Aldehyde Oxidoreductase Activity in *Desulfovibrio gigas: In Vitro* Reconstitution of an Electron-Transfer Chain from Aldehydes to the Production of Molecular Hydrogen†

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ABSTRACT: The molybdenum[iron–sulfur] protein, first isolated from *Desulfovibrio gigas* by Moura *et al.* [Moura, J. J. G., Xavier, A. V., Bruschi, M., LeGall, J., Hall, D. O., & Cammack, R. (1976) *Biochem. Biophys. Res. Commun. 72,* 782–789], was later shown to mediate the electronic flow from salicylaldehyde to a suitable electron acceptor, 2,6-dichlorophenolindophenol (DCPIP) [Turner, N., Barata, B., Bray, R. C., Deistung, J., LeGall J., & Moura, J. J. G. (1987) *Biochem. J. 243,* 755–761]. The DCPIP-dependent aldehyde oxidoreductase activity was studied in detail using a wide range of aldehydes and analogues. Steady-state kinetic analysis (K_M and V_{max}) was performed for acetaldehyde, propionaldehyde, benzaldehyde, and salicylaldehyde in excess DCPIP concentration, and a simple Michaelis–Menten model was shown to be applicable as a first kinetic approach. Xanthine, purine, allopurinol, and N^1-methylnicotinamide (NMN) could not be utilized as enzyme substrates. DCPIP and ferricyanide were shown to be capable of cycling the electronic flow, whereas other cation and anion dyes [O_2 and NAD(P)$^+$] were not active in this process. The enzyme showed an optimal pH activity profile around 7.8. This molybdenum hydroxylase was shown to be part of an electron-transfer chain comprising four different soluble proteins from *D. gigas*, with a total of 11 discrete redox centers, which is capable of linking the oxidation of aldehydes to the reduction of protons.

The molybdenum[iron–sulfur] protein isolated from *Desulfovibrio gigas* NCIB 9332, a sulfate-reducing organism, can be classified as a molybdenum hydroxylase that does not contain a flavin moiety (Moura *et al.,* (1978). The visible absorption spectrum of the protein is similar to those observed for the deflavo forms of xanthine and aldehyde oxidases (Moura *et al.,* 1978; Bray, 1975, 1980; Komai *et al.,* 1969; Rajagopalan, & Handler, 1964). Mössbauer and X- and Q-band EPR spectroscopic studies (Barata *et al.,* 1992; Bray *et al.,* 1991), completing previous CD and EPR[1] data (Moura *et al.,* 1976, 1978), revealed a [2Fe–2S] arrangement of the iron–sulfur cores. The molybdenum(V) "resting" and "slow" types of EPR signals (Moura *et al.,* 1978) and the EXAFS spectrum of the molybdenum center indicate that the predominant metal environment had close similarity to that found in desulfoxanthine oxidase (inactive) (Cramer *et al.,* 1984). A Mo(V) EPR signal (rapid type 2) centered at $g_{av} = 1.9750$, analogous to those observed for xanthine and aldehyde oxidases (Turner *et al.,* 1987), could be generated. The "rapid" EPR signals are physiologically significant, as they develop within the enzyme turnover time scale. These signals were obtained not only after a short-time chemical reduction (with dithionite) but also in the presence of salicylaldehyde (Turner *et al.,* 1987), suggesting that other substrates might also produce them. They were visualized after spectral subtraction, since they were obtained in a complex mixture of different Mo(V) EPR-active species. A molybdenum cofactor liberated from the protein was active in the nit-1 *Neurospora crassa* nitrate reductase assay. In view of this aldehyde–DCPIP oxidoreductase activity, it was then proposed that the protein was a form of aldehyde oxidase or dehydrogenase (Turner *et al.,* 1987).

These observations led us to consider in detail the catalytic properties of the *D. gigas* molybdenum[iron–sulfur] protein, particularly its apparent kinetic parameters (K_M and V_{max}) toward different aldehydes and other substrates typically utilized by the molybdenum hydroxylase enzyme group.

Sulfate-reducing bacteria belonging to the genus *Desulfovibrio* contain an extremely complex array of electron-transfer proteins, which have been thoroughly investigated individually. However, information is still missing concerning the way they specifically operate in a complex electron-transfer chain. We want to report here the reconstitution, piece by piece, of such a chain, the elements of which can be found in the cytoplasmic fraction of sulfate-reducing bacteria. The reconstituted electron-transfer chain is capable of producing molecular hydrogen from the oxidation of aldehydes using four components: the aldehyde oxidoreductase, flavodoxin, multiheme cytochromes, and hydrogenase. It is interesting to note that some sulfate-reducing organisms can use aldehydes as energy sources (Widdel, 1988; Fauque *et al.,* 1991).

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[1] Abbreviations: cyt *c*, horse heart cytochrome *c*; cyt c_3 (13 kDa), periplasmic tetraheme cytochrome; cyt c_3 (26 kDa), cytoplasmic octaheme cytochrome; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; HPLC, high-pressure liquid chromatography; HTP, hydroxylapatite; NMN, N^1-methylnicotinamide; NTB, nitro blue tetrazolium; PMS, phenazine methosulfate; TNBS, trinitrobenzenesulfonate.

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Table I: Purification of the Aldehyde Oxidase Activity in *D. gigas*[a]

purification step	total protein (mg)	specific activity[b] (units/mg)	total units	yield (%)	recovery
first	5744.3	0.04	231.4	100	N/A
DEAE-52	2314.8	0.10	230.3	99.5	2.47
DEAE-52	633.4	0.18	113.9	49.2	4.46
DEAE-Biogel	210.5	0.52	114.1	47.2	12.9
HTP	118.1	0.54	63.9	27.6	13.4
HPLC	53.1	1.17	62.0	26.8	29.0

[a] From 1500 g of wet cells. [b] 1 unit = 1 μmol of DCPIP reduced/min. $[DCPIP]_{final}$ = 35 μM, [benzaldehyde] = 100 μM.

Recently, the *in vitro* reconstitution of the electron-transfer chain from molecular hydrogen to sulfite, one of the bacteria respiratory substrates, was demonstrated (Chen *et al.*, 1993). These results, together with the ones reported here, enable us to define a complete electron-transfer scheme that is compatible with the hydrogen cycling hypothesis, as proposed by Odom and Peck (1981).

MATERIALS AND METHODS

Organism and Protein Purification. D. gigas NCIB 9332 cells were grown as indicated by LeGall *et al.* (1965) using a lactate–sulfate medium. A cell-free extract (crude extract), prepared as previously described, was obtained after cell disruption (using a French–Gaulin homogenizer) and centrifugation at 10 000 rpm for 30 min. This fraction was kept under argon until further utilization.

The molybdenum enzyme was purified from this cell-free crude extract using identical chromatographic methods, as previously described (Moura *et al.*, 1978), but additional HPLC chromatographic steps were introduced in order to refine the protein fraction in the last purification steps. After the dialyzed fraction was reduced to a minimum volume through pressure filtration and centrifugation (Amicon membranes and Centricon 30 microconcentrator), the protein solution was injected several times, in portions of 100 mg each (100 μL), into an HPLC-LKB chromatographic system using a DEAE (UltroPac TSK DEAE-3SW) column (21.5 × 150 mm) eluted with a gradient of microfiltered Tris-HCl buffer, pH 8.0, at a flow rate of 2 mL/min. Combined fractions provided 53 mg of purified enzyme, immediatly beaded into liquid nitrogen, from 1500 g (wet weight) of initial bacterial wet cells. All operations were carried out at 4 °C. The purification chart is shown in Table I.

The other *D. gigas* pure proteins [flavodoxin, tetra- and octaheme cytochromes c_3 (13 and 26 kDa, respectively), and hydrogenase] were purified as described in the literature (Moura *et al.*, 1978; Hatchikian *et al.*, 1969,1972; LeGall *et al.*, 1982).

Enzyme Assay. Protein determinations were performed using the Lowry method with the Folin reagent and with bovine serum albumin (Sigma) as a standard (Lowry *et al.*, 1951). Activities were determined aerobically at 25 °C by measuring the rate of DCPIP (2,6-dichlorophenol-indophenol) reduction at 600 nm (ϵ = 21 mM^{-1} cm^{-1}) in a 1 cm optical path length spectrophotometer quartz cell containing the following assay mixture, 100 mM Tris-HCl buffer (pH 7.6), 40 μM DCPIP, and 100 μM aldehyde, into which was added 20 μL of enzyme solution. Under these experimental conditions, 1 enzymatic unit corresponds to 1 μmol of DCPIP reduced per minute, and the specific activity is the number of units/mg of enzyme. For the Michaelis–Menten parameter determinations, the added volumes of the aldehydes (acetaldehyde, propionaldehyde, benzaldehyde, and salicylaldehyde) from different stock solutions were varied concomitantly with the buffer volume, so that the final value would always be 1 mL. The proportion, dye to protein, was kept constant. In all assays, the substrate was the last component to be mixed after a 2.5-min equilibration of the protein with the electron acceptor. Aldehydes were of the purest grade, redistilled when necessary, and were kept in dried ampules at −20 °C, and stock solutions were prepared just before use in the activity assay measurements. A pH profile of the enzyme activity was made in different buffer systems prepared at the same ionic strength (200 mM). These solutions were used to cover a pH range from 4 to 11 and to follow the relative initial rate activity of the enzyme using benzaldehyde as a substrate. The buffer systems were citrate, citrate/phosphate, phosphate, Tris-HCl, Tris/glycine, and glycine/NaOH. Overlapping pH ranges were made with these buffers; no significant difference in activity was found at the same pH that could be attributed to the nature of the buffer solution.

