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Title: Optimization of Bioconversion of Liquid and Solid
Residues.

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A. Xavier

Applicant Organization: Centro de Química Estrutural

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SIX MONTH REPORT (July 1 to December 31, 1986)

1. BIOCHEMICAL ENGINEERING

1.1. Mixed culture, phase separated work

Due to the lack of molasses slops in the whole of the country, we started using "synthetic" effluent (cf. previous report). It did not prove easy to test, as buffers and yeast extract additions were required for decent growth and attachment. Since last December the ethanol plants have restarted using molasses, we have gone back to molasses slops to be tested under batch as well as fixed bed fixed film systems with an automatic pH control recently bought and installed. Late in the year we expect to conduct completely stirred tank reactor studies for technical cultures in the acidogenic/sulphur reducing phase as a check. The methane reactor being used is still the fixed bed fixed film originally envisaged, but we are now using the sintered glass Raschig rings, supplied by Schott Mainz in West Germany (pore size 60 to 300 μ m for methane production and 60 to 100 μ m for acidogenic, the first allowing higher gas flow rates out of the adhered biofilm).

The changeover for the new supports took place while moving to a new laboratory, during September and October. Also, at the end of December the thermal conductivity detector, as well as its controls in the gas chromatography, have finally been assembled and, thus far, seem to be working correctly for the first time since we started up.

1.2. Pure cultures of *Propionibacterium acidipropionici*

The majority of the tests conducted under batch and CSTR

without pH control have now been carried out, propionic acid and vitamin B12 contents being assessed in relation to the operational conditions.

The CSTR tests with pH control are still half way through but have already shown a higher improvement in final acid content at pH 6, as one should expect.

Some batch tests conducted under growing glucose/xylose rations and controlled pH at 6 have allowed us to detect diauxic glucose/xylose utilisation and final consumptions of xylose at 12 g/l (or approximately 50% of initial concentration); this points to a possible fed-batch strategy as being probably appropriate to achieve an increase in xylose utilisation.

Concomitantly with the running up of the columnar tests preliminary tests with pH controlled CSTR and cell recycle over ultrafiltration inorganic membranes have been carried out. Recently, this tests were stopped as the acrylic glass column started leaking after approximately 7 months of continuous utilisations).

The results on propionic acid and vitamin B12, have been accepted for presentation at the 9th Symposium on Biotechnology for fuels and Chemicals (Boulder, Colorado, 5-8 May) and the 4th European Congress on Biotechnology (Amsterdam, 14-18 June), respectively. We enclose the abstracts and will provide copies of the presentations later.

2. SCREENING OF ELECTRON TRANSFER PROTEINS INVOLVED IN BIOCONVERSION PROCESSES (C.D.E., U.N.L., J.J.G.Moura, I.Moura, M.Teixeira, J.Lampreia, B.Barata, A.R.Lino and A.V.Xavier)

The screening of electron transfer proteins (ETP) and co-factors was extended to different bacterial groups.

2.1. SULFATE REDUCERS

Particular emphasis was given to the study of bacterial hydrogenases, their potential biotechnological applications and structure/activity relationships.

2.1.1. Bacterial Hydrogenases: a New Classification

The enzyme hydrogenase catalyzes the simplest reversible redox processes: $2H^+ + 2e^- = H_2$, and has been used in biotechnological processes, namely the production of hydrogen (water photolysis) using biological and artificial systems or for the use of hydrogen as a reductant in chemical synthesis. However the processes showed numerous experimental limitations due in particular to the instability of the biomolecules involved. The future developments of such processes depend on the screening of stable enzymes and on the detailed knowledge of the constitution and catalytic properties of their active centers.

We have screened, purified and characterized hydrogenases from sulfate reducing bacteria (*Desulfovibrio* sp.), where the metabolism of hydrogen is governed by a reversible enzyme. The hydrogenases isolated show that *Desulfovibrio* are choice organisms for the study of this complex process. At least three

different types of hydrogenases are now recognized within this bacterial group: [Fe] hydrogenases containing only iron-sulfur centers, one of them having unique properties (i.e., D.vulgaris), [NiFe] hydrogenases containing one nickel atom and iron-sulfur centers generally arranged as one 3Fe and two 4Fe clusters (i.e., D.gigas, D.desulfuricans 27774) and D.multispirans n.sp.); and [NiFeSe] hydrogenases containing nickel and selenium in equimolecular amounts and iron-sulfur centers (D.saxilegens and D.baculatus 9974).

2.1.2. [NiFe] hydrogenase from a Thermophilic Sulfate-Reducer: Desulfovibrio thermophilus

So far hydrogenases have been only purified from a few thermophilic organisms such as Bacillus achlegelii and Methanobacterium thermoautotrophicum strains H and Marburg. A hydrogenase has been partially purified from the thermophilic sulfate-reducing bacterium: Desulfovibrio (D.) thermophilus DSM 1276 grown at 65°C. A 22% purification yield was attained after three chromatographic steps with a purity index (A_{400}/A_{278}) of 0.16. The enzyme strongly binds to other proteins as seen by polyacrylamide gel electrophoresis making complete purifications extremely difficult to be achieved. The D.thermophilus hydrogenase appears to be 60% pure on SDS-gel electrophoresis with two major bands of molecular mass around 55 and 15 kDa. The hydrogenase fraction has a specific activity (micromoles per minute per mg protein) of the H₂ evolution assay at 60°C and of 52 in the D₂-H⁺ exchange reaction at 70°C. The EPR spectrum of the native enzyme detects the presence of Ni(III) as indicated by

the observation of a slow relaxing rhombic signal with g-values at 2.31, 2.27 and ~2.0 and suggests also the presence of a [3Fe-xS] oxidized center associated with an isotropic g=2.02 signal.

2.1.3. Model Studies

In order to further probe this complex enzymatic system, simpler model compounds are currently being studied. Metal substituted ferredoxins, isolated from *D. gigas* offer new synthetic possibilities. Novel mixed metal clusters have been produced: [Co, Fe₃-S₄] and [Zn, Fe₃-S₄]. Other small non-heme iron proteins (Rubredoxins - [FeCyS₄]) exhibit D₂/H⁺ exchange properties when nickel substitutes the native iron center. Most of the catalytic properties (exchange D₂/H⁺) have been determined in collaboration with Dr. P.A. Lespinat and Y.M. Berlier, A.R.B.S., C.E.A., Cadarache, France.

2.1.4. The cytochrome system of a nitrogen-fixing strain of a sulfate-reducing bacterium: *Desulfovibrio desulfuricans* strain Berre-Eau

Two c-type cytochromes were purified and characterized by electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) spectroscopic techniques, from the sulfate-reducer nitrogen-fixing organism *Desulfovibrio desulfuricans* strain Berre-Eau (NCIB 8387). The purification procedures included several chromatographic steps on alumina, carboxyethylcellulose and gel filtration. A tetrahaem and a monohaem cytochrome were identified. The multahaem cytochrome has

visible, EPR and NMR spectra with general properties similar of other low-potential bis-histidinyll axially bound haem proteins, belonging to the class of tetrahaem cytochrome c_3 isolated from other Desulfovibrio species. The monohaem cytochrome c_{553} is ascorbate-reducible and its EPR and NMR data are characteristic of a cytochrome with methionine-histidine ligation.

2.2. METHANE FORMING ORGANISMS

A thermophilic strain of Methanosarcina barkeri (strain MST) was grown in large scale fermenters (in collaboration with the University of Georgia, Department of Biochemistry, Profs. J. LeGall and H.D. Peck Jr.).

2.2.1. B_{12} - protein

A B_{12} protein which contains a corrinoid as a cofactor was isolated, and their properties compared to the B_{12} -enzyme previously isolated from the mesophile Methanosarcina barkeri, strains DSM 800 and 804. The molecular mass of the MST strain B_{12} protein as determined to be 38 kDa by gel electrophoresis.

The corrinoid extracted from the purified protein and from bacterial cells of the MST strain was identified as hydroxyl-benzimidazolyl-cobalamine (factor III) and corresponds to 90% of the total corrinoid content present in the bacteria. Both corrinoid and protein were reduced by sodium borohydride. Noteworthy is the fact that the B_{12} -protein from the MST strain was isolated in the methylated form (the enzyme isolated from the mesophile strain was present in the aquo form, but could be methylated with methyl iodide).

EPR spectra recorded at 77 K show typical signals from the

Co(II) low spin complexes. The hyperfine interactions between cobalt and the benzimidazol nitrogen atoms was observed, indicating that the base is coordinated to the metal atoms either in the free and the protein bound corrinoid (Figure 1). The physiological role of the B₁₂-proteins is still controversial, but the isolation of methylated-B₁₂-form from crude bacterial extracts further support the involvement of the enzyme in methyl transfer reactions (see previous reports).

