

Characterization of the Oxygen Tolerance of a Hydrogenase Linked to a Carbon Monoxide Oxidation Pathway in *Rubrivivax gelatinosus*

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A hydrogenase linked to the carbon monoxide oxidation pathway in *Rubrivivax gelatinosus* displays tolerance to O₂. When either whole-cell or membrane-free partially purified hydrogenase was stirred in full air (21% O₂, 79% N₂), its H₂ evolution activity exhibited a half-life of 20 or 6 h, respectively, as determined by an anaerobic assay using reduced methyl viologen. When the partially purified hydrogenase was stirred in an atmosphere containing either 3.3 or 13% O₂ for 15 min and evaluated by a hydrogen-deuterium (H-D) exchange assay, nearly 80 or 60% of its isotopic exchange rate was retained, respectively. When this enzyme suspension was subsequently returned to an anaerobic atmosphere, more than 90% of the H-D exchange activity was recovered, reflecting the reversibility of this hydrogenase toward O₂ inactivation. Like most hydrogenases, the CO-linked hydrogenase was extremely sensitive to CO, with 50% inhibition occurring at 3.9 μM dissolved CO. Hydrogen production from the CO-linked hydrogenase was detected when ferredoxins of a prokaryotic source were the immediate electron mediator, provided they were photoreduced by spinach thylakoid membranes containing active water-splitting activity. Based on its appreciable tolerance to O₂, potential applications of this hydrogenase are discussed.

Hydrogen is a clean fuel that addresses both adverse environmental impacts and periodically recurring energy crises. Industrial attention has been drawn to the microbial production of H₂ using hydrogenase enzymes because of the wide variety of acceptable feedstock which could substantially lower the cost of H₂ production. Hydrogenases catalyze the reversible reduction of protons into H₂, although most are committed to catalyze either in the H₂ oxidation direction or in the H₂ evolution direction based on the energy demands of the cells. This explains the existence of multiple hydrogenases within a living organism, each expressed under certain physiological conditions so that the organism can best meet its energy need (2, 3, 20). Potential applications of photosynthetic and fermentative microorganisms in the generation of H₂ have been reviewed extensively (9, 13, 29). Among these microorganisms, certain photosynthetic bacteria are unique in that they contain a CO oxidation pathway converting CO and H₂O into H₂ and CO₂. This reaction has been reported for *Rubrivivax gelatinosus* (19, 24, 25), *Rhodospirillum rubrum* (6, 20, 25), and *Rhodopseudomonas palustris* (16). Carbon monoxide is demonstrated to induce the de novo synthesis of a hydrogenase enzyme linking to the CO oxidation pathway in *Rhodospirillum rubrum* (7).

An ideal process to produce H₂ more economically would be water derived. Lacking photosystem II, photosynthetic bacteria cannot use water as the electron donor. Cyanobacteria, on the other hand, have both photosystems I and II and can therefore oxidize water to generate photoreductants. One potential solution is to express bacterial hydrogenase along with its physiological electron mediator(s) in a cyanobacterial host. However, most H₂-evolving hydrogenases are extremely sensitive to

O₂, which is an inherent byproduct of cyanobacterial photosynthesis. This sensitivity to O₂ was noted in reports of hydrogenases from *Clostridium pasteurianum*, *Desulfovibrio vulgaris*, and *Chlamydomonas reinhardtii*, where 50% of the initial evolution activities were lost from the reduced enzymes within several minutes of exposure to O₂ (1, 2, 11). Therefore, to establish a successful cyanobacterium-bacterium hybrid system using H₂O as the electron donor, one critical requirement is to use a hydrogenase that is not only tolerant to O₂ but also catalytically active in O₂.

