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Inactivation of Hydrogenase in Cell-free Extracts and Whole Cells of *Chlamydomonas reinhardi* by Oxygen[1]

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ABSTRACT

O_2 irreversibly inactivates hydrogenase from *Chlamydomonas reinhardi*. The mechanism for the inactivation involves the reaction of one molecule of hydrogenase with one molecule of O_2 (or two oxygen atoms) in the transition complex of the rate-limiting step. The second order rate constant for this reaction is 190 atmospheres^{-1} minute^{-1} (1.4×10^5 molar^{-1} minute^{-1}). At levels above 0.01 atmosphere O_2, the increased numbers of O_2 molecules may compete for the site of inactivation hindering the proper orientation for inactivation of any one O_2 molecule and resulting in lowered rates of inactivation.

CO is a reversible inhibitor of hydrogenase acting competitively against H_2. The K_i for CO is 0.0010 atmosphere. CO antagonizes O_2 inactivation. In a period when complete inactivation by O_2 would usually occur, the presence of CO greatly reduces the inactivation rate.

After 3 hours of adaptation in whole cells, the presence of H_2 lowers the rate of deadaptation of hydrogenase. Inasmuch as H_2 promotes increased O_2 uptake the cellular concentration of O_2 is likely to be lower. After 48 hours of adaptation O_2 uptake is reduced even when H_2 is present and the pattern of deadaptation under O_2 with and without H_2 and CO is qualitatively the same as observed for the inactivation of cell-free hydrogenase. The mechanism of inactivation of cell-free hydrogenase by O_2 may be the same as the mechanism for loss of hydrogenase during deadaptation in whole algal cells.

Since the initial observation of H_2 metabolism in green algae the sensitivity of algal H_2 metabolism to O_2 has been noted (5). Simultaneous uptake of H_2 and O_2 (the oxyhydrogen reaction) is inhibited when the O_2 level exceeds a specific concentration (6, 10). Also, the photoproduction of O_2 in algae will cause cessation of H_2 metabolism. Removal of photoproduced O_2 reversed the inhibition of hydrogenase (9). Such a loss of H_2 metabolism is termed deadaptation. Most studies on deadaptation have not directly determined the actual change in cellular hydrogenase concentration but have relied on over-all changes in H_2 metabolism. Such metabolism often involves variable levels of other enzymes, electron donors, and acceptors in addition to the hydrogenase itself.

Although both cell-free algal and bacterial hydrogenases are known to undergo irreversible inactivation by O_2 only a few studies have discussed the kinetics. Toai *et al.* (13) observed a biphasic time course for the inactivation of hydrogenases from *Chloropseudomonas ethylica* and *Thiocapsa roseopericina* in air and concluded that two forms of the enzyme exist, differing in susceptibility to O_2. Lappi *et al.* (11) reported an initial rapid loss of *Clostridium pasteurianum* hydrogenase activity in air followed by a slower rate of inactivation. They stated that this observation might imply either a two-step inactivation mechanism, two different mechanisms, or two different enzyme states with different O_2 sensitivities. Abeles (1) found that methylene blue reduction by hydrogenase from *Chlamydomonas* was inhibited irreversibly by low levels of O_2. With the exception of Abeles' report (1) there are none detailing the inactivation of cell-free hydrogenase at low levels of O_2.

Here, we compared the kinetics and mechanism of deadaptation in *Chlamydomonas reinhardi* cells with the O_2 inactivation of the cell-free hydrogenase. Some comparative data are included for *Chlamydomonas moewusii*. This kinetic behavior was observed over a 10,000-fold range of O_2 concentrations. Under conditions of deadaptation the actual levels of cellular hydrogenase concentration rather than over-all H_2 metabolism were monitored during the kinetic studies.

