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BIOLOGICAL H_2 FROM FUEL GASES AND FROM H_2O

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Abstract

When photosynthetic bacteria *Rhodocyclus gelatinosus* and *Rhodospirillum rubrum* were cultured under an atmosphere containing CO, a unique CO-linked pathway was quickly induced. These bacteria catalyze the water-gas shift reaction where CO (and H_2O) is oxidized to produce H_2 (and CO_2). The hydrogenase enzyme responsible for the H_2 production pathway is inducible only upon CO addition, and is a different enzyme from the formate-linked and the uptake hydrogenases in terms of induction criteria and electron mediator it can use. The CO-linked hydrogenase favors toward the H_2 evolution direction with a reaction rate-constant of greater than 60,000, therefore, is an ideal candidate for scale-up H_2 production. Water is ultimately the most abundant and inexpensive source of hydrogen on earth. A system of sustained H_2 production based on the photo-oxidation of water would be most economical. Since O_2 production is inherent in the oxidation of water, and the hydrogenase enzymes in most cyanobacteria and green algae are rapidly inactivated by O_2, the O_2-tolerant CO-linked hydrogenase enzyme is most suited if it can be transformed into a cyanobacterium where both O_2 and H_2 are produced simultaneously. To achieve this long-term goal of constructing a cyanobacterial-bacterial hybrid, we must devise assays to allow for the measurement of O_2 tolerance of the CO-linked hydrogenase with the simultaneous presence of O_2. This report documents an ongoing method development using a continuous sampling mass spectrometer to kinetically record the impact of O_2. Another issue needs to be addressed is the separation of the multiple hydrogenases in these organisms in order to specifically measure only the hydrogenase of interest. This report presents evidences on using the combinations of three strategies: via physiology, mutation and biochemical means, we achieve to spatially separate the three hydrogenase enzymes and to study the physiology of the enzymes and the electron mediators to which each can couple.

Introduction

Photosynthetic bacteria are versatile in their modes of H_2 metabolism. They have four terminal enzymes mediating the H_2 metabolism; they are nitrogenase, a classical uptake hydrogenase, a fermentative hydrogenase and a CO-linked hydrogenase. Nitrogenase is induced under N-free condition and catalyzes H_2 production while an abundance of energy is available (Stewart, 1973). This enzyme system is light-dependent and consumes 4 moles of ATP per mole of H_2 produced. A classical uptake hydrogenase has been isolated from various photosynthetic microbes and its main function is to break down H_2 to support CO_2 fixation (Colbeau *et al*., 1983). A hydrogenase linking to formate oxidation has also been identified and its main function is to dissipate excess electrons under fermentative dark growth mode (Gorrell and Uffen, 1977; Schultz and Weaver, 1982). This enzyme is extremely O_2 sensitive, with a half-life of less than 1 min when whole cells were stirred in full air, it equilibrates at 10% partial pressure of H_2 (Maness and Weaver, unpublished data), and is not suitable for scale-up application.

The fourth enzyme, the CO-linked hydrogenase, is the most unique among all hydrogenase reported. It was first reported by Uffen in two strains of photosynthetic bacteria to shift CO (and H_2O) in darkness to H_2 (and CO_2) (Uffen, 1981). We have since isolated 450 strains of photosynthetic bacteria possessing this water-gas shift pathway. Among them, *Rhodocyclus gelatinosus* CBS-2 is most unique in that its hydrogenase is highly resistant to O_2, with a half-life of 19 hours when whole cells were stirred under full air. When the partially purified hydrogenase devoid of membrane was stirred in full air, a half-life of 4.5 hr was measured, clearly indicating that its O_2 tolerance is due to the intrinsic nature of the enzyme conformation, not owing to higher respiratory rates. This enzyme has a rate constant greater than 60,000 and is energetically more favorable toward the H_2 evolution direction. Compared to most hydrogenase systems, the CO-linked pathway is most robust and has great potential for commercial applications.