2.2.2. Sulfite reductases

A new type of sulfite reductase was isolated from *M. barkeri* (DSM 800 and 804) and compared with a homologous protein isolated from a sulfur utilizing organism (*Desulfurmonas acetoxidans*). The enzyme contain siroheme and iron-sulfur centers. These three proteins reveal optical spectroscopic properties similar to siroheme containing enzymes: however the 314 nm band, typical of high spin Fe³⁺ isobacteriochlorine complexes is not present. The low spin (S=1/2) siroheme configuration in these proteins was confirmed through-EPR Spectroscopy. Spectra with g-values at 2.40, 2.30 and 1.86 (*M. barkeri*, DSM 800) and 2.44, 2.33 and 1.91 (*Desulfurmonas acetoxidans*) were obtained. External ligand binding to siroheme(S²⁻, CN⁻, CO) was observed under reducing conditions. Chemical analysis reveal the presence of one siroheme and one [4Fe-S] center for minimal molecular mass (~23 KDa). These enzymes represent a new class of low-molecular-weight/low-spin siroheme sulfite reductases.

2.3. ENZYMES INVOLVED IN NITROGEN METABOLISM - DENITRIFICATION

2.3.1. Cytochrome components of Wolinella succinogenes, a nitrate respiring organism

Two α -type cytochromes were isolated and purified from nitrate respiring *W. succinogenes*. One is a monohaem cytochrome (high-potential). The other is a two-haem cytochrome. The monohaem cytochrome has a minimum molecular weight of 8.2 kDa and contains 0.9 atom-% of iron and 0.95 haem μ per mole of protein. Visible spectroscopy suggest the presence of two ligand arrangements around the haem μ (i.e., a band at 695 nm indicates haem methionine coordination (low-spin form) coexisting with a high-spin form revealed by an absorption band at 619 nm and a shoulder at 498 nm). The redox potential as measured by visible redox titration in the presence of redox mediators is ± 100 mV. The binding of cyanide ($K_{\text{assoc}}^{\text{CN}} = 5 \times 10^5 \text{ M}^{-1}$) displaces the methionyl residue, and full conversion to a low-spin cyanide bound form occurs.

The structural features were studied by ^1H NMR spectroscopy in both the ferric and the ferrous states. In the oxidized state, the pH dependence of the haem methyl resonances (between pH 5 and 10) as well as the magnetic susceptibility measurements (using NMR) are consistent with the presence of a high-spin/low-spin equilibrium with a transition pK_a of 7.3. The spin equilibrium is fast in the NMR time scale. The haem methyl resonances present large down field chemical shifts. An unusually broad methyl resonance at 35 ppm (pH=7.5) was observed to be extremely temperature dependent ($\delta_{30^\circ\text{C}}^{\text{O}^+} - \delta_{0^\circ\text{C}}^{\text{O}^+} = 7.2$ p.p.m.)

and was assigned to the S-CH₃ group of the axial methionine. NMR studies indicate that in the ferrous state only a low-spin form is present. The haem mesoprotons, the methyl group and the methylene protons from the axial methionine were identified. Also the resonances from the aromatic residues (3 tyrosines and 1 phenylalanine) were assigned.

The NMR redox pattern of the monohaem cytochrome was analysed in detail from the fully reduced up to the fully oxidized state. The rate of the intermolecular electronic exchange process was determined to be approximately $6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at 303 K and pH=6.31.

The dihaem cytochrome has a molecular weight of 11 kDa and contains 2.43 Fe and 1.89 haem g/mole of cytochrome. No band at 695 nm was observed in the visible spectrum suggesting that methionine is not a ligand.

2.3.2. Nitrite Reductase from *Desulfovibrio desulfuricans* 27774

Nitrite reductase (hexaheme cytochrome) isolated from *D. desulfuricans* (27774) (a versatile sulfate reducer that can grow in nitrate) is a membrane-bound enzyme. The level of this enzyme was found to increase in bacteria grown with nitrate. The purified enzyme has a molecular weight of 66,000 and is capable of reducing nitrite to ammonia. It exhibits typical c-type cytochrome absorption spectrum and was determined to contain 6 c-type hemes by extinction coefficients measurement and iron-content determination. The dithionite-reduced hexaheme cytochrome was found to react with both nitrite and hydroxylamine; however, the ascorbate-reduced enzyme failed to

reduce either nitrite or hydroxylamine.

EPR study of this hexaheme cytochrome showed several heme species confirming the multiple heme nature of this enzyme. Figure 2 presents EPR spectra showing the effect of pH on the spin states of the heme groups. At pH 7.6 (Figure 2B), a distinct low-spin heme EPR signal was detected at $g=2.96$, 2.28 and 1.53.

This resonance was interpreted as a result of interactions between the heme groups rather than individual hemes. At low pH (Figure 2A), a high-spin ferric-heme-type signal appears at $g=6.0$ and $g=9.1$ species is also detected. The resonance at the $g=4$ region increases its intensity. At higher pH (Figure 2C), both the high-spin species and the $g=4$ resonance decrease their intensities substantially. Figure 2D shows an EPR spectrum of dithionite reduced hexaheme cytochrome reacted with nitrite. This spectrum shows that the " $g=2.96$ " heme is reoxidized to its native form. The signal at around $g=2$ region shows nitrogen hyperfine structure, and is typical of heme-NO complexes, indicating that some of the hemes have complexed NO.

In order to gain more information concerning the heme groups in nitrite reductases we start the study of redox equilibria and the determination of the redox potentials of these groups (Figure 3). The redox titration can therefore be followed by optical, EPR and Mossbauer techniques. However the hexaheme cytochromes, these experiments are difficult, since all six hemes exhibit identical optical spectra and not all the hemes are EPR detectable due to heme-heme interactions. However, interesting result occur upon varying the solution redox potential and new EPR signals appear as some of the heme become reduced and the heme-heme interactions

is disrupted. We also expect that these series of titrations may provide information concerning the roles these hemes play in the nitrite-reductase mechanism.

D. ISOTOPE ENRICHMENT

Labelling of enzymes with stable isotopes have been a very valuable approach for the spectroscopic studies performed. The labelling can be achieved by bacterial culture growth in enriched media or by reconstitution of active centers with the appropriated isotopes. ^{57}Fe and ^{61}Ni have been currently used.

E. SCIENTIFIC COLLABORATION

A.R.B.S., C.E.A., Cadarache, France
Drs. A.Lespinat, Y.M.Berlier, G.Fauque, R.Toci and Prof.J.LeGall

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Prof. E.Münck

CONFERENCES

1. CATALYTIC AND REDOX PROPERTIES OF THREE NICKEL-IRON-SULFUR-SELENIUM CONTAINING HYDROGENASES FROM Desulfovibrio baculatus STRAIN 9974
G.D.Fauque, M.Teixeira, B.Prickril, I.Moura, P.A.Lespinat, H.D.Peck, Jr., Y.Berlier, J.LeGall and J.J.G.Moura
72 nd Annual Meeting of SEB-ASM, Georgia, Athens, November 13-15, 1986.
2. PARTIAL PURIFICATION AND PROPERTIES OF A [NiFe] HYDROGENASE FROM THE THERMOPHILIC SULFATE-REDUCER: Desulfovibrio Thermophilus
G.D.Fauque, M.H.Czechowski, J.J.G.Moura and J.LeGall
Abstract ASM Meeting, Atlanta, 1-6 March, 1987.
3. PURIFICATION AND PARTIAL CHARACTERIZATION OF A FERREDOXIN FROM

THE DENITRIFIER Pseudomonas perfectomarina
G.D.Fauque, M.Y.Liu, J.J.G.Moura, I.Moura, W.J.Payne and J.LeGall
Abstract ASM Meeting, Atlanta, 1-6 March, 1987.

4. THE BIOLOGICAL ROLE OF VANADIUM

J.J.G.Moura
Cadarache, CEA, France, December 11, 1986.

5. STRUCTURAL ASPECTS OF NEWLY FORMED MIXED METAL CLUSTERS OF THE TYPE $[M-Fe_3S_4]$, M=Fe, Co and Zn - THEIR MAGNETISM AND REACTIVITY

J.J.G.Moura
Grenoble, CEN, Department de Recherche Fundamental, LBM, December 19, 1986.

6. STRUCTURAL STUDIES OF HAEM PROTEINS ISOLATED FROM SULPHATE REDUCERS AND DENITRIFIERS

J.J.G.Moura
Université de Paris VII, Faculté de Médecine, December 22, 1986.

7. PURIFICATION ET CARACTERIZATION D'UNE SULFITE-REDUCTASE ET D'UNE HYDROGENASE CHEZ METHANOSARCINA BARKERI (DSM 800)

G.Fauque, P.A.Lespinat, I.Moura, A.R.Lino, M.Ferreira, A.V.Xavier, J.J.G.Moura, D.V.DerVartanian, H.D.Peck Jr., and J.LeGall

1st Congress Société Française Microbiologie Toulouse, 3-5 April 1984.

