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Evidence for an [Fe]-type hydrogenase in the parasitic protozoan *Trichomonas vaginalis*

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The hydrogenase of the pathogenic protozoan *Trichomonas vaginalis* was extracted and partially purified. The catalytic and spectroscopic properties of the enzyme indicate that it belongs to the class of [Fe]-hydrogenases, rather than the [NiFe]-hydrogenases. The hydrogenase activity was highly sensitive to carbon monoxide, 50% inhibition being attained by 1 μM CO. The EPR spectrum of the most active fractions from chromatography, after reduction by hydrogen and partial reoxidation under argon, showed an EPR spectrum at $g = 2.10, 2.04, 2.00$. This unusual spectrum is characteristic of the 'H-cluster', as seen in [Fe]-hydrogenases of anaerobic bacteria such as *Clostridium* spp.

Pathogenic protozoa; EPR spectroscopy; Hydrogenase; Iron–sulfur protein; *Trichomonas vaginalis*

1. INTRODUCTION

Hydrogenases, the enzymes responsible for production and consumption of hydrogen gas, are widespread among the bacteria. The hydrogenases may be divided into three classes on the basis of their metal content. The [NiFe]-hydrogenases contain nickel centers and iron–sulfur clusters [1,2]; a sub-class of these, the [NiFeSe]-hydrogenases, also contain selenium which is coordinated to the nickel [3]. The [Fe]-hydrogenases contain iron–sulfur clusters, one of which, the H-cluster, is believed to represent the hydrogen-binding site [4]. On the basis of homologies in amino acid sequences, the [NiFe]- and [NiFeSe]-hydrogenases are closely related [5,6], but distinct from the [Fe]-hydrogenases [7]. A third class, recently discovered in methanogens, has methylenetetrahydromethanopterin as its other substrate and appears to contain no metal centers [8].

Among the eukaryotic organisms hydrogenases are confined to a few species. These include algae such as *Chlamydomonas reinhardtii* [9], fungi such as the rumen organism *Neocallimastix patriciarum* [10] and certain aerotolerant anaerobic protozoa such as *Trichomonas vaginalis* [11,12].

In protozoa, hydrogenase is localized in unusual microbody-like organelles called hydrogenosomes [10,13]. Under anaerobic conditions the hydrogenosomes produce energy at substrate level, and remove reducing equivalents as molecular hydrogen [14]. The process requires three iron–sulfur proteins: pyruvate: ferredoxin oxidoreductase [15], ferredoxin [16] and hydrogenase [17]. The origins of the hydrogenosomes are obscure. Their metabolism of pyruvate, forming acetate, is similar to that of Clostridia and other anaerobic bacteria. However the amino acid sequence and spectroscopic characteristics of the ferredoxin of *T. vaginalis* show it to be more similar to the [2Fe-2S]-proteins of the P-450 monooxygenase systems of aerobic bacteria and mitochondria [18]. It was of interest to determine if the hydrogenase is of the [Fe]-type, as found in Clostridia, or of the [NiFe]-type, which is most commonly found in aerotolerant bacteria.

So far, the evidence about the nature of the active site in eukaryotic hydrogenases has been indirect. The hydrogenase of the green alga *Chl. reinhardtii* has been isolated, and was reported to contain iron [9]. Lindmark et al. [17] have described the purification of hydrogenase from membranes of *Tritrichomonas foetus* hydrogenosomes. Estimation of the M_r by gel filtration HPLC gave a value of 100 kDa. On SDS-polyacrylamide gels, the preparation showed two bands of protein corresponding to M_r 58 and 28 kDa. An analogy was drawn to the [NiFe]-hydrogenases such as that from *Bradyrhizobium japonicum* [19], which comprise two subunits of M_r approx 60 and 30 kDa. If the hydrogenase of *Tr. foetus* proved to be of the [NiFe]-type, it would be the first such enzyme to be found in a eukaryote.

Electron paramagnetic resonance (EPR) spectros-

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Abbreviations: C., Clostridium; *Chl., Chlamydomonas*; *D., Desulfovibrio*; *T., Trichomonas*; *Tr., Tritrichomonas*; EPR, electron paramagnetic resonance

copy has proved a sensitive and discriminating technique for the characterization of the metal centers in hydrogenases and other iron–sulfur proteins. The iron–sulfur protein composition of hydrogenosomes from *Tr. foetus* and *T. vaginalis* has been examined by EPR [21,22]. In the latter case up to eight different EPR signals, provisionally labelled A–H, were resolved. Signal B was identified as due to ferredoxin. The iron–sulfur and nickel centers in hydrogenases have distinct EPR spectra, which are useful in distinguishing between the different types of enzyme.

