Requirements for Construction of a Functional Hybrid Complex of Photosystem I and [NiFe]-Hydrogenase

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The development of cellular systems in which the enzyme hydrogenase is efficiently coupled to the oxygenic photosynthesis apparatus represents an attractive avenue to produce H2 sustainably from light and water. Here we describe the molecular design of the individual components required for the direct coupling of the O2-tolerant membrane-bound hydrogenase (MBH) from Ralstonia eutropha H16 to the acceptor site of photosystem I (PS I) from Synechocystis sp. PCC 6803. By genetic engineering, the peripheral subunit PsaE of PS I was fused to the MBH, and the resulting hybrid protein was purified from R. eutropha to apparent homogeneity via two independent affinity chromatographical steps. The catalytically active MBH-PsaE (MBHPsaE) hybrid protein could be isolated only from the cytoplasmic fraction. This was surprising, since the MBH is a substrate of the twin-arginine translocation system and was expected to reside in the periplasm. We conclude that the attachment of the additional PsaE domain to the small, electron-transferring subunit of the MBH completely abolished the export competence of the protein. Activity measurements revealed that the H2 production capacity of the purified MBHPsaE fusion protein was very similar to that of wild-type MBH. In order to analyze the specific interaction of MBHPsaE with PS I, His-tagged PS I lacking the PsaE subunit was purified via Ni-nitrilotriacetic acid affinity and subsequent hydrophobic interaction chromatography. Formation of PS I-hydrogenase supercomplexes was demonstrated by blue native gel electrophoresis. The results indicate a vital prerequisite for the quantitative analysis of the MBHPsaE-PS I complex formation and its light-driven H2 production capacity by means of spectroelectrochemistry.

Molecular hydrogen (H2) is often discussed as an alternative source of energy (13, 22, 26, 41). It is a highly energetic, renewable, and zero-carbon dioxide emission fuel; however, it is produced mainly from fossil resources. One intriguing possibility for sustainable H2 production is the development of cellular systems in which the light-driven oxygenic photosynthesis is efficiently coupled to hydrogen production by hydrogenase (1, 21, 36).

During the process of oxygenic photosynthesis, photosystem II (PS II), a thylakoid membrane (TM)-embedded multiprotein complex, utilizes solar energy to oxidize water into dioxygen (O2), protons, and electrons. The electrons released by PS II are further conducted through an electron transport chain located at the acceptor site of PS I. The acceptor site is composed of plastoquinones, the cytochrome b6f complex, and the two additional cofactor-free extrinsic subunits PSaD and PsaE. In the final step, the electrons are transferred from F430 to the ferredoxin (PetF), which has a midpoint potential of ~412 mV (see Fig. 1B) (8, 9).

Hydrogenases of the NiFe and FeFe types catalyze the reversible cleavage of H2 into protons and electrons (18, 63). For most hydrogenases, this reaction is highly sensitive to O2 and leads to the reversible or even irreversible inactivation of the enzyme (49, 66, 67). A prominent exception is the oxygen-tolerant membrane-bound [NiFe]-hydrogenase (MBH) from Ralstonia eutropha H16, which catalyzes H2 conversion in the presence of O2 (42, 65). The MBH consists of large subunit HoxG (67 kDa), harboring the NiFe active site, and small subunit HoxK (35 kDa), bearing three FeS clusters (Fig. 1) (32). Both cofactor-containing subunits are completely assembled within the cytoplasm and become subsequently translocated through the cytoplasmic membrane by the twin-arginine translocation (Tat) system. This transport is guided by a specific Tat signal peptide that is located at the N terminus of small subunit HoxK (53). The MBH is then connected to the membrane via the hydrophobic C-terminal “anchor” domain of HoxK, which provides the electronic connection to the diheme cytochrome b, HoxZ (5, 57). All structural, accessory, and regulatory genes for the synthesis of active MBH are arranged in a large, megaplasmid-borne operon (7, 11, 14, 29, 33, 38, 58).

The concept of light-driven hydrogen production has been investigated in numerous studies (for reviews, see references 3, 21, and 23), including one involving direct electron transfer from PS I to the free form of hydrogenase in vitro (45). In a preliminary attempt, the MBH from R. eutropha was recently directly fused to PsaE (creating MBHPsaE) (28). The fusion protein was partially purified and subjected to in vitro recon-
stitution with PS I lacking PsaE (PS I<sub>PsaE<sub><sup>−</sup></sub></sub>) (54) for light-driven hydrogen production. This concept was based on the previous observation that PS I lacking the peripheral subunit PsaE is fully reconstituted in vitro simply by the addition of independently purified PsaE protein (12).

In the present communication, we describe a novel purification procedure for <i>R. eutropha</i> MBH<sub>PsaE</sub> that yields homogeneous, functionally active MBH<sub>PsaE</sub>. Additionally, a new method for efficient and fast purification of <i>Synechocystis</i> sp. PCC 6803 (hereafter referred to as <i>Synechocystis</i>) His-tagged PS I was established. Finally, the pure proteins MBH<sub>PsaE</sub> and PS I<sub>PsaE<sub><sup>−</sup></sub></sub> were successfully subjected to in vitro reconstitution.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. Strains carrying the initials HF are derived from <i>Ralstonia eutropha</i> H16. <i>R. eutropha</i> HSF632 is a derivative of the megaplasmid-free strain <i>R. eutropha</i> HF210 carrying plasmid pLO6 that harbors the complete MBH operon from <i>R. eutropha</i> and a single-copy <i>Phi(hok–luc<sub>2</sub>)</i> translational fusion inserted into the chromosome (39).<i>Synechocystis</i> sp. PCC 6803 is a kind gift from A. Wilde (Justus-Liebig University Giessen, Biology/Microbiology and Molecular Biology). The <i>E. coli</i>-free derivative of <i>Synechocystis</i> was a kind gift from H. Matthijs (University of Amsterdam, Faculty of Science/Department of Aquatic Microbiology/Institute for Biodiversity and Ecosystem Dynamics [IBED]).

