ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 12, pp. 1370-1376. © Pleiades Publishing, Inc., 2006.
Original Russian Text © L. T. Serebryakova, M. E. Sheremetieva, 2006, published in Biokhimiya, 2006, Vol. 71, No. 12, pp. 1683-1690.

Characterization of Catalytic Properties of Hydrogenase Isolated from the Unicellular Cyanobacterium *Gloeocapsa alpicola* CALU 743

L. T. Serebryakova* and M. E. Sheremetieva

Institute of Basic Biological Problems, Russian Academy of Sciences, Institutskaya ul. 2, 142292 Pushchino, Moscow Region, Russia; fax: (4967) 330-552; E-mail: sereb@issp.serpukhov.su

Received July 6, 2006
Revision received October 11, 2006

Abstract—The main catalytic properties of the Hox type hydrogenase isolated from the *Gloeocapsa alpicola* cells have been studied. The enzyme effectively catalyzes reactions of oxidation and evolution of H_2 in the presence of methyl viologen (MV) and benzyl viologen (BV). The rates of these reactions in the interaction with the physiological electron donor/acceptor NADH/NAD^+ are only 3-8% of the MV(BV)-dependent values. The enzyme interacts with $NADP^+$ and NADPH, but is more specific to NAD^+ and NADH. Purification of the hydrogenase was accompanied by destruction of its multimeric structure and the loss of ability to interact with pyridine nucleotides with retained activity of the hydrogenase component (HoxYH). To show the catalytic activity, the enzyme requires reductive activation, which occurs in the presence of H_2, and NADH accelerates this process. The final hydrogenase activity depends on the redox potential of the activation medium (E_h). At pH 7.0, the enzyme activity in the MV-dependent oxidation of H_2 increased with a decrease in E_h from –350 mV and reached the maximum at E_h of about –390 mV. However, the rate of H_2 oxidation in the presence of NAD^+ in the E_h range under study was virtually constant and equal to 7-8% of the maximal rate of H_2 oxidation in the presence of MV.

DOI: 10.1134/S0006297906120133

Key words: cyanobacterium, Hox type hydrogenase, hydrogenase activity, diaphorase activity, reductive activation

Cyanobacteria can synthesize two functionally different hydrogenases [1-3]. One is a membrane-bound Ni-Fe hydrogenase of the uptake type (HupSL), which is specific for nitrogen-fixing species. The function of this enzyme is reutilization of H_2 produced by nitrogenase [4, 5].

The other hydrogenase catalyzes *in vitro* both the formation and uptake of H_2 during the interaction with artificial donors or acceptors of electrons and, therefore, it is called reversible. The reversible hydrogenase has been found in different forms of cyanobacteria and seems to be the only enzyme responsible for physiological reactions of hydrogen metabolism in strains unable to fix molecular nitrogen [6-8]. The molecular biological properties of the enzyme have been studied in detail: the structural gene (*hoxEFUYH*) encoding the reversible hydrogenase in a relatively great number of cyanobacteria has been identified and completely sequenced [9-11]. HoxYH is a hydrogenase dimer carrying a bimetallic Ni-Fe active site. HoxEFU is a diaphorase moiety of the enzyme responsible for its interaction with $NAD(P)^+$/NAD(P)H. Notwithstanding abundant data on molecular biology of the reversible hydrogenase, its functional role in the metabolism of cyanobacteria is still under discussion. On the basis of high degree homology of the hydrogenase diaphorase subunits with three subunits of the respiratory NADH-dehydrogenase complex, it was suggested that the diaphorase moiety of the hydrogenase should simultaneously be a NADH-oxidizing moiety of this complex functioning in both the photosynthetic and respiratory electron transport chain (ETC) [9, 12, 13]. From the standpoint of this hypothesis, the physiological role of the reversible hydrogenase in cyanobacteria can be considered as a "valve" for an excess of reductive equivalents from ETC as H_2 [13, 14].

Abbreviations: BV, BV^{2+}, BV^+) benzyl viologen, its oxidized, and reduced form; E_h) redox potential of the medium; ETC) electron transport chain; MV, MV^{2+}, MV^+) methyl viologen, its oxidized, and reduced form; PSII) photosystem II; SD) sodium dithionite.

* To whom correspondence should be addressed.

