Stoichiometry of H_2 Production by an *in vitro* Chloroplast, Ferredoxin, Hydrogenase Reconstituted System

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Biophotolysis, H_2 Production, Hydrogenase

Studies of the rate and efficiency of H_2 production by a reconstituted system composed of washed chloroplasts, ferredoxin, and hydrogenase reveal that a significant proportion of the low potential electrons are leaked to H_2O_2 production via auto-oxidation of ferredoxin even in the presence of an oxygen trap (glucose and glucose oxidase). Under optimum conditions of ferredoxin concentration the leak can be minimized such that H_2 production is about 70% of the ferricyanide Hill reaction rate.

Introduction

The continuous production of molecular hydrogen by an *in vitro* reconstituted system composed of chloroplasts, ferredoxin and hydrogenase has recently been under study in several laboratories [1–3]. We reported [1] that such a system can continuously produce H_2 by a photosystem II driven reaction for 6–8 h when the reaction was carried out in the presence of O_2 (glucose and glucose oxidase) and hydrogen peroxide (ethanol and catalase) traps at a rate of 10–25 μmol H_2 produced per mg chlorophyll/h at 25–29 °C. This is only about 5–10% of the rate of the Hill reaction found with spinach chloroplasts under similar conditions *in vitro* but with other acceptors.

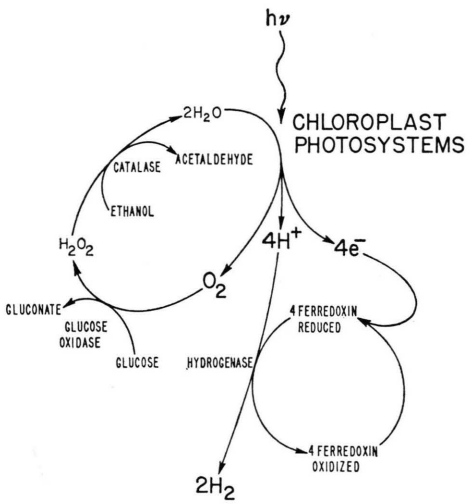

Fig. 1. Chloroplast, ferredoxin, hydrogenase coupled system for H_2 production from Fry *et al.* [1].

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In the present study we have further examined the stoichiometry of O_2 disappearance by assaying the acetaldehyde (CH_3CHO) production. This provides a measure of the H_2O_2 production by either glucose oxidase or by auto-oxidation of ferredoxin. These studies indicate that the glucose/glucose oxidase trap only accounts for 60–80% of the O_2 produced by water photolysis linked to H_2 production, the remainder being trapped by ferredoxin auto-oxidation. We have also made measurements of the Hill reaction rate under our conditions and found that the reconstituted system manages to use up to 72% of the potential electrons for H_2 production depending upon the ferredoxin concentration.

Materials and Methods

Experiments were carried out in 7 ml rubber stoppered vials using conditions as described previously [1]. Reaction mixtures in a total volume of 2 ml contained: type II spinach chloroplasts, 0.1 mg/ml; Spirulina ferredoxin (Fd), 0.1 mg/ml; *Clostridium pasteurianum* hydrogenase, 34 mμmol; glucose oxidase, 0.5 mg/ml; catalase, 0.1 mg/ml; glucose, 50 mM; ethanol, 2.5% v/v; bovine serum albumin, 0.5 mg/ml; HEPES buffer, 65 mM, pH 7.0. H_2 production was measured by withdrawing 50 μl aliquots from the gas phase and injecting into a Varian Model 920 GLC chromatograph with a molecular sieve column (5A mesh 30/60) and thermal conductivity detector. To estimate the Hill reaction rate, 20–50 μl additions of a 40 mM solution of potassium ferricyanide [$K_3Fe(CN)_6$] were injected into the vials, and the time during whiich cessation of H_2

production occurred was measured. H_2 uptake ceases because of the preferential shunting of electrons to ferricyanide [4]. Equal aliquots of $K_4Fe(CN)_6$ were added to duplicate vials as controls.

