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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 with CO in the gas phase, a CO-linked pathway was quickly induced. These bacteria perform a water-gas shift reaction with CO and H_2O, and produce H_2 and CO_2 in nearly stoichiometric quantities. The hydrogenase, a terminal enzyme of the linked pathway, is extremely O_2 resistant. In order to further investigate its tolerance to O_2, a mutant deficient in the uptake hydrogenase was isolated from a blue-green mutant of *R. rubrum* G9. Upon further purification, we obtained a fraction containing only the CO-linked hydrogenase. This allows for the determination of the inhibitory effect of O_2 on this hydrogenase without interference from uptake hydrogenase. Using a mass spectrometer to measure the H_2/D_2O exchange reaction, we confirmed that the hydrogenase was operative even with the simultaneous presence of up to 7% of O_2 for 20 min. More than 70% of the reaction rate was recovered upon the removal of O_2, a reversible property not normally observed by hydrogenases from most organisms. The addition of CO to a starved culture also enhanced the hydrogenase reaction rate significantly, although lesser effect was observed with the overall linked pathway. CO, or its oxidized product, may have a stimulatory effect on the longevity of the hydrogenase enzyme. This is not an issue in a scale-up bioreactor system where CO will be fed continuously. This report also proposes a scheme for the isolation of mutants deficient in various components of the CO-linked pathway in order to understand the reaction mechanism and the rate-limiting steps.

Introduction

Photosynthetic bacteria are versatile in their various modes of H_2 metabolism. They have four terminal enzymes that mediate their H_2 metabolism – nitrogenase, a classical uptake hydrogenase, a hydrogenase linking to fermentative pathway, and a CO-linked hydrogenase; all are involved in H_2 production under various growth conditions. Under photosynthetic conditions when nitrogen becomes limiting while an abundance of carbon substrates are available, the nitrogenase enzyme catalyzes the production of H_2 to dissipate excess reductants (Stewart, 1973). The nitrogenase-mediated system is light-dependent and consumes 4 moles of ATP per mole of H_2 produced. Long term growth of culture under limiting nitrogen condition also induce the nif$^-$ strains to emerge, therefore, losing the ability to produce H_2 for a prolonged period. A classical uptake hydrogenase has been isolated and purified from various photosynthetic microorganisms, and its main physiological function is to break down H_2 to support CO_2 fixation. This enzyme operates preferentially in the H_2 consumption direction (Colbeau *et al*., 1983). A third hydrogenase, the formate-linked hydrogenase, part of the formate-hydrogenlyase complex, has been reported in *Rhodospirillum rubrum* S1. This hydrogenase, induced when light becomes limiting (natural day/night cycle), is proposed to function under fermentative conditions when excess energy needs to be dissipated in order to maintain redox balance (Gorrell and Uffen, 1977; Schultz and Weaver, 1982). This hydrogenase is similar to the reversible hydrogenase reported in *Clostridium*, is highly O_2 sensitive and equilibrates at low partial pressure of H_2 (10%). To fully utilize this hydrogenase for industrial H_2 production mandates an absolute anaerobic condition, and the H_2 accumulated in the gas phase would have to be evacuated to ensure continuous production.

The CO-linked hydrogenase is unique among all hydrogenases reported. It was first reported by Uffen in two strains of photosynthetic bacteria that function only in darkness to shift CO (and H_2O) into H_2 (and CO_2), and the H_2 remained accumulated in the gas phase (Uffen, 1981). We have since isolated more than 450 strains of photosynthetic bacteria from local sites with elevated CO that perform the water-gas shift reaction. Among them, we have worked with *Rhodocyclus gelatinosus* CBS extensively. CBS and its variant, CBS-2, adapted for higher O_2 resistance, not only shift CO as Uffen had reported, but also quantitatively assimilate CO into new cell mass (Maness and Weaver, 1994). Upon being given 20,000 ppm of CO in the culture gas phase, a mere 0.1 ppm of CO remained at equilibrium, making this process suitable for producing high purity H_2 feeding directly to fuel cell applications. The CO-linked pathway has a rate constant of 60,000 and is energetically more favorable toward the evolution direction. The CO-linked hydrogenase is also unique in its O_2-resistant property. When whole cells of CBS-2 are stirred in 21% O_2 (full air) for 19 hours, the hydrogenase still retains 50% of its activity (Maness and Weaver, 1997). This membrane-bound hydrogenase has been purified 86 fold from other membrane proteins and exhibits a molecule weight of 58,000 daltons. When stirred in full air, this partially purified, membrane-free hydrogenase still exhibited a half-life of 4.5 hours, indicating that the resistance to O_2 is an intrinsic property of the enzyme itself, not owing to

higher respiratory rates of whole cells, nor its burial within cell membranes. Compared to most hydrogenase enzymes which have a half-life of 30 seconds in air and are irreversibly inactivated (Klibanov *et al*., 1978), this CO-linked system is most robust and has great potential for commercial scale-up of H_2 production from gasified biomass.