Reconstitution Experiments. Two types of experiments were performed in order to test the electron-transfer pathway from aldehyde to molecular hydrogen using cytoplasmic electron carriers: (i) spectrophotometric measurements were conducted in anaerobic cells (total volume 1 mL) in order to assess the extent of reduction of the electron carriers under different conditions; (ii) hydrogen evolution was determined by injecting appropriate volumes of the gas phase into a Varian 4600 gas chromatograph equiped with a 5-Å molecular sieve column maintained at 105 °C (6 ft, $^1/_8$ in., 45/60 μm). All experiments were conducted at 25 °C with 0.1 M Tris-HCl buffer (pH 7.6). Successive vacuum/argon cycles were made prior to the measurements, and all of the additions were made through gas-tight Hamilton syringes.

The following systems were tested (all concentrations indicated are final). *Experiment 1:* Flavodoxin (10 μM) and benzaldehyde (200 μM) were mixed. After a 10-min equilibration, the aldehyde oxidoreductase (20 μM) was added and the spectral alteration detected. *Experiment 2*: Flavodoxin (10 μM), periplasmic cytochrome c_3 (13 kDa) (2.4 μM) and benzaldehyde (200 μM) were mixed, and the evolution of the system was followed for a period of 10 min. Then, the aldehyde oxidoreductase (20 μM) was added and the time course of the spectral alterations measured. *Experiment 3*: An experiment similar to that described for experiment 2 was conducted replacing the cytochrome c_3 (13 kDa) (2.4 μM) with the cytochrome c_3 (26 kDa) (1.2 μM). *Experiment 4:* Hydrogen evolution was measured in 10-mL anaerobic flasks containing the following mixture in a total volume of 3 mL: 25 μM aldehyde oxidoreductase, 27 μM flavodoxin, 9 μM cytochrome c_3 (13 kDa) or 4.3 μM cytochrome c_3 (26 kDa), and 7.8 μM hydrogenase. The reaction was initiated by the addition of aldehyde to a final concentration of 250 or 500 μM (40 or 80 μL of a stock solution of aldehyde was added, respectively).

A set of parallel experiments was performed following the state of reduction of the different components by EPR, particularly, the rapid Mo(V) signal was generated on the aldehyde oxidoreductase in the presence of aldehyde, the formation of the semiquinone state of flavodoxin (in the presence and absence of the cytochromes), and the extention of reduction of cytochromes. The measurements were conducted on a Bruker ESP 300 EPR spectrometer equipped with an Oxford helium-transfer line.

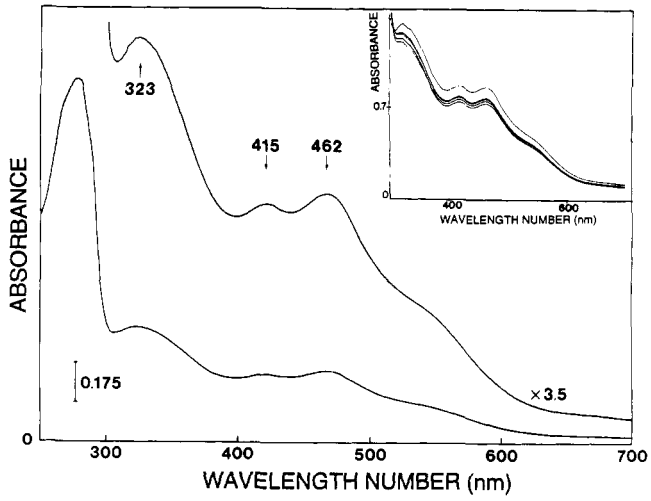


FIGURE 1: Absorption spectrum of the *D. gigas* Mo[Fe–S] protein after the final HPLC purification step. The protein (10 μM) was measured in 20 mM Tris-HCl, pH 7.8. The absorbance ratios at different wavelengths are as follows: $A_{280}/A_{323} = 3.17$; $A_{280}/A_{415} = 5.25$; $A_{280}/A_{462} = 5.05$; $A_{323}/A_{462} = 1.59$; $A_{462}/A_{550} = 1.97$. The inset indicates the extent of reduction of the [Fe–S] centers when reacted with benzaldehyde.

RESULTS

Purification of the Enzyme: Correlation with Aldehyde Oxidoreductase Activity. The purification procedure closely followed the one previously reported (Moura *et al.*, 1976,1978). Additional HPLC steps were introduced to improve the separation and recovery of secondary fractions from the DEAE-52 and HTP columns. The final enzyme preparation appeared to be homogeneous as revealed by the presence of a single electrophoretic band. From Table I, it is clear that the enzyme can be purified from the cell-free extract in large amounts with a high yield.

Typically, the enzyme fraction obtained after the first Biogel column (this activity is detected close to the fractions containing hydrogenase, APS reductase, and sulfite reductase) coeluted with cytochrome and flavin components. The cytochrome contamination was removed by an HTP column step and the flavin contamination by a second DEAE or Biogel column step. The final HPLC step allowed the effective separation of flavoprotein components and maximal expression of DCPIP-dependent aldehyde oxidoreductase activity. Figure 1 shows a typical spectrum of a pure native preparation. The visible spectrum previously described in Moura *et al.* (1976) showed a slightly different A_{280}/A_{322} ratio. Extinction coefficient ratios at characteristic wavelengths are indicated in the caption to Figure 1. The effect of the anaerobic addition of benzaldehyde is indicated in the inset. Aproximately 8% (after 1 min of reaction) and 11% (after 1 h) decreases in absorption were measured at 462 nm when the spectra were compared with the maximal bleaching obtained with dithionite.

Stability and Storage of the Enzyme. Invariant spectral features and higher activity were always found on samples that were kept at low temperatures (–30 °C) or after concentration and storage as beads in liquid nitrogen. Buffering systems were changed when necessary, to prevent damage through pH effects on freezing. Samples stored in phosphate buffer were fully active after they were thawed. Cooled, diluted samples (kept for 24 h) always showed an increase in the 415-nm spectral feature, inverting the absorption ratio between the 415- and 462-nm peaks, whereas the absorption at 323 nm was also blurred. Activity was lower after these diluted samples were thawed. Also, excessive protein concentration induced the appearance of a precipitate or slurry-like material.

Table II: Natural and Artificial Electron Acceptors Tested

electron acceptor[a]	E (mV)[b]	result (%)
DCPIP	+217	100
ferricyanide	+430	156
cytochrome *c* (horse heart)	+260	25
cytochrome c_3 (*D. gigas*)	–270[c]	20[d]
phenazine methosulfate	+40	0
methylene blue	+11	63
1,2-naphthoquinone 4-sulfonic acid	+215	0
1,2-naphthoquinone	+143	0
1,4-naphthoquinone	+60	0
5-hydroxy-1,4-naphthoquinone	+30	0

[a] [electron acceptor] = 40 μM, [benzaldehyde] = 100 μM, [Mo protein] = 0.45 μM, and 20 mM Tris buffer, pH 7.8. [b] Redox potential activity at pH 7.0. [c] The indicated number is a mean value for the four hemes. [d] Anaerobic assay (% of initial rate measurements).

Enzyme Activity and Kinetic Parameters. The exact conditions for the assays have been described in the Materials and Methods section. The specific activity of the pure enzyme was determined to be 1.17 units, using benzaldehyde as substrate and DCPIP as electron acceptor. This value is relatively constant for the different purified protein batches (10% variation). The efficient turnover of the substrate used, benzaldehyde, an aldehyde almost completely devoid of inhibitory hydrated forms, confirms that the unhydrated form is the true species reacting in the active center. Reproducible results were obtained using freshly diluted protein, previously beaded and stored in liquid nitrogen, following the activity assay during the same day and keeping the sample at 0 °C. Anaerobicity had no effect on the progression curve profiles nor on the rate determinations. Table II lists the electron acceptors tested under the previously described assay conditions (see Materials and Methods) using various aldehydes. They were selected according to their plausible reactivity either with the molybdenum or the iron–sulfur centers and/or the matching of redox potential involved (Palmer & Olson, 1980; Coughland, 1980). The anionic dyes (ferricyanide and DCPIP) were effective electron acceptors.

