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A Trimeric Supercomplex of the Oxygen-Tolerant Membrane-Bound [NiFe]-Hydrogenase from *Ralstonia eutropha* H16

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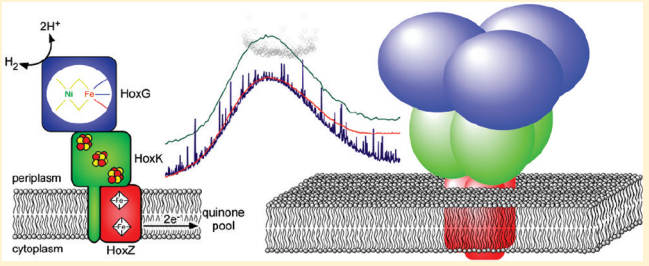
ABSTRACT: The oxygen-tolerant membrane-bound [NiFe]-hydrogenase (MBH) from *Ralstonia eutropha* H16 consists of three subunits. The large subunit HoxG carries the [NiFe] active site, and the small subunit HoxK contains three [FeS] clusters. Both subunits form the so-called hydrogenase module, which is oriented toward the periplasm. Membrane association is established by a membrane-integral cytochrome *b* subunit (HoxZ) that transfers the electrons from the hydrogenase module to the respiratory chain. So far, it was not possible to isolate the MBH in its native heterotrimeric state due to the loss of HoxZ during the process of protein solubilization. By using the very mild detergent digitonin, we were successful in isolating the MBH hydrogenase module in complex with the cytochrome *b*. H_2-dependent reduction of the two HoxZ-stemming heme centers demonstrated that the hydrogenase module is productively connected to the cytochrome *b*. Further investigation provided evidence that the MBH exists in the membrane as a high molecular mass complex consisting of three heterotrimeric units. The lipids phosphatidylethanolamine and phosphatidylglycerol were identified to play a role in the interaction of the hydrogenase module with the cytochrome *b* subunit.



Stability and prevention of disassembly is often the bottleneck in the purification of membrane protein complexes.[1,2] To liberate membrane proteins from their respective lipid environment, a detergent is added in order to solubilize the membrane and to bring the membrane proteins into solution.[3] During solubilization, lipids associated with membrane proteins can be removed, depending on the properties of the detergents, proteins, and lipids present in the sample. This so-called delipidation is often accompanied by the disruption of protein−protein interactions resulting in the loss of subunits of the protein complex.

A well-studied example is represented by the oxygen-tolerant membrane-bound [NiFe]-hydrogenase (MBH) from the *β*-proteobacterium *Ralstonia eutropha* H16.[4,5] The MBH is located at the periplasmic side of the cytoplasmic membrane, and the native complex consists of three proteins: a large subunit (HoxG, 67.2 kDa) comprising the [NiFe] active site, a small subunit (HoxK, 34.6 kDa) harboring three [FeS] clusters, and a membrane-integral cytochrome *b* (HoxZ, 27.6 kDa) carrying two heme groups (Figure 1). The H_2-activating module consists of HoxG and HoxK (HoxGK) and catalyzes the oxidation of H_2 into protons and electrons as well as the reverse reaction, the reduction of protons to H_2.[6] The electrons released from H_2 oxidation are transferred from the active site in HoxG via the [FeS] clusters in HoxK to the primary electron acceptor, the *b*-type cytochrome HoxZ. This membrane-integral component feeds the electrons via the quinone pool into the respiratory chain.[7] The periplasmic hydrogenases abundant in sulfate-reducing bacteria utilize cytochrome c_3 instead of cytochrome *b* as the primary electron acceptor.[8] The two hydrogenase subgroups can be distinguished according to the presence of a

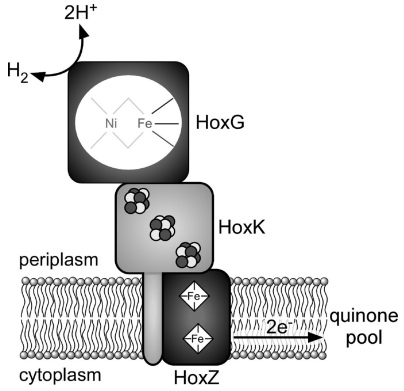


Figure 1. Schematic representation of the heterotrimeric membrane-bound hydrogenase. The large subunit HoxG oxidizes H_2 to protons and electrons. The latter are transferred via [FeS] clusters of the small subunit HoxK and the membrane integral cytochrome *b* (HoxZ) to the quinone pool.

