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# Purification and Characterization of a Benzylviologen-Linked, Tungsten-Containing Aldehyde Oxidoreductase from *Desulfovibrio gigas*

CHARLES M. H. HENSGENS,[1] WILFRED R. HAGEN,[2] AND THEO A. HANSEN[1]*

*Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, 9751 NN Haren,[1] and Department of Biochemistry, University of Wageningen, 6703 HA Wageningen,[2] The Netherlands*

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***Desulfovibrio gigas*** **NCIMB 9332 cells grown in ethanol-containing medium with 0.1 μM tungstate contained a benzylviologen-linked aldehyde oxidoreductase. The enzyme was purified to electrophoretic homogeneity and found to be a homodimer with a subunit $M_r$ of 62,000. It contained 0.68 ± 0.08 W, 4.8 Fe, and 3.2 ± 0.2 labile S per subunit. After acid iodine oxidation of the purified enzyme, a fluorescence spectrum typical for form A of molybdopterin was obtained. Acetaldehyde, propionaldehyde, and benzaldehyde were excellent substrates, with apparent $K_m$ values of 12.5, 10.8, and 20 μM, respectively. The natural electron acceptor is not yet known; benzylviologen was used as an artificial electron acceptor (apparent $K_m$, 0.55 mM). The enzyme was activated by potassium ions and strongly inhibited by cyanide, arsenite, and iodoacetate. In the as-isolated enzyme, electron paramagnetic resonance studies readily detected W(V) as a complex signal with *g* values in the range of 1.84 to 1.97. The dithionite-reduced enzyme exhibited a broad signal at low temperature with $g = 2.04$ and 1.92; this is indicative of a $[4Fe\text{-}4S]^{1+}$ cluster interacting with a second paramagnet, possibly the $S = 1$ system of W(IV). Until now W-containing aldehyde oxidoreductases had only been found in two *Clostridium* strains and two hyperthermophilic archaea. The *D. gigas* enzyme is the first example of such an enzyme in a gram-negative bacterium.**

*Desulfovibrio gigas* NCIMB 9332 is a sulfate-reducing bacterium which is able to grow with $H_2$, L- and D-lactate, $C_4$-dicarboxylic acids, and ethanol as energy sources (17, 35). *D. gigas* not only oxidizes ethanol (to acetate) but it can also store massive amounts of polyglucose (29) which under fermentative conditions can be degraded with ethanol as a major end product (26).

Recently, we characterized the NAD-dependent alcohol dehydrogenase from *D. gigas* and showed it to be an oxygen-labile, decameric enzyme (14). From lactate-grown cells, a molybdenum iron-sulfur protein (MOP) which had 2,6-dichlorophenol-indophenol (DCPIP)-linked aldehyde dehydrogenase activity (1, 31) was purified. In extracts of cells grown on lactate or ethanol, high levels of activity of a coenzyme A-independent, benzylviologen-linked aldehyde dehydrogenase (BV-AlDH) were measured (17). Because at that time only the MOP was known as an enzyme with aldehyde dehydrogenase activity in *D. gigas*, it was suggested that the BV-AlDH activity was catalyzed by the MOP. Recently, we found that the addition of 0.1 μM tungstate to the medium had a strongly stimulatory effect on the growth of *D. gigas* on ethanol. Omission of tungstate from the medium resulted in a decrease or absence of the BV-AlDH activity and an increase of the DCPIP-linked aldehyde dehydrogenase activity (13). These data and the different characteristics of the enzyme activities in cell extracts strongly suggested that the BV-AlDH and the MOP are two different enzymes.

In this report, we describe the purification and characterization of the BV-AlDH and provide evidence that this enzyme belongs to the group of molybdopterin- and tungsten-containing aldehyde:acceptor oxidoreductases.

