Polyol metabolism of *Rhodobacter sphaeroides*: biochemical characterization of a short-chain sorbitol dehydrogenase

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A sorbitol dehydrogenase (SDH; L-iditol: NAD⁺ 2-oxidoreductase; EC 1.1.1.14) was isolated from the phototrophic bacterium *Rhodobacter sphaeroides* strain M22, a transposon mutant of *R. sphaeroides* Si4 with the transposon inserted in the mannitol dehydrogenase (MDH) gene. SDH was purified 470-fold to apparent homogeneity by ammonium sulfate precipitation, chromatography on Phenyl-Sepharose, Q-Sepharose and Matrex Gel Red-A, and by gel filtration on Superdex 200. The relative molecular mass (M_r) of the native SDH was 61 000 as calculated from its Stokes' radius ($r_s = 3.5$ nm) and sedimentation coefficient ($S_{20,w} = 4.23S$). SDS-PAGE resulted in one single band representing a polypeptide with a M_r of 29 000, indicating that the native protein is a dimer. The isoelectric point of SDH was determined to be pH 4.8. The enzyme was specific for NAD⁺ and catalysed the oxidation of D-glucitol (sorbitol) to D-fructose, galactitol to D-tagatose and of L-iditol. The apparent K_m values were NAD⁺, 0.06 mM; D-glucitol, 6.2 mM; galactitol, 1.5 mM; NADH, 0.13 mM; D-fructose, 160 mM; and D-tagatose, 13 mM. The pH-optimum of substrate oxidation was 11.0 and that of substrate reduction 6.0–7.2. It was demonstrated that SDH is expressed in the wild-type strain *R. sphaeroides* Si4 together with MDH during growth on D-glucitol. Forty-four amino acids of the SDH N terminus were sequenced. This sequence exhibited 45–55% identity to the N-terminal sequence of 10 enzymes belonging to the short-chain alcohol dehydrogenase family.

Keywords: *Rhodobacter sphaeroides*, polyol metabolism, sorbitol dehydrogenase

INTRODUCTION

The phototrophic bacterium *Rhodobacter sphaeroides* Si4 induces a mannitol dehydrogenase (MDH) active on D-mannitol, D-glucitol (sorbitol) and D-arabitol, when grown in the presence of any of these substrates (Schneider & Giffhorn, 1989). When grown on ribitol or xylitol it induces a ribitol dehydrogenase (RDH) active on both polyols but lacking sorbitol dehydrogenase (SDH) activity (Kahle *et al.*, 1992). The gene encoding MDH (*mtlK*) has been cloned and sequenced (Schneider *et al.*, 1993) and used for constructing a strain that overproduces the enzyme (Schneider & Giffhorn, 1994). For cloning of *mtlK*, a transposon mutant, designated strain M22, was constructed that did not produce MDH. It was shown by sequence analysis that the transposon is inserted into *mtlK* (Schneider *et al.*, 1993). Unexpectedly, strain M22 still

possesses SDH activity which has been supposed to be a marginal activity of MDH. Since it is intended to use *R. sphaeroides* Si4 as a model for experimental enzyme evolution of polyol dehydrogenases with biotechnological significance (Schneider *et al.*, 1995), the origin of the SDH activity in the mutant M22 has to be elucidated.

This communication reports the detection, isolation and characterization of a new bacterial SDH (L-iditol : NAD⁺ 2-oxidoreductase; EC 1.1.1.14) from the *R. sphaeroides* mutant M22 and demonstrates its presence in the wild-type strain. On the basis of peptide sequence data and biochemical properties, SDH has been shown to be clearly distinct from *R. sphaeroides* Si4 MDH and RDH, but shows great similarities with enzymes of the short-chain alcohol dehydrogenase family.

METHODS

Organisms and growth conditions. The purple non-sulfur bacterium *Rhodobacter sphaeroides* strain Si4 (DSM 8371) is an

Abbreviations: MDH, mannitol dehydrogenase; RDH, ribitol dehydrogenase; SDH, sorbitol dehydrogenase.

isolate from this laboratory (Rode & Giffhorn, 1983). *R. sphaeroides* strain M22 is a transposon mutant of strain Si4 with Tn5 inserted in the MDH gene (*mtlK*) (Schneider *et al.*, 1993).

