The Enzymology of Nitrogen Fixation in Cell-free Extracts of Clostridium pasteurianum*

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SUMMARY

Extracts of Clostridium pasteurianum have been separated into five protein fractions, which are necessary for nitrogen fixation when pyruvate is used as the source of energy and electrons.

Pyruvate oxidase is shown to be heat-stable and to have gel filtration properties corresponding to a molecular weight of 100,000 or greater.

The function of ferredoxin as an electron carrier for nitrogen fixation is verified. In addition, it is shown here that reduced ferredoxin can act as the sole source of electrons for nitrogen fixation and for acetylene reduction.

Fraction C5S, which is necessary for both nitrogen fixation and acetylene reduction, has gel filtration properties corresponding to a molecular weight of 100,000 or greater. Its activity is stable at 0° but not at 60°, and is found in nitrogen-grown cells but not in ammonium sulfate-grown cells.

Fraction IVS, which is necessary for nitrogen fixation but not for acetylene reduction, is of a size corresponding to a molecular weight of 40,000. Its activity is present only in cells grown on nitrogen gas, and this activity is stable at 0° as well as at 60°.

Fraction IVS is necessary for both nitrogen fixation and acetylene reduction, and its size corresponds to a molecular weight of about 45,000. Its activity, which is found in cells grown on either nitrogen gas or ammonium sulfate, is stable at 60° but not at 0°.

The properties of these fractions are compared with those of similar fractions previously described.

Since the first description by Carnahan et al. (2) of cell-free systems that could fix nitrogen, these systems have come under intensive investigation in several laboratories. Although cell-free nitrogen fixation has been shown with several species of bacteria and with blue-green algae (3), enzymatic systems from the two bacteria, Azotobacter vinelandii and Clostridium pasteurianum, have been studied in greatest detail at the enzymatic level.

Cell-free extracts of C. pasteurianum will fix nitrogen, if provided either with hydrogen gas plus an ATP-generating system or with pyruvate (2, 4). Munson, Dilworth, and Burris (5) found that coenzyme A, ATP, and magnesium ions are required as cofactors if pyruvate is used as the substrate; Mortenson (4) demonstrated that an ATP-generating system must be supplied if hydrogen gas is used as the reducing agent.

From a study of the oxidation of pyruvate by cell-free preparations of C. pasteurianum, Mortenson, Valentine, and Carnahan (6) have shown that the products of the reaction are acetyl phosphate, carbon dioxide, and hydrogen gas. In addition, requirements for coenzyme A and phosphate were shown (6, 7). Munson et al. (5) have reported that the addition of thiamine pyrophosphate is necessary under certain conditions for nitrogen fixation, when pyruvate is used as the substrate. The protein, ferredoxin, has been shown to be required for pyruvate catabolism (8). It has also been shown to be needed for nitrogen fixation, if either pyruvate (9) or hydrogen gas (4) is used as substrate.

Mortenson, Mower, and Carnahan (10) reported the separation of cell-free extracts of C. pasteurianum into two fractions, both of which were necessary for nitrogen fixation. One was obtained by heat treatment of crude extract; the other by treatment of the unheated extract with protamine sulfate and calcium phosphate gel. Enzyme fractions have now been obtained by other procedures in Mortenson's laboratory (11–13).

In the course of my investigations of the enzymology of nitrogen fixation in cell-free extracts from C. pasteurianum I have also separated several protein fractions that are necessary for nitrogen fixation as measured by the 15N technique. The details of the fractionation procedure and of the properties of the various fractions are reported in this communication. The results are compared with those of experiments from other laboratories.
EXPERIMENTAL PROCEDURE

Materials

Hydrogen, nitrogen, acetylene, and argon were purchased from Air Reduction Company, Inc., Natiek, Massachusetts. Hydrogen was passed over palladium oxide in order to remove traces of oxygen; nitrogen and argon were purified by passage over hot copper. Nitrogen gas containing about 95% 15N was purchased from Isomet Corporation, Pulaskis Park, New Jersey. 

Sodium pyruvate, coenzyme A, reduced diphenophosphoryl nucleotide, creatine phosphate, and creatine kinase were purchased from Sigma. Lactate dehydrogenase and acetate kinase were purchased from Boehringer Mannheim, New York. Adenosine 5'-triphosphate was obtained from P-L Biochemicals, Milwaukee, Wisconsin. Acetyl phosphate was synthesized by the method of Stadtman (14), and its purity was established by reaction with hydroxylamine and by measurement of the absorbance of the free chloride-acetylyhydroxamate complex at 540 mμ with succinic anhydride as standard. Ferredoxin was purified by the method of Buchanan, Lovenberg, and Rabino-witz (16).