The time progression curves of the assay described in Materials and Methods, *i.e.*, by monitoring the DCPIP absorption decay at 600 nm in the presence of aldehyde, can be followed for a long period of time when the substrate is not limiting. Curves can be obtained 2–3 s after the final addition of aldehyde and rapid mixture by inversion of the optical cuvette. From the initial portion of the plot, the initial velocity was recorded. Since linearity of this activity with enzyme concentration was observed, the DCPIP concentration was varied and a maximal activity was found at 35 μM. While the DCPIP/aldehyde concentration ratio was varied and the aldehyde and enzyme concentrations were kept constant, the activity was followed until no further absorption variation was detected (usually 15 min).

These final absorption values were compared with the one corresponding to the full disappearance of oxidized DCPIP. This "completion ratio" was determined in order to verify the stoichiometry of the reaction between the enzyme and the electron acceptor. A titration curve was obtained which indicated that, up to a 1:1 ratio, full completion was achieved (Gutroo & Johns, 1971). The various initial rates for different aldehydes at variable substrate concentrations were determined. The experimental data were then fit using a simple Michaelis–Menten model. The kinetic parameters obtained are presented in Table IIIA for different aldehydes.

Table III: Apparent Kinetic Parameters of the Enzyme

A. For Different Reducing Substrates Using DCPIP As an Electron Acceptor[a]

aldehyde	K_m (μM)	V_{max} (μM min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	[IS][b] (μM)
acet-	13	31	1.14	7.0	500
propion-	15	12	0.44	1.8	250
benz-	52	39	1.43	1.7	1000
salicyl-	0.25	4.0	0.15	36	500

B. For Different Levels of DCPIP[c]

DCPIP (μM)	K_m (μM)	V_{max} (μM min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
16.5	42	114	4.2	6
35.0	52	39	1.43	1.7
47.0	31	35	1.59	3.1
76.0	19	31	1.11	3.5

[a] Other experimental conditions: [Mo(Fe–S)] = 450 nM, [DCPIP] = 35 μM, and [Tris] = 20 mM, pH 7.8. [b] [IS] is the typical inhibitory substrate concentration. [c] For benzaldehyde as reducing substrate.

These values are apparent parameters, valid for a DCPIP concentration of 35 μM. Extension of this analysis for a range of DCPIP values, using benzaldehyde as the reducing substrate, is presented in Table IIIB. CoA, Mg^{2+}, K^+, and $NAD(P)^+$ were not activators as far as initial rates are concerned, and the presence of important amounts of benzoate in the reaction mixture did not alter the rates or kinetic profiles of the standard enzymatic assay. The pH dependence of the activity was determined, and a curve was constructed using initial velocities as a function of pH. It has a bell shape centered at pH 7.8 without a large plateau and is independent of the nature of the buffer system used, as was also previously observed for xanthine oxidase (Coughland, 1980).

High concentrations of all of the reducing substrates inhibited the reaction. Depending on the aldehyde considered, this inhibition started at very different values, reflecting the K_M variation of the enzyme with substrate (Table IIIA) and also the extension of hydration of the different aldehydes. It is important to emphasize that the inhibitory behavior is fully developed at concentrations where xanthine and aldehyde oxidase are still expressing full uninhibited activity (Morphet, 1983). This fact can help us to accommodate and to understand the results shown in Table IV, which compares the reactivity of the *D. gigas* molybdenum[iron–sulfur] protein at approximately optimal substrate concentrations for xanthine oxidase (toward xanthine). This table gives evidence for the difference in specificity of these enzymes. Alcohols are inhibitors of the aldehyde conversion reactions (Bray, 1975, 1980); in particular, methanol was shown to be a competitive inhibitor in Mo hydroxylases for the aldehyde reactions (Coughland, 1980; Booth, 1938). The effect of methanol on *D. gigas* aldehyde oxidoreductase activity was studied in two ways: (i) the equilibration of *D. gigas* enzyme with different levels of methanol and (ii) the direct effect of the addition of methanol on the profile of DCPIP reduction in the presence of acetaldehyde. The effect of cyanide on the enzymatic activity was also measured in a similar way and is shown in parallel with the effect of methanol. The inhibitor effect of these two chemicals is presented in Figure 2 (panels A–C).

The best estimate of the functional performance of the enzyme is the detection of "rapid-type" signals generated in the presence of substrates. Three of these signals, detected after rapid anaerobic reaction of the *D. gigas* enzyme with benzaldehyde, salicylaldehyde, and acetaldehyde, are shown in Figure 3. The salicylaldehyde-generated, as well as the

Table IV: Specificity Comparison between Mo[Fe–S] Protein from *D. gigas* and Xanthine Oxidase[a]

	D. gigas protein		xanthine oxidase	
	A	B	A	B
acetaldehyde	100	59	21	24
propionaldehyde	35	32	10	19
glyceraldehyde	17	nd[b]	nd	nd
benzaldehyde	92	94	11	14
salicylaldehyde	19	8	7	44
furfuraldehyde	27	40	nd	nd
3-CBP[c]	159	73	11	27
2-CBP	0.2	25	13	57
NMN	0	0	0.1	9
xanthine	0.5	2	103	95
hipoxanthine	0	1	52	52
1-methylxanthine	0	0	170	150
allopurinol	0	0	120	182
purine	0	0	55	50
imidazole	0	0	3	4
pyrimidine	0	0	2	1
adenine	0	0	0.2	2
cafeine	0	0	0.1	2

[a] Assay conditions: [enzyme] = 450 nM (activity to flavin ratio = 125 for xanthine oxidase (Hart *et al.*, 1970)), [substrate] = 1 mM (A) and 100 μM (B), [DCPIP] = 35 μM, and [Tris-HCl] = 20 mM, pH 7.8; T = 25 °C. The initial rate of acetaldehyde/DCPIP activity was taken as 100 and all of the other rates are relative to this value. [b] nd, not determined. [c] CBP, pyridinecarboxaldehyde.

dithionite-generated, EPR-active species had previously been reported, which allowed us to place this enzyme in the group of the molybdenum hydroxylases (Turner *et al.*, 1987). All of the signals have identical parameters (see figure caption), and a spectral simulation is also added at the bottom of the figure.

Construction of an in Vitro Electron-Transfer Chain. Since it has been demonstrated that aldehydes are competent metabolites for sulfate reducing bacteria (Widdel, 1988; Fauque *et al.*, 1991), the definition of the electron acceptor(s) of aldehyde hydroxylase is important. As the redox potential of the pair aldehyde/carboxylate is very low, it was logical to determine whether the reduced aldehyde hydroxylase could transfer electrons to hydrogenase. Addition of benzaldehyde to a mixture of aldehyde oxidoreductase and hydrogenase did not induce the formation of molecular hydrogen, showing the necessity for other electron carrier proteins

The same reaction mixture in which tetraheme cytochrome c_3 was replacing hydrogenase showed a small (about 20%) reduction of the cytochrome (see Table II). It is to be noted that, in contrast to a previous report (Kremer *et al.*, 1988), the cytochromes present in the anaerobically dialyzed (against 10 mM Tris-HCl buffer, pH 7.6) crude extract were reduced after the addition of benzaldehyde. Flavodoxin was reduced to the level of semiquinone (90–100%) in the presence of benzaldehyde and aldehyde oxido-reductase Figure 4. After addition to such a system, both tetra- and octaheme cytochromes c_3 were now fully reduced Figure 5and flavodoxin was reduced to the hydroquinone level, as shown by the disappearance of both the fully oxidized (at 456 nm) and the semiquinone absorption peaks (at 590 nm) (see also the next EPR section). If aldehyde oxidoreductase and then benzaldehyde were added to a system containing both cytochrome and flavodoxin, the latter was reduced without the formation of the semiquinone form. A striking difference was found during the time course reduction of octaheme cytochrome when compared with the tetraheme protein: the α-band of the octaheme protein shifted about 4–5 nm, while there was practically no noticeable change in the same band in the case

