J Bioenerg Biomembr (2012) 44:495–502
DOI 10.1007/s10863-012-9450-3

Relationship of proton motive force and the F_0F_1-ATPase with bio-hydrogen production activity of *Rhodobacter sphaeroides*: effects of diphenylene iodonium, hydrogenase inhibitor, and its solvent dimethylsulphoxide

Lilit Hakobyan · Lilit Gabrielyan · Armen Trchounian

Received: 7 May 2012 / Accepted: 29 May 2012 / Published online: 12 June 2012

Abstract *Rhodobacter sphaeroides* MDC 6521 was able to produce bio-hydrogen (H_2) in anaerobic conditions under illumination. In this study the effects of the hydrogenase inhibitor—diphenylene iodonium (Ph_2I) and its solvent dimethylsulphoxide (DMSO) on growth characteristics and H_2 production by *R. sphaeroides* were investigated. The results point out the concentration dependent DMSO effect: in the presence of 10 mM DMSO H_2 yield was ~6 fold lower than that of the control. The bacterium was unable to produce H_2 in the presence of Ph_2I. In order to examine the mediatory role of proton motive force (Δp) or the F_0F_1-ATPase in H_2 production by *R. sphaeroides*, the effects of Ph_2I and DMSO on Δp and its components (membrane potential ($\Delta\psi$) and transmembrane pH gradient), and ATPase activity were determined. In these conditions $\Delta\psi$ was of –98 mV and the reversed ΔpH was +30 mV, resulting in Δp of –68 mV. Ph_2I decreased $\Delta\psi$ in concentrations of 20 μM and higher; lower concentrations of Ph_2I as DMSO had no valuable effect on $\Delta\psi$. The *R. sphaeroides* membrane vesicles demonstrated significant ATPase activity sensitive to *N,N′*-dicyclohexylcarbodiimide. The 10–20 μM Ph_2I did not affect the ATPase activity, whereas 40 μM Ph_2I caused a marked inhibition (~2 fold) in ATPase activity. The obtained results provide novel evidence on the involvement of hydrogenase and the F_0F_1-ATPase in H_2 production by *R. sphaeroides*. Moreover, these data indicate the role of hydrogenase and the F_0F_1-ATPase in Δp generation. In addition, DMSO might increase an interaction of nitrogenase with CO_2, decreasing nitrogenase activity and affecting H_2 production.

Keywords Proton motive force · ATPase activity · Bio-hydrogen production · Diphenylene iodonium · Dimethylsulphoxide · *Rhodobacter sphaeroides*

L. Hakobyan · L. Gabrielyan · A. Trchounian (✉)
Department of Biophysics, Yerevan State University,
0025 Yerevan, Armenia
e-mail: Trchounian@ysu.am

A. Trchounian
Department of Microbiology & Microbe and Plant Biotechnology,
Biology Faculty, Yerevan State University,
0025 Yerevan, Armenia

Introduction

Phototrophic microorganisms have been widely used in the energy conservation mechanism studies (Gabrielyan and Trchounian 2009a; Kapdan and Kargi 2006). The purple bacteria are perspective objects for biological hydrogen (H_2) production, which is connected with the absorption of light energy and hence can increase the efficiency of use of the solar radiation (Gabrielyan and Trchounian 2009a; Kapdan and Kargi 2006). These bacteria can use a wide variety of substrates for growth and H_2 production (Gabrielyan and Trchounian 2009a; Kapdan and Kargi 2006; Gabrielyan et al. 2010; Gabrielyan and Trchounian 2012; Hakobyan et al. 2012).

H_2 metabolism in phototrophic bacteria involves a coordinated action of two enzymes: nitrogenase and hydrogenase. However, the yield of H_2 in hydrogenase-catalyzed reaction is much higher than that of nitrogenase-catalyzed reaction (Gabrielyan and Trchounian 2009a; Basak and Das 2007; Das et al. 2006; Hallenbeck 2009). Photoproduction of H_2 by the phototrophic bacteria is suggested to be a nitrogenase-dependent reaction, whereas hydrogenases may be active for H_2 uptake in many photosynthetic bacteria; but it

depends on different factors (Kapdan and Kargi 2006; Basak and Das 2007; Das et al. 2006; Hallenbeck 2009).

According to the chemiosmotic theory of energetic coupling, the proton motive force (Δp) across the cytoplasmic membrane plays a key role in transformation of biological energy. It can be formed by carrier-mediated excretion of fermentation end products in the symport with H^+ and utilized with ATP synthesis (Abee et al. 1988; Boyer 1988). Also, in certain bacteria, the reaction can be reversed and the F_0F_1-ATPase can generate Δp, working as an H^+-pumping ATPase (Feniouk et al. 2007). The mechanisms for energy transformation in purple non-sulfur bacterium *Rhodobacter sphaeroides* are not clear. Moreover, these bacteria produce H_2 under reducing conditions upon the drop in oxidation-reduction (redox) potential (E_h), which could determine electron transfer within a bacterial membrane and generation of Δp (Hakobyan et al. 2011). A relationship between pH, E_h, Δp and H_2 production is proposed for these bacteria, but its nature is unclear.

