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A preliminary comparison between hydrogenase and oxygen as electron acceptors in irradiated aqueous dispersion of titanium dioxide

Giovanni M. Mura*, Maria L. Ganadu, Paolo Lombardi, Giuseppe Lubinu, Mario Branca, Valeria Maida

Dipartimento di Chimica, Università di Sassari, via Vienna 2, 07100 Sassari, Italy

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Abstract

Photocatalysis on titanium dioxide is dependent on the presence of suitable electron donors and acceptors in the system. In this paper, we tested *Pyrococcus furiosus*, *Acetomicrobium flavidum* and *Clostridium pasteurianum* hydrogenase as electron acceptors from irradiated titanium dioxide, with tris(hydroxymethyl)aminomethane as electron donor. The adsorption and correct orientation of the hydrogenases on the titanium dioxide surface, as well as their structural features, seems important for determining an efficient electron transfer from the irradiated powder to the enzyme. In order to obtain a comparison between oxygen and hydrogenase as electron acceptors, the hydrogen photoproduction in the presence of *P. furiosus* hydrogenase was compared with the oxygen reduction in the same conditions. The oxygen reduction followed a Langmuir–Hinshelwood kinetics. Hydrogenase turned out significantly faster than oxygen, with a turnover number of about 1300 min^{-1}.

Keywords: Titanium dioxide; Hydrogenase; Methylviologen; Oxygen; Photocatalysis

1. Introduction

Photocatalysis applied to environmental problems, in particular water depuration, is a rapidly expanding field of research [1,2]. Titanium dioxide (TiO_2) is one of the most widely studied photocatalysts used to carry out the partial or complete photooxidation of several organic molecules [3]. For this reason, heterogeneous photocatalysis with suspended or immobilised TiO_2 is considered a promising "advanced oxidation technology".

The photoreactivity of TiO_2 is primarily based on the generation of the electron/hole pair when a photon of sufficient energy is absorbed ($\lambda < 380$ nm). Electrons and holes are located in the conduction and valence bands, respectively. Due to their redox potential they are able to induce a wide variety of chemical reactions [4]. In particular, the oxidative degradation of organic substances, either directly or by hydroxy radical generation, is energetically favoured due to the strong oxidising potential of the holes (+2.8 V). The photooxidation rate under steady-state conditions is also affected by the corresponding rate of photoreduction in the presence of an electron acceptor and by the rate of electron/hole recombination [5,6].

There is a great interest in the literature in the anodic reactivity of TiO_2 with different electron donors, although the most commonly studied electron acceptor for the coupled cathodic reaction is dioxygen. The main reasons for this may be as follows: (i) the oxidant capacity of oxygen in the aqueous solutions subjected to photocatalytic treatments, (ii) the reactivity of the oxygen intermediate reduced species (O_2^-, $^\bullet$OOH) and (iii) its availability.

The possibility that oxygen photoreduction could be the rate-determining step and that oxygen reactivity on TiO_2 could affect the rate and the yield of photooxidation is a well known and discussed topic of debate. Gerischer and Heller [5], basing their work on theoretical models, considered oxygen photoreduction to be the rate controlling process. Kesselman et al. [6], studying TiO_2 photoelectrodes, concluded that no single rate constant can be stated to be "rate-determining", although Pt or Fe catalysis of electron transfer was reported to improve the photocatalysis. Sun and Bolton [7] observed a significant increase of the $^\bullet$OH generation when the electron acceptor hydrogen peroxide was added. Ferry and Glaze [8] studied the photocatalytic reduction of monosubstituted nitrobenzenes on TiO_2, and showed that the rate is controlled by

* Corresponding author. Tel.: +39-079-229542; fax: +39-079-229559.
E-mail address: billia@ssmain.uniss.it (G.M. Mura).

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the electron transfer process and not by the rate of the donor oxidation.

The investigation of the system by using electron acceptors other than oxygen is of general interest for the following reasons:

(a) the electron acceptors may be kinetically compared;
(b) the characterisation of different photodegradation pathways of organic molecules in the presence or absence of oxygen;
(c) the synthetic use of the reducing power of the conduction band electrons.