The photosynthetic bacterium *Rubrivivax gelatinosus* CBS contains an active CO-to-H₂ pathway (19), and this report documents the O₂ tolerance displayed by its CO-linked hydrogenase. Besides using reduced methyl viologen (MV) to measure hydrogenase activity, which precludes the addition of O₂ during the assay, it is ultimately important for us to determine whether this hydrogenase can also function in the simultaneous presence of O₂. Most hydrogenases catalyze a hydrogen-deuterium (H-D) exchange reaction in a hydrogen-deuterium oxide (D₂O) system, yielding HD (12, 15, 21, 30), and the isotopic exchange rate correlates positively with the H₂ production rate mediated by reduced MV (21). Because this reaction does not require a reducing agent such as sodium dithionite, O₂ can be included throughout the assay without being scavenged by the reducing agent. Since this assay does not involve any electron mediator, it becomes a direct assay of the enzyme itself. Consequently, any effect O₂ might have on the H-D exchange activity reflects the intrinsic nature of the enzyme. In this report, we describe findings of how O₂ affected the rate of the exchange reaction and its reversibility upon the removal of O₂. We also determined the sensitivity of this hydrogenase to CO once the bulk of the CO dehydrogenase (CODH) activity was removed. Finally, we constructed a spinach-ferredoxin-hydrogenase system and discuss its implication

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for the generation of H₂ from a cyanobacterium-bacterium hybrid system.

MATERIALS AND METHODS

Organism, media, and growth conditions. The photosynthetic bacterium used in this study was described previously and was originally classified as *Rhodobacter* sp. strain CBS (19). Based on 16S rRNA homology, it was reclassified as *Rubrivivax gelatinosus* CBS. Medium preparations, growth conditions, and cell dry weight determinations were as described previously (20) with the exception that the RCVBN medium (28) was modified to omit a carbon source and instead supplemented with 0.05% (wt/vol) yeast extract and 17% (vol/vol) CO.

Hydrogenase purification. A *Rubrivivax gelatinosus* CBS culture in the late log phase of growth (optical density at 660 nm, ~0.8 to 0.9) was harvested. Sodium dithionite (1 mM) and dithiothreitol (2 mM) were present throughout the preparation. The anaerobic preparation of the lysed spheroplast membrane, the extraction of CO-linked hydrogenase with 2% (wt/vol) CHAPS {3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate} detergent, and chromatography with DEAE-Sephacryl (Amersham Pharmacia Biotech) were described previously (20). Fractions containing active hydrogenase activity were pooled, diluted with 20 mM potassium phosphate buffer (pH 7.0) to reach a final NaCl concentration of 150 mM, and loaded onto a DEAE-Sephacryl column (1 by 6 cm). This column was washed with 150 mM NaCl in 20 mM potassium phosphate buffer (pH 7.0) prior to elution with 400 mM NaCl in 20 mM potassium phosphate buffer (pH 7.0). Fractions containing hydrogenase activity were pooled and loaded onto a Sephacryl S-200 column (3 by 36 cm; Amersham Pharmacia Biotech). Hydrogenase was eluted from the column with 20 mM potassium phosphate buffer (pH 7.0) without reducing agents. Protein was determined by the method of Lowry et al. (18). By following these steps, the hydrogenase was purified 27-fold, with a recovery of 5.7% compared to that of the activity of the lysed spheroplast membrane fraction.

Hydrogenase and CODH assays. CO-linked hydrogenase activity was determined by measuring H₂ evolution in a 2-ml assay mixture containing MV (2 mM) and Triton X-100 (0.1% [wt/vol]) in 25 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by addition of sodium dithionite (5 mM) and terminated by acidification with 0.1 ml of trichloroacetic acid (10%, wt/vol) at the end of a 15-min incubation at 30°C. The hydrogen produced was quantified by gas chromatography (20). Uptake hydrogenase activity was assayed by monitoring the decrease of H₂ coupled to the reduction of methylene blue in a Clark-type electrode system (20). CODH activity was determined by measuring the reduction of MV from CO oxidation at 578 nm (6) with a Cary 5E spectrophotometer (Varian).

