N₂ Fixation by Purified Components of the N₂-Fixing System of *Clostridium pasteurianum*

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**SUMMARY**

1. N₂ fixation, reductant-supported ATP utilization and ATP-dependent H₂ evolution are catalyzed by combined molybdoferredoxin and azoferredoxin, the two purified components of the N₂-fixing system of *Clostridium pasteurianum*. No additional protein components are needed. At this stage of purity, neither of the two components of the N₂-fixing system catalyzed any of these reactions without the other.

2. The utilization of ATP by the N₂-fixing system involves a single P₁ elimination; the rate of P₁ release from ATP is exactly matched by ADP formation. The product ADP inhibits each of the reactions catalyzed by the enzyme components.

3. Aided by a more sensitive colorimetric analysis for NH₃ with ninhydrin, N₂ fixation is routinely obtained with ATP as the sole source of energy. No ATP-generating system is needed.

4. With the purified components, the stoichiometry of P₁ released per electron pair for H₂ evolution and N₂ fixation is 4.

**INTRODUCTION**

Molybdoferredoxin and azoferredoxin, two protein components of the N₂-fixing system in *Clostridium pasteurianum*, have been partially purified from cells grown on N₂ (ref. 1). The same two protein components were previously shown to be required for ATP-dependent H₂ evolution and electron-dependent ATP utilization².

N₂ fixation by enzyme preparations requires a strong reducing agent and a source of ATP (refs. 3–5). In the clostridial system, the purified enzyme components have been assayed by reconstituting the crude extract². With an extract containing hydrogenase (H₂:ferredoxin oxidoreductase, EC 1.12.1.1) and acetate kinase (ATP: acetate phosphotransferase, EC 2.7.2.1), H₂ in the gas phase and added acetyl phosphate *plus* catalytic ATP, the requirements for electrons and ATP have been met³. But in studying the mechanism of fixation, this assay system has the disadvantage that most of the original contaminating enzymes are still present and characteristics of the system could be affected.

The use of dithionite (hydrosulfite) as an artificial electron donor in the place...
of reduced ferredoxin simplified the assay system in Azotobacter, particularly as this organism does not possess a hydrogenase capable of activating \( \text{H}_2 \) for \( \text{N}_2 \) reduction. Dithionite was equally effective as a substitute for reduced ferredoxin in the clostridial system. Early attempts to achieve \( \text{N}_2 \) fixation with substrate quantities of ATP yielded only slight activity, though significant ATP-dependent \( \text{H}_2 \) evolution was obtained with ATP as the sole source of energy using a partially purified \( \text{N}_2 \)-fixing system from Azotobacter. Recently, it was shown that acetylene reduction by the \( \text{N}_2 \)-fixing system in Clostridium could be supported by substrate quantities of ATP at similar initial rates as with an ATP generator.

This paper shows that good rates of \( \text{NH}_3 \) formation from \( \text{N}_2 \) are supported by ATP without an ATP generator. This was made possible by determining the correct reaction conditions and by improving the sensitivity of \( \text{NH}_3 \) estimation. The products of the reaction detected were ADP, \( \text{P}_1 \), and \( \text{NH}_3 \), plus an oxidized product of dithionite.

**METHODS**

**Reagents**

Sodium salts of ATP, ADP and AMP were obtained from Sigma Chemical Co. and solutions adjusted with KOH to pH 6.8 before use. Argon, \( \text{H}_2 \) and \( \text{N}_2 \) (purified grade) were obtained from AirCo and passed over heated copper to remove traces of \( \text{O}_2 \). TES (N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) buffer (CalBiochem) was neutralized with KOH solution. \( \text{Na}_2\text{S}_2\text{O}_4 \) (Fisher Chemical Co.) solution was prepared anaerobically in 0.05 M TES buffer.

**Preparation of nitrogenase components**

Molybdoferredoxin and azoferredoxin were prepared by protamine sulfate fractionation and Sephadex G-100 chromatography. Based on specific enzyme activity with an excess of azoferredoxin, molybdoferredoxin was purified an additional 2-fold over the Sephadex G-100 stage by gradient elution from DEAE-cellulose with Tris-HCl at pH 8.0, followed by adsorption chromatography on hydroxylapatite columns. Whereas a single well defined peak of molybdoferredoxin was observed in the ultracentrifuge, disc-gel electrophoresis (aerobically at pH 9.5) showed three bands; this is not regarded as certain evidence for inhomogeneity, since it is possible that dissociation may occur under the conditions of electrophoresis.

