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CHARACTERIZATION AND STABILITY OF HYDROGENASE FROM *CHROMATIUM*

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Summary

The absorption spectrum of the hydrogenase from *Chromatium*, which contains four iron atoms and four atoms of acid-labile sulfide, in 80% dimethylsulfoxide or hexamethylphosphoramide suggests the presence of a single [4Fe-4S] cluster. The EPR spectra of the oxidized enzyme in air, argon or carbon monoxide are the same with signals centered at $g = 2.01$. The enzyme reduced by hydrogen is EPR silent. The EPR spectrum is consistent with a [4Fe-4S] cluster. *Chromatium* hydrogenase and the hydrogenase from *Proteus vulgaris* show relative stability towards denaturation by sodium dodecyl.sulfate (SDS), urea, guanidine and organic solvents.

Introduction

The enzyme hydrogenase is of unique interest because of the simplicity of its substrate. In addition, hydrogenase plays a key role in various systems proposed [1] for the biophotolysis of water to hydrogen. Recent studies on the pure enzyme isolated from a variety of microorganisms (reviewed in Ref. 1) have established that hydrogenase is a non-heme iron-sulfur protein. Though different hydrogenases have different molecular weights, subunit composition, iron and acid-labile sulfide content, they share many common properties. All hydrogenases show a visible absorption peak near 400—410 nm and many show

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Abbreviation: DMSO, dimethylsulfoxide.

EPR spectra characteristic of iron-sulfur proteins.

With respect to the type of iron-sulfur cluster, the visible spectral data are consistent with either [2Fe-2S] clusters or [4Fe-4S] clusters [2] bound to the protein via iron-cysteine bridges. Since hydrogenase catalyzes heterolytic cleavage of hydrogen to form an enzyme hydride [1,3], it is essential to know the nature of the iron-sulfur cluster in order to deduce the molecular mechanism of hydrogen cleavage. In the case of the hydrogenases from *Clostridium pasteurianum* [4,5], and *Desulfovibrio gigas* [6] it has been established that the cluster is of the [4Fe-4S] type. This was determined by quantitative displacement of the iron-sulfur core by aromatic thiols with the protein denatured in organic solvents. The spectra of the displaced clusters were different for [4Fe-4S] and [2Fe-2S] types and could be used to characterize the clusters in the native proteins.

This laboratory has studied the hydrogenase from *Chromatium* [3] which has a molecular weight of 100 000 and contains four iron atoms and four acid-labile sulfides. The visible and EPR spectra established the presence of an iron-sulfur cluster in the enzyme. In this paper experiments are described which suggest that the cluster in this hydrogenase is of the [4Fe-4S] type. In the course of these studies the stability of *Chromatium* hydrogenase towards denaturation was observed and these studies are also described.

Experimental

All reagents were obtained from commercial sources and were of the highest purity available. *Chromatium* cells were grown as previously described [7] and stored as a frozen paste. Hydrogenase was purifed from these cells as previously reported [3] and final purity was determined by specific activity and by the ratio of absorbance at 280 nm (protein) to 410 nm (iron-sulfur chromophore). For the pure enzyme this ratio is 6.86 [3]. The specific activity was 82 μmol H_2/min per mg protein by the exchange assay, 320 by the methylene blue reduction assay and 35 by the methyl viologen oxidation assay [3,8]. The molecular weight of the enzyme determined by sucrose density gradient centrifugation was 99 000 as previously reported [3].

Hydrogenase activity was assayed by the deuterium exchange or tritium exchange methods [8] in 0.15 M phosphate buffer, pH 6.7. Bovine serum albumin (4 mg) was added to each assay. In the deuterium exchange assay, 1 unit of activity is defined as the appearance of 0.05% ^2H/h (with 10% ^2H_2O). In the tritium assay 1 unit is defined as an exchange rate of 1 mV/h under the standards assay conditions. 1 unit of exchange activity is equal to the activation of 0.287 μmol H_2/min.

