DISCOVERY OF AN ALTERNATIVE OXYGEN SENSITIVITY AND ITS POTENTIAL APPLICATION IN PHOTOSYNTHETIC H_2 PRODUCTION

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Abstract

We have discovered a new O_2 sensitivity in algal H_2 production that represents an alternative to the O_2 sensitivity of the hydrogenase per se. The principal findings serving as evidences of this alternative O_2 sensitivity are as follows: (1) the unexpected surge of H_2 photoevolution immediately after the onset of actinic illumination in the continued presence of low concentrations of O_2; (2) the relief effect of FCCP on O_2 inhibition of H_2 photoevolution; (3) the absence of tolerance to the continued presence of the background O_2 in National Renewable Energy Laboratory's O_2-tolerant mutants; and (4) the observation of residual hydrogenase activity at O_2 concentrations as high as 5000 ppm, where the steady-state H_2 photoevolution was inhibited almost completely.

The reported O_2 sensitivity is apparently linked to the photosynthetic H_2 production pathway that is coupled with proton translocation across the thylakoid membrane. Addition of the proton uncoupler (FCCP) can eliminate this mode of O_2 inhibition on H_2 photoevolution. This mode of inhibition is likely due to the background O_2 which apparently can serve as a terminal electron acceptor in competition with the H_2 production pathway for the photosynthetically generated electrons from water splitting. This O_2-sensitive H_2-production electron transport pathway can be inhibited by DCMU. Our experiments demonstrated that this alternative pathway is more sensitive to O_2 than the traditionally known O_2 sensitivity of the hydrogenase. These findings clarify the meaning of "oxygen tolerance" in algal H_2 production by demonstrating the two modes in which atmospheric oxygen interacts with the hydrogen evolution process. Future work will focus on mapping this alternative electron transport pathway and on applying it to enhancing the production of H_2.

Introduction

Algal photosynthetic hydrogen (H_2) production is a potential clean energy resource. In green algae, photoevolution of H_2 and O_2 occurs in the same cell, where the photosynthetically produced O_2 can inhibit the production of H_2.[1] Therefore, the application of green algae for H_2 production must addresses the problem of O_2 sensitivity. In the past, this O_2-sensitive phenomenon was generally interpreted as the direct O_2-inhibition effect on hydrogenase activity.[2] In the course of our investigations, we discovered that the classic interpretation of O_2 sensitivity needs to be revised. In recent experiments that characterized O_2 tolerance in H_2-producing wild-type *Chlamydomonas reinhardtii*, we observed a new O_2 sensitivity that is clearly distinct from that of the hydrogenase. This O_2 sensitivity indicates that there is an alternative electron transport pathway that can redirect electrons from the hydrogenase-catalyzed H_2-production pathway to O_2. Our experiments demonstrated that the alternative mechanism is more sensitive to O_2 than the classic O_2 sensitivity of hydrogenase. It is felt that these findings represent a significant progress in algal H_2 production studies. This paper reports the detailed experimental results.

Materials and Methods

Chlamydomonas reinhardtii wild-type strain 137c and O_2-tolerant mutants 155G6, 141F2, 76D4, and 104G5 were grown under light intensity of about 20 FE@m^{-2}@s^{-1} in minimal-plus-acetate medium. When the culture grew to a cell density of about10^6 cells/ml, the algal cells were harvested by gentle centrifugation (3000 RPM). The algal cells were then washed and re-suspensed in fresh minimal medium for O_2-tolerance hydrogen-production assays. The O_2-tolerance assays were performed under atmospheres of research-grade helium (purity >99.9999%, zero oxygen) and a series of O_2 concentrations [10, 100, 300, 1000 (0.1000%), 5000, and 10,000 ppm O_2] in helium using our unique dual-reactor-flow detection system. The 10, 100, 300, 1000, 5000, and 10,000 ppm O_2 in helium were primary standards purchased from Matheson Gas and Equipment, Inc.

As illustrated in Fig. 1, the assays were conducted using a laboratory-built dual-reactor flow detection system.[3] For each assay, 35 ml of algal sample (3 Fg Chl/ml) was placed and sealed in each of the two reactors that are water-jacketed and held at 20°C with a temperature-controlled water bath (Lauda RM6, Brinkmann Instruments, Germany). The algal sample was then purged by helium flow (50 ml gas/min) through the liquid reaction medium. The helium flow serves two purposes: (1) to remove O_2 from the algal sample to establish and maintain anaerobic conditions that are necessary for induction of the algal hydrogenase synthesis and production of H_2 and (2) to carry any H_2 gas product to the hydrogen sensors. After induction of hydrogenase and establishment of steady-state photoevolution of H_2 under the helium atmosphere (which normally required about 8 h or more), the primary standards 10, 100, 300, 1000, 5000, and 10,000 ppm O_2 in helium were introduced into the reactors by replacing the pure helium at the same flow rate (50 ml/min) to characterize the oxygen sensitivity of photoevolution of H_2. The actinic illumination at 100 uE@m$^{!2}$@s$^{!1}$ (about 5% of the full LED intensity) for the H_2 photoevolution assay was provided by an electronically controlled LED light source with its full (100%) intensity of about 2000

uE@m!2@s!1 at 670 nm. The actinic intensity was measured with an IL-1700 light meter. Both the rate of H_2 production and the actinic intensity were recorded simultaneously by a PC computer.

For the chemical inhibitor studies, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU) and carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Sigma Chemical Company. Stock (500 µM) solutions of these chemicals were made. Then, small aliquots of the stock solutions were injected into the reactors to give the final concentrations of 10 µM DCMU and 5 µM FCCP in the reaction medium as specified in the particular experiments.

