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Mechanism of Carbamyl Phosphate Inhibition of Nitrogenase of *Clostridium pasteurianum*

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Carbamyl phosphate caused a maximal inhibition of 50% of the in vitro nitrogenase activity measured by acetylene reduction and dinitrogen reduction. The addition of 1 mM carbamyl phosphate to a N_2-fixing culture caused a rapid decrease of 30% of the acetylene reduction activity and also repression of nitrogenase biosynthesis. However, carbamyl phosphate had no effect on the reductant-dependent adenosine triphosphate hydrolysis and H_2 evolution reactions catalyzed by nitrogenase. Studies on the binding of carbamyl phosphate to nitrogenase and each of its two components (azoferredoxin and molybdoferredoxin) indicated that optimal binding was obtained only in the presence of an operating nitrogenase system. Moreover, the binding seemed to be on the molybdoferredoxin component rather than azoferredoxin. From a Scatchard plot and a reciprocal plot of the data, the values of $n = 2$ and dissociation constant (K) of approximately 5×10^{-5} M were obtained. The value for the dissociation constant was of the same order of magnitude as the endogenous level of carbamyl phosphate in a N_2-fixing cell. The carbamyl phosphate pool in NH_3-grown cells was twice that of N_2-fixing cells.

The N_2-fixing system of *Clostridium pasteurianum* is regulated by NH_3 (4). When this organism is grown in the presence of excess NH_3, there is no detectable nitrogenase activity as measured by acetylene reduction and nitrogen fixation. In addition, Daesch and Mortenson (4) reported that, upon addition of NH_3 to a N_2-fixing culture, there is an abrupt cessation of nitrogenase biosynthesis. The nitrogenase already synthesized by such a culture is stable and diluted upon growth. Similar findings have been reported for *Azotobacter vinelandii* (20) and *Klebsiella pneumoniae* (12, 16, 23). None of these observations shows that NH_3 plays a direct role in the repression mechanism of nitrogenase. Moreover, its possible role as a feed-back inhibitor was eliminated because NH_3 did not affect the in vitro nitrogenase activity (3, 4).

Sorger reported the finding of two "gratuitous co-repressors" of nitrogenase, namely, methylamine and 2-methylalanine (19). Such compounds are convenient experimental tools for determining the mechanism of repression of nitrogenase. However, a physiological co-repressor has yet to be found. Gordon and Brill reported (7) studies with 2-methylalanine inhibition on *A. vinelandii* wild-type strain and a derepressed mutant. On the basis of the finding that both of these strains were similarly inhibited by 2-methylalanine, they suggested that this compound does not interact at the same regulatory site as NH_4^+. We reported recently (17) the findings that carbamyl phosphate not only inhibits nitrogenase activity, but also represses its synthesis. Carbamyl phosphate was also studied by Winter and Burris (American Society of Plant Physiologists meeting, 1964) as a possible intermediate of biological nitrogen fixation. With extracts of *C. pasteurianum*, they demonstrated that the activity of carbamate kinase was sufficient to hydrolyze carbamyl phosphate to produce NH_3 at a rate comparable to NH_3 formation during N_2-fixation. However, they ruled out carbamyl phosphate as an intermediate on the basis of the observation that no citrulline was formed when ornithine was added to the extract which had ornithine transcarbamylase activity. However, we found (results not shown) that carbamyl phosphate synthetase in clostridial extracts catalyzed the formation of carbamyl phosphate in the presence of glutamine, adenosine triphosphate (ATP), Mg^{2+}, and $KHCO_3$. The carbamyl phosphate produced was measured in an assay where, in the presence of added ornithine and ornithine transcarbamylase, citrulline was formed. The present communication describes

experiments undertaken to explain the mechanism of inhibition by carbamyl phosphate of nitrogenase in *C. pasteurianum*.

MATERIALS AND METHODS

Organisms. *C. pasteurianum* W5 was used as the N_2-fixing organism unless otherwise noted. PS19 was a *Salmonella typhimurium* strain (pyrA721, ara-9, leuD5046) kindly provided by M. Levinthal. We wish to thank W. J. Brill for providing the *A. vinelandii* OP strain.

