Localization of the Reversible Hydrogenase in Cyanobacteria

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

The reversible hydrogenase of the cyanobacterium *Anacystis nidulans* was purified 250-fold by classical methods. Activity staining on gels obtained by native PAGE allowed to identify two bands. Antibodies were raised against the electrophoretically homogeneous protein. The molecular weight of the hydrogenase was determined by SDS-PAGE followed by Western blot analysis with these antibodies. Crude extracts from the unicellular *Anacystis* and from heterocysts and vegetative cells of *Anabaena variabilis* showed precipitation bands of 56 and 17 kd. The reversible hydrogenase appeared to be composed of a larger and a smaller subunit as the NiFeS-hydrogenases from other bacteria. The hydrogenase subunits, particularly the smaller one, were subject to proteolysis. By application of the protein A-immuno-gold labeling technique the reversible hydrogenase was shown to associate with the cytoplasmic membrane in *Anacystis*. When *Anacystis* was grown microaerobically, the increase in the specific activity of the reversible hydrogenase paralleled the raise in the gold-labeling at the cell periphery as measured from the electron micrographs. It is concluded that *Anacystis* has two different hydrogenases: the reversible or bidirectional hydrogenase which is located exclusively at the cytoplasmic membrane of the cells, and a thylakoid-bound enzyme which catalyzes only the uptake of H_2.

Introduction

Both the conventional, Mo-containing nitrogenase [1−3], and the alternative, V-enzyme [4] of cyanobacteria catalyze the formation of H_2 simultaneous with the reduction of N_2 to NH_4^+. In the intact organisms, little H_2-production is normally seen, because the H_2 produced by nitrogenases is immediately reutilized by the uptake hydrogenase. Such reactions occur on the thylakoid membranes. H_2-utilization was established to proceed by two different pathways, namely the respiratory-linked and the photosystem I-dependent ones [5−9]. Uptake hydrogenase feeds in electrons to plastoquinone or a component near the plastoquinone site. Photosynthesis and respiration on the thylakoids share a common segment of electron carriers consisting of plastoquinone, the cytochrome-bf-complex and cytochrome-c_{553}, from where the electrons of H_2 are distributed to either the reaction center of photosystem I or the respiratory cytochrome oxidase [10−12]. The thylakoid-bound uptake hydrogenase may occur in two different forms [13, 14], but this aspect has not been studied in detail. The uptake hydrogenase is particularly active in heterocysts and is weakly [15] or not at all [3, 16] expressed in NH_4^+-grown and heterocyst-free filaments of cyanobacteria. In the heterocystous *Anabaena* 7119, H_2-consumption by the uptake hydrogenase is activated by thioredoxin [17].

Whilst there is little disagreement among the investigators about the two pathways of H_2-utilization at the thylakoid membranes, the existence of an additional hydrogenase, the "reversible" enzyme in cyanobacteria has been a matter of controversy since years. This enzyme is known from a number of bacteria, *e.g.* *Clostridium* or *Desulfovibrio* species, and was termed "bidirectional" hydrogenase [18]. Crude extracts from cyanobacteria were repeatedly described to contain a hydrogenase which catalyzed both the evolution of H_2 (with $Na_2S_2O_4$ and methylviologen as electron donors) and the uptake of H_2 (with electropositive acceptors like methylene blue, phenazinemethosulfate but not with ferredoxin) [19−21, 3]. Eisbrenner *et al.* [22] observed that the H_2-evolution activity increased more, the more drastic the method used to break the cells. They sug-

gested that the reversible hydrogenase is an artifact of cell-free preparations in cyanobacteria. Houchins and Burris [14, 23], however, were able to separate the reversible and uptake hydrogenases from each other by chromatography on DEAE-cellulose. The activity level of the reversible hydrogenase, but virtually not that of the uptake enzyme, was drastically enhanced by incubating intact cyanobacteria under anaerobic conditions [14, 23, 24]. Such findings were confirmed by our laboratory [17]. It remained mysterious that reversible hydrogenase could at best poorly support physiological reactions known to be H_2-dependent in other organisms. The rates of these reactions in cyanobacteria reflected only a small fraction of the total hydrogenase activity present [3, 17].

