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PURIFICATION AND SOME PROPERTIES OF THE SOLUBLE HYDROGENASE FROM *CHROMATIUM VINOSUM*

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Summary

A routine procedure for the growth and harvesting of large (600 l) batches of *Chromatium vinosum* and the isolation of hydrogenase (hydrogen : (acceptor) oxidoreductase, EC 1.12.—.—) are described. The enzyme is pure according to polyacrylamide gel electrophoresis, has a molecular weight of 61 000—63 000 and consists of a single polypeptide chain. The enzyme is stable in air but not active. Activity is obtained only after complete removal of oxygen.

EPR spectroscopy at 9 GHz shows a signal indicative for a $[4Fe\text{-}4S]^{3+(3+,2+)}$ cluster and in addition a rather complex signal of unknown origin. This additional signal completely disappears upon removal of oxygen, by incubation with 2-mercaptoethanol or by reduction with ferrocytochrome *c*. No EPR signals are detected in the enzyme reduced with H_2 or dithionite. The intensity of the EPR signal of the [4Fe-4S] cluster corresponds to one-quarter of the enzyme concentration, both in the untreated as well as in the He- or N_2-activated enzyme. If the enzyme is activated under He and then brought in contact with air the signal increases 4-fold and represents about one free spin/enzyme molecule. When measured at 35 GHz the line shape and peak positions of the additional signal change, indicating that the signal is not originating from a simple $S = \frac{1}{2}$ system.

None of the inhibitors of the hydrogenase activity has any effect on the shape or intensity of the EPR signal of the Fe-S cluster, ^2H_2O also has no effect. All EPR signals disappear after reduction with NADH or ascorbate in the presence of phenazine methosulphate. It is suggested that the Fe-S cluster is not the primary site of interaction of H_2 with the enzyme.

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Introduction

During the last decade there has been an increasing interest in research on methods to convert solar energy into readily utilizable non-polluting fuel sources. One of the approaches comprises the coupling of the photosynthetic machinery of plants to a hydrogenase, i.e. an enzyme that can combine protons and electrons to form hydrogen, resulting in a light-driven splitting of water into hydrogen and oxygen. Although such a reaction is experimentally possible, rates of hydrogen evolution reported thus far are rather low and the system deteriorates quickly (for review see Ref. 1), not only because of the inherent instability of the components making up the system, but also due to the production, by the chloroplasts, of oxygen which poisons the system by inactivation of the hydrogenase.

A number of approaches to solve these problems are now being studied in different laboratories. (1) A search for photosynthetic membranes with a higher photosynthetic capacity and, what is even more important, a higher stability in the light. (2) A search for more active and more stable hydrogenases and attempts to stabilize the enzyme, for instance by immobilization on a solid support. (3) The construction of a system in which oxygen and hydrogen are liberated in different compartments in order to shield the hydrogenase from the deteriorating action of oxygen. (4) Approaches aimed at replacing the biological components by stable non-protein catalysts.

This last possibility, however, requires a thorough understanding of the working mechanism of the enzymes and a detailed knowledge of the structure of the active centra. This is the principle aim of our work on hydrogenase. We have chosen to study the hydrogenase (hydrogen:(acceptor) oxidoreductase, EC 1.12.—.—) from the photosynthetic purple sulfur bacterium *Chromatium vinosum*. This enzyme contains 4 Fe and 4 labile S atoms [2] in contrast to a group of other hydrogenases that contain 12 Fe and 12 S atoms. Whereas this last group usually has a much higher activity, the enzymes having 4 Fe and 4 S seem simpler to study as they are generally more stable towards oxygen and have a smaller number of prosthetic groups.

Methods and Materials

Cell growth and harvesting

C. vinosum, strain D, was grown in a medium described by Hendley [3]. Medium-scale cultures were grown in a 60 l plexiglass vessel (100 × 60 × 10 cm) illuminated through the large side surfaces with eight 40 W fluorescent tubes (Philips, type 32). Large-scale cultures were grown in a 700 l glass tank (240 × 50 × 70 cm) illuminated through the bottom and the two large side surfaces by 12 250 W fluorescent lamps (AEG, power groove, type CWX). To ensure an even illumination of the contents of the tank a rapid circulation was induced in the culture by means of a liquid pump (capacity 60 l/min). The temperature of the culture was kept at 30°C by a large ventilator outside the tank, steered by a contact thermometer. Sterilization of both tanks was performed by incubating overnight with commercial bleach (0.15%), the remaining hypochlorite was decomposed under illumination and the Cl_2 formed was removed by gassing

with CO_2. Any remaining traces of the oxidants were finally removed by the thiosulfate present in the culture medium.

All cultures were continuously gassed with 95% N_2 plus 5% CO_2. The cell yield was generally 1—2 g of cells (wet weight) per l culture.

Cells of the medium-scale cultures were harvested by centrifugation for 1 h at 1400 × *g*, washed with 50 mM Tris-HCl buffer (pH 7.4) and stored at —20°C until use. Large-scale cultures were harvested by means of a Sharples MP4 continuous-flow centrifuge (Sharples Centrifuges Ltd., Chamberley, U.K.); harvesting of one 600 l batch takes about 2 h.