**Assays**

ATP-dependent \( \text{H}_2 \) evolution was measured under argon or CO as described by Burns and Bulen. The \( \text{O}_2 \)-sensitive nitrogenase components were added by syringe through a serum stop cap to the main compartment of the side-arm Warburg flasks and preincubated with \( \text{MgCl}_2 \) and dithionite. Reaction was initiated by addition of ATP from the side arm. Without ATP, no \( \text{H}_2 \) was evolved with molybdoferredoxin and azoferredoxin of the purity used here.

\( \text{N}_2 \) fixation and ATP hydrolysis were measured in 20-ml glass scintillation vials equipped with a rubber serum stopper at the base for addition of protein components and withdrawal of samples for \( \text{NH}_3 \) (0.5 ml) and \( \text{P}_1 \) (0.25 ml) analysis. The contents were agitated with a magnetic stirrer and high-purity \( \text{N}_2 \) passed over the reaction mixture, subsequent to sparging in dithionite solution to ensure anaerobiosis.

NH₃ synthesis from N₂ was measured by microdiffusion from 1.0 ml of saturated K₂CO₃ in micro-Conway dishes. After distillation for at least 7 h at room temperature, the acid NH₄⁺ sample (0.3 ml 0.1 M H₂SO₄) was diluted to 2.3 ml with NH₃-free water and 1.0-ml aliquots analyzed in duplicate with 1.0 ml of the ninhydrin–hydrindantin reagent developed by Moore and Stein for analysis of α-amino nitrogen. Following dilution with 4.0 ml of 50% ethanol, each μmole of NH₃ produced an extinction of 3.0–3.3 at 570 μM with 1.0-cm light path. The useful range of the method is 0.5–1 μg of NH₃-nitrogen.

P₁ was analyzed by the method of Taussky and Shorr. Protein was determined with the biuret reagent.

The reaction mixture was examined for nucleoside phosphate compounds by paper chromatography on Whatman No. 1 paper with isobutyric acid–1 M NH₄OH–0.2 M EDTA (100:60:0.8, by vol.) as the developing solvent, and analyzed by chromatography on Dowex-1 (AG-1, X8, Bio-Rad) columns, using a step-wise salt gradient to separate ATP, ADP and AMP.

RESULTS

*The time course of ATP-supported N₂ fixation*

The time course of P₁ release and NH₃ formation with purified molybdoferredoxin and azoferredoxin is shown in Fig. 1. A substrate level of ATP (15 mM)
was used, with dithionite as the electron donor. The initial rate of P\textsubscript{1} release and NH\textsubscript{3} formation was linear till about 30\% of the ATP was consumed, followed by a lesser rate. The specific rate of NH\textsubscript{3} formation (96 m\textsubscript{\mu}moles per min per mg molybdoferredoxin at 22\°) is lower than the possible maximum, for which a considerable excess of azoferredoxin is required (indicated below in Table I). Specific activities for ATP-dependent H\textsubscript{2} evolution up to 1100 m\textsubscript{\mu}moles H\textsubscript{2} per min per mg molybdoferredoxin at 22\° have been recorded (Fig. 3). Since the azoferredoxin fraction is less stable than molybdoferredoxin, the assays described have generally been performed with limiting molybdoferredoxin.

![Figure 2](image)

**Fig. 2.** Effect of ADP, AMP on (a) N\textsubscript{2} fixation, (b) H\textsubscript{2} evolution and (c) ATP utilization by molybdoferredoxin (MoFd) plus azoferredoxin (AzoFd). Reaction mixtures contained, in each ml: ATP, 15 mM; MgCl\textsubscript{2}, 7.5 mM; sodium dithionite, 17.5 mM; ADP, AMP, where indicated, 5 mM; molybdoferredoxin, 0.25 mg; azoferredoxin, 0.83 mg; Tris-HCl, 16 mM; TES, 25 mM, final pH 6.8; temp. 22\°. H\textsubscript{2} evolution was measured with an atmosphere of CO; N\textsubscript{2} fixation and P\textsubscript{1} formation in 15 ml reaction volume under N\textsubscript{2}. ••• ATP alone; △△△, + AMP; ■■■, + ADP.

