Multiple Forms of D(—)3-Hydroxybutyrate Dehydrogenase in Rhizobium

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SUMMARY

3-Hydroxybutyrate dehydrogenase was studied in 14 *Rhizobium* strains representing six species. Cell-free extracts of the bacteria were subjected to electrophoresis in starch or polyacrylamide gels and 3-hydroxybutyrate dehydrogenase was located *in situ* on the gels. Also, the rate of reaction of the enzyme, from different *Rhizobium* strains, with NAD was compared with its rate of reaction with various analogues of NAD. Most of the *Rhizobium* strains had characteristic 3-hydroxybutyrate dehydrogenase patterns on gel electropherograms and these patterns were highly reproducible. Multiple forms of 3-hydroxybutyrate dehydrogenase were detected in many of the *Rhizobium* strains.

INTRODUCTION

3-Hydroxybutyrate dehydrogenase (HBDH) (D-3-hydroxybutyrate:NAD oxidoreductase, EC 1.1.1.30) catalyses the reversible oxidation of 3-hydroxybutyrate to acetoacetate in the presence of NAD⁺. The enzyme occurs in micro-organisms (Shuster & Doudoroff, 1962) and in mitochondria of mammalian cells (Green, Dawan & Leloir, 1937). We recently showed, in a preliminary report (O'Hora & Fottrell, 1968), that multiple forms of HBDH could be detected in rhizobia with the aid of gel electrophoresis. The HBDH electrophoresis patterns, in some instances, were species and strain specific. In the present paper gel electrophoresis and coenzyme analogues (Kaplan & Ciotti, 1961) were used to compare HBDH from six *Rhizobium* species.

METHODS

Organisms. Fourteen *Rhizobium* strains representing six species were used. The bacteria, all of which nodulated their respective host plants, are listed in Table 1. All strains were streaked on yeast extract (Oxoid) mannitol agar (Allen, 1957), to confirm purity before being inoculated into growth medium. The bacteria were grown in 2-litre Erlenmeyer flasks containing yeast extract and mannitol, on an orbital shaker at 28°, for 4 days in the case of *Rhizobium leguminosarum*, *R. trifolii*, *R. meliloti* and *R. phaseoli* and for 8 days in the case of *R. japonicum* and *R. lupini*. The bacteria were harvested by centrifugation, washed with 0.9% NaCl and stored at -20° until required.

Soybeans (*Glycine max.*) were grown in nitrogen-free medium and inoculated with the appropriate *Rhizobium* strain as described previously (Fottrell, 1966). Bacteroids were isolated from soybean nodules as described by Bergersen (1960).

Preparation of cell-free extracts. Suspensions of bacteria and bacteroids (1 g. organisms/1.5 ml. buffer) were disrupted for 2 min. at a temperature less than 5° in 0.05 M-phosphate buffer ($\text{Na}_2\text{HPO}_4 + \text{NaH}_2\text{PO}_4$), pH 7.6, with an M.S.E. sonic disintegrator. HBDH was also extracted from organisms by grinding with alumina in 0.05 M-phosphate buffer (pH 7.6) for 30 min. at 4°. After disruption, the suspension was centrifuged for 90 min. at 35,000 g at 0° and the clear supernatant used for enzyme assays and electrophoresis. Approximately 85% of the total cellular HBDH activity was present in the supernatant after centrifugation.

Assay of HBDH. Enzyme activity was estimated spectrophotometrically at 340 m μ as described by Bergmeyer *et al.* (1967). Specific activity was expressed in international units per mg. protein (Bergmeyer, 1965). Protein was estimated by the biuret reaction (Gornall, Bardawill & David, 1949).

Table 1. *Physiological characteristics and source of Rhizobium strains*

Species	Strain no.	Test host	N-fixation*	Source†
<i>Rhizobium japonicum</i>	392	<i>Glycine max.</i>	+	A
	311b59	<i>Glycine max.</i>	+	C
	3401	<i>Glycine max.</i>	—	B
<i>R. leguminosarum</i>	1001	<i>Vicia hirsuta</i>	+	B
	1003	<i>Vicia hirsuta</i>	—	B
<i>R. trifolii</i>	7	<i>Trifolium pratense</i>	+	B
	6	<i>Trifolium pratense</i>	±	B
	5	<i>Trifolium pratense</i>	++	B
	4	<i>Trifolium pratense</i>	—	B
<i>R. phaseoli</i>	3601	<i>Phaseolus vulgaris</i>	+	B
	3602	<i>Phaseolus vulgaris</i>	—	B
<i>R. meliloti</i>	3 Doa30	<i>Medicago sativa</i>	+	C
<i>R. lupini</i>	3206	<i>Lotus corniculatus</i>	—	B
	3001	<i>Lotus corniculatus</i>	+	B

* ++, Highly effective in nitrogen fixation; +, normally effective; ±, poorly effective; —, ineffective.

† A, Agricultural Institute, Johnstown Castle, Wexford, Ireland; B, Rothamsted Experimental Station, Harpenden, Herts, England; C, United States Department of Agriculture, Beltsville, Md., U.S.A.

