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Purification and characterization of an alcohol dehydrogenase from 1,2-propanediol-grown *Desulfovibrio* strain HDv

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Abstract The sulfate-reducing bacterium *Desulfovibrio* strain HDv (DSM 6830) grew faster on *(S)*- and on *(R, S)*-1,2-propanediol (μ_{max} 0.053 h^{-1}) than on *(R)*-propanediol (0.017 h^{-1}) and ethanol (0.027 h^{-1}). From *(R, S)*-1,2-propanediol-grown cells, an alcohol dehydrogenase was purified. The enzyme was oxygen-labile, NAD-dependent, and decameric; the subunit mol. mass was 48 kDa. The N-terminal amino acid sequence indicated similarity to alcohol dehydrogenases belonging to family III of NAD-dependent alcohol dehydrogenases, the first 21 N-terminal amino acids being identical to those of the *Desulfovibrio gigas* alcohol dehydrogenase. Best substrates were ethanol and propanol (K_m of 0.48 and 0.33 mM, respectively). *(R, S)*-1,2-Propanediol was a relatively poor substrate for the enzyme, but activities in cell extracts were high enough to account for the growth rate. The enzyme showed a preference for *(S)*-1,2-propanediol over *(R)*-1,2-propanediol. Antibodies raised against the alcohol dehydrogenase of *D. gigas* showed cross-reactivity with the alcohol dehydrogenase of *Desulfovibrio* strain HDv and with cell extracts of six other ethanol-grown sulfate-reducing bacteria.

Key words *Desulfovibrio* strain HDv · Dissimilatory sulfate reduction · Alcohol dehydrogenase · 1,2-Propanediol dehydrogenase

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Abbreviations *ADH* Alcohol dehydrogenase · *DCPIP* 2,6-Dichlorophenolindophenol · *DTT* Dithiothreitol · *MTT* 3-(4′, 5′-Dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide

Introduction

Sulfate-reducing bacteria of the genus *Desulfovibrio* can use a diversity of alcohols as energy sources. Most data concern short primary alcohols such as ethanol and propanol or exceptionally methanol, but in recent years degradation of various diols (e.g., propanediols) and polyols such as glycerol has also received attention (Kremer and Hansen 1987; Nanninga and Gottschal 1987; Kremer et al. 1988; Tanaka 1990, 1992a, b; Qatibi et al. 1991a, b; Ouattara et al. 1992; Widdel and Bak 1992). Extracts of ethanol-grown *Desulfovibrio* strains contain high NAD-dependent ethanol dehydrogenase activities (Kremer et al. 1988). From *Desulfovibrio gigas*, an NAD-dependent, oxygen-labile, decameric alcohol dehydrogenase (ADH) has been purified, which has been shown to belong to family III of ADHs (Hensgens et al. 1993). Only few data concerning *Desulfovibrio* ADH enzymes involved in dehydrogenation of diols are available. The first step in 1,2-propanediol metabolism involves either a dehydration or a dehydrogenation, yielding propanal or lactaldehyde, respectively (Ouattara et al. 1992). In extracts of *Desulfovibrio* strain HDv, an organism able to grow with 1,2-propanediol as energy and carbon source, high NAD-dependent dehydrogenase activities are found with 1,2-propanediol and with ethanol as substrates. Propanediol is thought to be degraded to acetate via lactaldehyde, lactate, and pyruvate. The 1,2-propanediol dehydrogenase activity is approximately 50% of the ethanol dehydrogenase activity (Ouattara et al. 1992). Purified *D. gigas* ADH is only slightly active towards 1,2-propanediol (Hensgens et al. 1993). This raised our interest in the nature of the ADH enzyme(s) of *Desulfovibrio* HDv. The data of Ouattara et al. (1992) suggest the presence of an ADH with an activity ratio towards 1,2-propanediol and ethanol, which is

more similar to that of the 1,2-propanediol dehydrogenase of *Escherichia coli*, an enzyme which is also a family III ADH (Sridhara et al. 1969; Conway and Ingram 1989). Here we report the purification and characterization of the ADH that is most likely involved in 1,2-propanediol oxidation in *Desulfovibrio* strain HDv.

