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EFFECTS OF ACETYLENE ON HYDROGENASES FROM THE SULFATE REDUCING AND METHANOGENIC BACTERIA

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SUMMARY: The effect of acetylene on the activity of the three types of hydrogenase from the anaerobic sulfate reducing bacteria has been investigated. The (Fe) hydrogenase is resistant to inhibition by acetylene while the nickel-containing hydrogenases are inhibited by acetylene with the (NiFe) hydrogenase being 10-50 fold more sensitive than the (NiFeSe) hydrogenase. In addition the Ni(III) EPR signal (g~2.3) of the "as isolated" (NiFe) hydrogenase was significantly decreased in intensity upon exposure to acetylene.

Acetylene has been well established as an inhibitor of a number of microbial processes such as denitrification, nitrification, nitrogen fixation, methanogenesis, H_2 metabolism, methanotrophy and nitrate reduction to ammonia (1). It can form both π- and σ-bonded systems with transition metals and most of its target enzymes appear to be metalloproteins (2). Hyman and Arp (3) have recently studied the acetylene inhibition of the purified nickel-containing hydrogenase from Azotobacter vinelandii and observed that it is a slow, reversible, active site-directed process. The inactivation by acetylene was shown to be competitive with hydrogen and carbon monoxide and it was tentatively concluded that acetylene interacts with the active-site nickel.

Three different types of hydrogenases have been characterized from sulfate reducing bacteria belonging to the genus, Desulfovibrio: an "iron only" hydrogenase from D. vulgaris [(Fe) hydrogenase]; a nickel-iron-selenium hydrogenase from D. baculatus [(NiFeSe) hydrogenase]; and a nickel-iron hydrogenase from D. gigas [(NiFe) hydrogenase] (4). The genes encoding the three hydrogenases have been cloned and sequenced (5,6,7). The deduced amino acid sequences of the two subunits of this (NiFe) hydrogenase are highly homologous to that of the (NiFe) hydrogenases which have been recently cloned and sequenced from other bacteria (8,9,10). A F_{420}-reducing (NiFe) hydrogenase from a thermophilic strain of Methanosarcina barkeri (DSM 2905) and a F_{420}-reducing (NiFeSe) hydrogenase from Methanococcus voltae (DSM 1537) (11) have also been purified and characterized.

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We have been attempting to develop specific probes for the (Fe), (NiFe) and (NiFeSe) hydrogenases in order to determine their roles in the metabolism of the sulfate reducing bacteria and found that each hydrogenase has different inhibition patterns with CO, NO^-_2 and NO (12). It was therefore of interest to ascertain whether acetylene exhibited any differential effects on the activities of the three hydrogenases from the anaerobic sulfate reducing bacteria and the nickel-containing hydrogenases from methanogens.

MATERIALS AND METHODS

The purified enzymes were: the periplasmic [Fe] hydrogenase from D. vulgaris Hildenborough (NCIB 8303) (13,14); the periplasmic [NiFe] hydrogenase from D. gigas (NCIB 9332) (15); the periplasmic [NiFeSe] hydrogenase from D. baculatus (DSM 1743) (16); the F_{420}-reducing [NiFe] hydrogenase from a thermophilic strain of Methanosarcina MST-A1 (DSM 2905), and the F_{420}-reducing [NiFeSe] hydrogenase from Methanococcus voltae strain PS (DSM 1537) (11).

Hydrogen (purity 99.995%) and argon (purity 99.999%) were purchased from Selox Inc. Acetylene (purity 99.6%) was purchased from Airco Industrial Gases (Norcross, GA) and ethylene (purity 99.5%) was purchased from Matheson Gas Products. The hydrogen and argon were rendered oxygen-free by passing them over a heated copper catalyst. The acetylene was further purified according to Hyman and Arp (17).

Hydrogenase activity was assayed by the rate of H_2 evolution with sodium dithionite (15 mM) as electron donor and methyl viologen (1 mM) as mediator (18) and hydrogen was determined by means of a Varian 4600 gas chromatograph (15).

Protein concentration was measured by Bradford method (19), or by measuring absorbance at 400 nm, employing the extinction coefficient at 400 nm for D. vulgaris, D. gigas and D. baculatus hydrogenases, i.e. 48.3 (20), 46.5 (21) and 41.74 $mM^{-1}cm^{-1}$ (22) respectively.