C-terminal hydrophobic peptide extension, which, in the case of the membrane-bound enzymes, fulfills a bifunctional role. It anchors the protein to the lipid bilayer and provides the linker to the electron accepting cytochrome *b* subunit.[9]

Previous purification procedures for the MBH from *R. eutropha* led exclusively to the isolation of the heterodimeric HoxGK form.[5,10,11] The membrane integral cytochrome *b*

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subunit HoxZ was lost in the course of membrane solubilization that was accomplished with detergents such as Triton X, dodecylmaltoside (DDM), or octylglucoside. Similar effects were observed with closely related MBH complexes, e.g., Hyd-1 from *E. coli*.[12] Interestingly, two other hydrogenases, which are also related to the MBH, namely hydrogenase I from *Aquifex aeolicus* and the membrane-bound hydrogenase (HydABC) from *Wolinella succinogenes*, have previously been isolated as heterotrimers, using the detergents DDM and Triton X-100, respectively, for solubilization.[13−15] In order to investigate the MBH in its native composition, we selected conditions for the isolation of the entire hydrogenase complex. The basic features of the heterotrimeric MBH are discussed in context with previous characterizations of the hydrogenase module.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. *E. coli* JM109 was used as host for standard cloning procedures, and *E. coli* S17-1 served as donor in conjugative transfers. In order to fuse a sequence coding for a *Strep*-tagII to the 3′ end of the HoxZ gene, pCH460[16] was digested with *Xho*I, and the resulting 2613 bp fragment was ligated into pBluescript II KS+ that was previously digested with *Xho*I. The resulting plasmid pCH1284 served as template for an inverse PCR using primers 728 (CTAGCTAGCTGGAGCCACCCGCAGTTCGAAAAATGATGCGCAATGCAACCGAAGCGATCGCAGGCTTTCGTCG) and 729 (CTAGCTAGCGTCCTTAAAGGTCCGATAGCCCGAGACCATCGTGC). The resulting 5.3 kbp fragment was digested with *Nhe*I and was subsequently religated, resulting in plasmid pCH1285. pCH1285 was digested with *Xho*I, and the resulting 2346 bp fragment was ligated into the suicide vector pLO2[17] that was previously digested with *Xho*I. The resulting plasmid pCH1286 was used for homologous recombination using strain HF632,[18] resulting in HF655.

Media, Growth Conditions, Protein Purification, and Hydrogenase Activity Test. Media and growth conditions have been described.[10,11] Protein purification was essentially carried out as described,[10] except for the following changes. Sonication of the membrane fraction was omitted, and the final concentration of $K_3[Fe(CN)_6]$ was 1 mM. Solubilization was carried out using the following detergents at a final concentration of 1% (w/v) or at least a concentration of the CMC if this was greater than 1% (w/v) and incubation for 1.5 h at 4 °C: CHAPS, CHAPSO, CYMAL-5, escin, glycyrrhizic acid ammonium salt, MEGA-8, Thesit, Tween 20, Tween 80 (Sigma-Aldrich); lauryl maltose neopentyl glycol (Anatrace); digitonin, sodium deoxycholate, Triton X-114 (Serva); *n*-decyl-*β*-D-maltoside, *n*-dodecyl-*β*-D-maltoside, *n*-octyl-*β*-D-thioglucopyranoside (Applichem); Big CHAP, Deoxy Big CHAP, *n*-nonyl-*β*-D-glucopyranoside, *n*-octyl-*β*-D-glucopyranoside, sodium taurodeoxycholate (Merck). The concentration of each detergent was kept slightly above the cmc in all buffers following the solubilization step. Proteins were not frozen but used for further analyses directly after purification. Hydrogenase activity was measured as described.[10]

UV/vis Spectroscopy. Spectra were recorded from 200 to 800 nm at 25 °C on a Cary 5000 double-beam UV/vis spectrophotometer (Varian). The protein solution was filled into a 100 μL quartz cuvette with an optical path length of 10 mm. Buffer without protein was placed in the reference beam for baseline correction. The following buffers were used: HoxZ−50 mM K-PO_4 pH 7, 150 mM NaCl, 0.007% (w/v) DDM; heterotrimeric MBH−50 mM K-PO_4 pH 7, 150 mM NaCl, 0.07% (w/v) digitonin. HoxZ was reduced by adding Na-dithionite to a final concentration of 1 mM to the protein solution and to the reference buffer. Heterotrimeric MBH was reduced by sealing the cuvette with a rubber septum and flushing of the headspace with moisturized H_2 gas for 1.5 h.

