Stoichiometry of the Adenosine Triphosphate Requirement for 
N₂ Fixation and H₂ Evolution by a Partially Purified 
Preparation of Clostridium pasteurianum*

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SUMMARY

Cold ethanol or acetone precipitated about 75% of the N₂-fixing enzyme complex from extracts of Clostridium pasteurianum freed of nucleic acids. This simple preparation fixed about 45 nmoles of N₂ per mg of protein per min and is adequate for many studies of N₂ fixation. The preparation exhibited a dilution effect in N₂ fixation supported by Na₂S₂O₄ and an ATP-generating system. H₂ was evolved vigorously through an ATP-requiring, CO-insensitive reaction; the preparation evolved H₂ less vigorously in the absence of ATP through the CO-sensitive pathway unless ferredoxin was added. The N₂-fixing complex required 4.0 to 4.6 molecules of ATP per H₂ evolved or per third of a molecule of N₂ fixed throughout the time course of the reactions. At pH 6.0 the ATP/2e⁻ ratio approached 4.0, and it was near 4.6 at pH 8.0. Both N₂ fixation and ATP-dependent H₂ evolution had pH optima between pH 6.5 and 6.6.

The involvement of ATP in N₂ fixation was suggested by McNary and Burris (1), and it was later found that pyruvate could be replaced, as a requirement of cell-free fixation, by an ATP source and a reducing agent (2-4). There was no obligatory coupling of ATPase to N₂ reduction, since Mortenson (3) observed hydrolysis of ATP in the presence of H₂ regardless of the presence of N₂, and Bulen, Burns and LeComte (5) observed an evolution of H₂ requiring ATP and supported by dithionite (S₂O₄²⁻), a good reductant for N₂ fixation. The ATP-dependent H₂ evolution was associated with N₂ fixation in both Azobacter vinelandii and Clostridium pasteurianum extracts (4, 6, 7).

Several reports of the stoichiometry of the ATP requirement for N₂ fixation and H₂ evolution have been conflicting, being either 5 (8), 4 (9), or 2 (10) molecules of ATP per 2 electrons transferred. In this paper we report measurements of the stoichiometry of the ATP requirement in partially purified preparations from C. pasteurianum which indicate that the requirement varies with pH.

MATERIALS AND METHODS

C. pasteurianum, strain W-5, was maintained as a spore stock and cultured in the medium of Westlake and Wilson (11). The final culture was contained in a 150-liter, glass-lined reactor with a pH-stat to maintain the pH at 6.3, and the temperature was 35°. Cells were harvested, dried, and stored as described by Dilworth et al. (12).

Crude extracts were prepared by the method of Carnahan et al. (13). The crude extract was passed through an anionic column of Sephadex G-25 to remove dialyzable factors and to adjust the buffer concentration (14). Further treatments were done anaerobically by keeping the extract in rubber-capped serum bottles flushed with argon or hydrogen. Pre-gassed reagents were added through the rubber serum bottle cap with a hypodermic needle and syringe.

N₂ fixation was assayed by the formation of free ammonia (16). H₂ evolution supported by dithionite was measured in conventional Warburg respirometers. Reaction vessels were gassed with the appropriate gas mixtures by evacuation. Phosphocreatine hydrolysis was assayed by release of creatine, as measured by the method of Eggleton, Elsden, and Cough (16).

Solutions of dithionite were prepared by dissolving Na₂S₂O₄ in buffer or a predetermined amount of dilute NaOH to make a neutral solution. The solution was prepared anaerobically and used immediately or within 2 to 3 hours.

All reagents used were the highest purity commercial reagents, with the exception of phosphocreatine, which was synthesized by a modification (17) of the method of Ennor and Stocken.

RESULTS

Purification—The desalted, crude extract was treated with streptomycin sulfate to precipitate nucleic acids (18). Fig. 1

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Fig. 1. Effect of streptomycin sulfate on the $A_{260}/A_{280}$ ratio of an extract desalted with Sephadex G-25. Precipitation was performed by adding the appropriate amount of neutralized 10% streptomycin sulfate to 3.0 ml of the extract and diluting it to 4.0 ml. The precipitated nucleic acid was removed by centrifugation.

shows that the maximum $A_{260}/A_{280}$ ratio was obtained with 1.6 mg of streptomycin per mg of protein in the extract. The supernatant from this precipitation had the same protein content as the crude extract, and had virtually the same specific activity.

