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Redox properties of the iron-sulfur clusters in activated Fe-hydrogenase from *Desulfovibrio vulgaris* (Hildenborough)

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The periplasmic Fe-hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) contains three iron-sulfur prosthetic groups: two putative electron transferring [4Fe-4S] ferredoxin-like cubanes (two F-clusters), and one putative Fe/S supercluster redox catalyst (one H-cluster). Combined elemental analysis by proton-induced X-ray emission, inductively coupled plasma mass spectrometry, instrumental neutron activation analysis, atomic absorption spectroscopy and colorimetry establishes that elements with $Z > 21$ (except for 12–15 Fe) are present in 0.001–0.1 mol/mol quantities, not correlating with activity. Isoelectric focussing reveals the existence of multiple charge conformers with pI in the range 5.7–6.4. Repeated re-chromatography results in small amounts of enzyme of very high H_2-production activity determined under standardized conditions ($\approx$7000 U/mg). The enzyme exists in two different catalytic forms: as isolated the protein is 'resting' and O_2-insensitive; upon reduction the protein becomes active and O_2-sensitive. EPR-monitored redox titrations have been carried out of both the resting and the activated enzyme. In the course of a reductive titration, the resting protein becomes activated and begins to produce molecular hydrogen at the expense of reduced titrant. Therefore, equilibrium potentials are undefined, and previously reported apparent E_m and n values [Patil, D. S., Moura, J. J. G., He, S. H., Teixeira, M, Prickril, B. C., DerVartanian, D. V., Peck, H. D. Jr, LeGall, J. & Huynh, B.-H. (1988) *J. Biol. Chem. 263*, 18732–18738] are not thermodynamic quantities. In the activated enzyme an $S = 1/2$ signal ($g = 2.11, 2.05, 2.00$; 0.4 spin/protein molecule), attributed to the oxidized H cluster, exhibits a single reduction potential, $E_{m,7} = -307$ mV, just above the onset potential of H_2 production. The midpoint potential of the two F clusters (2.0 spins/protein molecule) has been determined either by titrating active enzyme with the H_2/H^+ couple ($E'_m = -330$ mV) or by dithionite-titrating a recombinant protein that lacks the H-cluster active site ($E_{m,7.5} = -340$ mV). There is no significant redox interaction between the two F clusters ($n \approx 1$).

The iron-sulfur protein hydrogenase catalyzes the reversible activation of molecular hydrogen, a process involving the transfer of two electrons. Most presently known hydrogenases are also nickel proteins. The nickel ion is generally assumed to be the redox-active catalytic center [1, 2]. A small subclass is formed by the Fe-hydrogenases; these enzymes presumably contain no other potentially redox active transition metals than iron [3]. By exclusion, this implies that the H_2 activation is located on an iron-sulfur cluster. Redox catalysis is not an established property of iron-sulfur clusters, therefore, the study of Fe-hydrogenase has an added fundamental relevance. Furthermore, of all hydrogenases, the *Desulfovibrio vulgaris* (Hildenborough) Fe-hydrogenase exhibits the highest specific activity in the hydrogen-production assay (cf. reviews [1, 3]). Therefore, knowledge of its active-site structure and mechanism has a potential technological relevance.

Fe-hydrogenases have two types of Fe/S clusters: one H cluster and two or four F clusters [3]. The activation of molecular hydrogen ($2e^-$ extraction) is thought to take place at the H cluster, an Fe/S cluster of unknown structure, which we have hypothesized to bear similarity to the [6Fe-6S] prismane model compounds [4]. Sequence comparison indicates that the protein coordination to the H cluster includes five Cys residues [5].

Reduction equivalents are channeled through the F clusters. These are presumably of the ferredoxin [4Fe-4S]$^{(2+;1+)}$ cubane type ($1e^-$ transfer). At present only four

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Abbreviations. PIXE, particle-induced X-ray emission; INAA, instrumental neutron activation analysis; ICP-MS, inductively coupled plasma mass spectrometry; AAS, atomic absorption spectrometry; NHE, normal hydrogen electrode.

Enzymes. Fe-hydrogenase or H_2: ferricytochrome-c_3 oxidoreductase (EC 1.12.2.1); Fe-hydrogenase or ferredoxin: H^+ oxidoreductase (EC 1.18.99.1).

Fe-hydrogenases have been purified to homogeneity. There are two F clusters in *D. vulgaris* Fe-hydrogenase [6], in *Megasphaera elsdenii* Fe-hydrogenase [7], and in *Clostridium pasteurianum* Fe-hydrogenase II [8]. The latter enzyme has a very low H_2-production activity. *C. pasteurianum* Fe-hydrogenase I probably contains four F clusters [5, 8]. The *D. vulgaris* Fe-hydrogenase has been crystallized repeatedly but internal crystal disorder has thus far precluded analysis by X-ray crystallography [9, 10].

With EPR spectroscopy six different signals have been identified from *D. vulgaris* hydrogenase: (a) the 'F-signal' from the two weakly dipole-coupled, reduced F clusters [11]; (b) the 'H signal', a rhombic signal (g = 2.11, 2.05, 2.00) [12]; (c) a 'second rhombic signal' (g = 2.07, 1.96, 1.89) [9]; (d) the '3Fe signal', a signal reminiscent of the EPR from the oxidized [3Fe-4S] cluster (g = 2.02) [11]; (e) an 'axial signal' (g = 2.06, 2.00) [13]; (f) a 'high-spin signal' (g = 5.0) [12]. Only two of these, the H signal and the F signal, have also been identified in all three other Fe-hydrogenases [14–16].

The redox properties associated with the first four signals have been studied by Patil et al. using 'resting' (or 'as-isolated', 'O_2-insensitive', 'de-activated') hydrogenase in EPR-monitored reductive titrations with sodium dithionite [17]. We have repeated these experiments and although we find the data of Patil et al. to be reproducible, we also find that they are not interpretable in a pure thermodynamic framework because the hydrogenase activates during the reductive titration. We have, therefore, extended our studies to pre-activated enzyme and to recombinant protein that lacks the H cluster. We have also re-examined some basic properties of *D. vulgaris* hydrogenase, *viz.* metal content, surface charge, and standardized specific activity.

MATERIALS AND METHODS

Strain, growth and harvesting

The sulfate-reducing anaerobe *Desulfovibrio vulgaris*, subspecies Hildenborough (NCIB 8303) was grown on Saunders' medium N [18] as stirred 240-l batch cultures in a home-built glass rectangular fermentor at 35 °C under nitrogen atmosphere. In the mid-to-late-log phase (absorbance at 600 nm ≈0.8) 200 g cells were harvested by continuous centrifugation (Sharples laboratory Super-centrifuge) at a flow rate of 0.4 l/min. After resuspension in 3 vol. water at 4 °C, the periplasm was extracted essentially according to [19] by gentle disintegration of the cell wall with EDTA (1:1 dilution to a final concentration of 50 mM Na_2 EDTA plus 50 mM Tris/HCl pH 9, raising the temperature to 32 °C and stirring until the onset of viscosity). The resulting suspension was immediately cooled and centrifuged (7000 g, 30 min, 4 °C). The pH of the supernatant was adjusted to 8.0 by gentle addition of 1 M KH_2PO_4 and thoroughly dialyzed against standard buffer (10 mM Tris/HCl pH 8.0, 4 °C). Minor precipitates formed during dialysis were removed by centrifugation (14000 g, 30 min, 4 °C).