Media, extraction of cells, and assays. *C. pasteurianum* was grown and harvested and extracts were prepared as described by Daesch and Mortenson (4). PS19 was grown in Vogel and Bonner minimal medium (21), supplemented with 20 μg each of L-arginine and L-leucine per ml, 5 μg of uracil per ml, and 0.4% glucose. PS19 cultures were harvested in Sorvall model RC-2 refrigerated centrifuge at 13,200 × *g* for 10 min. The resulting cells were washed by suspending them in 6 mM potassium phosphate buffer (pH 7.0) and centrifuging. Extracts were prepared by suspending cell pellets in 5 volumes of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.6) and disrupting them with a Branson Sonifier. The disrupted suspensions were centrifuged at 37,000 × *g* for 30 min.

A. vinelandii was grown and harvested and extracts were made by the method of Shah et al. (18).

Whole-cell acetylene reduction assays were performed by the modified method reported by Moustafa and Mortenson (14). Acetylene reduction and creatine phosphate hydrolysis with purified nitrogenase were performed at 30 C in 8.0-ml serum bottles containing 0.25 atm of C_2H_2 and 0.75 atm of argon. The reaction mixture of 2 ml contained 100 μmol of TES buffer (pH 7.0), 0.1 mg of creatine phosphokinase (specific activity 105 U/mg), 18 μmol of creatine phosphate, 10 μmol of ATP, 10 μmol of $MgCl_2$, 20 μmol of $Na_2S_2O_4$, 0.18 mg of molybdoferredoxin (222,000 daltons; MoFd) and 0.33 mg of azoferredoxin (15,000 daltons; AzoFd) at a 7-times molar excess over MoFd. At 2-min intervals, 30 μliters of each gas sample was removed and analyzed in a Varian 1520 gas chromatograph (15). For the creatine phosphate hydrolysis assay, 0.1 ml of each liquid sample was analyzed for creatine (6). Purified AzoFd and MoFd were kindly provided by M. Walker and G. Walker, respectively.

Dinitrogen fixation by nitrogenase was measured by NH_3 formation. The reaction mixture was similar to that for acetylene reduction except that the gas phase was 1 atm of N_2. The reaction was performed at 30 C. After 15 min, 1 ml of each reaction mixture was removed for the analysis of ammonia (13).

Incorporation of [^{14}C]carbamyl phosphate (specific activity 0.02 mCi/mmol) by a growing culture was measured by the radioactivity of trichloroacetic acid-precipitable materials. Samples (5 ml) of culture were removed at the indicated time and added to an equal volume of chilled 10% trichloroacetic acid. Samples were filtered through glass-fiber filters and washed twice with 5% trichloroacetic acid. Radioactivity was determined by submerging the filter in 5 ml of scintillation solution and counting this mixture with a Beckman LS-150 liquid scintillation counter. The scintillation solution, containing 5 g of 2,5-diphenyloxazole per liter and 100 g of naphthalene per liter, was made to 1 liter with dioxane as recommended by Beckman (see Beckman LS-150 liquid scintillation system manual).

Uptake of [^{14}C]carbamyl phosphate by a growing culture was measured by filtering 5-ml samples taken at various times through membrane filters (Millipore Corp.). The samples were filtered immediately and washed twice with cold 5 ml of 0.05 M phosphate buffer, pH 7. Radioactivity on the filters was determined as already described.

ATP-dependent H_2 evolution catalyzed by nitrogenase was measured using a Warburg apparatus. The reaction mixture was similar to that for acetylene reduction except that the gas phase was 1 atm of argon. MoFd (0.45 mg) and 0.60 mg of AzoFd were used for each activity determination.

Optical density of the cultures was measured at 550 nm in a cuvette with a 1-cm light path.

Protein was assayed by the biuret method (8).