8. PURIFICATION OF ADENYLYL SULFATE (APS) REDUCTASE AND DESULFOFUSCIDIN FROM A THERMOPHILIC STRAIN OF SULFATE REDUCER: DESULFOVIBRIO THERMOPHILUS

G.Fauque, M. Dzechowski, L.Kang-Lissolo, I.Moura, D.V.DerVartanian, J.J.G.Moura, J.Lampraia, A.V.Xavier and J.LeGall
Soc. for Indust.Microbiol., 10-15, Agosto 1986, San Francisco, U.S.A.

9. OXIDATIVE PHOSPHORYLATION LINKED TO THE DISSIMILATORY REDUCTION OF COLLOIDAL SULFUR BY DESULFOVIBRIO: A POSSIBLE FUNCTION FOR TETRA-HEME CYTOCHROME C_2

G.Fauque, L.L.Barton, J.J.G.Moura, R.Cammack and J.LeGall
XIV International Congress for Microbiology, 7-13 September 1986, Manchester, G.B.

10. PURIFICATION AND CHARACTERIZATION OF A NEW HIGH REDOX POTENTIAL RUBREDOXIN FROM DESULFOVIBRIO VULGARIS STRAIN HILDENBOROUGH

B.Prickril, I.Moura, J.J.G.Moura, B.H.Huynh, L.C.Sieker, A.V.Xavier and J.LeGall
72nd Ann.Meeting S.E.B.-A.S.M., 13-15 November 1986, Savannah, U.S.A.

PUBLICATIONS

1. $[Ni-Fe]$ HYDROGENASES FROM SULFATE REDUCING BACTERIA: NICKEL CATALYTIC AND REGULATORY ROLES

- J.J.G.Moura, M.Teixeira, I.Moura and J.LeGall
in: "Nickel in Biochemistry", J.R.Lancaster, ed., in press
2. ON THE ACTIVE SITES OF THE [Ni-Fe] HYDROGENASES FROM *D.GIGAS*:
MOSSBAUER AND REDOX TITRATION STUDIES
B.H.Huynh, D.S.Patil, I.Moura, M.Teixeira, J.J.G.Moura,
D.V.DerVartanian, M.H.Czechowski, B.C.Pickrill, H.D.Peck, Jr., and
J.LeGall
J.Biol.Chem., in press.
3. THE MOLYBDENUM IRON-SULFUR PROTEIN FROM *DESULFOVIBRIO GIGAS* AS
A FORM OF ALDEHYDE OXIDASE
N.Turner, B.Barata, R.C.Bray, J.Deistung, J.LeGall and
J.J.G.Moura
Biochem.J., in press.
4. PURIFICATION AND CHARACTERIZATION OF THREE PROTEIN FROM A
HALOPHILIC SULFATE-REDUCING BACTERIUM, *DESULFOVIBRIO SALEXIGENS*
M.Czechowski, G.Fauque, N.Galliano, B.Dimon, I.Moura,
J.J.G.Moura, A.V.Xavier, B.Barata, A.R.Lino and J.LeGall
J.of Indust.Microbiol., 1986, 1, 139-147.
5. RESONANCE RAMAN SPECTRA OF FERREDOXIN: NEW ASSIGNMENTS AND
VIBRATION COUPLING MECHANISM FROM IRON-54/IRON-56 ISOTOPE SHIFTS
AND VARIABLE WAVELENGTH EXCITATION. M.Czernuszewicz, J.LeGall,
I.Moura and T.G.Spiro, Inorg.Chem., 25, 695-700 (1986).
6. CHARACTERIZATION OF THE CYTOCHROME SYSTEM OF A NITROGEN FIXING
STRAIN OF A SULFATE-REDUCING BACTERIUM: *DESULFOVIBRIO*
DESULFURICANS, STRAIN BERRE-EAU
I.Moura, C.Fauque, J.LeGall, A.V.Xavier and J.J.G.Moura
Eur.J.Biochem., in press.
7. LOW-SPIN SULFITE REDUCTASES: A NEW HOMOLOGOUS GROUP OF NON-
HEME IRON-SIROHEME PROTEINS IN ANAEROBIC BACTERIA
I.Moura, A.R.Lino, J.J.G.Moura, A.V.Xavier, G.Fauque, H.D.Peck
Jr. and J.LeGall
Biochem.Biophys.Res.Comm., in press.
8. MOSSBAUER STUDY OF *D.GIGAS* FERREDOXIN II AND SPIN COUPLING
MODEL FOR Fe_3S_4 CLUSTER WITH VALENCE DELOCALIZATION
V.Papaefthymiou, J.J.Girerd, I.Moura, J.J.G.Moura and E.Münck
(1987) JACS submitted for publication.
9. EVIDENCE FOR THE FORMATION OF A $ZnFe_3S_4$ CLUSTER IN
DESULFOVIBRIO GIGAS FERREDOXIN II
K.Surerus, E.Münck, I.Moura, J.J.G.Moura and J.LeGall
(1987) JACS submitted to publication.

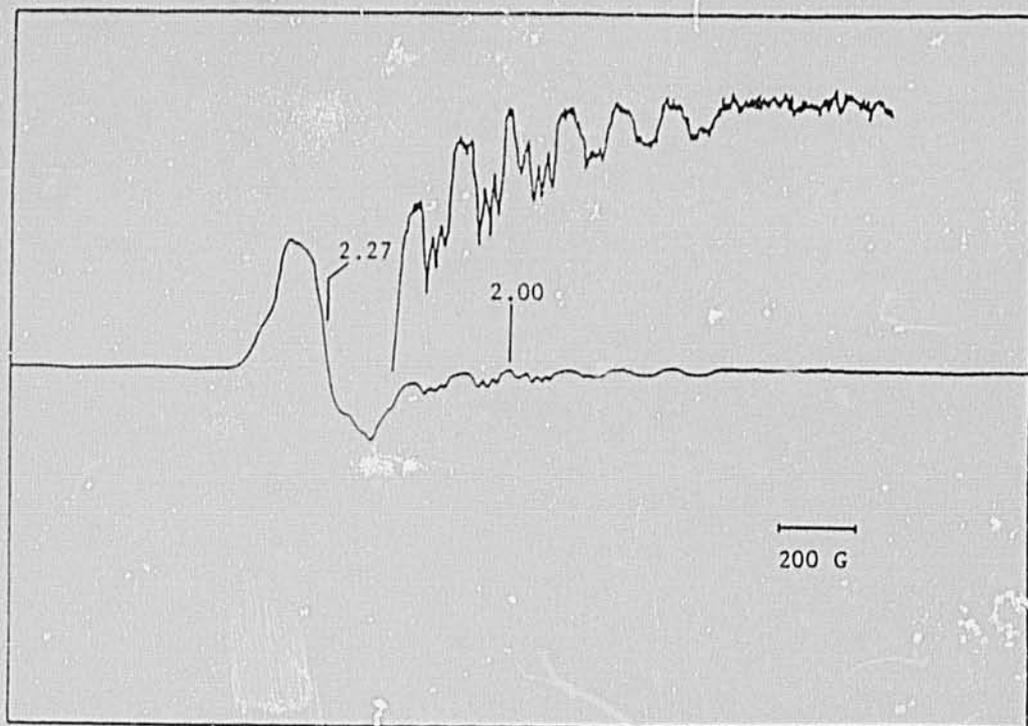


Figure 1. EPR spectra of the B₁₂-protein isolated from *M. bacillus* MST. The sample was illuminated after anaerobic reduction by sodium borohydride. Temperature 40 K, microwave frequency 9.52 GHz, microwave power 2 mW, amplitude modulation 1 mT.

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF IRON, NICKEL-IRON AND NICKEL-IRON-SELENIUM HYDROGENASES FROM 4 DESULFOVIBRIO SPECIES

Property	<i>D. vulgaris</i> Hildenborough	<i>D. gigas</i>	<i>D. baculatus</i> strain 9974	<i>D. salexigens</i> British Guiana
Molecular weight (kDa)	56	89.5	100	98
Subunits	2	2	2	2
Localization	periplasm	periplasm	periplasm	periplasm ^a
Metal content				
Non-heme iron	12	11	14	12
Nickel	0	1	1	1
Selenium	NR ^b	0	1	1
Specific activity ^c				
Uptake	50000	1500	120	1300
Production	4800	440	446	1830

a. At least 50 % of the hydrogenase was found to be located in the periplasm.

b. Not reported.

c. Expressed in micromoles H₂/min/mg protein.