An ideal process to produce H_2 more economically would be water-derived. However, lacking photosystem II, photosynthetic bacteria can not use water as the electron donor. One potential solution is to transfer the genes coding for hydrogenase enzyme, along with its physiological electron mediators into a cyanobacterial host to be expressed. Cyanobacteria have both photosystem I and II, and can therefore oxidize water and evolve O_2. The bacterial CO-linked hydrogenase is relatively O_2-resistant and will sustain. Cyanobacteria are phylogenetically closely related to photosynthetic bacteria, and thus most likely that their native electron mediators may couple to a bacterial system. This approach would require the cloning of the bacterial hydrogenase genes along with those coding for the redox mediators. Pursuing this also will facilitate our understanding of the regulation of the pathway and the amplification of overall rates. This report describes preliminary work on the isolation of a hydrogen uptake pathway (Hup^-) mutant and proposes a scheme for other mutant isolation.

Another criterion for economical H_2 production is to further enhance the O_2 resistance of the hydrogenase. Although we have determined previously that this hydrogenase is quite resistant to O_2 inactivation, the nature of the assay (using a reducing agent and methyl viologen) precludes the addition of O_2 during the assay. It is ultimately important for us to develop an assay system where the reaction rates of hydrogenase could be measured with the simultaneous presence of O_2. To achieve this, we take advantage of an exchange reaction between H_2 gas and heavy water yielding HD, catalyzed by most hydrogenase (San Pietro, 1957). Because this assay does not involve any electron donors and acceptors, it becomes a direct assay of the enzyme itself. Any resistance or inhibitory effect of O_2 on the enzyme thus is a direct reflection on the nature of the enzyme. We describe in this report findings of O_2 effect on the exchange reaction and the reversibility of the reaction upon the removal of O_2, as measured by a mass spectrometer.

Materials and Methods

Media and Growth Conditions

Rhodocyclus gelatinosus CBS-2 and *R. rubrum* P1 were cultivated in modified RCV defined medium (Weaver *et al.*, 1975) supplemented with 22 mM NH_4Cl as the nitrogen source to totally repress nitrogenase synthesis. Carbon sources were 30mM sodium malate for photoheterotrophic growth, or 16% CO along with 0.5% (w/v) yeast extract for growth on CO. The final pH of the growth medium was 7.2.

Media were prepared by boiling and dispensing to stoppered anaerobe tubes under a stream of argon gas and then sterilized in an autoclave press (Bellco Glass, Inc.). For larger cultures, media were sterilized aerobically, then bubbled with a stream of argon gas passing through a 0.2 μm pore size Acrodisc filter (Gelman Sciences, Inc.). For growth under CO, CO gas was added aseptically via syringe fitted with an Acrodisc filter (0.2 μm pore size).

Photosynthetic cultures were illuminated from above with 60W incandescent lamps. Light intensity reaching the culture surface was approximately 150 W/m^2 measured with a YSI-Kettering Radiometer (Model 65A).

Preparation of cell-free hydrogenase extract

To maintain anaerobiosis, most experimental procedures were carried out inside an anaerobic glove box (Coy, Inc.) and 2 mM dithiothreitol and 1 mM sodium dithionite were included throughout the preparation. Cells in mid-log phase of growth were harvested by centrifugation and the pellets were suspended into 50 mM Hepes buffer (pH 7.5) containing10 mM EDTA and 0.5 M glucose to a cell concentration equivalent to 8 mg cell dry weight/ml. Lysozyme was added to a final concentration of 0.5 mg lysozyme/mg cell dry wt., and incubated for 1 hr at 30 °C with occasional shaking. Formation of spheroplast was monitored by diluting an aliquot of the suspension 20-folds into distilled water and its lysis examined with phase-contrast microscopy. The spheroplast preparation was then centrifuged at 50,000 × g for 10 min. Each pellet resulted from 20 ml spheroplast suspension was subjected to an osmotic shock treatment by homogenizing with 35 ml of 1 mM Hepes buffer (pH 7.5). A spatula full of DNase and RNase was added to break down nucleic acids. The homogenates were allowed to stand for 20 min with occasional vigorous shaking followed by the addition of Hepes buffer (pH 7.5) to a final concentration of 20 mM. The suspension was centrifuged and washed once at 50,000 × g for 15 min. Membranes were suspended in 50 mM phosphate (pH 7.5) containing 1 mM EDTA and 15% glycerol and stored frozen at -70°C before detergent extraction. Preparations using this procedure contained no intact cells.

