BBA 45678

N_2 FIXATION BY PURIFIED COMPONENTS OF THE N_2-FIXING SYSTEM OF *CLOSTRIDIUM PASTEURIANUM*

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(Received February 6th, 1968)

SUMMARY

1. N_2 fixation, reductant-supported ATP utilization and ATP-dependent H_2 evolution are catalyzed by combined molybdoferredoxin and azoferredoxin, the two purified components of the N_2-fixing system of *Clostridium pasteurianum*. No additional protein components are needed. At this stage of purity, neither of the two components of the N_2-fixing system catalyzed any of these reactions without the other.

2. The utilization of ATP by the N_2-fixing system involves a single P_i elimination; the rate of P_i 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 NH_3 with ninhydrin, N_2 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 P_i released per electron pair for H_2 evolution and N_2 fixation is 4.

INTRODUCTION

Molybdoferredoxin and azoferredoxin, two protein components of the N_2-fixing system in *Clostridium pasteurianum*, have been partially purified from cells grown on N_2 (ref. 1). The same two protein components were previously shown to be required for ATP-dependent H_2 evolution and electron-dependent ATP utilization[2].

N_2 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 extract[2]. With an extract containing hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) and acetate kinase (ATP: acetate phosphotransferase, EC 2.7.2.1), H_2 in the gas phase and added acetyl phosphate *plus* catalytic ATP, the requirements for electrons and ATP have been met[3,5]. 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

Abbreviation: TES buffer, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

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of reduced ferredoxin simplified the assay system in Azotobacter[6], particularly as this organism does not possess a hydrogenase capable of activating H_2 for N_2 reduction. Dithionite was equally effective as a substitute for reduced ferredoxin in the clostridial system[7]. Early attempts to achieve N_2 fixation with substrate quantities of ATP yielded only slight activity[3,5], though significant ATP-dependent H_2 evolution was obtained with ATP as the sole source of energy using a partially purified N_2-fixing system from Azotobacter[8]. Recently, it was shown that acetylene reduction by the N_2-fixing system in Clostridium could be supported by substrate quantities of ATP at similar initial rates as with an ATP generator[9].

This paper shows that good rates of NH_3 formation from 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 NH_3 estimation. The products of the reaction detected were ADP, P_i and 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, H_2 and N_2 (purified grade) were obtained from AirCo and passed over heated copper to remove traces of O_2. TES (*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid) buffer (Cal-Biochem) was neutralized with KOH solution. $Na_2S_2O_4$ (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[1]. 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[1].

Assays

ATP-dependent H_2 evolution was measured under argon or CO as described by BURNS AND BULEN[10]. The O_2-sensitive nitrogenase components were added by syringe through a serum cap to the main compartment of the side-arm Warburg flasks and preincubated with $MgCl_2$ and dithionite. Reaction was initiated by addition of ATP from the side arm. Without ATP, no H_2 was evolved with molybdoferredoxin and azoferredoxin of the purity used here.

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 NH_3 (0.5 ml) and P_i (0.25 ml) analysis. The contents were agitated with a magnetic stirrer and high-purity N_2 passed over the reaction mixture, subsequent to sparging in dithionite solution to ensure anaerobiosis.

NH_3 synthesis from N_2 was measured by microdiffusion from 1.0 ml of saturated K_2CO_3 in micro-Conway dishes. After distillation for at least 7 h at room temperature, the acid NH_4^+ sample (0.3 ml 0.1 M H_2SO_4) was diluted to 2.3 ml with NH_3-free water and 1.0-ml aliquots analyzed in duplicate with 1.0 ml of the ninhydrin–hydrindantin reagent developed by MOORE AND STEIN[11] for analysis of α-amino nitrogen. Following dilution with 4.0 ml of 50% ethanol, each μmole of NH_3 produced an extinction of 3.0–3.3 at 570 mμ with 1.0-cm light path. The useful range of the method is 0.5–1 μg of NH_3-nitrogen.

P_i was analyzed by the method of TAUSSKY AND SHORR[12]. 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 NH_4OH–0.2 M EDTA (100:60:0.8, by vol.) as the developing solvent, and analyzed[13] 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 N_2 fixation

The time course of P_i release and NH_3 formation with purified molybdoferredoxin and azoferredoxin is shown in Fig. 1. A substrate level of ATP (15 mM)