This paper describes the partial purification of hydrogenase from hydrogenosomes of *T. vaginalis*. In order to determine which type of hydrogenase it is, we have examined two characteristic properties: its EPR spectra and its sensitivity to inhibition by carbon monoxide.

2. EXPERIMENTAL

2.1. *Growth and extraction of cells*

T. vaginalis, Bushby strain, was grown at 37°C and pH 6.2 in modified Diamond's medium containing 10% (v/v) heat-inactivated horse serum (Gibco, Paisley, Scotland) to a cell concentration of around 1.5×10^6 organisms·ml^{-1} [23]. 20 l of cell culture (grown in 1 l cultures) were harvested at 4°C by centrifugation at 4,060×*g* for 7 min. All further extraction and purification procedures were carried out in an anaerobic glovebox, in a nitrogen atmosphere containing less than 3 parts per million oxygen. Buffers were thoroughly degassed and bubbled with oxygen-free argon, and had 2 mM dithionite present. Harvested cells were resuspended in 50 mM Tris/HCl, pH 8.0, which contained 250 mM sucrose and 2 mM EDTA. Cells were washed at 4°C, in anaerobically sealed centrifuge tubes, by centrifugation at 16,000×*g* for 7 min. The supernatant was quickly decanted, and the soft pellet of cells agitated to form a slurry, which was poured into 50-ml polypropylene tubes. These were anaerobically sealed and frozen in liquid nitrogen.

2.2. *Purification of hydrogenase from T. vaginalis (Table I)*

The hydrogenase is associated with the membranes of the hydrogenosomes [24]. However, it was found in preliminary experiments that separating the large granule fraction which contains these organelles prior to isolation of the enzyme resulted in loss of yield with no significant improvement in purity. Therefore the enzyme was extracted from the membranes by treatment of whole cells with a detergent, sodium deoxycholate. All operations were carried out under nitrogen. The temperature was 20°C, except centrifugation, which was at 4°C. Column fractions were collected and stored on ice.

2.2.1. Preparation of solubilized crude extract

Cells from a 17-l culture (approximately 80 ml of slurry) were thawed from liquid nitrogen, and added to 100 ml of cell breakage medium, consisting of 0.1 M Tris-HCl, pH 8.2, 2% sodium deoxycholate, 1 mg/ml lysosyme, 0.1 mg/ml DNAse, 0.1 mg/ml catalase, 0.1 mg/ml glucose oxidase (all from Sigma). The suspension was incubated, with stirring, at room temperature for 1 h, then centrifuged under anaerobic conditions at 138,000×*g* for 1 h at 4°C. The supernatant (150 ml of solubilized crude extract) was diluted to 400 ml with 50 mM Tris, pH 8.0. The pellets were re-extracted with cell breakage medium, and recentrifuged. The resulting supernatant (40 ml) was added to the 400 ml of diluted crude extract.

2.2.2. Column chromatography

Crude extract (440 ml) was loaded onto a DEAE-cellulose anion-exchange column (Whatman DE52; 25 mm diameter×90 mm long) equilibrated in 50 mM Tris, pH 8.0. The unbound eluate, containing most of the hydrogenase activity, was stored overnight, in 200 ml fractions, in liquid nitrogen. The first 200 ml of DEAE-unbound extract eluted was thawed from liquid nitrogen. 1 M potassium phosphate buffer, pH 7.2, was added to a final concentration of 20 mM. The resulting solution was loaded onto a hydroxylapatite column (Bio-Rad; 25×75 mm) equilibrated with 20 mM potassium phosphate, pH 7.2. The column was washed with 50 ml of equilibration buffer. The tightly bound hydrogenase was eluted by washing the column with 45-ml volumes of phosphate, increasing the concentration in 0.1 M increments from 0.1 to 1.0 M, and 15 ml fractions were collected. Fractions with a high specific activity for hydrogenase were concentrated by centrifuging in Centricon-10 microconcentrators (Amicon), anaerobically sealed in 50 ml tubes, at 4°C. In order to avoid congestion on the membranes, the concentrate was gently mixed with a Pasteur pipette, at hourly intervals. The 50-fold concentrated samples (around 0.2 ml each) were added to EPR tubes and frozen in liquid nitrogen.

2.3. *Other methods*

Hydrogenase uptake activity was determined spectrophotometrically in septum-sealed cuvettes, measuring the rate of reduction of methyl viologen by hydrogen. The assay medium consisted of 10 mM methyl viologen, 5 mM dithiothreitol, in 50 mM Tris-HCl, pH 8.0, and was bubbled with hydrogen for 15 min. The assay was initiated by anaerobically adding between 1 and 10 μl of hydrogenase, and the rate of reduction of methyl viologen was monitored at 604 nm and at 20°C. The molar absorbance for methyl viologen at 604 nm was taken as 13,600 $M^{-1}\cdot cm^{-1}$. One unit of enzyme activity was defined as the amount of enzyme which gave a rate of 1 μM of product formed per minute under the conditions specified. Protein concentration was determined by the Bio-Rad Protein dye binding assay, calibrated with γ-globulin as the standard.