Thus, in this context, enzymes can be used as catalysts in redox processes photosensitised by inorganic semiconductors [9]. In particular, hydrogenase, which catalyses the reaction $2H^+ + 2e^- \rightarrow H_2$, is able to evolve hydrogen in irradiated suspensions of TiO_2, coupling the hydrogen production with the electron donor photooxidation [10]. Hydrogenase is also active with CdS, evolving hydrogen during formate photodecomposition [11]. Pedroni et al. [12,13] studied hydrogen production with TiO_2 and *Pyrococcus furiosus* hydrogenase and reported rates of up to 0.5 μmol H_2/min mg of TiO_2. With the same hydrogenase, Mura et al. [14] used lactose and dairy waste as electron donors to study the conditions for the coupling of hydrogen production and organic waste photooxidation.

In this work, hydrogenase was compared with oxygen as an electron acceptor from irradiated TiO_2. The rates of hydrogen production and oxygen consumption were compared with the same electron donor (tris(hydroxymethyl)-aminomethane) and in similar chemical and irradiation conditions.

2. Experimental

2.1. Chemicals

TiO_2 powder (P25, 50 m^2/g) was obtained from Degussa. Lyophilised *P. furiosus*, *Acetomicrobium flavidum* and *Clostridium pasteurianum* were purchased from DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). All the chemicals were of biochemical grade and purchased from Sigma. The chromatographic media and columns were from Pharmacia.

2.2. Growth of the cells and hydrogenase purification

P. furiosus was cultured at 85 °C in a 5 l vessel, inoculated with 0.5 l of pre-culture and mechanically stirred under an argon flow (40 ml/min). The composition of the growth medium was: yeast extract (1 g/l), triptone (5 g/l), cysteine (0.5 g/l) and synthetic sea water (30 g/l). The pH was adjusted to about 6.2 before inoculum. The cells were collected at a OD_{600} of about 0.5 after 24 h, centrifuged and stored at −80 °C. *P. furiosus* hydrogenase purification and activity assay were performed as previously described [15]. The *P. furiosus* hydrogenase activity was 42 U/mg, assayed at 70 °C in the following solution: Tris–Cl 50 mM, pH 7.5, $Na_2S_2O_4$ 30 mM, methylviologen (MV) 2 mM.

A. flavidum was grown and the hydrogenase was purified as previously described [16]. The *A. flavidum* hydrogenase activity was 16 U/mg, assayed at 58 °C in the following solution: Tris–Cl 50 mM, pH 7.5, $Na_2S_2O_4$ 30 mM, MV 2 mM.

C. pasteurianum was grown and the hydrogenase was purified as previously reported [17]. The *C. pasteurianum* hydrogenase activity was 28 U/mg, assayed at 30 °C in the following solution: Tris–Cl 50 mM, pH 7.5, $Na_2S_2O_4$ 30 mM, MV 2 mM.

One unit (U) of hydrogenase activity corresponds to 1 μmol H_2/min.

2.3. Gas chromatographic determination of hydrogen production and oxygen consumption in irradiated TiO_2 suspensions

The photoreactions were performed in a 14 ml Pyrex vial, jacketed and thermostated with circulating water, magnetically stirred and closed with silicone rubber stoppers, which was irradiated with an 80 W Hg lamp (Philips, XPL-N). The light was filtered by the Pyrex walls and the layer of circulating water.

The reaction mixtures (7 ml) for the hydrogen photoproduction were as follows: TiO_2 (4 mg/ml), hydrogenase (0–0.4 U/ml), tris(hydroxymethyl)aminomethane (50 mM, pH 7.5), MV (2 mM, when present), phosphate buffer (20 mM, pH 7.5, when present). Argon was bubbled for 10 min in the reaction mixture before the addition of hydrogenase and the reaction started with the exposure to the pre-warmed lamp. The temperature of the reaction mixture was 70, 58 and 30 °C when the *P. furiosus*, *A. flavidum* and *C. pasteurianum* hydrogenase, respectively, were present.

