Photosynthetic Hydrogen Evolution by Spinach Chloroplasts Coupled to a *Clostridium* Hydrogenase

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Photosystem I activity of isolated chloroplasts has been coupled to photosynthetic hydrogen evolution via methylviologen and a *Clostridium* hydrogenase. Rates of 125 μmol hydrogen evolved per mg chlorophyll and hour have been obtained. The conditions for such optimal activity suggest that a closed thylakoid vesicle is required in order to prevent side- and backreactions of reduced methylviologen, which compete with and reduce hydrogen evolution.

The coupling of the photosynthetic machinery of a higher plant to hydrogen evolution *i. e.* the coupling of the photosynthetic electron transport system to a hydrogenase reaction is of principal as well as of applied relevance. The possibility of a light driven bio-photo-electrolysis of water to oxygen and hydrogen has recently attracted new attention to the problem. The results in the past – reviewed by Lien and San Pietro [1] – had indicated that the theoretically expected coupling of photosystem I of a chloroplast preparation onto a bacterial hydrogenase is experimentally possible [2–7]. However, the rates of hydrogen evolution reported so far remained low. Even when photosystem II activity and oxygen evolution were blocked [2, 5] or evolved oxygen was immediately trapped [6] to prevent damage of the oxygen evolution were blocked [2, 5] or evolved oxygen far did not exceed 15 μmol H_2/mg chlorophyll·h, *i. e.* about one tenth of the photosynthetic capacity of the chloroplast system.

This paper presents some new data on optimizing hydrogen evolution by chloroplasts with a rate of 125 μmol hydrogen/mg chlorophyll·h. The results furthermore offer an explanation for the structural and functional requirements of the chloroplast system necessary to obtain high rates.

Methods

Spinach chloroplasts (type c = washed thylakoid membranes) were prepared as described by Selman and Bannister [8]. Digitonin particles D-1 were prepared according to the method of Nieman and Vennesland [9]; *i. e.* the chloroplasts were resuspended in 50 mM Tris/HCl buffer pH 7.6 and 1% digitonin to a chlorophyll concentration of 1 mg/ml. After 30 min incubation at 4 °C the pellet of a 200000 × *g* centrifugation were resuspended in 20 mM Tris/HCl buffer pH 7.6 containing 0.4 M sucrose, 10 mM KCl and 5 mM $MgCl_2$. Digitonin particles D-0.4 were prepared according to Hauska *et al.* [10].

Abbreviations: Chl, chlorophyll; DAD, diaminodurene; DTE, dithioerythritol; MV, methylviologen; PD, *p*-phenylenediamine; TMPD, N-tetramethyl-*p*-phenylenediamine.

Sonicated chloroplasts were prepared as described by Böhme *et al.* [11]; *i. e.* chloroplasts were resuspended in 20 mM Tris/HCl buffer pH 7.6 to a final chlorophyll concentration of 0.05 mg/ml and sonicated with the microtip of the Branson Sonifier at 4 – 4.5 A for 60 sec in ice. After sonication the pellet of a 200000 × *g* centrifugation was resuspended in 20 mM Tris/HCl buffer pH 7.6 containing 10 mM KCl, 5 mM $MgCl_2$ and 0.4 M sucrose.

Clostridium pasteurianum was grown on a medium as described in ref. [12]. For a homogenate of *Clostridium pasteurianum*, showing hydrogenase activity, 10 g of deep frozen cells were homogenized in 30 ml anaerobic water containing 20 mg lysozyme and 2 mg desoxyribonuclease. The suspension was incubated at 35 °C for 30 min under stirring. Cell fragments were centrifuged off at 12500 × *g* and the homogenate was frozen and stored under H_2. The preparation with 20 mg/ml protein had a hydrogenase activity of 300 μmol MV reduced/min·ml.

