The Expression of a Third Nitrogenase in the Cyanobacterium *Anabaena variabilis*

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Physiological experiments indicate that *Anabaena variabilis* can express either a V- or a Fe-nitrogenase in addition to the conventional, Mo-containing enzyme complex. The occurrence of the Fe-nitrogenase in *A. variabilis* can also be concluded from DNA-DNA hybridization experiments using cloned *anf*H or *nif*H probes coding for the smaller subunit (= nitrogenase reductase) of the Fe-nitrogenase from *Azotobacter vinelandii* or for the same subunit of the Mo-nitrogenase from *Klebsiella pneumoniae*. The cyanobacterium *A. variabilis* is the first phototroph found to contain all three nitrogenases which has been described only for *A. vinelandii* as yet. Vanadium cannot substitute for Mo in nitrate reduction of *A. variabilis*.

Introduction

Azotobacter vinelandii is the only organism so far reported to have the gene sets for three different classes of nitrogenases [1, 2]. In the presence of Mo in the medium, *A. vinelandii* expresses the conventional, Mo-nitrogenase which is encoded by the three structural genes *nif*HDK. When the medium is supplemented with V and lacks Mo, the bacterium synthesizes an alternative nitrogenase with a FeV-cofactor in the active centre. This enzyme complex produces more H_2 and reduces C_2H_2 with lower rates than the conventional, Mo-nitrogenase and forms low but significant amounts of C_2H_6. The production of C_2H_6 allows the occurrence of an alternative nitrogenase in an organism to be monitored by gas chromatography [3]. The V-nitrogenase is genetically determined by the four structural genes *vnf*HDGK. The V-nitrogenase has recently been shown to occur in the cyanobacterium *Anabaena variabilis* which grows in dependence on V in the medium under N_2-fixing conditions [4].

It has been established that *A. vinelandii* [5–7] possesses the gene set for a third nitrogenase encoded by *anf*HDGK which probably contains solely Fe-S centres in the prosthetic group [8]. This Fe-nitrogenase reduces N_2 and particularly C_2H_2 with low rates, produces H_2 with even higher rates than the V-enzyme and also forms small amounts of C_2H_6. The H-gene encoding the small subunit (also termed nitrogenase reductase) shows a high degree of sequence homology in all three nitrogenases. Therefore the H-gene probes from all three nitrogenases hybridize with the other two H-genes. This is not the case with the other structural genes which generally do not cross-hybridize [1, 6].

The present investigation was performed to characterize growth of *A. variabilis* under Mo-deficiency in more detail. These studies allow to conclude that this cyanobacterium possesses the Fe-nitrogenase in addition to the Mo- and V-enzymes.

Materials and Methods

Growth of the organism

The heterocystous *Anabaena variabilis* (ATCC 29413) was grown exactly as described in the preceeding publication [4]. Where used, the concentration of the trace elements was in μM: Na_2MoO_4 0.5; $VOSO_4$ 1, ReO_7 1, Na_2WO_4 1. Nitrate-media contained either 2 or 10 mM $NaNO_3$ and otherwise the same salts as for N_2-fixing cultures. Analytical grade chemicals (Merck A.G., Darmstadt) and double-distilled water without further purification were used for all the experiments.

Assay conditions and quantitative determinations

C_2H_2-reduction assays were performed in 7.0 ml Fernbach flasks exactly as previously [4]. The

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amounts of C_2H_4 and C_2H_6 formed from C_2H_2 were quantitatively determined in a gas chromatograph equipped with a flame ionization detector and a Carbosieve SII column at 210 °C and with N_2 as the carrier gas [4]. H_2 was measured in a gas chromatograph fitted with a thermal conductivity detector and a molecular sieve column [4]. Nitrate was quantified either colourimetrically with salicylic acid [9] or by its absorbance at 214 nm after separation by HPLC using PIC-A-reagent low UV (Waters-Millipore) [10]. Mo and V concentrations in the media were determined by atomic absorption spectrometry using a Perkin-Elmer model 3030 (kindly performed by Bayer A.G., Chemische Analytik, D-5090 Leverkusen). Protein from intact cells was quantified by a modification of the Lowry method [11].

