A novel type of Fe-hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetic electron transport chain.*,**

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**Running title:** A novel type of Fe-hydrogenase from *Scenedesmus obliquus*

**Summary:** Hydrogen evolution is observed in the green alga *Scenedesmus obliquus* after a phase of anaerobic adaptation. In this study we report the biochemical and genetical characterization of a new type of Fe-hydrogenase (HydA) in this photosynthetic organism. The monomeric enzyme has a molecular mass of 44.5 kDa. The complete hydA cDNA of 2609 bp comprises an open reading frame encoding a polypeptide of 448 amino acids. The protein contains a short transit peptide that routes the nucleus encoded hydrogenase to the chloroplast. Antibodies raised against the Fe-hydrogenase from *Chlamydomonas reinhardtii* react with both the isolated and in *E. coli* overexpressed protein of *S. obliquus* as shown by western blotting. By analyzing 5 kb of the genomic DNA, the transcription initiation site and 5 introns within hydA were revealed. Northern experiments suggest that hydA transcription is induced during anaerobic incubation. Alignments of *S. obliquus* HydA with known Fe-hydrogenases and sequencing of the N-terminus of the purified protein confirm that HydA belongs to the class of Fe-hydrogenases. The C-terminus of the enzyme including the catalytic site (H-cluster) reveals a high degree of identity to Fe-hydrogenases. However, the lack of additional Fe-S clusters in the N-terminal domain indicates a novel pathway of electron transfer. Inhibitor experiments show that the ferredoxin PetF functions as natural electron
donor linking the enzyme to the photosynthetic electron transport chain. PetF probably binds to the hydrogenase through electrostatic interactions.

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The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number AJ271546.

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The abbreviations used are: DSPD, disalicylidinepropandiamine; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region; TAP, Tris acetate phosphate; SDS, Sodium dodecyl sulfate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyle-p-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol; 1U = 1 µmol H₂/min

**Introduction**

Many prokaryotes and several eukaryotes have an enzyme complex in common catalyzing the reversible reduction of protons to molecular hydrogen. The diverse group of hydrogenases can be divided into three classes according to their metal composition in the active center (1). The NiFe-hydrogenases are widespread among all bacteria families and have been well characterized during the last 30 years (2). The iron sulfur proteins consist of one to four subunits and have an additional nickel atom in the catalytic site (3, 4). In contrast, the Fe-hydrogenases possess only [Fe-S] clusters and an Fe-cofactor with an unique structure of six
Fe-atoms (5, 6). The third class of hydrogenases lacks the iron sulfur clusters as well as additional metal atoms and was found only in methanogenic bacteria (7, 8).

Until now, Fe-hydrogenases have only been found in hydrogen-producing anaerobic bacteria and protozoa (9-13). The enzymes allow fermentative anaerobes to evolve H₂ without exogenous electron acceptors other than protons (14). They show a high specific activity which is about 100 fold higher compared to the NiFe-hydrogenases (15). Furthermore, all Fe-hydrogenases are extremely sensitive to oxygen and carbon monoxide. The structures of the Fe-hydrogenases from *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* have recently been investigated by X-ray crystallography (16, 17). The proteins consist of one or two subunits and have a remarkable Fe-cofactor (H-cluster) in the catalytic site. The H-cluster contains an unusual supercluster comprising a [4Fe4S] subcluster and a [2Fe] centre which are bridged together by a single cysteinyl sulfur (18). A number of conserved amino acids forms a hydrophobic pocket that shields the [2Fe] subcluster from the solvent. In all so far known Fe-hydrogenases at least eight conserved cysteines were found at the N-terminal site of the protein that coordinate two further [4Fe-4S] clusters (F-cluster). It is discussed that the F-clusters are responsible for the electron transfer from the surface of the protein to the active site (17, 19).

In green algae, Gaffron discovered a hydrogen metabolism 60 years ago (20). After anaerobic adaptation, he observed both H₂ uptake and hydrogen evolution dependent on the CO₂ partial pressure (21, 22). After bubbling the cells with an inert gas like argon, high rates of H₂-production can be measured in the light (23). Electrons are supplied either by photochemical water splitting at photosystem II which results in simultaneous production of hydrogen and oxygen, or by metabolic oxidation of organic compounds with release of CO₂ (24-27). Light dependent electron transport from organic substrate through the plastoquinone pool to the hydrogenase provides the cells with ATP under anaerobic conditions (28, 29).
From the unicellular green alga *Chlamydomonas reinhardtii* a monomeric Fe-hydrogenase with high specific activity has been isolated (30, 31). In contrast, a NiFe-hydrogenase was described for another well examined green alga, *Scenedesmus obliquus* (32, 33). The protein consists of two subunits of about 36 and 55 kDa and might be located in the chloroplast.