One requirement for economical H_2 production is to further enhance its O_2 tolerance in order to transform this enzyme system into a cyanobacterial hybrid, where H_2 is evolved using H_2O as the ultimate electron donor with the simultaneous production of O_2. Therefore, an ideal condition to quantify O_2 tolerance would be to measure the immediate impact of O_2 addition, and also to determine the rate and duration of H_2 production with O_2 present. Although we have determined previously that the CO-linked hydrogenase is quite tolerant to O_2, a drawback of the assay (using the Na-dithionite-reduced methyl viologen as mediator) prevents us from measuring activity while O_2 is present. Therefore we had routinely pre-exposed the hydrogenase to O_2 for a pre-determined amount of time, followed by the removal of O_2, and then conducted the assay using Na-dithionite as the reducing agent. One might also argue that the subsequent addition of a reducing agent such as Na-dithionite would re-activate the hydrogenase previously inactivated by O_2, as a result, an O_2 tolerance is falsely claimed. To take these issues into account, we must develop an assay that not only excludes a reducing agent, but also allows us to monitor the rate continuously. Hydrogenases from various sources have been reported to catalyze an H_2 and D_2O exchange reaction yielding HD without requiring a reducing agent (San Pietro, 1957; Fauque *et al*., 1988; Vignais *et al*., 1997). A continuous

sampling system using a mass spectrometer thus is an ideal technique to meet these criteria.

Another issue that complicates the O_2 tolerance determination is the existence of multiple hydrogenases, each having a varying degree of tolerance to O_2. Since multiple hydrogenases can be induced simultaneously under certain growth mode, it's confusing for one to conclude which hydrogenase is actually displaying the property. Ideally one would purify the hydrogenase of interest to physically insulate it. However, most hydrogenase is labile, the purification process is time-consuming, and the yield is low. Alternatively, one could devise growth condition where only one hydrogenase is preferentially induced to be studied. Most ideally will be to generate mutants lacking all hydrogenases except the one of interest. In this report, we document the induction conditions for each hydrogenase: through physiology by manipulating the growth condition, through mutant isolation to preclude uptake hydrogenase, and via biochemical means to partially purify the CO-linked hydrogenase. By combining all three strategies, we can selectively study the CO-linked hydrogenase without complications from others. We also report the on-going development of a kinetic mass spectrometer protocol to meet the goal of characterizing the O_2 tolerance of the CO-linked hydrogenase, applicable also to other hydrogenase enzyme systems.

Materials and Methods

Growth Conditions, Membrane Preparations and Assays

Rhodocyclus gelatinosus CBS-2, *Rhodospirillum rubrum* strains S1, G9 and P1 were cultivated in modified RCV medium (Weaver *et al*., 1975). P1 was a mutant derived from G9 deficient in the H_2-uptake pathway (Maness and Weaver, 1999). Carbon source were 30 mM Na-malate or 15 mM fructose for photoheterotrophic growth, 16% CO along with 0.5% (w/v) yeast extract for CO-supported growth, or 30 mM Na-pyruvate for fermentative growth. Media were prepared by boiling and dispensing into anaerobe tubes under a stream of argon gas. $NaCO_3$ at 0.1% final concentration was added just prior to autoclaving.

To adapt cells to using higher concentration of CO for the CO/hemoglobin binding assay, approximately 160-ml of cells from a malate-grown photosynthetic culture were added to a 885-ml Parr pressure reactor. The headspace was replaced with argon gas, followed by adding 100% CO to 40 psig, and then to 68 psig with argon gas. The initial optical density (OD, at 660 nm) is 0.5. The suspension was then incubated in darkness with constant stirring under pressure. An aliquot of the suspension was withdrawn from the reactor outlet and diluted into 50 mM Tris buffer (pH 8.7) to approximately 0.1 OD for the CO uptake reaction.

Photosynthetic cultures were illuminated with a band of 40W incandescent lamps. Light intensity reaching the surface of cultures is approximately 100 W/m^2.

The preparations of cell-free hydrogenase extract, its partial purification, the hydrogenase assays, and dry weight determination were according to the Methods described in Maness and Weaver (1999).

CO/Hemoglobin Binding Assay

CO determination for the CO uptake reaction was measured by the CO/hemoglobin binding assay (Bonam *et al*.,1984). Stoppered test tubes containing 2.5 ml hemoglobin solution was made anaerobic with argon gas. The CO uptake reaction was carried out inside a 5-ml glass syringe containing 2-ml cell suspension and pre-incubated inside a 45C water bath to reach equilibrium. Two milliliter of 20% CO gas was then withdrawn into the syringe and shook vigorously for 10 seconds before the headspace was expelled to enhance mass transfer. At various intervals, 0.2 ml aliquot of the cell suspension containing various amounts of CO was injected into the hemoglobin solution. The total spectral changes between the peak at 419 nm and the trough at 433 nm were recorded with a Cary 4E spectrophotometer. A total change of 1.0 OD corresponds to 13.5 nmole of dissolved CO.

