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COMPETITIVE INHIBITION OF THE MEMBRANE-BOUND HYDROGENASE OF ALCALIGENES EUTROPHUS BY MOLECULAR OXYGEN

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SUMMARY

The membrane-bound hydrogenase of Alcaligenes eutrophus H 16 was completely stable under air, but was irreversibly inactivated during incubation under oxygen-free or reducing conditions. Superoxide radicals could be ruled out as inactivating agents. The reduction of methylene blue was inhibited by oxygen in a competitive manner; the K_i was 17 µM.

INTRODUCTION

Since hydrogenases were first studied in anaerobic organisms these enzymes have been considered to be sensitive to molecular oxygen (1). However, a clear distinction between an inactivating effect of oxygen during storage and an inhibitory effect during the catalysis reaction was not generally made. Recent investigations on the oxygen sensitivity of various hydrogenases from widely different organisms (2 - 11) seem to question the general role of oxygen as a toxic agent for this class of enzymes.

The aerobic hydrogen bacterium Alcaligenes eutrophus has two hydrogenases (12), a soluble NAD-reducing one (5) and a membrane-bound one linked to the respiratory chain (13). In this paper, we provide evidence that the membrane-bound hydrogenase of this organism is stable during storage under air, but oxygen strongly inhibits the enzyme reaction in a competitive manner.

MATERIALS AND METHODS

Superoxide dismutase and xanthine oxidase were obtained from Boehringer, Mannheim, W. Germany, luminol (5-amino-2,3-dihydro-1,4-phthalazine dione) from Eastman Kodak, Rochester, New York, and adrenalin from Serva, Heidelberg, W. Germany.

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Cytoplasmic membranes were prepared from autotrophically grown cells of Alcaligenes eutrophus H 16 (DSM 428, ATCC 17 699) by lysozyme treatment followed by osmotic shock (14). Membranes were washed with and suspended in 50 mM potassium phosphate buffer, pH 7.0. Membrane-bound hydrogenase was solubilized and purified according to (13).
The activity of the membrane-bound hydrogenase was measured either spectrophotometrically (13) or manometrically (12) as hydrogen-dependent reduction of methylene-blue. Differential measurement of simultaneous uptake of hydrogen and oxygen by hydrogenase-methylene blue mixtures was carried out by gas chromatographical analysis (15,16). The reaction mixture was shaken (120 strokes/min) at 30 °C in 14 ml Warburg vessels sealed with rubber stoppers. Samples of the gas phase were taken in 5 to 10 min intervals by a gas-tight microliter syringe and applied to a Perkin-Elmer F 11 gas chromatograph [oven: 80°C, injection port: 120°C, thermal conductivity detector: 200°C, filament current:81 mA, stainless steel column (2 x 100 cm) filled with molecular sieve 5 Å, 18 - 50 mesh, carrier gas: argon, flow rate: 12 ml/min]. Hydrogen, oxygen, and nitrogen were separated completely under these conditions. The relative disappearance of hydrogen and oxygen were determined by an integrator (Autolab minigrator, Spectra physics, Darmstadt, W. Germany) and calculated to absolute uptake using nitrogen as internal standard. During test time (20 - 40 min) the total gas pressure decreased by 5 % at maximum. Gas mixtures were prepared by calibrated flow tubes or by calibrated mechanical pumps (Wösthoff, Bochum, W. Germany).
Four different methods were used for the detection of active oxygen species: hydroxylamine oxidation (17), adrenalin oxidation (18), luminol oxidation (19), and reduction of tetranitromethan (18). In all cases control experiments with xanthine and xanthine oxidase gave positive proof of superoxide production which could be abolished by superoxide dismutase.
Membrane protein was determined according to (20), hydrogenase protein according to (21).