The supernatant from the streptomycin precipitation was fractionated with either ethanol or acetone at $0^\circ$. Although each solvent gave almost the same fractionation, the recovery of activity from ethanol precipitation was about twice that from acetone precipitation. Table I shows the recovery of protein and enzymatic activity from a fractionation with ethanol. The precipitation of nucleic acid with streptomycin was essential, because ethanol fractionation of a crude extract was quite different. The enzymatic activity from crude extracts precipitated between 28 and 44.5% ethanol, and the specific activity was not increased, because the recovery of protein and activity were the same.

The active protein which precipitated from streptomycin-treated extracts in 13% ethanol or acetone was soluble in 0.05 M phosphate buffer, pH 6.7, and these solutions could be kept anaerobically for at least 24 hours at $15^\circ$ with little loss of activity. Freezing caused 20 to 50% inactivation, but there was little further loss on storage at $-20^\circ$ for at least 1 week.

The spectrum of the ethanol precipitate was virtually featureless in the visible light range, with a general rise of absorbance toward shorter wave lengths. The streptomycin supernatant showed a decrease of absorbance below 500 nm on reduction with Na$_2$S$_2$O$_4$, but this decrease was absent in the ethanol precipitate, suggesting a depletion of ferredoxin by the ethanol precipitation (19).

Attempts to purify the enzymatic activity further by chromatography on DEAE-cellulose or by gel filtration were unsuccessful, either because of denaturation of the active proteins or because of removal of an essential factor.

Effect of Protein Concentration—The specific activity of the purified preparation was dependent upon the protein concentration when assayed for $N_2$ fixation or for ATP-dependent $H_2$ evolution supported by dithionite. Fig. 2 shows the loss of activity (milliunits) is indicated as nanomoles of $N_2$ fixed per min, and specific activity as milliunits per mg of protein.

**Table I**

<table>
<thead>
<tr>
<th>Ethanol fraction</th>
<th>Protein</th>
<th>Initial activity</th>
<th>Activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0% (supernatant)</td>
<td>190.0</td>
<td>100</td>
<td>1410</td>
<td>100</td>
</tr>
<tr>
<td>0-10% (ppt.)</td>
<td>14.0</td>
<td>7.4</td>
<td>650</td>
<td>46.0</td>
</tr>
<tr>
<td>10-15% (ppt.)</td>
<td>17.5</td>
<td>9.2</td>
<td>106</td>
<td>7.5</td>
</tr>
<tr>
<td>15-20% (ppt.)</td>
<td>18.5</td>
<td>9.7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Fig. 2.** Effect of protein concentration on $N_2$ fixation by the 13% acetone precipitate. The assay mixture contained the following, in 1.0 ml: phosphocreatine, 50 μmoles; creatine kinase, 0.2 mg; ATP, 5 μmoles; MgCl$_2$, 10 μmoles; Na$_2$S$_2$O$_4$, 20 μmoles; and enzyme solution, 0.3 ml. Reaction mixtures were incubated for 20 min at 30°. Activity (milliunits) is indicated as nanomoles of $N_2$ fixed per min, and specific activity as milliunits per mg of protein.
activity on dilution. The fact that dilution and incubation for 30 min prior to assay did not cause greater loss of activity than dilution immediately before assay excludes enzyme inactivation as an explanation for this effect and indicates that at least two protein components were limiting the reaction.

