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Kinetics of the Oxyhydrogen Reaction in the Presence and Absence of Carbon Dioxide in *Scenedesmus obliquus*[1]

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ABSTRACT

The oxyhydrogen reaction in the presence and absence of CO_2 was studied in H_2-adapted *Scenedesmus obliquus* by monitoring the initial rates of H_2, O_2, and $^{14}CO_2$ uptake and the effect of inhibitors on these rates with gas-sensing electrodes and isotopic techniques. In the presence of 0.02 atmosphere O_2, the pH_2 was varied from 0 to 1 atmosphere. Whereas the rate of O_2 uptake increased by only 30%, the rate of H_2 uptake increased severalfold over the range of pH_2 values. At 0.1 atmosphere H_2 and 0.02 atmosphere O_2, rates for H_2 and O_2 uptake were between 15 and 25 micromoles per milligram chlorophyll per hour. As the pH_2 was changed from 0 to 1 atmosphere, the quotient H_2:O_2 changed from 0 to roughly 2. This change may reflect the competition between H_2 and the endogenous respiratory electron donors. Respiration in the presence of glucose and acetate was also competitive with H_2 uptake. KCN inhibited equally respiration (O_2 uptake in the absence of H_2) and the oxyhydrogen reaction in the presence and absence of CO_2. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone accelerated the rate of respiration and the oxyhydrogen reaction to a similar extent. It was concluded that the oxyhydrogen reaction both in the presence and absence of CO_2 has properties in common with components of respiration and photosynthesis. Participation of these two processes in the oxyhydrogen reaction would require a closely linked shuttle between mitochondrion and chloroplast.

The oxyhydrogen reaction in green algae, a process first noted and studied in depth by Gaffron (5), involves a simultaneous uptake of H_2 and O_2 under darkness. Using Warburg manometric techniques, he usually observed a quotient of H_2:O_2 equal to 1. In the presence of CO_2, uptake of CO_2 was observed and the quotient H_2:O_2 changed to 2. To account for these stoichiometries, perturbation of the quotient with inhibitors and the release of only traces of CO_2 during the oxyhydrogen reaction, Gaffron (5) concluded that little of the O_2 absorbed by the algal cell in the presence of H_2 was used for normal respiratory processes. Horwitz (11) used a mass spectrometer to monitor O_2 and CO_2 levels continuously and observed that both cellular respiration and the oxyhydrogen reaction coupled to CO_2 reduction had the same dependence on O_2 tension and that rates of O_2 uptake for the two reactions fell within the same range. The conclusion was reached that the oxyhydrogen reaction had some properties in common with respiration. Under oxyhydrogen reaction conditions, the reduction of CO_2 is known to involve the reductive pentose-P cycle (2, 14). Gibbs *et al.* (6) suggested a mechanism to account for CO_2 reduction coupled to the oxyhydrogen reaction. The proposed mechanism involves considerable intracellular transport between mitochondrion and chloroplast.

Here, we have studied the initial velocities of H_2, O_2, and CO_2 uptake in hydrogen-adapted *Scenedesmus obliquus* and the effect of inhibitors on these uptake rates. The rates of H_2 and O_2 uptake were monitored continuously during the first few min of the oxyhydrogen reaction using gas-sensing electrodes, whereas the assimilation of CO_2 was followed by isotopic methodology. Our work builds on that of Gaffron (5) and has uncovered aspects of the oxyhydrogen reaction not observable with manometric techniques.

MATERIALS AND METHODS

Culture of Scenedesmus. *S. obliquus* was grown on Tris-acetate-phosphate medium under fluorescent light and was harvested by centrifugation at logarithmic phase (7). The cells, after being washed and resuspended in 50 mM K-phosphate (pH 6.5), were evacuated three times and flushed with N_2. Before use in the experiments, the cells were adapted for 3 h under N_2 to induce hydrogenase activity.

Assays. H_2 and O_2 uptake were monitored simultaneously and continuously using electrodes contained in a Plexiglas vessel thermostated at 25 C. O_2 concentrations were determined using a YSI No. 5331 oxygen sensor and model 53 O_2 monitor. The H_2 electrode system was similar to that described by Wang *et al.* (16). The electrode chamber could be flushed with N_2. Reagents and cell suspensions were injected into the chamber via stopcocks equipped with serum stoppers.

Gas mixtures for the electrode work were prepared in stoppered 160-ml serum bottles. Ten ml buffer was evacuated and flushed with N_2 three times before H_2 or O_2 (in air) were introduced into the bottle at the appropriate partial pressure. The balance of the gas was N_2. Solubilities of 16.1 μl/ml H_2O for H_2 (3) and 28.3 μl/ml H_2O for O_2 (15) were used in calculations.