Hydrogenase samples used in cluster identification experiments were concentrated to give an absorbance of 1.0 or more at 410 nm in 50 mM Tris buffer, pH 8.5. The conditions for these experiments followed those developed by Gillum et al. [5]. All solvents and liquid thiols were distilled at reduced pressure under nitrogen. Hexamethylphosphoramide was distilled in the presence of sodium and the distillate stored frozen under nitrogen. *Chromatium* high potential iron protein (Hipip), containing a [4Fe-4S] cluster, was isolated as described [9] and spinach ferredoxin, containing a [2Fe-2S] cluster, was pur-

chased from Sigma Chemical Company. These proteins were used as references in spectral and displacement experiments. The results obtained using reduced Hipip and oxidized ferredoxin were comparable to the results previously reported [5]. For the displaced core of reduced Hipip, A_{458}/A_{550} values greater than 1.9 were routinely found. Such solutions were stable for up to 24 h, showing no further spectral change. All spectra were recorded on a Cary model 14 spectrophotometer.

EPR measurements were performed on a Varian E-9 EPR spectrometer at 10—15 K. Samples were cooled by a He vapor system (Air Products Co., Allentown, PA). Gas exchange was performed on EPR samples in a Thunberg-type EPR tube to facilitate equilibrium between solution and gas phases.

Results

Spectral properties of the iron-sulfur cluster

The visible absorption spectrum of oxidized *Chromatium* hydrogenase is shown in Fig. 1 along with the spectra of reduced Hipip [4Fe-4S] and oxidized spinach ferredoxin [2Fe-2S]. Hydrogenase shows a peak at 410 nm, Hipip at 388 nm and spinach ferredoxin at 420 and 465 nm. While the hydrogenase spectrum appears similar to that for Hipip (except for λ_{max}) and different from spinach ferredoxin, the spectrum of the native enzyme by itself is not sufficient to deduce the nature of the iron-sulfur cluster. However, it has been shown [5] that [4Fe-4S] proteins which exhibit dissimilar spectra near 400 nm, show the same characteristic spectrum when treated anaerobically with 80% DMSO or hexamethylphosphoramide. This spectrum, shown in Fig. 1 for Hipip consists of a shoulder near 400 nm with increasing absorption at shorter wavelengths and decreasing absorbance above 400 nm. In contrast, proteins containing [2Fe-2S] clusters (such as spinach ferredoxin, Fig. 1) give spectra only slightly different from that seen in aqueous solution with a maximum near 420 nm and a shoulder near 455 nm.

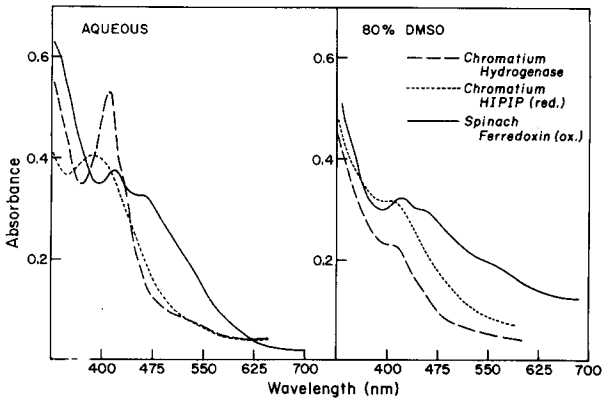

Fig. 1. Spectra of *Chromatium* hydrogenase and iron-sulfur proteins in aqueous and organic solvents. The left panel gives the spectra in 50 mM Tris buffer, pH 8.5, and the right panel shows the spectra in 80% DMSO/20% Tris buffer.