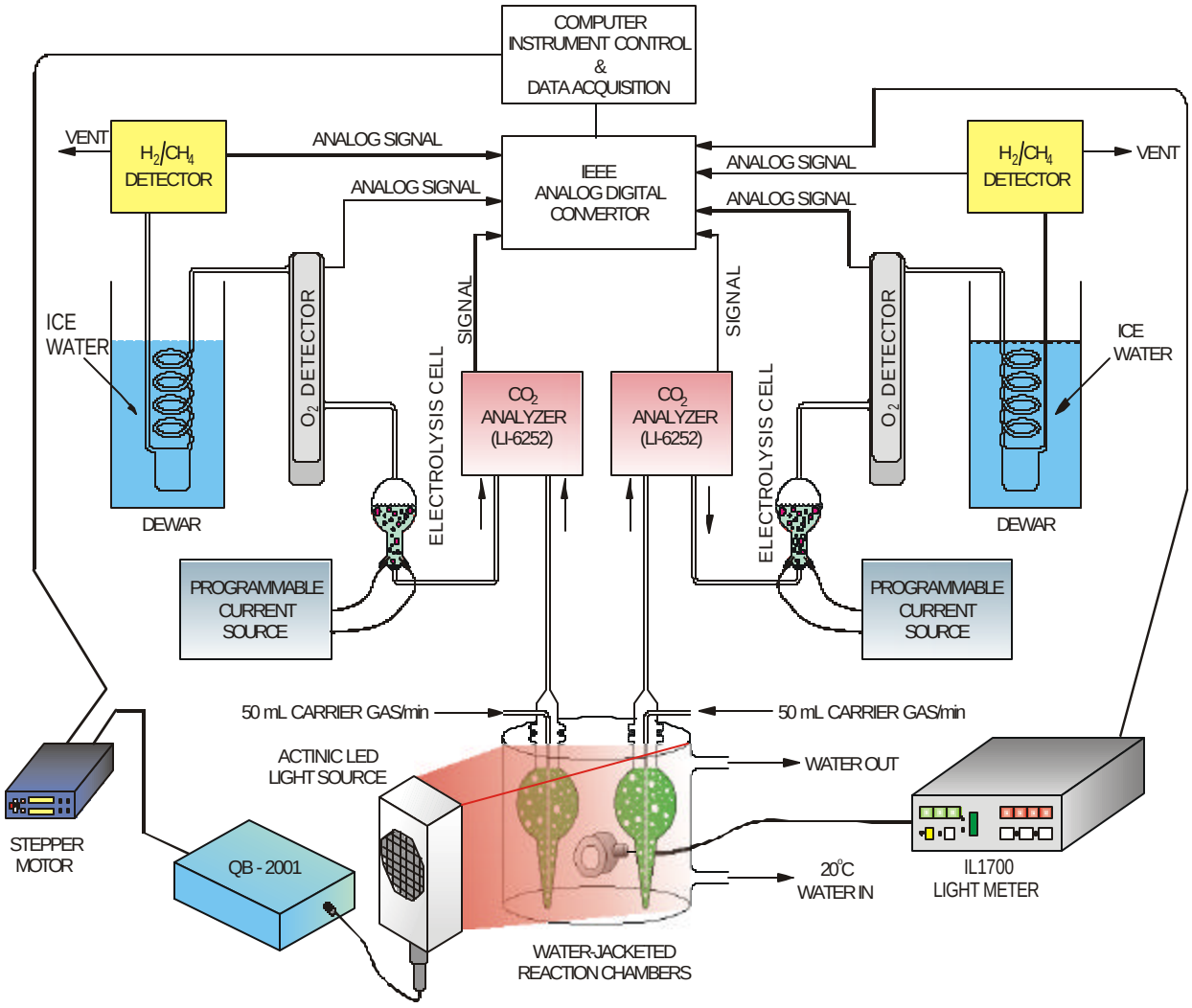

Figure 1. Schematic of a dual-reactor flow detection system for simultaneous detection of CO_2, H_2, and O_2.

Results and Discussion

First Evidence of a New O_2 Sensitivity in Algal H_2 Production

The results of the assays are very intriguing. As illustrated in Fig. 2, introduction of 0.1000% (1000 ppm) O_2 dramatically reduced the rate of algal H_2 photoevolution. The steady-state H_2 production rate in the presence of 0.1000% O_2 was 0.33 µmol H_2•mg Chl^{-1}•h^{-1} which is only about 2.8% of the full steady-state rate (12 µmol H_2•mg Chl^{-1}·h^{-1}) before the introduction of the 0.1000% O_2. In the past, this type of H_2 production decay was commonly interpreted as the inhibition of O_2 on hydrogenase activity. Our experimental results have now proved that this classic interpretation of oxygen sensitivity on algal H_2 production is not consistent with the data. According to the classic interpretation, the reduction of H_2 production after the introduction of 0.1000% O_2 is due to O_2 inhibition on hydrogenase per se: that is, hydrogenase activity would be the limiting factor for the rate of H_2 photoevolution. If this interpretation were correct, one would expect the rate of H_2 photoevolution to be no higher than the inhibited rate (0.33 µmol H_2•mg chl^{-1}•h^{-1}) after a brief dark period in the presence of 0.1000% O_2. However, the experimental data turned out to be very different from the classic expectation. As shown in Fig. 2, there was a

