The iron-sulfur composition of the active site of hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) deduced from its subunit structure and total iron-sulfur content

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The subunit composition and Fe/S content of nine different batches of *D. vulgaris* (H) hydrogenase have been determined. SDS-PAGE and FPLC show the enzyme to be an $\alpha\beta$ dimer consistent with the molecular mass of 46 + 13.5 kDa recently inferred from the nucleotide sequence. Based on this molecular mass and a protein determination calibrated on tryptophan + tyrosine content, the enzyme is found to contain 14–16 Fe and 12–14 S^{2-}, and $\varepsilon_{400} = 45\ mM^{-1}\cdot cm^{-1}$. It is suggested that the active site of this bidirectional hydrogenase is not a [4Fe-4S] cluster, but rather is a novel cluster comprised of approx. 6 Fe and 6 S^{2-}.

Hydrogenase *Iron-sulfur cluster* (Desulfovibrio vulgaris)

1. INTRODUCTION

Bidirectional hydrogenases catalyze both the oxidation and the production of molecular hydrogen at considerable rates [1]. According to the current point of view the enzymes from *Clostridium pasteurianum* [2,3], *Megasphaera elsdenii* [4] and *D. vulgaris* (strain Hildenborough) [5,6] have in common a content of approx. 12 mol of Fe and of acid-labile S^{2-} per mol protein. Ni^{2+} is thought not to be an intrinsic part of these bidirectional hydrogenases [6]. Until recently, the *D. vulgaris* (H) enzyme was considered to be a single-subunit protein [5] just as the other members of its group [2,4]. The molecular mass was estimated to be 50 kDa by gel filtration and SDS-polyacrylamide gel electrophoresis [5]. Related to this figure of 50 kDa are the reported values of some 12 Fe ions and 12 S^{2-} per enzyme molecule [5,6] and the quantitation of cluster extrusion experiments to approx. three [4Fe-4S] extruded per enzyme molecule [7]. Magnetic resonance studies [6] as well as the recently determined nucleotide sequence of the structural gene [8] indicate two of the clusters to be regular, electron-transferring, ferredoxin-type clusters. Additionally observed anomalous magnetic resonance properties [6,9] would then seem to imply (cf. [6]) the third cluster – the obvious candidate for the site of H_2 activation – to be an atypical [4Fe-4S] cluster.

Lately, the structural gene for *D. vulgaris* (H) hydrogenase was sequenced and found to encode a protein product of 45820 Da [8]. Immediately downstream from this gene another gene was found, encoding a 13493 Da protein [8]. Since SDS gel electrophoresis on some recent preparations of this enzyme exhibited a two-band pattern [10] corresponding in molecular mass to the above-mentioned gene products, it was proposed that the enzyme consists of two subunits [8]. This proposal, however, implies that the total molecular mass is 59313 Da and not the previously determined 50 kDa. This would mean that the protein concentration has hitherto been overestimated and that the reported Fe and S^{2-} content [5,6] should be corrected by +19% to an approximate

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stoichiometry of 14. Consequently, there would be some six, instead of four, Fe and S^{2-} to be accommodated in the hydrogenase active site.

We note that the approximate stoichiometries of 12 reported for the other bidirectional hydrogenases [2,4] are also based on estimated molecular masses as the primary structure of these proteins has yet to be determined. We have reinvestigated the subunit composition and the Fe and S^{2-} content of a representative set of *D. vulgaris* (H) hydrogenase preparations in order to establish a reliable number for the stoichiometry as a mandatory basis for subsequent spectroscopic and structural studies.