Similar reaction mixtures were prepared for determining the oxygen photoreduction, but in this case hydrogenase was not added and argon was not bubbled.

Hydrogen and oxygen were measured by using a Perkin Elmer 8500 gas chromatograph with a molecular sieve column (1 m, 1/16 in.). Gas carrier: argon (15 ml/min); detector: HWD range 1, temperature 200 °C; injector temperature: 200 °C; oven temperature: 35 °C. Samples (100 μl) for gas analysis were extracted with a gas-syringe from the headspace of the vial (7 ml).

2.4. Oxygraphic determination of oxygen consumption in irradiated TiO_2 suspensions

A YSI Model 5300 Oxygen Monitor (YSI Scientific, Yellow Springs Instrument) with a 5331 Standard Oxygen Probe (Clark electrode) was used to measure the oxygen consumption during the irradiation of TiO_2 suspensions. The vial and the experimental conditions were as reported

in the previous section, except that the vial was completely filled with the solution and the electrode was inserted into the tightly closed vial. Due to the absence of headspace, and with the vial tightly closed, the reactions were followed till the complete disappearance of the oxygen.

Calibration in air-saturated water solutions at known temperature, pressure and composition was necessary. The actual oxygen consumption rate was determined setting as 100% an air-saturated sample of aqueous solution, which contained 4.83 μl O_2/ml at pH 7.5, 1 atm and 40 °C. The output signal was sent to a personal computer by means of an I/O board. The experimental curves of the oxygen consumption were analysed using Microcal Origin 5.0. The derivative of the curve of oxygen consumption was obtained by means of a numerical method. The graphical representations of the oxygen concentration vs. its rate of disappearance were fitted, using the least-squares method, according to the Langmuir–Hinshelwwod
equation:

$$-\frac{d[O_2]}{dt} = \frac{kK[O_2]}{1 + K[O_2]}$$

3. Results and discussion

The photoreduction reactions studied in this work ($O_2 + e^- \rightarrow O_2^-$, $2H^+ + 2e^- \rightarrow H_2$) can occur both with direct or MV-mediated electron transfer from the photoexcited TiO_2, as reported in Scheme 1. The two electron acceptors are separately present in all the reactions reported, in other words when oxygen was present hydrogenase was not added, and when hydrogenase was present the reaction vial was degassed to remove oxygen.

Table 1
Hydrogenase activity with irradiated TiO_2 in the presence or in the absence of the electron carrier MV[a]

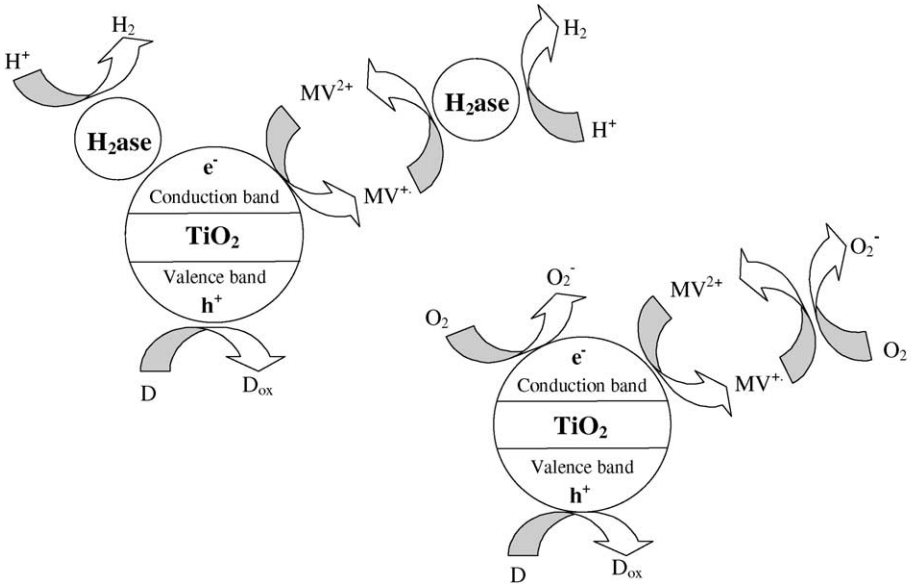
Hydrogenase	Activity[b] (U/mg)	TiO_2 + MV[c], dark	TiO_2, light	TiO_2 + MV[c], light
P. furiosus	42 ± 5	0	11 ± 2	40 ± 3
A. flavidum	16 ± 2	0	Traces	12 ± 4
C. pasteurianum	28 ± 4	0	26 ± 3	29 ± 4