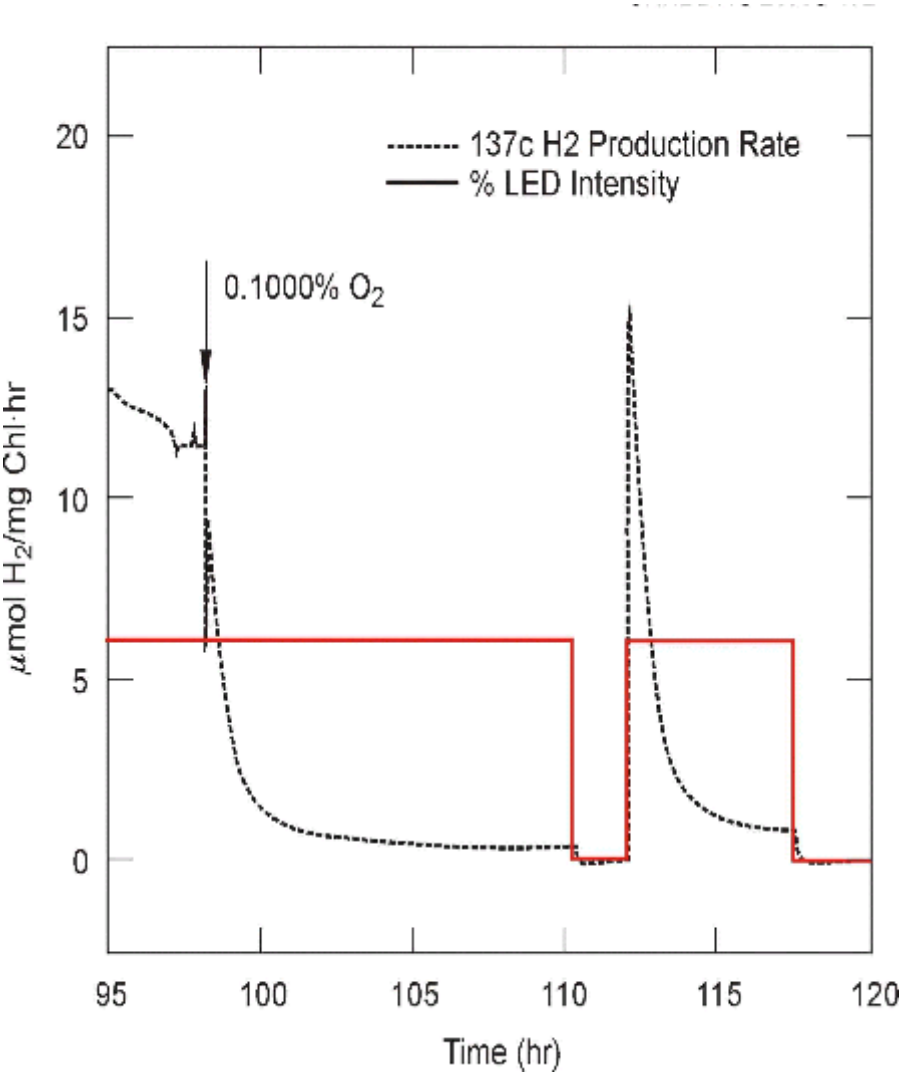

Figure 2. Observation of a new O_2 sensitivity to algal H_2 production in *Chlamydomonas reinhardtii.*

surge of H_2 photoevolution after a 2-h dark period *in the continued presence of 0.1000% O_2*. The peak rate of H_2 photoevolution was about 15 µmol H_2 mg Chl^{-1} h^{-1} which is about 45 times higher than the classically predicted rate (0.33 µmol H_2 mg Chl^{-1}·h^{-1}). This assay has now been repeated for more than 6 times and all the results were consistent with the observation presented in Fig. 2.

This observation clearly indicated that hydrogenase activity was not the limiting factor for H_2 photoevolution at this level of O_2. There must be an alternative electron transport pathway that takes the photogenerated electrons away from ferredoxin (Fd) to O_2. The observed reduction of H_2 production after the introduction of 0.1000% O_2 can be explained by such a pathway that competes for electrons with the Fd/hydrogenase-catalyzed H_2-production pathway. This is an important discovery since it fundamentally redefines the meaning of "oxygen tolerance" in algal H_2 production.

Evidence for a New O_2 Sensitivity from Studies with DCMU and FCCP

Our studies with the chemical inhibitor (DCMU) and proton uncoupler (FCCP) have yielded additional evidence for the new O_2 sensitivity. FCCP is a proton uncoupler that can dissipate the proton gradient across the thylakoid membrane in algal cells. As illustrated in Fig. 3, in the presence of 1000-ppm O_2 after the induction of the hydrogenase enzyme, the steady-state photoevolution of H_2 around the time of 20:00 hours was slightly less than 1 µmol H_2 mg Chl^{-1}·h^{-1}. After a brief dark period (from 20:20 to 22:20), a burst of H_2 photoevolution appeared, followed by an oscillation in the decay curve. Since both the actinic intensity and the background O_2 concentration (1000 ppm) remained the same, this oscillation also indicated that the decay (inhibition) in the rate of H_2 photoevolution resulted not from O_2 inhibition of the hydrogenase enzyme per se but from a kinetic effect of O_2 on the electron transport that is related to the H_2-production process. Addition of 5 µM FCCP produced a dramatic reversal of the O_2 inhibition on H_2 photoevolution. The rate of H_2 production rose to about 16 µmol mg Chl^{-1}·h^{-1}. This FCCP-stimulated H_2 production is clearly photo-dependent. As soon as the actinic light was turned off, the H_2 production stopped. The data (Fig. 3) also demonstrated that the FCCP-enhanced photoevolution of H_2 can last for more 4 hours with somewhat decay. The decay is due to a side effect of FCCP known as the ADRY effect in which FCCP gradually inhibits PSII activity