RESULTS

In intact cells as well as in membrane vesicles the membrane-bound hydrogenase of Alcaligenes eutrophus H 16 was stable when stored under air or hydrogen at 4°C, -18°C, or -196°C. Upon solubilization the hydrogenase was stable under air and lost only 30 % activity during two months at -18°C. However, storage of the solubilized or purified enzyme either under oxygen-free hydrogen or nitrogen or in the presence of reducing agents (mercaptoethanol, dithioerythritol) led to a rapid and complete loss of activity (Fig. 1). Reactivation of the enzyme by incubation under air or oxygen or by addition of ferricyanide was not possible.

The inhibitory effect of oxygen on hydrogenase activity and the hydrogen oxidase reaction was investigated using intact membrane vesicles as well as the purified enzyme. Oxygen concentrations higher than 60 µM (pO_2= 0.055 atm,

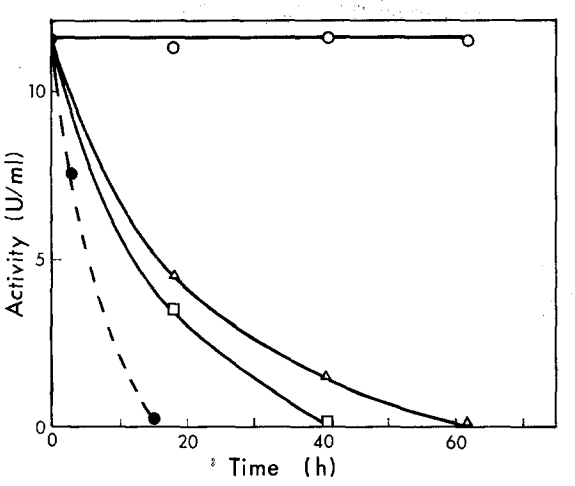

Fig. 1. Stability of solubilized membrane-bound hydrogenase of A. eutrophus. The enzyme solution (ca. 1.5 mg protein/ml) was stored in 50 mM potassium phosphate buffer, pH 7.0, at 4°C under air (○), oxygen-free nitrogen (△), or oxygen-free hydrogen (□). The dashed line refers to stability under air with 0.05 % mercaptoethanol. Activity was measured spectrophotometrically.

30°C) reversibly inhibited the hydrogen oxidase in membrane preparations (Fig. 2 A). This inhibition of the hydrogen oxidase was due to the oxygen sensitivity of the membrane-bound hydrogenase which constitutes the first enzyme of the hydrogen oxidase reaction chain. In its membrane-bound state at pH 7.0, the enzyme was 50 % inhibited at 67 µM oxygen (pO_2 = 0.06 atm). Upon solubilization the hydrogenase was much more sensitive (50 % inhibition at 35 µM O_2; Fig. 2 B). pH 7.0 and pH 5.5 are the optimal pH conditions for the membrane-bound and the solubilized enzyme, respectively. Removal of oxygen completely restored the original activity.

The generation of superoxide radicals by iron sulfur proteins (22) and the involvement of this active oxygen species in inhibition of respiratory enzymes (23 - 25) caused us to look for hydrogenase-catalyzed superoxide generation under hydrogen-oxygen gas mixtures. Intact membrane vesicles as well as the purified enzyme were incubated at different pH conditions (pH 5.5 - 8.5) under a hydrogen atmosphere containing various oxygen partial pressures (pO_2 = 0.015 - 0.1 atm). Using four different methods no superoxide radicals were detected. Also the addition of superoxide dismutase or xanthine and xan-