Methods

Growth and Harvest of Cells—The strain of C. pasteurianum originally isolated by Winogradsky (ATCC 6013) was secured from the American Type Culture Collection. The cells were grown to midlog phase, harvested, washed, and dried as described by Caruthers et al. (2). For the growth of cells on ammonia nitrogen, 17.2 mmoles of ammonium sulfate were added per liter of culture medium. For later experiments cells were grown in a 600-liter fermenter at the New England Enzyme Center. In the latter instance, calcium carbonate was not included in the medium for maintenance of hydrogen ion concentration since the pH of the fermentation was regulated automatically.

Preparation of Crude Extract—Dried cells were stored under vacuum at −20° until needed. A crude extract was prepared by autolysis of the cells in a buffered medium under hydrogen gas (2). After centrifugation of the crude autolysate, the supernatant liquid was adjusted to contain 25 mg of protein per ml at pH 6.6.

Assay for Nitrogen Fixation—Nitrogen fixation was measured by incubation of the components in question with nitrogen gas enriched with 15N. The incubation was conducted in stoppered, 25-ml Erlemeyer flasks equipped with a center well. A side arm on each flask was fitted into a gas manifold through which the flasks could be evacuated and filled with various, known amounts of hydrogen, argon, and 15N-enriched nitrogen gas. First, the air-stable components were added to the incubation flask. The gas phase was then constituted, and alkaline pyrogallol was added to the center well of each vessel. Finally, the air-labile components of the incubation mixture were injected through the stopper from an airtight syringe. The flasks were incubated at 30° with reciprocal shaking at a rate of 130 per min and an amplitude of 2 mm to ensure an adequate equilibration of gas with the liquid phase.

At the end of the incubation mixture was analyzed for total nitrogen by the Kjeldahl formula

\[ N_t = \frac{(N_{tot})(15N_t)}{(15N_a)} \]

where \( N_t \) = amount of nitrogen fixed, \( N_{tot} \) = total nitrogen in the liquid phase of the vessel, \( 15N_t \) = percentage of excess \( 15N \) in the liquid phase of the vessel, and \( 15N_a \) = percentage of excess \( 15N \) in the gas phase of the vessel.

Gas Chromatography—The separation of ethylene and acetylene was done according to the method described by Koch and Evans (17) with some modification. The separation was done at 130° on an Aerograph gas chromatograph equipped with a flame ionization detector at the end of an aluminum column, 6 feet × ¼ inch, packed with activated alumina, 48 to 100 mesh, through which carrier nitrogen flowed at a rate of 25 ml per min.

Assay for Acetylene Reduction—Acetylene reduction was assayed in a total volume of 2 ml buffered at pH 6.6 by 0.01 m potassium phosphate contained in the same vessels that were described for the assay for nitrogen fixation, and which had been calibrated with mercury in order to determine their total volume. First, the air-stable components were added to each vessel and then the vessels were evacuated and filled with 0.1 atm of acetylene and 0.9 atm of argon. Finally, the air-labile components of the reaction mixture were injected, and the vessels were incubated for the prescribed period of time. After the incubation the reaction was stopped by the injection of 1.0 ml of 20% HClO4 into each vessel. A measured volume of the gas phase of each vessel was injected into the gas chromatograph with a gas-tight syringe and needle.

Determination of Pyruvate and Pyruvate Catabolism—Pyruvate was determined in the presence of NADH and lactate dehydrogenase by measurement of the change of absorbance at 340 mμ according to a method described by Bücher et al. (18).

The activities of the pyruvate-catabolizing enzymes were assayed by incubation of vessels containing pyruvate, coenzyme A, ferredoxin, and the protein fraction being tested. The control vessel had the same composition except for the omission of the protein fraction. After the incubation, pyruvate was determined, and the concentration of pyruvate in each experimental vessel was subtracted from the concentration in the control vessel without enzyme. One unit of enzyme catalyzes a change in pyruvate concentration of 1.0 μ mole per ml per hour.

Anaerobic Chromatography—Columns for anaerobic chromatography were poured as well as developed in a system from which air could be excluded at all times. The details of the method have been previously described (1).

Protein Concentration—Protein concentration was determined either by the determination of the absorbance at 260 mμ or 280 mμ (19) or by the method of Lowry et al. (20).

RESULTS

Purification and Properties of Heat-labile Components for Nitrogen Fixation

Assay—The fractionation of crude extract was begun with the separation of its heat-stable and heat-labile components, which was accomplished according to the general procedure of Mortenson et al. (10). The heat-stable components necessary for nitrogen fixation are described further in a later section of this paper. They were contained in the supernatant solution, after centrifugation of the suspension resulting from the treat-
The heat-labile nitrogen-fixing activity was assayed as described in the text. A unit of activity is that amount of heat-labile fraction capable of catalyzing the reduction of 1.0 mmole of \( \text{N}_2 \) per 10 min. Protein concentration was determined by the method of Lowry et al. (20).

