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Properties of Stable Hydrogenase from the Purple Sulfur Bacterium *Lamprobacter modestohalophilus*

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Abstract—Some properties of a hydrogenase from the recently isolated phototrophic sulfur bacterium *Lamprobacter modestohalophilus* strain Syvash and its resistance to a number of inactivating factors have been investigated. The enzyme consists of two subunits, 64 and 30 kD; p*I* = 4.5. The optimal pH was 8.5-9.5 for hydrogen uptake and 4.0 for H_2 evolution. Hydrogenase preparations were resistant to the effects of O_2, CO, and temperature, revealing high stability under storage. A considerable inactivation of the enzyme was observed at temperatures above 80°C; the temperature optimum of methyl viologen reduction by H_2 was 85°C. Inhibitory effects of Ni^{2+}, Cd^{2+}, and Mg^{2+} on the hydrogenase activity were shown to be reversible and competitive with respect to methyl viologen in the hydrogen oxidation reaction.

Key words: hydrogenase, inhibition, CO, metal ions, purple sulfur bacteria

The ability of microorganisms to metabolize molecular hydrogen is dependent on the presence of hydrogenase, catalyzing hydrogen oxidation or production according to the reaction: $H_2 + A_{ox} = 2H^+ + A_{red}$.

Highly purified hydrogenase preparations have been isolated from microorganisms of different taxonomic groups. Some hydrogenases have been obtained in crystalline form and their three-dimensional structure determined [1]. The investigation of the properties and stability of hydrogenases from new sources is of great interest, both in theoretical and in practical aspects. Hydrogenases, having high activity and stability to the effect of inactivating agents, are promising hydrogen-activating catalysts for development of fuel cells and hydrogen sensors based on direct bioelectrocatalysis [2].

The hydrogenases of various microorganisms differ in molecular weight, quaternary structure, structure of the active site, specific electron carriers, and other properties. Three types of hydrogenases have been distinguished. Most contain nickel and iron (NiFe-hydrogenase) in the active site. Some hydrogenases contain only iron (Fe-hydrogenase). Hydrogenases of the third type do not contain metal, having their catalytic activity in the presence of an organic cofactor [3]. Hydrogenases isolated from the phototrophic bacteria *Thiocapsa roseopersicina* strain BBS, *Rhodobacter capsulatus*, and *Chlorobium limicola forma thiosulfatophilum* are NiFe-hydrogenases [4-6]. The enzymes from phototrophic bacteria are the most stable to various inactivating factors [7] as compared to hydrogenases from other sources.

The purple sulfur bacterium *Lamprobacter modestohalophilus* strain Syvash was isolated from algae-bacterial mat samples of lake Syvash [8]. Little-investigated pigment–protein complexes containing the carotenoid okenon have been found in this bacterium [9]. Interest in hydrogenase from *L. modestohalophilus* stems from the fact that cells of this bacterium have high hydrogenase activity, and their cell-free hydrogenase preparations are stable to various denaturing factors.

In this paper, we describe the properties and stability of hydrogenase isolated from the purple sulfur bacterium *L. modestohalophilus*.

MATERIALS AND METHODS

The cells *L. modestohalophilus* strain Syvash were grown under anaerobic conditions on Pfennig medium [10]. The following reagents were used in the present work: phenyl-Sepharose CL-4B from Pharmacia

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(Sweden), DEAE-cellulose DE_{52} from Whatman (Great Britain), methyl viologen from Sigma (USA), the reagents for polyacrylamide gel electrophoresis from Serva (Germany), solution of ampholytes Ampholine from LKB (Sweden). CO was obtained by decomposition of HCOOH (formic acid) by concentrated H_2SO_4 at 100°C. Other reagents used (of high purity) were from Russian suppliers.