The electrode chamber (1.25 ml) was filled with 50 mM K-phosphate (pH 6.5) which had been pre-equilibrated with O_2 or H_2 at specified partial pressures. Any other reagents were injected into the chamber. The reaction was initiated by addition of 0.1 ml of the cell suspension and was followed for 1 to 3 min. The reaction was linear for 30 s or more. Typical Chl concentration in the reaction mixture ranged from 40 to 60 μg/ml.

To determine CO_2 fixation rates, the reaction mixtures were incubated with $H^{14}CO_3^-$ under specified partial pressures of O_2 and H_2 (1 ml reaction mixture with 5 ml gas space above the liquid in stoppered serum bottles). The reaction was initiated by injection of 0.1 ml cell suspension and shaken at 120 reciprocal cycles/min. Sample aliquots (0.2 ml) were removed by syringe at 1-min intervals for up to 4 min and treated with 20 μl concentrated HCl. Samples were plated on aluminum planchets and, after drying, radioactivity fixed into acid-stable compounds was determined in an end window counter.

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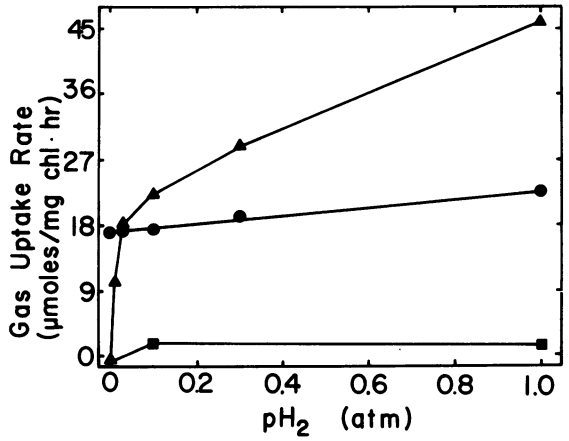

FIG. 1. Effect of pH_2 on O_2 and H_2 uptake rates. (●), O_2 uptake under 0.02 atm O_2; (▲), H_2 uptake under 0.02 atm O_2; (■), H_2 uptake in the absence of O_2.

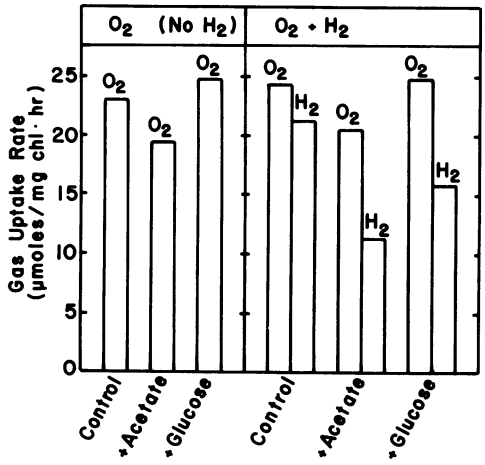

FIG. 2. Effect of acetate and glucose on O_2 and H_2 uptake rates. The gases (0.02 atm O_2, 0.10 atm H_2) present are indicated in the figure. Acetate and glucose concentrations were 10 mM.

In experiments where FCCP[3] was used, all samples (including controls) contained 1 μl ethanol/ml reaction medium. Chl was determined spectrophotometrically after extraction in hot 90% ethanol (10).

RESULTS AND DISCUSSION

OXYHYDROGEN REACTION IN ABSENCE OF CO_2

Gas Uptake. The bulk of O_2 uptake in algal cells is due to mitochondrial respiration (13). Mitochondrial electron flow monitored by O_2 uptake has been shown repeatedly not to be limited by the concentration of endogenous electron donors inasmuch as the addition of respirable substrates has little effect on the respiratory rate of freshly harvested cells (6). The addition of 1 atm H_2 increases the rate of O_2 uptake in adapted *Scenedesmus* by only 30% (Fig. 1). By contrast, the rate of H_2 uptake was highly dependent on the presence of O_2. At 1 atm H_2, the rate of H_2 consumption in the presence of 0.02 atm O_2 was 30 times the rate in the absence of O_2. At 0.1 atm H_2 and 0.02 atm O_2, rates for H_2 and O_2 uptake typically ranged between 15 and 25 μmol/mg Chl·h. Since H_2 uptake increased at a rate much greater than O_2 uptake with increasing pH_2, it seems that H_2 may compete successfully with endogenous electron sources in the mitochondrial reduction of O_2.

