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Measuring the pH Dependence of Hydrogenase Activities

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Abstract—The pH dependences of activities of homogenous hydrogenases of *Thiocapsa roseopersicina* and *Desulfomicrobium baculatum* in the reaction of hydrogen uptake in solution in the presence of benzyl viologen and the pH dependences of catalytic currents of hydrogen oxidation by electrodes on which these hydrogenases were immobilized were compared. Maximal activities of the hydrogenases from *T. roseopersicina* and *D. baculatum* in the reaction hydrogen uptake in solution were observed at pH 9.5 and 8.5, respectively. However, the steady-state current caused by catalytic uptake of hydrogen was maximal for the *T. roseopersicina* hydrogenase-containing electrode at pH 5.5-6.5 under overvoltage of 30-60 mV, whereas for electrodes with *D. baculatum* hydrogenase it was maximal at pH 6.0-6.5. Analysis of these data suggests that pH-dependent changes in the hydrogenase activities in solution during hydrogen uptake are due not only to the effect of proton concentration on the enzyme conformation or protonation of certain groups of the enzyme active center, but they are rather indicative of changes in free energy of the reaction accompanying changes in pH.

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Key words: hydrogenase, *Thiocapsa roseopersicina*, *Desulfomicrobium baculatum*, hydrogen electrode, pH effect

The activities of many enzymes are pH-dependent. This is because ionogenic groups in the enzyme active center are able to provide for catalytic activity only with a certain state of protonation. In this case, catalysis depends on the concentration of the enzyme active form and thus on pH of the medium. Methods have been developed for detection of the component of the enzyme reaction responsible for the character of the pH dependence of kinetic parameters in the pH effect on both the enzyme and its complexes [1]. In some cases, it is also possible to use experimental data on pH effect on enzyme activity for determination of the main surface groups responsible for stability of the protein globule [1]. It is supposed that in the course of investigation of the mechanism of pH effect on enzyme activity that the direction of the reaction used for studying pH dependence of enzyme activity is not essential because of reversibility of the enzyme action.

Hydrogenases are oxidoreductases (EC 1.12.-.-) catalyzing activation of molecular hydrogen. Their activity is measured in reactions of hydrogen release in the presence of a reduced donor of electrons (such as methyl viologen), of hydrogen uptake in the presence of oxidized electron acceptor (such as methyl viologen or benzyl viologen), as well as in the exchange reaction in the D_2/H_2O or H_2/D_2O systems by isotope appearance in the gas phase. The pH optima of activity of many hydrogenases (independently of their properties and metal content in their active centers) have been found in the acidic region for reactions of hydrogen release and in the alkaline region for reactions of its uptake [2-4]. Authors explain this by the fact that in the case of hydrogen release protons are a substrate whose concentration increases as pH becomes lower. On the contrary, in the case of hydrogen uptake the reaction becomes easier at decreased proton concentration and, owing to this, pH optimum is shifted to the alkaline region. However, there are examples when pH optimum for hydrogen uptake is in the acidic region [5, 6]. The pH optimum of hydrogenase activity, measured in the exchange reaction, is usually in the acidic region [3, 7, 8], but there are some exceptions for this case [8].

If a hydrogenase is immobilized on an electrode surface, an electrode can be designed capable of hydrogen oxidation and anode current generation. The current

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dependence on pH of such enzyme-containing hydrogen electrode with the *Thiocapsa roseopersicina* hydrogenase does not coincide with the pH dependence of both hydrogen release and uptake [9]. However, following the supposition concerning the role of concentration of protons, considered as products of the reaction of hydrogen oxidation by the electrode, then pH optimum for such reaction should be in the alkaline region like in the reaction of hydrogen uptake by an enzyme in solution, which is not supported by experimental data.

The goal of this work was to explain the apparent contradictions between data on the effect of pH on the hydrogenase activity in solution (in the reaction of hydrogen uptake) and on enzymes immobilized on an electrode surface (obtained by measuring the steady-state current during hydrogen uptake).