To partially purify the CO-linked hydrogenase, cell-free membranes were solubilized with 2% (w/v) CHAPS detergent for 1 hr at 25°C with continuous stirring. The solubilized fraction was obtained by centrifugation at 50,000 x g for 15 min, followed by 250,000 x g for 1.5 hr. The resulted supernatant was applied to a DEAE-Sephacel anion exchange column (1.6 cm x 13 cm) and eluted with a NaCl gradient from 150 mM to 400 mM in phosphate buffer (20 mM, pH 7.0). The collected fractions, in 5 ml size, were assayed for various hydrogenase activities.

Hydrogenase Assay

Hydrogenase activities were routinely assayed by the evolution of H_2 gas from methyl viologen reduced by sodium dithionite in 2-ml reaction mix. Reactions were carried out inside 13.5 ml

stoppered Wheaton vials shaken in a 30°C water bath. To measure linked pathway using whole cells, reaction mix contained (in final concentration) 50 mM phosphate buffer (pH 7.0), along with 17% CO gas for "CO-linked" pathway. For cell-free assay, 5 mM methyl viologen was the electron mediator reduced by 10 mM sodium dithionite. When whole cells were used instead of membranes to measure methyl viologen mediated reaction, Triton-X-100 (0.1%, w/v) was included in the reaction mix to facilitate methyl viologen permeation into whole cells. Reaction was started by the addition of electron donors and terminated by acidification with 0.1 ml of 10% (w/v) trichloroacetic acid solution. Samples in the gas phase were withdrawn and injected into a Varian 3700 series gas chromatograph equipped with a thermal conductivity detector and separated by a molecular sieve 5 Å column (60/80 mesh, 6' × 1/8"). H_2 was determined using argon as carrier gas and CO with helium as carrier gas.

H_2 uptake activity to methyl viologen (E_{578nm} = 9.7 mm^{-1} cm^{-1}) was conducted in a Hewlett-Packard 8450A spectrophotometer. Reactions (final volume 2.5 ml) in anaerobic Thunberg cuvettes contained in 20 mM Tris buffer (pH 8.7), 2.5 mM methyl viologen, the appropriate enzyme fraction, and the O_2-scrubbing system (Packard and Cullingford, 1978). Ten percent H_2 was present in the gas phase. Control cuvettes were gassed with argon and any non-specific reduction of the dye by endogenous reductants was subtracted to obtain H_2-dependent activities only.

H_2 uptake to methylene blue was carried out in a 2-ml water-jacket chamber fitted with a model 5331 Clark type electrode. The reaction mix was equilibrated with a 10% H_2 gas stream in 50 mM Tris buffer (pH 8.7) and initiated by injecting methylene blue (0.5 mM) as the electron acceptor.

Mutant Isolation

R. rubrum G9, a carotenoidless mutant of wild-type strain S1, was used to generate the H_2-uptake pathway mutant. *R. rubrum* G9 was cultured photoautotrophically on H_2 and CO_2 gas mixture (80:20) to maximally induce uptake hydrogenase enzyme and the H_2-uptake pathway. The fully-grown culture was later subjected to photokilling by bubbling an aliquot of the suspension vigorously with a stream of sterile air for 5 min while illuminating with a 150W flood lamp. The survivors were then serially diluted and plated onto RCV agar plates supplemented with malate and 0.5% (w/v) yeast extract for nonselective photosynthetic growth. Colonies emerged on the surface of agar plates were replica-plated with sterile velveteen cloth onto a set of RCV agar plates, one supplemented with malate as carbon source, the other with H_2 and CO_2 gas mixture. This strategy is to select those colonies that grow well photoheterotrophically, yet grow poorly or exhibit no growth under photoautotrophic condition. All colonies fitting those criteria were transferred into RCV-malate liquid medium and screened for uptake hydrogenase activities, coupling H_2 oxidation to either O_2 or methylene blue reduction.

