Pentitol metabolism of *Rhodobacter sphaeroides* Si4: purification and characterization of a ribitol dehydrogenase

**CORINNA KAHLE, KARL-HEINZ SCHNEIDER† AND FRIEDRICH GIFFHORN**

Institut für Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, D-3400 Göttingen, Germany

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The phototrophic bacterium *Rhodobacter sphaeroides* strain Si4 induced ribitol dehydrogenase (EC 1.1.1.56) when grown on ribitol- or xylitol-containing medium. This ribitol dehydrogenase was purified to apparent homogeneity by ammonium sulphate precipitation, affinity chromatography on Procion red, and chromatography on Q-Sepharose. For the native enzyme an isoelectric point of pH 6.1 and an apparent *M<sub>c</sub>* of 50 000 was determined. SDS-PAGE yielded a single peptide band of *M*<sub>c</sub> 25 000 suggesting a dimeric enzyme structure. The ribitol dehydrogenase was specific for NAD<sup>+</sup> but unspecific as to its polyol substrate. In order of decreasing activity ribitol, xylitol, erythritol, D-glucitol and D-arabitol were oxidized. The pH optimum of substrate oxidation was 10, and that of substrate reduction was 6.5. The equilibrium constant of the interconversion of ribitol to D-ribulose was determined to be 0.33 mM at pH 7.0 and 25 °C. The *K<sub>m</sub>* values determined for ribitol, ribulose, xylitol and NAD<sup>+</sup> (in the presence of ribitol) were 6.3, 12.5, 77 and 0.077 mM, respectively. Because of the favourable *K<sub>m</sub>* for ribitol, a method for quantitative ribitol determination was elaborated.

Introduction

Bacterial polyol dehydrogenases are currently being studied to test their potential use as selective reagents in enzymic analyses (Lunn et al., 1989; Schneider & Giffhorn, 1989, 1991; Scheper et al., 1989; Berezkenko & Sturgeon, 1991), as biocatalysts in syntheses of rare sugars from polyol precursors (Izumori et al., 1990), or as model systems of experimental enzyme evolution (Rigby et al., 1974; Wawzonek Thompson & Krawiec, 1983; Doten & Mortlock, 1985; Loviny et al., 1985). Recently, we reported growth of the facultative phototrophic bacterium *Rhodobacter sphaeroides* strain Si4 on D-glucitol, D-mannitol and D-arabitol, and the characterization of a new mannitol dehydrogenase (EC 1.1.1.67) involved in the metabolism of these substrates. The applicability of this mannitol dehydrogenase to quantitative determination of D-mannitol was demonstrated (Schneider & Giffhorn, 1989). In the same study it was found that *R. sphaeroides* also produced a polyol dehydrogenase active on xylitol when the organism was grown on xylitol-containing medium. Owing to the promising results with mannitol dehydrogenase with respect to application of the enzyme, we were interested in isolating this new *R. sphaeroides* polyol dehydrogenase and studying its properties. The results obtained are the subject of the present communication.

Methods

**Organism and growth conditions.** The bacterium used in this study was the purple non-sulphur bacterium *Rhodobacter sphaeroides* strain Si4, an isolate of this laboratory (Rode & Giffhorn, 1983). The cells were grown routinely under chemotrophic conditions in Erlenmeyer flasks at 30 °C with shaking (150 r.p.m.). To provide sufficient amounts of biomass for enzyme purification cells were grown under aerobic conditions in a 701 fermenter (provided by GBF, Braunschweig, FRG). Under these conditions a yield of 10 g wet cells l<sup>-1</sup> was achieved. The culture medium contained the following components in a final volume of 1 litre: xylitol, 9 g; KH<sub>2</sub>PO<sub>4</sub>, 1 g; NH<sub>4</sub>Cl, 1 g; MgSO<sub>4</sub>, 7H<sub>2</sub>O, 0.4 g; NaCl, 0.4 g; CaCl<sub>2</sub>, 2H<sub>2</sub>O, 0.05 g; trace element solution (tenfold concentrated), 1 ml (Pfenning & Lippert, 1966); vitamin solution (tenfold concentrated), 1 ml (Rode & Giffhorn, 1983). The pH was adjusted to 6.8 with NaOH. Solid media were prepared by addition of 1.5% (w/v) agar. Although the organism grew better with ribitol, we used the much cheaper xylitol as the carbon source.