## MATERIALS AND METHODS

**Organism, cultivation, and preparation of cell extract.** *D. gigas* NCIMB 9332 was cultivated on ethanol, harvested, washed, and stored as described earlier (14), with the following minor modifications. The washing buffer was 50 mM potassium phosphate (pH 7.5) containing 2 mM dithiothreitol (DTT) and 1 mM dithionite. Cell extract was prepared by three successive passages through a French pressure cell operated at 106 MPa and centrifugation (20 min at 48,000 × *g* and 4°C). The cell extract was then used for enzyme purification or stored at −20°C under $N_2$ until further use.

**Enzyme assays.** Assay systems in cuvettes were made anaerobic by bubbling with $N_2$ for at least 3 min and were then closed with grey butyl rubber stoppers. The standard assay contained 50 mM potassium phosphate buffer (pH 7.5) and 2 mM benzylviologen in a total volume (after the following additions) of 1 ml. Then, 5 μl of a freshly prepared dithionite solution (1.5 mM) and enzyme solution (usually 10 μl) were added with microsyringes and the reaction was started by adding acetaldehyde to a final concentration of 1 mM; oxygen was removed from acetaldehyde stock solutions in crimp-seal bottles by replacing the atmosphere with oxygen-free nitrogen twice. Addition of dithionite was necessary to prevent a lag phase in the reaction. Activities were measured spectrophotometrically at 30°C. The following electron acceptors were tested: BV (2 mM; $\varepsilon_{500} = 7.4\ mM^{-1}\ cm^{-1}$), methylviologen (2 mM; $\varepsilon_{578} = 9.6\ mM^{-1}\ cm^{-1}$), 3-(4′,5′-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) (2 mM; $\varepsilon_{550} = 8.1\ mM^{-1}\ cm^{-1}$), DCPIP (80 μM; $\varepsilon_{600} = 22\ mM^{-1}\ cm^{-1}$), Wursters blue (the free radical form of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; $\varepsilon_{578} = 9\ mM^{-1}\ cm^{-1}$ [assayed as described in reference 22]), and NAD(P) (5 mM; $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$). One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of aldehyde per min.

Potential inhibitors were preincubated for 5 min with the enzyme before the reaction was started with acetaldehyde in the standard assay system.

**Purification of BV-AlDH.** BV-AlDH activity in cell extracts of ethanol-grown *D. gigas* did not appear to be oxygen labile (17). However, attempts to purify BV-AlDH aerobically were unsuccessful and resulted in a total loss of activity after the first chromatography step. Therefore, all purification steps were carried out anaerobically in a glove box equipped with a palladium catalyst (BASF R0 0-20) with a gas atmosphere of approximately 90% $N_2$ and 10% $H_2$ at ambient temperature. Active fractions of BV-AlDH were stored on ice. Crude extract (45

* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31 50 3632163. Fax: 31 50 3632154. Electronic mail address: hansenta@biol.rug.nl.

ml) was applied (at 2 ml $min^{-1}$) onto a Q-Sepharose Fast Flow column (2.5 by 13 cm; Pharmacia) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM DTT, 1 mM dithionite, and 8.7% (vol/vol) glycerol. After the cell extract was applied, the column was washed with 127 ml of equilibration buffer. A linear gradient (400 ml; 1.9 ml $min^{-1}$) of 0 to 0.75 M KCl in 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM DTT, 1 mM dithionite, and 8.7% (vol/vol) glycerol was used to elute BV-AlDH. Activity was eluted at KCl concentrations between 0.3 and 0.5 M; active fractions were pooled (107 ml). After addition of KCl to a concentration of 0.75 M, the pooled fractions were loaded onto a Butyl-Sepharose 4 Fast Flow column (2.5 by 10 cm; Pharmacia) equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM DTT, 1 mM dithionite, 8.7% (vol/vol) glycerol, and 0.75 M KCl. Then, the column was washed with the equilibration buffer (90 ml; 1.9 ml $min^{-1}$) and a linear gradient of 0.75 to 0 M KCl in 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM DTT, 1 mM dithionite, and 8.7% glycerol (100 ml; 1.9 ml $min^{-1}$). An 8.7 to 43% (vol/vol) glycerol gradient in the above-mentioned buffer (200 ml; 1.9 ml $min^{-1}$) was used to elute BV-AlDH. Activity was eluted at glycerol concentrations between 16 and 41%. The pooled active fractions (148 ml; 1.9 ml $min^{-1}$) were applied onto a DEAE Memsep 1010 cassette (Millipore) equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM DTT, 1 mM dithionite, and 8.7% (vol/vol) glycerol. Then, the cassette was washed with equilibration buffer (50 ml; 1.9 ml $min^{-1}$) and BV-AlDH was eluted with a linear gradient of 0 to 0.375 M KCl in 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM DTT, 1 mM dithionite, and 8.7% (vol/vol) glycerol (400 ml; 1.9 ml $min^{-1}$); BV-AlDH eluted at KCl concentrations between 50 and 120 mM. Active fractions were tested for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); pure fractions were pooled and used for further characterization of the enzyme.