In order to investigate whether hydrogenases of the iron-only type also occur in green algae other than *C. reinhardtii*, we decided to look for the gene of a hydrogenase in *S. obliquus*. Interestingly, we isolated the protein and the gene encoding a monomeric Fe-hydrogenase (HydA). Although the H-cluster of the HydA protein of *S. obliquus* is very conserved, the N-terminal site is completely different compared to other Fe-hydrogenases. Further cysteines are not present. These cysteine residues coordinate the typical F-clusters which are necessary for the electron pathway in other Fe-hydrogenases. We performed physiological measurements of the hydrogen evolving activity in the present of chloroplast ferredoxin specific inhibitors as well as antibodies against this protein. The results clearly indicated that ferredoxin transfers electrons to the hydrogenase and links the enzyme to photosynthesis. The expression of the *hydA* gene is regulated at the transcriptional level. The mRNA is transcribed very rapidly during the process of anaerobic adaptation.
Experimental Procedures:

Algae strains, growth and anaerobic conditions

Wild-type *Scenedesmus obliquus* Kützing 276-6 was obtained originally from the culture collection of algae at the University of Göttingen. Cells were cultured photoheterotrophically (34) in batch cultures at 25 °C under continuous irradiance of 150 µmol photons x m⁻² s⁻¹. For anaerobic adaptation, 4 l cultures were bubbled vigorously with air supplemented with 5 % CO₂. After harvesting the cells in the mid exponential stage of growth the pellet was resuspended in fresh tris acetate phosphate (TAP)¹ medium. The algae were anaerobically adapted by flushing the culture with argon in the dark.

Hydrogen evolution assay

The *in vitro* hydrogenase activity was measured by using a gas chromatograph from Hewlett Packard (HP 5890, Series II) equipped with a thermal conductivity detector and a molecular sieve column. Methylviologen reduced by sodium dithionite was used as electron donor as described before (30). 1 U (unit) is defined as the amount of hydrogenase evolving 1 µmol H₂ x min⁻¹ at 25°C.

The *in vivo* activity in the presence of different inhibitors of the photosynthetic electron flow was determined as described (30). After anaerobic adaptation, cells were harvested, diluted in fresh TAP medium and transferred to sealed tubes. Inhibitors were added 1 h before H₂ evolving activity was measured. Cells were broken by sonification. Thylakoid membranes and photosynthetic electron transport chain remained intact as shown by O₂ polarography. Ferredoxin of *C. reinhardtii* and *S. obliquus* was isolated according to the method of Schmitter *et al.* (35).
RACE-PCR

RACE-PCR (36) was performed with the Clontech SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer’s recommendations except for modifications of the PCR and hybridization conditions. Starting material consisted of 1 µg mRNA from anaerobically adapted cells. The reverse transcription reaction was carried out with a M-MLV reverse transcriptase in two separate reaction tubes containing either the 5’- or the 3’-RACE-PCR specific primer from the kit. The cDNA of each sample served as template for the following PCR. For the 5´-RACE-PCR, a Universal Primer Mix (UPM) and the antisense primer Sc7 were used. The amplification of the 3´-cDNA end was performed with an UPM and the sense primer Sc6. To obtain more distinct PCR signals, the PCR was repeated for both reactions with nested universal primers and designed primers (inverse Sc6 and inverse Sc7, respectively) using a dilution of the products of the first PCR as template.

Primer extension

RACE-PCR was also implemented to map the transcription initiation site of the hydA mRNA (37). A gene specific primer (Sc17) was used to carry out the first-strand cDNA synthesis with the Supercript II reverse transcriptase (Life Technologies, Rockville, MD, USA) and 200 ng of mRNA as template. PCR was performed using either Sc12 or Sc27 and the SMART™ specific adapter primer UPM. Two different DNA fragments of 234 bp and 183 bp were amplified under standard PCR conditions. Both fragments were cloned into the pGem®-T-Easy vector (Promega, Madison, WI, USA) and sequenced using primers from the polylinker of the vector.
Genome Walking with genomic DNA