Enzyme purification

The first part of the purification scheme is a slight modification and an order-of-magnitude scaling-up of the original procedure described by van der Westen et al. [19]. All steps were carried out at 4 °C except for the final purification with FPLC (ambient temperature). The dialyzed extract was applied to a column (5 cm × 20 cm) of DEAE-Sephacel (Pharmacia) pre-equilibrated with standard buffer. After application, the column was washed with 1000 ml standard buffer with 20 mM NaCl and eluted with a linear gradient (1600 ml) of 20–400 mM NaCl in standard buffer. Active fractions were pooled, dialyzed against standard buffer, and concentrated on a small (2.5 cm × 8 cm) column of DEAE-Sephacel. The concentrate (≈15 ml) was applied to a column (3.5 cm × 100 cm) of Sephadex G-150 equilibrated and eluted with 25 mM potassium phosphate pH 7.5. Fractions were pooled on the basis of activity and a limiting purity index, $f = A_{400}/A_{280} > 0.25$. The pool was applied to a column (2.5 cm × 9 cm) of hydroxyapatite (Bio-Gel HT, Bio-Rad) pre-equilibrated with 25 mM potassium phosphate pH 7.5. This column was eluted with a linear gradient (600 ml) of 25–300 mM potassium phosphate pH 7.5. Fractions with $f >$ 0.33 were pooled, dialyzed against standard buffer, and concentrated on YM 30 filters in Diaflo Amicon concentrators (50 and 10 ml). The concentrate was split-up in portions of 1–2 mg and in subsequent runs applied to a preparative MonoQ HR 5/5 anion-exchange column attached to a Pharmacia FPLC system. The column was eluted with a 40-ml gradient of 0–500 mM NaCl in standard buffer. The eluate was fractionated according to the peak pattern of on-line monitored absorbance at 280 and 436 nm and subsequently checked for their f factor and activity. Fractions with $f > 0.350$ were pooled and Na_2EDTA was added to a concentration of 0.5 mM. The final preparation was frozen in liquid nitrogen after dialysis against 5 mM Tris/HCl pH 8.0 and concentration on Centricon devices with YM-30 filter. The results of a typical purification are summarized in Table 1; the last step is discussed in Results.

Table 1. Purification of *D. vulgaris* (Hildenborough) periplasmic hydrogenase. The periplasmic extract was prepared from freshly harvested cells (178 g wet mass) according to Materials and Methods. Activity was measured with the Chen and Mortenson [28] manometric hydrogen-production assay.

Fraction	Volume	Protein	Activity	Specific activity
	ml	mg	kU	U/mg
Periplasmic extract	1130	5850	181.5	31
Extract after dialysis	1550	5810	136.5	23
DEAE-Sephacel	209	743	112.2	238
Sephadex G-150	74	134	83.3	622
Hydroxyapatite	91	26.0	57.0	2192
FPLC MonoQ	2.0	10.7	35.2	3290
MonoQ two-fold rerun	0.6	0.6	4.2	6900

Recombinant (H-cluster-lacking) protein

Recombinant *D. vulgaris* (Hildenborough) Fe-hydrogenase was isolated and purified from the TG1 derivative of *Escherichia coli*, strain JM101, transformed with plasmid pHV150 (plasmid pUC9 with an insert consisting of a *lac* promotor plus the two structural genes for hydrogenase *hydA* and *hydB*). The construction of the clone, isolation from five 1-l cultures and purification of the recombinant protein have previously been described [20]. TG1(pHV150) produces about ten times more hydrogenase subunits/cell protein than *D. vulgaris*; however, only some 10% is soluble and properly assembled into an $\alpha\beta$ dimer [21], from which the recombinant hydrogenase is purified. Its protein part is almost indis-

tinguishable from wild-type hydrogenase, however, the F clusters are present substoichiometrically and the H cluster is completely absent [20]. The specific activity is lower than 0.1 U/mg in the hydrogen-production assay. The Fe/S clusters in the recombinant protein do not react with molecular hydrogen; they can be reduced with dithionite.

Megasphaera elsdenii Fe-hydrogenase

The fermentative rumen bacterium *Megasphaera elsdenii*, strain LC 1 [22], was grown as in [23]. The Fe-hydrogenase was isolated and purified anaerobically as previously described [7].

Hydrogen-production activity

No general agreement exists at this time on the standard conditions for assaying H_2-production activity (1 unit $\equiv$ 1 μmol H_2/min at 30 °C). Therefore, direct comparison of literature data is in general not possible. The reaction studied is usually the methyl-viologen semiquinone cation radical: H^+ oxidoreductase activity, with the radical produced by reduction of the viologen with excess sodium dithionite. The major problem is the choice of pH. It affects: (a) the substrate concentration, (b) the $2\,H^+ + 2e \rightleftarrows H_2$ equilibrium, (c) the extent of reduction of the methyl viologen via the pH dependence of the midpoint potential of the $S_2O_4^{2-}/SO_3^{2-}$ couple, (d) the stability of the enzyme and of the methyl-viologen semiquinone and (e) the catalytic performance of the enzyme itself. In addition to the pH problem there are other problems related to the viologen concentration, the method of H_2 detection, the purity of sodium dithionite and the stimulating or inhibiting effects of certain salts and buffers.

The activity has no well-defined pH optimum. For all Fe-hydrogenases it increases continuously with decreasing pH down to the range in which enzyme inactivation sets in [24–26], probably by destabilization of [Fe-S] centres. In their initial description of the assay, Peck and Gest prescribed a 'buffer of appropriate pH' and subsequently used 63 mM potassium phosphate pH 6.5 [27]. Also, they used 13 mM methyl viologen, a concentration at which dimerization of the semiquinone is considerable (the dissociation constant is typically 2 mM, depending on the ionic strength; see [23] and references quoted therein). The assay was considerably modified in the proposal of Chen and Mortenson: 50 mM Tris/HCl pH 8 and 1 mM methyl viologen [28]. Although the complication of significant semiquinone dimerization is avoided, these modified conditions result in significantly suboptimal activity numbers because the pH is high, the viologen concentration is low, and Tris buffer has an inhibitory effect on at least some Fe-hydrogenases, in particular the ones from *M. elsdenii* [15] and from *D. vulgaris* (our unpublished observations).

An additional complication is in the detection of the molecular hydrogen produced. This is conveniently done manometrically in a Warburg apparatus [27, 28] or by gas chromatography with catharometric detection [29]. The assay conditions of Chen and Mortenson combined with H_2 detection in the Warburg were specifically designed as a convenient method to determine activity on undiluted samples all the way through a purification procedure [28]. Suitable amounts of hydrogenase for this manometric hydrogen production assay are 0.01–2 U for the conventional 13–22 ml Warburg vessels. Gas chromatography has a larger dynamic range combined with a much lower H_2-detection limit. With a Varian 3400 gas chromatograph (0.5-nm molecular sieve column 150 cm × 0.6 cm, catharometric detection) progression curves can easily be recorded for 0.0001–10 U hydrogenase by injection of 10–250-μl volumes of the 6-ml headspace, sampling every 0.2–90 min. The gas chromatograph is calibrated with diluted and pure hydrogen gas (10–500 μl). The calibration of the head-space volume is checked by standard addition of hydrogen to the head-space of the vials.