H-D exchange assay. A glass vessel containing 1 ml each of the partially purified hydrogenase (~0.7 mg of protein ml⁻¹ in 20 mM potassium phosphate buffer [pH 7.0]) and D₂O was attached to the manifold of an ultrahigh-vacuum chamber equipped with an Uti mass spectrometer, the configuration of which was described previously (10). The anaerobic hydrogenase suspension was frozen in liquid N₂, followed by several cycles of evacuation and purging with N₂. Hydrogen and various amounts of O₂ were then introduced. The hydrogenase suspension was then warmed to 30°C with continuous stirring. At the end of 15 min, the hydrogenase suspension was again frozen in liquid N₂, and the gas reaction products containing HD were released to the mass spectrometer. Mass spectra of 0 to 50 amu were recorded every 2 s by using a Labview program (National Instruments, Austin, Tex.).

Spinach-ferredoxin-hydrogenase system. Membranes containing CO-linked hydrogenase activity were prepared by suspending the pellet of a late-log-phase culture of *Rubrivivax gelatinosus* CBS in 50 mM Tris-HCl buffer (pH 7.7) and subjecting it to sonication for 5 min (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Unbroken cells were removed by centrifugation at 50,000 × g for 15 min, and the membrane-enriched supernatant was used as the source of hydrogenase. Spinach thylakoid membrane was prepared according to the method of Chantakis and Babcock (8), and its O₂ evolution activity was determined polarographically by coupling H₂O photolysis to 2,5-dichloro-*p*-benzoquinone (0.5 mM), an artificial electron acceptor, in 1.5 ml of K4 buffer (400 mM sucrose, 15 mM NaCl, 5 mM MgCl₂, 20 mM potassium phosphate buffer [pH 7.0]) in a Clark-type oxygen electrode system. Chlorophyll was extracted with 80% acetone, and its presence was determined spectrophotometrically (4). Ferredoxins were from a *Spirulina* sp. and *C. pasteurianum* (Sigma Chemical Co.). The photolinkage reaction was performed with 3-ml stoppered anaerobic cuvettes containing, in a final volume of 2.2 ml, 1.5 ml of K4 buffer, glucose (25 mM), glucose oxidase (5 U ml⁻¹), catalase (500 U ml⁻¹), ethanol (1% [vol/vol]), hydrogenase (2.2 mg of protein ml⁻¹), spinach thylakoids (86 μg of chlorophyll ml⁻¹), and various amounts of ferredoxin (with final concentrations indicated in parentheses). The cuvettes were incubated at 30°C for 30 min while being illuminated with a band

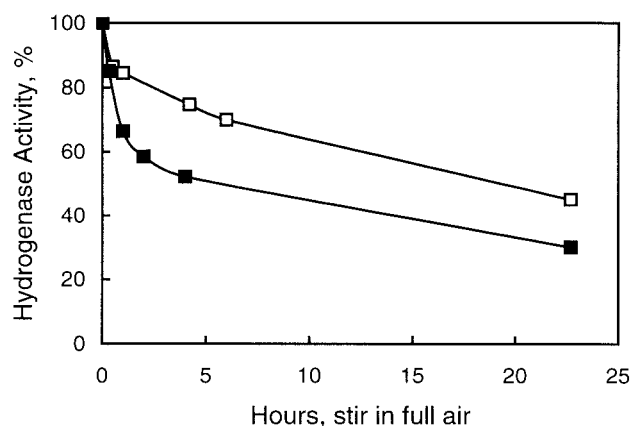


FIG. 1. Inhibition of MV-mediated hydrogenase activity by O₂. *Rubrivivax gelatinosus* CBS whole cells in late log phase of growth (□) or partially purified hydrogenase (■) were stirred in full air. At various intervals, an aliquot of the aerated suspension was injected into the MV-containing anaerobic assay mixture and then gassed with argon for 5 min to remove residual O₂. The reaction was then initiated with sodium dithionite. The assay samples contained 0.18 mg (dry weight) of cells ml⁻¹ or 0.04 mg of protein ml⁻¹ for the whole-cell or cell-free fractions, respectively. The rates corresponding to 100% activity were 263 nmol of H₂ min⁻¹ mg (dry weight) of cells⁻¹ for whole cells and 268 nmol of H₂ min⁻¹ mg of protein⁻¹ for partially purified hydrogenase. Samples were run in duplicate, and data represent the average results for at least three independent experiments.

of 30-W flood lamps yielding a light intensity of 130 microeinsteins s⁻¹ m⁻². Hydrogen produced in the gas phase was determined by gas chromatography (20).