Isolation of the genomic DNA and Southern hybridization

The hybridization protocol was essentially the same as in [6]. *A. variabilis* (1 l culture) was harvested by centrifugation, washed twice with 10 mM phosphate-buffer pH 7.0 and resuspended in 25 ml of 50 mM glucose, 25 mM Tris-HCl buffer pH 8.0, 10 mM EDTA and 2 mg/ml lysozyme. After incubating at 30 °C for 2 h, 10 ml 250 mM EDTA and 3.5 mg proteinase K were added followed by a further incubation for 10 min. After adding 5 ml 10% SDS, the suspension was kept at 50 °C for 2 h. DNA was extracted twice with phenol/chloroform and dialysed against 10 mM Tris-HCl pH 7.0, 1 mM EDTA overnight. DNAs (10 µg per slot) were digested with restriction endonucleases, electrophoresed on 0.8% agarose gels in Tris-acetate-EDTA and transferred to nylon membranes (Hybond-N, Amersham-Buchler, Braunschweig) following the manufacturer's instructions. ^{32}P-labeled DNA-probes were synthesized by nick translation and hybridization was done in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na-citrate), 0.5% SDS, 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinyl pyrrolidone and 40% formamide at 42 °C (see [12]). The probes used were for *anf*H: 0.9 kb *Sph*I/*Eco*RI fragment of pLWH 3 containing the *anf*H gene from *A. vinelandii* cloned in pUC 18 (kindly supplied by Dr. P. Bishop, Raleigh, U.S.A.) and for *nif*H(D): 1.8 kb *Bam*HI/*Eco*RI fragment of pHK 8 containing the complete *nif*H and part of the *nif*D gene from *Klebsiella pneumoniae* (kindly supplied by Dr. R. N. Pau, Brighton, G.B.).

Results

In earlier investigations [13, 14] tungsten had been shown to be incorporated into nitrogenase of *A. vinelandii* which, however, was catalytically inactive. Growth of an unidentified strain of the cyanobacterium *Nostoc muscorum* was affected by W when either N_2 or NO_3^- served as the N-source [15]. Surprisingly, spontaneous mutants of this *Nostoc* strain required W for the growth under N_2-fixing or NO_3^--assimilating conditions [15]. As expected, the addition of tungsten to cultures performing N_2-fixation by the Mo-nitrogenase severely suppressed both C_2H_4- and H_2-formation activities by *A. variabilis* (Table I). In contrast, the addition of W to cultures which had expressed V-nitrogenase enhanced C_2H_2-reduction activity, left H_2-formation unaltered and diminished C_2H_6-production. Tungsten is likely incorporated into the FeV-cofactor of this enzyme which may cause not easily explainable changes in the catalytic activities.

Rhenium caused a parallel decrease of C_2H_2-reduction and H_2-formation activities by both Mo- and V-nitrogenase finally resulting in the death of the cultures. Likewise, Re could not substitute for Mo in *Anabaena variabilis* cultures growing on nitrate. Methionine sulfoximine (MSX) is known to

Table I. The effect of W on N_2-fixing *Anabaena variabilis* which had expressed either Mo- or V-nitrogenase.

Culture condition	Formation of C_2H_4	H_2	C_2H_6
A) Mo-culture:			
a) control culture grown with Mo	598	0.5	0
b) medium with 10 µM W	155	0.3	0
c) medium with 100 µM W	10	0.3	0
B) V-culture:			
a) control culture grown with V	280	5.0	2.8
b) medium with 10 µM W	310	4.5	1.4
c) medium with 100 µM W	340	5.0	0

Anabaena had been grown with either Mo or V in the medium and had fully expressed Mo- or V-nitrogenase. Mo or V was then substituted by $Na_2WO_4 \times H_2O$ and the activities were determined after 11 d. Rates are given in nmol × h^{-1} × mg $protein^{-1}$.

Table II. The addition of nitrate to N_2-fixing cultures of *Anabaena variabilis* grown with either Mo or V.

	h after addition of 10 mM $NaNO_3$			
	Mo-culture		V-culture	
	0	72	0	72
C_2H_2-reduction rate (nmol × h^{-1} × mg protein^{-1})	380	186	122	120
protein content (mg/ml)	0.5	1.0	0.35	1.0
nitrate content (mM)	10	6.5	10	9.8
% heterocysts within the filaments	7	3.5	7	7

A. variabilis grown under N_2-fixing conditions was diluted with medium containing $NaNO_3$ and incubated in the light at 30 °C and an atmosphere of air +5% CO_2.

specifically affect glutamine synthetase also in cyanobacteria [16]. As shown by the inhibition pattern with MSX, both Mo- and V-grown cells incorporated ammonia formed by nitrogenase *via* the glutamine synthetase/glutamate synthase pathway. The inhibitor caused an increase in the C_2H_2-reduction activity particularly in V and to some extent also in Mo-cultures (not documented).