For enzyme production, cells were grown heterotrophically in a 10 l bioreactor at 32 °C with aeration (0.3 v.v.m.), agitation (200 r.p.m.) and pH regulation. The medium contained the following components in a final volume of 1 l: D-glucitol, 9 g; KH_2PO_4 , 1.0 g; NH_4Cl , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; NaCl , 0.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g; 10 × trace element solution SL4 (Pfennig & Lippert, 1966), 1.0 ml; 10 × vitamin solution (Rode & Giffhorn, 1983), 1 ml. The pH was adjusted to 6.8 with NaOH. For growth of *R. sphaeroides* M22, kanamycin was added to the medium at a concentration of 25 $\mu\text{g ml}^{-1}$.

Enzyme assays and protein determination. Polyol dehydrogenase activities were determined as described previously (Schneider & Giffhorn, 1989). Protein concentrations were determined by the method of Goa (1953) with crystalline bovine serum albumin as standard.

Purification of SDH. Wet cells (78 g) were suspended in 230 ml Tris/HCl buffer (20 mM, pH 7.0) and 10 mg DNase I was added. Cells were disintegrated by two passages through a French press at 11000 N cm^{-2} . Subsequently the cell debris was removed by centrifugation at 4 °C and 53000 g for 30 min.

Unless otherwise specified all following operations were carried out at 4 °C.

Step 1. During stirring on ice, saturated $(\text{NH}_4)_2\text{SO}_4$ solution was added dropwise to the crude extract until 35 % saturation was reached. The precipitated proteins were removed by centrifugation at 53000 g for 30 min. The supernatant was filtered through a nitrocellulose 0.45 μm pore size filter.

Step 2. The protein solution from step 1 was applied to a C 16/20 Phenyl Sepharose column (Pharmacia), containing 23 ml of the resin, equilibrated with potassium phosphate buffer (20 mM, pH 7.0) with 30 % $(\text{NH}_4)_2\text{SO}_4$ saturation. The proteins were eluted with a linear $(\text{NH}_4)_2\text{SO}_4$ gradient (30–0 % saturation) in 500 ml potassium phosphate buffer (20 mM, pH 7.0) and 7 ml fractions were collected. Fractions containing high SDH activities were pooled and dialysed against Bistris/HCl buffer (20 mM, pH 6.0).

Step 3. The dialysed enzyme solution was applied to a XK 50/30 Q-Sepharose column with a bed volume of 80 ml, equilibrated with Bistris/HCl buffer (20 mM, pH 6.0). The enzyme was eluted with a linear NaCl gradient (0–0.3 M) in 500 ml Bistris/HCl buffer and 7 ml fractions were collected. Fractions containing high enzyme activity were pooled and dialysed against Tris/HCl buffer (20 mM, pH 7.5).

Step 4. The dialysed enzyme solution was applied to a C 16/20 Matrex Gel Red A affinity chromatography column with a bed volume of 22 ml, equilibrated with Tris/HCl buffer (20 mM, pH 7.5). The enzyme was eluted with a linear KCl gradient (0–0.4 M) in 400 ml Tris/HCl buffer and 5 ml fractions were collected. Fractions containing high SDH activity were pooled and concentrated by ultrafiltration through a YM 10 membrane (Amicon) to a volume of 2 ml.

Step 5. The enzyme preparation of step 4 was further purified by FPLC gel filtration on a Superdex 200 column equilibrated with potassium phosphate buffer (20 mM, pH 7.0, containing 0.1 M NaCl). Fractions of 2 ml were collected at a flow rate of 1 ml min^{-1} . Fractions containing polyol dehydrogenase activities were pooled and concentrated to a volume of 1 ml.

Determination of Stokes' radius and sedimentation coefficient. The Stokes' radius of the enzyme was determined using a Sephacryl S-300 column and known standards: catalase,

5.22 nm; aldolase, 4.81 nm; bovine serum albumin, 3.55 nm; ovalbumin, 3.05 nm. The sedimentation coefficient was determined by sucrose gradient centrifugation (6–30 %, w/v, sucrose) at 140000 g for 5 h with aldolase ($S_{20,w} = 7.35\text{S}$), bovine serum albumin (4.31S) and ovalbumin (3.55S) as standards (Siegel & Monty, 1966).