The present communication describes the purification of the reversible hydrogenase. It will be shown by the immuno-gold labeling technique that the enzyme resides at the cytoplasmic membrane in *Anacystis nidulans*.

Materials and Methods

General

Anacystis nidulans (= *Synechococcus leopoliensis* [25]) was the no. 1402-1 of the Sammlung von Algenkulturen of the Pflanzenphysiologisches Institut, Universität Göttingen, F.R.G., and *Anabaena variabilis* ATCC 29413 was kindly made available to us by Professor C. P. Wolk, East Lansing, U.S. The cyanobacteria were grown either aerobically (air/$CO_2 = 95/5$ by vol.) or microaerobically under continuous flushing with $N_2/CO_2 = 95/5$ in Allen and Arnon's medium under conditions described previously [26]. *Anabaena variabilis* was grown without combined nitrogen [26]. To get enough cells for the purification of hydrogenase, *Anacystis* was grown in 5 l Penicillin flasks in which the medium was supplemented with 12 μM $NiCl_2$ and 20 μM EDTA. The preparation of extracts from vegetative cells and heterocysts of *Anabaena variabilis* was described [22]. H_2-uptake with either phenazinemethosulfate or methylene blue as electron acceptor was measured amperometrically with the H_2-probe exactly as described previously [17]. 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 gaschromatography [17]. Protein content was measured by the Bradford method [27].

Purification of the reversible hydrogenase

9 l *Anacystis* culture in the logarithmic growth phase were centrifuged ($4000 \times g$, 5 min), suspended in 50 mM K-phosphate buffer pH 6.5 and flushed with argon for 20 min. All the following steps were done under a continuous stream of argon. The cells were passed twice through a French Press at 137,000 KPa, and debris was removed by centrifugation ($48,000 \times g$, 30 min). The supernatant with a protein content of 300−400 mg was subjected to 20% $(NH_4)_2SO_4$ precipitation and centrifuged ($48,000 \times g$, 20 min). The pellet was discarded, and the supernatant was dialysed overnight against 50 mM phosphate buffer pH 6.5, 0.15 M KCl and 1 mM phenyl-methyl-sulfonyl-fluoride (as protease inhibitor). The dialysate was given onto a Whatman DE-52 cellulose column (20×4.2 cm) which had been preequilibrated with 0.15 M KCl dissolved in 50 mM phosphate buffer pH 6.5. The column was washed with this buffer until the eluate was free of protein. Hydrogenase was eluted from the column by using a linear KCl-gradient (0.15−0.4 M KCl, 300 ml altogether) in 50 mM phosphate buffer pH 6.5. Hydrogenase fractions (eluting around 0.29 M KCl) were pooled and given onto an Octyl-Sepharose CL-4B column (Pharmacia, 13×1.6 cm) preequilibrated with 0.3 mM KCl dissolved in 20 mM Tris HCl buffer pH 7.5. The column was washed with the same buffer and then with only 20 mM Tris HCl. Hydrogenase could be eluted from the column by 10% ethanol in 20 mM Tris HCl pH 7.5. Proteins eluting with 40% or 90% ethanol had no hydrogenase activity. Hydrogenase fractions were concentrated about twofold in dialysis tubes by incubation with polyethylenglycol 20,000 and subjected to a discontinuous gel electrophoresis on 7.5% polyacrylamide gel as described by Blackshear [28]. Separation under denaturating conditions was performed on 12% polyacrylamide gels and 0.1% SDS. Proteins were stained with Coomassie brilliant blue R-250 (Serva) or with silver [29] if a more sensitive staining was necessary.

Preparation of the antibodies and immuno-gold labeling

For preparing polyclonal antiserum, the upper hydrogenase band of the native gel electrophoresis with about 200 μg protein (see Fig. 1) was cut out and suspended in 500 μl phosphate buffered saline (see [30]). The hydrogenase preparation was mixed

with the same volume of Freund's complete adjuvant and injected subcutaneously into a rabbit. A booster shot of the same amount of hydrogenase in Freund's incomplete adjuvant was given after 2 weeks. Twenty ml blood were taken from the ear vein 10 days after the second injection, stored at 4 °C overnight, centrifuged ($4,000 \times g$, 10 min), frozen in liquid nitrogen and stored at -20 °C. Prior to use, intact IgG antibodies were purified by affinity chromatography on a protein-A-Sepharose CL-4B column (15×1 cm, Pharmacia). The IgG-fraction eluted from the column with 100 mM glycine pH 3.0 and was made up to pH 6.5 with phosphate buffer. The specificity of the antibodies was tested by the Western blot technique [31]. The hydrogenase-antibody interactions on the nitrocellulose membrane were detected by using a secondary antibody, a goat-anti-rabbit IgG-alkaline phosphatase conjugate (Sigma).