Preparation of cellular extracts and purification of hydrogenase. The cells were separated from the medium at the end of the logarithmic phase of their growth. The cell paste (100 g) was resuspended in distilled water (1 : 1) and poured into 1.2 liters of acetone cooled to −20°C. The mixture was incubated for 30 min with stirring. The precipitate was collected on a Buchner funnel, washed with cooled acetone, and dried under vacuum. The acetone powder was resuspended in distilled water (1 : 20) and sonicated using a UZDN-1 disintegrator (22 kHz, three times for 10 min, 10°C). Unbroken cells and cell fragments were precipitated by centrifugation (14,000*g*, 40 min, 4°C). The precipitate was resuspended in 150 ml of distilled water and reprocessed again by ultrasonic treatment. The precipitation procedure was repeated under the same conditions. The supernatants obtained after two precipitations were combined and fractionated with ammonium sulfate up to 20% saturation to remove pigment–protein complexes. After centrifugation, the supernatant was heated for 5 min at 75°C to remove thermolabile proteins. Denatured proteins were removed by centrifugation (5000*g*, 30 min). Further additional purification stages were carried out as described previously [11]. The hydrogenase preparation was purified additionally by preparative electrophoresis in 7% polyacrylamide gel according to the method described earlier [12].

Hydrogenase assay. The enzymatic activity of hydrogenase was determined by reduction of methyl (benzyl) viologen with hydrogen using a spectrophotometric method in a Thunberg cuvette [13]. The reaction mixture (total volume 2 ml) contained 50 mM Tris-HCl buffer, pH 7.0 (9.0), 4 (1) mM methyl viologen (benzyl viologen), and 5-10 μg hydrogenase protein. Hydrogenase activity was measured by hydrogen evolution from reduced methyl viologen with dithionite [14]. The activity of the enzyme was measured at 30°C. To calculate the enzymatic activity, we used the molar absorbance coefficient of reduced methyl viologen (ε_{600} = 13,000 $M^{-1}\cdot cm^{-1}$) [15] and benzyl viologen (ε_{555} = 7550 $M^{-1}\cdot cm^{-1}$) [16]. The enzymatic activity was expressed in μmol H_2/min per mg protein.

Isoelectric focusing was carried out by the Rigly method with modifications in the instrument for disk electrophoresis. Ampholine (40%, pH 3.5-5.0) was used for preparation of the working solution [17]. Upon completion of isoelectric focusing, which was determined by the decreasing current, the gels were extracted from the tubes, washed out in distilled water and assayed for hydrogenase activity. To determine the hydrogenase isoelectric point, 3 mm bands corresponding to the bands of hydrogenase activity were cut from gels obtained in parallel. The cut bands were pressed through a glass syringe and resuspended in distilled water. The pH was determined in 24 h on OP-264/1 pH meter.

Determination of quaternary structure. Subunit composition of the hydrogenase and estimation of subunit molecular weight were determined by electrophoresis in 12.5% polyacrylamide gel in the presence of 0.1% SDS by the method of Laemmli [18]. The samples for electrophoresis containing 5-10 μg protein were desalted on Centricon filters and boiled for 10 min in the buffer with SDS. The protein bands in gels were stained after fixation with 0.05% solution of Coomassie R-250. The protein concentration was determined by the method of Bradford [19].

RESULTS AND DISCUSSION

Hydrogenase isolated from *L. modestohalophilus* strain Syvash, like purified preparations of hydrogenase from other phototrophic bacteria [7], catalyzes both the direct and the reverse reaction of hydrogen oxidation. The specific activity of the hydrogenase from *L. modestohalophilus* was similar to that of the enzyme from *T. roseopersicina* [12], being 40-50 μmol H_2/min per mg protein. Much of the hydrogenase activity measured in the reaction of hydrogen oxidation after disintegration of *L. modestohalophilus* cells remained in a soluble fraction (~80%). Similar data for determination of hydrogenase activity localization were obtained for *T. roseopersicina*, *Rhodospirillum rubrum* [20], and *C. limicola forma thiosulfatophilum* [6].

