Inactivation and Reactivation of the Hydrogenases of the Green Algae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*

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The hydrogenases of the green algae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* were activated under anaerobic conditions. Exposure of whole cells and cell-free homogenates to air lead to a complete inactivation of the hydrogenases. The inactivation in whole cells of *Scenedesmus* is faster than the inactivation of the cell-free homogenate. Inactivation of the hydrogenases could be reversed by anaerobic readaptation in whole cells. The inactivation of the hydrogenase in homogenates seems to be irreversible. Neither the removal of oxygen nor the addition of ATP, NAD(P)H, sodium dithionite, dithiothreitol, ferredoxin and thioredoxin to homogenates facilitated the reactivation of the hydrogenase.

The occurrence of a hydrogenase regulating factor is discussed.

Introduction

Scenedesmus obliquus was the first green alga in which a hydrogen metabolism was detected. During experiments with algae under anaerobic conditions Hans Gaffron found the ability of green algae to reduce CO_2, with molecular hydrogen [1, 2]. Only a few years later he described the evolution of hydrogen in light under an anaerobic atmosphere [3]. The uptake of hydrogen was called "photoreduction", the opposite process "photohydrogen evolution". In subsequent years a wide variety of green algae with hydrogen metabolism were reported [4–6]. Intensive studies on photohydrogen evolution were also conducted with cyanobacteria [7–10]. The enzymes catalyzing these reactions are called "hydrogenases" [11, 12].

Prerequisite for the activity of the hydrogenases in green algae is the adaptation under anaerobic conditions. The duration of this process depends on the organism [5, 13–16]. The active hydrogenases both in whole cells and in cell-free crude homogenates are very sensitive against oxygen [17]. Exposure to air leads to a total inactivation of the hydrogenase. If these inactivated whole cells are placed in an anaerobic atmosphere, the hydrogenase activity reappears.

In a previous paper, the reactivation of whole cells of *Scenedesmus obliquus* under anaerobiosis was studied in the presence and absence of ribosomal inhibitors [18]. In this paper we investigate the inactivation and reactivation processes particularly of cell-free preparations of the green algae *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* under various conditions.

Abbreviations: ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); SDT, sodium dithionite; U, units.

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Experimental

Organisms and growth conditions

Cells of the wild type of the green alga *Scenedesmus obliquus* strain D_3 [1–3] were grown heterotrophically in the dark at 33 °C as described by Senger and Bishop [19]. The inorganic medium was supplemented with 0.5% glucose and 0.25% yeast extract. *Chlamydomonas reinhardtii* strain WT 11–32(+) was grown photoautotrophically in inorganic medium as described by Kuhl [20, 21]. Cultures were aerated with 3% CO_2 in air and illuminated with fluorescent white light (4 Wm^{-2}).

Determination of hydrogen

Hydrogen evolution was measured polarographically with a Micro-Clark electrode (Gilson Medical Electronics, Middleton, Wisconsin, U.S.A.) at 28 °C under saturating incandescent light (400 Wm^{-2}). The electrode was calibrated with a gas mixture containing 2% H_2 (Messer,

Griesheim) and the solubility of H_2 in aqueous solution calculated with solubility data from the literature [22].

In cell-free homogenates reduced methylviologen (10 mM) was used as electron donor.

Protein and PCV determination

Protein was quantitated by the method of Bradford [23].

Packed cell volume (PCV) was determined by centrifugation of aliquots of the cell suspension in hematocrite tubes for 5 min at 1400 × *g*.

Hydrogenase activation

Cells were harvested by centrifugation and washed twice with buffer (see below). They were resuspended in the same buffer, placed in 50 ml Schlenk-bulbs and sealed with serum stoppers. The suspensions were degassed by flushing with nitrogen. During adaptation cells were shaken in darkness at 30 °C.

Scenedesmus-buffer: 100 mM phosphate pH 6.3
Chlamydomonas-buffer: 60 mM phosphate pH 7.4

Preparation of cell-free samples

Anaerobically adapted cells were disrupted with a vibrogen cell mill (Bühler, Tübingen) [24] and subsequently centrifuged for 30 min at 48000 × *g* at 1 °C. Hydrogenase activity was measured with methylviologen as electron donor in the supernatant, which had a protein content of about 5 mg ml^{-1}.

All preparation steps were done under nitrogen and dim green safe light.

Materials

Ferredoxin from spinach and thioredoxin from *Spirulina platensis* were obtained from Sigma, St. Louis; NADPH from Fluka, Buchs (Switzerland); sodium dithionite from Roth, Karlsruhe; all other chemicals were analytical reagent grade and purchased from Merck, Darmstadt.

Results and Discussion

Activation of the hydrogenases

Photohydrogen evolution could be detected 15 min after degassing of the algal cells. The maximum yield of photohydrogen evolution was reached after a 4–5 h period of anaerobiosis for both algae (Fig. 1). Subsequent aereation of the whole cells leads to the total inactivation of the hydrogenases. This process could be reversed by repeated degassing and adaptation. The time of readaptation and the maximum rate of photohydrogen evolution were similar to the primary adaptation process. For *Chlamydomonas* this is consistent with data from the literature [25]. For *Scenedesmus,* a shorter activation process was described for autotrophically grown cells than observed in the present experiments. This may be the result of different culture conditions and larger sample volume [19, 26, 27].