Gel electrophoresis. Analytical PAGE was carried out in 10 % (w/v) slab gels using the Tris–Glycine buffer system. The gels were run at a current of 20 mA. Proteins were visualized by silver staining (Merrill *et al.*, 1981). For activity staining of the SDH, the gels were incubated for 5 min in a staining solution containing the following components in a final volume of 10 ml: 0.2 M Tris/HCl (pH 9.0), 5 ml; 90 mM NAD^+ -solution, 100 μl ; nitro-blue tetrazolium chloride (10 mg ml^{-1}), 100 μl ; 5-methylphenazonium methylsulfate (1 mg ml^{-1}), 100 μl ; D-glucitol solution (1.5 M), 1000 μl .

SDS-PAGE was performed in 15 % slab gels (Laemmli, 1970). The gels were run at a current of 10 mA per gel. For molecular mass determination of protein subunits, aldolase (M_r 39200), triose-phosphate isomerase (26000), trypsin inhibitor (20100) and lysozyme (14300) were used as standards.

Determination of the isoelectric point. Isoelectric focussing was performed in polyacrylamide gels (5 %) containing Servalyt Precotes pH 3–10 (Serva). Focussing was carried out at 400 V for 3 h. The standard test mixture with proteins of known pI included amyloglucosidase (pH 3.5), ferritin (pH 4.4), albumin (pH 4.7), β -lactoglobulin (pH 5.3), conalbumin (pH 5.9), horse myoglobin (pH 7.3), whale myoglobin (pH 8.3), ribonuclease (pH 9.45) and cytochrome c (pH 10.65).

Determination of the reaction products. The sugars produced by oxidation of different polyols by SDH were identified with HPLC. To produce the sugars for product analysis, enzyme reactions were performed in a volume of 500 μl . The reaction mixture contained the following components: 100 mM Tris/HCl (pH 8.5), 9 mg polyol, 10 mg sodium pyruvate, 1.4 μmol NAD^+ , 5 U lactate dehydrogenase, and 0.22 U SDH. After incubation at 28 °C for 3 h, the sugar produced was identified by ligand exchange chromatography with HPLC on a Ca^{2+} -loaded cation exchange matrix (Carbohydrate Ca^{2+} , 300 × 7.8, BC-100, Benson). The column temperature was 85 °C. Water was used as mobile phase with a flow rate of 0.8 ml min^{-1} . Detection was performed at 35 °C with a refractive index detector (Beckman). The retention times were compared with those for authentic sugars, which were 9.5 min for fructose and 11.6 min for tagatose.

In addition D-fructose concentration in the reaction mixture was determined by an enzymic assay (Beutler, 1984).

Chemicals and enzymes. The coenzymes, enzymes and molecular mass standards were purchased from Boehringer Mannheim. Sugars and polyols were obtained from Serva or Fluka, except L-iditol which was from Aldrich. Phenyl-Sepharose, Q-Sepharose, Sephacryl S 300 and Superdex 200 were from Pharmacia, and Matrex Gel Red A from Amicon.

RESULTS

Purification of SDH from *R. sphaeroides* M22

To obtain sufficient cell material for enzyme purification *R. sphaeroides* M22 was grown in a 10 l bioreactor on minimal medium with D-glucitol as carbon source. The growth rate was 0.22 h^{-1} ($t_d = 3.15$ h). Cells were harvested at the transition to stationary phase. A yield in dry mass of 1.8 g l^{-1} was obtained. Enzyme purification

Table 1. Purification of SDH from *R. sphaeroides* M22

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Purification (-fold)	Yield (%)
Crude extract	339	3685	0.09	1.0	100
Ammonium sulfate precipitation	319	1649	0.19	2.1	94
Chromatography on Phenyl Sepharose	216	203	1.1	12	64
Chromatography on Q-Sepharose	113	17	6.6	73	33
Chromatography on Matrex Gel Red-A	71	3.8	18.7	208	21
Gel filtration on Superdex 200	55	1.3	42.3	470	16

was started from 78 g wet cells. Details of the purification procedure are described in Methods and the results are summarized in Table 1. Accordingly, SDH was purified 470-fold to a specific activity of 42.3 U mg⁻¹ with a recovery of 16%.

