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Isolation and first EPR characterization of the [FeFe]-hydrogenases from green algae

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ABSTRACT

Hydrogenase expression in *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 [FeFe]-hydrogenase (HydA1) and develop a 25-fold higher *in vitro* hydrogenase activity. Based on this efficient induction principle we have established a novel purification protocol for the isolation of HydA1 that can also be used for other green algae. From an eight liter *C. reinhardtii* culture 0.52 mg HydA1 with a specific activity of 741 μmol H_2 min^{-1} mg^{-1} was isolated. Similar amounts were also purified from *Chlorococcum submarinum* and *Chlamydomonas moewusii*. The extraordinarily large yields of protein allowed a spectroscopic characterization of the active site of these smallest [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 H_{ox}-CO state of the active site from [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.

1. Introduction

Hydrogenases catalyze the reversible redox reaction $2\,H^+ + 2\,e^- \rightleftharpoons H_2$. 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 [Fe]-hydrogenases, which do not contain iron–sulphur clusters and are only found in methanogenic archaea [1]. In the second group are the [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 [FeFe]-hydrogenases, which harbour a dinuclear [FeFe]-cluster linked to a [4Fe–4S]-cubane [3,4]. They are also found in eukaryotes [3–5]. In particular [FeFe]-hydrogenases are extremely sensitive to oxygen and thus are only expressed under anaerobic conditions [5–7]. Numerous prokaryotic and some eukaryotic [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 [FeFe]-hydrogenases from two organisms, *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* (ATCC 7757), are known from X-ray crystallography. The periplasmic heterodimeric [FeFe]-hydrogenase DdH from *D. desulfuricans* contains the H-cluster as the active site. This consists of a [4Fe–4S]-cluster bridged by the sulphur atom of a cysteine residue to a binuclear iron subcluster and two classical [4Fe–4S]-cubanes (F-clusters); in addition to these structure elements the cytoplasmic monomeric [FeFe]-hydrogenase CpI from *C. pasteurianum* contains one [4Fe–4S]-cluster with a histidine ligand and one [2Fe–2S]-cluster [10,11]. Since 2001 seven green algal hydA genes have been discovered, initiating a new subgroup of extraordinarily small [FeFe]-hydrogenases, which only contain the catalytic iron–sulphur center and no accessory Fe–S clusters [12–16]. Since the other [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 [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 [FeFe]-hydrogenases DdH of *D. desulfuricans* and CpI of *C. pasteurianum*, the structures of these algal type [FeFe]-hydrogenases have not yet been characterized. Because of their small size, their monomeric form and their high specific activity, these [FeFe]-hydrogenases are especially suited for analysis of maturation, structure and catalytic mechanism. In the green alga *Chlamydomonas reinhardtii* two genes, hydA1 and hydA2, coding for two similar [FeFe]-hydrogenases, have been identified [15]. The [FeFe]-hydrogenase HydA1 of *C. reinhardtii* was purified and characterized by Happe and Naber as a

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48 kDa enzyme with a specific activity for hydrogen evolution of around 935 μmol min^{-1} mg^{-1} [6]. Hydrogenase expression was induced by anaerobic adaptation of the cells. Under these conditions 1.4 μg HydA1 per liter algal culture was isolated. Unfortunately, this yield was not sufficient to analyse the structure or to characterize the mechanism of H_2-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). Algae strains were grown photoheterotrophically in Tris-Acetate-Phosphate (TAP) medium at 20 °C under continuous illumination at 150 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 anaerobically 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 final chlorophyll concentration of 20 μg ml^{-1} or TAP-S medium, containing 75 μM $MgSO_4$ per liter, was directly inoculated with cells grown as above (5 ml l^{-1}). Algal cultures were incubated under continuous illumination at ~50 μmol photons m^{-2} s^{-1} for up to eight days in gas tight suba sealed glass bottles [18].

