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Hydrogen evolution by subchloroplast preparations of photosystem II from pea and spinach

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Hydrogen (H_2) evolution rates were measured by the gas chromatographic technique upon illumination of different subchloroplast preparations of higher plants without exogenous hydrogenase under anaerobic conditions. Subchloroplast preparations enriched in photosystem II (PS II) in the presence of an electron donor TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) are shown to have higher H_2-evolution rates (up to 30 nmol/mg Chl per h) than preparations enriched in PS I under the same conditions. The data on the suppression of H_2 evolution by well-known inhibitors of PS II (dinoseb, atrazine) prove that the H_2 photoproduction is sensitized by PS II reaction centers.

Photosystem II; Hydrogen evolution; (Pea, Spinach)

1. INTRODUCTION

Recently, hydrogen evolution by photosynthetic organisms has been intensively studied. It has been recently shown that isolated chloroplasts of higher plants are able to produce hydrogen under illumination after inactivation of the oxygen-evolving system [1]. From analysis of the effects of electron-transport inhibitors and thermoinactivation, it has been suggested that photosystem (PS) II participates in hydrogen photoevolution by intact chloroplasts of higher plants.

It has been found recently [2] that intact cells of *Chlamydomonas reinhardii* mutants lacking the chlorophyll-protein complex of PS I reaction centers (RCs) are capable of photosynthetic production of molecular hydrogen. Electron transfer from the reduced pheophytin ($E_0' = -610$ mV), functioning in the RCs of PS II as an intermediary electron acceptor, to the typical acceptors of PS I ($NADP^+$, nitrate, nitrite, benzyl viologen, methyl viologen) with redox potentials about -400 mV under anaerobic conditions [3,4] is a prerequisite to this work.

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2. MATERIALS AND METHODS

Experiments were carried out on 'heavy' oxygen-evolving particles of PS II (DT-20) isolated from pea and spinach chloroplasts pretreated with 1% digitonin and 0.1% Triton X-100 [5,6]. These particles, termed 'initial' DT-20, contain 80–100 Chl molecules per PS II reaction center and evolve O_2 upon illumination in the presence of 1 mM ferricyanide at a rate of 100–120 μmol/mg Chl per h. The P700 content of the particles is 70–80-times less than in chloroplasts (1 P700 per 14000–15000 chlorophyll molecules). In addition, a 'light' fraction of PS II (sedimented at 60000 × *g*) as well as preparations enriched in PS I (144000 × *g*) containing 120–140 Chl molecules per P700 [6] were used. 'Complete' (>95%) removal of manganese from DT-20 particles was carried out as in [5]. These are designated 'Mn-lacking' particles. However, it should be noted that the treatment of PS II particles with Tris-HCl buffer of high molarity is known to lead to removal of both manganese ions and polypeptides of molecular mass 10, 17, 23 and 33 kDa [5].

The activity of PS II was determined from the values of reversible photoinduced changes in Chl fluorescence yield (ΔF)

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related to photoreduction of the PS II primary electron acceptor Q_A as well as from the values of photoinduced absorbance changes (ΔA) at 685 nm with respect to photoreduction of pheophytin (Pheo) [3–6]. The activity of PS I was assayed using the ΔA at 700 nm related to photooxidation of the primary electron donor P700. ΔF and ΔA were measured on a phosphoroscopic set-up [3–6].

Molecular hydrogen was determined according to [7]. Experiments were carried out in 14-ml vessels sealed hermetically and placed horizontally on a shaker. The volume of the reaction mixture and the gas phase sampled from the vessels with a gas-tight syringe was 8 ml and 200 μl, respectively. The flasks were illuminated with DRLF lamps (220 V, 400 W) for 30 min through a heat filter. The light intensity at the flask surface was 1.5×10^{-2} J/cm^2 per s. In the dark, experiments were carried out under the same conditions but flasks were wrapped in 2 layers of aluminium foil. Anaerobic conditions in the reaction medium (330 mM mannitol, 50 mM KCl, 1 mM $MgCl_2$, 30 mM Hepes-KOH buffer; pH 7.0) were achieved by employing the following system: glucose oxidase (30 U/ml), glucose (10 mM), catalase (approx. 1000 U/ml). Chlorophyll concentration was 30–50 μg/ml in the reaction medium. The reduced form of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) from Serva was used in experiments.