**Table I**

**Purification of heat-labile nitrogen-fixing activity**

The heat-labile nitrogen-fixing activity was assayed as described in the text. A unit of activity is that amount of heat-labile fraction capable of catalyzing the reduction of 1.0 mmole of \( \text{N}_2 \) per 10 min. Protein concentration was determined by the method of Lowry et al. (20).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity (units/mL)</th>
<th>Specific Activity</th>
<th>Relative Specific Activity</th>
<th>Total Activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_1 )</td>
<td>15.0</td>
<td>25.0</td>
<td>918</td>
<td>36.7</td>
<td>13,700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( P_1 )</td>
<td>27.0</td>
<td>9.2</td>
<td>518</td>
<td>51.8</td>
<td>14,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( C_1 )</td>
<td>25.0</td>
<td>2.4</td>
<td>457</td>
<td>191.0</td>
<td>11,400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( C\text{S}_1 )</td>
<td>22.0</td>
<td>0.9</td>
<td>429</td>
<td>477.0</td>
<td>9,400</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Stability of Heat-labile Nitrogen-fixing Activity—In order to test its stability to air, calcium phosphate fraction was dialyzed against 0.05 M potassium phosphate, pH 6.6, overnight with a loss of 92% of activity for nitrogen fixation, whereas an identical sample was dialyzed anaerobically for the same time with no loss of activity. Various attempts to prevent the aerobic inactivation of the enzyme by the addition of reducing reagents have been uniformly unsuccessful.

Since heat-stable fractions and the \( C\text{S}_1 \) fraction were both isolated from crude extract, and since heated extract will not fix nitrogen unless Fraction \( C\text{S}_1 \) is added, it was concluded that this latter activity was destroyed by heat treatment of the crude extract.

In contrast to the properties of another enzyme fraction to be reported, the heat-labile nitrogen-fixing activity is more stable for anaerobic chromatography. The column was developed with 0.01 M potassium phosphate, pH 6.6. A fraction was collected at the protein front and designated as \( "C\text{S}_1" \). It can be seen from the data in Table I that the above steps resulted in a total purification of the enzyme activity of about 13-fold with a recovery of 69%.

Also, as shown in Fig. 1, the nitrogen-fixing activity of Fraction \( C\text{S}_1 \) is eluted with the protein front from a column of Sephadex G-100. Therefore, the size of the protein responsible for the nitrogen-fixing activity in this fraction is probably 100,000 or higher.

**Fig. 1.** Elution of the nitrogen-fixing activity of calcium phosphate fraction from Sephadex G-100. Crude extract, containing 40 mg per ml, was prepared by autolysis of dried cells. Then, 2.5 ml of 1% protamine sulfate were added to the crude extract and the resulting suspension was allowed to stand for 5 min, was centrifuged, and used for anaerobic gel filtration at 11°C. The protamine sulfate solution (1% in potassium phosphate buffer, pH 6.0) was added with stirring until the protamine to protein ratio (w/w) was 1.3. After the resulting suspension was allowed to equilibrate under anaerobic conditions for 5 min, it was centrifuged and used for anaerobic gel filtration at 11°C. The column was developed with 0.01 M potassium phosphate at pH 6.6, and fractions were collected anaerobically as indicated on the figure. Protein concentration was assessed by measurement of the absorbance at 280 nm and at 260 nm. Nitrogen-fixing activity of the heat-labile components was assayed as described in the text.
Further Fractionation of Heat-stable Components

Small Molecular Weight Components—The experiments leading to the identification of the cofactors necessary for nitrogen fixation, which are present in the heat-stable fraction, yielded results confirming those of concurrent work published from Burris’s laboratory (5). Since results from both laboratories demonstrated that ATP, magnesium ion, and CoA were necessary for nitrogen fixation, these compounds were added to the incubation mixture in the assays to be described subsequently in this paper.

Protein Components of Heat-stable Fraction—Three protein fractions, which are necessary for nitrogen fixation with pyruvate as the substrate, have been obtained from the heat-stable components of the crude extract by fractionation on Sephadex G-100. One of these fractions contains the enzyme activities that catalyze the metabolism of pyruvate (Fig. 2) when exogenous ferredoxin is added. The enzymes of this fraction are eluted from the column with the protein front and, therefore, are of a molecular size corresponding to a molecular weight of 100,000 or greater.

Ferredoxin is required both for pyruvate metabolism and nitrogen fixation. It is present in unfraccionated, heat-stable fraction and is separated from the pyruvate-catabolizing enzymes mentioned above by the gel filtration procedure.

The third heat-stable component necessary for nitrogen fixation is separable from the pyruvate-catabolizing enzymes on Sephadex G-100 and has been designated “Fraction IV.” Thus, the four protein components so far recognized are Fraction C1S1, the pyruvate-catabolizing enzymes, ferredoxin, and Fraction IV. Evidence that the activity of Fraction IV is not the same as the nitrogen-fixing activity in Fraction C1S1 is presented in Table II. Both Fractions IV and C1S1 are necessary for nitrogen fixation; neither will substitute for the other.