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% N_2 and 1% H_2. Unless otherwise indicated all buffers contained 10 mM sodium dithionite to prevent deactivation of the hydrogenase by residual oxygen. For the isolation of hydrogenase eight liter sulphur-deprived cultures were harvested by centrifugation in the presence of 25 mM sodium dithionite. The resulting pellet was stored at −80 °C suspended in a small volume of 50 mM Tris–HCl pH 8, 10% glycerin, 25 mM sodium dithionite or directly suspended in 0.015 vol. 50 mM Tris–HCl pH 8.5, and 100 mM sodium dithionite. Cells were lysed by adding 1–2% Triton X-100 (final concentration) and stirring for 25 min. Cell debris was pelleted by centrifugation and the protein supernatant was precipitated with ammonium sulfate in the presence of 150 mM sodium dithionite. After the initial step (40% saturation) the protein solution was centrifuged (1 h, 6000 rpm, 4 °C) and the supernatant saturated to 75% ammonium sulfate followed by further centrifugation. The pellet was suspended in 15 ml 50 mM Tris–HCl pH 8.5 and dialysed against 100 vol. of the same buffer overnight. The solution was centrifuged (10 min, 10,000 rpm) and loaded on a Q-Sepharose fast-flow column (30 mm×60 mm) (Amersham Bioscience, München, Germany), previously equilibrated with 50 mM Tris–HCl pH 8.5. After washing with the same buffer, hydrogenase was eluted with a pH gradient (50 mM Tris–HCl pH 8.5 to 50 mM potassium phosphate pH 7) and washed through the column with 4 vol. of 50 mM potassium phosphate pH 7. Fractions with hydrogenase were pooled and diluted 1.5-fold with 50 mM Tris–HCl pH 8.5. The solution was loaded on a second Q-Sepharose fast-flow column (20 mm×35 mm) and the hydrogenase was finally eluted with a linear salt gradient (0–500 mM KCl). Active fractions were pooled and concentrated by centrifugation to a volume of 1 ml using a Vivaspin 6 (Sartorius, Göttingen, Germany). The concentrated sample was applied to a HiLoad 16/60 Superdex-75 gel filtration column (16 mm×600 mm) (GE Healthcare, München, Germany), previously equilibrated with 50 mM Tris–HCl pH 7.5, 150 mM KCl, 1 mM sodium dithionite or 50 mM Tris–HCl pH 8.5, 1 mM sodium dithionite, and eluted with the same buffer. Fractions, containing the hydrogenase, were concentrated and stored strictly anaerobically at −80 °C.

Table 1
List of primers used for the identification of the *hydA* genes from *C. moewusii* and *C. submarinum*

Primer name	Primer sequence
CmI5A	AATCTGCCGCTCAGCAAACA
CmI5B	CATCATCTGAGGCGACTTGC
CmII5A	TCAATGCCACGCTCAGTCAG
CmII5B	CTTCGGCGAAGAAGTTCTTG
AL5	TGCAAGTCGCCCCAGATGATG
AL9i	GCCGGGGCAGGCCATGACCTC
Cs3A	GAACGACAGCAGCAAGTTCA
Cs3B	CTCAACATCCGCATCGCAGT
Cs3C	ACGCCAAGAAGCTCATCACC
Cs5A	ATGACGGACACCAGGCTGAT
Cs5B	GACACCAGGCTGATCTCAGT
Cs5C	TCGGCGAAGAAGCTCTTCAC

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 methylviologen reduced by sodium dithionite in a gas chromatograph (GC-2010 Shimadzu, Duisburg, Germany). Gas tight suba sealed tubes, containing a final volume of 2 ml 60 mM potassium phosphate pH 6.8, 100 mM sodium dithionite, 10 mM methylviologen and 2–10 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 algal cultures, the assays contained 100 μl cell culture in addition to 1% Triton X-100 for cell lysis.

SDS Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [20], using a 6% stacking and a 15% separating gel. Proteins were stained with Coomassie Brilliant Blue.