When cells were disrupted anaerobically as described under "experimental" the supernatant from the adapted cells retained the hydrogenase activity (Table I). The activity values of cell-free hydrogenase activity have to be multiplied by a factor of 12 according to the protein content of 12 mg ml^{-1} (PCV). The hydrogenase activity in the

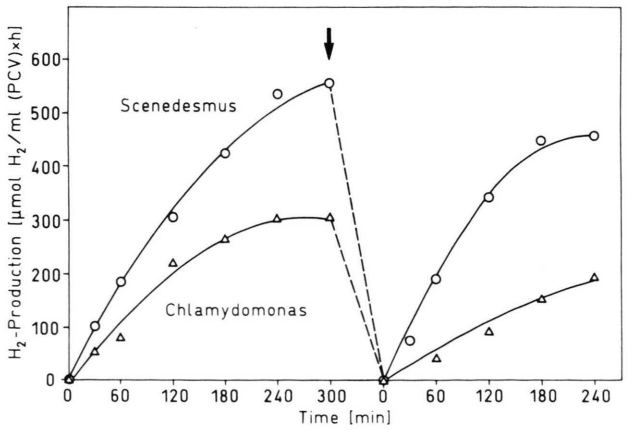

Fig. 1. Development of photohydrogen evolution during adaptation and readaptation of *Scenedesmus obliquus* (–○–) and *Chlamydomonas reinhardtii* (–△–). *Scenedesmus* cells were grown 3 d heterotrophically in the dark, *Chlamydomonas* cells 5 d autotrophically in the light. Algae were harvested as described in the Experimental section and degassed by flushing 30 min with nitrogen (arrow indicates aeration). The photohydrogen evolution was measured by transferring aliquots of the algal suspension containing 50 µl PCV in 2 ml phosphate buffer into the reaction chamber of the electrode. Samples were illuminated by saturating incandescent light (400 Wm^{-2}).

Table I. Hydrogen evolution of whole cells and cell-free preparations of *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*. Note that the parameters for hydrogen rates are different. 1 ml PCV contains about 12 mg of soluble protein.

	Whole cells [μmol H_2/ ml(PCV) h]	Cell-free homogenate [μmol H_2/ mg(protein) h]
Scenedesmus obliquus	560	205
Chlamydomonas reinhardtii	310	200

cell-free system is 4- to 7-fold higher than the activity in the whole cells after this calculation. This may be due to the high efficiency of the artificial electron donor used in the *in vitro* assay.

Inactivation of the hydrogenases

To study the inactivation process, anaerobically adapted algal cells or cell-free homogenates were gassed with air. In both algae, *Scenedesmus* and *Chlamydomonas*, aeration leads to a complete inactivation of the hydrogenase activity within 5 min in suspensions of whole cells and in cell-free homogenates of *Chlamydomonas*. In *Scenedesmus*, the inactivation time of the homogenate was longer. The activity was 1% after 30 min of aeration, the whole cell suspension had only 0.1% of the activity (relative to the non-aereated homogenate) after 2.5 min (Fig. 2).

From the different kinetics for inactivation one might draw the conclusion, at least for *Scenedesmus*, that a factor regulating inactivation might have been lost during preparation of cell-free extracts.