For studies on the inhibition of hydrogenase, acetylene was directly injected into assay-vials in an anaerobic chamber and the vials were shaken for 30 min. Appropriate amounts of hydrogenases were then injected into the assay vials and the H_2-evolution activity was determined.

The hydrogenase samples for EPR studies (50 μM in 100 mM potassium phosphate buffer, pH 7.6) were prepared employing a specially designed glass manifold equipped with a vacuum pump and H_2/Ar gas source. Enzyme samples in EPR tubes sealed with serum-stoppers were attached to the manifold through a syringe needle. For the preparation of oxidized hydrogenase samples, the EPR tubes were purged and evacuated several times with Ar. For the preparation of H_2-reduced samples, the EPR tubes were purged with purified hydrogen for 60 min. with occasional shaking, and then the hydrogen-filled EPR tubes were placed in a hydrogen filled cylinder until the hydrogenase became reduced as indicated by decolorization. The time for hydrogen reduction was about 30 min. for the (Fe) and (NiFeSe) hydrogenases, while the full reduction of (NiFe) hydrogenase required 24-48 hrs. For studies with acetylene, oxidized (Ar) and reduced (H_2) hydrogenase samples were flushed with purified acetylene (17) for 30 min. and placed in an acetylene-containing cylinder overnight. The EPR tubes were then frozen in liquid nitrogen. EPR measurements were performed with a Varian E-109 spectrometer interfaced with a Hewlett Packard Model 9816 microcomputer and equipped with an air products APD-E automatic temperature controller for low temperature spectroscopy.

RESULTS AND DISCUSSION

The effect of acetylene on the activity of the (Fe), (NiFe) and (NiFeSe) hydrogenases from the sulfate reducing bacteria is shown in Fig. 1. In order to compare the effect of acetylene on the three hydrogenases, each enzyme was

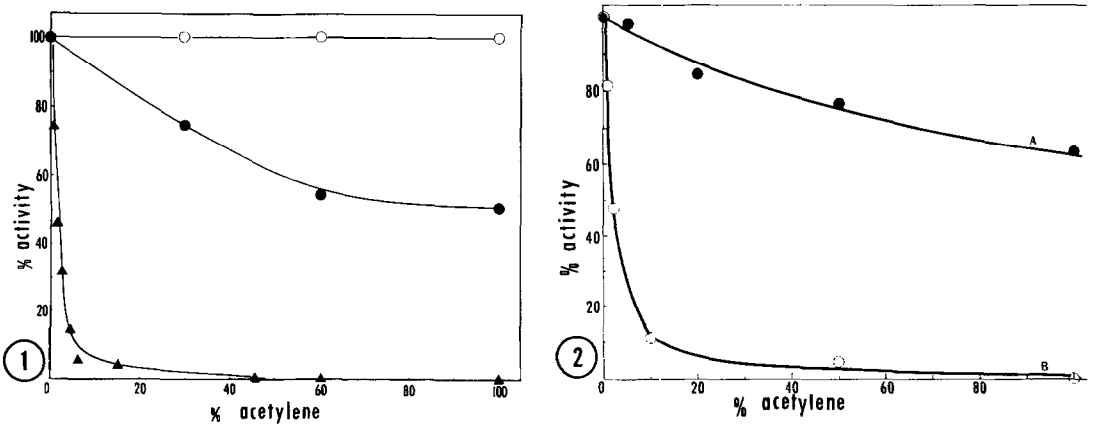

Figure 1. The effect of acetylene on the three types of hydrogenases from the sulfate reducing bacteria.
(Fe) hydrogenase from D. vulgaris, -o-.
(NiFe) hydrogenase from D. gigas, -▲-.
(NiFeSe) hydrogenase from D. baculatus, -●-.

Figure 2. The acetylene inhibition of the F_{420}-reducing hydrogenases from Mc. voltae and Methanosarcina MST.
A. The (NiFeSe) hydrogenase from Mc. voltae.
B. The (NiFe) hydrogenase from Methanosarcina MST.

incubated with acetylene for 30 min. and a portion of the enzyme was removed for the determination of activity. Acetylene exerts no inhibitory effect on the periplasmic (Fe) hydrogenase of D. vulgaris and is in contrast to CO which is highly inhibitory for the (Fe) hydrogenase (12). This observation may explain some earlier reports which indicated that acetylene does not inhibit hydrogenase activity (23); however, the failure to detect acetylene inhibition could equally as well have been due to contaminating hydrogen in their acetylene.