Electrophoresis. Polyacrylamide gels, containing 5.5% (w/v) polyacrylamide were prepared with 0.05 M-tris + glycine buffer (pH 8.4) in 6 × 64 mm. glass tubes as described by Davis (1964). Vertical starch gels were prepared with 14% starch (Connaught Laboratories, Toronto) in 5 mm-tris + maleate buffer (pH 8.4) (Smithies, 1959). Occasionally starch gels were prepared in 5 mm-tris + maleate buffer (pH 7.4).

Extracts were assayed for HBDH before being added to the gels. Depending on the activity of the extract, 20 μ l. to 30 μ l. was added to polyacrylamide gels and 40 μ l. to 50 μ l. to vertical starch gels. In a typical experiment a 20 μ l. sample of extract contained 12 mg. protein and 0.32 i.u. of HBDH per ml.

For polyacrylamide gels, 50 v was applied for 90 min. which gave 2.5 m.amps per glass tube. Vertical starch gel electrophoresis was carried out at 4 v per cm. of gel for 16 to 20 hr.

After electrophoresis, the enzyme was located by immersing the gels in a solution containing the following reagents:

(a) 1.0 ml. DL-3-hydroxybutyric acid (M) (British Drug Houses Ltd.); (b) 1.0 ml. NAD (10 mg./ml.) (Boehringer & Son, Mannheim); (c) 0.25 ml. phenazine methosulphate (1.0 mg./ml.) (Sigma Chemical Co.); (d) 2.5 ml. nitro-blue tetrazolium (1 mg./ml.) (Sigma Chemical Co.); (e) 2.5 ml. phosphate buffer (0.5 M, pH 7.4); (f) 1.0 ml. MgCl_2 (5 mM); (g) 1.0 ml. NaCl (0.1 M).

About 20 min. at 37° was usually allowed for development of the gels and after staining was completed starch gels were stored in the dark in methanol+acetic acid+water (5+1+5) and polyacrylamide gels in 7.5% (v/v) acetic acid.

For detecting non-specific proteins, starch gels were stained with Coomassie Blue (G. T. Gurr Ltd., London) for 15 min. and then rinsed in methanol+acetic acid+water (5+1+5). Coomassie Blue was more efficient than Naphthalene Black 10B for revealing protein bands.

Coenzyme analogues. The rate of reaction of HBDH from different *Rhizobium* strains was measured with NAD and with various analogues of NAD. The ratio of the rate of reaction with NAD/coenzyme analogue was determined for the following analogues: Acetylpyridine NAD, Pyridinealdehyde NAD, Deamino NAD and Acetylpyridine Deamino NAD (Sigma Chemical Co.).

Table 2. *Relative mobilities of major 3-hydroxybutyrate dehydrogenase bands from various Rhizobium strains*

Strain no.	HBDH bands									
	a	b	c	d	e	f	g	h	i	j
1001	—	—	0.41*	—	—	0.76	0.83	0.88	0.94	1.00
1003	—	—	—	—	0.62	0.77	—	—	—	—
4	0.19	—	—	—	0.67	0.78	—	—	—	—
5	—	—	—	—	—	0.78	—	—	—	—
6	—	—	—	0.53	—	0.78	—	—	—	—
7	0.19	—	—	—	0.67	0.78	—	—	—	0.97
3601	—	—	—	—	—	—	—	—	—	1.10
3602	—	—	—	—	—	0.80	—	—	—	—
3001	—	—	—	—	—	0.79	—	—	—	1.08
3206	—	—	—	—	0.65	—	0.82	—	—	—
3D0a30	—	—	—	—	—	—	—	0.87	—	—
3401	—	—	—	—	—	—	—	—	—	1.40
311b59	—	—	—	0.53	0.62	—	—	—	—	—
311b59B**	—	—	—	0.52	0.68	—	—	—	—	—
392	—	0.30	0.44	—	—	—	—	—	—	—
392B**	—	0.26	0.44	—	—	—	—	—	—	—

* Extracts were separated by vertical starch gel electrophoresis at pH 8.4. Strain 1001 was used as a reference strain and the mobilities of the other bands were expressed as a fraction of the mobility of band j of 1001, which was given an arbitrary value of 1. Each figure represents a mean measurement for three separate extracts.

** Bacteroids isolated from nodules of soybeans inoculated with either 311b59 or 392.

RESULTS

HBDH patterns of Rhizobium strains. A qualitative comparison of HBDH from 14 *Rhizobium* strains by polyacrylamide gel electrophoresis showed that various degrees of complexity existed in the HBDH patterns of different strains. Multiple forms of HBDH were detected in several strains, e.g. strains 7, 1001 and 392. Vertical starch gel electrophoresis, at pH 8.4, was used to compare electrophoretic mobilities

of major bands because it was possible to compare ten extracts under identical conditions. Strain 1001 was used as a reference strain and the electrophoretic mobility (distance in cm. from the origin) of the most rapidly migrating band of HBDH from this strain was given an arbitrary value of 1. Mobilities of major HBDH bands from extracts of other strains were expressed as a ratio of this value (Table 2). The values obtained for relative mobilities of HBDH bands, by this procedure, were very reproducible when different extracts of a given strain were compared. The data in Table 2 also showed that individual *Rhizobium* strains had characteristic patterns although strains of *Rhizobium trifolii* (e.g. 4 and 7) and *R. leguminosarum* (e.g. 1003) showed some resemblance to each other. It was also interesting that the HBDH patterns of strains 392 and 311b59 persisted in bacteroids isolated from soybean nodules. Results similar to those in Table 2 were also obtained when extracts from *Rhizobium* strains were compared at pH 7.4 instead of pH 8.4, with the exception that extracts of strains 392 and 311b59 were difficult to separate at pH 7.4. Protein patterns of individual *Rhizobium* strains, in contrast to HBDH patterns, did not show a similar degree of strain specificity.