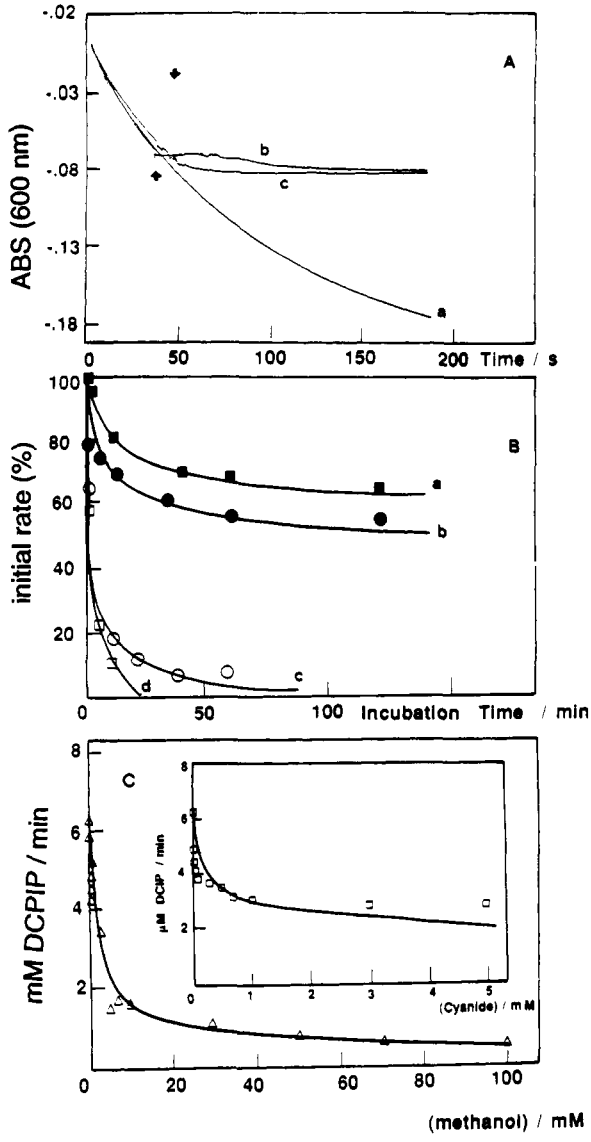


FIGURE 2: Inhibition effect of methanol and cyanide on *D. gigas* aldehyde oxidoredutase acetaldehyde/DCPIP activity in Tris-HCl buffer (200 mM, pH 7.6). (A) Trace a indicates the uninhibited assay. Effect of the addition (arrow) of cyanide (50 mM) and methanol (50 mM) (b and ctraces, respectively) on the time course reduction of DCPIP (35 μM) by acetaldehyde (100 μM) with 100 nM enzyme is shown. (B) Initial rates were also studied as a function of incubation time for two different concentration levels of cyanide and methanol. These were added to 400 μM enzyme, and samples of these mixtures were taken after increasing the incubation time and assayed as in A. Traces correspond to 200 nM enzyme for (a) 50 μM cyanide, (b) 50 μM methanol, (c) 50 mM cyanide, and (d) 50 mM methanol. (C) The protein was incubated with increasing concentrations of methanol (or cyanide; see insert) for 2 min prior to the addition of acetaldehyde, and activities were recorded as previously described. Experimental conditions were the same as in trace a of panel A.

of the tetraheme protein. This could be explained by the fact that the hemes of the octaheme cytochrome are in more unequivalent environments than they are in the tetraheme one.

These results were also confirmed by anaerobic EPR experiments conducted in similar situations. As described previously (Barata *et al.*, 1992; Turner *et al.*, 1987), when benzaldehyde was added to the oxidoreductase, the Mo(V) rapid-type signal was generated (see Figure 3) and a partial reduction of the iron–sulfur centers detected (Figure 6, panel A, spectrum i). After the addition of flavodoxin to the system, the spectrum was dominated by an intense radical due to the semiquinone state (Figure 6, panel A, spectrum ii), while the

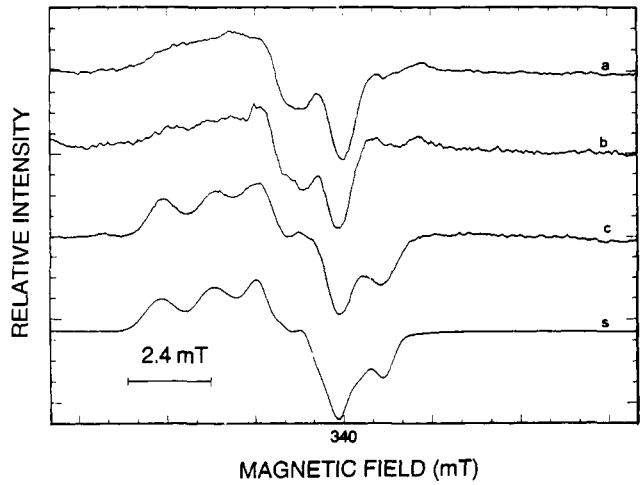


FIGURE 3: Rapid type Mo(V) EPR signals obtained by reacting the enzyme with benzaldehyde (a), salicylaldehyde (b), and acetaldehyde (c). The lower trace is a simulation of the spectral data using the following X-band parameters: g_1 = 1.9895 (0.85, 1.4, 0.35), g_2 = 1.9715 (0.6, 1.3, 0.5), g_3 = 1.9640 (0.6, 0.7, 1.3), and g_{av} = 1.9750. Values in brackets indicate the line widths (2Γ) and the two protons hyperfine splittings (A_i), respectively, in millitesla. Experimental EPR conditions: frequency, 9.381 GHz; modulation amplitude, 1 mT; microwave power, 10 mW; and temperature, 105 K.

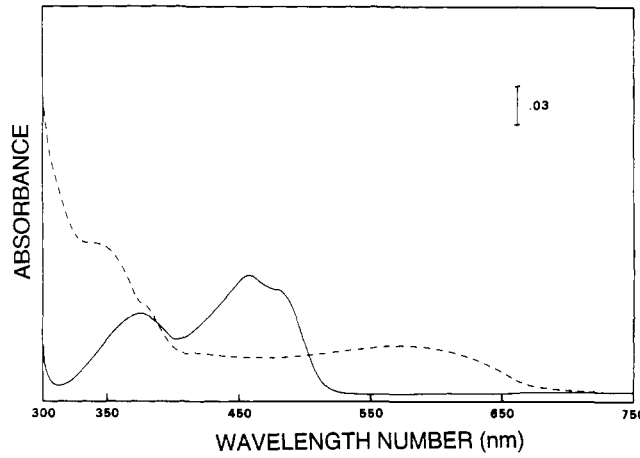


FIGURE 4: Reduction of flavodoxin to the level of semiquinone (90–100%) in the presence of benzaldehyde and aldehyde oxidoreductase. Flavodoxin (10 μM) and benzaldehyde (200 μM) were mixed in 50 mM Tris-HCl buffer (pH 7.8), and after 10 min of equilibration, the aldehyde oxidoreductase (AO) (20 μM) was added and the spectral alteration followed at 590 nm. Electron-transfer scheme: aldehyde → Mo protein → flavodoxin.

reduction level of the aldehyde oxidoreductase iron–sulfur centers remained unchanged. In the same panel, spectrum iii shows the radical species reduced 10-fold. Another experiment using the ternary system aldehyde oxidoreductase, flavodoxin, and cytochrome c_3 (13 kDa) shows that almost quantitative reduction of the heme protein was attained after aldehyde addition. The level of the semiquinone form at this stage was quite low (Figure 6, panel B), when compared with the situation previously described where the heme protein is absent.

Since cytochromes c_3 are considered to be direct electron donors to hydrogenase, it was expected that this system would be fully functional after the addition of the latter enzyme. Indeed, when benzaldehyde was added to a complete system (aldehyde oxidoreductase, flavodoxin, tetra- or octaheme cytochrome c_3, and hydrogenase), all of the proteins were reduced and hydrogen could be detected in the head space of the anaerobic spectrophotometric cuvette. Time course experiments were performed using hydrogen detection by gas

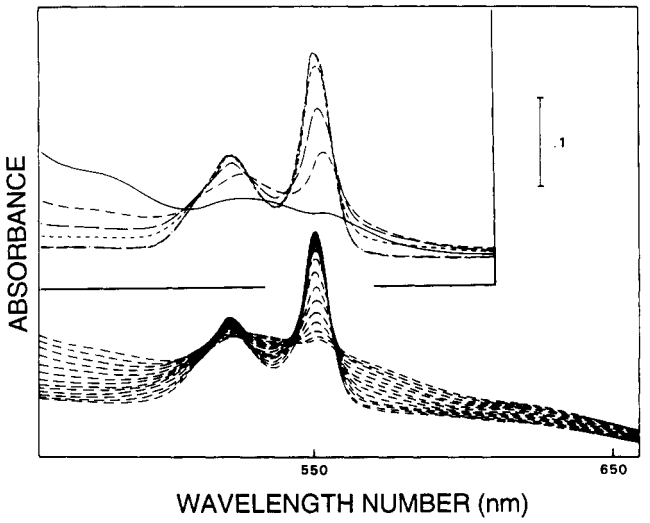


FIGURE 5: Full reduction of both tetra- and octaheme cytochromes c_3 (see inset). Flavodoxin was reduced to the hydroquinone level. The same concentrations were used as those given in the caption to Figure 1 plus 2.4 μM periplasmic cytochrome c_3. The components were mixed previously and the evolution of the system was detected for 10 min. Then, the aldehyde oxidoreductase was added and the time course of the spectral alterations followed. Electron-transfer scheme: aldehyde → Mo protein → flavodoxin → cyt c_3 (13 or 26 kDa).

chromatography, as indicated in Figure 7. The evolution of hydrogen was detected under standard conditions (0.8 μM aldehyde) in the presence of cytochrome c_3 (13 or 26 kDa), and a control without the molybdenum protein was also performed. The rate of hydrogen production was very slow at very low ionic strengths. The ratios of hydrogen produced to the benzaldehyde added were 0.3 (with 1.6 μmol of added aldehyde) and 0.21 (with 0.8 μmol of added aldehyde). This ratio could be explained by thermodynamic reasons since the redox potentials of the two couples (benzaldehyde/benzoic acid and H_2/H^+) are very close (Lang *et al.*, 1961; Thauer *et al.*,1977), and its variation could be explained by the inhibitory effect of benzaldehyde at high concentrations. It is important to stress that no hydrogen was evolved by the system when cytochrome or aldehyde oxidoreductase was absent. Molecular hydrogen was also produced in a similar system after benzaldehyde was replaced by acetaldehyde.