Enzyme assays

Hydrogenase activity was measured as H_2 consumption at 30°C with methylene blue as acceptor essentially as described by Feigenblum and Krasna [4]. The main compartment of a Warburg flask contained 50 mM Tris-HCl buffer (pH 7.4), 100 μM benzyl viologen, 2 mM mercaptoethanol, the enzyme and, with low protein concentrations, 0.3 mg/ml bovine serum albumin in a total volume of 2.1 ml. The side arm contained 0.1 ml 0.1 M methylene blue. Vessels were flushed for 10 min with H_2 freed from O_2 by passage through a column (150 × 4 cm) filled with palladium catalyst (Degussa, type E236P). They were then closed and shaken for another 3 h before tipping in the methylene blue.

H_2 evolution was measured as described by Peck and Gest [5].

Analytical procedures

Protein was determined according to the method of Lowry et al. [6] using bovine serum albumin as a standard.

Ultracentrifugation analyses were performed in a MSE analytical ultracentrifuge Model MK2, equipped with an ultraviolet scanning system. Sedimentation-equilibrium measurements for determination of the molecular weight were made by the 3 mm column method of Van Holde and Baldwin [7] using the absorption optic system at 280 nm.

EPR measurements were performed at 4.2—30 K on a Varian E-9 spectrometer or on a Varian E-3 spectrometer adapted for work at low microwave powers. Digitizing and simulation of spectra was performed as described by Albracht et al. [8].

Gel electrophoresis in the presence and in the absence of sodium dodecyl sulfate (SDS) was performed in 8—12% polyacrylamide gels, using the buffer system of Laemmli [9]. About 30 μg protein were applied per tube. Bovine serum albumin, catalase, ovalbumin and aldolase were used as molecular weight standards. Gels were run for approx. 3—4 h at 2 mA per tube. Analysis for hydrogenase activity in the gel was performed as described by Ackrell et al. [10].

Optical spectra were recorded on a Cary-17 spectrophotometer.

Results

Localization and solubilization of the enzyme

None of three treatments that are known to render the outer membrane permeable and release periplasmic proteins (treatment with Tris-EDTA (pH

TABLE I

EXTRACTION OF HYDROGENASE FROM *CHROMATIUM VINOSUM* CELLS

1. Cells were suspended at 25 mg/ml in a 50 ml vessel and sonicated by means of a Branson B12 sonifier, operating at 150 W for 20 min in 30-s periods with intermittent cooling, to keep the temperature below 8°C. 2. Cells were suspended at 25 mg/ml and disrupted by a single passage at 0°C through a Ribi RM-cell fractionater operating at a pressure of about 1000 Bar. 3. Cells were ground in a mechanical mortar with sand (weight (g) equal to about 4-times vol. (ml) cells) for the times indicated. 'Soluble' hydrogenase activity is determined in the supernatant after centrifugation for 90 min at 180 000 × *g*. No loss of activity was observed with any of the procedures used.

Method	'Soluble' hydrogenase (% of total)
1. Sonication	11
2. French press	17
3. Grinding for (h)	
0.5	11—15
1	14—20
1.5	25—30
2	35—40
3	45—50

9.0) at 21°C and 37°C, with Tris-EDTA (pH 8.0) in the presence of 1% toluene at 37°C and osmotic shock according to Heppel [11]), released any hydrogenase activity in the supernatant after centrifugation (10 min, 6000 × *g*) of the treated cell suspension. It is concluded, therefore, that, in contrast to, for instance, the hydrogenase from *Desulfovibrio vulgaris* [12], the enzyme in *C. vinosum* is not a periplasmic enzyme.

The two remaining possibilities for the localization of the enzyme, i.e. membrane-bound and soluble, were tested by breaking open the cells using three different methods: (1) sonication, (2) French-press treatment and (3) grinding with sand in a mechanical mortar. 'Soluble' was defined operationally as activity remaining in solution after centrifugation for 90 min at 180 000 × *g*. The results are shown in Table I. It is clear that the amount of hydrogenase found in the soluble fraction of the cell is rather variable and depends on the method used for cell disruption. This may be part of the explanation for the different values given in the literature, ranging from totally insoluble [4] to completely soluble [13]. Differences in growth conditions may also be of influence.

The amount of hydrogenase solubilized increases with increasing grinding time but levels off at about 50% after 3 h. Since the solubilized hydrogenase activity is retarded on a Ultrogel AcA-44 column, which has an exclusion limit of about 130 000 daltons, it may be concluded that the enzyme is truly solubilized and not bound to small membrane fragments. No increase in yield was found by grinding with 1 M NaCl in the presence of 10 mM EDTA, a treatment found effective with *Rhodospirillum rubrum* [14].