[a] TiO_2: 4 mg/ml; donor: tris(hydroxymethyl)aminomethane 50 mM, pH 7.5.
[b] Measured with $Na_2S_2O_4$ and MV as reported in Section 2. One unit (U) of hydrogenase activity corresponds to 1 μmol H_2/min.
[c] 2 mM (when present).

3.1. Electron transfer between irradiated TiO_2 and hydrogenase

In Table 1 the rate of hydrogen production in irradiated suspensions of TiO_2, using three different hydrogenases, is reported. In the absence of the electron carrier MV, *A. flavidum* hydrogenase is inactive, while *P. furiosus* and *C. pasteurianum* hydrogenase are able to produce hydrogen. Comparing this with the activity measured under the usual assay conditions ($Na_2S_2O_4$, MV, dark) it can be seen that *C. pasteurianum* hydrogenase retains its total activity. With *P. furiosus* hydrogenase the activity is significantly reduced. This different behaviour is not related to a different inactivation of the three proteins in the suspensions of TiO_2. Indeed, in the presence of MV they show approximately the entire activity, as measured in the usual assay.

Some structural information is available for these hydrogenases. *P. furiosus* hydrogenase is a hyperthermophilic protein, with a molecular mass of 153 kDa, made up of four different subunits, containing at least six Fe–S centres and the NiFe active site [15,18]. *A. flavidum* hydrogenase is a



Scheme 1. Reactions in irradiated TiO_2 with hydrogenase (top) or with oxygen (bottom) in the presence or absence of methylviologen.

dimeric protein (75 kDa), with a small subunit shorter than the usual small subunits of the NiFe hydrogenase, and thus with only one Fe–S centre [16]. *C. pasteurianum* hydrogenase is a monomeric protein (60 kDa). The X-ray structure of this enzyme was recently determined and it contains four Fe–S clusters and the Fe–Fe active site, located near the surface of the protein [19]. A comparison of these structural features with the activity of the three hydrogenases reported in Table 1 strongly suggests that the ability of the hydrogenase to transfer electrons directly from the irradiated TiO_2 depends on the number of Fe–S clusters (with respect to their molecular mass) and their proximity to the surface of the protein. In particular, in the case of *C. pasteurianum* hydrogenase, at least two Fe–S clusters seems involved in the interaction with alternative electron donors [20]. Since the enzyme is fully active in irradiated TiO_2, even when MV is absent, the electron transfer seems to occur through the whole surface of the protein, irrespective of its orientation on the surface of the semiconductor. In the case of *P. furiosus* hydrogenase the activity without the electron carrier (MV) is only a fraction (about 25%) of the total activity. The enzyme is about 2.5 times larger than the *C. pasteurianum* hydrogenase. Furthermore, only two (α and δ) of the four subunits catalyse the hydrogenase activity [21]. Probably, only a part of the enzyme surface is involved in the electron transfer from external electron donors towards the active hydrogenase site. As a consequence, the percentage of activity without MV (∼25%) is an indirect measure of the fraction of the *P. furiosus* hydrogenase adsorbed with a correct orientation.

3.2. Comparison between hydrogenase and oxygen

The initial rate of hydrogen production without MV was measured as a function of the hydrogenase concentration. The results obtained with the *P. furiosus* hydrogenase are shown in Fig. 1. At a concentration of hydrogenase between 0.05 and 0.25 μM the rate of hydrogen production shows a linear dependence on the concentration of the protein, indicating that the photocatalytic activity is limited by the concentration of the hydrogenase. The maximum initial rate obtained with hydrogenase of 0.25 μM was 322 ± 25 nmol H_2/min ml.

