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In vivo Inactivation of Soluble Hydrogenase of *Alcaligenes eutrophus*

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Abstract. The soluble, NAD^+-reducing hydrogenase in intact cells of *Alcaligenes eutrophus* was inactivated by oxygen when electron donors such as hydrogen or pyruvate were available. The sole presence of either oxygen or oxidizable substrates did not lead to inactivation of the enzyme. Inactivation occurred similarly under autotrophic growth conditions with hydrogen, oxygen and carbon dioxide. The inactivation followed first order reaction kinetics, and the half-life of the enzyme in cells exposed to a gas atmosphere of hydrogen and oxygen (8:2, v/v) at 30°C was 1.5 h. The process of inactivation did not require ATP-synthesis. There was no experimental evidence that the inactivation is a reversible process catalyzed by a regulatory protein. The possibility is discussed that the inactivation is due to superoxide radical anions (O_2^-) produced by the hydrogenase itself.

Key words: *Alcaligenes eutrophus* – Soluble hydrogenase – Autotrophic growth – Oxygen-dependent inactivation

Alcaligenes eutrophus strain H16 is a facultatively chemolithoautotrophic Gram-negative bacterium which is able to grow in a minimal salts medium under a gas atmosphere of hydrogen, oxygen and carbon dioxide. Under normal autotrophic growth conditions, the oxygen concentration is 10% (v/v). Hydrogen is activated in *A. eutrophus* by a membrane-bound hydrogenase and a soluble NAD-reducing hydrogenase. Both enzymes have been purified and extensively studied (Schink and Schlegel 1979; Schneider and Schlegel 1976). The soluble hydrogenase (hydrogen: NAD^+ oxidoreductase, EC 1.12.1.2) exhibited a high stability during storage under air (Schneider and Schlegel 1976). Recent experiments carried out with the purified soluble hydrogenase from *A. eutrophus* in the presence of oxygen and electron donors such as molecular hydrogen or reduced pyridine-nucleotide revealed an irreversible inactivation by superoxide radicals produced by the enzyme itself (Schneider and Schlegel 1981).

Occasionally it has been found that the specific activity of soluble hydrogenase in cells grown autotrophically in batch cultures varied significantly, however, the reasons remained unexplained. In the course of regulatory studies on autotrophic metabolism it was necessary to examine the stability of hydrogenase in intact cells of *A. eutrophus*. Evidence is presented that the soluble hydrogenase is continuously inactivated under autotrophic growth conditions. The results strongly suggest that the inactivation in vivo is due to the formation of superoxide radicals, as described for the purified enzyme (Schneider and Schlegel 1981). This observation may also be relevant in view of increasing interest in a hydrogen uptake system for enhancing the energy efficiency of nitrogen fixation (Andersen et al. 1980), and in solar energy conversion utilizing hydrogenase (Pow and Krasna 1979).

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Materials and Methods

Chemicals. Rifampicin and Antimycin A were products of Boehringer (Mannheim, FRG), Amytal, 2,4-dinitrophenol and chloramphenicol were purchased from Serva (Heidelberg, FRG).

Growth Conditions. Alcaligenes eutrophus strain H16 (ATCC 17699, DSM 428) was grown at 30°C in a minimal salts medium described by Schlegel et al. (1961) with 0.2% NH_4Cl as nitrogen source. Cells were routinely grown in 500 ml side-arm flasks using a volume of 100 ml culture liquid on a rotary shaker at 30°C. Under heterotrophic conditions, an organic carbon source was added as indicated and cells were incubated under air. Autotrophic growth was performed similarly under a gas atmosphere of 80% (v/v) H_2, 10% (v/v) O_2 and 10% (v/v) CO_2 in the absence of any organic carbon source. Growth was monitored with a Klett-Summerson Colorimeter equipped with a 520–580 nm filter. 100 Klett units were equivalent to an optical density of 1.0 measured with a PL4 Zeiss-spectrophotometer at 436 nm.