The absorption spectrum of hydrogenase preparations from *L. modestohalophilus* measured in Tris-HCl buffer, pH 7.0, at 30°C containing 10 μg protein was similar to that of hydrogenase from *T. roseopersicina*, having absorption maxima at 280 nm and in the range of 400-410 nm, typical for proteins containing non-heme iron [12].

According to the data available, the molecular mass of 100 kD is typical for hydrogenases of phototrophic bacteria [7]. For example, hydrogenase with monomeric structure and molecular weight of approximately 66 kD was detected for *C. limicola* [6]; hydrogenase isolated from *T. roseopersicina* is a heterodimer consisting of large and small subunits with molecular weights of 64 and 34 kD, respectively [14, 21]. The electrophoretic mobility in native PAGE and isoelectric point of hydrogenase isolated from *L. modestohalophilus* (p*I* = 4.5) are similar to those of the enzyme from *T. roseopersicina* [22], suggesting similarity in their structures. Homogeneous hydrogenase preparations from *L. modestohalophilus* in SDS-PAGE showed two protein bands corresponding to 64 and 30 kD

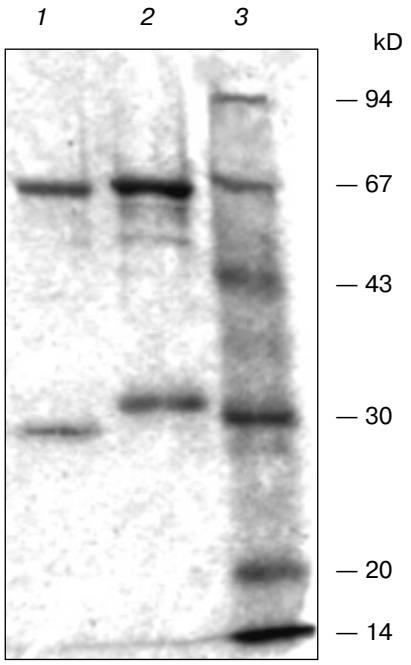

Fig. 1. Electrophoresis in 12.5% polyacrylamide gel in the presence of 0.1% SDS: *1*) hydrogenase from *L. modestohalophilus*; *2*) hydrogenase from *T. roseopersicina*; *3*) standard marker proteins.

(Fig. 1). Thus, the enzyme isolated from *L. modestohalophilus* is a heterodimer consisting of two subunits.

Stability of the hydrogenase on storage. As with hydrogenase from *T. roseopersicina* [23], high stability is typical for the enzyme from *L. modestohalophilus* during storage. To clarify conditions influencing the stability of the hydrogenase from *L. modestohalophilus*, the kinetics of enzymatic inactivation was studied on storage under various gas phases at different temperatures (Fig. 2). The temperature has a more significant effect on the stability of the enzyme than the composition of a gas phase. The period of semi-inactivation of hydrogenase during storage at 4°C under air was almost 5 times higher, and under argon and hydrogen almost 2 times higher than for storage at 20°C. On storage under argon, the stability of the hydrogenase preparations was approximately 1.5 times higher compared to storage under air and hydrogen. Storage of the hydrogenase preparation from *L. modestohalophilus* at –70°C did not reduce the activity of the enzyme.

Thermal stability of the hydrogenase. The hydrogenase from *L. modestohalophilus* has high thermal stability. To study the thermal stability of the enzyme, hydrogenase preparations from *L. modestohalophilus* were incubated for 1.5 min at a set temperature in medium containing 50 mM Tris-HCl buffer (pH 7.0). Hydrogenase catalytic activity was further determined in the hydrogen oxidation reaction (pH 9.0) at 30°C. Considerable inactivation of the enzyme was observed at temperatures above 80°C (Fig. 3a). To study the influence of the temperature on the activity of hydrogenase from *L. modestohalophilus*, hydrogenase preparations (2-5 μl) were added to the previously heated reaction mixture and the enzymatic activities were measured in the hydrogen oxidation reaction. Temperature optimum of methyl viologen reduction was 85°C (Fig. 3b). The activity of hydrogenase increased more than 10 times on increasing the temperature from

