NITROGENASES AND HYDROGENASES IN CYANOBACTERIA

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A. Nitrogenases

Apart from the conventional, Mo-containing nitrogenase, the cyanobacterium *Anabaena variabilis* can express at least two alternative N_2-fixing enzyme complexes. This cyanobacterium grows with V in a Mo-deficient medium. The nitrogenase then expressed reduces C_2H_2 partly beyond C_2H_4 to C_2H_6 and produces more H_2 than the Mo-enzyme (Kentemich et al., 1988; Yakunin et al., 1991). Genes for this V-nitrogenase, *vnf*DGK (Thiel, 1993) and *nif*B (commonly used for both nitrogenases) (Lyons and Thiel, 1995) have subsequently been cloned, mapped and sequenced. It is generally agreed that *A. variabilis* contains a V-nitrogenase. In addition, there is physiological evidence that *A. variabilis* also contains an Fe-only nitrogenase (Kentemich et al., 1991). After an extensive state of N-deprivation where the cells totally bleached due to the utilization of phycobilins as N-reserve, *A. variabilis* restarted to grow slowly. The cultures reduced C_2H_2 partly to C_2H_6 and produced H_2 with a high rate. Determination by atomic absorption spectrometry showed that the concentration of Mo in the medium was <10 nM and of V <20 nM and likely too low for the expression of a Mo- or a V-nitrogenase. The cells showed an outburst in C_2H_6- and H_2-formations 3 h after the addition of Mo to the medium which typically happens with the Fe-only hydrogenase from *Azotobacter vinelandii* (Pau et al., 1989). Hybridizations of the *anf*H and *nif*H probes with genomic DNA from *A. variabilis* gave at least two distinct bands (Kentemich et al., 1991). Supporting evidence for the existence of an Fe-only nitrogenase came from Ni et al., (1990). The gene set coding for this enzyme complex has, however, not yet been found which leaves, of course, doubts about the meanings of these physiological results.

A new development in the field has recently been forwarded independently by two groups (Thiel, 1994; Schrautemeier, 1994; Schrautemeier et al., 1995). Concomitantly with a second copy of the *fdx*H gene (its product probably is the immediate electron carrier to nitrogenase), a second gene set, termed *nif*HDK2, could be detected in *A. variabilis*. These genes *fdx*H2 and *nif*HDK2 are transcribed exclusively under anaerobic conditions, early (< 4 h) after N-depletion in vegetative cells and apparently

independently of heterocyst differentiation. With respect to its O_2-sensitivity, this alternative nitrogenase resembles the enzyme complex of the filamentous, non-heterocystous *Plectonema boryanum*. In *A. variabilis*, the expression of this alternative nitrogenase is apparently independent of a Mo-depletion in the medium, and the gene cluster apparently does not contain the G gene. Therefore this alternative nitrogenase is suggested to contain Mo in the active center, although it has not been characterized biochemically. Its discovery is somewhat surprising, since earlier experiments with *A. cylindrica* using nitrogenase antibodies (Murry et al., 1984) and with *Anabaena* PCC 7120 utilizing promoter$_{nif}$HDK-*lux*AB reporter fusions (Elhai and Wolk, 1990) indicated the location of nitrogenase exclusively in heterocysts. With respect to its N_2-fixing capabilities, *A. variabilis* could well be totally different from other studied cyanobacteria (*A.* 7120, *Anabaena cylindrica*), since the V-nitrogenase also occurs only in *A. variabilis* (Kentemich et al., 1988) and presumably in the *Anabaena* sp. isolated from the fern *Azolla* (Thiel, 1993).

B. Hydrogenases

Despite the fact that isolated nitrogenases from all organisms produce H_2 with substantial rates, no or marginal net productions are generally observed in experiments with intact cyanobacteria. This is due to the fact that the H_2 evolved by nitrogenases is immediately recycled by hydrogenases. Cyanobacteria possess at least two different of such enzymes catalyzing the reaction $H_2 \Rightarrow 2\ H^+ + 2\ e^-$. One enzyme is called uptake hydrogenase, as it catalyzes only H_2-consumption with methylene blue (= MB) or phenazine methosulfate (= PMS) as electron acceptor, but virtually not the evolution of the gas. It is particularly (Tel-Or et al., 1978; Eisbrenner et al., 1981), if not exclusively (Peterson and Wolk, 1978), active in heterocysts of cyanobacteria. Independent evidence by Peschek (1979) and Eisbrenner et al. (1978), however, indicated that an uptake hydrogenase also occurs in the unicellular, non-N_2-fixing *Anacystis nidulans* (= *Synechococcus leopoliensis*). Currently it is not clear whether *Anabaena* and *Anacystis* contain two unrelated uptake hydrogenases, since these enzymes have not been characterized biochemically.