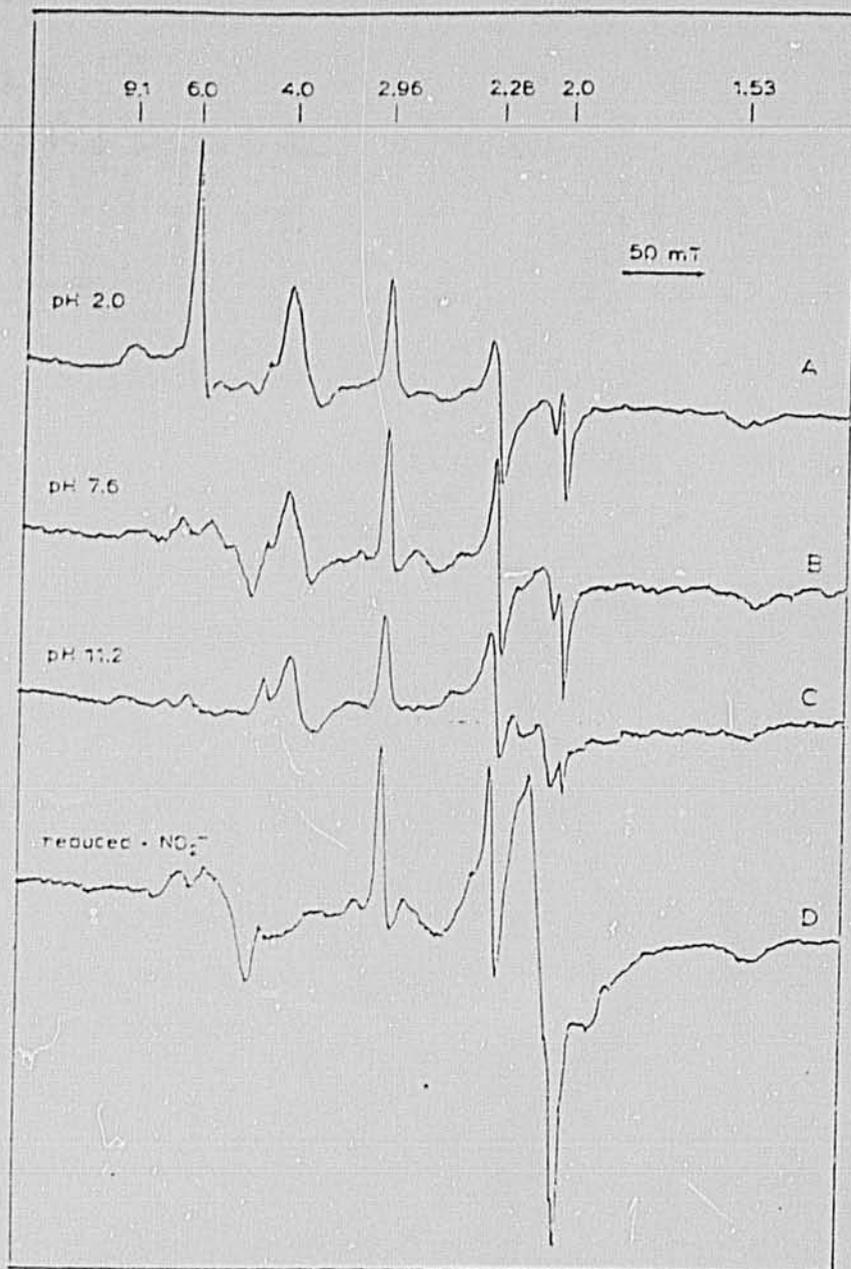


Figure 2. EPR spectra of hexaheme cytochrome from *D. desulfuricans* (27774) showing the effect of pH and the formation of NO complex. Experimental conditions are 2 mW at 9.530Hz and 10 K with 1 mT modulation, and for (A): 4×10^5 gain, for (B), (C) and (D): 6.3×10^5 gain.



Figure 3. EPR Redox Titration of nitrite reductase.

ABSTRACT SUBMISSION FORM
Ninth Symposium on Biotechnology for Fuels and Chemicals

Title Production of Propionic Acid using a xylose utilizing Propionibacterium

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Type your 150- to 200-word abstract on this sheet (single space). Abstracts are due by December 15, 1986. Papers will be selected prior to January 15, 1987, and authors will be notified of acceptance by February 1, 1987. Mail this form to: Charles D. Scott, Ninth Symposium on Biotechnology, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, Tennessee 37831.

The kinetics of *P. acidipropionici* (ATCC 25562), a xylose-utilizing microorganism, have been examined for the conversion of xylose and glucose to acetic and propionic acids.

Concomitant utilization of both sugars is observed, with slower consumption of xylose. No inhibition effect is apparent for the substrates or acetic acid produced, but propionic acid inhibition leads to a peak production of 12g prop./l in batch tests without pH control. Controlling pH at 6.0 maximum concentrations of 27g prop./l and 8,5g acetic/l were obtained.

Acid yields are close to the theoretical maximum (82%) at economically favorable molar ratios of 3:1 propionic: acetic as compared to 2:1 theoretical ratio. High cell mass yield coefficients of 0,26g cell/g subst. lowered the propionic yield to 0,45g propionic/g substrate.

High cell density systems are under study using either immobilized cell reactors (ICR) or cell recycling on tangential ultrafiltration membrane systems (UF).

ICR studies are performed utilizing cell growing adsorption processes solid supports, and UF experiments were conducted using mineral membranes (zirconium oxide) with a nominal molecular weight cut off of 500.000 Dalton. As expected, much higher productivities are obtained, namely for the cell recycle systems; sugar utilization, cell mass densities and product concentrations obtained at different regimes are also presented and compared with batch results. During UF experiments the broth rheological behaviour was studied and modelized.

REFERENCES

1. E.C. Clausen et al., Chem. Engrg. Prog., 80 (1984) n912, 59-63
2. M.J. Payne, Propionic Acid, in: M.Moo Young (Ed.) Comprehensive Biotechnology, Vol.3, Sec.3, Pergamon Press, London, 1985
3. A.E. Humphrey, Chem. Engrg. Prog., 73 (1977) n95, 85-91
4. O.Fond et al., Biotechnol. Bioeng., 28 (1986) 160-175

PROPIONIC ACID AND VITAMIN B₁₂ PRODUCTION USING A XYLOSE UTILIZING BACTERIUM AND DIFFERENT BIOREACTORS

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Propionic acid production by fermentation might be required for utilization in foods, animal feed and grain preservation; also, weight yields to propylene are substantially larger than those from the ethylene from fermentation ethanol route (1-3). Industrial vitamin B₁₂ production utilizes Propionibacterium freundenrichii, namely subsp. shermanii, the bacteriostatic effect of the acid produced being responsible for decreased contamination (4,5).

The kinetics of P.acidipropionici (ATCC 25562), a xylose utilizing rumen microorganism, have been studied to assess its use to propionic acid and vitamin B₁₂ production from cellulose - hemicellulose hydrolysates.

Concomitant utilization of xylose and glucose is observed up to 50 g/l total sugars; at higher sugar concentrations, a repression of xylose utilization similar to that described for C.acetobutlicum (6) seems to occur. No inhibition is apparent for glucose or acetic acid produced but propionic acid inhibition leads to a peak production of 12 g prop./l in batch tests without pH control; controlling pH at 6.0 maximum concentrations of 27 g prop./l and 8.5 g acetic/l were obtained. Acid yields are close to the theoretical maximum (82%) at economically favorable molar ratios of 3:1 propionic:acetic as compared to 2:1 theoretical ratio. High cell mass yield coefficients of 0.26 g cell/g subst., although advantageous for vitamin B₁₂ production, lowered the product yield to 0.45 g product/g substrate.

Low cell growth strategies for acid production and faster autocatalytic processes for vitamin production were followed using either immobilized or fixed cell reactors as well as cell recycling on ultrafiltration membrane systems. Productivities, sugar utilization, cell mass densities and final product (acids and vitamin) concentrations obtained at different regimes are presented and compared with the batch results.

REFERENCES

1. E.C.Clausen et al., Chem.Engrg Prog., 80 (1984) n.12, 59-63.
2. M.J.Playne, Propionic Acid, in: M.Moo Young (Ed.) Comprehensive Biotechnology, Vol.3, Sec. 3, Pergamon Press, London, 1985.
3. A.E.Humphrey, Chem.Engrg Prog., 73 (1977) n.5, 85-91.
4. L.J.Florent and F.M.Ninet, Vitamin B₁₂, in: H.Peppler and D. Perlman (Eds.) Microbial Technology, Vol.1, Academic Press, New York, 1979, 497-519.
5. H.C.Friedman and C.M.Cagen, Microbial Biosynthesis of B₁₂ like compounds, Ann.Rev.Microbiol., 24 (1970) 159-208.
6. O.Fond et al., Biotechnol.Bioeng., 28 (1986) 160-175.

