A Novel Type of Iron Hydrogenase in the Green Alga *Scenedesmus obliquus* Is Linked to the Photosynthetic Electron Transport Chain*

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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 iron hydrogenase (HydA) in this photosynthetic organism. The monomeric enzyme has a molecular mass of 44.5 kDa. The complete *hydA* cDNA of 2609 base pairs 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 iron hydrogenase from *Chlamydomonas reinhardtii* react with both the isolated and in *Escherichia coli* overexpressed protein of *S. obliquus* as shown by Western blotting. By analyzing 5 kilobases of the genomic DNA, the transcription initiation site and five introns within *hydA* were revealed. Northern experiments suggest that *hydA* transcription is induced during anaerobic incubation. Alignments of *S. obliquus* HydA with known iron hydrogenases and sequencing of the N terminus of the purified protein confirm that HydA belongs to the class of iron hydrogenases. The C terminus of the enzyme including the catalytic site (H cluster) reveals a high degree of identity to iron 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.

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 nickel-iron 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 iron hydrogenases possess only [Fe-S] clusters and an iron cofactor with an unique structure of six iron 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, iron 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 that is about 100 fold higher compared with the nickel-iron hydrogenases (15). Furthermore, all iron hydrogenases are extremely sensitive to oxygen and carbon monoxide. The structures of the iron 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 iron cofactor (H cluster) in the catalytic site. The H cluster contains an unusual supercluster comprising a [4Fe-4S] subcluster and a [2Fe] center, which are bridged together by a single cysteiny1 sulfur (18). A number of conserved amino acids forms a hydrophobic pocket that shields the [2Fe] cluster from the solvent. In all known iron 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).

In green algae, Gaffron (20) discovered a hydrogen metabolism 60 years ago. 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 iron hydrogenase with high specific activity has been isolated (30, 31). In contrast, a nickel-iron 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.

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 iron hydrogenase (HydA). Although the H cluster of the HydA protein of *S. obliquus* is very conserved, the N-terminal site is completely different compared with other iron hydrogenases. Further cysteines are not present. These cysteine residues coordinate the typical F clusters that are necessary for the
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Electron pathway in other iron 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 S. obliquus Kützing 276-6 was obtained originally from the culture collection of algae at the University of Göttingen. Cells were cultured phototetehorotropically (34) in batch cultures at 25 °C under continuous irradiance of 150 μmol photons × m 2 × s 1. For anaerobic adaptation, 4-liter cultures were bubbled vigorously with air supplemented with 5% CO 2. After harvesting the cells in the mid exponential stage of growth, the pellet was resuspended in fresh Tris acetate phosphate 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 was used as electron donor as described before (30). 1 unit is defined as the amount of hydrogenase evolving 1 μmol H 2 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 Tris acetate phosphate medium, and transferred to sealed tubes. Inhibitors were added 1 h before H 2 evolving activity was measured. Cells were broken by sonification. Thylakoid membranes and photosynthetic electron transport chain remained intact as shown by O 2 polarography. Ferredoxin of C. reinhardtii and S. obliquus was isolated according to the method of Schmitter et al. (35).

**Rapid Amplification of cDNA Ends—Polymerase Chain Reaction—**RACE-PCR (36) was performed with the SMART 2nd-Choice 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 of mRNA from anaerobically adapted cells. The reverse transcription reaction was carried out with a Moloney murine leukemia virus 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, Universal Primer Mix (CLONTECH) and the antisense primer Sc7 were used. The amplification of the 3’-cDNA end was performed with Universal Primer Mix 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 a 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 Superscript II reverse transcriptase (Life Technologies, Inc.) and 200 ng of mRNA as template. PCR was performed using either Sc12 or Sc27 and the SMART 2nd-choice specific adapter primer Universal Primer Mix. Two different blunt end containing末端 leader primers (Novl, Drol, Poul1, HincII, and EcoRV) and by adapter ligation at the ends of the resulting DNA fragments. Both fragments were cloned into the pGem®T-Easy vector (Promega, Madison, WI) and sequenced using primers from the poly linker of the vector.

**Genome Walking with Genomic DNA—**Applying the CLONTECH Genome Walker Kit, genomic libraries from S. obliquus were generated by digestion of different blunted ends using enzymes (Nael, DraI, PvuII, HincII, and EcoRV) 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 and 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.e., Sc10 and 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 dye deoxy chain termination method (39).