For the immuno-gold labeling, *Anacystis* was washed three times in phosphate buffered saline and chemically fixed by incubation in buffered glutaraldehyde (0.2%, w/v) / formaldehyde (0.3%, w/v) in ice for 1 h. The cells were then washed three times with 10 mM glycine in buffer and embedded in the low temperature resin Lowicryl K4M. Details of the procedure have been published [32, 33, 30]. The post-embedding labeling of hydrogenase was performed on ultrathin sections with protein A-colloidal gold as also described in the same publications [32, 33, 30]. The gold particles used had a diameter of 14 nm, and 20 *Anacystis* cell sections of comparable size were counted for each growth condition. Label closer than 50 nm to the periphery was counted as being associated with the cytoplasmic membrane, and the rest of label in the cells was defined as being located in the cytoplasm. The amount of background label (5–9 gold particles/cell) as determined by incubation without hydrogenase antibodies was substracted in all cases.

Results

When *Anacystis* was grown aerobically, reversible hydrogenase had a specific activity of less than 10 nmol H_2 evolved $min^{-1} \times mg\ protein^{-1}$ in the crude extract. Any enzyme purification is problematic with such low activity in the starting material. Attempts to purify reversible hydrogenase by affinity chromatography with reactive-red-120 agarose as done with the enzyme from *Bradyrhizobium japonicum* [34] or with Azur A-Sepharose CL-4B failed with the *Anacystis* enzyme (see [30]). Reversible hydrogenase from this cyanobacterium could, however, be enriched 250-fold by the use of the classical methods ion exchange chromatography with DEAE-Cellulose and hydrophobic chromatography with Octyl-Sepharose CL-4B (Table I). There are

Table I. Purification of the reversible hydrogenase from *Anacystis nidulans*.

Purification step	Specific activity	Enrich-ment	Total activity ($\times 10^3$ U)	Yield [%]
1. crude extract	855	1.0	329	100
2. supernatant after 20% $(NH_4)_2SO_4$ addition	929	1.1	254	77
3. linear KCl gradient on DEAE-cellulose fraction no 10	2631	3.0	69	21
11	3993	4.6	110	33
12	2170	2.5	24	7
4. Octyl-Sepharose CL-4B chromatography fraction no 4	16136	18.9	53	16
5	214408	250.7	85	26

The specific activity is given in nmol $H_2/(h \times mg\ protein)$ in the $Na_2S_2O_4$ and methyl viologen dependent evolution assay. The total activity refers to a 9 l culture which was concentrated to 90 ml crude extract. 1 U corresponds to 1 nmol H_2 evolved/h.

claims in the literature [35, 36] that cyanobacterial hydrogenases are damaged by oxygen, but the opposite has also been reported [14, 19]. This aspect has not been studied systematically in the present investigation. The O_2-sensitivity of the hydrogenase may differ with the oxidation state of the enzyme, and the oxidation state of the enzyme may change within the isolation protocol [3, 35]. Therefore all purification steps were routinely performed under argon. All manipulations were done at room temperature where the enzyme had higher stability than at 4 or 0 °C.

Hydrogenase retains 30—40% of the H_2-evolving activity on the gels after native polyacrylamide gel electrophoresis (= PAGE). When the 250-fold purified hydrogenase preparation was electrophoretically separated on 7.5% polyacrylamide gels, two bands showed hydrogenase activity (Fig. 1). The upper band was cut out and used for the preparation of antibodies. Western blot analysis gave the somewhat surprising result that antibodies correlated only with the lower band (Fig. 1). Reversible hydrogenase is known to aggregate [3]. To explain the Western blot data, it is assumed that the upper band consists of a hydrogenase polymer in which the antigenic sites are buried within the molecule. However, attempts to transform the upper band hydrogenase into the lower band protein and vice versa failed so far.

