HupW Protease Specifically Required for Processing of the Catalytic Subunit of the Uptake Hydrogenase in the Cyanobacterium *Nostoc* sp. Strain PCC 7120

Pia Lindberg, Ellenor Devine, Karin Stensjö, and Peter Lindblad

Department of Photochemistry and Molecular Science, The Ångström Laboratories, Uppsala University, Uppsala, Sweden

The maturation process of [NiFe] hydrogenases includes a proteolytic cleavage of the large subunit. We constructed a mutant of *Nostoc* strain PCC 7120 in which *hupW*, encoding a putative hydrogenase-specific protease, is inactivated. Our results indicate that the protein product of *hupW* selectively cleaves the uptake hydrogenase in this cyanobacterium.

The [NiFe] hydrogenases are multisubunit enzymes that undergo an extensive maturation process carried out by an array of accessory proteins. Independent of the number of subunits, the core of the hydrogenases is always made up of a small subunit, containing FeS clusters for electron transport, and a large subunit, which contains the [NiFe] active site. The maturation process for the large subunit has been elucidated most extensively in *Escherichia coli* (1, 6), where a group of enzymes called the Hyp proteins are responsible for assembly of the active site of the hydrogenase, which includes the incorporation of the iron and nickel metals as well as CN and CO ligands. This assembly is followed by proteolytic cleavage of the C-terminal end of the large subunit, performed by a hydrogenase-specific protease. The protease binds to the hydrogenase after insertion of nickel into the active site and cleaves off around 20 amino acids, depending on the type of hydrogenase and strain (12, 21). The cleavage takes place after the conserved amino acid sequence DPCXXCXXH/R, making the histidine or arginine residue the new C terminus (7, 12, 15, 18). For bacterial strains that possess more than one [NiFe] hydrogenase, one set of Hyp proteins may be responsible for maturation of the active site of the different hydrogenases in the cell. For the proteolytic cleavage, however, different proteases that will specifically cleave only one type of hydrogenase are generally present (1).

In cyanobacteria, three key enzymes may be involved in the hydrogen metabolism: nitrogenase, which produces hydrogen as a by-product of nitrogen fixation; a heterodimeric uptake hydrogenase (HupSL), the role of which is to reoxidize hydrogen gas released by the nitrogenase; and a pentameric bidirectional hydrogenase (HoxYHEFU), which can take up or produce hydrogen depending on conditions (14, 19). *Nostoc* sp. strain PCC 7120 is a nitrogen-fixing strain that possesses all three enzymes (10).

Independent of the number of hydrogenases (uptake and/or bidirectional hydrogenases), cyanobacteria usually contain only one copy of the *hyp* genes whose protein products perform the maturation process for the large subunit of both the bidirectional and the uptake hydrogenases (19). This seems not to be the case for genes encoding putative hydrogenase-specific proteases, whose number in a cyanobacterial strain will correspond to the number of hydrogenases (3, 23). *In silico* studies have indicated a specificity among these proteases with respect to the cleavage of different hydrogenases, i.e., one type of hydrogenase-specific protease will cleave only one type of hydrogenase (1, 3, 23), but no *in vivo* studies have been performed in cyanobacteria.

In *Nostoc* PCC 7120, two genes that may encode hydrogenase-specific proteases, *hoxW* and *hupW*, have been identified (23). In order to examine the function of these genes and the respective encoded proteins further, with a focus on the putative specificity of cyanobacterial hydrogenase-specific proteases, we constructed a *hupW* mutant knockout strain of *Nostoc* PCC 7120. This is the first study where the function and specificity of putative hydrogenase specific proteases are investigated in cyanobacteria.

