Eur. J. Biochem. 233, 266–276 (1995)

Molecular biological analysis of a bidirectional hydrogenase from cyanobacteria

Oliver SCHMITZ[1], Gudrun BOISON[1], Ralf HILSCHER[1], Barbara HUNDESHAGEN[1], Wolfgang ZIMMER[1], Friedrich LOTTSPEICH[2] and Hermann BOTHE[1]

[1] Botanisches Institut, Universität zu Köln, Germany
[2] Max-Planck-Institut für Biochemie, Martinsried, Germany

(Received 24 May/3 July 1995) – EJB 95 0829/2

An 8.9-kb segment with hydrogenase genes from the cyanobacterium *Anabaena variabilis* has been cloned and sequenced. The sequences show homology to the methyl-viologen-reducing hydrogenases from archaebacteria and, even more striking, to the NAD^+-reducing enzymes from *Alcaligenes eutrophus* and *Nocardia opaca* as well as to the $NADP^+$-dependent protein from *Desulfovibrio fructosovorans*. The cluster from *A. variabilis* contains genes coding for both the hydrogenase heterodimer (*hoxH* and *hoxY*) and for the diaphorase moiety (*hoxU* and *hoxF*) described for the *A. eutrophus* enzyme. In *A. variabilis* the gene cluster is split by two open reading frames (between *hoxY* and *hoxH* and between *hoxU* and *hoxY*, respectively), and a probably non-coding 0.9-kb segment in an unusual way. The *hoxH* partial sequence from *Anabaena* 7119 and *Anacystis nidulans* was amplified by PCR. Using the labeled segment from *A.* 7119 as probe, Southern analysis revealed homologous gene segments in the cyanobacteria *A.* 7119, *Anabaena cylindrica*, *Anacystis nidulans* and *A. variabilis*. The bidirectional hydrogenase from *A. nidulans* was purified and digests were sequenced. The amino acid sequences obtained showed partial identities to the amino acid sequences deduced from the DNA data of the 8.9-kb segment from *A. variabilis*. Therefore the 8.9-kb segment contains the genes coding for the bidirectional, reversible hydrogenase from cyanobacteria. Crude extracts from *A. nidulans* perform NAD(P)H-dependent H_2 evolution corroborating the molecular biological demonstration of the $NAD(P)^+$-dependent hydrogenase in cyanobacteria.

Keywords: bidirectional hydrogenase; $NAD(P)^+$-reducing [NiFeS] hydrogenase; cyanobacteria; *Anabaena variabilis; Anacystis nidulans*.

Many microorganisms contain more than one hydrogenase (EC class 1.12) performing the reaction: $2\ H^+ + 2\ e^- = H_2$ [1, 2]. In cyanobacteria, one enzyme catalyzing only H_2 utilization in the presence of electron acceptors like methylene blue or phenazine methosulfate ('uptake hydrogenase') has been shown to reside mainly [3, 4] or exclusively [5] in heterocysts of N_2-fixing filamentous forms. In these cells, it is apparently an integral protein of the thylakoid membranes [6]. Such an uptake hydrogenase seemingly occurs also in the unicellular, non-N_2 fixing *Anacystis nidulans* [7, 8]. Cyanobacteria contain a further hydrogenase which can be separated from the uptake enzyme in crude extracts [9]. This second enzyme catalyzes H_2 formation (using the electron donors $Na_2S_2O_4$ and methyl viologen) as well as H_2 uptake and is, therefore, termed bidirectional or reversible hydrogenase. Immunogold-labeling experiments with antibodies raised against this enzyme from *A. nidulans* demonstrated its location at the cytoplasmic membrane [10]. As the H^+ gradient in cyanobacteria is directed outwards and as this enzyme has a high affinity for H_2, it probably functions in cleaving H_2 at the periplasmic side. It might allocate electrons to the respiratory chain which was shown to reside in the cytoplasmic membrane [11].

Correspondence to H. Bothe, Universität zu Köln, Gyrhofstr. 15, D-50931 Köln, Germany

Abbreviations. $PhMeSO_2F$, phenylmethylsulfonyl fluoride; ORF, open reading frame.

Note. The DNA sequence of the whole 8927-base segment published here has been deposited with the EMBL sequence data bank and is available under the accession number X79285.

Despite the potential of cyanobacterial H_2 evolution for practical applications ('bioconversion of solar energy'), hydrogenases from cyanobacteria have not yet been characterized in much detail biochemically due to their low specific activity in the cells and their instability upon purification. Moreover, attempts to isolate hydrogenase genes were consistently unsuccessful mainly because heterologous probes failed to hybridize [12]. However, a search for conserved regions within the DNA sequences of various organisms and the synthesis of oligonucleotide primers of such regions has now enabled us to amplify a DNA segment from *A. variabilis* and other cyanobacteria which showed sequence homologies to hydrogenases from other microorganisms. The whole gene set coding for the bidirectional hydrogenase from *A. variabilis* has subsequently been isolated. The present communication analyzes the genes of this hydrogenase, which, unexpectedly for a cyanobacterial enzyme, is related to the enzymes from archaebacteria and *A. eutrophus*.

MATERIALS AND METHODS

Strains and growth conditions. *Anabaena variabilis* ATCC 29413, *Anabaena* PCC 7119, *Anacystis nidulans* (= *Synechococcus leopoliensis*) SAUG 1402-1 and *Anabaena cylindrica* SAUG 1403-2 were purchased from the collections. The cyanobacteria were grown aerobically in BG-11 medium with nitrate under continuous gassing with a mixture of air/CO_2 (95/5, by vol.) as described [13].

Escherichia coli strain TG1, grown in Luria-Bertani broth or solid media, was used as the host for plasmid constructions, following standard cloning procedures [14]. For the propagation of bacteriophage λ clones, *E. coli* strain LE392 was grown in Luria Bertani medium supplemented with 0.2% maltose and 10 mM $MgSO_4$, using agarose instead of agar in the solid medium [14]. The concentrations of the antibiotics for plasmid selection were 100 and 50 μg/ml for ampicillin and kanamycin, respectively.

Sequence alignment and analysis. The alignments of the protein sequences were obtained using the CLUSTAL V multiple sequence alignment program. A second program [15] was devised to define conservative regions within the protein sequences. The EMBL data library and the GenBank database were used for sequence database searches.

Synthesis of oligonucleotides and polymerase chain reaction. The oligonucleotides were synthesized by a Pharmacia LKB Gene Assembler Plus. After removal from the column, they were purified by separation on a 1.5-ml NAP™-10 column (Pharmacia). The assay conditions for the PCR reaction were the same as described earlier [16]. In order to define the optimal annealing temperature for each combination of oligonucleotides, primers were annealed at 56, 52, 50, 48, 44 or 40°C.

DNA sequencing. The amplified PCR products were cloned into pCR™II (TA Cloning™, Invitrogen, San Diego, USA) and were subsequently cloned into bacteriophages M13mp18 and M13mp19 (Life Technol., Eggenstein). Bacteriophage λ DNA, containing inserts of *A. variabilis* genomic DNA, was subcloned into pBluescript™ II SK(-) phagemid (Stratagene, Heidelberg) and into M13mp18/M13mp19, allowing the sequencing of both strands by the dideoxy-mediated chain-termination technique using the TAQuence™ sequencing kit (U.S. Biochem. Corp.). Sequential series of overlapping clones for DNA sequencing were generated using the Cyclone System (International Biotechnologies, New Haven, USA). Sequencing was performed with M13(-40) forward primer and with specific 17-base primers directed against internal sequences of the insert DNA, respectively.

Construction of *Anabaena variabilis* genomic DNA bank in bacteriophage λ. Genomic DNA of *A. variabilis* (5 μg) was partially digested with 1/8 U *Sau*3AI for 1 h at 37°C. 0.5 μg of the partially restricted DNA with an average fragment size of 15 kb was ligated with 0.5 μg *Bam*HI-restricted λGEM™-11 vector DNA (Promega) in a total volume of 10 μl at 14°C overnight using 1 U T4 DNA ligase (Boehringer). The DNA was transferred into *E. coli* strain LE392 using the Gigapack™ II gold packaging extract (Stratagene). The infected bacteria were mixed with 3.5 ml prewarmed Luria Bertani broth (48°C) containing 0.7% agarose and plated on Terrific Broth agar plates which were incubated overnight at 37°C.

DNA labeling and hybridization. PCR products were labeled with 800 pmol digoxigenin-11-dUTP/50 μl PCR assay (Boehringer Mannheim). Electroeluted DNA restriction fragments [14] were labeled by random priming using the digoxigenin DNA labeling and detection kit (Boehringer Mannheim). The hybridization and staining procedures were performed as recommended by the manufacturer using hybridization buffer without formamide at 68°C. Hydrogenase genes of *A. variabilis* were isolated by screening the genomic DNA bank cloned into bacteriophage λ and by screening partial genomic DNA banks generated by restriction of genomic DNA with *Eco*RI/*Eco*RV and *Hin*dIII/*Eco*RV, respectively. Subsequently, fragments of the appropriate size were cloned into M13mp18/M13mp19.