For some years we have been studying the hydrogen metabolism in unicellular non-nitrogen-fixing cyanobacterium *Gloeocapsa alpicola* CALU 743. This microorganism can produce and uptake molecular hydrogen due to functioning in the cells of a Hox type reversible hydrogenase [15, 16]. Besides acetate and CO_2, H_2 is major product of the fermentation of glycogen stored during photosynthesis [17, 18]. H_2 is intensively oxidized by hydrogenase in the photosynthesizing cells and can be the only donor of electrons in anoxygenic photosynthesis [18, 19]. We have found that the hydrogenase synthesis is not regulated on the transcriptional level, although the hydrogenase activity of the cells increases several-fold in cultures growing under nitrogen (nitrate) limiting conditions, when photosystem II (PSII) is inactivated and the intracellular medium becomes more reduced [15, 16]. A high sensitivity of the reversible hydrogenase to oxygen makes difficult purification of the enzyme and its *in vitro* investigation. Reversible hydrogenases from the unicellular cyanobacteria *Synechocystis* sp. PCC 6803 and *Anacystis nidulans* isolated and purified in an anaerobic box have been characterized most comprehensively [11]. However, there are only a few data on the catalytic properties of the enzyme; they are fragmentary and often contradictory.

This work presents the results of studies on the catalytic behavior of the hydrogenase isolated from *G. alpicola*: its stability and activation, and optimal conditions for catalysis of H_2 evolution and H_2 oxidation on the interaction with artificial and physiological donors and acceptors of electrons.

MATERIALS AND METHODS

The hydrogenase was isolated from *Gloeocapsa alpicola* GALU 743 cells, strain Fitzgerald 1051, cultured in a periodic regimen on BG_0 medium [20] supplemented with the growth-limiting concentrations of nitrate (2-4 mM) [18].

During the enzyme purification, the hydrogenase activity of the cells and preparations was determined amperometrically [18] in the reaction of H_2 evolution from the sodium dithionite (SD)-reduced MV^+ (methyl viologen, reduced form) and expressed in μmol/min per mg protein. The H_2 evolution and oxidation catalyzed by the hydrogenase on interaction with various donors and acceptors of electrons were determined spectrophotometrically [21, 22] and amperometrically [18] and expressed in μmol/min per ml or μmol/min per mg protein. The following values of the molar absorption coefficient were used in the calculations: $\varepsilon_{600} = 12{,}300\ M^{-1}\cdot cm^{-1}$ for methyl viologen (MV), $\varepsilon_{555} = 7550\ M^{-1}\cdot cm^{-1}$ for benzyl viologen (BV), and $\varepsilon_{340} = 6300\ M^{-1}\cdot cm^{-1}$ for NADH. The pH dependence of the hydrogenase activity was determined in 20 mM citrate-phosphate (pH 3.0-7.5) and glycine-phosphate (pH 6.5-10.5) buffers. In acidic reaction mixtures (pH < 6.0) metallic zinc was used instead of SD for reduction of MV.

For reductive activation each hydrogenase preparation (~1-3 mg protein per ml) was placed into the side-arm of a 20-ml glass vessel deoxygenated by several cycles of evacuation and re-equilibration with H_2 or Ar. To remove traces of oxygen and monitor the anaerobic medium, 1 ml of 2 mM SD-reduced MV was placed into the central part of the vessel. The necessary additions were injected into the hydrogenase preparation with a microsyringe. Samples of hydrogenase were also taken with a microsyringe and transferred anaerobically into the reaction system.

To purify the hydrogenase, nitrate-limited cyanobacterium cells were used, which possessed high hydrogenase activity and low content of phycobilin pigments. The cell suspension in 0.05 M potassium-phosphate buffer (pH 7.0) was ultrasonicated with constant cooling, and the homogenate was centrifuged for 40 min at 14,000*g*. The supernatant (a cell-free extract) was subjected to stepwise chromatography on a column (3 × 15 cm) with DEAE-cellulose DE_{52}, which was washed in 0.05 M potassium-phosphate buffer and then 0.1 M NaCl in the same buffer, and the hydrogenase was then eluted with 0.5 M NaCl in the same buffer. The resulting fraction retained 100% of the hydrogenase activity of the cell-free extract. This fraction was supplemented with $(NH_4)_2SO_4$ to the concentration of 0.6 M and placed onto a column (1 × 5 cm) with phenyl-Sepharose CL 4B equilibrated with 0.8 M $(NH_4)_2SO_4$. The column was washed with 0.25 M $(NH_4)_2SO_4$, and the hydrogenase was eluted with 0.05 M potassium-phosphate buffer (pH 7.0). The resulting preparation was chromatographed on a column (1 × 10 cm) with Fractogel DEAE 650 (S). The proteins were eluted in a linear concentration gradient of NaCl (0.1-0.8 M) in 0.05 M potassium-phosphate buffer (pH 7.0). The central fractions with the hydrogenase activity were combined, concentrated on a small column (0.3 × 2 cm) with DEAE-cellulose DE_{52}, and subjected to gel filtration on a column (1 × 100 cm) with Sephacryl S-300 equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl.