The activity of the (NiFe) hydrogenase of D. gigas in the evolution assay (Fig. 1) is inhibited by 30 min. exposure to relatively low concentrations of acetylene (50% inhibition in the presence of 0.69 mM acetylene). Similar inhibition of the homologous (NiFe) hydrogenase from A. vinelandii in the H_2 utilization assay with methylene blue has been reported by Hyman and Arp (3). The (NiFe) hydrogenases are around 20 fold less sensitive to inhibition by CO than the (Fe) hydrogenases (12,24,25) and the inhibition of both hydrogenases by CO is a rapid process involving the reduced forms of both types of hydrogenase (26,27). As these observations suggested that acetylene is a specific inhibitor of nickel-containing hydrogenases, it was of interest to determine the inhibitory effect of acetylene on the activity of the (NiFeSe) hydrogenase from D. baculatus. The (NiFeSe) hydrogenase differs from the (NiFe) hydrogenase in two aspects: first, it lacks the $[Fe_3S_4]$ non-heme iron center (28) and second, one of the cysteinyl ligands to the nickel is replaced by a selenocysteinyl ligand (29,22). As

shown in Fig. 1, the (NiFeSe) hydrogenase is much less sensitive to inactivation by acetylene than the (NiFe) hydrogenase (50% inactivation in the presence of 23.2 mM of acetylene in 30 min). As the [Fe_3S_4] non-heme iron center in the hydrogenase of D. gigas does not appear to be directly involved in the activation of hydrogen (30), it appears highly probable that the selenium ligand to the nickel-containing active site is responsible for this differential sensitivity to inactivation by acetylene.

These initial observations were extended by determining the effect of acetylene on the inactivation of the F_{420}-reducing (NiFe) hydrogenase from the thermophilic strain of Methanosarcina and the F_{420}-reducing (NiFeSe) hydrogenase from Mc. voltae. The overall pattern of acetylene inactivation (Fig. 2) is similar to that described for the corresponding nickel-containing hydrogenase from the sulfate reducing bacteria.

Our results indicate that inactivation by acetylene can be utilized to distinguish between hydrogenase activities due to (Fe) hydrogenases and nickel-containing hydrogenases in the sulfate reducing bacteria. Acetylene can also be utilized to distinguish between hydrogenase activities due to (NiFe) and (NiFeSe) hydrogenases from the sulfate reducing and methanogenic bacteria. However, all nickel-containing hydrogenase do not exhibit these characteristic responses to acetylene (3).

As carbon monoxide induces significant changes in the EPR spectrum of the partially reduced (NiFe) hydrogenase (26), it was therefore of interest to determine whether exposure to acetylene induced any changes in the EPR spectra of the enzyme. When the reduced forms of the (NiFe) and (NiFeSe) hydrogenases were exposed to acetylene (1 atm) for 30 min., there was no change in the EPR spectra. As carbon monoxide also interacts in some fashion with the oxidized (as isolated) (NiFe) hydrogenase, the effect of acetylene on the as isolated (NiFe) hydrogenase was investigated. In Fig. 3A, the characteristic EPR signal for the Ni(III) species found in the "as isolated" (NiFe) hydrogenase with g values at 2.31 and 2.2 (0.61 spin per mole) is shown. The EPR signal (g=2.02) originating from the (3Fe-4S) non-heme iron cluster (0.9 spin per mole) is also evident. After incubation at 0° under an atmosphere of freshly prepared acetylene (Fig. 3B), the intensity of the Ni(III) signal is reduced by 60% to 0.36 spin/mole; however, the g=2.02 signal remains essentially unchanged. When the acetylene-treated (NiFe) hydrogenase was subjected to high vacuum for 30 min. followed by anaerobic incubation under argon for several hrs., the Ni(III) EPR signal returned to its original intensity indicating that the effect of acetylene is reversible. No effect on the EPR signal was observed when the (NiFe) hydrogenase was reacted under similar conditions with either ethylene or acetonitrile. These results demonstrate that acetylene reacts in a specific and reversible fashion with the nickel