Various inhibitors suppress both H_2 uptake and production by purple bacteria (Gabrielyan and Trchounian 2009a). Magnani with co-workers (Magnani et al. 2000) have shown inhibition of *Rhodobacter capsulatus* hydrogenase by diphenylene iodonium (Ph_2I), a known inhibitor of mitochondrial complex I and various monooxygenases (Gatley and Sheratt 1976; Ellis et al. 1988; Doussiere and Vignais 1992). The action mode of Ph_2I on hydrogenase is not fully clarified, but a study of the effect of Ph_2I on H_2 production by purple bacteria can be helpful for identifying of responsible enzymes, understanding of mechanisms and pathways of H_2 production.

As known, Ph_2I is dissolved in a water-miscible solvent such as dimethylsulphoxide (DMSO), which has wide applications in cell biology and biochemistry. DMSO itself affects the membrane stability (Gordeliy et al. 1998; Yu and Quinn 1998), and bacterial growth properties (Horne and McEwan 1998; Markarian et al. 2002). DMSO at low concentration serves as an electron donor for an anaerobic respiratory chain stimulating *Escherichia coli* growth and survival through the modulation the H^+-K^+-exchange and H_2 production activity (Markarian et al. 2002).

In our previous studies, we have shown that *R. sphaeroides* strains isolated from mineral springs in Armenia were able to grow and produce H_2 in anaerobic conditions upon illumination using various carbon and nitrogen sources (Gabrielyan et al. 2010; Gabrielyan and Trchounian 2012; Hakobyan et al. 2012). To investigate the role of Δp in H_2 production by *R. sphaeroides*, Δp and its components (the membrane potential ($\Delta\psi$) and the transmembrane pH gradient (ΔpH)) were determined (Hakobyan et al. 2011).

In this study the effect of Ph_2I and its solvent DMSO on growth properties (such as specific growth rate), external medium characteristics (pH, E_h, etc.) and H_2 production by *R. sphaeroides* str. MDC 6521 was investigated. In order to examine the mediatory role of Δp or the F_0F_1-ATPase in H_2 production by *R. sphaeroides*, the effects of Ph_2I on Δp and its components ($\Delta\psi$ and ΔpH), and ATPase activity was studied. This study was, therefore, important for understanding of mechanisms for biological effects of DMSO and Ph_2I on bacterial growth properties and H_2 production, and for revealing a role of hydrogenase in these processes.

Materials and methods

Bacterial strain and growth conditions

The purple non-sulfur bacterium *R. sphaeroides* MDC 6521 (Microbial Depository Center, Armenia, WDCM803) used in this study was isolated from Arzni mineral springs in Armenia (Gabrielyan and Trchounian 2009b).

R. sphaeroides MDC 6521 was grown in anaerobic conditions in Ormerod medium at 30 ± 2^0 C under illumination of approximately 1500 lux (Gabrielyan et al. 2010; Gabrielyan and Trchounian 2009b). Succinate (3.54 gL^{-1}) and yeast extract (2 gL^{-1}) were used as sole carbon and nitrogen sources (Hakobyan et al. 2012). The concentration of Ph_2I ranged from 1, 5, 10, 20 to 40 μM; the concentration of DMSO in the growth medium ranged from 10, 30 to 50 mM.

The medium initial pH was adjusted to 7.0 by means of 0.1 M HCl or NaOH. The pH of growth medium was measured at certain time intervals (0–96 h) by a pH-potentiometer (HANNA Instruments, Portugal) with selective pH electrode (HJ1131B) (Gabrielyan et al. 2010; Gabrielyan and Trchounian 2012; Hakobyan et al. 2012; Gabrielyan and Trchounian 2009b). The bacterial growth was monitored by a Spectro UV-Vis Auto spectrophotometer (Labomed, USA) by changes in absorbance of cell suspension at 660 nm (Gabrielyan and Trchounian 2009b). Specific growth rate (h^{-1}) was determined as described (Gabrielyan et al. 2010; Gabrielyan and Trchounian 2012; Hakobyan et al. 2012). The absorption spectra of *R. sphaeroides* suspension were recorded at the wavelength region of 400 to 1000 nm on a programmable Spectro UV-Vis Auto spectrophotometer (Labomed, USA) (Gabrielyan and Trchounian 2012; Hakobyan et al. 2012). For obtaining comparable data the original spectra were subtracted of the scattering and normalized to the same cell concentration.

Redox potential determination and hydrogen production assays

The redox potential (E_h) of bacterial growth medium was measured at certain time intervals (0–96 h) by using a platinum (Pt) electrode (EPB-1, Gomel State Enterprise of

Electrometric Equipment (GSEEE), Gomel, Belarus, or PT42BNC, HANNA Instruments, Portugal) (Gabrielyan and Trchounian 2012; Hakobyan et al. 2012; Trchounian et al. 2011a).

H_2 production was assayed by using a pair of redox Pt and titanium-silicate (Ti-Si) (EO-02, GSEEE) electrodes as described (Gabrielyan and Trchounian 2012; Hakobyan et al. 2012; Trchounian et al. 2011a). This determination is close to the method using a Clark-type electrode employed by different authors (Eltsova et al. 2010; Piskarev et al. 2010): a strong correlation between E_h and H_2 production yield in liquid media is shown. The H_2 yield was calculated by the decrease of E_h to low negative values as described by Piskarev and co-workers (Piskarev et al. 2010) and expressed in mmol L^{-1}.