2. MATERIALS AND METHODS

D. vulgaris (strain Hildenborough NCIB 8303) was grown and its hydrogenase was assayed and purified by standard techniques [5]. The final preparation was dialyzed twice for 12 h against 50 mM Tris buffer and 5 mM EDTA (pH 7.5) and twice for 12 h against 25 mM potassium phosphate (pH 7.5). Subunit composition was studied by SDS-PAGE [11] and by FPLC (Pharmacia) on a Superose 12 column in the presence of 6 M guanidine hydrochloride. Sample preparation for the latter technique was as in [10]. Iron was determined as in [12] and inorganic sulphide was determined according to [13]. Protein was determined from the A_{280} after precipitation with 11% trichloroacetic acid and overnight resolubilization in 3% NaOH at ambient temperature. The method was calibrated using the known Tyr and Trp content of *D. vulgaris* (H) hydrogenase [8] and bovine serum albumin [14] and the extinction coefficients of Tyr and Trp in alkaline solution [15]. Protein was also determined with the microbiuret [16], Lowry et al. [17] and Bradford or Coomassie [18] method.

3. RESULTS

The subunit composition of purified *D. vulgaris* (H) hydrogenase was found to be invariant over nine different batches. In SDS-PAGE all samples show a two-band pattern of apparently constant intensity ratio (fig.1), with apparent molecular masses of approx. 44 and 13.5 kDa. This result is consistent with the previous suggestion from

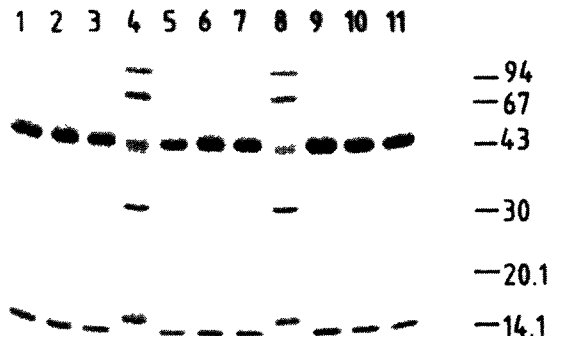

Fig.1. SDS-PAGE on a 17.5% acrylamide gel of hydrogenase purified from nine batches of *D. vulgaris* (H). Lanes 4 and 8 are markers of molecular mass 94, 67, 43, 30, 20.1 and 14.4 kDa, respectively. Lanes 1–3, 5–7, 9–11 are 12.5 μg hydrogenase and correspond, respectively to the nine entries in tables 1 and 2.

nucleotide sequencing work [8] that *D. vulgaris* (H) hydrogenase is a two-subunit enzyme. As an additional corroboration the concentration ratio of the two subunits was established to be unity: the subunits were dissociated in 6 M guanidine hydrochloride and the Cys residues were subsequently carboxylated (cf. [10]). The subunits were then separated by FPLC at pH 8 monitoring the A_{280} (fig.2). Normalizing on the Tyr and Trp content, n_i, as calculated from the proposed two-product operon [8], using the extinction coefficients, ϵ_i, of alkaline Tyr and Trp [15], the relative subunit concentration is obtained by integration of the A_{280} elution peak (cf. fig.2A):

$$[S] = \int A_{280}\, dA/(n_{Tyr}\epsilon_{Tyr} + n_{Trp}\epsilon_{Trp})$$

Using this proportionality, the concentration ratio of the larger over the smaller subunit was found to be 1 : 1.1. Thus, it is now firmly established that *D. vulgaris* (H) hydrogenase is expressed, and can be subsequently purified to homogeneity, as a two-subunit protein of 59313 Da total molecular mass. We can now proceed by redetermining the iron and acid-labile sulfur content of this enzyme related to its protein concentration on the basis of this corrected molecular mass.

