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Short Communication

An Electron Donor System For Nitrogenase-Dependent Acetylene Reduction by Extracts of Soybean Nodules

Robert V. Klucas[1] and Harold J. Evans

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331

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The catalysis of nitrogen reduction by extracts of nitrogen-fixing organisms requires an ATP-generating system and a source of electrons (1, 2, 5, 11, 13). The phosphoroclastic reaction provides both ATP and electrons for the reduction of nitrogen by cell-free extracts of *Clostridium pasteurianum* (2, 5, 13) and ferredoxin functions as the natural electron carrier from the phosphoroclastic reaction to nitrogenase (12). Some evidence has been reported (2) that borohydride, hydrogen, NADH or NADPH, in presence of ferredoxin, will serve as a reductant for nitrogen fixation by the clostridial system. Cell-free extracts of *Azotobacter vinelandii* (1) and bacteroids from soybean nodules (11) also catalyze the reduction of N_2 to NH_3 but an exogenous source of both reductant and ATP must be furnished. Probably the physiological source of reductant and ATP for nitrogen fixation by legume nodules and azotobacter is the respiratory electron transport chain with its associated oxidative phosphorylation. All efforts however to couple nitrogen fixation by cell-free extracts of these organisms to naturally occurring oxidation-reduction reactions have failed. There are no reports of the identification in azotobacter or legume nodules of a low potential electron carrier analogous to ferredoxin.

Using the reduction of acetylene to ethylene as a measure of nitrogenase activity (3, 14), evidence has been obtained that NADH generated by the β-hydroxybutyrate dehydrogenase reaction or high concentrations of NADH supplied directly function as a source of electrons for acetylene reduction by extracts of nodule bacteroids or azotobacter. Activity also is dependent upon an ATP-generating system and a suitable dye.

Cell-free extracts of soybean nodules were prepared by the method of Koch, Evans, and Russell (10, 11) with minor changes to improve the nitrogenase activity. Ethylene production from acetylene was measured by a method described by Kelly, Klucas, and Burris (8) with the exception that the volume of each reaction bottle was 21 ml instead of 5 ml. The assay conditions are described in table I.

Acetylene reduction occurs in reactions containing a crude cell-free extract of bacteroids, an ATP-generating system and a reducing system composed of β-hydroxybutyrate, NADH and benzyl viologen (table I). The rate of reduction in the complete reaction is 8% of that observed when 40 μmoles of sodium hydrosulfite is supplied instead of NADH and β-hydroxybutyrate. Absolute requirements for the dye and the ATP system are demonstrated. No reaction occurred unless active enzyme was present. Stimulations of 4-fold (64–268) and 13-fold (21–268) were observed by the addition of 0.1 M β-hydroxybutyrate and 0.5 mM NADH, respectively. In other experiments NAD effectively substituted for NADH in the reaction but NADP failed to function. The addition of NADH at a concentration of 25 mM instead of the NADH-generating system consisting of β-hydroxybutyrate, endogenous β-hydroxybutyrate

Table I. *Requirements for Acetylene Reduction by the Nodule Bacteroid System*

The complete reaction mixture in a final volume of 2 ml contained: an ATP-generating system (75 μmoles of creatine phosphate, 7.5 μmoles of ATP, 10 μmoles of $MgCl_2$, 0.2 mg creatine phosphokinase); 200 μmoles DL-β-hydroxybutyrate, 0.5 μmoles of benzylviologen; 1.0 μmole NADH, 120 μmoles *N*-tris-(hydroxymethyl)-2-aminoethane sulfonic acid (Tes) buffer at pH 7.5 and 0.2 ml of soybean nodule bacteroid extract containing 6.8 mg of protein. The gas volume (19 ml) was composed of 0.1 atmosphere of acetylene and 0.9 atm of argon.

Reaction	C_2H_4 formed
	nmoles/20 min
Complete	268
Without ATP system	0
Without benzylviologen	0
Without NADH	21
Without β-hydroxybutyrate	64

[1] United States Public Health Service Fellow (GM-35-348-01)

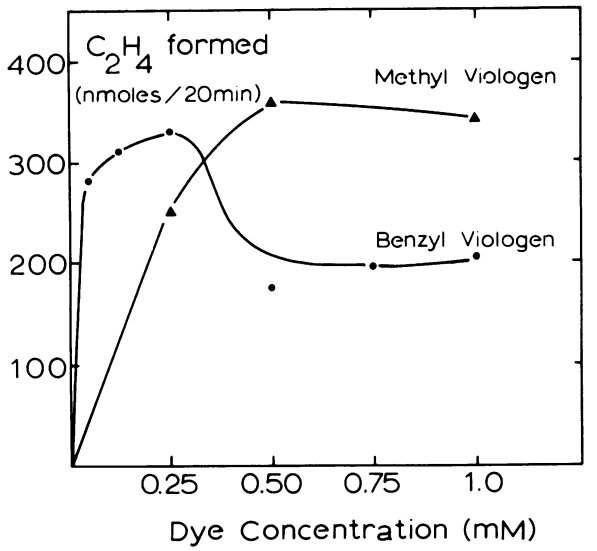

FIG. 1. The effect of concentrations of benzyl and methyl viologens on the rate of acetylene reduction. The experimental conditions were the same as those described for the complete reaction in table I with the exceptions that each reaction contained 0.2 ml (8.4 mg protein) of crude soybean nodule bacteroid extract and concentrations of dyes as indicated.

dehydrogenase and NADH at a concentration of 0.5 mM resulted in a rate of acetylene reduction comparable to that observed with the complete reaction (table I).