MATERIALS AND METHODS

Culture of *Chlamydomonas* and Enzyme Preparation. *C. reinhardi* was grown on Tris-acetate-phosphate medium (7) under fluorescent light and harvested at late log phase (A_{550} was 0.3–0.4). The cell paste (200 g) was resuspended in 800 ml of Tris-Cl (pH 8.0) and 10 μM DCMU and adapted under H_2 for 3 h at 25 C. Continuing under anaerobic conditions the cells were broken by freezing in liquid N_2 and thawing at 40 to 50 C. The following steps were carried out at 25 C. $(NH_4)_2SO_4$ was dissolved in the suspension at the level of 200 g/l. After centrifugation at 15,000*g* for 1 h the supernatant was decanted and saturated with $(NH_4)_2SO_4$ by addition of 700 g/l. The precipitated hydrogenase was recovered by centrifugation and redissolved in Tris-Cl (pH 8.0). After desalting on a Sephadex G-25 column the enzyme was further purified by passage through a DEAE-cellulose (DE52, Whatman) column eluted with 0.10 M and 0.20 M KCl in the Tris buffer. The hydrogenase was eluted with the 0.20 M KCl while the dark ferredoxin band remained on the column. The enzyme was concentrated using the Immersible Molecular Separators (Millipore Corp.).

C. moewusii was grown in minimal medium plus acetate (7). The hydrogenase was prepared as above.

Assays. Protein was assayed by the biuret method (8) and Chl was assayed spectrophotometrically in 80% acetone (2). The enzyme was assayed by MV[3] reduction followed on a Gilford recording spectrophotometer at 605 nm. Hydrogenase solution was injected into a cuvette with 2.0 ml of anaerobically prepared 10 mM MV in 50 mM Tris-HCl (pH 8.0) under 1.0 atm H_2 at 25 C. The extinction coefficient for reduced MV was determined to be 9.6 mM^{-1} cm^{-1} by reduction with excess sodium dithionite at pH 8.0. One unit of hydrogenase activity is defined as the reduction of 1.0 μmol MV/min.

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[3] Abbreviation: MV: methyl viologen.

O_2 uptake rates were monitored using a Yellow Springs Instrument electrode system in a closed 1.25-ml Lucite chamber.

Inhibition Studies with Partially Purified Hydrogenase. In the O_2 studies, 1.5 ml of the hydrogenase solution [redissolved $(NH_4)_2SO_4$ precipitate] was rapidly equilibrated with O_2 in a bottle (volume, 157 ml; internal surface area, 160 cm^2) by vigorous shaking. Shaking was continued during the course of inactivation. The enzyme concentration was measured by MV reduction. Injection of the sample into assay cuvettes having a small quantity of reduced MV removed any O_2. Once the O_2 was removed no further inactivation of the hydrogenase was observed.

CO inhibition was observed by MV reduction after injection of hydrogenase (purified through the DEAE-cellulose step) into cuvettes containing concentrations of CO, H_2, and MV specified under "Results."

In checking the reversibility of CO and O_2 inhibition or the effects of combinations of these compounds on hydrogenase, 5 to 10 ml of enzyme solution was incubated in 157-ml bottles with shaking. At specified times 1-ml aliquots were removed and frozen in liquid N_2. When all aliquots were removed they were rapidly thawed and repeatedly evacuated and flushed with N_2 to remove all CO and O_2. The samples were then assayed by MV reduction.

Deadaptation Studies. Cultures of *C. reinhardi* were harvested aseptically and then adapted under N_2 in 50 mM Tris-Cl (pH 8) (with 10 μM DCMU) for 3 to 48 h in the dark. Aliquots of cell suspension (15 ml containing 50–200 μg Chl/ml) were injected into 1.2-liter vacuum flasks containing various combinations of O_2 (0.01 atm), CO (0.10 atm), and H_2 (0.90 atm) with N_2 (to 1.1 atm final pressure). The gas mixtures were prepared after the flasks had been evacuated and flushed with N_2 five times over a 10-min period. The flasks were shaken at 100 reciprocal cycles/min (5-cm strokes) at room temperature (25 C). One-ml aliquots were removed by syringe at specified time intervals and frozen in liquid N_2. The samples were thawed under vacuum to remove O_2 and CO and then kept under N_2. Thawing also broke the cells to release the hydrogenase into solution (sonication or a combination of both disruption techniques would release the same quantity of enzyme). The thawed suspensions were assayed for hydrogenase by MV reduction.

Calculations. The primary measurement of gaseous substrates and inhibitors was in atm. When presented as μM the solubility coefficients of 21.4 μl CO/ml and 28.3 μl O_2/ml at 25 C were used for the conversion (14).

The data were analyzed using the linear regression program of the Texas Instruments 58 calculator. Correlation coefficients for the linear plots were greater than 0.995 and generally greater than 0.998. The use of ± signs in presenting kinetic parameters represents the maximum deviations from the average of two or more values.