The protein concentration on the solution and on the powder was measured after centrifuging. The hydrogenase was found to be almost completely adsorbed on the TiO_2. In these conditions the turnover number of the enzyme (i.e. the number of reactions occurring per molecule of catalyst in the time unit) can be calculated and the fitting of the data reported in Fig. 1 gave a value of $1325 \pm 95\ min^{-1}$.

The rate of oxygen reduction was studied in the same conditions, without hydrogenase and without degassing the sample, by means of oxygraphic measures. As the reaction takes place in tightly-sealed and completely filled vials, the photoreduction of oxygen was observed in its entirety. Due to the absence of head space, diffusive type phenomena with a gaseous phase were not present.

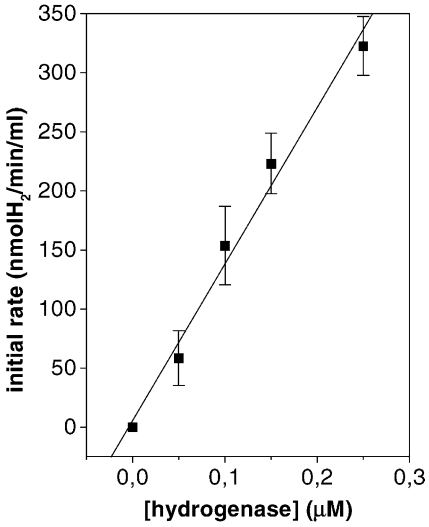


Fig. 1. Initial rate of the hydrogen production in irradiated suspensions of TiO_2 with different concentrations of *P. furiosus* hydrogenase. Conditions: TiO_2 4 mg/ml, tris(hydroxymethyl)aminomethane 50 mM, pH 7.5, *T* 70 °C. The initial rate was calculated on the first 30 min of reaction and reported per millilitre of reaction mixture.

A typical measure of the oxygen disappearance and the treatment of the data are shown in Fig. 2. The kinetics of oxygen photoreduction depends on the presence of light, TiO_2 and tris(hydroxymethyl)aminomethane. The initial reaction rate increases with the growth of the donor concentration (data not shown) and depends on the quantity of TiO_2. The Langmuir–Hinshelwood (LH) equation was used to analyse the whole curve:

$$-\frac{d[O_2]}{dt} = \frac{kK[O_2]}{1 + K[O_2]}$$

The rate of oxygen disappearance was calculated at every concentration of oxygen from the oxygraphic measure (Fig. 2a), and the data were minimised according to the LH equation (Fig. 2b).

The values calculated for the LH constants (*k* and *K*) with different quantities of TiO_2 are reported in Table 2. The apparent adsorption constant (*K*) has an average value of $3.8 \pm 1.2 \times 10^4\ M^{-1}$. The initial rate of oxygen reduction increased with increasing quantities of TiO_2 up to 47 μM/min.

A preliminary evaluation of hydrogenase and oxygen as electron acceptors can be performed on the basis of the ini-

Table 2
Kinetic parameters for the LH analysis of the oxygen consumption in irradiated suspensions of TiO_2[a]

TiO_2 (mg/ml)	*k* (μM/min)	*K* (M^{-1})	COD(R^2)
0	2.81	–	–
0.44	21.04	4.0×10^4	0.985
0.87	29.25	4.0×10^4	0.993
1.75	34.54	2.1×10^4	0.982
4.00	46.69	5.1×10^4	0.998

[a] Donor: tris(hydroxymethyl)aminomethane 50 mM, pH 7.5.