<i>Escherichia coli</i> JM109 was used as the recipient in standard cloning procedures, and <i>E. coli</i> S17-1 was used for conjugal plasmid transfer between <i>E. coli</i> and <i>Synechocystis</i> (61, 69).

A His<sub>6</sub> tag fusion to the C terminus of the MBH small subunit HoxK was constructed as follows. Using primers 11 and 12 (Table 2) and pCH497 as the template, a 170-bp fragment was amplified by PCR (Venton-Polymerase, New England Biolabs) (47). A 153-bp NdeI-BglII fragment of the PCR product was inserted into the equanimously cut plasmid pCH1352, resulting in pCH1417. Subsequently, a 724-bp Acc65I-SmaI fragment from pCH1417 was cloned into pCH1351 (Acc65I-SmaI), yielding pCH1418. Finally, a 2.18-kbp Scal fragment was transferred to the Pmel site of suicide vector pLO1 (7.32 kbp) to give pCH1419.

For the establishment of a <i>hoxK–PsaE</i> fusion (for the protein HobK–PsaE<sub>−</sub>) on pLO6, a conditionally lethal suicide plasmid was constructed as follows: an 890-bp MscI fragment from pCH1365 (carrying a truncated <i>hoxK</i> gene encoding HoxK<sub>H9262</sub>–Strep-tag II [HoxK<sub>H9262</sub>]) was cloned into the SmaI–Ecl136II sites of pLTMU282 to give pCH1408 (HoxK<sub>H9262</sub>). Using Pf polymerase (Invitrogen) and primers 1 and 2 (Table 2), a psaE-containing fragment (243 bp) was amplified from <i>Synechocystis</i> genomic DNA. The PCR amplification was cut with BglII, and the resulting 231-bp fragment was cloned into BglII-digested pCH408, yielding pCH4109. From pCH4109, a 805-bp SmaI–Acc65I fragment was transferred to the equally cut plasmid pCH1352, resulting in pCH1417. Subsequently, a 2.26-kbp Scal fragment derived from pCH1410 was cloned into the Pmel site of the suicide vector pLO1 to give pCH1411. In the resulting pCH1412, a Scal fragment was reinserted into the BglII-EcoRI sites of pCH1417, resulting in pCH1418. Finally, a 2.18-kbp Scal fragment was transferred to the Pmel site of suicide vector pLO1 (7.32 kbp) to give pCH1419.

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Protein purification. (i) Purification of MBH<sub>PsaE</sub>P<sub>H9004</sub> (for the protein HobK–PsaE<sub>−</sub>) on pLO6, a conditionally lethal suicide plasmid was constructed as follows: an 890-bp MscI fragment from pCH1365 (carrying a truncated <i>hoxK</i> gene encoding HoxK<sub>H9262</sub>–Strep-tag II [HoxK<sub>H9262</sub>]) was cloned into the SmaI–Ecl136II sites of pLTMU282 to give pCH1408 (HoxK<sub>H9262</sub>). Using Pf polymerase (Invitrogen) and primers 1 and 2 (Table 2), a psaE-containing fragment (243 bp) was amplified from <i>Synechocystis</i> genomic DNA. The PCR amplification was cut with BglII, and the resulting 231-bp fragment was cloned into BglII-digested pCH408, yielding pCH4109. From pCH4109, a 805-bp SmaI–Acc65I fragment was transferred to the equally cut plasmid pCH1352, resulting in pCH1417. Subsequently, a 2.26-kbp Scal fragment derived from pCH1410 was cloned into the Pmel site of the suicide vector pLO1 to give pCH1411. In the resulting pCH1412, a Scal fragment was reinserted into the BglII-EcoRI sites of pCH1417, resulting in pCH1418. Finally, a 2.18-kbp Scal fragment was transferred to the Pmel site of suicide vector pLO1 (7.32 kbp) to give pCH1419.

(ii) Purification of MBH<sub>PsaE</sub>P<sub>H9004</sub>–Strep-tagged PsaE. The strains R. <i>eutropha</i> HSF653 and HSF678 were cultivated at 30°C in 12 baffled flasks, each filled with 160 ml FGN medium. Strain R. <i>eutropha</i> HSF771 was grown at 30°C in 4
Synechocystis PCC 6803

Plasmid

LITMUS28
Ap' lacZ' ColE1 ori
New England Biolabs

pBluescript II KS(+) Ap' lacZ' T7 gene 10 promoter, f1 ori
Stratagene Cloning Systems

pDONR221 Clh' Kan' ColE1 ccdB attP1 attL1 attP2 attL2
Invitrogen

pEDY309

Te' RK2 ori Mob '+
Plo1
Km' sacB RP4 oriT ColE1 ori
This work

pLO6
MBH overexpression plasmid (pEDY309 derivative carrying the MBH operon)
This work

pL011
Te' RK2 ori Mob '+' overexpression vector for Strep-tagged fusion proteins in R. eutropha
This work

pCH497
2-kbp BamHI fragment (Klenow fragment treated) containing hoxKG' in pBluescript II SK(+) 7

pCH1351
8.96-kbp PslE-Ecl136II fragment carrying hoxKGMLOQRTV in LITMUS28
This work

pCH1352
Derivative of pCH497 containing the hoxK-Strep-tag II fusion
This work

pCH1353
9.00-kbp PslE-Ecl136II fragment carrying hoxKGMLOQRTV with a Strep-tag II coding sequence fused to the 3' end of hoxK in LITMUS28 harboring a disrupted KpnI site
This work

