Isolation and first EPR characterization of the \([\text{FeFe}]\)-hydrogenases from green algae

Christina Kamp, Alexey Silakov, Martin Winkler, Edward J. Reijerse, Wolfgang Lubitz, Thomas Happe

Ruhr-University Bochum, Department of Biochemistry of Plants, 44780 Bochum, Germany
Max-Planck-Institute for Bioorganic Chemistry, 45470 Mülheim an der Ruhr, Germany

A B S T R A C T

Hydrogenase expression in \(\text{Chlamydomonas reinhardtii}\) can be artificially induced by anaerobic adaptation or is naturally established under sulphur deprivation. In comparison to anaerobic adaptation, sulphur-deprived algal cultures show considerably higher expression rates of the \([\text{FeFe}]\)-hydrogenase (HdA1) and develop a 25-fold higher \textit{in vitro} hydrogenase activity. Based on this efficient induction principle we have established a novel purification protocol for the isolation of HdA1 that can also be used for other green algae. From an eight liter \(\text{C. reinhardtii}\) culture 0.52 mg HdA1 with a specific activity of 741 \(\mu\)mol \(\text{H}_2\) min\(^{-1}\) mg\(^{-1}\) was isolated. Similar amounts were also purified from \(\text{Chlorococcum submarinum}\) and \(\text{Chlamydomonas moewusii}\). The extraordinarily large yields of protein allowed a spectroscopic characterization of the active site of these smallest \([\text{FeFe}]\)-hydrogenases for the first time. An initial analysis by EPR spectroscopy shows characteristic axial EPR signals of the CO inhibited forms that are typical for the \(\text{H}_2\)-CO state of the active site from \([\text{FeFe}]\)-hydrogenases. However, deviations in the \(g\)-tensor components have been observed that indicate distinct differences in the electronic structure between the various hydrogenases. At cryogenic temperatures, light-induced changes in the EPR spectra were observed and are interpreted as a photodissociation of the inhibiting CO ligand.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Hydrogenases catalyze the reversible redox reaction \(2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\). They are subdivided into three groups, characterized by the specificity and structure of the metal–sulphur cluster forming the active center. The first group comprises \([\text{Fe}]-\)hydrogenases, which do not contain iron–sulphur clusters and are only found in methanogenic archaea [1]. In the second group are the \([\text{NiFe}]-\)hydrogenases, which are widely distributed among the prokaryotic organisms; characteristic of this group is a dinuclear nickel–iron center in the active site [2]. In the third group are the catalytically more efficient \([\text{FeFe}]-\)hydrogenases, which harbour a dinuclear \([\text{FeFe}]-\)cluster linked to a \([4\text{Fe}–4\text{S}]-\)cubane [3,4]. They are also found in eukaryotes [3–5]. In particular, \([\text{FeFe}]-\)hydrogenases are extremely sensitive to oxygen and thus are only expressed under anaerobic conditions [5–7]. Numerous prokaryotic and some eukaryotic \([\text{FeFe}]-\)hydrogenase genes have been described, coding for proteins, which are divided into a more or less variable N-terminal part (F-domain) and a catalytically relevant H-domain at the C-terminus [8,9]. So far structures of \([\text{FeFe}]-\)hydrogenases from two organisms, \(\text{Clostridium pasteurianum}\) and \(\text{Desulfovibrio desulfuricans}\) (ATCC 7757), are known from X-ray crystallography. The periplasmic heterodimeric \([\text{FeFe}]-\)hydrogenase DdH from \(\text{D. desulfuricans}\) contains the H-cluster as the active site. This consists of a \([4\text{Fe}–4\text{S}]-\)cluster bridged by the sulphur atom of a cysteine residue to a binuclear iron subcluster and two classical \([4\text{Fe}–4\text{S}]-\)cubanes (F-clusters); in addition to these structure elements the cytoplasmic monomeric \([\text{FeFe}]-\)hydrogenase Cpl from \(\text{C. pasteurianum}\) contains one \([4\text{Fe}–4\text{S}]-\)cluster with a histidine ligand and one \([2\text{Fe}–2\text{S}]-\)cluster [10,11]. Since 2001 seven green algal hydA genes have been discovered, initiating a new subgroup of extraordinarily small \([\text{FeFe}]-\)hydrogenases, which only contain the catalytic iron–sulphur center and no accessory Fe-S clusters [12–16]. Since the other \([\text{FeFe}]-\)hydrogenases are so much more complex, it is of great importance to study these small algal molecules to gain a better understanding of the active sites and the catalytic process. Green algal \([\text{FeFe}]-\)hydrogenases are nuclear encoded enzymes localized in the chloroplast and linked to the photosynthetic electron transport chain with ferredoxin as their natural electron donor [13,14]. In contrast to the previously investigated \([\text{FeFe}]-\)hydrogenases DdH of \(\text{D. desulfuricans}\) and Cpl of \(\text{C. pasteurianum}\), the structures of these algal type \([\text{FeFe}]-\)hydrogenases have not yet been characterized. Because of their small size, their monomeric form and their high specific activity, these \([\text{FeFe}]-\)hydrogenases are especially suited for analysis of maturation, structure and catalytic mechanism. In the green alga \(\text{Chlamydomonas reinhardtii}\) two genes, hydA1 and hydA2, coding for two similar \([\text{FeFe}]-\)hydrogenases, have been identified [15]. The \([\text{FeFe}]-\)hydrogenase HdA1 of \(\text{C. reinhardtii}\) was purified and characterized by Happe and Naber as a
48 kDa enzyme with a specific activity for hydrogen evolution of around 935 μmol min⁻¹ ng⁻¹ [6]. Hydrogenase expression was induced by anaerobic adaptation of the cells. Under these conditions 14 μg HydA1 per liter algal culture was isolated. Unfortunately, this yield was not sufficient to analyse the structure or to characterize the mechanism of H₂-turnover in this hydrogenase. To achieve sufficient quantities of active native protein for such biophysical investigations, we used the technique of sulphur deprivation to optimize the expression rate of hydrogenase in C. reinhardtii and developed a new purification procedure for the isolation of HydA1, which is faster and far more efficient than previous methods. Additionally, we succeeded in using this procedure to isolate two thus far undescribed [FeFe]-hydrogenases; HydA from the brackish water green alga Chlorococcum submarinum, which has potential for biotechnological applications [17], and HydA1 from Chlamydomonas moewusii, which was earlier shown to exhibit an extraordinary in vitro hydrogen evolution activity, exceeding the activity of similarly treated C. reinhardtii cultures by a factor of three [16]. This extraordinary improvement in the yield of the native protein has enabled us, for the first time, to perform structural characterization of the active center from all three green algal type hydrogenases using electron paramagnetic resonance (EPR) spectroscopy.

