HoxE—a subunit specific for the pentameric bidirectional hydrogenase complex (HoxEFUYH) of cyanobacteria

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Abstract

NAD(P) + -reducing hydrogenases have been described to be composed of a diaphorase (HoxFU) and a hydrogenase (HoxYH) moiety. This study presents for the first time experimental evidence that in cyanobacteria, a fifth subunit, HoxE, is part of this bidirectional hydrogenase. HoxE exhibits sequence identities to NuoE of respiratory complex I of Escherichia coli. The subunit composition of the cyanobacterial bidirectional hydrogenase has been investigated. The oxygen labile enzyme complex was purified to close homogeneity under anaerobic conditions from Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 6301. The 647-fold and 1290-fold enriched purified enzyme has a specific activity of 46 A mol H2 evolved (min mg protein)−1 and 15 A mol H2 evolved (min mg protein)−1, respectively. H2-evolution of the purified enzyme of S. sp. PCC 6803 is highest at 60 °C and pH 6.3. Immunoblot experiments, using a polyclonal anti-HoxE antibody, demonstrate that HoxE co-purifies with the hydrogenase activity in S. sp. PCC 6301. SDS-PAGE gels of the purified enzymes revealed six proteins, which were partially sequenced and identified, besides one nonhydrogenase component, as HoxF, HoxU, HoxY, HoxH and, remarkably, HoxE. The molecular weight of the native protein (375 kDa) indicates a dimeric assembly of the enzyme complex, Hox(EFUYH)2. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cyanobacteria; Bidirectional hydrogenase; Hydrogen metabolism; HoxE; NADH dehydrogenase

1. Introduction

In cyanobacteria, up to two different hydrogenases are expressed [1–3]. One is the dimeric membrane-bound uptake hydrogenase, which is mainly confined to heterocysts and functions in reutilising the H2-gas produced by the nitrogenase. Substantial evidence for this function has recently been obtained by mutational analysis [4]. The second enzyme, the so-called bidirectional hydrogenase, catalyses in vitro H2-formation as well as H2-uptake in the presence of artificial electron donors or acceptors, respectively. It is widely, though not ubiquitously, distributed amongst cyanobacteria in both heterocysts and vegetative cells [5–8].

The molecular biological characterisation of this enzyme provided new insights on its possible function. Five genes have been described to form the enzyme complex [9–13]. Four of them are homologous to the genes encoding the heterotetrameric NAD + -reducing hydrogenase of Ralstonia eutropha [14]: hoxYH coding for the hydrogenase dimer of the enzyme and hoxFU for its diaphorase part. Extracts of Synechococcus sp. PCC 6301, indeed, performed H2-dependent NAD(P) + -reduction as well as NAD(P)H-dependent H2-evolution [15]. However, in contrast to the soluble enzyme of R. eutropha, the gene cluster of the bidirectional hydrogenase contains a further ORF (hoxE), which might encode a third diaphorase subunit [11,12]. The diaphorase subunits Hox(E)FU have been discussed to serve as the NADH oxidising part of complex I either active in respiration or cyclic electron transport around photosystem I [6,11,16,17], mainly because of significant sequence similarities to three subunits of the mitochondrial

Abbreviations: MV, methyl viologen; APPS, advanced protein purification system
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complex I (NADH:Q oxidoreductase). It has been also suggested that the hydrogenase is linked to photosystem I working as electron valve for an excess of reductans [17]. According to the first model, HoxE is the homologue to NuoE of Escherichia coli, one of the three subunits constituting the hydrophilic part of complex I. However, it has not yet been demonstrated that HoxE is part of the hydrogenase complex.

Although information about the physiology [1,5] and the molecular biology of the bidirectional hydrogenase is available [2,7,17,18], the enzyme is only poorly characterised biochemically. Inactivation of the bidirectional hydrogenase in vitro by oxygen complicates purification and further investigation. The enzyme has been purified to near homogeneity from Synechocystis sp. PCC 6301 [9]. By sequencing peptides derived of a LysC digest of the purified enzyme, only the large subunits of both moieties, HoxH and HoxF were identified. Additional protein bands, which might represent by size HoxE, HoxY and HoxU, were present in the purified enzyme preparations. The spectroscopic characterisation of the partially purified enzyme from Anabaena variabilis [19] corroborated the existence of an FMN molecule and several FeS-clusters, in line with sequence information obtained for HoxF [9].