With purified components catalyzing the reaction, each of the activities almost ceased before the ATP was consumed, presumably from ADP inhibition. In Fig. 2 it is shown that ADP but not AMP inhibits ATP utilization, ATP-dependent H\textsubscript{2} evolution and N\textsubscript{2} fixation, each to a similar extent.

**The product of ATP utilization**

As shown in Fig. 1, ADP was the product of ATP metabolism by purified molybdoferredoxin plus azoferredoxin. The rate of ADP formation coincided exactly with the rate of P\textsubscript{1} release, and the same relative rate of NH\textsubscript{3} formation was maintained throughout the reaction period (approx. 30 ATP per N\textsubscript{2} fixed). No ultraviolet-absorbing species other than material with the same chromatographic properties as ADP and residual ATP, was detected, either by paper chromatography or ion-exchange chromatography.

**Mg\textsuperscript{2+} requirement**

Based on initial rates of reaction, a ratio of Mg\textsuperscript{2+}/ATP of 0.5–1.0 was required for maximum enzyme activity. However, as shown in Fig. 3 for H\textsubscript{2} evolution, ratios of Mg\textsuperscript{2+}/ATP greater than 1.0 produced some inhibition of the initial rate of enzyme
activity. For $P_1$ formation, $H_2$ evolution and $NH_3$ formation, the rate of activity decreased sooner in reactions with excess $Mg^{2+}$ than in reactions with a lower $Mg^{2+}$ concentration but at a similar or even lower initial rate. This is consistent with inhibition by a greater $Mg^{2+}$/ATP ratio, but could also indicate that sensitivity to ADP is increased with more $Mg^{2+}$. Together with progressive ADP inhibition and a decreasing ATP concentration, development of a less favorable $Mg^{2+}$/ATP ratio would contribute to the declining rate of activity with time. However, the initial rates of activity are linear and extrapolate to zero time and are therefore a valid measure of enzyme activity.

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**Fig. 3.** The effect of $Mg^{2+}$/ATP ratio on the initial rate of ATP-dependent $H_2$ evolution. Reaction mixtures contained, in 2.0 ml final volume under CO: ATP, $MgCl_2$, as indicated; sodium dithionite, 17.5 mM; Tris-HCl, 6 mM; TES, pH 6.8 (final pH), 40 mM; 0.5 mg molybdoferredoxin; 3.3 mg azoferredoxin; temp. 22°C. $\bullet$—$\bullet$, 5 mM ATP; $\triangle$—$\triangle$, 10 mM ATP; $\circ$—$\circ$, 15 mM ATP.

**Fig. 4.** ADP inhibition of ATP-dependent $H_2$ evolution. Reaction mixture as in Fig. 3 with 15 mM ATP; 7.5 mM $MgCl_2$, 0.5 mg molybdoferredoxin and 2.5 mg azoferredoxin; temp. 30°C. Duplicate experiments on the same enzyme material are shown.

**ADP inhibition of $H_2$ evolution**

The curve for inhibition of ATP-dependent $H_2$ evolution with a constant level of ATP and varying ADP concentration is shown in Fig. 4. A possible argument that ADP inhibits by binding $Mg^{2+}$ is shown to be invalid in Fig. 5, which illustrates the effect of varying $MgCl_2$ concentration on the extent of inhibition by two levels of ADP.

**Relative rates of ATP utilization and $N_2$ fixation**

The relative rate of $P_1$ released for each $N_2$ reduced with purified components varied from one experiment to another within the range 18–35. As a stoichiometric relationship between ATP utilization and electron transfer in the system, these values
are too high, since more than 40% of the electrons which could possibly reduce $N_2$ were lost as $H_2$, similar to results obtained with purified $N_2$-fixing components from Azotobacter\textsuperscript{14}. Conditions which would provide the minimum loss of electrons as ATP-dependent $H_2$ evolution are not known. Neither the $Mg^{2+}$/ATP ratio nor the relative amounts of molybdoferredoxin and azoferredoxin appears to be a factor (Table I). Possibly the integrity of the $N_2$-fixing system is dependent on an additional factor(s), allowing complete coupling between ATP utilization and $N_2$ reduction, but such a component is not required for activity, and the leakage of electrons could also be caused by partial damage to the enzyme components during their preparation.

![Graph](image)

**Fig. 5.** Effect of $Mg^{2+}$ concentration on ADP inhibition of ATP-dependent $H_2$ evolution. Conditions as in Fig. 4: •—•, minus ADP; △—△, 5.0 mM ADP; ■—■, 7.5 mM ADP.