RESULTS

Effect of O₂ on MV-mediated hydrogenase activity. Figure 1 shows the effect of O₂ on hydrogenase activity when whole cells were preexposed to full air (21% O₂, 79% N₂) for a period of 23 h with continuous stirring and then measured for H₂ production under anaerobic conditions from reduced MV. The half-life of the reaction (the point at which 50% of the inhibition occurred) is approximately 20 h, clearly revealing that this organism contains an O₂-tolerant hydrogenase not reported previously. To rule out the possibilities that high respiratory activity in whole cells may scavenge O₂ and that cell membranes may serve as a barrier to O₂ permeation, thus contributing to the observed O₂ tolerance, a similar measurement was performed with a partially purified, membrane-free hydrogenase fraction. Data from Fig. 1 show that although the half-life of a partially purified hydrogenase is reduced to 6 h after being stirred in full air, it still displays tolerance to O₂.

Effect of O₂ on H-D exchange activity. In order to determine if the hydrogenase is catalytically active in the presence of O₂, and to rule out the possibility that the addition of a reducing agent such as sodium dithionite might reactivate a previously inactivated enzyme, we developed a mass spectrometric method to measure H-D exchange activity. Figure 2 shows that, like most hydrogenases, CO-linked hydrogenase could catalyze an H-D exchange reaction when it was suspended in D₂O in an H₂ atmosphere. When the enzyme suspension was exposed to 3.3% O₂ in H₂ for 15 min with continuous stirring, more than 80% of the H-D exchange activity was retained.

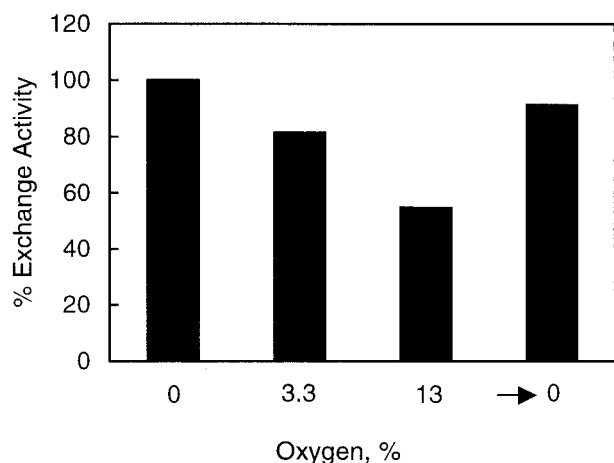


FIG. 2. Effect of O₂ on H-D exchange activity. Hydrogenase was partially purified from *Rubrivivax gelatinosus* CBS as described in Materials and Methods. The ion current corresponding to HD with an atomic mass unit of 3 was recorded, and 100% activity represented a rate of 1.7×10^{-7} A min⁻¹ mg of protein⁻¹. An arrow indicates the return of the exchange assay to an anaerobic environment for a 15-min assay following exposure to various levels of O₂ for 30 min.

Following the 15-min exposure to 3.3% O₂, the same enzyme solution was subsequently exposed to 13% O₂ in H₂ for an additional 15 min. Nearly 60% of the H-D exchange activity was retained at the end of the second exposure. After a total of 30 min of exposure to various levels of O₂, this enzyme suspension was returned to an anaerobic condition in an atmosphere of H₂ and N₂. At the end of a 15-min incubation, more than 90% of the original H-D exchange activity was restored, revealing the reversibility of this hydrogenase toward O₂ inactivation. Since no reducing agent was present during the entire assay, no O₂ scavenging or reactivation of the enzyme was expected.