Acetaldehyde was measured using a modified 2,4-thiobarbituric acid (TBA) assay [5]. The reaction was stopped by adding 0.5 ml trichloroacetic acid to each vial, the contents were centrifuged and a 1 ml aliquot of the supernatant added to 1.4 ml of a TBA solution (0.71 g TBA, 0.7 ml 1 M NaOH made up to a final volume of 100 ml distilled water) and heated in a boiling water bath for 20 min. The absorbance measurements were made using an American Instruments DW-2 spectrophotometer in the dual beam mode at 499 – 600 nm. Under the conditions employed, with both CH_3CHO and glucose present, absorption at 499 nm is proportional to the concentration of CH_3CHO [6].

Results

After flushing the reaction mixture with N_2 gas, the initial rates of H_2 production and acetaldehyde production in the complete coupled system were determined in the absence of glucose oxidase (Fig. 2 a) or hydrogenase (Fig. 2 b). In the absence of hydrogenase there was some CH_3CHO formation which ceased after about 1 h. This indicates that O_2 and/or H_2O_2 were present in the reaction mixture. Thereafter acetaldehyde production paralleled H_2 production. If glucose oxidase was omitted CH_3CHO formation continued. This indicates that glucose oxidase was not the only pathway present for formation of H_2O_2 from O_2. Reduced ferredoxin is known to be auto-oxidized to form H_2O_2 (7) *viz.*;

$$4\,Fd_{ox} + 2\,H_2O \xrightarrow[Chl]{h\nu} O_2 + 4\,Fd_{red} + 4\,H^+ \quad (1)$$

$$O_2 + Fd_{red} \longrightarrow O_2^- + Fd_{ox} \quad (2)$$

$$2\,H^+ + O_2^- + Fd_{red} \longrightarrow H_2O_2 + Fd_{ox} \quad (3)$$

or

$$2\,H^+ + 2\,O_2^- \longrightarrow H_2O_2 + O_2 \quad (4)$$

sum of (2) + (3) or 2(2) + (4):

$$2\,H^+ + O_2 + 2\,Fd_{red} \longrightarrow H_2O_2 + 2\,Fd_{ox} \quad (5)$$

$$H_2O_2 + Ethanol \xrightarrow{catalase} CH_3CHO + 2\,H_2O \quad (6)$$

$$\text{Overall } 2\,Ethanol + O_2 \longrightarrow 2\,CH_3CHO + 2\,H_2O \quad (7)$$

When glucose oxidase was absent (Fig. 2 a) H_2 production was slightly slower probably due to a smaller pool of reduced ferredoxin or a slightly higher steady state level of O_2 partially inhibiting the enzyme. In the absence of glucose oxidase, with ferredoxin consuming O_2 the expected ratio of CH_3CHO: O_2 is 2. Therefore, the expected ratio of CH_3CHO: H_2 would be 1, which was observed (Figs 2 a, b). When glucose oxidase was present the observed ratio of CH_3CHO: H_2 was found to be between 0.6 and 0.7, not 0.5 as predicted by the scheme in Fig. 1. This corresponds to 60 – 80% of the O_2 released by water photolysis during H_2 production being trapped by glucose oxidase. Our previous studies [1] showed that glucose disappearance (equal to O_2 trapped) was also found to be approximately 70% of the value expected according to the scheme in Fig. 1. The remaining O_2 (30%) is trapped by

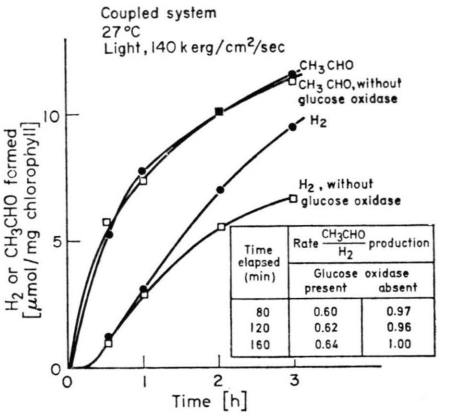

Fig. 2 a.

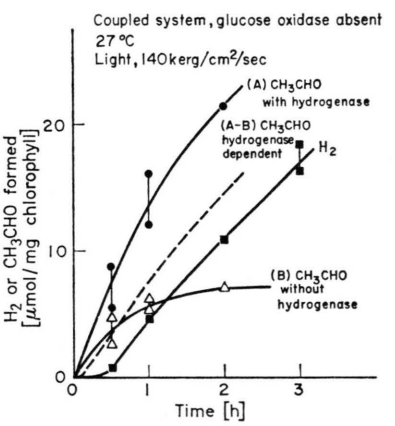

Fig. 2 b.