Fractions were stored under $N_2$ at −20°C between the different purification steps.

**Molecular weight determinations.** The native $M_r$ of BV-AlDH was determined by gel filtration over two different columns: a Sephacryl S-300 column (40 by 3 cm; 1 ml $min^{-1}$) and a Superose 12 HR 10/30 column (Pharmacia; 0.5 ml $min^{-1}$). Thyroglobulin ($M_r$, 670,000), gamma globulin ($M_r$, 158,000), ovalbumin ($M_r$, 44,000), myoglobin ($M_r$, 17,000), and vitamin B-12 ($M_r$, 1,350) were used as molecular weight standards.

**Gel electrophoresis.** SDS-PAGE was carried out by the method of Laemmli (18); β-galactosidase ($M_r$, 116,400), fructose-6-phosphate kinase ($M_r$, 85,200), glutamate dehydrogenase ($M_r$, 55,600), aldolase ($M_r$, 39,200), triose phosphate isomerase ($M_r$, 26,600), trypsin inhibitor ($M_r$, 20,100), and lysozyme ($M_r$, 14,300) were used as molecular weight markers; gels were stained with Coomassie brilliant blue R-250. For nondenaturing PAGE, 4 to 20% gradient gels were used. Activity gels were run and stained in the glove box according to the method of White et al. (34), with 1 mM BV and 1 mM acetaldehyde in 50 mM potassium phosphate buffer (pH 7.5). The reduced BV band was stabilized with neotetrazolium chloride (1 mM).

**Analytical methods.** The spectrum of purified BV-AlDH (0.12 mg $ml^{-1}$) was recorded on an SLM Aminco DW-2000 spectrophotometer. The acid iodine oxidation method as described by Yamamoto et al. (36) was used to liberate form A of the molybdopterin from purified BV-AlDH. Fluorescence spectra were recorded on a Perkin Elmer LS 50B luminescence spectrometer. Protein was determined according to the method of Bradford (3), with bovine serum albumin as a standard. Tungsten and molybdenum were determined colorimetrically (4). For analysis of Fe and labile S, the methods of Fish (10) and Chen and Mortenson (6) were used. The N-terminal amino acid sequence of purified BV-AlDH was determined by automated Edman degradation on a pulse-liquid sequenator (model 477A; Applied Biosystems); stepwise released phenylthiohydantoin-amino acid derivatives were analyzed with an on-line reverse phase high performance liquid chromatography unit (model 120A; Applied Biosystems).

**EPR studies.** Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ER-200 D spectrometer with peripheral equipment and data handling as described before (22). The W(V) spectrum was simulated as a sum of S = 1/2 components subject to inhomogeneous broadening by *g* strain as detailed in reference 12.

## RESULTS

**Purification of BV-AlDH.** BV-AlDH was purified to electrophoretic homogeneity in three successive chromatography steps (Table 1). The enzyme proved to be oxygen labile; therefore, all purification steps were performed in an anaerobic glove box. Addition of dithionite to the buffers was essential for preventing a considerable loss of activity. The purified enzyme was not very stable at 4°C; storage at this temperature resulted in a decrease of 89% of the activity in 8 days. Storage at −20°C for 1 month resulted in a decrease of 16% of the activity. The concentrated BV-AlDH had a reddish brown color and could be seen after the first purification step as a brown band on the column. Activity staining of the purified enzyme after nondenaturing PAGE resulted in a band at the same position as the band obtained with Coomassie brilliant blue R-250 (data not shown).