2. Materials and methods

All chemicals and reagents used were of the highest commercially available purity.

2.1. Algae strains and culture conditions

The wild type strains of C. reinhardtii 137 (SAG 11-32a), C. submarinum (SAG 2.96) and C. moewusii (SAG 24.91) were originally obtained from the Culture Collection of Algae at Göttingen (SAG) (Germany). Algal strains were grown phototrophically in Tris-Acetate-Phosphate (TAP) medium at 20 °C under continuous illumination at 30 μmol photons m⁻² s⁻¹ up to a chlorophyll concentration of 20 μg ml⁻¹. For anaerobic adaptation, cells were harvested by centrifugation, suspended in 0.1 vol. of fresh TAP medium and anaerobiically adapted by flushing the culture with argon for several hours in the absence of light [6]. For sulphur deprivation, cells were either harvested by centrifugation and suspended in TAP-S medium (TAP, in which all sulfate salts were replaced by the chloride equivalents) at a concentration of 5 μM MgSO₄ per liter algal culture was isolated. Unfortunately, this yield was not sufficient to analyse the structure or to characterize the mechanism of hydrogenase activity of all cultures, the assays contained 100 μl cell culture in addition to 1% Triton X-100 for cell lysis.

2.2. Purification of hydrogenase

All purification steps were carried out under strict anaerobic conditions in a glove box (Coy Laboratories, Detroit, USA) containing an atmosphere of 99% N₂ and 1% H₂. Unless otherwise indicated all buffers contained 10 mM sodium dithionite to prevent deactivation of the hydrogenase by residual oxygen. For the isolation of hydrogenase unless otherwise indicated all buffers contained 10 mM sodium dithionite to prevent deactivation of the hydrogenase by residual oxygen.

