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EFFECT OF UNCOUPLERS ON ANAEROBIC ADAPTATION OF HYDROGENASE ACTIVITY IN C. REINHARDTII

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SUMMARY

An anaerobic incubation period of varying duration is required to induce hydrogenase activity in C. reinhardtii. Inclusion of sodium acetate, a metabolizable carbonaceous substrate, in the medium during anaerobic incubation accelerates the activation process. Thus, in the presence of sodium acetate, hydrogen photoproduction is detected within 7 to 15 minutes after the onset of anaerobiosis. On the contrary, if an uncoupler of phosphorylation, such as CCCP or sodium arsenate, is present during anaerobic incubation, little activation of the hydrogenase is observed even after hours of anaerobic adaptation. Since the uncouplers had no inhibitory effect on hydrogen photoproduction by the alga when added to previously activated cells, they are not inhibitors of activated hydrogenase. The uncouplers interfere, most likely, with the activation of hydrogenase. Similar effects of uncouplers on the hydrogenase activation process were obtained using a cell-free assay of hydrogenase activity. These observations provide strong evidence that anaerobic activation of the hydrogenase is an energy requiring process.

In various species of green algae, the enzyme hydrogenase is catalytically inactive under aerobic conditions. Thus, both production and utilization of H_2 by these algae requires an anaerobic adaptation period of varying duration during which the catalytic activity of hydrogenase is induced (1-3). However, the enzymatic systems, the biochemical events and the reaction mechanisms involved in the induction of hydrogenase activity in algal cells during anaerobic incubation are poorly understood at present. In view of the high sensitivity of the enzyme to oxygen, and by analogy to the activation process for certain procaryotic hydrogenases (4,5), it was suggested that activation of algal hydrogenases (2,6) is due to reductive and deoxygenation processes occurring under anaerobic conditions. In contast, Hartman and Krasna (7,8) reported that addition of dithionite or sulfide during anaerobic adaptation of Scenedesmus effectively inhibited the activation of hydrogenase. However, these reagents do not inhibit the catalytic activity of the activated hydrogenase. Since dithionite is a strong reductant which can either reduce or deoxygenate

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the enzyme rapidly, it is obvious that simple reduction or deoxygenation of the inactive enzyme is not sufficient to convert it into its active form. Based on their experiments, Hartman and Krasna suggested the possibility that conversion of protein disulfide groups into dithiols may be involved in the activation of the enzyme (8). In this article we report recent observations on the anaerobic adaptation of hydrogenase activity in another unicellular green alga, Chlamydomonas reinhardtii.

MATERIALS AND METHODS

C. reinhardtii strain 137 C (+) was grown photoautotrophically in a high salt minimal medium (ref. 9, p. 95) which was vigorously stirred in a three-liter spinner flask (Belco, No. 1967-03000) and aerated with compressed air containing 0.2-0.3% CO_2 at room temperature. The cultures were illuminated with banks of cool white fluorescent lamps providing a light intensity of approximately 600 foot candles (measured at the surface of the culture flask). The algal cells were harvested in the early stationary phase of growth by centrifugation at 5000 rpm for 10 minutes (Beckman Model J21 centrifuge using Type JA-14 Rotor). The cell pellets were washed once by resuspending in an appropriate volume (e.g., 0.25 ml of buffer per 1 ml of the original culture suspension) of either Tris-Cl buffer or potassium phosphate buffer (both at 60 mM concentration and pH 7.4) and then centrifuged as described above. The washed cell pellets were resuspended in the above mentioned buffer solutions and adjusted to a cell density such that 1 ml of the cell suspension contained 60-120 µg of total chlorophylls. Determination of total chlorophylls was carried out as previously described (10). Anaerobic incubation or adaptation of the alga was performed by continuous bubbling of argon gas through the cell suspension in the dark at room temperature.

Oxygen and hydrogen contents in the experimental algal suspension (having a cell density equivalent to 15 to 25 µg of total chlorophylls per ml) were monitored simultaneously with two Clark type polarographic electrodes (YSI Model 5331) in a water jacketed (25°C) reaction chamber having a total sample volume of 1.65 ml. For measurement of oxygen, the Clark type electrode was operated in the normal fashion, i.e., the Pt-electrode was polarized negatively (0.7V) with respect to the Ag/AgCl electrode. For measurement of hydrogen, the second Clark electrode polarized the Pt-electrode positively (+0.6V) with respect to the Ag/AgCl electrode as described by Wang et al. (11).