Stability of Hydrogenase. Cells were grown heterotrophically in minimal medium with 0.1% (w/v) sodium pyruvate as carbon source for 48 h. At the end of this incubation the pH of the medium had increased from 6.8 to 7.1, and cells had reached an optical density of 180 Klett units and contained a specific activity of soluble hydrogenase of approximately 500 U/g of protein. Cells were diluted with minimal medium of pH 7.1 to an optical density of 100 Klett units and incubated in 500 ml side-arm flasks on a rotary shaker at 30°C. The desired gas mixtures were supplied continuously. The gas flow was 50 to 100 ml/min, measured with a gas flowmeter (Fischer and Porter, Göttingen, FRG).

Cell samples of 10 ml were taken at time intervals as indicated within an incubation period of 5 h. They were rapidly cooled down to 1–4°C, washed once with 50 mM potassium phosphate buffer, pH 7.0, and resuspended in the same buffer to an optical density of 30, measured at 436 nm with a PL4 Zeiss-spectrophotometer. Thus only intact unlysed cells were used for the determination of soluble hydrogenase.

Enzyme Assays. The activity of soluble hydrogenase was routinely determined with fresh whole cells as previously described (B. Friedrich et al. 1981), the activity of soluble hydrogenase in cell-free extracts was determined as described (Schneider and Schlegel 1976). A unit of enzyme activity is the amount of protein which catalyzes the formation of 1 µmol of product per min. Specific activity is defined as one unit of enzyme activity per g of protein. Protein of whole cells and cell extracts was determined by the method of Lowry et al. (1951).

Preparation of Cell Extracts. Cell extracts were prepared by suspending the cells in 50 mM potassium phosphate buffer, pH 7.0, to an optical

density of about 100 (436 nm). Cells were disrupted by sonication, and the cell debris was removed by centrifugation at 5000 × *g* for 20 min.

Results

Inactivation of Hydrogenase in the Presence of Oxygen

Cells of *Alcaligenes eutrophus* strain H16 grew heterotrophically with pyruvate as the sole carbon source with a doubling time of 1.5 h. After 8 h of growth in minimal medium containing 0.1 % pyruvate, the cells had reached the stationary phase of growth and were further incubated for 40 h. During exponential growth almost no hydrogenase activity was detectable (CG Friedrich et al. 1981). Only after growth had ceased, did hydrogenase activity increase continuously up to a level of 500 U/g of protein, which was comparable to the hydrogenase activity normally determined in autotrophically grown cells (CG Friedrich, personal communication).

The hydrogenase in cells grown to pyruvate exhaustion was stable when the cells were incubated in minimal medium under air. A change of the gas atmosphere from air to 80 % H_2 and 20 % O_2 led to a rapid decrease of hydrogenase activity. The semilogarithmic plot of the activity versus incubation time, resulted in a straight line and indicated first order reaction kinetics. A half-life of 90 min for hydrogenase was deduced from this plot. The presence of chloramphenicol did not affect inactivation. However, when the cells were kept under an atmosphere of pure hydrogen, the enzyme activity remained stable as observed under air (Table 1). On the other hand, hydrogenase was inactivated under air when the cells were transferred to a minimal medium which contained pyruvate as an energy and carbon source. The removal of oxygen from the atmosphere resulted in a stable hydrogenase even in the presence of pyruvate (Table 1).

Apparently oxygen was required for the inactivation of hydrogenase. However, inactivation by oxygen occurred only when a suitable electron donor such as pyruvate or hydrogen was present. This led to the question whether oxygen was directly responsible for the inactivation of hydrogenase or whether it acted through an energy-dependent process.

Effect of Inhibitors of the Respiratory Chain on the Inactivation of Hydrogenase

Provided the loss of hydrogenase activity was due to an energy-dependent process the inhibition of respiratory chain phosphorylation should prevent the enzyme from being inactivated. Most of the inhibitors examined were ineffective. Antimycin A and amytal did not inhibit or only very poorly inhibited growth. Other inhibitors such as 2,4-dinitrophenol or cyanide not only inhibited the growth but also severely inhibited the catalytic activity of hydrogenase as shown by experiments with the purified enzyme. Sodium azide did not affect the catalytic function of hydrogenase as significantly (K. Schneider, personal communication) but proved to be a potent inhibitor of growth due to its inhibition of the respiratory chain (Ishaque and Aleem 1970).