MATERIALS AND METHODS

The hydrogenase preparations were isolated from cells of the purple sulfur bacterium *T. roseopersicina* BBS and sulfate-reducing bacterium *D. baculatum* 9974. The hydrogenase from *T. roseopersicina* was purified using the previously developed technique [10]. The enzyme from *D. baculatum* was purified by a modified method [11]. After isolation of the hydrogenase from *D. baculatum* cells, it was purified by successive column chromatography on DEAE-cellulose and phenyl-Sepharose. In this case highly purified, nearly homogeneous preparations were obtained, the specific activity of which by hydrogen uptake was ~60 μmol H_2/min per mg protein for *T. roseopersicina* hydrogenase and ~500 μmol H_2/min per mg protein for the enzyme from *D. baculatum*.

The effect of pH on hydrogenase activity in the reaction of H_2 uptake in the presence of benzyl viologen (molar absorption coefficients ε_{555} = 7550 $M^{-1}\cdot cm^{-1}$ and ε_{600} = 12,300 $M^{-1}\cdot cm^{-1}$) was studied spectrophotometrically in 50 mM Tris-HCl buffer [10]. To measure pH-dependence of immobilized hydrogenase activity, separate fibers of carbon tissue with hydrogenase were placed into the spectrophotometric cell with the same buffer. After active mixing the kinetics of changes in absorption were registered. In both cases, the reaction mixture contained 1-5 μg enzyme. To compare data for the solution and immobilized enzyme activity, the values were graphed in percent, the highest specific activity being taken as 100%.

The hydrogen electrode used in the experiments consisted of carbon tissue with polypyrrole viologen and immobilized hydrogenase, which was connected in an electric circuit via a gold wire. The hydrogenases were immobilized on the surface of LShG-240 carbon tissues with polypyrrole viologen as described previously [12].

To study the effect of pH on the steady-state currents of hydrogenase hydrogen electrodes, we have used a device assembled in our laboratory (Fig. 1) consisting of a cell connected with a IPC-compact LLC Volta potentiostat (Russia) following the three-electrode scheme, peristaltic pump, recycler, and hydrogen generator. To maintain a high partial pressure of H_2 in solution, a buffer solution (20 mM potassium phosphate buffer in 100 mM KCl), saturated with hydrogen, was steadily pumped from the recycler. All liquid lines were connected by vacuum tube, and Tygon-type tubes with minimal coefficient of gas diffusion were used for the peristaltic pump. Preliminary experiments showed that even at maximal current levels (1.2 mA) the rate of buffer recycling of 100 ml/min was saturating. That is why this rate was used in the experiments. Solution from the cell was returned into the vessel, through which pure H_2 passed from the ELDIS 15MD hydrogen generator (additionally purified free from traces of oxygen by a palladium filter). The cell was made of glass with internal space partitioned by 230 μm thick naphionic membrane (a membrane of MF4 CK perfluorosulfonic polymer, a Teflon analog with some fluorine atoms replaced by sulfo groups, produced in PLC Plastpolymer, St. Petersburg) into two equal parts of 15 × 15 × 10 mm^3 each. The hydrogenase hydrogen electrode (10 × 10 mm^2) and the AgCl reference electrode were placed at one side of the membrane, where the hydrogen-saturated solution was drawn. An auxiliary electrode was placed at the opposite side of the membrane. This space was filled with the same buffer and connected with the environment. The membrane was used to prevent penetration into the space with hydrogenase electrode of reaction products released at the auxiliary electrode. Owing to its high proton conductivity, the membrane had practically no effect on electrochemical reaction.