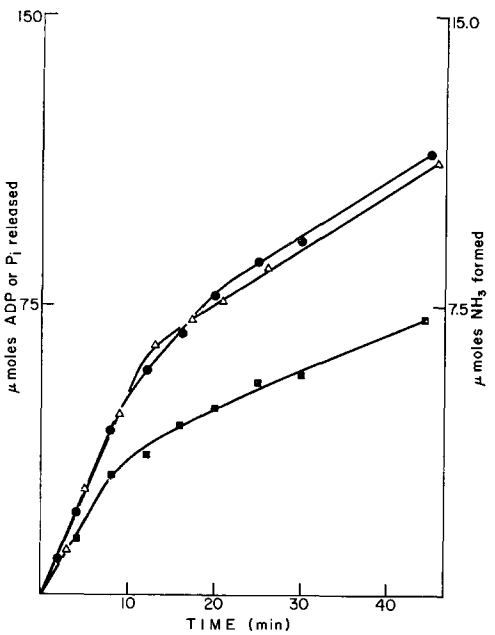

Fig. 1. Time course of P_i release and N_2 fixation with a substrate level of ATP; ADP formation. Reaction mixture contained, in 15.0 ml: ATP, 15 mM; $MgCl_2$, 7.5 mM; sodium dithionite, 17.5 mM; molybdoferredoxin, 4.5 mg; azoferredoxin, 10.0 mg; 18 mM Tris–HCl; 25 mM TES, final pH 6.8; temp. 23°. Samples were withdrawn by syringe and the reaction was terminated as follows: P_i, addition to 10% trichloroacetic acid; NH_3, addition to satd. K_2CO_3; ADP, boiling for 30 sec. ■—■, NH_3 formed; ●—●, P_i released; △—△, ADP formed.

was used, with dithionite as the electron donor. The initial rate of P_i release and NH_3 formation was linear till about 30% of the ATP was consumed, followed by a lesser rate. The specific rate of NH_3 formation (96 mμmoles 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_2 evolution up to 1100 mμmoles H_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.

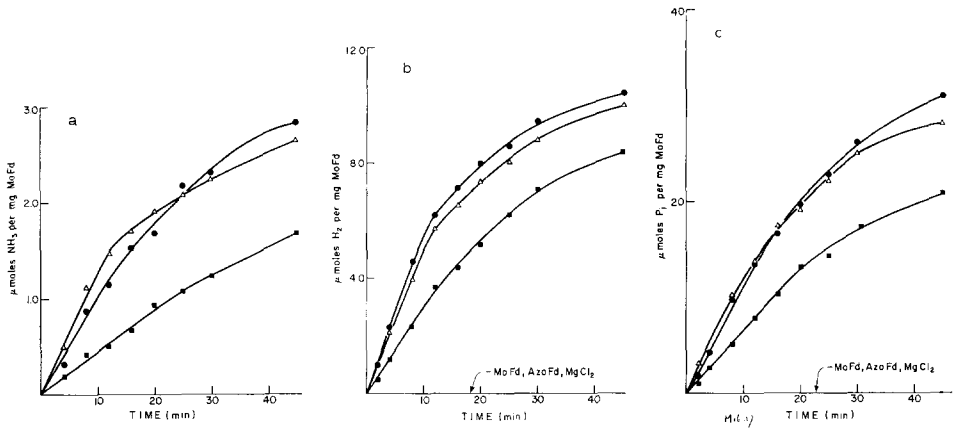

Fig. 2. Effect of ADP, AMP on (a) N_2 fixation, (b) H_2 evolution and (c) ATP utilization by molybdoferredoxin (MoFd) *plus* azoferredoxin (AzoFd). Reaction mixtures contained, in each ml: ATP, 15 mM; $MgCl_2$, 7.5 mM; sodium dithionite, 17.5 mM; ADP, AMP, where indicated, 5 mM; molybdoferredoxin, 0.25 mg; azoferredoxin, 0.83 mg; Tris–HCl, 16 mM; TES, 25 mM, final pH 6.8; temp. 22°. H_2 evolution was measured with an atmosphere of CO; N_2 fixation and P_i formation in 15 ml reaction volume under N_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_2 evolution and N_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_i release, and the same relative rate of NH_3 formation was maintained throughout the reaction period (approx. 30 ATP per N_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^{2+} requirement

Based on initial rates of reaction, a ratio of Mg^{2+}/ATP of 0.5–1.0 was required for maximum enzyme activity. However, as shown in Fig. 3 for H_2 evolution, ratios of Mg^{2+}/ATP greater than 1.0 produced some inhibition of the initial rate of enzyme

activity. For P_i 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.