The *hupW* mutant strain of *Nostoc* PCC 7120 was constructed by the introduction of a neomycin (Nm) resistance cassette (from the pUCK4 vector, GenBank accession no. X06404) into *hupW* using standard cloning procedures and transfer of the interrupted gene into *Nostoc* PCC 7120 by triparental conjugation and homologous recombination according to established procedures (2, 5, 9) (Fig. 1A). The cargo vector used was pRL271 (GenBank accession no. L05081). The isolation of a fully segregated *hupW* mutant strain was confirmed by PCR (Fig. 1B) using primers alr1423-F and -R (5'-TGCTGTAGGCGTAATCATCG-3' and 5'-TTTATTTAATTGAGGCGGCG-3', respectively), resulting in a 1,629-bp product corresponding to the insertion of the Nm cassette into the gene *hupW*, while no shorter products were observed. A second primer pair, 2-alr1423-F and -R (5'-CTCGAGCATCACTCAACCCAAAAGCA-3' and 5'-GAGCTCTTGCCAGAGATTGTGCTG-3', respectively), gave similar results.

A hydrogen electrode was used to measure *in vivo* aerobic hydrogen evolution from nitrogen-fixing cultures, grown in BG11₀ medium (17) under continuous illumination (40 $\mu\text{mol photons s}^{-1} \text{ m}^{-2}$) at 25°C, of *Nostoc* PCC 7120 wild type and a *hupW* mutant strain, as previously described (16). Figure 2 shows the signal from the hydrogen electrode in one representative experiment for each strain. It was found that the *hupW* mutant strain released hydrogen gas at an average rate of 3.3 $\mu\text{mol H}_2 \text{ mg chlorophyll } a \text{ (Chl } a)^{-1} \text{ h}^{-1}$ (Table 1), while the wild-type strain released only small amounts of hydrogen (Fig. 2; Table 1). From these results, it is clear that disrupting the *hupW* gene in *Nostoc*

Received 23 June 2011 Accepted 12 October 2011

Published ahead of print 21 October 2011

Address correspondence to Peter Lindblad, peter.lindblad@fotomol.uu.se.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.05957-11

A *Nostoc* sp. strain PCC 7120

FIG 1 Illustration demonstrating the genomic region of the *hupW* gene in *Nostoc* PCC 7120, interrupted by the Nm resistance cassette, and the identification of positive clones by PCR. (A) The *hupW* gene in *Nostoc* PCC 7120, including primers used for PCR verification: 1, alr1423-F/R; 2, 2-alr1423-F/R; and 3, pUC4KF/R. Marked on the picture are also the transcriptional start sites (arrows) and NtcA binding sites, together with $-10/-35$ regions. (B) Result from PCR verification of the *hupW* mutant. The numbers refer to the primers shown in panel A. The gels were loaded in the following order: genomic DNA from *hupW* mutant as template, negative control for the PCR with no genomic DNA, and genomic DNA from wild type (WT) as template.

PCC 7120 had a direct effect on the function of the heterodimeric uptake hydrogenase (HupSL). The function of the uptake hydrogenase is to reoxidize hydrogen produced as a by-product during nitrogen fixation by the nitrogenase (19). When the uptake hydrogenase is inactivated, by interruption of the structural genes as well as the accessory genes, hydrogen will be released into the atmosphere and can easily be detected, as has previously been shown in several species and strains, such as *Anabaena variabilis*, *Nostoc*

FIG 2 *In vivo* hydrogen production in nitrogen-fixing cultures. The figure shows hydrogen electrode measurements of H_2 evolution in nitrogen-fixing cells of wild-type *Nostoc* PCC 7120 and *hupW* mutant strains, in one representative experiment for each strain. Cells were first incubated in the dark for 2 min, and the arrow indicates the time point when light was turned on.

TABLE 1 Hydrogen evolution during nitrogen fixation and bidirectional hydrogenase activities of wild type and the *hupW* mutant strain^a

Strain	Hydrogen evolution during nitrogen fixation ($\mu\text{mol } H_2 / [\text{mg Chl } a \cdot \text{h}]$)	Bidirectional hydrogenase activity ($\text{nmol } H_2 / [\text{mg Chl } a \cdot \text{h}]$) according to growth condition:	
		Non-nitrogen fixing	Nitrogen fixing
<i>Nostoc</i> PCC 7120	0.44 ± 0.08	8.5 ± 2.0	88.4 ± 13.9
<i>Nostoc</i> PCC 7120 <i>hupW</i> mutant	3.31 ± 1.14	14.2 ± 3.3	110.8 ± 10.1