For Western blot analysis samples were taken anaerobically at the indicated times. The pelleted cells were suspended in a small volume of sample buffer, containing 7% SDS, followed by incubation at 95 °C for 10 min. The resulting extract was separated by a 15% SDS Polyacrylamide gel after loading the lanes with samples, and an equal amount of chlorophyll (10 μg/lane). Proteins were blotted onto a nitrocellulose membrane and incubated with a specific HydA1-antibody.

2.3. Identification of the hydA-cDNA sequences from C. submarinum and C. moewusii

Genomic DNA for PCR experiments was isolated from green algal cultures according to the method of Newman [21]. For genomic PCR the *PfuUltra* Hotstart DNA Polymerase from Stratagene (Amsterdam, Netherlands) was used, while RT-PCR experiments were done with the One-Step-RT-PCR-Kit from Quiagen (Hilden, Germany). RACE-PCR was performed, using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Saint-Germain-en-Laye, France) following the instructions of the manufacturer's manual, with mRNA, isolated from anaerobically induced culture of *C. moewusii* or *C. submarinum*. The sequences of genomic and cDNA fragments were determined, using the dideoxynucleotide chain termination method performing a cycle sequencing reaction with the BigDye-Sequencing Kit (Applied Biosystems, Darmstadt, Germany). Separation and detection of the labelled amplification products was done by a capillary sequencer (ABI Prism 310). Sequence analysis and homology alignments were performed with the Sci Ed Central program package 5.2. Leader peptide prediction analysis was carried out with the ChloroP server 1.1 of the Technical University of Denmark. Primers used for the identification of the *hydA* genes from *C. moewusii* and *C. submarinum* are described in Table 1.

2.4. Identification of the isolated proteins

For protein identification 500 ng of the purified protein was separated by a SDS-PAGE and stained with Coomassie Brilliant Blue. The gel band, containing the purified protein, was cut out and washed with acetonitrile and ammonium bicarbonate buffer. After drying of the gel fragment, the protein was digested with Trypsin (10 ng μl^{-1}) and the digestion products finally extracted from the gel matrix with ammonium bicarbonate buffer, acetonitrile and formic acid, using an ultrasonicator. The supernatant was reduced to a volume of 10 μl and analysed by Q-TOF mass spectrometry [22].

2.5. EPR measurements

All sample preparations were performed under anaerobic conditions in a glove box containing an atmosphere of 99% N_2 and 1% H_2. Protein samples were concentrated by centrifugation to a final concentration of 100 μM, using a Vivaspin 500 (Sartorius, Göttingen, Germany), transferred into EPR tubes and frozen in liquid nitrogen. Treatment of the samples with gas was performed outside the glove box, using gas tight suba sealed tubes. EPR spectra were obtained at Q-band frequencies using the 2 pulse electron spin echodetected EPR technique [23,24]. In this experiment an electron spin echo (ESE) is detected as a function of the external magnetic field after two

Table 2
Purification of hydrogenase from *C. reinhardtii*, *C. submarinum* and *C. moewusii*

Sample	*C. reinhardtii*		*C. submarinum*		*C. moewusii*	
	Yield (%)	Specific activity (μmol H_2 min^{-1} mg^{-1})	Yield (%)	Specific activity (μmol H_2 min^{-1} mg^{-1})	Yield (%)	Specific activity (μmol H_2 min^{-1} mg^{-1})
Crude extract (8 l of cultures)	100	13.8	100	3	100	11
$(NH_4)_2SO_4$ precipitation (40–75%)	47.8	33.9	47	8	67	32
First anion-exchange chromatography (pH gradient)	47.5	218	46	145	58	694
Second anion-exchange chromatography (KCl gradient)	24.5	355	33	268	28	717
Gel filtration chromatography (Superdex 75)	21.6	741	28	639	23	1598
Total protein (mg)	0.52		0.4		0.32	

