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The role of Hox hydrogenase in the H_2 metabolism of *Thiocapsa roseopersicina*

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Abstract

The purple sulfur phototrophic bacterium *Thiocapsa roseopersicina* BBS synthesizes at least three NiFe hydrogenases (Hox, Hup, Hyn). We characterized the physiological H_2 consumption/evolution reactions in mutants having deletions of the structural genes of two hydrogenases in various combinations. This made possible the separation of the functionally distinct roles of the three hydrogenases. Data showed that Hox hydrogenase (unlike the Hup and Hyn hydrogenases) catalyzed the dark fermentative H_2 evolution and the light-dependent H_2 production in the presence of thiosulfate. Both Hox^+ and Hup^+ mutants demonstrated light-dependent H_2 uptake stimulated by CO_2 but only the Hup^+ mutant was able to mediate O_2-dependent H_2 consumption in the dark. The ability of the Hox^+ mutant to evolve or consume hydrogen was found to depend on a number of interplaying factors including both growth and reaction conditions (availability of glucose, sulfur compounds, CO_2, H_2, light). The study of the redox properties of Hox hydrogenase supported the reversibility of its action. Based on the results a scheme is suggested to describe the role of Hox hydrogenase in light-dependent and dark hydrogen metabolism in *T. roseopersicina* BBS.

Keywords: H_2 metabolism; Hydrogenase Hox; Hup; Hyn; *Thiocapsa roseopersicina*

1. Introduction

Significant biodiversity of hydrogenases has been demonstrated in different groups of organisms including archaea, bacteria and eukaryotes [1]. An increasing number of microbes have been described of synthesizing several hydrogenases having similar sequences and structures. Peculiarities of their synthesis, function and interaction are still not fully understood.

The purple sulfur bacterium *Thiocapsa roseopersicina* is known to synthesize several NiFe hydrogenases [2]. The membrane-bound HynSL hydrogenase is characterized by unusual gene organization as the *hynSL* genes are separated by two open reading frames (*isp1* and *isp2*). This enzyme is remarkably stable and is therefore very promising for practical applications. The second membrane-bound hydrogenase, HupSL, encoded in the *hupSLCDHIR* operon, is extremely labile. It has properties typical for other uptake hydrogenases supposed to play role in recycling of H_2. It is notable that, even though *T. roseopersicina* contains genes of H_2 sensing and regulation, the expression of *hupSL* is hydrogen independent [3]. The third hydrogenase (HoxEFUYH) is a cytoplasmic NAD^+-reducing enzyme analogous to the heteropentameric cyanobacterial bidirectional hydrogenases. Considerable attention has been paid recently to the examination of cyanobacterial Hox hydrogenases. Long-term H_2 production in the darkness, short-term H_2 production upon illumination and following H_2 uptake due to CO_2 assimilation were demonstrated [1,4]. It has been proposed that in some cyanobacteria H_2 uptake proceeds through the complex I and Hox hydrogenase is a part of complex I or connected to it via common subunits [4,5]. In purple bacteria there are no data indicating the structural relation of Hox hydrogenase and complex I. It was shown recently that in the phototrophic culture of *T. roseopersicina* this enzyme is

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responsible for H_2 evolution under nitrogenase repression and for H_2 consumption under nitrogenase-derepression [6]. It is unclear which electron donors (acceptors) are invilved in the light-dependent H_2 evolution (consumption) and if there is any dark H_2 production and/or consumption involving Hox hydrogenase.

The aim of this study is detailed characterization of physiological functions of Hox hydrogenase in *T. roseopersicina* in light and dark conditions as compared to HynSL and HupSL hydrogenases. For this purpose we examined H_2 consumption/evolution reactions by cell suspensions of mutants with deletions of structural genes of two hydrogenases in various combinations and, thus, with expression of a single hydrogenase. Influence of some factors (light/dark conditions, availability of physiological donors/acceptors, growth conditions) was tested. This approach enabled us to demonstrate the diversity and peculiarities of functions of Hox hydrogenase compared to other hydrogenases in purple sulfur bacteria.