**Enzyme assays.** Ribitol dehydrogenase activity was measured spectrophotometrically by following the absorbance change of NADH at 265 nm (ε = 3.4 × 10<sup>3</sup> mol<sup>-1</sup> cm<sup>-1</sup>). The standard assay mixture for substrate oxidation contained in a final volume of 1 ml at 30 °C: 100 µmol piperazine hydrochloride buffer (pH 9.0), 1.8 µmol NAD<sup>+</sup>...
and 0.02-0.2 U ribitol dehydrogenase. The reaction was started by the addition of 150 μmol ribitol.

The standard assay mixture for substrate reduction contained in a final volume of 1 ml at 30 °C: 100 μmol potassium phosphate buffer (pH 6.5), 0.28 μmol NADH and 0.02-0.2 U ribitol dehydrogenase. The reaction was started by the addition of 100 μmol D-ribulose.

One unit (U) of enzyme activity was defined as the amount of enzyme required to reduce/oxidize 1 μmol of NAD+/NADH per min under standard assay conditions.

**Protein determination.** Protein was determined by the method of Goeh (1953). Crystalline bovine serum albumin was employed as the standard.

**Purification of the ribitol dehydrogenase.** Potassium phosphate buffer (20 mm, pH 7.4) was used as the standard buffer. For enzyme purification 30 g frozen cells were thawed and suspended in 80 ml standard buffer to which 0.1 mg DNase ml⁻¹ was added, and disintegrated by passage through a French pressure cell at 15 500 N cm⁻². Cell debris was removed at 5 °C by centrifugation at 27 500 g for 1 h.

Unless specified otherwise all following operations were carried out at room temperature.

**Step 1.** During stirring on ice, saturated ammonium sulphate solution was added dropwise to the crude extract up to a final concentration of 30% saturation. The precipitate was removed by centrifugation at 27 500 g, and supernatant was dialysed at 4 °C against standard buffer.

**Step 2.** The dialysed protein solution from step 1 was applied to an affinity column (Procion red, 1.6 cm) equilibrated with standard buffer. The ribitol dehydrogenase was eluted with 500 ml of a linear KCl gradient (0-2 M) in standard buffer. Fractions containing high ribitol dehydrogenase activities were combined, concentrated by ultrafiltration using a PM 10 membrane (Amicon Corp.) and dialysed at 4 °C against standard buffer.

**Step 3.** The concentrated enzyme solution was applied to a Q-Sepharose column (1.6 × 7 cm) equilibrated with standard buffer. The enzyme was eluted using 400 ml of a linear KCl gradient (0-0.6 M in standard buffer). Fractions containing high enzyme activity were pooled, concentrated and dialysed as specified above.

**Determination of Mₘ.** The Mₘ of ribitol dehydrogenase was determined by gel filtration on a Superose 12 column with FPLC (Pharmacia). Standards of known Mₘ, were catalase (240 000), aldolase (158 000), bovine serum albumin (68 000) and ovalbumin (45 000).

**Gel electrophoresis.** Analytical PAGE was carried out in 7.5% (w/v) continuous gels (0.5 × 8 cm) using the Tris-barbituric acid system (Maurer, 1968). The gels were run for 2 h at a current of 2 mA per tube. For activity staining of the ribitol dehydrogenase the gels were incubated for 30 min in a staining solution containing the following components in a final volume of 10 ml: 1 mmol Tris/HCl (pH 9.0), 18 μmol NAD⁺, 1 mg nitro-blue tetrazolium chloride, 0.01 mg 5-methylphenazonium methylsulphate, 1 nmol ribitol (Gabriel, 1971).

**SDS-PAGE.** SDS-PAGE was performed in 7.5% (w/v) vertical slab gels (Laemmli, 1970). The gels were run for 2 h at a current of 25 mA. For determination of Mₘ, cytochrome c (Mₘ, 12 500), chymotripsinogen A (Mₘ, 25 000), ovalbumin (Mₘ, 45 000) and bovine serum albumin (Mₘ, 68 000) were used as standards.