The number of iron atoms and acid-labile sulfur atoms per protein mass of 59313 Da are given in table 1 for the nine different preparations. In sum-

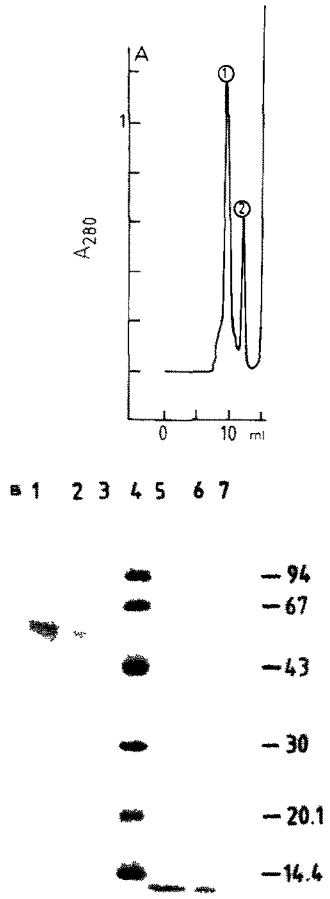

Fig.2. (A) FPLC on Superose 12 of carboxymethylated subunits of hydrogenase from *D. vulgaris* (H) in the presence of 6 M guanidine-hydrochloride. 4 mg total protein was applied in a volume of 200 μl. Fractions 1 and 2 were identified as the 46 and 13.5 kDa subunit by SDS-PAGE on a 17.5% acrylamide gel (B). Lane 1–3 are 5, 2 and 1 μg fraction 1; lanes 5–7 are 5, 2 and 1 μg fraction 2, respectively. Lane 4 contains the molecular mass markers.

mary, the enzyme contains some 14–16 Fe and some 12–14 S atoms. Specifically, it seems reasonable to conclude tentatively that 14 Fe and 14 S^{2-} make up the Fe/S clusters of the enzyme, since one would expect the iron determination to give an upper limit of intrinsic iron (because of possible contamination from chemicals and glassware) and one would expect the sulfur determination to result in a lower limit of S^{2-} (because no significant contamination is anticipated).

Table 1

Iron and acid-labile sulfur content and light extinction of hydrogenase from nine batches of *D. vulgaris* (H)

Preparation	Fe/mol	S^{2-}/mol	ϵ_{400}	A_{400}/A_{280}
A	14.6	12.2	43.4	0.321
D	14.8	12.8	41.8	0.321
E	14.4	13.0	40.9	0.320
F	15.5	14.8	45.8	0.329
L	17.1	14.0	50.9	0.339
H	15.0	12.7	44.6	0.347
N	17.6	15.1	54.9	0.315
T	14.0	11.2	39.7	0.297
O	14.4	11.9	43.2	0.334
Mean	15.3	13.1	45.0	0.325
(s)	(1.3)	(1.3)	(4.9)	(0.015)

Note: Protein concentration was determined with the microbiuret method using a molecular mass of 59313 Da

The previously reported extinction coefficient at 400 nm of 46 $mM^{-1} \cdot cm^{-1}$ [5] was based on an incorrect molecular mass of 50 kDa. Therefore, we also report in table 1 the ϵ_{400} of the nine preparations, based on the correct molecular mass. Remarkably, the average value of ϵ_{400} = 45 $mM^{-1} \cdot cm^{-1}$ does not significantly deviate from the previously reported value.

Determination of Fe/S stoichiometries does not depend only on an accurate knowledge of the protein molecular mass, but equally well on an accurate protein concentration determination. The Lowry et al. and Coomassie color reactions have hitherto been used for hydrogenases without proper calibration of the absorption of the stained hydrogenase with respect to the stained standard, usually bovine serum albumin. Thus, the advantage of relatively high sensitivity of these methods loses significance because of a systematic error of unknown magnitude. We have used the less sensitive microbiuret method of protein backbone staining to minimize color differences related to specific secondary and tertiary structure. As an independent check we have also determined protein concentration of acid-denatured and subsequently alkalinized protein from its absorption at 280 nm using the extinction coefficients of alkaline Tyr and Trp. The ratio of the two determinations is given in table 2. Determinations by the Lowry et

Table 2

Ratio of apparent protein concentration of hydrogenase from nine batches of *D. vulgaris* (H) as determined by different methods compared to the microbiuret method

Preparation	A_{280}	Lowry et al.	Coomassie
A	1.09	1.22	0.84
D	1.06	1.26	0.89
E	1.02	1.28	0.88
F	1.12	1.21	0.77
L	1.21	1.29	0.95
H	1.03	1.11	0.82
N	1.31	1.35	–
T	1.05	1.21	0.96
O	1.06	1.23	0.95
Mean	1.11	1.24	0.88
(s)	(0.10)	(0.07)	(0.07)

al. and Coomassie methods are also included to allow for a comparison with literature data.