2. Materials and methods

Three mutants of *T. roseopersicina* BBS were used in this study: GB1121 (Δ*hupSL*, Δ*hynSL*), GB1131(Δ*hynSL*, Δ*hoxEFUYH*) and GB2131(Δ*hupSL*, Δ*hoxEFUYH*) [6]. The strains were grown in Pfennig`s media with 12 mM acetate, 24 mM $NaHCO_3$, 19 mM NH_4Cl, 6.3 mM thiosulfate. In some cases (as indicated) thiosulfate concentration was increased to 25 mM. The incident light intensity was approximately 60–80 μE m^{-2} s^{-1} (unless otherwise stated). Routinely, 100–200 ml of bacterial culture was harvested, washed with 25 mM K-phosphate buffer pH 7.0 with 17 mM NaCl and suspended in this buffer. For redox measurements cell suspension was sonicated at 4 °C, centrifuged for 15 min at 15,000×*g*. The supernatant was used as cell-free extract.

Hydrogen evolution was assayed in 12 ml sealed vials with 2 ml of reaction mixture containing 25 mM K-phosphate buffer pH 7.0 with 17 mM NaCl, cell suspension (45–90 μg bacteriochlorophyll as specified) with or without additions (glucose, pyruvate, thiosulfate as indicated in the tables and figures). The vials were repeatedly evacuated, refilled with Ar and incubated at 30 °C with shaking under light (approximately 130 μE m^{-2} s^{-1}) or dark conditions. H_2 concentration was analyzed by gas chromatography. For H_2 consumption study reactions were performed as above but without additions. After refilling with Ar, an appropriate volume of air or CO_2 (as specified) and 130 μl H_2 was introduced. Initial and residual (after 3 and 7 h incubation) H_2 concentration was analyzed by gas chromatography. H_2 consumption rates ~20±10 μl h^{-1} mg^{-1} Bchl were presented as "tr". These values hardly exceed values obtained in control experiments without bacterial cells. Hydrogenase-independent H_2 "consumption" could result from some diffusion of H_2 out of vials, dilution effect due to the sampling and replacing gas phase with argon, etc.

Fluorescence was recorded with the excitation at 366 nm and emission at 465 nm. Reaction was performed in sealed 5-ml tubes containing 3 ml of washed cell suspension and 10 mM glucose, gas phase — argon. Tubes were incubated under light or dark conditions (as described above) and fluorescence was measured at regular intervals.

The dependence of hydrogenase activity in cell-free extracts on redox potential was studied in a chamber with controlled E_h and hydrogen electrode as described earlier [7]. Redox potentials were measured against saturated calomel electrode and expressed versus normal hydrogen electrode by adding +240 mV.

Bacteriochlorophyll (Bchl) and elemental sulfur S^0 content was estimated spectrophotometrically in methanol extracts using extinction coefficients 84.1 g^{-1} l cm (772 nm) and 23.9 g^{-1} l cm (260 nm) [8]. During S^0 estimation the absorption at 260 nm was corrected for bacteriochlorophyll absorption. Total carbohydrate content was estimated with the anthrone reagent after extraction of S^0 from cell pellet [9].

3. Results

3.1. Hydrogen evolution and consumption by cell suspension of the mutant GB1121(Hox$^+$) as compared to mutants GB1131 (Hup$^+$) and GB2131(Hyn$^+$)

Table 1 compares the ability of the three mutants for endogenous or substrate-dependent H_2 evolution/consumption. Note that all activities were measured in cells grown at standard conditions: 2–3 days with 6.3 mM thiosulfate at light intensity 60–80 μE m^{-2} s^{-1}. The growth medium contained 19 mM NH_4^+ for repression the nitrogenase synthesis. (Some effects of cultivation parameters on reactions of H_2 metabolism are considered below).

Significant rates of H_2 evolution in the darkness were found in cell suspension of the mutant GB1121(Hox$^+$) (Table 1). H_2 evolution was accelerated upon addition of glucose, pyruvate, fumarate (2–40 mM), but not acetate. Appreciable H_2 evolution was observed without added substrates and was supported evidently by endogenous carbohydrate, glycogen. Under the given conditions accumulation of polyhydroxyalkanoate was believed to be insignificant [10]. Values of dark H_2 production by other mutants did not exceed 1.5% of that by GB1121 and were considered as insignificant. Consequently, dark H_2 production was mediated by Hox-hydrogenase.