Effect of CO on Reactions—As shown in Fig. 3, the enzyme evolved $H_2$ in the presence of dithionite without an ATP source at about 20% of the rate with an ATP source. The low rate of $H_2$ evolution without ATP was evidently catalyzed by hydrogenase (hydrogen:acceptor oxidoreductase, EC 1.12.1.1), because it was totally inhibited by CO. However, CO only slightly inhibited $H_2$ evolution in the presence of ATP, suggesting that the ATP-independent reaction was not operating in the presence of ATP. Transfer of electrons from $Na_2S_2O_4$ to ferredoxin and thence to hydrogenase to support $H_2$ evolution should be unaffected by ATP. However, because the CO-sensitive $H_2$ evolution was suppressed by ATP, and because ferredoxin was absent or greatly depleted in the preparation, it is possible that the first electron carrier in the ATP-dependent reaction was able to donate electrons (independent of ferredoxin) to hydrogenase at a low rate when the ATP-dependent route was blocked by the omission of ATP.

Addition of ferredoxin to the extracts therefore should abolish the apparent competition between these two $H_2$-evolving reactions and establish the electron transfer route $S_2O_4^{2-} \rightarrow$ ferredoxin $\rightarrow$ hydrogenase $\rightarrow H_2$. Fig. 4 shows that addition of ferredoxin enhanced the total $H_2$ evolution in a reaction mixture containing ATP to approximately the same extent as ferredoxin addition enhanced $H_2$ evolution in the absence of ATP. Furthermore, CO inhibited both reactions to about the same extent. The addition of ferredoxin thus abolished the competition between the ATP-dependent and ATP-independent $H_2$-evolving reactions and allowed them to run independently.

**TABLE II**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$N_2$ fixed (A)</th>
<th>$H_2$ required to fix A (B)</th>
<th>Gas evolved (C)</th>
<th>$A + B + C$</th>
<th>$H_2$ evolved in argon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.3</td>
<td>43.0</td>
<td>31.4</td>
<td>88.7</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>50.0</td>
<td>90.0</td>
<td>42.3</td>
<td>162.3</td>
<td>177 (129 + CO)</td>
</tr>
<tr>
<td>3</td>
<td>23.8</td>
<td>71.4</td>
<td>44.0</td>
<td>139.2</td>
<td>140 (134 + CO)</td>
</tr>
</tbody>
</table>
ATP Requirement for H₂ Evolution and N₂ Fixation—The
hydrolysis of ATP was determined by assaying the creatine
released from phosphocreatine, since the equilibrium of the creatine
kinase reaction is such that nearly all of the adenine nucleotide is
kept in the form of ATP (20). Electron transfer from dithionite
could be followed directly by the evolution of H₂ in an argon
atmosphere, or by the combined fixation of N₂ and evolution of
H₂ in a N₂ atmosphere. Table II shows that the total electron
flow to N₂ and H₂ in an N₂ atmosphere is closely equivalent to the
total H₂ evolution in an argon atmosphere. The ATP require-
ment for energy-dependent electron flow under different at-
mospheres is shown in Table III. With argon alone, some ATP-
independent H₂ evolution may have occurred, so that the
apparent ATP requirement was lower than that observed when
CO was present to inhibit the hydrogenase reaction. Under N₂,
the majority of electrons went to reduce N₂ to ammonia, but,
when the observed gas evolution was corrected for the N₂ fixed,
the ATP requirement was essentially the same as under argon.
When CO was present, N₂ fixation was blocked, so that all the
electrons were again removed as H₂, and the ATP requirement
was nearly the same as under argon.

The pH dependence of H₂ evolution, N₂ fixation, and ATP
requirement was determined, and the results are shown in Fig. 5.
The similar pH dependence of H₂ evolution and N₂ fixation lends
further support to the hypothesis that they are manifestations of
the same enzymatic reaction. It is interesting to note that the
ATP/2e⁻ ratio increased with increasing pH. Since the H₂
evolution as an index of electron flow was observed in the pres-
ence of CO, and the ATP hydrolysis was corrected for hydrolysis
in the absence of a reductant, this ratio should represent the true
ATP requirement for the ATP-dependent electron flow from
dithionite to H₂. The fact that this ratio rose from a value of 4
at pH 6 to greater than 4.5 at pH 8 suggests that part of the ATP
requirement involves the generation of protons.