pCH1365
Derivative of pCH497 containing the HoxKstop gene–Strep-tag II fusion
This work

pCH1408
890-bp MscI fragment carrying parts of HoxKstop gene–Strep-tag II and hoxG
This work

pCH1409
235-bp BglII pse + PCR fragment in pCH1408
This work

pCH1410
805-bp Msal-Acc65I fragment of pCH1409 in pCH1351
This work

pCH1411
2,260-bp Scal fragment of pCH1410 in pL01 (PmeI)
This work

pCH1412
2,274-bp MscI-Scal fragment of pCH466 in pL01 (PmeI)
This work

pCH1413
946-bp EcoRI-BglII fragment of pCH1412 containing hoxQ-His tag in pCH1412
This work

pCH1414
1,421-bp EcoRI-XbaI PCR fragment of Synechocystis genomic DNA in pBluescript II KS(+) 58

pCH1415
1,311-bp EcoRI-Xhol PCR fragment of Synechocystis genomic DNA in pCH1414
This work

pCH1416
1,053-bp chloramphenicol resistance-conferring HincII cassette from pDONR221 in pCH1415 (Klenow fragment-filled HindIII) 7

pCH1417
153-bp pCH497 PCR fragment (Ndel-BglIII) in pCH1352
This work

pCH1418
724-bp Acc65I-Smal fragment from pCH1418 in pL01 (PmeI)
This work

pCH1419
2,179-bp Scal fragment from pCH1418 in pL01 (PmeI)
This work

pGE636
21.60-kbp SpeI-XbaI fragment carrying the R. eutropha H16 MBH operon with a Strep-tag II coding sequence fused to the 3’ end of hoxK in pEDY309
This work

pGE638
21.4-kbp SpeI-XbaI fragment from pCH1367 in pEDY309
This work

pGE659
235-bp BglII pse + PCR fragment in pLO11
This work

baffled flasks, each containing 500 ml FGN medium. Gene expression in R. eutropha HF771 was induced after 24 h by the addition of acetoin to a final concentration of 2 mM. After a total cultivation time of 48 h, all strains reached an OD630 of 8 to 10 and were harvested by low-speed centrifugation (4,000 × g at 4°C for 20 min). For protein purification, the cell pellet (~12 g) was resuspended in 12 ml of buffer B (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, one tablet of complete EDTA-free protease inhibitor cocktail [Roche], 10 µg DNase I [Roche], and the cells were disrupted by two passages through a chilled French pressure cell (SLS Aminco). The soluble protein fraction was separated from the cell debris and membranes by ultracentrifugation (88,000 × g at 4°C for 45 min). The resulting soluble extracts were applied onto Strep-Tactin Superflow columns (1-ml bed volume; IBA, Göttingen, Germany) in 2.5-ml columns (MoBiTec, Germany). The columns were washed with 5 BVs of buffer B, and the bound proteins were eluted with elution buffer (buffer B plus 5 mM desthiobiotin). The protein-containing fractions were pooled and concentrated using a centrifugal filter device (Amicon Ultra-15 [PL-30]). For concentration of Strep-tagged PsaE, a Centricron device with a 3,000-molecular-weight cutoff was used (Millipore). For the improved isolation procedure for MBHPsaE from R. eutropha HF769, an...
additional purification step was established. The soluble extract was applied onto a 10-ml MoBiTec column containing 4 ml of Ni-NTA Superflow (Qiagen, Germany) on which all His-tagged proteins were immobilized. The resulting flowthrough was then directly loaded onto the Strep-Tactin Superflow column.

All purification steps were performed at 4°C. Purified proteins were stored at −80°C in 50 mM Tris-HCl, pH 8, 50 mM NaCl, and 20% glycerol.

The BCA method (Pierce) was applied for protein quantification using bovine serum albumin as the standard. The purity of the samples was estimated by visual inspection of SDS-polyacrylamide gels stained with Coomassie brilliant blue G-250 (37). For detection of Streptagged proteins, a Strept-Tactin alkaline phosphatase conjugate (IBA, Göttingen, Germany) was used. For immunological detection of MBH-related proteins, the following antisera were applied in the indicated dilutions: anti-HoxK serum (1:5,000), anti-HoxG serum (1:10,000), anti-HoxO serum (1:10,000), anti-HoxR serum (1:10,000), and anti-HoxQ serum (1:10,000).

Preparation of thylakoid membranes. Cultures of Synechocystis were harvested in the logarithmic growth phase by centrifugation for 15 min at 4,000 g at 4°C. The BCA method (Pierce) was applied for protein quantification using cattle serum albumin as the standard. The purity of the samples was estimated by visual inspection of SDS-polyacrylamide gels stained with Coomassie brilliant blue G-250 (37). For detection of Streptagged proteins, a Strept-Tactin alkaline phosphatase conjugate (IBA, Göttingen, Germany) was used. For immunological detection of MBH-related proteins, the following antisera were applied in the indicated dilutions: anti-HoxK serum (1:5,000), anti-HoxG serum (1:10,000), anti-HoxO serum (1:10,000), anti-HoxR serum (1:10,000), and anti-HoxQ serum (1:10,000).

Preparation of thylakoid membranes. Cultures of Synechocystis were harvested in the logarithmic growth phase by centrifugation for 15 min at 4,000 g and 4°C. Thylakoids were prepared according to the method of Dühring et al. (16).