Purification

All steps in the purification were performed at 4°C under aerobic conditions. After grinding the cells in a mechanical mortar, as described above, sand and cell walls were removed by low-speed centrifugation (10 min, 1400 × *g*). A subsequent high-speed centrifugation (90 min, 180 000 × *g* for small quantities or 4 h, 70 000 × *g* for larger quantities) sedimented all membraneous material leav-

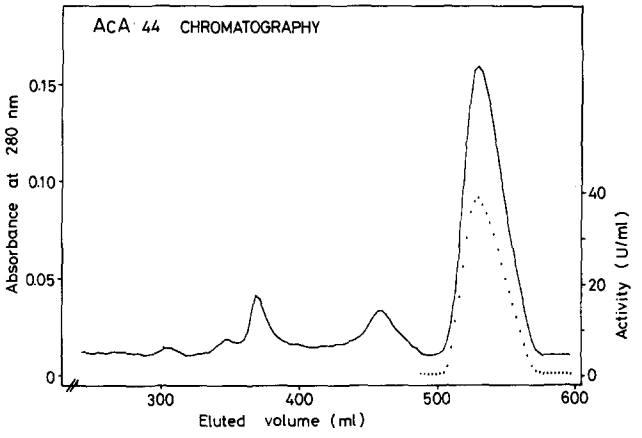

Fig. 1. Elution pattern of hydrogenase from Ultrogel AcA-44 column (2.5 × 160 cm). A_{280} (———), hydrogenase activity (· · · · ·).

ing about 50% of all hydrogenase activity in solution. The supernatant after the high-speed centrifugation was then brought to 20% saturation with solid $(NH_4)_2SO_4$, keeping the pH at 7.4 by addition of concentrated ammonia. The suspension was centrifuged at 70 000 × *g* for 20 min and the supernatant brought to 60% saturation with $(NH_4)_2SO_4$, after which the hydrogenase was sedimented by centrifugation for 20 min at 70 000 × *g*. The pellet was dissolved in 50 mM Tris-HCl buffer (pH 7.4) and brought on to a Phenyl-Sepharose CL-4B (Pharmacia Final Chemicals AB) column equilibrated with 50 mM Tris-HCl buffer (pH 7.4). After elution of the non-binding material with two column volumes of starting buffer, the hydrogenase bound to the column was eluted with a linear gradient of 0—70% ethylene glycol. Since the hydrogenase comes off the column just before the bulk of the bound proteins, a high purification is achieved in this single step. The collected fractions from the Phenyl-Sepharose column, without concentration or dialysis, were brought on to a DEAE-cellulose (Whatman, DE-52) column in 50 mM Tris-HCl buffer (pH 7.4) and eluted with a linear gradient of 0—0.4 M NaCl in the same buffer.

As a final step in the purification, gel filtration on an Ultrogel AcA-44 column (LKB-produkter AB) was used (Fig. 1), after concentration of the DEAE-eluate to about 5 ml on an Amicon PM 30 filter. Gel filtration on a Sephadex G-100 column gave results inferior to those obtained with an Ultrogel AcA-44 column.

In this way we were able to obtain a preparation with a specific activity of about 425 μmol H_2 consumed/min per mg in a 13% yield. A summary of the above purification is given in Table II.

This procedure is, however, not suitable for isolations on a somewhat larger scale. Applying the above procedure to the cell paste of a single 600 l culture (i.e., about 0.6—1.2 kg cells) results in a hydrogenase preparation that is only about 25% pure, according to its specific activity, so that the following two additional steps are required. (1) Heat denaturation: after incubation for 10 min at 62—64°C, either directly before or after the $(NH_4)_2SO_4$ precipitation,

TABLE II

PURIFICATION OF HYDROGENASE FROM *CHROMATIUM VINOSUM*

The hydrogenase activities in the table are based on H_2 consumption with methylene blue as electron acceptor [4]. The rate of H_2 production with dithionite plus methyl viologen [5] was about one-tenth of these values.

	Total protein (mg)	Specific activity (μmol H_2 consumed/ min per mg)	Purification	Yield (%)
Cell-free extract	34 534	0.13	1	100
High-speed supernatant	6 062	0.33	2.5	52
20% $(NH_4)_2SO_4$ supernatant	3 457	0.53	3.8	40
60% $(NH_4)_2SO_4$ pellet	1 204	1.6	12.3	41
Phenyl-Sepharose eluate	17.2	44.2	340	28
DEAE-cellulose eluate	9.7	91.7	705	20
Ultrogel AcA-44 eluate	1.5	425	3269	13

the resultant precipitate is removed by centrifugation (20 min, 180 000 × *g*). The purification factor of this step is usually around 2-fold and when it is carried out in the presence of a high salt concentration (1 M NaCl) the yield is at least 80%. (2) In addition to the three columns described above a second Phenyl-Sepharose chromatography is now employed as a last purification step. Recovery of activity is usually around 55%, the purification factor being about 1.8.

In this way preparations of 60—80% purity can be obtained in an overall yield of about 10%. Since for most physico-chemical work a 100% pure enzyme is not absolutely necessary, we have not attempted to further improve these large scale isolations.

Purity and subunit structure

Electrophoresis on 8 and 12% acrylamide gels of the preparation obtained after column chromatography over Ultrogel AcA-44 in the small-scale isolation (see Table II), shows a single protein band running at the same height as the activity band, indicating that the preparation is pure.

Electrophoresis on 12% acrylamide gels in the presence of 0.1% SDS gave a single band an R_F value indicating a subunit molecular weight of 61 000—63 000. The molecular weight of the active enzyme was estimated by means of high-speed equilibrium sedimentation in an analytical ultracentrifuge. Assuming a partial specific volume of 0.734 cm^3/g [2] the molecular weight was calculated to be 62 000. From the elution behaviour of the enzyme on Ultrogel AcA-44, a molecular weight for the native protein in the range between 60 000 and 70 000 can be determined. Thus the enzyme consists of a single polypeptide chain of about 62 000 daltons.