H_2/D_2O Exchange Assay

A schematic of the ultra high vacuum (UHV) chamber equipped with an Uti mass spectrometer has been given previously (Dillon *et al.,* 1995). The UHV chamber is pumped both by a 60 l/s and a 110 l/s Balzers turbomolecular pump enabling operating pressures of 1-2 x 10^{-8} Torr to be obtained without baking. The 60 l/s pump is positioned directly behind the mass spectrometer. An ion gauge and a capacitance manometer are employed to monitor the pressure of the chamber. A four component mechanically-pumped gas manifold is also attached to the chamber. Gas admission is controlled with a variable conductance leak valve. Isolation gate valves separate the main chamber during high-pressure gas exposures. For these experiments a glass cold finger containing 2 ml hydrogenase sample in 2 ml D_2O stirred under a H_2 atmosphere was attached to the manifold. The finger was frozen with liquid nitrogen while the manifold was evacuated. This freeze-pump-thaw procedure was performed several times prior to beginning experiments. The cold finger was then warmed to room temperature and H_2 gas with varying amount of O_2 was bled into the chamber at a pressure of 5 x 10^{-6} Torr and reacted for 20 min under continuous stirring. Only the gate valve leading to the 60 l/s pump was left open so that all of the gas molecules were passed by the mass spectrometer. Mass spectra were recorded from 0 – 50 a.m.u. with a National Instruments Labview program every 2 seconds.

Dry Weight Measurement

Turbidity of cell suspensions was determined by measuring optical density at 660 nm in a Spectronic 21 Colorimeter (Bausch & Lomb, Inc.). The measurement was then related to dry weight from a prepared calibration curve.

Results and Discussion

Identification of a Hup^- Mutant and its Hydrogenase Activities

In order to determine O_2 resistance of the CO-linked hydrogenase without interference from the uptake hydrogenase, mutants deficient in H_2-uptake pathway were isolated. A carotenoidless blue-green mutant such as *R. rubrum* G9 is very susceptible to photokilling in the presence of O_2, and therefore, an ideal candidate to generate Hup^- mutants (Fig. 1). Our photokilling selection procedure resulted in 9 isolates that showed good photoheterotrophic growth on malate, yet were unable to grow photoautotrophically on H_2 and CO_2. These isolates were then screened for any residual uptake hydrogenase activity coupling H_2 oxidation to O_2 or methylene blue reduction. One mutant, designated as *R. rubrum* P1, consistently yielded no detectable levels of H_2-uptake activity coupling to O_2 assay, and the rate of H_2-dependent methylene blue reduction was only 3% of the parental strain G9. Fig. 2A shows that the induction of uptake hydrogenase activity in

the parental strain G9 paralleled cell growth, consistent with previous observation in wild-type photosynthetic organisms (Maness and Weaver, in preparation). However, under identical growth conditions, the levels of uptake hydrogenase activity in mutant strain P1 was negligible throughout the entire growth cycle (Fig. 2B). Both G9 and P1 exhibited similar growth rates under photoheterotrophic condition.

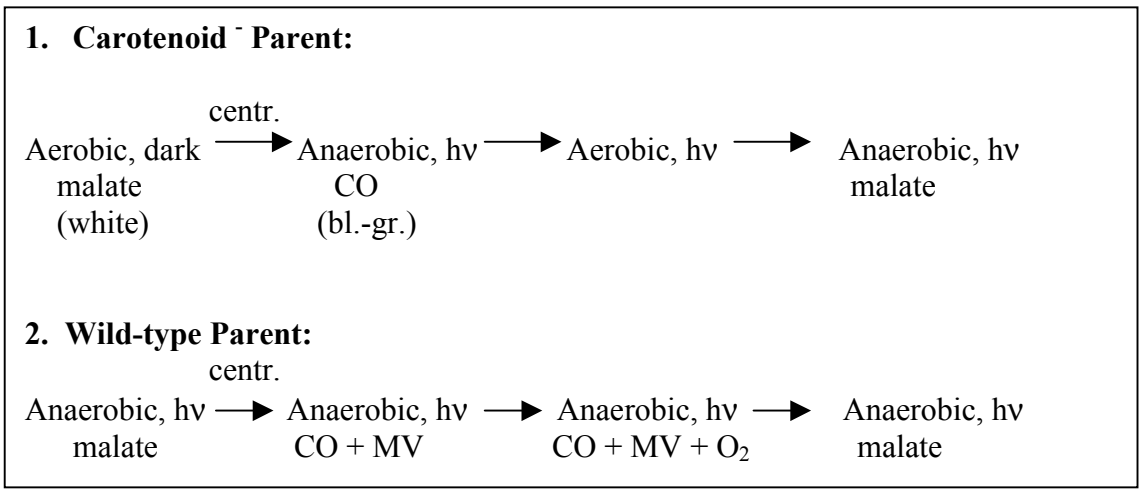

Figure 1. Schemes for mutant selection

Hydrogen-dependent methylene blue reduction assay is a direct assay of the uptake hydrogenase itself, and mutant strain P1 expressed only negligible amounts of uptake hydrogenase enzyme activity. CO-linked hydrogenase from mutant P1 was, therefore, chosen to determine whether it can functionally evolve H_2 while O_2 was simultaneously present using a D_2O/H_2 exchange assay.