Electrophoresis of the hydrogenase preparations was performed under nondenaturing conditions in 7.5% polyacrylamide gel [23], and the activity zones were developed by incubation of the gels in 0.025 M Tris-HCl buffer (pH 8.0) containing 1 mM MV in an atmosphere of H_2 at 30°C.

The protein concentration was determined by the Bradford method [24].

The following reagents were used: Tris, MV, BV, NAD^+, NADH, $NADP^+$, NADPH, and SD from Serva (Germany); DEAE-cellulose DE_{52}, phenyl-Sepharose CL-4B, and Sephacryl S-300 from Pharmacia (Sweden); Fractogel DEAE 650 (S) from Merck (Germany). Other

reagents were of chemical purity and special purity, domestic production.

RESULTS

Purification of the hydrogenase. The hydrogenase was purified under aerobic conditions using four column chromatographies (Table 1). The preparation with maximal specific activity of 32 μmol H_2/min per mg protein was obtained at the gradient chromatography stage on a column with Fractogel DEAE. Gel filtration of this preparation caused a sharp decrease in the hydrogenase activity and stability and the loss of its ability to interact with $NAD(P)^+$ and NAD(P)H. The molecular weight of the hydrogenase assessed by gel filtration of the final preparation on a calibrated column with Sephacryl S-300 was 100 kD, whereas the M_r of the native hydrogenase complex (HoxEFUYH) was about 180 kD [11]. Electrophoresis in polyacrylamide gel of the resulting hydrogenase under nondenaturing conditions gave an activity zone with higher mobility than electrophoresis of the cell-free extract of the cyanobacterium (Fig. 1). These data suggest that the native multimeric enzyme is destroyed (or dissociated) during the isolation, but the structure retained is sufficient to exhibit activity in the reactions with MV. Therefore, catalytic characteristics of the native hydrogenase from *G. alpicola* were determined on the preparations obtained after ion-exchange chromatography on the column with Fractogel. The hydrogenase purity in these preparations was sufficiently high, because for these works the biomass was grown under conditions of nitrogen limitation.

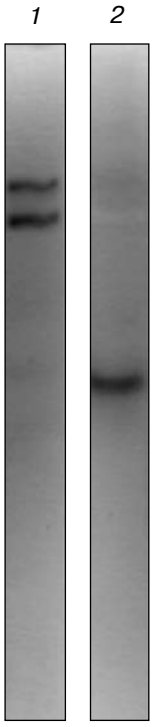

Fig. 1. Electrophoregram of preparations of the hydrogenase from *Gloeocapsa alpicola*: *1*) cell-free extract; *2*) hydrogenase preparation after gel filtration on a column with Sephacryl S-300. The activity zones were developed in the reaction of H_2-dependent reduction of BV.

Catalyzed reactions. The interactions of the hydrogenase with artificial (MV and BV) and physiological $NAD(P)H/NAD(P)^+$ donors/acceptors of electrons were characterized (Table 2). The hydrogenase effectively catalyzed reactions of evolution and oxidation of H_2 with the artificial electron carriers. The rate of NAD(P)H-dependent evolution of H_2 was only 3-6% of the rate with MV because of the lower value of the MV redox potential (E'_o = –440 mV) compared to that of NAD(P)H (E'_o = –320 mV). However, in the reactions of H_2 oxidation the hydrogenase was also considerably more active with MV

Table 1. Purification of the hydrogenase from *Gloeocapsa alpicola* cells grown under conditions of nitrate limitation

Preparation	Total protein, mg	Total activity*, μmol H_2/min	Specific activity*, μmol H_2/min per mg protein	Yield, %	Purification factor
Initial extract	300	135.0	0.45	100	1
Stepwise chromatography on DEAE-cellulose DE_{52}	54	135.0	2.5	100	5.6
Hydrophobic chromatography on phenyl-Sepharose CL-4B	9.2	89.0	9.7	66	21.6
Gradient chromatography on Fractogel DEAE 650	2.4	78.0	32.3	58	71.8
Gel filtration on Sephacryl S-300	0.8	7.8	9.8	5	21.8

* The hydrogenase activity was determined in the reaction of H_2 evolution from MV^+ reduced by SD.