Applying the Clontech GenomeWalker Kit (Clontech Laboratories), genomic libraries from *S. obliquus* were generated by digestion with different blunt-end cutting endonucleases (*Nae*I, *Dra*I, *Pvu*II, *Hinc*II and *Eco*RV) and by adapter ligation at the ends of the resulting DNA fragments. These libraries were utilized as independent templates in five different PCR reactions (38). Two gene specific primers (Sc27, Sc35) derived from the *hydA* cDNA sequence of *S. obliquus* were used in combination with a kit adapter primer (AP1) in a first PCR reaction. Subsequently, 1 µl of the first PCR served as a template in a secondary PCR, applying two nested gene specific primers (i-Sc10, Sc32) along with a nested kit adapter primer (AP2). The resulting products were cloned into pGem®T-Easy and sequenced. Sequencing was performed by the dideoxy chain termination method (39).

Purification of the Fe-hydrogenase

40 l cultures of *Scenedesmus obliquus* were grown heterotrophically. After centrifugation (10 min, 5000 x g) the pellet was resuspended in 200 ml TAP medium. The cells were anaerobically adapted by flushing the solution with argon for 1 hour in the dark. All further purification steps were performed in an anaerobic chamber (Coylab, MI, USA). The cells were disrupted in a 50 mM Tris/HCl buffer pH 8.0, 10 mM sodium dithionite by vortexing 3 min with glass beads. The further purification steps were made as described earlier for the isolation of the Fe-hydrogenase of *Chlamydomonas reinhardtii* (30). Automated Edman degradation of the N-terminal site of the protein was performed with an Applied Biosystem model 477A sequencer with online analysator model 120 A.
RNA blot hybridization

Total RNA of *S. obliquus* was isolated according to the method described earlier (40). Equal amounts (20 µg) were separated electrophoretically on 1.2 % agarose gels containing formaldehyde (41). The RNA was transferred onto nylon membranes (Hybond+, Amersham) and hybridized with RNA probes labeled with DIG-dUTP using the *in vitro* transcription method. A 1.3 kb *Eco*RI cDNA fragment was used to detect transcripts of the *hydA* gene while a DIG-dUTP labeled cDNA, encoding constitutively expressed plastocyanin (42), was used as control. Hybridization reactions were carried out using protocols supplied by the manufacturer (Roche Diagnostics, Mannheim, Germany).

Sequence analysis software

Nucleic acid and protein sequences were analyzed with the programs Sci Ed Central (Scientific Educational Software) and ClustalW (43). The Blast server (44) of the National Center for Biotechnology Information (Bethesda, MD) was used for database searches.

Recombinant expression in *E. coli*

The *hydA* open reading frame was amplified by PCR using the primer pair Sc29 and Sc30 containing flanking *Nde*I-*Bam*HI sites. The PCR product was cloned into the pGem®-T-Easy vector. After digestion with *Nde*I-*Bam*HI, the *hydA* gene was cloned into the corresponding site of the pET9a expression vector (Promega) producing pLF29.2. The insert of pLF29.2 was sequenced confirming that the fragment contains the exact full coding region of the hydrogenase without transit peptide. *E. coli* strain BL21(DE3)pLysS was transformed with pLF29.2. Expression was induced with 1 mM isopropyl-thio-β-D-galactoside at an OD₆₀₀ of 0.3. Pelleted cells were resuspended in lysis buffer (100 mM Tris/HCl; 4 mM EDTA; 16 % Glycine; 2 % SDS; 2 % Mercaptoethanol; 0.05 % Bromphenolblue; 8 M Urea). After heating,
the protein extract was separated by 10 % SDS-PAGE and blotted onto a PVDF membrane. Western blot analyses were performed using antisera against the Fe-hydrogenase of *Chlamydomonas reinhardtii* at 1:1000 dilution as described earlier (31).