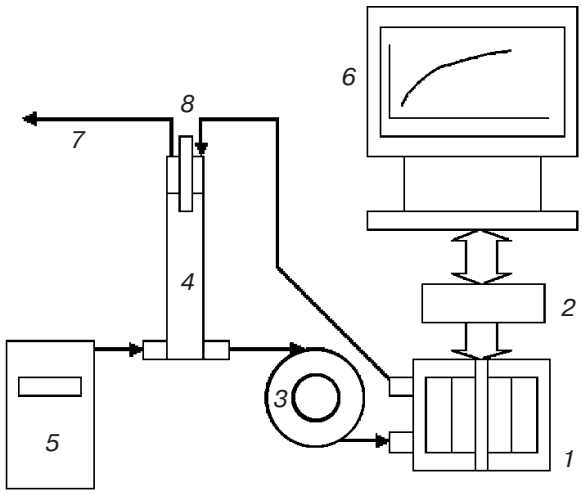

Fig. 1. Scheme of the device for measuring polarograms of hydrogen electrodes at different pH values: *1*) electrochemical cell; *2*) potentiostat; *3*) peristaltic pump; *4*) recycling chamber with buffer; *5*) hydrogen generator; *6*) computer; *7*) outlet for excess hydrogen; *8*) pH electrode with pH meter.

All electrochemical measurements began with the buffer pH 7.0. After switching on the device and liquid saturation with hydrogen before beginning measurements, it was necessary to wait for stabilization of the potential on the hydrogenase electrode (−628 to −643 mV for different electrodes relative to the AgCl electrode). This potential was taken as −412 mV (Fig. 2). Then the dependence of current on voltage was measured (under galvanostatic conditions). The current value was considered as steady-state if it did not change for 1 min. After that the current was fixed at zero and pH was changed first towards higher values by addition of 0.5 M NaOH, and then to lowering values with 0.5 M HCl. After pH stabilization, the polarogram was measured at fixed pH. If upon return of pH to 7.0 the polarogram was from the initial one, further measurements with this electrode were not carried out. Most often, this happened in the case of continuous measurement of the current, when no steady-state condition appeared. Since different hydrogenase electrodes for the same hydrogenase were characterized by significant scattering of maximal current values (0.4-1.2 mA at pH 7.0), the latter for each electrode were expressed as current percent at pH 7.0 and overvoltage of 50 mV and data for different electrodes with the same hydrogenase were averaged. The dependence of current on pH, also expressed in percent, was determined on the basis of polarograms for electrodes with the two different hydrogenases. Potentials on electrodes are given relative to the normal hydrogen electrode and overvoltage (the difference between potential of reaction equilibrium at given pH and potential on an electrode) was expressed relative to the equilibrium potential at given pH (potential of hydrogen electrode at this pH). It should be noted that currents for both hydrogenase electrode in the absence of hydrogen and electrodes with polyviologens in the presence of hydrogen would be more than two orders of magnitude below those in the presence of hydrogenase and hydrogen, and owing to this their values were not considered.