A ferredoxin free hydrogenase preparation was obtained from 5 ml homogenate after centrifugation for 10 min at 1200 × *g*. The anaerobic supernatant containing 2 mM DTE was adjusted to pH 8.0 by adding Tris base and then run through a column ϕ 1 cm of 1 g Dowex 2/acetate and 1 cm DE 52, equilibrated with anaerobic 0.2 mM DTE in 0.2 M Tris/acetate buffer pH 8.0. The hydrogenase preparation, collected from the column as a golden fraction and free of ferredoxin, was adjusted to pH 5.7 by anaerobic 1 N acetic acid. After an incubation for 10 min at 60 °C under H_2 denaturated proteins were centrifuged off at 1200 × *g*. The hydrogenase preparation obtained was frozen and

stored under H_2. This preparation with 4.4 mg protein/ml had a hydrogenase activity of 116 μmol MV reduced/min·ml.

Anaerobic photoreduction of MV by chloroplasts and reoxidation by the hydrogenase preparation from *Clostridium pasteurianum* was measured under N_2 at 604 nm in a Zeiss PM Q II modified for side illumination. The cuvettes were fitted with a rubber stopper and were made anaerobic by evacuating and flushing with N_2 several times via an appropriate manifold. The cuvette was illuminated with white light of 10^5 erg/mg·sec. The general reaction mixture contained, in 2 ml : 200 mM hepes/NaOH buffer pH 7.3, 10 mM KCl, 5 mM $MgCl_2$, 30 μM DCMU, 15 μg gramicidin, 2 mM DTE, chloroplasts with 20 μg chlorophyll and the additions in the Tables and figures.

Photoevolution of H_2 by chloroplasts and the hydrogenase preparation of *Clostridium pasteurianum* was measured gaschromatographically as well as manometrically. For gaschromatography the reaction was carried out in small test tubes of a volume of 6 ml, determined before each run. The tubes were fitted with a rubber stopper and were made anaerobic like the cuvettes above. The reaction mixture was the same as above, except for chloroplasts with 100 μg chlorophyll in 2 ml. After a preillumination of 5 min the hydrogenase preparation was added to the tube with a Hamilton syringe. After an illumination with white light of 30000 lx for 10 min at 20 °C the assays were stopped with 0.5 ml anaerobic 1 N $HClO_4$. Hydrogen content was determined by injecting 0.3 cm^3 of gas in a Varian Aerograph 920 equipped with a molecular sieve 18 – 50 mesh column (length: 210 cm, ϕ 2 mm). Argon was used as carrier gas, the column temperature was 50 °C, the temperature of the thermal conductivity detector 95 °C (filament current: 115 mA). Hydrogen was detected after a retention time of 0.4 min.

To measure H_2 photoevolution manometrically Warburg vessels were used with two sidearms. One was fitted with a serum rubber cap, the other one with a glass vent. The reaction mixture was the same as above except for 3 mM TMPD, 120 mM sodium ascorbate, 0.2 mM MV and chloroplasts with 100 μg chlorophyll and 50 μl homogenate. The vessels were flushed with N_2 for 20 min and preilluminated with white light of 30000 lx for 5 min before adding the homogenate.

Photophosphorylation was measured by the method of Conover *et al.* [13]. The general reaction mixture above contained in addition 5 mM ADP and 3.3 mM $^{32}P_i$ instead of gramicidin in 200 mM tricine/NaOH buffer pH 8.0.

MV and triquat catalyzed light dependent oxygen consumption by chloroplasts (pseudocyclic electron flow) was measured in a Clark type teflon covered oxygen electrode. The reaction mixture was the same as the one to measure photoreduction of MV, except for 5 mM MV, 5 mM sodium ascorbate and 0.1 mM sodium azide and no DTE.