Table 2. Catalytic parameters of reactions catalyzed by the hydrogenase from *G. alpicola*

Reactions*	*V*, μmol/min per mg protein	K_m, μM	pH optimum	Temperature optimum, °C
Evolution of H_2 in the presence of MV^+ (2 mM MV, 6 mM SD, 20 μg hydrogenase)	30.6	77.0 (MV^+)	4.3	65
Evolution of H_2 in the presence of NADH (3 mM NADH, 6 mM SD, 0.2 mg hydrogenase)	2.3	83.0 (NADH)	6.8	45
Evolution of H_2 in the presence of NADPH (3 mM NADPH, 6 mM SD, 0.2 mg hydrogenase)	0.7	476.0 (NADPH)	6.8	45
Oxidation of H_2 in the presence of MV^{2+} (1 mM MV, 20 μg hydrogenase)	37.5	103.0 (MV^{2+})	9.0	70
Oxidation of H_2 in the presence of BV^{2+} (1 mM BV, 20 μg hydrogenase)	57.8	105.0 (BV^{2+})	9.0	70
Oxidation of H_2 in the presence of NAD^+ (1 mM NAD^+, 0.2 mg hydrogenase)	3.5	110.0 (NAD^+)	8.5	50
Oxidation of H_2 in the presence of $NADP^+$ (1 mM $NADP^+$, 0.2 mg hydrogenase)	1.1	no determinations	8.5	50

* Reactions of H_2 evolution were performed in 50 mM potassium-phosphate buffer (pH 7.0) at 30°C; reactions of H_2 oxidation were performed in 50 mM Tris-HCl buffer (pH 8.5) at 30°C.

and BV than with pyridine nucleotides. These differences can be explained by polyfunctionality of the hydrogenase native molecule: the MV(BV)-dependent reactions are catalyzed by the hydrogenase moiety (HoxYH) itself, whereas the interaction with NAD(P)H/$NAD(P)^+$ needs the involvement of both hydrogenase and diaphorase moieties of the enzyme, and in the latter case the diaphorase activity seems to be limiting. It should be noted that for the hydrogenase from *G. alpicola* NAD^+ and NADH were more specific than $NADP^+$ and NADPH. The dependence of the H_2 evolution and H_2 oxidation on the enzyme concentration was studied. It showed that on the interaction with NAD^+ and NADH the specific activities of the hydrogenase were maximal at its relatively high contents (Fig. 2, curves *1* and *2*). Hence, there is an optimal concentration when all (or the majority) of the molecules of the enzyme retain the structure capable of catalyzing these processes. The decrease in the specific activity of the hydrogenase with its dilution in the reaction system seems to be caused by destruction of the native structure of the enzyme or its dissociation. In the reactions with MV, the dilution of the hydrogenase resulted in increase in its specific activity (Fig. 2, curves *3* and *4*). This effect was not associated with the probable dissociation of the native hydrogenase, but indicated only the functioning of hydrogenase dimer. The tendency for increase in the specific activity of the hydrogenase in the reactions with MV with a decrease in its content in the reaction system was also observed for other highly purified Ni-Fe hydrogenases [25, 26]. This phenomenon was studied in the case of hydrogenase from *Thiocapsa roseopersicina* [25], and the authors concluded that it was due to the nature of the hydrogenase reaction (phase heterogeneity, reversibility of the catalytic action) and specific features of the measurement of the hydrogenase activity. Therefore, in our experiments we sought to standardize the conditions of measurement of catalytic parameters of the hydrogenase, in particular using its near optimal concentrations in the corresponding reactions.

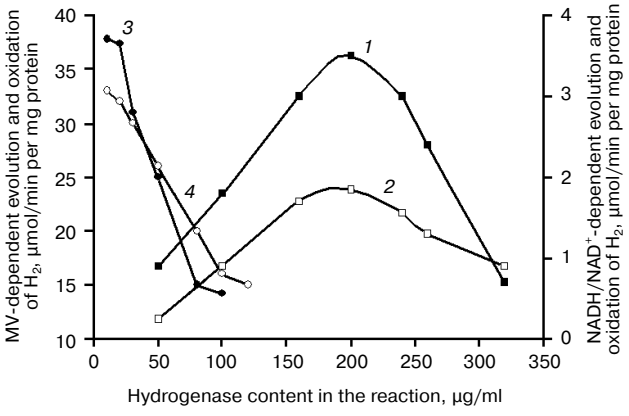

Fig. 2. Dependence of the rate of H_2 evolution and oxidation catalyzed by the hydrogenase from *Gloeocapsa alpicola* on the enzyme content: *1*, *2*) NAD^+/NADH-dependent reactions; *3*, *4*) MV^{2+}/MV^+-dependent reactions; *1*, *3*) oxidation of H_2; *2*, *4*) evolution of H_2.