Working with purified samples of *D. vulgaris* Fe-hydrogenase, we have compared the two methods in parallel experiments. In both methods we detected H_2 evolution from 3.25 g/l dithionite (> 87% purity obtained from Merck) with 1 mM methyl viologen as redox mediator, 0.5 mg/ml bovine serum albumin (Sigma) as stabilizer, in 50 mM Tris/HCl pH 8.0 at 30 °C in 2-ml volume. The vessels were shaken at 150 strokes of ≈4 cm/min. For both assays, linearity of hydrogen production was observed during the production of 0–15 μmol hydrogen with 0.05–2 U (Warburg) and 0.0005–5 U (gas chromatograph) of hydrogenase. It is obvious that the buffering capacity of 100 μmol Tris buffer is insufficient to keep the pH at 8.0 during the generation of up to 15 μmol of the acidic HSO_3^- from dithionite. The pH of reaction mixtures just after the production of 1–15 μmol hydrogen was usually in the range of 7.9–7.2. As the Fe-hydrogenase exhibits a higher activity at lower pH [25], both assays must have an artificial linearity during the production of 1–15 μmol hydrogen. This could result from a balancing effect between the activity increase due to the lower pH and an activity decrease due to hydrogen inhibition [25] or to lowering of the methyl-viologen-semiquinone concentration by production of sulfite [25]. Our comparison between the results of the manometric and gas chromatographic procedure applies to a constant pH 8.0 during the assay. Although the assay conditions were exactly identical and linearity and calibration of the manometric and gas-chromatographic procedure were thoroughly checked, resulting activity numbers were not mutually consistent. The gas chromatographic method systematically gives 30–50% higher values. We have presently no explanation for this discrepancy.

We have elaborated, above, on the multiple complications that arise in attempts to compare Fe-hydrogenase activity data taken under non-identical conditions. We emphasize their importance for a meaningful comparison of our present work with other studies on hydrogenase. Specifically, we intend to contrast our previous and present work with that from Huynh and collaborators, who have been working on what appears to be the same enzyme from the same bacterial source [13, 17, 30]. The dispute concerns difference in specific activity (cf. the comparison of the two preparations in [1]), difference in iron stoichiometry [4, 13], and difference in redox properties (see below). All activity data reported in Results and Discussion and in all our previous work, refer to measurements with the, intentionally, suboptimal manometric assay of Chen and Mortenson [28]. Contrarily, activities of the preparations described in [13, 17, 30] refer to the Peck and Gest assay [27], or to a modification thereof using 100 mM potassium phosphate pH 7.6 [30], and subsequent gas chromatographic detection of H_2. An apparent increase in (specific) activity, by at least 30%, ensues from each of the following substitutions: (a) Tris/HCl by potassium phosphate; (b) initial pH 8.0 by pH 7.6; (c) manometric by gas chromatographic detection. Furthermore, the use of 13 mM [27] instead of 1 mM methyl viologen [28] in our experiments results in a striking 2–2.5-fold increase of the apparent (specific) activity (cf. Fig. 5 in [25]) both with the Warburg and the gas chromatographic procedure.

Hydrogenase pre-activation

Resting, O_2-insensitive protein was converted to active, O_2-sensitive enzyme by means of a complete argon, hydrogen, argon redox cycle (cf. [12, 31]). For the hydrogen activation the protein was incubated under 120 kPa hydrogen pressure with continuous stirring for 30 min at ambient temperature. Subsequently the H_2 was replaced with argon (120 kPa pressure) in 6–8 vacuum/argon cycles under continuous stirring. For the reductive dye-mediated redox titrations the hydrogenase was activated by adding the anaerobic resting enzyme to a pre-reduced mediator mixture.

Redox titrations

Reductive titrations with buffered sodium dithionite and oxidative titrations with potassium ferricyanide or 2,6-dichloroindophenol were carried out at 25 °C in an anaerobic cell under argon. The potential of the stirred solution was measured at a platinum electrode (Radiometer P-1312) with respect to the potential of a saturated calomel electrode (Radiometer K-401). All potentials have been recalculated with respect to the normal hydrogen electrode (NHE). Redox mediator dyes (40 μM each) were *N,N,N'N'*-tetramethyl-*p*-phenylenediamine, 2,6-dichloroindophenol, phenazine ethosulfate, methylene blue, resorufin, indigo disulfonate, 2-hydroxy-1,4-naphthoquinone, anthraquinone-2-sulfonate, phenosafranine, safranine o, neutral red, benzyl viologen, methyl viologen.

Titrations with the H_2/H^+ couple were done by stirred incubation of the protein under H_2 pressures of 10–120 kPa for 30 min at ambient temperature. The H^+ concentration was pre-set by passing the protein over a small Bio-Gel P-6DG molecular sieve column (2-ml bed volume) equilibrated with 100 mM buffer of variable pH, using piperazine (pH 4.5 –5.1), Mes (pH 5.3–6.0), Hepes (pH 6.3–8.9), or Ches (pH 9.3–10.2). The resulting bulk potential was calculated (at 25 °C) according to:

$$E = -0.05915\,[\mathrm{pH} + 1/2 \log (p_{H_2}/p_0)], \qquad (1)$$

with $p_0 \equiv 101.3$ kPa (i.e. atmospheric pressure).

Elemental analysis

Colorimetry

The procedures for determination of protein, iron and molybdenum/tungsten with the microbiuret, ferene and dithiol methods, respectively, were as outlined in [32] and the references therein.

PIXE

Multi-elemental analysis was performed with particle-induced X-ray emission (PIXE) at the Eindhoven University of Technology. Sample application to the Millipore MF SCWP 8-μm filters was according to [32, 33]. Energy-dispersive X-ray emission spectra were digitally recorded during the irradiation with a 3-MeV proton beam from a cyclotron and analyzed with the software as described in [34]. Iron as determined by the ferene method served as internal standard for the determination of other elements.

ICP-MS

Inductively coupled plasma mass spectroscopy (ICP-MS) [35] was carried out with a VG Instruments PlasmaQuad at the Koninklijke/Shell-Laboratorium (Amsterdam). Data were analyzed with the software supplied by the manufacturer. Samples were mixed with 1% HNO_3 prior to analysis. Controls were included of buffer blanks and of samples with known concentrations of relevant elements. We note that the argon plasma, the aqueous nitric acid matrix, and the polypeptide backbone (or, more likely its decomposition products) produce some artificial peaks in the mass spectrum. For most elements interferences can easily be excluded or quantitated by checking the isotope composition of the elements. The element of interest that is most seriously affected by background interference is Fe. Contributions of the background (i.e. $^{40}Ar^{16}O$) were 2–16% for the nominal mass of 56 Da. With a careful correction for the usually slowly drifting background, reliable quantitative determination of Fe was possible.