Optical absorption spectrum

The absorption spectrum in the 250—550 nm region, given in Fig. 2, shows in addition to the 280 nm band (ϵ = 57 200 $M^{-1} \cdot cm^{-1}$) a very broad absorption band around 410 nm (ϵ = 13 200 $M^{-1} \cdot cm^{-1}$), which is rather common for iron-sulphur proteins [15]. Addition of dithionite causes a slight decrease in the absorption around 410 nm (data not shown).

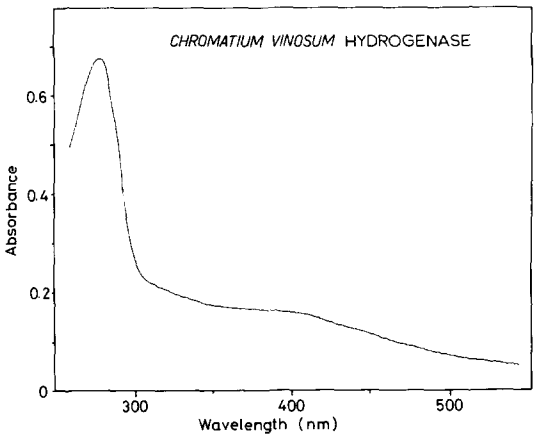

Fig. 2. Optical absorption spectrum of pure hydrogenase from *C. vinosum* (0.76 mg protein/ml).

EPR properties

The EPR spectrum of the purified enzyme at X-band (9 GHz) is shown in Fig. 3. Upon reduction with dithionite (Fig. 3B) or H_2 (data not shown) all signals completely disappear. In this respect, our preparation differs from that of Gitlitz and Krasna [2] who found weak signals at $g = 2.2$ and 2.06, after reduction. When the enzyme is activated, but not reduced, by removal of O_2 using repeated evacuation and flushing with O_2-free He or N_2 (containing less

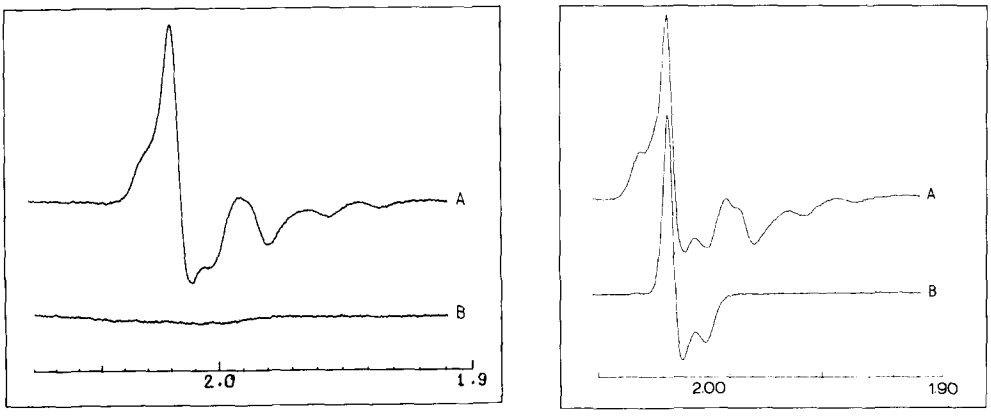

Fig. 3. EPR spectrum of pure hydrogenase from *C. vinosum*. (A) Enzyme as isolated. (B) After reduction with sodium dithionite. EPR conditions: Microwave frequency, 9337 MHz; temperature, 12 K; microwave power, 0.38 mW; modulation amplitude 1 mT; scanning rate, 12.5 mT/min. The modulation frequency in this and all other EPR spectra was 100 KHz. The scale at the bottom of this and the other EPR figures refers to the *g* value.

Fig. 4. Effect of removal of oxygen on the EPR spectrum of hydrogenase. The enzyme used for this and the following figures was not pure (specific activity 64 μmol H_2 consumed/min per mg), but no foreign EPR signals could be detected in this preparation. (A) Enzyme as isolated. (B) After repeated evacuation and gassing with the He and incubation under He for 15 h. EPR conditions: microwave frequency, 9338 MHz; temperature, 12.5 K; microwave power, 0.15 mW; modulation amplitude, 0.4 mT; scanning rate, 5 mT/min.

than 1 ppm of O_2), a remarkable change in the EPR spectrum was observed (compare Fig. 4A and 4B). The lines at $g = 1.94$, 1.96 and 1.98 as well as the shoulder at $g = 2.03$ have disappeared and the remaining spectrum has a shape and EPR properties specifically indicative for a $[4Fe\text{-}4S]^{3+(3+,2+)}$ cluster, and is comparable to that reported by Gitlitz and Krasna [2]. The lines at $g = 1.94$, 1.96 and 1.98 and the shoulder at $g = 2.03$ will be referred to as the 'additional signal'.