H_2 evolution was confirmed chemically as described (Gabrielyan and Trchounian 2012; Trchounian et al. 2011a). This method was suggested for detecting enhanced H_2 production (Maeda and Wood 2008).

Determination of proton motive force

Δp was calculated as a sum $\Delta\psi$ and ΔpH according to $\Delta\mu_{H+}/F = \Delta\psi - Z\Delta pH$ (Zakharyan and Trchounian 2001), where Z is RT/F equal to 60 mV at 30 ^{0}C. $\Delta\psi$ was determined from the tetraphenylphosphonium cation (TPP^+) distribution between the cytoplasm and the external medium as described (Hakobyan et al. 2011; Zakharyan and Trchounian 2001; Grinus et al. 1980). 1 μM TPP^+ was added in the assay medium, containing 150 mM Tris-phosphate buffer, then the bacteria were added and changes of the concentration of this probe was determined by using a TPP^+-selective electrode as described (Hakobyan et al. 2011; Zakharyan and Trchounian 2001; Grinus et al. 1980). The intracellular volume of $3.75 \cdot 10^{-9}$ μl $cell^{-1}$ was employed. The ΔpH was calculated from the external ($[pH]_{out}$) and intracellular ($[pH]_{in}$) pH values. The $[pH]_{in}$ was determined by the quenching of fluorescence of 9-aminoacridine (9-AA) as described (Hakobyan et al. 2011; Zakharyan and Trchounian 2001; Puchkov et al. 1983), which was measured with a Cary Eclipse spectrofluorimeter (Varian, USA) with excitation at 390 nm and emission at 460 nm. The uptake of 9-AA by bacterial cells was determined from the disappearance of 9-AA from the assay media. For protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) effects studies, cells were incubated with the reagent (2 μM) for 10 min.

ATPase activity assay

Bacterial membrane vesicles were obtained from lysozyme treated cells as described (Gabrielyan and Trchounian 2009b; Trchounian and Vassilian 1994). ATPase activity in the membrane vesicles was measured by the liberation of inorganic phosphate (P_{in}) in the reaction with ATP by the method of Taussky and Shorr using a spectrophotometer Spectro UV-Vis Auto (Labomed, USA) (Gabrielyan and Trchounian 2009b; Trchounian and Vassilian 1994). ATPase activity was expressed in μM P_{in} min^{-1} μg^{-1} protein. Protein was determined by the Lowry method (Gabrielyan and Trchounian 2009b). For *N,N′*-dicyclohexylcarbodiimide (DCCD) inhibition studies, cells were incubated with the reagent (0.5 mM) for 10 min.

Reagents and data processing

Yeast extract (Carl Roths GmbH, Germany), succinic acid (Unichem, China), ATP (Tris salt), CCCP, DCCD, DMSO, Ph_2I (Sigma, Aldrich, USA), and the other reagents used were of analytical grade; used acid was neutralized by NaOH. The average data are represented from three independent experiments; the standard errors were calculated as described (Lakin 1992) and did not exceed 5 % if not indicated.

Results and disscussion

The effects of DMSO and Ph_2I on *R. sphaeroides* growth properties

The growth characteristics were determined and pH changes were monitored during phototrophic growth of *R. sphaeroides* MDC 6521 in the presence of various concentrations of DMSO and Ph_2I.

Both reagents affected the cell specific growth rate. When Ph_2I in various concentrations was added into the growth medium, the cells yield was lower than that of the control, indicating that 1, 5 and 10 μM Ph_2I could inhibit the growth of these bacteria (see Fig. 1). It should

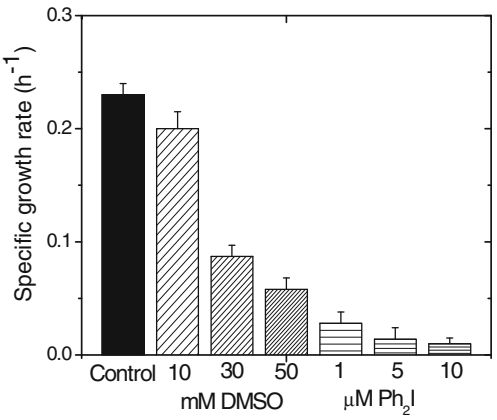

Fig. 1 The effects of DMSO and Ph_2I at various concentrations on *R. sphaeroides* MDC 6521 specific growth rates. This growth property was determined as described in Materials and methods

be noted that *R. sphaeroides* was unable to grow in medium with 20–40 μM Ph_2I (not shown). For understanding the inhibitory effect of Ph_2I was also studied was the effect of its solvent DMSO on the cell specific growth rate. In the presence of 10 mM DMSO this rate was the same as the control (no additions) (Fig. 1). The high concentration of DMSO (30–50 mM) suppressed the growth of bacterium: the specific growth rate decreased to ~3–4 fold in comparing with the control (see Fig. 1). These data were similar to the results on DMSO effects obtained for *E. coli* (Markarian et al. 2002).

Formation of the photosynthetic apparatus takes place during phototrophic growth of *R. sphaeroides*; it consists of two light-harvesting (LH) complexes (B800-850 and B875) surrounding a photochemical reaction center (RC) (Horne and McEwan 1998; Kim et al. 2006). The LH complexes consist of a combination of polypeptides and pigments, which include carotenoids and bacteriochlorophyll *a* (Bchl *a*) (Gabrielyan and Trchounian 2009a). As known, B875 complex accumulates and transfers the light energy to a RC (Kim et al. 2006).