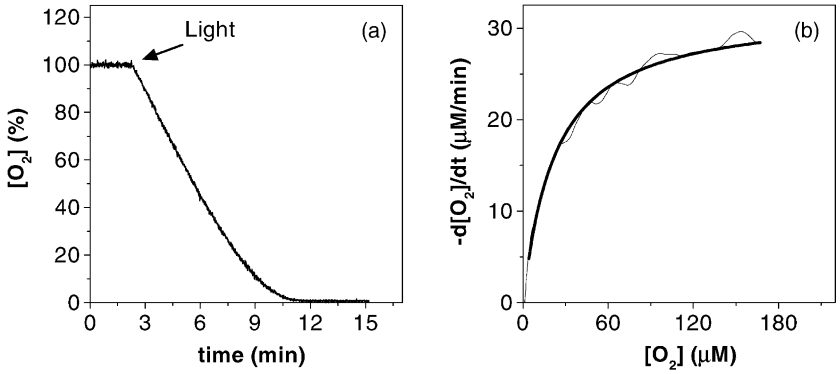


Fig. 2. Analysis of the oxygen consumption in irradiated suspensions of TiO_2 according to the LH equation: (a) experimental curve of the oxygen consumption (TiO_2 0.87 mg/ml, tris(hydroxymethyl)aminomethane 50 mM, pH 7.5); (b) plot of the oxygen consumption rate vs. the oxygen concentration and the corresponding fitting.

tial rates measured in the same condition. The rate of hydrogen photoproduction (322 ± 25 μM/min) was about 7-fold higher than the rate of oxygen reduction, measured with the same TiO_2 quantity (4 mg/ml) and donor concentration (tris(hydroxymethyl)aminomethane 50 mM).

Another comparison can be obtained at equal and rate-limiting concentration of both acceptors, according to the following considerations. In the LH model, a first-order dependence of the rate of oxygen disappearance from the oxygen concentration itself can be observed when the oxygen concentration meet the condition $K[O_2] \ll 1$, i.e. at a sufficiently low oxygen concentration. In this condition, the LH equation can be approximated using the following expression:

$$-\frac{d[O_2]}{dt} = kK[O_2]$$

and the product kK has the meaning of a pseudo first-order rate constant (k'_{O_2}). The value calculated from the data reported in Table 2 can be compared with the turnover number calculated above for the hydrogenase. In the same experimental condition (TiO_2 4 mg/ml, tris(hydroxymethyl)aminomethane 50 mM) the value calculated for the (k'_{O_2}) is 2.4 min^{-1}, about 2–3 orders of magnitude lower than the turnover number of the hydrogenase ($1325 \pm 95\ min^{-1}$).

One must bear in mind the limits of this estimation for comparing hydrogenase and oxygen, mainly because hydrogenase is a catalyst while oxygen is a substrate. Nevertheless, the high difference between the two values indubitably indicates that the electron transfer from irradiated TiO_2 to hydrogenase is significantly faster than that to oxygen, under rate-limiting concentration of the two acceptors.

Previous results on the enhancement of the rate of electron transfer, both catalysing the oxygen reduction or using alternate electron acceptors, have been reported. Pd and Pt incorporation in TiO_2 increases the rate of oxygen photoreduction [21,22]. In general, transition metal ions can be efficiently reduced in irradiated TiO_2 and, as electron acceptors, can influence the rate of photooxidation, reviewed in [23]. The comparison between hydrogenase and oxygen supports the evidence that the rate of electron transfer can be significantly increased.

3.3. Effect of phosphate and MV

The hydrogen photoproduction and the oxygen photoreduction were evaluated in the presence of phosphate buffer (20 mM, pH = 7.5), with tris(hydroxymethyl)aminomethane as electron donor and without MV. The results are shown in Fig. 3. In the presence of phosphate the *P. furiosus* hydrogenase activity is completely suppressed, while there is only a slight effect on oxygen photoreduction. The concentration of the protein in the solution and in the powder was measured after centrifuging. In the presence of phosphate the hydrogenase was recovered on the solution, and so was not adsorbed on the TiO_2 surface.