Saturating white light illumination (1.2×10^6 erg cm^{-2} sec^{-1}) of the sample was obtained from a tungsten-halogen lamp filled with a fiber optic illuminator (Fiber-Lite, Model 170-D, Dolar-Jenner Industries, Inc.) Cell free assay of the hydrogenase activity in a cell-free extract was performed by measuring the rate of H_2 production (using the polarographic assay) after lysing the anaerobically adapted algal suspension in the deoxygenated buffer solution containing 0.2% Triton X100 and 5 to 10 mM sodium dithionite. Hydrogen production was initiated by injection of 5 to 10 µl of methyl viologen (MV) to give a final concentration of 150 to 300 µM.

RESULTS AND DISCUSSION

Since hydrogen metabolism in green algae is catalyzed solely by hydrogenase, both production and utilization of H_2 by the algal cells requires

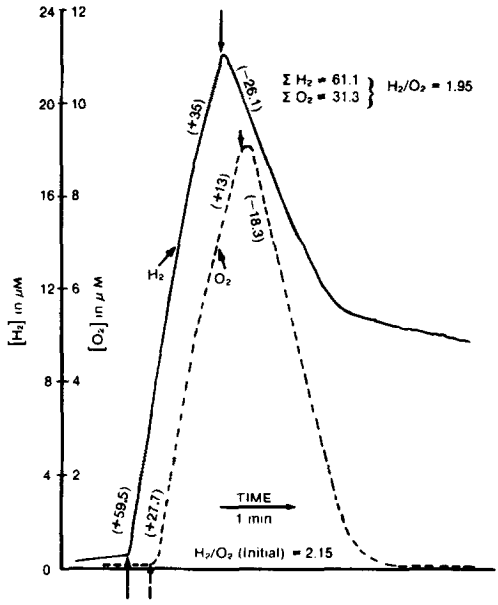

FIGURE 1. Simultaneous Photoproduction of H_2 and O_2 and the Oxyhydrogen Reaction by a Whole Cell Suspension of C. reinhardtii.

The sample contained anaerobically adapted (3 hr) cells of C. reinhardtii in 60 mM PO_4 buffer (pH 7.4) at a cell density equivalent to 14 μg Chl/ml. Saturating intensity of white-light illumination was provided as indicated by the upward arrows and terminated as indicated by the downward arrows. The traces for H_2 and O_2 are offset slightly horizontally for clarity. Numbers in parentheses represent the rate of net production or net consumption of the gases in μmoles/mg Chl hr.

an active hydrogenase. Thus, photoproduction of hydrogen in vivo provides a convenient means of monitoring the time course of hydrogenase activation during the adaptation period. However, the assay must be of short duration to prevent inactivation of the enzyme by the molecular oxygen which accumulates during illumination.

The simultaneous production of both hydrogen and oxygen in a molar ratio closely approximating the theoretical value of 2:1 by an anaerobically adapted (3 hr. incubation) suspension of C. reinhardtii is shown in Figure 1. These data indicate that water is the major source of reductant for hydrogen production. Furthermore, it is clear that oxygen concentrations of less than 20 μM exhibit little inhibitory effect on the activity of hydrogenase. This conclusion is based on the facts that: (1) the initial rate of net hydrogen production at the onset of illumination is essentially equal to the gross rate of hydrogen production at the moment

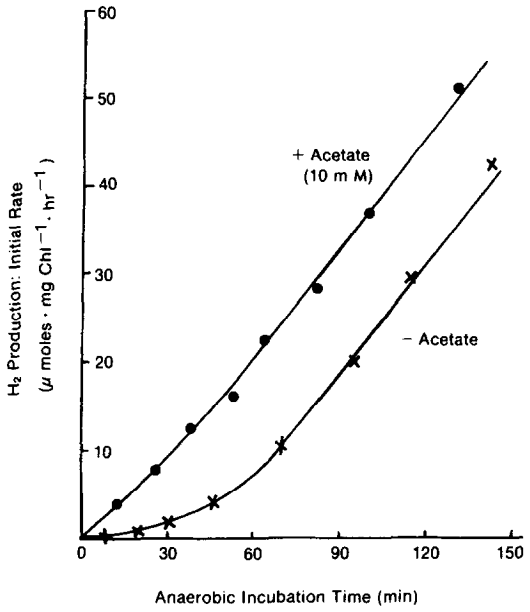

FIGURE 2. Effect of Sodium Acetate on the Time Course of Hydrogenase Activation.

The initial rate of H_2 production measured by the polarographic method (see text) was used to monitor the appearance of hydrogenase activity. The washed cells were incubated anaerobically in 650 mM PO_4 buffer (pH 7.4) at a cell density equivalent to 35 μg Chl/ml both in the absence of sodium acetate (x-x-x) and in the presence of 10 mM sodium acetate (o-o-o).

when illumination was terminated; and (2) the presence of an active hydrogen consuming reaction (oxyhydrogen reaction) during the microaerobic phase of the dark period immediately following a brief illumination. In the following, we relate the initial rate of hydrogen production to the activation of hydrogenase.