^a Hydrogen evolution was measured using a hydrogen electrode in the light. Bidirectional hydrogenase activities were measured in anaerobically induced cells in the dark, with headspace samples analyzed by gas chromatography. Hydrogen evolution measurements are averages of four cultures with duplicate samples for each culture. Bidirectional hydrogenase activities are averages of two cultures with duplicate samples for each culture. Standard deviations are given.

punctiforme ATCC 29133, *Nostoc* PCC 7120, and *Nostoc* PCC 7422 (8, 11, 13, 24). The rate at which hydrogen is produced in our *hupW* mutant strain is in the same range as the rate measured for a *hupL* mutant strain of *Nostoc punctiforme*, 7 to 14 $\mu\text{mol } H_2 / (\text{mg Chl } a \cdot \text{h})$ (11). Comparisons with the other *hupL* mutant strains mentioned above cannot easily be made, since in those studies, hydrogen produced by the cultures was measured under an argon atmosphere, which will cause much higher hydrogen evolution from the nitrogenase.

In *Nostoc* PCC 7120, it has previously been shown that the bidirectional Hox hydrogenase cannot replace the function of the uptake hydrogenase (13). Instead, it was shown that a *hoxH* mutant in *Nostoc* PCC 7120 will actually produce less hydrogen than the wild type during nitrogen fixation and that the nitrogenase activity, as measured by acetylene reduction activity, will be slightly lower (13). Thus, our results showing enhanced hydrogen evolution from nitrogen-fixing cells of the *hupW* mutant strain, together with previous studies, suggest that this release of molecular hydrogen in the *hupW* mutant strain cannot be coupled to inactivation of the large subunit of the bidirectional hydrogenase but is the result of inactivation of the uptake hydrogenase.

A potential cleavage site for proteolytic processing analogous to the corresponding sites in other organisms has been identified at a position 16 amino acids (aa) from the C-terminal end of the protein in the sequence of *Nostoc* PCC 7120 HupL, immediately after the sequence DSCLVCTVH (23). A cleavage at that position will result in a processed protein with a molecular mass of 58.4 kDa, compared to the full-length, unprocessed HupL, which has a calculated molecular mass of 60.2 kDa. SDS-PAGE followed by Western immunoblotting using standard procedures was used to detect the HupL protein in cell extracts of nitrogen-fixing cultures of both wild-type *Nostoc* PCC 7120 and the *hupW* mutant strain (Fig. 3). Anti-HupL antibodies were produced using a synthetic peptide with the amino acid sequence LAHEDKYQKPTIEGR, corresponding to a part of HupL from *Nostoc punctiforme* ATCC 29133, as antigen for the generation of specific polyclonal antibodies (Agrisera, Sweden).

The immunoblot results show a band in protein extracts from the *hupW* mutant strain that is larger than that in the wild type. The size difference is in agreement with cleavage of an 1.8-kDa

FIG 3 Western immunoblot assay of HupL protein. (Top) Western blot analysis using HupL-specific antibodies. Lane 1 (M), protein molecular mass marker; lane 2, wild-type (WT) *Nostoc* PCC 7120; lane 3, the *hupW* mutant strain. (Bottom) Corresponding SDS-PAGE gel showing equal loading of the lanes. Cell extract containing 1 μ g of Chl *a* was loaded in each lane.

portion of HupL, corresponding to cleavage at the predicted site. This result clearly points to HupW being the protease responsible for cleavage of HupL in *Nostoc* PCC 7120. Furthermore, as seen in Fig. 3 (upper panel), the amount of HupL protein is substantially smaller in the *hupW* mutant strain than in the wild type. This effect could be explained by a lower stability of the unprocessed form of HupL. When the protein is not properly processed, it may be prevented from forming a stable complex with the small subunit HupS and thus may be targeted for degradation to a larger extent than the properly matured HupL in the wild type. In a proteomics study on the previously mentioned HupL[−] strain of *Nostoc punctiforme*, it was observed that when HupL is missing from the cells, the small subunit of the uptake hydrogenase, HupS, is greatly reduced in abundance as well (4). This may be due to a similar degradation on the small subunit in the absence of a complete and stable hydrogenase.