When cells grown under standard conditions with 0.1 % of pyruvate were transferred to a minimal medium containing 1 mM azide under an atmosphere of H_2 and O_2 inactivation of hydrogenase proceeded as fast as in the control without azide. However, when the azide concentration was increased to 5 mM inactivation of hydrogenase was significantly retarded (Table 1).

Table 1. Stability of soluble hydrogenase in whole cells of *Alcaligenes eutrophus*

Condition of incubation[a]		Hydrogenase activity[b] (%)	Half-life[c] (h)
Gas atmosphere	Additions		
Air	–	109	stable
H_2	–	100	stable
$H_2 + O_2$	–	10	1.5
Air	Pyruvate + Cm[d]	65	8
N_2	Pyruvate + Cm[d]	100	stable
$H_2 + O_2$	1 mM azide	10	1.5
$H_2 + O_2$	5 mM azide	27	2.7

[a] Cells were grown heterotrophically with 0.1 % of pyruvate, transferred to a minimal medium and incubated at 30°C as indicated. Gas mixtures of oxygen and hydrogen were in a ratio of 20:80

[b] Hydrogenase activity was determined by the whole cell assay at the beginning of the experiment and after 5 h of incubation, expressed as % of original activity

[c] Specific activity of hydrogenase plotted logarithmically against incubation time resulted in a straight line from which the half-life was calculated

[d] Pyruvate was added at a concentration of 0.2 % (w/v) and chloramphenicol (Cm) was present at a concentration of 120 µg/ml

Effect of ATP on Hydrogenase Inactivation in Cell Extracts

The experimental data with azide did not allow to draw a firm conclusion on a possible energy dependence of the inactivation process. Thus the effect of ATP on soluble hydrogenase activity in cell extracts was examined. In agreement with the results obtained in vivo, hydrogenase activity in extracts derived from pyruvate-exhausted cells was fairly stable under air at 30°C. After 4 h of incubation 84 % of the initial activity was still present. On the contrary, the hydrogenase activity in extracts from cells grown with glycerol as carbon source (C Friedrich et al. 1981) exhibited a high degree of instability. After 4 h of incubation only 7 % of the initial activity was recovered. The addition of 2 mM ATP to both extracts did not enhance the inactivation of hydrogenase.

Effect of Carbon Dioxide on the Inactivation of Hydrogenase

The energy required for autotrophic carbon dioxide fixation is solely derived from the oxidation of molecular hydrogen. The essential function of hydrogenase in autotrophic metabolism suggested that the soluble hydrogenase might be stable in cells incubated under autotrophic growth conditions, i.e. hydrogen, oxygen and carbon dioxide. In fact, the specific activity of the enzyme was relatively constant under those conditions. However, when growth of the cells was not permitted using protein synthesis inhibitors such as chloramphenicol or rifampicin, the hydrogenase activity decreased

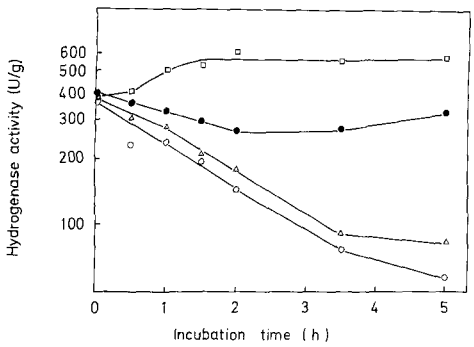

Fig. 1. Inactivation of hydrogenase in intact cells of *Alcaligenes eutrophus* grown autotrophically. Cells were grown in minimal salts medium under a gas atmosphere of 80% H_2, 10% O_2, and 10% CO_2. Cells were diluted as described in Materials and Methods, and the autotrophic incubation was continued for 1.5 h. Then (indicated as zero time) the incubation conditions were changed as follows, and hydrogenase activity was determined with washed intact cells: Addition of 120 µg/ml chloramphenicol (○——○), and 20 µg/ml rifampicin (△——△); substitution of the autotrophic gas atmosphere by 100% hydrogen (□——□). The control was incubated unter H_2, O_2 and CO_2 without additions to the medium (●——●)

rapidly (Fig. 1). The half-life of approximately 95 min was in accordance with the half-life of hydrogenase determined under hydrogen and oxygen without carbon dioxide (Table 1).