Results

The photoreduction of methylviologen by photosystem I in isolated chloroplasts is usually measured under *aerobic* conditions via the oxygen uptake upon the autooxidation of reduced methylviologen. Rates of such a pseudocyclic electron flow system, catalyzed by methylviologen, are very high (in the order of 500 to 1000 μequ/mg chlorophyll·h), though of course depending on the particular chloroplast preparation and assay conditions employed. Oxygen uptake is coupled to stoichiometric ATP formation with a P/e_2 ratio of one *i. e.* 1 mol ATP/2 equ of MV or O_2. The assay of photosystem I activity under *anaerobic* conditions by measuring at the wavelength of absorption of the blue reduced methylviologen has been suggested (see ref. 14). The rate in such a system, where the substrate accumulation of reduced methylviologen is measured, depends, of course, very much on very strict anaerobic conditions, but even then the rates are lower than in the aerobic pseudocyclic system. As Table I indicates, the rates of anaerobic MV reduction are 278 μequ MV reduced/mg·h and are therefore about half of the expected pseudocyclic rate (compare with Table IV). The fact, that the photosynthetic potential is, indeed, higher, becomes apparent by comparing the measured P/2e ratio of 3 for the complete system in Table I, with the usually observed P/2e ratio of 1 under aerobic conditions.

Table I. Coupling of anaerobic MV photoreduction to ATP formation by isolated thylakoid vesicles. The samples were illuminated and absorption changes at 604 nm were followed in the spectrophotometer. The complete system contained in the general reaction mixture 5 mM MV, 3 mM DAD and 60 mM Na ascorbate.

	μequ MV red / mg Chl·h	μmol ATP / mg Chl·h	P/e_2
complete system	278	487	3.5
minus ascorbate	20	347	34.7
minus DAD	59	42	1.4
minus MV	—	23	

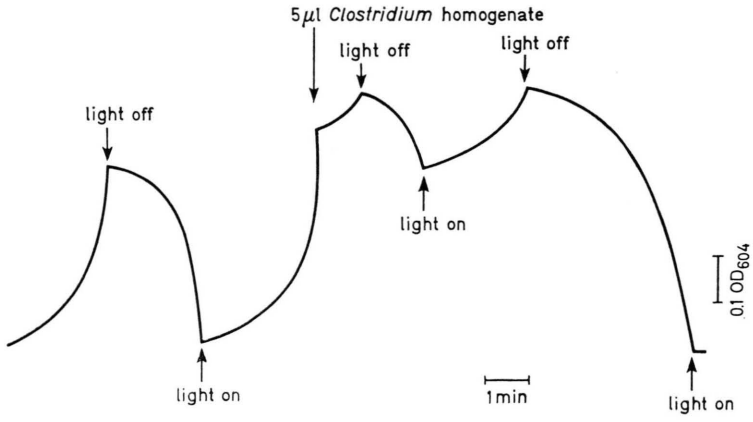

Fig. 1. Formation of reduced MV by chloroplasts in the light and its reoxidation by a *Clostridium* hydrogenase. Conditions and measurements as in Table I except for 0.05 mM MV and hepes buffer pH 7.3. 5 µl homogenate of *Clostridium* (with a hydrogenase activity of 1.5 µmol MV reduced/min) was added where indicated.

The rate of ATP formation suggests a rate of electron flow through photosystem I of 974 µequ. The "disappearance" of electrons might be due to some oxygen left in the system or, more likely, indicates that a cyclic electron flow system is superimposed. In both cases reduced MV is consumed. In the absence of ascorbate noncyclic electron flow is practically abolished in favor of the cyclic pathway as indicated by the high photophosphorylation rate (Table I). In the absence of the donor DAD the system practically stops. Though the results of Table I indicate a loss of electrons, when MV reduction is measured under anaerobic conditions, the rate is still appreciable and thus anaerobic MV reduction may be used to follow the coupling of a hydrogenase onto the chloroplast via MV. As Fig. 1 is to indicate, the photoreduction of 0.05 mM MV by chloroplasts, measured at 604 nm, levels off at about 80% reduction. Upon light off, a new steady state level is approached. The cycle might be repeated. Addition of a crude hydrogenase preparation from *Clostridium* leads to a fast consumption of reduced MV, indicated by the decrease in absorption. Light on rereduces MV, but because of the presence of hydrogenase a new steady state of about 60% reduced MV is obtained.