The successful isolation of the Hup^- mutant also proves that our mutagenesis/selection technique will work for the isolation of other mutants deficient in various components of the overall CO to H_2 metabolic pathway (Fig. 1). Mutagenized cells, either by 1-methyl-3-nitro-1-nitrosoguanidine or ethyl methanesulfonate, can be cultured in darkness under aerobic conditions to repress the synthesis of photosynthetic pigments. A subsequent photosynthetic subculture into CO under anaerobic condition induces the water-gas shift pathways and chlorophyll synthesis among wild-type cells. Only those cells deficient in the water-gas shift pathway, thus pigmentless, will survive the photo-oxidative killing. A complete library of mutants could be generated based on this extreme sensitivity of the blue-green mutant to photo-oxidative killing conditions. Any mutants deficient in the CO shift reaction will elucidate the induction mechanism of the CO-linked pathway. Mutants altered in their redox mediators allow for their biochemical characterization and better determination of any rate-limiting steps in the overall

reaction. Subsequently, a scheme to obtain constitutively overly-expressed mutants can be derived from these mutants.

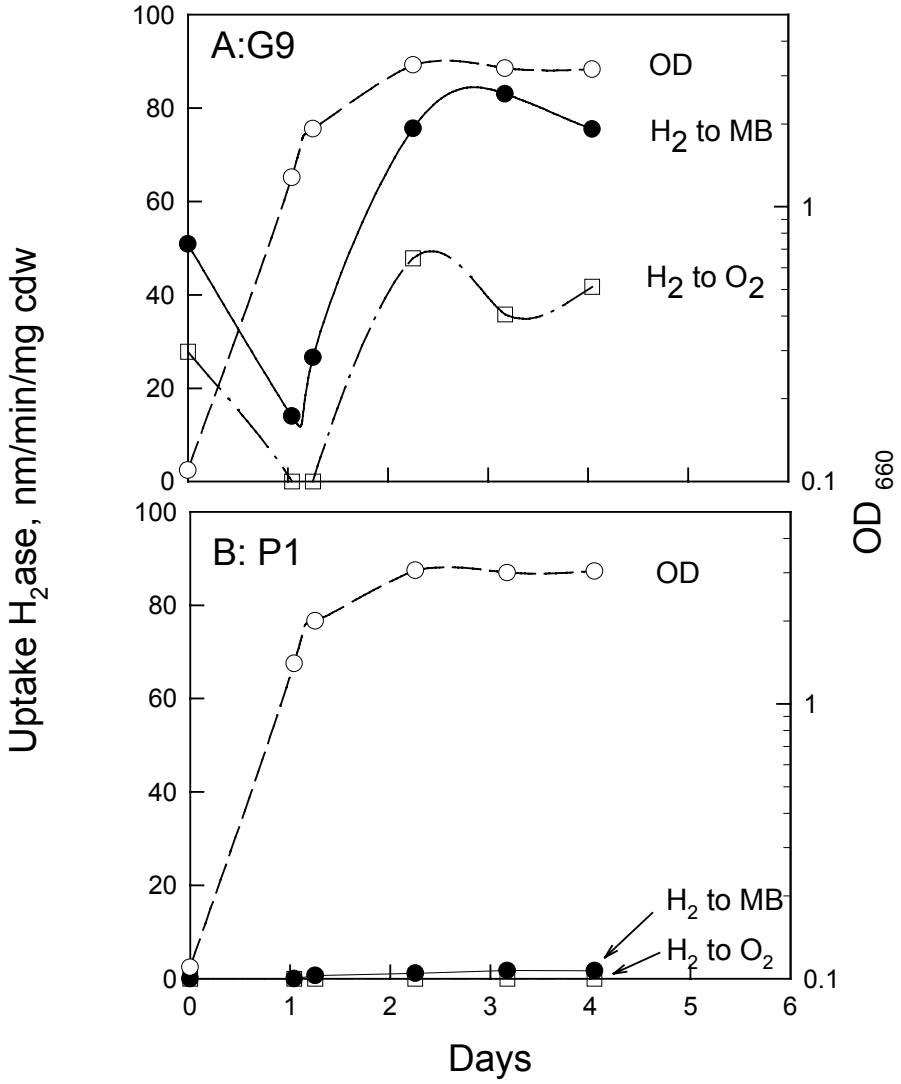

Figure 2. The induction of uptake hydrogenase in both *R. rubrum* G9 parent (A) and mutant P1 (B). Methylene blue (MB) is the electron acceptor where indicated.