Materials and methods

Organisms, cultivation, and cell extract preparation

The following strains were used: *Desulfovibrio gigas* NCIMB 9332, *D. carbinolicus* EDK82 (DSM 3852^T; laboratory collection), *D. alcoholovorans* SPSN (DSM 5433^T; kindly provided by Dr. A. I. Qatibi, Marseilles), *Desulfovibrio* strain HDv (DSM 6830), *D. vulgaris* Hildenborough (NCIMB 8303), *D. fructosovorans* JJ (DSM 3604^T; kindly provided by Dr. A. I. Qatibi, Marseilles), *Desulfobulbus propionicus* 1pr3 (DSM 2032^T). Unless otherwise mentioned, the strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), or the National Collection of Industrial and Marine Bacteria (Aberdeen, UK).

The bacteria were grown in the bicarbonate-buffered mineral medium of Pfennig et al. (1981), which was supplemented with trace elements (Pfennig and Lippert 1966), 0.1 mM sodium tungstate, 0.1 mM sodium selenite, vitamin solution (Stams et al. 1983), 0.02% (w/v) yeast extract (Difco, Detroit, Mich., USA) and Na_2S (2 mM). Substrates were *(R, S)*-1,2-propanediol (15 mM) for *Desulfovibrio* strain HDv (unless otherwise indicated) and ethanol (20 mM) for the other strains. *Desulfovibrio* strain HDv was grown at 37° C; the other strains were grown at 30° C.

For purification of the ADH, *Desulfovibrio* strain HDv was grown on 15 mM *(R, S)*-1,2-propandiol in 20-l batch cultures; the harvesting of the cells and cell extract preparation were performed as described by Hensgens et al. (1993) except that the washing buffer was changed to 50 mM potassium phosphate (pH 7.0) and the cells were suspended in the same buffer containing 2 mM dithiothreitol (DTT). Cell extract was used for enzyme purification or stored under N_2 at –20° C. Small amounts of cell extracts were prepared by ultrasonic disintegration of cells under a stream of N_2.

Growth rates (μ_{max}) were determined in Bellco screw-capped Hungate tubes that could be inserted in a Biotron colorimeter for measuring the optical density at 660 nm. Growth yields were determined after Stams et al. (1984).

Alcohol dehydrogenase purification

ADH was purified in an anaerobic chamber (approx. 90% N_2/10% H_2) equipped with a palladium catalyst (0 R020; BASF, Ludwigshafen, Germany). Fractions from the columns were tested for 1,2-propanediol and ethanol dehydrogenase activity. Active fractions were cooled on ice. Enzyme preparations were stored at –20° C. Cell extract (50 ml; 2.5 ml min^{-1}) was applied on a Q-Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden; 17 × 2.6 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 2 mM DTT (buffer A). The column was eluted with 90 ml buffer A and a linear gradient of 0–0.5 M KCl (200 ml) in buffer A. The activity eluted in two peaks: the first peak eluted just before and at the beginning of the KCl gradient, the second peak, which contained 7% of the total activity, eluted at approximately 200 mM KCl. Active fractions of the first peak were pooled (65 ml), and ammonium sulfate was added to 1 M final concentration. This preparation was loaded onto a Butyl-Sepharose CL-4B column (Pharmacia; 1.6 × 16 cm; 1.5 ml min^{-1}) equilibrated previously with 1 M ammonium sulfate in buffer A. The column was eluted with a linear gradient (400 ml) from 1 to 0 M ammonium sulfate in buffer A and then washed with buffer A. The activity eluted at the end of the gradient in a total volume of 74 ml. The preparation thus obtained was electrophoretically nearly homogeneous and was used for ADH characterization.