This indicates that chloroplasts and hydrogenase may be coupled to form H_2 in the light. The following Tables and figures show some properties of a system, composed of an artificial electron donor and photosystem I of chloroplast thylakoid vesicles coupled onto *Clostridium* hydrogenase. Evolved H_2 gas may be measured either by a gas chromatograph or even manometrically, since the rate turned out to be high enough. Fig. 2 is to indicate the optimal MV concentration for H_2 evolution, Fig. 3 the neces-

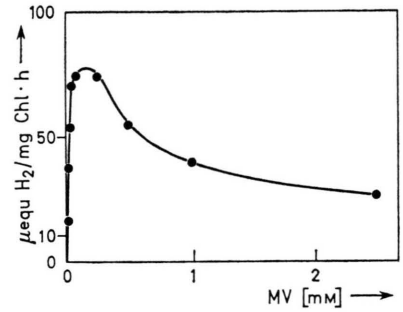

Fig. 2. The dependence of light driven hydrogen evolution by chloroplasts + hydrogenase on MV concentration. The reaction was run in anaerobic test tubes; hydrogen evolved was measured gaschromatographically. The general chloroplast reaction mixture contained 3 mM DAD, 60 mM Na ascorbate and 20 µl *Clostridium* homogenate.

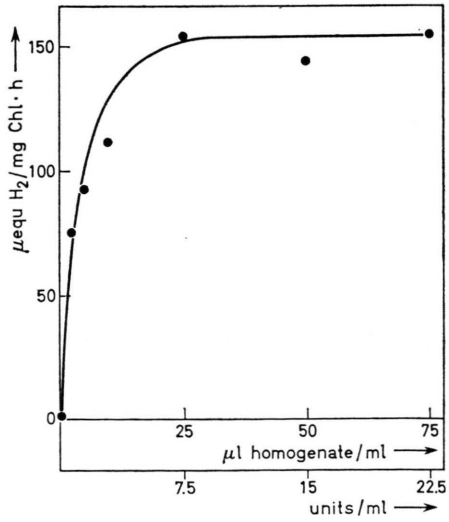

Fig. 3. Dependence of light driven hydrogenase evolution by chloroplasts on the homogenate concentration. Conditions and measurements as in Fig. 2; 0.2 mM MV and 120 mM Na ascorbate in the chloroplast reaction mixture 1 unit hydrogenase = µequ MV reduction/min.

sary amount of hydrogenase preparation and Fig. 4 the effect of ascorbate concentration. In Table II hydrogen evolution with different electron donors is compared, TMPD turns out to be the best. From these results, an optimal system of photosystem I

Table II. Comparison of different electron donors for photosystem I in the reduction of MV and evolution of hydrogen by illuminated chloroplasts + *Clostridium* hydrogenase. The reaction was run in anaerobic cuvettes. MV reduction was measured at 604 nm and hydrogen evolution gaschromatographically. The general chloroplast mixture contained 120 mM Na ascorbate and 5 mM MV in the MV reduction experiment, but 0.2 mM MV and 50 µl *Clostridium* homogenate in the H_2-evolution experiment.

Donor	MV reduction [µequ/mg Chl·h]	H_2 evolution [µequ/mg Chl·h]
DAD	489	177
TMPD	1566	250
PD	434	171
indamin	494	177
no donor	87	74
TMPD		198
N-dimethyl-PD		155
N-trimethyl-PD		186
DAD		144
dimethyl-indoanilin		144
no donor		59

Table III. Comparison of MV, triquat and spinach ferredoxin in light dependent H_2 evolution by chloroplasts + hydrogenase. Conditions and measurements as in Fig. 2. 3 mM TMPD instead of DAD and 50 µl purified *Clostridium* homogenate.

	µequ H_2/mg Chl·h
0.05 mM methylviologen	110
0.2 mM methylviologen	168
1 mM methylviologen	145
0.05 mM triquat	84
0.2 mM triquat	135
1 mM triquat	166
5 µM ferredoxin	99
10 µM Ferredoxin	108
no mediator	26

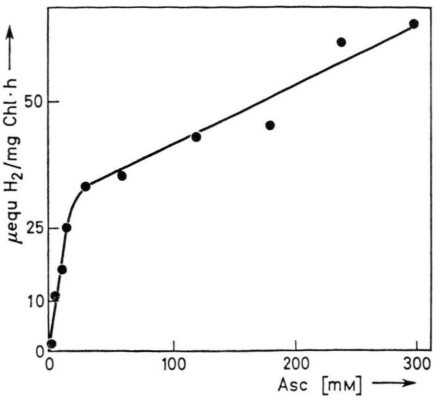

Fig. 4. Dependence of light driven hydrogen evolution by chloroplasts + hydrogenase on ascorbate concentration. Conditions and measurements as in Fig. 2. 0.2 mM MV and 20 µl *Clostridium* homogenate in the general reaction mixture.