The first ratio being close to unity lends credit to the notion that our protein determination is sufficiently accurate to establish, e.g. the number of Fe and S^{2-} within the 10% confidence range. Note also that, for the combination of this particular hydrogenase and the BSA standard, the Lowry et al. method gives a significant overestimation of protein (and thus an underestimation of Fe/S^{2-}). This is to be expected on the basis of the higher Tyr and Trp content of the hydrogenase [8] over that of BSA [14], as these residues usually account for about one half of the intensity in the Lowry et al. color reaction [19]. The Coomassie method results in a – relatively smaller – underestimation of protein.

4. DISCUSSION

Van der Westen et al. [5] reported the presence in *D. vulgaris* (H) hydrogenase of 12.5 Fe and 11.5 S^{2-} by chemical analysis. Corrected for the right molecular mass (× 1.19) and for protein overestimation by the Lowry et al. method (× 1.24) these values become 18.4 Fe and 16.9 S^{2-}. In contrast, Huynh et al. [6] found 11 Fe by plasma emission spectroscopy. Corrected for the right molecular mass (× 1.19) and for protein underestimation by the Coomassie method (× 0.88) this number becomes 11.5 Fe. Thus, these two single determinations are mutually inconsistent even under the assumption that only [4Fe-4S] clusters are present. We now find 14–16 Fe and 12–14 S^{2-}. We consider this at present to be the most accurate determination of Fe/S^{2-} in bidirectional hydrogenase since, in contrast to previous work, our values are based on (i) an exact number for the molecular mass; (ii) a checked protein determination; (iii) an average over nine preparations. In spite of this, the magnitude of the propagated error is not small enough to pin down exactly the number of Fe and S^{2-}. Thus the only conclusion that we can rigorously draw is that the number of Fe and S^{2-} in bidirectional hydrogenases (and, for that matter, in all hydrogenases) is not exactly established. More specifically, there is no present justification for the common practice to simplify the available experimental results on Fe and S^{2-} content to the number 12, or, for hydrogenases in general, to a multiple of 4 (cf. [1,20,21]).

We tentatively interpret our results to indicate that *D. vulgaris* (H) hydrogenase contains 14 Fe and S atoms in Fe/S clusters. We do not at present wish to exclude the possibility that an additional 1–2 intrinsic Fe atoms are present in an non-Fe/S environment. Cluster extrusion has been reported to result in essentially quantitative release of Fe/S as [4Fe-4S] clusters [7]. There are two likely interpretations for 14 Fe/S extruding as [4Fe-4S] clusters: (i) the enzyme can contain up to four [4Fe-4S] clusters but all preparations contain some apoenzyme; (ii) the enzyme contains a different cluster that is extruded as a [4Fe-4S] cluster. In support of the last possibility we recall the existence of metastable [6Fe-6S] model clusters that easily, and quantitatively rearrange into [4Fe-4S] clusters [22]. Note also that 6Fe/S and 4Fe/S clusters are not discriminated by Mössbauer spectroscopy [22] and that unusual EPR [6,9] and MCD [23] data have been reported.