**Purification of the Iron Hydrogenase—**40-liter cultures of S. obliquus were grown heterotrophically. After centrifugation (10 min at 5000 × g), the pellet was resuspended in 200 ml of Tris acetate phosphate medium. The cells were anaerobically adapted by flushing the solution with argon for 1 h in the dark. All further purification steps were performed in an anaerobic chamber (Coylab, Ann Arbor, MI). 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 iron hydrogenase of C. reinhardtii (30). Automated Edman degradation of the N-terminal site of the protein was performed with an Applied Biosystem model 477A sequencer with online analyzer 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 Pharmacia Biotech) and hybridized with RNA probes labeled with DIG-dUTP using the in vitro transcription method. A 1.3-kilobase EcoRI cDNA fragment was used to detect transcripts of the hydA gene, whereas 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 Molecular Biochemicals).

**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 data base searches.

**Recombinant Expression in Escherichia coli—**The hydA open reading frame was amplified by PCR using the primer pair Sc29 and Sc30 and flanking Ndel-BamHI sites. The PCR product was cloned into the pGEM®T-Easy vector. After digestion with Ndel-BamHI, the hydA gene was cloned into the corresponding site of the pET7a 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)pLyS8 was transformed with pLF29.2. Expression was in-
the Southern blot experiments are mentioned.

The sequencing strategy; each letted cells were resuspended in lysis buffer (100 mM Tris/HCl, 4 mM EDTA, 16% glycerol, 2% SDS, 2% mercaptoethanol, 0.05% bromophenol blue, 8 mM urea). After heating, the protein extract was separated by 10% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Western blot analyses were performed using antiserum against the iron hydrogenase of *C. reinhardtii* at 1:1000 dilution as described earlier (31).

**RESULTS**

**Induction of Hydrogenase Activity and Purification of the Iron Hydrogenase Protein**—Anaerobic adaptation is the most efficient way to induce hydrogenase activity in *S. obliquus*. Bubbling the alga culture in the dark with argon led to a dramatic increase (10-fold) of hydrogenase activity during the first 2 h. We purified the enzyme of *S. obliquus* to homogeneity by successive column chromatography. Because 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 (AGPTAECDRFPAPAPKAITHWWQ) is, except for two amino acids, identical to the amino acid sequence deduced from the DNA data (AGPTAECDCPFPAPKAPHHWWQ). 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 iron 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 Iron 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 μmol O₂/mg Chl × 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 PSI was blocked by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. In contrast, addition of 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone 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 2,6-dichlorophenoldioxidin. In this reaction, 2,6-dichlorophenoldioxidin 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-disalicylidinepropanediamine (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, and 350 units/mg protein with *S. obliquus*, *C. reinhardtii*, and spinach ferredoxin, respectively. No hydrogen production could be measured with other possible electron donors like cytochrome c₅₅₃ and NADPH. In *D. desulfuricans* the iron hydrogenase was reported to catalyze both hydrogen production and uptake with low potential multiheme cytochromes like cytochrome c₃ (17).

**Molecular Characterization of hydA Encoding an Iron Hydrogenase**—To isolate the gene encoding a iron hydrogenase in *S. obliquus*, we isolated poly(A)⁺ RNA from cell cultures after 1 h of anaerobic adaptation. Isolated RNA was transcribed and...
amplified by reverse transcription-PCR using oligonucleotides derived from conserved regions within the C. reinhardtii hydA gene. 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'-untranslated region 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 poly(A) sequence (45).

In an effort to examine the exon-intron structure and the promoter region of the hydA gene, about 5 kilobases of the genomic DNA from S. obliquus were sequenced. The gene comprises five introns with a total size of 1310 bp (Fig. 1) whose 5' and 3' ends 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 11. As described for other green algae genes, a highly conserved TATA box element upstream of the transcription starting point 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

2 T. Happe, unpublished results.

Fig. 3. Comparison of the deduced HydA protein sequence with other iron hydrogenases. The protein alignment was done by using the Vector NTI program (InforMax). White letters with black background indicate amino acids identical to the HydA protein. Black letters with gray background indicate conserved changes of the amino acids. S. o., S. obliquus (this work); M. e., Megasphaera elsdenii (13); D. d., D. desulfuricans (17); T. v., Trichomonas vaginalis (10); C. p., C. pasteurianum (9); T. m., T. maritima (12); N. o., N. ovalis (11).
involved in gene expression.

