PURIFICATION OF AZOTOPHORE MEMBRANES CONTAINING THE NITROGENASE FROM AZOTOBACTER VINELANDII*

Dan W. Reed, Robert E. Toia, Jr. and Dan Raveed
Charles F. Kettering Research Laboratory
Yellow Springs, Ohio 45387

Received March 5,1974

SUMMARY: Azotophore membranes containing nitrogenase have been purified in high yield from A. vinelandii by differential and sucrose density gradient sedimentation. The purified preparations appeared as uniform vesicular membranes of 40-75 nm diameter containing the majority of the nitrogenase activity from these cells and were readily separated from the intracytoplasmic membranes containing the cytochromes of the respiratory electron transfer system. The yield and specific activity of azotophores from cells broken by mechanical or osmotic treatment were similar.

INTRODUCTION: Nitrogenase catalyzes nitrogen fixation by reducing atmospheric nitrogen to ammonia. This enzyme requires nitrogen, a strong reductant and ATP for substrates as well as an anaerobic environment to maintain catalytic activity. These rather stringent requirements of the nitrogenase present important limitations on attempts to extend biological nitrogen fixation.

Both conformational and respiratory protection of the highly oxygen-sensitive nitrogenase have been proposed as mechanisms which function in azotobacter during growth at high oxygen concentrations (1). The molecular organization necessary for the operation of these mechanisms has not been established. Reports have indicated that nitrogenase occurred in a particulate oxygen-insensitive form after rupture of A. vinelandii (2) or A. chroococcum (3) in a French pressure cell but osmotic lysis by glycerol treatment produced a "soluble" oxygen-sensitive preparation (4).

In this paper we report the isolation of small vesicular nitrogenase-containing membranes from A. vinelandii which have been named "azotophores". Results are presented which indicate that the azotophore membranes can be isolated in high yield and purity from cells broken either by mechanical or osmotic treatment.

*Contribution No. 513 from the Charles F. Kettering Research Laboratory

METHODS: Nitrogenase activity in cell-free extracts was determined by measuring the ethylene formed from the reduction of acetylene essentially as described by Bulen et al (2). Reaction mixtures of 2.0 ml in 25 ml serum vials containing 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM ATP (Sigma), 50 mM creatine phosphate (Boehringer) and 0.12 mg/ml creatine phosphokinase (Boehringer) were flushed three times by evacuating and filling with 10% acetylene in argon (Matheson) and brought to 30° with magnetic stirring before addition of dithionite, 50 mM, and enzyme fraction containing 2-10 mg protein. Linear reaction rates were determined by removing gas samples at several different times and measuring the ethylene by a Varian series 2400 gas chromatograph equipped with a Porpak N column operated at 50°. The activity of several protein aliquots were measured to establish the proportionality to enzyme concentration.

Protein was measured by the biuret procedure of Gornall et al. (5) using bovine serum albumin (Sigma) as standard and dispersing the membrane fractions in 1% sodium deoxycholate. A Cary 14 recording spectrophotometer equipped with a scattered transmission attachment was used to measure absorption spectra and cytochrome b concentrations were calculated using the differential extinction coefficient of 17.3 mM^{-1} cm^{-1} (6) for the difference in 560 nm absorbance between ferricyanide-oxidized and anaerobic $Na_2S_2O_4$-reduced samples. Methyl viologen was also added to the azotophore fraction.

Azotobacter vinelandii strain OP (ATCC #13705) was grown at 30° in a modified Burk's nitrogen-free medium (7). Cultures were maintained by periodic growth of 250 ml from a 10% innoculum shaken at 150 rpm for 16 hr in a 500 ml Erlenmeyer flask. Two liter cultures were grown in 4 liter bottles with aeration at 7 liters per min through gas diffusers. After 16 hr of logarithmic growth, the cultures were rapidly cooled and fractions were maintained at 4° during all subsequent steps unless otherwise specified. Bacteria were harvested by centrifugation for 5 min at 10,000 x g, rinsed with 10 volumes of 0.1 M Tris-HCl buffer, pH 8.0, and used immediately.