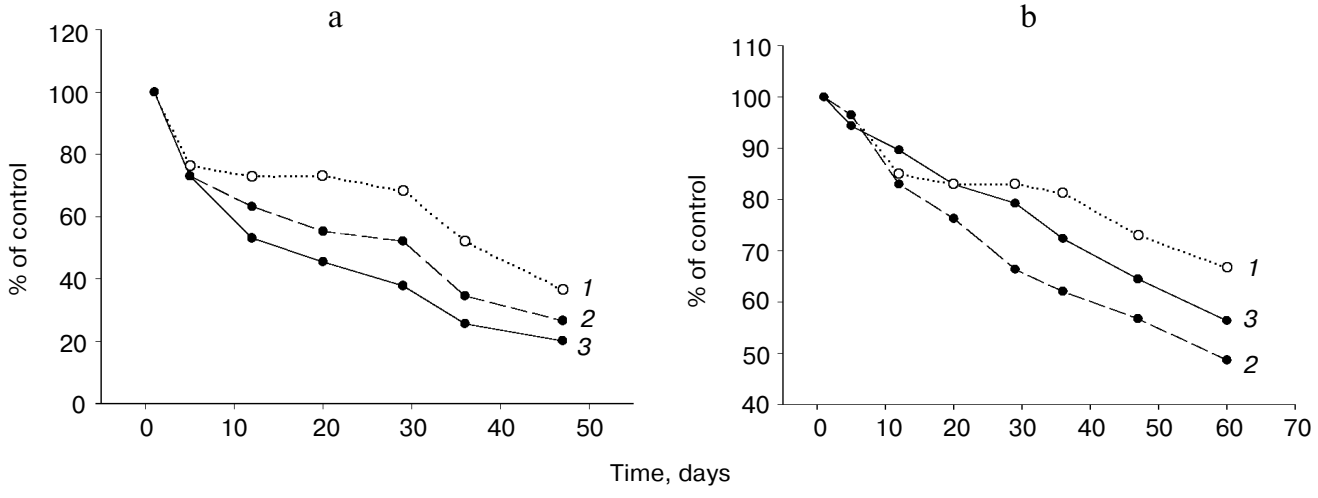

Fig. 2. Stability of hydrogenase from *L. modestohalophilus* on storage at 20°C (a) or 4°C (b) under various atmospheres: *1*) argon; *2*) hydrogen; *3*) air. Hydrogenase preparations (~100 μg protein) were incubated in medium consisting of 50 mM Tris-HCl buffer (pH 7.0) and 0.02% NaN_3. The hydrogenase activity of 40 μmol H_2/min per mg protein measured in the hydrogen oxidation reaction in 50 mM Tris-HCl buffer (pH 9.0) at 30°C was taken as 100% enzymatic activity.