SDS-gel electrophoresis was performed on 12.5% (w/v) polyacrylamide slab gels in Tris/glycine buffer. Gels were stained for protein with Coomassie brilliant blue G-250.

EPR spectra were recorded either on a Varian E4 spectrometer, or on a Bruker ESP300 spectrometer with an Oxford Instruments ESR900 helium flow cryostat.

3. RESULTS

3.1. *Properties of the isolated hydrogenase*

The specific activity of the purified hydrogenase in hydrogen:methyl reductase activity is relatively low, but comparable, for example, with V_{max} for the hydrogenase II of *Clostridium pasteurianum*, 120 μmol H_2 consumed/min/mg protein, measured at 30°C [25]. It should be noted that the activities of enzymes with different artificial electron acceptors are quite variable. The rate of

Table I

Purification of *T. vaginalis* hydrogenase

Step	Total activity (U)	Specific activity (U·mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	7,156	4.59	100	1.0
DEAE-cellulose anion exchange	1,434	23.90	20	5.2
Hydroxylapatite chromatography	794	661.77	11	144.2

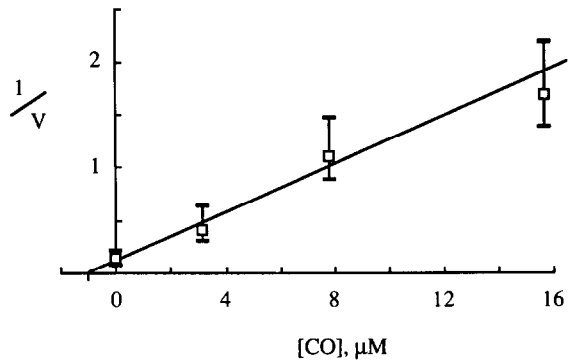

Fig. 1. Inhibition of hydrogen:methyl viologen reductase activity of *T. vaginalis* hydrogenase by carbon monoxide. Error bars represent 95% confidence limits for the rate, extrapolated to infinite hydrogen concentration.

hydrogen:methyl viologen reductase activity was measured in the pH range of 5.0 to 8.6, and showed a pH optimum at 7.0. The K_m for hydrogen in the standard assay was estimated to be 0.152 mM. The K_m for methyl viologen was 1.4 μM. These values are comparable with those of iron-containing hydrogenases.

SDS-PAGE showed a major polypeptide band with M_r ≈64,000 kDa.

3.2 *Inhibition by carbon monoxide*

The [Fe]- and [NiFe]-hydrogenases differ in their sensitivity to carbon monoxide [2]. The [NiFe]-hydrogenases are generally less sensitive, and some not sensitive at all [26], while the [NiFeSe]-hydrogenases are intermediate in their properties. Inhibition is complex, with both short-term reversible effects and progressive irreversible loss of activity [4]. The extent of inhibition is often defined in terms of the concentration for 50% inhibition. This ranges between 0.1 and 2 μM for the [Fe]-hydrogenases.

The inhibition of the hydrogenase of *T. vaginalis* was determined by measuring the rate of methyl viologen reduction as a function of carbon monoxide and hydrogen concentrations. The concentration of gases was adjusted by adding various concentrations of buffer saturated with the respective gases. The rates were extrapolated to infinite hydrogen concentration, and plotted as a Dixon plot, $1/V$ vs. CO concentration. The results are shown in Fig. 1. The K_i for carbon monoxide is estimated to be 1.0 μM, a value which is within the range found for [Fe]-hydrogenases [4].

3.3. *EPR spectroscopy*

EPR spectra of the hydrogenase are shown in Fig. 2. In the hydrogen-reduced enzyme the low-temperature spectrum contains components from a number of iron–sulfur clusters. The partially purified hydrogenase was enriched in iron–sulfur clusters D and F [22]. On partial reoxidation by flushing out the hydrogen with argon gas, the iron–sulfur clusters are partially oxidized (signal D remains mainly reduced, indicating a potential around −270 mV [22]). A new, narrow EPR spectrum is observed at g = 2.10, 2.04, 2.00. This spectrum is very similar, both in g-factors and linewidths, to those observed on partial oxidation of the [Fe]-hydrogenases from *C. pasteurianum* hydrogenase I and II [4], *Desulfovibrio vulgaris* (Hildenborough strain) [27,28] and *D. desulfuricans* (strain ATCC 7757) [29]. The spectrum was detectable at temperatures up to 100 K, unlike the spectra of typical [4Fe-4S]-clusters, which are generally not detectable above 50 K.