CO inhibition. Carbon monoxide is a potent inhibitor of many hydrogenases, and K_i values in the range of 5.8 to 40 μM CO have been reported (2, 11, 14, 22, 23). Similar to the CO oxidation system in *Rhodospirillum rubrum* (7), CO also induces both the CODH and hydrogenase activities in *Rubrivivax gelatinosus* CBS (data not shown), with the former enzyme's primary function being the oxidation of CO. Therefore, in order to determine the sensitivity of the CO-linked hydrogenase to CO, the bulk of the CODH would have to be removed to prevent it from scavenging CO to a very low level. Figure 3 shows such a CO inhibition profile with a partially purified hydrogenase fraction from which the majority of the CODH activity was removed, as determined by a CO-supported MV reduction activity of 11.5 nmol of MV reduced min⁻¹ mg of protein⁻¹, compared to that of 5.1 μmol of MV reduced min⁻¹ mg of protein⁻¹ for the lysed spheroplast membrane fraction. The result clearly indicates that when a sufficient amount of CODH is removed, the CO-linked hydrogenase, like most hydrogenases, becomes extremely sensitive to CO, with 50% of the inhibition occurring at 3.9 μM dissolved CO. It is not known whether the CO-linked hydrogenase would have become even more sensitive to CO had CODH been removed completely.

Hydrogen from water photolysis. Hydrogen evolution from an illuminated chloroplast-ferredoxin-hydrogenase system has

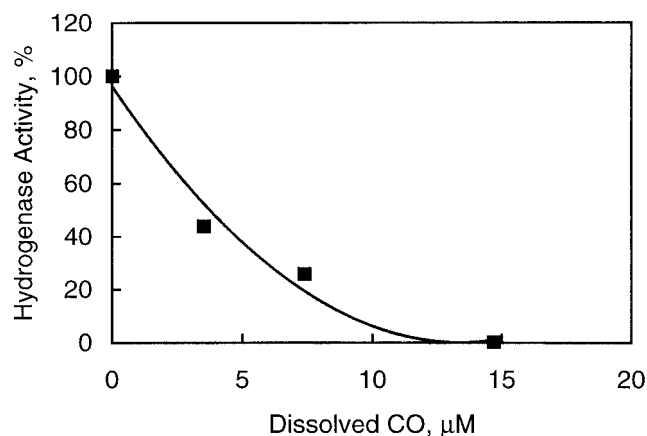


FIG. 3. Inhibition of the CO-linked hydrogenase activity by CO. Hydrogenase was partially purified from *Rubrivivax gelatinosus* CBS as described in Materials and Methods. Various amounts of gaseous CO were included in the H₂ evolution assays mediated by dithionite-reduced MV, and the assay sample contained 0.04 mg of protein ml⁻¹. The rate corresponding to 100% activity was 210 nmol of H₂ min⁻¹ mg of protein⁻¹. Dissolved CO was calculated based on the assumption that each microliter of CO-saturated buffer contains 0.702 nmol of CO at 30°C and under a 0.82 atm. Samples were tested in duplicate, with a standard deviation of less than 10%.

been established previously (5). In order to determine if CO-linked hydrogenase can also couple to a photoreduced ferredoxin generated from H₂O photolysis, we constructed an in vitro system as described in Materials and Methods. Data from Fig. 4 show that in this chloroplast-hydrogenase system, the rate of H₂ production correlates positively with an increased concentration of the *Spirulina* ferredoxin. This result implies that reduced ferredoxin of a cyanobacterial source can mediate electron flow to the CO-linked hydrogenase to produce H₂. In a similar assay, photoreduced ferredoxin from *C. pasteurianum* is also able to mediate H₂ production, although at a much lower rate (33% of the rate of the *Spirulina* ferredoxin). No H₂ was detected in darkness.

