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# ATP SYNTHETASE ASSOCIATED WITH THE NITROGENASE OF AZOTOBACTER VINELANDII

Lucille S. Morowitz and Harold J. Morowitz

Department of Molecular Biophysics and Biochemistry
Yale University, New Haven, Conn. 06520

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SUMMARY: Preparations of nitrogenase from Azotobacter vinelandii show an ATP synthetase activity when incubated in the presence of ADP, phosphate, ammonium chloride and an oxidizing agent. The synthesis is linked to an oxidation-reduction and the activity parallels nitrogenase activity through purification and in a step gradient sedimentation. The reductive dephosphorylation of nitrogen fixation may possibly be reversed to yield an oxidative phosphorylation.

All nitrogenase preparations show an absolute requirement for ATP (1) in nitrogen fixation or other reductive activity. In this context we may regard the reaction as reductive dephosphorylation and raise the question as to whether the system can be driven backward in solution phase oxidative phosphorylation. The usual laboratory reaction consists of mixing the enzyme with ATP, a reducing agent such as sodium dithionite and an oxidized substrate such as $N_2$ or $C_2H_2$. In the experiments reported in this paper we mixed the enzyme with ADP, phosphate, ammonium chloride, and ferricyanide as an oxidant. To assay for ATP formation as well as to drive the reaction in the appropriate direction we included hexokinase and glucose to form glucose 6-phosphate and regenerate ADP from ATP formed in the system. All active preparations of nitrogenase showed the ability to catalyze ATP synthesis under these conditions. This synthesis is redox linked and appears to be catalyzed by the same enzyme complex that is involved in nitrogen fixation.

## MATERIALS AND METHODS

The organism used was Azotabacter vinelandii (ATCC #13705). The cells were grown under vigorous aeration in a medium consisting of 0.2 g $KH_2PO_4$, 0.0025 g $Na_2MoO_4 \cdot 2H_2O$, 0.3 g $FeCl_3$, 0.045 g $CaCl_2$ (Anhydrous), 0.2 g $MgSO_4$, 0.8 g $K_2HPO_4$, 20 g sucrose and 1000 ml $H_2O$. This method of enzyme purification was essentially that described by Burns and Hardy (2) and involves: cell breakage in a French press, removal of cell fragments by centrifugation, precipitation of nucleic acids by protamine sulfate, heat treatment and centrifugation to remove denatured proteins, precipitation of nitrogenase with protamine sulfate and resolubilization. The supernatant from the heat inactivation stage is designated Δ60 and the resolubilized protamine sulfate precipitate is designated PS-2. These two fractions were used in this study.

Assays are carried out in TES buffer.[1] Nitrogenase activities are deter-

[1]Abbreviations used: TES buffer, 0.05 mol N-tris [Hydroxymethyl] methyl-2-aminoethane Sulfonic Acid, BSA, bovine serum albumin.

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mined by the reduction of acetylene to ethylene. ATP generator solution consists of 5 mg BSA, 27.6 mg ATP, 63.7 mg phosphocreatine, 1 mg creatine phosphokinase (Sigma from rabbit muscle), 1.5 ml TES buffer, 0.5 ml of 0.1 mol $MgCl_2$ in TES buffer. To a 5 ml serum bottle are added 0.2 ml of ATP generator solution and 0.4 ml of TES buffer. The bottle is sealed, evacuated, and filled to 0.8 atmospheres with argon and 0.2 atmospheres with acetylene. The reaction is initiated by adding 0.2 ml of enzyme solution and 0.2 ml of sodium dithionite solution (174 mg of sodium dithionite dissolved in 10 ml of TES saturated with $H_2$). The bottle is placed in a 30°C shaking water bath for 30 minutes following which the reaction is terminated by the addition of 0.3 ml of 5N $H_2SO_4$. Samples of the gas phase are then examined chromatographically for the ratio of ethylene to acelylene. We used a 1/8 inch x 6 foot column of carbosieve B (Supelco). The ATP synthetase assay uses the following solutions:

| Solution A | Solution B |
|---|---|
| 20 mg hexokinase (Sigma type IV from yeast) | 15 mg potassium ferricyanide |
| 10 mg glucose | 10 mg $NH_4Cl$ |
| 10 mg $K_2HPO_4$ | 7.5 mg ADP |
| 0.9 ml TES buffer | 1 ml TES |
| 0.1 ml 0.1 mol $MgCl_2$ in TES | 5 μcurie [$^{32}$P] phosphate |

Assays are performed by first mixing 0.2 ml of solution A, 0.2 ml of solution B, and 0.4 ml of TES in a 5 ml serum bottle which is then sealed, evacuated, and filled to one atmosphere with argon. The reaction is initiated with the addition of 0.2 ml of enzyme solution. After 30 minutes at 30°C, 3.8 ml of 0.19 M tricholoracetic acid are added and the mixture is centrifuged at 5000 rpm for ten minutes. Samples from the supernatant are then analyzed for the ratio of [$^{32}$P] glucose 6-phosphate to [$^{32}$P] phosphate using the method of Watkins and Lehninger (3). Protein determinations are carried out with the biuret reaction.