INAA

Instrumental neutron activation analysis (INAA) [36] was performed at the Interuniversitair Reactor Centrum, Delft. Samples were desalted by gel-filtration on BioGel P-6DG equilibrated with demineralized water, and lyophilized by freeze-drying in the polyethylene INAA vials. INAA is not very sensitive for Fe and therefore has an inherently large relative error of 20–3% for our samples containing quantities of 1–27 μg Fe/vial. We have, nevertheless, used the Fe content as an internal standard to correct for possible losses during the gel-filtration and lyophilization pre-treatment.

AAS

Nickel was determined by atomic absorption spectroscopy (AAS) at 231.4 nm with a Perkin-Elmer atomic absorption spectrometer (ICP 5500) equipped with an HG-400A graphite furnace atomizer. Samples were successively mixed with HNO_3 and ethanol to final concentrations of 5% and 20% (by vol.), respectively. Prior to injection into the graphite furnace samples were spun (7000 *g*, 10 min) to remove denatured protein. The recovery of Ni added as $NiCl_2$ to the protein was > 95%.

Isoelectric focussing

Flat-bed isoelectric focussing on Serva Precotes (pI 5–7) was performed on a LKB Ultrophor electrophoresis unit at 4 °C. Markers were parvalbumin (rabbit muscle), carbonic anhydrase (bovine erythrocytes) and alcohol dehydrogenase (horse liver), with isoelectric points at 4 °C of 5.3, 5.8 and 6.8, respectively [37]. Activity staining was according to [38].

Spectroscopy

Ultraviolet/visible data were obtained with a DW-2000 spectrophotometer. EPR spectroscopy was done with a Bruker EPR 200 D spectrometer with peripheral equipment and data handling as described in [39]. The modulation frequency was always 100 kHz.

RESULTS

Metal content of *D. vulgaris* and *M. elsdenii* Fe-hydrogenase

The metal content of Fe-hydrogenases has been for several years a matter of debate and will continue to be so. There are

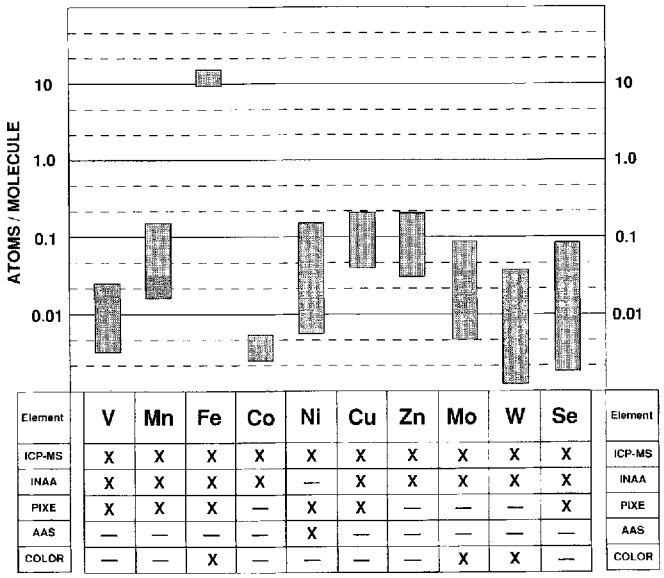

Fig. 1. Elemental analysis of *D. vulgaris* Fe-Hydrogenase. The bars indicate the range of observed amounts of the elements, analyzed with the tagged techniques. The diagram summarizes the results from determinations on preparations with specific activities of 2100–6900 U/mg. For elements other than Fe the lower end of the bars indicates the limit of detection for 0.1–1 mg amounts of Fe-hydrogenase per determination. Ferene (Fe) and dithiol (Mo and W) colorimetric (COLOR) techniques, and the ICP-MS, INAA, PIXE and AAS methods were according to Materials and Methods.

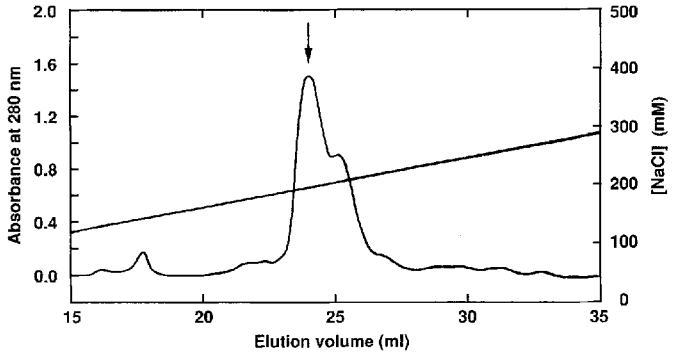

Fig. 2. Charge conformers of *D. vulgaris* Fe-hydrogenase partially resolved by shallow-gradient preparative anion-exchange FPLC. The hydrogenase preparation (3.2 mg) applied onto the HR 5/5 MonoQ column had a specific activity of 3300 U/mg. After two reruns of the fraction indicated by the arrow, 0.6 mg hydrogenase with a specific activity of 6900 U/mg was obtained.

three aspects to this problem. The first question is whether or not the enzyme contains metal ions other than iron [11, 13]. The second question is how many iron ions are contained in the protein molecule and how are these distributed over the different Fe/S clusters (cf. [3] and references quoted therein). A third problem is what percentage of purified protein is apoprotein with respect to each of the different Fe/S clusters [7, 20].

A number of hydrogenase preparations was subjected to multi-elemental analysis with a range of suitable techniques. Fig. 1 summarizes the combined results on various Fe-hydrogenase preparations with specific activities ranging over 2000–6900 U/mg. Note that quantitative analysis of substoichiometric amounts consumes 10^2-10^5 more enzyme than quantitative analysis of the Fe content. Substoichiometric amounts of V, Mn, Co, Ni, Cu, Zn, Mo, W, and Se could be quantitated (Fig. 1). In a previous determination by PIXE significant (i.e. almost stoichiometric) amounts of Ni, Cu, and Zn were reported [11]. The present PIXE data were obtained with support filters thoroughly washed with EDTA (in order to remove Zn), with severalfold higher protein concentration, and with improved data analysis [36]. These three factors combined resulted in a substantially lower background and more reliable background correction. Even with proper precautions to exclude contamination and after an extensive EDTA dialysis, preparations of the Fe-hydrogenase are inevitably contaminated with 0.04–0.09 ion Cu and/or Zn/molecule. Nickel and selenium were usually present at much lower levels. At least five high-activity preparations contained < 0.01 mol/mol amounts of Ni and Se. The content of these and the other elements presented in Fig. 1 had no correlation with the specific activity of the hydrogenase preparations used. This strongly suggests that these elements are not functional in *D. vulgaris* Fe-hydrogenase. We have also taken the opportunity to establish that the selenium content of *M. elsdenii* Fe-hydrogenase is < 0.2 mol/mol, as this datum has not previously been reported.

Variations in pI, specific activity and Fe content

Higuchi et al. have reported that the solubilized nickel-hydrogenase from *D. vulgaris* (Miyazaki F), purified to homogeneity according to SDS/PAGE, resists crystallization unless it is separated with HPLC into three hydrogenase subfractions [40]. These components were not distiguishable in terms of activity or spectral properties (*ibidem*). It is possible that they represent species with minor differences in surface charge. A determination of the chemical modification involved was not reported. One could imagine oxidation of a non-cluster-bound cysteine to cysteinate or deamidation of glutamine or asparagine residues. Incited by the difficulties that we encountered in the crystallization of the *D. vulgaris* (Hildenborough) Fe-hydrogenase, we searched for inhomogeneity in this protein similar to that of the Miyazaki enzyme.