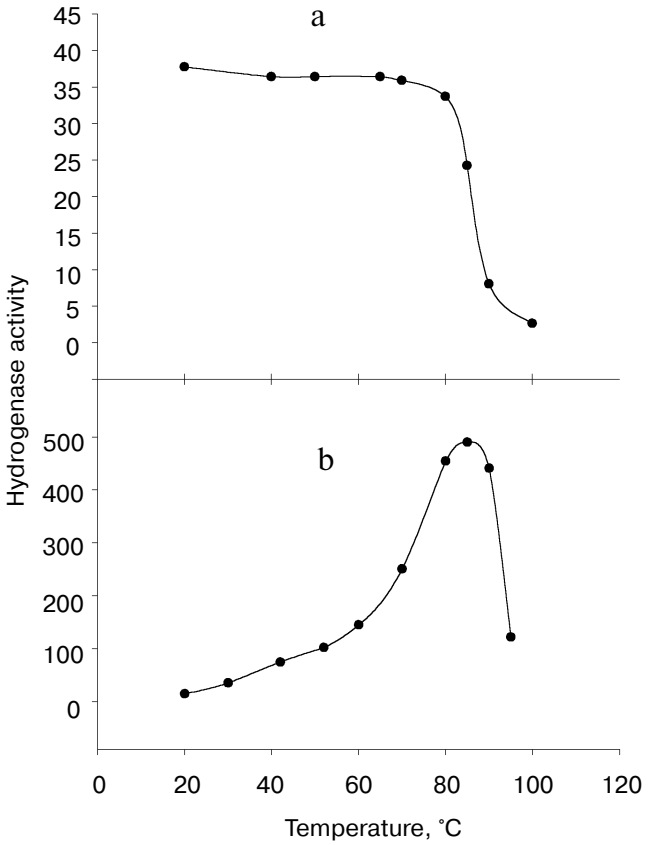

Fig. 3. Thermal stability (a) and temperature optimum (b) of the hydrogenase from *L. modestohalophilus* in the hydrogen oxidation reaction.

30 to 80°C, reaching 531 μmol H_2/min per mg protein. High activity and thermal stability make this enzyme promising for practical use as a hydrogen activating biocatalyst [1, 2].

Influence of pH on the enzyme activity. The influence of pH on hydrogenase activity from *L. modestohalophilus* demonstrated that the activity of the enzyme in H_2 oxidation and H_2 evolution was constant in the initial period at every pH value studied. The protons of the medium and reduced methyl viologen are substrates of hydrogenase in H_2 evolution or the products of hydrogen oxidation reaction. Therefore, the hydrogenase activity in hydrogen evolution should be maximal at low pH and in hydrogen oxidation at high pH values of the medium. The pH optimum in hydrogen evolution was in fact pH 4.0, and for H_2 oxidation it was in the range of 8.5-9.5 (Fig. 4). The same optimal pH values were typical for hydrogenases from other phototrophic and chemotrophic bacteria [6, 14].

Influence of CO on hydrogenase activity. Carbon monoxide is known to be an inhibitor of the hydrogenases from a number of phototrophic bacteria [24]. It has been found that in the reaction of hydrogen oxidation CO is a noncompetitive with respect to oxidized methyl viologen and competitive with respect to hydrogen inhibitor of hydrogenase from the phototrophic sulfur bacterium *T. roseopersicina* [25]. The dependence of the initial rate of H_2 absorption by hydrogenase from *L. modestohalophilus* on CO concentration at various concentrations of oxidized methyl viologen represented parallel straight lines in coordinates $\{1/v; [I]_0\}$ (data not presented). Therefore, CO is a noncompetitive inhibitor with respect to oxidized methyl viologen. The dependence of the rates of H_2 oxidation by methyl viologen catalyzed by the hydrogenase from *L. modestohalophilus* on CO concentration at various concentrations of H_2 in coordinates $\{[S]/v; [I]_0\}$ presented parallel straight lines, suggesting that CO is a competitive inhibitor with respect to H_2. To determine the inhibition constant the same data are presented in coordinates $\{1/v; [I]_0\}$ (Fig. 5). The inhibition constant for hydrogenase from *L. modestohalophilus* is 7.0 μM, two times higher than that for the hydrogenase from *T. roseopersicina* (3.5 μM) [25]. It is obvious that hydrogenase from *L. modestohalophilus* is more stable to CO than that from *T. roseopersicina*. High stability of hydrogenase from *L. modestohalophilus* to carbon monoxide is an important property for possible application of this enzyme for developing new sorts of fuel cells [2].