The EPR spectrum indicates that the Fe-S cluster is close to axial symmetry with a $g_z < g_{x,y}$. This could be confirmed by a computer simulation of the spectrum (see Fig. 5). This specific feature is uncommon for $[4Fe\text{-}4S]^{3+}$ clusters described so far. The classical $[4Fe\text{-}4S]^{3+}$ cluster of high potential iron protein (Hipip) from *C. vinosum*, for example, gives an EPR signal with $g_z > g_{x,y}$ [16].

On increasing the temperature to 30 K all signals disappear due to relaxation broadening.

The power-saturation behaviour of the 'additional signal' is similar to that of the Fe-S signal, except for a slight difference in the $P_{1/2}$ values (data not shown).

The additional signal is present in all enzyme preparations throughout the whole purification procedure with a constant intensity relative to that of the Fe-S signal. After incubation of the hydrogenase for 4 h in 2-mercaptoethanol (2 mM) it disappears, as it also does, within 2 min, after addition of either NADH (2 mM) or ascorbate (20 mM) in the presence of phenazine methosulfate (1 and 5 μM, respectively). After removal of the additional signal by incubation under He, only about 10% of the signal is regained after 22 h in air. An

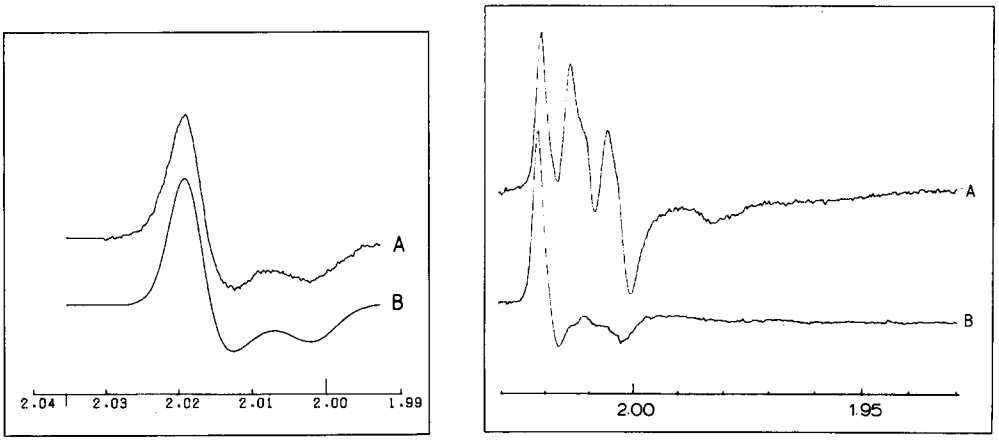

Fig. 5. Comparison of the EPR spectrum of hydrogenase after incubation under He for 16 h, and the simulated spectrum. (A) Experimental spectrum; (b) Simulated spectrum. Parameters used in simulation: $g_{x,y,z}$ = 2.0183, 2.01639, 2.0019, and widths (x, y, z) = 0.68, 1.1, 1.05 mT. EPR conditions: microwave frequency 9316 MHz; temperature, 4.2 K; microwave power, 1.5 mW; modulation amplitude, 0.4 mT; scanning rate, 10 mT/min.

Fig. 6. EPR spectra of hydrogenase at Q-band frequency. (A) Enzyme as isolated, (B) after incubation in 2 mM 2-mercaptoethanol for 4 h. EPR conditions: microwave frequency, 35 039 MHz; temperature, 19 K; microwave power, 2.2 mM; modulation amplitude, 0.63 mT; scanning rate, 20 mT/min.

TABLE III

TITRATION OF H_2-REDUCED HYDROGENASE WITH FERRICYTOCHROME *c*

Hydrogenase in the indicated amounts (based on quantitative amino acid analysis and M_r 62 000), dissolved in 1.8 ml 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 nmol $K_3Fe(CN)_6$ (as mediator), was placed in the main compartment of a Thunberg cell. The side arm contained excess fericytochrome *c* (15.0 nmol in Expt. 1; 25.9 nmol in Expt. 2). Both compartments were cooled in ice and evacuated to boiling. O_2-free H_2 was then admitted. This evacuation and flushing cycle was repeated five times. After 16 h, the H_2 was replaced by He by the same procedure. Evaporation was allowed for by determining loss in weight during this procedure. After mixing the two compartments, the $A_{550\ nm}$ was measured. No change took place in 10 min immediately after mixing. The tube was then opened, excess $K_3Fe(CN)_6$ added and the absorbance measured again. From the difference in $A_{550\ nm}$, the amount of ferricytochrome *c* reduced by the hydrogenase was calculated ($\Delta_{550\ nm}$ (red-ox) = 21 $mM^{-1} \cdot cm^{-1}$).