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Purification and characterization of three proteins from a halophilic sulfate-reducing bacterium, *Desulfovibrio salexigens*

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SUMMARY

Hydrogenase, desulfoviridin and molybdenum proteins have been isolated from a halophilic sulfate-reducing bacteria, *Desulfovibrio salexigens* strain British Guiana. At least 50% of the hydrogenase was found to be located in the periplasm. The hydrogenase has a typical absorption spectrum, a 400/280 nm ratio of 0.28, a molecular weight by sedimentation equilibrium of 81 000 and is composed of two subunits. It has one nickel, one selenium and 12 iron atoms per molecule. The sulfite reductase has a typical desulfoviridin absorption spectrum, a molecular weight of 191 000 and iron and zinc associated with it. The molybdenum-iron protein is gray-green in color and exhibits an absorption spectrum with peaks around 612, 410, 275 nm and a shoulder at 319 nm. It is composed of subunits of approximately 13 250 and has an approximate molecular weight of 110 000. Three molybdenum and 20 iron atoms are found associated with it.

An extensive study of these three proteins will allow a better understanding of the function of these enzymes and also of their possible role in microbially caused corrosion.

INTRODUCTION

Sulfate-reducing bacteria have been implicated in the phenomenon of microbially caused corrosion in neutral anaerobic environments [13]. Hydrogen consumption and sulfate reduction contribute to this corrosion process. Hydrogen is consumed by the hydrogenase enzyme from either the metal surface [58] or from the iron sulfide film on the metal [28]. Hydrogen sulfide is produced by the sulfate reduction system and acts as an anodic reactant

[59], a cathodic reactant [12] or in the formation of a reactive phosphorous compound [26].

Desulfovibrio (D.) salexigens strain British Guiana is the only well known halophilic strain in the genus *Desulfovibrio* and only studies on its cytochrome C, [16] flavodoxin and rubredoxin [41] have been published.

Hydrogenases have been purified to apparent homogeneity from many bacterial species, including anaerobic microorganisms such as sulfate reducers [46] and methanogenic bacteria [5,18,27].

They are involved either in the hydrogen consumption in which hydrogen is used as a reductant for CO₂ fixation or for energy generation via electron transport or in hydrogen production which enables bacteria to dispose of excess electrons [1,50].

An important enzyme in sulfate reduction is dissimilatory sulfite reductase or bisulfite reductase [47]. This enzyme has been purified from the cytoplasm of many sulfate-reducing bacteria [46] and is believed to be involved in ATP production in these micro-organisms [44]. Reduction of sulfite either involves a cyclic scheme, utilizing intermediates trithionate and thiosulfate to sulfide [3] or a direct six electron reduction to sulfide [46].

A molybdenum protein has been found in some species of sulfate reducers from the genus *Desulfovibrio* (*D.*) *gigas* [40], *D. africanus* [21], *D. desulfuricans* strains Berre Eau [7] and Berre Sol (our unpublished results). It is characterized by not only containing molybdenum but also iron and labile sulfide. Its function is still unknown.

A purification scheme and partial characterization of hydrogenase, desulfoviridin (bisulfite reductase) and molybdenum protein from *D. salexigens* is reported. A study of these proteins may help elucidate the phenomenon of microbially caused corrosion.

MATERIALS AND METHODS

Growth of cells. *Desulfovibrio salexigens* strain British Gutana (NCIB 8403) was grown at 37°C on lactate/sulfate medium [54] with 3.0% NaCl. Cells for localization studies were harvested by centrifugation and immediately used. Cells (250 g) for enzyme purification were harvested, resuspended in 10 mM Tris-HCl (pH 7.6) lysed with a French press and then frozen at -80°C until used.

Assay and metal determinations. Hydrogenase activity was measured at 32°C either by the hydrogen evolution assay from dithionite-reduced methyl viologen [45] using an Aerograph A-90 P3 gas chromatograph or by hydrogen consumption with benzyl viologen as electron acceptor using Warburg respirometry [8]. One unit of hydrogenase activity

is defined as the amount of enzyme which catalyses the evolution or the consumption of 1 μmol H₂/min. Dissimilatory sulfite reductase activity was measured by a manometric assay at 32°C [31] using pure hydrogenase from *D. gigas* to reduce methyl viologen under H₂. The initial rate of hydrogen utilization is proportional to the amount of sulfite reductase. Protein was determined by a modification of the Lowry method as proposed by Markwell et al. [38]. Iron and nickel were determined by plasma emission spectroscopy using the Jarrel-Ash Model 75 atomcomp.

Optical spectra. Ultraviolet and visible absorption spectra were recorded on a Beckman DU 7 spectrophotometer.

Electrophoresis and molecular weight determination. Purity of the enzymes was established by polyacrylamide disc electrophoresis [11] and by comparison of published absorption ratios for similar type pure proteins. Subunit structure was determined by SDS-polyacrylamide gel electrophoresis [17]. Molecular weight was determined by SDS electrophoresis, gel filtration using a TSE 3000 SW analytical column (high-pressure liquid chromatography) or sedimentation equilibrium [49].

RESULTS

Location of the hydrogenase

Lysing the cells with the French press and centrifuging a 120 000 × g for 70 min released about 80% of the hydrogenase as soluble protein. The cells were washed in a 1:1 w/v buffer solution (pH 8.0) of 50 mM Tris-HCl/10 mM EDTA/500 mM glucose/3% NaCl, incubated in the same buffer at a 1:50 w/v ratio with 8 mg of lysozyme/ml and then centrifuged. This results in 70-80% of the hydrogenase being found in the supernatant. A visible spectrum showed 30% of the desulfoviridin was also found in the supernatant. Further experiments using MgCl₂·6H₂O instead of NaCl found almost 50% of the hydrogenase in the supernatant and less than 5% of desulfoviridin. This seems to indicate a periplasmic origin for at least 50% of the hydrogenase enzyme. It is important to add NaCl or

Table 1
Purification of hydrogenase from *D. salzigens*

Fraction	Protein (mg)	Total activity* ($\mu\text{mol H}_2/\text{min}$)	Specific activity* ($\mu\text{mol H}_2/\text{min per mg}$)
Crude extract	10750	47290	4.4
DEAE Bio-Gel A column	562	24670	42.7
Hydroxylapatite column	28	15552	561
Gel exclusion by HPLC	3.9	7170	1830

* in H_2 evolution.

$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to the washing or incubating buffers or the cells clump together. When Na_2SO_4 was substituted at the same concentration, the cells clumped together and there was some cell lysis.

Purification of hydrogenase, desulfoviridin and molybdenum-iron sulfur protein

All purification procedures were carried out in air at 4°C and the pH of the buffers, Tris-HCl and phosphate (KPB), were 7.6 (measured at 5°C).

The lysed cells were slowly defrosted and centrifuged for 1.5 h at $20\,000 \times g$. The supernatant was centrifuged at $120\,000 \times g$ for 2 h, dialyzed against 10 vol. of 10 mM Tris-HCl for 24 h and then centrifuged at $120\,000 \times g$ for 1 h. The cen-

trifuge^d extract was loaded onto a DEAE Bio-Gel A column (5×25 cm) equilibrated with 10 mM Tris-HCl and the column washed with 500 ml of the same buffer. A gradient of 1500 ml 10 mM Tris-HCl and 1500 ml 400 mM Tris-HCl was set up. A molybdenum-iron protein, gray in color, came off at 100–125 mM Tris-HCl. The hydrogenase and desulfoviridin eluted off together at about 200–250 mM Tris-HCl. The hydrogenase activity recovered in the H_2 evolution was 51%.

This last fraction was loaded onto a Bio-Rad hydroxylapatite column (HTP) (4.3×26 cm) equilibrated with 250 mM Tris-HCl. The column was washed with 250 mM Tris-HCl and a reverse gradient of 400 ml 250 mM Tris-HCl and 400 ml 10 mM Tris-HCl was set up. The column was then washed with 200 ml of 10 mM KPB. A gradient of 1500 ml 10 mM KPB and 1500 ml 500 mM KPB was set up. The desulfoviridin eluted off at about 200 mM to 250 mM KPB and had a 409/630 nm ratio of 2.80 and a 279/630 nm ratio of 4.7. The hydrogenase band (brown) began to migrate at about 450 mM KPB and was collected at 500 mM KPB. The activity was 65% of that from the DEAE Bio-Gel column. The sample was concentrated in a diaflow apparatus using a YM 30 membrane. It was loaded onto a high-pressure liquid chromatography gel exclusion column which was equilibrated with 500 mM KPB (pH 7.5). Different fractions were analyzed spectroscopically and those with the highest 400/280 nm ratios were combined. Gel electrophoresis revealed one major band for the hydrogenase and the protein was estimated to be 95%

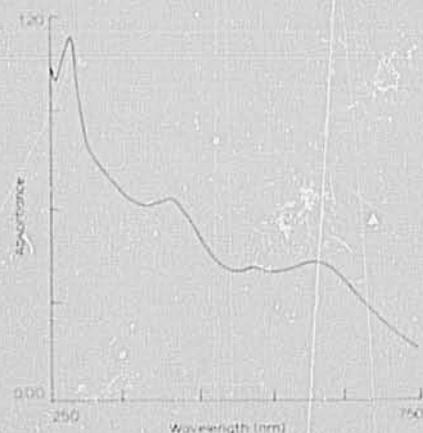


Fig. 1. Electronic absorption spectrum of oxidized molybdenum-iron protein from *Desulfovibrio salzigens* recorded at 25°C .

pure. The final yield of hydrogenase was 15.2%. A summary of the hydrogenase purification is found in Table 1.