Alternatively, wild-type strains with carotenoid pigments can be incubated with CO and methyl viologen (Fig. 1). Upon exposure to O_2, those cells having the capability to reduce methyl viologen by CO will immediately generate superoxide radicals, which are lethal to living cells. The survivors without the water-gas shift pathway can then be cultured on malate medium for further analysis.

O_2-Resistance Measurement

Mutant P1 still contains 3% of the uptake hydrogenase of its parental strain. We therefore proceeded to partially purify the CO-linked hydrogenase according to the Method. Figure 3 shows an elution profile of the CO-linked hydrogenase (•) and the uptake hydrogenase (▲) against a NaCl salt gradient (■). Two fractions contain high activity of the CO-linked hydrogenase without the interference from uptake hydrogenase activity. These two fractions were pooled together and used for the subsequent D_2O/H_2 exchange assay. These results also provide direct evidence that the CO-linked hydrogenase does not couple to a high potential dye such as methylene blue (E_m at pH 7.0 = - 11 mV) as an electron acceptor. Therefore, the reduction of methylene blue becomes a specific assay for the classical uptake hydrogenase enzyme only. In photosynthetic organisms where multiple hydrogenases exist, methylene blue assay becomes a useful tool to identify the presence of uptake hydrogenase specifically.

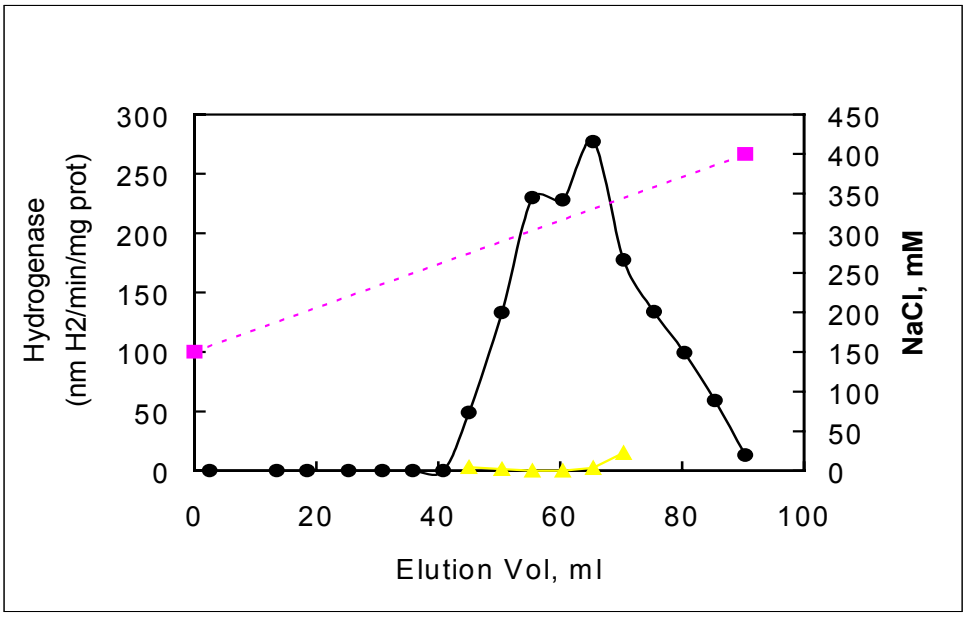

Figure 3. Purification of CO-linked hydrogenase from *R. rubrum* mutant P1

Fig. 4 shows that with 1% O_2 in the gas phase and stirring for 20 min, the hydrogenase enzyme still retained more than 50% of its deuterium exchange activity. At 7% O_2, the enzyme was about 15% active. However, even after exposure to various levels of O_2 for 40 min, more than 70% of the activity was still recovered upon the subsequent removal of O_2. These data indicated that the hydrogenase was still partially operative under aerobic conditions and a majority of the activity is reversible upon returning to anaerobic condition. A parallel study using crude membranes containing the bulk of the CO-linked hydrogenase also confirms its O_2 resistance. Upon stirring the membrane fraction with 5% O_2 in the gas phase, the hydrogenase still retained 40% of its activity after 2 hours (Fig. 5).