In the absence of exogenous acceptor no appreciable dark H_2 uptake was found in three mutants (Table 1). Addition of O_2 resulted in stimulation of H_2 uptake in suspensions of the mutant GB1131(Hup$^+$) only. The mutant GB1121(Hox$^+$) did not reveal substantial H_2 consumption in the dark in the presence or absence of O_2. Thus, Hox hydrogenase did not participate in dark H_2 consumption under the conditions tested. It should be emphasized that cells were grown under anaerobic conditions.

None of the mutants demonstrated light-dependent endogenous H_2 production (Table 1) if grown in the presence of 6.3 mM

Table 1
Hydrogen evolution/consumption rates in suspensions of the mutants GB1121 (Hox$^+$), GB1131(Hup$^+$) and GB2131(Hyn$^+$) depending on the presence of the electron donor/acceptor and light/dark conditions

Process	Additions	H_2 evolution/consumption, μl h^{-1} mg^{-1} Bchl		
		GB1121 (Hox$^+$)	GB1131 (Hup$^+$)	GB2131 (Hyn$^+$)
H_2 evolution, dark [a]	no	79±19	tr	tr
	glucose, 5 mM	323±43	tr	tr
H_2 evolution, light [a]	no	tr	tr	tr
	thiosulfate, 25 mM	59±13	tr	tr
H_2 consumption, dark [b]	no	tr	tr	tr
	3.7% O_2	tr	116±34	tr
H_2 consumption, light [b]	no	61±18(21±14) [c]	90±26	tr
	10% CO_2	58±20(126±34) [c]	148±48	tr

Values represent the average of 5–25 experiments ±95% confidential interval. Cultures were grown photoheterotrophically with 6.3 mM thiosulfate.
[a] Bchl content in reaction mixture was approx 45 μg.
[b] Bchl content in reaction mixture was approx 90 μg.
[c] Cultures were grown with 25 mM thiosulfate.

thiosulfate. However, upon the addition of thiosulfate (2–25 mM) to suspensions of the mutant GB1121(Hox$^+$) light-dependent H_2 production was observed. Exogenous sulfide, sulfite or hydrophilic elemental sulfur resulted in light-dependent H_2 production comparable to that generated from thiosulfate. Furthermore, endogenous H_2 production under illumination (27±12 μl h^{-1} mg^{-1} Bchl) was found in Hox$^+$ cells grown at thiosulfate excess and contained stored S^0. Thiosulfate-dependent H_2 evolution under the light was absent from the cell suspension of the mutants GB1131(Hup$^+$) and GB2131(Hyn$^+$).

Appreciable rate of light-dependent endogenous (without added acceptors) H_2 uptake was observed in the suspensions of the mutants GB1131(Hup$^+$) and GB1121(Hox$^+$) (Table 1). In GB2131(Hyn$^+$) these values hardly exceed the background level of dark H_2 consumption ("traces"). Addition of CO_2 resulted in significant stimulation of H_2 consumption by the mutant GB1131(Hup$^+$) indicating that light-dependent H_2 consumption was related to the synthesis of glycogen and other C-compounds. But in the case of GB1121(Hox$^+$) we did not observe stimulation by CO_2 (using cells grown with 6.3 mM thiosulfate). This could be attributed to the preferred utilization of available endogenous electron acceptors.

Grown with elevated thiosulfate concentration (25 mM) when the cells contained stored S^0, however, this mutant demonstrated markedly decreased level of endogenous H_2 consumption and substantial stimulation by CO_2 (Table 1).

So, the Hox$^+$ mutant demonstrated the ability both to produce and to consume H_2 depending on the actual physiological conditions of the cells. Furthermore, the relative capacity to evolve/produce H_2 under the same reaction conditions appeared to be dependent on cultivation conditions. For instance, cells grown under high light intensity (~100 μE m^{-2} s^{-1}) demonstrated ~4-fold higher H_2 consumption capacity in the presence of CO_2 than the cells grown under low intensity (~30 μE m^{-2} s^{-1}) (Fig. 1). For the H_2 evolving capacity, this ratio between high-light and low-light grown cells was approximately 0.5 (Fig. 1). Thus, cells of the mutant GB1121(Hox$^+$) grown under high light intensity demonstrated a lower ratio of H_2 evolution/consumption, i.e., a lower potential for H_2 production than the cultures cultivated under low light intensity.