**TABLE I**

<table>
<thead>
<tr>
<th>RELATIVE RATES OF $P_I$ RELEASE AND $N_2$ FIXATION</th>
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<tbody>
<tr>
<td>Reaction in 10 ml final volume with conditions as described in Fig. 1, with 1.0 mg molybdoferredoxin (varying azoferredoxin) and 2.5 mg molybdoferredoxin, 16.5 mg azoferredoxin (varying $MgCl_2$); temp. 22°C.</td>
</tr>
<tr>
<td>$Azoferredoxin$ (mg/10 ml)</td>
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<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>1.25</td>
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<tr>
<td>2.5</td>
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<td>5.0</td>
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<td>7.5</td>
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<table>
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<tr>
<th>$N_2$ fixed (mμmoles/min per mg molybdoferredoxin)</th>
<th>$P_I$ released (mμmoles/min per mg molybdoferredoxin)</th>
<th>$P_I/N_2$ ratio</th>
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<td>1372*</td>
</tr>
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</tr>
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<td>72.0*</td>
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</tr>
<tr>
<td>50.0*</td>
<td>58.4*</td>
<td>27*</td>
</tr>
<tr>
<td>* Measured at 3-9-min period.</td>
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</table>

*Biochim. Biophys. Acta, 153 (1968) 777–786*
Clearly, examination of the ATP cost for N₂ reduction must take account of electrons lost as H₂. When this was done, the relative rate of Pi released for each electron pair transferred to N₂ was about 4, equal to the relative rate for ATP-dependent H₂ evolution with an atmosphere of argon (see Table II). A correction was made for H₂ evolution by measuring net gas evolution with an atmosphere of N₂, plus the N₂ uptake calculated by direct analysis for NH₃. An alternative method for measuring N₂ fixation is to determine the difference in gas evolution between reaction mixtures incubated with a gas phase of argon and reaction mixtures incubated under N₂. The difference equals the N₂ fixed plus 3 equiv of H₂ used in its reduction. This method agreed with direct analysis for NH₃.

**TABLE II**

**Relative Rates of ATP Utilization and N₂ Reduction**

Reactions were performed under N₂ in Warburg flasks at 22°C containing, in 2 ml: ATP, 15 mM; MgCl₂ 7.5 mM; sodium dithionite, 17.5 mM; Tris-HCl, 6 mM; TES (pH 6.8), 40 mM; plus, Expt. 1: molybdoferredoxin, 0.2 mg; azoferredoxin, 2.2 mg; Expt. 2: molybdoferredoxin, 0.5 mg; azoferredoxin, 1.7 mg; Expt. 3: molybdoferredoxin, 0.2 mg; azoferredoxin, 2.2 mg.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Time (min)</th>
<th>Pi (μmoles)</th>
<th>ze⁻/equiv (3N₂ + H₂)</th>
<th>Pi/ze⁻ as H₂</th>
<th>Percentage as H₂</th>
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<td>1</td>
<td>12</td>
<td>8.1</td>
<td>1.72</td>
<td>4.7</td>
<td>51</td>
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<td>9.6</td>
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<td>4.0</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>9.5</td>
<td>2.40</td>
<td>4.0</td>
<td>100*</td>
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</tbody>
</table>

* Argon atmosphere.

Are molybdoferredoxin and azoferredoxin enzymes?

At the stage of purity reported here, neither molybdoferredoxin nor azoferredoxin catalyzed any reaction without the other component of the system. Because of this, perhaps they should not be regarded as separate enzymes in the classical sense of catalytic proteins, unless it should be subsequently shown that one or the other is able to catalyze a particular reaction. In earlier reports, it was suggested that molybdoferredoxin was able to react directly with ATP or ADP in a non-catalytic reaction, to form a product of molybdoferredoxin and part of the nucleoside phosphate molecule. This is now refuted, since the molybdoferredoxin of improved purity used in this study no longer bound radioactivity from [³⁵C]ATP or ADP. Following Sephadex G-100 chromatography, molybdoferredoxin still contained about 10% of its maximum ATPase and N₂-fixing activity as when recombined with an excess of azoferredoxin. This activity is from residual traces of azoferredoxin, which are removed with further purification. It is not clear whether the extra purification removed an essential factor in N₂ fixation which is returned when the azoferredoxin fraction is added, or whether a contaminant foreign to the N₂-fixing system was responsible for the reaction observed; at this time, we assume the latter.
Metabolic inhibitors