Enzyme assays

The enzyme assays were done essentially as described in Hensgens et al. (1993); the reaction was started with the substrate instead of the enzyme. The standard dehydrogenation assays contained 50 mM Tris-HCl (pH 9.0), 10 mM ethanol or 100 mM *(R, S)*-1,2-propanediol, 5 mM NAD, and enzyme solution (in a total volume of 1 ml). The reverse reaction was assayed with 50 mM Tris-HCl (pH 7.5), 2 mM acetaldehyde, 0.2 mM NADH, and enzyme solution (in a total volume of 1 ml). Dye-linked alcohol dehydrogenase (EC 1.1.99.8) was tested under N_2 in 50 mM Tris-HCl (pH 7.5), 50 mM Hepes-KOH (pH 7.5), 50 mM potassium phosphate (pH 7.5), or 100 mM Tris-HCl (pH 9.0) containing 50 mM NH_4Cl. The following electron acceptors were used: 0.07 mM 2,6-dichlorophenolindophenol (DCPIP; ε_{600} 19 mM^{-1} cm^{-1}) and 1.2 mM 3-(4′,5′-dimethylthiazol-2-yl)-2,4-diphenyltetrazo lium bromide (MTT; ε_{578} 13 mM^{-1} cm^{-1}). The reactions were started by injecting either ethanol or 1,2-propanediol to a final concentration of 10 mM and 100 mM, respectively.

The influence of metal chelators and sulfhydryl blocking reagents was tested by preincubation of the enzyme with the compound for 5 min. The reaction was started by injecting ethanol to a final concentration of 10 mM. The effect of metal ions was tested by preincubation with the enzyme for 3 min. Ethanol (10 mM) was used to start the reaction.

Other biochemical methods

Polyclonal antibodies were elicited by injecting rabbits four times at intervals of 10 days subcutaneously with 0.3 mg *D. gigas* ADH in Freund's complete adjuvant. *D. gigas* ADH was purified as described previously (Hensgens et al. 1993); the protein was blotted onto nitrocellulose, which was dissolved with dimethylsulfoxide prior to injection (Harlow and Lane 1988). Cell extracts and purified proteins were subjected to SDS-PAGE (12.5%) according to Laemmli (1970); proteins were stained with Coomassie Brilliant Blue R-250. Western blotting was carried out according to Kyhse-Andersen (1984), using a semi-dry electroblotter. Protein was measured according to Bradford (1976) with bovine serum albumin as a standard. N-terminal amino acid analysis was carried out on a 477A Applied Biosystems sequenator by Eurosequence (Groningen, The Netherlands).

Electron microscopy, metal analysis, and other methods

Anoxically stored *Desulfovibrio* strain HDv ADH was applied to a grid covered with a carbon-coated Formvar film. Further electron microscopy techniques and image analysis were as described by Vonck et al. (1991). Co, Fe, Mg, and Zn were determined in 4.3 mg of purified ADH by instrumental neutron activation analysis at the Interfacultair Reactor Instituut (Delft, The Netherlands). Alcohols were determined according to Laanbroek et al. (1982).

Chemicals

All chemicals used were of the highest purity commercially available. *(S)*-1,2-propandiol and *(R, S)*-1,2-propanediol were obtained from Merck (Darmstadt, Germany); *(R)*-1,2-propanediol was obtained from Merck and Fluka (Buchs, Switzerland).

Results

Growth experiments and ADH purification

The specific growth rates of *Desulfovibrio* strain HDv with ethanol, *(S)*-, *(R)*-, and *(R, S)*-1,2-propanediol were

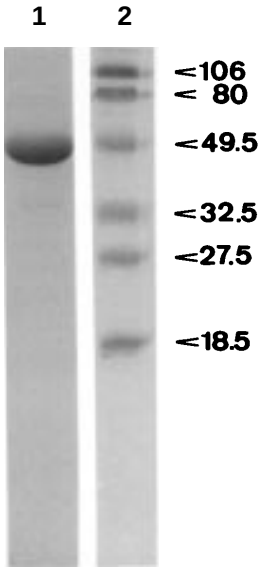

Fig. 1 SDS-PAGE (12.5%) of *lane 1* ADH (28 μg) of *Desulfovibrio* strain HDv and *lane 2* mol. mass markers (kDa): phosphorylase B (106), bovine serum albumin (80), ovalbumin (49.5), carbonic anhydrase (32.5), soybean trypsin inhibitor (27.5), and lysozyme (18.5)