This final enzyme preparation was apparently homogeneous as shown by analytical PAGE and SDS-PAGE, each showing one single protein band (Fig. 1a, b).

Properties of SDH

The M_r mass of the native SDH was calculated to be 61000 on the basis of the determined values for the Stokes' radius ($r_s = 3.5$ nm) and the sedimentation coefficient ($S_{20,w} = 4.23S$) (Siegel & Monty, 1966). Since SDS-PAGE resulted in one single protein band (Fig. 1b) whose mobility corresponded to a M_r of 29000, it was concluded that SDH is a dimer of identical subunits. The isoelectric point of SDH was determined to be pH 4.8 by isoelectric focussing.

With respect to the coenzyme, SDH was specific for NAD⁺. In Table 2 the relative enzyme activities against various polyol and sugar substrates are shown. With respect to the polyols, D-glucitol is the preferred substrate of the enzyme. Lower activities could be found with L-idoitol and galactitol. For the reduction of sugars, D-fructose, the product of D-glucitol conversion, is the main substrate of SDH. Of the other sugars tested, only D-tagatose is converted with an activity higher than 5% of that found for D-fructose.

The reaction rates were determined in the standard assay systems at pH 9.0 (oxidation) and pH 6.5 (reduction). The saturation curves for all substrates tested were hyperbolic and the corresponding double-reciprocal plots were linear. The apparent K_m values determined for D-glucitol, galactitol and NAD⁺ (in the presence of 100 mM D-glucitol) were 6.2 mM, 1.5 mM and 0.06 mM, respectively. The K_m values for D-fructose, D-tagatose, and NADH (in the presence of 200 mM D-fructose) were 160 mM, 13 mM and 0.13 mM, respectively.

The products of D-glucitol and galactitol oxidation with SDH were identified to be D-fructose and D-tagatose, respectively, by HPLC. In addition, D-fructose was estimated by enzymic analysis (see Methods).

The optimal pH for substrate oxidation by SDH was pH 11.0 as determined in 100 mM piperazine/HCl buffer. Fifty percent of this activity was found at pH 9.2 and 50% at pH 11.6. Since both enzyme and NADH were not stable at the pH optimum, all assays monitoring substrate oxidation were performed at pH 9.0 in 100 mM Tris/HCl buffer. Under these conditions, 48% of the maximal enzyme activity was obtained. For sugar reduction, SDH exhibited a broad pH optimum between pH 6.0 and 7.2 with 50% of this activity at pH 5.3 and 50% at pH 7.8.

The stability of SDH was tested in 20 mM potassium phosphate buffer (pH 7.0) in the presence and absence of additives. When incubated at room temperature, SDH lost 50% of the initial enzyme activity after 35 d without the addition of additives, after 9 d in the presence of 1 mM DTT, and after 140 d in the presence of 30% sucrose. After incubation for 3 months at 4 °C without additives as well as with 1 mM DTT and with 30% sucrose, 95, 29 and 97% of the initial enzyme activity were found, respectively. Accordingly, SDH is stabilized by sucrose but is inactivated by DTT. Thermal stability of the enzyme was investigated in the optimal buffer potassium phosphate (20 mM, pH 7.0) plus 30% sucrose in which the enzyme lost 50% of its initial activity during incubation at 30, 40 and 50 °C after 8 d, 18 h and 2 min, respectively.

In addition to DTT, N-bromosuccinimide and iodoacetic acid were inhibitory on SDH. The activity of SDH was not stimulated by MgSO₄, MnSO₄, CoCl₂, NiCl₂, CuSO₄ or ZnSO₄, each at a concentration of 1 mM. The chelating agents EDTA and α,α' -dipyridyl did not inhibit the enzyme at a concentration of 1 mM. These results indicate that divalent metal ions are not required for catalytic activity.