TABLE 1. Purification of BV-AlDH

| Purification step | Total protein (mg) | Total activity (U) | Sp act[a] (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Cell extract | 702 | 1,123 | 1.6 | 100 | 1 |
| Q-Sepharose | 173 | 813 | 4.7 | 72.4 | 2.9 |
| Butyl-Sepharose | 19.2 | 531 | 27.6 | 47.3 | 17.3 |
| DEAE Memsep 1010 | 11.4 | 437 | 38.5 | 38.9 | 24 |

[a] Activity determined with BV (2 mM) and acetaldehyde (1 mM).

Prolonged storage of Q-Sepharose under anaerobic conditions with the buffer system used resulted in a column material that could no longer be used for the purification of BV-AlDH because it led to a total loss of activity.

**Molecular weight and subunit size.** Native BV-AlDH had $M_r$s of 120,000 ± 10,000 and 126,000 ± 8,000 as determined by size exclusion chromatography over a Sephacryl S-300 column and a Superose 12 HR 10/30 column, respectively. The subunit $M_r$ determined by SDS-PAGE (Fig. 1) was 62,000, which is interpreted as showing that the enzyme is a homodimer.

**Catalytic properties.** Acetaldehyde, propionaldehyde and benzaldehyde were excellent substrates for the enzyme (Table 2). Acetaldehyde concentrations of up to 5 mM had no negative effect on the activity. As can be seen in Table 2, the level of activity decreases with increasing length of the aliphatic aldehydes. In the assay with acetaldehyde, the artificial electron acceptors BV, methylviologen, MTT, Wursters blue, and DCPIP had relative activities of 100, 19, 5, 0, and 1.3%, respectively; NAD and NADP showed no activity. The best artificial electron acceptor (BV) had an apparent $K_m$ of 0.55 mM.

The reaction with BV as the electron acceptor and acetaldehyde as the substrate assayed in a potassium phosphate buffer

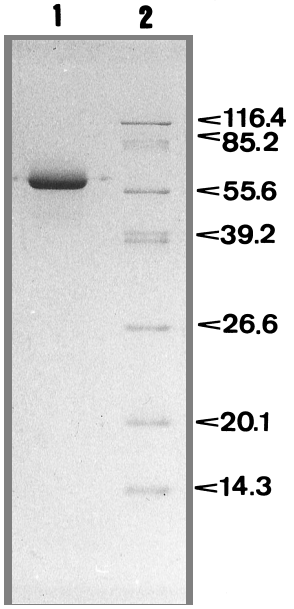


FIG. 1. SDS-PAGE of purified BV-AlDH from *D. gigas* (lane 1) and $M_r$ markers (in thousands) (lane 2). The gel was stained with Coomassie brilliant blue R-250.

TABLE 2. Substrates and $K_m$ values of BV-AlDH

| Aldehyde (1 mM) | Relative activity (%) | $K_m$ (μM) |
|---|---|---|
| Formaldehyde | 16 | |
| Acetaldehyde | 100 | 12.5 |
| Propionaldehyde | 117 | 10.8 |
| Butyraldehyde | 16 | |
| Pentanal | 9 | |
| Hexanal | 6 | |
| Octanal | 6 | |
| Benzaldehyde | 93 | 20 |
| Salicylaldehyde | 14 | |
| *p*-Anisaldehyde | 19 | |
| *m*-Anisaldehyde | 5 | |
| L-Glyceraldehyde | 0 | |
| D-Glyceraldehyde | 0 | |
| Glutaraldehyde | 20 | |
| Glycolaldehyde | 23 | |
| Furfural | 77 | 30.8 |
| Phenylacetaldehyde | 31 | |