microwave pulses (π/2 and π). The delay between the MW pulses was fixed to τ=360 ns. The length of the π/2 MW pulse was set to 36 ns and the π 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 TE_{011} 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 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 l^{-1} reaches a maximum of hydrogenase activity of around 70 μmol H_2 min^{-1} mg^{-1} chlorophyll after 48 h. The *in vitro* activity maximum of a self-limiting culture is at around 75 μmol H_2 min^{-1} mg^{-1} chlorophyll. This activity develops after about eight days, when the culture has reached a chlorophyll concentration of 20–25 mg l^{-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 H_2 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 H_2 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 H_2 min^{-1} mg^{-1} and from a similar amount of *C. moewusii*, 0.32 mg HydA1 with a specific activity of 1598 μmol H_2 $min^{-1}mg^{-1}$ 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

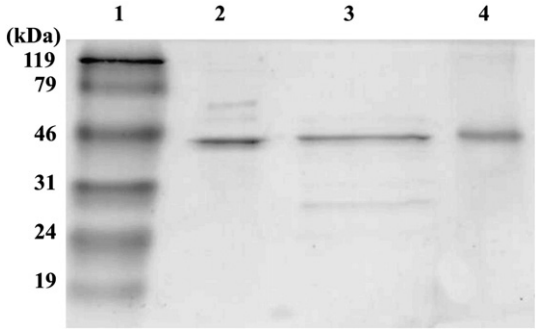

Fig. 1. SDS-PAGE of purified hydrogenases from three different green algal species. Lane 1: marker proteins; lane 2: purified hydrogenase from *C. submarinum*; lane 3: purified hydrogenase from *C. moewusii*; lane 4: purified hydrogenase from *C. reinhardtii*. The lanes were each loaded with 500 ng protein.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