Size Exclusion Chromatography and Light Scattering Analyses. Size exclusion chromatography (SEC) was carried out on an ÄKTApurifier 10 system equipped with a Superdex 200 HR 10/30 column that was calibrated with the calibration kits MWGF200 and MWGF1000 from Sigma-Aldrich. The buffer system was 50 mM K-PO_4 pH 7, 150 mM NaCl, 0.07% (w/v) digitonin.

SEC-light scattering (SEC-LS) was carried out according to ref 19. Thyroglobulin was used as reference protein with an absorption coefficient A_{280} of 0.914 mL mg^{-1} cm^{-1} to determine the buffer-specific constant K (203 879), which is needed for calculating the molecular mass of proteins. For the MBH an absorption coefficient A_{280} of 0.53 mL mg^{-1} cm^{-1} was used. Prior to SEC-LS analysis, a preparative SEC was carried out, and subsequently 500 μL of the first peak was applied to SEC-LS analysis.

Miscellaneous. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to ref 20. Blue native gel electrophoresis (BN-PAGE) was essentially carried out as described,[21] except that purified protein samples were mixed 1 + 1 with sample buffer (50 mM Bis-Tris pH 7, 1 M *ε*-aminocaproic acid, 5 mM EDTA). Finally per 100 μL of sample 5 μL of 5% Coomassie dissolved in sample buffer was added and incubated for 5 min on ice. For 2D-BN/SDS-PAGE prior to loading a lane of the BN-PAGE to the second dimension the gel piece was incubated at 40 °C for 5 min in SDS-PAGE electrophoresis buffer containing 1% SDS and 0.1% *β*-mercaptoethanol for denaturation. Coomassie and silver staining of SDS gels followed published protocols.[22,23] Hydrogenase activity-specific staining of gels was carried out according to ref 16. Reference lipids were purchased from Avanti Polar Lipids. Lipids from *R. eutropha* membranes and purified protein samples were isolated according to a modified protocol.[24] Lipid-containing supernatants that are discarded in the original protocol were collected and protein precipitates were discarded instead. Thin-layer chromatography of lipids was carried out according to ref 25, and lipids were visualized by a short incubation of the TLC plate in 5% H_2SO_4 and subsequent charring at 250 °C. Peptide mass fingerprint analysis of HoxZ followed published protocols.[26] All other methods followed published protocols.[27]

RESULTS

Conditions for the Isolation of the Membrane-Bound Hydrogenase as a Heterotrimer. To screen for detergents sustaining heterotrimeric MBH complexes, a *Strep*-tag II was genetically fused to the C-terminal end of HoxZ, which allows mild purification by affinity chromatography. Using $HoxZ_{Strep}$ as bait, 21 detergents covering different surfactant classes (listed in Experimental Procedures) were included in a solubilization approach aimed at the isolation of the entire MBH complex consisting of the subunits HoxGKZ. Treatment of the membranes with digitonin resulted in the isolation of the heterotrimeric hydrogenase complex (Figure 2). The identity of HoxZ in the electrophoretically separated proteins (band at ∼23 kDa) was verified by mass fingerprint analysis (data not shown). The discrepancy between the apparent molecular mass of $HoxZ_{Strep}$ observed in the SDS gel and its calculated mole-

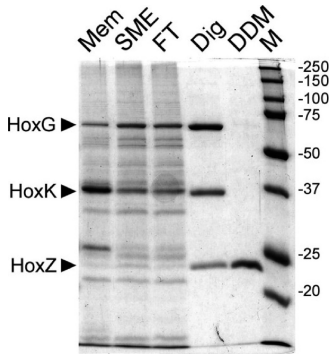

Figure 2. Purification of the MBH heterotrimer. 10 μg protein of extracts and 2 μg of purified protein were separated on a 12.5% acrylamide SDS-PAGE. Molecular masses are given in kDa, and the positions of HoxG, HoxK and HoxZ are indicated. Mem = membrane extract, SME = solubilized membrane extract, FT = flow through of affinity chromatography, Dig = purified hydrogenase heterotrimer solubilized with digitonin, DDM = cytochrome *b* HoxZ solubilized with dodecylmaltoside, and M = molecular mass marker.

cular mass of 28.8 kDa can be explained by altered mobility of membrane proteins as frequently observed in SDS-PAGE.[28] Treatment with all other detergents employed in these experiments led exclusively to the isolation of HoxZ and the loss of the hydrogenase module as demonstrated representatively for DDM in Figure 2.