For breakage in the French pressure cell, washed bacteria were suspended in 3 volumes of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.001 M $MgCl_2$ (Tris-Mg buffer). The mixture was immediately flushed with 10% hydrogen in argon which has passed through a Deoxo gas purified cartridge (Englehard Industries, East Newark, NJ) to remove traces of oxygen. Sodium dithionite, 0.2 mg/ml, and approx. 0.1 µg/ml of DNAse (Sigma, DN100) were added. The mixture was passed through a French pressure cell at 15,000 psi and collected under hydrogen-argon.

Bacteria were osmotically lysed by glycerol treatment similar to the method of Robrish and Marr (8). Cells were suspended in 1 volume of 3 M glycerol, incubated for 10 min at 25° and diluted into 10 volumes of the Tris-Mg buffer. The mixture was immediately flushed with hydrogen-argon. Sodium dithionite and DNAse were added and the mixture was incubated for 20 min at 25°.

Cell-free lysates were prepared by centrifuging the lysates prepared by either procedure at 20,000 x g for 10 min. Membrane fractions were isolated from these cell-free lysates by successive centrifugations at 120,000 x g for 90 min and at 160,000 x g for 5 hr. The pellets were resuspended separately in Tris-Mg buffer. All fractions were flushed with hydrogen-argon and stored at 4°.

Gradient separations were performed by layering 4.0 ml samples onto linear gradients prepared from 16 ml each of 0.5 M and 2.0 M sucrose in Tris-Mg buffer and centrifuging for 1.5 hr at 110,000 x g (25,000 rpm in a Spinco SW27 rotor).

RESULTS: The distribution of nitrogenase from A. vinelandii in the subcellular fractions isolated by differential centrifugation is summarized in Table I. The membranes which sedimented from the cell-free lysate during the first 90 min ultracentrifugation contained the cytochrome components of the respiratory electron transfer system and very little nitrogenase activity. The respiratory membrane preparations from French pressure cell lysis con-

TABLE I

ISOLATION OF AZOTOPHORE MEMBRANES FROM A. vinelandii

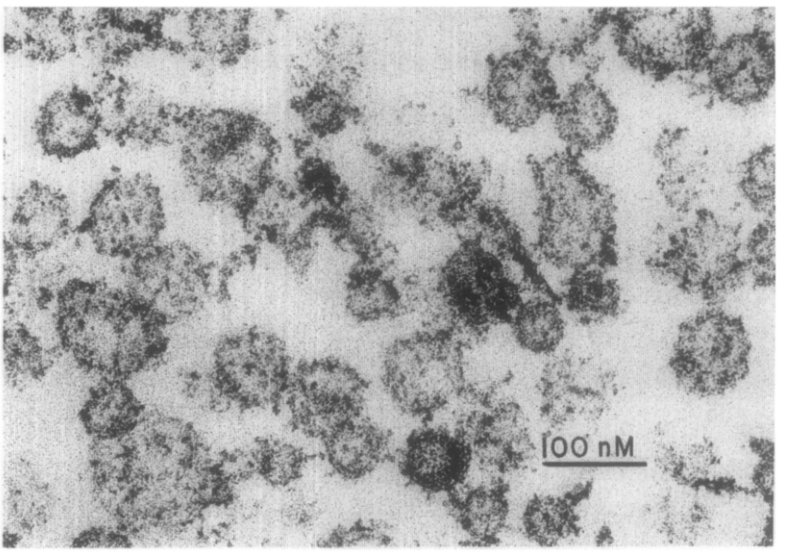
FRENCH PRESSURE CELL LYSIS

FRACTION	VOLUME (ml)	PROTEIN (mg)	NITROGENASE (nmoles/min)	SPECIFIC ACTIVITY (nmoles/min/mg)
Cell-free lysate	58	1943	17,300	8.9
Respiratory membrane	11.6	776	38.8	0.05
Azotophore membrane	11.4	394	18,900	48
Soluble	60	780	24.8	0.03
OSMOTIC GLYCEROL LYSIS				
Azotophore membrane	9.0	306	10,700	35

tained 900 mg protein/μmole cytochrome b. After osmotic lysis with glycerol, the respiratory membranes sedimented more readily with the large bacterial ghosts during the initial ultracentrifugation and these preparations also contained very low nitrogenase activity.

The additional 5 hr centrifugation sedimented the azotophore membrane fraction containing the great majority of the nitrogenase activity, approx. 20% of the protein from the cell-free lysate and no detectable cytochromes. The azotophore fraction contained small vesicular membranes shown in Fig. 1. French pressure cell lysis and isolation of azotophore membranes in Tris-Mg buffer containing additional 0.25 M sucrose or 0.005 M $MgCl_2$ did not significantly change the yield of this fraction.

