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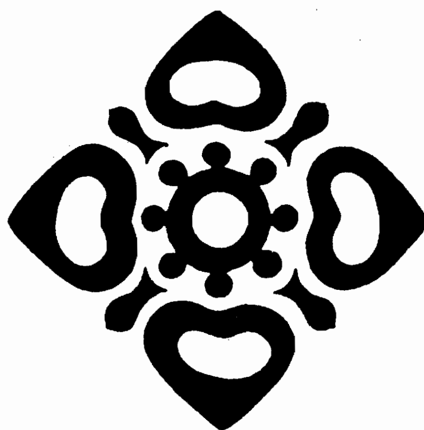

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Molecular biological aspects of the nitrogenase-hydrogenase relationship

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The cyanobacterium *Anabaena variabilis* can express three different nitrogenases. Under normal growth conditions, in the presence of sufficient amounts of Mo in the medium, cells catalyze the reaction: $8\ H^+ + 8\ e^- + N_2 = 2\ NH_3 + H_2$ by the conventional, Mo-containing hydrogenase. When Mo is substituted by V in the medium, *A. variabilis* synthesizes an alternative, V-containing enzyme, which reduces C_2H_2 partly beyond C_2H_4 to C_2H_6 and produces more H_2 than the Mo-containing enzyme complex(1,2). The genes of the V-nitrogenase from *A. variabilis* have recently been cloned and characterized(3). In the absence of either V or Mo in the medium, cells of *A. variabilis* still continue to grow and apparently express a third nitrogenase which probably contains only Fe-S centres in the prosthetic group(4). This enzyme reduces C_2H_2 with lower activity, but partly also to C_2H_6 and produces also relatively high amounts of H_2(4). Thus *A. variabilis* is the second organism after *Azotobacter vinelandii*(5) which has been shown to have the capability of synthesizing three different nitrogenases.
In intact cells of the autotrophically growing, N_2-fixing cyanobacteria at best little H_2-production can be detected, due to the fact that the H_2 evolved is immediately recycled by hydrogenases catalyzing the reaction: $H_2 \rightarrow 2\ H^+ + 2e^-$. Cyanobacteria possess at least two different hydrogenases(6). One enzyme catalyzes only H_2-uptake and is particularly active in heterocysts(7) but also occurs in the unicellular, non-N_2-fixing cyanobacterium *Anacystis nidulans*(8,9). Thylakoid preparations of heterocysts from *Anabaena* 7119 (formerly *Nostoc muscorum*) catalyze the photoreduction of $NADP^+$ in dependence of H_2(10) indicating that this uptake hydrogenase must be an integral membrane protein in these specialized cells. The protein has poorly been characterized biochemically(6). A second hydrogenase which can be separated from the other one in crude extracts(11,12) catalyzes both the uptake (with methylene blue or PMS as electron acceptor) and evolution (with $Na_2S_2O_4$ and methyl viologen as electron donor system) of H_2 and is therefore called reversible or bidirectional enzyme(6). Immunogold-labeling experiments with antibodies against this enzyme from *A. nidulans* showed its location at or near the cytoplasmic membrane(13) from which it is readily solubilized in *A. nidulans*(14). As the H^+-gradient is directed outwards in cyanobacteria, the reversible enzyme probably splits H_2 at the periplasmic face(13,14). The situation may, however, be different in *A. variabilis* where the reversible hydrogenase was found to reside inside the cytoplasm and in particularly high concentration at the thylakoids, which was concluded also from immunogold-labeling experiments(15).
The molecular biological characterization of hydrogenases is well advanced in many microorganisms(16,17), but is still at infancy with the cyanobacterial enzymes. This is partly due to the fact that DNA-hybridizations with heterologous probes from

various microorganisms consistently failed(18). A potential subunit of the reversible hydrogenase from *Anabaena cylindrica*(19) and also from *Synechococcus*(20) has been sequenced. This 42 kDa protein, surprisingly, catalyzed only the tritium exchange reaction, independently of any other protein. Recent evidence(21,22) suggests this protein to be more likely an amino transferase.

DNA sequences of the smaller subunit were recently compared for conserved regions by computer analysis in this laboratory. One region, coding for a putative iron-sulfur binding site, served to synthesize an oligonucleotide-primer with the sequence

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5´ GTT GTA GGT GGT AGG ACC CTT GCA ACC 3´
           T   T   T       T   A
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which allowed to amplify by PCR a 329 bp segment with this primer at both ends. The sequence analysis of this segment revealed strong homologies to the corresponding part of the *nifJ* gene from *Klebsiella pneumoniae* and *Enterobacter agglomerans* coding for a pyruvate:ferredoxin (flavodoxin) oxidoreductase(23). This Fe-S protein catalyzes the cleavage of pyruvate and coenzyme A to acetylcoenzyme A and CO_2, and the remaining two electrons are transferred to either ferredoxin or flavodoxin. Its occurrence in cyanobacteria has amply been demonstrated biochemically in a series of papers(24-26) which, however, was not really accepted in the literature(27,28). In the meantime, an 1.1 Kb segment of this gene has been amplified using inverse PCR, cloned and sequenced (O. Schmitz, unpublished). The occurrence of this gene has recently been reported independently from our laboratory(29). DNA-DNA hybridizations as well as DNA amplifications by PCR with oligonucleotide-primers showed the occurrence of this gene in *A. variabilis* and *A.* PCC 7119 but not in *A. nidulans* which apparently cleaves pyruvate by the lipoic acid dependent pyruvate dehydrogenase complex as inferred from enzyme measurements and whole cell studies(30).