The calculations for the analysis of the O_2 inhibition data are presented under "Discussion." These analyses require that the hydrogenase concentration be less than the O_2 concentration. Estimates of the concentration of hydrogenase may be made by assuming that purified *C. reinhardi* and *C. pasteurianum* hydrogenases have similar specific activities. Purified *C. pasteurianum* hydrogenase has a specific activity of 4,300 units/mg (3). For the O_2 studies the *C. reinhardi* enzyme activity was 13 units/ml. Using a mol wt of 49,000 (obtained by gel filtration) the concentration of *C. reinhardi* hydrogenase may be estimated at 0.010 μM. At the lowest pO_2 used (0.0001 atm) the O_2 concentration would be 0.130 μM. It is difficult to say how valid this estimate is since the kinetic reaction parameters (K_m values for H_2 and MV) differ as much as 10-fold between the two enzymes although the gross physical properties (mol wt and elution pattern on DEAE-cellulose) are similar.

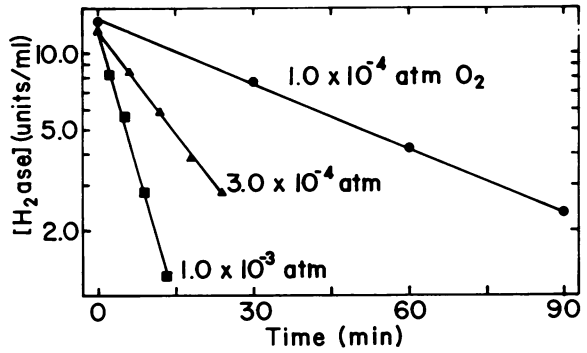
RESULTS

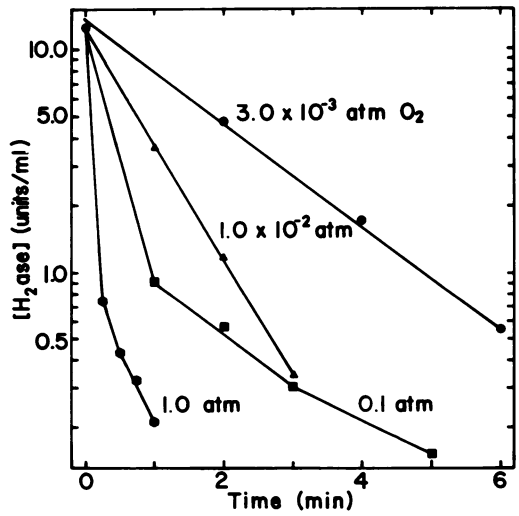
O_2 Inhibition of Partially Purified Hydrogenase. The inhibition of *C. reinhardi* hydrogenase by O_2 was irreversible. Neither removal of O_2 by repeated evacuation and H_2 or N_2 flushing nor addition of hydrogenase plus H_2 or dithionite would restore activity to O_2-inactivated hydrogenase. Figures 1 and 2 are plots of hydrogenase concentration *versus* time. At less than 0.10 atm O_2 the plots are linear indicating a pseudo-first order reaction with respect to hydrogenase. Pseudo-second, third, or fourth order plots were not linear. At 0.10 and 1.0 atm O_2 the plots were curved. The plot of log k_{obs} *versus* O_2 concentration (Fig. 3) has a slope of 0.94 which is equal to the number of O_2 molecules participating in the rate-limiting step (see under "Discussion"). The second order rate constant for the reaction of one molecule of hydrogenase and the equivalent of one molecule of O_2 is 190 ± 21 atm^{-1} min^{-1}.

The addition of H_2 (0.9–1.1 atm) had only a small effect on inactivation by O_2 at 0.001 and 0.010 atm O_2. With H_2 present the inactivation rates ranged from 85 to 115% of the rates observed in the absence of H_2.

Also under 0.001 atm O_2 and 1.0 atm N_2 the observed constant for inhibition of *C. moewusii* hydrogenase was 0.082 ± 0.014 min^{-1}.