DNA isolation, restriction and blotting. Cyanobacterial DNA was isolated as described by Adams [17]. 5 μg DNA was digested by 10 U restriction endonucleases (from Life Technologies) for 2 h. The DNA restriction fragments were separated on a 0.7% (mass/vol.) agarose gel and blotted onto Qiabrane nylon plus membrane (Qiagen, Hilden). After 16 h of transfer, the DNA was immobilized by incubating the filters at 80°C for 1 h. For phage and colony lifts Hybond™-C nitrocellulose membranes (Amersham Buchler) were used. These membranes were treated with 0.1% (mass/vol.) SDS, 0.3 M NaCl, 30 mM sodium citrate for 2 h at 68°C, proteinase K (20 μg/ml) for 1 h at 37°C and phenylmethylsulfonyl fluoride ($PhMeSO_2F$, 0.2 mM) for 5 min at room temperature after the immobilization step in order to remove cellular debris (DIG-System User's Guide, Boehringer). Transformation, plasmid preparation, restriction analysis and ligation were performed by conventional techniques [14].

Hydrogenase activity measurements and purification of the bidirectional hydrogenase from *A. nidulans*. H_2 evolution rates with methylviologen reduced by $Na_2S_2O_4$ as electron donor were determined in 7-ml Fernbach flasks, and the H_2 formed was quantified by gas chromatography [6, 8, 10]. The purification protocol described earlier [10] was modified including additional purification steps using an advanced protein purification system (Waters, Milford, USA). To obtain sufficient biomass for the purification, *A. nidulans* was grown in 5-l penicillin flasks at 40°C; 20 l culture of the logarithmic growth phase was centrifuged (4000×*g*, 8 min), suspended in 50 mM Tris/HCl pH 7.5, containing 2 mM 1,4-dithiothreitol and 1 mM $PhMeSO_2F$ and then flushed with argon for 20 min. Since cyanobacterial hydrogenases are sensitive to O_2 upon purification, all the following steps were performed under a continuous stream of N_2 or argon. The cells were passed twice through a French press at 137 MPa, and cell debris were removed by centrifugation (48000×*g*, 20 min). The supernatant was subjected to $(NH_4)_2SO_4$ precipitation (20%, mass/vol.). After stirring (20 min) and centrifuging (48000×*g*, 20 min), the supernatant was dialysed overnight against 10 l 50 mM Tris/HCl pH 7.5, 0.15 M KCl; 1 mM dithiothreitol was added to the dialysate which could be frozen in liquid nitrogen and stored at −80°C for at least a month without substantial loss (<10%) of activity after rethawing. The dialysate was absorbed on a DEAE-Sepharose CL-6B column (2.6×25 cm, Pharmacia LKB) equilibrated with 200 ml 50 mM Tris/HCl pH 7.5, 0.15 M KCl. The column was washed free of protein with this buffer. Hydrogenase eluted from the column in 10-ml fractions using a linear KCl gradient (0.15−0.4 M KCl, 250 ml total volume) in 50 mM Tris/HCl pH 7.5; dithiothreitol (1 mM, final concentration) was added to each fraction. Fractions eluting at around 0.3 M KCl were pooled and chromatographed on an octyl-Sepharose CL-4B column (1.6×15 cm, Pharmacia LKB), equilibrated with 150 ml 50 mM Tris/HCl pH 7.5, 0.3 M KCl. The column was washed with 80 ml of the same buffer and then with 30 ml 50 mM Tris/HCl pH 7.5. Hydrogenase was eluted from the column with 10% ethanol in 50 mM Tris/HCl pH 7.5 and was absorbed on a Protein Pak glass DEAE-5PW column (Millipore) using the advanced protein purification system. After washing the column with 10 ml 20 mM triethanolamine pH 7.2, a linear 90-ml gradient of 0−0.6 M KCl in 20 mM triethanolamine pH 7.2 was applied to the column. Hydrogenase fractions eluting at 0.32 M KCl were diluted with 5 vol. 50 mM Tris/HCl pH 8.5 to reduce the KCl concentration and to adjust the pH to 8.5. Hydrogenase was further purified by using the same column but with a linear 40-ml gradient of 0.25−0.5 M KCl in 50 mM Tris/HCl pH 8.5; 1 M triethanolamine pH 5.0 (0.1 vol.) was immediately added to each fraction to adjust the pH to 7.5, a necessary step to stabilize the enzyme activity. The enzyme eluted from the column at 0.38 M KCl and was desalted and concentrated by ultrafiltration using a Centricon™-3 unit (Amicon). Purities of the hydrogenase frac-

tions were checked by separation on a 12% polyacrylamide gel with 0.1% SDS. Proteins were stained with Coomassie brilliant blue R-250 (Serva) or, more sensitively, with silver.

Protein sequencing. The hydrogenase subunits of *A. nidulans* were separated by SDS gel electrophoresis, stained with Coomassie brilliant blue and cleaved directly in the gel with endoproteinase LysC (Boehringer) [18]. Resulting peptides were separated by reverse-phase HPLC and sequenced using a 477A pulsed liquid-phase sequencer equipped with an on-line 120A phenylthiohydantoin analyser (both Applied Biosystems) according to the instructions of the manufacturer.

Determinations of H_2-evolution activities. For NAD(P)H- and reduced methylviologen-dependent H_2 evolution, extracts of cells, incubated under $N_2/H_2/CO_2$ (75/20/5, by vol.) overnight were prepared by passage twice through a French press at 137 MPa followed by centrifugation (20 min, 48000×*g*). The assays were performed in 7.0-ml Fernbach flasks containing, in 3 ml, 250 mM Tris/HCl pH 7.5, 0.8 mM methylviologen, 12 mM $Na_2S_2O_4$ or 0.8 mM NADH, generated in the assay flasks from 10 mM galactose and 40 mU β-galactosidase or 0.8 mM NADPH, generated from 10 mM glucose-6-*P* and 175 mU glucose-6-phosphate dehydrogenase, and supernatant of the centrifugation containing 0.1–4 mg protein. H_2 formation in the gas phase was determined after 1–3 h by gas chromatography [6, 8]. The detection limit for H_2 was < 0.1 nmol · h^{-1} · mg $protein^{-1}$.

RESULTS AND DISCUSSION

Strategy employed for obtaining the 8.9-kb segment with the genes of the bidirectional hydrogenase. A computer analysis for conserved regions was performed with the sequences of the large subunit from members of the three hydrogenase classes I, II and IV [2], totally from 12 bacteria. Selecting for regions with five identical amino acid residues in a block of nine, the matrix gave several stretches of dots (not shown). Two regions could successfully be employed for primer synthesis and gene amplification by PCR. One region with the motif (F/Y)DPCXXC represents the putative C-terminal Ni-binding site and is strongly conserved in all [NiFe] hydrogenases and also in the [NiFeSe] enzymes (in the latter with the substitution of the first cysteine with selenocysteine [2]). The other region (= PXTRXEGH) represents a consensus sequence of hydrogenases of classes I, II and IV but is also found in proteins of unrelated genes [2]. Taking into account the codon usage of cyanobacteria [19], the following primers were synthesized: 5′ CCT GT(A/T) AC(A/C) CG(C/T) AT(C/T) GA(A/G) GG(A/C/G/T) CA 3′ and 5′ CAA GCA AA(A/G) CA(A/G/T) GG(A/G) TC(A/G) (A/T)A 3′, which allowed the amplification of an 1.35-kb segment of the predicted size by using *Anabaena* 7119 DNA as template (Fig. 1). The segment was cloned and sequenced; a comparison with the protein sequences of the EMBL data bank indicated about 40% identity and 65% similarity to the corresponding part of the large subunit of either the NAD^+-reducing hydrogenase from *Alcaligenes eutrophus* or the methylviologen-reducing enzymes from *Methanococcus voltae*. The same primers could also be used to amplify a segment with DNA from *Anacystis nidulans* (Fig. 1). Primer 1 alone gave non-specific PCR bands with DNA only from *A. nidulans* (Fig. 1) suggesting that this region does not specifically code for hydrogenases [2]. Amplification of an 1.35-kb segment, however, required both primers (Fig. 1). This segment strongly hybridized with the 1.35-kb digoxigenin-labeled probe from *Anabaena* 7119 (not shown).