3. RESULTS

Fig.1a shows a chromatogram of a mixture of hydrogen, oxygen and nitrogen in argon (in all subsequent chromatograms the nitrogen peaks are not shown). Hydrogen is demonstrated in the form of a distinct peak, the sensitivity of registration being 1.3 nmol H_2 in 200 μl of the introduced sample that corresponds to a chromatographic peak height of 10 cm. Hydrogen, oxygen and nitrogen were retained in a column for 30, 65 and 185 s, respectively. At the moment of sampling (indicated by the arrow) a shift of the zero line (fig.1a, M) is observed in all chromatograms. This results from pressure changes in the column.

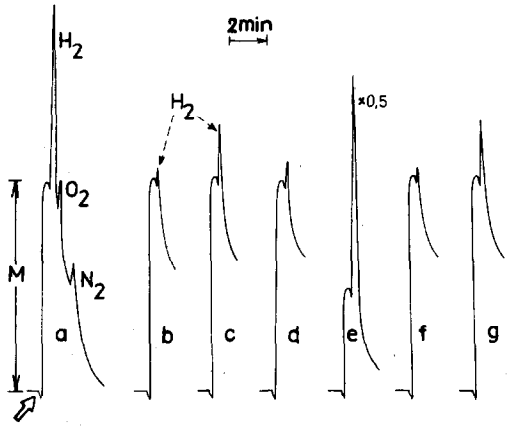

Fig.1. Chromatograms of an artificial gas mixture of H_2, O_2 and N_2 in argon containing 1.0 nmol H_2 (a); of a gas phase of the reaction vessels containing the initial particles without additions (b); the same as (b) plus 0.1 mM TMPD (c); manganese-lacking particles without additions (d); manganese-lacking particles plus 0.1 mM TMPD (reduced by half) (e); PS I particles plus 0.1 mM TMPD (f); initial DT-20 particles in the presence of methyl viologen (150 μM) reduced by dithionite (1 mg/ml) in the dark (g).

Analysing the gas phase of the reaction vessels after 30 min illumination of the initial DT-20 particles under anaerobic conditions, an H_2 peak corresponding to a rate of 1.4 nmol H_2/mg Chl per h is registered (fig.1b). Addition of 0.1 mM TMPD, a donor-mediator of electron transfer, to DT-20 particles activates H_2 photoproduction 5 times (fig.1c).

Inactivation of the PS II donor side by treating the initial particles with Tris-HCl buffer of high molarity, resulting in the removal of manganese and a number of proteins from the water-oxidizing complex [5], leads to a considerable (10-fold) increase in H_2 photoevolution (fig.1e). The maximal rate of H_2 photoproduction by manganese-lacking particles is about 30 nmol/mg Chl per h in the presence of TMPD (fig.1e). In the absence of TMPD hydrogen photoevolution does not exceed 3.5% of this value (table 1 and fig.1d). Addition of 1 μM $MnCl_2$ to the manganese-lacking particles has no effect on the rate of H_2 photoevolution (not shown).