Assay for Nitrogen-fixing Activity of Fraction IV—The nitrogen-fixing activity of Fraction IV was assayed in the presence of 160 μmoles of sodium pyruvate, 9.0 μmoles of MgSO4, 1.7 μmoles of ATP, 1.1 μmoles of CoA, 30 μmoles of phosphate buffer (pH 6.6), 620 μg of purified ferredoxin, and 1.0 to 1.2 mg of protein as Fraction C1S1 in a total volume of 3.0 ml under a gas phase containing 0.1 atm of nitrogen gas enriched with 15N and 0.9 atm of argon. In some assays the pyruvate-catabolizing activity was added as a separate component, whereas later it was found that Fraction C1S1 contained a sufficient amount of this activity, and therefore the former was not added to the assay mixture. The reaction was carried out in a single incubation for 30 min at 30° after which the reaction products were analyzed for 15N. A unit of heat-stable enzyme activity is defined as that amount of protein capable of catalyzing the reduction of 1 μmole of N2 per 30 min. The activity of Frac-

![Figure 2. Elution of Fraction IV from Sephadex G-100. Heat-stable fraction and calcium phosphate fraction (C1) were prepared as described in the text. The heat-stable fraction was applied to a column of Sephadex G-100, 2.5 x 50 cm, which was prepared for anaerobic gel filtration. The column was developed with 0.05 M potassium phosphate, pH 6.6, and fractions were collected as indicated in the figure. Protein concentration was assayed by the measurement of the absorbances at 280 nm and 260 nm. Pyruvate catabolism was measured in vessels containing a final volume of 0.2 ml of 0.05 M potassium phosphate buffer at pH 6.6. Each vessel contained 10 μmoles of sodium pyruvate, 0.23 μmole of CoA, 62 μg of ferredoxin, and 0.1 ml of the particulate column fraction being tested. The vessels were incubated for 1 hour at 30°, and the reaction was stopped by the addition of 0.65 ml of 6% HClO4 to each. After centrifugation, the pyruvate concentration in the supernatant solution was measured as described previously in the text. Nitrogen fixation was assayed in a final volume of 3.0 ml containing 0.05 M potassium phosphate buffer at pH 6.6. Each vessel contained 100 μmoles of sodium pyruvate, 9.0 μmoles of magnesium sulfate, 1.1 μmoles of CoA, 1.7 μmoles of ATP, 620 μg of purified ferredoxin, and 0.24 mg of protein as calcium phosphate fraction. Each vessel also contained 1.0 ml of the column fraction being tested for nitrogen fixation under a gas phase with nitrogen gas enriched with 15N. The vessels were incubated at 30° for 30 min, after which a measured volume from each vessel was prepared for 15N analysis.

<table>
<thead>
<tr>
<th>System</th>
<th>Nitrogen fixation (μmoles N fixed/30 Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>148</td>
</tr>
<tr>
<td>Minus Fraction IV</td>
<td>28</td>
</tr>
<tr>
<td>Minus Fraction C1S1</td>
<td>19</td>
</tr>
</tbody>
</table>

Table II

Requirement of Fraction IV and Fraction C1S1 for nitrogen fixation

Fractions IV and C1S1 were prepared as described in the text. Nitrogen fixation was assayed in 3.0 ml of 0.05 M potassium phosphate buffer at pH 6.6. Each vessel contained: sodium pyruvate, 160 μmoles; magnesium sulfate, 9.0 μmoles; ATP, 1.7 μmoles; CoA, 1.14 μmoles; ferredoxin, 620 μg, and pyruvate-catabolizing enzymes, prepared as described in the legend to Fig. 2. 2.4 mg. The gas phase consisted of 0.9 atm of argon as an inert gas and 0.1 atm of nitrogen enriched for 15N. The complete reaction mixture contained 2.8 mg of protein as Fraction C1S1 and 0.61 mg of protein as Fraction IV. The incubation was at 30° for 30 min, after which a measured volume from each vessel was subjected to 15N analysis.
Fraction C\(\text{S}_{\text{II}}\) and Fraction IV were prepared as described in the text and each contained 2.0 mg of protein per ml. The pyruvate-catabolizing enzymes were prepared as described in the legend to Fig. 2. After Fraction IV was assayed for nitrogen-fixing activity, portions of it were stored under the conditions listed in the table for the period of time indicated. The stored Fraction IV was then assayed for nitrogen-fixing activity in the same manner as before.