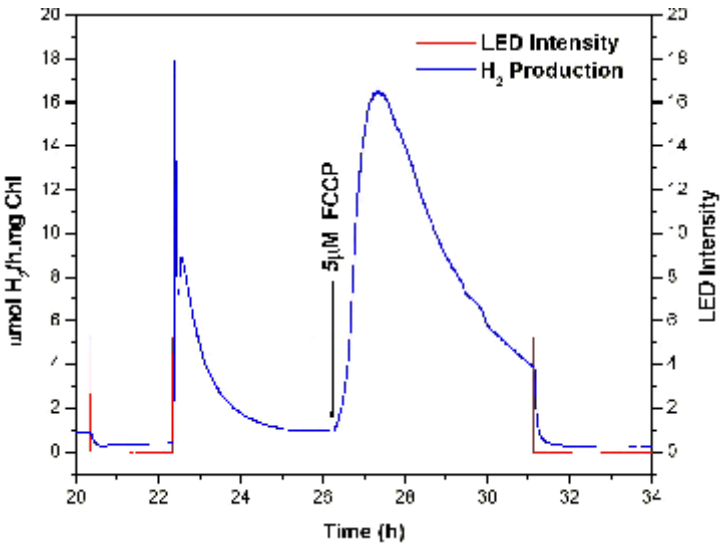

Figure 3. Evidence of a new O_2 sensitivity from H_2 production assays with proton uncoupler FCCP under continued presence of 1000-ppm O_2 in *C. reinhardtii* 137c.

by deactivation of the photosynthetic water-splitting complex in the S_2 and S_3 states.[4] However, FCCP does not have any known effect on the hydrogenase enzyme per se. Therefore, the observed stimulation of H_2 photoevolution by FCCP in the presence of 1000 ppm O_2 is additional evidence for a new O_2 sensitivity in algal H_2 production that is alternative to the oxygen sensitivity to hydrogenase per se. The result clearly demonstrated that the newly discovered O_2-sensitive electron transport pathway requires the presence of a proton gradient (or ATP) to operate.

DCMU is a chemical inhibitor that binds at the Q_B site of photosystem II (PSII) and thus blocks the transport of electrons acquired from PSII water splitting to photosystem I (PSI). The experimental data (Fig. 4) showed that addition of DCMU inhibited the burst of H_2 photoevolution after a dark period in the presence of 1000-ppm background O_2. This result indicated that over 90% of the electrons that are used in the photoproduction of H_2 are derived from PSII water splitting. Therefore, water is the main source of electrons for H_2 production even after the dark period in the presence of 1000 ppm O_2. Organic reserves are not the main source of electrons in this mode of H_2 production.

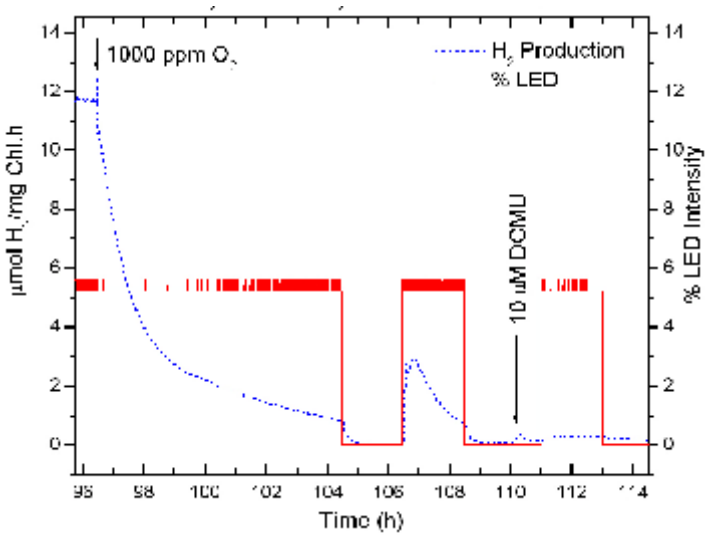

Figure 4. O_2-tolerance assay in algal H_2 production with DCMU.

Evidence for a New O_2 Sensitivity from Studies with O_2-Tolerant Mutants

There is an active DOE research effort to produce H_2-producing, O_2-tolerant mutants of green algae *Chlamydomonas reinhardtii*.[5, 6] Using classical chemical mutagenesis and metronidazole-selection techniques, a number of H_2-producing O_2-tolerant mutants of *Chlamydomonas reinhardtii* (e.g., 155G6, 141F2, 76D4, and 104G5) have been recently generated at National Renewable Energy Laboratory (NREL). According to the short-term O_2-tolerance assays that were conducted at NREL, mutants 76D4 and 141F2, respectively, had 4 and 9 times higher O_2 tolerance than did the wild-type parental strain. Mutant strains 104G5 and 155G6 reportedly have an O_2 tolerance 13 times higher than that of the wild-type parental strain.[7] In a collaborative effort with Drs. M. Seibert and M. Ghirardi of NREL, all of these mutants were sent to our Oak Ridge National Laboratory (ORNL) for O_2-tolerance assays with continuous exposure to low concentrations of O_2. The experimental results showed that none of the mutants possess any sustained O_2 tolerance under the steady-state assay conditions. A typical experimental result is presented in Fig. 5 for mutant strain 155G5 and its wild-type parent 137c. When the carrier gas was

shifted from pure helium to 1000-ppm O_2 in helium, H_2 photoevolution decayed at essentially the same rate—from about 13 to 1 µmol H_2/mg Chl^{-1}·h^{-1}—in both mutant155G6 and the wild type137c. This result indicates that mutant 155G6 does not possess improved O_2-tolerant properties under these steady-state conditions. However, the spike (the initial rate) of H_2 production immediately followed the onset of the actinic illumination after a 2-h dark period was higher in 155G6 than in 137c. The observed higher initial rate in 155G6 compared with that of 137c seems consistent with results of NREL's short-term O_2-tolerance assays, which may reflect the O_2 tolerance of the hydrogenase per se. The H_2 photoevolution (Fig. 5) showed that the spike was followed by a quick oscillation and then a quite steady decay in the rate of H_2 production in both 155G6 and 137c. Also, both of the strains responded to the addition of 5 µM FCCP and resulted in dramatic relief of O_2 inhibition on the H_2 photoevolution. These similarities between155G6 and 137c in the response to the presence of the continuous background (1000-ppm) O_2 and to the addition of FCCP also suggested that the observed inhibition in the stready-state H_2 photoevolution was due to the newly discovered oxygen sensitivity that is alternative to the O_2 sensitivity to the hydrogenase per se.