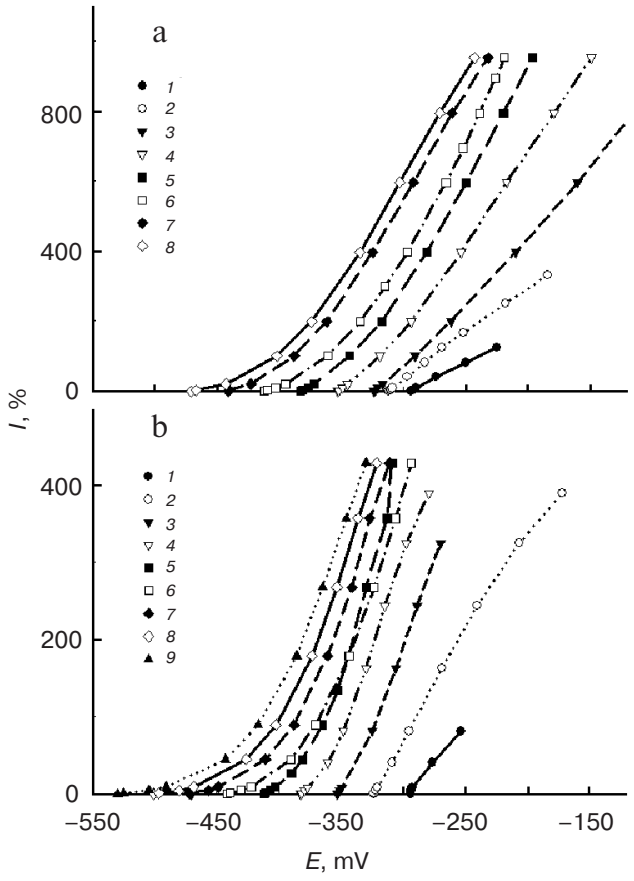

Fig. 2. An example of a hydrogen electrode polarogram: a) with hydrogenase of *T. roseopersicina* (*1-8* correspond to pH 5.0, 5.3, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, respectively); b) with *D. baculatum* hydrogenase (*1-9* correspond to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, respectively).

RESULTS AND DISCUSSION

Maximal activity of homogeneous *T. roseopersicina* hydrogenase in the hydrogen uptake reaction in solution was observed at pH 9.5 (Fig. 3a, curve *1*) and for the homogeneous enzyme from *D. baculatum* at pH 8.5 (Fig. 3b, curve *1*). Both the pH increase and decrease from the mentioned values resulted in decreased activities of the two hydrogenases. The values of pH optima coincide with those described in the literature for hydrogenases of *T. roseopersicina* [3] and *D. baculatum* [8].

The steady-state current of electrodes with *T. roseopersicina* hydrogenase, caused by catalytic hydrogen uptake (Fig. 4a), was maximal at pH 5.5-6.5 and overvoltage of 30-60 mV about equilibrium potential at this pH. This is in line with the previously described data [9]. With overvoltage of 100-150 mV, optimal range of pH was narrowed to 6.0-6.5. It should be noted that at pH < 5.5 electrodes with this hydrogenase became unstable, as was revealed by retarded achievement of steady-state current and irreversible inactivation under increased overvoltage. This can be due to the fact that the activity of *T. roseopersicina* hydrogenase depends on redox potential of the medium; it increases as redox potential decreases and reaches 50% activity at −100 to −250 mV as compared with the normal hydrogen electrode [13, 14]. The steady-state current of electrodes with *D. baculatum* hydrogenase had pH optimum 6.0-6.5 and it became more pronounced when overvoltage was increased (Fig. 4b). Thus, the pH optima of steady-state currents of electrodes are shifted to the acidic region as compared with pH optima of reaction of uptake by over 3 units for *T. roseopersicina* hydrogenase and ~2 units for the enzyme of *D. baculatum*.

It could be supposed that such a shift of pH optimum in measurement of steady-state currents was caused by immobilization of the hydrogenases. To check this possi-

bility, the activities of the hydrogenases immobilized on carbon tissue were measured at different pH values in the same reaction of hydrogen uptake in the presence of benzyl viologen (Figs. 2a and 2b). The pH optima of the immobilized hydrogenases of *T. roseopersicina* (Fig. 2a, curve *2*) and *D. baculatum* (Fig. 2b, curve *2*) were shifted from the pH optima of the soluble hydrogenases to acidic region by 0.5 pH unit, which cannot explain differences in pH optima of the uptake reaction and steady-state currents. Taking into account that the surface of carbon tissue has a positive charge (in the form of two positively charged quaternary amines per unit of immobilized polypyrrole viologen), one should expect that due to repulsion of hydroxonium ions, local pH near the surface will somewhat exceed that of the solution. This explains the small shift in pH optimum for the immobilized hydrogenases. Thus, it can be assumed that immobilization on the surface of the electrode (i.e., of carbon tissue with polypyrrole viologen) has practically no effect on the pH profile of the enzyme.