### Table III: Stability of Fraction IV

<table>
<thead>
<tr>
<th>Gas phase</th>
<th>Temperature</th>
<th>Storage time</th>
<th>Treatment prior to assay</th>
<th>Nitrogen-fixing activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>20°</td>
<td>12 hr</td>
<td>None</td>
<td>25% initial activity</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>20°</td>
<td>12 hr</td>
<td>None</td>
<td>132% initial activity</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>-20°</td>
<td>12 hr</td>
<td>None</td>
<td>110% initial activity</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>0°</td>
<td>12 hr</td>
<td>1-hr under hydrogen at 30°</td>
<td>67% initial activity</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>0°</td>
<td>12 hr</td>
<td>None</td>
<td>126% initial activity</td>
</tr>
</tbody>
</table>

Fraction IV is linear with respect to protein concentration up to 143 units under the conditions of the assay.

### Properties of Fraction IV and Stability of Its Nitrogen-fixing Activity

The molecular weight of the protein of Fraction IV was roughly estimated (21) from its elution volume from the Sephadex column of Fig. 2. Interpolation of the plot of molecular weight versus elution volume from a Sephadex G-100 column provided an estimate of 40,000 to 50,000 for the molecular weight of the enzymatic components of Fraction IV.

The results of an investigation of the stability of the nitrogen-fixing activity of Fraction IV are presented in Table III. Although this activity was unstable in air, it was stable under hydrogen at 20° and at -20°. However, the activity was lost at 0° under hydrogen and could be recovered by incubation at 30° under hydrogen. Cold inactivation and subsequent reactivation at higher temperature under hydrogen gas have been previously noted in the crude extracts (22, 23). Fraction IV has been stored for as long as 3 weeks at -20° under hydrogen with loss of about half of the nitrogen-fixing activity.

### Role of Protein Fractions Required for Nitrogen Fixation

In this section attempts have been made to determine whether Fraction C\(\text{S}_{\text{II}}\) and Fraction IV have a unique relation to the process of nitrogen fixation and to elucidate further the role of ferredoxin in the fixation process.

**Assay for Nitrogen Fixation in Extracts of Cells Grown on Ammonium Sulfate**—Whatever specific reactions these two fractions, C\(\text{S}_{\text{II}}\) and IV, may catalyze, they each contain some activity uniquely concerned with nitrogen fixation (Table IV). When attempts were made to replace either fraction in the assay for nitrogen fixation with a crude extract of cells that had been grown in the presence of ammonium sulfate, no reduction of nitrogen was observed. These data support the conclusion that at least one necessary component in each fraction is produced when cells are grown with nitrogen gas as the sole source of nitrogen-containing compounds and that one or both are directly concerned with the specific reaction with molecular nitrogen.

**Role of Reduced Ferredoxin in Reduction of Nitrogen**—Since ferredoxin is necessary for the generation of secoyl phosphate from pyruvate and inorganic phosphate (7), it is presumably necessary for the generation of ATP from pyruvate. It is possible, therefore, that ferredoxin is necessary in nitrogen fixation, when pyruvate is the source of electrons and energy, solely because of its function as an electron sink for the generation of ATP from the catabolism of pyruvate. If this were the only function of ferredoxin, then it should be possible to replace ferredoxin with ATP-generating systems known to be active in nitrogen fixation. The results reported in Table V show that ferredoxin is not thus replaced.

The results of the above experiments indicate, therefore, that ferredoxin not only serves as an electron sink for the generation of ATP from the catabolism of pyruvate but also functions in the electron transport chain between pyruvate and nitrogen gas. Since ferredoxin is also known to be required for the enzymatic reduction of nitrogen by hydrogen (4), it must, therefore, be the branch point of the electron transport chains concerned with the reduction of protons and nitrogen gas by pyruvate.

In the following experiments, efforts have been made to separate these two functions of ferredoxin by supplying an ATP-generating system and by using reduced ferredoxin as the sole reducing source. Under these circumstances it should be possible to reduce small amounts of nitrogen gas with the participation of the two fractions, C\(\text{S}_{\text{II}}\) and IV.

The data presented in Table VI indicate that reduced ferredoxin can support nitrogen fixation as the only source of electrons. Ferredoxin was reduced enzymatically in a system containing pyruvate and the pyruvate-metabolizing enzymes and was isolated by anaerobic chromatography of the incubation mixture on a column of Sephadex G-75. The reduced ferredoxin...
Attempts to replace ferredoxin with ATP-generating system

Fractions IV and C,S, were prepared as described in the text. Nitrogen fixation in Experiment 1 was assayed in 3.0 ml of 0.05 M potassium phosphate buffered at pH 6.6. The vessel with the complete system contained 160 μmoles of sodium pyruvate, 12 μmoles of magnesium sulfate, 1.7 μmoles of ATP, 1.1 μmoles of CoA, 620 μg of purified ferredoxin, 1.2 mg of protein as Fraction C,S, and 2.0 mg of protein as Fraction IV. The gas phase was composed of 0.9 atm of argon as an inert gas and 0.1 atm of nitrogen enriched with ¹⁵N. Dilithium acetyl phosphate, 150 μmoles, was added as the ATP-generating system. The incubation was at 30° for 30 min, after which analysis for ¹⁵N was carried out on a measured volume from each vessel. In Experiment 2, the vessel with the complete system contained 160 μmoles of sodium pyruvate, 12.0 μmoles of magnesium sulfate, 1.7 μmoles of ATP, 1.4 μmoles of CoA, 0.48 mg of purified ferredoxin, 1.2 mg of protein as Fraction C,S, and 2.0 mg of protein as Fraction IV. Creatine phosphate, 67.5 μmoles, plus creatine kinase, 200 μg, were added to the vessel with the ATP-generating system. The incubation was at 30° for 30 min, after which analysis for ¹⁵N was done on a measured volume from each vessel.