The protein concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The hydrogenase activity was determined by measuring the amount of hydrogen evolved from methyleneblue reduced by sodium dithionite in a gas chromatograph (GC-10 Shimadzu, Duisburg, Germany). Gas tight stuba sealed tubes, containing a final volume of 2 ml 60 mM potassium phosphate pH 6.8, 100 mM sodium dithionite, 10 mM methyleneblue and 2–30 μl protein solution, were flushed with argon and incubated in a shaker at 37 °C for 20 min. For measuring the in vitro hydrogenase activity of all cultures, the assays contained 100 μl cell culture in addition to 1% Triton X-100 for cell lysis.

Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CnI5A</td>
<td>AATCTGCCCTGCTGCAAAACA</td>
</tr>
<tr>
<td>CnI5B</td>
<td>CATCATGAGGAGCAGTGC</td>
</tr>
<tr>
<td>CnI5A5</td>
<td>TAATGCAAGGCCTGTCGAG</td>
</tr>
<tr>
<td>CmI5B</td>
<td>CTGGTGACCAAGGTTTGTC</td>
</tr>
<tr>
<td>A5S</td>
<td>TGGAGTGGGGCCAGGATGAT</td>
</tr>
<tr>
<td>A5H</td>
<td>GGCCGGGGCAGCCATGACCT</td>
</tr>
<tr>
<td>C5A</td>
<td>GAGAACAGACAGAAGGTC</td>
</tr>
<tr>
<td>C5B</td>
<td>CTCAATTACACTGACGAGCT</td>
</tr>
<tr>
<td>C5A</td>
<td>ATGCCGACCAAGCCGTTAGAT</td>
</tr>
<tr>
<td>C5B</td>
<td>GACACCCGTGTTGCTCAGT</td>
</tr>
<tr>
<td>C5B</td>
<td>TGGGCAAGATGCGTTCTAC</td>
</tr>
</tbody>
</table>

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.

The concentration was determined spectrophotometrically by the method of Bradford [19], using a protein assay kit (Bio-Rad, München, Germany) with bovine serum albumin as a standard.
microwave pulses (n/2 and n). The delay between the MW pulses was fixed to τ = 360 ns. The length of the n/2 MW pulse was set to 36 ns and the n pulse to 68 ns. All pulse Q-band EPR measurements were performed on a Bruker ELEXSYS E580 Q-band spectrometer with the SuperQ-FT microwave bridge, working at ~34 GHz and a temperature of 20 K. For the measurements we used a home-built slightly overcoupled cylindrical TESI resonator [25] with a construction similar to that described by Sienkiewicz et al. [26]. Cryogenic temperatures were achieved using an Oxford CF935 continuous flow cryostat. During the photodissociation experiments samples were illuminated inside the cryostat at 40 K within the EPR setup. Illumination was performed using an OPO laser system (Quantel, Brilliant Series), tuned to 531 nm with 8 mJ/pulse.

3. Results

3.1. Induction of hydrogenase activity

Hydrogenase expression in *C. reinhardtii* can be induced by an anaerobic adaptation, in which the culture is flushed with argon for several hours [6], or by incubating the algae under sulphur-deprived conditions [27–29]. For this method cells from a sulphur-replete culture were harvested by centrifugation and suspended in sulphur-free medium. Alternatively, a medium, containing a small initial concentration of sulphur (75 μM), was used. In both cases the cultures were incubated under continuous illumination for several days [18]. To obtain the highest yield of hydrogenase protein we tested both procedures and measured the hydrogenase activity in *vitro*. Under sulphur deprivation induced by an exchange of medium after several days of growth, a *C. reinhardtii* culture of 20 mg chlorophyll **1** reaches a maximum of hydrogenase activity of around 70 μmol H₂ min⁻¹ mg⁻¹ chlorophyll after 48 h. The in *vitro* activity maximum of a self-limiting culture is at around 75 μmol H₂ min⁻¹ mg⁻¹ chlorophyll. This activity develops after about eight days, when the culture has reached a chlorophyll concentration of 20–25 mg g⁻¹ (data not shown). In both cases a 25-fold higher hydrogenase activity in comparison to the anaerobic adaptation procedure used by Happe and Naber [6] could be measured. This result is also confirmed by western blot analyses of total protein extract from *C. reinhardtii*, showing that more hydrogenase protein is expressed under sulphur deprivation than under anaerobic adaptation (data not shown). Similar examinations of the two other algal species, *C. submarinum* and *C. moewusii*, indicated that both strains also develop high in *vitro* activities under sulphur deprivation. *C. moewusii* shows a hydrogenase activity, which is comparable to that of *C. reinhardtii*, while *C. submarinum* reaches half of the activity of the other two cultures (data not shown).