CO Inhibition. Inhibition of partially purified *C. reinhardi* hydrogenase by CO was fully reversible by removal of the CO with repeated evacuation and flushing with N_2. In determining the type of inhibition that CO offered *versus* H_2 the assay medium contained a saturating concentration of 150 mM MV. The double reciprocal plot (Fig. 4) indicates a competitive inhibition for CO *versus* H_2. The K_i for CO is 0.0010 ± 0.0009 atm (0.9 ± 0.8 μM).





FIGS. 1 and 2. Semilogarithmic plot of inactivation time course for hydrogenase incubated under O_2. A solution of hydrogenase (11.3 mg protein/ml) was equilibrated with a gas phase composed of 1.0 atm N_2 and the indicated pO_2.

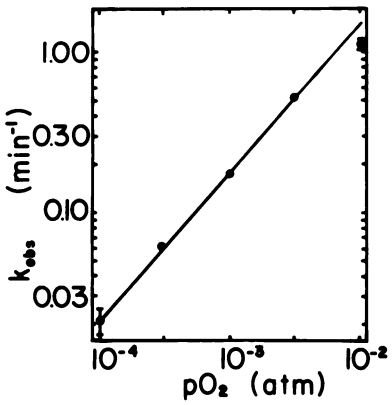


FIG. 3. k_{obs} *versus* pO_2 plotted on logarithmic axes.

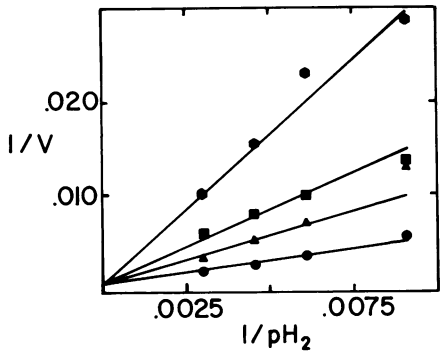


FIG. 4. Double reciprocal plot of 1/v *vs.* $1/pH_2$. 1/v is in mg protein/unit and $1/pH_2$ is in atm^{-1}. CO levels: (●—●), no CO; (▲—▲), 0.0011 atm; (■—■), 0.0022 atm; (⬢—⬢), 0.0043 atm.

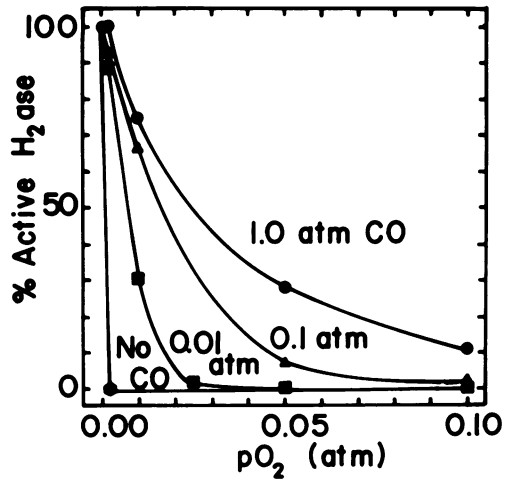


FIG. 5. Active hydrogenase remaining after 30-min incubation under several combinations of pCO and pO_2. N_2 was used to maintain a total pressure of 1.1 atm.

CO *versus* O_2 Inhibition. When partially purified *C. reinhardi* hydrogenase was incubated in a mixture of CO and O_2 a protective effect was exerted by the CO. Increasing the CO levels resulted in a progressive lowering of hydrogenase inactivation rates by O_2 (Fig. 5). At 1.0 atm CO, no loss of hydrogenase activity was observed after 30-min incubation in 0.001 atm O_2 (second solid circle on the curve) while no activity remained when CO was absent. The addition of H_2 to the CO-O_2 gas phase partially counteracted the protective effect of the CO (Fig. 6).

Deadaptation. When *C. reinhardi* cell suspensions (adapted 3 h) were placed under conditions of deadaptation (0.010 atm O_2) there was a rapid decline in cellular hydrogenase content (Fig. 7). Addition of 0.01 atm CO greatly reduced the inactivation rate. The addition of H_2 had little effect on the inactivation rate under CO plus O_2 but did retard inactivation when O_2 alone was present.

Considering this finding the 60% stimulation of O_2 uptake by the presence of H_2 is to be noted (Fig. 8). CO tends to depress O_2 uptake somewhat. Also O_2 uptake is less at 48 h than at 3 h.