With H_2 as the substrate, the heterotrimeric MBH was able to reduce the electron acceptors methylene blue, phenazine methosulfate, menadione, duroquinone, and ubiquinone Q_0. Reduction of ferricyanide was not observed. In order to compare the hydrogenase activity of the heterotrimer with that of the HoxGK module, the specific activities were determined with methylene blue as the electron acceptor (Table 1)

Table 1. H_2 Oxidation Activities of Heterodimeric and Heterotrimeric MBH Forms after Different Purification Steps[a]

specific activity	U/mg	U/nmol
HoxGK Aff[b]	140 ± 10.8	14.4 ± 1.1
HoxGKZ Aff	66 ± 18.5	8.6 ± 2.4
HoxGKZ SEC	143.5 ± 20.5	18.7 ± 2.7

[a]Specific enzyme activities were determined by H_2-dependent methylene blue reduction. One unit (U) represents the amount of H_2 in μmol consumed per minute. Presented are mean values of at least three measurements ± SD in U/mg and for better comparison also in U/nmol. [b]Values are taken from ref 10. HoxGK Aff = hydrogenase module after affinity purification, HoxGKZ Aff = heterotrimeric MBH after affinity purification, and HoxGKZ SEC = heterotrimeric MBH after size exclusion chromatography.

commonly used in MBH assays.[5] After affinity purification the heterotrimeric MBH displayed specific hydrogenase activity approximately half the level of that observed for the heterodimeric hydrogenase module. However, further purification by size exclusion chromatography (SEC, see also Figure 5) gave rise to a significant increase of the specific activity that was comparable to the value determined for the HoxGK module. This increase in specific activity is due to the removal of excess HoxZ protein not bound to the hydrogenase module which does not contribute to the hydrogenase activity. For an appropriate comparison the specific activities are also expressed in units per nmol of protein which takes the mass of the cytochrome *b* subunit of the MBH into account.

HoxZ Is a Cytochrome b_{562}. The oxidation state of the primary electron acceptor HoxZ can be easily monitored spectroscopically on the basis of the characteristic redox properties of the heme cofactors and allows for testing the activity of the heterotrimeric MBH in the absence of artificial electron acceptors. Therefore, the spectral properties of $HoxZ_{Strep}$ were characterized in its isolated form (Figure 2) using *n*-dodecyl-β-D-maltoside (DDM) as solubilizing agent. DDM was chosen because this detergent does not induce any spectral changes in *b*-type cytochromes.[29] Absorption spectra of the "as-isolated" (oxidized in air) and Na-dithionite reduced forms of HoxZ were recorded by UV/vis spectroscopy. Negative absorption in the region from 290 to 350 nm resulted from addition of Na-dithionite to the sample as well as to the buffer in the reference beam. Upon reduction, the Soret band shifted from 416 to 429 nm and the α- and β-bands were detected at 562 and 532 nm, respectively (Figure 3A).

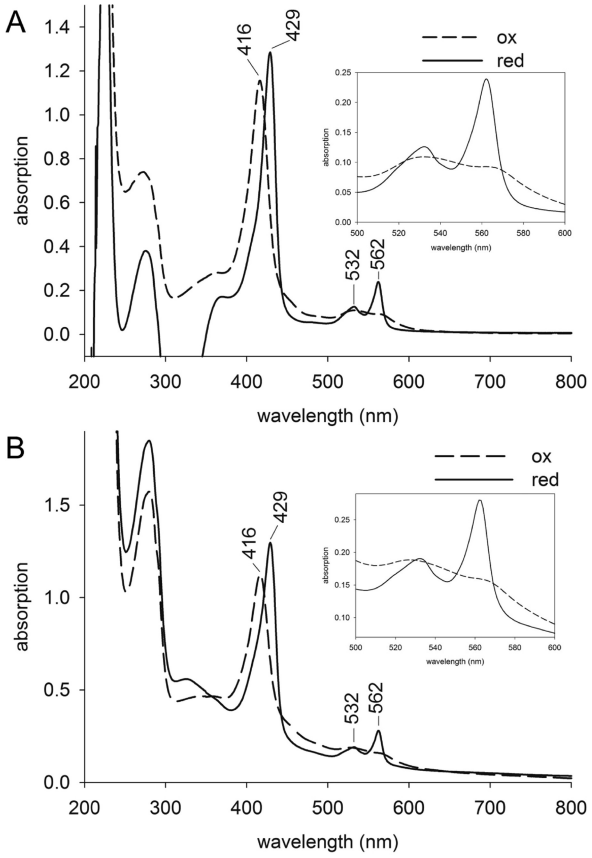

Figure 3. Dithionite and H_2-mediated reduction of the cytochrome *b*. UV/vis spectra of the oxidized "as-isolated" forms (dashed lines; ox) and reduced forms (solid lines; red) of (A) purified HoxZ protein (0.48 mg/mL) and (B) purified heterotrimeric MBH (0.8 mg/mL). Peak maxima of the Soret and α/β-bands are given. HoxZ was reduced by Na-dithionite (A) while the heterotrimeric MBH was reduced by H_2 gas (B).