	Expt. 1	Expt. 2
Hydrogenase (nmol)	5.69	7.59
Ferricytochrome *c* reduced (nmol)	12.95	16.56
Correction for $Fe^{II}(CN)_6$ (nmol)	0.5	0.5
Ferricytochrome *c* reduced by hydrogenase (nmol)	12.45	16.16
mol ferricytochrome *c* reduced per mol hydrogenase	2.19	2.13

enzyme preparation incubated in 2-mercaptoethanol does not regain the signal after removal of the 2-mercaptoethanol by dialysis, whether or not ferricyanide is added. Reoxidation by air of a H_2-reduced enzyme (after exchange of excess H_2 by He) causes the reappearance of the Fe-S signal with hardly any additional signal. Oxidation with ferricytochrome *c*, however, does cause the additional signal to return. Two molecules of ferricytochrome *c* are required (see Table III), suggesting that the additional signal is caused by a redox component. Fig. 6A shows the spectrum of the isolated enzyme at Q-band frequency (35 GHz) and Fig. 6B shows the spectrum after incubation in 2-mercaptoethanol, i.e., when the additional signal has disappeared and only the signal of the [4Fe-4S] cluster can be seen. Both the line shape of the [4Fe-4S] cluster and the *g* values are independent of the microwave frequency. This is to be expected for the spectrum of a Fe-S cluster, since the line widths are governed by *g* strain [17] and are thus proportional to the microwave frequency. Since the splitting of the *g* values is also proportional to the frequency, the overall appearance of such spectra is usually rather similar at X- and Q-band. This does not, however, hold true for the other signals seen in Fig. 6A. Especially the line at $g = 2.03$ (X-band) shifts to $g = 2.015$ in Q-band and an intense line at $g = 2.005$ (Q-band) is seen that is not detected in the X-band. The lines at $g = 1.94$, 1.96 and 1.98 in X-band spectra do not seem to be appreciably shifted in Q-band spectra. We conclude, therefore, that the additional signal does not arise from a single paramagnetic species. Moreover, since the *g* values of a number of the extra lines change upon going from 9.3 GHz to 35 GHz (compare Figs. 3A and 6A) we conclude that at least some of the species responsible for these signals are not simple $S = 1/2$ systems.

The intensity of the EPR signal of the [4Fe-4S] cluster in the isolated enzyme, quantitated with respect to a standard of copper perchlorate, corresponds to a $S = 1/2$ concentration equal to one quarter of the enzyme concentration (based on M_r 62 000). This apparent underestimation of the amount of free spins/molecule is frequently observed in other hydrogenases. In our case it is apparently not caused by a partial reduction of the enzyme, since ferri-

cyanide, either in the presence or absence of the redox mediator phenazine methosulfate, had no effect on the intensity of the EPR spectrum. We have, however, encountered two different conditions in which higher values for the amount of free spins/molecule can be obtained. (1) An enzyme preparation activated under He and then brought in contact with air shows a slow and steady increase in the height of the [4Fe-4S] signal. The ultimate signal, obtained after 5 h, accounts for almost one free spin/enzyme molecule. (2) Immediately after reduction with NADH or ascorbate, both in the presence of phenazine methosulfate, there is an increase in signal height of the [4Fe-4S] cluster, followed by a much slower decay (see Fig. 7A and 7B). As mentioned above, the additional signal also disappears within a few minutes after addition of the reducing agents but whether the increase in signal height of the [4Fe-4S] cluster exactly corresponds to the disappearance of the additional signal must still be examined.

Replacement of water by ^2H_2O has no effect on the line shape of the EPR signal of the [4Fe-4S] cluster. From this observation it may be concluded that no easily exchangeable protons in magnetic interaction with the Fe-S cluster are present.

Effect of inhibitors

None of the inhibitors of the enzyme listed in Table IV had any effect on shape or intensity of the EPR signals. The lack of effect of CO is in contrast to its marked effect on the $[4Fe\text{-}4S]^{3+(3+,2+)}$ cluster and the two $[4Fe\text{-}4S]^{2+(2+,1+)}$ clusters in the hydrogenase from *Clostridium pasteuranium* [18, 19]. The inhibiting action of *N*-bromosuccinimide, which modifies tryptophan residues, might mean that one or more tryptophan residues are essential for enzyme activity, especially since a gross conformational change of the protein is unlikely in view of the lack of effect on the EPR signal of the Fe-S cluster.

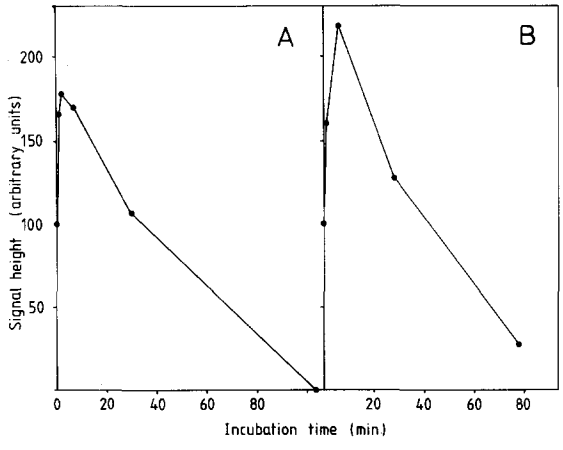

Fig. 7. Time course of the effect of NADH/phenazine methosulfate (A) and ascorbate/phenazine methosulfate (B) on the height of the $g = 2.01$ signal. NADH (2 mM) and phenazine methosulfate (1 μM), or ascorbate (20 mM) and phenazine methosulfate (5 μM), were added to hydrogenase preparations in an EPR tube. Incubation was carried out at room temperature in a single EPR tube with intermittent freezing and thawing.

TABLE IV

INHIBITION OF HYDROGENASE ACTIVITY

Hydrogenase activity was based on H_2 consumption with methylene blue as electron acceptor [4]. All inhibitors were present at a final concentration of 1 mM except CO that was present in a 1 : 1 ratio with H_2.