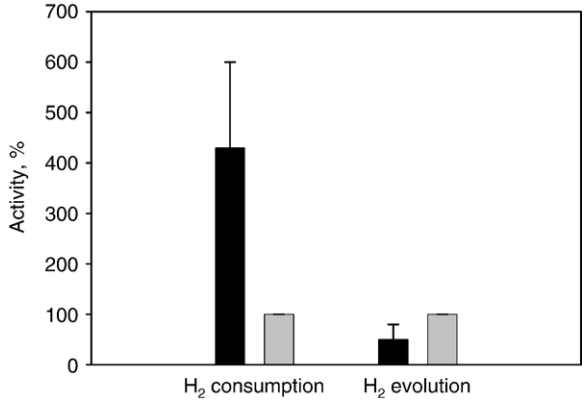

Fig. 1. Relative capacity to light-dependent H_2 consumption (in presence of CO_2) and evolution (in presence of thiosulfate) by the mutant GB1121(Hox$^+$) grown under different incident light intensity: 100 μE m^{-2} s^{-1} (dark bar) and 30 μE m^{-2} s^{-1} (grey bar). Rate of H_2 consumption by cells grown under 30 μE m^{-2} s^{-1} was taken as 100%. Data represent average values for 4 independent experiments ±95% confidential interval.

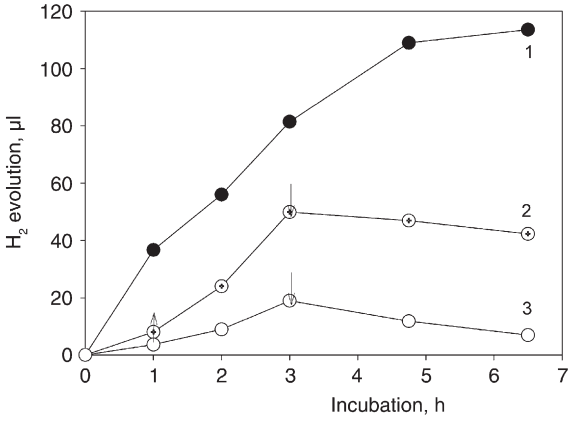

Fig. 2. Hydrogen evolution from pyruvate (10 mM) by cell suspension of the mutant GB1121(Hox$^+$) depending on dark/light conditions: (1) darkness through the process; (2) light (10 μE m^{-2} s^{-1}), 1st arrow—light turn off, 2nd arrow—light turn on; (3) light (80 μE m^{-2} s^{-1}), 1st arrow—light turn off, 2nd arrow—light turn on. Data represent results of a typical experiment. Repetition of individual data sets showed the same tendency but scattering in absolute values.

3.2. Effect of light on H_2 evolution and on the redox state of pyridine nucleotides

Pyruvate-dependent or endogenous H_2 production via Hox-hydrogenase was inhibited by light unlike the thiosulfate-dependent process. Inhibition increased with increasing light intensity (Fig. 2). Switching off the light restored (at least partially) the capacity for hydrogen production, the next light-on reversed the process from H_2 production to H_2 uptake. The trace of H_2 evolution/consumption by H_2 electrode showed that the response to light off/on was not prompt.

Addition of glucose in the dark resulted in a slow increase in the specific fluorescence indicating an increasing NADH/NAD^+ ratio (redox state). Turning on light after dark incubation decreased the ratio of NADH/NAD^+ (Fig. 3). Thus, dark incubation was favorable for the higher redox state of pyridine nucleotides and the higher H_2-producing capacity as well. The lower pyridine nucleotide fluorescence in the light in the presence of glucose might be attributed to the restricted capacity of *T. roseopersicina* to use organic compounds as photosynthetic electron donor for NAD^+ reduction.

Light-dependent H_2 evolution via Hox hydrogenase is probably only one of the possible ways for NADH consumption. Redistribution of NADH utilization could influence H_2 production. In line with this, a stimulation (up to 3-fold) of thiosulfate-dependent H_2 production was observed following the addition of 5–20 μM chloramphenicol, a protein synthesis inhibitor. These data suggest the occurrence of competition for reductant (NADH) between H_2 production and other processes.