**Determination of the isoelectric point.** Isoelectric focussing was performed in tubes (0.5 × 10 cm). The gels, 7.5% (w/v) in acrylamide, contained 6.3% (w/v) Servalyt 3-10 (Laas & Fast-Johansson, 1979). The anode solution was 10 mM-imidodiacetic acid, the cathodic solution was 10 mM-ethylenediamine. After focussing at a current of 1 mA per tube for 30 min, 5 μg ribitol dehydrogenase was applied per tube. Focussing was carried out at 500 V for 6 h. One gel was stained (Vesterberg et al., 1977) and a reference gel was cut into 1 cm pieces. The pH gradient was determined by eluting the amphylole from each piece with 1 ml of a 10 mM-KCl solution. Subsequently, the pH of the eluant was measured.

**Thin layer chromatography.** Ascending thin layer chromatography was performed on silica gel 60 slabs (Merck) with butanol/ethanol/water (10:1:2 by vol.) as solvent (Spiro, 1966). Products of the enzymic conversions were identified by co-chromatography with authentic sugars and staining with silver nitrate reagent (Dawson et al., 1986). Samples (10 μl) containing about 1.5 μg D-ribulose were withdrawn from reaction mixtures (30 min) consisting of 1 μmol ribitol, 20 U ribitol dehydrogenase and 15 μmol NAD⁺ in the standard assay buffer.

**Chemical and enzymes.** The coenzymes, enzymes and molecular mass standards were purchased from Boehringer Mannheim. D-Ribulose and D-xylulose were obtained from Fluka. The polyols and other sugars, Serva blue G and Servalyt 3-10 were purchased from Serva; Q-Sepharose and Superose 12 were from Pharmacia; Procion red from Deutsche ICI. The other chemicals were of analytical grade and obtained from Merck.

**Results**

**Growth of R. sphaeroides Si4 on xylitol and ribitol and detection of a ribitol dehydrogenase activity**

Fig. 1 illustrates growth of the facultative phototrophic bacterium *R. sphaeroides* Si4 in mineral salts media on the pentitols xylitol and ribitol with rates of 0.15 h⁻¹ and 0.22 h⁻¹, respectively. Apparently, growth on xylitol was significantly slower than growth on ribitol and it was preceded by an extended lag-phase of approximately 10 h when inocula from D-glucitol-grown cultures were used. Cell extracts from either culture revealed the presence of both xylitol and ribitol dehydrogenase in similar ratios of activities of about 1:5. This finding led to the assumption that both activities were expressions of the same enzyme.

**Purification of the ribitol dehydrogenase**

Enzyme purification was started from a batch of 30 g wet cells. Details of the purification procedure are described in Methods, and the results are summarized in Table 1. Accordingly, the enzyme was purified 310-fold to a specific activity of 59 U (mg protein)⁻¹ with a recovery of 45%. The mannitol dehydrogenase was separated from the ribitol dehydrogenase by affinity chromatography during which the bulk of the mannitol dehydrogenase was eluted in the washing buffer (see Methods). Final purification of the ribitol dehydrogenase was achieved by chromatography on Q-Sepharose. This enzyme preparation was homogeneous as shown by native-PAGE (Fig. 2) and SDS-PAGE (data not shown).
Ribitol dehydrogenase of Rhodobacter sphaeroides

Fig. 1. Growth of R. sphaeroides on ribitol (■) and xylitol (●). The organism was grown chemotrophically in 300 ml Erlenmeyer flasks at 30 °C with shaking (150 r.p.m.). A mineral medium containing 2 g polyol 1−1 was used.