This study aims at investigating the subunit composition of the bidirectional hydrogenase in view of HoxE as the putative fifth subunit of the complex. This was performed on the one hand by an immunological approach using an antibody specifically directed against HoxE from Synechocystis sp. PCC 6301 [13]. On the other hand, the hydrogenase of the totally sequenced Synechocystis sp. PCC 6803 [13] was purified, and all subunits of the enzyme were N-terminally sequenced.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Synechocystis sp. PCC 6803 was grown in BG11 medium with 17.7 mM nitrate [20] at 34 °C under continuous illumination with an incident light intensity of 250 μE m−2 s−1 and by sparking with air enriched by 1% CO2. Nitrogen limitation was achieved in BG11 medium containing only 1 mM nitrate. Synechococcus sp. PCC 6301 (=Anacystis nidulans SAUG 1402-1) was cultivated as described elsewhere [9].

E. coli M15 (NalR, StrR, RifR, Lac−, Ara−, Gal−, Mfd−, RecA+, UvrA+, Lon+) was used for HoxE overexpression and grown in standard DYT (double yeast tryptone) medium with 100 μg ampicilllin ml−1 and 25 μg kanamycin ml−1.

2.2. Measurement of hydrogen evolution

Hydrogenase activity of Synechocystis sp. PCC 6803 was determined in vitro with reduced MV using a GC (Hewlett Packard 5890 A Series II, column: molecular Sieve 5 Å, Mesh 60/80). The assay, containing in a final volume of 2 ml: PIPES pH 6.8 (20 mM), MgCl2 (10 mM), Na2S2O4 (5 mM), MV (2.5 mM), was incubated anaerobically at 34 °C for 10 min. The H2-evolution of Synechocystis sp. PCC 6803 was measured as described [9].

2.3. Purification of the bidirectional hydrogenase

Frozen Synechocystis sp. PCC 6803 cells were suspended in 100 ml phosphate buffer (20 mM, pH 6.8) containing 100 mM Na2S2O4. All subsequent steps were carried out at room temperature anaerobically by flushing all solutions and preparational devices with argon. The cell suspension was passed twice at 20,000 psi through a French Press 40K cell (Amicon, USA) and centrifuged (20 min, 8000 × g) to remove cell debris resulting in the crude extract. All subsequent steps were performed in anaerobic chamber (Coylab, USA) at room temperature. Hydrogenase was first enriched by (NH4)2SO4 precipitation. The fraction precipitating between 30% and 50% was centrifuged (12 min, 20,000 × g), dissolved in 80 ml buffer A (20 mM potassium phosphate, pH 6.8) and dialysed overnight against the same buffer. The solution was passed through a POROS 50 HQ anion exchange column (1.6 × 10 cm; PerSeptive Biosystems, USA). Bound hydrogenase was eluted by a linear NaCl gradient (0–500 mM, 20 column volumes) in buffer A with a flow rate of 12 ml min−1. Fractions showing activities in the Na2S2O4/MV dependent H2-evolution assays were combined, solved in buffer B (10 mM potassium phosphate, pH 6.8; 1 M (NH4)2SO4) and loaded onto a pre-equilibrated hydrophobic interaction PORUS PE column (1.6 × 6.5 cm, PerSeptive Biosystems). The column was washed with 5 column volumes buffer B and hydrogenase was eluted with a linear (NH4)2SO4 gradient (1.0–0.0 M, 20 column volumes). Active fractions were concentrated to 200 μl with Centricron 30K (Amicon), loaded on a Sephacryl S-300 HR column (1.6 × 40 cm, Pharmacia, Sweden) followed by elution with phosphate buffer (20 mM, pH 7.5) and 150 mM NaCl at a flow rate of 30 ml h−1. The fraction with the highest activity eluted after about 44 ml. Protein was quantified by the Bradford method [21] with BSA as standard. The enzyme was stable for about 3 days at 4 °C and for several weeks at −80 °C, if kept under strict anaerobic conditions. The bidirectional hydrogenase of Synechocystis sp. PCC 6301 was purified as described [9]. Protein content of the Synechocystis sp. PCC 6301 enzyme was determined by the Micro BCA Protein assay (Pierce) with exception of the last purification step in which the protein content was calculated from Coomassie Brilliant Blue stained SDS-PAGE with respect to a BSA standard.