Fig. 2 gives the elution profile (280 nm) of a typical preparative FPLC anion-exchange chromatography run according to the one-to-last step in the purification scheme of Table 1. All peaks in the 20–33-ml elution range exhibited a normal two-band (45.8+10.1 kDa) pattern on SDS/PAGE. The hydrogenase main peak eluting at 200 mM NaCl (23–26 ml) appears to consist of two components. The top fraction (arrow) does not significantly differ in its purity factor, $f = 0.355 \pm 0.005$, from most of the other bands in the 20–33-ml range. However, it has a higher specific H_2-production activity. By repeating this anion-exchange chromatography step, fractions are obtained with specific activities in the manometric H_2-production assay ranging from very low to very high values: 50–6900 U/mg.

In isoelectric focussing the fractions are found to differ significantly (Fig. 3). The high-activity fraction mainly consists of a diffuse band at pI $\approx$ 6.2. The other fractions exhibit a number of well-focussed, intense bands at pI 5.7–6.4. When duplicate gels were stained for H_2-uptake activity with benzyl viologen and triphenyl tetrazolium chloride [38], initially only the diffuse band at pI $\approx$ 6.2 would color. However, upon prolonged incubation eventually a second band with pI $\approx$ 5.8 became colored (indicated in Fig. 3). Native electrophoresis of hydrogenase preparations (not shown) confirmed that the

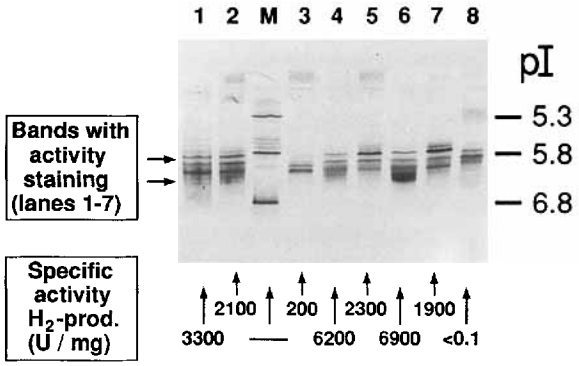

Fig. 3. Isoelectric focussing of multiple charge conformers of *D. vulgaris* Fe-hydrogenase. Fe-hydrogenase with indicated specific activity was separated on flat-bed isoelectric focussing. Lanes 1 and 2, Fe-hydrogenase obtained with the purification procedure described in Materials and Methods; lanes 3–5 and 6–7, Fe-hydrogenase fractions obtained by repeated shallow gradient anion-exchange chromatography of the preparations of lanes 1 and 2, respectively; lane 8, inactive (H-cluster-lacking) *D. vulgaris* Fe-hydrogenase expressed in *E. coli*; lane M, marker mixture of proteins with pI indicated on the right. The lanes contain 3–6 μg protein. Activity staining with H_2, triphenyltetrazolium chloride and benzyl viologen at pH 7 [38] was observed for the two bands indicated with horizontal arrows.

multiple bands are not an artifact of the isoelectric focussing technique. The nature and significance of the diffuseness of the pI ≈ 6.2 band is at present not understood.

A logical step towards resolution of the different charge conformers was to try chromatofocussing. The hydrogenase emerged from the MonoP HR 10/30 FPLC chromatofocussing column at pH values below 5. This seriously affected the recovery of activity, probably due to the lability of the [Fe-S] centres at low pH. When the pH of the fractions eluting from the column was immediately adjusted to pH 8, the recovery of activity was about 70%. The resolution of charge conformers was, in the best runs, comparable to the separation on the MonoQ anion-exchange column. In one experiment we checked the performance of different charge conformers in both the H_2-production and H_2-consumption manometric assay. Within experimental error, the various species had the typical H_2-consumption/H_2-production ratio of 10 (cf. [19]).

After incubation under H_2 of fractions enriched in the main peak and in the right shoulder (cf. Fig. 2), drastically different EPR spectra resulted. The main high-activity form showed a fully developed F signal. The lower activity shoulder showed a faint F signal. After reduction by dithionite, the EPR spectrum (F signal) of the fractions was identical (not shown). The behaviour of the 'shoulder' fraction is reminiscent of the inactive *D. vulgaris* hydrogenase expressed in *E. coli* (cf. Fig. 6 in [20]).

We interpret the combined results, above, as follows. Purified *D. vulgaris* Fe-hydrogenase occurs in a number of forms with different pI, probably reflecting heterogeneity of charge. The protein can be enriched in the individual charge conformers by anion-exchange chromatography, chromatofocussing, native electrophoresis and isoelectric focussing. The charge conformers also differ in their specific H_2-production and H_2-consumption activity and in their H_2-reducibility (monitored on the EPR F signal). Thus, it appears that the charge heterogeneity reflects not just surface charge modification but also differences in a catalytically vital part of the protein.

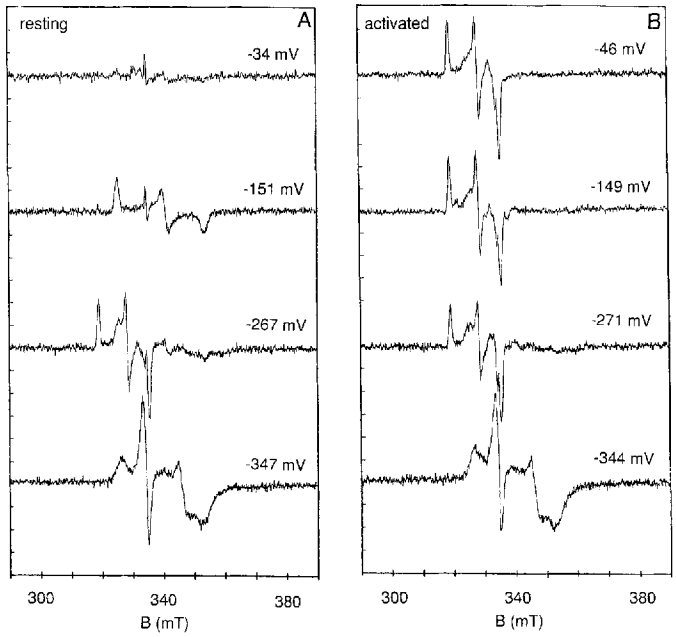

Fig. 4. A comparison of resting (A) and pre-activated (B) *D. vulgaris* Fe-hydrogenase by EPR-monitored, reductive titration. The enzyme, 60 μM protein, in the presence of 40 μM each of redox mediators, in 100 mM Tris/HCl pH 7.0, was titrated to the indicated potentials with sodium dithionite in the same buffer. The hydrogenase was pre-activated with a H_2/Ar cycle as described in Materials and Methods. EPR conditions: microwave frequency, 9.33 GHz; microwave power, 13 μW; modulation amplitude, 1.0 mT; temperature, 13 K.

We have previously reported on the presence of 13–15 Fe mol/mol in *D. vulgaris* hydrogenase [4]. If the last FPLC step in the purification scheme (Fig. 2 and Table 1) is omitted, the Fe number is lower (9–11 Fe ions/molecule). The colorimetrically determined iron content of highly active hydrogenase always was 12–15 mol/mol. Results of iron determination with colorimetry were within experimental error identical to those obtained with ICP-MS and INAA methods.