DISCUSSION

A comprehensive comparison of *D. gigas* aldehyde oxidoreductase's physicochemical properties and its kinetic performances with those of the group of hydroxylases is attempted using data obtained through steady-state experiments. Aldehyde and xanthine oxidases were taken as prototypes. Most of the molybdenum hydroxylases exist as dimers displaying an active center composition of one molybdenum per four iron atoms, arranged as two [2Fe–2S] centers, and one FAD per monomer. The existence of a deflavo form, together with naturally occuring demolybdo and desulfo (or inactive) forms in crude preparations, is commonly found during purification (Bray, 1975; Waud *et al.*, 1975).

It was possible to generate EPR data similar to those obtained for xanthine/aldehyde oxidases using either a reducing substrate (aldehyde) or dithionite, namely Mo(V) signals (rapid type 2 and slow) and [iron–sulfur] reduced centers (types I and II) (Moura *et al.*, 1976, 1978; Turner *et al.*, 1987; Barata *et al.*, 1992). The so-called "very rapid" type Mo(V) signal has not yet been obtained, not only because

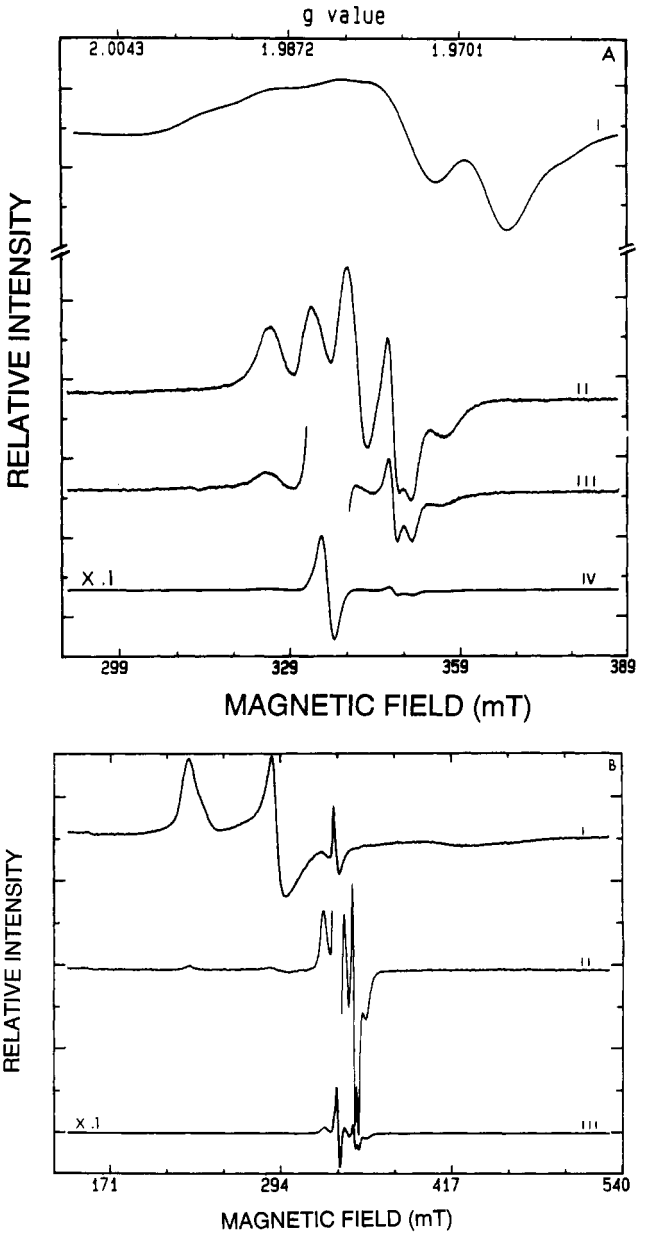


FIGURE 6: Anaerobic EPR experiments conducted in situations similar to those described previously in Figures 4 and 5 (see text for experimental conditions). Final concentrations used: aldehyde oxidoreductase, 163 μM; flavodoxin, 147 μM; cytochrome c_3 (13 kDa), 197 μM; and the reaction was initiated by the addition of 1 mM benzaldehyde. (A) Spectra i and ii:aldehyde oxidoreductase plus aldehyde. Spectra iii and iv: After the addition of flavodoxin to the previous mixture. (B) Spectrum i: cytochrome c_3, flavodoxin, plus aldehyde. Spectra ii and iii: After the anaerobic addition of aldehyde oxidoreductase. Experimental conditions: microwave frequency, 9.42 GHz; gain 1.25×10^4; spectral windows, 0.1 T (panel A, spectra ii–iv) and 0.01 T (spectrum i) centered at 340 mT and 0.4 T (panel B) centered at 210 mT; modulation amplitude, 10 G_{pp} and $T = 12$ K for all spectra, except 1 G_{pp} and $T = 84$ K for spectrum i, panel A.

xanthine or xanthine-like derivatives were not substrates but also because large quantities of the enzyme would be necessary in order to reach the required time scale in the fast flow apparatus (Olson *et al.*, 1974a,b). EPR rapid signals in the presence and in the absence of the electron acceptor are virtually the same and can be attributed to the enzyme–aldehyde complex.

Moura *et al.*(1978) have shown that the redox potentials of the different centers were comparable to the equivalent couples in xanthine/aldehyde oxidases (Palmer & Olson,

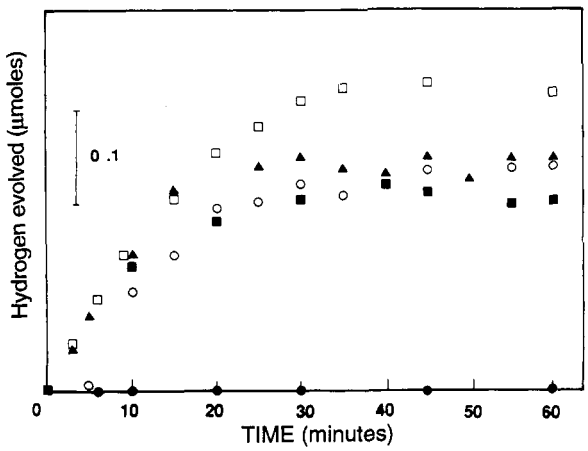


FIGURE 7: Time course experiments for the detection of hydrogen production from aldehydes. The evolution of hydrogen was detected under standard conditions (0.8 μM aldehyde) in the presence of cytochrome c_3 (13 or 26 kDa), and a control without the molybdenum protein was also performed. See text for details on the experimental conditions. Hydrogen evolution was measured in 10-mL anaerobic flasks containing the following mixture in a total volume of 3 mL: 25 μM aldehyde oxidoreductase, 27 μM flavodoxin, 9 μM cytochrome c_3 (13 kDa) or 4.3 μM cytochrome c_3 (26 kDa), and 7.8 μM hydrogenase. The reaction was initiated by the addition of aldehyde to a final concentration of 250 or 500 μM (40 or 80 μL of a stock solution of aldehyde were added, respectively). Electron-transfer scheme: aldehyde → Mo protein → flavodoxin → cyt c_3 (13 or 26 kDa) → hydrogenase → H_2. Symbols: □, complete system, 80 μL of aldehyde; ○, complete system, 40 μL of aldehyde; ▲, complete system, 40 μL of aldehyde but twice the molybdenum protein concentration; ■, complete system, 40 μL of aldehyde using cyt c_3 (26 kDa); ●, control, complete system without molybdenum protein.

1980). The existence of a deflavo form in *D. gigas* enzyme preparations raises questions concerning the identity of the native protein, namely, whether it is a purification artifact or a new kind of molybdenum [iron–sulfur] protein intrinsically devoid of the flavin moiety as a cofactor. As expected, the protein does not use O_2 or NAD(P)$^+$. Addition of flavin to the preparation does not increase the DCPIP aldehyde-dependent activity. This limits the utilization of other natural or artificial electron acceptors that could react at either the Mo or Fe/S center. Unlike other molybdenum hydroxylases, of the possible candidates (DCPIP, Me Blue, TNBS, and PMS) acting on the molybdenum or Fe/S center (ferricyanide, possibly NBT, cytochrome *c*, cytochrome c_3, and quinone derivatives), only DCPIP, ferricyanide, and cytochrome c_3 (partially and under anaerobic conditions) succeeded in accepting electrons (Table II) under the selected conditions.