Table 1. Purification of a ribitol dehydrogenase from R. sphaeroides Si4

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity* (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg−1)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>245</td>
<td>1321</td>
<td>0.19</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>248</td>
<td>650</td>
<td>0.38</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Chromatography on Q-Sepharose</td>
<td>231</td>
<td>52.4</td>
<td>4.4</td>
<td>23</td>
<td>94</td>
</tr>
<tr>
<td>Chromatography on Procion red</td>
<td>111</td>
<td>1.9</td>
<td>59.0</td>
<td>310</td>
<td>45</td>
</tr>
</tbody>
</table>

* Enzyme activity was assayed with ribitol as the substrate.

each yielding a single protein band. Since the xylitol dehydrogenase activity was co-purified in a constant ratio of about 1:5 with the ribitol dehydrogenase activity it was concluded that both activities were properties of the same enzyme.

Properties of the ribitol dehydrogenase

The M₉ of the native ribitol dehydrogenase was determined to be 50000 by gel filtration on Superose 12 with FPLC. Since SDS-PAGE gave a single band whose mobility corresponded to a M₉ value of 25000, it was concluded that the R. sphaeroides Si4 ribitol dehydrogenase is a dimeric enzyme. The isoelectric point was estimated to be pH 6-1.

The stability of ribitol dehydrogenase was tested in standard buffer at 4, 25, 40, 50 and 60 °C. Fifty percent of the initial enzyme activities were retained after incubation of 15 d, 25 h, 210, 27 and 1 min, respectively. During storage of frozen cells at −20 °C the ribitol dehydrogenase lost about 50% of its initial activity within 4 months.

The pH optimum of the enzyme with ribitol as the substrate was assayed in 0.1 M-glycylglycine/piperazine/NaOH buffer in a range from pH 8.0–11.5. The ribitol dehydrogenase exhibited a plateau of optimal activity from pH 9.0–10.3 with 50% activity at pH 8.0 and 10.7. When D-ribulose was used as the substrate assays were carried out in 0.1 M-potassium phosphate buffer in a range from pH 6.0–8.0. For D-ribulose reduction an optimal pH of 6.5 was determined. The temperature optimum of ribitol dehydrogenase was assayed in the standard buffer for ribitol oxidation and was found to be 45 °C. Metal salts such as MgSO₄, MnSO₄ or ZnSO₄ did not stimulate the activity of the ribitol dehydrogenase when added at concentrations of 1 mM. Equally, no inhibitory effect of EDTA at concentrations between 1 and 10 mM was observed.

The ribitol dehydrogenase was highly specific for NAD⁺ but it was relatively unspecific with respect to various polyol and sugar substrates (Table 2). Apparently, the enzyme showed a clear catalytic preference for ribitol compared to xylitol. With regard to the sugars, D-ribulose was the preferred substrate followed by D-xylulose. The product of ribitol conversion was identified as D-ribulose by co-chromatography with authentic D-ribulose (see Methods).

The rates of substrate oxidation and reduction were determined in the standard assay system at pH 9.0 and 6.5, respectively. Referring to the substrates below, all
substrate specificity of the ribitol dehydrogenase from R. sphaeroides Si4

Table 2. Substrate specificity of the ribitol dehydrogenase from R. sphaeroides Si4

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribitol</td>
<td>100</td>
</tr>
<tr>
<td>Xyitol</td>
<td>21</td>
</tr>
<tr>
<td>Erythritol</td>
<td>12</td>
</tr>
<tr>
<td>D-Glucitol</td>
<td>10</td>
</tr>
<tr>
<td>D-Arabinol</td>
<td>10</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>0</td>
</tr>
<tr>
<td>Galactitol</td>
<td>0</td>
</tr>
<tr>
<td>m-Inositol</td>
<td>0</td>
</tr>
<tr>
<td>Glycerin</td>
<td>0</td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td>0</td>
</tr>
<tr>
<td>D-Ribulose</td>
<td>100</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>60</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>3</td>
</tr>
<tr>
<td>D-Erythrose</td>
<td>3</td>
</tr>
<tr>
<td>D-Arabinoose</td>
<td>1</td>
</tr>
<tr>
<td>D-Rbose</td>
<td>0</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>0</td>
</tr>
<tr>
<td>D-Tagatose</td>
<td>0</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>0</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>0</td>
</tr>
<tr>
<td>D-Sorbose</td>
<td>0</td>
</tr>
</tbody>
</table>