Reductive titration of resting and activated hydrogenase

We have repeated the mediated, reductive titration of resting Fe-hydrogenase with sodium dithionite, as previously reported by Patil et al. [17]. We have quantitatively confirmed their results in terms of apparent midpoint potentials, apparent *n* values, and EPR integrated intensities. Specifically, we find (cf. Fig. 4A) the following sequence of events upon lowering the bulk potential with dithionite: (a) the second rhombic signal (g = 2.07, 1.96, 1.89) appears to a maximum S = 1/2 spin intensity of ≈ 0.45; (b) the second rhombic signal disappears with an apparent $E_{m,7}$ ≈ −250 mV, concomitant with the appearance of the H signal (a rhombic signal with g = 2.11, 2.05, 2.00) and the H signal increases to a maximum S = 1/2 spin intensity of ≈ 0.33; (c) around E ≈ −300 mV the H signal collapses and the F signal from the two regular cubanes appears with a steeply sloping titration curve, suggesting strong redox interaction between the two cubanes (i.e. n ≈ 2; cf. [17]).

Patil et al. have proposed an interpretation of these three events in terms of redox transitions under thermodynamic equilibrium conditions [17]. We argue against this proposal on the basis of the following observations. The sequence of events is not reversible. During the first reducing cycle the

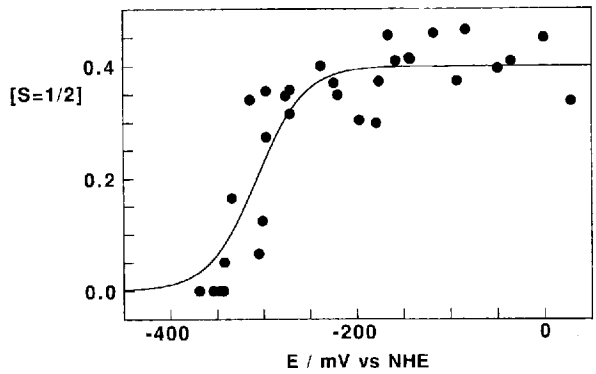

Fig. 5. Oxido-reductive titration of the H signal ($g = 2.10$) from hydrogen-pre-activated *D. vulgaris* Fe-hydrogenase. The protein and EPR conditions were as in Fig. 4. The data points were obtained from three independent titrations of two Fe-hydrogenase preparations. The solid line is a least-squares fit to all data points assuming a Nernstian $n = 1$ system with $E_m = -307$ mV.

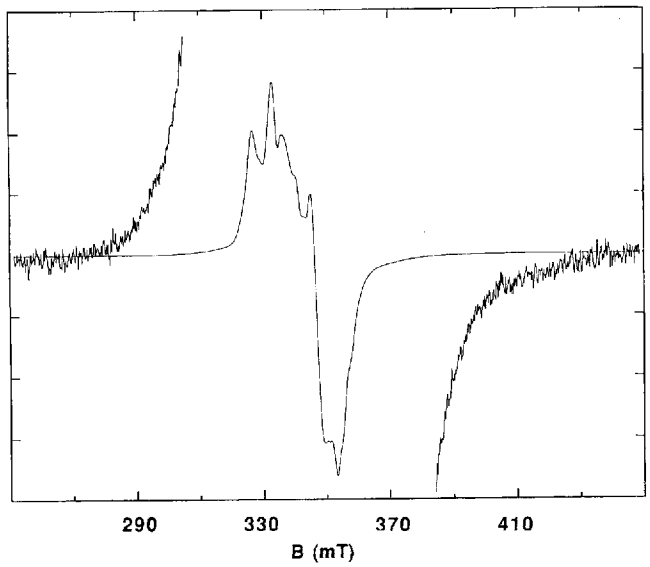

Fig. 6. Broad-wing EPR interaction spectrum from the two F clusters in very concentrated, hydrogen-reduced *D. vulgaris* Fe-hydrogenase. The enzyme, 2 mM protein in 200 mM Hepes pH 7.0, was reduced by stirred incubation under 120 kPa H_2 pressure at ambient temperature for 20 min. The extent of the wings from dipole–dipole broadening are emphasized by a 100-fold blow up. Limits for proper spin quantitation by double integration should, therefore, span almost the full 200-mT scan range. EPR conditions: microwave frequency, 9.325 GHz; microwave power, 2 mW; modulation amplitude, 0.8 mT; temperature, 15 K.

hydrogenase activates. Upon reoxidation the second rhombic signal does not reappear. The titration curve of the H cluster is no longer bell-shaped. In Fig. 4B the equivalent of the three-event titration is presented but for H_2/Ar-cycle-activated hydrogenase. The pattern is considerably simplified and, in fact, is now very similar to that observed with *M. elsdenii* Fe-hydrogenase [7]. Only the H signal and the F signal are observed. The H signal now has a single transition potential. We conclude that the responses of the second rhombic signal to lowering of the bulk potential represent transients in the reductive activation process. Only the H signal and the F signal represent redox-active species in the active enzyme.

Oxido-reductive titration of the H signal in activated hydrogenase

The hydrogenase was activated with a H_2/Ar cycle, diluted with a mixture of redox mediators, and then titrated reductively with sodium dithionite and/or oxidatively with potassium ferricyanide. As only relatively small amounts of highly active enzyme can be obtained, the enzyme-consuming titration experiments reported in this paper were performed with samples with intermediate specific activities of 2400–4000 U/mg. The integrated intensity of the resulting $S = 1/2$, H cluster, EPR signal is plotted in Fig. 5 versus the bulk potential. The H signal shows a single, reversible reduction-oxidation step at $E_{m,7.0} \approx -307$ mV. The titration curve does not show the artifactual abrupt cut off that was observed in the reductive titration of resting enzyme [17]. Nevertheless, the data points at low potentials are probably somewhat influenced by the H_2 production from solvent protons and dithionite electrons. This inherent uncertainty precludes an exact determination of the curvature of the Nernst plot; therefore, an n value can not be assigned to the H cluster. We have drawn an $n = 1$ Nernst curve in Fig. 5 for convenience, not for interpretational purposes. The $S = 1/2$ intensity of the fully oxidized H signal does not exceed 0.4 spin.

Titration with molecular hydrogen

There is only one possible way to redox-titrate an active hydrogenase enzyme while at the same time circumventing the kinetic complication of H_2 production at the expense of added reducing equivalents. The use of molecular hydrogen as the reductant (and solvent proton as the associated oxidant) ensures that bulk electron transfer is limited to within the Nernstian system: H_2/H^+ plus oxidized/reduced enzyme. We have titrated the Fe-hydrogenase with the H_2/H^+ couple by applying a hydrogen pressure of around 120 kPa to enzyme solutions of varying pH. At neutral pH this produces a potential of -416 mV (cf. Eqn 1). Therefore, the H signal ($E_m^7 \approx -300$ mV) is not observable in these experiments.