3.2. Purification of hydrogenase

For the isolation of hydrogenase, eight liters of self-limiting TAP-S cultures were incubated under continuous illumination for around eight days. After reaching the maximum in *vitro* hydrogenase activity the enzyme was isolated under anaerobic conditions. The isolation procedure is summarized in Table 2.

After lysing the cells using Triton X-100 the protein supernatant was precipitated with ammonium sulfate to remove chlorophyll and larger cell components. Subsequently, the protein solution was separated by two Q-Sepharose anion-exchange columns. In the first case the hydrogenase was eluted with a pH gradient using a range from 8.5–7. In the second case a linear salt gradient from 0–500 mM was applied. Finally the proteins were separated by size using a gel filtration chromatography column and the hydrogenase was purified 54-fold to homogeneity (95–100%) with 21% recovery of total activity from the crude extract. A maximum yield of 0.52 mg HydA1 with a specific activity of 741 μmol H₂ min⁻¹ mg⁻¹ was obtained (see Table 2). The isolation procedure was repeated ten times with independent cell cultures. Using this purification protocol an average of 40 μg hydrogenase per liter of algal culture with a specific activity of 730 μmol H₂ min⁻¹ mg⁻¹ could be purified. The experimental standard deviation for measuring the specific activity for hydrogen evolution of the enzyme using the procedure described in Materials and Methods is 20%. The purification factor of 54 is comparatively low since in the sulphur-deprived culture the portion of hydrogenase enzyme with respect to the total amount of protein is approximately 92-fold higher than in a culture induced by anaerobic adaptation.

Using this new purification protocol we were further able to isolate comparative amounts of hydrogenase from *C. submarinum* and *C. moewusii* (see Table 2 and Fig. 1). From eight liters of *C. submarinum* culture, 0.4 mg hydrogenase with a specific activity of 639 μmol H₂ min⁻¹ mg⁻¹ and from a similar amount of *C. moewusii*, 0.32 mg HydA1 with a specific activity of 1598 μmol H₂ min⁻¹ mg⁻¹ could be obtained. In both cases minor impurities of other proteins are visible on the SDS-PAGE (see Fig. 1).

3.3. Identification of hydA-cDNA sequences from *C. moewusii* and *C. submarinum*

For characterizing the hydA sequence of *C. submarinum* a set of mismatch primers was designed (data not shown), derived from

---

**Table 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>C. reinhardtii</em></th>
<th><em>C. submarinum</em></th>
<th><em>C. moewusii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (%)</td>
<td>Specific activity (μmol H₂ min⁻¹ mg⁻¹)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>Crude extract (8 l of cultures)</td>
<td>100</td>
<td>13.8</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation (40–73%)</td>
<td>47.8</td>
<td>33.9</td>
<td>47</td>
</tr>
<tr>
<td>First anion-exchange chromatography (pH gradient)</td>
<td>47.5</td>
<td>21.8</td>
<td>46</td>
</tr>
<tr>
<td>Second anion-exchange chromatography (KCl gradient)</td>
<td>24.5</td>
<td>355</td>
<td>33</td>
</tr>
<tr>
<td>Gel filtration chromatography (Superdex 75)</td>
<td>21.6</td>
<td>741</td>
<td>28</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>0.52</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** SDS-PAGE of purified hydrogenases from three different green algal species. Lane 1: marker proteins; lane 2: purified hydrogenase from *C. submarinum*; lane 3: purified hydrogenase from *C. moewusii*; lane 4: purified hydrogenase from *C. reinhardtii*. The lanes were each loaded with 500 ng protein. The gel was stained with Coomassie Brilliant Blue.
analysed by Q-TOF mass spectrometry. In the case of