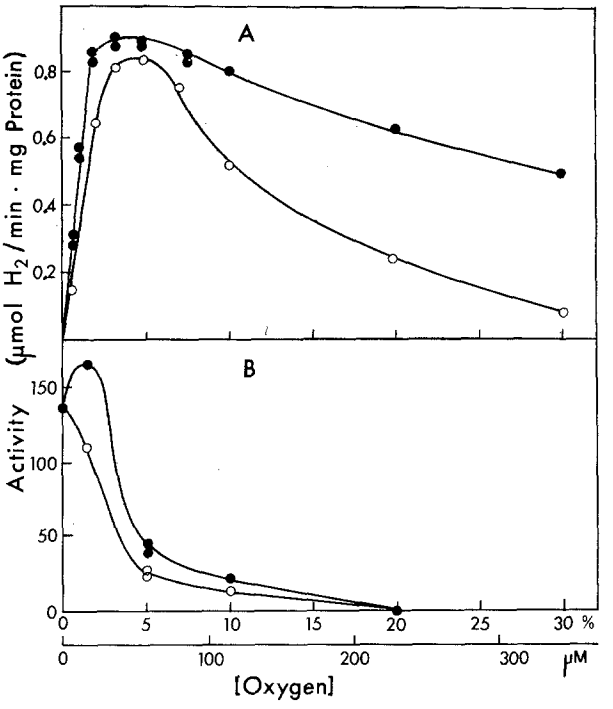

Fig. 2. Effect of oxygen on hydrogen oxidation rates. Gas uptake rates were measured manometrically.
A. Activity of hydrogen oxidase in membrane vesicles in 50 mM potassium phosphate buffer, pH 7.0 (●), or pH 7.6 (○). Protein content: 0.33 mg / assay.
B. Activity of purified hydrogenase in 50 mM potassium acetate buffer, pH 5.5; 0.8 µg protein, 10 µmol methylene blue/assay. (●) gas uptake rates as experimentally determined; (○) net hydrogen uptake rates calculated assuming immediate total reoxidation of reduced methylene blue.

thine oxidase as a superoxide generating system did not change the inhibitory effect of oxygen. Therefore, it seems very unlikely that superoxide radicals play a significant role as an inhibitory agent.

Hydrogen saturation curves at two different oxygen concentrations showed a competitive inhibition of the hydrogen oxidase by oxygen (Fig. 3). Studies on the type of inhibition of the free hydrogenase enzyme were difficult because thus far the membrane-bound hydrogenase only reacts with a few artificial electron acceptors such as methylene blue which are all spontaneously reoxidized in the presence of oxygen (see also Fig. 2 B). Therefore, the reaction rates obtained from manometric or spectrophotometric measurements were always altered by the reoxidation of the electron acceptor. Since a calibration of this

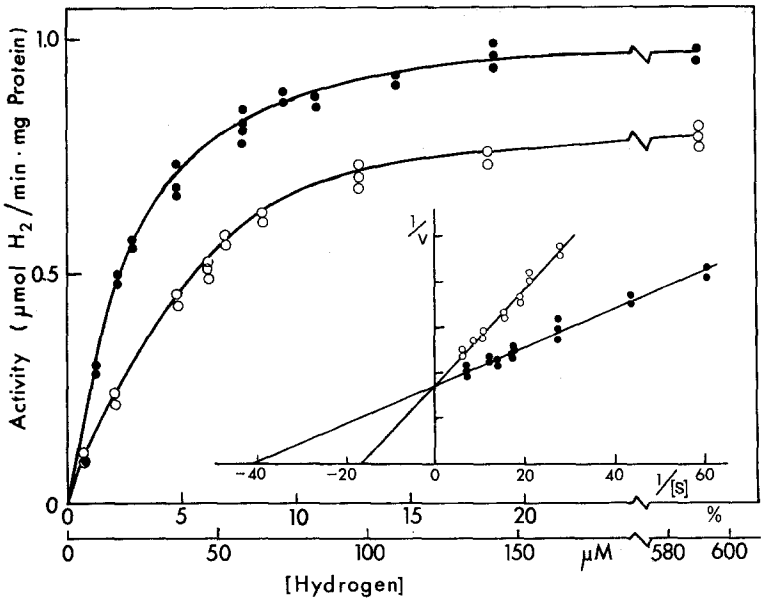

Fig. 3. Hydrogen saturation curves of hydrogen oxidase in intact membrane vesicles. Gas uptake rates were measured manometrically at pH 7.0 under an atmosphere of varying concentrations of hydrogen in nitrogen, with (●) 5 % and (○) 10 % oxygen as electron acceptor. Protein content: 0.4 mg/assay. Inset: Double-reciprocal plot.

reoxidation reaction was impossible it was necessary to differentiate between hydrogen and oxygen uptake by gas chromatographical analysis. The hydrogen saturation curves obtained with four different oxygen concentrations confirmed the assumption that oxygen inhibits the membrane-bound hydrogenase of A. eutrophus H 16 in a competitive manner (Fig. 4). The K_i value for oxygen was 17 µM (pO_2 = 0.015 atm).