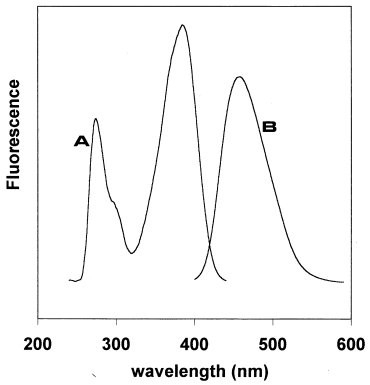


FIG. 3. Fluorescence spectra after acid iodine oxidation of purified BV-AlDH (0.12 mg of protein $ml^{-1}$). The sample was adjusted to pH 12 with NaOH. The excitation spectrum (trace A) was measured at an emission wavelength of 455 nm; the emission spectrum (trace B) was measured at an excitation wavelength of 380 nm.

(or in 50 mM Tris-HCl) had a broad pH optimum around pH 8. The level of activity increased with increasing temperature, but from 48°C the initial velocity remained constant (approximately 2.2 times the value at 30°C). The reaction remained linear for several minutes, but at 65°C the level of activity began to decrease noticeably after 3 min.

Preincubation of the enzyme for 5 min with possible inhibitors resulted in the following relative activities (control without addition is 100%): iodoacetate (5 mM), 0%; KCN (5 mM), 36% (which decreased during the assay); arsenite (5 mM), 0%; EDTA (5 mM), 100%; methanol (50 mM), 100%; and methanol (1 M), 38%.

BV-AlDH was stimulated by potassium ions when added to the assay system; desalted BV-AlDH from which the potassium ions were removed showed a 45% lower level of activity. The optimal level of activity was reached at 10 mM $K^+$ when assayed with sodium phosphate buffer (pH 7.5), BV, and acetaldehyde. Other monovalent cations (25 mM) such as $NH_4^+$, $Li^+$, $Na^+$, $Rb^+$, and $Cs^+$ (all added as chloride salts) did not have an effect on the reaction rate. Neither did the anions $Cl^-$, $SO_4^{2-}$, and $PO_4^{3-}$.

**Effect of air on enzyme activity.** Exposure of enzyme preparations to air led to inactivation. The exposed enzyme showed a lag phase before the reaction started (in an anaerobic assay), and the final maximal activity decreased upon prolonged incubation (Fig. 2). When an enzyme preparation was made anaerobic after air exposure and dithionite was added (1 mM), the maximal activity was reached without a lag phase; however, no complete reactivation of the enzyme occurred.

**Metals, labile sulfur, and cofactor determination.** The enzyme was found to contain 0.68 ± 0.08 tungsten, 4.8 iron, and 3.2 ± 0.2 labile sulfur per subunit (from four determinations using two different enzyme preparations). Molybdenum was not present in significant quantities.

Acid iodine oxidation of the purified enzyme led to formation of a compound with a fluorescence spectrum similar to that of the form A molybdopterin derivative (Fig. 3; cf. reference 16).

**UV-visible spectra.** The UV-visible spectrum of the red-brown colored BV-AlDH is shown in Fig. 4. No indications for the presence of a flavin were found in the spectrum. Air-oxidized BV-AlDH showed an absorption shoulder around 425 nm, which is typical for Fe/S clusters. Addition of acetaldehyde (1 mM) or acetate (1 mM) to the purified enzyme did not result in clear spectral changes.

**N-terminal amino acid sequence.** The N-terminal amino acid sequence as determined by automated Edman degradation was (tentative assignments in parentheses) Met-Asp-Lys-Ile-Leu-Arg-Ile-Asp-Val-Gly-Ala-Glu-Gly-Gly-Pro-Lys-(Leu)-(Thr)-(Thr)-(Leu). No similarity with known N-terminal amino acid sequences of W-containing aldehyde dehydrogenases is apparent (20).