Isolation of PS I-His$_{2}$tag from Synechocystis. Purification of Synechocystis His-tagged PS I was performed according to a protocol developed for PS I from Thermoanaerobacter elongatus (E. A. R., El-Mohssney and M. Rüniger, unpublished data) with slight modifications. Thylakoid membranes (TM) were homogenized in buffer A (1 mg Chl per ml 20 mM HEPES, pH 7.5, 10 mM MgCl$_{2}$, 0.5 mM mannitol, 0.05% beta-DM) by homogenization and centrifugation (20 min at 4°C and 5,000 g). The supernatant was then subjected to 15 to 20 min at room temperature, followed by resuspension in extraction buffer B (0.6 mM ammonium sulfate, 20 mM HEPES, pH 7.5, 10 mM MgCl$_{2}$, 10 mM MgCl$_{2}$, pH 7.5) to a final Chl concentration of 1 mg ml$^{-1}$. After stirring for 15 to 20 min at room temperature, 0.9% beta-DM was added and stirring was continued for another 20 min. Solubilized proteins were separated from the membrane by ultracentrifugation (1 h at 4°C and 257,000 g). A chelating Sepharose fast-flow column (Pharmacia) was charged with a nickel solution (100 mM NiCl$_{2}$, 10% acetic acid in H$_{2}$O) and equilibrated by two column volumes (CV) of buffer C (50 mM MES [morpholinopentane-N-sulfonic acid], pH 6.5, 300 mM NaCl, 10 mM MgCl$_{2}$, 10 mM CaCl$_{2}$, 0.25 M mannitol, 0.03% beta-DM, 1 mM histidine). Hist$_{2}$PS I was isolated by elution with a linear gradient of 1 to 100 mM histidine. The pooled PS I fractions were concentrated and desalted by overnight dialysis in histidine-free buffer C. After the addition of 3 M (NH$_{4}$)$_{2}$SO$_{4}$ solution to a volumetric ratio of 1:2 (eluate to (NH$_{4}$)$_{2}$SO$_{4}$ solution), the protein solution was loaded onto a hydrophobic interaction chromatography (HIC) column (POROS 50 G), which had been equilibrated with buffer D (20 mM HEPES, pH 7.5, 10 mM MgCl$_{2}$, 10 mM CaCl$_{2}$, 0.5 mM mannitol, 0.03% beta-DM). PS I$_{s}$(PsaE) protein was eluted by a linear gradient of 1.5 to 0 M (NH$_{4}$)$_{2}$SO$_{4}$. Desalted and concentrated PS I$_{s}$(PsaE) protein solutions were frozen and stored at −70°C. The PS I$_{s}$(PsaE) protein complexes (3 to 5 µg Chl) were further purified and separated into trimeric and monomeric PS I$_{s}$(PsaE) fractions by size exclusion chromatography (TSK 3000 column) using running buffer D (20 mM HEPES, pH 7.5, 10 mM MgCl$_{2}$, 10 mM CaCl$_{2}$, 0.03% beta-DM) for isocratic elution. Fluorescence spectroscopy at 77 K (Aminco Bowman series 2 spectrophotometer) was used to check the purity and integrity of the isolated monomeric and trimeric PS I$_{s}$(PsaE) subspecies. Upon excitation at 440 nm, Chl fluorescence emission was recorded in the range from 630 to 730 nm. PS I$_{s}$(PsaE) samples were adjusted to 3 to 5 µg Chl ml$^{-1}$ and frozen in liquid nitrogen.

**RESULTS**

Construction of an expression system for purification of the MBH-PsaE fusion protein. The MBH of *Ralstonia eutropha* shows the common structural composition of [NiFe]-hydrogenases, a large subunit, HoxG, that accommodates the NiFe region of HoxK to a membrane-integral b-type cytochrome, thereby facing the periplasm (5, 7, 58). For light-driven H$_{2}$ production, Ibara and coworkers have used only heterogeneous preparations of HoxG-HoxK-PsaE (MBH-PsaE) fusion protein purified by anion-exchange and size exclusion chromatography (28). In order to obtain pure MBH$_{PsaE}$ for biochemical and electrochemical studies, we used an established expression system designed for overproduction of MBH proteins in *R. eutropha* (39). For construction of MBH$_{PsaE}$, the C-terminal “anchor” sequence encoded at the 3' end of hoxK was replaced by an Arg-Ser linker followed by Strep-tag II (MBH$_{stop}$). In a second

<table>
<thead>
<tr>
<th>Primer</th>
<th>No.</th>
<th>DNA sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>psaE rev + BglII</td>
<td>1</td>
<td>ACGAGATTTTGTGCAGCGTTGCACAAATCCAAATCC</td>
</tr>
<tr>
<td>psaE fw + BglII</td>
<td>2</td>
<td>CCGAGCTCGGCGGGGGTCTTAAATCGTGGTGACAAAAGTTAGAATC</td>
</tr>
<tr>
<td>hoxO fw + EcoRI</td>
<td>3</td>
<td>TAGAATTCAATCATACATCAACTACATTTCCCTCATTCGGCTGTCGTCGTCGCC</td>
</tr>
<tr>
<td>hoxO rev + BglII</td>
<td>4</td>
<td>CACACGAGATTTTGCAGCGTTGCACAAATCCAAATCC</td>
</tr>
<tr>
<td>psaE fw + NcoI</td>
<td>5</td>
<td>CTTCATGGCCCTTAAATCCTGTTG</td>
</tr>
<tr>
<td>psaE rev + BglII</td>
<td>6</td>
<td>TTGAGCTTTGTCGCCGCTT</td>
</tr>
<tr>
<td>PS I His rev + EcoRI</td>
<td>7</td>
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<tr>
<td>PS I His rev + XbaI</td>
<td>8</td>
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<tr>
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<td>9</td>
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</tr>
<tr>
<td>PS I His rev + Xhol</td>
<td>10</td>
<td>TGACTCGAGAAGACATCCTCGCCAAAGCCAGGGAAAA</td>
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<td>11</td>
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<tr>
<td>MBH His rev + NdeI</td>
<td>12</td>
<td>GCCCATATGACCCCTGACAGGTTGGACGG</td>
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| TABLE 2. Oligonucleotide primers used in this study |
step, a sequence coding for the Synechocystis PS I subunit psaE equipped with an N-terminal Gly-Gly linker was introduced between hoxK and the Strep-tag II sequence (Fig. 1). Subsequently, the hoxK allele on the expression plasmid pGE638 was exchanged by the hoxK-psaE–Strep-tag II allele through double homologous recombination (40). As expected, the resulting recombinant strain HF768 (MBHPsaE) failed to sustain H₂-dependent autotrophic growth (data not shown) due to the fact that the MBHPsaE protein was incapable of establishing a proper connection to the b-type cytochrome, HoxZ.