The respiratory and azotophore membranes were also readily separated by sedimentation on linear sucrose density gradients. The purified respiratory membrane preparations from French press lysis sedimented as a major band near the middle of the gradient (ca. 1.6 M sucrose). A second minor band near the



Purified azotophore membranes, thin section (X 171,000). The sample was fixed in cold 1% OsO_4, 2% glutaraldehyde in 10 mM K phosphate, 0.5 mM $MgCl_2$, 125 mM sucrose, pH 7.2, embedded in Spurr's mixed epoxy resin and post-stained with 1% uranyl acetate and Reynold's lead citrate. The preparation contains primarily vesicular structures of 40-75 nm diameter which are bound by thin membranes. Some vesicles are connected and appear as strings.

top of the gradient (ca. 0.6 M) contained nitrogenase and approx. 10% of the protein in most preparations. In the purified azotophore membrane preparations nearly 90% of the protein and the nitrogenase sedimented as a single upper band at the same position as the minor band from the respiratory membrane preparations. Cell-free French press lysates sedimented directly on gradients gave two membrane bands whose protein content and positions correspond to those of the individual membrane fractions purified by differential centrifugation. Although only approximately 50% of the nitrogenase activity was recovered after the aerobic sucrose centrifugation, nitrogenase was only present in the band containing azotophore membranes.

DISCUSSION: The results presented here indicate that the nitrogenase in A. vinelandii is bound to membranes which can be isolated as uniform preparations of small vesicles called azotophores. The occurrence of nitrogenase in membranes is consistent with the previous reports of a particulate enzyme in

French pressure cell extracts (2-4) and similar preparations are sedimented from glycerol lysates by this longer centrifugation than that used previously (4) as the basis for the conclusion that glycerol produced a soluble nitrogenase. The purified azotophore preparations contain the majority of the nitrogenase and a substantial amount of the total protein from cells having high nitrogen fixation activity.

The similar sedimentation of azotophore membranes on sucrose density gradients both from unpurified cell-free lysates and from preparations purified in different buffers indicates that these membranes are not formed by aggregation of smaller components during sedimentation. The azotophore membranes containing the nitrogenase also have different properties from the larger respiratory membranes containing the cytochrome components and these two membranes are readily separated by differential sedimentation or on density gradients.

The release of the azotophores by osmotic lysis under conditions where the respiratory cytochromes remain associated with the bacterial ghost suggests that the azotophores may exist within the cell as a small vesicular membranes separate from the respiratory membrane which is contiguous with the cytoplasmic membrane (4,8). This type of subcellular organization would be important for the respiratory protection of nitrogenase from oxygen inactivation in this aerobic bacterium. However, ultrastructural studies are necessary to establish the ontogeny of the azotophores within bacterial cells. The localization of nitrogenase in A. vinelandii by binding of the ferritin conjugate of rabbit gamma globulin which reacts specifically with the iron-molybdenum protein of nitrogenase have been reported in brief form (9) and details will be reported elsewhere. Studies to characterize other components in azotophore membranes which may function in electron transfer associated with nitrogen fixation are currently in progress.

REFERENCES:

1. Drozd, J. and Postgate, J.R. (1970) J. Gen. Microbiol. 63, 63-73.

2. Bulen, W.A., Burns, R.C. and LeComte, J.R. (1965) Proc. Nat. Acad. Sci. U.S. 53, 532-539.

3. Kelly, M. (1969) Biochim. Biophys. Acta 191, 527-540.

4. Oppenheim, J., Fisher, R.J., Wilson, P.W. and Marcus, L. (1970) J. Bacteriol. 101, 292-296.

5. Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751-766.

6. Tissieres, A. (1956) Biochem. J. 64, 582-589.

7. Bulen, W.A., LeComte, J.R. and Lough, S. (1973) Biochem. Biophys. Res. Comm. 54, 1274-1281.

8. Robrish, S.A. and Marr, A.G. (1962) J. Bacteriol. 83, 158-168.

9. Raveed, D., Reed, D.W. and Toia, R.E. (1973) J. Cell Biol. 59, 282a.