**Results:**

**Induction of hydrogenase activity and purification of the Fe-hydrogenase protein**

Anaerobic adaptation is the most efficient way to induce hydrogenase activity in *Scenedesmus obliquus*. Bubbling the alga culture in the dark with argon led to a dramatic increase (10-fold) of hydrogenase activity during the first 2 hours. We purified the enzyme of *S. obliquus* to homogeneity by successive column chromatography. Since the enzyme is irreversible inactivated by lowest oxygen levels, all purification steps were performed under strictly anaerobic conditions and in the presence of reducing agents (dithionite). The purification scheme resulted in a 5200-fold purification of HydA with 5% recovery (data not shown). The most powerful step for purifying the protein was a Q-Sepharose high performance column chromatography with pH gradient elution. Gel filtration chromatography of hydrogenase on a calibrated Superdex-75 column resulted in a single activity peak corresponding to a molecular mass of 45 kDa. The monomeric structure of the enzyme could also be shown on a SDS polyacrylamide gel after Coomassie-blue staining (data not shown). The N-terminal sequence of HydA was determined by Edman degradation. The protein sequence (AGPTAECDRPPAPAPKAXHWQ) is, except for two amino acids, identical to the amino acid sequence deduced from the DNA data (AGPTAECDPPAPAPKAPHWQ). In the course of our purification procedure we never found a hint for a second hydrogenase in *S. obliquus* because the hydrogenase activity was never separated in several distinct fractions. Biochemical data show a high similarity of HydA to the Fe-hydrogenase from *C. reinhardtii* (Table I). The enzymes have a high temperature optimum of about 60 °C, are strongly
inhibited by O₂ and CO and catalyze the H₂-evolution with a typical high specific activity. Experiments with inhibitors of translation on ribosomes (data not shown) and analysis of the gene structure (see below) show that HydA from S. obliquus is translated in the cytoplasm and then transported into the chloroplast.

**Ferredoxin is the natural electron donor of the Fe-hydrogenase**

Hydrogenase activity was determined in intact and broken cells after anaerobic adaptation. The integrity of the photosynthetic electron transport in the sonified cell preparation was demonstrated by the rate of oxygen evolution (154 µmole O₂/mg Chl x h). This rate corresponds to 85% of the oxygen evolution measured with intact Scenedesmus cells. In S. obliquus, the hydrogen evolution is linked to the photosynthetic electron transport chain through PSI. As shown in Table II, the cells were still able to photoproduce hydrogen when electron flow of PSII was blocked by DCMU. In contrast, addition of DBMIB resulted in inhibition of the H₂-production, thus giving evidence of the involvement of PSI in the supply of electrons to hydrogenase. The electron transport from PSI to ferredoxin was inhibited using the artificial electron acceptor DCPIP. In this reaction, DCPIP is reduced instead of ferredoxin and the electron transfer to hydrogenase is interrupted.

Hydrogenase activity was dramatically reduced (up to 30-fold) by the ferredoxin antagonist sulfo-DSPD (Table II). Similar results were achieved with α-PetF-antibodies that specifically recognize the ferredoxin protein. In both cases, the hydrogenase enzyme can not evolve hydrogen, thus demonstrating the role of ferredoxin as the obligatory electron donor for the hydrogenase reaction.

The electron transfer properties of different plant-type ferredoxins were measured *in vitro* with dithionite as reducing reagent. The ferredoxin proteins of spinach, C. reinhardtii and S. obliquus were comparable regarding their capability to reduce purified S. obliquus hydrogenase. In this assay, we obtained H₂-evolving activities of 420, 390 und 350 U/mg protein with S. obliquus,
*C. reinhardtii* and spinach ferredoxin, respectively. No hydrogen production could be measured with other possible electron donors like cytochrome and NADPH. In *D. desulfuricans* the Fe-hydrogenase was reported to catalyse both hydrogen production and uptake with low potential multiheme cytochromes like cytochrome c₃ (17).

**Molecular characterization of hydA encoding a Fe-hydrogenase**

In order to isolate the gene encoding a Fe-hydrogenase in *S. obliquus*, we isolated polyA⁺ RNA from cell cultures after one hour of anaerobic adaptation. Isolated RNA was transcribed and amplified by RT-PCR using oligonucleotides derived from conserved regions within the *C. reinhardtii hydA* gene (Happe, unpublished results). The complete cDNA clone of 2609 bp was obtained by 5'- and 3'-RACE PCR. It contains an open reading frame of 1344 bp encoding a polypeptide of 448 amino acids (Fig. 1) followed by an extensive 3' UTR of about 1100 bp. The coding region of *S. obliquus hydA* exhibits features common to other green algae such as high GC content (64.2 %) and a characteristic putative polyadenylation signal, TGTAA, 15 bp upstream of the polyA⁺ sequence (45).

In an effort to examine the exon-intron structure and the promoter region of the *hydA* gene, about 5 kb of the genomic DNA from *S. obliquus* were sequenced. The gene comprises 5 introns with a total size of 1310 bp (Fig. 1) whose 5'- and 3'-end contain typical plant splice donor and acceptor sites that follow the GT/AG rule.