Results and Discussion

Variation in Hydrogenase Activities during Growth

A hydrogenase enzyme linking to CO oxidation has been reported by Uffen (1981) and Bonam *et al*. (1984) in two photosynthetic bacteria. We have observed a similar hydrogenase in both *Rhodospirillum rubrum* S1 and *Rhodocyclus gelatinosus* CBS-2 upon adding CO to the culture headspace. The appearance of this enzymes peaked when cells were in the mid-log phase of growth. No activity was measured when CO was not added. This CO oxidation pathway is postulated to play a role in either CO detoxification or in a novel CO-supported dark growth mode with the possible generation of energy (Champine and Uffen, 1987; Kerby *et al*., 1995). A second hydrogenase activity was also detected in both organisms coupling to the reduction of methylene blue. This uptake hydrogenase activity paralleled cell growth and leveled off at the stationary-phase of cell growth. This uptake hydrogenase is postulated to be involved in the H_2-supported CO_2 fixation. A third hydrogenase has been identified in *R. rubrum* S1 when light was limiting, as the culture became denser. This activity was linked to formate oxidation, it appeared later, and peaked at early-stationary phase of cell growth. This enzyme was proposed to dissipate excess energy during fermentative growth in darkness (Schultz and Weaver, 1982). The appearance of all three enzymes at difference phases of growth strongly suggests that they are three separate enzymes, each has its own physiological function. The presence of multiple hydrogenases also complicates their characterization as one can harvest cells at any stage of growth and one or more enzymes will be present.

Growth Conditions to Induce Individual Hydrogenase Activities

In order to characterize the CO-linked hydrogenase of interest to us without complications from the other hydrogenases, we have to understand the criteria for their induction in order to devise conditions where only the CO-linked hydrogenase is induced. This is especially important for the quantification of O_2 tolerance since the uptake hydrogenase is known to be highly tolerant to O_2. If uptake hydrogenase is present along with the CO-linked hydrogenase, then the former enzyme could attribute some of the O_2 resistance measured. Formate-linked hydrogenase is known to be extremely sensitive to O_2 and could also confuse the measurement. We therefore carried out experiments to investigate the induction criterion of each hydrogenase activity. A general approach is to add a suspected enzyme inducer along with a protein-synthesis inhibitor to see if *de novo* protein synthesis is involved. If the result is positive, then we have identified the inducer for each enzyme. Once the identity of an inducer is known, we can add an inhibitor to prevent its synthesis during growth such that the specific enzyme is not made.

Fig. 1A shows that when exogenous formate was added, a hydrogenase activity linking to formate oxidation appeared rapidly when assayed by the formate-linked H_2 production pathway. A very low background activity was detected in the control culture where formate was not added. Formate produced through normal fermentative metabolism probably accounts for the low levels of activity in this culture. Chloramphenicol, an inhibitor at the protein translation level, immediately stopped the appearance of formate-induced hydrogenase activity when added along with formate as shown in Fig.1A. This indicates that formate is required to induce a hydrogenase involved in formate oxidation. Thereby, formate is the inducer for this pathway. In theory, if an inhibitor is added to inhibit formate synthesis completely during growth, formate-linked hydrogenase will not be synthesized at all.

Identical culture supplemented with CO exogenously similarly synthesized a chloramphenicol-sensitive, CO-dependent, H_2 production activity (Fig. 1B). The result suggests that CO is the inducer in expressing a hydrogenase enzyme that is linked to CO oxidation. Similar results of a CO-inducible hydrogenase in *R. rubrum* had been reported previously by Bonam *et al*. (1989). Since CO is not a natural byproduct of normal cell metabolism, at least not at any measurable quantity, CO-linked hydrogenase is generally not present during growth. Consequently, the appearance of CO-linked hydrogenase can be easily manipulated.