Influence of metal ions on hydrogenase activity. As in the case with hydrogenase from *T. roseopersicina*, inhibitory effect of metal ions on hydrogenase from *L. modestohalophilus* was manifested to the most extent for hydrogen absorption [13]. Inhibition of the enzyme by nickel, cadmium, and magnesium ions was reversible and decreased substantially as the concentration of the substrate, oxidized methyl viologen, was

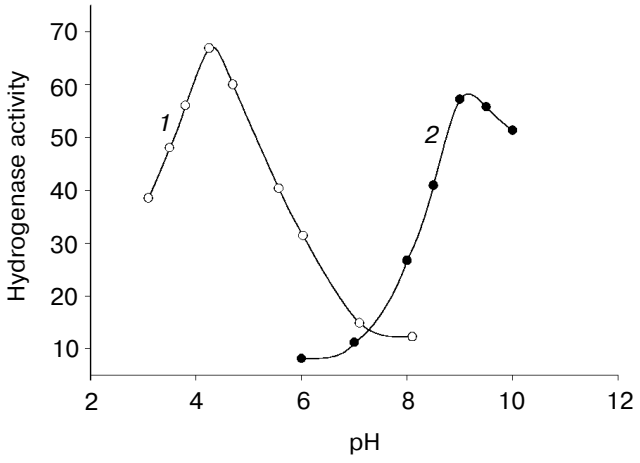

Fig. 4. pH optimum of the hydrogenase from *L. modestohalophilus* in the reactions of hydrogen evolution (*1*) and oxidation (*2*).

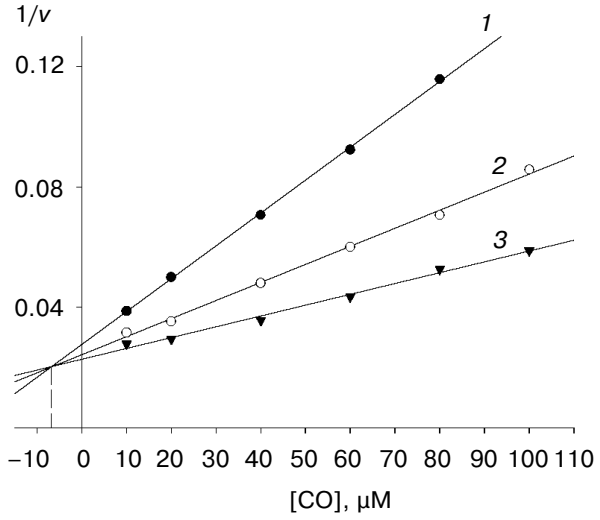

Fig. 5. Dependences of the reciprocal value of the rate of hydrogen oxidation by *L. modestohalophilus* hydrogenase ($1/v$) on CO concentration at various fixed concentrations of H_2 (M): *1*) $4 \cdot 10^{-5}$; *2*) $8 \cdot 10^{-5}$; *3*) $1.6 \cdot 10^{-4}$ (K_i = 7.0 μM).

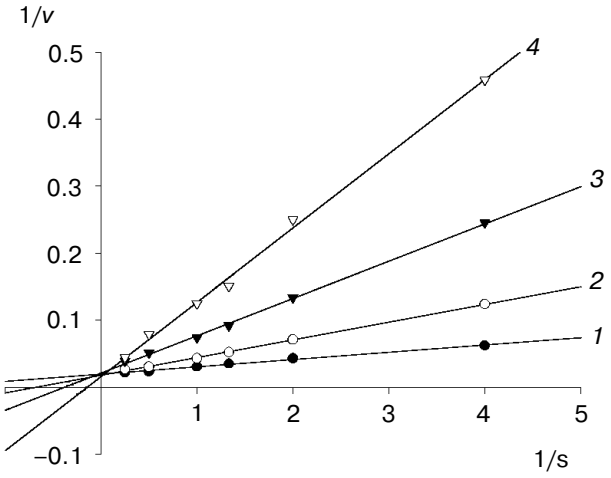

Fig. 6. Dependences of the rate of hydrogen oxidation by *L. modestohalophilus* hydrogenase on methyl viologen concentration (mM) (in double reciprocal values) at various fixed concentrations of $NiCl_2$: *1*) in the absence of $NiCl_2$; *2*) 0.5 mM $NiCl_2$; *3*) 1.0 mM $NiCl_2$; *4*) 1.5 mM $NiCl_2$.