Replacing the membrane anchor of the MBH small subunit, HoxK, with the PsaE protein affects the export competence of the MBH. The MBHPsaE hybrid protein lacks the HoxK anchor peptide responsible for membrane attachment. Thus, we expected that the protein could be purified from the soluble protein fraction. It has been shown previously that the cytoplasmic, premature HoxK protein (preHoxK) copurifies with the accessory HoxO and HoxQ proteins. The preHoxK protein carries an N-terminal signal peptide of 43 amino acids that is cleaved off during translocation of the MBH through the Tat translocation apparatus (58). Thus, C-terminally Strep-tag II-tagged HoxK-PsaE was expected to occur in two protein complexes upon affinity chromatography, the mature HoxG–HoxK-PsaE and the premature preHoxK-PsaE–HoxO/Q. It was therefore our intention to isolate MBHPsaE from the periplasmic fraction that is generally devoid of any premature MBH forms. In order to examine whether the MBHPsaE protein was appropriately translocated through the membrane to the periplasm, an immunological analysis was conducted using cytoplasmic, membrane, and periplasmic fractions from various R. eutropha cell preparations (Fig. 2) (6). Tat signal peptide-bearing precursors of the respective HoxK variants were detected in the cytoplasmic fraction of strains HF768 (MBHPsaE), HF632 (MBHwt), and HF689 (H9004 hoxG). However, the total amount of preHoxK-PsaE was significantly smaller than that of wild-type HoxK (Fig. 2A). A signal for preHoxK-PsaE was also obtained for the membrane fraction (Fig. 2B) but was absent in the periplasmic preparation (Fig. 2C). Mature HoxK-PsaE was not detectable in the periplasm or in the membrane. This contrasts with the situation for the wild type, in which the vast majority of mature HoxK protein was found in the membrane (Fig. 2B and C). Owing to the MBH overproduction in control strain HF632, both the mature and precursor forms of HoxK as well as HoxG were found in the cytoplasm and in the periplasm. This phenomenon is a result of cross-contamination during the preparation process of the individual fractions and was described previously (58).

Unexpectedly, a HoxK form whose size corresponded to that of the mature small subunit was observed in strain HF689, which does not synthesize the large subunit. The cofactor-containing preform of HoxK is not translocated through the membrane in the event of blocked HoxG maturation (8).
The fact that pre-HoxK-PsaE was found in the cytoplasm and in considerable amounts in the membrane, whereas mature HoxK-PsaE was absent from the periplasm, led to the conclusion that the modification of the C terminus strongly affects the Tat-dependent transport competence of the fusion protein. The replacement of the anchor by the PsaE domain obviously had the same effect as the removal of the large MBH subunit by deletion of the \( \text{hoxG} \) gene (Fig. 2). In both cases, the respective HoxK precursors were retained in the cytoplasm or stalled in the membrane, probably within the Tat translocon.

Purification and properties of the MBH\textsubscript{PsaE} fusion protein. The experiments described in the previous section demonstrated that the MBH\textsubscript{PsaE} protein had to be purified from the soluble, cytoplasmic fraction that was “contaminated” by MBH precursor forms. Thus, soluble extract was prepared from \( \text{R. eutropha} \) HF768 cells grown under hydrogenase-derepressing conditions and applied to a one-step affinity chromatography using \( \text{Strep-Tactin Superflow} \). After a washing step, matrix-bound protein was eluted using buffer containing desthiobiotin. The eluate, designated MBH\textsubscript{PsaE}* was concentrated and subsequently subjected to SDS-PAGE (Fig. 3). Coomassie blue staining revealed five distinct protein bands as shown in Fig. 3A, lane 3. Concomitant immunological analysis identified four of these proteins as the MBH large subunit, HoxG (67 kDa), the premature, nontranslocated preHoxK-PsaE (44 kDa), and the chaperones HoxQ (31 kDa) and HoxO (18 kDa) (Fig. 3B, lane 3). Furthermore, the HoxK antibody detected a protein of ~43 kDa. Since this protein also reacted with the \( \text{Strep-Tactin alkaline phosphatase (AP) conjugate} \), we conclude that this “truncated” HoxK harbors an intact C terminus of HoxK-PsaE but has lost at least a significant part of its N-terminal signal peptide during the purification process presumably due to proteolysis of this presumably exposed part of the protein. The identity of the ~35-kDa protein (Fig. 3, \( \nabla \)) remains unclear. A His\textsubscript{6}-tagged version of wild-type MBH and the MBH\textsubscript{stop} protein, in which the anchor peptide was replaced...
TABLE 3. \( \text{H}_2 \) uptake and evolution activities

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity (( \mu \text{mol H}_2/\text{min/mg of protein} ))(^b)</th>
<th>( \text{MV}_{\text{red}}-\text{H}_2 )</th>
<th>( \text{H}<em>2-\text{MB}</em>{\text{ox}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBH(_{\text{wt}})</td>
<td>0.5</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>MBH(_{\text{stop}})</td>
<td>0.3</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>MBH(_{\text{stop}})(^*)</td>
<td>0.21</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>MBH(_{\text{stop}})</td>
<td>0.44</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Activities of hydrogenase-photosystem fusion proteins compared to wild-type MBH purified either from the soluble extract or from the membrane (see text for details).