The spectrum of *Chromatium* hydrogenase in 80% DMSO (Fig. 1) is nearly identical to that of Hipip in the same solvent and decidedly different from that of spinach ferredoxin. Identical results were obtained in 80% hexamethylphosphoramide. These results suggest that the four irons and four acid-labile sulfides in *Chromatium* hydrogenase are present as a single [4Fe-4S] cluster. The hydrogenase of *C. pasteurianum* which contains three [4Fe-4S] clusters showed a spectrum in 80% hexamethylphosphoramide [5] similar to that shown in Fig. 1 for *Chromatium* hydrogenase.

The displacement of the iron-sulfur cluster of *Chromatium* hydrogenase by aryl thiols, with the protein in 80% non-aqueous solvent, was attempted after first demonstrating that the method worked as reported [4—6,10] for Hipip and spinach ferredoxin. *Chromatium* hydrogenase in 80% DMSO or 80% hexamethylphosphoramide with 20% 0.05 M Tris buffer, pH 8.5, was treated anaerobically with a 10—100-fold excess of benzenethiol. No change in the spectrum was observed suggesting that the iron-sulfur cluster was not displaced from *Chromatium* hydrogenase under these conditions. As the thiol/iron ratio was increased above 100, a precipitate formed making further spectral observations impossible. When hydrogenase was added directly to DMSO or hexamethylphosphoramide containing a 100-fold excess of benzenethiol, a precipitate appeared immediately. No such precipitation was observed with Hipip or spinach ferredoxin. Use of tetramethylurea or *N*-methylformamide as solvent or *p*-methoxybenzenethiol as thiol gave similar results, i.e., no extrusion below a 100-fold excess of thiol and precipitation above a 100-fold excess.

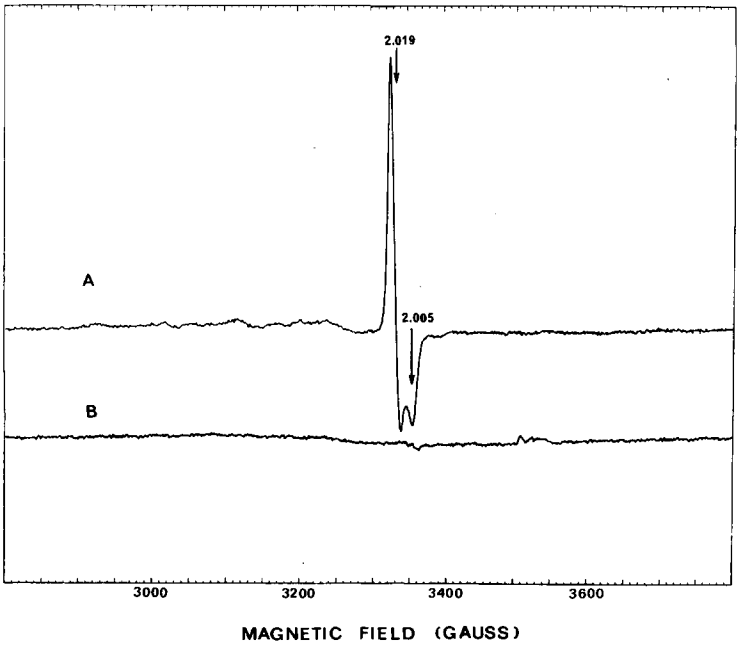

Fig. 2. X-band EPR spectra of *Chromatium* hydrogenase at 11 K. The enzyme ($1.6 \cdot 10^{-5}$ M) was in 0.05 M Hepes buffer (pH 7.1) containing 0.1 M KCl. EPR operating conditions: microwave power, 5 mW; modulation amplitude, 10 G; microwave frequency, 9.402 GHz; spectrometer gain, $2.5 \cdot 10^3$. A, oxidized hydrogenase; B, enzyme reduced with H_2.