The F clusters are presumably two ferredoxin-type cubanes [6] and they are subject to mutual weak dipolar interaction [11]. The F signal is a complex interaction spectrum but its double integral should still be proportional to the cubane concentration. A practical problem is that the magnetic interaction creates broad wings of low amplitude to both sides of the spectrum so that the limits of integration are difficult to determine. We have prepared an Fe-hydrogenase sample of very high concentration (2 mM), reduced the enzyme with H_2 at pH 7, and taken the EPR spectrum in order to obtain a very low-noise F signal. This spectrum is given in Fig. 6. From a 100-fold blow up, it is evident that the integration limits should span a field scan more than twice the width of the main spectral feature.

The $S = 1/2$ spin intensity of the F signal as a function of the potential, poised with the H_2/H^+ couple, is presented in Fig. 7. At low potential the signal quantitatively accounts for two $[4Fe\text{-}4S]^{1+}$ clusters. The solid line in Fig. 7 is a Nernst curve for two independent $n = 1$ acceptors of identical reduction potentials, $E'_m = -330$ mV. The slope in the data appears to be slightly less than that expected for $n = 1$, and this may reflect a slight difference between the reduction potentials of the two clusters (< 20 mV). In contrast to the suggestion brought forward by Patil et al. [17], there is no sign of positive redox interaction between the two clusters;

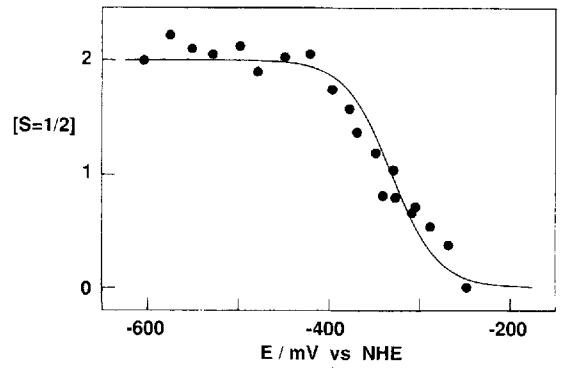

Fig. 7. Redox titration with the H_2/H^+ couple of the F signal from *D. vulgaris* Fe-hydrogenase by variation of the pH at constant H_2 pressure. The enzyme, 50–70 μM protein in 100 mM buffer of variable pH (detailed in Materials and Methods), was incubated under H_2 and then measured by EPR as in Fig. 4. On the ordinate axis the spin quantitation is plotted as determined by double integration. The abscissa axis gives the potential calculated for the H_2/H^+ couple according to Eqn (1). The solid line is a least-squares fit to all data points assuming two identical and independent $n = 1$ acceptors each with $E'_m = -330$ mV.

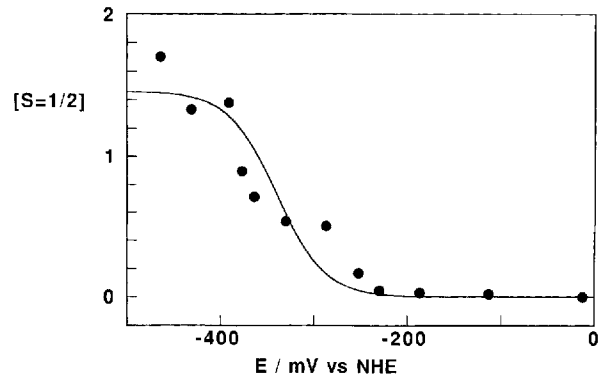

Fig. 8. Reductive titration of the F signal from inactive (H-cluster-lacking) *D. vulgaris* Fe-hydrogenase expressed in E. coli. The recombinant protein was 50 μM with 40 μM each of the redox mediators in 50 mM Hepes pH 7.5. The reductant was buffered sodium dithionite. EPR conditions were as in Fig. 4. The solid line is a least-squares fit to all data points assuming two identical and independent $n = 1$ acceptors each with $E'_m = -340$ mV. Addition of excess dithionite in buffer to the inactive hydrogenase in absence of mediators also gives a limiting 1.5 spin/protein.

specifically, the cluster pair does not at all behave as a single, two-electron acceptor.

Redox titration of the H-cluster-free recombinant protein

Cloning and expression of the two structural genes (the *hydA* + *hydB* operon) of *D. vulgaris* Fe-hydrogenase in *E. coli* allows for the isolation and purification of an inactive 'recombinant' protein that is almost indistinguishable from wild-type hydrogenase except that the F clusters are present for only ≈60–80% and the H cluster appears to be completely absent [20]. The EPR F signal does not show up upon incubation under H_2 but it does develop upon reduction with sodium dithionite. Because the recombinant protein does not interact with hydrogen, a dye-mediated equilibrium redox titration is not disturbed by enzyme activation and/or H_2-production activity. Thus, the potential of the F clusters are readily determined. From the data in Fig. 8 it can be estimated that the two clusters (73% incorporation) each titrate with $E_{m,7.5} \approx -340$ mV and $n \approx 1$.

DISCUSSION

The protein and the metal ions

The site of hydrogen activation in Fe-hydrogenases is, or encompasses, to all likelihood an iron-sulfur cluster. Its structure is not known; its elemental stoichiometry is not firmly established. A problem of normalization has clouded the field for several years. We have initiated the debate by comparing four different methods of protein determination for the *D. vulgaris* enzyme. This work led us to conclude that: (a) the 'easy' determinations (in particular the Lowry and the Bradford methods) bore systematic errors of significance when applied to Fe-hydrogenase; and that (b) the total number of Fe ions/molecule of protein was greater than previously assumed, namely, approximately 14 [4, 7]. This revised stoichiometry was based on the Biuret protein determination, because this polypeptide-bond-oriented complexation method should be less prone to deviations from the average absorption coefficient for a particular protein.

Subsequently, Adams and his collaborators re-determined the protein concentration of *C. pasteurianum* Fe-hydrogenase I and II samples (hitherto determined with the Lowry method) by amino-acid analysis, and they found that Fe/S numbers for both these proteins had to be corrected upwards by an astounding factor of two [8]. The same method was then applied by Patil et al. on the *D. vulgaris* Fe-hydrogenase and, combined with elemental analysis by plasma emission spectroscopy, the result was some 10 Fe ions/protein molecule[17]. In the hands of these workers protein concentration determined with the biuret method versus the amino-acid analysis differed by 10% (*ibidem*). Since the molecular mass of the mature protein is exactly known [6, 41], and since the accuracy of the iron determination is not questioned, it is implied that the difference in Fe contents reported by us [4, 7] and by Patil et al. [17] (i.e. ≈14 versus ≈10) is a real one. On the assumption that 8 Fe atoms are in the F clusters, the remaining difference (i.e. ≈6 versus ≈2) would apply to the H-cluster active site.

In view of this gross difference, we have again evaluated the metal content of a number of *D. vulgaris* Fe-hydrogenase preparations. We have not limited our analyses to iron, because in early investigations from this laboratory significant amounts of Ni, Cu, Zn were found with PIXE [11]. We have now combined a methodologically improved PIXE with ICP-MS, INAA, AAS, and colorimetry to arrive at the conclusion that the name 'Fe-hydrogenase' is appropriate as the highly active protein contains only small amounts of any metal other than iron.