Comparable concentrations of the antibodies blocked the evolution and uptake of H_2 to approximately the same extent in crude extracts of *Anacystis*. A residual activity of 40—50% remained, however, unaffected in both assays (Fig. 2).

The molecular weight of the "reversible" hydrogenase was estimated by SDS gel electrophoresis followed by Western blot analysis using conventional marker proteins (Fig. 3). The 250-fold purified hydrogenase preparation gave a band at 53 kDa and weaker one at 56 kDa. Crude extracts from *Anacystis* showed bands at 56, 17 kDa and a weaker one at 53 kDa. The antibodies against hydrogenase from *Anacystis* crossreacted also with the enzyme from the heterocystous *Anabaena variabilis* (Fig. 3). Extracts from heterocysts of this cyanobacterium gave bands at 56 kDa and 17 kDa. Multiple bands were obtained with extracts from vegetative cells, stronger ones at 56, 43 and 16 kDa, and weaker ones at 59 and 53 kDa. It cannot yet be excluded that this latter
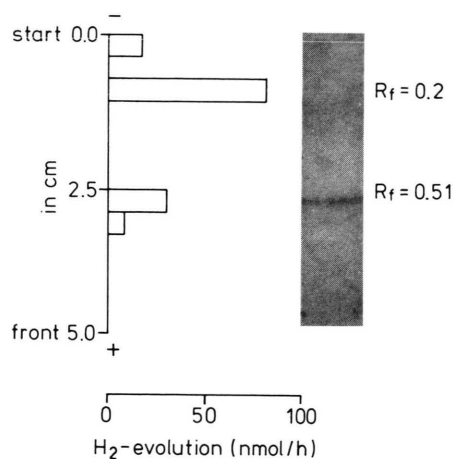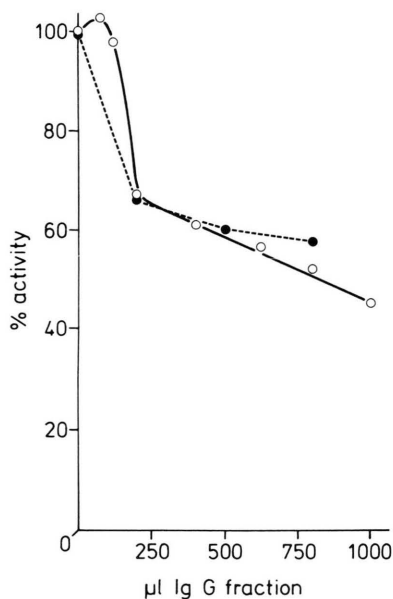
Fig. 1. Western blot analysis of the reversible hydrogenase in crude extracts of *Anacystis nidulans*. The hydrogenase fractions in the crude extract were separated from each other by a non-denaturing 7.5% polyacrylamide gel electrophoresis. One part of the gel was cut into 0.5 cm pieces which were assayed for $Na_2S_2O_4$ and methyl viologen dependent H_2-evolution activity. The other part was taken for the Western blot analysis.

Fig. 2. Inhibition of the evolution and uptake of H_2 by hydrogenase IgG antibodies. O———O $Na_2S_2O_4$ and methyl viologen dependent H_2-evolution activity. The assay was performed with the crude extract of 0.57 mg protein/assay in Fernbach flasks. 100% activity corresponded to 200 nmol H_2 evolved/(h × mg protein); ●-----● H_2-uptake with phenazinemethosulfate in the crude extract. The assay was performed with a total amount of 1 mg protein in the H_2-electrode. 100% activity corresponded to 525 nmol H_2 consumed/(h × mg protein). For both assays the purified IgG antibodies had a concentration of 1.6 mg/ml.

weaker bands in vegetative cells are due to unspecific precipitations.