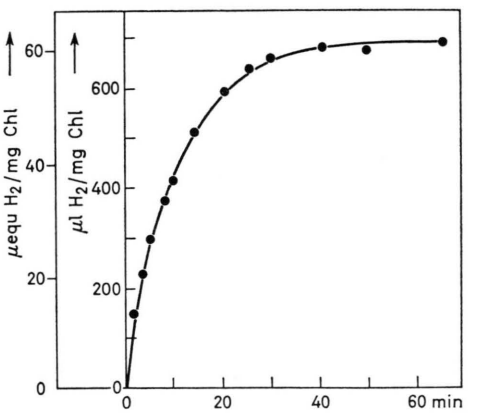

Fig. 5. Kinetics of light driven hydrogen evolution by chloroplasts + hydrogenase. Conditions as in Fig. 2. Measurement of hydrogen evolution manometrically. The general chloroplast reaction mixture contained 0.2 mM MV, 3 mM MTPD, 120 mM Na ascorbate and 50 µl *Clostridium* homogenate.

Table IV. Comparison of the rate of hydrogen evolution and of pseudocyclic electron flow in fragmented chloroplasts. Conditions and measurements of hydrogen evolution gaschromatographically as in Fig. 2, oxygen uptake by the oxygen electrode and anaerobic MV reduction and ATP formation as in Table I. 2 µM plastocyanin added in sonicated and digitonin fragmented chloroplasts.

	H_2 evolution [µequ/mg Chl·h]	Aerobic oxygen uptake via MV [µequ/mg Chl·h]	Anaerobic MV reduction [µequ/mg Chl·h]	ATP formation [µmol/mg Chl·h]
type c chloroplast (washed thylakoids)	212	500	—	200
sonicated chloroplasts	70	852	82	—
0.4% digitonin chloroplasts	81	1000	282	57
1% digitonin chloroplasts	none	1890	2.7	none

driven hydrogen evolution is constructed with a rate of 125 μmol of hydrogen/mg chlorophyll and hour in the first 10 min of illumination (Fig. 5). Table III compares the effect of three different mediators between photosystem I and hydrogenase. Triquat is not better than MV, whereas spinach ferredoxin supports only a somewhat lower rate of hydrogen evolution. By comparison with the forgoing data purification of the hydrogenase from the homogenate does not increase the effectivity of the system.

Table IV is to show hydrogen evolution by different types of chloroplast preparations. Isolated chloroplast thylakoid vesicles (type c chloroplasts) as well as fragmented chloroplasts have appreciable electron flow activity, as measured by aerobic MV reduction, *i. e.* oxygen uptake. Actually, fragmented chloroplasts have even larger electron flow rates, as known in the literature and indicated also in Table IV. Hydrogen evolution and anaerobic MV reduction, however, decrease appreciable, when the chloroplasts are fragmented. In sonicated and 0.4% digitonin fragmented chloroplasts only 70 to 80 μequ H_2 evolution/mg chlorophyll·h remains, whereas pseudocyclic electron flow actually increases, compared with type c chloroplasts. In 1% digitonin particles no hydrogen evolution is observed anymore but pseudocyclic electron flow is high. By comparing also photophosphorylation Table IV seems to indicate that the activity of hydrogen evolution runs parallel to the ATP forming capacity, which is possible only in intact thylakoid vesicles.