**HydA Is a Novel Type of Iron 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 a Val-Xaa-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 iron hydrogenases as shown in Fig. 3. The homology in the C-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* iron 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 FTSC-CPGW (343–350), TGTVMEAALR (474–483), and MACPG-GCXGGGQQP (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 iron hydrogenases. The protein sequences of the other enzymes comprise at least two [4Fe-4S] ferredoxin-like domains (called F cluster) that are necessary for the electron transport from the electron donor to the catalytic center. The iron 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 iron hydrogenase, the hydA clone was expressed in the heterologous system *E. coli*. One band of recombinant HydA protein was observed on SDS-PAGE at ~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 cross-react with other iron hydrogenases but not with nickel-iron 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 iron 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 iron hydrogenases.

**Rapid Induction of hydA mRNA during Anaerobic Adaptation**—The regulation of the hydA gene expression was examined by Northern blot analysis and reverse transcription-PCR. Aerobically grown cells of *S. obliquus* did not show a hydrogenase activity (Fig. 5A). Total RNA and also mRNA were isolated from cells that were induced by argon bubbling for 0, 1, and 4 h. Northern blot analysis and reverse transcription-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 and C). The full length of the hydA cDNA clone was confirmed by the transcript signal (2.6 kilobases) on the Northern blot.

**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 *S. obliquus* that we present in this work belong to the class of iron hydrogenases.

Iron 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 (Met₁–Ala₁₆) of the amino acid sequence derived from the cDNA sequence are supposed to function as a short transit peptide that routes the nuclear encoded protein to the chloroplast. Several positively charged amino acids that describe a typical feature for algal transit peptides (47) are found in HydA. The three terminal residues of the signal sequence, Val-Xaa-Ala, constitute the consensus sequence for stromal peptidases (48).

The hydrogenase of *S. obliquus* represents a novel type of iron hydrogenase. The monomeric enzyme of 448 amino acids and a calculated molecular mass of 44.5 kDa for the processed protein is the smallest iron hydrogenase isolated so far. The protein sequence consists of an unusual N-terminal domain and a large C-terminal domain containing the catalytic site. The structurally important C terminus of the *S. obliquus* HydA sequence is very similar to that of other iron hydrogenases. Four cysteine residues at positions 120, 175, 336, and 340 coordinate the special [6Fe] cluster (H cluster) of the active site (Fig. 6). A number of additional residues define the environment of the catalytic center. Peters et al. (16) postulated 12 amino acids in *C. pasteurianum* to form a hydrophobic pocket around the cofactor. Ten residues are strictly conserved, while two amino acids vary within the iron hydrogenase family (Ser₁²⁻ and Ile²⁰⁸ in *C. pasteurianum*, Ala¹¹⁹ and Thr¹³⁵ in *T. vaginalis*, and Ala²⁴⁴ and Thr²⁶³ in *S. obliquus*). A small insertion of 16 amino acids is noted in *S. obliquus*, but this addition occurs in an external loop of the protein and probably has no special function (Fig. 6).

Until now, all iron hydrogenases possessed a ferredoxin-like domain in the N terminus coordinating two [4Fe4S] clusters (FS4A and FS4B; Refs. 10 and 13 and 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 *S. obliquus* protein is strongly reduced compared with other iron 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 *S. obliquus* (32), we could neither detect the postulated two subunits of a potential nickel-iron hydrogenase nor find a nickel dependence related to the hydrogenase activity. Francis reported about two forms of hydrogenases in *S. obliquus* (52), but although we used the same algal 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 *S. obliquus* (25, 26), we measured PSII independent H2 production that is not influenced by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The electrons required for H2 evolution come from redox equivalents of the fermentative metabolism and are supplied into the photo-
synthetic electron transport chain via the plastochinone pool.

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

The analysis of the three-dimensional structure of the iron 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 iron hydrogenase from *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* iron 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*; Ref. 58) was published. The structure revealed negatively charged amino acids like aspartate and glutamate near the [2Fe-2S] cluster. The [Fe-S] center 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). Reverse transcription-PCR and Northern blot analyses with mRNA of aerobic and anaerobically adapted cells from *S. obliquus* showed an increased level of hydA transcript after 1 h 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 microaerobic conditions during the RNA isolation procedure. Alternatively, a low level of hydA transcript might be constitu-
tively present in the cell and is only drastically increased after anaerobic adaptation.

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