Genomic DNA from *A.* 7119, *A. nidulans* (Fig. 2A), *A. variabilis* and *A. cylindrica* (not documented) was digested with

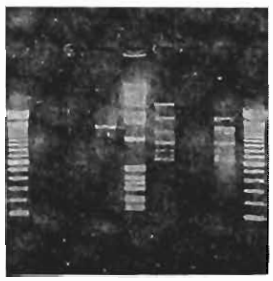

Fig. 1. PCR amplification of a segment of the hydrogenase large subunit with DNA from *A.* 7119 and *A. nidulans* with the oligonucleotide primers 1 and 7. For primers, see Fig. 1. Lanes 1–3, *A.* 7119; lanes 4–6: *A. nidulans*; S1, 1-kb ladder (Gibco); S2, 100-bp ladder (Gibco); primer combinations: lanes 1 and 4, primer 1; lanes 2 and 5, primer 2; lanes 3 and 6, both primers 1 and 2. For primers, see text. PCR conditions: touchdown of annealing temperature from 60°C to 52°C in steps of 2°C (two cycles for each temperature), then 30 cycles more at 56°C.

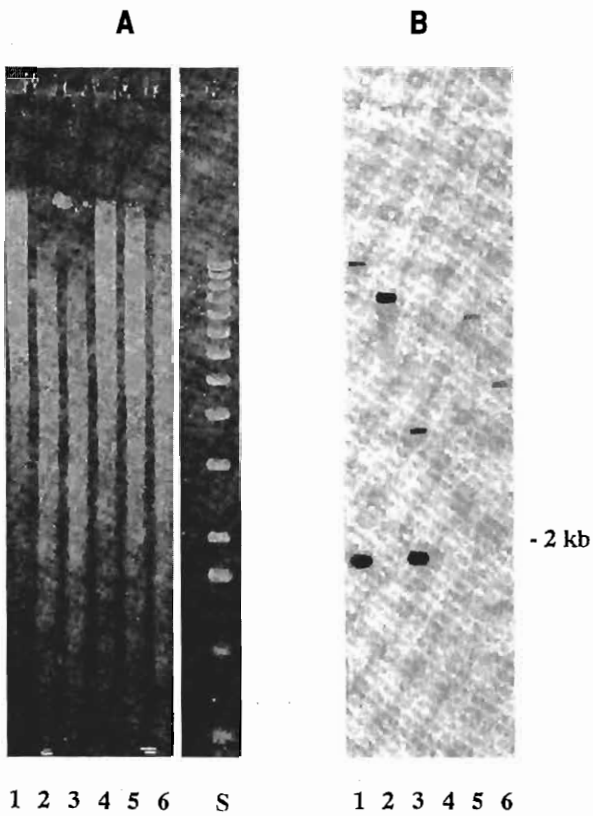

Fig. 2. Restriction patterns of (A) *A.* 7119 and *A. nidulans* DNA and (B) hybridization signals using the digoxigenin-labeled PCR segment from the hydrogenase large subunit of *A.* 7119. Lanes 1–3, *A.* 7119; lanes 4–6, *A. nidulans;* S, 1-kb ladder; 5 µg genomic DNA was restricted with *Eco*RI (lanes 1 and 4), *Hin*dIII (lanes 2 and 5) or *Eco*RI + *Hin*dIII (lanes 3 and 6).

*Eco*RI and *Hin*dIII; Southern hybridization with the digoxigenin-labeled 1.35-kb segment from *A.* 7119 gave strong bands in all cases (Fig. 2B) indicating the occurrence of this hydrogenase segment in the four cyanobacteria assayed. Sequencing of the segment from *A. variabilis* revealed identities with the *A.* 7119 segment of 93% on the DNA and 97% on the amino acid level. To obtain the sequence of the complete locus coding for hydrogenase, approximately 20000 clones of the λ gene bank

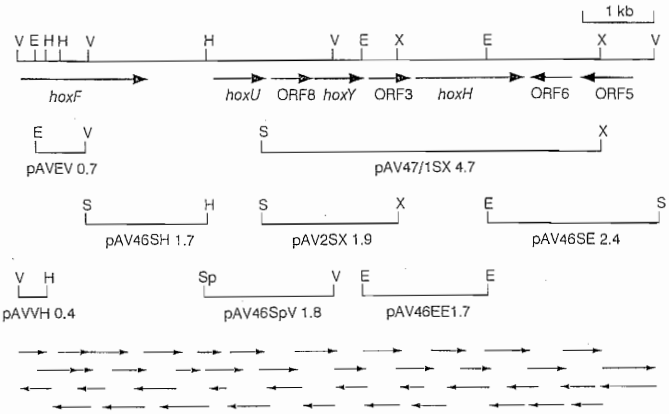

Fig. 3. Map of the *hox* gene locus of *A. variabilis*. The localization of plasmid inserts, used for sequencing and hybridizations, are shown above the sequencing strategy indicated by small arrows.

representing the *A. variabilis* genome about 50-fold were screened using the 1.35-kb *A. variabilis* segment as probe. Among the 16 positive λ clones obtained, 6 (which were overlapping) were chosen for further subcloning into Bluescript and M13mp18/M13mp19 and sequencing. The strategy employed and the plasmids used to obtain the complete sequences of the locus are outlined in the legend to Fig. 3. Since the 5′-region of the gene termed *hoxF* could not immediately be obtained due to the lack of suitable clones in the gene bank (hybridization with pAV46SH1.7 did not provide an overlapping clone, see Fig. 3), this region of the genomic DNA was characterized by restriction analysis, followed by hybridization of the genomic DNA with pAV46SH1.7 and subcloning of the corresponding restriction fragment into pAVEV0.7. Further hybridizations of the genomic DNA with this plasmid resulted in the isolation of pAVVH0.4 (see Fig. 3) to complete the sequence of the 8.9-kb region. The origin of these fragments and all other subclones coming from the λ gene bank was verified by Southern hybridization with genomic DNA from *A. variabilis* in each case.

The 8.9-kb segment of the *A. variabilis* genome sequenced (Fig. 3) contains four genes which show strong similarity to genes coding for the NAD^+-reducing hydrogenase from *A. eutrophus* but have relatively little similarity to the two structural genes *hup*L and *hup*S of the membrane-bound NiFe hydrogenases (Table 1). Therefore the genes are denoted *hoxH*, *hoxY*, *hoxU* and *hoxF* as in the case of the *A. eutrophus* soluble hydrogenase [20]. The hydrogenase gene cluster from *A. variabilis* contains the ORF3 (corresponding to a protein of 199 amino acids, 22.5 kDa) between *hoxH* and *hoxY*. The deduced protein sequence showed 31% sequence identity to a hypothetical 17.7-kDa protein of unknown function in the BPS 3′-region of the archaebacterium *Desulfurolobus ambivalens* (W. Kletzin, EMBL accession no. P32987). Additionally, ORF8 (198 amino acids, 22.4 kDa) separates *hoxU* and *hoxY* but this ORF has no counterpart in the data banks. *HoxF* and *hoxU* are separated by a 0.95-kb region with a series of stop codons in all three reading frames on both strands. This region may code for several small proteins, the largest containing maximally 70 amino acids. However, no part of the three reading frames scored to any protein deposited in either the EMBL or GENBANK databanks. Therefore this 0.95-kb sequence is more likely to be a non-coding region of unusual length. Recently, the uptake hydrogenase genes from *Anabaena* 7120 have been shown to undergo rearrangement prior to expression during heterocyst differentiation [21, 22]. No gene part within the 8.9-kb locus (ORF8, ORF3 or others) shows similarity to the recombinases XisA (involved in *nifHDK* rearrangement), XisF (for *fdxN*) or XisC (for *hupL*) [22], and the consensus sequence for a *NtcA*-binding site observed upstream of *xisA* is also not present. Southern blots with different probes of the 8.9-kb segment do not give additional hybridization signals, either from heterocyst or vegetative cell DNA. The whole 8.9-kb segment sequenced has a G+C content of 43% and codon usage is typical for *Anabaena* [19].

Characterization of the genes coding for the hydrogenase heterodimer. The *hoxH* gene of *A. variabilis* (54.8 kDa, 487 amino acids including 8 Cys residues) has characteristics typical of a large subunit of [NiFe] hydrogenases. No conventional Shine-Dalgarno sequence is seen upstream of the start codon. Sequence alignment with the genes from *M. voltae* and *A. eutrophus* (Fig. 4A), however, indicates that the protein starts with the first Met observed after the stop codon of ORF3. This is located 8 amino acids upstream of the sequence PXTRXEGH underlined in Fig. 4A and used to develop one primer for PCR. The next possible start codon, GTG encoding Val, resides downstream of this motif which is strongly conserved in hydrogenases.