For the first time the absorption spectra of *R. sphaeroides* intact cells grown with DMSO and Ph_2I was investigated in order to reveal pigments synthesis. As shown in Fig. 2, in the control cells absorption spectrum it was observed the several peaks in wavelength region of 400 to 1000 nm, which are typical for this bacterium and are indicators for their LH components (Gabrielyan and Trchounian 2012; Hakobyan et al. 2012; Horne and McEwan 1998; Paronyan 2002). These peaks could be assigned to carotenoids (450, 478 and 510 nm) and Bchl *a* (590, 800, 850 and 875 nm), respectively.

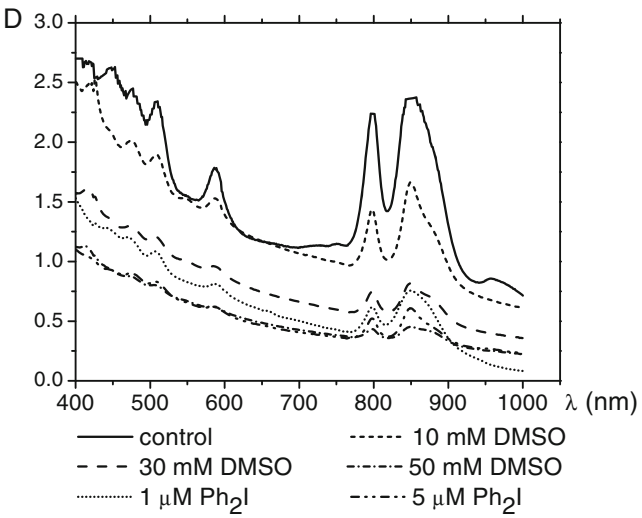

Fig. 2 Absorption spectra of *R. sphaeroides* MDC 6521 bacterial suspension, grown in the presence of 10–50 mM DMSO and 1–5 μM Ph_2I, which is recorded as described in Materials and methods (D was optical density)

R. sphaeroides pigments have been found to be significantly sensitive to the content of the growth medium (see Fig. 2). By addition of DMSO and Ph_2I a decrease in the level of B800-850 and B875 complexes was observed. Absorption peaks typical for carotenoids and Bchl *a* gradually disappeared in the presence of 30–50 mM DMSO and 1–5 μM Ph_2I (see Fig. 2). Similar data were reported by Horne and McEwan (Horne and McEwan 1998), showing that the addition of DMSO caused a decrease in the level of LH complexes and a decrease in the level of carotenoids.

The effect of DMSO and Ph_2I on *R. sphaeroides* external pH, E_h and H_2 production

The external pH and E_h are indicative parameters for bacterial growth under various conditions (Hustede et al. 1993; Vassilian and Trchounian 2009). Both reagents used affected external pH and E_h during the *R. sphaeroides* anaerobic growth upon illumination (Fig. 3). In the presence of 10 mM DMSO pH changes during bacterial growth were similar to the control (no addition): pH increased to 8.5–8.9 during the growth up to 96 h (Fig. 3a). The other kinetics of pH change were observed in the presence of Ph_2I: the pH value increased to ~7.20 in the presence of 1 μM Ph_2I, and decreased to ~6.50 by addition of 5–10 μM Ph_2I (Fig. 3a).

Bacterial anaerobic growth has been shown to be connected with E_h falling from positive down to low negative values (Gabrielyan et al. 2010; Hakobyan et al. 2012), indicating the enhancement of reduction processes related to the formation of fermentation end-products, the production of amino acids and the synthesis of proteins and other compounds; this is typical for the metabolic processes under anaerobic conditions (Vassilian and Trchounian 2009).

E_h measured by a Pt electrode gradually decreased during the growth with 10–50 mM DMSO (Fig. 3b): potential drop was more intensive in the presence of 10 mM DMSO (up to –465 mV) (Table 1). Such decrease in E_h might indicate enhanced H_2 production, which also was confirmed by chemical assay (see Materials and methods). In the presence of Ph_2I E_h was not changed much (see Fig. 3b). By addition of 10 μM Ph_2I, E_h decreased less – to~–15 mV (see Table 1).

The H_2 yield of 0.85 mmol L^{-1} was obtained for *R. sphaeroides* in the medium with 10 mM DMSO, which was ~6 fold lower than that of the control (5.24 mmol L^{-1}) (Table 1). The bacterium was unable to produce H_2 in the presence of 30–50 mM DMSO and Ph_2I. These results indicated that DMSO could inhibit H_2 production depending on its concentration.

The production of H_2 and CO_2 from various organic substrates by purple bacteria is observed in anaerobic conditions under illumination (Gabrielyan and Trchounian 2009a; Kapdan and Kargi 2006; Golomysova et al. 2010).