Electron paramagnetic resonance spectra

The EPR spectrum of *Chromatium* hydrogenase, as isolated, is shown in Fig. 2A. This spectrum was taken with the enzyme exposed to air. No precautions were taken to exclude oxygen, since the enzyme is known to be reversibly inhibited by oxygen [1] and its possible effect on the EPR spectrum was of interest. The sample was then evacuated, flushed with oxygen-free argon, and the EPR spectrum was recorded after incubation in an argon atmosphere for 30 min. No changes were observed in the spectrum. The g values of the observed axial signal were the same as previously reported [3]: i.e., $g_{\perp} \simeq 2.019$ and $g_{\parallel} \simeq 2.005$. The hydrogenase was then flushed with hydrogen and incubated with hydrogen for 30 min. The oxidized signals completely disappeared (Fig. 2B) and no new signals were observed demonstrating reduction of the enzyme by its substrate. Addition of sodium dithionite did not change the spectrum of the reduced enzyme. The previously reported [3] weak EPR signal in the reduced enzyme could be observed, but only after extensive manipulation of the sample, including repeated freeze-thaw cycles. Its low intensity and g values (uncharacteristic of other reported iron-sulfur proteins) suggest that this species is not a normally accessible state of the native enzyme and may, in fact, arise from a degradation product of the enzyme.

After incubation with carbon monoxide, the H_2-reduced enzyme still gave no detectable EPR signal. The characteristic oxidized spectrum could be regenerated by exposure to air or addition of ferricyanide. The latter did not increase the intensity of the oxidized signal. Carbon monoxide had no effect on the oxidized signal.

Effect of denaturants on Chromatium hydrogenase

The first indication that hydrogenase activity was relatively stable was the observation that bands containing enzyme activity were detected after polyacrylamide gel electrophoresis in the presence of SDS or urea. A study was then undertaken of the quantitative effect of denaturants on hydrogenase activity as measured by deuterium exchange and is summarized in Table I. SDS at a concentration of 0.1% had little effect on hydrogenase activity. When the SDS concentration was raised to 0.5—1.0%, half the activity remained if the assay was conducted in one-tenth the concentration of denaturant while only 10% of the activity was retained if the assay was run in 0.5—1.0% SDS. These results indicate that SDS denaturation is partially reversible. The presence of mercaptoethanol (0.1—1.0%) during SDS denaturation did not affect the extent of inactivation. The relative stability of *Chromatium* hydrogenase to SDS has also been reported by Kakuno et al. [11] while the enzyme from *Desulfovibrio vulgaris* was inactivated by SDS [12].

Table I shows that *Chromatium* hydrogenase was relatively insensitive to denaturation by urea at concentrations up to 5 M. Even at 7.5 M urea, the enzyme still retained 18% activity which could be appreciably reversed by dilution. This stability to urea denaturation has also been observed in other laboratories [11,12]. Guanidine at concentrations below 2 M had no appreciable effect on hydrogenase activity while at 6.2 M the enzyme was irreversibly inactivated. In both cases, the presence of mercaptoethanol had little effect on the degree of inactivation.

TABLE I

EFFECT OF DENATURANTS ON *CHROMATIUM* HYDROGENASE ACTIVITY

For each experiment the enzyme was incubated at 25°C for the indicated period in the presence of the denaturant in a total volume of 0.5 ml. A control was diluted to the same extent with buffers and incubated for the same period of time. The assay was run in the presence of the same concentration of denaturant or in the absence of additional denaturant. HMPA, hexamethylphosphoramide.

Reagent	Concentration of denaturant		Incubation time (h)	Activity remaining (%)
	During incubation	During assay		
SDS	0.1%	0.01%	3	100
	0.1%	0.1%	3	71
	0.5%	0.05%	1	42
	0.5%	0.5%	1	11
	1.0%	0.1%	3	40
	1.0%	1.0%	1	8
Urea	5.2M	0.6M	3	100
	5.0M	5.0M	3	41
	7.5M	1.35M	3	76
	7.5M	7.5M	3	18
Guanidine	2.0M	0.2M	3	100
	2.1M	2.1M	3	41
	6.2M	1.2M	3	0
DMSO	50%	5%	1	92
	80%	8%	0.5	0
HMPA	50%	5%	1	93
	80%	8%	1	0