The analytical data reexamined recently indicated that demolybdo forms were present (Barata *et al.*, 1992) in purified samples. Also, in order to analyze the specific activity data, the question of the percent of functionality was addressed, taking into consideration the specific activity measurements, the intensity of the generated Mo(V) slow signal, the metal analysis, and the proportion of maximum anaerobic bleaching of the visible spectra of the enzyme in the presence of substrates. The values indicated in Table IIIA suggest a maximum of 20% functionality of the enzyme for benzaldehyde, if we assume a specific activity of the order of 5 (Bray, 1975) for 100% functional molybdenum hydroxylases. This number aggrees with the high intensity of the slow signal observed in these preparations.

The activity parameter measurements for the aldehydes tested (see Table IIIA) show remarkable differences in reactivity and affinity of the *D. gigas* enzyme toward those simple substrates. Activity was linear with enzyme concentration (Figure 2) and showed an optimal pH of 7.8 with an optimal DCPIP concentration of 35 μM, representing an excess in relation to the protein concentration and the K_M values.

As the oxidation of aldehydes to acids is a two-electron process, but the Mo(V) rapid signal producing process is a one-electron "image" of the actual Mo(IV) catalytic species (Bray, 1980), we can in principle accept the idea that only one molecule of DCPIP per catalytic (Mo) center is needed during turnover. Note that ferricyanide interacts with the iron–sulfur centers and not with the molybdenum site (Coughland, 1980); its reduction rate, higher than the one observed for DCPIP, is consistent with rapid electron transfer from the molybdenum center to the iron–sulfur centers (Palmer & Olson, 1980). Michaelis–Menten-type behavior could be followed without pronounced substrate inhibition in the benzaldehyde case (Table III). Very low K_M values were obtained when compared with the ones reported for the aldehyde/xanthine oxidase acting on aldehydes (Poels *et al.*, 1987; Massey, 1973; Krenetsky *et al.*, 1972; Morphet, 1983; Booth, 1938; Palmer, 1962a,b). The turnover numbers calculated for the *D. gigas* protein are also definitely lower. As the mechanism of action of DCPIP at the molybdenum center appears to be the same for the deflavo and complete aldehyde/xanthine oxidase proteins (using NMN as the reducing substrate and irrespective of the presence of superoxide dismutase) (Bray, 1980; Branzoli & Massey, 1974), we do not attribute the lower turnover numbers to the lack of flavin, but rather to the presence of both demolybdo and desulfo molecules in our preparations.

An important difference in substrate specificity is also noted when the reactivity of the *D. gigas* enzyme is examined with xanthine oxidase. Almost complementary performances are observed, in spite of the assay conditions not being optimal for the *D. gigas* enzyme (Table IV) (Moura *et al.*, 1976; Turner *et al.*, 1987; Booth, 1938). Similar studies undertaken by several authors show that different molybdenum proteins have different specificities, according to their origin and that of the oxidizing substrate used (Bray, 1980; Moura *et al.*, 1976; Coughland, 1980; Morphet, 1983; Olson *et al.*, 1974a,b; Palmer, 1962a,b); substrate inhibition was recognized early by these authors and was interpreted as deriving from the lower value of the catalytic velocity corresponding to the process of dissociation of the enzyme–product complex (Palmer & Olson, 1980). Our results are similar to those obtained with xanthine oxidase.

Until recently, only two types of NAD(P)$^+$-dependent aldehyde dehydrogenases have been shown to be present in mammalian (Evces, & Lindhal, 1989; Johansson *et al.*, 1988) and bacterial (aerobic or anaerobic) systems utilizing and degrading alcohols (Loomes *et al.*, 1990; Yan & Chen, 1990; Smith & Kaplan, 1980; Jendrossek *et al.*, 1987). These enzymes are dimeric in nature and present no real prosthetic groups. The enzymes isolated in the anaerobic eubacteria have a catalytically active cysteine residue and are capable of coupling the aldehyde oxidation to CoA, an acylating and energy-conserving step. There is also a report on a different kind of NAD$^+$-dependent aldehyde dehydrogenase, possibly tetrameric and with no "real" cofactor, isolated from bacteria and able to utilize the mandelate pathway (Chalmers & Fewson, 1989). Other kinds of NAD(P)$^+$-independent, although heme-containing, bacterial enzymes have been reported before, but the first report on a bacterial, NAD(P)$^+$-independent, molybdenum-containing aldehyde dehydrogenase was made by Poels *et al.* (1987) in *Pseudomonas*

testosteroni. The enzyme was described as a new type of molybdenum hydroxylase, with respect to its cofactor content [one FAD, one molybdenum, four iron atoms per 92 kDa] and the electron acceptors used [cationic dyes were utilized such as Wuster's blue, phenazine methosulfate and thionine, but not anionicones such as DCPIP or ferricyanide nor O_2 or $NAD(P)^+$].

The aldehyde oxidoreductase from *D. gigas* is distinct from these last enzyme groups since it is inhibited in the presence of alcohols. Xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase are molybdenum containing enzyme, with wide substrate specificity, capable of oxidizing purines as well as aldehydes. It is also unique in lacking a constitutive FAD moiety and in containing one molybdenum and four iron atoms arranged as two [2Fe–2S] clusters. As for other molybdenum-containing enzymes, methanol inactivaction is reversible, progressive, and probably turnover-dependent, and the site of attack is the active molybdenum center. In these proteins, EPR studies have also shown the development of a distinct signal from the Mo(V) state, the "inhibited signal".

D. gigas is the only sulfate reducer which has been found, so far, to contain a single gene encoding system for an [Ni–Fe] hydrogenase (Li *et al.*, 1987). Studies conducted using spheroplasts have shown the presence of hydrogenase activity in both periplasmic and cytoplasmic fractions [Bell *et al.*, 1979; Odom & Peck, 1981b; Fauque *et al.*, 1988; Fourest *et al.*, unpublished results in Fauque *et al.*, 1988)]. Also, the same enzymatic preparation is obtained whether the protein is purified from the periplasmic or the cytoplasmic fraction. It should be noted that regulatory mechanisms have been described in which a single gene codes for identical enzymes in different cellular compartments (Wu & Tzagoloff, 1987; Carlson & Boldstein, 1982; Natsoulis *et al.*, 1986). Both flavodoxin and ferredoxin from *D. gigas* are found in the cytoplasmic fraction and are active in the reconstitution of sulfite reduction from molecular hydrogen (LeGall & Dragoni,1966) and in the production of hydrogen from pyruvate (Hatchikian & LeGall, 1970; Moura *et al.*, 1977).

Tetra- and octaheme cytochromes c_3 have been found in *D. gigas* (LeGall *et al.*, 1965; Hatchikian *et al.*, 1969). The tetraheme protein is periplasmic, whereas extraction procedures are in favor of the cytoplasmic location of the octaheme protein found in the cytoplasm. In *D. gigas*, the two cytochromes are not active in the reduction of sulfite from molecular hydrogen. However, the octaheme cytochrome can couple the reduction of thiosulfate from this gas (Hatchikian *et al.*,1969, 1972; Xavier *et al.*, 1979; LeGall & Peck, 1987; LeGall & Fauque, 1988).

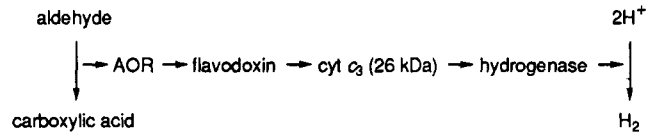
The redox potentials of the active sites of the isolated electron carriers are compiled in Table V. Aldehyde oxidoreductase alone (in the presence of aldehyde) is not capable of fully reducing the cytochrome; this is attained only after addition of flavodoxin to the system. Moreover, the aldehyde oxidoreductase leads the flavodoxin mainly to a semiquinone state without significant reduction to the hydroquinone state. A possible interpretation is that the complex flavodoxin–cytochrome c_3, which has been shown to be formed at low ionic strength by both NMR and molecular modeling methods (Stewart *et al.*, 1988a,b), results in a new system in which the midpoint potentials of the redox centers of each protein have been significantly modified.

The proposed cytoplasmic physiological electron-transfer chain, based on the known localization of the different proteins implicated (Odom & Peck, 1981a,b; LeGall & Peck, 1987; Bell *et al.*,1974), is defined as follows:

Table V: Active Site Midpoint Redox Potential of Relevant Proteins Involved in the Electron-Transfer Chain between Aldehyde and Molecular Hydrogen[a]

protein	center	redox potential (mV)	pH
aldehyde oxidoreductase	2Fe–2S I	–280	
	2Fe–2S II	–285	7.6
	Mo(VI)/(V)	–450	
	Mo(V)/(IV)	–530	
flavodoxin	oxid/SQ	–140	5.5
		–200	7.0
		–225	7.9
	SQ/reduc	–355	5.5
		–395	7.0
		–440	7.9
cytochrome c_3 (tetraheme)	hemes 1–4	–180 to –360	var
hydrogenase [Ni–Fe]	Ni(III)/(II) inactive	–220	8.5
	Ni(III)–H intermed	–360/–400	
	Ni(III)–[Fe–S] cat.	–360/–400	
	[3Fe–4S] inactive/active	–70/–290	
	[4Fe–4S]	ca. –320	7.0

[a] References: Moura *et al.* (1977, 1978), Fauque *et al.* (1988), and Coletta *et al.* (1991).