At 48 h the kinetics of cellular hydrogenase inactivation (Fig. 9) closely resembled those of the partially purified enzyme (Fig. 6). O_2 alone rapidly inactivated the enzyme (with little protection through the addition of H_2) whereas CO protection against O_2 inactivation was diminished by the presence of H_2.

The rates for deadaptation in whole cells and for cell-free hydrogenase inactivation by O_2 were compared. Deadaptation rates of 3-h-adapted cells at 0.001 and 0.010 atm O_2 tended to be lower or equal to the rates of inactivation of cell-free hydrogenase. For 48-h-adapted cells, however, the deadaptation rate of 0.010 atm O_2 varied from 50 to 150% of the cell-free inactivation rates and at 0.001 atm O_2 the range was even greater at 50 to 300%. We did not have sufficient data to establish a correlation of these fluctuations with varying O_2 uptake rates.

The anaerobic incubation altered the appearance of the algal cells. Within 10 min of preparation under N_2 the cell wall re-

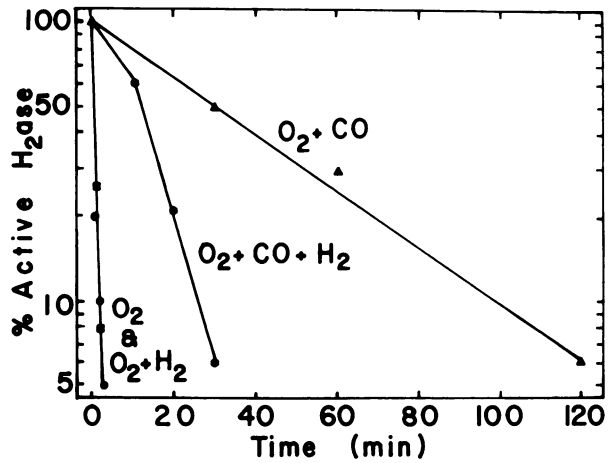


FIG. 6. Time course for hydrogenase inactivation under O_2, CO, and H_2. Partial pressures used were: O_2, 0.010 atm; CO, 0.10 atm; H_2, 0.90 atm; N_2, balance to a total of 1.1 atm.

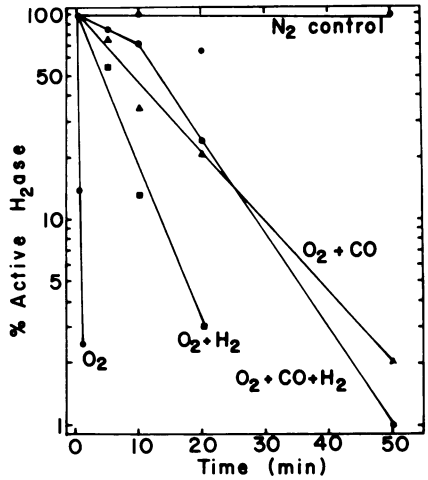


FIG. 7. Time course for deadaptation of hydrogenase in whole algal cells adapted for 3 h under N_2. O_2, CO, and H_2 concentrations were the same as in Figure 6.

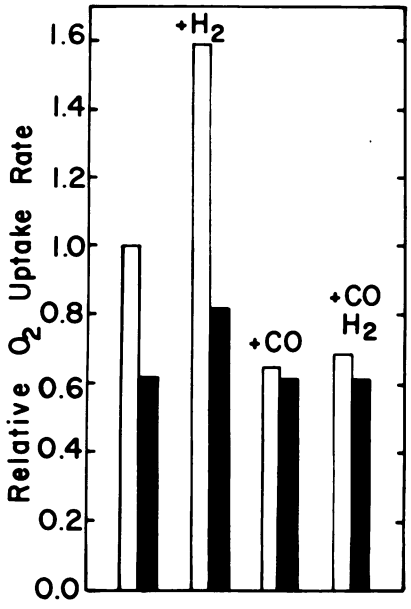


FIG. 8. Relative rates of O_2 uptake in 3-h- and 48-h-adapted cells under 0.010 atm O_2. O_2 ± CO (0.10 atm) or H_2 (0.90 atm). (□): 3-h adaptation; (■): 48-h adaptation.