Attempts have been made to characterize hydrogenase from cyanobacteria by DNA-DNA hybridization experiments. Cloned DNA of the membrane-bound uptake hydrogenase from *Bradyrhizobium japonicum* (plasmid pHU 52) and of the reversible hydrogenase from *Desulfovibrio vulgaris* (plasmid pHU 150) was kindly made available to us by Prof. H. J. Evans, Corvallis, Oregon and Dr. G. Voordouw, Cambridge, U.K., respectively. Using the Southern hybridization technique with [^{32}P]dATP nick translated DNA, positive results could not be obtained with DNA isolated from *Anacystis nidulans*, or *Anabaena variabilis* even under low stringencies. Controls with DNA from the bacterium *Azospirillum brasilense* Sp7 were, however, positive with the probe from *Bradyrhizobium japonicum* (for details, see [30]).

Fig. 4 shows representative electron micrographs obtained by the application of the post-embedding protein A-gold labeling technique on ultrathin sections of *Anacystis nidulans*. When *Anacystis* was grown aerobically, the label was concentrated along the cell periphery in close vicinity of the cytoplasmic

Fig. 3. Molecular weight determination of the reversible hydrogenase by SDS-gel electrophoresis followed by Western blot analysis.
1: purified hydrogenase from *Anacystis nidulans*;
2: crude extract from *Anacystis nidulans*;
3: extract from heterocysts of *Anabaena variabilis*;
4: extract from vegetative cells of *Anabaena variabilis*;
5: marker proteins with the following bands and molecular weights (in kDa):
   a) phosphorylase B 92.5;
   b) bovine serum albumin 66.2;
   c) ovalbumin 45;
   d) carbonic anhydrase 31;
   e) trypsin inhibitor 21.5;
   f) lysozyme 14.4.
Marker proteins were obtained from Biorad Lab. Munich. For experimental details see Materials and Methods.

membrane (Fig. 4a, b). Growth under microaerobic conditions increased the labeling along the periphery (Fig. 4c, d). Control experiments without hydrogenase antibodies did not exhibit any specific deposition of gold particles (data not shown). A specific labeling of the thylakoid membrane or of hydrogenase inclusion bodies [32] within the cytoplasm could not be detected in any of the electron micrographs. A number of cell thin sections of approximately the same size were counted to get statistically sound data (Table II). This evaluation confirmed the visual impression that the gold particles were associated with the periphery. When the cultures were grown microaerobically, the increase in the label along the cytoplasmic membrane paralleled the increase in the specific activity, whereas the number of the colloidal gold particles within the cytoplasm did not change (Table II).

Table II. Localization of the reversible hydrogenase in aerobically and microaerobically grown *Anacystis nidulans* by the immuno-gold labeling technique.

	Cells grown Aerobically	Microaerobically
specific H$_2$-evolution activity	269	408
gold label associated with the cytoplasmic membrane	65 ± 11	108 ± 9
gold label associated with the cytoplasm	26 ± 11	28 ± 8

The specific H$_2$-evolution activity is given in nmol H$_2$ evolved/(h × mg protein). The immuno-gold labeling is described under Materials and Methods. Twenty cell sections of comparable size for each growth condition were evaluated, and standard deviations were calculated.
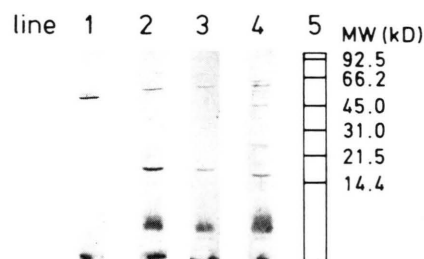
Discussion