With the identification of the different inducers for various hydrogenases, we can then devise growth condition where only the hydrogenase of interest is selectively induced. Under light-limiting condition, the cellular fermentative pathway produces trace amounts of formate, responsible for the induction of the low-level of formate-linked hydrogenase. When higher level of formate-linked hydrogenase is desirable, we can supplement the culture medium with formate to induce it to a much higher level. To repress this enzyme entirely, we must use an inhibitor to repress the synthesis of formate. Sodium hypophosphite (HPP), a formate analog, has been reported as a potent inhibitor of the

enzyme responsible for the cellular production of formate (Gorrell and Uffen, 1977; Thauer *et al*., 1972). With the addition of HPP, therefore, no formate should be produced. HPP had no effect on the formate-hydrogenlyase reaction in *R. rubrum* (Gorrell and Uffen, 1978). As expected, we have not detected any formate-linked hydrogenase activity in *R. rubrum* whenever 10 mM HPP was included during growth. The CO-linked hydrogenase activity can be selectively induced by supplementing 20% CO to the culture gas phase in the presence of HPP. Uptake hydrogenase activity can be maximally induced by growing cells photoautotrophically under H_2 and CO_2 in the presence of HPP. For all the experiments described below, cells were cultured under the appropriate condition (referred to as "induced" condition) so that each hydrogenase is singly induced. Table 1 summarized the induction conditions used to maximally induce the individual hydrogenase of interest through physiology manipulations.

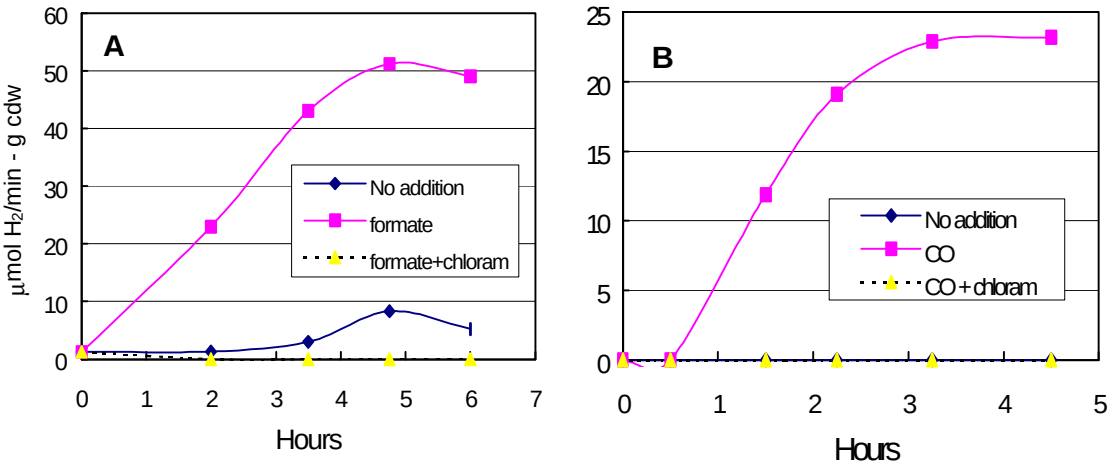

Figure 1 - The Effect of Chloramphenicol (chloram) on the Induction of the Formate-linked (A) and CO-linked (B) Hydrogenases

Table 1. Growth Conditions to Induce Individual Hydrogenase Activity

Hydrogenase	**Induction Condition**
CO-linked Hydrogenase	Add CO to a photosynthetic culture with Na-hypophosphite
Formate-linked Hydrogenase	Fermentative conditions, or add formate to a photosynthetic culture
Uptake Hydrogenase	Malate or H_2/CO_2 gas mixture with the presence of Na-hypophosphite

Electron Donor/Acceptor Mediating Various Hydrogenase Activities

All three hydrogenases can use methyl viologen (-450 mV vs. NHE) to mediate both the production and uptake of H_2. Uptake hydrogenase is known to use methylene blue (+11 mV vs. NHE) as the terminal electron acceptor (Adams and Hall, 1977; Colbeau and Vignais, 1981). Yet it is not known whether the CO-linked hydrogenase can also use methylene blue as the electron acceptor. To examine this, we partially purified the CO-linked hydrogenase from *R. rubrum* P1, a mutant from which nearly 98% of the uptake hydrogenase has been removed previously, and obtained fractions enriched in CO-linked hydrogenase (Maness and Weaver, 1999). These fractions were then assayed for activity linking to various electron mediators. As expected, we detected good rate of H_2 evolution from reduced methyl viologen, low rate of H_2 uptake to methyl viologen (its backward reaction); yet no H_2 uptake to methylene blue was detected at all (Fig. 2). Identical results were obtained when we repeated the experiment. These data suggest that an evolving hydrogenase preferentially couples to a low-redox mediator to produce H_2 whereas an uptake hydrogenase favors a high-redox mediator such as methylene blue for the uptake of H_2. The methylene-blue reduction activity can thus be used to specifically detect the presence of the uptake hydrogenase activity only. We can, therefore, use this procedure to obtain only the CO-linked hydrogenase and study its O_2 tolerance without interference from the uptake hydrogenase, which is known for its higher O_2 tolerance. Any observed tolerance to O_2 can consequently be attributed entirely to the CO-linked hydrogenase only.