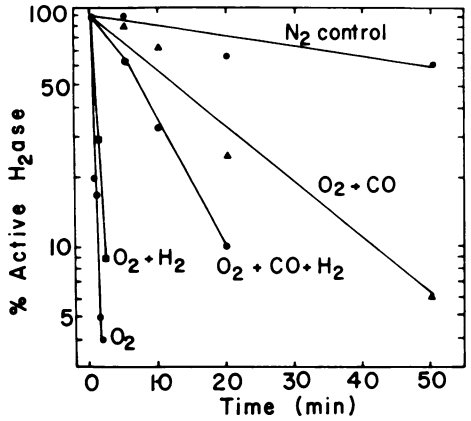


FIG. 9. Time for deadaptation of hydrogenase in whole algal cells adapted for 48 h under N_2. Gas partial pressures: O_2, 0.010 atm; CO, 0.10 atm; H_2, 0.90 atm.

mained well defined, the flagella were easily observed, and at least 90% of the cells were motile. After 48-h adaptation under N_2 the cell wall lacked definition and neither flagella nor motility was observed in any cell. These cells looked very much like those which had been frozen in liquid N_2 and thawed. Unlike those frozen and thawed cells no hydrogenase was released to the supernatant; instead the enzyme activity remained with the cells sedimented for 10 min at 10,000*g*.

DISCUSSION

Inactivation of Cell-free Hydrogenase by O_2. The inhibition of partially purified *C. reinhardi* hydrogenase by O_2 was irreversible. That neither physical removal of O_2 nor treatment with strong reducing agents such as dithionite or H_2 and hydrogenase would restore activity implies that more than a simple oxidation or oxygenation is involved.

In quantitating and analyzing the inactivation of cell-free *C. reinhardi* hydrogenase by O_2 a reaction equation can be set up in which x mol of hydrogenase reacts with y mol of O_2 to yield inactive hydrogenase:

$$x\ H_2ase + y\ O_2 \rightarrow \text{inactive } H_2ase$$

The rate of reaction (in derivative form) may be written as:

$$-d[H_2ase]/dt = k[H_2ase]^x[O_2]^y \quad (1)$$

in which $[H_2ase]$ is the hydrogenase concentration; $[O_2]$ is the concentration of O_2 in the gas phase in equilibrium with the hydrogenase solution; t is time; and k is the reaction rate constant. When the dissolved O_2 concentration is greater than the hydrogenase concentration, then the previous equation may be written as:

$$-d[H_2ase]/dt = k_{obs}[H_2ase]^x \quad (2)$$

in which:

$$k_{obs} = k[O_2]^y \quad (3)$$

If only one molecule of hydrogenase reacts in the rate-limiting transition complex, *i.e.* x = 1, equation 2 can be rearranged and integrated to:

$$\ln[H_2ase]_t = -k_{obs}t + \ln[H_2ase]_0 \quad (4)$$

At less than 0.10 atm O_2 all plots of $\ln[H_2ase]$ *versus* time are linear (Figs. 1 and 2). When the data are plotted using an integrated equation for x equal to 2, 3, or 4, the plots are not linear.

Equation 3 may be rearranged:

$$\log k_{obs} = y \log [O_2] + \log k \quad (5)$$

Plotting the data according to this equation (Fig. 3) yielded a slope, y, equal to 0.94. This value is the equivalent number of O_2 molecules participating in the rate-limiting step.

These results indicate that the rate-limiting step for inactivation of *C. reinhardi* hydrogenase by O_2 involves one molecule of hydrogenase and the equivalent of one molecule of O_2 (which may be O_2 or two oxygen atoms) with a second order rate constant of 190 atm^{-1} min^{-1}. This analysis has not addressed the possibility of participation by H_2O, H^+, or electrons in the inactivation process.

Above 0.01 atm O_2 a second reaction interferes with O_2 inactivation (Fig. 2). At 0.1 atm and 1.0 atm O_2 the rates of inactivation decrease with time. A possible explanation is that a number of O_2 molecules converge on the hydrogenase molecule and attempt to bind at the site of inactivation but no single O_2 molecule can achieve the steric orientation necessary to elicit inactivation of the hydrogenase.

A comparison of O_2 inactivation at 0.001 atm O_2 shows that the hydrogenase from *C. moewusii* loses activity at half the rate of the *C. reinhardi* enzyme (0.082 min^{-1} *versus* 0.176 min^{-1}). Comparisons of reaction parameters for H_2 activation and physical structure of hydrogenases from organisms with unequal O_2 inactivation rates might be of some interest in determining the factors involved in O_2 inactivation.