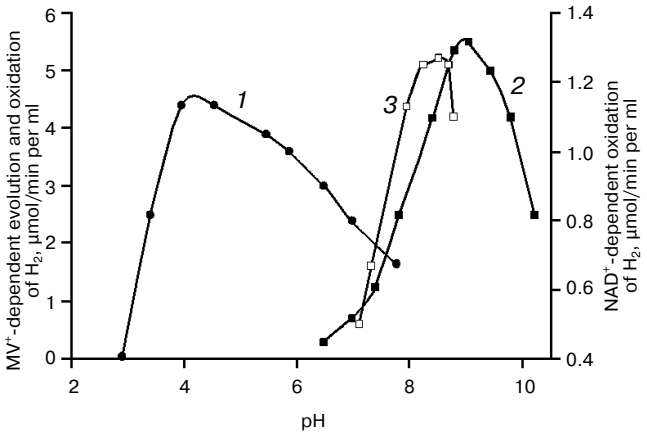

Fig. 3. Effect of pH on the rates of reactions catalyzed by the hydrogenase from *Gloeocapsa alpicola*: *1*) MV^+-dependent evolution of H_2; *2*) MV^{2+}-dependent oxidation of H_2; *3*) NAD^+-dependent oxidation of H_2.

The hydrogenase from *G. alpicola* is characterized by a high affinity for H_2, $K_m^{H_2} = 38$ μM. As a metal-containing enzyme, it was inhibited by carbon monoxide. The inhibition was strictly competitive with respect to the substrate (H_2), with $K_i = 42$ μM, which indicated a relative resistance of this hydrogenase to CO. Optimum pH values of the reactions catalyzed by the hydrogenase depended on the role of the protons as a substrate or a product: in the MV-dependent evolution of H_2 (proton reduction) pH optimums were in the acidic region, in the oxidation of H_2 they were in the alkaline region (Fig. 3). The pH dependence of the H_2 evolution from reduced pyridine nucleotides was characterized by unclear maximums in the pH range of 6.4-7.2 (not shown). This seemed to be associated with a low stability of these substrates in the acidic medium. The difference between the pH optimums for the MV- and NAD^+-dependent oxidation of H_2 was insignificant. The hydrogenase from *G. alpicola* was rather resistant to heating: incubation of the enzyme at 60°C for 10 min did not decrease its activity. No influence of O_2 on the rate of the hydrogenase thermal inactivation was revealed. The values of temperature optimums of the catalyzed reactions also suggest high thermostability of the enzyme (Table 2).

Activation of the hydrogenase. To show the catalytic activity, the hydrogenase isolated under aerobic conditions needed reductive activation. In the reactions of H_2 evolution, SD (which was present in the anaerobic reaction system) acted as a reducer. To catalyze oxidation of molecular hydrogen, the hydrogenase was fully activated during incubation of the preparations in an H_2 atmosphere for 40-50 min and retained the maximal activity level in both H_2 and argon atmospheres (Fig. 4). A transient exposure of the activated enzyme in air resulted in its fast but reversible inactivation. Long-term incubation of the enzyme in air resulted in its irreversible inactivation ($\tau_{1/2} = 20$ h). In these experiments the presence of NADH in the activation medium did not influence the hydrogenase activation. Addition of the glucose oxidase system (5 mM glucose, 40 units of glucose oxidase, 500 units of catalase), which removed oxygen traces from the samples incubated in the H_2 atmosphere, allowed us to observe the hydrogenase activation during the direct monitoring of the H_2-dependent MV-reduction (Fig. 5). The figure shows that in this case the addition of small quantities of NADH (0.3 mM) accelerated the enzyme activation shortening of the lag-period of the reaction. The stimulating effect of NADH was realized through the stage of MV reduction by the diaphorase moiety of the enzyme, as indicated by the virtual coincidence of the first stages (0-8 min) of the MV reduction kinetics in the presence of NADH in both the H_2 and Ar atmosphere (curves *2* and *3*). The maximal level of the hydrogenase activity did not depend on the presence of NADH in the reaction system, as shown by similar slopes of the terminal parts of the kinetic curves *1* and *2*.