3.4. Identification of the isolated HydA proteins

To identify the purified proteins, concentrated samples were analysed by Q-TOF mass spectrometry. In the case of C. submarinum three oligo-peptides were identified, clearly belonging to the HydA polypeptide sequence determined as described above (see Fig. 2). As for C. moewusii two HydA genes were identified both of which are induced under anaerobic conditions (data not shown); the purified protein may consist of either one or a mixture of both HydA iso-enzymes. The Q-TOF analysis enabled identification of two oligo-peptides clearly belonging to a chlorophycean-type [FeFe]-hydrogenase, whereas one identifies the protein band as HydA1 (see Fig. 2). Similar examinations led to an unequivocal identification of HydA1 in the case of the protein purification from sulphur-deprived C. reinhardtii cultures. Although in both cases the polypeptide sequences of HydA1 and HydA2 show a significant similarity (see Fig. 2) and the expression of the hydA2 genes could be demonstrated in earlier examinations [15], only the respective HydA1 enzyme was isolated during the purification procedure.

3.5. The paramagnetic states and their characterization by EPR spectroscopy

The following figures (Figs. 3–5) show the first EPR spectra obtained of the active sites of the algal [FeFe]-hydrogenases HydA1 from C. reinhardtii and C. moewusii as well as HydA from C. submarinum in comparison to the spectra of DdH from D. desulfuricans [30–35].

Measurements of the as-isolated sample of HydA1 from C. reinhardtii reveal a complex EPR spectrum, which is a mixture of different signals (see Fig. 3B). One of these signals can be identified as a rhombic spectrum with principal g-values 2.102, 2.040 and 1.998 (see Table 3), which most probably corresponds to the oxidized form of HydA1 ($H_{ox}$). Another contribution to the EPR spectrum is an axial signal with g-values 2.052 and 2.007. Although no CO was added to this particular sample, there is an indication of the presence of the CO inhibited state of the H-cluster: measurements of the as-isolated sample after flushing with CO reveal only this axial spectrum (see Fig. 3A). In this case CO ligands, dissociated from oxidatively damaged H-clusters, probably bind as free CO molecules to intact H-clusters forming the $H_{ox}-CO$ species [32]. Flushing the as-isolated sample with hydrogen ($H_2$) leads to the reduced state of the protein. This is expected to be EPR silent and indeed shows no prominent spectrum (see Fig. 3C). Some weak signals can be detected after prolonged accumulation, which is assigned to oxidatively damaged H-clusters. In contrast to DdH, treatment of this HydA1 sample...
with argon does not change the signal. Furthermore the addition of thionine, which was successfully used to form the $H_{ox}$ state of the hydrogenase Cpl from *C. pasteurianum* [36], had no effect on HydA1 of *C. reinhardtii* (data not shown).

Similar spectra were obtained from HydA of *C. submarinum*. The as-isolated sample (Fig. 3E) shows an EPR signal, which is characterized by a mixture of the rhombic ($H_{ox}$) and the axial ($H_{ox}$-CO) signals. The CO inhibited form shows only the characteristic axial EPR signal with $g$-values of 2.056 and 2.008 (see Fig. 3D). EPR experiments on HydA1 of *C. moewusii* after treatment with CO show an intense axial EPR spectrum, similar to that, obtained from HydA1 of *C. reinhardtii*.

![Fig. 3. Q-band pulse EPR spectra of the [FeFe]-hydrogenase from *C. reinhardtii* (A, B, C), *C. submarinum* (D, E) and *C. moewusii* (F). A. HydA1 $H_{ox}$-CO state, 30 μM in 50 mM Tris–HCl pH 8.5 as-isolated after flushing with CO for 20 min. B. HydA1 as-isolated, 100 μM in 50 mM Tris–HCl pH 8.5. C. HydA1 as-isolated, 100 μM in 50 mM Tris–HCl pH 8.5 after flushing with $H_{2}$ for 20 min and with Ar for 35 min. D. HydA $H_{ox}$-CO state, 100 μM in 50 mM Tris–HCl pH 7.5, 150 mM KCl, 1 mM sodium dithionite as-isolated after flushing with CO for 20 min. E. HydA as-isolated, 100 μM in 50 mM Tris–HCl pH 7.5, 150 mM KCl, 1 mM sodium dithionite. F. HydA1 $H_{ox}$-CO state, 100 μM in 50 mM Tris–HCl pH 7.5, 150 mM KCl, 1 mM sodium dithionite as-isolated after flushing with CO for 20 min. The spectrum is shown together with components of the simulations (in color). The red simulation represents a contribution by the $H_{ox}$ state and the blue simulation the $H_{ox}$-CO state. All measurements were performed at 20 K. The $g$-tensor components of the individual spectra are given above of each set of experimental spectra (blue: $H_{ox}$-CO; red: $H_{ox}$). (see Table 3).