The inhibition by CO *versus* H_2 for *C. reinhardi* hydrogenase was competitive with a K_i of 0.0010 atm CO (Fig. 4). Competitive inhibitions were also observed for hydrogenases from *P. vulgaris*, *Desulfovibrio desulfuricans*, and *C. pasteurianum* with K_i values of 18, 3, and 5.6 μM, respectively (3, 12). The K_i (0.9 ± 0.8 μM CO) for *C. reinhardi* hydrogenase is somewhat lower than for the hydrogenases from nonphotosynthetic organisms.

There are three possible and different explanations for the protection by CO against O_2 inactivation of hydrogenase: (*a*) both CO and O_2 bind to the same site or sites thereby excluding one when the other is present; (*b*) they bind at different sites and CO exerts an allosteric effect which precludes the binding of O_2; (*c*) they bind with different forms of the enzyme which are in rapid equilibrium with each other. Because of the equilibrium the binding of an inhibitor molecule would cause a shift in the concentrations of the various enzyme forms including the one

necessary for binding of the other inhibitor. Such enzyme forms might be a series of different oxidation states as is known to exist for the *C. pasteurianum* hydrogenase (4). That H_2 antagonizes the protective effect of CO against O_2 inactivation of hydrogenase and is competitive in CO inhibition may support a hypothesis that CO and O_2 bind to the active site.

The irreversible inactivation by O_2 presents serious problems in the handling of algal hydrogenase even when using prepurified gases which may contain 10 μl/l or less of O_2. Even at these low levels the half-life of cell-free *C. reinhardi* hydrogenase is 1 to 2 h. This means that in the course of a working day a stock of active hydrogenase could be reduced to zero in the absence of additional protective measures. CO (in the absence of H_2) can afford protection against inactivation by O_2 during the preparation of hydrogenase. Removal of CO by evacuation is much simpler than the measures required for removal of other protective agents such as dithionite. Unfortunately CO inhibits the enzyme and cannot be used during the study of a fully active enzyme.

Inactivation of Hydrogenase in Whole Cells by O_2. We have followed deadaptation in whole cell *C. reinhardi* as a function of hydrogenase concentration determined by MV reduction. As shown in Figure 7, 0.01 atm O_2 inactivates the enzyme in 3-h-adapted cells in a matter of min. This reaction is irreversible in that neither evacuation nor the presence of dithionite or reduced MV would reactivate the hydrogenase. As occurs with the cell-free hydrogenase 0.10 atm CO protects against inactivation by O_2. The presence of 0.90 atm H_2 does lower the whole cell deadaptation rate in contrast to the cell-free findings. This may be attributed to O_2 uptake rates enhanced by the presence of H_2 (Fig. 8). Presumably the oxyhydrogen reaction is occurring and electrons from H_2 are supplied via hydrogenase to a system reducing O_2 to H_2O. This reaction would lower both the O_2 concentration within the cell and the rate of hydrogenase inactivation. After 48-h adaptation O_2 uptake rates are lower than at 3 h and H_2 fails to protect the hydrogenase in the presence of O_2. Also, H_2 promotes a higher rate of hydrogenase inactivation by O_2 in the presence of CO. These findings are qualitatively the same as those observed for cell-free hydrogenase inactivation. We conclude that the fate of the hydrogenase molecule during deadaptation is similar to that of the cell-free enzyme exposed to O_2.

We have tried to compare observed rate constants for cell-free and whole cell inactivation of hydrogenase. After 3-h adaptation whole cell hydrogenase exhibits inactivation rates which are equal to or less than the rates for the cell-free enzyme. After 48-h adaptation the observed rates for inactivation of whole cell hydrogenase varied from 50 to 300% of the cell-free inactivation rates. Although O_2 uptake was less at 48 h than 3 h (Fig. 8) there were insufficient data to establish a meaningful correlation between O_2 uptake rates and hydrogenase inactivation rates. We speculated that in cases where the whole cell rates were higher than the cell-free rates a chemical derivative of O_2 (such as H_2O_2) was formed by the reduced conditions of the anaerobic cells and promoted a more rapid inactivation of hydrogenase than O_2 did.

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