Photoevolution of H_2 by the manganese-lacking particles is suppressed (inhibited) by 97% with sodium ascorbate in the presence of the reduced

Table 1

Effect of TMPD on H_2 photoproduction by PS II manganese-lacking particles

Experimental conditions	Hydrogen evolution (% of control)
+ TMPD	100
− TMPD	3.3
− TMPD + Mn^{2+}	3.9
+ TMPD + asc	2.8
+ TMPD + MV	72.2
+ TMPD + asc + MV	1.4

TMPD at 0.1 mM; Mn^{2+}, 1 μM $MnCl_2$; asc, 10 mM sodium ascorbate; MV, 150 μM methyl viologen. 100% refers to 30.0 nmol H_2/mg Chl per h

form of TMPD. Oxidized methyl viologen (150 μM) added to the reaction mixture decreases the rate of H_2 photoevolution by 30% in the presence of TMPD and sodium ascorbate (table 1). In the dark, hydrogen evolution from methyl viologen reduced by dithionite (1 mg/ml) ('hydrogenase activity') was 5 nmol/mg Chl per h.

When 10 μM diuron, a compound which blocks electron transfer on the PS II acceptor side between plastoquinones Q_A and Q_B, was added to particles containing no manganese, hydrogen photoevolution was practically unchanged (fig.2b). In contrast, 10 μM dinoseb (a well-known inhibitor of PS II reactions [8]) resulted in 95% suppression of this process (fig.2c). The same effect was observed upon addition of 10 μM K-15, a new inhibitor of electron transfer in PS II, a dinitrobenzene derivative [9]. The other inhibitors of electron transfer in PS II suppress the process of H_2 photoevolution less efficiently: 10 μM atrazine and 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) caused 30 and 25% suppression, respectively (fig.2e,f).

During all the experiments studying the effect of inhibitors on H_2 photoevolution from the initial and manganese-lacking particles, the reaction of pheophytin photoreduction in PS II RCs was determined after H_2 measurements on a gas chromatograph. The results showed that there was a correlation between inhibition of the pheophytin photoreduction reaction and the decrease in hydrogen photoevolution rate. For example, in the presence of 10 μM dinoseb H_2 photoevolution and pheophytin photoreduction were suppressed by 95 and 80%, respectively.

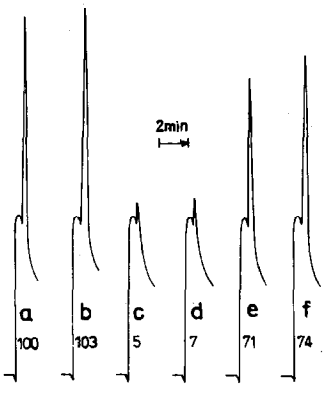

Fig.2. Effect of PS II inhibitors (10 μM) on hydrogen photoproduction by PS II manganese-lacking particles in the presence of 0.1 mM TMPD. (a) Control (19 nmol/mg Chl per h), (b) plus diuron, (c) plus dinoseb, (d) plus K-15, (e) plus atrazine, (f) plus CCCP. Values beside curves give the % of control.

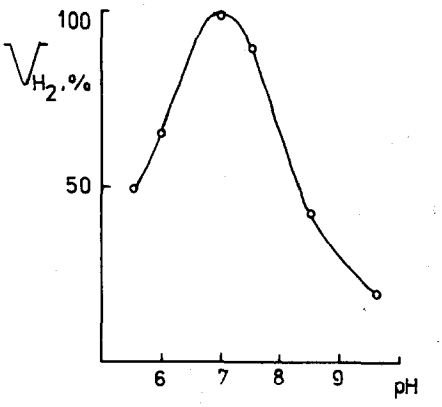

Fig.3. Dependence of H_2 photoevolution by initial DT-20 particles on the reaction mixture pH: 5.5, 6.0 (30 mM Mes); 7.0, 7.5 (30 mM Hepes); 8.6 (30 mM Tricine); 9.65 (30 mM Tris).

The light intensity that we used for illumination of the samples to study hydrogen evolution was 1.5×10^{-2} J/cm^2 per s, which was saturating for oxygen evolution as measured by gas chromatography in the presence of 1 mM ferricyanide and 0.5 mM dimethyl benzoquinone as electron acceptors. At the same light intensity no obvious saturation of H_2 photoproduction was observed.