The $g$-values for this signal obtained by simulation are 2.050 and 2.008. This EPR spectrum also contains a minor contribution of a rhombic signal (see Fig. 3F). The simulation reveals that this component of the EPR spectrum is similar to the signals measured for the as-isolated samples of HydA1 from *C. reinhardtii* and HydA.

![Fig. 4. Illumination of the [FeFe]-hydrogenase HydA1 from *C. reinhardtii* in the $H_{ox}$-CO state at 20 K. A. Q-band pulse EPR spectrum of the $H_{ox}$-CO state before illumination. B. Q-band pulse EPR spectrum after laser illumination (531 nm) for 3 h together with spectral simulations: the red line represents a simulation of the EPR spectrum of the $H_{ox}$ state, and the blue line of the $H_{ox}$-CO state. The respective $g$-tensor components are given above (see Table 3).](image)

![Fig. 5. Illumination of the [FeFe]-hydrogenase HydA from *C. submarinum* in the $H_{ox}$-CO state at 20 K. A. Q-band pulse EPR spectrum of the $H_{ox}$-CO state before illumination. B. Q-band pulse EPR spectrum after laser illumination (531 nm) for 3 h together with spectral simulation: the blue line represents a simulation of the EPR spectrum of the $H_{ox}$-CO state, the red line the $H_{ox}$ state (LI1) and the green line the second light-induced state (LI2). The respective $g$-tensor components are given above (see Table 3).](image)
from C. submarinum. Therefore, it is assigned to the oxidized state of the H-cluster (Hox). However, the measured principal g-values 2.103, 2.038 and 1.998 deviate slightly from those measured for the other species. The presence of this signal most probably indicates that CO inhibition was not 100% efficient and some of the sample remained in the as-isolated state.

3.6. Light sensitivity of the CO inhibited [FeFe]-hydrogenases

In bacterial [FeFe]-hydrogenases the CO ligand can reversibly dissociate upon illumination at cryogenic temperatures. The final product of this photodissociation process is the Hox state [32]. In addition, a second light-induced intermediate state has been detected during illumination, most likely caused by the dissociation of the bridging CO ligand, while the external, inhibiting CO is still present [34,35]. In this study these effects have been used to support our interpretation of the observed EPR signals of the algal [FeFe]-hydrogenases. Figs. 4 and 5 show the EPR spectra of the Hox-CO state of the hydrogenases from C. reinhardtii and C. submarinum before and after illumination. After illumination for 3 h at 20 K, the axial signal of the Hox-CO state of the [FeFe]-hydrogenase from C. reinhardtii significantly decreases, while the rhombic signal with g-values 2.102, 2.040 and 1.998 appears (see Fig. 4). Both, the axial and the rhombic spectra, have g-values identical to those of the as-isolated species. The EPR spectrum of the CO inhibited form of the hydrogenase from C. submarinum also contains the rhombic signal, which increases during illumination (see Fig. 5). Moreover, during illumination a light-induced intermediate state with g-values 2.230, 2.115 and 2.052 has been detected. These g-values are similar to the second light-induced state of the [FeFe]-hydrogenase from D. desulfuricans (LI2). After illumination the sample tube was placed in cold ethanol (ca. 200 K) for 10 min, frozen again in liquid nitrogen and subsequently measured by EPR. Remarkably, the resulting spectrum was identical to that, measured before illumination, indicating a complete recovery to the Hox-CO state (data not shown). Table 3 gives an overview of the g-values obtained from measurements of different hydrogenase species. The EPR spectra of the algal and bacterial [FeFe]-hydrogenases in their various states seem quite similar. Nevertheless, the small deviations in their g-values indicate distinct differences in the electronic structures of their active sites.

4. Discussion

The isolation and purification to homogeneity of the [FeFe]-hydrogenase from C. reinhardtii was first published by Happe and Naber [6]. Although the interest in these “photosynthetic hydrogenases” was high, it transpired out that the isolation of active hydrogenases from green algae is very difficult and requires a large amount of technical equipment. Over the last ten years, three further enzymes from S. obliquus, C. fusca and Chlorococcum littorale have been isolated from dark adapted algal cultures [12,14,17]. In all of the preparations either the quantity of functional hydrogenase obtained or the yield of the isolated protein was low, impeding further biochemical or biophysical experiments.