<table>
<thead>
<tr>
<th>Nitrogen fixation</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg moles N₂/vessel</td>
<td>489</td>
<td>411</td>
</tr>
<tr>
<td>Minus ferredoxin</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>Minus ferredoxin plus ATP-generating system</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Acetyl phosphate system</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatine phosphate system</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nitrogen fixation with reduced ferredoxin

Fraction IV, Fraction C,S, and heat-stable fraction were prepared by procedures described in the text. Heat-stable fraction, 7 ml, was incubated with 1.6 μmoles of sodium pyruvate under argon at 30° for 1 hour, after which the reaction mixture was applied to a column of Sephadex G-75 (2.5 × 25 cm) for anaerobic gel filtration. The column was developed with 0.01 M potassium phosphate buffered at pH 6.6. The brown band of protein eluted within a range expected for ferredoxin was collected anaerobically and was used as reduced ferredoxin. The ATP-generating system consisted of 1.7 μmoles of ATP, 10.2 μmoles of magnesium sulfate, 120 μmoles of dilithium acetyl phosphate, and 1.0 mg of protein as purified, Escherichia coli acetate kinase. The incubation vessels contained, in a total volume of 3.0 ml, 0.05 M potassium phosphate buffer, pH 6.6, protein fractions, and the indicated ATP generating system under a gas phase consisting of 0.9 atm of argon as an inert gas and 0.1 atm of nitrogen enriched with ¹⁵N. Incubation was at 30° for 30 min, after which a measured volume from each vessel was used for ¹⁵N analysis.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Reduced ferredoxin (μg/vessel)</th>
<th>Fraction C,S, (μg protein/vessel)</th>
<th>Fraction IV (μg protein/vessel)</th>
<th>ATP-generating system</th>
<th>Nitrogen fixed (mg moles N₂/vessel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>2.0</td>
<td>+</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.16</td>
<td>1.2</td>
<td>2.0</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>0.16</td>
<td>1.2</td>
<td>2.0</td>
<td>+</td>
<td>70</td>
</tr>
</tbody>
</table>

Acetylene-reducing Activity of Fraction C,S, and Fraction IV

Since workers in several laboratories (24-26) have found that the requirements for nitrogen fixation seem to be the same as those for the reduction of acetylene to ethylene, the fractions described above were used in assays for the latter activity. When the assay for Fraction C,S, was done with acetylene in the gas phase instead of nitrogen gas, the results reported in Fig. 3 were obtained. It can be seen that Fraction C,S, is necessary for acetylene reduction and that the activity is linear with the amount of Fraction C,S, under 1.8 units of activity, when 1 unit of activity is defined as the amount of protein that catalyzes the reduction of 1.0 μ mole of acetylene in 10 min. However, in distinction to the requirements for nitrogen fixation the data in Table VII demonstrate that those for acetylene reduction can be satisfied by Fraction C,S, plus the components of a crude extract of cells grown on ammonium sulfate. Al-
though the greatest activity is seen in the presence of pyruvate, quite comparable activity is shown when reduced ferredoxin plus an ATP-generating system are the sources of electrons and energy, respectively. The requirement for the crude extract of cells grown on ammonium sulfate is also demonstrated when reduced ferredoxin plus an ATP-generating system are used as the source of electrons and energy. Since significant activity is demonstrated in the absence of both pyruvate and reduced ferredoxin, it seems likely that the extract of cells grown on ammonium sulfate is also providing electrons from reduced ferredoxin.