Sequence similarities to the large subunit of membrane-bound uptake hydrogenases, including the uptake enzyme from heterocysts of *A.* 7120 [21, 22], are only 20–25% and thus lower than to the enzymes from *M. voltae* and *A. eutrophus* (Table 1). The large subunit of [NiFe] hydrogenases contains two strictly conserved motifs $RGXEX_{16}RXCGXCXXXH$ (block B) and DPCX(A/S)CXXH (block F) [1, 2, 23]. The block B of the N-terminus is also found in the protein from *A. variabilis*, with the exception that the last His is replaced by a Pro. This His might not be essential, as it is also substituted, by a Thr, in HupL from *Azotobacter chroococcum* [24]. The motif DPCX-(A/S)CXXH (block F) is, in *A. variabilis*, strictly the same as in all other hydrogenases but not in the large subunit of the *A.* 7120 uptake enzyme where the region is DSCLVCTVH [22]. The triplet TGA coding for selenocysteine in NiFeSe hydrogenases at the position of the first Cys of this motif is not present in the *A. variabilis* sequence. The HX_6L (2L) and the GX_4PRGX_3H (3L, see [23]) patterns also occur in the *A. variabilis* protein. Given these similarities, it seems highly likely that the sequenced gene from *A. variabilis* codes for the large subunit of a [NiFe] hydrogenase. Previous physiological experiments [25, 26] already indicated that the expression of the bidirectional hydrogenase from cyanobacteria requires Ni in the medium.

Processing of the large subunit of hydrogenases proceeds at the carboxy-terminus of the His of the strongly conserved motif DPCXXCXXH [27–29] with the exception of *E. coli* hydrogenase 3 where this His is substituted by Arg [29]. This region of the *hoxH* sequence of *A. variabilis* is homologous to those of *A. eutrophus* and *M. voltae* and utilizes a His in this position (Fig. 4A). So the formation of the mature protein in *A. variabilis* might necessitate the cleavage of a fairly long stretch of 29 amino acids. This cleavage might be influenced by the availability of nickel and its subsequent liganding to the large subunit [27–29]. A signal peptide at the N-terminus of HoxH is not apparent.

The *hoxY* gene (coding for the smaller subunit of hydrogenase, 22.5 kDa, 205 amino acids, 5 Cys) might begin immediately after the stop codon of ORF8 with the Val triplet GTG (Fig. 4B). A putative ribosome-binding site GGAG resides in front of the gene. The 5′-region contains five tandem repeats of the heptamer CCCCAAT. Such short tandemly repeated sequences have been described for other cyanobacterial DNA sequences [30, 31]. The tandemly repeated motif CCCCA(A/G)T could represent a typical cyanobacterial signature. The repeats

Table 1. Comparison of HoxH, HoxY, HoxU and HoxF of *A. variabilis* bidirectional hydrogenase with [NiFe], [NiFeSe], and [Fe] hydrogenases of other organisms and with related proteins. For each pair of related proteins, the percentage of identical/similar amino acids is given, referred to *Anabaena* protein length. Where sequence similarities are confined to certain domains, the amino acid positions of start and end of similarity are given in brackets. Protein sequences were from SWISS-PROT database: *A. eutrophus* HoxFUYH (P22317/P22318/P22319/P22320), *R. capsulatus* HupSL (P15283/P15284), *D. baculatus* HupSL (P13063/P13065), *C. pasteurianum* HydB (P29166), *C. pasteurianum* [2Fe2S] ferredoxin (P07324); translated from GenBank: *A.* 7120 HupSL (U08013), *D. fructosovorans* HndCD (U07229); and translated from EMBL: *M. voltae* VhcGA (X61203), *E. coli* NuoBDEFG (X68301).

Organism	Protein	Gene product		Identical/similar amino acids to *A. variabilis* product			
		name	length (amino acids)	HoxH (487)	HoxY (205)	HoxU (238)	HoxF (535)
				%			
Alcaligenes eutrophus	NAD^+-reducing [NiFe] hydrogenase	HoxH	487	43/66			
		HoxY	209		33/62		
		HoxU	233			32/59	
		HoxF	602				38/63 (113–535)
							23/51 (1–112)
Methanococcus voltae	methylviologen-reducing [NiFe] hydrogenase	VhcA	470	39/65			
		VhcG	289 (1–180)		32/57		
Rhodobacter capsulatus		HupL	597	27/57			
		HupS	358 (22–228)		21/47		
Anabaena PCC 7120	uptake hydrogenase	HupL	531	26/52			
		HupS	>51[a]		–[a]		
Desulfovibrio baculatus	[NiFeSe] hydrogenase	HupL	513	26/52			
		HupS	315 (10–235)		21/51		
Desulfovibrio fructosovorans	$NADP^+$-reducing [Fe] hydrogenase	HndD	585 (1–247)			34/60	
		HndC	490				59/79 (113–535)
Clostridium pasteurianum	[Fe] hydrogenase I	HydB	574 (1–236)			26/55	
Clostridium pasteurianum	[2Fe-2S] ferredoxin	–	102				27/47 (14–112)
Escherichia coli	NADH:Q-oxidoreductase (= NADH dehydrogenase I)	NuoD	407	17/37			
		NuoB	220		20/46		
		NuoG	820 (1–272)			26/55	
		NuoE	166 (67–166)				15/40 (1–95)
		NuoF	445				39/69 (113–535)

[a] Only the C-terminal part of HupS (51 amino acids) is sequenced not showing significant similarity to HoxY from *A. variabilis*.

often reside in intergenic regions but also occur within a gene (*nifJ*) [32]. Several repeats are found in other intergenic regions (three TTTTGAA between *hoxY* and ORF3 and four (T/A)TAGGGGT between *hoxH* and ORF6) within the 8.9-kb hydrogenase locus. Therefore HoxY may start with a Met located 24 amino acids downstream of the Val and immediately after the tandem repeats. It is noteworthy that the protein from *A. eutrophus*, but not the one from *M. voltae*, possesses a stretch in the same region upstream of the Met, albeit with a dissimilar amino acid composition (Fig. 4B). If HoxY were, indeed, to start with the Met, the protein would have a molecular mass of 19.9 kDa and 181 amino acids. Such a value would closely match with the 17 kDa determined by SDS-gel electrophoresis for the *Anacystis* protein [10]. On the other hand, a typical Shine-Dalgarno sequence would be missing in the vicinity of the start Met. Alignments with the sequences of related genes (Fig. 4B) rule out that codons other than those mentioned above could act as start.

There is no evidence that the *hoxY* gene expresses a leader sequence with the typical block RRXFXK nor is the C-terminal hydrophobic region, common to the membrane-binding HupS protein [2], present. This negative evidence suggests that *hoxY* could code for a soluble protein.

HoxY is more closely related to the corresponding subunit of the NAD^+-reducing hydrogenase from *A. eutrophus* and of the methylviologen-reducing enzymes from *M. voltae* than to any other protein. In particular, the *hoxY* sequence from *A. variabilis* shows no similarities to the sequenced part of the small subunit of the uptake hydrogenase from *A.* 7120 [21, 22], corroborating the statement that this cyanobacterium can express two totally different hydrogenases. HoxY contains only five Cys residues whereas other hydrogenase small subunits contain 9–12 [2, 23]. The motifs GCSGCHMS (corresponding to motif b, possibly also involved in Ni coordination, see [2]), GXCAXXG (c) and PGCPP (d), corresponding to the $CXXCX_nGXCXXXGX_mGCPP$ pattern of Albracht [23], are present as in the proteins from *M. voltae* and *A. eutrophus* (Fig. 4B). In contrast, motif e, including its highly conserved sequence CIGC [2, 23], probably representing an Fe-S cluster binding site, is missing as in the soluble hydrogenase from *A. eutrophus*. The latter is common in many