2.4. Polyacrylamide gel electrophoresis

SDS-PAGE was performed with the conventional buffer system [22]. For Synechocystis sp. PCC 6803 proteins, 10–15%
linear gradient gels (1.5 mm) were used. S. sp. PCC 6301 proteins and E. coli extracts with overexpressed HoxE were separated by nongradient SDS-PAGE (12% and 15% gels, respectively) as described [9].

2.5. Amino acid sequence analysis

The subunits of the hydrogenase from S. sp. PCC 6803 were separated by SDS-PAGE and transferred to PVDF membranes. The excised protein bands were sequenced using an Applied Biosystems model 477A amino acid sequencer with an on-line analyser model 120A according to the instructions of the manufacturer.

2.6. Overexpression of HoxE and antibody generation

HoxE of S. sp. PCC 6301 was fully amplified out of pBgX7 [18] by PCR [Pfu-Polymerase (Promega); 3 min 95 °C; 31 cycles: 1 min at 95 °C, 30 s at 61 °C (first cycle) or 58 °C (all other cycles), 3 min at 72 °C; final elongation at 72 °C for 5 min] using the sequence-specific primers E60NI GCTGAGTCAGATGGCTACTTCTGGAAAC and E60BII CCCAAATTTCTCCAATAGATC-TGTGCCTCTCCTGTC, which were slightly modified (basepairs marked in bold) from hoxE [genebank Y13471] to generate restriction sites (marked in italics) for further cloning.

After cutting with NcoI/BglII, the PCR product was cloned into the expression vector pQE60 (C-terminal His-tag; Qiagen) and the identity of the insert was checked by sequencing. HoxE was overexpressed in E. coli strain M15 and purified from IPTG-induced cultures under denaturing conditions using Ni-NTA slurry (1/1, by vol., Qiagen) following the protocols of the manufacturer (Qiagen). The size of the purified His-tagged protein was determined on a 15% SDS-PAGE gel to be 21 kDa and was thus slightly larger than HoxE (18 kDa) itself. The eluted His-tagged HoxE was desalted by dialysis three times against PBS (3 h each) and was, after removing debris by centrifugation, concentrated twice using Centricon-3 units (Amicon) to a final protein content of 1–2 mg ml⁻¹. This fraction served to generate the polyclonal antibody from rabbit (performed by Biogenes, Berlin). Specificity of the antibody preparation was increased by standard affinity purification using the antigen HoxE blotted on PVDF-membrane (Biorad). The serum was incubated with the membrane-bound antigen and the antibody eluted twice from the membrane with 5 mM glycine/0.5 mM NaCl-buffer (1st elution: pH 2.8; 2nd elution: pH 2.0). The eluates were neutralised with Tris/HCl pH 8.5, stabilised with BSA and stored at 4 °C. Serum incubation and elution of the purified antibody were repeated eight times. The fractions, pooled according to their original pH, were concentrated to 1/10th volume using Centricon-3 units (Amicon). After affinity purification, the background in Western blots was reduced by a factor of at least five.

2.7. Western blotting, staining procedure and quantification of immunosignals

The antigen was transferred with a semi dry blot device (Schleicher-Schuell) in 1.5–2 h from SDS-PAGE gels onto Hybond-C nitrocellulose membranes (Amersham) using the RotiBlot buffer system (Roth). Blocking, application of the affinity-purified Anti-HoxE and the alkaline phosphatase conjugated secondary antibody (anti rabbit IgG f(ab')₂; Bioropa) was performed with standard techniques. The membrane was stained with NBT/X-phosphate (Roche) and the signal strength was quantified using the NIH Image software.