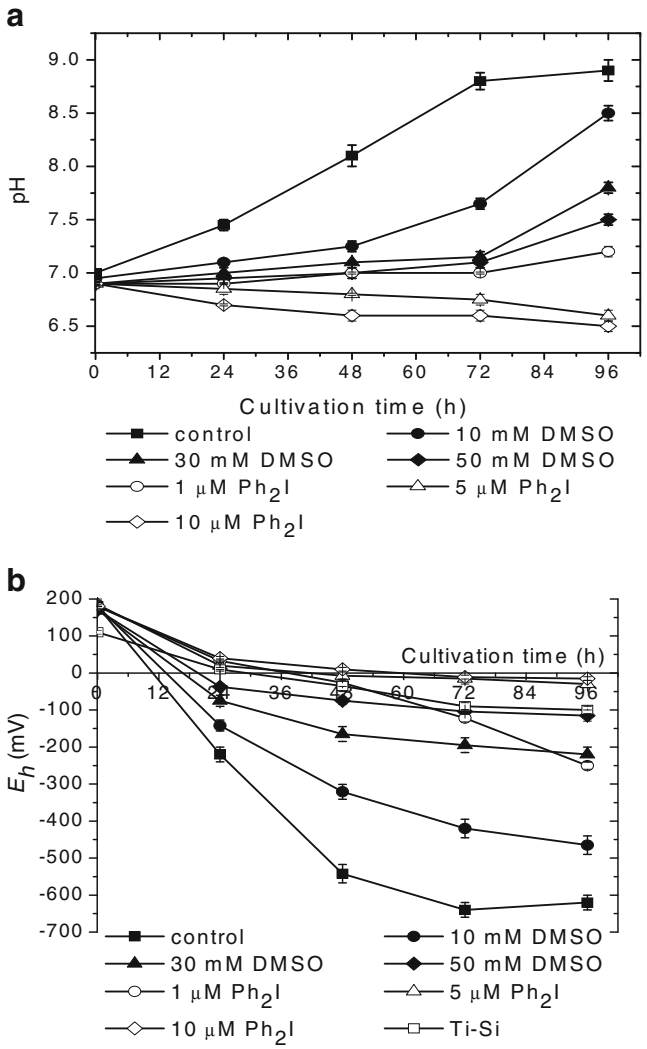

Fig. 3 The pH (**a**) and E_h (**b**) changes of *R. sphaeroides* MDC 6521 during the anaerobic growth at DMSO and Ph_2I at various concentrations. pH was measured at regular intervals by using a pH-potentiometer; E_h was measured at regular intervals using Pt and Ti-Si electrodes (see Materials and methods)

Table 1 The effects of DMSO and Ph_2I at various concentrations on H_2 yield of *R. sphaeroides* MDC 6521 during growth up to 96 h in phototrophic anaerobic conditions and its correlation with E_h

Reagents	H_2 yield[a] (mmol L^{-1})	Final E_h (mV)
Control (no addition)	5.24	−620±20
DMSO (10 mM)	0.85	−465±25
DMSO (30 mM)	–	−220±20
DMSO (50 mM)	–	−115±15
Ph_2I (1 μM)	–	−250±10
Ph_2I (5 μM)	–	−30±10
Ph_2I (10 μM)	–	−15±5

[a] The mean values calculated by decrease in E_h (see Materials and methods) are represented

Minus (−) sign represented absence of H_2 production

The substrate is oxidized in the tricarboxylic acid cycle with the derived electron passing through an electron transfer pathway via NAD/NADH and ferredoxin, before being recombined with proton by a nitrogenase to produce H_2 (Golomysova et al. 2010). This process is thermodynamically uphill and requires ATP hydrolysis, which is produced by the photosynthetic proton gradient. High concentration of DMSO might increase an interaction with CO_2, decreasing nitrogenase activity, whereas Ph_2I affects the formation of photosynthetic pigments and hydrogenase synthesis.

The effects of Ph_2I on intracellular pH and $\Delta\psi$ in *R. sphaeroides*

The effect of DMSO and Ph_2I on growth properties and H_2 production observed under anaerobic conditions may be explained by the variation of the bioenergetics characteristics and ATP yields for these conditions.

Two components of $\Delta p - \Delta pH$ and $\Delta\psi$ were measured in *R. sphaeroides*, grown in anaerobic conditions under light. The distribution of 9-AA between external and intracellular spaces in the bacterial cells reflects the pH gradient across the cytoplasmic membrane. Dependence of 9-AA fluorescence on the medium pH without bacteria is presented in Fig. 4a: the intensity of fluorescence remained constant and decreased insignificantly at pH below 7.50. The fluorescence quenching by addition of *R. sphaeroides* occurred when $[pH]_{out}$ was higher than $[pH]_{in}$ (Fig. 4b). The $[pH]_{in}$ measured by the 9-AA quenching was 7.00±0.05 (Hakobyan et al. 2011). The fluorescence quenching was eliminated by addition of 2 μM CCCP (Fig. 4b). The internal pH was not sensitive to Ph_2I addition (not shown).

At pH 7.50 the $\Delta\psi$ in *R. sphaeroides* was –98 mV (Table 2). $\Delta\psi$ was compensated for by a reversed ΔpH of +30 mV (Hakobyan et al. 2011), resulting in the Δp of –68 mV (Table 2). The addition of succinate (30 mM) resulted in an increase of $\Delta\psi$ to –120 mV. In the presence of protonophore CCCP $\Delta\psi$ was ~2 fold lower compared with the control (Table 2). The F_0F_1-ATPase inhibitor DCCD did not affect $\Delta\psi$. The addition of Ph_2I decreased $\Delta\psi$ at concentrations of 20 μM and higher; lower concentrations of Ph_2I have no valuable effect on $\Delta\psi$, as well as Ph_2I solvent DMSO (Table 2).