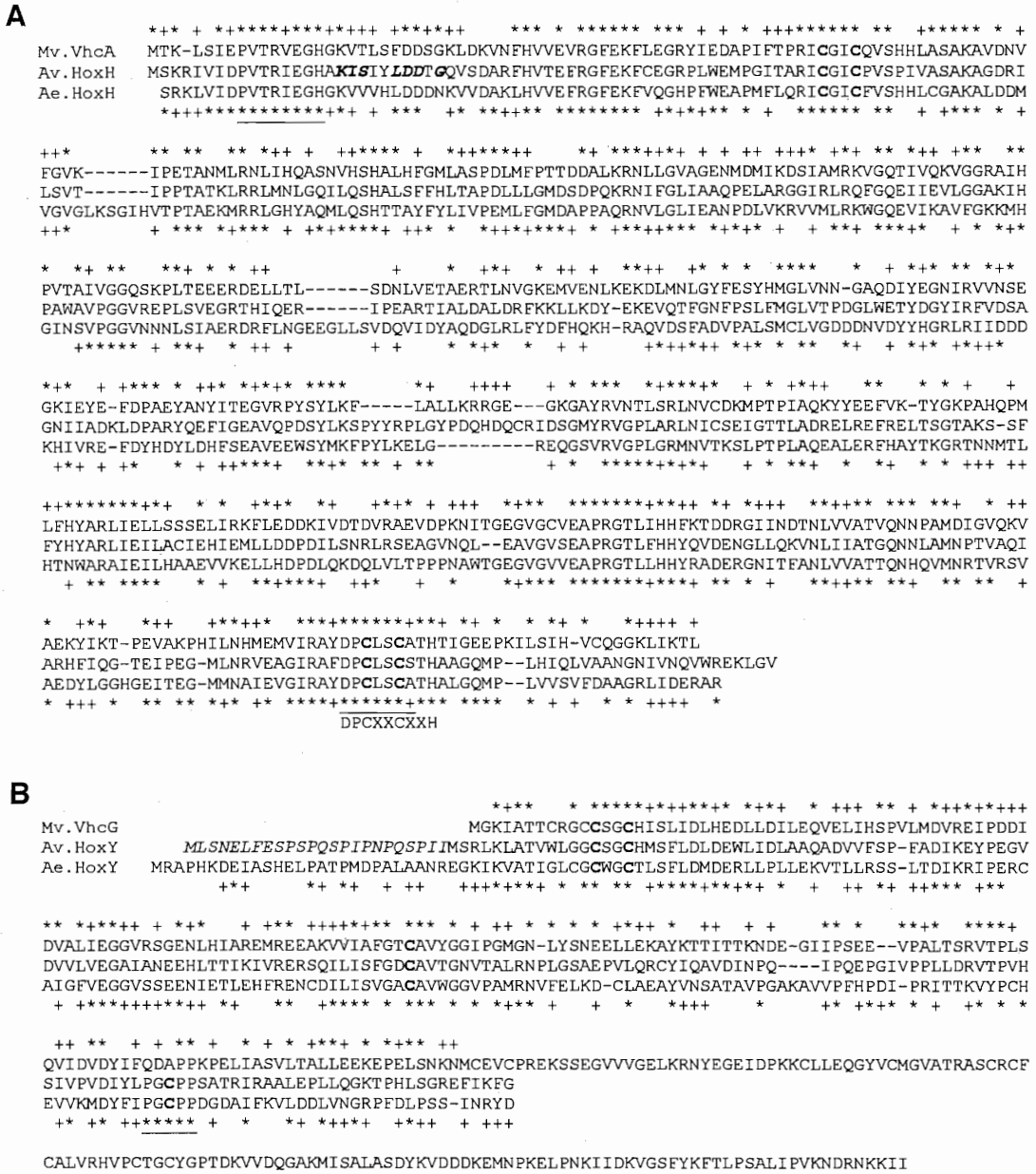

Fig. 4. Sequence alignment of hydrogenase (A) large and (B) small subunit. The amino acid sequence of the *A. variabilis* hydrogenases compared to the *M. voltae* and *A. eutrophus* enzymes. Amino acids identical or similar to the *A. variabilis* sequence are indicated by asterisks or crosses, respectively. Cysteines conserved in almost all of the [Ni-Fe] hydrogenases are shown in bold letters. Oligonucleotide primers for the PCR were developed from the underlined sequences in the large subunit. The consensus sequences DPCXXCXXH and (P/A/Q)GCPP (see text) are also underlined. Mv. VhcA/Mv. VhcG, large/small subunit of the *M. voltae* methylviologen-reducing, selenium-non-containing hydrogenase; Av. HoxH/Av. HoxY, large/small subunit of *A. variabilis* bidirectional hydrogenase; Ae. HoxH/Ae. HoxY, large/small subunit of *A. eutrophus* NAD^+ reducing hydrogenase (for accession numbers see legend to Table 1). The CLUSTAL V program was used for alignments.

[FeS] proteins and this region has been used to amplify a gene segment of pyruvate:ferredoxin oxidoreductase from *A. variabilis* [16].

Characterization of the genes coding for the diaphorase part of the hydrogenase. Whereas two subunits have been described for the methylviologen-reducing hydrogenase from archaebacteria, the NAD^+-dependent multimeric enzyme from *A. eutrophus* consists of two further genes coding for the diaphorase part (*hoxU* with the gene product γ, HoxF = α-subunit). These diaphorase genes are also present in the hydrogenase locus of *A. variabilis* (Figs 3, 5, 6). Their sequences show homologies to those of the corresponding genes from *A. eutrophus*, but, even more striking, to the $NADP^+$-reducing hydrogenase from *Desulfovibrio fructosovorans* [33]. The *hoxU* gene (238 amino acids, 26.9 kDa, 15 Cys) of *A. variabilis* has a putative ribosome-binding site 4 bp upstream of the start codon (or alternatively, but less likely, 27 bp upstream of the start). The sequence homologies of HoxU to the first approximately 275 amino acids of the N-terminus of the 75–78-kDa subunit of the mitochondrial complex I has repeatedly been pointed out [34–36]. Likewise, 25–35% similarity to the N-terminal part of Fe hydrogenases from *Clostridium pasteurianum* [37], *C. acetobutylicum* (Santangelo et al., Genbank accession no. U09760), *Desulfovi-*

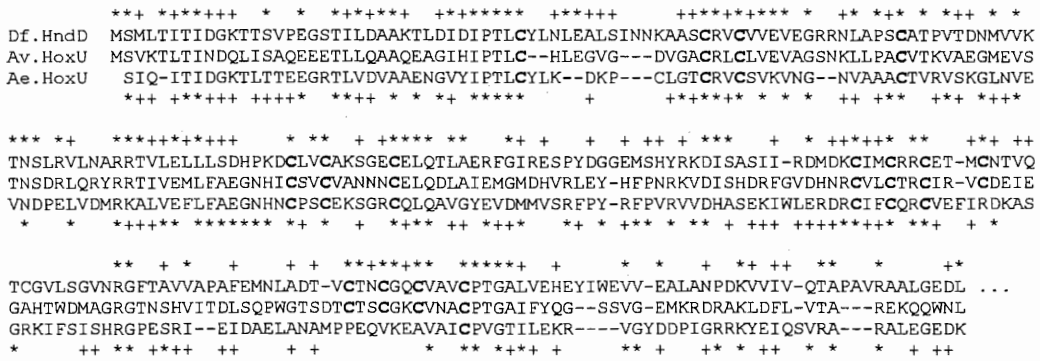

Fig. 5. Comparison of the HoxU sequence from *A. variabilis* with HoxU from *A. eutrophus* and the N-terminal part of HndD from *D. fructosovorans*. Df. HndD, *D. fructosovorans* [Fe] hydrogenase, HndD (only the first 247 amino acids are shown); Av. HoxU, *A. variabilis* bidirectional hydrogenase, HoxU; Ae. HoxU, *Alcaligenes eutrophus* NAD^+-reducing hydrogenase, HoxU.

brio vulgaris [38] and *D. fructosovorans* [33] has been noted previously which is also the case for the gene product from *A. variabilis*. The motif $CX_{10-15}CRXCX_{11-18}CX_{35-43}CX_2CX_5$-$CX_{38-40}CX_2CX_2CX_{43-47}CP$ is conserved in the HoxU subunit of *A. variabilis*, *A. eutrophus*, *D. fructosovorans* (Fig. 5) and also in the mitochondrial 75−78-kDa protein (see Fig. 3 in [39], also [40, 41]). The *hoxU* gene product from *A. eutrophus* [42] and *Nocardia opaca* [43] is believed to harbour one [4Fe-4S] cluster and possibly a further either [4Fe-4S] or [2Fe-2S] center. One CXXCXXC motif is present in the 75−78-kDa mitochondrial subunit and in all hydrogenase *hoxU* genes (including the one from *A. variabilis*); these three Cys might provide ligands for a tetranuclear Fe-S center as in bacterial ferredoxins. The fourth Cys ligand is usually some distance away in the primary structure and is followed by a Pro. This ligand might be the proximal conserved Cys in the 75−78-kDa mitochondrial protein [39] and the last conserved Cys in the HoxU of *A. variabilis* and *A. eutrophus*. There are two other CXXC blocks and further separated Cys residues strongly conserved in all sequences (Fig. 5) which could well function as binding sites for [2Fe-2S] clusters [41]. Additionally, the *hoxU* genes of *A. variabilis* and *D. fructosovorans* (but not of *A. eutrophus*) contain a further CXXCXXC motif preceded by a conserved Cys located 34 amino acids upstream which may coordinate an additional [4Fe-4S] cluster.