3. Results

3.1. Purification of the bidirectional hydrogenase complex from the two unicellular, non-N₂-fixing cyanobacteria S. sp. PCC 6301 and S. sp. PCC 6803

In the past, cyanobacterial hydrogenase was purified with lower specific activity [15]. The purification is hampered by low enzyme activity of the crude extracts, instability of the isolated protein and contamination of the enzyme with phycobiliproteins [23]. The present study aimed at purifying the bidirectional hydrogenase from two cyanobacterial species to determine the subunit composition of the enzyme complex. Both procedures are based on the increased stability of the enzyme in response to lowered oxygen tension and the presence of reducing agents. The procedures involved cell disruption using the French Press and precipitation with ammonium sulfate, followed by three liquid column chromatographies. Details of the purification steps are described in Materials and methods. The increase in the specific activities are listed in Table 1.

In the case of S. sp. PCC 6301, the final purification step using an advanced protein purification system (APPS; Millipore) was most effective. This column chromatography led to an additional increase of hydrogenase activity by a factor of about 50. The specific activity of the 1290-fold enriched highly purified hydrogenase enzyme was 15 μmol H₂ evolved min⁻¹ mg protein⁻¹ (Table 1) and thus 3.5 times higher than previously reported [9]. The low yield of 2.6% determined for the highly purified hydrogenase fraction results from enzyme instability and particular high loss of activity during DEAE column separation, the latter of which had to be accepted to optimise separation of hydrogenase enzyme from contaminant pigments.

Bidirectional hydrogenase of the cyanobacterial model organism S. sp. PCC 6803 was purified in a modified way. The enzyme was isolated from cells grown under nitrate limitation, which increased hydrogenase activity in the crude extract and strongly reduced phycobiliprotein content (data not shown). Using a gel filtration chromatography (Sephacryl S-300HR column) in the last purification step, a
<table>
<thead>
<tr>
<th>Sample</th>
<th>Total activity [μmol H₂ × min⁻¹]</th>
<th>Total protein [mg]</th>
<th>Yield [%]</th>
<th>Specific activity [μmol H₂ × min⁻¹ × mg prot⁻¹]</th>
<th>Purification factor [-fold]</th>
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<td>45.8</td>
<td>647</td>
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Hydrogen evolution was measured with reduced MV. All activities refer to pooled fractions. For details about different steps, see Materials and methods. Lanes marked in white refer to Synecocystis sp. PCC 6803, lanes marked in grey refer to Synecococcus sp. PCC 6301.
molecular weight of 375 kDa was estimated, referred to molecular standards. The 647-fold enriched hydrogenase of *S. sp. PCC 6803* had a specific activity of about 46 μmol H₂ evolved min⁻¹ mg protein⁻¹. Moreover, a yield of nearly 10% was obtained during this purification procedure and 0.023% of the total protein in the crude extract were isolated as hydrogenase enzyme during the last separation step. This was higher than the 0.002% achieved for *S. sp. PCC 6301*. This difference may be due to the fact that *S. sp. PCC 6803* was cultivated under nitrogen-deprived conditions and that during the purification of the hydrogenase from *S. sp. PCC 6803*, all manipulations were performed in an anaerobic chamber. Another purified protein sample, which was used for further biochemical characterisation, had even a higher specific activity of 67 μmol H₂ evolved min⁻¹ mg protein⁻¹.

### 3.2. Presence of HoxE in purified hydrogenase fractions from *S. sp. PCC 6301* as detected by Western blot experiments

Western blot experiments using affinity-purified polyclonal antibodies directed against HoxE from *S. sp. PCC 6301* should show whether HoxE is part of the hydrogenase complex in this cyanobacterium. Hydrogenase activity, as determined by the MV/Na₂S₂O₄-dependent H₂-evolution, eluted from the first liquid chromatography (DEAE column) in a broad peak within several fractions under the conditions employed. Western blot analyses showed that the amount of HoxE strongly correlated with the hydrogenase activity (Fig. 1). This result indicates that HoxE elutes together with the hydrogenase complex. Furthermore, the amount of HoxE was quantified during the complete purification procedure and was plotted against the hydrogenase activity determined for these fractions (Fig. 2). The perfectly linear correlation indicates that HoxE co-purifies with the active bidirectional hydrogenase complex up to the last separation step.