Of a number of possible inhibitors tested, only mersalyl affected the enzyme activity of combined molybdoferredoxin and azoferredoxin (see Table III). Inhibitors known to operate on ATP metabolism in other systems (ouabain, fluoride, arsenate) had no effect. Iron-binding reagents (α,α'-dipyridyl, disodium-1,2-dihydroxybenzene-3,5-disulfonate (Tiron)) were also ineffective at 1 mM concentration, even though a pink color characteristic of iron-chelation was obtained with α,α'-dipyridyl. The mercurial, mersalyl, which removes the iron and acid-labile sulfur from ferredoxin17, inhibited both ATP-utilizing and H₂-evolving activity, suggesting the need for structural integrity of the metallo-prosthetic groups for each function of the system. When incubated with 4 mM mersalyl, the brown color of the azoferredoxin fraction was rapidly bleached and its iron content 95% dissociated, whereas the metal content of molybdoferredoxin remained almost intact for a 5-min period (mersalyl was removed by passing the mixture through a column of Sephadex G-25).

DISCUSSION

Previous attempts to achieve significant N₂ fixation with substrate quantities of ATP were hampered probably because an incorrect Mg²⁺/ATP ratio was chosen and the low amounts of NH₃ produced with the ATP-supported system required a more sensitive detection method. Apparent inhibition by high ATP concentration of N₂ fixation in Azotobacter⁸ and of azide reduction in Clostridium¹⁸ can be at least partly explained as a result of an excessive ATP/Mg²⁺ ratio. The use of substrate levels of ATP, with dithionite as the electron donor, is an important simplification of the assay system for N₂ fixation, since only the enzyme components being studied need now be added to the reaction mixture.

That ADP is the product of ATP utilization by the N₂-fixing system was inferred from indirect evidence in the Azotobacter system. This paper contains the first direct evidence that the energy requirement for N₂ fixation is satisfied by a single P₁ elimination from ATP, forming ADP.

Inhibition by ADP of each of the reactions catalyzed by the N₂-fixing system, previously shown with crude extracts for N₂ fixation in Azotobacter, reductant-supported ATP utilization in Clostridium and with nitrogenase components for acetylene reduction in Clostridium, shows that the ADP effect is a basic one on the activities of the enzyme system, possibly resulting from a conformational change in either molybdoferredoxin or azoferredoxin, or a change in combination of the two. Evidence that the acetylene-reducing system requires interaction with more than one molecule of ATP has been obtained and a suggestion made that ADP is a negative modifier for the N₂-fixing system. The sigmoidal shape of the ADP inhibition curve for H₂ evolution in Fig. 4 indicates that ADP inhibition also involves binding at more than one site.

The reactive species of ATP appears to be a complex with Mg²⁺. At the pH of the reaction, Mg–ATP⁻ is the predominating Mg²⁺ complex, for which a stability constant of 10⁴⁺ was found. Calculating from a stability constant of 10⁴, the concentration of free ATP (with an initial 15 mM) would be 7.6, 1.18 and 0.10 mM for 7.5, 15 and 30 mM MgCl₂ in the reaction mixture, respectively. Thus, in the range of Mg²⁺ concentration giving maximum activity, more than 90% of the ATP would be complexed with Mg²⁺ (99.3% with 30 mM MgCl₂). Conversely, with less than 15 mM MgCl₂, nearly all the Mg²⁺ would be complexed to ATP. The observed moderate inhibition of enzyme activity at greater Mg²⁺ concentration is consistent with competition between Mg²⁺ and Mg–ATP⁻ for catalytic binding sites, particularly since evidence that molybdoferredoxin binds Mg²⁺ has been reported.

It has not been possible to assign a specific role to either molybdoferredoxin or azoferredoxin in the utilization of ATP and N₂ reduction. Inhibitor studies for this and the Azotobacter system indicate that the ATPase activity of the N₂-fixing system is probably a unique type. The results reported here are in general agreement with published hypotheses of N₂ fixation, confirming the close similarity of purified N₂-fixing systems from Clostridium and Azotobacter.

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REFERENCES

19 P. T. Bui, Thesis, 1968, Purdue University, Ind.