According to the absorption maximum of the α-band at 562 nm and the NC-IUB guidelines,[30] HoxZ can be assigned as a cytochrome b_{562}.

HoxZ Is Electronically Connected to the Hydrogenase Module. To test whether the cytochrome b_{562} is properly

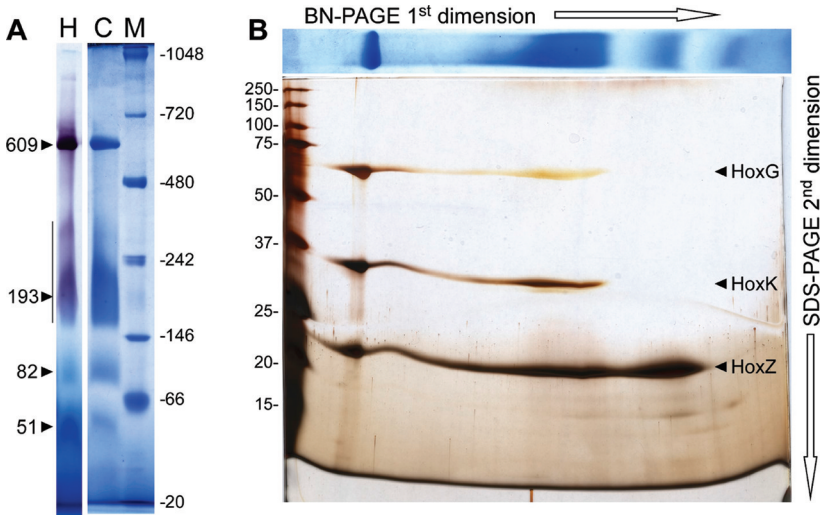

Figure 4. BN-PAGE and 2D-BN/SDS-PAGE analysis of the isolated MBH. (A) BN-PAGE analysis of purified heterotrimeric MBH. 20 μg of protein was loaded for the hydrogenase activity stain (H) and 40 μg for Coomassie stain (C). Molecular masses of the marker (M) and calculated masses of MBH complexes are given in kDa. (B) One lane of the BN-PAGE containing 10 μg of MBH was subjected to an SDS-PAGE as second dimension. The Coomassie stained lane from (A) is depicted on top for comparison. The SDS gel was silver stained. The molecular masses of reference proteins, positions of MBH subunits, and the migration directions of the two gels are indicated.

attached to the HoxGK hydrogenase module upon solubilization with digitonin, the isolated heterotrimeric complex was reduced with H_2 and analyzed by UV/vis spectroscopy (Figure 3B). The spectral changes were essentially identical to those observed for isolated HoxZ (Figure 3A), demonstrating that the hydrogenase module oxidized H_2 and the released electrons were transmitted to the heme groups of HoxZ. This result is consistent with a recent spectroelectrochemical investigation of the heterotrimeric MBH in which the enzyme was immobilized on an electrode and analyzed by resonance Raman spectroscopy.[31] These observations clearly demonstrate that the hydrogenase module is properly connected to the cytochrome b_{562}.

The MBH Heterotrimer Assembles to a High Molecular Mass Complex. To examine the oligomeric state of the MBH, the purified heterotrimeric complex was first subjected to blue native-PAGE (BN-PAGE),[32] and in a second step, blue native/SDS two-dimensional gel electrophoresis (2D BN/SDS-PAGE) was applied (Figure 4). The BN-PAGE revealed four major Coomassie-stained bands (Figure 4A, lane C). The apparent molecular masses were determined as 51, 82, 126–308 (with a peak at 193), and 609 kDa. Hydrogenase activities of the complexes were qualitatively visualized by using an in-gel activity staining assay (Figure 4A, lane H). Bands displaying hydrogenase activity developed purple color that can be easily distinguished from the Coomassie blue-stained background of the BN-PAGE. Most of the hydrogenase activity was found in the 609 kDa band. Enzymatic activity was also observed in the poorly resolved region of 126–308 kDa.