The effect of organic solvents on hydrogenase activity is also shown in Table I. DMSO or hexamethylphosphoramide at 50% concentration had no significant effect on hydrogenase activity when the assay was run at one-tenth solvent concentration. These solvents at 80% concentration completely inactivated the enzyme as measured by deuterium exchange. However, some residual activity was detected by the colorimetric reduction of benzyl viologen by hydrogen. Chen and Mortenson [13] reported that the hydrogenase of *C. pasteurianum* in 80% DMSO could oxidize ferredoxin and reduce methyl viologen but could not catalyze the reverse reactions.

These results establish the relative stability of *Chromatium* hydrogenase to denaturation. The changes in the visible absorption spectra of hydrogenase were then monitored under denaturing conditions which led to appreciable loss of catalytic activity, i.e., 1% SDS, 5.6 M guanidine, and 6.7 M urea (Fig. 3). In all cases the intensity of the 410 nm peak due to the iron-sulfur chromophore was decreased and the extent of decrease was in the order: guanidine > urea > SDS. However, even under conditions where no exchange activity was detectable, there was still some absorption at 410 nm indicating a perturbation of the chromophore but not its complete destruction. This flattening of the 410 nm peak into a shoulder is similar to that seen when hydrogenase was placed in 80% DMSO or hexamethylphosphoramide under anaerobic conditions.

Another indication of the stability of *Chromatium* hydrogenase was the ob-

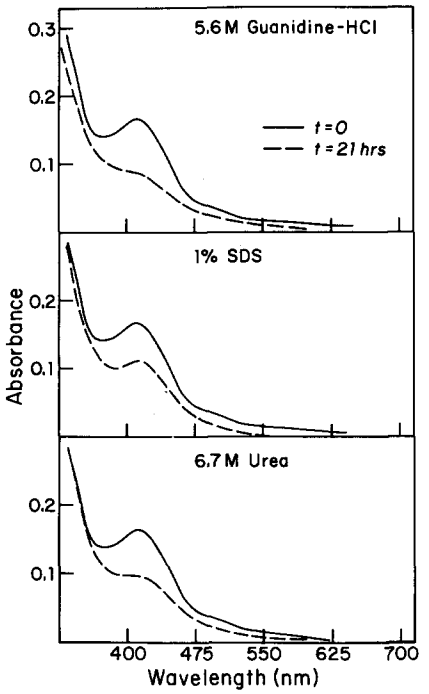

Fig. 3. Spectra of *Chromatium* hydrogenase in the presence of denaturants. All samples were buffered in 50 mM Tris, pH 8.5.

servation that the enzyme retained full catalytic activity after being treated with pronase for trypsin for 19 h at 25°C. The effect of the denaturants SDS, urea, guanidine, DMSO and hexamethylphosphoramide on the activity of the partially purified hydrogenase from *Proteus vulgaris* [14] was the same as that found for the *Chromatium* enzyme.

Discussion

The evidence presented here strongly suggests that a single [4Fe-4S] cluster forms the active site of *Chromatium* hydrogenase. The spectral changes observed in 80% non-aqueous solvent are characteristic of [4Fe-4S] clusters [5] and are quite distinct from those seen for [2Fe-2S] clusters. The failure to displace the cluster with thiol may be attributable to inaccessibility of the active site, despite the changes in protein structure suggested by the spectral changes induced in non-aqueous solvents. The activity of the enzyme in the presence of a number of common denaturants, as reported here, provides additional evidence for stability of the active site to such disruption.

Further evidence for the [4Fe-4S] nature of the cluster comes from the EPR signal of the oxidized enzyme. Similar signals, centered at $g = 2.01$, have been observed in oxidized clostridial ferredoxins [15,16], oxidized four-iron ferredoxins [17,18], oxidized four-iron hydrogenases [4,19—21], and several other iron-sulfur proteins all of which contain no fewer than four iron atoms and four inorganic sulfides (Ref. 15 and Refs. therein). Detailed EPR and chemical

studies of oxidized *C. acidi-urici* eight-iron ferredoxin suggest that this signal arises from [4Fe-4S] clusters in the (3+) oxidation state, a so-called super-oxidized ferredoxin [15].