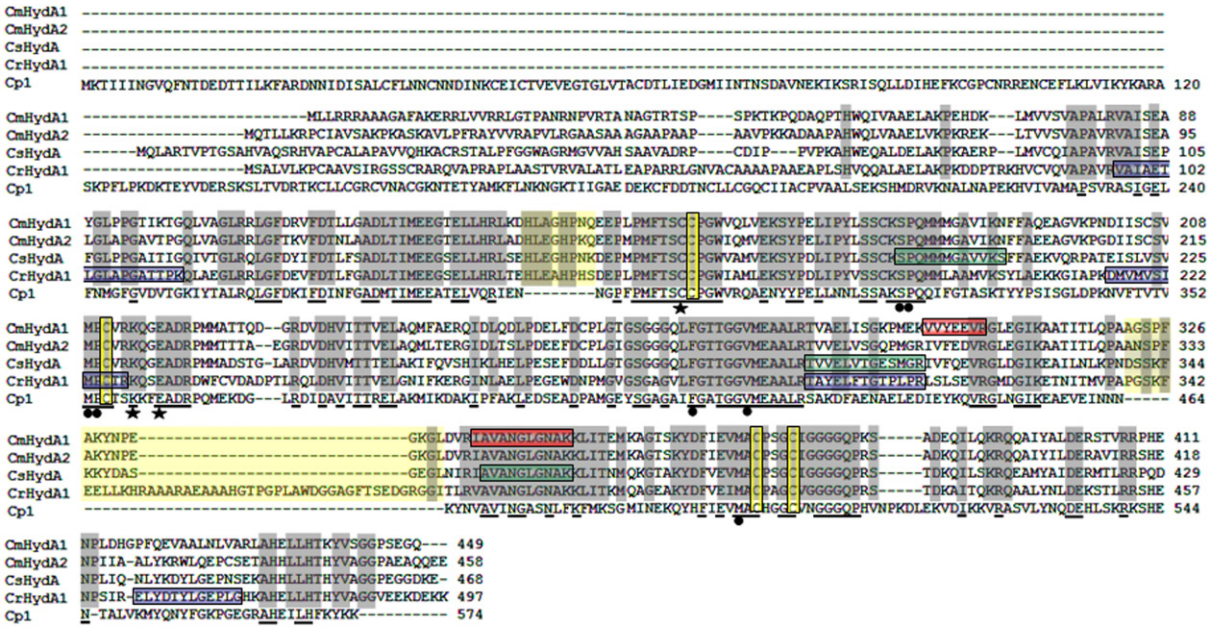

Fig. 2. Amino acid alignment of the HydA sequences from *C. reinhardtii* (HydA1), *C. moewusii* (HydA1 and HydA2), *C. submarinum* (HydA) and CpI of *C. pasteurianum*. Sequence regions conserved among the green algal proteins are shaded in grey. Residues conserved in all sequences including CpI are underlined by black bars. Sequence regions within the homologous C-terminal part of the hydrogenases only found in the green algal proteins (sequence insertions) are shaded in yellow. The highly conserved cysteine residues involved in H-cluster binding are highlighted by yellow boxes. Residues probably involved in the proton transfer pathway are marked by an asterisk. Residues participating in the structural integration of the active center pocket are marked by a dot. Sequence parts identified by Q-TOF mass spectrometry to verify the identity of the purified HydA proteins, are presented in red (*C. moewusii*), green (*C. submarinum*) or blue (*C. reinhardtii*) boxes within the respective alignment position.

a multiple sequence alignment and comprising the *hydA*-cDNA sequences of *C. reinhardtii*, *Scenedesmus obliquus* and *Chlorella fusca*. By performing PCR on the genomic DNA of *C. submarinum*, using the mismatch primers AL5 and AL9i (see Table 1), a 1430 kb fragment was amplified. Polypeptide sequence comparisons performed with the putative translation product, using the BLAST server database of NCBI, indicated homologies of up to 80% with several [FeFe]-hydrogenases [12–16]. The coding sequences of the genomic *hydA* fragment served as a basis for deriving the RACE-PCR primers Cs3A, Cs3B, Cs5A and Cs5B. 3′RACE-PCRs with Cs3A, Cs3B and Cs3C lead to a 1.8 kb fragment of the *hydA* cDNA, while 5′RACE-PCRs performed with the gene specific primers Cs5A, Cs5B, Cs5C gave a 0.78 kb product at the 5′ end of *hydA*. Both products overlapped with the complete cDNA sequence of 1962 bps. As described earlier, 1.2 kb of the *hydA1* cDNA and 0.8 kb of the *hydA2* cDNA from *C. moewusii* were isolated performing genomic and RT-PCR techniques [16]. RACE-PCRs with the gene specific primers CmII5A and CmII5B (5′RACE) lead to identification of the 5′ terminus of *hydA2*. Thus the complete cDNA of *hydA2* comprises 1850 bps, consisting of a 74 bps 5′ untranslated region (UTR), an open reading frame of 1374 bps and a 3′UTR of 402 bps [AY578072]. Using the gene specific primers CmI5A and CmI5B the identifiable section of *hydA1* could be extended to 1178 bps, consisting of a 1017 bps region, coding for 339 amino acids of HydA1, followed by the 3′ UTR of 161 bps. Compared to most [FeFe]-hydrogenases already identified HydA1 and HydA2 of *C. moewusii* and HydA of *C. submarinum* possess homology values between 50 and 80% sequence similarity.

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 isoenzymes. The Q-TOF analysis enabled identification of two oligopeptides 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 of *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 CpI 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*.