To determine the subunit composition of the various protein complexes (Figure 4A, lane C), the sample was further analyzed by SDS-PAGE in the second dimension (Figure 4B). This analysis clearly showed that the large complex of 609 kDa consists of the three subunits HoxG, HoxK, and HoxZ at almost equimolar stoichiometry. The broad band around 193 kDa uncovered all three subunits of the MBH. However, an unbalanced band ratio indicated the occurrence of disassembled fragments of the large entire complex. Disassembly was probably induced by binding of Coomassie molecules, as commonly observed in BN-PAGE.[33] The BN-PAGE band at 82 kDa was assigned to cytochrome b_{562}. Hence, the preparation contains excess cytochrome b_{562} resulting from the affinity purification via HoxZ. The smallest band at 51 kDa did not show detectable amounts of protein and presumably represents empty detergent micelles that bind Coomassie blue.

In the following approach, size exclusion chromatography (SEC) was used to determine the molecular mass of the MBH complex (Figure 5). The analysis revealed three peaks with

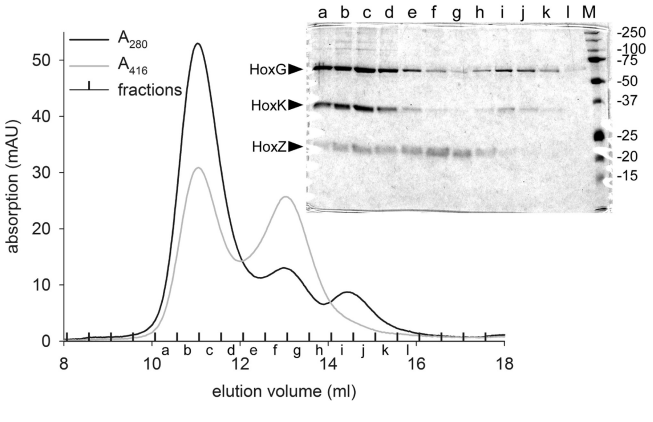

Figure 5. Size exclusion chromatography of the MBH complex. The MBH was separated via size exclusion chromatography. UV/vis absorption at 280 nm (protein) and 416 nm (cytochrome b_{562}) were recorded. The total protein of 200 μL from fractions a–l was precipitated and subjected to SDS-PAGE analysis and Coomassie staining. M = molecular mass marker.

apparent molecular masses of 500, 150, and 90 kDa. Absorption at 416 nm is indicative for the presence of HoxZ. Thus, only the first two peaks contained cytochrome b_{562} (Figure 5, gray trace). Comparison of the absorptions at 280 and 416 nm indicates that the protein content per heme molecule was much higher in the first than in the second peak. This was corroborated by further investigation of the SEC fractions by

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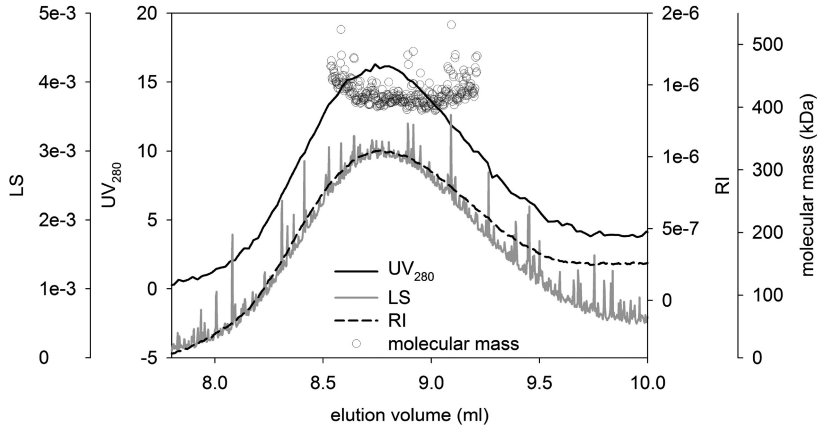

Figure 6. The MBH forms a high molecular mass complex. A fraction from a preparative size exclusion chromatography was subjected to size exclusion chromatography in combination with static light scattering. UV absorption at 280 nm (UV_{280}), the refractive index (RI), and static light scattering (LS) were continuously recorded and used to calculate the molecular mass of the protein complex. The "smiling effect" of the calculated molecular masses results from band broadening between the individual detectors and is frequently observed in SEC-LS analyses.[19]

SDS-PAGE (Figure 5, inset). The first peak (Figure 5, fractions a–c) comprised all three subunits of the MBH while the second peak (Figure 5, fractions f, g) contained only cytochrome b_{562}. Traces of HoxG and HoxK in the latter fractions presumably reflect contaminations due to peak tailing. The third peak (Figure 5, fractions i–k), however, contained exclusively the hydrogenase module HoxGK.