In the present study, the reversible hydrogenase of *Anacystis nidulans* is shown to be associated with the cytoplasmic membrane by the immuno-gold labeling technique. The enzyme is clearly different from the uptake hydrogenase which couples to the segment of electron carriers shared by photosynthesis and respiration in cyanobacteria [10−12, 37]. It had been demonstrated that H$_2$ supports the respiratory O$_2$-uptake and the photoreduction of NADP$^+$ in particles from *Anacystis* [8] and from heterocysts of *Anabaena* 7119 [22]. The latter reaction is partic-
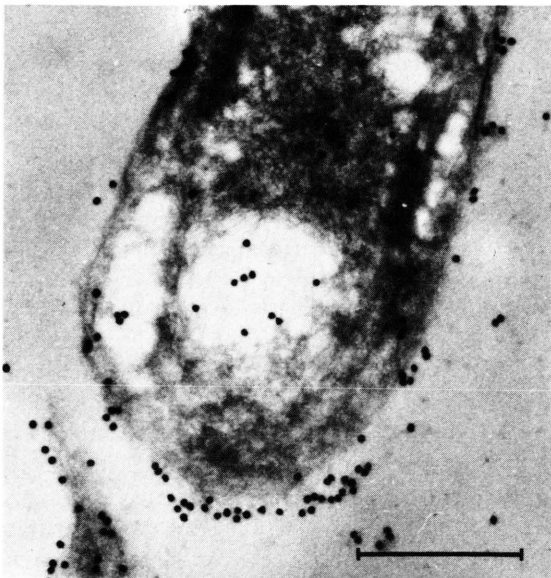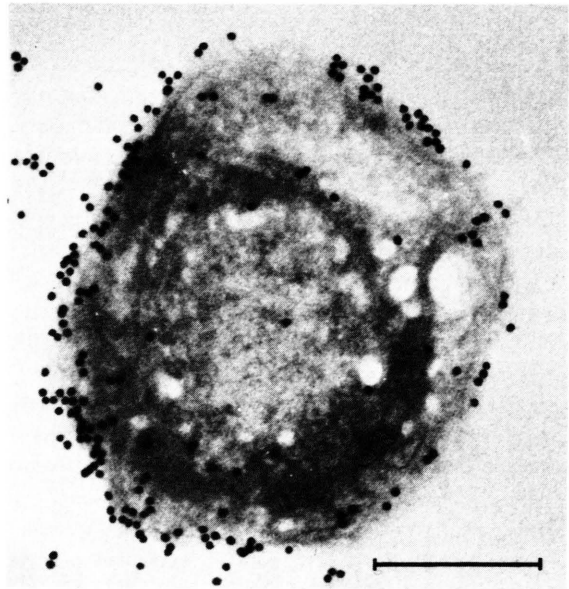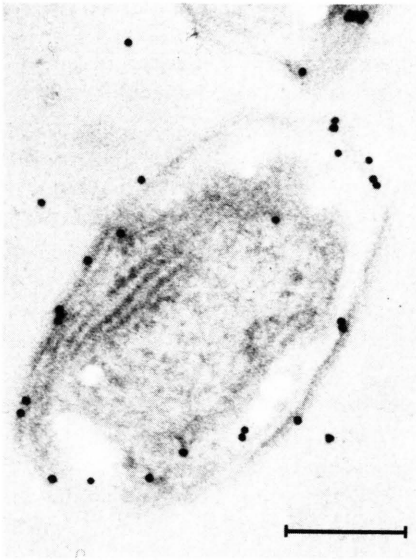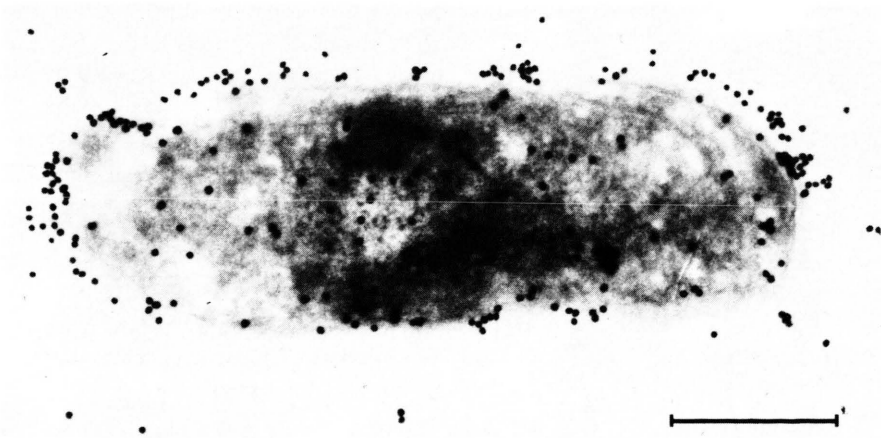
Fig. 4. Localization of the reversible hydrogenase by immuno-gold labeling in *Anacystis nidulans*. a, b Aerobically grown cells; c, d cells grown under nitrogen gas (= micro-aerobically grown cells). The magnification was 78,000 × in a), and 110,000 × in b), c) and d). The bars correspond to 1 μm. For details see Materials and Methods.