The effect of Ph_2I on ATPase activity of *R. sphaeroides* membrane vesicles

R. sphaeroides MDC 6521 membrane vesicles demonstrated ATPase activity, which was inhibited by various inhibitors (Hakobyan et al. 2011; Gabrielyan and Trchounian 2009b). The incubation of membrane vesicles of this bacterium with Ph_2I caused at marked inhibition in ATPase activity: the inhibition revealed concentration dependence (Fig. 5). The inhibition in the ATPase activity by the concentrations of

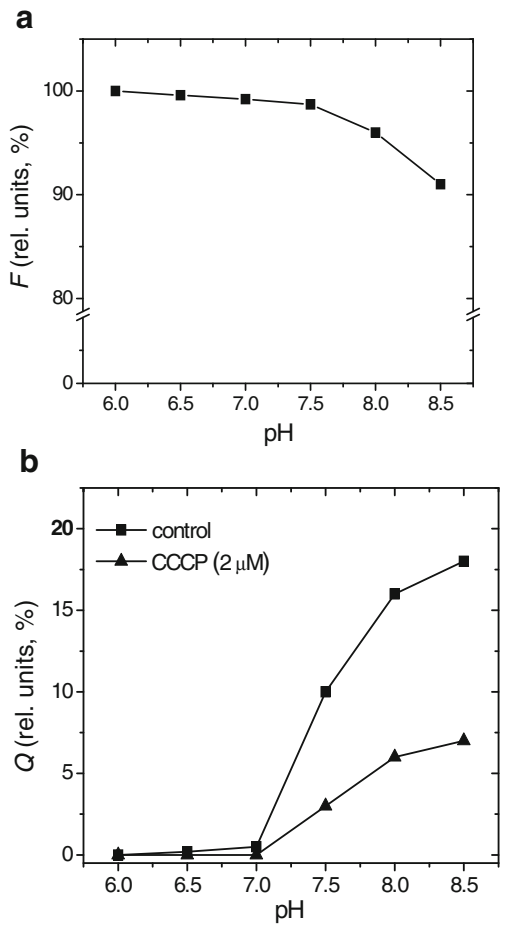

Fig. 4 pH dependence of 9-AA fluorescence (**a**) and its quenching (**b**) induced *R. sphaeroides* MDC 6521. The fluorescence intensity (*F*) in relative units at pH 6.0 was taken as 100 %, the fluorescence quenching (*Q*) was expressed in % of the initial value of *F*. 2 μM CCCP added eliminated the quenching

Ph_2I lower than 20 μM did not overreach 10 %. Valuable inhibition in the ATPase activity was observed in the presence of 40 μM Ph_2I (~40 %). DMSO did not affect the ATPase activity, whereas the F_0F_1-ATPase inhibitor DCCD

Table 2 Effect of various reagents on the membrane potential and proton motive force in *R. sphaeroides* MDC 6521

Reagents	$\Delta\psi$ (mV)	Δp[a] (mV)
Control (no additions)	−98±7	−68±7
Succinate (30 mM)	−120±3	−90±3
CCCP (2 μM)	−57±5	−27±5
DCCD (0.5 mM)	−105±7	−30±7
DMSO (10–50 mM)	−96±6	−66±6
Ph_2I (1 μM)	−96±7	−66±7
Ph_2I (10 μM)	−90±2	−60±2
Ph_2I (20 μM)	−70±4	−40±4

[a] Δp calculated as a sum $\Delta\psi$ and reversed ΔpH of +30 mV

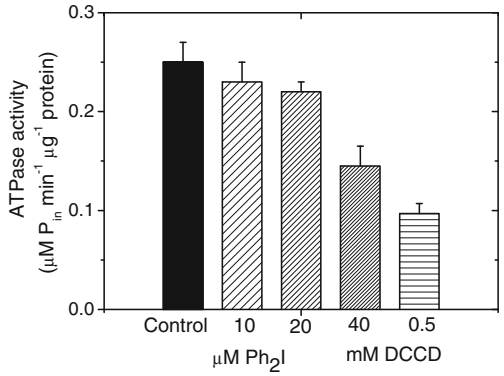

Fig. 5 The ATPase activity of *R. sphaeroides* strain MDC 6521 membrane vesicles in the presence of 10–40 μM Ph_2I. The ATPase activity was calculated by colorimetric determination of inorganic phosphate (P_{in}) liberation per time and protein upon adding ATP adding (see Materials and methods)

suppressed the ATPase activity by 60 %. The results obtained confirm the role of photosynthetic pigments and hydrogenase but not ATPase in the H_2 production process in this bacterium.

Concluding remarks

R. sphaeroides have been shown to grow well in anaerobic conditions at 30 ^{0}C under illumination, using succinate as carbon source and yeast extract as nitrogen source (Hakobyan et al. 2012). During bacterial photosynthesis the carbon source—organic acid can be completely decayed up to 2H and CO_2 in anaerobic conditions whereas nitrogen source is used for synthesis of nucleic acids and proteins (Gabrielyan and Trchounian 2009a; Kapdan and Kargi 2006; Golomysova et al. 2010). During electron transport by photosynthesis, H^+ is pumped through the membrane, H^+-gradient is generated, and the proton-translocating F_0F_1-ATPase uses it to make ATP (Golomysova et al. 2010). The latter serves as energy source for many processes but it is important that ATP is used for the transfer of electrons to ferredoxin. At the same time, electrons and H^+ form H_2 via hydrogenase or nitrogenase but appropriate mechanisms and regulation are not clear. Thus, H_2 production by *R. sphaeroides* is interesting in two aspects at least: (1) which enzyme—nitrogenase or hydrogenase is responsible for H_2 production depending on the conditions, (2) what is a role of the F_0F_1-ATPase.