The above interpretation may be incorrect or oversimplified. As things stand, however, it is at least as likely as the common view that bidirectional hydrogenases contain only [4Fe-4S] clusters. Thus the structure and composition of the active site in *D. vulgaris* (H) hydrogenase is an unsolved problem. This conclusion holds a fortiori for the bidirectional hydrogenases from *C. pasteurianum* and *M. elsdenii* of which the reported iron and

acid-labile sulfur stoichiometries (12 and 12) are not based on the exact molecular mass. Furthermore, in both these enzymes unusual EPR [24,25] and MCD [25,26] signals have been detected similar to those of the *D. vulgaris* enzyme.

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REFERENCES

[1] Adams, M.W.W., Mortenson, L.E. and Chen, J.-S. (1981) Biochim. Biophys. Acta 594, 105–176.
[2] Chen, J.-S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta 371, 283–298.
[3] Gillum, W.O., Mortenson, L.E., Chen, J.-S. and Holm, R.H. (1977) J. Am. Chem. Soc. 99, 594–595.
[4] Van Dijk, C., Mayhew, S.G., Grande, H.J. and Veeger, C. (1978) FEBS Lett. 86, 122–126.
[5] Van der Westen, H.M., Mayhew, S.G. and Veeger, C. (1978) FEBS Lett. 86, 122–126.
[6] Huynh, B.H., Czechowski, M.H., Krüger, H.-J., Dervartanian, D., Peck, H.D. and LeGall, J. (1984) Proc. Natl. Acad. Sci. USA 81, 3728–3732.
[7] Grande, H.J., Dunham, W.R., Averill, B., Van Dijk, C. and Sands, R.H. (1983) Eur. J. Biochem. 136, 201–207.
[8] Voordouw, G. and Brenner, S. (1985) Eur. J. Biochem. 148, 515–520.
[9] Hagen, W.R., Van Berkel-Arts, A., Krüse-Wolters, K.M., Dunham, W.R. and Veeger, C. (1986) FEBS Lett. 201, 158–162.
[10] Voordouw, G., Walker, J.E. and Brenner, S. (1985) Eur. J. Biochem. 148, 509–514.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Massey, V. (1957) J. Biol. Chem. 229, 763–770.
[13] Brumby, P.E., Müller, R.W. and Massey, V. (1965) J. Biol. Chem. 240, 2222–2228.
[14] Peters, T. and Hawn, C. (1967) J. Biol. Chem. 242, 1566–1573.
[15] Sober, H.A. (1970) Handbook of Biochemistry, The Chemical Rubber Co., Cleveland, OH.
[16] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951) J. Biol. Chem. 193, 267–275.
[18] Bradford, M.N. (1976) Anal. Biochem. 72, 248–254.
[19] Peterson, G.L. (1979) Anal. Biochem. 100, 201–220.
[20] LeGall, J., Moura, J.J.G., Peck, H.D. and Xavier, A.V. (1982) in: Iron-Sulfur Proteins (Spiro, T.C. ed.) pp.177–248, Wiley, New York.
[21] Adams, M.W.W., Johnson, M.K., Zambrano, I.C. and Mortenson, L.E. (1986) Biochimie 68, 35–41.
[22] Kanatzidis, M.G., Hagen, W.R., Dunham, W.R., Lester, R.K. and Coucouvanis, D. (1985) J. Am. Chem. Soc. 107, 953–961.
[23] Stephens, P.J., Devlin, F., McKenna, M.C., Morgan, T.V., Czechowski, M., Dervartanian, D.V., Peck, H.D. and LeGall, J. (1985) FEBS Lett. 180, 24–28.
[24] Van Dijk, C., Grande, H.J., Mayhew, S.G. and Veeger, C. (1980) Eur. J. Biochem. 107, 251–261.
[25] Adams, M.W.W., Johnson, M.K., Zambrano, I.C. and Mortonson, L.E. (1986) Biochimie 68, 35–41.
[26] Thomson, A.J., George, S.J., Richards, A.J.M., Robinson, A.E., Grande, H.J., Veeger, C. and Van Dijk, C. (1985) Biochem. J. 227, 333–336.