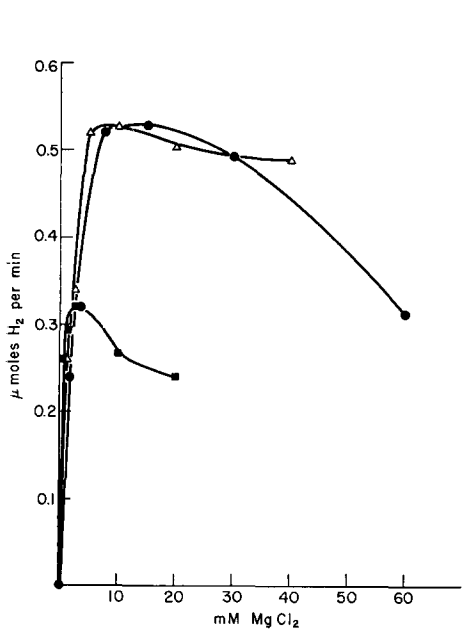

Fig. 3. The effect of Mg^{2+}/ATP ratio on the initial rate of ATP-dependent H_2 evolution. Reaction mixtures contained, in 2.0 ml final volume under CO: ATP, $MgCl_2$, as indicated; sodium dithionite, 17.5 mM; Tris–HCl, 6 mM; TES, pH 6.8 (final pH), 40 mM; 0.5 mg molybdoferredoxin; 3.3 mg azoferredoxin;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

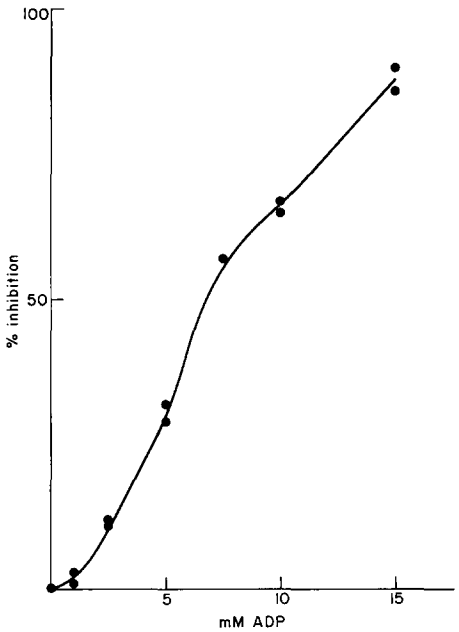

Fig. 4. ADP inhibition of ATP-dependent H_2 evolution. Reaction mixture as in Fig. 3 with 15 mM ATP; 7.5 mM $MgCl_2$, 0.5 mg molybdoferredoxin and 2.5 mg azoferredoxin; temp. 30°. Duplicate experiments on the same enzyme material are shown.

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_i 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[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.

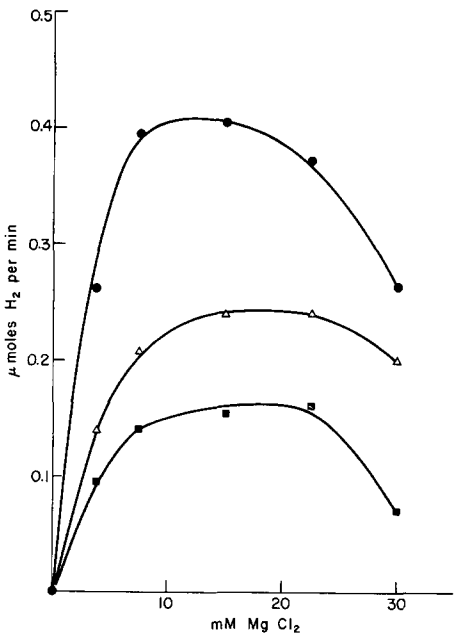

Fig. 5. Effect of Mg^{2+} concentration on ADP inhibition of ATP-dependent H_2 evolution. Conditions as in Fig. 4; ●—●, *minus* ADP; △—△, 5.0 mM ADP; ■—■, 7.5 mM ADP.

TABLE I

RELATIVE RATES OF P_i RELEASE AND N_2 FIXATION

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°.

	Azoferredoxin (mg/10 ml)					*Mg^{2+}/ATP ratio*			
	1.25	*2.5*	*5.0*	*7.5*	*12.5*	*0.25*	*0.5*	*1.0*	*2.0*
N_2 fixed (mμmoles/min per mg molybdoferredoxin)	40.0	75.0	125.0	147.5	162.5	50.0*	72.0*	58.4*	50.0*
P_i released (mμmoles/min per mg molybdoferredoxin)	900	1550	—	2700	3000	1432*	1972*	1648*	1372*
P_i/N_2 ratio	22.5	20.7	—	18.3	18.4	28.6	27.4	28.2	27.4

* Measured at 3–9-min period.

Clearly, examination of the ATP cost for N_2 reduction must take account of electrons lost as H_2. When this was done, the relative rate of P_i released for each electron pair transferred to N_2 was about 4, equal to the relative rate for ATP-dependent H_2 evolution with an atmosphere of argon (see Table II). A correction was made for H_2 evolution by measuring net gas evolution with an atmosphere of N_2, *plus* the N_2 uptake calculated by direct analysis for NH_3. An alternative method for measuring N_2 fixation is to determine the difference in gas evolution between reaction mixtures incubated with a gas phase of argon and reaction mixtures incubated under N_2. The difference equals the N_2 fixed *plus* 3 equiv of H_2 used in its reduction. This method agreed with direct analysis for NH_3.