Although coupling well with reduced methyl viologen in the H_2-evolution direction, it is not known whether the formate-linked hydrogenase can couple to methylene blue. We have previously determined that this fermentative hydrogenase is extremely sensitive to CO, while the uptake hydrogenase is not. We decided to study the formate-linked hydrogenase from *R. rubrum* P1 since the bulk of its uptake hydrogenase has been removed by us. By culturing P1 with Na-pyruvate in darkness, the fermentative hydrogenase can be selectively induced. This culture still contains a low level of uptake hydrogenase coupling to methylene blue. We then determined that 14.8% of CO inhibits nearly 85% of the hydrogenase activity in the evolution direction using reduced methyl viologen; yet the traces of H_2 to methylene blue activity are not affected at all, even at 28% of CO. If the formate-linked hydrogenase were able to couple to methylene blue, CO would have affected its activity to the same extent. This result implies that the fermentative hydrogenase does not normally couple to methylene blue as the electron mediator. Since uptake hydrogenase is always induced in a growing culture, this finding allows one to use methylene blue as the electron mediator to specifically detect its presence. Table 2 summarizes the various electron mediators to which each hydrogenase couples.

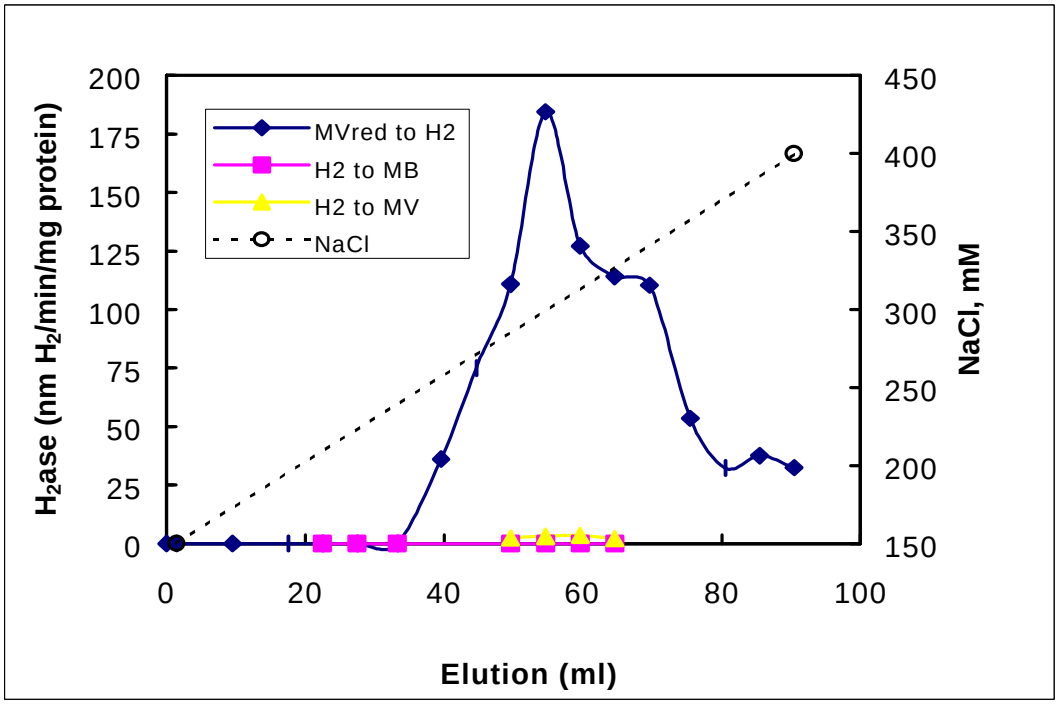

Figure 2 - Partial Purification of CO-linked Hydrogenase from *R. rubrum* P1

Table 2. Electron Mediators Mediating Various Hydrogenase Activities

Hydrogenase	MV_{red} to H_2	H_2 to MV	H_2 to MB
CO-linked	+	+	—
Formate-linked	+	+	—
Uptake	+	+	+