increased. The data presented in Figs. 6 and 7 show that Ni^{2+} is a competitive inhibitor with respect to oxidized methyl viologen. The inhibition constant for cadmium ions, as well as for nickel ions, decreased by ~8 times on increasing the pH from 7.0 to 9.0 (Fig. 7, a and b), indicating the increase in enzymatic affinity to metal ions. Total negative charge of hydrogenase increased with increasing pH, since the p*I* of hydrogenase from *L. modestohalophilus* is 4.5. Taking into consideration that the inhibition is reversible and competitive with respect to methyl viologen, we suggest that metal cations interact electrostatically with the acceptor (methyl viologen) binding center of the hydrogenase.

Thus, the data show that the hydrogenase from the phototrophic sulfur bacterium *L. modestohalophilus* is very close on its properties to the enzyme isolated from *T. roseopersicina*. However, the hydrogenase from *L. modestohalophilus* is more stable to the effect of high temperature and carbon monoxide. Therefore, the hydrogenase from *L. modestohalophilus* is rather interest-

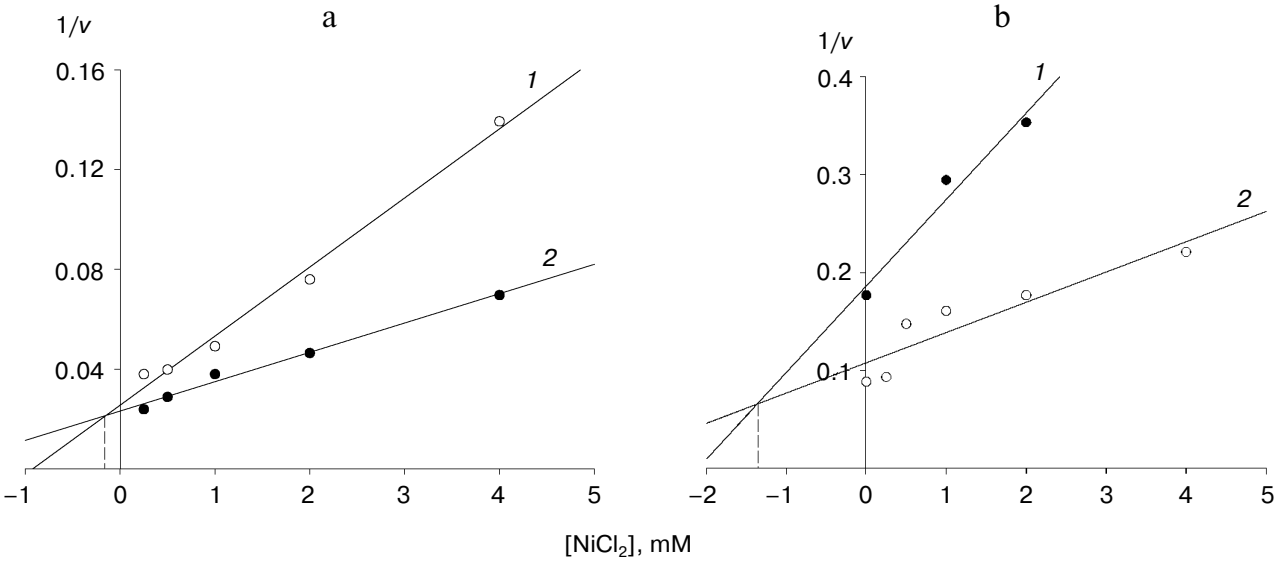

Fig. 7. Dependences of the reciprocal value of the rate of hydrogen oxidation by *L. modestohalophilus* hydrogenase ($1/v$) on $NiCl_2$ concentration at various fixed concentrations of oxidized methyl viologen (mM): *1*) 1; *2*) 4; a) pH 9.0; b) pH 7.0; K_i = 0.16 (a) or 1.35 mM (b).

ing from the point of view of practical application in various systems of energy conversion based on molecular hydrogen.

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