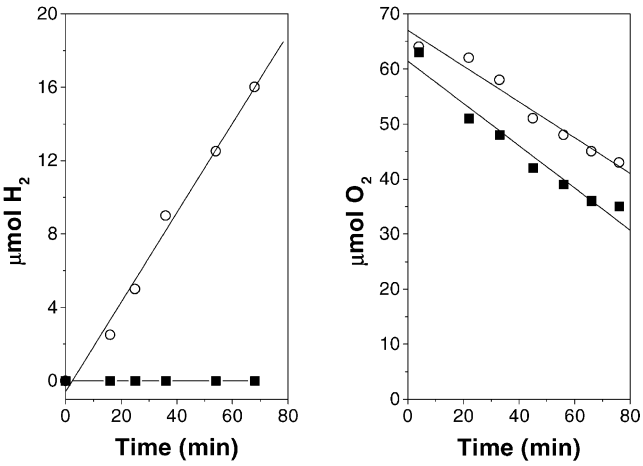


Fig. 3. Gas chromatographic measures of hydrogen production (left) and oxygen consumption (right) in the presence (squares) or in the absence (circles) of phosphate buffer 20 mM, pH 7.5. TiO_2 4 mg/ml, *P. furiosus* hydrogenase (left): 0.2 U/ml. Reaction volume 7 ml; headspace 7 ml.

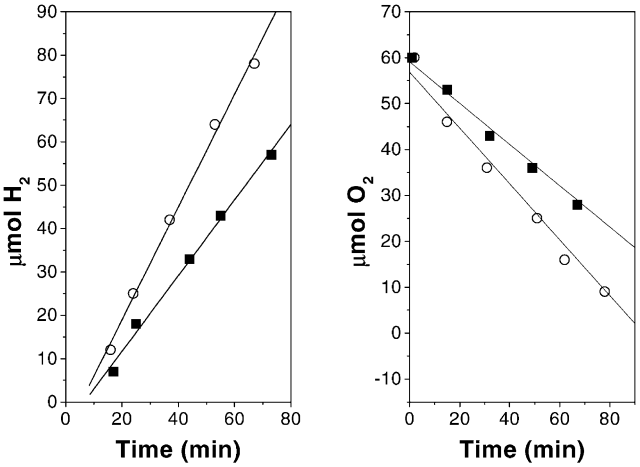


Fig. 4. Gas chromatographic measures of hydrogen production (left) and oxygen consumption (right) in the presence of MV 2 mM and with (squares) or without (circles) phosphate buffer 20 mM, pH 7.5. TiO_2 4 mg/ml, *P. furiosus* hydrogenase (left): 0.2 U/ml. Reaction volume 7 ml; headspace 7 ml.

The suppression of hydrogen photoproduction from irradiated TiO_2 in the presence of phosphate was previously observed by Nikandrov et al. [24] with *T. roseopersicina* hydrogenase. In order to verify if the hydrogenase is still active after the addition of phosphate, the reactions were conducted in the presence of MV, as shown in Fig. 4. The hydrogenase was active also when phosphate was present. Our results agree with those of Nikandrov et al. [24] that the adsorption of hydrogenase seems a necessary condition for direct electron transfer between the irradiated semiconductor and the enzyme.

It can be seen in Fig. 4 that when phosphate is present, the rates of both hydrogen production and oxygen reduction are lower than in the absence of phosphate. This effect of phosphate is probably due to the well-known adsorption of the phosphate ions at the oxidation sites on the TiO_2 strongly affecting the rate of photocatalysis [25].

4. Conclusions

Hydrogen photoproduction in irradiated TiO_2 was studied using three different hydrogenases purified from *P. furiosus*, *A. flavidum* and *C. pasteurianum*. The catalytic efficiency of the hydrogenase was determined by its adsorption and correct orientation on the surface of the semiconductor.

P. furiosus hydrogenase catalyses the hydrogen photoproduction with a rate about 7 times higher than the rate of oxygen reduction. The enzyme has a turnover number of $1325 \pm 95\,min^{-1}$.

Since hydrogenase, in catalytic concentrations, is an efficient electron acceptor and its reduction product (H_2) goes out from the reaction environment, in our opinion could also be a useful tool to better understand TiO_2 photocatalysis in anaerobic conditions.

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