The fact that the periplasmic tetraheme cytochrome c_3 replaces the cytoplasmic octaheme cytochrome c_3 can be explained by the lack of specificity toward hydrogenase of this type of cytochromes. For example, the triheme cytochrome c_3 (*i.e.*, cytochrome c_7) isolated from *Desulforomonas acetoxidans*, an organism devoid of hydrogenase, is reduced by this enzyme (Moura *et al.*, 1991). In the scheme, all of the proteins are cytoplasmic. In this case, the only physiologically active cytochrome is the octaheme which is located in the cytoplasm, and hydrogen cycling, *via* periplasmic hydrogenase and tetraheme cytochrome c_3, is compulsory for energy conservation. Thus, thanks to the remarkable specificity of protein/protein interactions, four soluble proteins, representing a total of 11 discrete redox centers (see Table V), can operate together without the necessity of any extra "matrix" molecules. It is also to be noted that this electron transfer chain is constituted by a combination of dehydrogenating and hydrogenating reactions.

We now turn to the physiological significance of the *D. gigas* molybdenum protein other than as a scavenger of environmental aldehydes. The existence of both ethanol dehydrogenase and aldehyde oxidase in sulfate-reducing bacteria (Widdel, 1988) has been previously demonstrated, as well as the possibility of *D. gigas* utilizing internal polyglucose (Stams *et al.*,1983) and the observation of the utilization of aldehydes by anaerobic bacteria (White *et al.*, 1989; Mukund & Adams, 1990, 1991). We need to stress again that benzaldehyde was chosen from among other aldehydes because it is a more convenient substrate, not because it could have special physiological relevance. Although there are reports on the utilization of aromatic aldehydes by sulfate-reducing bacteria (SRB) (Zellner *et al.*, 1989, 1990; Brune *et al.*, 1983; Folkers *et al.*, 1989), which are significant from the point of view of wood degradative pathways, growth of *D.*

gigas in the presence of benzaldehyde has not been demonstrated. The fact that acetaldehyde is also a good substrate for the molybdenum protein, as shown in Table IIIA, deserves some comments concerning the endogenous generation of aldehydes. *D. gigas* is able to grow with ethanol as substrate and to convert it into acetate with stoichiometric reduction of sulfate into sulfide (Kremer *et al.*,1988). Acetaldehyde dehydrogenase activity was detected in crude extracts, and the authors pointed out that this activity possibly could be due to the molybdenum protein. However, when *D. gigas* was utilizing ethanol without sulfate, in the presence of a methanogenic bacterium, the latter grew through hydrogen interspecies transfer, but *D. gigas* did not.

This system is reminiscent of the one described in a previous report by Bryant *et al.* (1977) and Reddy *et al.* (1972a,b) using the S organism isolated from the mixed culture *Methanobacterium omelianskii*, which ferments ethanol to acetate and hydrogen in a ferredoxin-dependent pathway. However, in this case and in contrast with *D. gigas*, the *S* organism was able to grow in the mixed culture. Our results are compatible with these observations since, in the absence of sulfate, hydrogen can be produced in a purely cytoplasmic electron-transfer chain, which is then unable to conserve energy in the absence of sulfates. However, in their presence, hydrogen can be reutilized by the periplasmically located hydrogenase and thus give rise to ATP formation through hydrogen cycling (Odom & Peck, 1981a,b, 1984), a hypothesis which is supported by the recent report showing that reconstitution of the electron-transfer chain, from molecular hydrogen to sulfite, could be achieved using either the tetraheme cytochrome c_3 or a new, high molecular weight cytochrome *c* solubilized from membrane preparations (Chen *et al.*, 1991, 1993).

Glyceraldehyde, another substrate used by *D. gigas* aldehyde oxidoreductase, is of special interest because of the observation (Stams *et al.*, 1983) confirming earlier results (Thomas, 1972) stating that *D. gigas* accumulates large amounts of polyglucose under normal growth conditions. This molecule has been viewed as a storage polymer, since it can be degraded into acetate and hydrogen in the absence of sulfate, and as an energy source for sulfate-reducing bacteria. Recently, *in vivo* NMR studies indicated that nucleotide triphosphate is formed from the degradation of polyglucose in the presence of oxygen in *D. gigas* (Santos *et al.*, 1993). In the absence of oxygen, and when glycolytic enzymes are inhibited with fluoride, 3-phosphoglycerate and glycerol 3-phosphate acumulate in the cells, suggesting the importance of glyceraldehyde in this pathway. Observations by Mukund and Adams (1991) show that a tungsten-containing aldehyde ferredoxin oxidoreductase from *Pyrococcus furiosus* could be involved in a glycolytic pathway, through which glucose is converted into acetate. The enzyme converts glyceraldehyde into glycerate with ferredoxin-linked hydrogen production through hydrogenase. *D. gigas* aldehyde oxidoreductase could play a similar role and be involved in the degradation of glyceraldehyde. However, the electron-transfer chain present in *D. gigas* is far more complicated than that in *P. furiosus*, which requires only ferredoxin as an intermediate electron carrier, as we have discussed earlier.

Thus, *D. gigas* is in a special situation since it is lacking an uptake system for glucose and/or hexokinase, preventing it from growing directly on glucose (Stams *et al.*, 1983), but it is deriving glyceraldehyde from internal polyglucose. This anaerobic variation of the Entner–Doudoroff pathway (Mukund & Adams, 1991), which was proposed to be operative in *P. furiosus*, could exist in *D. gigas* because all of the activities of the enzymes which are necessary to phosphorylate glycerate (obtained from the $NAD(P)^+$-independent molybdenum-containing aldehyde oxidoreductase reaction) and transform it into pyruvate (3-phosphoglycerate mutase, enolase, and pyruvate Kinase) are present at significant levels in the cell-free extracts of lactate-grown bacteria (Kremer & Hansen, 1987). However, a more classical interpretation cannot be excluded.

This mechanism of energy conservation with aldehyde oxidation through an *in vitro* electron-transfer chain is conceptually possible *in vivo* and abides with other experimental observations in *Desulfovibrio* (Tsuji & Tatsuhiko, 1980; Peck & LeGall, 1982). Optimization, stimulation, and coupling of this chain to pyridine nucleotides or nucleotide phosphates are currently in progress.

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REFERENCES

Barata, B. A. S., Liang, J., Moura, I., LeGall, J., Moura, J. J. G., & Huynh, B. H. (1992) *Eur. J. Biochem. 204*, 773–778.

Bell, G. R., LeGall, J., & Peck, H. D., Jr. (1974) *J. Bacteriol. 120*, 994–997.

Booth, V. H. (1938) *Biochem. J. 32*, 503–507.

Branzoli, U., & Massey, V. (1974) *J. Biol. Chem. 249*, 4346–4349.

Bray R. C. (1975) in *The Enzymes* (Boyer, P. D., Ed.) Vol. XII, pp 299–419.

Bray, R. C. (1980) *Adv. Enzymol. 51*, 107–165.

Bray, R. C., Turner, N. A., LeGall, J., Barata, B. A. S., & Moura, J. J. G. (1991) *Biochem. J. 280*, 817–820.

Brune, G., Schoberth, S. M., & Sahm, H. (1983) *Appl. Environ. Microbiol. 46*, 1187–1192.

Bryant, M. P., Campbell, L. L., Reddy, C. A., & Crabill, M. R. (1977) *Appl. Environ. Microbiol. 33*, 1162–1169.

Carlson, M., & Bolstein, D. (1982) *Cell 28*, 145–154.

Chalmers, R. M., & Fewson, C. A. (1989) *Biochem. J.263*, 913–919.

Chen, L., Liu, M.-Y., & LeGall, J. (1991) *Biochem. Biophys. Res. Commun. 180*, 238–242.

Chen, L., Liu, M.-Y., & LeGall, J. (1993) *Arch. Biochem. Biophys. 303*, 44–50.

Coletta, M., Catarino, T., LeGall, J., & Xavier, A. V. (1991) *Eur. J. Biochem. 202*, 1101–1106.

Coughland, M. (1980) in *Molybdenum and Molybdenum containing enzymes* (Coughland, M., Ed.) pp 119–185, Pergamon Press, Oxford, U.K.

Cramer, S. P., Moura, J. J. G., Xavier, A. V., & LeGall, J. (1984) *J. Inorg. Biochem. 20*, 275–280.

Duine, J. A., Frank, J. J., & Jongejan, J. A. (1987) *Adv. Enzymol. 59*, 169–212.

Evces, S., & Lindhal, R. (1989) *Arch. Biochem. Biophys. 274*, 518-524.

Fauque, G., Peck, H. D., Jr., Moura, J. J. G., Huynh, B. H., Berlier, Y., DerVartanian, D. V., Teixeira, M., Przybyla, A. E., Lespinat, P. A., Moura, I., & LeGall, J. (1988) *FEMS Microbiol. Rev. 54*, 299–344.

