization, Brisbane, Australia; PA, Plan Agropecuario, Montevideo, Uruguay; DATA, Department of Agriculture, Tasmania, Australia; WAU, Western Australia University; DANZ, Department of Agriculture, New Zealand; DMKA, Department of Microbiology, Kensington, Australia.

[b] Data from Martínez-Drets and Arias (9).

with yeast glucose 6-phosphate dehydrogenase (Boehringer). Enzymatic production of $^{14}CO_2$ from 6-phospho[1-^{14}C]gluconate was measured by a modification of the method of Brown and Wittenberger (3). The assays were performed in a 10-ml Warburg vessel that has a center well and two lateral arms. The main compartment contained: tris(hydroxymethyl)aminomethane buffer, pH 7.5, 50 μmol; sodium 6-phospho[1-^{14}C]gluconate, 2.5 μmol (166,000 cpm); ethylenediaminetetraacetic acid, tetrasodium salt, 1 μmol; and partially purified enzyme preparation, 0.7 U, in a volume of 0.8 ml. One lateral arm contained NAD⁺ or NADP⁺, 2 μmol, and the other lateral arm contained 0.2 ml of 5 N H_2SO_4. The center well contained 0.2 ml of 3.4 N KOH. The vessels were connected with a Warburg manometer and incubated for 5 min at 30°C to permit temperature equilibration. The reaction was begun by the addition of NAD⁺ or NADP⁺ from the lateral arm to the main compartment. The vessels were incubated for 1 h at 30°C; after this time, the reaction was stopped by adding H_2SO_4 from the second lateral arm. To complete the total liberation of $^{14}CO_2$ and its trapping in the center well, the vessels were further incubated for 1 h at 37°C in a shaker bath. The total content of the center well was transferred to a centrifuge tube, transformed into $Ba^{14}CO_3$, and separated by centrifugation, and its radioactivity was measured in a thin-window gas-flow G-M counter.

Pentoses were determined by the cysteine-carbazole method (1) and the orcinol method (6). Ribulose (Calbiochem) was identified by paper chromatography by using a solvent system of *n*-butanol–acetic acid–water (120:30:50) and spraying the paper chromatograms with ammoniacal silver nitrate, phloroglucinol reagent, or anisidine phthalate (17). 6-Phosphogluconate was determined by following the formation of NADPH enzymatically (12) in the presence of limiting amounts of the substrate. Protein was determined by Bucher's method, which was standardized with bovine serum albumin (4).

RESULTS

Activity of NAD⁺-6PGD. NAD⁺-6PGD activity was detected in cell-free extracts from eight strains of slow-growing rhizobia, and no activity of NADP⁺-6PGD was found in the same extracts (Table 1, top eight lines). NAD⁺-6PGD activity was found in eight strains of fast-growing rhizobia but not in two fast-growing strains belonging to the group of *R. meliloti*; NADP⁺-6PGD was found, as previously (9), in all the fast-growing strains of rhizobia studied (Table 1, bottom eight lines). Little if any NADH or NADPH oxidase was detected in each extract. Mg^{2+} had no apparent effect on the 6PGD activities and was omitted in later experiments.

Table 2 shows that, for *R. japonicum* 5006, there was some variation in the NAD⁺-dependent activity depending on the carbon source; in no case was there NADP⁺-dependent activity.

Purification of NAD⁺-6PGD. NAD⁺-6PGD was partially purified from a cell-free extract of *R. japonicum* 5006 (Table 3). The cell-free extract was treated with 2% protamine sulfate in 0.05 M sodium phosphate buffer (pH 7.0) (0.114 mg of protamine per mg of protein) and centrifuged after 30 min at 0°C; the precipitate was discarded. The supernatant (protamine fraction) solution was heated in a water bath at 80°C for 2 min and centrifuged at 10,000 × *g* for 10 min; the precipitate was discarded. The supernatant solution (heated fraction) was taken to 55% saturation with solid ammonium sul-