ularly indicative for the location of the uptake hydrogenase on the thylakoid membranes. Thus the earlier statement that the reversible hydrogenase is an artifact of cell-free preparations [22] is no longer valid. The reversible hydrogenase is released into the soluble fraction after breaking the cells, and it was deduced from this observation that it is not an integral membrane protein [3]. However, such a conclusion needs not be right, because the cell envelope in cyanobacteria appears to be particularly fragile. This can be concluded from the observation that cyanobacterial cells readily lose phycobilins at least partly after freezing or even after washing and centrifugation at high speed. An inverse correlation between the $Na_2S_2O_4$ and methyl viologen dependent H_2-evolution activity and the integrity of the membrane fractions had earlier been found in extracts from cyanobacteria [22], which could indicate that the reversible hydrogenase is a membrane protein in reality. The enzyme from *Anacystis* is not unique in the aspect that it can be purified as soluble protein, although membrane-associated. Such properties have also been reported for the hydrogenases from *Methanococcus voltae* [38] and from *Alcaligenes eutrophus* [32]. In contrast to the membrane-bound enzyme from *Alcaligenes* [32], reversible hydrogenase from *Anacystis* does not undergo reversible membrane association, at least under the growth conditions tested. The orientation of the reversible hydrogenase towards the cytoplasmic or periplasmic face of the cytoplasmic membrane remains to be elucidated.

The purified reversible hydrogenase of *Anacystis* has a molecular weight of around 55,000, and this value has also been found for the enzyme from *Anabaena* 7120 [14] and from *Spirulina maxima* [35]. The higher molecular weights determined for the reversible hydrogenase from cyanobacteria, *e.g.* 113,000 and 165,000 for the enzyme from *Anabaena* 7120 [14] and 230,000 for that from *Anabaena cylindrica* are likely due to the occurrence of aggregation forms [3]. The enzyme from *Anacystis* investigated in the present study has also the tendency to aggregate, as seen in the native gel electrophoresis. More interestingly, a second immuno-precipitation band with hydrogenase antibodies was observed at 17 kDa in Western blots with crude extracts from *Anacystis nidulans* or *Anabaena variabilis*. It is tempting to assume that this band at 17 kDa is the smaller subunit of the reversible hydrogenase. There is now agreement among most of the investigators that the

NiFeS-hydrogenases from the different organisms are composed of two subunits of molecular weights of around 60,000 and 25,000 [39]. The smaller subunit which carries the transit peptide is particularly sensitive to proteolysis [39]. This smaller subunit is seen as 17 kDa protein in crude extracts and might be completely degraded during the purification of the enzyme from *Anacystis nidulans*. The molecular weight of 56,000 might be the true one for the larger subunit, and the 53,000 band is presumably a proteolysis product in *Anacystis*. The reversible hydrogenase from cyanobacteria likely belongs to the class of NiFeS-hydrogenases, because the expression of both evolution and uptake of H_2 was shown to be dependent on Ni in the growth medium [40, 41]. Strict proof for this must, however, come from a biochemical characterization which is still missing for both the reversible and the uptake hydrogenase in cyanobacteria.

The function of the reversible hydrogenase associated with the cytoplasmic membrane remains obscure. There is agreement that the cytoplasmic membrane of *Anacystis* does not contain chlorophyll [37]. Because of the absence of chlorophyll, ferredoxin might also not occur. It is now understandable why all isolated reversible hydrogenase preparations did not couple with ferredoxin [3]. The cytoplasmic membrane of *Anacystis* was reported to possess a respiratory electron transport chain with quinones and cytochrome oxidase [37], but the opposite for the latter compound was also communicated [42]. The high affinity of the reversible hydrogenase for H_2 [3] indicates that the enzyme operates in the H_2-utilization under physiological conditions. It remains to be shown whether H_2-uptake by the reversible hydrogenase is used by the cells for the generation of the proton motive force across the cytoplasmic membrane. The present investigation confirms that the activity of the reversible hydrogenase and the number of copies per cell drastically increase by incubating the cells microaerobically. The reversible hydrogenase is possibly nothing else than a useless relict of ancient times when the earth had an anaerobic or microaerobic atmosphere.

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