The desulfoviridin appeared to be pure by the absorbancy ratios which were identical to that obtained for pure desulfoviridin from *D. gigas* [30] and *D. vulgaris* Hildenborough [31]. Before staining the electrophoresis gel there was one green band about 25% down the gel and a red band which migrated near the bromophenol blue. After staining, one major band was found in the location of the green band. The location of the red band stained but slowly dissipated after 24 h. This band is believed to be a siroheme which is part of the desulfoviridin [51]. The enzyme was judged to be about 95% pure.

The gray protein from the DEAE Bio-Gel column was loaded onto a HTP column (5 × 24 cm) equilibrated with 150 mM Tris-HCl. The column was developed with a KPB gradient up to 1 M and very little of the gray protein came off. The column was then washed with 2 M KPB and the majority of the protein eluted off the column. The 278,612 nm ratio is 2.92. The absorbance spectrum of this protein (Fig. 1) appears to be very similar to the molybdenum-iron-sulfur protein spectrum of *D. africanus* [21] and has the same 278.5:615 nm ratio.

Characterization of hydrogenase

The hydrogenase of *D. salaxigens* is brown in color and its native form exhibits a typical hydrogenase UV/visible absorption spectrum with a broad shoulder around 400 nm (Fig. 2). SDS gel electrophoresis showed that it is composed of two different subunits of molecular weight $62\,000 \pm 5\,000$ and $35\,000 \pm 3\,000$. The total molecular weight by addition of the subunits is 97 000 but the molecular weight found by sedimentation equilibrium is 81 000. The ratio of 400/280 nm is 0.28 and the extinction coefficient at 400 nm using a molecular weight of 81 000 is $46\text{ mM}^{-1}\text{ cm}^{-1}$.

A metal content of 12.3 iron atoms, 0.8 nickel atoms and 0.86 selenium atoms per molecule of hydrogenase was determined. The hydrogenase does not exhibit an activation phase before maximum H_2 evolution activity. The specific activity in the H_2

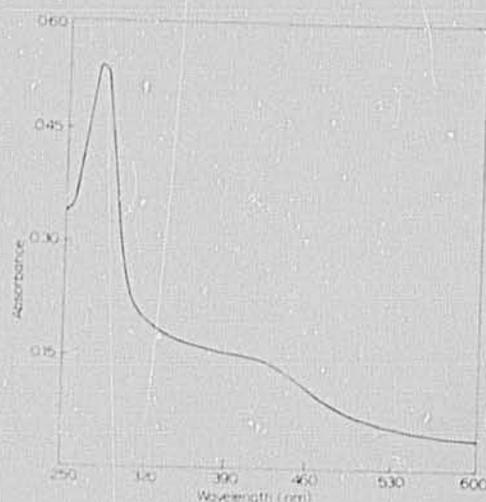


Fig. 2. Electronic absorption spectrum of oxidized *Desulfovibrio salaxigens* hydrogenase at 25°C.

evolution is $1830\ \mu\text{mol/min}$ per mg protein and is $1300\ \mu\text{mol/min}$ per mg protein in H_2 consumption.

Characterization of desulfoviridin

The optical spectrum of desulfoviridin shows absorption bands at the following wavelengths (nm): 630 (0.595), 584 (0.374), 409 (1.668), 391.5 (1.544) and 279 (2.79); relative intensities are indicated in parenthesis. The molecular weight, determined by sedimentation equilibrium, is 191 000. The metal content is 31 iron atoms and 1.4 zinc atoms per molecule. The specific activity is $136\text{ nmol H}_2\text{ consumed/min}$ per mg protein. The activity was found to be greater at pH 6.0 than at pH 7.6 which indicates that bisulfite is the substrate [42]. An end product of the reaction was sulfide, appearing as a yellow precipitate of cadmium sulfide, which was formed in the center well of the reaction vial.

Characterization of molybdenum protein

The molybdenum-iron protein is gray-green in color and exhibits an absorbance spectrum with peaks around 612, 410, 275 nm and a shoulder at 319 nm (Fig. 1). Denaturing gel electrophoresis

Table 2
Properties of assimilatory (A) and dissimilatory (D) sulfite reductases from anaerobic bacteria

Sulfite reductase	Mr ($\times 10^{-3}$)	Activity ^a	Active center	Reference
<i>Desulfuromonas acetoxidans</i> (A)	23.5	906	Sir + Fe-S(α)	[35]
<i>D. vulgaris</i> (A) Hildenborough	27	900	Sir + Fe-S(α)	[25]
<i>M. burkeri</i> (DSM800) P 590 (A)	23	2790	Sir + Fe-S(α)	[42]
<i>Desulfatovacuum nigrificans</i> P 582 (D)	145	65*	Sir + Fe-S	[4]
<i>D. gigas</i> desulfoviridin (D)	200	632	Sir + Fe-S($\alpha_2\beta_2$)	[30]
<i>D. vulgaris</i> Hildenborough desulfoviridin (D)	226	260	Sir + Fe-S($\alpha_2\beta_2$)	[31]
<i>D. desulfuricans</i> Norway 4 desulforubidin (D)	225	430	Sir + Fe-S($\alpha_1\beta_1$)	[32]
<i>D. baculatus</i> 9974 desulforubidin (D)	n.d.	198	Sir + Fe-S($\alpha_2\beta_2$)	Fauque (unpublished)
<i>Thermodesulfobacterium commune</i> desulfotascidin (D)	167	2000	Sir + Fe-S($\alpha_2\beta_2$)	[22]

^a Activity expressed in nmol H₂ consumed/min per mg protein at pH 6 at 30°C except for *Thermodesulfobacterium commune* at 65°C

* partially purified P-582

Sir, siroheme; Fe-S, iron sulfur center.

n.d., not determined

yielded bands of molecular weights of approximately 13 250, 26 000, 42 500 and 64 300. These results suggest a protein of several subunits of molecular weight of 13 250. The conditions were probably not sufficient to completely dissociate the protein. The molecular weight as determined by high-pressure liquid chromatography analytical gel exclusion column is approximately 110 000. The enzyme has three molybdenum atoms and 20 iron atoms for 110 000 molecular weight. The amount of molybdenum protein in the bacterium was large. The final amount is about 50 mg per 250 g of cells.

DISCUSSION

The location of a hydrogenase in the periplasmic space of *D. salicigenis* is not unusual. Periplasmic

origin of hydrogenase is common in sulfate-reducing bacteria of the genus *Desulfovibrio*. *D. vulgaris* strains Hildenborough [57], Marburg [6], Miyazaki [2], *D. gigas* [9], *D. desulfuricans* (NRC 49001) [39], and *D. baculatus* strain 9974 [55].

The existence of more than one hydrogenase in the same species of the genus *Desulfovibrio* has been postulated [43].

The need for chloride at high concentration (> 2%) in order to prevent the clumping of cells from *D. salicigenis* may be related to some function in the outer membrane or in response to the use of a high concentration of EDTA. It has been found that *D. salicigenis* has an absolute requirement for chloride [37] and the cells in our buffer conditions exhibited an abnormal response to less than 2% of it.

It has been proposed that a periplasmic location

of hydrogenase in anaerobic bacteria is a specific adaptation important for utilization of low level of H_2 and for interspecies H_2 transfer [9]. The periplasmic location of hydrogenase would be important in hydrogen utilization from metal surfaces or iron sulfide films. However, attempts to show direct correlation between hydrogenase activity and bacterial corrosion have shown mixed results. [19]. Recently, a marine strain of a sulfate-reducing bacterium has been found to utilize cathodically produced hydrogen from a metal surface [20]. Conclusions concerning 'hydrogenase-less' sulfate-reducing bacteria will have to be revised because many of these strains are now known to have hydrogenase activity, e.g., *Desulfotomaculum orientis* [34] and *Desulfovibrio desulfuricans*. El Agheila Z (our own unpublished results).