Isolation of the SDH from the wild-type strain *R. sphaeroides* Si4

It had been shown previously that *R. sphaeroides* Si4 expresses a MDH during growth on D-glucitol, D-mannitol or D-arabitol (Schneider & Giffhorn, 1989). Since MDH oxidizes each of these polyols, it was concluded that this enzyme enables *R. sphaeroides* to utilize D-glucitol and D-mannitol as well as D-arabitol. However, the existence of a SDH in the mutant strain M22 raises

Fig. 1. PAGE of isolated SDH from *R. sphaeroides* M22. (a) Analytical PAGE with an enzyme sample of the final purification step. 1, Silver stain; 2, activity stain. (b) SDS-PAGE with purified SDH (lane 2) and marker proteins (lane 1). The marker proteins are (from top to bottom): aldolase ($M_r = 39200$), triose-phosphate isomerase (26000), trypsin inhibitor (20100) and lysozyme (14300).

the question of whether SDH also occurs in the wild-type strain during growth on D-glucitol. Therefore, purification of SDH was performed from cells of *R. sphaeroides* Si4 grown on D-glucitol using the same procedure as for the purification of SDH from strain M22. A separation of MDH from SDH could be obtained during chromatography on Matrex Gel Red A. MDH did not adsorb to the column and was directly eluted while SDH bound to the gel material and could be eluted with 0.4 M NaCl. The isolated SDH from *R. sphaeroides* Si4 was identical to the SDH isolated from the mutant strain M22 with regard to molecular mass, substrate spectrum and migration in polyacrylamide gels, indicating that it is the same enzyme. The study also showed that SDH is induced along with MDH when *R. sphaeroides* Si4 is grown on D-glucitol.

Table 2. Substrate specificity of SDH from *R. sphaeroides*

Enzyme activities in the direction of oxidation and reduction were measured in the standard assay systems at pH 9.0 and pH 6.5, respectively. The assays each contained 0.07 U purified SDH and the substrates at saturating concentrations. These were 100 mM for the polyols except galactitol (20 mM), and in the reverse direction 200 mM for all sugars except D-xylulose (100 mM) and lactose (50 mM). The percentage (100% corresponded to 0.07 U) values were calculated from mean values of duplicate determinations. The following compounds were not substrates for SDH: ribitol, *m*-erythritol, myo-inositol, glycerol, 1,2-pentanediol, *n*-butanol, ethanol and methanol (substrate oxidation); and L-ribulose, D-glucose, D-mannose, D-galactose, D-arabinose, L-arabinose, D-xylose, D-ribose, lactose, melobiose, maltose, raffinose and D-turanose (substrate reduction).

Polyol oxidation		Sugar reduction	
Substrate	Relative enzyme activity (%)	Substrate	Relative enzyme activity (%)
D-Glucitol	100	D-Fructose	100
L-Iditol	58	D-Tagatose	17
Galactitol	16	D-Xylulose	3
L-Arabitol	8	L-Sorbose	2
D-Mannitol	4		
Xylitol	4		
D-Arabitol	< 1		

Determination of the N-terminal sequence of SDH and sequence analysis

SDH was subjected to a partial amino acid sequence analysis starting from the N-terminus. Thus, 44 amino acids could be determined by automated sequencing with a 473A sequencer from Applied Biosystems. The amino acid sequence is shown in Fig. 2.

A data bank search (GenBank, EMBL Data Library, SwissProt and NBRF PIR protein database) was performed using the BLAST search algorithm. Several proteins were found that showed strong homology in their N-terminal amino acid sequence to the SDH sequence. Each of these proteins belonged to the protein family of short-chain alcohol dehydrogenases (Persson *et al.*, 1991; Neidle *et al.*, 1992). A sequence alignment of SDH with the 10 proteins exhibiting the highest degree of homology as well as with other polyol dehydrogenases belonging to this enzyme family is shown in Fig. 2. No gaps had to be introduced into the amino acid sequence of any of the proteins to get a proper alignment with SDH. The 10 proteins with the highest degree of homology exhibited a 45–55% identity to SDH over the total length of 44 amino acids, and 30–39% sequence identity was found with the polyol or polyol phosphate dehydrogenases. Therefore, SDH from *R. sphaeroides* is likely to be a member of the protein family of short-chain alcohol dehydrogenases, although only the N-terminal part of the amino acid sequence is so far known.