The Development of a H_2 – D_2O Exchange Assay

Using a mass spectrometer to measure a hydrogenase by its H_2-D_2O exchange activity, we have previously determined that the partially purified CO-linked hydrogenase from *R. rubrum* P1 was partially functional in the presence of 1% and 7% of O_2. The inhibition was more than 70% reversible upon the subsequent removal of O_2 from the headspace (Maness and Weaver, 1999). However, the assay was conducted during a 20-min incubation at batch mode with or without O_2. It will provide very valuable information if we were to develop a kinetic assay such that the impact of O_2 can be measured

immediately upon its addition. Knowing how to separate the three hydrogenases from each other, this year we set out to develop a kinetic assay. Preliminary work with a direct sampling mass spectrometer sampled at 5 second interval showed evidence of exchange, but at a very low level. This was partly due to the high flow rates of H_2 required (5 ml/min) for adequate sampling introduction, which dilute the reaction products in the headspace. The other explanation is that our CO-linked hydrogenase has a very high forward/backward ratio of reaction rates with regard to methyl viologen. The initiation of the exchange reaction mandates the H_2 molecule to be broken down first, a rate-limiting step for our hydrogenase, prior to exchange with the protons or deuterium ions in the medium to yield HD. Similar observations have also been recorded where a hydrogenase more favorable for H_2 production was reported to have a lower rate of D_2 exchange activity (Arp and Burris, 1981; Fox *et al*.,1996). To circumvent, we are presently developing techniques using a mass spectrometer equipped with a capillary leak for sample introduction. The capillary leak provides continuous sampling of reactor headspace at ambient pressure. An initial H_2 concentration of 3% was included in the headspace so that no continuous bubbling of H_2 is required. The work is currently in progress.

The Enhancement of the CO Shift Activity

To make this process for H_2 production more economical, one of our goals is to increase the rate of CO shift to H_2. We accomplished this by measuring the shift activity at very low cell density (< 0.1 OD) using cells adapted to higher CO concentration, by increasing mass transfer, and by conducting the assay at its optimal temperature of 45C using the CO/hemoglobin binding assay. Fig. 3 shows the kinetics of CO uptake by *R. gelatinosus* CBS-2 with a rate of 8.3 mmole/min-g cell dry weight, calculated from the data points between the 4 and 10 second interval. Zero time measurement was determined separately where no cells were added. Previously we determined that the *Ks* of the shift reaction is around 5.9 μM of CO. Therefore the rate derived is an underestimation since even at 10 seconds the CO substrate is already at near the rate-limiting level. In future experiments, we will conduct multiple assays and for each we only measure CO concentration at one time interval. Kinetics are then constructed accordingly to a get more accurate rate.

Conclusion

Based on the data from cell physiology, biochemical purification and genetic studies, we have demonstrated that in *R. rubrum*, there are at least three separate hydrogenases, and each can be maximally induced at a different stage of growth. The induction study using a protein synthesis inhibitor chloramphenicol provides evidence that the induction initiates a *de novo* protein synthesis. Based on the induction conditions acquired for each hydrogenase, we were able to devise growth conditions to preferentially induce one hydrogenase over the others in order to characterize various physical and biochemical properties specific to the hydrogenase of interest without interferences from the others. The results of the mutant study of *R. rubrum* P1 further confirm that the induction of both the CO-linked and the formate-linked hydrogenases do not rely on the presence of the

uptake hydrogenase enzyme. Since the uptake hydrogenase co-induces with the CO-linked hydrogenase during the entire growth cycles on CO, and the uptake hydrogenase also co-purifies with the CO-linked hydrogenase, prior removal of the uptake hydrogenase in mutant P1 is critical in order to study the CO-linked hydrogenase only. Indeed, when the CO-linked hydrogenase is partially purified from P1, we were able to determine that the CO-linked hydrogenase does not couple to methylene blue, a high-redox dye known to be readily reducible by the uptake hydrogenase. This finding gives us a specific assay for the uptake hydrogenase and allows us to determine the purity of the CO-linked hydrogenase from the uptake hydrogenase enzyme.