Acetylene-reducing Activity in Extracts of Ammonium Sulfate-grown Cells—Since the above results are somewhat different from those of similar experiments in which nitrogen fixation was measured, the properties of the necessary activity present in the crude extract of cells grown on ammonium sulfate were examined more closely. The results of experiments to test the cold stability of the necessary activities are recorded in Fig. 4, where it can be seen that, with pyruvate as the source of energy and electrons, at least one of the acetylene-reducing activities contained in extracts of cells grown on ammonium sulfate is cold-labile. In addition, the following experiment also demonstrates the cold lability when an ATP-generating system plus reduced ferredoxin are provided instead of pyruvate. Incubations were performed with reduced ferredoxin and an ATP-generating system as described for Table VII except that one vessel contained extract of ammonium sulfate-grown cells treated at 0°, whereas the second contained the same amount of the same original extract treated at 20° as in the experiment of Fig. 4. In the first vessel, 2.0 μmoles of ethylene were produced whereas in the second vessel 89.5 μmoles of ethylene were produced. For the heat treatment each of several tubes containing a fresh, crude extract of ammonium sulfate-grown cells was incubated at a different temperature under hydrogen for 10 min. After centrifugation, a measured amount of the supernatant solution was added to the corresponding reaction vessel. After incubation for 30 min, analysis for ethylene production was done as described under "Methods." Treatment at temperatures up to 65° resulted in a loss of only 8% of the activity and about a 2-fold increase in specific activity over that of the crude extract.

In order to establish the relationship between the acetylene-reducing activity present in extracts of ammonium sulfate-grown cells and the nitrogen-fixing activity of Fraction IV, the heat-stable components of an extract of cells grown on nitrogen gas were stored overnight at 0° before assaying for nitrogen fixation both in the presence and the absence of a fresh extract of cells grown on ammonium sulfate. The results of Fig. 5 show that the extract of ammonium sulfate-grown cells can replace the cold-labile component present in extracts of cells grown on nitrogen gas. Since, under the conditions of the assay, the only heat-stable components necessary for nitrogen fixation are those ordinarily present in Fraction IV, and since the cold lability of the nitrogen-fixing activity of Fraction IV has been demonstrated, there must be at least one necessary component present both in Fraction IV and extracts of cells grown on ammonium sulfate. However, Fraction IV activity for nitrogen fixation could not be replaced by a crude extract of cells grown on ammonium sulfate. Therefore, Fraction IV must contain more than one component that is necessary for nitrogen fixation.

The results of an experiment to test this hypothesis are presented in Fig. 6. The heat-stable components of an extract of

<table>
<thead>
<tr>
<th>Components present</th>
<th>Acetylene reduction (μmole C₂H₂ produced/assay vessel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic system</td>
<td>0.15</td>
</tr>
<tr>
<td>Minus ATP-generating system</td>
<td>0</td>
</tr>
<tr>
<td>Minus C₄S₁</td>
<td>0</td>
</tr>
<tr>
<td>Minus extract of (NH₄)₂SO₄-grown cells</td>
<td>0</td>
</tr>
<tr>
<td>Plus reduced ferredoxin</td>
<td>0.67</td>
</tr>
<tr>
<td>Minus extract of (NH₄)₂SO₄-grown cells, plus reduced ferredoxin</td>
<td>0</td>
</tr>
<tr>
<td>Minus ATP generating system plus pyruvate (320 μmoles)</td>
<td>0.86</td>
</tr>
</tbody>
</table>
cells grown on nitrogen gas were subjected to anaerobic, gel filtration on Sephadex G-100, and several fractions were collected with an elution volume corresponding to that of Fraction IV. Each fraction was assayed for acetylene reduction in the presence of a cold-treated extract of cells grown on ammonium sulfate and for nitrogen fixation in the presence of an untreated extract of cells grown on ammonium sulfate. Although the two activities are not clearly separated, the fact that their ratio is not the same in each of the column fractions indicates that they represent at least two different proteins. The first protein, designated Fraction IVa, is present in extracts of cells grown on either ammonium sulfate or nitrogen gas. Its activity, which is heat-stable and cold-labile, is necessary both for the reduction of acetylene and for the fixation of nitrogen. Although it is polydisperse on gel filtration, its maximum molecular size corresponds to a molecular weight of about 45,000. The second protein, which is designated “Fraction IVN,” is present only in extracts of cells grown on nitrogen gas. Its activity is necessary for nitrogen fixation but not for acetylene reduction and is stable to both heat and cold. The molecular size of Fraction IVN corresponds to a protein with a molecular weight of about 40,000.

The above information brings the number of fractions necessary for nitrogen reduction by reduced ferredoxin to three: Fraction CIS1, Fraction IVN, and Fraction IVa. One enzyme, Fraction IVN, is a normal constituent of cells grown on ammonium sulfate or nitrogen gas was divided into two parts, which were subsequently treated, respectively, at 0° and at 20° as in Fig. 4. The heat-stable components of cells grown on nitrogen gas were prepared as described previously and chromatographed as described in Fig. 2. In the assay for acetylene reduction, each vessel received 320 pmoles of sodium pyruvate, 12 pmoles of MgSO₄, 1.7 pmoles of ATP, 1.14 pmoles of CaO₄, 0.25 μg of purified ferredoxin, 1.06 μg of protein as Fraction CIS1, 9.3 μg of protein as a crude extract of ammonium sulfate-grown cells, which had been stored at 0° overnight, and the appropriate column fraction as indicated on the figure. The gas phase consisted of 0.1 atm of acetylene and 0.9 atm of argon. In the assay for nitrogen fixation, each vessel contained the same components except that, in the latter case, the crude extract of ammonium sulfate-grown cells had been treated at 20° overnight instead of at 0°, and the gas phase of each vessel contained 15N-enriched nitrogen instead of acetylene. Incubation of both sets of vessels was for 30 min at 30° and both were analyzed for their respective reaction products as described previously.