Both BN-PAGE and SEC analyses gave clear evidence for a high molecular mass MBH complex. These methods, however, do not discriminate between protein and detergent content of the analyzed particles. Since the protein:detergent ratio of a given complex is an individual parameter, an additional method that overcomes this problem was applied, namely static light scattering in combination with size exclusion chromatography (SEC-LS).[19] For this purpose the MBH was subjected to a preparative SEC run, and a monodisperse fraction of the first peak containing all three subunits of the MBH was subsequently subjected to SEC-LS (Figure 6). According to the SEC-LS analysis, the molecular mass of the MBH complex was 415 kDa, which is lower than the estimated masses derived from BN-PAGE and SEC. Based on a calculated molecular mass of 130.6 kDa, the data indicate that the heterotrimeric MBH forms a tripartite high molecular mass complex.

The MBH Complex Contains Lipids. Since lipids are often involved in oligomerization of membrane proteins, the isolated MBH complex was analyzed via thin layer chromatography (TLC) for the presence of lipids (Figure 7). Purified lipids from *E. coli* served as reference for identifying the major lipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). According to the TLC analysis, the total lipid extract from *R. eutropha* contained mainly PE and PG lipids. Dissection of the purified MBH sample revealed that PE and PG are also associated with the MBH complex in addition to the detergent digitonin, which can be identified as a slowly migrating strong signal (Figure 7).

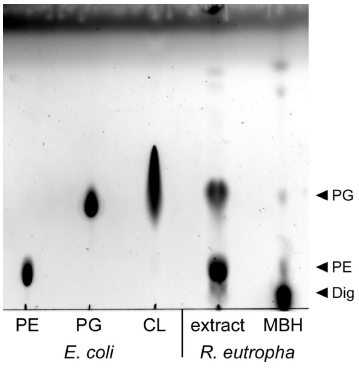

Figure 7. Lipids are bound to the MBH complex. Lipids were extracted from total *R. eutropha* membranes (extract) and from purified MBH protein complexes (MBH) and separated by thin layer chromatography. Pure lipids from *E. coli* served as reference (50 μg each). PE = phosphatidylethanolamine, PG = phosphatidylglycerol, CL = cardiolipin, and Dig = digitonin.

DISCUSSION

Investigations of [NiFe]-hydrogenases often focus on the core protein, a dimeric module consisting of the bimetal cofactor which is deeply embedded in the large subunit and interacts with a minimum of one [FeS] cluster in the small subunit for the intramolecular electron transfer.[34] Activity assays are usually based on the application of artificial electron acceptors and donors, respectively. To get deeper insights into the coupling of the membrane bound hydrogenase of *R. eutropha* with its native electron acceptor a cytochrome b_{562}, the primary goal of this study was screening for an appropriate detergent which allows isolation of a HoxGKZ MBH heterotrimer. Among 21 detergents tested exclusively digitonin proved to be the detergent of choice.

So far it was not possible to determine experimentally which one of the two quinones present in *R. eutropha* membranes, namely menaquinone and ubiquinone Q_8,[35] is utilized by the MBH as natural electron acceptor. The heterotrimeric MBH transfers electrons to menadione as well as ubiquinone Q_0, implying that the MBH can use both quinones *in vivo*. However, it is quite obvious that ubiquinone is preferred by the MBH under aerobic conditions *in vivo* because the menaquinone level is dominant only under oxygen limiting conditions.[36] Furthermore, the midpoint potential of menaquinone is much lower (−74 mV) than the midpoint potentials of the heme groups of HoxZ in the membrane (+10/+166 mV), and menaquinone is noncatalytically oxidized by O_2, which is not the case for ubiquinone.[7,37,38] On the basis of these considerations, we conclude that ubiquinone is the dominant

electron acceptor of the MBH under chemolithoautotrophic growth conditions.