Fig. 2. H_2 and H_2O_2 production by the coupled system a. absence of oxygen trap (glucose oxidase), b. absence of oxygen trap and hydrogenase.

ferredoxin, but since O_2 is evolved in the reduction of ferredoxin the percentage of the total O_2 production trapped by ferredoxin was about 40%.

The Hill reaction rate under the usual conditions employed (0.1 mg/ml chlorophyll, light intensity of 140 k ergs/cm^2), as measured by the time required for $K_3Fe(CN)_6$ to be consumed, was 55 – 60 μmol/h/mg chlorophyll (Fig. 3). This was accompanied by an initial rate of H_2 production of 9.8 mmol H_2/h/mg chlorophyll or 33% of the Hill reaction rate. Addition of $K_4Fe(CN)_6$ did not affect the rate of H_2 production, but an addition of 1 μmol of O_2 caused an inhibition of H_2 production similar to that caused by the addition of $K_3Fe(CN)_6$. This suggests that the oxygen produced by the reduction of the $K_3Fe(CN)_6$ is responsible for the slight inhibition of H_2 production after the $K_3Fe(CN)_6$ has been all reduced. Fig. 4 shows that increasing the ferredoxin concentration to 0.55 mg/ml increased the rate of H_2 production to 72% of the Hill reaction rate.

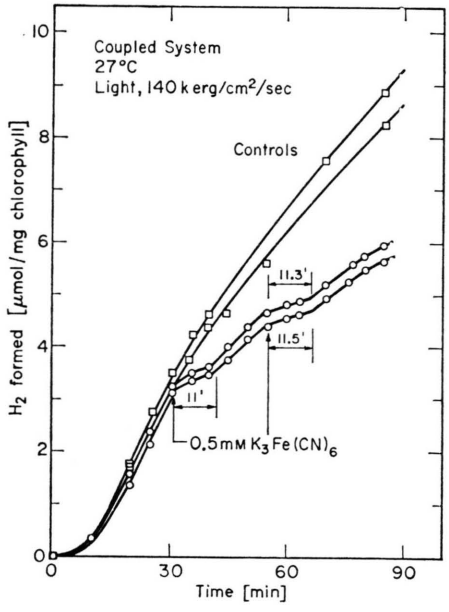

Fig. 3. Estimation of the Hill reaction rate under conditions used in the complete coupled system for H_2 production.

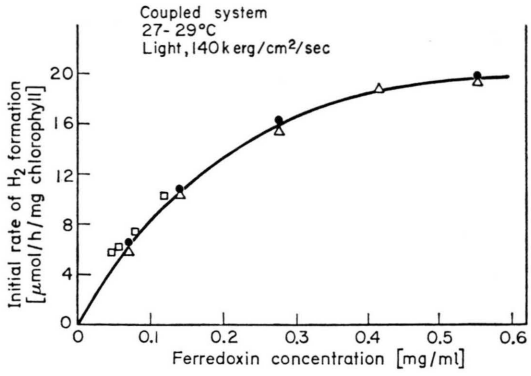

Fig. 4. Ferredoxin dependence of H_2 production by the coupled system.

Discussion

The manner in which low potential electrons produced by chloroplast photosystems are partitioned between NADPH production, electrons for cyclic photophosphorylation, auto-oxidation by oxygen of ferredoxin, has been studied in various laboratories which has established that *in vivo* NADPH production successfully competes for low potential electrons [4]. However, in the present investigation, we have studied a reconstituted system using washed chloroplasts plus added ferredoxin and hydrogenase, in which NADP and the enzymes for NADP reduction are absent. In this system the only other reaction which can compete for low potential electrons produced by the photosystems, other than hydrogenase, is the auto-oxidation by oxygen of ferredoxin (1) which produces hydrogen peroxide (2 – 5). Assays for hydrogen peroxide production (2 – 6) show that reactions (2 – 4) significantly compete for electrons. At higher concentrations of ferredoxin more electrons are diverted to production which can be up to 72% of the $K_4Fe(CN)_6$ Hill reaction rate. Thus photolysis of water by a coupled system composed of chloroplasts, ferredoxin and hydrogenase under certain conditions can function with great efficiency for H_2 production.

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