\(^b\) \( \text{MV}_{\text{red}} \)-reduced methyl viologen; \( \text{MB}_{\text{ox}} \)-oxidized methylene blue.

by Strep-tag II, were purified as controls. Whereas MBH\(_{\text{wt}}\) was isolated from the membrane fraction (as described in Materials and Methods) (Fig. 3A, lane 1), the MBH\(_{\text{stop}}\) protein was purified from soluble extract of strain HF653. The resulting eluate of the latter revealed a similar protein pattern as for MBH\(_{\text{PsaE}}\) (Fig. 3A, lane 2). Due to the lack of the anchor peptide, the corresponding HoxK derivatives were found at positions corresponding to 34 kDa (preHoxK\(_{\text{stop}}\)) and 29 kDa (HoxK\(_{\text{stop}}\)) respectively.

In order to obtain pure MBH\(_{\text{PsaE}}\), it was necessary to efficiently separate premature preHoxK-PsaE–HoxO/Q complex from the desired preHoxK-PsaE–HoxG dimers. Therefore, hoxQ was equipped with a hexahistidine-encoding sequence at its 5’ end. This genetic construct was then established in addition to hoxK-psiE–Strep-tag in strain HF769. In a first step, soluble extract of HF769 was subjected to immobilized metal-affinity chromatography (IMAC) using a Ni-nitritroleic acid (NTA) Superflow column. This resulted in the retardation of any proteins complexed with the HoxQ-His\(_9\) fusion. In a second step, the IMAC flowthrough was loaded onto a Strep-Tactin Superflow column. As shown in Fig. 3A, lane 5, the subsequent addition of desthiobiotin resulted in the elution of homogeneous preHoxK-PsaE–HoxG (MBH\(_{\text{PsaE}}\)).

In order to analyze the proteins bound to the Ni-NTA Superflow column via HoxQ-His\(_9\), the proteinaceous material was eluted with imidazole. The resulting eluate contained six distinct protein bands (not shown). To examine if these proteins form a stable complex, the IMAC eluate was applied to Strep-Tactin affinity chromatography. The resulting protein pattern obtained after electrophoretic separation of the Strep-Tactin eluate (Fig. 3A, lane 4) still displayed the six proteins, confirming their tight association. According to immunoblot analysis (Fig. 3B), the proteins were assigned to preHoxK-PsaE (44 kDa), an N-terminally truncated version of preHoxK-PsaE (39 to 40 kDa), HoxQ (31 kDa), HoxO (18 kDa), and a protein of unknown origin at approximately 40 kDa. The largest protein (67 kDa) was identified as HoxG, strongly indicating that the MBH large subunit interacts with the preHoxK-PsaE–HoxO/Q complex (Fig. 3A, lane 4).

Activity measurements showed the functionality of the preHoxK-PsaE–HoxO/Q complex, which exhibited an \( \text{H}_2 \)-oxidizing activity of 10 U/mg of protein (ca. 25% of wild-type activity) and an \( \text{H}_2 \)-evolving activity of 0.03 U/mg of protein (ca. 6% of wild-type activity).

\( \text{H}_2 \)-evolving activity of purified MBH\(_{\text{PsaE}}\): The \( \text{H}_2 \)-evolving activity of the purified MBH variants was determined using dithionite-reduced methyl viologen as the electron donor. The MBH\(_{\text{PsaE}}\) protein purified by IMAC and Strep-Tactin affinity chromatography showed high specific activity which was more than twice as high as the level of activity of the MBH\(_{\text{PsaE}}\) protein complex that was purified just on basis of the Strep-tag II and still contained the maturation proteins HoxO and HoxQ (Table 3). The highest \( \text{H}_2 \) production activity was observed for MBH\(_{\text{wt}}\) and MBH\(_{\text{stop}}\) displayed a comparatively low level of activity. These results show that H2 catalysis was maintained in all MBH variants. However, modification of the C-terminal “anchor” region of HoxK affected the \( \text{H}_2 \) uptake activity to a greater extent than it did \( \text{H}_2 \) evolution (Table 3).

Rapid purification of PS I-His\(_{\text{10}}\) from Synechocystis sp. PCC 6803. For fast and easy purification of PS I derivatives, a decahistidine-encoding sequence was genetically fused to the 5’ end of the psaF gene encoding a membrane-integral subunit of PS I. The His\(_{\text{10}}\)-psaF allele was transformed into the Syn-echocystis PS I\(_{\text{wt}}\) and PS I\(_{\text{A,psiE}}\) strains and established in the respective genomes by segregation. Both mutant strains maintained their capability for photoautotrophic growth (not shown).

Purification of His-tagged PS I was done according to a protocol developed for PS I from Thermosynechococcus elongatus (E. A. R. El-Mohsnawy and M. Rögener, unpublished data) with slight modifications. To monitor PS I recovery during the purification procedure, the chlorophyll (Chl) contents of the samples were determined routinely after each purification step by UV-visible (UV-Vis) spectroscopy. Starting with thylakoid membranes containing approximately 21 mg Chl, solubilization, IMAC, and HIC resulted in recoveries of 36%, 16%, and 8% Chl, respectively. After Ni-NTA chromatography, the His-tagged PS I displayed a single, obviously homogenous protein peak (data not shown). PS I-containing fractions were pooled and applied to HIC, yielding two distinct peaks (Fig. 4). Analysis by subsequent size exclusion chromatography clearly showed that the first peak of the HIC column represents trimeric PS I and the second peak monomeric PS I (Fig.)
was conducted by mixing the purified proteins in a ratio of 10:2648 SCHWARZE ET AL. APPL. ENVIRON. MICROBIOL.