Fauque, G., LeGall, J., & Barton, L. L. (1991)in *Variations in Autotrophic Life* (Shively, J. M., & Barton, L. L., Eds.), pp 271–337, Academic Press Limited, London.

Folkers, M., Ney, U., Kneifel, H., Stackebrandt, E., Witte, E. G., Forstel, H., Schoberth, S. M., & Sahm, H. (1989) *Syst. Appl. Microbiol. 11*, 161–169.
Gutroo, H. L., & Johns, D. C. (1971) *J. Biol. Chem. 246*, 286–293.
Hart , L. I., McGartoll, M., Chapman, H. R., & Bray, R. C. (1970) *Biochem. J. 116*, 851–864.
Hatchikian, E. C., & LeGall, J. (1970) *Ann. Inst. Pasteur (Paris) 118*, 288–301.
Hatchikian, E. C., Bruschi, M., LeGall, J., & Dubourdieu, M. (1969) *Bull. Soc. Fr. Physiol. Veg. 15*, 381–390.
Hatchikian, E. C., LeGall, J., Bruschi, M., & Dubourdieu, M. (1972) *Biochim. Biophys. Acta 258*, 701–708.
Jendrossek, D., Steinbuchel, A., & Schlegel, H. G. (1987) *Eur. J. Biochem. 167*, 541–548.
Johansson, J., Bahr-Lindstrom, H., Jeck, R., Woenckhaus, C., & Jornvall, H. (1988) *Eur. J. Biochem. 172*, 527–533.
Komai, H., Massey, V., & Palmer, G. (1969) *J. Biol. Chem. 244*, 1692–1700.
Kremer, D. R., & Hansen, T. A. (1987) *Arch. Microbiol. 147*, 249–256.
Kremer, D. R., Nienhuis, H. E., & Hansen, T. A. (1988) *Arch. Microbiol. 150*, 552–557.
Krenetsky, T. A., Neal, S. M., Elion, G. B., & Hitchings, G. M. (1972) *Arch. Biochem. Byophis. 150*, 535–599.
Lang, C., King, E.J., & Sperry, W. M. (1961) in *Free Energy Data and Oxidation-Reduction Potentials-Biochemist's Handbook*, SPON, Oxford, U.K.
LeGall, J., & Dragoni, N. (1966) *Biophys. Biochem. Res. Commun. 23*, 145–149.
LeGall, J., & Peck, H. D., Jr. (1987) *FEMS Microbiol. Rev.46*, 35–40.
LeGall, J., & Fauque, G. (1988) in *Biology of Anaerobic Microorganisms* (Zehnder, A. J. B., Ed.) pp 587–639, Wiley Interscience, John Wiley and Sons, New York.
LeGall, J., Mazza, G., & Dragoni, N. (1965) *Biochim. Biophys. Acta 99*, 385-387.
LeGall, J., Ljungdal, P. O., Moura, I., Peck, H. D., Jr., Xavier, A. V., Moura, J. J. G., Teixeira, M., Huynh, B. H., & Dervartanian, D. V. (1982) *Biophys. Biochem. Res. Commun. 106*, 610–616.
Li, C., Peck, H. D., Jr., LeGall, J., & Przybyla, A. E. (1987) *DNA 6*, 539–551.
Loomes, K. M., Midwinter, G. G., Blackwell, L. F., & Buckley, P. D. (1990) *Biochemistry 29*, 2070–2075.
Lowry, O. H., Rosebrough, N. J., Tan, A. C., & Randal, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Morphet, F. F. (1983) *Biochim. Biophys. Acta 774*, 328.
Moura, J. J. G., Xavier, A. V., Bruschi, M., LeGall, J., Hall, D. O., & Cammack, R. (1976) *Biochem. Biophys. Res. Comm. 72*, 782–789.
Moura, J. J. G., Xavier, A. V., LeGall, J., & Cabral, J. M. P. (1977a) *J. Less Common Met. 4*, 555–562.
Moura, J. J. G., Xavier, A. V., Hatchikian, E. C., & LeGall, J. (1977b) *FEBS Lett. 89*, 177–179.
Moura, J. J. G., Xavier, A. V., Cammack, R., Hall, D. O., Bruschi, M., & LeGall, J. (1978) *Biochem. J. 173*, 419–425.
Moura, J. J. G., Costa, C., Liu, M.-Y., & LeGall, J. (1991) *Biochim. Biophys. Acta 1058*, 61–66.
Mukund, S., & Adams, M. W. W. (1990) *J. Biol. Chem. 265*, 11508–11516.
Mukund, S., & Adams, M. W. W. (1991) *J. Biol. Chem. 266*, 14208–14216.
Natsoulis, G., Hilger, F., & Fink, G. R. (1986) *Cell 46*, 235–245.
Odom, J. M., & Peck, H. D., Jr. (1981a) *FEMS Lett. 12*, 47–50.
Odom, J. M., & Peck, H. D., Jr. (1981b) *J. Bacteriol. 147*, 161–169.
Odom, J. M., & Peck, H. D., Jr. (1984) *Annu. Rev. Microbiol. 38*, 551–592.
Olson, J. J., Ballou, D. P., Palmer, G., & Massey, V. (1974a) *J. Biol. Chem. 249*, 4350–4362.
Olson, J. J., Ballou, D. P., Palmer, G., & Massey, V. (1974b) *J. Biol. Chem. 249*, 4363–4382.
Palmer, G. (1962a) *Biochim. Biophys. Acta 56*, 444–459.
Palmer, G. (1962b) *Biochim. Biophys. Acta 64*, 135–148.
Palmer, G., & Olson, J. S. (1980) in *Molybdenum and Molybdenum containing enzymes* (Coughland, M., Ed.) pp 187–220, Pergamon Press, Oxford, U.K.
Peck, H. D., Jr., & LeGall, J. (1982) *Philos. Trans. R. Soc. London B 298*, 443–466.
Poels, P. A., Groen, B. W., & Duine, J. A. (1987) *Eur. J. Biochem.166*, 575-579.
Rajagopalan, K. V., & Handler, P. (1964) *J. Biol. Chem. 239*, 2022–2026.
Reddy, C. A., Bryant, M. P., & Wolin, M. J. (1972a) *J. Bacteriol. 109*, 539–545.
Reddy, C. A., Bryant, M. P., & Wolin, M. J. (1972b) *J. Bacteriol. 110*, 133–138.
Santos, M. H., Fareleira, P., Xavier, A. V., Chen, L., Liu, M.-Y., & LeGall, J. (1993) *Biochem. Biophys. Res. Commun. 195*, 551–557.
Smith, L. T., & Kaplan, N. O. (1980) *Arch. Biochem. Biophys. 203*, 663–675.
Stams, F. J. M., Veenhuis, M., Weenk, G. H., & Hansen, T. A. (1983) *Arch. Microbiol. 136*, 54–59.
Stams, F. J. M., Hoekstra, L. G., & Hansen, T. A. (1986) *Arch. Microbiol. 145*, 272–276.
Stewart, D. E., LeGall, J., Moura, I., Moura, J. J. G., Peck, H. D., Jr., Xavier, A. V., Weiner, P. K., & Wampler, J. E. (1988a) *Biochemistry 27*, 2444–2450.
Stewart, D. E., LeGall, J., Moura, I., Moura, J. J. G., Peck, H. D., Jr., Xavier, A. V., Weiner, P. K., & Wampler, J. E. (1988b) *Eur. J. Biochem. 185*, 695–700.
Thauer, R. K., Jungermann, K., & Decker, K. (1977) *Bacteriol. Rev. 41*, 100–180.
Thomas, P. (1972) *J. Microsc. (Paris) 13*, 349–360.
Tsuji, K., & Tatsuhiko, Y. (1980) *Arch. Microbiol. 125*, 35–42.
Turner, N., Barata, B. A. S., Bray, R. C., Deinstung, J., LeGall, J., & Moura, J. J. G. (1987) *Biochem. J. 243*, 755–761.
Waud, W. R., Brady, F. O., Wiley, R. O., & Rajagopalan, K. V. (1975) *Arch. Biochem. Biophys. 169*, 695–701.
White, H., Strobl, G., Feicht, R., & Simon, H. (1989) *Eur. J. Biochem. 184*, 89–96.
Widdel, F. (1988) in *Biology of Anaerobic Microorganisms*, (Zehnder, A. J. B., Ed.) pp 469–586, Wiley Interscience, John Wiley and Sons, New York.
Wu, M., & Tzagoloff, A. (1987) *J. Biol. Chem. 262*, 12275–12282.
Xavier, A. V., Moura, J. J. G., LeGall, J., & Dervartanian, D. V. (1979) *Biochimie 61*, 689–695.
Yan, R. T., & Chen, J. S. (1990) *Appl. Environ. Microbiol. 56*, 2591–2599.
Zellner, G., Messner, P., Kneifel, H., & Winter, J. (1989) *Arch. Microbiol. 152*, 329–334.
Zellner, G., Kneifel, H., & Winter, J. (1990) *Appl. Environ. Microbiol. 56*, 2228-2233.