(a)	
SDH-Rs	MRLDGKTALITGSARGIGRAFAEAYVREGARVAIADINLEAARE
PGDH-Hs	MHVNGKVALVTGAAQGIGRAFAEALLKGAVALVDWNLEAGVQ
ADH-Zm	(31) SHHGWDGNGAVPTPMPKRLDGKVAIVTGGARGIGEAIVRLFAGKHGARVVIADIDDAAGEA
AMDH-Fs	TTAGVSRRPGRLAGKAAIVTGAAGGIGRATVEAYLREGASVVMADLAPRLAAT
DBDDH-Pp	MRLEGEVALVTGGGAGLGRAIVDRYVAEGARVAVLDKSAAGLEA
7HSDH-Ec	MFNSDNLRLDGKCAIITGAGAGIGKEIAITFATAGASVVVSDINADAANH
BDDH-Ps	MKLKGEAVLITGGASGLGRALVDRFVAEGAKVAVLDKSAERLAE
KACPR-Ec	MNFEKGIALVTGASRGIGRAIAETLAARGAKVIGTATSENGARI
DCHDDH-Pp	MSDLSGKTIIVTGGSGIGRATVELLVASGANVPVADINDEAGEA
3BHDH-Ct	TNRLQGKVALVTGGASGVGLEVVKLLLGEGAKVAFSDINEAAGQQ
20BHDH-Sh	MNDLSGKTVIITGGARGLGAEAAARQAVAAGARVVLADVLDEEGAA
	++ ++ ++ ** + +++++ + +++ + + +
(b)	
SDH-Rs	MRLDGKTALITGSARGIGRAFAEAYVREGARVAIADINLEAARE
ArDH-Ca	MDSAYWSYDNIVPSFRLDGKLVILTGGSGGLAAVVSRALLAKGADVALVDMNLEREQ
RDH-Kp	MKHSVSSMNTSLSGKVAAITGAASGIGLECARTLLGAGAKVVLIDREGEKLNK
GPDH-Kp	MNTWNLNLDKNVIVTGGASGIGLAIVDELLSQGAHVQMIDIHGGDRHH

Fig. 2. Alignment of the N-terminal amino acid sequence of SDH from *R. sphaeroides* and homologous proteins identified by a database search. (a) Alignment of *R. sphaeroides* SDH and the 10 proteins exhibiting the highest degree of homology. *, Identity of an amino acid in all 10 proteins; +, identity of an amino acid in five or more proteins. The amino acid sequence of alcohol dehydrogenase from *Zea mays* begins at position 31; all other sequences start at the N terminus. (b) Alignment of *R. sphaeroides* SDH with other polyol or polyol phosphate dehydrogenases classified as members of the short-chain alcohol dehydrogenase family. SDH-Rs, SDH from *R. sphaeroides* (this study); PGDH-Hs, human 15-hydroxyprostaglandin dehydrogenase (Krook *et al.*, 1990); ADH-Zm, alcohol dehydrogenase from *Zea mays* (Delong *et al.*, 1993); AMDH-Fs, *N*-acylmannosamine 1-dehydrogenase from *Flavobacterium* sp. (Yamamoto-Otake *et al.*, 1991); DBDDH-Pp, *cis*-1,2-dihydrobenzene-2,2-diol dehydrogenase from *Pseudomonas putida* (Neidle *et al.*, 1992); 7HSDH-Ec, 7 α -hydroxysteroid dehydrogenase from *Escherichia coli* (Yoshimoto *et al.*, 1991); BDDH-Ps, biphenyl-2,3-dihydrodiol dehydrogenase from *Pseudomonas* sp. (Hofer *et al.*, 1993); KACPR-Ec, ketoacyl-acyl carrier protein reductase homologue from *E. coli* (Verwoert *et al.*, 1992); DCHDDH-Pp, 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase from *Pseudomonas paucimobilis* (GenBank accession no. D14595); 3BHDH-Ct, 3 β -hydroxysteroid dehydrogenase from *Comamonas testosteroni* (Yin *et al.*, 1991); 20BHDH-Sh, 20 β -hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (Marekov *et al.*, 1990); ArDH-Ca, arabinitol dehydrogenase from *Candida albicans* (Wong *et al.*, 1993); RDH-Kp, RDH from *Enterobacter aerogenes* (Dothie *et al.*, 1985); GPDH-Kp, D-glucitol-6-phosphate dehydrogenase from *Klebsiella pneumoniae* (EMBL accession no. S23835).