A genomic southern blot was probed with a 750 bp PCR fragment to determine the copy number of the *hydA* gene (Fig. 2). Single bands were observed in lanes with samples digested with *HincII*, *EcoRV* and *NdeI* and a double band in the lane containing genomic DNA digested with *SacI*. The band migration positions matched the sizes predicted from the sequence of the *hydA* gene, indicating that HydA is encoded by a single copy gene (Fig. 2). The same hybridization pattern was observed even under low stringency conditions (hybridization temperature 50 °C; data not shown). The transcription start position was
determined by primer extension using RACE-PCR and was found 139 bp upstream of the
ATG start codon. We designed several primers within 100 bp of the 5´-end of the known
hydA cDNA to confirm the accuracy of the transcription initiation site. All of the sequenced
PCR clones had the same 5´-ends at position +1. As described for other green algae genes, a
highly conserved TATA box element upstream of the transcription startpoint is absent (46).
However, the TACATAT motive at position –25 in a GC rich region shows similarities to
other TATA motives in C. reinhardtii and therefore might be involved in gene expression.

**HydA is a novel type of Fe-hydrogenase**

The polypeptide derived from the cDNA sequence has a length of 448 amino acids and a
predicted molecular mass of 48.5 kDa (44.5 kDa without transit peptide, respectively);
consequently, HydA is the smallest hydrogenase protein known so far. The N-terminus of
HydA is basic and contains numerous hydroxylated amino acids and an Val-X-Ala motive at
position 35, a characteristic feature of chloroplast transit peptides (47, 48).

The processed HydA protein is compared with four bacterial and two eukaryotic Fe-
hydrogenases as shown in Figure 3. The homology in the carboxy-terminal region of all
proteins is quite striking. For example, the *S. obliquus* HydA protein shows 44 % identity and
57 % similarity to the *C. pasteurianum* Fe-hydrogenase (9). The H-cluster in *S. obliquus*
might be coordinated by four cysteine residues at positions 120, 175, 335 and 340. Other
strictly conserved amino acid structures like FTSCPGW (343-350), TGGVMEAALR (474-
483) and MACPGGCXXGGGQP (576-589) probably define a pocket surrounding the active
center as shown by the structural data of *C. pasteurianum* and *D. desulfuricans* (16), (17). On
the other hand, the N-terminal region is completely different from all other Fe-hydrogenases.

The protein sequences of the other enzymes comprise at least two [4Fe-4S] ferredoxin like
domains (called F-cluster) which are necessary for the electron transport from the electron
donor to the catalytic center. The Fe-hydrogenases of *C. pasteurianum, Thermotoga maritima*
and *Nyctotherus ovalis* (9, 12, 11) contain an extra [4Fe-4S] cluster and one [2Fe-2S] center. This N-terminal domain with the F-cluster or other [Fe-S] centers is completely lacking in HydA of *S. obliquus*. This indicates that there is a direct electron transport pathway from the exogenous donor to the H-cluster.

To verify that the isolated cDNA encodes a Fe-hydrogenase, the *hydA* clone was expressed in the heterologous system *E. coli*. One band of recombinant HydA protein was observed on SDS-PAGE at approximately 44 kDa, in agreement with the molecular mass of the polypeptide predicted from the cDNA sequence. Antibodies raised against the HydA protein of *C. reinhardtii*, which crossreact with other Fe-hydrogenases but not with NiFe-hydrogenases (data not shown), were applied in Western blot analysis. One distinct signal with the overexpressed HydA protein of *S. obliquus* was obtained (Fig. 4).

The lysate of induced *E. coli* cells exhibited no hydrogenase activity. This result corresponds to observations by Voordouw *et al.* (50) and Stokkermans *et al.* (51) who also detected no H₂-production of recombinant Fe-hydrogenases in *E. coli* cells. The reason for that might be that the bacterial cells do not have the ability to assemble the special H-cluster of Fe-hydrogenases.

**Rapid induction of *hydA* mRNA during anaerobic adaptation**

The regulation of the *hydA* gene expression was examined by Northern blot analysis and RT-PCR. Aerobically grown cells of *S. obliquus* did not show a hydrogenase activity (Fig. 5A). Total RNA and also mRNA were isolated from cells which were induced by argon bubbling for 0, 1, and 4 hours. Northern blot analysis and RT-PCR demonstrated that the *hydA* gene is expressed after anaerobic adaptation. There is a very weak signal without adaptation (t=0), but strong signals of the transcript could be detected after anaerobic induction(Fig. 5 B,C). The full length of the *hydA* cDNA clone was confirmed by the transcript signal (2.6 kb) on the Northernblot.
Discussion

In green algae, the occurrence of a hydrogen metabolism induced by anaerobic conditions is well established. Despite the great interest in hydrogen evolution for practical applications (‘biophotolysis’), the hydrogenase genes from green algae have not yet been isolated. The hydA gene and the isolated HydA protein of Scenedemus obliquus that we present in this work belong to the class of Fe-hydrogenases.