In order to investigate whether inactivation of HupW affects the bidirectional hydrogenase in *Nostoc* PCC 7120, measurements of bidirectional hydrogenase activity were carried out. Methyl viologen was used as an artificial electron donor to the hydrogenase. The assays were performed as previously described (20), with the following modifications: Cells were sparged with pure argon gas for 3 h in the dark before addition of 10 mM methyl viologen and 30 mM sodium dithionite. The amount of evolved hydrogen was determined by gas chromatography using a Perkin-Elmer Clarus 500 gas chromatograph (GC) equipped with a thermal conductivity detector (TCD). In assays using cells harvested from non-nitrogen-fixing cultures, grown in BG11 medium (17), it was found that the *hupW* mutant strain produced amounts of H₂ similar to, or even slightly larger than, those produced by the wild-type *Nostoc* PCC 7120 (Table 1). Experiments were also performed using cultures grown under nitrogen-fixing conditions, resulting in observed activities that were higher than those for non-nitrogen-fixing cultures but still similar between wild type

and the *hupW* mutant strain (Table 1). It is known that the assay used is specific for the bidirectional hydrogenase (10), and thus, the observed activities under nitrogen-fixing conditions cannot stem from the uptake hydrogenase. These results indicate that inactivating HupW has no effect on the bidirectional hydrogenase, and therefore, the HupW activity is specific for the uptake hydrogenase.

By producing a *hupW* mutant in *Nostoc* PCC 7120, we can now examine the function and specificity of hydrogenase-specific proteases in detail in cyanobacteria. It is known from studies in other organisms that a protease performs a proteolytic cleavage on the large subunit of [NiFe] hydrogenases (1, 12, 21). A phylogenetic study has shown that the proteases can be divided into groups comparable to the phylogenetic groups identified for the hydrogenases (3), and that HupW and HoxW, like the uptake and the bidirectional hydrogenase, belong to two different clades within the tree (3, 22). The same study also revealed, by *in silico* studies, a HoxW-specific sequence, the HOXBOX, which may be part of the mechanism(s) behind the observed specificity. The HOXBOX is situated in a part of the protease which is conserved within all hydrogenase-specific proteases. While this conserved part in other groups of hydrogenase-specific proteases consists of the sequence D(G/C)GT, in members of the HoxW group it is replaced by an HQL sequence (aa 42 to 44 in HoxW of *Nostoc* PCC 7120) (3). This is the only reported difference in amino acid levels between groups of hydrogenase-specific proteases. Since the hydrogenase-specific proteases in *Nostoc* PCC 7120 belong to two different groups, with and without this HOXBOX, this strain will be ideal for further studies on the importance of this amino acid sequence for protease function and/or substrate recognition.

This is the first time that the function of a cyanobacterial hydrogenase-specific protease has been demonstrated experimentally. By inactivation of the *hupW* gene, we were able to show that the protein product of *hupW* is specific for the uptake hydrogenase in *Nostoc* PCC 7120. In the *hupW* mutant strain, the uptake hydrogenase is dysfunctional due to an incomplete maturation process, while the bidirectional hydrogenase retains full activity. This study may form the basis for further studies of the substrate recognition (possibly by the HOXBOX) and function of hydrogenase-specific proteases in cyanobacteria.

ACKNOWLEDGMENTS

This work was supported by the Swedish Energy Agency, the Knut and Alice Wallenberg Foundation (project M.SES) and the European Union/Energy FP7 project SOLAR-H2 (contract no. 212508).