Influence of redox potential of the medium. The maximal activity of the hydrogenase achieved during the reductive activation by molecular hydrogen significantly depended on the H_2 concentration. In the presence of the hydrogenase and the chosen concentrations of H_2 (0.033-100%), a certain redox potential (E_h) was created calculated by the Nernst equation [27], and the hydrogenase activity in this E_h region was determined (Fig. 6). At pH 7.0, the hydrogenase activity in the reaction of MV-

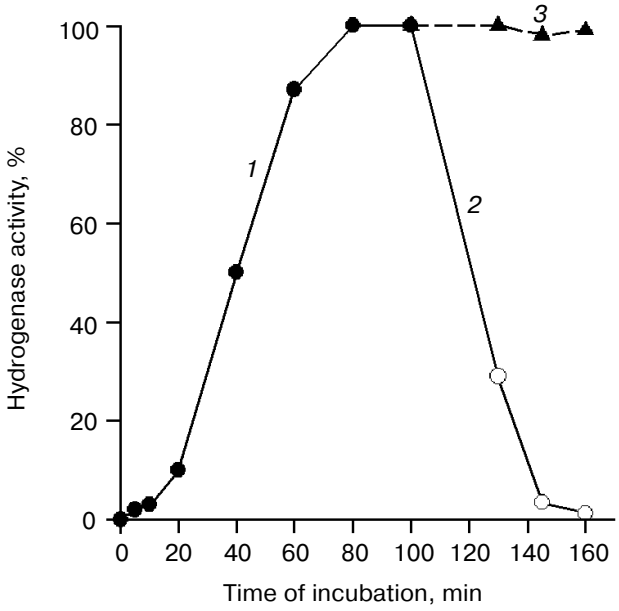

Fig. 4. Activation of the hydrogenase from *Gloeocapsa alpicola* by molecular hydrogen: *1*) incubation in H_2 atmosphere; *2*) inactivation in air; *3*) incubation in Ar atmosphere. The hydrogenase activity was measured in the reaction of MV^{2+}-dependent oxidation of H_2. The 100% activity corresponded to 28.5 μmol/min per ml.

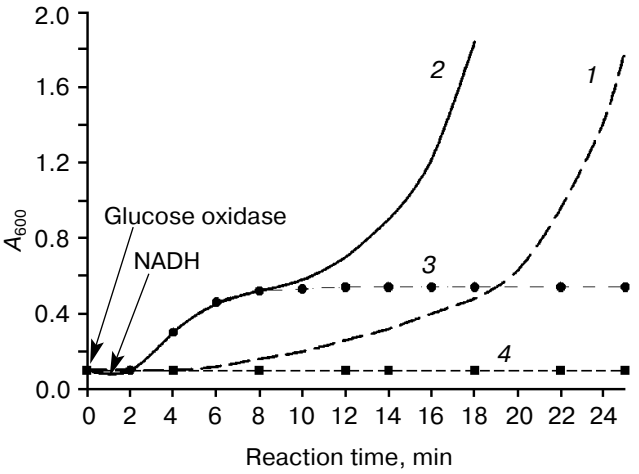

Fig. 5. The influence of NADH on the H_2-dependent reduction of MV^{2+} catalyzed by the hydrogenase from *Gloeocapsa alpicola*: *1*) reaction kinetics in the presence of H_2; *2*) reaction kinetics in the presence of H_2 and NADH (0.3 mM); *3*) reaction kinetics in the presence of NADH (0.3 mM) and Ar; *4*) reaction kinetics in the presence of Ar (control). The reaction systems (2 ml) contained: the enzyme preparation (50 μg), 1 mM MV^{2+}, 5 mM glucose, 40 units of glucose oxidase, 500 units of catalase in 0.05 M Tris-HCl buffer (pH 8.5). Reduction of MV^{2+} was recorded spectrophotometrically at 600 nm.

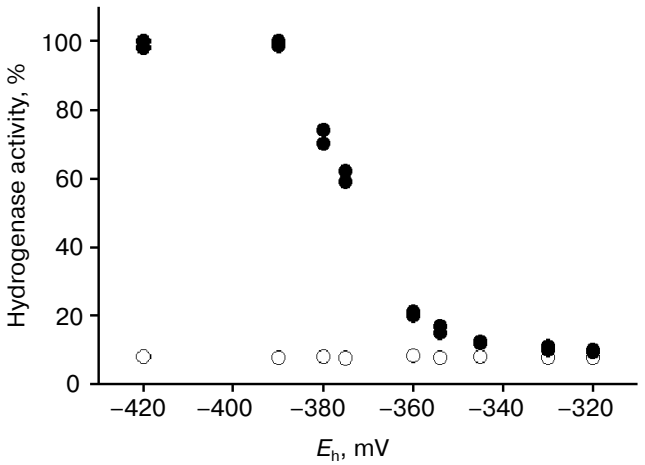

Fig. 6. Influence of E_h of the activation medium on activity of the hydrogenase from *Gloeocapsa alpicola*. The hydrogenase activity was determined in the reactions of MV^{2+}-dependent (•) and NAD^+-dependent (o) oxidation of H_2. The reaction system (2 ml) contained: 1 mM MV^{2+} or 0.5 mM NAD^+ in 0.05 M Tris-HCl buffer (pH 8.5), H_2. The reactions with MV were performed in the presence of ~20 μg hydrogenase, and in the reactions with NAD^+ ~300 μg hydrogenase was used. The 100% activity corresponded to 24.5 μmol/min per mg protein.