**Fig. 5.** Replacement of a cold-labile activity necessary for nitrogen fixation by a crude extract of ammonium sulfate-grown cells. Fractions CIS1, crude extract of ammonium sulfate-grown cells, and the heat-stable components of nitrogen-grown cells were prepared as described in the text. The heat-stable, cold-stable components of nitrogen-grown cells were prepared by treatment of the heat-stable components at 0° overnight under hydrogen. Assay for nitrogen fixation was conducted as described under “Methods” in a volume of 2.0 ml of 0.05 M potassium phosphate buffered at pH 6.6. Added to each reaction flask were 320 μmoles of sodium pyruvate, 12 μmoles of MgSO₄, 1.7 μmoles of ATP, 1.1 μmoles of CaO₄, 0.25 μg of purified ferredoxin, 1.32 mg of protein as Fraction CIS1, 6.4 mg of protein as the heat-stable, cold-stable components of an extract of cells grown on nitrogen gas, and amounts of untreated crude extract of ammonium sulfate-grown cells as indicated above. After an incubation of the vessels at 30° for 30 min, the contents of each vessel were analyzed for 15N as described previously.

**Fig. 6.** Demonstration of more than one activity in Fraction IV. A crude extract of cells grown on ammonium sulfate was divided into two parts, which were subsequently treated, respectively, at 0° and at 20° as in Fig. 4. The heat-stable components of cells grown on nitrogen gas were prepared as described previously and chromatographed as described in Fig. 2. In the assay for acetylene reduction, each vessel received 320 pmoles of sodium pyruvate, 12 pmoles of MgSO₄, 1.7 pmoles of ATP, 1.14 pmoles of CaO₄, 0.25 μg of purified ferredoxin, 1.06 μg of protein as Fraction CIS1, 9.3 μg of protein as a crude extract of ammonium sulfate-grown cells, which had been stored at 0° overnight, and the appropriate column fraction as indicated on the figure. The gas phase consisted of 0.1 atm of acetylene and 0.9 atm of argon. In the assay for nitrogen fixation, each vessel contained the same components except that, in the latter case, the crude extract of ammonium sulfate-grown cells had been treated at 20° overnight instead of at 0°, and the gas phase of each vessel contained 15N-enriched nitrogen instead of acetylene. Incubation of both sets of vessels was for 30 min at 30° and both were analyzed for their respective reaction products as described previously.

**DISCUSSION**

Reports from other laboratories have described the separation of nitrogen-fixing extracts of several organisms into at least two necessary protein fractions. Bulen and LeComte (27) described the separation of extracts of A. vinelandii into two fractions, which are necessary for nitrogen fixation when dithionite was used as the source of electrons. Kelly, Kluas, and Burris (24) reported a similar separation and demonstrated that the fractions were also necessary for acetylene reduction. The evidence in the latter report indicates that at least one of the fractions is cold-labile. Mortenson, Morris, and Jeng (13) were the first to report a similar separation from extracts of C. pasteurianum. Although such comparisons cannot be rigorously made until more is known concerning the role of each enzyme fraction in nitrogen fixation, it would appear that Fraction CIS1 described in this communication corresponds to molybdoferredoxin from Mortenson's laboratory. Supporting the probability of their identity are the facts that Fraction CIS1 and molybdoferredoxin have very similar molecular size and stability, and that their distribu-
tion in ammonium sulfate-grown and nitrogen-grown cells is the same. In addition, from the data concerning its cold lability and its molecular weight, it would appear that Fraction IV reported in this communication corresponds to Mortenson's azoferredoxin. This suggestion is supported by the fact that Fraction IV and azoferredoxin are each required for both acetylene reduction and nitrogen fixation (26). However, the data reported in this communication demonstrate that Fraction IV contains at least two fractions necessary for nitrogen fixation when reduced ferredoxin is used as the source of electrons. Some of the properties of Fraction IV belong to Fraction IVa. Although it cannot be rigorously proven from the published data, it would appear likely that azoferredoxin also contains at least two fractions necessary for nitrogen fixation.

The fact that acetylene reduction does not require the same components as does nitrogen fixation should be borne in mind in the interpretation of experiments in which the former activity has been used as an assay for nitrogen fixation. It seems likely that these two processes share some of the same mechanism but that the mechanism of nitrogen fixation is somewhat more complicated than that of acetylene reduction. Experiments to describe more precisely the mechanisms of these two processes are currently in progress.

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Kenneth B. Taylor


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