Molybdenum is also a component of NO_3^--reductase and can apparently not be substituted by V (Table II). The addition of nitrate to cells which had expressed Mo-nitrogenase resulted in a decrease of C_2H_2-reduction activity and heterocyst frequency by some 50% after 72 h. Such a culture utilized nitrate and continued to grow. In contrast, V-grown cells performed C_2H_2-reductions with unaltered rates after the addition of nitrate. The heterocyst frequency remained unchanged, and the filaments could not reduce nitrate and grew by performing N_2-fixation (Table II). No evidence for an occurrence of an alternative, V-containing nitrate reductase was obtained also from a whole series of similar experiments.

In these experiments, a control culture was kept in a medium containing neither Mo nor V nor nitrate. This culture was diluted every second day with fresh Mo and V deficient medium (40% fresh medium, 60% culture, v/v). After approximately 30 d it had lost its blue colour due to phycobilin degradation. Phycobilins are known to serve as an N-reserve under N-deprivation [17]. The culture restarted growth after approximately 50 d. Growth determinations between days 50 and 135 showed a 2–4-fold increase within 4 d which is almost as fast as in a Mo-culture. After about 80 d the culture had recovered phycobilin content and heterocyst frequency and the filaments looked healthy. Such a culture reduced C_2H_2 with about 10 times lower activity than Mo-grown cells but produced more H_2 (Table III). This growth experiment was repeated with essentially the same results. In addition, a culture which had expressed these activities could be stored on agar slants.

Determination by atomic absorption spectrometry showed that the concentration of Mo and V in the medium was below the detection limit which was 10 nM for Mo and 20 nM for V. To determine the amount of Mo required for half-maximal expression of the conventional nitrogenase, a V-grown, N_2-fixing culture was supplemented with different concentrations of Mo (Fig. 1). Measurements of the increase in C_2H_2-reduction and decrease in H_2-formation rates gave half-maximal expression of the Mo-nitrogenase at 100 nM. Similarly, previous experiments [4] had shown that the half maximal expression of V-nitrogenase required

Table III. Nitrogenase activity of a culture growing 100 d without V or Mo. Evidence for the occurrence of the Fe-nitrogenase in *A. variabilis*.

	Formation of		
	C_2H_4	H_2	C_2H_6
without addition	49.2	1.3	0.3
activity 3 h after the addition of 0.5 μM Mo	90.6	2.8	1.1

Rates are given in nmol × h^{-1} × mg protein^{-1}.

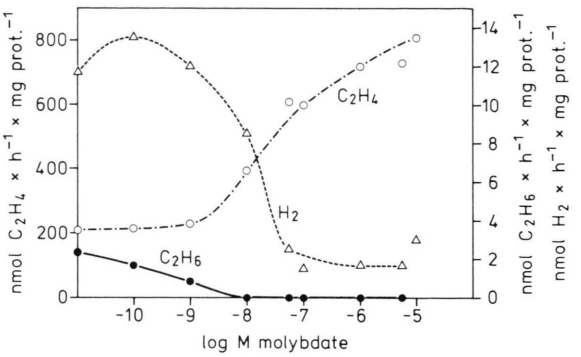

Fig. 1. Nitrogenase activities in V-grown cultures 4 days after the addition of Mo to the cultures. V-cultures in the logarithmic growth phase were diluted with fresh V-medium (20% culture/80% medium) and supplemented with the different concentrations of molybdate as indicated in the abscissa of the figure. Nitrogenase activities were determined after 4 days of growth. For other details see Materials and Methods.

at least 100 nM also of this element. Therefore nitrogenase activities in the Mo- and V-free medium is unlikely due to contaminants in the water or minerals salts.

The conclusion that activity is due to a third, Mo- and V-independent nitrogenase is corroborated by the observation that the addition of Mo to such cultures increased H_2 and C_2H_6 formations within 2–4 h (Table III). The latter gas was unequivocally demonstrable only by such treatment within the detection limits of the assay conditions employed. Similar rapid increases after the addition of Mo are features of only the Fe-nitrogenase of *Azotobacter vinelandii* [6]. They might be due to the incorporation of the FeMo-cofactor into the Fe-nitrogenase [2, 6] which may also happen with the *Anabaena variabilis* enzyme. It should be noted that similar rapid increases in the H_2- and C_2H_6-formation did not occur within 2–4 h when the V-grown cultures of Fig. 1 were supplemented with Mo (not documented).