The *hoxF* gene of the bidirectional hydrogenase from *A. variabilis* (α-protein, 535 amino acids, 57.9 kDa, 15 Cys) has a potential ribosome-binding site 13 bp (or a less typical one 7 bp) upstream of the putative start Met. Alternative start codons could be two GTGs (coding for Val or Met) residing 11 amino acid residues upstream or 19 amino acids downstream, respectively, of this Met (Fig. 6); however, potential Shine-Dalgarno sequences would then be at least 20 bp distant from these starts. In *A. eutrophus* and *Nocardia opaca*, the α-subunit is believed to be a fusion of homologues of the 24-kDa and 51-kDa subunits of the respiratory complex I or the *E. coli* NADH:ubiquinone oxidoreductase [35, 42, 43]. The amino acids 1−188 of HoxF from *A. eutrophus* are related to the 24-kDa mitochondrial protein, and the remainder to the 51-kDa subunit. The carboxyl-terminal region of the α-protein from *A. variabilis* is also closely related to the 51-kDa mitochondrial protein. In particular, the highly conserved domains block A, B, and C [40] of the 51-kDa protein are seen in the α-subunits of the hydrogenases from *A. eutrophus*, *D. fructosovorans* and *A. variabilis* (Fig. 6). Block A possesses the Gly-rich fingerprint motif GXGXXGXXXG which is generally assumed to bind the adenine-nucleotide moiety of $NAD(P)^+$. The second conservative region (B in Fig. 6) is also rich in Gly and might represent the FMN-binding site as in the other proteins mentioned. The typical motif CXXCXXC of block C [40] might provide ligands for a [4Fe-4S] cluster. The fourth ligand is likely the conserved Cys residue located 40 amino acid residues further C-terminal in the 51-kDa protein and in the α-subunit of the $NAD(P)^+$-reducing hydrogenases including the *A. variabilis* protein. In contrast, the first 112 amino acid residues of the α-protein from *A. variabilis* bear no significant sequence similarity to the 24-kDa mitochondrial protein, to that part of the *A. eutrophus* α-subunit [20, 42] nor to any sequence from *D. fructosovorans* [33]. Sequence similarities are, however, scored to the [2Fe-2S] ferredoxin from *Clostridium pasteurianum* [44]. The N-terminus of the *A. variabilis* protein is characterized by the block $CCX_4CX_{31}CX_3CC$. Although the typical motif CX_2C is not present as in [2Fe-2S] ferredoxins, the distribution of the Cys in this block somewhat resembles that seen in the [2Fe-2S] ferredoxin from *C. pasteurianum* [44]. It is noteworthy that the motif $CX_4CX_{31-35}CX_3C$ is also present in the mitochondrial 24-kDa protein [39]. The N-terminus of the *A. variabilis hoxF* gene product may harbour at least one additional, possibly unusually liganded [Fe-S] center not present in the *A. eutrophus* protein.

Transcription and termination signals. General consensus sequences for cyanobacterial promoters have not been determined [45]. The consensus recognition sequences for the *E. coli* σ^{70} or σ^{54} factors are not seen upstream of the *hoxF* gene or at any other position in the 8.9-kb hydrogenase locus of *A. variabilis*. Termination of transcription apparently occurs downstream of *hoxH* since two ORFs (5 and 6) are transcribed on the opposite strand (Fig. 3). Whereas ORF5 does not show significant similarity to any other protein sequence deposited as yet, ORF6 is slightly similar (23% on the protein basis) to an ORF upstream of the *vnfA* gene from *Azotobacter vinelandii* [46]. The proteins encoded by ORF5 and ORF6 are, therefore, presumably unrelated to hydrogenase subunits or regulators. Termination of transcription is also indicated by an inverted repeat residing between *hoxH* and ORF6. The region from the nucleotides 6979 to 7044 (see EMBL data bank accession no. X79285) is characterized by a G+C-rich stem (ΔG [25°C] ≈ -189 kJ/mol). Another termination signal is seen upstream of the *hoxF* gene. The inverted repeats could well form a hairpin loop between the nucleotides 2−83 (ΔG of ≈ -124 kJ/mol). A further inverted repeat resides between *hoxU* and ORF8 (nucleotides 3469−3515, ΔG ≈ -109 kJ/mol). However, such possible stem and loop structures downstream of coding regions could also act as barriers against exonuclease activities instead of serving as termination signals [45]. Expression studies are needed to show whether the

Fig. 6. Sequence alignment of HoxF from *A. variabilis* with HoxF from *A. eutrophus* and HndC from *D. fructosovorans*. Conserved sequence motifs, probably indicating the binding sites for NAD(H), FMN and a [4Fe4S] cluster, are underlined and marked with A, B, and C, respectively. Four Cys, most likely involved in binding of the [4Fe4S] cluster, and a further four Cys in the N-terminal part of HoxF from *A. variabilis*, discussed as potential binding sites for a FeS cluster, are printed in bold letters. Amino acid identities between the deduced *A. variabilis* protein sequence and fragments of the LysC-digested bidirectional hydrogenase of *A. nidulans* are also shown in bold letters and printed in italics. Df. HndC, *D. fructosovorans* [Fe] hydrogenase, HndC; Av. HoxF, *A. variabilis* bidirectional hydrogenase, HoxF; Ae. HoxF, *A. eutrophus* NAD^+-reducing hydrogenase, HoxF.

hydrogenase heterodimer genes (*hoxH* and *hoxY*) and the genes coding for the diaphorase part (*hoxFU*) form separate transcriptional units.

Identity of the gene products with the bidirectional hydrogenase. As mentioned in the introduction, cyanobacteria can express at least two different hydrogenases, and the 8.9-kb locus may code for either, though not both. To solve this question, the bidirectional enzyme was purified and proteolytic digests were sequenced by Edman degradation. As the hydrogenase activities in extracts particularly from filamentous cyanobacteria are very low and as hydrogenases are highly unstable, the enzyme was purified from the unicellular fast-growing *A. nidulans* [10]. The purified preparations had the relatively low specific activity of ≈250 µmol H_2 evolved $\times h^{-1} \times$mg protein^{-1}. With each of the different preparations, SDS-gel electrophoresis consistently revealed several bands (Fig. 7) of which some could be subunits, precursors or proteolytic digests. The different bands were digested with the endopeptidase LysC in the gels, and the products were sequenced. The two fragments (a) and (b) showed strong similarity between the protein sequences and the amino acid sequences deduced from the DNA data.

a) 47-kDa band (HoxH = β-subunit)

```
(K)(I)(S) V F L D D Q G N A E      (amino acid sequence)
 |  |  |  · · |  |  |  | ·
 K  I  S  I Y L D D T G Q V S      (derived from DNA sequence)
```

b) 60-kDa band (HoxF = α-subunit)

```
   (K)V F A L T G K          L I E V P M      (amino acid sequence)
    | | | | | · | |          | | | | | |
G T K V F A L A G K I R N T G L I E V P M G T  (derived from DNA sequence)
```

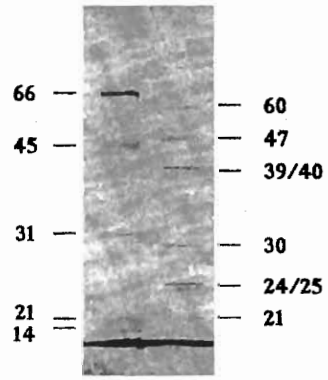

Fig. 7. SDS/PAGE of the purified bidirectional hydrogenase from *A. nidulans*. The gel is silver-stained. Lane 1, molecular mass standards (Bio-Rad), 100 ng of each protein, values in kDa; lane 2, fraction of bidirectional hydrogenase, purified by the advanced protein purification system (see Materials and Methods), ≈400 ng protein.

Table 2. H_2-evolution from NAD(P)H or reduced methyl viologen in extracts from *Anacystis nidulans*. Specific activity was measured as rate of H_2 evolution/mass protein.

Reductant	Specific activity	Relative activity
	nmol · h^{-1} · mg^{-1}	%
1. Methylviologen reduced by $Na_2S_2O_4$	1515	100
2. NADH plus $Na_2S_2O_4$	48.3	3.2
3. NADPH plus $Na_2S_2O_4$	9.3	0.6
4. $Na_2S_2O_4$ alone	7.5	0.5
5. NADH alone	5.9	0.4
6. NADPH alone	2.1	0.1
7. Without any reductant	<0.1	0.0

Thus, although the DNA sequence (from *A. variabilis*) and the protein data (from *A. nidulans*) have been obtained from different organisms, the sequence comparison indicates that the 8.9-kb locus characterized contains genes coding for the bidirectional hydrogenase. On the SDS gels (Fig. 7), the 60-kDa band appears to be HoxF. The two bands at 46/47 kDa seem to be the mature HoxH and possibly its precursor. In sequencing, the N-terminus of this protein was blocked. The band at 30 kDa could represent HoxU whereas HoxY could be in the band seen at 24/25 kDa or at 21 kDa. The presence of HoxF on the SDS gels indicates that the diaphorase part copurifies with the hydrogenase heterodimer (HoxYH). Other proteins seen on the gels could be digests as extracts from cyanobacteria are known to be sensitive to proteolysis. The deviations of the molecular masses determined on the SDS gels from the true values obtained from the sequence data are not unusual for Fe-S proteins. The complexity of this multimeric enzyme may explain the relatively low specific activities of the purified preparations.

NAD(P)H-dependent H_2 – evolution in extracts from *A. nidulans*. The activity of the bidirectional hydrogenase, assayed by the $Na_2S_2O_4$ and methylviologen-dependent H_2 evolution, is low in crude extracts from *A. nidulans* (Table 2) compared to the rates measured with the purified enzyme from *Alcaligenes eutrophus* [47]. Due to the unfavourable thermodynamic conditions, NAD(P)H-dependent H_2 evolutions are even lower, but unambiguously detectable in *A. nidulans*. The relative activities for NADH-dependent, NADPH-dependent and reduced-methylviologen-dependent H_2 evolutions (Table 2) are very similar to those for the *A. eutrophus* enzyme [47]. The activity measurements corroborate the sequence data that the NAD(P)H-dependent hydrogenase occurs in cyanobacteria.