0.027, 0.053, 0.017, and 0.053 h^{-1}, respectively. The growth yield on *(R, S)*-1,2-propanediol was 8 g dry weight mol^{-1}. Extracts of ethanol-grown *Desulfovibrio* HDv had a lower ethanol dehydrogenase activity [0.8 μmol NADH formed min^{-1} (mg protein)$^{-1}$] than cells grown on *(R, S)*-1,2-propanediol [4.9 μmol NADH formed min^{-1} (mg protein)$^{-1}$]. NAD-dependent activities with 1,2-propanediol (10 mM) as a substrate were considerably lower than with ethanol (only approximately 1.4% of the latter value at 10 mM; at 100 mM, only 7% of the value) in extracts of 1,2-propanediol-grown cells; the ratio of the activities in extracts was far lower than found by Ouattara (1992). We did not detect activities of ethanol- or 1,2-propanediol-dehydrogenating enzymes with artificial electron acceptors such as DCPIP and MTT.

The NAD-dependent ADH of *Desulfovibrio* strain HDv was purified nearly to homogeneity (Fig. 1; Table 1). After the first chromatography step, two activity peaks were found. The second peak contained about 7% of the total activity and did not differ from the first peak with respect to substrate use; the ratios of the NAD-dependent dehydrogenase activities with 1,2-propanediol and ethanol were the same. The pooled fractions of the main peak were used for further purification. The Butyl-Sepharose chromatography step resulted in a considerable loss of activity, but also in a good purification, as judged by electrophoresis. The enzyme constituted over 5% of the protein in the cell extract (see Table 1).

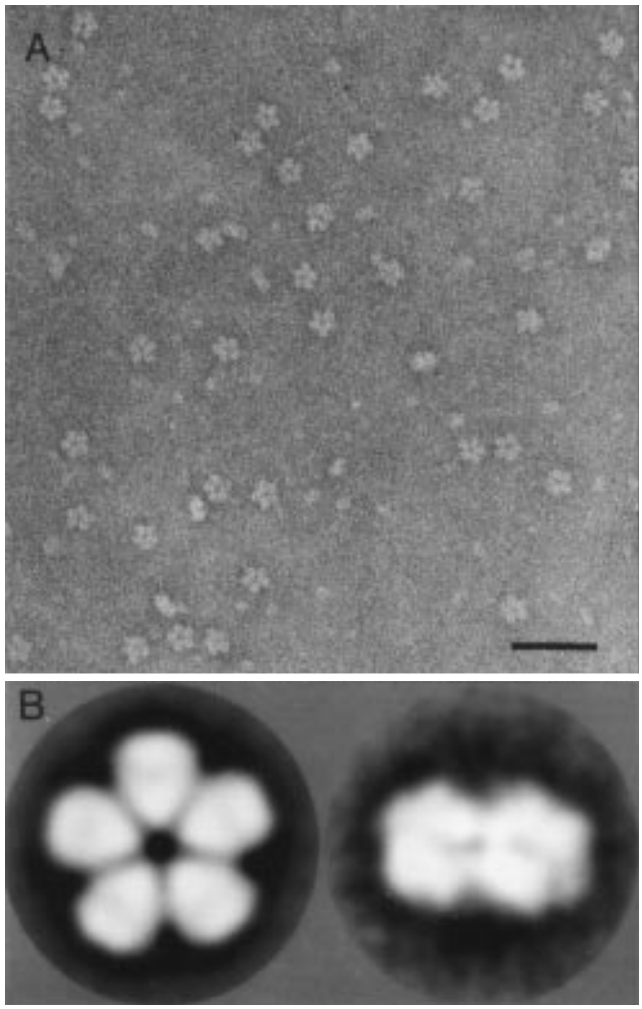

Fig. 2 **A** Electron micrograph of *Desulfovibrio* strain HDv ADH, negatively stained with 1% uranyl acetate, *bar* 50 nm. **B** Computer images of the *Desulfovibrio* strain HDv ADH; *left* top view and *right* side view