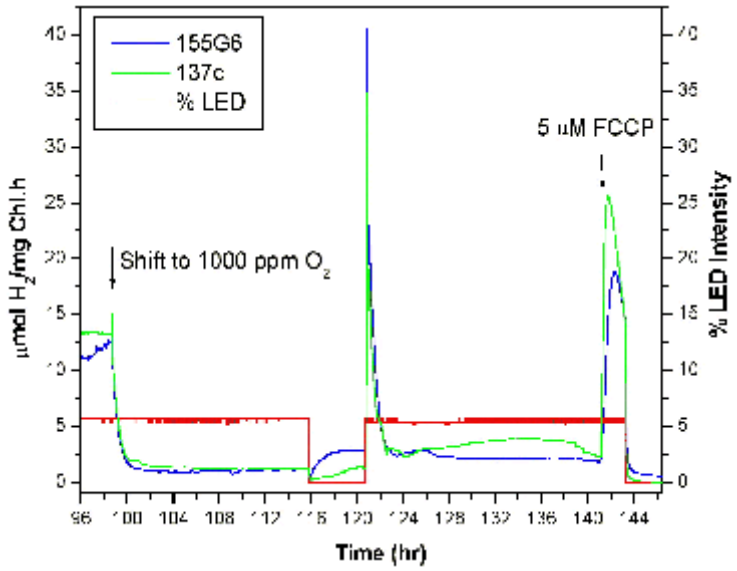

Figure 5. O_2-tolerance assay with NREL mutant 155G6 and the wild-type parental strain 137c.

Kinetic Characterization of the New O_2 Sensitivity

This newly discovered O_2 sensitivity was further characterized by using a series of different O_2 concentrations. The experimental results showed that introduction of 100-ppm O_2 had no significant effect on the steady-state rate of H_2 photoevolution in 137c (Fig. 6). However, addition of 300-ppm O_2 began to show some inhibitory effect on H_2 production (Fig. 7). When the O_2 concentration was raised to 5000 ppm, the inhibition on H_2 production was dramatic and the rate H_2 photoevolution decreased to nearly zero (Fig. 8). However, the

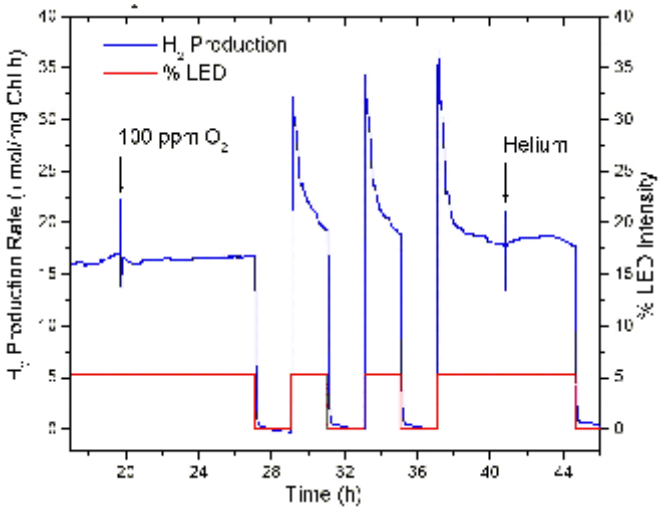

Figure 6. Wild-type algal H_2 production can tolerate 100-ppm O_2.

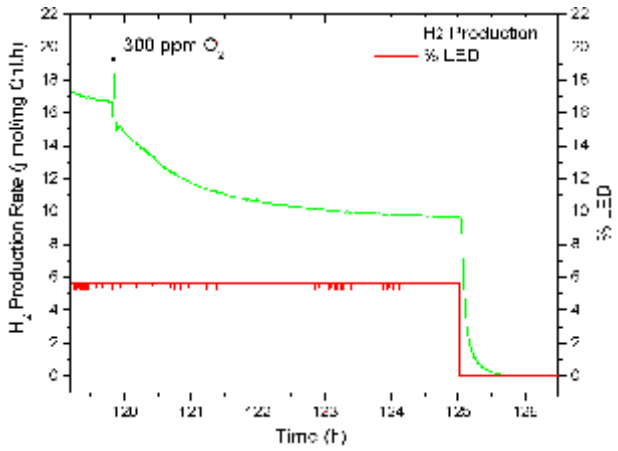

Figure 7. Introduction of 300-ppm O_2 leads to partial inhibition of algal H_2 production.

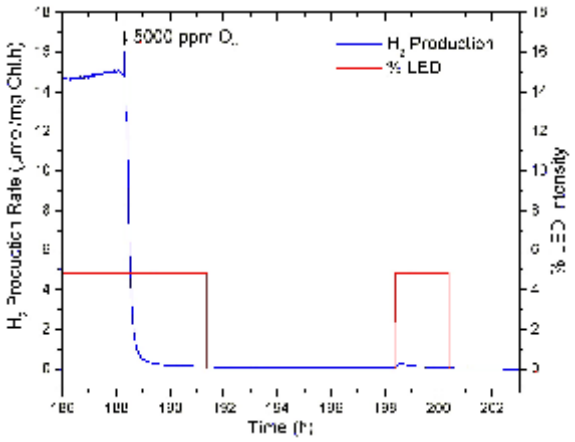

Figure 8. Introduction of 5000-ppm O_2 has dramatic inhibition of H_2 production, but the hydrogenase remains active.

hydrogenase in the algal cells was still active even after the continued presence of 5000 ppm O_2 for more than 10 h. When the actinic was turned on again after hour 198, a small peak of H_2 photoproduction was observed. As illustrated in the expanded scale (Fig. 9), this H_2 photoproduction peak was clearly above the background noise and/or dark-H_2 signal, indicating the presence of active hydrogenase in the algal cells. Therefore, the hydrogenase in the wild-type cells can tolerate up to 5000 ppm of O_2.