dependent oxidation of H_2 increased with the decrease in E_h value from –350 mV and reached the maximum at E_h ~ –390 mV. However, the rate of H_2 oxidation in the range of E_h values under study was virtually constant and equal to 7-8% of the maximal oxidation rate of H_2 in the presence of MV.

DISCUSSION

The data obtained evidence that the complicated multimeric structure of the isolated hydrogenase from *G. alpicola* is unstable and destroyed during purification under aerobic conditions with the loss of the diaphorase activity. This can be due to oxidative destruction of the hydrogenase complex, because in anaerobically purified preparations of hydrogenases from *Synechocystis* sp. PCC 6803 and *Anacystis nidulans* electrophoresis under denaturing conditions revealed all five structural units [11]. However, dissociation of a tetrameric hydrogenase of the Hox type is quite specific for purified enzymes from some hydrogen-oxidizing bacteria [28, 29]. So, the molecular weight of the NAD^+-reducing hydrogenase from *Alcaligenes eutrophus* Z-1 determined by gel filtration decreased from 160 to 90 kD depending on the enzyme concentration in the samples used [27]. This was accompanied by the loss of ability of the enzyme preparation to reduce both NAD^+ and MV. In contrast, the Hox-hydrogenase from *Alcaligenes eutrophus* H16 (*Ralstonia eutropha*) is a very stable enzyme, which retains the tetrameric structure and NAD^+-reducing activity even during electrophoresis in polyacrylamide gel. Hydrogenases isolated from *Rhodococcus opacus* MR22 and MR11 easily produce two dimeric components: the hydrogenase itself (βδ) and NADH:acceptor-oxidoreductase (αγ) in low molarity buffer solutions, and Ni^{2+} stabilizes these hydrogenases [29]. It should be noted that, similarly to our case, the hydrogenase dimer functioned in interactions with viologen dyes. However, the stability of the hydrogenase dimer from *G. alpicola* sharply decreased in the absence of the diaphorase moiety. This was indicated by the threefold decrease in the specific hydrogenase activity upon gel filtration (Table 1), and the activity continued to decrease even during storage under optimal conditions. Obviously, retention of the native structure is a significant stabilizing factor for the hydrogenase component of the enzyme molecule.

To show the catalytic activity the enzyme from *G. alpicola,* like other known Ni-Fe hydrogenases isolated under aerobic conditions, needs to be reductively activated in an atmosphere of H_2. It has been shown earlier [30] that, as in the case of Hox type hydrogenases of hydrogen bacteria [31], NADH and NADPH can physiologically activate this process in the H^+/D_2 exchange reaction. In the present work, we have shown that NADH stimulates the hydrogenase activation in the reaction of the H_2-dependent reduction of MV through the stage of its NADH-dependent reduction via the diaphorase compo-

nent of the hydrogenase complex. The resulting reduced MV is involved in the reduction of the H_2-binding Ni-Fe site of the hydrogenase and, thus, shortens the lag-period, which is due to the autocatalytic stage of the reaction cycle [26].

The dependence of the activity of *G. alpicola* hydrogenase on the redox potential of the activation medium determined in the reaction of H_2 oxidation with MV as an electron acceptor is typical for reversible type hydrogenases [21]. Note that the rate of the H_2-dependent reduction of NAD^+ was virtually unchanged in the E_h region under study. It seems that this is the case of activation of just the hydrogenase moiety of the enzyme molecules, which is responsible for the reaction with MV. The NAD^+ reduction requires the functioning of both hydrogenase and diaphorase moieties of the enzyme, and the action of diaphorase moiety determines the reaction rate. Therefore, in the E_h region chosen by us (< –320 mV), changes in the activity of the hydrogenase moiety did not affect the rate of the interaction of hydrogenase with NAD^+, because even its minimal activity was sufficient for providing the diaphorase moiety with electrons.

Thus, we have studied the main catalytic properties of the hydrogenase from *G. alpicola*. The catalytic action of the isolated enzyme has strictly reversible character, thus, the physiological role of the hydrogenase seems to be equally associated with both formation and uptake of H_2, depending on the redox state of the intracellular medium.