Physical and catalytic properties

The subunit mol. mass of the *Desulfovibrio* strain HDv ADH was 48,000 Da, as determined by SDS-PAGE (Fig. 1); the band had virtually the same position as the *Desulfovibrio gigas* ADH subunit with an estimated mol. mass of 43,000 Da (Hensgens et al. 1993). Metal analysis revealed the presence of 0.7 Zn and 0.05 Co per subunit. The native mol. mass of the *Desulfovibrio* HDv enzyme was estimated to be 263,000 and 251,000 Da by size-exclusion chromatography over a Sephacryl S-300 and a Superose-6 column, respectively; calibration with mol. mass

Table 1 Purification of ADH from *Desulfovibrio* strain HDv. Activities were measured with 10 mM ethanol. One unit is the amount of enzyme that forms 1 μmol NADH min^{-1}

Purification step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein)$^{-1}$]	Yield (%)	Purification (fold)
Cell extract	295	1446	4.9	100	1
Q-Sepharose	71	1043	14.7	72	3.0
Butyl-Sepharose	18	311	17.2	22	3.5

Table 2 Comparison of the relative activities of *Desulfovibrio* strain HDv ADH and *Desulfovibrio gigas* ADH. *D. gigas* data are from Hensgens et al. (1993), except for the data on 1,2-propanediols (*n.d.* not determined)

Substrates alcohols (10 mM)[a] and aldehydes (2 mM)[b]	*Desulfovibrio* strain HDv ADH (%)	*D. gigas* ADH (%)
Ethanol	100	100
1-Propanol	106	105
Butanol	39	26
Ethanediol	10	n.d.
1,3-Propanediol	8	7
(R, S)-1,2-propanediol	1.4	6.8
(R, S)-1,2-propanediol (100 mM)	7	20
(S)-1,2-propanediol	2.0	22.6
(S)-1,2-propanediol (100 mM)	12	46
(R)-1,2-propanediol	0.9	4
(R)-1,2-propanediol (100 mM)	2.4	7.3
2,3-Butanediol	<1	4
Glycerol	<1	6
2-Propanol	3	3
Formaldehyde	8	8
Acetaldehyde	100	100
Propanal	98	250
Butyraldehyde	14	37
Glycolaldehyde	11	

[a] Unless indicated otherwise
[b] Aldehyde-reducing activity

markers (Bio-Rad) was carried out in accordance with instructions of the manufacturer. These figures indicate a pentameric structure for the native enzyme. To verify this, purified ADH was analyzed by electron microscopy. Images showed molecules attached in top- and side-view position to the support film (Fig. 2A). For image analysis, we selected 1267 top views and 361 side views from 22 of such electron micrographs. After alignment and subsequent classification of the projections, the best 560 top views and 62 side views were summed (Fig. 2B). The average top view shows a 5-fold symmetrical projection with a diameter of 16.4 nm; the side view shows a two-layered structure with a height of 11 nm. Taken together, these two views show that the enzyme has a decameric quaternary structure consisting of two stacked pentameric rings.

The N-terminal amino acid sequence of the first 41 amino acids was (parentheses indicate less certainty): Ala-Val-Arg-Glu-Gln-Val-Tyr-Gly-Phe-Phe-Ile-Pro-Ser-Val-Thr-Leu-Ile-Gly-Ile-Gly-Ala-Ala-Lys-Ala-Ile-Pro-Glu-Lys-Ile-Lys-Ala-Leu-Gly-(Gly)-(Ser)-(Lys)-(Pro)-(Leu)-(Ile)-(Val)-(Thr). The first 21 amino acids were identical to the N-terminal amino acid sequence of the *D. gigas* ADH (see Hensgens et al. 1993).

Purified ADH was clearly oxygen-labile, but less so than the *D. gigas* ADH. When exposed to air at 0° C, the enzyme lost 40% of its activity in 1 h. After 3 h, 70% of the activity was lost. The remaining activity was gradually lost in approximately 4 days when stored under air at –20° C. Enzyme preparations were still active even after 3 months when stored under N_2 at –20° C. The pH optimum was around pH 9.5 in the dehydrogenation assay with ethanol (10 mM) and 1,2-propanediol (100 mM) as substrate. For the reverse reaction with acetaldehyde (2 mM) as a substrate, the pH optimum was around pH 5.5. Highest activity was found at 45° C in the standard dehydrogenation assay. Above this temperature, inactivation was considerable.