TABLE 2. *6PGD activity in extracts of R. japonicum 5006 grown on different carbon sources*

Carbon source in growth medium	Sp act (×10³)	
	NAD⁺	NADP⁺
D-Glucose	66.0	<0.5
Glycerol	37.4	<0.5
D-Sodium gluconate	30.0	<0.5
L-Arabinose	18.9	<0.5

TABLE 3. *Purification of NAD+-6PGD*[a]

Fraction	Vol (ml)	Total U (×10³)	Total protein (mg)	Sp act (×10³)	Yield (%)
(1) Sonic extract	28	35,840	19.3	66	100
(2) Protamine fraction	30	43,200	13.8	104	120
(3) Heated fraction	26	36,140	2.6	534	101
(4) Ammonium sulfate	3	22,460	7.2	1,040	62.8
(5) Sephadex G-100	2.5	16,000	1.1	5,818	44.6

[a] Values shown are for 16 g of bacterial cells (wet weight).

fate, and the precipitate was collected after 10 min by centrifugation, dissolved in 0.005 M sodium phosphate buffer (pH 7.0), and dialyzed overnight against the same buffer (ammonium sulfate fraction). The dialyzed solution was chromatographed on a Sephadex G-100 column (1.3-cm diameter by 14.5 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The column was eluted at a rate of 30 ml/h with 0.01 M sodium phosphate buffer (pH 7.0). One peak of NAD+-6PGD activity was obtained, precipitated with ammonium sulfate to 80% saturation, and dissolved in 0.005 M sodium phosphate buffer (pH 7.0) (Sephadex G-100 fraction). All operations were performed at 0°C unless otherwise stated.

Gluconate dehydrogenase, which was present in the crude extract, had disappeared after heat treatment. Appreciable Entner-Doudoroff pathway activity in the cell-free extract was totally separated by the passage through the Sephadex G-100 column.

Properties of NAD+-6PGD from *R. japonicum* 5006. (i) Stability. The cell-free extract was kept frozen for more than 2 months without losing its activity. Partially purified extract was kept for 1 month at −20°C with a loss of about 30% of its activity.

(ii) Effect of pH on reaction rate. The optimal pH was between 7.5 and 8.0, with about 30% activity at pH 8.7. The buffers used for the different pH determinations were sodium acetate (pH 4.8 to 6.0), sodium phosphate (pH 6.0 to 8.0), and tris(hydroxymethyl)aminomethane (pH 7.5 to 9.0).

(iii) Effect of substrate and coenzyme concentration. The Michaelis constants were determined following the method of Lineweaver-Burk. Values for 6-phosphogluconate and NAD+ were 1.2×10^{-4} and 9.1×10^{-5} M, respectively.

(iv) Enzymatic reaction mechanism. It was necessary to establish the nature of the reaction of the enzyme (fraction 5, Table 3) and to determine whether NAD+-6PGD from *R. japonicum* 5006 had decarboxylating as well as oxidative activities.

Decarboxylating activity. The product of enzymatic decarboxylation of 6-phosphogluconate was determined by measuring the $^{14}CO_2$ formed. In Table 4, it is shown that by incubating NAD+ with 6 phospho[1-^{14}C]gluconate there was stoichiometric production of $^{14}CO_2$. No significant amount of $^{14}CO_2$ was produced by substituting NADP+ for NAD+.

Oxidative activity. 6-Phosphogluconate oxidation was followed by: (i) measuring the formation of NADH, and (ii) determining the final products formed. Formation of NADH was followed spectrophotometrically, and the products of 6-phosphogluconate oxidation were determined by colorimetric and chromatographic methods (17). For this determination, a mixture of NAD+, 6-phosphogluconate, and the enzyme preparation was added to a system able to reoxidize the formed NADH. The incubation mixture contained 6-phosphogluconate, 25 μmol; NAD+, 4 μmol; sodium pyruvate, 40 μmol; rabbit muscle lactic dehydrogenase (Sigma), 0.75 mg; glycylglycine buffer (pH 8.0), 100 μmol; and partially purified enzyme (1.9 U), to a final volume of 2.0 ml. Two controls lacked NAD+ or contained boiled enzyme. The mixture was incubated at room temperature for 1 h. At 0, 30, and 60 min, portions were taken to