Fe-hydrogenases have been isolated only from certain anaerobic bacteria and some anaerobic eukaryotes as well as from the anaerobically adapted green alga C. reinhardtii (30). The enzymes are found to exist in monomeric (9, 13, 53, 54), dimeric (17) and multimeric (12) forms; however, in eukaryotes only monomeric proteins have been isolated (10, 11).

The HydA protein of S. obliquus is synthesized in the cytoplasm. The first 35 residues (M1 to A35) of the amino acid sequence derived from the cDNA sequence are supposed to function as a short transit peptide which routes the nuclear encoded protein to the chloroplast. Several positively charged amino acids which describe a typical feature for algal transit peptides (47) are found in HydA. The three terminal residues of the signal sequence, Val-X-Ala, constitute the consensus sequence for stromal peptidases (48).

The hydrogenase of S. obliquus represents a novel type of Fe-hydrogenase. The monomeric enzyme of 448 amino acids and a calculated molecular mass of 44.5 kDa for the processed protein is the smallest Fe-hydrogenase isolated so far. The protein sequence consists of an unusual N-terminal domain and a large carboxyterminal domain containing the catalytic site.

The structurally important C-terminus of the S. obliquus HydA sequence is very similar to that of other Fe-hydrogenases. Four cysteine residues at positions C120, C175, C336 and C340 coordinate the special [6Fe] cluster (H-cluster) of the active site (Fig. 6). A number of additional residues define the environment of the catalytic centre. Peters et al. postulated twelve amino acids in C. pasteurianum to form a hydrophobic pocket around the cofactor (16). Ten residues are strictly conserved while two amino acids vary within the Fe-
hydrogenase family (S$^{232}$, I$^{268}$ in \textit{C. pasteurianum}, A$^{119}$, T$^{155}$ in \textit{T. vaginalis} and A$^{44}$, T$^{80}$ in \textit{S. obliquus}). A small insertion of 16 amino acids is noted in \textit{S. obliquus} but this addition occurs in an external loop of the protein and has probably no special function (Fig. 6).

Until now, all Fe-hydrogenases possess a ferredoxin like domain in the N-terminus coordinating two [4Fe4S] clusters (FS4A, FS4B, (10, 13) Fig. 6). The iron sulfur clusters facilitate the transfer of electrons between external electron donors or acceptors and the H-cluster. The N-terminus of the \textit{S. obliquus} protein is strongly reduced compared to other Fe-hydrogenases and no conserved cysteines are found. Therefore we postulate that all accessory Fe-S clusters (FS2, FS4A, FS4B and FS4C) are missing. No hints for a second subunit have been observed during purification of the protein.

In contrast to earlier observations in \textit{S. obliquus} (32), we could neither detect the postulated two subunits of a potential NiFe-hydrogenase, nor could we find a Ni-dependency related to the hydrogenase activity. Francis reported about two forms of hydrogenases in \textit{S. obliquus} (52), but although we used the same alga strain and identical adaptation conditions we were not able to detect a second hydrogenase activity during the purification steps.

Physiological studies have shown that the hydrogen evolution is coupled to the light reaction of the photosynthesis (24-26). In contrast to earlier observations in \textit{S. obliquus} (25, 26), we measured PSII independent H$_2$-production that is not influenced by DCMU. The electrons required for H$_2$-evolution come from redox equivalents of the fermentative metabolism and are supplied into the photosynthetic electron transport chain via the plastoquinone pool.

For the first time we demonstrate that the ferredoxin PetF functions as the \textit{in vivo} electron donor of the Fe-hydrogenase from \textit{S. obliquus}. Hydrogenase activity can be specifically blocked by addition of the ferredoxin antagonist sulfo-DSPD (55) and antibodies raised against the PetF protein. \textit{In vitro}, a hydrogen evolution by HydA was only measured with plant-type [2Fe-2S] ferredoxins like PetF of \textit{S. obliquus}, \textit{C. reinhardtii} and spinach as
electron mediators. Bacterial Fe-hydrogenases are known to be reduced by [4Fe-4S] ferredoxins and do not accept electrons from plant-type proteins (56).