**Table I**

**ATP requirement for total electron flow**
The complete reaction system was the same as in Table I; the
gas phase is indicated. Reactions were terminated after 30 min
with 2 ml of saturated K₂CO₃ to liberate ammonia formed and to
stop further phosphocreatine hydrolysis. Protein concentration
was 1.4 mg per reaction vessel.

<table>
<thead>
<tr>
<th>Gas phase</th>
<th>Phosphocreatine hydrolyzed*</th>
<th>H₂ evolved</th>
<th>N₂ reduced</th>
<th>H₂ equivalents</th>
<th>ATP/H₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon</td>
<td>31.0</td>
<td>7.80</td>
<td>7.80</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Argon + CO (100 mm)</td>
<td>30.5</td>
<td>7.00</td>
<td>7.00</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>N₂</td>
<td>30.5</td>
<td>3.35*</td>
<td>1.35</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>N₂ + CO (100 mm)</td>
<td>31.7</td>
<td>7.16</td>
<td>7.16</td>
<td>4.4</td>
<td></td>
</tr>
</tbody>
</table>

* Values have been corrected for 5.5 μmoles of phosphocreatine hydrolyzed in the absence of Na₂S₂O₄.
  * Value has been corrected for uptake of N₂.

**Fig. 5.** Effect of pH on H₂ evolution, N₂ fixation, and ATP/2e⁻ ratio. Reaction conditions were the same as in Fig. 3, except
that a mixed buffer, containing 100 μmoles of 2-(N-morpholino)-
ethanesulfonic acid and 100 μmoles of N₂ hydroxymethylpiper-
zine-N'-2-ethanesulfonic acid, at the indicated pH, was added.
H₂ evolution (2-electron transfer) was assayed manometrically in
argon + CO. N₂ fixation colorimetrically by N₂H₄ formation under
N₂, and ATP hydrolysis by creatine released from phosphocrea-
tine. Protein concentration was 2.5 mg per reaction vessel.

Although the protein precipitated with ethanol had a sub-
stantial increase in specific activity over the crude extract, it still
was relatively impure. From the observed nitrogen content of
the dried cells and the bacterial growth rate, it can be calculated
that the minimum specific activity of the total cell protein should
be approximately 33 milliunits per mg. Even if the N₂-fixing
proteins were 10% of the total cell protein, the specific activity
should be at least 330. Based on these assumptions, at least a
6-fold purification of the ethanol precipitate should be possible.
However, this preparation has the distinct advantages that it
can be made rapidly and that relative to crude preparations it
supports little ATP-independent H₂ evolution or reductant-
independent ATPase activity.

Hardy, Knight, and D'Eustachio (7) have presented a scheme
for electron flow in N₂ fixation which implies that ATP-inde-
pendent H₂ evolution can occur only via ferredoxin. Our ob-
servations suggest that electron flow also may occur from reduced
X (their terminology) to hydrogenase in the absence of ferredoxin
when the ATP-dependent route is inoperative.

Investigators have experienced great difficulty in verifying the
claim of Hardy and Knight (10) that "a maximum of 2 mole-
cules of ATP are required to activate two electrons for the evolution
of 1 molecule of H₂." Our results and those of Mortenson (9)
suggest that 4 molecules of ATP are required per 2 electrons
transferred, and Bulen and LeComte (8) have reported a re-
quirement of 5.

At the higher pH values tested, an ATP requirement greater
than 4 molecules/2 electrons transferred (Fig. 5) may be con-
cerned in a pH-dependent reaction, possibly acid hydrolysis of the
product from a complex at the site of reduction. As the pH rises,
hydrolysis of ATP may become necessary to supply protons.

The idea that ATP hydrolysis may supply a proton for hy-
drolysis of an imido complex was suggested by Brintzinger (21) following his study of a model system based on the Vol’pin Shur reaction (22). It is particularly attractive to speculate (as have Hardy and Knight (10)) that the active site of N₂ reduction may be protected from the aqueous environment in vivo, because protons reacting with low potential electrons would support evolution of H₂ and thus waste the energy required to induce the low potential required for N₂ fixation.

REFERENCES

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