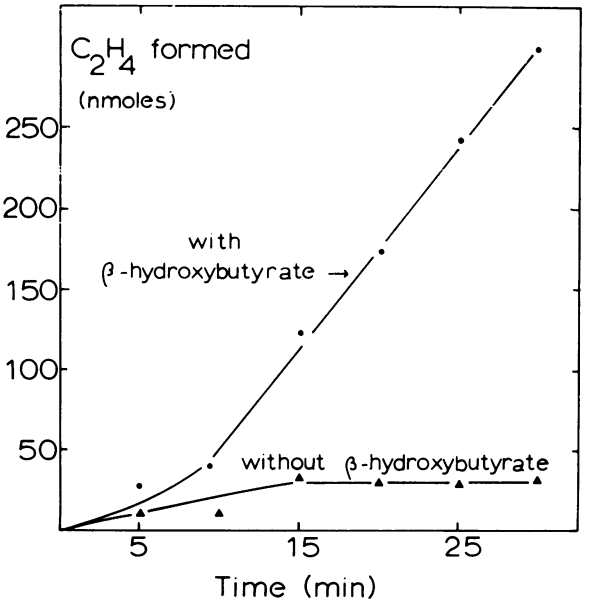

FIG. 2. Time course for the reduction of acetylene in the presence and absence of β-hydroxybutyrate. The experimental conditions are the same as those of the complete reaction (table I) with the exceptions that the period of incubation varied and β-hydroxybutyrate was omitted as indicated.

Either benzyl viologen or methyl viologen functions effectively as the electron carrier for the acetylene reduction system (fig 1). The optimum concentrations of the dyes are 0.25 and 0.5 mM for benzyl viologen and methyl viologen respectively. Experiments in which *Scenedesmus* ferredoxin (25 or 250 μM), clostridial ferredoxin (25 or 40 μM), or B_{12} coenzyme (10 or 50 μM) replaced benzyl viologen or methyl viologen in the assay yielded negative results.

In figure 2, time courses for the reduction of acetylene are shown with and without exogenously supplied β-hydroxybutyrate. Without the addition of β-hydroxybutyrate the reaction proceeded at a slow rate because a low concentration of NADH was added and endogenous β-hydroxybutyrate was present. When β-hydroxybutyrate was included in the complete reaction, a brief lag occurred before a linear rate of reduction was obtained. This very likely was caused by traces of oxygen in reaction vessels.

Strong evidence (3, 6, 11, 14) supports the conclusion that both nitrogen fixation and acetylene reduction are catalyzed by nitrogenase. This information together with the results reported here provide the basis for a scheme of electron transport for nitrogen fixation by soybean nodule bacteroids (fig 3). *Rhizobium* species have been reported (7) to contain poly-β-hydroxybutyrate and Mr. Peter Wong, in our laboratory, has shown that up to 40 % of the dry weight of nodule bacteroids may be accounted for as poly-β-hydroxybutyrate. Also bacteroid extracts exhibit an unusually high activity of β-hydroxybutyrate dehydrogenase. A NADH dehydrogenase (diaphorase) which catalyzes the reduction of benzyl viologen and other dyes has been identified in extracts of soybean nodule bacteroids (4). The natural factor represented by X in the scheme (fig 3) has not been identified but benzyl

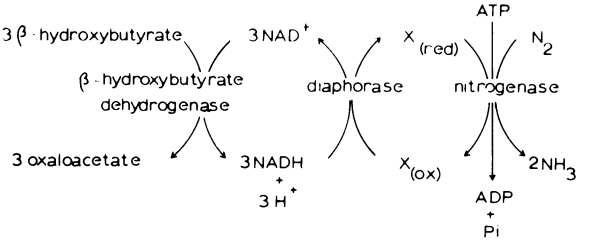

FIG. 3. A scheme of electron transport from β-hydroxybutyrate dehydrogenase to nitrogenase.

or methyl viologen effectively substitutes for the postulated natural carrier. Since benzyl viologen (redox potential of —0.36 volt) functions effectively in the coupled reactions it seems unnecessary to search for a natural carrier with a lower potential. The natural carrier could have a redox potential similar to that of flavodoxin (—0.28 volt) which has been isolated from iron deficient *C. pasteurianum* (9).

Other experiments in our laboratory have shown that extracts of *Azotobacter vinelandii* also catalyze acetylene reduction provided that NAD, β-hydroxybutyrate, benzyl or methyl viologen and an ATP-generating system are supplied. The optimum level of dye is considerably less than that found for the reaction catalyzed by the nodule bacteroid system. These results will be presented elsewhere.

Acknowledgment

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