DISCUSSION

With regard to their sensitivity to oxygen, hydrogenases of aerobic hydrogen bacteria reflect the taxonomic heterogeneity of this physiological group of microorganisms. While some previous studies on hydrogenases of hydrogen bacteria reported these enzymes to be inactivated by oxygen (26, 27, 28), more recent investigations revealed good stability to oxygen in some cases (5, 6, 10, 29). The two hydrogenases of A. eutrophus H 16 were even more stable in the presence than in the absence of oxygen or under reducing conditions (5, 13). Whereas the soluble enzyme tolerates oxygen concentrations up to 0.6 atm in the

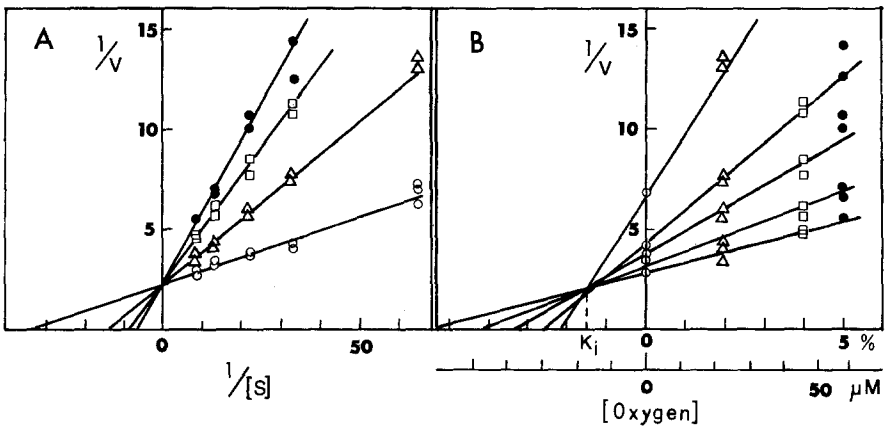

Fig. 4. Hydrogen saturation curves of purified hydrogenase. Hydrogen uptake was determined by gas chromatographical analysis. The assay contained 0.9 µg enzyme protein and 10 µmol methylene blue in 2 ml 50 mM potassium phosphate buffer, pH 5.5. The gas atmosphere contained hydrogen, nitrogen, and (○) 0 % oxygen, (△) 2 % oxygen, (□) 4 % oxygen, (●) 5 % oxygen. A. Double-reciprocal plot. B. Dixon plot.

test assay during reduction of NAD (30) the membrane-bound enzyme is inhibited by oxygen at low concentrations. The mechanism of this inhibition was shown in the present paper to be competitive suggesting that oxygen might bind to the active center of this enzyme and, thereby, stabilize it during storage.

Only few data exist so far about interference of oxygen with hydrogenases of other bacteria. The hydrogenase of Proteus vulgaris was reversibly inhibited by oxygen (31) whereas the membrane-bound hydrogenase of Chlamydomonas reinhardii was rapidly, irreversibly inactivated (32). The inactivating effect of oxygen on the soluble hydrogenase of Clostridium pasteurianum could be diminished by complexing agents suggesting that in the inactivation of this enzyme heavy metal ions are involved which probably catalyze random oxidation of sulfhydryl groups (33). Therefore, although the active centers of all hydrogenases studied so far were characterized as iron sulfur clusters of rather similar structure the interference of hydrogenases with oxygen seems to be rather different with respect to stabilization and inhibition mechanisms.

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