If such competition exists one would expect to decrease the thiosulfate-dependent H_2 production in the mutant GB1121 (Hox$^+$) upon the addition of C-substrates (CO_2 or acetate) to the

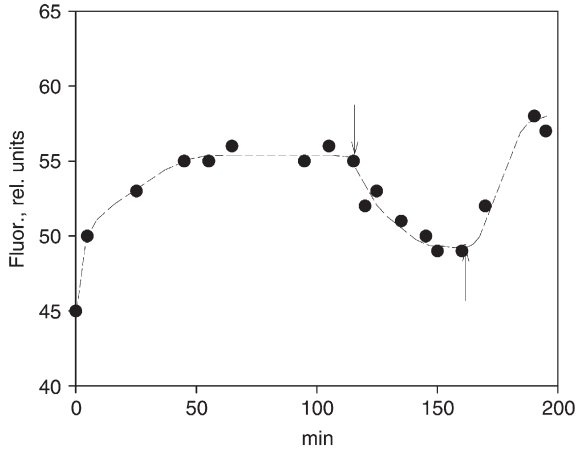

Fig. 3. Relative fluorescence in suspension of the mutant GB1121(Hox$^+$) depending on dark/light conditions with 10 mM glucose under argon. 1st arrow—light turn on, 2nd arrow—light turn off. Data represent results of a typical experiment. Repetition of individual data sets showed the same tendency but scattering in absolute values.

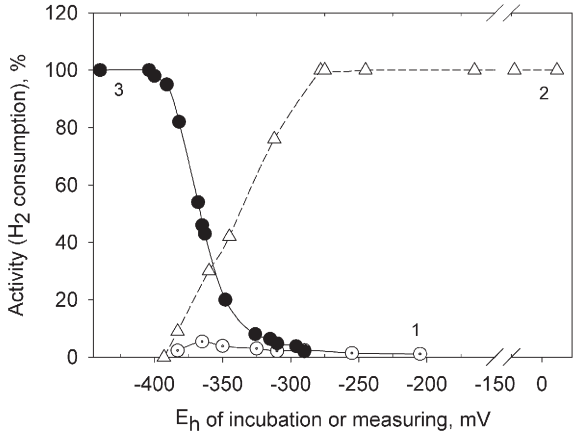

Fig. 5. Redox titration of Hox hydrogenase in cell-free extracts of the mutant GB1121: (1) H_2 consumption was measured at the same E_h as E_h of incubation (indicated in the figure); (2) E_h of measuring was as indicated in the figure, but cell-free extract was preliminary activated under H_2 atmosphere; (3) H_2 consumption was measured at −200 to −250 mV, E_h of incubation was as indicated in the figure. Data represent average values for 3 independent experiments.

cell suspensions. Indeed, the inhibitory effect was observed for several hours after addition of high concentration of C-substrates (10% CO_2 or 20 mM acetate). By contrast, addition of low concentration of C-substrates caused a two-phase effect: at first inhibition, then stimulation of H_2 production. So, upon addition of 5% CO_2 the H_2 production activity during the first hour was 30± 10% (100%=control without CO_2 addition), which increased during the 2nd to 8th hour up to 160±40% (7 experiments). Apparently the stimulatory effect appeared as C-substrate was consumed. Kinetics of CO_2 consumption and H_2 evolution (Fig. 4) indicated the interplay of two sequential processes.

Remarkably the addition of C substrates in the presence of thiosulfate did appreciably facilitate the accumulation of S^0 during incubation (data not shown). This pointed to the acceleration of thiosulfate utilization and presumably activation of photosynthetic processes and glycogen synthesis as long as CO_2 was available.

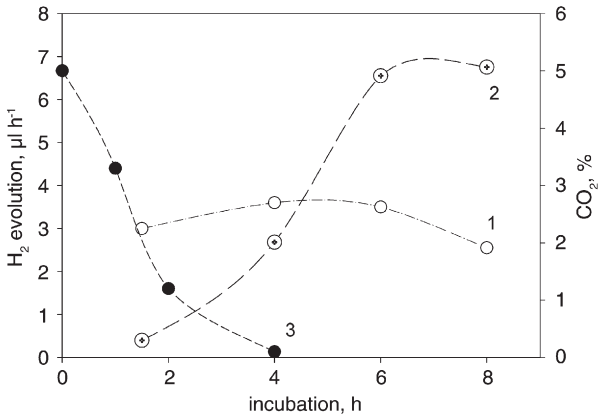

Fig. 4. Influence of 5% CO_2 addition on the rate of thiosulfate-dependent H_2 photoevolution by the mutant GB1121(Hox$^+$) on the 3rd day of cultivation. (1) Control rate of H_2 evolution; (2) H_2 evolution rate in presence of CO_2; (3) CO_2 concentration in the gas phase. Data represent results of a typical experiment. Repetition of individual data sets showed the same tendency but scattering in absolute values.