Concluding remarks. Earlier studies showed by biochemical means that cyanobacteria possess an uptake and a bidirectional hydrogenase with different functions [6, 9]. It was, therefore, expected that the DNA sequences coding for the uptake [21, 22] and bidirectional (this communication) hydrogenase are dissimilar. The demonstration that cyanobacteria possess the gene set for a $NAD(P)^+$-dependent hydrogenase with properties similar to the enzyme from *A. eutrophus* [42], *Nocardia opaca* [43] and *D. fructosovorans* [33] is surprising. Cyanobacteria have not been shown to grow chemoautotrophically at the expense of H_2 as the electron donor and energy source in contrast to *A. eutrophus* [42]or *B. japonicum* [48]. NAD(P)H-dependent H_2 evolution catalyzed by the bidirectional hydrogenase can, however, be measured in assays performed in the dark (Table 2). Previous experiments showed that preparations from heterocysts perform H_2-dependent reduction of $NAD(P)^+$ catalyzed by uptake hydrogenase in a light-(photosystem I)-dependent reaction [4].

Since the immunogold labeling experiments suggested that the bidirectional hydrogenase from *A. nidulans* resides at the cytoplasmic membrane with a presumable orientation to the periplasmic face [10], leader sequences or membrane spanning regions should be expected in some of the products of the genes coding for this enzyme. The diaphorase part (*hoxFU* gene products) might be orientated towards the cytoplasmic face of the cytoplasmic membrane, and the reduction of $NAD(P)^+$ might proceed in the cytoplasm. It remains to be shown how the HoxYH proteins are integrated into or transferred across the cytoplasmic membrane. The amino acids processed from the carboxy-terminus of the immature HoxH possibly play a role in this insertion, as suggested for the *D. gigas* hydrogenase [28]. For comparison, the NAD^+-reducing hydrogenase from *A. eutrophus* resides in the cytoplasm [49], but the coenzyme-F_{420}-reducing enzymes from *M. voltae* [50, 51] and from *Methanosarcina barkeri* [52], though lacking signal peptides [50], appear to be at least partly associated with the cytoplasmic membrane as shown by immunogold labeling. Experiments with an antibody different from ours suggested that the bidirectional and uptake hydrogenases from *A. variabilis* occur in the cytoplasm and with a higher labeling at the thylakoids of both heterocysts and vegetative cells [53]. The bidirectional hydrogenases in *A. nidulans* and *A. variabilis* may be structurally different, although this is unlikely because of their sequence similarities. As the cytoplasmic membrane from unicellular cyanobacteria contains the respiratory complex I [11, 54], an association of the $NAD(P)^+$-reducing hydrogenase to this membrane appears to be probable. The findings that the diaphorase is a component of the bidirectional hydrogenase rules out suggestions that the low-potential cytochrome c_{549} is the electron acceptor for this enzyme [55].

The bidirectional hydrogenase characterized here and also the uptake enzyme [21, 22] are unrelated to a 'soluble hydrogenase' described from *Anabaena cylindrica* [56]. Interestingly enough, the smaller subunit (42-kDa protein) of this enzyme is solely required for catalyzing the tritium exchange reaction, whereas the larger subunit alone is claimed to perform the $Na_2S_2O_4$-dependent H_2 evolution. It has been suggested that the smaller subunit of this enzyme from *A. cylindrica* represents a discrete class of hydrogenases [2, 57], as its sequence is totally unrelated to any other hydrogenase genes including the cyanobacterial ones. Recent evidence suggests that the gene of the

42-kDa protein has remote homology to amino acid transferases [58, 59]. The tritium exchange reaction may represent an artificial side reaction of this enzyme otherwise unrelated to hydrogenases.

This work was kindly supported by a grant from the BMFT within the program *Biologische Wasserstoffgewinnung* (FE 9342A/H2). The authors are indebted to Dr M. G Yates, (Brighton, England) for correcting the English.

REFERENCES

1. Przybyla, A. E., Robbins, J., Menon, N. & Peck, H. D. Jr (1992) Structure-function relationships among nickel-containing hydrogenases, *FEMS Microbiol. Rev. 88*, 109–136.
2. Wu, L.-F. & Mandrand, M. A. (1993) Microbial hydrogenases: Primary structure, classification, signatures and phylogeny, *FEMS Microbiol. Rev. 104*, 243–270.
3. Tel-Or, E., Luijk, L. W. & Packer, L. (1978) Hydrogenase in N_2-fixing cyanobacteria, *Arch. Biochem. Biophys. 185*, 185–194.
4. Eisbrenner, G., Roos, P. & Bothe, H. (1981) The number of hydrogenases in cyanobacteria, *J. Gen. Microbiol. 125*, 383–390.
5. Peterson, R. B. & Wolk, C. P. (1978) Localization of an uptake hydrogenase in *Anabaena*, *Plant Physiol. 61*, 688–691.
6. Papen, H., Kentemich, T., Schmülling, T. & Bothe, H. (1986) Hydrogenase activities in cyanobacteria, *Biochimie 68*, 121–132.
7. Peschek, G. A. (1979) Evidence for two functionally distinct hydrogenases in *Anacystis nidulans*, *Arch. Microbiol. 123*, 81–92.
8. Eisbrenner, G., Distler, E., Floener, L. & Bothe, H. (1978) The occurrence of the hydrogenase in some blue-green algae, *Arch. Microbiol. 118*, 177–184.
9. Houchins, J. P. & Burris, R. H. (1981) Comparative characterization of two distinct hydrogenases from *Anabaena* sp. strain 7120, *J. Bacteriol. 146*, 215–221.
10. Kentemich, T., Bahnweg, M., Mayer, F. & Bothe, H. (1989) Localization of the reversible hydrogenase in cyanobacteria, *Z. Naturforsch. 44c*, 384–391.
11. Kraushaar, H., Hager, S., Wastyn, M. & Peschek, G. A. (1990) Immunologically cross-reactive and redox-competent cytochrome b_6f complexes in the chlorophyll-free plasma membrane of cyanobacteria, *FEBS Lett. 273*, 227–231.
12. Kentemich, T. & Bothe, H. (1990) Localization of the reversible hydrogenase in cyanobacteria, in *Nitrogen fixation, achievements and objectives* (Gresshoff, P. M., Evans Roth, L., Stacey, G. & Newton, W. E., eds) p. 547, Chapman & Hall, New York and London.
13. Neuer, G. & Bothe, H. (1985) Electron donation to nitrogenase in heterocysts of cyanobacteria, *Arch. Microbiol. 143*, 185–191.
14. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning*. 2nd edn, vol. 1–3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
15. Vingron, M. & Argos, P. (1991) Motif recognition and alignment for many sequences by comparison of dot matrices, J. *Mol. Biol. 218*, 33–43.
16. Schmitz, O., Kentemich, T., Zimmer, W., Hundeshagen, B. & Bothe, H. (1993) Identification of the *nifJ* gene coding for pyruvate: ferredoxin oxidoreductase, *Arch. Microbiol. 160*, 62–67.
17. Adams, D. G. (1988) Isolation and restriction analysis of DNA from heterocysts and vegetative cells of cyanobacteria, *J. Gen. Microbiol. 134*, 2943–2949.
18. Eckerskorn, C. & Lottspeich, F. (1989) Internal amino acid sequence analysis of proteins separated by gel electrophoresis after tryptic digestion in polyacrylamide matrix, *Chromatographia 28*, 92–94.
19. Tandeau de Marsac, N. & Houmard, J. (1987) Advances in cyanobacterial molecular genetics, in *The Cyanobacteria* (Fay, P. & van Baalen, C., eds) pp. 251–302, Elsevier, Amsterdam, New York, Oxford.
20. Tran-Betcke, A., Warnecke, U., Böcker, C., Zaborosch, C. & Friedrich, B. (1990) Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16, *J. Bacteriol. 172*, 2920–2929.
21. Matveyev, A. V., Rutgers E., Söderbäck, E. & Bergman, B. (1994) A novel genome rearrangement involved in heterocyst differentiation of the cyanobacterium *Anabaena* sp. PCC 7120, *FEMS Microbiol. Lett. 116*, 201–208.
22. Carrasco, C. D., Buettner, J. A. & Golden, J. W. (1995) Programmed DNA rearrangement of a cyanobacterial *hupL* gene in heterocysts, *Proc. Natl Acad. Sci. USA 92*, 791–795.
23. Albracht, S. P. J. (1993) Intimate relationships of the large and the small subunits of all nickel hydrogenases with two nuclear-encoded subunits of mitochondrial NADH: ubiquinone oxidoreductase, *Biochim. Biophys. Acta 1144*, 221–224.
24. Ford, C. M., Garg, N., Garg, R. P., Tibelius, K. H., Yates, M. G., Arp, D. J. & Seefeldt, L. C. (1990) The identification, characterization, sequencing and mutagenesis of the genes (*hupSL*)encoding the small and large subunits of the H_2-uptake hydrogenase of *Azotobacter chroococcum*, *Mol. Microbiol. 4*, 999–1008.
25. Daday, A. & Smith, G. D. (1983) The effect of nickel on the hydrogen metabolism of the cyanobacterium *Anabaena variabilis*, *FEMS Microbiol. Lett. 20*, 327–330.
26. Bothe, H., Nelles, H., Kentemich, T., Papen, H. & Neuer, G. (1983) Recent aspects of heterocyst biochemistry and differentiation, in *Compartments in algal cells and their interaction* (Wiessner, W., Robinson, D. G. & Starr, R. C., eds) pp. 218–232, Springer, Berlin, Heidelberg, New York.
27. Sorgenfrei, O., Linder, D., Karas, M. & Klein, A. (1993) A novel very small subunit of a selenium containing NiFe hydrogenase of *Methanococcus voltae* is posttranscriptionally processed by cleavage at a defined position, *Eur. J. Biochem.* 213, 1355–1358.
28. Menon, N. K., Robbins, J., Der Vartanian, M., Patil, D., Peck, H. D. Jr, Menon, A. L., Robson, R. L. & Przybyla, A. E. (1993) Carboxy-terminal processing of the large subunit of [NiFe] hydrogenases, *FEBS Lett. 331*, 91–95.
29. 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.
30. Holland, D. & Wolk, C. P. (1990) Identification and characterization of *hetA*, a gene that acts early in the process of morphological differentiation of heterocysts, *J. Bacteriol. 172*, 3131–3137.
31. Mazel, D., Houmard, J., Castets, A. M. & Tandeau de Marsac, N. (1990) Highly repetitive DNA sequences in cyanobacterial genomes, *J. Bacteriol. 172*, 2755–2761.
32. Bauer, C. C., Scappino, L. & Haselkorn, R. (1993) Growth of the cyanobacterium *Anabaena* on molecular nitrogen: NifJ is required when iron is limited, *Proc. Natl Acad. Sci. USA 90*, 8812–8816.
33. Malki, S., Saimmaime, I., De Luca, G., Rousset, M., Dermoun, Z. & Belaich, J.-P. (1995) Characterization of an operon encoding an NADP-reducing hydrogenase in *Desulfovibrio fructosovorans*, *J. Bacteriol. 177*, 2628–2636.
34. Pilkington, S. J., Skehel, J. M., Gennis, R. B. & Walker, J. E. (1991) Relationship between mitochondrial NADH-ubiquinone reductase and a bacterial NAD-reducing hydrogenase, *Biochemistry 30*, 2166–2175.
35. Weidner, U., Geier, S., Ptock, A., Friedrich, T., Leif, H. & Weiss, H. (1993) The gene locus of the proton-translocating NADH: ubiquinone oxidoreductase in *Escherichia coli*, *J. Mol. Biol. 233*, 109–122.
36. Patel, S. D., Aebersold, R. & Attardi, G. (1991) cDNA-derived amino acid sequence of the NADH-binding 51-kDa subunit of the bovine respiratory NADH dehydrogenase reveals striking similarities to a bacterial NAD^+-reducing hydrogenase, *Proc. Natl Acad. Sci. USA 88*, 4225–4229.
37. Meyer, J. & Gagnon, J. (1991) Primary structure of hydrogenase I from *Clostridium pasteurianum*, *Biochemistry 30*, 9697–9704.
38. Stokkermans, J., van Dongen, W., Kaan, A., van den Berg, W. & Veeger, C. (1989) Hyd, a gene from *Desulfovibrio vulgaris* (Hildenborough) encodes a polypeptide homologous to the periplasmic hydrogenase, *FEMS Microbiol. Lett. 58*, 217–222.
39. Preis, D., Weidner, U., Conzen, C., Azevedo, J. E., Nehls, U., Röhlen, D., Van der Pas, J., Sackmann, U., Schneider, R., Werner, S. & Weiss, H. (1991) Primary structures of two subunits of NADH: ubiquinone reductase from *Neurospora crassa* concerned