Binding studies with carbamyl phosphate and nitrogenase. Binding experiments were performed in 8-ml serum bottles under an atmosphere of N_2. The various reactants, indicated in the appropriate experiment below, were added such that the total volume was 1 ml. After 1 min of incubation, 0.5 ml of the incubation mixture was loaded anaerobically onto a Sephadex G-100 column (0.6 by 38 cm) previously equilibrated with 0.05 M Tris-hydrochloride (pH 7) and 1.5 mM dithionite. The flow rate of the column was regulated by a Buchler polystaltic pump at 7 ml/h. The column was eluted with the same buffer, and 1-ml fractions were collected anaerobically. Protein was determined by the Lowry method (11), and radioactivity was determined.

Measurement of the endogenous level of carbamyl phosphate. The method for measurement of carbamyl phosphate (endgenous level) was adopted from Williams et al. (22). A crude extract from PS19 was used as the source of ornithine transcarbamylase. The crude extract was filtered through a Bio Gel P100 column (2.4 by 6 cm) equilibrated with 0.02 M Tris-hydrochloride, pH 7.

Chemicals. [^{14}C]carbamyl phosphate (specific activity 16.5 mCi/mmol) and DL-[5-^{14}C]ornithine were purchased from New England Nuclear Corp. Creatine phosphokinase, creatine phosphate, ATP, and carbamyl phosphate were purchased from Sigma Chemical Co.

RESULTS

Inhibition and repression of the dinitrogen-fixing system. It has been established that nitrogenase can reduce numerous substrates in an in vitro assay system, namely acetylene, N_2, cyanide, azide, and isocyanate (5, 9, 10). We have reported previously that carbamyl phosphate inhibited the reduction of acetylene up to 50% (17). Our recent data show that it similarly

inhibits N_2 fixation (Table 1). Like the results with acetylene reduction, the inhibition reached a maximum of 50% at the higher concentrations of the inhibitor. This confirmatory result suggested that the inhibition phenomenon was not an artifact with specificity for certain substrates. Figure 1 shows that the inhibition of acetylene reduction by 5 mM carbamyl phosphate was approximately 50%. Inhibition was observed within 2 min after the addition of carbamyl phosphate to the reaction mixture.

The above results with nitrogenase of *C. pasteurianum* have been confirmed with *A. vinelandii*. We found that 7 mM carbamyl phosphate caused a decrease of acetylene reduction activity by a crude preparation of *Azotobacter* nitrogenase from 48.0 to 19.7 nmol of C_2H_4 per min per mg. Like the studies with *C. pasteurianum* nitrogenase, lower concentrations of carbamyl phosphate (1, 3, and 5 mM) caused less inhibition. These results show that the inhibitory effect of carbamyl phosphate is not strain specific, i.e., carbamyl phosphate inhibits the reduction of acetylene and dinitrogen by the nitrogenase of both *C. pasteurianum* and *A. vinelandii*.

One could argue that carbamyl phosphate inhibited phosphocreatine kinase rather than nitrogenase and that inhibition of substrate reduction occurred as a consequence of the lack of a sufficient ATP supply. This possibility was ruled out based on two lines of evidence. First, carbamyl phosphate had no effect on the hydrolysis of creatine phosphate coupled to acetylene reduction by nitrogenase (Table 2). Data not shown here also showed that reductant-dependent ATP hydrolysis when measured directly was not inhibited by carbamyl phosphate. Secondly, when the activity of phosphocreatine kinase was measured in the presence of various concentrations of carbamyl phosphate, no inhibition was observed.