In the oxyhydrogen reaction, as studied manometrically by Gaffron (5), the total uptake of H_2 in the majority of experiments was equal to the volume of O_2 introduced into the Warburg vessel. However, some experiments approached a maximum of 3 volumes, namely, $2H_2{:}1O_2$ or the formation of water. We also noted this variability. As can be calculated from Figure 1, this quotient varied from 0 to roughly 2, depending upon the pH_2. We interpret this variable ratio to reflect the competition between H_2 and the endogenous electron sources with H_2 as the major reductant at 1 atm.

Effect of Glucose and Acetate. We were interested to see if H_2 uptake in the oxyhydrogen reaction would be affected by exogenous substrates. We supplied our algal cells with 10 mM glucose or 10 mM acetate during respiration and the oxyhydrogen reaction and the rates of H_2 and O_2 uptake are compared in Figure 2. Consumption of O_2 was slightly inhibited by acetate, whereas glucose caused a small stimulation.

In spite of the small effect of glucose and acetate on O_2 uptake, isotopic studies indicate that these substrates are readily metabolized in freshly harvested algae (6, 13). H_2 uptake was inhibited by glucose and to an even greater extent by acetate. This inhibition of H_2 uptake has been noted previously with the suggestion that glucose competes with H_2 as a hydrogen donor (5, 11).

We can speculate on how H_2 competes with carbon substrates as electron donor for the reduction of O_2. Hydrogenase seems to be located in the chloroplast. This proposal is based on the observation that there are a number of light-stimulated H_2 reactions and that the chloroplastic, soluble ferredoxin appears to be the physiological acceptor of electrons from hydrogenase (12). During the oxyhydrogen reaction, electrons removed from H_2 might be used to reduce NADP in the chloroplast. This reaction would be facilitated by hydrogenase, ferredoxin, and NADP-reductase (1, 4). Reduced pyridine nucleotide might be used to reduce oxaloacetate to malate with malate exported from the chloroplast in a dicarboxylate shuttle system such as that described for higher plants (9). These exported electrons then would be conveyed to the mitochondrion as malate for respiratory uptake of O_2. Glucose and acetate might compete with H_2-derived reductant at this point by supplying oxidizable carbon into the citric acid cycle.

Effect of KCN and FCCP. We compared the effects of KCN and FCCP on both respiration and the oxyhydrogen reaction. Respiration (*i.e.* O_2 uptake at pH_2 equal to 0 atm) and O_2 and H_2 uptake in the oxyhydrogen reaction are nearly equally affected by KCN (Fig. 3). The activity of the hydrogenase as measured by the reduction of benzoquinone with H_2 by the *Scenedesmus* cells was inhibited by less than 5% at 200 μM KCN (data not shown).

The effects of varied FCCP concentrations on respiration and the oxyhydrogen reaction are summarized in Figure 4. The uncoupling effect of FCCP accelerated the rate of O_2 uptake in respiration, indicating that energy conservation, rather than a supply of endogenous electron donors, limited mitochondrial electron transport. The rates of H_2 and O_2 uptake in the oxyhydrogen reaction followed the same pattern as that of cellular respiration, namely, an increasing rate up to 5 μM FCCP.

The similar response of respiration and the oxyhydrogen reaction to cyanide may indicate that the primary terminal oxidase in both processes was cytochrome oxidase (8). The findings with FCCP suggest that the oxyhydrogen reaction may be coupled to ATP formation, presumably through mitochondrial electron transport.

[3] Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

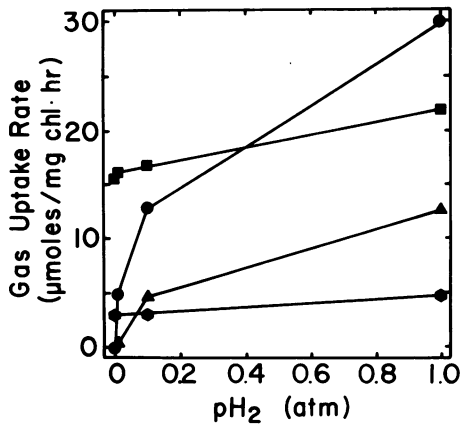

FIG. 3. Effect of KCN on O_2 and H_2 uptake rates at various pH_2 values. pO_2 was held at 0.02 atm while the pH_2 was varied. (■), O_2 uptake in the absence of KCN; (●), H_2 uptake in the absence of KCN; (◉), O_2 uptake in the presence of 200 μM KCN; (▲), H_2 uptake in the presence of 200 μM KCN.