3.3. Redox titration

Initially the redox titration of the cell-free extracts of the mutant GB1121(Hox$^+$) was performed by incubation at fixed E_h for 20 min followed by the determination of the activity at the same E_h. Very low activity was observed in the range of E_h −350 to −380 mV (Fig. 5, curve 1). This redox potential is close to the equilibrium potential of H_2 electrode. Since the rates of H_2 consumption and evolution are close to equal at this region, the determination of hydrogenase activity is difficult. By using hydrogenase activated under hydrogen prior to the measurement (curve 2), it could be demonstrated that H_2 uptake activity was constant upon lowering E_h from 0 to −300 mV. Then the activity decreased and disappeared at about −390 mV, this potential is in rough agreement with the equilibrium potential of H_2 electrode at pH 8.0 and 4% H_2 (our experimental conditions). It confirmed the inadequacy of the initial approach. Actually, curve 1 showed interplaying of two processes upon the lowering of E_h (from 0 to −390 mV): activation of the enzyme resulting in the increase of activity and the decrease of the actually measured activity. Because of this the process of activation could be studied only after incubation at desired E_h and activity measured in the E_h region above −300 mV, where the activity is held constant. By applying this approach, we demonstrated (curve 3) that E_0' for Hox hydrogenase was about −365 mV (H_2 consumption process).

4. Discussion

Results of our investigation demonstrate the functionally distinct role of Hox hydrogenase (as compared to other hydrogenases) in H_2 metabolism of purple sulfur bacterium *T. roseopersicina*. Only Hox hydrogenase can participate in light-dependent and dark H_2 evolution (Fig. 6). In darkness H_2 is produced due to fermentation but light-dependent H_2

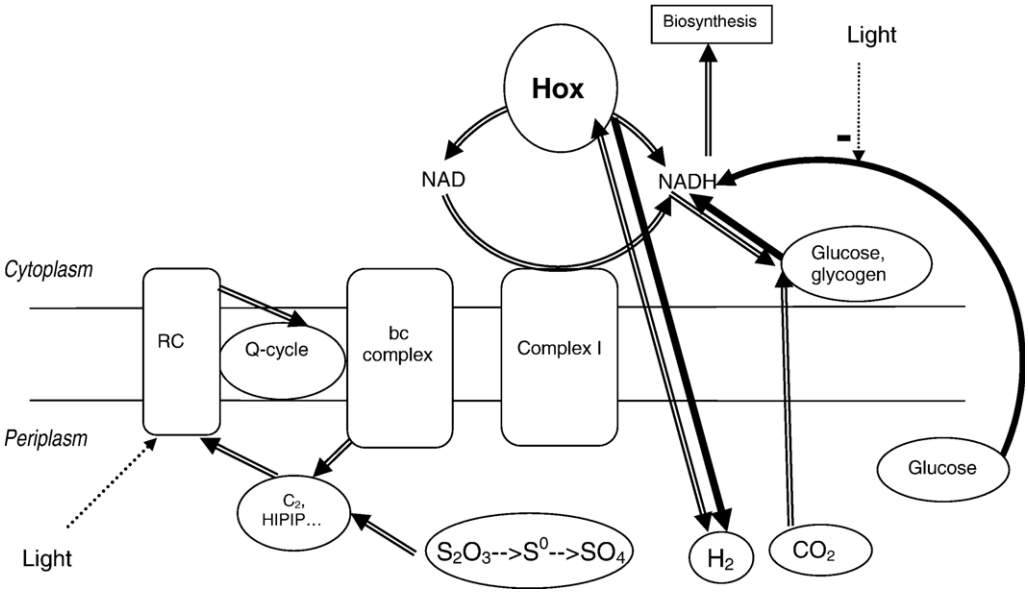

Fig. 6. Involvement of Hox hydrogenase in evolution and consumption of H_2 by *T. roseopersicina* under the light (light arrows) and in the darkness (dark arrows).