In this work a cultivation procedure for the eucaryotic green alga C. reinhardtii is described, which leads to a 25-fold higher production rate of [FeFe]-hydrogenases compared to earlier protocols [6]. The method is based on sulphur deprivation of the algal cultures [27–29] and causes a significantly higher rate of hydrogenase expression than anaerobic culture adaptation. The development of a new isolation procedure for HydA1 (see Table 2) enabled us to purify around 40 μg hydrogenase per liter of algal culture with a specific activity for hydrogen evolution of around 730 μmol min⁻¹ mg⁻¹; 30-fold more protein than previous culture adaptation studies and only 50% less than the amount which could be isolated with similar specific activity after heterologous expression in Clostridium acetobutylicum [6,37]. Using the new cultivation and isolation procedure we were further able to isolate comparable amounts of two other interesting [FeFe]-hydrogenases that were so far undescribed: HydA1 from C. moewusii and HydA from C. submarinum. The brackish water green alga C. submarinum is especially interesting for biotechnological applications since it can be cultivated in a medium with a higher salt concentration [17,38]; this diminishes the risk of contamination compared to freshwater algae. The specific activity for hydrogen evolution of HydA1 from C. moewusii was found to be two-fold higher than for HydA1 from C. reinhardtii and HydA from C. submarinum. This finding is interesting and should be further investigated in the future.

Sequence alignments of different [FeFe]-hydrogenases show that all sequence motifs characteristic for the H-domain of [FeFe]-hydrogenases are conserved (see Fig. 2). Besides the four highly conserved cysteine residues necessary for binding the catalytic H-cluster, residues postulated to establish the catalytic proton transfer pathway and the hydrophobic pocket, protecting the H-cluster against the outer medium, are identified [11,39,40]. Similar to [FeFe]-hydrogenases of other green algae HydA1 from C. moewusii and HydA of C. submarinum only consist of the catalytically relevant H-domain, usually located in the C-terminal part following the F-domain of other [FeFe]-hydrogenases [9]. Thus, besides the H-cluster, there is no indication of any additional iron–sulphur cluster being integrated into the HydA apo-protein, as already demonstrated for HydA1 from C. reinhardtii [6,41].

The relatively large amount of isolated [FeFe]-hydrogenase opened the possibility, for the first time, to characterize this hydrogenase using spectroscopic methods. In contrast to algal type [FeFe]-hydrogenases, whose only prosthetic group is the H-cluster (active site), the [FeFe]-hydrogenase DdH of D. desulfuricans was found to be two-fold higher than for HydA1 from C. moewusii and HydA from C. submarinum. This finding is interesting and should be further investigated in the future.

Table 3

<table>
<thead>
<tr>
<th>Species</th>
<th>Hox-CO</th>
<th>Hox</th>
<th>LI2</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. desulfuricans</td>
<td>2.065, 2.007, 2.001</td>
<td>2.100, 2.040, 1.999</td>
<td>2.221, 2.135, 2.048</td>
</tr>
<tr>
<td>C. reinhardtii</td>
<td>2.052, 2.007, 2.007</td>
<td>2.102, 2.040, 1.998</td>
<td>2.230, 2.115, 2.052</td>
</tr>
<tr>
<td>C. submarinum</td>
<td>2.056, 2.008, 2.008</td>
<td>2.100, 2.040, 1.998</td>
<td>2.230, 2.115, 2.052</td>
</tr>
<tr>
<td>C. moewusii</td>
<td>2.052, 2.008, 2.008</td>
<td>2.103, 2.038, 1.998</td>
<td></td>
</tr>
</tbody>
</table>
examinations in the future. More comprehensive pulsed EPR studies as well as FTIR experiments of the various states of the algal [FeFe]-hydrogenases are currently in progress in our laboratories.

Acknowledgments

This work was supported by the European Commission (6th FP, NEST STRP SOLAR-H contract 516510), the BMBF (Bio-H2) and the Max-Planck-Gesellschaft. C. Kamp, M. Winkler and T. Happe were further supported by the Deutsche Forschungsgemeinschaft (SFB 480).

References