The maximal rate of hydrogen evolution by manganese-lacking particles of PS II is observed at pH 7.0 and is decreased by 50% at pH 5.5 and 8.0 (fig.3). A similar dependence was observed in hydrogen photoevolution by chloroplasts in our previous work [1].

Hydrogen photoevolution by subchloroplast particles enriched in PS I at the same chlorophyll concentration was not greater than 10% of the hydrogen evolution by PS II particles both in the light in the presence of TMPD (fig.1f) and in the dark in the presence of methyl viologen reduced by dithionite. No H_2 production is observed in the supernatant after sedimentation of PS I particles at 144000 $\times$ *g* either in the dark in the presence of methyl viologen reduced by dithionite or in the light in the presence of TMPD.

4. DISCUSSION

Our results show that subchloroplast preparations enriched in PS II have considerably greater rates of hydrogen production in the presence of an electron donor than those enriched in PS I under the same conditions. This effect probably correlates with the fact that our PS II particles have a greater total molecular mass than PS I particles and that during isolation 'hydrogenase' is located in this fraction. It is interesting to note that PS II particles have been shown to have carbonic anhydrase activity [10] and amino acid oxidase activity [11].

We have previously shown that H_2 and O_2 photoevolution by chloroplasts has a competitive character, i.e. the suppression of O_2 by various methods results in an increase in H_2 evolution [1]. In our experiments where PS II particles are used, their treatment with Tris-HCl buffer leads to a considerable increase in H_2 photoevolution. The possibility cannot be excluded that H_2 can be decomposed in the manganese system and that manganese removal stimulates the process of H_2 evolution. Tris-buffer treatment results in the simultaneous removal of manganese ions, proteins of 10, 17, 24 and 34 kDa and probably some other components. It is not excluded that the removal of these proteins eliminates hydrogen consumption which we have reported in chloroplasts [1].

Suppression of H_2 production is probably caused by the enhancement of cyclic electron transport in PS II. According to the results listed in table 1, H_2 photoevolution is suppressed in the presence of sodium ascorbate. It is likely to be connected with the fact that the oxidized form of ascorbate (dehydroascorbate) can induce cyclic electron flow as has been shown for model systems using PS I particles [12].

We observed maximal rates of H_2 photoevolution when TMPD was used as an electron donor. TMPD is known to reduce the components of the photosynthetic water-oxidation system in the S_2 state [13]. In the paper cited, it is also shown that TMPD can reduce the oxidized secondary electron donor Z^+ in PS II particles pretreated with Tris-HCl buffer.

The data on suppression of the H_2 photoevolution process by such well-known inhibitors of PS II as dinoseb, atrazine and K-15 (fig.2) indicate that this reaction is sensitized by PS II where pheophytin with $E_0' = -610$ mV is an electron acceptor [3,4,6]. These results are in agreement with the developing 'alternative' scheme of electron transport from water to $NADP^+$ without participation of PS I [14].

It remains unclear as to which component of DT-20 particles has hydrogenase activity, providing for H_2 evolution due to interaction with photoreduced pheophytin or with the components reduced by it. The data on H_2 evolution in the dark by such particles indicate that this process is not brought about by the manganese system. DT-20 particles only contain such metalloorganic complexes as iron-containing cytochrome *b*-559 and plastoquinone complex with iron being in the content of PS II electron acceptors. The possibility cannot be excluded that these components account for the hydrogenase activity of DT-20 particles, although this requires further investigation. It should be noted that the ability of PS II preparations to evolve hydrogen is unlikely to be connected with bacterial contamination, since practically no H_2 photoevolution is observed in PS I particles isolated from the same chloroplasts. Previously, we have shown that chloroplasts isolated from the leaves of higher plants grown under sterile conditions can evolve hydrogen [1]. Thus, our data indicate that PS II particles isolated from pea and spinach chloroplasts are able to evolve molecular hydrogen in the light and that the process of H_2 photoevolution is probably sensitized by the reaction centers of PS II.

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