...heterologously using the expression vector pLO11, on which R. eutropha Strep. For this purpose, the acoX in MBHPsaE, the PsaE protein was also purified individually from /H9004 and PS I approximately 700 photochemical activity in the presence of methyl viologen was By the artificial electron donor system ascorbate-DCPIP, the coupling of MBHPsaE to PS I /H9004 PsaE domain. In vitro coupling of MBH_psaE to PS I_psaE. Blue native gel analysis is a suitable tool for the analysis of complex formation between membrane-bound proteins or between membrane-bound and soluble proteins (56). In order to collect data for the specificity of a potential complex between PS I_psaE and MBH_psaE, the PsaE protein was also purified individually from R. eutropha. For this purpose, Strep-tagged PsaE was produced heterologously using the expression vector pLO11, on which the Strep-tagged PsaE gene expression was under the control of the acoX promoter and the hoxF ribosome binding site (Fig. 6).

Formation of specific PS I_psaE–MBH_psaE supercomplexes was conducted by mixing the purified proteins in a ratio of 10 pmol PS /I_psaE trimer, 40 pmol MBH-wt/MBH_psaE/MBH_stop, and 400 pmol free PsaE and incubation for 30 min at 20°C. Subsequently, the preparation was loaded on a blue native gel. Figure 7 shows that MBH_psaE interacts specifically with PS I_psaE. The two proteins formed a complex that remained stable during blue native electrophoresis. No complex formation was observed for PS I_psaE together with MBH-wt or MBH_stop. Also, there was no interaction detectable for any of the MBH variants with PS I_psaE as the bait. In the presence of a 10-fold excess of free PsaE, reconstitution of MBH_psaE was competitively inhibited, strongly indicating that the complex formation between PS I_psaE and MBH_psaE is based on the PsaE domain.

**DISCUSSION**

The idea of using microorganisms for the production of H₂ from light and water became extremely attractive as a consequence of the energy crises in the 1970s. In these years, the artificial and indirect coupling of chloroplast and thylakoids preparations to hydrogenases isolated from various sources was established and then pursued in the next decades (4, 27, 34, 45). Although successful, these efforts died down because of the limited efficiency and—most importantly—due to the limited availability of molecular genetics and genomic information. Today, the situation has changed dramatically, and synthetic biology offers new possibilities to create microorganisms possessing synthetic functions. Direct coupling of a hydrogenase to PS I is one of the attractive new options to enhance light-driven H₂ production. This strategy was first designed by Ihara et al. (in 2006), who fused the PsaE subunit of PS I from *Thermosynechococcus elongatus* to the C terminus of the electron-transferring subunit of the membrane bound [NiFe]-hydrogenase from *Ralstonia eutropha* (28). The resulting MBH_psaE fusion protein was combined in vitro with purified PS I from *Synechocystis* sp. PCC 6803 lacking the peripheral PsaE subunit yielding a PS I-hydrogenase hybrid complex capable of light-driven H₂ production using ascorbate as an artificial electron donor. In these experiments, the H₂ production rate was rather low and it was not possible to quantify and specify the interaction of hydrogenase and PS I. One likely reason for the low H₂ production rate might be the fact that the MBH-psaE fusion was only partially purified and likely contaminated by various immature variants of the MBH small subunit HoxK (58). Since the PsaE domain was fused to HoxK, all copurified non-catalytically active precursor forms are supposed to interact with the PsaE-free PS I, leading to consider-
ably smaller amounts of PS I-hydrogenase complexes capable of \( \text{H}_2 \) production. Therefore, it was impossible to relate the \( \text{H}_2 \) production rate quantitatively to the amount of active hydrogenase bound specifically to PS I.

In order to obtain homogenous starting material for quantitative interaction studies, we have developed a new purification strategy for the MBH\(_{\text{PsaE}}\)-fusing protein and PS I\(_{\text{PSaE}}\). The new approach not only yielded pure and active fusion protein, it also gave new insights into the complex maturation process of MBH. One important result is based on the observation that the MBH\(_{\text{PsaE}}\) protein had to be purified from the cytoplasmic fraction. It was our initial intention to isolate the hybrid protein from the periplasm. After metallocenter assembly, which takes place in the cytoplasm, the wild-type MBH undergoes Tat-dependent translocation through the cytoplasmic membrane completed by coupling of the protein to the membrane-integral cytochrome \( b \). Whereas the membrane transport is guided by the specific Tat signal peptide located N-terminally relative to MBH small subunit HoxK, the attachment of the MBH to the membrane crucially depends on the C-terminal anchor peptide of HoxK (5, 6, 58). Hence, we simply expected that the exchange of the C-terminal anchor by the Psae domain would lead to the detachment of the fusion protein from the membrane without affecting the Tat translocation. Surprisingly, the catalytically active MBH\(_{\text{PsaE}}\)-hybrid was found exclusively in the cytoplasm (Fig. 2). Also, most of the small subunit of purified MBH\(_{\text{top}}\) protein that lacks the C-terminal membrane anchor resided in the precursor form still containing the Tat signal sequence (Fig. 3). We therefore conclude that modifications of the C-terminal anchor peptide strongly affect the Tat translocation efficiency of the MBH heterodimer. It is conceivable that the hydrophobic tail participates at least to some extent in the formation of the export competent form of the MBH. A similar observation has been made for the model Tat substrate TorA-PhoA. The removal of 33 amino acids from the C terminus of the PhoA moiety converted the fusion protein to a weak Tat substrate, although the formation of structure-determining disulfide bonds was not affected in the mutant protein (44).