The substrate profile of ADH of *Desulfovibrio* strain HDv was similar to that of *D. gigas* ADH (Table 2). The best substrates were ethanol and propanol for the dehydrogenation reaction and acetaldehyde and propanal for the reverse reaction. The ADH had relatively low activities towards 10 and 100 mM *(R, S)*-1,2-propanediol, even when compared with *D. gigas* ADH. ADH showed a clear preference for *(S)*-1,2-propanediol. Apparent K_m values were: ethanol, 0.48 mM; 1-propanol, 0.33 mM; *(R, S)*-1,2-propanediol, 166 mM; *S*-1,2-propanediol, 39 mM; acetaldehyde, 1.0 mM; glycolaldehyde, 63 mM; NAD^+ (with 10 mM ethanol), 0.2 mM; NADH (with 2 mM acetaldehyde), 0.026 mM. NAD^+ and NADH were used as electron acceptor and donor, respectively; other electron acceptors (DCPIP, MTT, $NADP^+$) were not active or nearly inactive. NADP(H) had activities of 3.3 and <1% of the values with NAD(H), with ethanol and acetaldehyde as substrates.

Addition of metal chelators and sulfhydryl blocking agents resulted in the following activities (control without addition was considered as 100%): EDTA (10 mM), 94%; 1,10-phenanthroline (0.6 mM), 63%; 2,2-bipyridyl (3 mM), 99%; *p*-chloromercuribenzoate (0.25 mM), 58%; *N*-ethylmaleimide (2 mM), 4%. The presence of Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} (all at 2 mM) resulted in relative activities of 57, 14, 58, and 2%, respectively; Zn^{2+} (1 mM) and Cu^{2+} (5 mM) resulted in relative activities of 9 and 78%, respectively. Ca^{2+} and Mg^{2+} (2 mM) had virtually no effect.

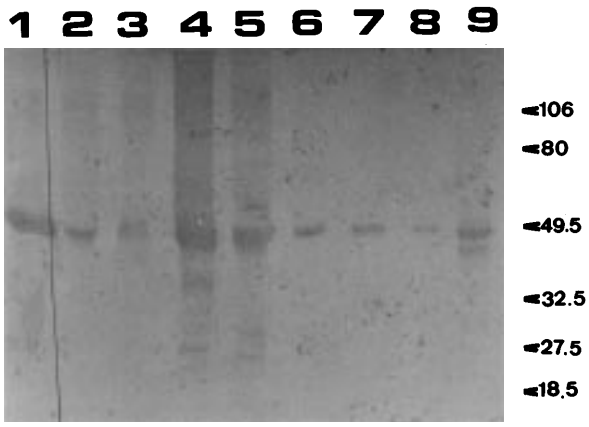

Fig. 3 Western blot of cell extracts (approximately 10 mg) from *1 Desulfovibrio gigas*, *2 D. carbinolicus*, *3 D. fructosovorans*, *4 D. vulgaris*, *5 D. alcoholovorans*, *6 Desulfovibrio* strain HDv, *9 Desulfobulbus propionicus*, and of *7* purified *Desulfovibrio* strain HDv ADH (3 μg), and *8* material from second activity peak of *Desulfovibrio* strain HDv (15 μg). Antibodies raised against *D. gigas* ADH were used. Mol. mass markers are indicated in kDa

Alcohol dehydrogenases in other sulfate reducers

Cell extracts of six other sulfate-reducing bacteria (see Materials and methods) grown on ethanol had substrate profiles similar to the ADHs of *D. gigas* and *Desulfovibrio* strain HDv (data not shown). Activities with ethanol ranged from 0.03 to 1 mmol NADH formed min^{-1} (mg protein)$^{-1}$. Cell extracts of these strains were subjected to SDS-PAGE. Western blots showed that the antibodies raised against purified *D. gigas* ADH cross-reacted with *Desulfovibrio* strain HDv ADH and with the extracts of all of the strains tested (Fig. 3). The mol. mass values were similar; in the case of *Desulfobulbus propionicus* DSM 2032, two bands were found that reacted with the antibodies against *D. gigas* ADH.