In a parallel experiment the autotrophically grown cells were exposed to a gas atmosphere of pure hydrogen (Fig. 1). As already evident from results presented in Table 1, hydrogenase activity remained constant.

Discussion

The results presented in this communication allow the conclusion that the soluble hydrogenase of *Alcaligenes eutrophus* strain H16 is inactivated under autotrophic conditions and to a lesser extent under heterotrophic conditions of growth. This indicates that at least during autotrophic growth the cells have to compensate for the loss of catalytic activity by a high differential rate of hydrogenase synthesis. Several lines of evidence support the assumption that the inactivation of hydrogenase observed in the presence of oxygen and an electron donor such as H_2 or pyruvate, is not mediated by an energy-dependent enzymatically catalyzed reaction: (i) The addition of chloramphenicol to cells incubated under inactivation conditions did not prevent hydrogenase from being inactivated. This suggests that no inactivating protein was formed specifically under these conditions. (ii) An inactivation of hydrogenase by an enzymatically catalyzed chemical modification should be reversible. Under no experimental conditions examined so far, could a significant reactivation of hydrogenase ever be observed. (iii) The inhibition of ATP regeneration by azide did not overcome the inactivation process. (iv) Even in the presence of carbon dioxide in the gas atmosphere, the inactivation of hydrogenase proceeded at a continuous rate, although considerable differences in pool sizes of metabolites such as ATP under hydrogen and oxygen and hydrogen, oxygen and carbon dioxide have been reported (Cook et al. 1976). (v) The addition of ATP to cell-free extracts did not affect hydrogenase stability. We, therefore, conclude that the inactivation of hydrogenase is due to oxygen itself and not a result of an energy-mediated process in the cell.

The inactivation followed first order reaction kinetics and was in accordance with the data obtained for the purified hydrogenase (Schneider and Schlegel 1981).

The half-life of the enzyme under hydrogen and oxygen was determined as 90 min for intact cells, and 30 min for the purified hydrogenase. Furthermore, the inactivating conditions in vivo were similar to those in vitro. Besides oxygen, an electron donor such as hydrogen or NADH was also required for inactivation in vitro. The instability of hydrogenase under heterotrophic growth conditions and in cell-free extracts derived from glycerol-grown cells is explained by the presence of NADH. On the other hand, the enzyme in carbon- and energy-starved cells, i.e. under pyruvate exhaustion, exhibited a marked stability presumably due to a lower capacity of reducing power.

The inactivation of the purified hydrogenase was previously identified as a result of self-produced superoxide radicals (Schneider and Schlegel 1981). The present work is the first study in vivo showing that the high degree of instability of hydrogenase is apparently caused by the same self-destructive mechanism which we would prefer to designate as "autogenous" inactivation. The fact that higher concentrations of azide retarded the inactivation may either indicate that azide competes with oxygen for the binding site of the hydrogenase or inhibits the catalytic function of the enzyme. Both modes of action could lead to an inhibition of superoxide radical formation.

Obviously the hydrogenase is not well protected against the inactivation by superoxide radicals in vivo. A possible protective role is assigned to the superoxide dismutase. However, the results obtained with the purified enzyme revealed that the protection by superoxide dismutase is limited (Schneider and Schlegel 1981). A respiratory protection against an irreversible inactivation by oxygen has been discussed for nitrogenase from *Azotobacter vinelandii* (Shah et al. 1973). Such a mechanism could also be considered relevant for hydrogenase of *A. eutrophus*. Nitrogenase of *Klebsiella pneumoniae* is not only inactivated by oxygen but its formation is severely repressed in the presence of oxygen (Eady et al. 1978; John et al. 1974). Apparently the formation of hydrogenase in *A. eutrophus* is not directly regulated by the supply of oxygen. The experimental results lead to the assumption that the cells have to compensate for the continuous loss of hydrogenase activity by a high differential rate of enzyme synthesis.

The properties of hydrogenase in vivo described in this communication have to be considered in future studies on the regulation and catalytic function of this enzyme in hydrogen bacteria and nitrogen fixing rhizobia.

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