REFERENCES

1. Houchins, J. P. (1984) *Biochim. Biophys. Acta*, **768**, 227-255.
2. Appel, J., and Schulz, R. (1998*) J. Photochem. Photobiol. B: Biol.*, **47**, 1-11.
3. Vignais, P. M., Billoud, B., and Meyer, J. (2001) *FEMS Microbiol. Rev.*, **25**, 455-501.
4. Happe, T., Schutz, K., and Bohme, H. (2000) *J. Bacteriol.*, **182**, 1624-1631.
5. Tamagnini, P., Axelsson, R., Lindberg, P., Oxefelt, F., Wunschiers, R., and Lindblad, P. (2002) *Microbiol. Mol. Biol. Rev.*, **66**, 1-20.
6. Papen, H., Kentemich, T., Schmulling, T., and Bothe, H. (1986) *Biochimie*, **68**, 121-132.
7. Serebryakova, L. T., Zorin, N. A., and Gogotov, I. N. (1992) *Mikrobiologiya*, **61**, 175-182.
8. Boison, G., Bothe, H., and Schmitz, O. (2000) *Curr. Microbiol.*, **40**, 315-321.
9. Appel, J., and Schulz, R. (1996) *Biochim. Biophys. Acta*, **1298**, 141-147.
10. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosava, M., Sigura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) *DNA Res.*, **30**, 185-209.
11. Schmitz, O., Boison, G., Salzmann, H., Bothe, H., Schutz, K., Wang, S., and Happe, T. (2002) *Biochim. Biophys. Acta*, **1554**, 66-74.
12. Schmitz, O., and Bothe, H. (1996) *Naturwissenschaften*, **83**, 525-527.
13. Appel, J., Phunpruch, S., Steinmuller, K., and Schulz, R. (2000) *Arch. Microbiol.*, **173**, 333-338.
14. Cournac, L., Guedeney, G., Peltier, G., and Vignais, M. (2004) *J. Bacteriol.*, **186**, 1737-1746.
15. Serebryakova, L. T., Sheremetieva, M., and Tsygankov, A. A. (1998) *FEMS Microbiol. Lett.*, **166**, 89-94.
16. Sheremetieva, M., Troshina, O. Yu., Serebryakova, L. T., and Lindblad, P. (2002) *FEMS Microbiol. Lett.*, **214**, 229-233.
17. Troshina, O. Yu., Serebryakova, L. T., Sheremetieva, M., and Lindblad, P. (2002) *Int. J. Hydrogen Energy*, **27**, 1283-1289.
18. Serebryakova, L. T., Sheremetieva, M. E., and Lidblad, P. (1999) *Microbiologiya*, **68**, 293-298.
19. Serebryakova, L., Novichkova, N., and Gogotov, I. N. (2002) *Int. J. Photoenergy*, **4**, 169-173.
20. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) *J. Gen. Microbiol.*, **111**, 1-61.
21. Zorin, N. (1986) *Biochimie*, **68**, 97-101.
22. Serebryakova, L. T., Medina, M., Zorin, N., Gogotov, I. N., and Camack, R. (1996) *FEBS Lett.*, **383**, 79-82.
23. Serebryakova, L. T., Troshina, O. Yu., and Gogotov, I. N. (1995) *Biochemistry (Moscow)*, **60**, 373-378.
24. Bradford, M. (1976) *Analyt. Biochem.*, **72**, 248-251.
25. Bagyinka, Cs., Zorin, N., and Kovacs, K. (1984) *Analyt. Biochem.*, **142**, 7-15.
26. Osz, J., Bodo, G., Branca, R. M. M., and Bagyinka, Cs. (2005) *Biophys. J.*, **89**, 1957-1964.
27. Lissolo, T., Pulvin, S., and Thomas, D. (1984) *J. Biol. Chem.*, **259**, 11725-11729.
28. Popov, V., Berezin, I., Zaks, A., Gazaryan, I., Utkin, I., and Egorov, A. (1983) *Biochim. Biophys. Acta*, **744**, 298-303.
29. Grzeszik, C., Ros, K., Reh, M., and Schlegel, H. G. (1997) *Arch. Microbiol.*, **167**, 172-176.
30. Vignais, P. M., Cournac, L., Hatchikian, T. C., Elsen, S., Serebryakova, L., Zorin, N., and Dimon, B. (2002) *Int. J. Hydrogen Energy*, **27**, 1441-1448.
31. Hyman, M. R., Fox, C. A., and Arp, D. (1988) *Biochem. J.*, **254**, 463-468.