Addition of organic substrates can, in some cases, shorten the duration of the anaerobic adaptation period (12-14). As shown in Figure 2, the presence of sodium acetate in the medium during anaerobic incubation accelerated the appearance of hydrogenase activity during the first hour of adaptation. Since sodium acetate is an effective carbonaceous substrate for the heterotrophic growth of C. reinhardtii, it may improve the energy supply of the cells during adaptation and thereby accelerate the rate of hydrogenase activation provided the latter is an energy requiring process.

TABLE I. Effect of Uncouplers on the Activation of Hydrogenase in C. reinhardtii

Medium for Anaerobic Incubation		Initial Rate of H_2 Production (μmoles $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$)	
		1.5 hr Anaerobiosis	4.0 hr Anaerobiosis
Expt. 1A:	In PO_4 Buffer (60mM, pH 7.4)	26.0	106.0
B:	A + 2.5 μM CCCP	3.7	12.0
C:	A + 5 μM CCCP	0.8	7.0
D:	A + 10 μM CCCP	0.2	0.7
Expt. 2A:	In Tris-Buffer (60mM, pH 7.4)	37.0	120.0
B:	A + 5 mM Sodium Arsenate	0.3	0.5

Experiments 1 and 2 were performed with two different batches of autotrophically grown cells on separate dates. In both cases, the cells were harvested and incubated anaerobically under identical conditions. The cell density during incubation and assay was kept within the range of 20-30 μg Chl per ml. (See Materials and Methods for details of assay.)

To ascertain further the energy requirement of the hydrogenase activation process in C. reinhardtii, we examined the effect of uncouplers (of phosphorylation) on the activation of hydrogenase during anaerobic incubation (Table I). Clearly, CCCP (an effective uncoupler of phosphorylation coupled to electron transport) at concentrations ranging from 2.5 μM to 10 μM greatly inhibited hydrogen production by the algal cell suspension. Similarly, addition of sodium arsenate (5 mM) during the anaerobic incubation (in a phosphate-free medium) resulted in a nearly complete inhibition of hydrogen production. The finding that sodium arsenate, a well known competitive inhibitor of ATP production by either substrate level phosphorylation or electron transport coupled phosphorylation, caused such a pronounced inhibition on H_2 further demonstrates the energy requirement of the activation process. This interpretation of the inhibitory effect of CCCP and sodium arsenate on the activation of hydrogenase activity of C. reinhardtii is consistent with the well established fact that uncouplers are not inhibitors of hydrogenase activity when they are introduced directly into suspensions of preactivated algae. The insensi-

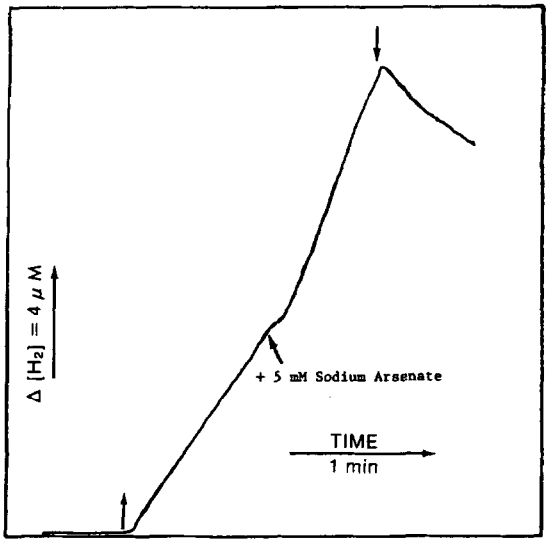

FIGURE 3. Lack of Inhibitory Effect by Sodium Arsenate on the Rate of Hydrogen Photoproduction in Preactivated Cells of C. reinhardtii.

An anaerobically activated cell suspension (3 hr under anaerobiosis) in 60 mM Tris-Cl, pH 7.4, at a cell density equivalent to 14 μg Chl/ml was illuminated with saturating white light (1.2×10^6 ergs cm^{-2} sec^{-1}) as indicated by the upward arrow. Sodium arsenate was added at approximately 1 minute after the onset of illumination. The downward arrow indicated the termination of illumination.

tivity of hydrogen photoproduction by adapted algae toward the inhibitory action of CCCP is well documented (11, 15, 16). The lack of effect of sodium arsenate on the rate of hydrogen production by anaerobically adapted C. reinhardtii is illustrated in Figure 3. As with CCCP, sodium arsenate exerts no inhibitory action. Instead, a significant (about two-fold) stimulation on the rate of hydrogen production is obtained.