The hybridization using the cloned gene of *nif*H (specific probe for the small subunit of the Mo-nitrogenase) or *anf*H (specific probe for the small subunit of the Fe-nitrogenase) as probes gave two strong bands at 9 and 4.8 kb and a weak one at >12 kb in each case when the DNA from *Anabaena variabilis* digested with *Eco*RI but not with HindIII was assayed (Fig. 2). Such an experiment adds to the informations on the existence of more than one nitrogenase in *Anabaena variabilis*. The faint hybridization signal at >12 kb could indicate the existence of three *nif*H like genes. However, artifacts cannot be excluded with such weak signals.

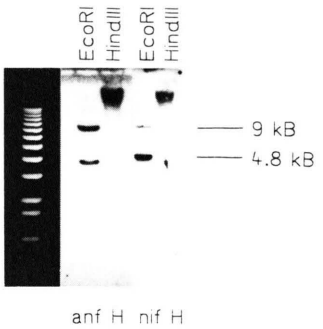

Fig. 2. DNA-DNA hybridization using the digested total DNA from *Anabaena variabilis* and the nitrogenase *nif*H and *anf*H genes as probes. For experimental details see Materials and Methods. Lane 1 shows the molecular weight markers (1 kb ladder).

Discussion

The physiological evidence of this and the preceeding [4] publication indicates the occurrence of all three nitrogenases in *A. variabilis*. The Mo-nitrogenase is expressed when the medium contains Mo. The existence of the V-nitrogenase can be deduced from the findings that *A. variabilis* can be grown in dependence on V in the medium and that C_2H_2-reduction of such cultures is 2/3 lower than in Mo-grown cells [4]. In addition, Mo-deficient and V-grown cultures express V-nitrogenase without a transient state of N-deprivation as observed for Fe-nitrogenase. Arguments for the occurrence of the Fe-nitrogenase are the extensive transient state of N-deprivation (for which no explanation is available) and the low C_2H_2-reduction rate which is approximately 1/10 of that for Mo-nitrogenase. The different pattern in the formations of H_2 and C_2H_6 observed in +V and in −V −Mo grown cultures observed 2–4 h after the addition of Mo also indicates the existence of two alternative nitrogenases.

The hybridization experiments with the *nif*H(D) probe only point to the existence of at least two

different nitrogenases in *A. variabilis*. It had been shown by sequencing that the 9 kb *Eco*RI fragment of *A. variabilis* genomic DNA contains the whole linked *nif*H and *nif*D genes [18]. Therefore the second Southern hybridization band is not due to splitting within these genes at an *Eco*RI restriction site. Two *nif*H like sequences of unknown function had been detected in the cyanobacterium *Anabaena* 7120 [19] and even three in *Calothrix* [20]. The third hybridization band observed in the present study is so weak that any conclusion about the existence of a third H gene is not permitted. Hybridization with a *vnf*H probe was not attempted because the percentage identity between *nif*H and *vnf*H is high (1). Final proof for the occurrence of three nitrogenases in *A. variabilis* might only come from the construction of deletion mutants which cannot be achieved easily but is a long-term goal at present.

As compared to H_2-formation by alternative nitrogenases from *A. vinelandii*, H_2-evolutions by these enzymes from *A. variabilis* were small. This is not a special feature of the alternative nitrogenases from *A. variabilis* but is due to the fact that the experiments were performed with intact cells. Cyanobacteria possess two active hydrogenases which effectively reutilize most of the H_2 produced by nitrogenase. As shown for the non-N_2-fixing *Anacystis nidulans*, one of these enzyme (the "uptake" hydrogenase) resides on the thylakoid membrane and the other one (the "bidirectional" hydrogenase) is located at the cytoplasmic membrane [21].

This is the first report for an organism other than *A. vinelandii* to possess three nitrogenases. Currently the distribution of alternative nitrogenases among organisms is puzzling. *Azotobacter chroococcum* which is closely related to *A. vinelandii* has only the Mo- and the V-enzymes [7]. The phototroph *Rhodobacter capsulatus* apparently can express the Fe-nitrogenase in addition to the Mo-protein [22] and the archaebacterium *Methanosarcina barkeri* 227 is reported to possess two different Mo-nitrogenases [23]. A survey of the distribution of the enzymes among cyanobacteria is lacking. In another *Anabaena* species, *Anabaena cylindrica*, the V-nitrogenase is apparently missing because the addition of V to a Mo-deficient culture caused an amplification of the symptoms of Mo-deficiency [24]. Like the distribution, the ecological role of these enzymes is currently mysterious.

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