TABLE 1. *Effect of carbamyl phosphate on N_2 fixation catalyzed by nitrogenase*[a]

Condition	NH_3 (μmol)	μmol of NH_3/min/mg of MoFd
Complete system	1.20	0.170
Complete system plus		
1 mM CP	1.12	0.158
3 mM CP	0.90	0.127
5 mM CP	0.63	0.089
7 mM CP	0.64	0.090
Complete system with 7 mM CP but minus nitrogenase	0.00	

[a] The activity determinations were performed in 8.0-ml serum bottles containing 1 atm of N_2. The reaction mixtures of 2 ml contained 0.86 mg of AzoFd and 0.47 mg of MoFd, the reactants described in Materials and Methods, and the indicated amount of carbamyl phosphate (freshly prepared). The reaction mixtures were incubated for 15 min at 30 C. The reaction was terminated by removing 1 ml of the reaction mixtures and adding it to an equal volume of saturated K_2CO_3. NH_3 analysis by the Conway method was immediately performed on the mixtures (13).

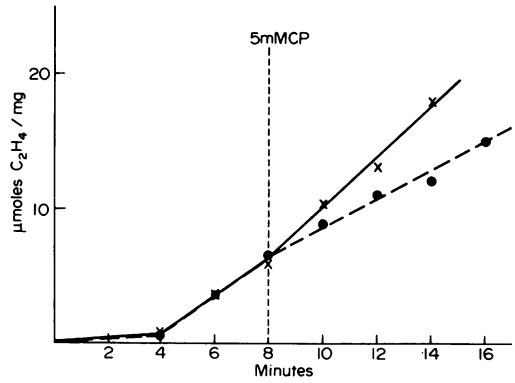

FIG. 1. *Kinetics of inhibition by carbamyl phosphate (5 mM) on the in vitro acetylene reduction catalyzed by nitrogenase; 0.33 mg of AzoFd and 0.18 mg of MoFd were used in each activity determination. The control vessel (×) contained the complete reaction mixture with $Na_2S_2O_4$ as the reductant and the ATP generating system (see Materials and Methods). The experimental vessel (●) contained an identical reaction mixture and, at the indicated time, 5 mM carbamyl phosphate (freshly prepared) was added to the mixture. Gas samples (30 μliters) were removed at the indicated intervals to determine the amount of ethylene produced.*

TABLE 2. *Effect of carbamyl phosphate on creatine phosphate utilization coupled to nitrogenase*[a]

Addition	nmol of creatine/min/mg of MoFd
None	5,220
Carbamyl-P	
1 mM	5,220
3 mM	5,000
5 mM	5,000
7 mM	5,020

[a] The indicated concentrations of carbamyl phosphate were added to the assay mixtures immediately before the start of the reactions; 0.34 mg of AzoFd and 0.18 mg of MoFd were used in each activity determination. The reaction mixtures (see Materials and Methods) were incubated in 8-ml serum bottles under an atmosphere of argon. At 2-min intervals, 0.1-ml samples were removed and added directly to 0.9 ml of 0.1 N NaOH, and the amount of creatine was determined (6).

To study the effect of carbamyl phosphate in vivo, it was necessary to show that carbamyl phosphate permeates the cell. Evidence that this occurred was obtained when it was shown that *C. pasteurianum* was able to incorporate [^{14}C]carbamyl phosphate (1 mM) added to the medium. In 30 min, approximately 7.35 nmol (per 1.32×10^9 cells) of carbamyl phosphate was incorporated into trichloroacetic acid-precipitable materials. From the cell dimensions of 1 μm in diameter and 3 μm in length, we calculated the cell volume for 1.32×10^9 cells to be approximately 3.12×10^{-6} *l*. Therefore, the concentration of carbamyl phosphate incorporated (7.35×10^{-9} mol/3.12×10^{-6} *l*) was found to be 2.36 mM. However, considerably more carbamyl phosphate was transported into the cells. It was found that 25.7 nmol was taken up by 1.32×10^9 cells in 2 min; the internal concentration was 8.24 mM. The cells are capable of concentrating exogenous carbamyl phosphate more than eightfold, an amount sufficient to bring about the in vitro inhibition previously described.