The effect of the uncoupler, CCCP, on the anaerobic activation of hydrogenase was examined in greater detail and the results are summarized in Figure 4. In these experiments, the appearance of hydrogenase activity was assayed by two independent methods: (a) by the in vivo assay, as described earlier, using the initial rate of hydrogen production accompanying a brief and saturating illumination of a whole-cell suspension of the alga; and (b) by an in vitro assay using the rate of light-independent hydrogen production with a detergent-treated cell-free sample in the presence of sodium dithionite and methyl viologen. In both assays, the rate of hydrogen production measured at 4 hours after anaerobic incubation was

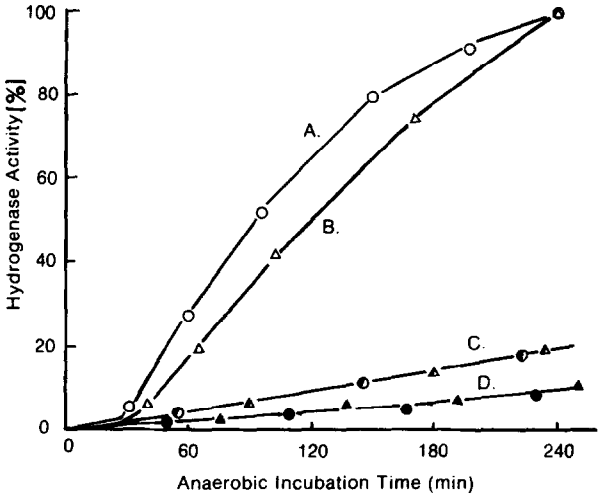

FIGURE 4. The Effect of CCCP on the Activation of Hydrogenase Activity as Measured by In Vivo and Cell-Free Assays.

Three samples of a washed whole-cell suspension of C. reinhardtii were incubated anaerobicly at a cell density equivalent to 31 µg Chl/ml in 60 mM PO_4 buffer, pH 7.6, in the presence of 0, 2.5 and 5.0 µM CCCP (Curves A or B, C, and D, respectively). At the indicated time after anaerobiosis, aliquots from each sample were withdrawn and analyzed for hydrogenase activity. The enzyme activity obtained with the control sample (i.e., CCCP = 0 µM) taken at 4 hours after anaerobic incubation was arbitrarily set at 100% hydrogenase activity for both the in vivo and in vitro (cell-free) assays. The values of the 100% activity in the in vivo assay was 92 µmoles of $H_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$, while the 100% value for the cell-free assay in the presence of 0.30 mM methyl viologen and 5 mM sodium dithionite was 53 µmoles $H_2 \cdot hr^{-1}$.

O Whole cell assay: CCCP = 0 µM
◐ Whole cell assay: CCCP = 2.5 µM
● Whole cell assay: CCCP = 5.0 µM
△ Cell-free assay: CCCP = 0 µM
▲ Cell-free assay: CCCP = 2.5 ΔM
▲ Cell-free assay: CCCP = 5.0 µM

arbitrarily taken as 100% of hydrogenase activity. Curves C and D indicate that in the presence of CCCP (2.5 µM and 5 µM, respectively), the time course of hydrogenase activation measured by the in vivo assay is indistinguishable from that obtained by the in vitro assay. In the control samples (i.e., in the absence of CCCP), a very similar time course of

activation was obtained by the two assay methods (see Curves A and B). A minor difference is that the initial rise (curvature) of the *in vivo* activation curve (Curve A) is higher than that obtained by the cell free assay (Curve B). This discrepancy is probably due to the fact that, in the *in vivo* assay, the rate of electron donation to hydrogenase cannot keep up with the catalytic activity of the enzyme present in the cells obtained after 3 to 4 hours of anaerobic incubation. Thus, with fully activated samples, the rate of electron donation to hydrogenase rather than the catalytic activity of the enzyme is the limiting parameter determining the maximal rate of hydrogen production in the *in vivo* assay. Therefore, with samples containing highly activated hydrogenase, the *in vitro* assay (in the presence of saturating amounts of dithionite and MV) is a preferred assay procedure.

In conclusion, using the initial rate of hydrogen production accompanying brief, saturating illumination to monitor the activity of hydrogenase *in vivo* we have demonstrated acceleration of the hydrogenase activation process by the addition of a metabolizable substrate. In contrast, introduction of uncoupling agents, such as CCCP and sodium arsenate, abolished the activation of the enzyme during anaerobic incubation. The inhibition of hydrogenase activation by uncouplers was confirmed with an *in vitro* assay of hydrogenase activity using detergent treated cell-free preparations.

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