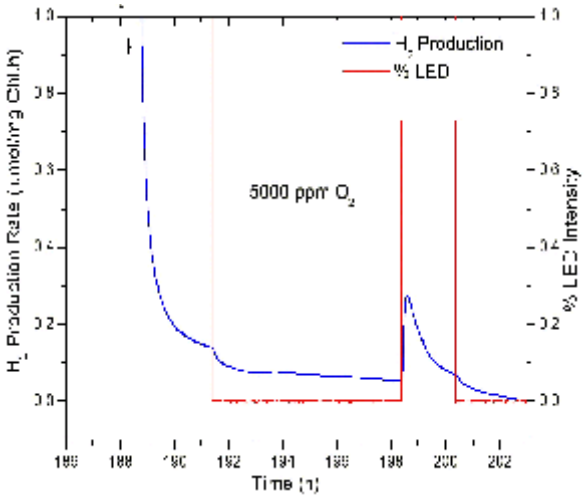

Figure 9. Expanded scale of Fig. 8, showing a clear peak of H_2 photoevolution after 10 h of continued presence of 5000-ppm O_2.

Figure 10 plots percentage of steady-state H_2-production rate against the background O_2 concentrations in the mutant 141F2 and the wild-type 137c. This result indicates that compared with the wild type, mutant 141F2 does not possess improved O_2-tolerant properties under these steady-state conditions. Thus, with respect to this newly discovered O_2 sensitivity, mutant 141F2 showed no more tolerance to O_2 than its wild-type parental strain137c. The O_2 concentration that gave 50% inhibition of H_2 photoevolution was about 500 ppm for both the mutant and the wild type.

Conclusions

We have discovered a new O_2 sensitivity in algal H_2 production that is different from the O_2 sensitivity of the hydrogenase. The principal findings serving as evidence of this new O_2 sensitivity are as follows: (1)

the unexpected surge of H_2 photoevolution immediately after the onset of actinic illumination in the continued presence of low concentration of O_2; (2) the relief effect of FCCP on O_2 inhibition of H_2 photoevolution; (3) the absence of tolerance to the continued presence of the background O_2 in NREL's O_2-tolerant mutants; and (4) the observation of hydrogenase activity at O_2 concentrations as high as 5000 ppm, where the steady-state H_2 photoevolution was inhibited almost completely.

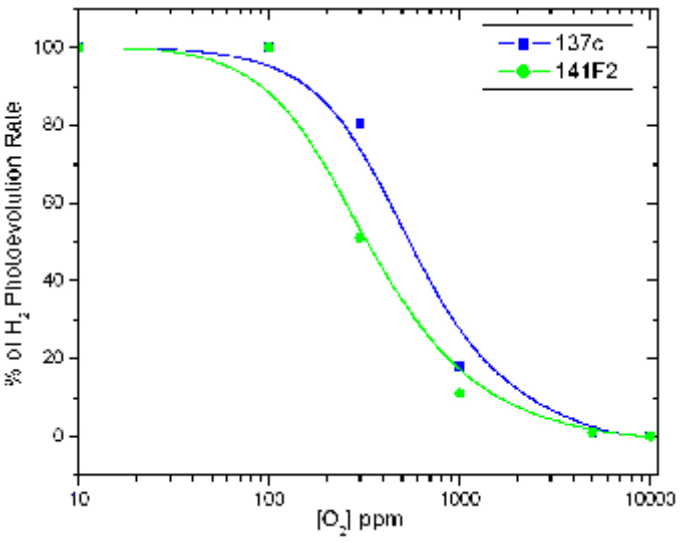

Figure 10. Effect of background O_2 concentrations on steady-state H_2 photoevolution.

This newly discovered O_2 sensitivity is apparently linked to the photosynthetic H_2 production pathway that is coupled with proton translocation across the thylakoid membrane. Addition of the proton uncoupler (FCCP) can eliminate this mode of O_2 inhibition on H_2 photoevolution. This mode of O_2 inhibition on H_2 production is likely due to the background O_2, which apparently can serve as a terminal electron acceptor in competition with the H_2 production pathway for the photosynthetically generated electrons from water splitting. This O_2-sensitive H_2-production electron transport pathway can be inhibited by DCMU. Our experiments demonstrated that this alternative mechanism is more sensitive to O_2 than the O_2 sensitivity of the hydrogenase. These findings redefine oxygen tolerance in algal H_2 production. Future work should focus on mapping this alternative electron transport pathway and on developing a technique to control this pathway to enhance the production of H_2.

Future Work

Our future work will focus on mapping this alternative electron transport pathway and on developing a new approach to enhance the production of H_2 based on the new findings.

