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

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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_r of 50 000 was determined. SDS-PAGE yielded a single peptide band of M_r 25 000 suggesting a dimeric enzyme structure. The ribitol dehydrogenase was specific for NAD⁺ 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 nM at pH 7.0 and 25 °C. The K_m -values determined for ribitol, ribulose, xylitol and NAD⁺ (in the presence of ribitol) were 6.3, 12.5, 77 and 0.077 mM, respectively. Because of the favourable K_m 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.*, 1991; Berezenko & 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 70 l fermenter (provided by GBF, Braunschweig, FRG). Under these conditions a yield of 10 g wet cells l⁻¹ was achieved.

The culture medium contained the following components in a final volume of 1 litre: xylitol, 9 g; KH₂PO₄, 1 g; NH₄Cl, 1 g; MgSO₄·7H₂O, 0.4 g; NaCl, 0.4 g; CaCl₂·2H₂O, 0.05 g; trace element solution (tenfold concentrated), 1 ml (Pfennig & 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 ($\epsilon = 3.4 \times 10^3$ l mol⁻¹ cm⁻¹). 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⁺

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and 0.02–0.2 U ribitol dehydrogenase. The reaction was started by the addition of 150 μ mol ribitol.

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

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

Protein determination. Protein was determined by the method of Goa (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 DNAase 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 the 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 × 14 cm) equilibrated with 20 mM-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. During chromatography on Procion red, the bulk of mannitol dehydrogenase appeared in the washing buffer while ribitol dehydrogenase was eluted with the gradient.

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 M_r . The M_r of ribitol dehydrogenase was determined by gel filtration on a Superose 12 column with FPLC (Pharmacia). Standards of known M_r 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 μ mol NAD⁺, 1 mg nitro-blue tetrazolium chloride, 0.01 mg 5-methylphenazonium methylsulphate, 1 mmol ribitol (Gabriel, 1971).

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 M_r , cytochrome c (M_r 12 500), chymotrypsinogen A (M_r 25 000), ovalbumin (M_r 45 000) and bovine serum albumin (M_r 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-iminodiacetic acid, the cathodic solution was 10 mM-ethylenediamine. After prefocussing at a current of 1 mA

per tube for 30 min, 5 μ g 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 ampholyte 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 μ l) containing about 1.5 μ g D-ribulose were withdrawn from reaction mixtures (30 min) consisting of 1 μ mol ribitol, 20 U ribitol dehydrogenase and 15 μ mol NAD⁺ in the standard assay buffer.

Chemicals 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),

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 l⁻¹ was used.

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

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

* 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 M_r of the native ribitol dehydrogenase was determined to be 50 000 by gel filtration on Superose 12 with FPLC. Since SDS-PAGE gave a single band whose mobility corresponded to a M_r value of 25 000, 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.

Fig. 2. Analytical PAGE of various fractions of the *R. sphaeroides* ribitol dehydrogenase purification. Gels (7.5%) in tubes were loaded with about 20 µg of protein each and run at a current of 2 mA (for further details see Methods). (a) Activity staining of the gel with nitroblue tetrazolium (Gabriel, 1971), (b) ion exchange chromatography on Q Sepharose, (c) affinity chromatography on Procion red A, (d) ammonium sulphate precipitation and (e) cell extract. Gels b-d were stained for protein with Serva blue G.

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

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

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.

Substrate	Relative enzyme activity (%)
Ribitol	100
Xylitol	21
Erythritol	12
D-Glucitol	10
D-Arabitol	10
D-Mannitol	0
Galactitol	0
<i>m</i> -Inositol	0
Glycerin	0
2,3-Butandiol	0
D-Ribulose	100
D-Xylulose	60.7
D-Fructose	3.6
D-Erythrose	3.6
D-Arabinose	1.7
D-Ribose	0
D-Xylose	0
D-Tagatose	0
D-Glucose	0
D-Mannose	0
D-Sorbose	0

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-xylulose 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 mM 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

Fig. 3. Conversion of varying concentrations of ribitol by the *R. sphaeroides* ribitol dehydrogenase. The substrate conversions were performed in the standard assay system at pH 9 (see Methods), and the change in NADH A_{340} ($\epsilon = 6.3 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}$) was recorded. The assay contained (in 1 ml) 2 U ribitol dehydrogenase, and 100 (A), 75 (B), 50 (C) and 25 (D) nmol ribitol.

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 M_r values of about 110 000 (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 aribitol dehydrogenase of *Mycobacterium smegmatis* (Wojtkiewicz *et al.*, 1988) that exhibit marginal activities with ribitol.

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