The analysis of the 3D-structure of the Fe-hydrogenase from *C. pasteurianum* (CpI) gave evidence that the interaction with external electron donors might occur at the accessory [Fe-S] clusters in the N-terminal domain (14). Based on the X-ray structure of CpI, we modeled the Fe-hydrogenase of *S. obliquus* (57). As shown in Fig. 7, a region of positive surface potential is observed within HydA based on a local concentration of basic residues. In contrast to the docking position of ferredoxin in CpI, these charged amino acids in the *S. obliquus* Fe-hydrogenase are located within the C-terminal domain, forming a niche for electron donor fixation.

The known algal ferredoxin proteins exhibit high degrees of sequence identity (over 85 %) and the charged amino acids are strictly conserved. The petF sequence of *S. obliquus* is unknown, but very recently the X-ray model of the ferredoxin from another *Scenedesmus* species (*Scenedesmus vacuolatus*, 58) was published. The structure revealed negatively charged amino acids like aspartate and glutamate near the [2Fe-2S]-cluster. The [Fe-S] centre and the H-cluster of the hydrogenase probably come into close proximity through electrostatic interactions. This geometry is consistent with efficient electron transfer among these prosthetic groups.

As already shown in various studies, a correlation exists between the duration of time of the anaerobic adaptation and the increase of hydrogen production (30, 32). RT-PCR and Northern blot analyses with mRNA of aerobic and anaerobically adapted cells from *S. obliquus* showed an increased level of *hydA* transcript after one hour of induction. Correspondingly, hydrogen evolution was only measured after a short time of anaerobic adaptation. These results suggest that the expression of the *hydA* gene is regulated at the transcriptional level. The small amount of transcript that was detected at t=0 may be due to transcript synthesis induced by microanaerobic conditions during the RNA isolation procedure. Alternatively, a low level of
hydA transcript might be constitutively present in the cell and is only drastically increased after anaerobic adaptation.

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Tables and Figures

Fig. 1. **Schematic representation of S. obliquus hydA genomic and cDNA structures.**
A: The coding region of the hydA cDNA is illustrated as large arrow with the transit peptide shown in black. The untranslated 5´- and 3´-sequences are marked as lines. The arrows below indicate the sequencing strategy; each arrow represents an independent sequence determination. TSP, transcription start point; ATG, startcodon.
B: The mosaic structure of hydA is indicated by grey (exons) and white boxes (introns). The S2 probe and different restriction enzymes that were used in the Southern blot experiments are mentioned.

Fig. 2. **The hydA gene is located in the genome of Scenedesmus obliquus as a single copy gene.**
Southern analysis was carried out by digesting genomic DNA of S. obliquus with four different restriction endonucleases (SacI, HincII, EcoRV, Ndel). 10 µg of DNA was loaded per lane. The S2 DNA-Probe (750 bp) was used for the hybridisation as indicated in Fig. 1.

Fig. 3. **Comparison of the deduced HydA protein sequence with other Fe-hydrogenases.**
The protein alignment was done by using the Vector NTI programme (InforMax). Black highlited letters indicate amino acids identical to the HydA protein. Grey underlaid amino acids indicate conserved changes of the amino acids. The abbreviations of the organisms are: S. o., *Scenedesmus obliquus* (this work), M. e., *Megasphaera elsdenii* (13), D. d., *Desulfovibrio desulfuricans* (17), T. v., *Trichomonas vaginalis* (10), C. p., *Clostridium pasteurianum* (9), T. m., *Thermotoga maritima* (12), N. o., *Nyctotherus ovalis* (11).
Fig. 4. Recombinant expressed HydA reacts with antibodies raised against *C. reinhardtii* Fe-hydrogenase.

The hydA gene coding region corresponding to amino acid 36 to residue 448 was cloned Ndel-BamHI into pET9a. HydA protein was expressed upon induction with IPTG. 

Lane1: Recombinant expressed HydA protein from *S. obliquus*; 
Lane 2: Recombinant expressed Fe-hydrogenase from *C. reinhardtii*; 
Lane 3: Purified hydrogenase from *C. reinhardtii*; 
Lane 4: Total proteins from induced *E. coli* cells without plasmid; 

A: (SDS-PAGE) M: molecular mass marker (Bio-Rad) indicating relative molecular masses in kDa. SDS-PAGE stained with Coomassie Blue. B: Western blot probed with HydA antibody. The recombinant proteins of lane 1 and 2 from figure A were diluted 1:10.

Fig. 5. Induction of the hydrogenase activity and differential expression of the hydA gene during anaerobic adaptation.