A further computer search for conserved sequences was then performed with the published data for the larger subunit of hydrogenases from different organisms. The matrix obtained from all these data gave two short, but distinct regions located close to each end of this gene. One of these coded for the Ni-binding site. These regions were used to synthesize two oligonucleotide primers (a 23mer, 128-fold degenerated and a 20mer, 48-fold degenerated). These were, indeed, suited to give an amplificate in the range of the predicted size of 1.35 Kb with DNA from *Anabaena* 7119 and *A. nidulans*. Sequencing revealed significant homologies to the corresponding subunit of either the NAD^+-reducing hydrogenase from *Alcaligenes eutrophus* and to the MV-reducing enzymes from *Methanococcus voltae*. A genomic bank from *A. variabilis* cloned in λGEM11 served to identify adjacent gene regions also coding for hydrogenase (Fig.1). The small subunit, of 22.5 kDa (deduced from the DNA-sequence), also showed distinct homologies to the corresponding part (δ) of the NAD^+-reducing enzyme from *Alcaligenes eutrophus* and to the MV-reducing proteins from archaebacteria. It was, however, unusually separated from the large subunit by an ORF of 0.6 kb (Fig. 1). A search in the EMBL-data bank revealed

homologies of this gene only to one from the archaebacterium *Desulforolobus* coding for a protein of unknown function. This intergenic DNA region does not appear to be related to the intragenic regions found in nitrogenase and excised during gene rearrangement when heterocysts differentiate(31). Additional sequences have now been identified also showing singificant homologies to the genes coding for the diaphorase part of the *Alcaligenes* NAD^+-reducing hydrogenase (Fig. 1). The corresponding γ-subunit (16) has completely been sequenced whereas DNA-data for more than half of the α-subunit are available currently. In *A. variabilis*, these two subunits were also separated by DNA-regions (an ORF between the δ and γ part and a non-coding region between the γ and the α subunit). The functions of these intervening DNA-regions are unknown at present. Gene expression as well as mutant studies have to show whether they code for essential functions within this hydrogenase gene cluster in cyanobacteria.
Two non-identified ORFs downstream of the large subunit were also detected (Fig. 1). They coded in the opposite direction indicating that the hydrogenase operon ended with the large subunit. A promoter region and transit sequence has not yet been identified in any of the genes sequenced.

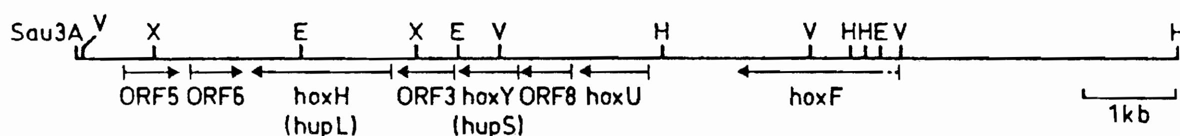

Fig. 1: Arrangement of the gene cluster of the reversible hydrogenase from *Anabaena variabilis* (*hox*H codes for the β-subunit, *hox*Y= δ, *hox*U=γ, *hox*F=α).

The codon usage as well as the tandemly repeated heptamers and octamers are typical for cyanobacterial genes. In addition, the special regions interspersed in the hydrogenase structural genes have not yet been described for any other organism. The occurrence of a hydrogenase gene cluster showing homologies in all parts to the NAD^+-reducing enzyme from *Alcaligenes eutrophus* is somewhat surprising. Cyanobacteria do not grow chemoautotrophically which H_2 as source for energy and electron donors and, therefore, have no need for a H_2-dependent NADH formation. There are no reports in the literature for $NAD(P^+)$-reduction with H_2 in the dark. As said, the corresponding α-subunit has not yet been fully sequenced. Thus flavin and NAD^+-binding sites are not known at present. It is therefore premature to say that this part is modified in cyanobacteria. The reversible hydrogenase from cyanobacteria was, however, reported to transfer electrons to the low potential cytochrome c_{550}(32).

All the evidence thus far obtained clearly indicates that the hydrogenase sequenced codes for the reversible, bidirectional protein from *A. variabilis*. It is closely related to the NAD^+-reducing hydrogenase from *Alcaligenes* and to a recently discovered NAD^+-reducing enzyme from *Desulfovibrio fructosovorans*(33), which are also soluble enzymes, but the gene arrangement is different. Hydrophobicity plots do not give indications for a membrane-bound protein. More convincingly, the protein from *Anacystis* has been purified. SDS-gels with the more recent preparations showed 5-6 prominent bands with apparent molecular weights of 60, 47, 39, 30 and 24 kDa. These bands have been subjected to Lys-C digestion and some of the digests were partly sequenced (kindly performed by Prof. F. Lottspeich, Munich). Two fragments (6 and 5 amino acids) of the 60 kDa protein gave identities to the corresponding amino acid sequence deduced from the DNA-sequence of the α-subunit of the *A. variabilis* gene. A further sequence of the 47 KDa has also been found to be homologous with a region on the sequence of the 56 kDa protein coding for the β-subunit. The discrepancy in the molecular weight (47 kDa on the SDS-gels, 56 kDa from amino acid sequence deduced from the DNA-sequence) is not worrying. The smaller and larger subunit of the hydrogenase have been found to be particularly sensitive to proteolysis in crude extracts, and a molecular weight of 56 kDa has also been determined for this protein on SDS-bands in earlier experiments(13). This subunit from *Alcaligenes* is known to be processed(16) which may also occur with the cyanobacterial enzyme.
In conclusion, although cyanobacteria are systematically unrelated to archaebacteria and to Alcaligenes, they have a NAD^+-reducing hydrogenase in common.

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