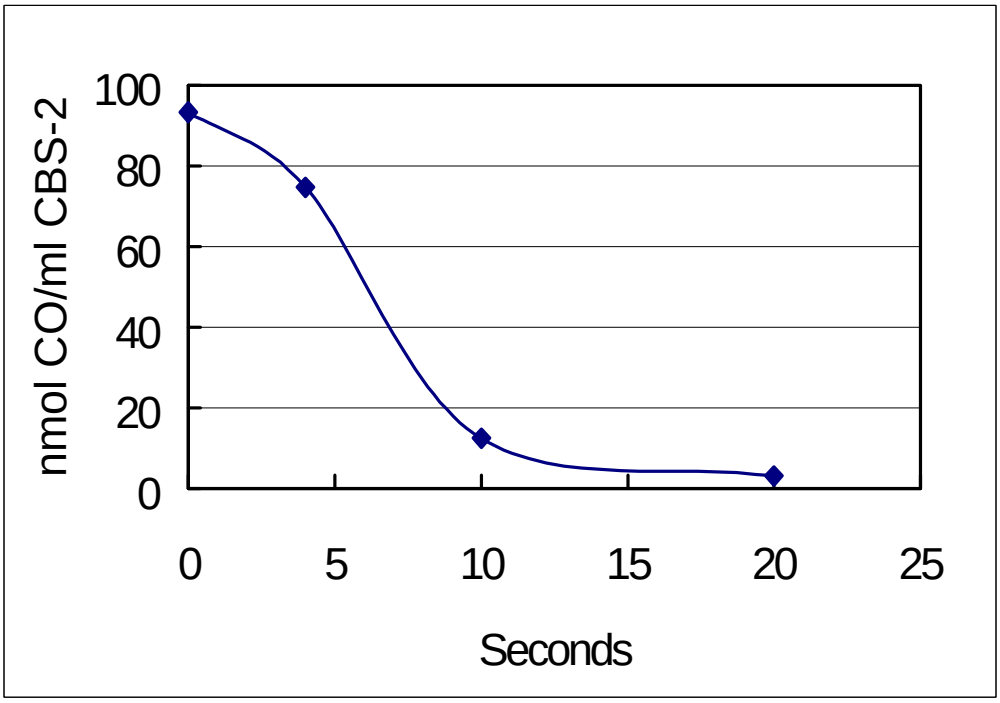

Figure 3 – Kinetics of CO Uptake by *Rhodocyclus gelatinosus* CBS-2

The development of a H_2-D_2O exchange assay using a mass spectrometer is essential for us to determine whether a hydrogenase can function in the presence of O_2. Knowing how to obtain a CO-linked hydrogenase fraction without complications from the other hydrogenases, this report documents preliminary findings on the exchange reaction. Due to the irreversible nature of the CO-linked hydrogenase favoring more in the H_2 evolution direction, this hydrogenase may not be the ideal candidate for the exchange assay. However, we can perhaps overcome this limitation by using a capillary mass spectrometer that provides continuous sampling of the reactor headspace without the constant flowing of a H_2 gas stream, which dilutes the signals significantly. This work is currently in progress.

Future Work

The long term goals of this project is to characterize the bacterial CO to H_2 shift reaction at both the biochemical and genetic levels so that both the reaction rates and durability of H_2 production can be enhanced. The characterization of the genetic systems of the hydrogenase complex also facilitates its subsequent transfer and expression into a cyanobacterium so that both H_2 and O_2 can be simultaneously produced from the water-splitting reaction. To enhance reaction rates and durability, we need to understand the regulation and physiology of the overall CO to H_2 pathway at the biochemical and physiological levels. By identifying the individual components involved, we can then determine the rate-limiting step. At the genetic levels, we plan to improve mutant selection/enrichment techniques in order to obtain a series of mutants deficient in the various components of the CO to H_2 pathway. By examining their revertants, we can then seek out those overly expressed mutants that synthesize the CO-linked hydrogenase enzyme constitutively. We will also initiate work in identifying and cloning the CO-linked hydrogenase gene in order to understand its regulation at the molecular genetics levels. This will allow us to manipulate the genetic capacity for H_2 production activity by making multiple copies of the hydrogenase gene. Cloning the hydrogenase gene will also enable us to construct a plasmid carrying this gene and later transform the plasmid into a cyanobacterium to meet our long-term goals of producing H_2 from the water-splitting reaction.

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