To reveal factors responsible for differences in pH-dependences of the enzyme activities in the hydrogen uptake reaction in solution and of enzyme-containing electrode, it is necessary to analyze conditions of these processes. Hydrogen uptake proceeds in the presence of only a single component with redox characteristics of benzyl viologen measured at the start of the reaction (optical density of 1-cm layer of solution is 0.05-0.5). Knowing the extinction coefficient of benzyl viologen, its redox potential ($E_0 = -359$ mV), which is independent of pH [15], and total concentration in solution (4 mM), one can estimate the redox potential range under which the reaction takes place. It is −215 to −280 mV about the normal hydrogen electrode and is independent of pH. The equilibrium potential (E_0) of the hydrogen electrode is pH-dependent and equal to −412 and −530 mV at pH 7.0 and 9.0, respectively. Thus, the change in the reaction free energy, proportional to that of electrochemical potential, in the solution kinetics is pH-dependent, because the potential deviations from the equilibrium value at pH 7.0 and 9.0 are equal to 130-200 and 250-315 mV, respectively, i.e., the change in the reaction free energy becomes larger as pH is increased.

In electrochemical kinetics the current of the hydrogen electrode is equalized under constant overvoltage [9], i.e., at constant level of potential relative to the hydrogen electrode potential at given pH (Fig. 4). This is done in

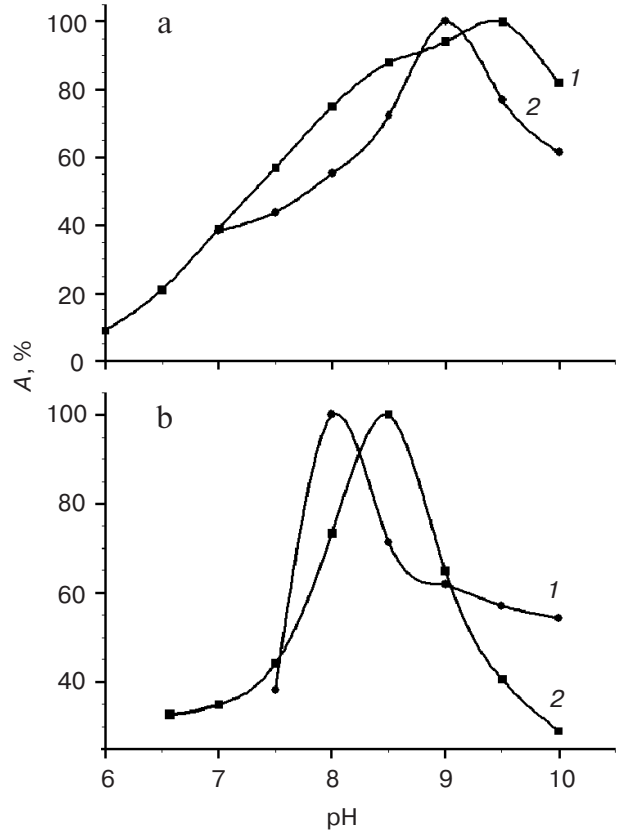

Fig. 3. Effect of pH on activity of hydrogenases from *T. roseopersicina* (a) and *D. baculatum* (b) in solution (*1*) and in immobilized state (*2*) in reaction of hydrogen uptake in the presence of benzyl viologen.

