Cyanobacterial H_2 Metabolism: Knowledge and Potential/ Strategies for a Photobiotechnological Production of H_2

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ABSTRACT

Molecular hydrogen is an environmentally clean source of energy that may be a valuable alternative to the limited fossil fuel resources of today. For photobiological H_2 production, cyanobacteria are among the ideal candidates since they have the simplest nutritional requirements: they can grow in air (N_2 and CO_2), water (electrons and reducing agents), and simple mineral salts with light as the only source of energy. In N_2-fixing cyanobacteria, H_2 is mainly produced by nitrogenases, but its partial consumption is quickly catalyzed by a unidirectional uptake hydrogenase. In addition, a bidirectional (reversible) enzyme may also oxidize some of the molecular hydrogen. Filamentous cyanobacteria have been used in bioreactors for the photobiological conversion of H_2O to H_2. However, the conversion efficiencies achieved are low because the net H_2 production is the result of H_2 evolution via a nitrogenase and H_2 consumption mainly via an uptake hydrogenase. Consequently, the improvements of the conversion efficiency are achieved through the optimization of the conditions for H_2 evolution by the nitrogenase and through the production of mutants deficient in H_2 uptake activity. Symbiotic cells are of fundamental interest since they *in situ* "function as a bioreactor", possess a high metabolism and there is transfer of metabolite(s) from symbiont to host, but almost no growth. This communication presents the general knowledge about hydrogen metabolism/ hydrogenases in filamentous cyanobacteria, outline strategies for improving the capacity of H_2 production by filamentous strains, and stresses the importance of international cooperations and networks.

Keywords: biotechnology, cyanobacteria, H_2 evolution/uptake, hydrogenase, *Nostoc*

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RESUMEN

El hidrógeno molecular constituye una fuente de energía limpia y una alternativa potencial frente a los limitados recursos de combustibles fósiles. Las cianobacterias son candidatos ideales para la producción fotobiológica de H_2, ya que tienen los requerimientos nutricionales más simples: pueden crecer en aire (N_2 y CO_2), agua (electrones y agentes reductores) y sales minerales, con la utilización de la luz solar como única fuente de energía. En las cianobacterias fijadoras de N_2, el H_2 es producido fundamentalmente por nitrogenasas. Sin embargo, su consumo parcial es rápidamente catalizado por una hidrogenasa unidireccional. Además, una enzima bidireccional (reversible) también puede oxidar parte del H_2 molecular. Las cianobacterias filamentosas han sido empleadas en biorreactores para la conversión fotobiológica de H_2O en H_2. Sin embargo, las eficiencias de conversión logradas han sido bajas debido a que la producción neta de H_2 resulta de la evolución de H_2 a través de una nitrogenasa y el consumo de H_2 fundamentalmente a través de una hidrogenasa. En consecuencia, para incrementar la eficiencia de conversión se requiere, por un lado, la optimización de las condiciones de evolución de H_2 por la nitrogenasa y, por otro, producir mutantes deficientes en la actividad de asimilación de H_2. Las células simbióticas son de especial interés porque "funcionan como biorreactores" *in situ*, presentan un metabolismo intenso y hay transferencia de uno o más metabolitos del simbionte al hospedero sin crecimiento prácticamente. Este trabajo presenta una revisión sobre el conocimiento general que se tiene del metabolismo del H_2 y las hidrogenasas en cianobacterias filamentosas, destaca estrategias para mejorar la capacidad de producción de H_2 por cepas filamentosas, y hace énfasis en la importancia del intercambio y la cooperación internacionales.

Palabras claves: biotecnología, cianobacterias, evolución/consumo de H_2, hidrogenasa, *Nostoc*

Introduction

Molecular hydrogen is a future energy source/carrier that may be a valuable alternative to the limited fossil fuel resources of today. Its advantages as fuel are numerous: it is environmentally clean, efficient, renewable, and during its generation e.g. no CO_2 and at most only small amounts of NOx are produced. An attractive possibility is the direct splitting of water for the generation of H_2 using solar radiation. This splitting can be achieved either in photochemical fuel cells, or by applying photovoltaics, which directly utilizes solar radiation for electrolysis of water into H_2 and O_2. The third and most challenging option is the production of hydrogen by photosynthetic microorganisms. For photobiological H_2 production, cyanobacteria are among the ideal candidates since they have minimal nutritional requirements: they can thrive on air (N_2 and CO_2), water (electrons and reducing agents) and mineral salts, with light as the only energy source. Cultivation is therefore simple and relatively inexpensive. Filamentous cyanobacteria may contain at least three enzymes directly involved in H_2 metabolism: a) a nitrogenase, evolving H_2 during N_2 fixation; b) an uptake hydrogenase, reutilizing this H_2; and c) a bidirectional (reversible) hydrogenase [1-5].