The overall result of the new iron determinations is not different from our previous work, i.e. there are some 12–15 Fe ions/highly purified protein molecule. Thus, the controversy over the Fe stoichiometry remains. However, our present work provides a new clue to the nature of varying Fe numbers. The protein appears to exist in some four slightly different forms as judged from isoelectric focussing, from native PAGE and from FPLC. This inhomogeneity is reflected in unsatisfactory crystallization results, in a varying lag time for activation to H_2-uptake activity (not shown), in differential H_2-reducibility (and related specific activity and EPR response).

We have previously hypothesized that the purified Fe-hydrogenase from *M. elsdenii* exists as a mixture of fully active holoenzyme and inactive protein still carrying the two F clusters but deficient in the H cluster [7]. We now propose to carry over this hypothesis to the *D. vulgaris* enzyme.

This may provide a framework for the interpretation of spectroscopic data, however, it does not fully resolve the difference in reported iron numbers. We propose that the relative content of apo-protein is directly related to the protein isolation procedures. In the original scheme of van der Westen et al. (of which our procedure is an upscaling and extension) the hydrogenase is extracted from the periplasm of freshly harvested cells by partial digestion of the outer membrane with EDTA at 32°C [19]. When this is done in Tris/HCl buffer of pH 9.0 the hydrogenase de-activates to a resting, O_2-insensitive, form. The molecular mechanism of this process is not known but it works equally well on purified, H_2-activated enzyme [31]. Contrarily, in the procedure described by Huynh et al. harvested cells are suspended in Tris/HCl buffer of pH 7.6 and then stored for two days at −80°C. They are subsequently defrosted for 20 h and after centrifugation the hydrogenase is purified from the supernatant [13]. It is not clear to what extent this procedure results in de-activated enzyme; when applied to purified protein it does not work [31]. It is likely that the freezing/defreezing causes rupture of the periplasmic membrane. For the isolation of cytoplasmic proteins we have disrupted freshly harvested *D. vulgaris* cells in a chilled Manton-Gaulin press [32, 39]. When the Fe-hydrogenase is isolated from this starting material and purified to homogeneity by using an identical purification procedure as outlined in this paper, the specific activity is usually between 50 − 200 U/mg in the Chen and Mortenson H_2-production assay. One should compare this finding with the observation that purification starting with a periplasmic extract and a final FPLC MonoQ step yields hydrogenase with ≈ 7000 U/mg in addition to low-activity species. Apparently, the specific activity of Fe-hydrogenase can differ over some two or three orders of magnitude without changes in the usual purity index (≈ 0.36) or SDS/PAGE purity (> 99%).

Patil et al. reported a specific H_2-production activity of 4000 U/mg when measured in the gas chromatograph and apparently under the conditions according to the Peck and Gast procedure [13, 17, 30, 42]. We find 7000 U/mg in the standard manometric assay. A comparison of these determinations would require our number to be multiplied by a factor of 2 − 3. This suggests that the specific activity of preparations purified according to our current procedure is significantly higher.

In order to begin to work towards a resolution (or at least a normalization) of the problems of the specific activity and (in)homogeneity of Fe-hydrogenases and the derived problems of interpreting, e.g., spectroscopic data, we propose that all future work should contain the following information: (a) a protein determination according to the micro-biuret method and/or amino-acid analysis; (b) a H_2-production activity determination according to exactly defined conditions; (c) an iron stoichiometry number, e.g. according to the ferene colorimetric determination.

Redox properties of the clusters

Patil et al. EPR-monitored a dithionite titration of resting *D. vulgaris* hydrogenase and deduced a complicated scheme of redox events: two components titrated with bell-shaped response curves, a third component was subject to 'a strong cooperative effect' [17]. We have shown here that all these responses reflect non-equilibrium transients in the reductive activation of the enzyme. When the protein is pre-activated with its substrate H_2, which is subsequently removed by substitution with argon, the results of an ensuing titration experiment point to a much simpler scheme. There are just two components each with a single redox transition.

From the H_2/H^+ titration of activated enzyme and the dithionite/ferricyanide titration of inactive, recombinant protein it can be concluded that the F clusters are independent (i.e. non-interacting), indistinguishable, $n = 1$ electron-transferring groups with a reduction potential $E_m \approx -335$ mV. There is no indication that this potential is dependent on the pH. Double integration of the EPR from activated enzyme at high pH, therefore at high H_2/H^+ ratios, gives two $S = 1/2$ systems/enzyme molecule. Thus, on the very reasonable assumption that the F clusters are cubanes [6], eight of the Fe ions have been accounted for.

The rhombic signal with $g = 2.11, 2.05, 2.00$ is ascribed to the H cluster [3]. At neutral pH the signal titrates with a reduction potential $E_m = -307$ mV. The maximally developed signal has a double integral corresponding to ≈ 0.4 spin $S = 1/2$ systems/enzyme molecule. This substoichiometric number can be interpreted in two ways: either the remaining spins are not EPR-detectable, or the samples are partially apoprotein with respect to the H cluster. Validity of the latter interpretation exclusively would imply that the H cluster encompasses a very large number of iron ions. It is possible that a small percentage of the preparations lack the H cluster and, therefore, that the observed total Fe content is slightly low. Stephens et al. have reported on indications from magnetic CD spectroscopy that the EPR H signal is accompanied by an EPR-silent high-spin component of unidentified spin [43]. We have previously reported on a very unusual high-spin signal (a single line with $g_{eff} = 5.0$) in H_2/Ar-cycled hydrogenase [4]. The relation, if any, between the magnetic CD high-spin signal and the EPR $g = 5$ signal is presently still obscure. In the experiments described in this paper (specifically that of Fig. 5) no $g = 5$ EPR signal was observed. In our view the crucial remaining problem is the low intensity of the H-cluster EPR signal. It is now abundantly clear that the signal can not be related to metals other than iron. It also appears that missing intensity is not in half-integer superspins ($S \geq 7/2$), because we have found none for hydrogenase where we have been successful with several other Fe/S proteins (nitrogenase [44], sulfite reductase [39], CO dehydrogenase [45], the prismane protein [46]). Clearly, the resolution of this problem requires further spectroscopic studies on normalized protein samples.

The two redox potentials that we have found are both significantly more positive than the potential of the hydrogen electrode. The half-maximal hydrogen production activity of Fe-hydrogenases as a function of the applied potential is approximately equal to the hydrogen electrode potential [24, 25]. This would seem to imply that in order for the *D. vulgaris* (Hildenborough) Fe-hydrogenase to operate in hydrogen production, a considerable reductive power must be continuously exerted by the enzyme's natural electron donor system. It would also seem to imply that the oxidized form of the H cluster, associated with the rhombic EPR H signal, is not necessarily a catalytically competent redox state.

Elemental analysis by ICP-MS was carried out by Dr A. A. van Heuzen at the Koninklijke/Shell-Laboratorium, Amsterdam. Elemental analysis by INAA was carried out by Th. G. van Meerten and A.

van der Meer at the Interuniversitair Reactor Centrum, Delft (The Netherlands). Mr I. Walinga helped us with the atomic absorption spectroscopy at the Department of Soil Science and Plant Nutrition, Wageningen. Mr L. C. de Folter helped us with the PIXE spectroscopy at the Cyclotron Laboratory in Eindhoven (The Netherlands). This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) and by the National Institutes of Health (GM 12176 & 32785 to RHS).

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