Uptake hydrogenase both in heterocysts and in unicellular forms is an integral thylakoid membrane protein. The enzyme feeds in electrons from H_2 either to a cytochrome (b or c) or directly to plastoquinone. The electrons are then transferred to the cytochrome b,f complex commonly utilized for photosynthesis and respiration on the thylakoids and are then allocated to either the respiratory complex IV or photosystem I (Eisbrenner et al., 1981). The enzyme is activated by the thioredoxin system on the thylakoids (Dai et al., 1992).

Attempts to find the genes coding for uptake hydrogenase by heterologous DNA-probing or by PCR consistently failed in the past. The genes have recently been found per chance in a screening program for DNA that undergoes rearrangement during heterocyst differentiation and prior to expression (Matveyev et al., 1994; Carrasco et al., 1995) similar to *nif* genes in *Anabaena* 7120. Late during heterocyst differentiation, a 10.5 kb

DNA segment is excised between 16 bp direct repeats that flank the element within the *hup*L gene coding for the larger subunit of this [NiFeS]-hydrogenase. The site specific recombinase XisC is encoded on this 10.5 kb element. The genes *hup*L and partly *hup*S have been sequenced (Carrasco et al., 1995).

Cyanobacteria possess at least a further hydrogenase, the so-called reversible or bidirectional enzyme, catalyzing both H_2-uptake (with PMS or MB) and the $Na_2S_2O_4$ and methylviologen (=MV)-dependent evolution of the gas (Houchins, 1984, Papen et al., 1986). This enzyme occurs both in heterocysts and vegetative cells of heterocystous cyanobacteria and also in unicellular forms. The enzyme is not activated by thioredoxin but its activity levels are enhanced by anaerobic incubation of the cells. Immunogold-labeling experiments with antibodies raised against the enzyme from *A. nidulans* indicated its location at or close to the cytoplasmic membrane (Kentemich et al., 1989). As the H^+-gradient is directed outwards in cyanobacteria and as the enzyme has a high affinity for H_2, it probably serves in recycling the gas at the periplasmic face of the cytoplasmic membrane.

The gene set coding for the bidirectional hydrogenase has now been localized on a 8.9 kb segment from *A. variabilis* (Schmitz et al., 1995). The cluster contains the two structural genes *hox*H and *hox*Y of this NiFeS-hydrogenase which, surprisingly, show strong homologies to the corresponding genes of the $NAD(P)^+$-reducing hydrogenases from *Alcaligenes eutrophus, Desulfovibrio fructosovorans* and the MV-reducing enzymes from archaebacteria. The 8.9 kb segment also contains the two genes *hoxU* and *hox*F genes coding for the diaphorase part of the $NAD(P)^+$-reducing hydrogenases. This gene cluster is interspersed with two open reading frames and with an 0.95 kb apparently non-coding region in an unusual way. Their function, if any, and the transcription units in this gene cluster have not yet been elucidated. The findings that *A. variabilis* (and also *A. PCC 7120, A. cylindrica* and *Anacystis nidulans*) possess the genes coding for such a hydrogenase is somewhat surprising, since extracts from cyanobacteria have never been described to catalyze a NAD(P)H-dependent H_2-evolution or a H_2-dependent reduction of $NAD(P)^+$ and since these organisms do not grow photoautotrophically with H_2 as reductant. Both NAD(P)H-dependent H_2-evolution (Schmitz et al., 1995) and H_2-uptake with $NAD(P)^+$ (Schmitz, unpublished) could now be demonstrated in extracts from *Anacystis nidulans*. Protein sequences of digests obtained from the purified bidirectional hydrogenase from *A. nidulans* matched with amino acid sequences translated from the DNA codons of the A. *variabilis* gene, leaving no doubt that the 8.9 kb segment encoded the bidirectional hydrogenase (Schmitz et al., 1995). These findings rule out earlier suggestions that the electron acceptor for this hydrogenase is a low potential cytochrome (Morand et al., 1994).