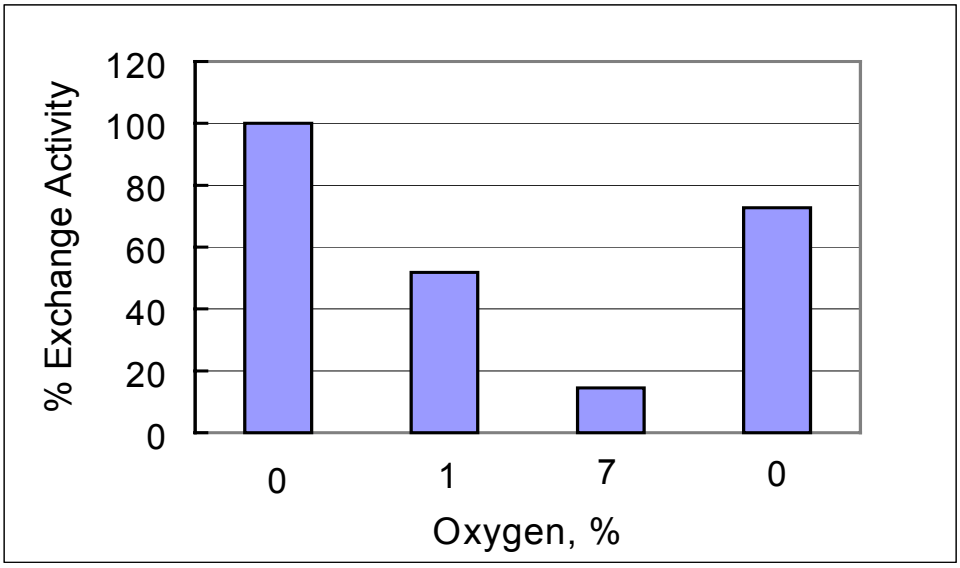

Figure 4. Effect of oxygen on the H_2 – deuterium exchange reaction of *R. rubrum* P1 hydrogenase

We had reported previously that the CO-linked hydrogenase from *R. gelatinosus* CBS-2 is also relatively O_2 resistant (Weaver, *et al*., 1998). The partially purified hydrogenase was 55% active in the presence of up to 13% O_2 for the duration of the deuterium exchange assay (20 min). The system is more than 90% reversible upon returning to the anaerobic condition. The O_2 resistance property could be unique among hydrogenases linking to CO oxidation. This hydrogenase can serve as an ideal model to investigate structure-function relationships. An insight into the amino acids around its catalytic site will yield important information regarding its relatively O_2-resistant property. Either biochemical purification of the hydrogenase enzyme, or cloning of its DNA sequence will elucidate this unique property. This information can then be used to design other proteins to become more O_2-tolerant.

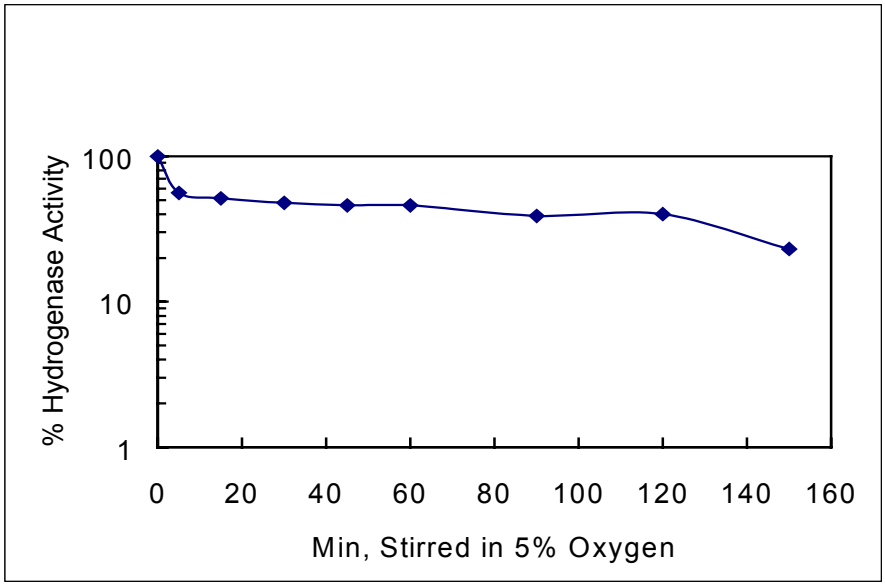

Figure 5. Oxygen sensitivity of CO-linked hydrogenase from the lysed spheroplast membrane of *R. rubrum* P1

Effect of CO on the Longevity of the CO-linked Pathway

Carbon monoxide or its derivative is the inducer for the water-gas shift pathway among various photosynthetic bacteria and the appearance of the activities is due to *de novo* protein synthesis (Uffen, 1981; Bonam et al., 1989; Maness & Weaver, in preparation). However, there is no report to date on the duration or life time of the enzymes once induced, nor on whether CO or its immediate product has to be present continuously to prevent the existing enzymes from turning over. This concerns long-term biological shift activity in an applied bioreactor system.