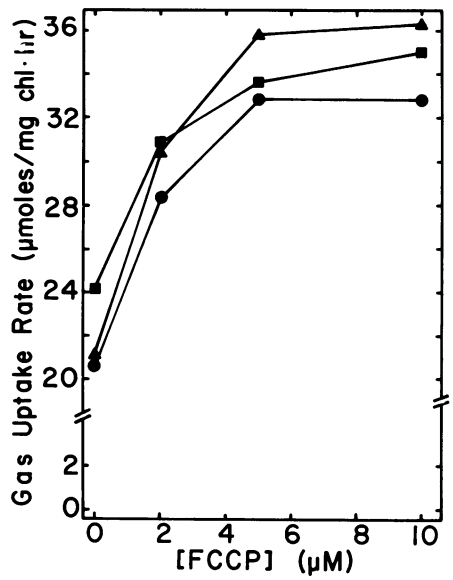

FIG. 4. Effect of FCCP on O_2 and H_2 uptake rates. (●), O_2 uptake measured under 0.02 atm O_2 but in the absence of H_2; (▲), O_2 uptake measured in the presence of 0.02 atm O_2 and 0.10 atm H_2; (■), H_2 uptake measured in the presence of 0.02 atm O_2 and 0.10 atm H_2.

OXYHYDROGEN REACTION IN PRESENCE OF CO_2

Gas Uptake. H_2 stimulated CO_2 fixation by roughly 2-fold when added in the presence of O_2 (compare O_2, CO_2, -KCN with O_2, H_2, CO_2, -KCN in Fig. 5). However, the presence of CO_2 did not perturb the H_2 to O_2 uptake rate given in Figure 5 (H_2, O_2, -KCN). The rates of CO_2 uptake were less than 20% (and often less than 5%) of the rates for either H_2 or O_2 consumption. Clearly, the chemosynthetic reduction of CO_2 was coupled to the oxyhydrogen reaction but, inasmuch as it apparently made little demand upon that reaction, the uptake rates of H_2 and O_2 remained undisturbed.

Gaffron (5) observed that the addition of CO_2 did influence the quantities of H_2 and O_2 consumed. Combining our observations with those of Gaffron indicates that H_2-related metabolism in *Scenedesmus* may change with time and with the growth conditions used for the algae.

Effect of KCN. Figure 3 indicates that 200 μM KCN was a strong inhibitor of respiration and the oxyhydrogen reaction. It was of interest to determine if the chemosynthetic reduction of CO_2 was similarly inhibited by cyanide. Figure 5 illustrates the effect of 200 μM KCN on $^{14}CO_2$ uptake in the presence or absence of H_2. In the presence of O_2 alone, the CO_2 fixation rate was diminished by less than 20%, whereas under oxyhydrogen conditions, 200 μM cyanide caused an 80% inhibition of the CO_2 fixation rate. The relative insensitivity of CO_2 fixation to cyanide under O_2 alone may be due to two factors: (*a*) fixation was supported by ATP and reduced pyridine nucleotide that had been accumulated during anaerobic adaptation or (*b*) fixation proceeded by cytoplasmic carboxylation of P-enolpyruvate and not by the reductive pentose-P cycle of the chloroplast. Most likely, the first mechanism was functioning since the reductive pentose-P cycle has been shown to be the pathway for assimilation of $^{14}CO_2$ under oxyhydrogen reaction conditions (2, 14).

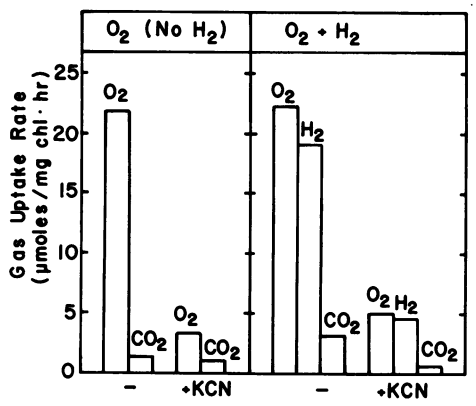

FIG. 5. Effect of KCN on the oxyhydrogen reaction in the presence of CO_2. Where used, the KCN concentration was 200 μM, [^{14}C]bicarbonate (60 μCi/μmol) was 10 mM, pO_2 was 0.02 atm, and pH_2 was 0.10 atm.

We conclude that the oxyhydrogen reaction has properties in common with components from both respiration and photosynthesis. This would require a closely linked relationship between mitochondrion and chloroplast. Final judgement on the validity of this conclusion awaits separation of the algal cell into organelles which can be investigated for their respective roles.

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