Photosynthetic microorganisms will, under natural conditions, produce (and evolve) no or very small amounts of H_2. Through specific incubations and/or treatments, a substantial induction of H_2 production

may occur. Previous studies using small scale bioreactors demonstrated a capacity for photoproduction of H_2 by several filamentous heterocystous cyanobacteria. However, the conversion efficiencies are low. In order to achieve significant H_2 production rates over a long period, the following needs to be considered: 1) the strains used must be selected for their specific hydrogen metabolism, 2) the selected strains must be genetically engineered in order to produce large amounts of H_2 and 3) the overall conditions for cultivation in bioreactors must be improved. The potential, problems, and prospects of H_2 production by cyanobacteria/hydrogen biotechnology have recently been reviewed [1-5].

The structural genes coding for hydrogenases have been sequenced and characterized in many microorganisms representing several different taxonomic groups [6]. However, molecular studies concerning cyanobacterial hydrogenases are scarce. In 1995, a developmental genome rearrangement for *Anabaena* sp. strain PCC 7120 was described [7]. It is present in addition to the known *nifD* [8] and *fdxN* [9] rearrangements also taking place during the differentiation of a photosynthesizing vegetative cell into a nitrogen-fixing heterocyst. This third rearrangement occurs within a gene *(hupL)* that exhibits homology to genes coding for the large subunits of membrane-bound uptake hydrogenases. A 10.5 kbp element is excised late in the heterocyst differentiation process, indicating that the gene encoding HupL in *Anabaena* sp. PCC 7120 is expressed in heterocysts only [7].

The bidirectional/reversible hydrogenase catalyzes both H_2 production and consumption [1-4]. It is believed to be a common cyanobacterial enzyme, and its presence is not linked to nitrogenase. The structural genes *(hox)* coding for a bidirectional hydrogenase have been sequenced in *Anabaena variabilis* [10] and in the unicellular non N_2-fixing *Anacystis nidulans* [11]. Nucleotide sequence comparisons showed that there is a high degree of homology between the *hox* genes of cyanobacteria and the genes coding for the NAD^+-reducing hydrogenase from the chemolithotrophic H_2-metabolizing bacterium *Alcaligenes eutrophus,* as well as methyl viologen-reducing hydrogenases from species of the archaebacterial genera *Methanobacterium, Methanococcus* and *Methanothermus*. Moreover, the sequence of a $NADP^+$-reducing hydrogenase operon of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 has been determined [12].

The present communication will discuss the general knowledge about hydrogen metabolism/hydrogenases in filamentous cyanobacteria, outline strategies for improving the capacity of H_2 production by filamentous strains, and stress the importance of international cooperations and networks.

Nostoc punctiforme strain PCC 73102 as the model organism

At present, the authors' group concentrates its studies on the filamentous heterocystous cyanobacterium *Nostoc* sp. strain PCC 73102, a free-living strain originally isolated from coralloid roots of the Australian cycad *Macrozamia*. *Nostoc* sp. strain PCC 73102 is proposed as the type strain of the species *Nostoc punctiforme* in the Pasteur's Culture Collection (Paris, France). It is very important to describe and characterize in detail all hydrogenases and H_2 metabolism, including regulations, in a particular cyanobacterial strain. With this knowledge, further molecular experiments, e.g. the construction of specific mutants with desired hydrogen metabolism, can be both performed and correctly evaluated. Growth conditions and some additional strains used in these studies have been discussed [13]. Immunological, physiological and molecular experiments for the analysis of cyanobacteria have been described previously [13-16].

Number of hydrogenases—immunological studies

Immunological studies [14] using polyclonal antisera directed against hydrogenases purified from *Bradyrhizobium japonicum, Azotobacter vinelandii, Methanosarcina barkeri* and *Thiocapsa roseopersicina* demonstrated the presence of two native enzymes/isoforms in N_2-fixing cells of *Nostoc* sp. strain PCC 73102, with at least one common subunit of approximately 58 kDa. Moreover, two additional polypeptides with molecular masses of about 34 and 70 kDa were recognized with some of the antisera used. The antigens were localized in both the N_2-fixing heterocysts and the photosynthetic vegetative cells, with considerable higher antigen content in the latter cell type.