The hydrogenases known for purple non sulfur bacteria belong to the class of [Ni-Fe]–hydrogenases which are suggested to act as H_2-uptaking or oxidizing hydrogenases (Basak and Das 2007; Das et al. 2006; Colbeau et al. 1993). However, H_2-uptaking hydrogenases frequently coexist with H_2-producing hydrogenases or nitrogenases and can recycle H_2 within bacteria, greatly decreasing overall H_2 yield (Gabrielyan and Trchounian 2009a). Reversibility of

hydrogenases is suggested for different bacteria (Trchounian et al. 2011b; Trchounian et al. 2012) so the hydrogenase in *R. sphaeroides* might be responsible for H_2 production. The results with inhibitory effects of DMCO and Ph_2I on H_2 production and ATPase activity in *R. sphaeroides* provide novel evidence for involvement or responsibility of hydrogenase and DCCD-inhibited F_0F_1-ATPase in H_2 production by this bacterium. Moreover, these data indicate their involvement or role in Δp generation and utilization. This is likely with an idea about a relationship between hydrogenase and the F_0F_1-ATPase in H_2 production and H^+ transport by *R. sphaeroides* (Gabrielyan and Trchounian 2009b). Moreover, this could mean that *R. sphaeroides* hydrogenase has a role in regulation of pH like [Ni-Fe]–hydrogenases in other bacteria (Trchounian et al. 2011b; Trchounian et al. 2012). Besides, DMSO might increase an interaction of nitrogenase with CO_2, decreasing nitrogenase activity and affecting H_2 production.

Acknowledgments This work was supported by the Ministry of Education and Science of the Republic of Armenia, Research grant to AT (#11-1 F202) and Young scientists support program to LH (#11A-1f052) as well as by a research grant from the Armenian National Science and Education Fund (ANSEF) based in New York, USA, to LG (NS-Biotechnology-2704).

References

Abee T, Hellingwerf KJ, Konings WN (1988) Effects of potassium ions on proton motive force in *Rhodobacter sphaeroides*. J Bacteriol 170(12):5647–5653

Basak N, Das D (2007) The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art. World J Microbiol Biotechnol 23(1):31–42

Boyer PD (1988) Bioenergetic coupling to proton motive force: should we be considering hydronium ion coordination and not group protonation? Trends Biochem Sci 13(1):5–7

Colbeau A, Richaud P, Toussaint B, Caballero FJ, Elster C, Delphin C et al (1993) Organisation of the genes necessary for the hydrogenase expression in *Rhodobacter capsulatus*. Sequence analysis and identification of two hyp regulatory mutants. Mol Microbiol 8 (1):15–29

Das D, Dutta T, Nath K, Kotay ShM, Das AK, Veziroglu TN (2006) Role of Fe-hydrogenase in biological hydrogen production. Curr Sci 90(12):1627–1637

Doussiere J, Vignais PM (1992) Diphenylene iodonium as an inhibitor of the NADPH oxidase complex of bovine neutrophils. Eur J Biochem 208(1):61–71

Ellis JA, Mayer SS, Jones OTG (1988) The effect of the NADPH oxidase inhibitor diphenyleneiodonium on aerobic and anaerobic microbicidal activities of human neutrophils. Biochem J 251 (3):887–891

Eltsova ZA, Vasilieva LG, Tsygankov AA (2010) Hydrogen production by recombinant strains of *Rhodobacter sphaeroides* using a modified photosynthetic apparatus. Appl Biochem Microbiol 46 (5):487–491

Feniouk BA, Suzuki T, Yoshida M (2007) Regulatory interplay between proton motive force, ADP, phosphate, and subunit β in bacterial ATP synthase. J Biol Chem 282(1):764–772

Gabrielyan L, Trchounian A (2009a) Purple bacteria and cyanobacteria as potential producers of molecular hydrogen: an electrochemical and bioenergetic approach. In: Trchounian A (ed) Bacterial membranes. Ultrastructure, bioelectrochemistry, bioenergetics and biophysics. Research Signpost, Kerala, pp 233–273

Gabrielyan L, Trchounian A (2009b) Relationship between molecular hydrogen production, proton transport and the F_OF_1-ATPase activity in *Rhodobacter sphaeroides* strains from mineral springs. Int J Hydrogen Energ 34(6):2567–2572

Gabrielyan L, Trchounian A (2012) Concentration dependent glycine effect on the photosynthetic growth and bio-hydrogen production by *Rhodobacter sphaeroides* from mineral springs. Biomass Bioenergy 36:333–338

Gabrielyan L, Torgomyan H, Trchounian A (2010) Growth characteristics and hydrogen production by *Rhodobacter sphaeroides* using various amino acids as nitrogen sources and their combinations with carbon sources. Int J Hydrogen Energ 35 (22):12201–12207

Gatley SJ, Sheratt SA (1976) The effect of diphenylene iodonium on mitochondrial reactions. Biochem J 158(2):307–315