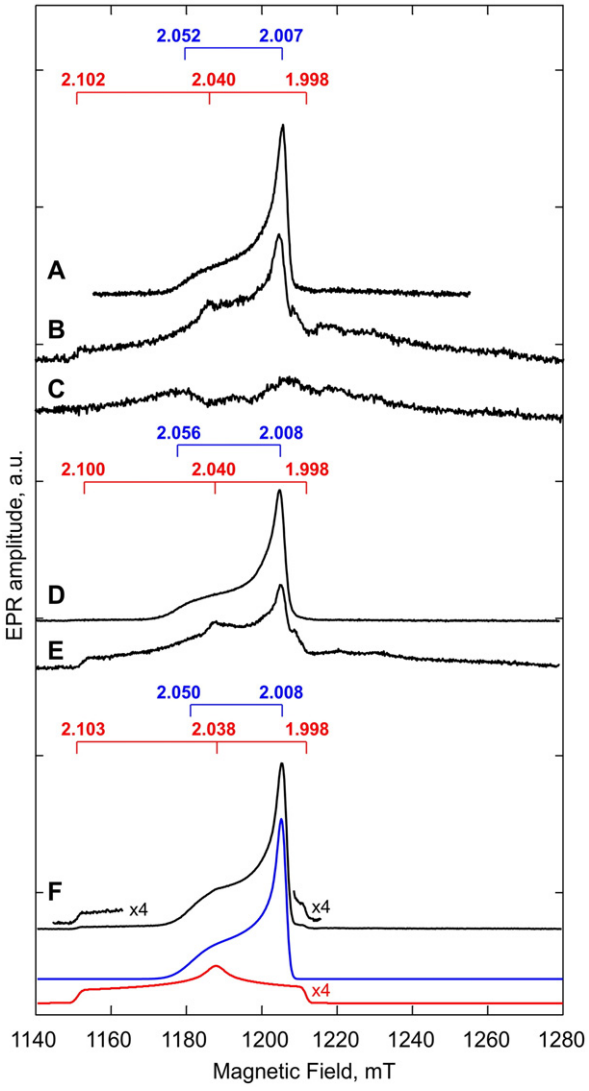

Fig. 3. Q-band pulse EPR spectra of the [FeFe]-hydrogenase from *C. reinhardtii* (A, B, C), *C. submarinum* (D, E) and *C. moewusii* (F). A. HydA1 H_{ox}-CO state, 30 μM in 50 mM Tris–HCl pH 8.5 as-isolated after flushing with CO for 20 min. B. HydA1 as-isolated, 100 μM in 50 mM Tris–HCl pH 8.5. C. HydA1 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).

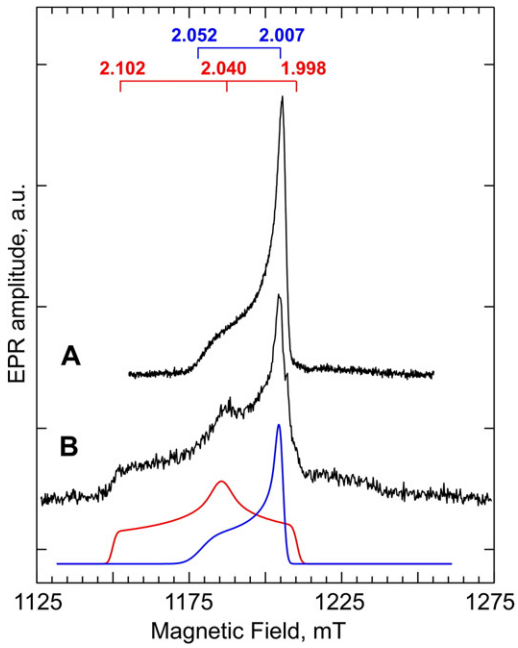

Fig. 4. Illumination of the [FeFe]-hydrogenase HydA1 from *C. reinhardtii* in the H_{ox}-CO state at 20 K. A. Q-band pulse EPR spectrum of the H_{ox}-CO state before illumination. B. Q-band pulse EPR spectrum after laser illumination (531 nm) for 3 h together with spectral simulations: the red line represents 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).