Uptake hydrogenase

Nostoc sp. strain PCC 73102 has the capacity to take up atmospheric hydrogen [15]. This uptake is stimulated by light, and positively regulated by the substrate (added either directly as H_2 from a cylinder or indirectly through the action of the nitrogenase). Additionally, the *in vivo* nitrogenase and hydrogen uptake activities appear to be co-regulated when exposing nitrogen-fixing cells to either combined nitrogen or organic carbon sources [16]. We have cloned and sequenced two overlapping fragments together encoding a complete *hupSL* homologue, with upstream and downstream flanking regions. *Nostoc* PCC 73102 *hupS* and *hupL* encode two proteins with calculated molecular masses of 34 917 and 60 157 Da, respectively. This correlates with the polypeptides recognized in the immunological study [14]. In Southern blot hybridizations using both nitrogen-fixing and non-fixing cells of *Nostoc* PCC 73102 and different probes from within *hupL,* it was clearly demonstrated that in contrast to *Anabaena* PCC 7120, there is no rearrangement within *hupL*. Moreover, the non-coding region between *hupS* and *hupL* is longer in *Nostoc* PCC 73102 as compared to *Anabaena* PCC 7120 and most other microorganisms. A general comparison of the uptake hydrogenase sequences shows that *Nostoc* PCC 73102 and *Anabaena* PCC 7120 are very similar but that they differ considerably in relation to other microorganisms. Recent transcriptional studies using RT-PCR indicate a regulation on the gene [17].

Bidirectional hydrogenase

Using both molecular and physiological techniques no evidence for either *hox* genes, or corresponding bidirectional enzyme activities in *Nostoc* sp. strain PCC 73102 were found. The same techniques clearly showed the presence of a bidirectional enzyme and corresponding structural genes in *N. muscorum, Anabaena* sp. strain PCC 7120 and *A. variabilis* [13].

Hyp genes

Hydrogenases contain nickel in their active site, and the maturation of the individual subunits is believed to be nickel-dependent. In *Nostoc* sp. strain PCC 73102, it was possible to demonstrate a stimulatory effect of niquel ions on the *in vivo*, light-dependent H_2-uptake activity [15]. In *Anabaena* PCC 7120, a cluster consisting of the *hupB, A, E* and *D* genes homologous to the corresponding regulatory *hyp* genes known in other bacteria, was identified upstream of *hupSL*, but nothing is known about their functions. Taking into account that hydrogenase-related genes are often found in the neighborhood of genes encoding functional hydrogenases, two genes have been cloned revealing striking homology to *hypF* and *hypC* in other bacteria using parts of the *hupSL* genes as probes to screen a cosmid library of *Nostoc* PCC 73102 DNA (Hansel A, Lindblad P; unpublished). An almost complete open reading frame (ORF) encoding a *hypF* homologue (ca. 750 aa), followed by a *hypC* homologous ORF (expected size approximately 95 aa) starting 90 bp downstream, were detected on the 30-kbp insert of one cosmid. Homologues to *hypF* and *hypC* are present in organisms such as *Rhodobacter capsulatus, Escherichia coli, Azotobacter, Methanococcus, Alcaligenes eutrophus, Rhizobium leguminosarum, Bradyrhizobium japonicum* [18-26], and also in the *Synechocystis* PCC 6803 genome. In this unicellular cyanobacterium, the *hyp* genes are not clustered, and no *hup* genes encoding an uptake hydrogenase are present. The organization of *hypF* and *hypC* resembles that in *R. leguminosarum* and *B. japonicum*, where these genes are part of the *hypABFCDE* operon following the *hupSLCD(E)F-GHIJK* gene cluster. The N-terminus of the HypF sequence includes two typical zinc finger motifs [C-X2-C-X18-C-X2-C], which are interspersed by 24 aa. These two motifs are more likely to be involved in nickel binding. The products of *hypF* and *hypC* may play a role in the synthesis, processing and/or insertion of metal clusters in the active center of most hydrogenases.

Biotechnological potential

Nostoc sp. strain PCC 73102 seems to be an unusual cyanobacterium. Both a nitrogenase [15, 27] and an uptake hydrogenase [15-17] are clearly present in the cells, whereas there is no evidence for the presence of a bidirectional enzyme [13]. In addition, this strain *in situ* exhibits a high metabolic activity with almost no growth—a natural bioreactor. A *hupL*$^-$ mutant has been described in *Rhodobacter* [28], and other *hup* mutants used in biotechnological experiments [28-31]. We are presently constructing mutants lacking a functional *hupL* gene. Such a mutant, compared to the wild type, could give an insight into the role of the uptake hydrogenase in *Nostoc* sp. PCC 73102. From a biotechnological point of view, the mutant(s) should produce H_2 through the action of the nitrogenase, should have no functional uptake hydrogenase, and reveal no potential uptake or regulatory effect(s) by a bidirectional enzyme.