Compound tested	Inhibition (%)
CO	100
$CuSO_4$	100
$CdSO_4$	87
$ZnSO_4$	96
$CoCl_2$	37
$MnSO_4$	0
N-Bromosuccinimide	100
p-Hydroxymercuribenzoate	0
Mersalyl	0
$NaAsO_2$	0
KCN	0
NaN_3	0

Well-known inhibitors of metalloenzymes like cyanide and azide and -SH inhibitors such as *p*-hydroxymercuribenzoate and Mersalyl have no effect.

The finding that none of the inhibitors of the enzyme had any effect on the EPR spectrum indicates that they either cause very small changes in protein structure and/or that these changes are far away from the Fe-S cluster, since it is known that Fe-S clusters generally respond very sensitively to small changes in their environment.

Discussion

Comparison of soluble with membrane-bound enzyme

In our results, hydrogenase activity is found in both the soluble and the membrane fractions. This result disagrees with those of Feigenblum and Krasna [4] and Gitlitz and Krasna [2] who find that the enzyme is membrane-bound, and Weaver et al. [13] who find it completely soluble, but agrees with Buchanan and Bachofen [20] who have reported that 25% of the enzyme is soluble. These differences may be due both to differences in growth conditions and the fact that hydrogenases might readily form strong complexes with other cellular proteins [1].

The membrane-bound form of *C. vinosum* hydrogenase has been isolated by Gitlitz and Krasna [2] and by Kakuno et al. [21]. The preparation of Kakuno et al. with a specific activity of 1.9 μmol H_2 evolved/min per mg, equivalent to about 19 μmol H_2 consumed/min per mg, is much less active than ours (425 μmol H_2 consumed/min per mg). The activity of our preparation is similar to that isolated by Gitlitz and Krasna [2], as is the EPR spectrum in the presence of 2-mercaptoethanol, which was used by these authors in the isolation. The weak signals found by Gitlitz and Krasna [2] after reduction with $Na_2S_2O_4$ were absent in our preparation.

We found a molecular weight for the native protein of about 62 000, both by sedimentation-equilibrium measurements and gel filtration. Since the value ob-

tained after electrophoresis on polyacrylamide gels in the presence of SDS and 2-mercaptoethanol is also 61 000—63 000, we conclude that the enzyme consists of a single polypeptide chain. Gitlitz and Krasna [2] found a molecular weight of 100 000 for the membrane-bound hydrogenase, the molecule being built from two subunits of 50 000 daltons. Although these data suggest, at first, that the two enzymes are different proteins, the marked similarity in the EPR spectra indicates against this. The higher molecular weight for the membrane-bound protein found by Gitlitz and Krasna [2] could well be due to binding of deoxycholate used in the isolation procedure, since bound detergent can seriously influence the hydrodynamic properties of proteins. The different results on the determination of the subunit molecular weight by polyacrylamide electrophoresis in the presence of SDS cannot be explained simply.

EPR spectroscopy

The EPR spectrum indicates that the enzyme, as isolated, contains a [4Fe-4S]$^{3+(3+,2+)}$ cluster with nearly axial symmetry. In addition to the signal of this cluster we find in the oxidized enzyme a number of other lines, the origin of which is not yet known. From a comparison of spectra taken at X-band and Q-band frequencies we may conclude that the 'additional' signal does not arise from a single paramagnetic species. It is, furthermore, very likely that some of the species responsible for these lines are not simple S = 1/2 systems, since the *g* values of a number of the lines change upon going from 9 GHz to 34 GHz (compare Figs. 3A and 6A).

Although the 'additional' signal disappears upon removing oxygen, neither oxygen itself nor an interaction of oxygen with the enzyme can be the cause of the signals, since it is regained under anaerobic conditions, after anaerobic reoxidation of the enzyme with ferricytochrome *c*. However, the interesting possibility remains that the 'additional' EPR signal is in some way related to the state of activity of the enzyme.

When isolated hydrogenase is reduced anaerobically by NADH or ascorbate in the presence of phenazine methosulfate, there is a rapid disappearance of the 'additional' EPR signal and a more than doubling of the signal intensity of the Fe-S cluster (see Fig. 7). From this observation we can draw two conclusions: (1) the 'additional' EPR signal is caused by redox species with a rather high (around 0 mV) redox potential, or the additional signal is caused by an interaction of two paramagnets one (or perhaps both) of which has a high redox potential, and (2) the [4Fe-4S] cluster also has a high redox potential.

As yet we have no explanation of the increase in signal intensity of the Fe-S cluster. If the 'additional' signal is caused by an interaction of two paramagnets, one of these could in principle be the [4Fe-4S] cluster. However, the observation that removal of oxygen results in the disappearance of the 'additional' signal without any effect on the signal intensity of the [4Fe-4S] cluster makes this less likely, but not impossible, since we have never observed the 'additional' signal together with the complete (i.e., one free spin/molecule) Fe-S signal.

As to the quantification of the [4Fe-4S] cluster signal there still remain some problems. The isolated oxidized enzyme shows 0.26 free spin/molecule after removal of the additional signal; oxidation with ferricyanide has no effect.