## RESULTS AND DISCUSSION

To establish the ATP synthetase activity, the assay was carried out on a PS-2 preparation and showed an activity of 7.2 nanomoles of ATP synthesized per minute per mg of protein. (We will define a unit of ATP synthetase activity as one nanomole per minute per mg of protein.) For controls the assay was run without enzyme, without ADP, and with enzyme heat treated at 100°C for ten minutes. The first two controls had no activity while the heated material showed less than one percent of the ATP synthesis compared to the untreated enzyme preparation. This established that the phosphorylation was due to an ADP linked heat labile catalyst associated with the nitrogenase. A Δ60 preparation was then made which showed an ATP synthetase activity of 1.59 units and a nitrogenase activity of 36 activity units. The net synthesis was run as function of protein concentration and the results are shown in Table I. At a fixed contration of protein (1.4 mg), 0.260 μmol ATP were synthesized in 60 minutes as compared with 0.137 μmol in 30 minutes. The time and concentration behavior were consistent with an enzyme-catalyzed reaction.

The next step was to test if the phosphorylation was indeed redox linked. The oxidizing agent present in the assay solution is ferricyanide while dithionite is normally used as the reducing agent in the forward reaction. Various

TABLE I

ATP synthesis as a function of amount of protein in sample

| Protein per sample | ATP produced in 30 min |
|---|---|
| 1.4 mg | .133 μmol |
| 2.8 mg | .286 μmol |
| 5.6 mg | .337 μmol |
| 8.4 mg | .518 μmol |

mixtures of potassium ferricyanide and sodium dithonite were used to ascertain the effect on ATP synthesis. The results are shown in Table II. The reaction is redox linked which further supports the idea that the synthetase may be the nitrogenase operating in reverse. At this stage we did not pursue the stoichiometry of ATP formation and ferricyanide reduction because of the presence in the preparation of some material with uncharacterized oxidizing and reducing groups (4).

The further similarity of the two activities was tested by layering 2 ml of an enzyme solution over 3 ml of TES buffer made up in 33% $D_2O$. This step gradient was made up in a cellulose nitrate centrifuge tube and spun at 40,000 rpm for 7 hours in a swinging bucket head. The gradient was then collected in 5 fractions each of which was assayed for both activities. The results are shown in the figure. The central peak is made up of single nitrogenase complexes while the rise at sample 1 is due to aggregates, some of which pellet. The activity ratio is seen to vary somewhat across the gradient. It has been reported (2) that a sigmoidal activity-concentration curve is frequently observed for the nitrogenase assay and this might be responsible, in part, for the variation. In a complex reaction sequence of this nature operating far from equilibrium, the forward and reverse reactions may be rate-limited by different steps. In any case the figure demonstrates the close association of the two activities.

One clear result which emerges from these experiments is the existence of a soluble enzyme system of Azotobacter vinelandii which catalyzes the synthesis of ATP from ADP and phosphate in the presence of a suitable oxidizing agent. The synthetase parallels nitrogenase in its behavior toward protamine sulfate precipitation, its resistance to thermal inactivation at 60°C and its sedmentation behavior on a step gradient. It seems reasonable to suggest that the ATP synthetase may be the nitrogenase enzyme being run in reverse. This would indicate that the hydrolysis of ATP is the source of the protons in nitrogen fixation, a concept which relates the action of nitrogenase to the ATP synthetase of oxidative phosphorylation. When run in reverse the reaction of reductive dephosphorylation involves protons from ATP (5).

TABLE II

ATP synthesis as a function of amounts of oxidant and reductant

| Sample | Ferricyanide | Dithionite | ATP produced |
|---|---|---|---|
| 1 | 9.0 mmol | 0 | .633 μmol |
| 2 | 4.5 mmol | 8.5 mmol | .159 μmol |
| 3 | 0 | 17.0 mmol | .046 μmol |
| 4 | 0 | 34.0 mmol | 0 μmol |

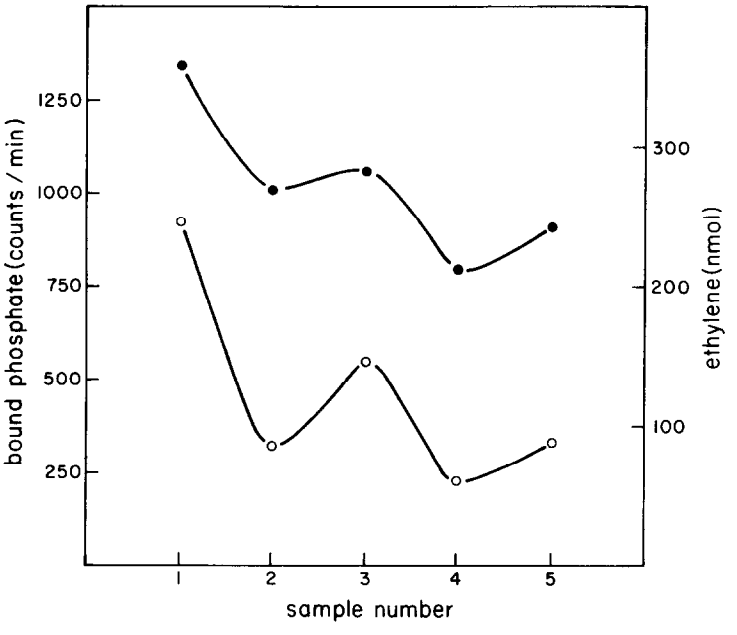


Figure 1

Fractions from a step gradient are assayed for nitrogenase and ATP synthetase. Fraction 1 is at the bottom of the tube fraction 5 at the top. ATP synthetase activities shown in dots and nitrogenase activity shown in open circles.

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