TABLE 4. *Decarboxylating activity of NAD+-6PGD from R. japonicum 5006*

Incubation mixture	$^{14}CO_2$		Pentose formed (μmol)
	cpm	Amt (μmol)	
Complete	138,000	2.07	1.65
Minus NAD+	4,500	0.08	0.08
Minus NAD+ + NADP+[a]	5,600	0.10	ND[c]
Minus enzyme	2,800	0.05	ND
Minus enzyme + boiled enzyme[b]	4,500	0.08	0.10

[a] NAD+ was substituted by NADP+ (also 2 μmol) in the incubation mixture.

[b] Enzyme was denatured by heating at 100°C for 5 min. (For details, see Materials and Methods.)

[c] ND, Not determined.

measure disappearance of 6-phosphogluconate. There was total disappearance of the 6-phosphogluconate by 1 h. The reaction was stopped by heating the incubation mixture at 100°C for 5 min. The percentage of pentose phosphate formed from 6-phosphogluconate was 87%. The preparation was then centrifuged, and the supernatant fraction was taken to pH 5.5, treated with potato acid phosphatase (7 U, Sigma) for 4 h at 30°C, and deproteinized by heating at 100°C for 5 min. The supernatant portion was passed through a mixed column of Amberlite IR-45 (OH^-)-IR 120 (H^+) (2.3 by 7.5 cm), and the unabsorbed fraction, together with a water wash, was shaken for 30 min with activated carbon, evaporated to dryness, and dissolved in 0.5 ml of water. Pentose recovered after dephosphorylation and purification was 58%. Paper chromatography (Materials and Methods) gave a single spot of reducing sugar, which, by comparison with standards (D-glucose, D-gluconate, D-ribose, D-ribulose, D-xylose, L-arabinose, D-fructose, DL-glyceraldehyde, sodium lactate, sodium pyruvate, and dihydroxyacetone) was coincident with D-ribulose.

DISCUSSION

6PGD is a widely distributed enzyme that has been isolated from different sources, and it is generally found to be specific for $NADP^+$: sheep liver (16), human erythrocytes (11), *Neurospora crassa* (15), *Escherichia coli* (14), *Candida utilis* (13), and brewer's yeast (5). However, it has been found that some bacteria have 6PGD with activity associated with both NAD^+ and $NADP^+$. Thus, 6PGD isolated from glucose-adapted cells of *Streptococcus faecalis* was $NADP^+$ specific, whereas gluconate-adapted cells possessed an NAD^+-specific 6PGD in addition to the $NADP^+$-linked enzyme (3). The data reported here suggest the existence of an NAD^+-linked 6PGD in the eight slow-growing rhizobia studied and absence of $NADP^+$-6PGD in the same extracts. The NAD^+-6PGD partially purified from *R. japonicum* 5006, which was freed from the Entner-Doudoroff pathway and gluconate dehydrogenase, catalyzes the transformation of 6-phosphogluconate by an oxidative decarboxylation mechanism, with the formation of CO_2 and ribulose phosphate. It is unlikely that the oxidative decarboxylation detected in *R. japonicum* was due to the action of two different enzymes, a dehydrogenase and a decarboxylase, as already reported in *Leuconostoc mesenteroides* BO7 (19), because the addition of semicarbazide (10 mM) to the complete incubation mixture (Table 4) had no effect on the decarboxylating activity of NAD^+-6PGD. Bowien and Schlegel (2) reported the existence in *Pseudomonas facilis* of an oxidative decarboxylating 6PGD specific for NAD^+, which seems to be similar to NAD^+-6PGD isolated from *R. japonicum* 5006. Similarity between NAD^+-6PGD from rhizobia and from the pseudomonad is new evidence supporting their phylogenetic relationship (18).

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