Activation of the enzyme under He or N_2 gas, followed by re-entry of air, causes an increase in the amount of clusters detected per molecule to about one. Neither this finding nor the experiments discussed above, in which the amount of Fe-S signal increases upon reduction with NADH/phenazine methosulfate or ascorbate/phenazine methosulfate, can be explained at the moment.

The function of the iron-sulfur cluster

Up to now all investigators working on hydrogenases have implicitly or explicitly suggested that the Fe-S cluster(s) in hydrogenase is (are) directly involved in the H_2-binding (releasing) reaction in the enzyme [2,18,22]. The following evidence indicates that this is not the case in the hydrogenase from *C. vinosum*: (1) experiments with inhibitors of the enzyme indicate that a severe inhibition of the enzyme is not reflected in the EPR spectrum of the Fe-S cluster, although this spectrum is very sensitive to small changes in the protein conformation in the vicinity of the cluster. (2) It is very likely that the active center contains a site that can bind and release protons. Exchange of water for ^2H_2O will lead to $^2H^+$ binding and, therefore, if this deuteron is in magnetic interaction with the Fe-S cluster, to a change in the EPR spectrum of the cluster. No changes were, however, observed. (3) The Fe-S cluster can be reduced completely by ascorbate ($E_0' \sim 50$ mV [23]), indicating that the redox potential of the Fe-S cluster must be around 0 mV, which is so high that a direct involvement in the H_2 production reaction is not likely since the H^+/H_2 couple has an E_0' of −420 mV.

C. vinosum, like many other photosynthetic bacteria, can grow under H_2/CO_2 in the light [24,1]. Under these conditions hydrogenase transfers reducing equivalents from H_2 to the natural, yet unknown, acceptor of the enzyme. We propose that the Fe-S cluster is involved in electron-transfer from the reduced enzyme to the acceptor, a function fulfilled by Fe-S clusters in many dehydrogenases in bacterial and mitochondrial electron-transfer systems. Since the Fe-S cluster in *C. vinosum* hydrogenase has a relatively high potential, energy is produced when electrons pass through the enzyme from H_2 to the acceptor. It is likely that this energy is conserved for the cell in a suitable form. This then implies that another redox group is involved at the site where electrons are withdrawn from H_2. This is the subject for further investigations.

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References

1 Weaver, P.F., Lien, S. and Seibert, M. (1980) Sol. Energy 24, 3—45
2 Gitlitz, P.H. and Krasna, A.I. (1975) Biochemistry 14, 2561—2568

3 Hendley, D.D. (1955) J. Bacteriol. 70, 625—634
4 Feigenblum, E. and Krasna, A.I. (1970) Biochim. Biophys. Acta 198, 157—164
5 Peck, H.D., Jr. and Gest, H. (1957) J. Bacteriol. 73, 569—580
6 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
7 Van Holde, K.E. and Baldwin, R.L. (1958) J. Phys. Chem. 62, 734—743
8 Albracht, S.P.J., Dooijewaard, G., Leeuweik, F.J. and Van Swol, B. (1977) Biochim. Biophys. Acta 459, 300—317
9 Laemmli, U.K. (1970) Nature 227, 680—685
10 Ackrell, B.A.C., Asato, R.N. and Mower, H.F. (1966) J. Bacteriol. 92, 828—838
11 Heppel, L.A. (1971) in Structure and Function of Biological Membranes (Rothfield, L.I., ed.), pp. 223—247, Academic Press, New York
12 Van der Westen, H.M., Mayhew, S.G. and Veeger, C. (1978) FEBS Lett. 86, 122—126
13 Weaver, P., Tinker, K. and Valentine, R.C. (1965) Biochem. Biophys. Res. Commun. 21, 195—201
14 Kakuno, T., Hiura, H., Yasmashita, J., Bartsch, R.G. and Horio, T. (1978) J. Biochem. (Tokyo) 1649—1651
15 Orme-Johnson, W.H. (1973) Ann. Rev. Biochem. 42, 159—204
16 Antanaitis, B.C. and Moss, T.H. (1975) Biochim. Biophys. Acta 405, 262—279
17 Fritz, J., Anderson, R., Fee, J., Palmer, G., Sands, R.H., Tsibris, J.C.M., Gunsalus, I.C., Orme-Johnson, W.H. and Beinert, H. (1971) Biochim. Biophys. Acta 253, 110—133
18 Erbes, D.L., Burris, R.H. and Orme-Johnson, W.H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4795—4799
19 Chen, J.S., Mortenson, L.E. and Palmer, G. (1976) in: Iron and Copper Proteins (Yasunobu, K.T., Mower, H.F. and Hayaishi, O., eds.), pp. 68—82, Plenum Plublishing Corp., New York
20 Buchanan, B.B. and Bachofen, R. (1968) Biochim. Biophys. Acta 162, 607—610
21 Kakuno, T., Kaplan, N.O. and Kamen, M.D. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 861—863
22 Chen, J.S. (1978) in Hydrogenases: Their Catalytic Activity, Structure and Function (Schlegel, H.G. and Schneider, K., eds.), pp. 57—81 Goltze, Göttingen
23 Ball, E.G. (1937) J. Biol. Chem. 118, 219—239
24 Roelofsen, P.A. (1934) Proc. Royal. Acad. Sci. (Amsterdam) 37, 660—669