Table 3. *Ratios of reaction rates of 3-hydroxybutyrate dehydrogenase from various Rhizobium strains with NAD and analogues of NAD*

Strain no.	Specific* activity	NAD. PyAldNAD	NAD. AcPyNAD	NAD. AcPyDeNAD
1001	0.03	4.8	4.3	6.7
1003	0.10	10.9	6.7	11.5
7	0.05	11.9	6.0	12.2
4	0.06	11.9	6.5	> 100
5	0.05	13.0	7.1	23.0
3601	—	5.3	10.2	> 100
3602	0.07	15.5	5.9	10.0
3D0a30	0.08	11.8	18.8	> 100
392	0.10	9.0	10.3	42.0
392B**	0.08	4.6	10.0	30.2
311b59	0.14	12.8	10.0	53.4
3002	0.05	> 100	21.7	> 100
3206	0.07	14.1	38.4	40.0

* Units, per mg. protein (determined with NAD).

** Bacteroids isolated from nodules of soybeans inoculated with 392.

Each figure represents the mean of at least three separate assays on different extracts of the same strain.

Coenzyme analogues. The values of the ratios for the rates of reaction with NAD and with three coenzyme analogues, for HBDH from different *Rhizobium* strains, are given in Table 3. These analogues were active with HBDH from all the *Rhizobium* strains whereas deamino NAD was inactive with enzyme from several strains. In some instances, however, the rates of reaction with the analogues were relatively low, e.g. *Rhizobium lupini*, strains 3002 and 3206 (Table 3). In common with the electrophoresis results, each *Rhizobium* strain had characteristic values for the coenzyme analogue ratios. In view of the similarity of the electrophoresis patterns of some strains of *R. leguminosarum* and *R. trifolii* it was interesting that one strain from each of these species (i.e. 1003 and 7) had very similar coenzyme analogue ratios. Other workers have also detected similarities between some strains of *R. trifolii* and *R. leguminosarum*.

by serological tests (Graham, 1963), DNA homology tests (Heberlein, De Ley & Tijtgat, 1967) and numerical analysis (Graham, 1964; 'tMannetje, 1967).

DISCUSSION

Gel electrophoresis of soluble proteins followed by histochemical methods for locating enzymes *in situ* on gels is becoming an increasingly popular method for characterizing micro-organisms (Cann & Willox, 1965; Williams & Bowden, 1968; Peberdy & Turner, 1968).

Kaplan and co-workers (Kaplan, 1963) have also shown that coenzyme analogues can be used to compare functionally similar enzymes in different strains of the genus *Bacillus* and of the Enterobacteriaceae. These present results show that gel electrophoresis and coenzyme analogues can be used to compare HBDH from different *Rhizobium* species. Although only 14 *Rhizobium* strains were studied, interesting findings emerged on the relationship between some strains of *R. trifolii* and *R. leguminosarum*.

HBDH electrophoresis patterns together with coenzyme analogue ratios could be useful procedures for characterizing micro-organisms which contain poly-3-hydroxybutyric acid. The polymeric ester of D(-)3-hydroxybutyric acid is the principal storage product in a wide variety of bacteria (Dawes & Ribbons, 1964) including nitrogen-fixing bacteria and blue-green algae (Carr, 1966). HBDH is one of the enzymes responsible for the conversion of poly-3-hydroxybutyrate to utilizable cell material (Shuster & Doudoroff, 1962). In some *Rhizobium* species where poly-3-hydroxybutyrate can account for 40% of the dry weight of the bacteria (Hayward, Forsyth & Roberts, 1959; P. F. Fottrell and A. O'Hora, unpublished), the polymer probably serves as a source of energy and reducing power during nitrogen fixation (Klucas & Evans, 1968).

Although multiple forms of HBDH probably exist in *Rhizobium* great care was taken to avoid some of the artifacts which can arise in studies of this nature (Kaplan, 1968; Heidrich, 1968). For example, it was possible that multiple forms of HBDH might result from the formation of disulphide bridges in the enzyme molecule during the extraction procedure. However, identical electrophoresis patterns were obtained when *Rhizobium* strains were extracted by different procedures in the presence and absence of mercaptoethanol.

The physiological significance of the multiple forms of HBDH in *Rhizobium* is not known at present. Five forms of HBDH were found in extracts of human brain by Van der Helm (1962) and recently in a heterozygote of *Paramecium aurelia* by Tait (1968).

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