Mapping Out the Pathway

As discussed previously, this newly discovered O_2 sensitivity apparently represents a new pathway in the photosynthetic H_2 production that is coupled with proton translocation across the thylakoid membrane. As illustrated in Fig. 11, the site for the reduction of O_2 could be at the RuBisco enzyme, which can serve as both a RuDP carboxylase and/or a RuDP oxygenase in the Calvin cycle. Under the conditions for H_2 photoevolution where CO_2 is not present and ATP is abundant owing to the associated photophosphorylation, the Calvin-cycle enzymes are fully activated and RuBisco could act as a strong oxygenase. This hypothesis can explain how FCCP mitigates the O_2 inhibition of H_2 photoevolution, since

the operation of the Calvin cycle requires formation of ATP using the proton gradient across the thylakoid membrane. Another possible site for the O_2 interaction could be at Ferredoxin (Fd), which could also serve as the electron donor to O_2 (as in the classic Mehler reaction). More experimental studies are needed to completely elucidate the O_2-sensitive H_2-production electron transport pathway. We plan to elucidate this pathway by using more chemical inhibitors and a RuBisco-deficient mutant of *Chlamydomonas reinhardtii* in our experimental studies. RuBisco-deficient mutants, such as strain 76-5EN, which was generated by chemical mutagenesis, are now available. We will obtain RuBisco-deficient mutant 76-5EN from the *Chlamydomonas* Genetic Center at Duke University. Use of this mutant in our future assays should make it possible to confirm whether RuBisco is indeed the site where the background O_2 could act as a terminal sink for the photosynthetically generated electrons and thus reduce H_2 production because of the nonproductive drainage of the reducing power (electrons). If RuBisco is indeed the site where the background O_2 enters the pathway, the H_2 photoevolution in a RuBisco-deficient mutant is expected to have a sustained tolerance to the background O_2.

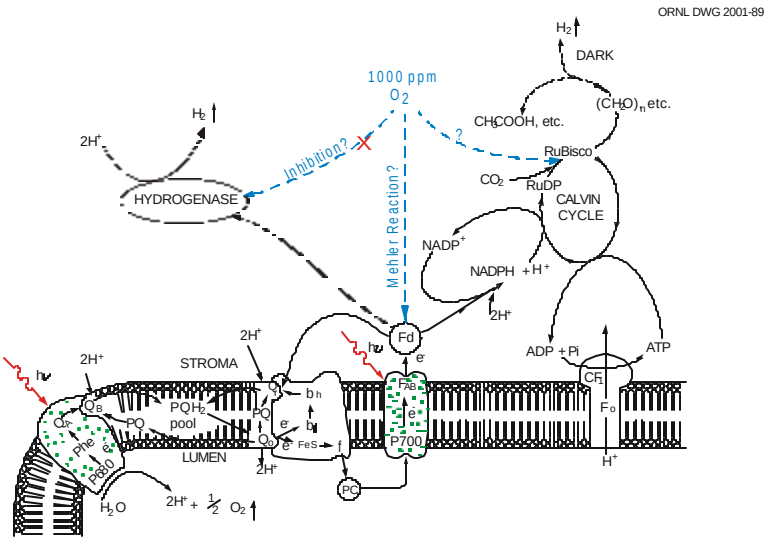

Figure 11. The newly discovered O_2 sensitivity is likely due to the background O_2 (at about 1000 ppm levels) acting as a terminal sink, in competition with the Fd/hydrogenase H_2 production pathway, for photosynthetically generated electrons.

Developing a New Approach to Enhance H_2 Production

Our experimental studies have demonstrated that addition of the proton uncoupler FCCP can enhance H_2 photoevolution by eliminating or reducing the activity of the newly discovered O_2 sensitivity. If the site of O_2 entry is indeed at the RuBisco enzyme, then a RuBisco-deficient mutant could, theoretically, also be free of this O_2 sensitivity for H_2 production. However, a RuBisco-deficient mutant cannot grow photoautotrophically and requires organic substrate for its photoheterotrophic growth. Moreover, it lacks the ability to overcome the problem of proton-gradient buildup that impedes the linear electron transport from PSII water splitting to the Fd/hydrogenase in H_2 photoevolution.[8] Therefore, it is probably impractical to use such a RuBisco-deficient mutant for large-scale H_2 production.

Addition of proton uncouplers such as FCCP could eliminate both the problems of back-proton accumulation and the newly discovered O_2 sensitivity. However, most of these chemical proton uncouplers, such as FCCP, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and anilinothiophene,

have undesirable side effects such as the ADRY effect that can damage the photosynthetic activity in algal cells. Also, these chemicals are hazardous materials that are environmentally unacceptable for large-scale applications. Therefore, we propose a technique to overcome these problems in a more effective way.

This new approach is to insert a piece of DNA that contains a hydrogenase promoter and a CF1 suppressor (or a proton-channel gene) into a host alga as illustrated in Fig. 12. This DNA insertion can be achieved by using the techniques of gene transformation. The CF1-suppressor or proton-channel gene will not be expressed under aerobic conditions since the hydrogenase promoter is activated only under anaerobic conditions. Therefore, the transformed alga should be able to perform autotrophic photosynthesis using ambient air CO_2 as the carbon source and grow normally under aerobic conditions such as in an open pond. When the algal culture is grown and ready for H_2 production, the CF1-suppressor or proton-channel gene can then be expressed simultaneously with the induction of the hydrogenase enzyme under anaerobic conditions. The expression of the CF1 suppressor should create CFo, which could act as a free proton channel without the CF1 cap, thus dissipating the proton gradient across the thylakoid membrane without ATP formation (Fig. 13). The effect of the free CFo channel on H_2 photoevolution could be twofold. (1) The accumulation of proton gradient that impedes the photosynthetic electron transport from water to Fd/hydrogenase could be prevented; and (2) The newly discovered O_2 sensitivity could be avoided by