It is also possible that the C-terminal peptide provides an interaction domain for a chaperone during the maturation process. One potential candidate is the HoxT protein that is encoded in many gene clusters of MBH-like hydrogenases (64). By means of the yeast two-hybrid system, it has been shown that the HoxT homologue in \( E. \) coli, designated HybE, coordinates the assembly and export of both the small and large subunits of hydrogenase 2 (15, 19, 60). The HybE crystal structure revealed a hydrophobic cleft that could accommodate hydrophobic peptides such as parts of the Tat signal peptide or the C-terminal membrane anchor (49).

Two other chaperones, HoxO and HoxQ, have been shown to form stable complexes with the small-subunit precursor of the MBH (58). Homologous proteins are involved in the maturation of \( E. \) coli hydrogenase 1 (15, 46) and the MBH of \( \text{Rhizobium leguminosarum} \) (43). Mutant analysis showed that HoxO and HoxQ also interact with a HoxK version that lacks the C-terminal anchor peptide, supporting the notion that these chaperones are associated with the signal peptide. This is supported by the fact that HoxO and HoxQ interact specifically with synthetically produced signal peptides (58). In this study, we showed that HoxO and HoxQ form a catalytically active MBH\(_{\text{PsaE}}\) protein located in the cytoplasm (Fig. 3). This observation can be interpreted in two ways. The first is that such a complex is part of the regular MBH maturation pathway and has been overlooked previously (58). The second is that maturation is severely delayed because of the modified C terminus of the small subunit, leading to ineffective removal of HoxO and HoxQ from the preHoxK-PsaE-\( \text{HoxG} \) protein, which in turn prevents membrane translocation. However, the specifically designed purification procedure described in this study yielded highly pure, HoxO- and HoxQ-free MBH\(_{\text{PsaE}}\) protein isolated from the cytoplasm, which seemingly excludes the second hypothesis.

Our purification strategy allowed the efficient removal of contaminating premature MBH forms and yielded homogenous preHoxK-PsaE-\( \text{HoxG} \) (Fig. 3, Table 3). The MBH\(_{\text{PsaE}}\) showed an \( \text{H}_2 \) evolution activity that was comparable to the wild-type MBH protein purified from the membrane. However, the \( \text{H}_2 \) uptake activity was decreased to about 50% of that of the wild-type control. A similar decrease in \( \text{H}_2 \) uptake activity was also observed for the MBH\(_{\text{top}}\) protein, which lacks the C-terminal anchor peptide of small subunit HoxK. This finding points out the significance of the HoxK C terminus for channeling the electrons from \( \text{H}_2 \) oxidation to the protein surface. On the other hand, proton reduction seems to be less affected.

In this study, we also developed a rapid method for the isolation of PS I derivatives from \( \text{Synechocystis} \) sp. \( \text{PCC} \) 6803 via Ni-NTA affinity chromatography. Similarly, as previously described for PS II from \( \text{Synechocystis} \), a decahistidine tag was fused to one of the membrane-intrinsic subunits of \( \text{Synechocystis} \) PS I (10). His-tagged versions of PS I are already described in the literature (50, 62). Tang and Chitnis attached His\(_5\) tags to the C-terminal ends of the Psak and \( \text{PsaL} \) subunits of PS I from \( \text{Synechocystis} \) (62). The His\(_5\) peptides were, however, accessible only after harsh urea treatment and could not be used for purification of catalytically active PS I particles. The attachment of a His\(_{10}\) tag to the N terminus of PsaA from the green alga \( \text{Chlamydomonas reinhardtii} \) yielded an active PS I version that could be purified via IMAC (25). Since the His\(_{10}\) tag was located at the cytoplasmic side of PS I, a linkage to NTA-modified surfaces would hide the acceptor site of the protein.

In this study, a His\(_{10}\) tag was attached to the N terminus of PsaF, which is exposed to the luminal side of thylakoid membranes (30). This strategy allows an oriented immobilization of the PS I onto modified gold electrodes (see below). The attachment of the decahistidine peptide had no effect on photocatalytic growth. Purified PS I\(_{\text{PsS}}\) and PS I\(_{\text{PSaE}}\) displayed final activities of ca. 700 \( \mu \text{mol} \text{O}_2/\text{mg chlorophyll} /\text{h} \), a value that is comparable to activities described for PS I purified without Ni-NTA affinity chromatography (51, 70).

By analyzing the protein-protein interaction, we clearly showed that MBH\(_{\text{PsaE}}\) and PS I\(_{\text{PSaE}}\) form a supercomplex (Fig. 7). Complex formation was competitively inhibited by the addition of an excess of free PsaE, underlining the specificity of the PS I\(_{\text{PSaE}}\)-MBH\(_{\text{PsaE}}\) supercomplex formation.

The results presented in this study provide the basis for further studies on the light-driven \( \text{H}_2 \) production capacity of the PS I-MBH fusion protein. The His\(_{10}\) tag attached to the
luminal side of PS I enables oriented immobilization onto Ni-NTA-modified electrode surfaces (2). This strategy allows the quantitative characterization of the structure, composition, and light-mediated H2 evolution activity of the respective protein monolayer by spectroelectrochemical methods. In fact, it has been shown very recently that this PS I-MBH fusion protein immobilized onto a modified gold surface exhibits the highest light-driven H2 production rates ever observed for comparable systems (35).

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REFERENCES