When 1 mM carbamyl phosphate was added to both a N_2-fixing chemostat culture and to its medium reservoir, there was a 30% decrease in acetylene reduction activity within 5 min of the addition. The activity continued to decrease for the duration of the experiment. This observation suggested that the addition of carbamyl phosphte not only inhibited nitrogenase activity but also caused a repression of nitrogenase synthesis. Growth was unaffected for 15 min, after which time growth decreased at a rate comparable to that of nitrogenase activity (Fig. 2a). If carbamyl phosphate decomposed or was converted to NH_3, the growth rate of the culture should return to normal or increase. This suggests that carbamyl phosphate is not decomposed enough to allow restoration of growth. It is possible that carbamyl phosphate is metabolized further to yield a product that controls nitrogenase.

The experiment in Fig. 2a is to be compared with the results of Fig. 2b in which 1 mM $(NH_4)_2SO_4$ was added to a batch culture growing under N_2-fixing conditions. There was an increase in growth rate after the addition—similar to previously reported results (4). This is in contrast to the drastic decrease in growth that resulted from the addition of carbamyl phosphate shown in Fig. 2a. Nitrogenase activity measured by acetylene reduction decreased gradually after the NH_4^+ addition. The decrease in activity in both cases is a result of repression of nitrogenase biosynthesis.

We have yet to show that the repression

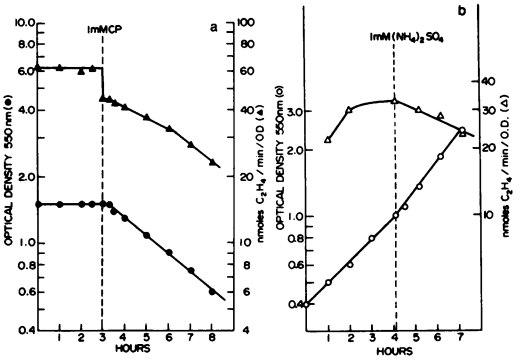

FIG. 2. *(a) Effect of carbamyl phosphate (1 mM) on the growth and nitrogenase activity of a N_2-fixing chemostat culture of Clostridium pasteurianum. After inoculation, the sucrose-limiting chemostat culture was grown for 48 h to establish equilibrium. The generation time of the culture was 1.7 h, and the corresponding k (growth constant) was 0.41. Carbamyl phosphate (1 mM) was added to both the growth vessel and the medium reservoir at the time indicated by the dash line. Samples were taken at predetermined intervals for growth and activity determinations. (b) Effect of ammonium ions on the growth and nitrogenase activity of a N_2-fixing batch culture of C. pasteurianum. A 200-ml culture medium was inoculated from a N_2-fixing chemostat culture. $(NH_4)_2SO_4$ (1 mM) was added to the culture at the time indicated by the dash line. Samples were removed at intervals for growth and activity determinations.*

occurred because NH_4^+ was metabolized to carbamyl phosphate or to a modified form of carbamyl phosphate. However, it is clear that the effect of carbamyl phosphate did not result from its decomposition of NH_3, as demonstrated by the greatly different effects on growth and nitrogenase activity. In addition, Allen and Jones (1) reported that ammonia release from carbamyl phosphate is favored by acidic pH. The pH of the culture media in our experiments was buffered at pH 6. At this pH, Allen and Jones found no significant carbamyl phosphate decomposition to ammonia in 5 min. Therefore, the 30% decrease in activity shown in Fig. 2a could not have resulted from the action of ammonia on nitrogenase. After 2 h at pH 6, a decomposition to ammonia of approximately 33% was reported (1). This amount of ammonia, if produced, could not account for the rate of activity decrease observed in Fig. 2a and, in fact, should have led to an increase in the growth of the culture. In the in vitro experiments, carbamyl phosphate decomposition to ammonia was even less likely to occur since the reaction mixtures were buffered at pH 7. More importantly, ammonia, unlike carbamyl phos-

phate, does not inhibit in vitro nitrogenase activity.