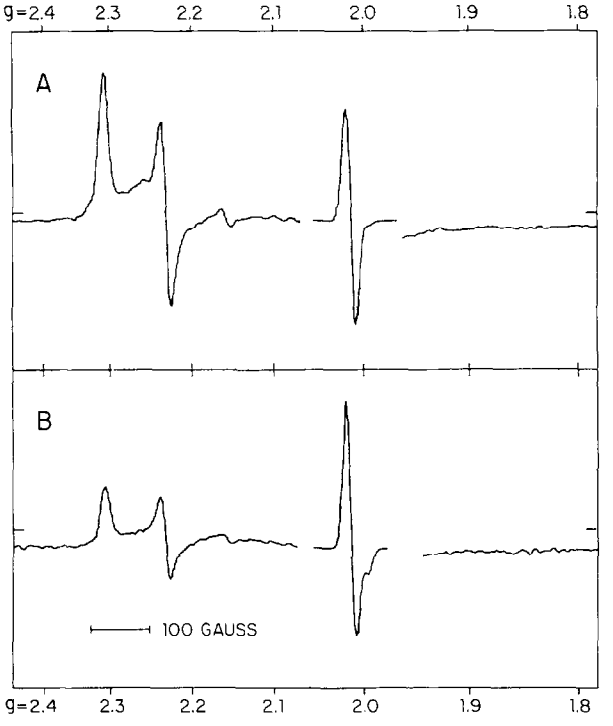

Figure 3. The effect of acetylene on the EPR spectra of the "as isolated" (NiFe) hydrogenase from D. gigas. A. [NiFe] Hydrogenase as isolated. EPR conditions: temperature, 6K; microwave frequency, 9.241 GHz; scanning rate, 250 Gauss per minute; time constant, 0.129 sec; modulation amplitude, 10 gauss. In g=2.3 region, the microwave power was 50 μwatt and the gain 3200, while in the g=2.0 region, the microwave power was 20 μwatt and the gain 1250. B. As (A) but treated with acetylene as described in the Methods Section for 30 minutes. EPR conditions as in (A).

(III) species responsible for the g=2.31 and 2.2 EPR signal from the (NiFe) hydrogenase; however, it is not known whether this is the only site at which acetylene reacts with this hydrogenase as it has recently been reported that the (NiFe) hydrogenase from Azotobacter vinelandii only reacts with acetylene in its catalytically active form (32).

The nickel (III) EPR signals (g~2.3) have been proposed to represent inactive or unready forms of the (NiFe) hydrogenase (33,34) which can be activated by H_2 or CO (31,21) and with hydrogen, produces an EPR "silent" state. Our observation that the (Fe) hydrogenase is not inhibited by acetylene is consistent with the idea that nickel is the site of acetylene inhibition. It should be noted that the intensity of the Ni(III) EPR signal (g~2.3) is 10-50 fold greater in the (NiFe) hydrogenase than in the (NiFeSe) hydrogenases. Activation of the (NiFe) hydrogenase may require 2-3 hours for full activation and is correlated with the loss of the g~2.3 EPR signals (35). The rapid activation of the (NiFeSe) hydrogenase is not correlated with the loss of Ni (III) (g~2.3) EPR signal and only requires the presence of hydrogen (36). Thus, the high susceptibility of the (NiFe) hydrogenase to

inhibition by acetylene may be due to the presence of a stable catalytically inactive nickel species characterized by the g-2.3 EPR signals.

Studies on the inhibition of hydrogenase activity by acetylene have produced contradictory results due to either competition with hydrogen added as a component of the assay system or as a contaminant in acetylene or the different sensitivities of hydrogenases to acetylene inhibition as shown in this report. Definitive evidence for the presence of multiple hydrogenase activities have been reported for a number of aerobic and anaerobic bacteria, i.e. E. coli (37), D. vulgaris (38), Alcaligenes eutrophus (39), C. pasteurianum (40), Mb. thermoautotrophicum (41) and Acetobacterium woodii (Chatelus, C., J. Le Gall, A.E. Przybyla and H.D. Peck, Jr., unpublished results). Hydrogenases function in both respiratory and fermentative processes and acetylene may prove to be a useful probe for determining the type of hydrogenase involved in these processes in different microorganisms.

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