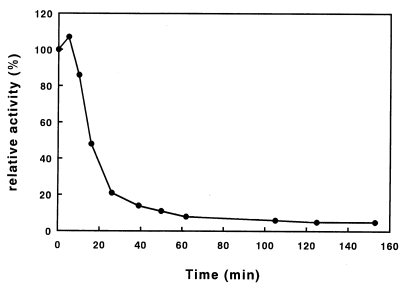


FIG. 2. Effect of exposure to air on BV-AlDH. A vial with BV-AlDH in 10 mM potassium phosphate buffer containing 1 mM dithionite, 2 mM DTT, and 8.7% (vol/vol) glycerol (1 ml) was opened and incubated on ice without stirring. Enzyme activities were monitored (anaerobic assays), and the enzyme activity (value at 10 min after start of the reaction) was plotted against exposure time.

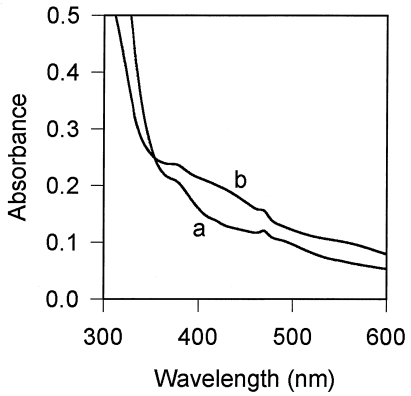


FIG. 4. UV-visible spectrum of purified BV-AlDH (0.36 mg · $ml^{-1}$) in 10 mM potassium phosphate buffer containing 1 mM dithionite, 2 mM DTT, and 8.7% (vol/vol) glycerol (trace a) and of BV-AlDH after oxidation with air (trace b).

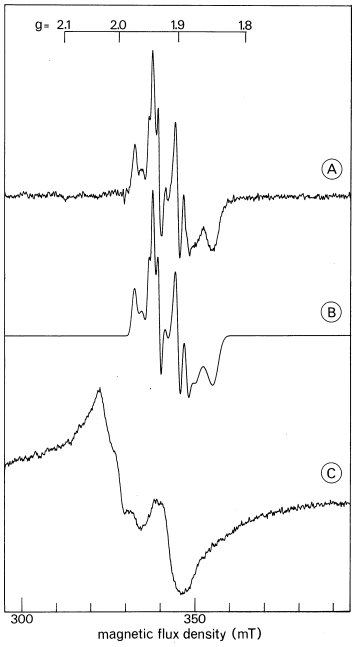


FIG. 5. Electron paramagnetic resonance spectra of tungsten and iron-sulfur in BV-AlDH. Trace A is W(V) from 28 mg · $ml^{-1}$ protein in 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM dithionite, 2 mM DTT, and 8.7% glycerol. EPR conditions: microwave frequency, 9,184 MHz; modulation frequency, 100 kHz; microwave power, 2 mW; modulation amplitude, 0.32 mT; temperature, 55 K. Trace B is a computer simulation of trace A based on the parameters given in Table 3. The simulation was based on 40 × 20 molecular orientations. Trace C is the Fe/S signal from protein that was diluted 1:4 anaerobically in a solution of 5 mM dithionite in 200 mM 2-*N*-cyclohexylaminoethanesulfonic acid buffer, pH 9.5. EPR conditions: microwave power, 3.2 mW; modulation amplitude, 1.0 mT; temperature, 15 K.

**EPR data.** In the partly reduced enzyme as isolated, tungsten was readily detected by EPR spectroscopy (Fig. 5, trace A). The lack of lifetime broadening at 55 K indicates a relatively slow spin-lattice relaxation, and this, together with the observation that all *g* values were less than 2.00, is consistent with the $5d^1$ configuration of W(V). Remarkably, the signal is quite complex, and this is indicative of some form of inhomogeneity at the tungsten side of the enzyme. A simulation of the spectrum as a sum of S = 1/2 components, assuming *g*-strain broadening colinear with the *g* tensor (see reference 12), required at least four different components. The simulation is shown in Fig. 5 (trace B), and the simulation parameters are given in Table 3. The nature of the inhomogeneity is presently unknown.