Discussion

Our data strongly suggest that *Desulfovibrio* strain HDv uses an ADH that has relatively poor kinetics towards 1,2-propanediol, as compared to ethanol and 1-propanol, for the dehydrogenation of 1,2-propanediol. During the first purification step, we detected a second activity peak. This contained, however, only about 7% of the total activity and had the same substrate profile as was found for the first peak. Protein from this second activity peak reacted to the antibodies raised against purified *D. gigas* ADH in a way similar to that of the purified enzyme; Western blots indicated a very similar subunit mol. mass. This second peak might be a still active degradation product of the decameric ADH with a different chromatographic behavior. Other ADHs were not detected.

The large discrepancy between our results and those of Ouattara et al. (1992) with respect to the specific activity towards 1,2-propanediol in cell extracts of *Desulfovibrio* HDv cannot be explained easily. The ratio of the growth rates on the isomers of 1,2-propandiol was consistent with the ratio of the activities of *(S)*-1,2-propanediol and *(R)*-1,2-propanediol at 10 mM. This and the fact that no other alcohol dehydrogenase activities were detected make it plausible that the isolated enzyme is indeed the enzyme that acts as the 1,2-propanediol dehydrogenase during growth of *Desulfovibrio* HDv with 1,2-propanediol. Furthermore, the 1,2-propanediol dehydrogenating activity of the ADH in the cell extract [70 nmol min^{-1} (mg protein)$^{-1}$ at 10 mM propanediol when assayed at 30° C; approximately 30% higher at 37° C] was almost high enough to account for the growth rate. With a molar growth yield of 8 g mol^{-1} and a specific growth rate on 15 mM 1,2-propanediol at 37° C of 0.053 h^{-1}, the specific substrate consumption rate in cultures (μ/Y) was approximately 110 nmol min^{-1} (mg dry weight)$^{-1}$. The 1,2-propanediol dehydrogenase from *E. coli* is specific for the *(S)* form of 1,2-propanediol and, in this respect, resembles *Desulfovibrio* HDv ADH with its preference for the *(S)* form, but unlike the latter enzyme, it has a K_m for 1,2-propanediol lower than for ethanol (Sridhara et al. 1969; Cocks et al. 1974).

The ADH from *Desulfovibrio* strain HDv strongly resembles the ADH from *Desulfovibrio gigas* in physical and catalytic properties and in N-terminal amino acid sequence. Both enzymes contained Zn, but were also strongly inhibited by Zn^{2+}. These similarities are striking because *Desulfovibrio* strain HDv grows well on 1,2-propanediol, whereas *D. gigas* does not grow at all with this substrate. The biochemical difference between the two strains that underlies this phenomenon is, therefore, most likely not the nature of the ADH, but possibly the lower expression of the ADH in *D. gigas* or a lower activity of the aldehyde dehydrogenase towards lactaldehyde or a combination of both factors. The cross-reaction of antibodies raised against purified *D. gigas* ADH with cell extracts of other *Desulfovibrio* strains and *Desulfobulbus propionicus* indicates that this type of ADH is widespread among the sulfate-reducing bacteria.

The low-resolution quaternary structure of *Desulfovibrio* HDv ADH, as revealed by electron microscopy and image analysis, was similar to the structures found for the NAD-dependent ADHs from *D. gigas* (Hensgens et al. 1993) and *Bacillus methanolicus* (Vonck et al. 1991) and for other (m)ethanol-dehydrogenating enzymes from *Amycolatopsis methanolica* and *Mycobacterium gastri* (Bystrykh et al. 1993). On the basis of N-terminal amino acid sequences or complete gene sequences, these enzymes belong to family III of ADHs; the number of known family III ADHs is rapidly increasing (Bruchhaus and Tannich 1994; Nair et al. 1994; Reid and Fewson 1994).

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