DISCUSSION

This and a previous study (Schneider & Giffhorn, 1989) show that *R. sphaeroides* Si4 concomitantly produces SDH and MDH both active on D-glucitol when grown in the presence of D-glucitol. However, the affinity of SDH for D-glucitol ($K_m = 6.2$ mM) and its catalytic efficiency ($K_{cat}/K_m = 7.0 \times 10^3$ M $^{-1}$ s $^{-1}$) are significantly higher than those of MDH ($K_m = 32$ mM; $K_{cat}/K_m = 1.3 \times 10^3$ M $^{-1}$ s $^{-1}$). From these data the conclusion can be drawn that both enzymes are involved in *R. sphaeroides* Si4 D-glucitol metabolism, but SDH is the predominant enzyme at low substrate concentrations. This is in agreement with the observation that both *R. sphaeroides* wild-type and the mutant strain M22 grow on D-glucitol with the same growth rate of 0.22 h $^{-1}$.

From the biochemical data it is evident that SDH is distinct from the two other polyol dehydrogenases so far isolated from *R. sphaeroides* Si4 (Schneider & Giffhorn,

1989; Kahle *et al.*, 1992). It is also different to *Bacillus subtilis* SDH which is a tetrameric zinc enzyme with a M_r of 150 000 (Ng *et al.*, 1992) as well as to the most complex SDH of *Cephalosporium chrysogenum* (M_r 300 000) which is composed of 10 identical subunits (Birken & Pisano, 1976). However, the *R. sphaeroides* SDH resembles the SDH from *Pseudomonas* sp. (M_r 65 000) with respect to size, subunit composition, the absence of a metal requirement and some kinetic properties (Schneider & Giffhorn, 1991). Unlike the mammalian SDHs which generally require zinc for catalytic activity and share a high degree of structural similarity (Jeffery & Jörnval, 1988; Maret & Auld, 1988; Karlsson & Höög, 1993), microbial SDHs are much more heterogeneous. On the basis of sequence data, mammalian SDHs (Jeffery *et al.*, 1984; Karlsson *et al.*, 1989, 1991) have been assigned to the group of zinc-containing medium-chain alcohol dehydrogenases (Jörnval *et al.*, 1987). Only the SDH of *B. subtilis* (Ng *et al.*, 1992) and *Saccharomyces cerevisiae*

(Sarchy *et al.*, 1994) have been included in the same enzyme family and share 36 and 42% homology with the sequences of the mammalian SDHs, respectively.

However, other microbial polyol dehydrogenases have been classified on the basis of sequence data as members of the short-chain alcohol dehydrogenase family which comprises a group of relatively small enzymes exhibiting no metal requirements (Persson *et al.*, 1991). Enzymes of this family are RDH from *Enterobacter aerogenes* (Dothie *et al.*, 1985), arabinitol dehydrogenase from *Candida albicans* (Wong *et al.*, 1993) and D-glucitol-6-phosphate dehydrogenase from *Klebsiella pneumoniae* (EMBL accession no. S23835). Although only a relatively short amino acid sequence of the R. *sphaeroides* SDH is known, the comparative N-terminal amino acid sequences of SDH and MDH show a significant identity between the enzymes (Schneider *et al.*, 1995), and it will be the subject of further studies to elucidate whether SDH and MDH are evolutionarily related. There is also a high degree of homology between the N-terminal parts of SDH and those of other short-chain dehydrogenases. The NAD⁺-binding domain with the consensus sequence GXXXGXG (Wierenga *et al.*, 1985) is located in the N-terminal part of these enzymes (Persson *et al.*, 1991). This motif has also been found in the sequenced part of the SDH from R. *sphaeroides* at position 12–18 (Fig. 2). On the basis of the low molecular mass, the absence of a metal requirement and the sequence homology, we consider the SDH from R. *sphaeroides* Si4 to be a member of the short-chain dehydrogenase family.

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