As mentioned above, the homologous MBHs from *Aquifex aeolicus* and *Wolinella succinogenes* have previously been isolated as heterotrimers. This raises the question whether there are major differences between more stable heterotrimeric hydrogenases and enzymes like the MBH and for instance Hyd-1 from *E. coli* that tend to disassemble. According to the crystal structure of the formate dehydrogenase-N from *E. coli*, which contains a cytochrome *b* that is highly similar to several hydrogenase-associated *b*-type cytochromes,[39] complex formation results from hydrophobic interactions as well as an H-bond between H275, located in the C-terminal membrane anchor of the small electron-transferring subunit, and Y171 of the cytochrome *b*.[40] Corresponding amino acid residues (H305, Y202) have been demonstrated to be essential for functionality and probably even structural stability of the MBH from *W. succinogenes*.[15] However, these residues cannot solely account for the differential sensitivity toward certain detergents because these particular amino acids are conserved in the MBH from *R. eutropha* and Hyd-1 from *E. coli*. As indicated above, hydrophobic interactions also contribute to the heterotrimer formation, yet no major difference in the hydrophobicity patterns of the C-terminal membrane anchors of the small hydrogenase subunits was observed (data not shown). Therefore, we suggest that the lipid composition of the hydrogenase complex is involved in maintenance of the quaternary structure and hence plays a significant role in the sensitivity toward certain detergents. In the case of the formate dehydrogenase-N, a cardiolipin molecule was found to be involved in the formation of the heterotrimer.[40] This specific lipid was not detected in the MBH complex; however, the lipids PE and PG discovered in the preparation may take over this adhesive function as reported for other membrane protein complexes.[41] Since these lipids comprise only two fatty acyl chains per molecule rather than four present in cardiolipin, PG and PE might be more prone to the delipidation process, resulting in a less stable complex. Accordingly, hydrogenase I from *A. aeolicus* was shown to be further stabilized in the presence of lipids.[37]

Our data indicate that three heterotrimeric MBH complexes form a tripartite supercomplex which is in line with other membrane protein complexes described previously.[42] The formate dehydrogenase-N from *E. coli* was crystallized as a homotrimer,[40] and since the cytochrome *b* present in this complex shares homology with the cytochrome b_{562} HoxZ, a similar oligomerization pattern is likely for the MBH.

However, the physiological impact of this oligomerization is unclear. There are two possible roles in case of the MBH. First, each cytochrome *b* subunit comprises only one quinone binding site. Upon oligomerization, the individual quinone binding sites are faced toward the hydrophobic core of the membrane whereby a considerable proportion of the protein surface is no longer available for the interaction with quinones, which diffuse freely in the membrane. This increases the probability of productive interactions between quinones and cytochrome-derived binding sites. Second, an electronic cross-talk between the single hydrogenase modules within a trimeric assembly is also conceivable. The recently solved crystal structure of the MBH hydrogenase module shows that the [FeS] cluster in the position distal to the [NiFe] active site is located very close to the protein surface with distances of 5−7 Å.[43] Consequently, in an MBH oligomer, these [FeS] clusters could be located in respect to each other within a physiologically relevant edge-to-edge distance for direct electron tunnelling of 14 Å.[44] Interestingly, the crystal structures of the membrane-bound hydrogenases from *Allochromatium vinosum* and *Hydrogenovibrio marinus* revealed dimers of two hydrogenase modules, which are considered to have biological relevance.[45,46] In these structures, the distal [FeS] centers indeed display edge-to-edge distances of 13.9 and 12.6 Å, respectively. Although our data imply a trimeric rather than a dimeric supercomplex, the crystal structures demonstrate that the distal clusters can be located in close proximity to each other. Whether this is of relevance for the *A. vinosum* hydrogenase is not known, but such an interaction of the hydrogenase modules could be beneficial especially for O_2-tolerant hydrogenases for the reactivation of active sites occupied by oxygen species. The current mechanistic model for O_2 tolerance of certain membrane-bound hydrogenases, including the MBH from *R. eutropha*, relies on the reductive removal of O_2 from the [NiFe] active site.[10,43,47,48] In this scenario, the electrons flow reversibly from the quinone pool to the active site.[4] In a trimeric enzyme complex these electrons could instead be delivered from a neighboring MBH unit that has not been inactivated and delivers electrons derived from H_2 oxidation.

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ABBREVIATIONS

MBH, membrane-bound hydrogenase; HoxZ, cytochrome *b* subunit of the MBH; HoxG, large subunit of the MBH; HoxK, small subunit of the MBH; HoxGK, hydrogenase module of the MBH; DDM, dodecylmaltoside; SEC, size exclusion chromatography; SEC-LS, SEC-light scattering; TLC, thin layer chromatography; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; RI, refractive index; CMC, critical micelle concentration.

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