4. DISCUSSION

Both the iron- and nickel-containing hydrogenases give characteristic EPR spectra when they are reduced with hydrogen and then partially reoxidized. On careful oxidation the [Fe]-hydrogenases give rise to an EPR signal with principal g-factors 2.10, 2.04 and 2.001 [2,30]. This signal has been assigned to the 'H-clusters'. The best estimate, based on analytical and spectroscopic data, is that the H-cluster consists of six iron atoms of two different types, possibly with non-sulfur coordination to the protein [2]. The nickel-containing hydrogenases give a signal, designated Ni-C, in the partially reduced state, with g-factors 2.19, 2.14, 2.01 for the [NiFe]-hydrogenases [1,2], and 2.23, 2.17, 2.01 in the [NiFeSe]-hydrogenases [31]. The consistency of the g-factors and linewidths of these spectra in hydrogenases from a variety of microbial species means that they

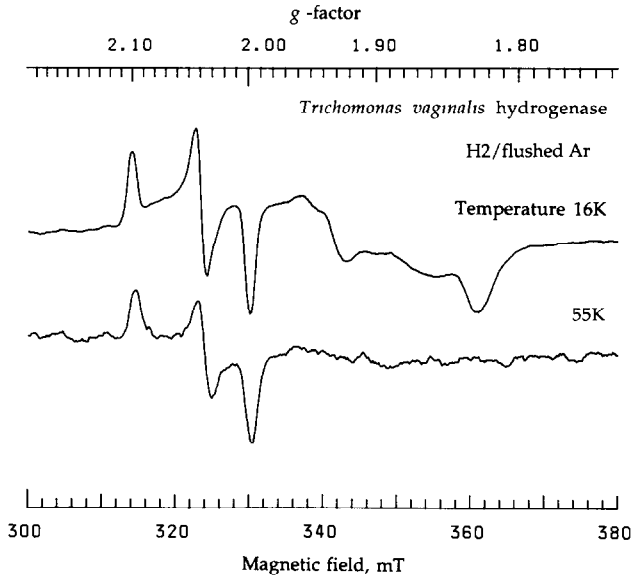

Fig. 2. EPR spectra of *T. vaginalis* hydrogenase: most active fraction from hydroxylapatite chromatography. The sample was reduced under hydrogen gas, then flushed with argon for 20 min at 20°C. The temperature of measurement are indicated. Other conditions of measurement: microwave frequency, 9.236 GHz, power 20 mW, modulation amplitude 1 mT.

represent a discriminating criterion for the type of hydrogenase present. The EPR signal observed in *T. vaginalis* hydrogenase strongly resembles that from the [Fe]-hydrogenases in its shape and *g*-factors, and is distinctly different from that of the [NiFe]-enzymes. This evidence, taken with the sensitivity of the enzyme to carbon monoxide, strongly suggests that the eukaryotic enzyme is a [Fe]-hydrogenase.

The [Ni]- and the [Fe]-hydrogenases also contain ferredoxin-type $[4Fe\text{-}4S]^{1+}$ clusters, which appear to act as electron carriers. In [Fe]-hydrogenases such as those from *C. pasteurianum* they are known as 'F-clusters', to distinguish them from the catalytic H-clusters [2]. The EPR spectra of the partially purified *T. vaginalis* hydrogenase, reduced with hydrogen and recorded at temperatures below 30 K, showed the presence of iron–sulfur proteins (Fig. 2a). Their spectra may be compared with those identified in the hydrogenosomal fraction of *T. vaginalis* by Chapman et al. [22]. The EPR spectra of reduced hydrogenase resembled signals D (g = 2.085, 1.88, 1.83, detectable below 25 K) and F (g = 2.05, 1.95, 1.874, detectable below 25 K). Other signals in the hydrogenosomes may be due to pyruvate:ferredoxin reductase. In reduced, partially purified pyruvate: ferredoxin reductase, EPR spectra were observed which resembled signals E (g = 2.05, 1.95, 1.89, detectable below 40 K) and F (g = 2.05, 1.95, 1.874, detectable below 25 K) (A. Chapman, R. Cammack and K. Williams, cited in [32]). The observation of EPR signal F in both enzymes might indicate that they contain two different clusters with similar EPR properties. Alternatively the two enzymes might form complexes with the same iron–sulfur protein. The definitive assignment of the EPR signals of iron–sulfur proteins of hydrogenosomes can only be resolved by the complete purification of both enzymes.

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