Cyanobacteria might possess further hydrogenases. As said, the uptake enzymes from heterocysts and from the unicellular *A. nidulans* are presumably not related to each other. G. Smith and coworkers (Ewart and Smith, 1989, Ewart et al., 1990) characterized a soluble hydrogenase from *A. cylindrica*. The larger subunit (50 kDa protein) was reported to catalyze the dithionite and MV dependent H_2-evolution without the

involvement of the smaller subunit which would be rather unusual for hydrogenases. The smaller subunit (42 kDa protein) was reported to catalyze the tritium exchange reaction, also alone. The gene coding for this smaller subunit was found to represent a new class of hydrogenases (Wu and Mandrand, 1993). Recent evidence suggest that the 42 kDa protein has sequence homologies to amino acid transferases (de Zoysa and Danpure, 1993; Ouzonis and Sander, 1993). The tritium exchange reaction may represent an interesting artificial side reaction of this enzyme otherwise unrelated to hydrogenases.

C. Cyanobacterial H_2-formations and potential applications in solar energy conversions

There is continuous interest to exploit cyanobacteria for solar energy conversion programs. Cyanobacteria have the simplest nutrient requirements in Nature. They thrive on photosynthetically in simple inorganic salt media and can meet their N-demand by N_2-fixation. It is the hope that the photosynthetically generated "reducing power" can be coupled to hydrogenases or nitrogenases to efficiently produce molecular H_2. Up-to date, formations by cyanobacteria are usually low, perhaps 1 µmol/(h x mg dry weight of cells) (Morand et al., 1994). Rates can partly be enhanced by immobilizing the cells (Gisby et al., 1987). However, the system suffers from its inherent instability, since H_2-production proceeds maximally few days. In addition, the solar enery conversion efficiency is generally under 1 %. Striking exceptions to this have been reported for the marine strain Miami BG7 (Mitsui and Kumazawa, 1977) and for the non-heterocystous *Lyngbya* (Kuwada et al., 1988) which showed high and sustained H_2-productions with a solar energy conversion efficiency of some 8 %. It is not yet clear whether any biological material can ever compete with the photovoltaic or any other established system in the generation of fuels.

In the isolates just mentioned and also in all other generally studied cyanobacteria (*A. variabilis, A.. cylindrica, A.* 7120), all H_2-formations come from nitrogenases. The maximal rate reported for H_2-formation by hydrogenase in *Anabaena* was 2 µmol x h^{-1} and g dry $weight^{-1}$ (Hallenbeck et al., 1981) and thus extremely low. The situation may, however, be different in *Oscillatoria limnetica* where H_2S can serve as the photosynthetic electron donor (Belkin and Padan, 1978). H_2-production by fermentation has also been reported to occur in some cyanobacteria (Heyer et al., 1989; Heyer and Krumbein, 1991; van der Oost et al., 1989). The physiological function may be to dispose of excess of reductant under strict anaerobic conditions. It is not yet clear whether the pathway of this formation involves pyruvate:formate lyase and formate:hydrogen lyase as in the Enterobacteriaceae. Alternatively, H_2 could be generated from NAD(P)H and the bidirectional hydrogenase. The rates of this NAD(P)H-dependent formation in crude extracts from *A. nidulans* were, however, only about 1 % of the $Na_2S_2O_4$ and MV dependent activity (Schmitz et al., 1995) and thus very low. Clearly, H_2-production by fermentation in cyanobacteria cannot be exploited for solar energy conversion. It had been pointed out earlier that it is energetically more favourable to ferment carbohydrates to CH_4 than to H_2 (Bothe, 1982).

To maximize H_2-photoproductions catalyzed by nitrogenase in the autotrophic cyanobacteria, genetic manipulations of the H_2-utilizing hydrogenases seem to be prerequisital. Both the bidirectional hydrogenase of the cytoplasmic membrane and the uptake hydrogenase of the thylakoids appear to function in the utilization of the gas. Recent work with hydrogenase mutants from *A. variabilis* (Mikheeva et al., 1995) showed that H_2-productions can, indeed, be enhanced by affecting these enzymes.

Acknowledgements. The authors are indebted to B. Schrautemeier, Bonn, for communicating his results on the second Mo-nitrogenase prior to acceptance of his paper. Our work was supported by the Bundesministerium für Forschung and Technology, both by grants to H. B. and for a cooperative work between the Moscow State University (Prof. S. Shestakov) and the Cologne laboratory.

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