To investigate the short-term effect of CO on previously induced enzymes, we added CO to a culture that had starved for CO for 24 hours, and followed the overall CO-linked activity and the hydrogenase enzyme activity. Figure 6 shows that upon feeding 16% CO at time zero, there is a near 2.2 fold increase in the hydrogenase activity. The rate returned to a lower steady-state level as CO was consumed to near zero. With a second feeding of 16% CO after 24 hours, the hydrogenase activity was again elevated by 1.6 fold. A less dramatic effect was observed with the overall linked pathway. Chloramphenicol, an inhibitor for new protein synthesis, yet has no impact on any existing protein, was added along with CO to determine if the increase in rates was due to *de novo* synthesis. We observed a similar pattern of increases when both CO and

chloramphenicol were included, indicating that the enhancement was due to an activation of the existing enzymes, not due to new protein synthesis (data not shown). CO, or its metabolized product, may stimulate the hydrogenase enzyme directly, or alleviate the regulation of the enzyme. However, we can not explain the less dramatic effect CO had on the overall CO to H_2 linked pathway. Perhaps one of the rate-limiting steps is still regulated. Our proposal to isolate mutants deficient in various components of the linked pathway shall elucidate the regulations of the overall pathway and facilitate in the scale up of the bacterial water-gas shift system.

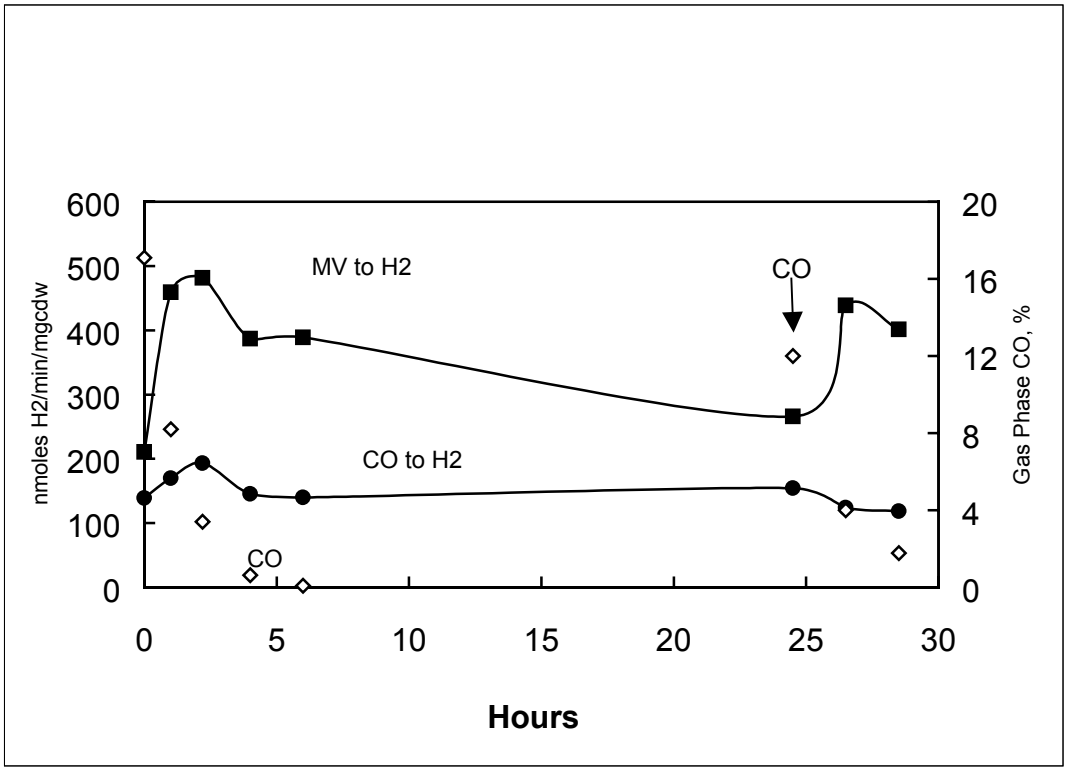

Figure 6. Effect of CO additions on the activities of previously-induced enzymes of the CO to H_2 pathway in *R. gelatinosus* CBS-2

Acknowledgement

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