Enzyme activities given are mean values of duplicate determinations. The tests were carried out in the standard assay systems at pH 9.0 and 6.5, respectively (see Methods). The assays contained 1 µg (corresponding to 0.06 U which is equivalent to 100%) purified ribitol dehydrogenase and the substrates indicated at saturating concentrations: 150 mM for the polyols except galactitol (60 mM) and 100 mM for the sugars.

saturation curves were hyperbolic, and the corresponding double reciprocal plots were linear. The apparent $K_m$ values determined for ribitol, xylitol and NAD$^+$ (in the presence of 100 mM-ribitol) were 6.3, 77 and 0.077 mM, respectively. The values for D-ribulose and D-xylose were 1.1 and 12.5 mM, respectively.

The equilibrium constant for the conversion of ribitol was determined spectrophotometrically at 340 nm in 100 mM-potassium phosphate buffer (pH 7.0) at 25 °C by measuring changes in the NAD$^+$/NADH ratio. The calculation of the equilibrium constant was based on four independent determinations in which the concentrations of NAD$^+$ (0.2–2 mM) and of ribitol (1–10 mM) were varied. From the data a mean value of 0.33 nM with a standard deviation of 0.12 was calculated.

Evaluation of a method for quantitative determination of ribitol

Quantitative conversion of varying amounts of ribitol with the ribitol dehydrogenase from R. sphaeroides Si4 is possible and is illustrated in Fig. 3. The reaction was complete within 15 min and the change in absorbance was strictly proportional to the amount of ribitol added. All endpoints were in agreement with the theoretical values calculated on the basis of the extinction coefficient of NADH at 340 nm. With the customary photometric equipment the assay was sensitive enough to estimate ribitol accurately down to 10 nmol ml$^{-1}$.

Discussion

A ribitol dehydrogenase (ribitol: NAD$^+$ 2-oxidoreductase, EC 1.1.1.56) was isolated by a convenient method with good yields from R. sphaeroides strain Si4. The enzyme was induced by both ribitol and xylitol but it exhibited a significantly higher activity with ribitol, which was converted to D-ribulose. The R. sphaeroides ribitol dehydrogenase was reasonably stable and could be used as a biocatalyst for quantitative determination of ribitol. However, its stability must be significantly improved prior to use it as a biocatalyst for the preparative synthesis of D-ribulose. To our knowledge, ribitol dehydrogenases have been purified to homogeneity and characterized from only the enterobacteria.
Aerobacter aerogenes (Fromm, 1958; Nordlie & Fromm, 1959; Fromm & Bietz, 1966) and Klebsiella aerogenes wild-type and mutant strains (Taylor et al., 1974; Burleigh et al., 1974). Therefore, we refer only to the properties of the ribitol dehydrogenases isolated from these organisms. The main differences of the ribitol dehydrogenases concern the enzyme structures. Whilst the R. sphaeroides ribitol dehydrogenase has a dimeric structure, the corresponding enzymes of A. aerogenes and K. aerogenes are tetramers with M₅ values of about 110000 (Fromm & Bietz, 1966; Taylor et al., 1974). On the other hand, the kinetic properties of the ribitol dehydrogenases from the different microbial sources are similar. A thorough kinetic analysis of the K. aerogenes ribitol dehydrogenase has been performed by Burleigh et al. (1974). The \( K_m \) values for ribitol range from 5 to 8.3 mM and the high \( K_m \)-values for xylitol indicate the poor affinities of the enzyme towards this substrate. The slow growth of R. sphaeroides observed on xylitol may reflect the poor affinity of the ribitol dehydrogenase for this substrate. In this respect growth of R. sphaeroides on xylitol resembles that of a K. aerogenes mutant which metabolizes xylitol by using a weak side activity of the ribitol dehydrogenase (Wu et al., 1985; Taylor et al., 1974). Finally, it should be noted that there are a number of polyol dehydrogenases such as xylitol dehydrogenase of Serratia marcescens (Doten & Mortlock, 1985) and arithitol dehydrogenase of Mycobacterium smegmatis (WOJTKIEWICZ et al., 1988) that exhibit marginal activities with ribitol.

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References