Discussion

The reducing potential of the primary electron acceptor of photosystem I of the photosynthetic electron transport chain of chloroplasts is well above the redox potential of ferredoxin or MV. Plant ferredoxin is photosynthetically reduced and distributes reducing equivalents in the chloroplasts to various light dependent reductions, like $NADP^+$, nitrate or sulfate reduction. Also bacterial ferredoxin is reduced by chloroplasts in the light [15]. Hydrogenases evolve or consume hydrogen and may be coupled via ferredoxin or MV. Therefore it is to be expected that a hydrogenase system and hydrogen evolution should easily be coupled to the light driven electron transport system. Indeed hydrogen evolution by chloroplasts plus a bacterial hydrogenase (*Chromatium*) has been described already in 1961 by Arnon *et al.* [2]. An electron donor system for photosystem I had been used and oxygen evolution had been blocked in order to prevent inactivation of the oxygen sensitive hydrogenase. The rate of the system described by Arnon *et al.* [2] were low but the principle accomplishment of the coupling was the purpose of the experiment. But also in more recent experiments the rates of hydrogen evolution in chloroplasts plus hydrogenase system remained low [3–7]. Low rates can be explained if the purpose of experiment was to show oxygen and hydrogen evolution simultaneously, but not if a donor system for photosystem I is used or oxygen is trapped by glucose/glucose oxidase under strict anaerobic conditions.

The experiments reported here do show high rates of hydrogen evolution in the light by a chloroplast system coupled to a *Clostridium* hydrogenase and a donor system for photosystem I. 125 μmol hydrogen gas were obtained per mg chlorophyll and hour. The hydrogen evolved was identified in the gas chromatograph; the rates were high enough to follow the reaction in a manometer. The reason for the high rates of hydrogen evolution in our experiment becomes clear from the requirement for optimal rates: strict anaerobiosis, high concentration of ascorbate, TMPD as donor and a closed thylakoid vesicle system. This suggests that back reactions = cycling of electrons around photosystem I, are the main reason for the failure in the past to observe high rates. As Fig. 6 is to summarize, reduced MV (or ferredoxin) produced by photosystem I in the light is an excellent electron donor not only for the hydrogenase system, but also for the endogenous

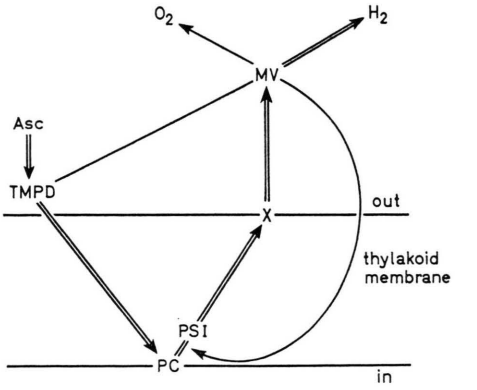

Fig. 6. Scheme of photosynthetic electron flow via photosystem I, indicating possible back reaction diminishing the flux from ascorbate to hydrogen.

primary electron donor for photosystem I. This back reaction is avoided in an intact vesicle because it prevents access of the exogenous hydrophilic MV to the endogenous donor on the inside of the membrane. Another undesired cycling occurs when the reduced MV rereduces the exogenous electron donor for photosystem I. This is prevented by excess ascorbate to keep the donor reduced and by using TMPD as electron donor, because TMPD is positively charged in the oxidized form as is MV. Further possibilities of undesired electron cycling via the donor (which in the oxidized form is also an acceptor of photosystem I) is also diminished by excess ascorbate.

This main evidence for the argument of cycling is that in ruptured chloroplast particles photosystem I activity and MV reduction measured by oxygen uptake remain high, whereas the rate of hydrogen evolution drops to zero. In fragmented chloroplasts the rate of hydrogen evolution runs parallel to the rate of ATP formation coupled to MV reduction, the later capability being an expression of the intactness of the vesicle.

The finding that the donor side of photosystem I has to be shielded from a back reaction by the mediator (MV) to the hydrogenase seems to be a setback for the hope that purified (and thus stabilized) photosystem I preparations without protection of the sidedness of the membrane would be a way to establish a hydrogen evolving photosynthetic system for applied purposes.

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