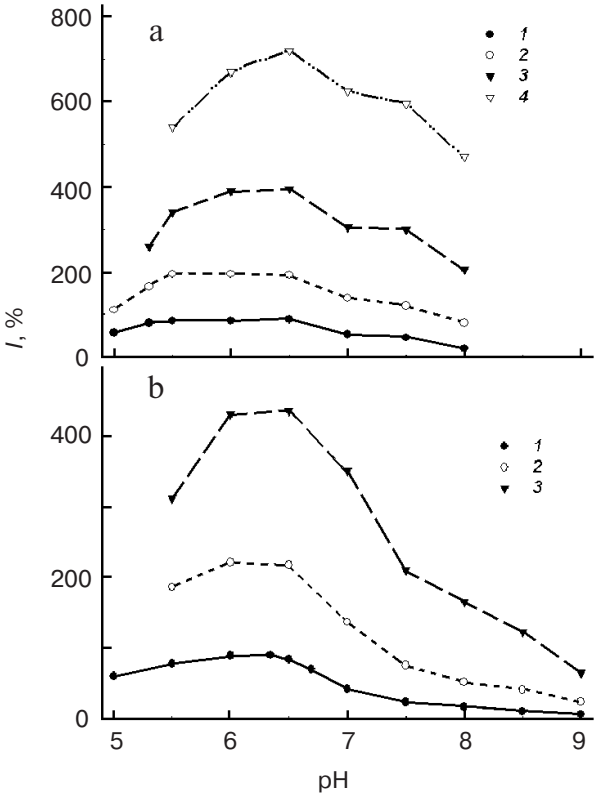

Fig. 4. Effect of pH on current of hydrogen electrode with hydrogenases of *T. roseopersicina* (a) and *D. baculatum* (b) at overvoltage of 30 (*1*), 60 (*2*), 100 (*3*), and 150 mV (*4*) about equilibrium potential at given pH.

order to achieve a constant change in the reaction free energy and so that found dependences would be indicative of the enzyme activity proper. It can be assumed that just this is mainly responsible for differences in pH optima of hydrogenase activities in the processes under study.

This supposition can be checked by plotting the pH dependence of the hydrogen oxidation current at constant potential (but not under overvoltage), for instance, at −250 mV about the normal hydrogen electrode (Fig. 5, curve *1*). The current measured in this way for *T. roseopersicina* hydrogenase increased as pH was increased from 5.5 to 8.0. It should be noted that activity of this hydrogenase in the hydrogen uptake reaction increased 8-fold if pH was changed from 6.0 to 8.0 (Fig. 2a, curve *1*), whereas steady-state current at −250 mV increased only 2.3 times for the same change in pH (Fig. 5). This may be so because at high levels of the current it could be regulated by the rate of hydrogen diffusion towards the electrode surface rather than by the activity of the hydrogenase. Unfortunately, no steady-state currents were established for *T. roseopersicina* hydrogenase at −250 mV about the normal hydrogen electrode, and at pH optimal for the reaction of hydrogen uptake, steady-state currents were not established as well, because at all pH values steady-state currents were not obtained at overvoltage of −250 mV due to the irreversible loss of the electrode catalytic activity (data not shown). Possibly just the high difference between the equilibrium potential and redox potential of the medium during reaction at pH 10 (380 mV) is one of the factors that caused decrease in both enzyme activity and the uptake reaction.

Our data do not allow us to reveal the pH effect on steady-state currents at −250 mV for *D. baculatum* hydrogenase, immobilized on the electrode surface, because it is possible to establish a steady-state current at this potential only at pH 5.0 (Fig. 2b). Increasing pH from 5.0 to 6.5 at −280 mV resulted in 10-fold increase in steady-state currents of electrodes with this hydrogenase (Fig. 5, curve *2*). Carrying out the reaction of hydrogen uptake by this hydrogenase at pH < 6.5 was difficult due to very low hydrogenase activity. Because of this, the direct comparison of pH effect on activity of *D. baculatum* hydrogenase in solution and on steady-state currents under steady potential on the electrode is impossible. Nevertheless, the increase in activity and steady-state currents upon pH increase at least to 6.5 is also characteristic of this hydrogenase. It should be noted that electrodes with this hydrogenase were less stable at higher overvoltage levels than those with the enzyme from *T. roseopersicina* (this is shown indirectly in the polarograms in Fig. 2b). Since activity of this hydrogenase in reaction of hydrogen uptake in the presence of benzyl viologen also began to decrease at pH below that for hydrogenase from *T. roseopersicina*, it can be assumed that the *D. baculatum* hydrogenase is more sensitive than the hydrogenase of *T. roseopersicina* to the higher difference between the hydrogen electrode potential under these conditions and potential of hydrogenase (redox potential of the medium). However, this mechanism should be considered separately.