### 3.3. Molecular composition of the purified bidirectional hydrogenase complex as determined by protein sequencing

The molecular composition of the purified bidirectional hydrogenase complex from *S. sp. PCC 6301* and *S. sp. PCC 6803* was analysed by SDS-PAGE. In the case of *S. sp. PCC 6301*, six predominant protein bands with estimated molecular weights of 60, 47, 39, 30, 25, and 21 kDa, respectively, were separated on SDS-PAGE (Fig. 3A). In a previous investigation [9], internal sequences of the upper three protein bands were obtained, identifying the 60 and 47 kDa proteins as HoxF and HoxH, respectively. The 39 kDa protein shares sequence similarity to a putative xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (slr0453) from *S. sp. PCC 6803*. The corresponding gene is not

![Fig. 1. Presence of HoxE in partially purified bidirectional hydrogenase fractions. Different DEAE fractions of partially purified bidirectional hydrogenase of *S. sp. PCC 6301* were analysed for the presence of HoxE by Western blots (bottom lane) using a polyclonal anti-HoxE antibody: HoxE co-elutes with total bidirectional hydrogenase activity measured as MV/Na₂S₂O₄ dependent H₂-evolution (n=1; top lane).](image-url)
Fig. 2. Co-purification of HoxE and bidirectional hydrogenase. The presence of HoxE in partially purified bidirectional hydrogenase complex of \textit{S. sp. PCC 6301} was investigated in Western blots using a polyclonal anti-HoxE antibody. The amount of HoxE in these fractions as quantified by determining the signal strength in the Western blot ($n=3$) correlated with the specific activity of bidirectional hydrogenase measured as MV/Na$_2$S$_2$O$_4$-dependent H$_2$ evolution. (▲ DEAE; ■ Octyl-Sepharose; ▲ APPS=DEAE-5PW-column; pH 7.2).

Fig. 3. Electrophoresis of purified hydrogenase. SDS-PAGE of purified hydrogenase from (A) \textit{S. sp. PCC 6301} and (B) \textit{S. sp. PCC 6803}. Hydrogenase fractions were separated on (A) a 12\% polyacrylamide gel, (B) a 10–15\% linear polyacrylamide gradient gel. Proteins were (A) silver-stained, (B) stained with Coomassie Brilliant Blue. Additional protein bands correspond to (A) xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (39 kDa) and (B) glutamine synthetase (57 kDa). Lane M: protein standards with (A) 66, 44, 31 and 21 kDa and (B) 67, 42, 25, 20 and 16 kDa; molecular weight of protein bands given in kDa.
located within the hydrogenase locus. This protein elutes differently from the remaining five proteins when the hydrogenase containing fractions were subjected to an additional separation step via APPS at pH 8.5 (data not shown). This indicates that the 39 kDa protein is not part of the hydrogenase complex. However, further purification of hydrogenase was accompanied by loss of activity, probably caused by a further increase of oxygen sensitivity of the purified enzyme. The 21 kDa band was identified as HoxE by the Western blot experiments just described. As concluded by their sizes, the remaining two proteins migrating at 30 and 25 kDa might represent the diaphorase subunit HoxU and the hydrogenase subunit HoxY, respectively. However, sequences for the three smaller proteins were not obtained because protein concentrations were too low.