Golomysova A, Gomelsky M, Ivanov PS (2010) Flux balance analysis of photoheterotrophic growth of purple nonsulfur bacteria relevant to biohydrogen production. Int J Hydrogen Energ 35 (23):12751–12760

Gordeliy VI, Kiselev MA, Lesieur P, Pole AV, Teixeira J (1998) Lipid membrane structure and interactions in dimethylsulphoxide/water mixtures. Biophys J 75(5):2343–2351

Grinus LL, Daugelavichus RJ, Alkmavichus GA (1980) Study of the membrane potential of *Bacillus subtilis* and *Escherichia coli* cells by the penetration ion method. Biokhimia 45(9):1609–1618 (in Russian)

Hakobyan L, Gabrielyan L, Trchounian A (2011) Proton motive force in *Rhodobacter sphaeroides* under anaerobic conditions in the dark. Curr Microbiol 62(2):415–419

Hakobyan L, Gabrielyan L, Trchounian A (2012) Yeast extract as an effective nitrogen source stimulating cell growth and enhancing hydrogen photoproduction by *Rhodobacter sphaeroides* strains from mineral springs. Int J Hydrogen Energ 37(8):6519–6526

Hallenbeck PC (2009) Fermentative hydrogen production: principles, progress, and prognosis. Int J Hydrogen Energ 34(17):7379–7389

Horne I, McEwan AG (1998) Dimethylsulfoxide enhances phototrophic growth of *Rhodobacter sphaeroides* in blue light. FEMS Microbiol Lett 168(2):283–288

Hustede E, Steinbüchel A, Schlegel HG (1993) Relationship between the photoproduction of hydrogen and the accumulation of PHB in non-sulphur purple bacteria. Appl Biochem Microbiol 39(1):87–93

Kapdan IK, Kargi F (2006) Bio-hydrogen production from waste materials. J Enzym Microb Tech 38(5):569–582

Kim E-J, Kim J-S, Kim M-S, Lee JK (2006) Effect of changes in the level of light harvesting complexes of *Rhodobacter sphaeroides* on the photoheterotrophic production of hydrogen. Int J Hydrogen Energ 31(4):531–538

Lakin GF (1992) Biometry. Vishaya shcola, Moscow [in Russian]

Maeda T, Wood TK (2008) Formate detection by potassium permanganate for enhanced hydrogen production in *Escherichia coli*. Int J Hydrogen Energ 33(9):2409–2412

Magnani P, Doussiere J, Lissolo T (2000) Diphenylene iodonium as an inhibitor for the hydrogenase complex of *Rhodobacter capsulatus*. Evidence for two distinct electron donor sites. Biochim Biophys Acta 1459(1):169–178

Markarian SA, Poladyan AA, Kirakosyan GR, Trchounian AA, Bagramyan KA (2002) Effect of diethylsulphoxide on growth, survival and ion exchange of *Escherichia coli*. Lett Appl Microbiol 34(6):417–421

Paronyan AKh (2002) Consumption of organic carbon sources and biosynthesis of lactic acid by the photosynthetic bacterium *Rhodobacter* sp.D-4. Appl Biochem Microbiol 38(1):53–57

Piskarev IM, Ushkanov VA, Aristova NA, Likhachev PP, Myslivets TS (2010) Establishment of the redox potential of water saturated with hydrogen. Biophysics (Moscow) 55(1):13–17

Puchkov EO, Bulatov IS, Zimchenko VP (1983) Investigation of intracellular pH in *Escherichia coli* by 9-aminoacridine fluorescence measurements. FEMS Microbiol Lett 20(1):41–45

Trchounian AA, Vassilian AV (1994) Relationship between the F0F1-ATPase and K+ transport system within the membrane the anaerobically growth *Escherichia coli*. N, N′-Dicyclohexylcarbodiimide-sensitive ATPase activity in mutants with defects in K+-transport. J Bioenerg Biomembr 26(5):563–571

Trchounian K, Sanchez-Torres V, Wood KT, Trchounian A (2011a) *Escherichia coli* hydrogenase activity and H_2 production under glycerol fermentation at a low pH. Int J Hydrogen Energ 36 (7):4323–4331

Trchounian K, Sanchez-Torres V, Wood KT, Trchounian A (2011b) Dependence on the F_0F_1-ATP synthase for the activities of the hydrogen-oxidizing hydrogenases 1 and 2 during glucose and glycerol fermentation at high and low pH in *Escherichia coli*. J Bioenerg Biomembr 43(6):645–650

Trchounian K, Poladyan A, Vassilian A, Trchounian A (2012) Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: Dependence on fermentation substrate, pH and F_0F_1-ATPase. Crit Rev Biochem Mol Biol 47(3):236–249

Vassilian A, Trchounian A (2009) Environment oxidation-reduction potential and redox sensing of bacteria. In: Trchounian A (ed) Bacterial membranes. Ultrastructure, bioelectrochemistry, bioenergetics and biophysics. Research Signpost, Kerala, pp 163–195

Yu ZW, Quinn PJ (1998) The modulation of membrane structure and stability by dimethylsulphoxide. Mol Membr Biol 15(2):59–68

Zakharyan E, Trchounian A (2001) K^+ influx in *Escherichia coli* is accompanied by a decrease in H^+ efflux. FEMS Microbiol Lett 204(1):61–64