Desulfovibrio salexigenis hydrogenase is similar to the recently purified Fe-Ni-Se hydrogenases found in *D. desulfuricans* Norway 4 [48] and *D. haerulatus* strain 9974 [55]. The only known selenium-containing hydrogenase without nickel is from *Methanococcus vannielii* [60]. The function of selenium in this enzyme is not known. Other nickel-containing hydrogenases are present in sulfate-reducing bacteria of the genus *Desulfovibrio*, i.e., *D. gigas* [33], *D. desulfuricans* ATCC 27774 [29] and *D. multispicans* [15]. The *D. salexigenis* hydrogenase does not exhibit an activation phase as found in two nickel-containing hydrogenases from *Desulfovibrio* [10, 15, 36]. There is no indication in the published results of an activation phase in the nickel-selenium-containing hydrogenases. The specific activity in hydrogen evolution of *D. salexigenis* hydrogenase is the highest presently known value for nickel or nickel-selenium hydrogenases from *Desulfovibrio*. This specific activity is higher than previously reported with the hydrogenase isolated following a different purification procedure [14]. The native, partially reduced and fully reduced states, using hydrogen or dithionite as electron donors, of the *D. salexigenis* hydrogenase have been analyzed by electron paramagnetic resonance spectroscopy [56].

The dissimilatory sulfite reductases of *Desulfovibrio* are complex structures ($\alpha_2\beta_2$ subunit structures) with molecular weights of about 200 000,

Fe-S clusters and sirohemes (Table 2). The absorption spectrum and molecular weights of *D. salexigenis* desulfoviridin are similar to that found in *D. gigas* [30] and *D. vulgaris* Hildenborough [31]. The specific activity of *D. salexigenis* desulfoviridin is lower than other dissimilatory type sulfite reductases from *Desulfovibrio* and of assimilatory type sulfite reductases from sulfate or sulfur reducers and methanogenic bacteria (Table 2). Comparison of sulfite reductase activity from lysed cell extracts of different *Desulfovibrio* showed that *D. salexigenis* had also the lowest activity (our unpublished results). Dissimilatory sulfite reductases may under different assay conditions form trithionate and thiosulfate in addition to sulfide. Desulfoviridin from this bacterium was able to qualitatively form sulfide in our assay conditions.

The optical absorbance spectrum and molybdenum/iron content of the *D. salexigenis* molybdenum/iron protein are very similar to the one found in *D. africanus* [21]. Similarity between cytochrome c_3 of *D. africanus* Benghazi and *D. salexigenis* British Guiana have also been found [52]. However, antiserum to *D. africanus* Benghazi cells reacts weakly to *D. salexigenis* British Guiana cells [53].

The function of the molybdenum protein in *Desulfovibrio* is unknown. Molybdenum in bacteria is found associated with the nitrogenase, formate, dehydrogenase and nitrate reductase enzymes [24]. The amount of molybdenum protein in this bacterium is large as is found in *D. africanus* (30 mg/250 μ g wet weight cells) and implies an important function. In *D. salexigenis* the amount of cytochromes is low and perhaps the molybdenum protein may function as an electron transport protein. The high molecular weight and the subunit structure may also imply a metal storage capacity for this protein. Further studies on the physiological function of this protein are in progress.

Metal corrosion in sea water is an important problem for the off-shore oil industry. *Desulfovibrio salexigenis* is an obligative halophilic sulfate-reducing bacterium and may be used as a reference organism. Hydrogenase, sulfite reductase, and molybdenum protein (metal storage protein) which can be key enzymes in anaerobic bacterial corrosion

deserve to be well defined in this bacterium. A study of these important enzymes will allow a better understanding of how these enzymes function, their role in microbially caused corrosion, and a more rational development of biocides to control these bacteria.

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REFERENCES

- Adams, M.W.W., L.E. Mortenson and J.S. Chen. 1981. Hydrogenase. *Biochim. Biophys. Acta* 594: 105-176.
- Aketagawa, J., K. Kobayashi and M. Ishimoto. 1983. Characterization of periplasmic hydrogenase from *Desulfovibrio vulgaris* Miyazaki. *J. Biochem.* 93: 755-762.
- Akagi, J.M. 1981. Dissimilatory sulphate reduction, mechanistic aspects. In: *Biology of Inorganic Nitrogen and Sulfur* (Bothe, H. and A. Trebet, eds), pp. 169-177. Springer-Verlag, Berlin.
- Akagi, J.M. and V. Adams. 1973. Isolation of a bisulfite reductase activity from *Desulfotomaculum nigrificans* and its identification as the carbon monoxide-binding pigment, P₅₄₂. *J. Bacteriol.* 116: 392-298.
- Albracht, S.P.J., E.G. Graf and R.K. Thauer. 1982. The EPR properties of nickel in hydrogenase from *Methanobacterium thermoautotrophicum*. *FEBS Lett.* 140: 311-313.
- Badziong, W. and R.K. Thauer. 1980. Vectorial electron transport in *Desulfovibrio vulgaris* (Marburg) growing on hydrogen plus sulfate as sole energy source. *Arch. Microbiol.* 125: 167-174.
- Barata, B.A.S., I. Moura, A.V. Xavier, G. Fauque, J. LeGall and J.J.G. Moura. 1984. Molybdenum containing iron-sulfur protein from *Desulfovibrio desulfuricans* (Berre-Eau) - EPR spectroscopic characterization of the redox centers. PC3 in Setimo Encontro Anual da Sociedade Portuguesa de Quimica, 9 a 14 de Julho, 1984, Lisboa.
- Bell, G.R., J.P. Lee, H.D. Peck, Jr. and J. LeGall. 1978. Reactivity of *Desulfovibrio gigas* hydrogenase towards artificial and natural electron donors. *Biochimie* 60: 315-320.
- Bell, G.R., J. LeGall and H.D. Peck, Jr. 1974. Evidence for the periplasmic location of hydrogenase in *Desulfovibrio gigas*. *J. Bacteriol.* 120: 994-997.
- Berlier, Y.M., G. Fauque, P.A. Lespinat and J. LeGall. 1982. Activation, reduction and proton-deuterium exchange reaction of the periplasmic hydrogenase from *Desulfovibrio gigas* in relation with the role of cytochrome c₁. *FEBS Lett.* 140: 185-188.
- Brewer, J.M. and R.B. Ashworth. 1969. Disc electrophoresis. *J. Chem. Educ.* 46: 41-45.
- Costello, J.A. 1974. Cathodic depolarization by sulfate reducing bacteria. *S. Afr. J. Sci.* 70: 202-204.
- Crombie, D.J., G.J. Moody and J.D.R. Thomas. 1980. Corrosion of iron by sulfate-reducing bacteria. *Chem. Indust.* 21: 500-504.
- Czechowski, M., G. Fauque, Y. Berlier, P.A. Lespinat and J. LeGall. 1985. Purification of an hydrogenase from an halophilic sulfate reducing bacterium: *Desulfovibrio salicigenis* strain British Guiana. *Rev. Port. Quim.* 27: 196-197.
- Czechowski, M.H., S.H. He, M. Nacro, D.V. DerVartanian, H.D. Peck, Jr. and J. LeGall. 1984. A cytoplasmic nickel-iron hydrogenase with high specific activity from *Desulfovibrio multispirans* sp. N., a new species of sulfate reducing bacterium. *Biochem. Biophys. Res. Commun.* 108: 1388-1393.
- Drucker, H., E.B. Trousil and L.L. Campbell. 1970. Purification and properties of cytochrome c₁ from *Desulfovibrio salicigenis*. *Biochemistry* 9: 3395-3400.
- Fairbanks, G., T.L. Steck, and D.F.H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10: 2600-2617.
- Fauque, G., M. Teixeira, I. Moura, P.A. Lespinat, A.V. Xavier, D.V. DerVartanian, H.D. Peck, Jr., J. LeGall and J.J.G. Moura. 1984. Purification, characterization and redox properties of hydrogenase from *Methanosarcina barkeri* (DSM 800). *Eur. J. Biochem.* 142: 21-28.
- Hamilton, W.A. 1985. Sulphate-reducing bacteria and anaerobic corrosion. *Annu. Rev. Microbiol.* 39: 195-217.
- Hardy, J.A. 1983. Utilization of cathodic hydrogen by sulfate-reducing bacteria. *Br. Corros. J.* 18: 190-193.
- Hatchikian, E.C. and M. Bruschi. 1979. Isolation and characterization of a molybdenum iron-sulfur protein from *Desulfovibrio africanus*. *Biochem. Biophys. Res. Commun.* 86: 725-734.
- Hatchikian, E.C. and J.G. Zeikus. 1983. Characterization of a new type of dissimilatory sulfite reductase present in *Thermodesulfobacterium commune*. *J. Bacteriol.* 153: 1211-1220.
- Hatchikian, E.C., M. Bruschi and J. LeGall. 1978. Characterization of the periplasmic hydrogenase from *Desulfovibrio gigas*. *Biochem. Biophys. Res. Commun.* 82: 451-461.
- Hinton, S.M. and L.E. Mortenson. 1985. Identification of molybdoproteins in *Clostridium pasteurianum*. *J. Bacteriol.* 162: 477-494.