TABLE II

RELATIVE RATES OF ATP UTILIZATION AND N_2 REDUCTION

Reactions were performed under N_2 in Warburg flasks at 22° containing, in 2 ml: ATP, 15 mM; $MgCl_2$ 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.

Expt.	*Time (min)*	P_i *(μmoles)*	$2e^-$ *μequiv* $(3N_2 + H_2)$	$P_i/2e^-$ *ratio*	*Percentage as* H_2
1	12	8.1	1.72	4.7	51
	20	10.7	2.50	4.3	53
2	30	6.3	1.64	3.9	47
3	12.5	6.7	1.70	4.0	43
	25	9.6	2.38	4.0	47
	25	9.5	2.40	4.0	100*

* 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[15,16] 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 [^{14}C]ATP or ADP. Following Sephadex G-100 chromatography[1], molybdoferredoxin still contained about 10 % of its maximum ATPase and N_2-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 N_2 fixation which is returned when the azoferredoxin fraction is added, or whether a contaminant foreign to the N_2-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

TABLE III

EFFECT OF INHIBITORS

Conditions as in Tables I and II. Reaction was initiated by addition of ATP after 5 min or less preincubation of all other components. In Expt. 3 rates given are averaged over first 10 min.

Expt.	*Components*	*Activity (mμmoles/min per mg molybdoferredoxin)*		
		P_i released	*N_2 fixed*	*H_2 evolved*
1	Control	1350	95.0	—
	NaF (10 mM)	1300	102.0	—
	α,α′-Dipyridyl (1 mM)	1300	105.0	—
	Sodium arsenate (1 mM)	1250	96.0	—
2	Control	1440	—	—
	Ouabain (25 mg/ml)	1400	—	—
3	Control	700	—	160
	Tiron (1 mM)	700	—	160
	Mersalyl	150	—	0

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 ferredoxin[17], inhibited both ATP-utilizing and H_2-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 N_2 fixation with substrate quantities of ATP were hampered probably because an incorrect Mg^{2+}/ATP ratio was chosen and the low amounts of NH_3 produced with the ATP-supported system required a more sensitive detection method. Apparent inhibition by high ATP concentration of N_2 fixation in Azotobacter[6] and of azide reduction in Clostridium[18] can be at least partly explained as a result of an excessive ATP/Mg^{2+} ratio. The use of substrate levels of ATP, with dithionite as the electron donor, is an important simplification of the assay system for N_2 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 N_2-fixing system was inferred from indirect evidence in the Azotobacter system[8]. This paper contains the first direct evidence that the energy requirement for N_2 fixation is satisfied by a single P_i elimination from ATP, forming ADP.

Inhibition by ADP of each of the reactions catalyzed by the N_2-fixing system, previously shown with crude extracts for N_2 fixation in Azotobacter[6], reductant-supported ATP utilization in Clostridium[19] and with nitrogenase components for acetylene reduction in Clostridium[9], 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 N_2-fixing system[9]. The sigmoidal shape of the ADP inhibition curve for H_2 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 Mg^{2+}. At the pH of the reaction, Mg–ATP^{2-} is the predominating Mg^{2+} complex, for which a stability constant of $10^{4.6}$ was found[20]. Calculating from a stability constant of 10^4, 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 $MgCl_2$ in the reaction mixture, respectively. Thus, in the range of Mg^{2+} concentration giving maximum activity, more than 90% of the ATP would be complexed with Mg^{2+} (99.3% with 30 mM $MgCl_2$). Conversely, with less than 15 mM $MgCl_2$, nearly all the Mg^{2+} would be complexed to ATP. The observed moderate inhibition of enzyme activity at greater Mg^{2+} concentration is consistent with competition between Mg^{2+} and Mg–ATP^{2-} for catalytic binding sites, particularly since evidence that molybdoferredoxin binds Mg^{2+} has been reported[1].

It has not been possible to assign a specific role to either molybdoferredoxin or azoferredoxin in the utilization of ATP and N_2 reduction. Inhibitor studies for this and the Azotobacter system[8] indicate that the ATPase activity of the N_2-fixing system is probably a unique type. The results reported here are in general agreement with published hypotheses of N_2 fixation[2,8], confirming the close similarity of purified N_2-fixing systems from Clostridium and Azotobacter[14,21].

ACKNOWLEDGEMENTS

This work was supported by grant (GB 05004) from the National Science Foundation. We are grateful to Mrs. C. POLIS for excellent technical assistance and to Drs. E. MOUSTAFA and P. T. BUI for helpful discussion.

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