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

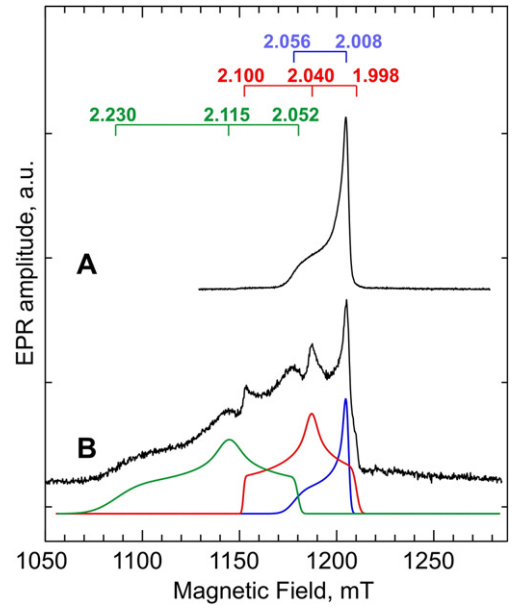

Fig. 5. Illumination of the [FeFe]-hydrogenase HydA from *C. submarinum* in the H_{ox}-CO state at 20 K. A. Q-band pulse EPR spectrum of the H_{ox}-CO state before illumination. B. Q-band pulse EPR spectrum after laser illumination (531 nm) for 3 h together with spectral simulation: the blue line represents 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).

Table 3
Overview of the measured *g*-values of the different species

Species	H_{ox}-CO	H_{ox}	LI2
D. desulfuricans	2.065, 2.007, 2.001	2.100, 2.040, 1.999	2.221, 2.135, 2.048
C. reinhardtii	2.052, 2.007, 2.007	2.102, 2.040, 1.998	
C. submarinum	2.056, 2.008, 2.008	2.100, 2.040, 1.998	2.230, 2.115, 2.052
C. moewusii	2.052, 2.008, 2.008	2.103, 2.038, 1.998	

from *C. submarinum*. Therefore, it is assigned to the oxidized state of the H-cluster (H_{ox}). 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 H_{ox} 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 H_{ox}-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 H_{ox}-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 H_{ox}-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 specific activity of the purified 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 apoprotein, 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* is characterized as a heterodimer, containing the H-cluster and, additionally, two accessory iron–sulphur clusters (F-clusters) [10]. The active oxidized state (H_{ox}) of the H-cluster gives rise to a characteristic rhombic EPR signal with *g*-values of 2.100, 2.040 and 1.999 (see Table 3) [30–32]. This H_{ox} state can be obtained from the reduced state after treatment with argon (Ar). Also characteristic for [FeFe]-hydrogenases is the reaction of the enzyme with carbon monoxide (CO). CO binds to the H-cluster, forming the so-called CO inhibited H_{ox}-CO state, which gives a typical axial EPR signal with *g*-values of 2.065, 2.007 and 2.001 for DdH [30–34]. The EPR spectra presented here demonstrate that the [FeFe]-hydrogenases from the aerobic eukaryotic green algae contain the H-cluster active site characteristic for [FeFe]-hydrogenases usually found in anaerobic living organisms (see Figs. 3–5) [4,8–11]. Furthermore, it has been verified that HydA, unlike other [FeFe]-hydrogenases, possesses no accessory iron–sulphur clusters in addition to the H-cluster. The structures of the H-clusters from these green algal type [FeFe]-hydrogenases seem to be similar to each other and to the active sites of the [FeFe]-hydrogenases from *D. desulfuricans* and *C. pasteurianum* however, differences in the *g*-values indicate that the electronic structure of the active sites is not exactly the same (see Table 3). Preliminary proton ENDOR experiments of the algal [FeFe]-hydrogenases reveal a hyperfine interaction with the methylene protons of the cysteine residues coordinating the cubane subcluster of the active site (data not shown). In addition these signals bear remarkable similarity with those found for the bacterial [FeFe]-hydrogenases, although small but distinct differences are also observed here. Moreover, the reaction of the enzymes to the treatment with thionine or argon shows interesting differences worthy of more detailed

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.

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