REFERENCES

1. Bock A, King PW, Blokesch M, Posewitz MC. 2006. Maturation of hydrogenases. *Adv. Microb. Physiol.* 51:1–71.
2. Cohen MF, Meeks JC, Cai YA, Wolk CP. 1998. Transposon mutagenesis of heterocyst-forming filamentous cyanobacteria. *Methods Enzymol.* 297:3–17.
3. Devine E, Holmqvist M, Stensjö K, Lindblad P. 2009. Diversity and transcription of proteases involved in the maturation of hydrogenases in *Nostoc punctiforme* ATCC 29133 and *Nostoc* sp. strain PCC 7120. *BMC Microbiol.* 9:53.
4. Ekman M, et al. 2011. Metabolic adaptations in a H₂ producing heterocyst-forming cyanobacterium: potentials and implications for biological engineering. *J. Proteome Res.* 10:1772–1784.
5. Elhai J, Wolk CP. 1988. Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol.* 167:747–754.
6. Forzi L, Sawers RG. 2007. Maturation of [NiFe]-hydrogenases in *Escherichia coli*. *Biomaterials* 20:565–578.

7. Gollin DJ, Mortenson LE, Robson RL. 1992. Carboxyl-terminal processing may be essential for production of active NiFe hydrogenase in *Azotobacter-Vinelandii*. FEBS Lett. 309:371–375.
8. Happe T, Schutz K, Bohme H. 2000. Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. J. Bacteriol. 182:1624–1631.
9. Heidorn T, et al. 2011. Synthetic biology in cyanobacteria: engineering and analyzing novel functions. Methods Enzymol. 497:539–579.
10. Houchins JP, Burris RH. 1981. Occurrence and localization of two distinct hydrogenases in the heterocystous cyanobacterium *Anabaena* sp. strain 7120. J. Bacteriol. 146:209–214.
11. Lindberg P, Schutz K, Happe T, Lindblad P. 2002. A hydrogen-producing, hydrogenase-free mutant strain of *Nostoc punctiforme* ATCC 29133. Int. J. Hydrogen Energ. 27:1291–1296.
12. Magalon A, Böck A. 2000. Dissection of the maturation reactions of the [NiFe] hydrogenase 3 from *Escherichia coli* taking place after nickel incorporation. FEBS Lett. 473:254–258.
13. Masukawa H, Mochimaru M, Sakurai H. 2002. Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp PCC 7120. Appl. Microbiol. Biotechnol. 58:618–624.
14. McIntosh CL, et al. 2011. The [NiFe]-hydrogenase of the cyanobacterium *Synechocystis* sp. PCC 6803 works bidirectionally with a bias to H₂ production. J. Am. Chem. Soc. 133:11308–11319.
15. Menon NK, et al. 1993. Carboxy-terminal processing of the large subunit of [NiFe] hydrogenases. FEBS Lett. 331:91–95.
16. Oxelfelt F, Tamagnini P, Salema R, Lindblad P. 1995. Hydrogen uptake in *Nostoc* strain PCC 73102—effects of nickel, hydrogen, carbon and nitrogen. Plant Physiol. Biochem. 33:617–623.
17. Rippka R, et al. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111:1–61.
18. Rossmann R, Sauter M, Lottspeich F, Böck A. 1994. Maturation of the large subunit (Hyce) of *Escherichia coli* hydrogenase 3 requires nickel incorporation followed by C-terminal processing at Arg537. Eur. J. Biochem. 220:377–384.
19. Tamagnini P, et al. 2007. Cyanobacterial hydrogenases: diversity, regulation and applications. FEMS Microbiol. Rev. 31:692–720.
20. Tamagnini P, et al. 1997. Hydrogenases in *Nostoc* sp. strain PCC 73102, a strain lacking a bidirectional enzyme. Appl. Environ. Microbiol. 63:1801–1807.
21. Theodoratou E, Huber R, Böck A. 2005. [NiFe]-hydrogenase maturation endopeptidase: structure and function. Biochem. Soc. Trans. 33:108–111.
22. Vignais PM, Billoud B, Meyer J. 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol. Rev. 25:455–501.
23. Wüschiers R, Batur M, Lindblad P. 2003. Presence and expression of hydrogenase specific C-terminal endopeptidases in cyanobacteria. BMC Microbiol. 3:8.
24. Yoshino F, Ikeda H, Masukawa H, Sakurai H. 2007. High photobiological hydrogen production activity of a *Nostoc* sp. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. Mar. Biotechnol. 9:101–112.