Strategies for improving cyanobacterial strains for H_2 production

Additional strains

Thorough studies on H_2 uptake and/or evolution have until now focused on only a few filamentous cyanobacteria, e.g. different *Anabaena* and *Nostoc* strains. However, other cyanobacteria (e.g. *Oscillatoria)* are able to fix N_2 without forming heterocysts with the strategy of time-separating the O_2-sensitive nitrogen fixation and the O_2-evolving photosynthesis. Such strains deserve a thorough examination concerning their H_2 metabolism. Considering the versatility of cyanobacteria and their ability to survive under many different environmental conditions, more strains originating from different habitats have to be studied with respect to their applicability in biohydrogen production. Of specific interest might be isolates originating from nitrogen-fixing associations. The situation of the symbiotic cyanobacteria is similar to steady state cultures in bioreactors: cells almost do not grow, they have a high nitrogen fixation and thus a high H_2 production rate, and they export metabolite(s) to the host.

The author's group is presently screening numerous cyanobacterial strains (obtained from a broad variety of sources) for the presence of DNA sequences similar to *Anabaena* sp. strain PCC 7120 and *Nostoc* sp. strain PCC 73102 *hup* genes (uptake hydrogenase) and *Anabaena variabilis* ATCC 29413 *hox* genes (bidirectional hydrogenase). DNA sequences similar to *hup* genes seem to be present in all N_2-fixing strains tested, while DNA sequences similar to *hox* genes have an irregular pattern of occurrence/absence (Tamagnini P, Lindblad P; unpublished).

Genetic engineering

Genetic engineering has become possible with the establishment of molecular biology tools and techniques for cyanobacteria. A few unicellular strains, including *Synechoccus* PCC 6301 and PCC 7942 as well as *Synechocystis* PCC 6803, are naturally transformable. Protocols and vector systems useful for the transfer of DNA into different cyanobacteria are available for non-transformable strains. These methods have been successfully used with filamentous genera such as *Anabaena* and *Nostoc,* which might be interesting candidates for future photobiotechnological applications [32].

Several strategies are available for improving existing cyanobacterial strains for the biotechnological production of H_2. Inactivation of a gene encoding an uptake hydrogenase might lead to mutants that are not able to recycle the H_2 evolved by the nitrogenase under N_2-fixing conditions. As a consequence, the H_2 produced through the action of a nitrogenase will either be oxidized by some other hydrogenase or, if the latter is not present, will evolve from the cells. The absence of a bidirectional enzyme in *Nostoc* PCC 73102 makes it an interesting candidate for such inactivation experiments. Identification/engineering of an oxygen-stable H_2-evolving hydrogenase might result in a photosynthesizing microorganism evolving H_2. Moreover, overproducing mutants might be obtained by providing genes encoding a selected hydrogenase on a suitable expression vector. Coupling the genes to a promoter of a gene strongly expressed in heterocysts, such as the *nif* genes, might lead to an increased amount of the hydrogenase and thus increased levels of H_2 production in the organism. Similarly, overexpression might also be used for increasing the nitrogenase activity. A thorough examination of the genes involved in the regu-

lation of hydrogenase expression might generate knowledge leading to further strategies for improving H_2 production rates in cyanobacteria. In the completely sequenced genome of *Synechocystis* PCC 6803, several ORFs homologous to regulatory genes of other bacterial hydrogenases were identified. However, nothing is known about their interaction with the structural genes in this strain or in any other cyanobacterial strain.

International cooperation/networks

At present, two major initiatives can be recognized [1]. In the international program of IEA (http://www.iea.org), Hydrogen Implementing Agreement, Annex 15: "Photobiological hydrogen production", the main objectives are to investigate and to develop processes and equipment for the production of hydrogen by direct conversion of solar energy. In the European program COST 8.41 "Biological and Biochemical Diversity of Hydrogen Metabolism" (http://www.h2-ase.szbk.u-szeged.hu), the main objective is to pool interrelated European expertise in order to understand the molecular basis of the functions, as well as the factors that influence the activity and stability of hydrogenase enzymes. For further information see reference 1.

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