- 25 Huynh, B.H., L. Kang, D.V. DerVartanian, H.D. Peck, Jr. and J. LeGall. 1984. Characterization of a sulfite reductase from *Desulfovibrio vulgaris*. Evidence for the presence of a low-spin heme and an exchange coupled heme-heme (4Fe-4S) unit. *J. Biol. Chem.* 259: 15373-15376.
- 26 Iverson, W.P. 1974. Microbial corrosion of iron. In: *Microbial Iron Metabolism* (Neilands, J.B., ed.), pp. 475-513. Academic Press, New York.
- 27 Jacobson, F.S., L. Daniels, J.A. Fox, C.T. Walsh and W.H. Orme-Johnson. 1982. Purification and properties of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from *Methanobacterium thermoautotrophicum*. *J. Biol. Chem.* 257: 3385-3388.
- 28 King, R.A. and J.D.A. Miller. 1971. Corrosion by the sulfate-reducing bacteria. *Nature* 233: 491-492.
- 29 Kruger, H.J., B.H. Huynh, P.O. Ljungdahl, A.V. Xavier, D.V. DerVartanian, J. Moura, H.D. Peck, Jr., M. Teixeira, J.J.G. Moura and J. LeGall. 1982. Evidence for nickel and three iron center in the hydrogenase of *Desulfovibrio desulfuricans*. *J. Biol. Chem.* 257: 14620-14623.
- 30 Lee, J.P. and H.D. Peck, Jr. 1971. Purification of the enzyme-reducing bisulfite to trithionate from *Desulfovibrio gigas* and its identification as desulfoviridin. *Biochem. Biophys. Res. Commun.* 45: 583-589.
- 31 Lee, J.P., J. LeGall and H.D. Peck, Jr. 1973. Isolation of assimilatory and dissimilatory-type sulfite reductase from *Desulfovibrio vulgaris*. *J. Bacteriol.* 115: 529-542.
- 32 Lee, J.P., C.S. Yi, J. LeGall and H.D. Peck, Jr. 1973. Isolation of a new pigment desulfoirubidin from a *Desulfovibrio desulfuricans* (Norway strain) and its role in sulfite reduction. *J. Bacteriol.* 115: 451-455.
- 33 LeGall, J., P.O. Ljungdahl, I. Moura, H.D. Peck, Jr., A.V. Xavier, J.J.G. Moura, M. Teixeira, B.H. Huynh and D.V. DerVartanian. 1982. The presence of redox-sensitive zinc periplasmic hydrogenase from *Desulfovibrio gigas*. *Biochem. Biophys. Res. Commun.* 106: 610-616.
- 34 Lospinat, P.A., G. Denariac, G. Fauque, R. Toci, Y. Berlier and J. LeGall. 1985. Fixation de l'azote atmosphérique et métabolisme de l'hydrogène chez une bactérie sulfato-réductrice sporulante, *Desulfotomaculum orientis*. *C.R. Acad. Sci. Paris, T. 301, série III* n.16: 707-710.
- 35 Lino, A.R., J.J.G. Moura, A.V. Xavier, I. Moura, G. Fauque and J. LeGall. 1985. Characterization of two low-spin bacterial heme proteins. *Rev. Port. Quim.* 27: 215.
- 36 Lissolo, T., S. Pulvin and D. Thomas. 1984. Reactivation of the hydrogenase from *Desulfovibrio gigas* by hydrogen. *J. Biol. Chem.* 259: 11725-11729.
- 37 Littlewood, D. and J.R. Postgate. 1957. Sodium chloride acid the growth of *D. sulfobacterium desulfuricans*. *J. Gen. Microbiol.* 17: 378-389.
- 38 Markwell, M.K., S.M. Haas, L.L. Bieber and N.E. Töbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein in samples. *Anal. Biochem.* 87: 206-210.
- 39 Martin, S.M., P.R. Glick and W.G. Martin. 1980. Factors affecting the production of hydrogenase by *Desulfovibrio desulfuricans*. *Can. J. Microbiol.* 26: 1209-1213.
- 40 Moura, J.J.G., A.V. Xavier, M. Bruschi, J. LeGall, D.O. Hall and R. Cammack. 1976. A molybdenum containing iron-sulfur protein from *D. gigas*. *Biochem. Biophys. Res. Commun.* 75: 1037-1044.
- 41 Moura, I., J.J.G. Moura, M. Bruschi and J. LeGall. 1980. Flavodoxin and rubredoxin from *Desulfovibrio salexigens*. *Biochim. Biophys. Acta* 591: 1-8.
- 42 Moura, J.J.G., I. Moura, H. Santos, A.V. Xavier, M. Scandellari and J. LeGall. 1982. Isolation of P-590 from *Methanosaeta barkeri*: evidence for the presence of sulfite reductase activity. *Biochem. Biophys. Res. Commun.* 108: 1002-1009.
- 43 Odom, J.M. and H.D. Peck, Jr. 1981. Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria *Desulfovibrio* sp. *FEMS Lett.* 12: 47-50.
- 44 Peck, H.D., Jr. 1960. Evidence for oxidative phosphorylation during the reduction of sulfate with hydrogen by *Desulfovibrio desulfuricans*. *J. Biol. Chem.* 235: 2734-2738.
- 45 Peck, H.D., Jr. and H. Gest. 1956. A new procedure for assay of bacterial hydrogenase. *J. Bacteriol.* 71: 70-80.
- 46 Peck, H.D., Jr. and J. LeGall. 1982. Biochemistry of dissimilatory sulfate reduction. *Phil. Trans. R. Soc. Lond. B* 298: 443-466.
- 47 Postgate, J.R. 1956. Cytochrome c_3 and desulfoviridin, pigments of the anaerobic *Desulfovibrio desulfuricans*. *J. Gen. Microbiol.* 14: 545-572.
- 48 Rieder, R., R. Cammack and D.O. Hall. 1984. Purification and properties of the soluble hydrogenase from *Desulfovibrio desulfuricans* (strain Norway 4). *Eur. J. Biochem.* 145: 637-643.
- 49 Schachman, H.K. 1979. *Ultracentrifugation in Biochemistry*. Academic Press, New York.
- 50 Schlegel, H.G. and K. Schneider. 1978. Distribution and role of hydrogenases in microorganisms. In: *Hydrogenases: their catalytic Activity, Structure and Function*. (Schlegel, H.G. and K. Schneider, eds.), pp. 15-44. E. Goltz KG, Göttingen.
- 51 Siegel, L.M. 1978. Structure and function of heme and heme enzymes. In: *Mechanisms of Oxidizing Enzymes* (Singer, T.P. and R.N. Oudarza, eds.), pp. 201-214. Elsevier, New York.
- 52 Singleton, R., J. Denis and L.L. Campbell. 1984. Antigenic diversity of cytochromes c_3 from the anaerobic sulfate-reducing bacteria, *Desulfovibrio*. *Arch. Microbiol.* 139: 91-94.
- 53 Singleton, R., J. Denis and L.L. Campbell. 1985. Whole cell antigens of members of the sulfate-reducing genus *Desulfovibrio*. *Arch. Microbiol.* 141: 195-197.
- 54 Starkey, R.L. 1938. A study of spore formation and other morphological characteristics of *Vibrio desulfuricans*. *Arch. Mikrobiol.* 8: 268-304.
- 55 Teixeira, M., I. Moura, A.V. Xavier, J.J.G. Moura, G. Fauque, B. Prickril and J. LeGall. 1985. Nickel-iron-sulfur-selenium containing hydrogenases isolated from *Desulfovibrio bacillatus* strain 9974. *Rev. Port. Quim.* 27: 194-195.

56. Teixeira, M., I. Moura, G. Fauque, M. Czechowski, Y. Berlier, P.A. Lespinat, J. LeGall, A.V. Xavier and J.J.G. Moura. 1986. Redox properties and activity studies on a nickel-containing hydrogenase isolated from a halophilic sulfate reducer *Desulfovibrio salaxigens*. *Biochimie* 68: 75-84.
57. Van der Westen, H.M., S.G. Mayhew and C. Veeger. 1978. Separation of hydrogenase from intact cells of *Desulfovibrio vulgaris*, purification and properties. *FEBS Lett.* 86: 122-126.
58. Von Wolzogen Kuhr, C.A.M. and I.S. Van der Vlugt. 1934. The graphitization of cast iron as an electrochemical process in anaerobic soils. *Water (The Hague)*, 16: 147.
59. Wanklyn, J.N. and C.J.P. Spence. 1952. Influence of sulphate-reducing bacteria on the corrosion potential of iron. *Nature*, 169: 928-929.
60. Yamazaki, S. 1982. A selenium-containing hydrogenase from *Methanococcus vannielii*. Identification of the selenium moiety as a selenocysteine residue. *J. Biol. Chem.* 257: 7926-7929.

28-