production could be driven by thiosulfate, H_2S, SO_3^{2-} and S^0. In purple sulfur bacterium sulfur compounds function eventually as photosynthetic electron donors with following NAD^+ reduction. The electrons from sulfur compounds can be transferred initially either to a periplasmic cytochrome, or HIPIP, or directly to the quinone pool within the membrane with participation of flavocytochrome c (as sulfide dehydrogenase) or sulfide-quinone reductase [11–14]. Reduced NADH generated in complex I by reversed electron transport could be further oxidized by Hox hydrogenase accompanied by H_2 evolution similarly to dark H_2 evolution during fermentation (Fig. 6). In both cases H_2 evolution is a sustained process that lasts for several days or weeks. Unlike it, in cyanobacterium *Synechocystis* only very short Hox-mediated H_2 production was recorded upon illumination followed by rapid H_2 uptake for CO_2 fixation [4].

The close examination of the utilization of reducing power has been carried out in continuous cultures of *Allochromatium vinosum* [15]. The allocation of the reducing power (hydrogen sulfide) in storage products (sulfur, glycogen) has been shown to depend on light conditions. It is of interest that under light limitation about 40% of reducing power was not found in storage products or in cell material. This fact could be probably interpreted in view of our data: the diminished value of incident light intensity is favorable for H_2 production (Fig. 1). So, hydrogen could be the missing product in the *A. vinosum* study [15].

The reports on dark H_2 production by purple sulfur bacteria are few in number or seem to escape notice [10]. However, an alternative pathway for utilization of reducing agent arising from dark glycogen degradation, namely sulfide production, was considered as a main and energetically more favorable option [10]. It is reasonable that capacities for either H_2 or H_2S production depend on the S^0 content in cells. In any case the ability of purple sulfur bacterium for dark anaerobic growth with organic substrates is open to question yet.

According to our data both Hox and Hup hydrogenase are able to mediate light-dependent H_2 consumption (Table 1, Fig. 6). But H_2 consumption via Hox hydrogenase was stimulated by CO_2 addition in cells grown with high thiosulfate concentrations (and, consequently, containing S^0) only. We can speculate that the presence of S^0 can accelerate an exhaustion of endogenous acceptors thus enabling the CO_2 assimilation with H_2 consumption. Another explanation is that utilization of H_2 as an electron donor (via Hox hydrogenase) is insufficient for CO_2 fixation. It requires the presence of S^0 (or thiosulfate) as the main photosynthetic electron donor. Further studies are necessary to clarify this point.

Light-dependent H_2 consumption can be important under nitrogen-fixing conditions (H_2 recycling). Hox hydrogenase of *T. roseopersicina* was shown earlier to play an important role in this process [6]. It is agreed that purple sulfur bacteria are able to oxidize H_2 as a single electron donor for photoautotrophic growth. It seems likely that Hup hydrogenase (and probably Hox hydrogenase) could support the growth of this bacterium in presence of H_2 as an electron donor (Table 1).

Hox hydrogenase was not able to mediate oxygen-dependent H_2 consumption unlike to Hup hydrogenase (Table 1). It is unclear whether this process has any physiological significance for this bacterium or not. It is surprising that Hyn hydrogenase displayed no activity in these reactions. Apparently its function is related to other growth conditions and other physiological reactions (besides indicated in Table 1).

The reversibility of Hox-type hydrogenases is well known [1]. Our data confirmed this feature of Hox hydrogenase in *T. roseopersicina* in both redox titration study (Fig. 5) and experiments with cell suspensions (Figs. 1 and 2). Activation of Hox hydrogenase in cell-free extracts occurred at approximately −365 mV favorable for H_2 consumption. Such value of E_0' is in agreement with the assumption that Hox hydrogenase is related to pyridine nucleotide pool and can be activated due to

the increasing reduction level of NADH (E_0'=−320 mV for NADH/NAD^+). At E_h below −420 mV this hydrogenase maintained its activity and catalyzed H_2 evolution. Similar redox properties were described for other Hox-type hydrogenases, for example, in hydrogen-oxidizing bacteria *R. eutropha* [16], cyanobacterium *Anabaena variabilis* [17]. In cell suspensions the direction of the H_2 evolution/consumption process was governed by combination of different factors: availability and ratio of glucose (glycogen), CO_2, H_2, S compounds and light.

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