Similarly, six bands were obtained for the purified hydrogenase complex of S. sp. PCC 6803 (Fig. 3B). The apparent molecular weights were 62, 57, 51, 26, 23, and 18 kDa, respectively. All six protein bands were identified upon sequencing their N-terminal parts by Edman degradation. The proteins with the deduced molecular weights of 62, 51, 26 and 23 kDa are the four subunits HoxF, HoxH, HoxU, and HoxY (Table 2), previously described as subunits of the bidirectional hydrogenase complex from cyanobacteria [9,11]. The N-terminal sequence obtained for the 57 kDa band corresponded to the protein glutamine synthetase, the level of which is increased in cells grown under nitrogen-starved conditions [24]. Remarkably, the 18 kDa band represented HoxE (Table 2). Thus, in agreement with the results obtained for S. sp. PCC 6301, the bidirectional hydrogenase complex purified from S. sp. PCC 6803 is also composed of five subunits.

### Table 2

<table>
<thead>
<tr>
<th>Subunits</th>
<th>Length (aa)</th>
<th>Deduced from DNA sequence</th>
<th>1st MW (kDa)</th>
<th>Derived from purified hydrogenase</th>
<th>2nd MW (kDa)</th>
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<td>62.5</td>
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<tr>
<td>HoxH</td>
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<td>MSKTVIPDVT-</td>
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<td>HoxU</td>
<td>238</td>
<td>MSSLVTIDDK-</td>
<td>26.2</td>
<td>VFTLTDIHK-</td>
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<td>HoxY</td>
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<td>22.0</td>
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<td>23</td>
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<tr>
<td>HoxE</td>
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<td>MTVATDRQTVLP-</td>
<td>18.8</td>
<td>MTVATDRQTVLP-</td>
<td>18</td>
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</table>

The length of the different subunits in amino acids (aa) is derived from the DNA sequences [13]. The molecular weight of the subunits was deduced from the DNA sequences (1st MW) and determined experimentally by SDS-PAGE (2nd MW).

### 3.4. Biochemical characterisation of the purified hydrogenase complex from S. sp. PCC 6803

The optimal temperature for H2-evolution from reduced methyl viologen (MV), catalysed by the purified hydrogenase complex from S. sp. PCC 6803, was 60 °C (data not shown). The specific activity of the enzyme at this temperature was four times higher than H2 evolution under the standard assay condition of 34 °C. Significant inactivation of the enzyme was only detected above 75 °C. The enzyme shows maximum activity under slightly acidic conditions (pH 6.3; data not shown). H2-evolution was determined with various physiological electron carriers, with highest hydrogenase activity (67 μmol H2 evolved min⁻¹ mg protein⁻¹) obtained in the presence of reduced MV (Table 3). The apparent Kₘ value for MV in the reaction was 89 μM, as deduced from Lineweaver–Burk plots (data not shown). Potential natural electron donors are NADH and NADPH. With both reductants, a significant H2-production was detected, even though the rates were strongly reduced. The activity with NADPH was only slightly higher than that observed for the artificial donor Na2S2O4. Reduced ferredoxins and flavins (Table 3) as electron donors showed no H2 evolution.

### 4. Discussion

The genome sequencing project of S. sp. PCC 6803 [13] unambiguously revealed that the bidirectional hydrogenase is the only hydrogenase present in this unicellular non-nitrogen fixing cyanobacterium. In crude extracts, the specific activities of the bidirectional hydrogenase of cyanobacteria are low compared to the activities of the homologous NAD⁺-reducing hydrogenase of R. eutropha [9,15,25]. In the present study, the S. sp. PCC 6803 enzyme was purified 647-fold to close homogeneity exhibiting a specific activity of 46 μmol H2 evolved min⁻¹ mg protein⁻¹ using reduced MV (MV_red) as electron donor. For the purified soluble hydrogenase of R. eutropha, an MV_red-dependent specific activity of 48 μmol H2 evolved min⁻¹ mg protein⁻¹ has been reported [25]. The now achieved specific activity of the purified S. sp. PCC 6803 enzyme was higher compared to previous cyanobacterial hydrogenase preparations ranging from 1 to 10 μmol H2 evolved min⁻¹ mg protein⁻¹ using reduced MV (MV_red).
evolved min$^{-1}$ mg protein$^{-1}$ [9,26–28], presumably because the culture was grown under nitrogen deprivation and the purification procedure was performed in an anaerobic chamber. The low H$_2$-evolution activity with the physiological electron donor NAD(P)H (2.1–4.2% activity of the maximal activity obtained with MV$_{red}$) was also reported for the enzymes from *Rhodococcus opacus* [29] and *R. eutropha* [25] and is explained by the unfavourable thermodynamic conditions. In view of the relative specific activities determined for the purified enzyme from *S. sp. PCC 6803*, NAD(H) rather than NADP(H) seems to be at least in vitro the more favoured electron mediator for the reaction. This is in agreement with results previously obtained for crude extracts of *S. sp. PCC 6301* [15], indicating that the bidirectional hydrogenase in cyanobacteria is an NAD(P)$^+$-reducing enzyme.