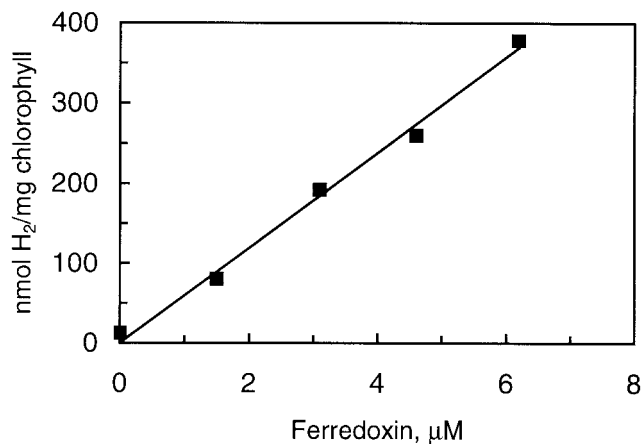


FIG. 4. Ferredoxin requirement of H₂ production in a spinach thylakoid-hydrogenase system. Ferredoxin was from a *Spirulina* sp. The spinach thylakoid membrane has an O₂ evolution rate of 649 nmol of O₂ min⁻¹ mg of chlorophyll⁻¹. Hydrogenase reaction rates were calculated based on the accumulation of H₂ at the end of 30 min in light. Results are the averages from two independent experiments.

DISCUSSION

By both the sodium dithionite-MV assay and the H-D exchange assay to measure a hydrogenase activity, CO-linked hydrogenase from *Rubrivivax gelatinosus* CBS demonstrates both tolerance and reversibility toward O₂ inactivation. Until our recent work, no H₂-producing hydrogenase that would function for any appreciable period of time in the presence of more than a small concentration of O₂ has been described. Using a membrane-free, partially purified hydrogenase fraction, we ruled out any protective mechanisms provided by whole cells or membranes to scavenge O₂ (Fig. 1 and 2). Using the H-D exchange assay, we also eliminated the possible reactivation by sodium dithionite of a previously inactivated hydrogenase (Fig. 2). This would have been the case with the hydrogenases from both *Desulfovibrio gigas* (27) and *Methanosarcina barkeri* (17), both of which can be purified aerobically and then activated with H₂ in a reducing environment to regain full activity. *Rubrivivax gelatinosus* CBS contains an active uptake hydrogenase enzyme (data not shown). Uptake hydrogenase is known to catalyze an H-D exchange reaction, which is quite resistant to O₂ (26). In the present report, we used a partially purified hydrogenase fraction from which the bulk of the uptake hydrogenase activity was removed, as determined by a lack of methylene blue-dependent H₂ uptake activity, to rule out any O₂ tolerance attributed to the uptake hydrogenase. Therefore, without complications from respiration, reactivation, and the uptake hydrogenase, all the HD accumulated at the end of a 15-min reaction must have resulted solely from the CO-linked hydrogenase functioning in the presence of O₂ (Fig. 2). The configurations of the mass spectrometer used in this experiment allowed for only the batch detection of signals at the end of incubation. Therefore, no information as to the immediate impact of O₂ addition on H-D exchange rates, whether the reaction had occurred at a constant but lower rate or whether it proceeded at a higher initial rate followed by a gradual decline, etc., is available. To obtain this information, a mass spectrometer with a continuous-sampling capability is needed.

The O₂ tolerance exhibited by the CO-linked hydrogenase renders it a viable candidate to be used in a scaled-up bioreactor system which, due to its size, may otherwise be impractical as a means to remove traces of residual O₂. Another potential application is to express this hydrogenase in a cyanobacterium, which will constantly evolve O₂ as a byproduct of oxygenic photosynthesis. Data from Fig. 4 strongly suggest that this hydrogenase may be able to use the host ferredoxin of the cyanobacterium-bacterium hybrid system to mediate H₂ production from water photolysis. Work is under way to clone the hydrogenase gene and to further purify the hydrogenase protein in order to elucidate whether any unique protein structure had contributed to its appreciable tolerance to O₂. If verified, this knowledge could be used as a model to engineer other hydrogenases to become more O₂ tolerant.

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