A: *S. obliquus* cells were anaerobically adapted by flushing the culture with argon in the dark. After removing cell samples at the indicated times, the algae were broken by Triton X-100 treatment. The *in vitro* hydrogenase activity was measured as described under Experimental Procedures. 

B: Northern hybridization was performed with the hydA specific probe. Adapted cells were harvested at 0, 1 and 4 hours, and the RNA was isolated. 20 µg of total RNA was loaded per lane. C: The same RNA was hybridized with a constitutive expressed gene (plastocyanin) as control.
Fig. 6. **Schematic alignment of the conserved cysteine residues and other important amino acids of the H-cluster.**

The protein is illustrated as a large grey arrow. Small arrows indicate parallelograms which demonstrate conserved amino acids in the protein. Cysteines participating at the coordination of the [Fe-S]-clusters are grey shaded while identical amino acids are black shaded. An insertion of 16 amino acids in the *S. obliquus* protein is illustrated as a spotted bar. FS4 = [4Fe-4S]-cluster, FS2 = [2Fe-2S]-cluster.

Fig. 7. **Schematic view of the structures of *S. obliquus* HydA (A) and the electron donor ferredoxin (B).**

The figure shows the alpha carbons and the side chains of charged residues which might be important for the electron transfer reaction or the interaction between HydA and the ferredoxin from *Scenedesmus vacuolatus* (58). The 16 amino acid insertion of the hydrogenase appears as external loop and is distinguished as dotted line.

The amino acid sequence of the mature HydA protein (His^{19}\text{-}Tyr^{404}) was submitted to the SWISS-MODEL server (59). We generated a model of HydA with the known 3D-structure of the Fe-hydrogenase from *C. pasteurianum* (16) as template, sharing 57% sequence identity with the submitted sequence. The PDB-file was visualized by the Swiss-Pdb-viewer (57).
Fig. 1. Schematic representation of *S. obliquus* hydA genomic and cDNA structures.

Fig. 2. The hydA gene is located in the genome of *Scenedesmus obliquus* as a single copy gene.
Fig. 3. Comparison of the deduced HydA protein sequence with other Fe-hydrogenases.
Fig. 4. Recombinant expressed HydA reacts with antibodies raised against *C. reinhardtii* Fe-hydrogenase.

Fig. 5. Induction of the hydrogenase activity and differential expression of the *hydA* gene during anaerobic adaptation.
Fig. 6. Schematic alignment of the conserved cystein residues and other important amino acids of the H-cluster.
Fig. 7. Schematic view of the structures of *S. obliquus* HydA (A) and the electron donor ferredoxin (B)
### Table I. Biochemical data comparison of purified Fe-hydrogenases from *C. reinhardtii* and *S. obliquus*.

<table>
<thead>
<tr>
<th></th>
<th><em>Chlamydomonas reinhardtii</em></th>
<th><em>Scenedesmus obliquus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td>49 kDa</td>
<td>44.5 kDa</td>
</tr>
<tr>
<td><strong>Specific activity</strong></td>
<td>935 U/mg protein</td>
<td>700 U/mg protein</td>
</tr>
<tr>
<td><strong>Temp. optimum</strong></td>
<td>60 °C</td>
<td>60 °C</td>
</tr>
<tr>
<td><strong>pH optimum</strong></td>
<td>6.9</td>
<td>7.3</td>
</tr>
<tr>
<td><strong>Localization</strong></td>
<td>chloroplast stroma</td>
<td>chloroplast</td>
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<tr>
<td><strong>Coding site</strong></td>
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<td>nuclear</td>
</tr>
<tr>
<td><strong>pI value</strong></td>
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<td>5.17</td>
</tr>
<tr>
<td><strong>K_M value (MV)</strong></td>
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<td>800 µM</td>
</tr>
<tr>
<td><strong>K_M (ferredoxin)</strong></td>
<td>35 µM</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Table II. **Effects of different photosynthetical inhibitors on hydrogenase activity**

After anaerobic adaptation, cells were harvested, diluted in fresh TAP medium and incubated with inhibitors as described in Experimental Procedures.

Inhibitors: α-PetF-antibody was raised against spinach ferredoxin. DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; Sulfo-DSPD, sulfo-disalicylidinepropanediamin; DCPIP, 2,6-dichlorophenolindophenol; 1U = 1 µmol H₂/min.
A novel type of Fe-hydrogenase in the green alga Scenedesmus obliquus is linked to the photosynthetic electron transport chain
Lore Florin, Anestis Tsokoglou and Thomas Happe

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