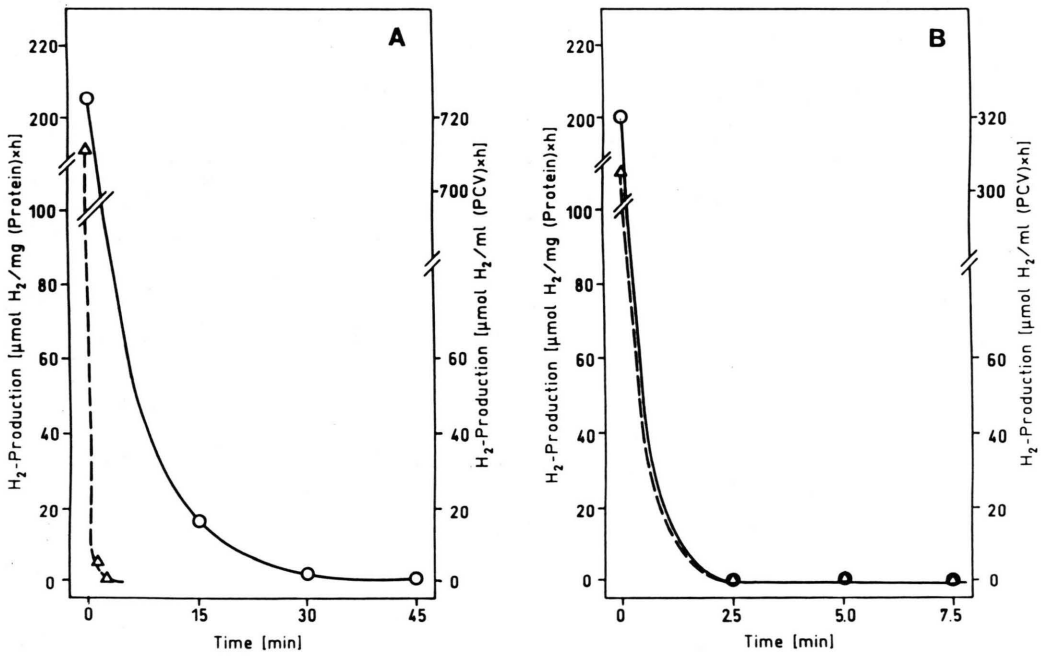

Fig. 2. Inactivation of hydrogenase activity in cell-free homogenates (−○−) and whole cells (−△−) of (A) *Scenedesmus* and (B) *Chlamydomonas*. Algae were adapted, anaerobically, disrupted and centrifuged 30 min at 48,000 × *g*. Inactivation occurred by intensive stirring in air. Hydrogen evolution was measured as described in the Experimental section.

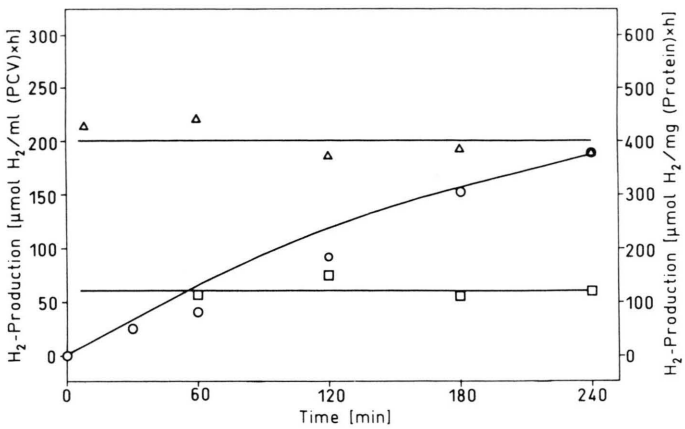

Fig. 3. Addition of equal amounts of homogenates with active hydrogenase from *Chlamydomonas* and (−□−) homogenates with inactivated hydrogenase and (−○−) with buffer alone. (−△−) shows the readaptation of whole cells of *Chlamydomonas*. Results obtained from *Scenedesmus* were identical to those obtained for *Chlamydomonas*.

Reactivation experiments in vitro

To elaborate whether the reactivation occurs in cell-free homogenates, adapted cells were broken and the hydrogenase was completely inactivated by gassing with air. The removal of oxygen by degassing (repeated evacuating and reflushing with nitrogen) or by addition of 10 U glucose oxidase, 4 U catalase and 12.5 mM glucose [29] did not lead to an activation of the hydrogenase.

To test whether the activation process is an energy requiring process, the influence of ATP (10 mM) was tested, but no activity could be detected after incubation for 6 h. Activation of the soluble hydrogenase from *Alcaligenes eutrophus* H 16 requires NAD(P)H [29]. In *Scenedesmus* and *Chlamydomonas* no activation occurred with 10 mM NAD(P)H, neither in nitrogen nor in hydrogen atmosphere. This confirms results obtained from *Chlamydomonas* [25].

Most successful reagents for the reactivation of procaryotic hydrogenases are reducing agents [30]. However, neither SDT (20 mM) nor DTT (20 mM) nor ferredoxin (0.01−0.05 mg/ml) were able to activate the hydrogenases of *Scenedesmus* and *Chlamydomonas*. Also combinations of thioredoxin (0.1−0.2 mg/ml) with 20 mM SDT or 20 mM DTT [31] failed to convert the inactive hydrogenase to an active enzyme. Furthermore, the combination of equal amounts of homogenates with active hydrogenase and homogenates with inactivated hydrogenase did not lead to a higher than expected hydrogenase activity. The activity remained constant (Fig. 3).

The true nature of the reactivation process remains yet unknown. From the inactivation kinetics of the green alga *Scenedesmus obliquus* we can presume the presence of an activating/deactivating principle in whole cells. A direct and simple oxygenation process of the hydrogenases of *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* could be ruled out, because there is no reactivation by removing the oxygen from the homogenate. The results rather indicate that there is an irreversible oxydation of the hydrogenase or that the reactivation process is dependent on a structural component. According to inhibition studies [18] we suppose that *de novo* synthesis of proteins plays an important role in the (re-)establishing of the hydrogenase activity. This interpretation is in accordance with results of Roessler and Lien, obtained for *Chlamydomonas reinhardtii* 137C(+) [25]. The activating/deactivating principle discussed above as the primary target of oxygen inhibition could not be proven to be a soluble factor, since the addition of active cell-free preparations could not stimulate hydrogenase activity in inactivated solubilized hydrogenase.

Acknowledgements

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