In contrast, no well-characterized two-iron ferredoxin has been shown to give a $g = 2.01$ EPR signal in any oxidation state [2]. Instead these proteins all produce the '$g = 1.94$' EPR signal, and only when reduced.

Recent theoretical studies of the magnetic properties expected of spin-coupled iron tetrads makes the identification of the $g = 2.01$ EPR signals with oxidized (3+ state) [4Fe-4S] clusters all the more credible [22]. Specifically, these calculations predict that a spin state with a small g-tensor anisotropy (i.e., $g_\perp - g_\parallel \simeq 0.015$) and with $g_\perp > g_\parallel$ (the reverse of the order of $g_\parallel$ and $g_\perp$ for *Chromatium* Hipip [22,23]) can be the ground state of an oxidized [4Fe-4S] cluster. These are precisely the properties that characterize the well-resolved axial EPR spectrum of *Chromatium* hydrogenase (see Fig. 2).

Though the weight of empirical evidence as well as theoretical considerations point to an active site for *Chromatium* hydrogenase consisting of a single [4Fe-4S] cluster, recent studies on aconitase [24] indicate that a $g = 2.01$ EPR signal in an oxidized enzyme may not, by itself, constitute a diagnostic signature of cluster type. Designation of cluster type must, therefore, come from the composite picture constructed from all the available data (see above and below).

The EPR signal observed in the present study was the same for the oxygenated and deoxygenated forms of the oxidized enzyme [1] and was not affected by CO. Apparently, binding of the inhibitory gaseous ligands, O_2 and CO, to the enzyme does not significantly change the electronic configuration of the iron-sulfur cluster though the cluster can no longer be reduced by H_2 in their presence. Van Heerikhuizen et al. [25] reported EPR spectra for the soluble hydrogenase from *Chromatium* which are very similar to the spectra reported here for the enzyme purified from the particulate fraction of *Chromatium*. In both cases the oxidized enzymes exhibit strong signals near $g = 2.01$ while the reduced enzymes are apparently EPR silent. The spectrum of the soluble enzyme in air [25] had extra lines below $g = 2.00$ which were shown to be due to oxygen and were not seen in the presence of 2-mercaptoethanol. (In the present work the enzyme was isolated in the presence of 2-mercaptoethanol.)

The hydrogenases from *Chromatium* [this study, Refs. 3, 25], *Proteus mirabilis* [19] and *Rhodospirillum rubrum* [21] all show similar EPR spectra with a characteristic signal near $g = 2.01$ in the oxidized state and no signal in the reduced state. This type of signal has been observed in Hipip [22,23] and suggests that these hydrogenases function by cycling between the oxidized (3+) and reduced (2+) states. If this is so, then the enzyme hydride intermediate [1] could be formed by transferring two electrons sequentially since the (1+) oxidation state has not been observed with these hydrogenases.

Hydrogenases from *Desulfovibrio vulgaris* [26,27], *C. pasteurianum* [4] and *Alcaligenes eutrophus* [20] display somewhat different EPR signals. The oxidized enzymes show weak Hipip-like signals, whereas the reduced enzymes show two or more sets of strong signals of the $g = 1.94$ type. It has been suggested [4] that these hydrogenases could function as either one- or two-electron transfer agents since both paramagnetic (3+) and (1+) oxidation states have been observed. In these hydrogenases the signal in the reduced enzyme is

10-times greater than in the oxidized enzyme. This result suggests that these enzymes function primarily by cycling between the oxidized (2+) state and reduced (1+) states in contrast to other hydrogenases (see above) that utilize the (3+) and (2+) oxidation states.

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