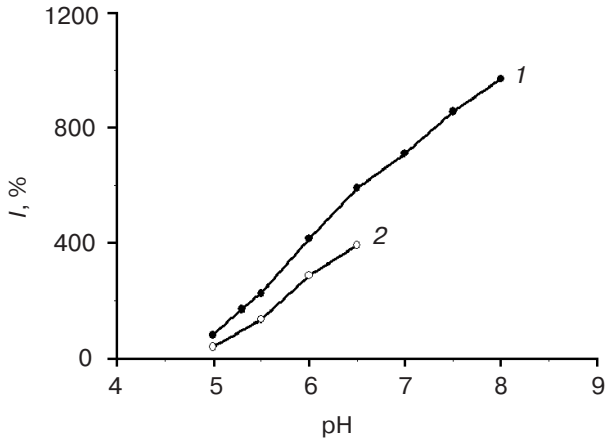

Fig. 5. Effect of pH on the current of hydrogen electrode with hydrogenases of *T. roseopersicina* (*1*) and *D. baculatum* (*2*) at constant potentials of −250 mV (*1*) and −280 mV (*2*) about the normal hydrogen electrode.

Experiments on the effect of pH on hydrogenase activities in reaction of hydrogen uptake in the presence of electron acceptor, equilibrium potential of which is also pH dependent, could serve as an additional control of our hypothesis concerning the effect of changes in free energy of reaction upon studies of pH dependence. An appropriate acceptor for this purpose is methylene blue, the equilibrium potential of which at pH 7.0 is +11 mV and at pH above 6.0 it changes by −30 mV per pH unit [16]. Unfortunately, the hydrogenases under study exhibited very low activity in the presence of methylene blue. However, there are data in the literature like those for membrane-bound hydrogenase of *Paracoccus denitrificans*. It was shown that hydrogen uptake by solubilized preparations of this hydrogenase in the presence of benzyl viologen proceeded at maximal rate at pH ~9.0, and changing the pH from 6.0 to 8.0 resulted in approximately 7-fold increase in its activity [17]. However, the solubilized membranes with this hydrogenase exhibited maximal activity in the same reaction but in the presence of methylene blue at pH 6.5-7.5, and changing the pH from 5.0 to 9.0 caused no more than doubling of enzyme activity (it decreased at pH >7.5 or <6.5) [18].

Another example is the hydrogenase of *Chromatium vinosum*. The pure enzyme preparations exhibited pH optimum 6.8-7.5 in the hydrogen uptake reaction in the presence of benzyl viologen [19] and 5.5-8.5 in the presence of methylene blue [20]. Thus, when along with the change in pH there is an insignificant change in the difference between redox potential, at which reaction is carried out, and equilibrium potential (potential of hydrogen electrode at given pH), like in the case of methylene blue, pH-dependence of enzyme activity is much smoother,

which supports our hypothesis. It should be also noted that exceptions from the general features of the manifestation of optimal pH for the reaction of hydrogen uptake in the alkaline region are described in the literature [5, 6] for reactions in the presence of methylene blue, which is an additional fact supporting our hypothesis.

In reaction of hydrogen release from reduced methyl viologen, which is usually used for determination of hydrogenase activities, pH optimum for all studied hydrogenases is in the acidic region. If the redox potential of methyl viologen is considered to be –446 mV and pH-independent [16], then according to our hypothesis, along with pH decrease, the reaction free energy increases in proportion to the difference between potential at which the reaction takes place (~ –500 mV in the case of methyl viologen reduction by sodium dithionite) and equilibrium potential. This explains the shift of the pH optimal for this reaction to the acidic region.

Thus, the change in hydrogenase activities in solution in the hydrogen uptake reaction depending on pH is not only the result of the effect of proton concentration on enzyme conformation or protonation of certain groups of the enzyme active center, but it is also indicative of changes in the reaction free energy. This should be considered in interpreting the pH dependence of hydrogenase activities.

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