with NADH oxidation. Relationship to a soluble NAD-reducing hydrogenase of *Alcaligenes eutrophus*, *Biochim. Biophys. Acta 1090*, 133–138.
40. Runswick, M. J., Gennis, R. B., Pernley, I. M. & Walker, J. E. (1989) Mitochondrial NADH:ubiquinone reductase: Complementary DNA sequence of the import precursor of the bovine 75-kDa subunit, *Biochemistry 30*, 9697–9704.
41. Walker, J. E. (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains, *Q. Rev. Biophys. 25*, 253–324.
42. Friedrich, B. & Schwartz, E. (1993) Molecular biology of hydrogen utilization in aerobic chemolithotrophs, *Annu. Rev. Microbiol. 47*, 351–383.
43. Schneider, K., Cammack, R. & Schlegel, H. G. (1984) Content and localization of FMN, Fe-S clusters and nickel in the NAD-linked hydrogenase of *Nocardia opaca* 1b, *Eur. J. Biochem. 142*, 75–84.
44. Meyer, J., Bruschi, M. H., Bonicel, J. J. & Bovier-Lapierre, G. E. (1986) Amino acid sequence of [2Fe-2S] ferredoxin from *Clostridium pasteurianum*, *Biochemistry 25*, 6054–6061.
45. Houmard, J. (1994) Gene transcription in filamentous cyanobacteria, *Microbiology 140*, 433–441.
46. Joerger, R. D., Jacobson, M. B. & Bishop, P. E. (1989) Two *nifA*-like genes required for expression of alternative nitrogenases by *Azotobacter vinelandii*, *J. Bacteriol. 171*, 3258–3267.
47. Schneider, K. & Schlegel, H. G. (1976) Purification and properties of soluble hydrogenase from *Alcaligenes eutrophus* H16, *Biochim. Biophys. Acta 452*, 66–80.
48. Sayavedra-Soto, L. A., Powell, G. K., Evans, H. J. & Morris, R. O. (1988) Nucleotide sequence of the genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase, *Proc. Natl Acad. Sci. USA 85*, 8395–8399.
49. Johannssen, W., Gerberding, H., Rohder, M., Zaborosch, C. & Mayer, F. (1991) Structural aspects of the soluble NAD-dependent hydrogenase isolated from *Alcaligenes eutrophus* H16 and *Nocardia opaca* 1b, *Arch. Microbiol. 155*, 303–308.
50. Halboth, S. & Klein, A. (1992) *Methanococcus voltae* harbors four gene clusters potentially encoding two NiFe and two NiFeSe hydrogenases, each of the cofactor F_{420}-reducing or F_{420} non-reducing types, *Mol. Gen. Genet. 233*, 217–224.
51. Muth, E. (1988) Localization of the F_{420}-reducing hydrogenase in *Methanococcus voltae* cells by the immuno-gold technique, *Arch. Microbiol. 150*, 205–207.
52. Lünsdorf, H., Niedrig, M. & Fiebig, K. (1991) Immuno-cytochemical localization of the coenzyme F_{420}-reducing hydrogenase in *Methanosarcina barkeri* Fusaro, *J. Bacteriol. 173*, 978–984.
53. Serebriakova, L., Zorin, N. A. & Lindblad, P. (1994) Reversible hydrogenase in *Anabaena variabilis* ATCC 29413, *Arch. Microbiol. 161*, 140–144
54. Berger, S., Ellersiek, U., Kinzelt, D., Steinmüller, K. (1993) Immunopurification of a subcomplex of the NAD(P)H-plastoquinone oxidoreductase from the cyanobacterium *Synechocystis* sp. PCC 6803, *FEBS Lett. 326*, 246–250.
55. Alam, J., Sprinkle, J., Hermodson, M. A. & Krogmann, D. W. (1984) Characterization of cytochrome c_{550} from cyanobacteria, *Biochim. Biophys. Acta 766*, 317–321.
56. Ewart, G. D. & Smith, G. D. (1989) Purification and properties of soluble hydrogenase from the cyanobacterium *Anabaena cylindrica*, *Arch. Biochem. Biophys. 268*, 327–337.
57. Ewart, G. D., Reed, K. C. & Smith, G. D. (1990) Soluble hydrogenase of *Anabaena cylindrica.* Cloning and sequencing of a potential gene encoding the tritium exchange subunit, *Eur. J. Biochem. 187*, 215–223.
58. De Zoysa, P. A. & Danpure, C. J. (1993) Possible evolutionary relationship between mammalian alanine:glyoxylate aminotransferase 1 and the 42-kDa subunit of cyanobacterial soluble hydrogenase, *Mol. Biol. Evol. 10*, 704–706.
59. Ouzounis, C. & Sander, C. (1993). Homology of the NifS family of proteins to a new class of pyridoxal phosphate-dependent enzymes, *FEBS Lett. 322*, 159–164.