Reduction with sodium dithionite at pH 7.5 resulted in the virtual disappearance of the tungsten signal, which indicates that the reduction potential for the W(V)/W(IV) couple is $\geq -0.4$ V. Concomitantly, a weak, broad, complex signal was detected at 15 K, but not at 55 K. Upon additional incubation with dithionite at pH 9.5, this signal further increases by a factor of 4.3 to approximately 0.2 spin per 62-kDa subunit. Thus, the signal appears to be associated with a reduction potential of $< -0.5$ V. The spectrum has approximate apparent *g* values of 2.04 and 1.92 (cf. Fig. 5, trace C), indicative of reduced [2Fe-2S] or [4Fe-4S] clusters. The broadening of the signal beyond detection at 55 K argues against [2Fe-2S]. The shape of the spectrum, especially the broad wings at low and high fields, suggests magnetic dipolar interaction. The observation that the shape is invariant to the extent of reduction (pH 7.5 versus pH 9.5) indicates that the magnetic interaction is not between two [4Fe-4S] clusters, as, e.g., is found in bacterial 8Fe ferredoxins, because unbroadened spectra of individual, noninteracting clusters are not detected upon partial reduction. Therefore, the spectrum must be from an Fe/S cluster interacting with a different paramagnet that does not change redox state during the reduction of the Fe/S cluster. A possible candidate for the second paramagnet would be the $5d^2$, S = 1 system from W(IV).

TABLE 3. Simulation parameters for the W(V) EPR signal of the aldehyde dehydrogenase from *D. gigas*

| Component no. | $g_x$ | $g_y$ | $g_z$ | $W_x$[a] | $W_y$ | $W_z$ | Ratio |
|---|---|---|---|---|---|---|---|
| 1 | 1.850 | 1.902 | 1.943 | 9.0 | 3.7 | 3.0 | 1.00 |
| 2 | 1.869 | 1.932 | 1.974 | 11.5 | 2.4 | 4.8 | 0.63 |
| 3 | 1.844 | 1.889 | 1.949 | 7.5 | 3.6 | 2.8 | 0.59 |
| 4 | 1.874 | 1.921 | 1.962 | 5.6 | 4.5 | 5.0 | 0.25 |

[a] The line widths are the diagonal elements of a *g*-strain tensor colinear with the *g* tensor; they are expressed as *g*-value units times $10^{-3}$ (see reference 12 for details).

TABLE 4. Comparison of aldehyde oxidoreductases of *D. gigas*

| Characteristic of: | |
|---|---|
| BV-AlDH[a] | MOP[b] |
| Oxygen-sensitive | Not oxygen-sensitive |
| Active with BV | Active with DCPIP |
| Subunit $M_r$, 62,000; dimer | Subunit $M_r$, 100,000; dimer |
| Molybdopterin present | Molybdopterin present |
| W present | Molybdenum present |
| No effect of 50 mM MeOH[c] | Inhibitory effect of 50 mM MeOH |
| No inhibition by 5 mM acetaldehyde | Strong inhibition by 5 mM acetaldehyde |
| 1 [4Fe-4S] cluster per subunit | 2 [2Fe-2S] clusters per subunit |

[a] As determined in study.
[b] As determined in references 1, 25, and 31.
[c] MeOH, methanol.

## DISCUSSION

The BV-AlDH is clearly different from the other aldehyde oxidoreductase found in *D. gigas*, the MOP, with respect to catalytic and physical data and N-terminal amino acid sequence (cf. references 1, 25, 30, and 31; Table 4). We now have definite proof that the BV-AlDH activity first detected by Kremer et al. (17) is not due to the MOP; the latter enzyme is only active with anionic electron acceptors such as DCPIP (1) and uses flavodoxin as the natural electron acceptor, whereas BV was the best electron acceptor for our purified enzyme. One of the questions that remain concerns the nature of the natural electron acceptor of BV-AlDH. *D. gigas* contains at least two low-potential redox carriers that might function as natural electron acceptors, namely, ferredoxin and flavodoxin (9). Furthermore, flavoredoxin ($E^{\circ\prime} = -348$ mV [7]) also deserves further study as a potential acceptor. *D. gigas* is not the only organism with both a W-containing aldehyde oxidoreductase and a molybdenum-containing aldehyde oxidoreductase. This is also true for *Clostridium formicoaceticum* (15, 32, 33).