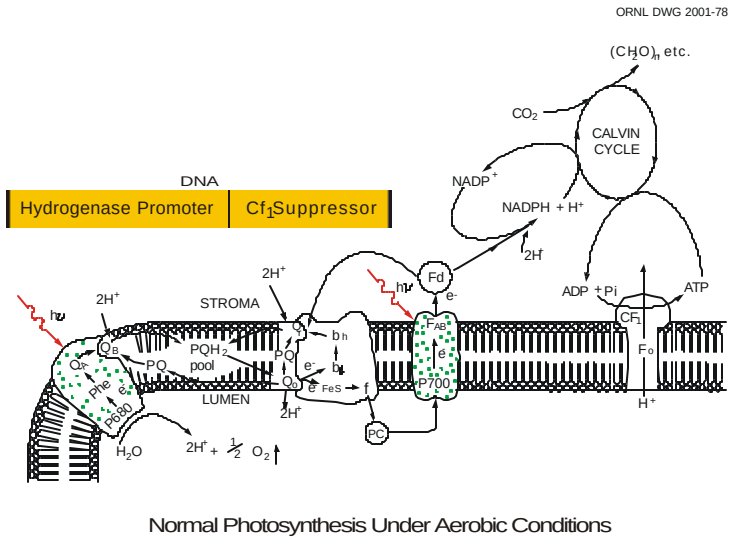

Figure 12. A transformed alga that contains the DNA of a hydrogenase promoter and a CF1 suppressor could grow photoautotrophically using ambient air CO_2.

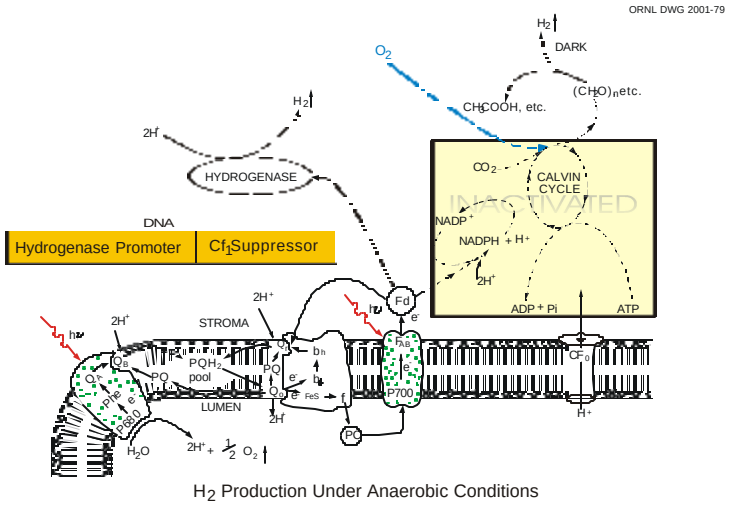

Figure 13. Expression of the CF1 suppressor to create free CFo (without CF1 cap) simultaneously with induction of the hydrogenase in the transformed alga. CFo could serve as a proton channel to dissipate the proton gradient to enhance H_2 production.

eliminating the photophosphorylation that is required for the activation of the Calvin cycle and the RuDP-oxygenase activity of the RuBisco enzyme (assuming that the entry of the background O_2 is indeed at the RuBisco).

Membrane proton channels made of synthetic polypeptides have already been demonstrated. From the known polypeptide sequence, a corresponding DNA sequence for a polypeptide proton channel can now be constructed. Therefore, construction and transformation of a vector that contains the hydrogenase promoter and a synthetic DNA sequence for the polypeptide proton channel are probably more achievable. Expression of such a polypeptide proton channel (Fig. 14) should provide the same benefits to enhance H_2 photoevolution as those from the free CFo channels (Fig. 13). Our collaborator, Prof. Laurie Mets of the University of Chicago, has already cloned a hydrogenase promoter. In our future work, we will focus on the construction and transformation of a vector that contains the hydrogenase promoter and a synthetic DNA sequence for the polypeptide proton channel into *Chlamydomonas reinhardtii* DSP521. DSP521 is a genetically modified strain for which we have experimentally demonstrated the higher overall photon-utilization efficiency which is attributed to its smaller chlorophyll-antenna size in comparison with that of its wild-type parental strain DES15.[9] Coordinated expression of the polypeptide proton channel (or CF1 suppressor) simultaneously with the induction of the hydrogenase would transform algal cells into inexpensive, effective, and environmentally friendly machines for production of H_2 by photosynthetic water splitting.

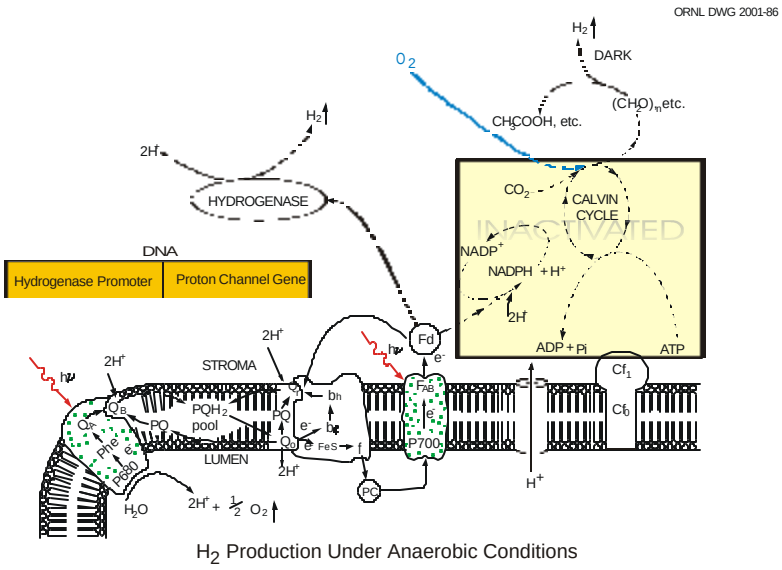

Figure 14. Development of an efficient algal H_2-production system by construction and transformation of a vector that contains the hydrogenase promoter and a piece of synthetic DNA for a polypeptide proton channel. The transformed alga could grow normally using ambient air CO_2 under aerobic conditions without the polypeptide proton channel, which could be expressed only with the induction of the hydrogenase under anaerobic conditions when its function is needed for enhanced H_2 production.

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