The molecular weights of bidirectional hydrogenases in various microorganisms are in the range of 42 to 230 kDa [1,30]. Gel filtration chromatography indicates that the hydrogenase complex from *S. sp. PCC 6803* is larger and was estimated to be about 375 kDa, which indicates a dimeric assembly of the native protein with the five subunits HoxEFUYH constituting a pentamer of around 180 kDa. Up till now, it has only been suggested that HoxE is a subunit of the NAD(P)$^+$-dependent bidirectional hydrogenase in the unicellular cyanobacteria studied so far [12,31], but even circumstantial evidence was missing. The present study provides clear-cut experimental data for this assumption, i.e. HoxE was found to be part of the hydrogenase complex in both *S. sp. PCC 6301* and *S. sp. PCC 6803* by immunological detection as well as protein purification and sequencing. Previous investigations demonstrated already that hoxE is located upstream of and is cotranscribed with hoxF in *S. sp. PCC 6301* [7]. However, the level of transcription for both genes was found to be considerably lower compared to the third diaphorase gene hoxU in the closely related *S. sp. PCC 7942* [18]. HoxE is the least conserved of all structural genes of the hydrogenase complex and could not be detected by Southern hybridisation in the filamentous strain *A. variabilis* [6]. Nevertheless, it was identified in the course of the genome sequencing project of the genetically closely related *Anabaena* sp. PCC 7120 [32,33], suggesting that the bidirectional hydrogenase *may* consist of five subunits also in filamentous cyanobacteria. Different open reading frames that are interspersed in the hox gene clusters of several cyanobacterial strains [9,13,32,33] are unlikely to be components of the bidirectional hydrogenase for several reasons. None of the proteins with significant sequence similarities to the putative products of these ORFs is, as far as known, related to hydrogen metabolism. Furthermore, number, order and identity of the open reading frames are not alike in the different strains. The preparations of the bidirectional hydrogenase of the two unicellular strains described here contained both one contaminating protein, in *S. sp. PCC 6803* glutamine synthetase and in *S. sp. PCC 6301* xylulose-5-phosphate/fructose-6-phosphate phosphoketolase. These enzymes are not known to be related to hydrogen metabolism. The contaminants were neither the same nor encoded in the hox cluster of the two cyanobacteria.

The pentameric structure of the cyanobacterial hydrogenases investigated in this study stands in contrast to the NAD$^+$-reducing hydrogenases described for organisms other than cyanobacteria, e.g. *R. eutropha* and *R. opacus*, where the native hydrogenase complex is built up by only four subunits [34,35]. In these organisms, the hydrogenase is a soluble enzyme, whereas it has been described to be membrane associated in *S. sp. PCC 6803*, *A. variabilis* and *S. sp. PCC 6301* [17,28,36]. In this context, bidirectional hydrogenase has been suggested to be linked in cyanobacteria to NADH:Q oxidoreductase (complex I) and/or to electron transport from photosystem I [11,16,17]. HoxE may be involved as a bridging subunit in membrane attachment. In addition, a functional role in electron transport directed to membrane components could be considered, as sequence motifs for binding of an additional [2Fe–2S] cluster are present in the gene which are missing in the soluble tetrameric enzyme from *R. eutropha* [9]. The function of HoxE in the complex has to be addressed in future work.

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**References**