Whether there is another specific metabolic role of either of the coenzyme A-independent aldehyde oxidoreductases is not known. The fermentative production of ethanol from storage polyglucose by the strain indicates that the cells can carry out the reduction of acetate to acetaldehyde and thus can re-

verse the acetaldehyde dehydrogenase reaction; alternatively, one must assume the presence of a different pathway. The molybdenum-containing aldehyde oxidoreductase of *C. formicoaceticum* and the tungsten-containing aldehyde oxidoreductases of *Clostridium thermoaceticum* and *Pyrococcus furiosus* have been shown to catalyze the reduction of acetate to acetaldehyde (5, 32–34).

Tungsten is not a very common element in active enzymes; it has only been found in some formylmethanofuran dehydrogenases of thermophilic methanogens (2, 27), in a few formate dehydrogenases from acetogenic *Clostridium* strains (36), in aldehyde oxidoreductases from the same strains (34) and from hyperthermophilic archaea (19, 20), and in a glyceraldehyde-3-phosphate ferredoxin oxidoreductase from *P. furiosus* (21). All of these enzymes have a molybdopterin cofactor (16, 23). The aldehyde oxidoreductases all are able to reduce viologen dyes and are oxygen labile. Recently, N-terminal sequence homology between the aldehyde oxidoreductase from *P. furiosus*, the formaldehyde oxidoreductase from *Thermococcus litoralis*, and the carboxylic acid reductases from *C. thermoaceticum* and *C. formicoaceticum* was detected (20). Despite the similar characteristics of *D. gigas* BV-AlDH, the first example of a tungsten-containing protein from a gram-negative bacterium, such a sequence homology was not found with this enzyme. Complete sequences of the genes coding for the enzymes are required for a proper comparison. They were reported to be under way for the thermophilic archaea (5); the sequence of the *P. furiosus* aldehyde oxidoreductase gene has been deposited (accession no., X79777). The MOP gene sequence has been published (30).

The tungsten is readily detected in the as-isolated *D. gigas* BV-AlDH by EPR as the S = 1/2 system from W(V) with all *g* values below the free electron value of 2.00, as is expected for a $d^1$ configuration. This observation is remarkable in view of previously reported data on naturally occurring tungsten enzymes. Highly unusual *g* values were reported for W(V) in *C. thermoaceticum* formate dehydrogenase ($g = 2.10$ [8]) and W(V) in *Methanobacterium wolfei* formylmethanofuran dehydrogenase ($g = 2.05$ [28]). It is also interesting that the tungsten in *P. furiosus* aldehyde oxidoreductase has been reported to be of extremely low potential, the W(VI)/W(V) couple having a reduction potential of $< -500$ mV (11), while we find for the *D. gigas* aldehyde dehydrogenase that the W(V)/W(IV) couple has an $E_{m,7.5}$ of $> -400$ mV. An unusual aspect of the signal reported here is its apparent inhomogeneity: four different S = 1/2 signals in nonstoichiometric ratios are required to reproduce the signal by simulation, although each individual component forms a normal W(V) signal. The significance of this inhomogeneity is not known. Note that a similar inhomogeneity in the formylmethanofuran dehydrogenase from *M. wolfei* has been assigned to an unexpectedly large hyperfine splitting from $^{183}$W hyperfine interaction (28). Further studies are required to elucidate the nature of these inhomogeneities.

A single [4Fe-4S]$^{(2+;1+)}$ cluster per 62-kDa subunit is consistent with the EPR data and roughly in agreement with the analytical data. Intra- or intermolecular magnetic interaction between similar Fe/S clusters is excluded because the EPR spectral shape is independent of the degree of reduction. It is possible that the broad wings of the Fe/S EPR spectrum reflect interaction with W(IV) which is an S = 1 system. This would imply a distance on the order of 1 nm between the tungsten and the Fe/S cluster. Such a distance between the Fe/S cluster and the W site was also reported for the *Pyrococcus* aldehyde oxidoreductase (5).

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