Effect of carbamyl phosphate on the H_2 evolution activity catalyzed by nitrogenase. The results described above established that carbamyl phosphate inhibited acetylene and N_2 reduction but did not inhibit ATP hydrolysis. The specificity is similar to that of CO inhibition. One possible explanation is that electron flow through the nitrogenase system is inhibited but ATP utilization is not. Results shown in Table 3 eliminated this possibility since ATP-dependent H_2 evolution was unaffected. Thus, the inhibition observed with acetylene and N_2 reduction cannot be accounted for by the lack of ATP utilization or by the lack of at least part of the electron transport system. Electron transfer from the "site" of H_2 evolution to the "site" for N_2 reduction could be inhibited.

Binding of carbamyl phosphate to nitrogenase. Since the effect of carbamyl phosphate on nitrogenase was specifically on the reduction of N_2 and acetylene but not H^+ and since MoFd was suggested to contain the substrate reduction site (2), it was of interest to determine whether carbamyl phosphate complexed with either protein. Table 4 shows that carbamyl phosphate binds to MoFd in the presence of 5 mM Mg^{2+} and 10 mM ATP. From the amount of radioactivity associated with the protein peak, it was determined that 0.07 mol of carbamyl phosphate was bound to 1 mol (tetramer) of MoFd. Little difference in binding was observed in the absence of ATP or Mg^{2+}.

Since other functions of nitrogenase required the presence of both its protein components, Mg^{2+}, ATP, and reductant, the binding of carbamyl phosphate to nitrogenase was also examined with both components present (4.6:1 molar ratio of AzoFd-MoFd) as well as Mg^{2+}, ATP, and reductant. As shown in Fig. 3, radioactivity was associated with the protein but the peak of radioactivity was at the front of the protein peak eluted from Sephadex G-100. Unbound AzoFd eluted from the column at the end of the peak (in fractions 4 and 5), whereas the AzoFd-MoFd complex eluted at the front of the protein peak and free MoFd eluted at the center of the peak. This differential elution accounted for the skewed profile of the protein peak and suggested that the radioactivity was with the complex and MoFd. With the complete system present (AzoFd, MoFd, 5 mM Mg^{2+}, and 10 mM ATP) 0.45 mol of carbamyl phosphate was bound for each mole (tetramer) of MoFd. ATP and Mg^{2+} were required for optimal binding (Table 4). In the absence of ATP and Mg^{2+}, only 0.09 mol of carbamyl phosphate was bound. Since in the absence of AzoFd only 0.1 mol of carbamyl phosphate was bound per mol of MoFd, most of the radioactivity seems to be associated with the MoFd-AzoFd complex.

Carbamyl phosphate did not bind to AzoFd. Only 0.006 mol of carbamyl phosphate bound to 1 mol (dimer) of AzoFd, an amount one-tenth that with MoFd alone. Table 4 summarized the results from [^{14}C]carbamyl phosphate-binding experiments performed under different experimental conditions. ATP, Mg^{2+}, MoFd, and AzoFd are all required for optimal binding of

TABLE 3. *Effects of carbamyl phosphate on H_2 evolution catalyzed by nitrogenase*[a]

Addition	nmol of H_2/min/mg of MoFd
None	848
Carbamyl-P	
1 mM	848
3 mM	840
5 mM	856

[a] The determinations were performed with 0.60 mg of AzoFd and 0.45 mg of MoFd. All solutions were degassed and flushed with argon. The following reactants (the amounts indicated in Materials and Methods) were added to the Warburg flask: TES buffer (pH 7), creatine phosphokinase, creatine phosphate, ATP, Mg^{2+}, $Na_2S_2O_4$, and carbamyl phosphate (concentrations as indicated in the table). The center well contained 0.1 ml of 40% KOH. Nitrogenase was added to the side arm. The reaction was started by tipping the nitrogenase into the main compartment. At 30-s intervals, readings were taken to record the amount of H_2 evolved.

TABLE 4. *Summary of results from various binding experiments with carbamyl phosphate and nitrogenase*

Expt	Conditions[a]	mol of CP bound/mol of MoFd[b]
1	MoFd (2.05 mg), 5 mM CP	0.10
2	MoFd, 5 mM CP, 10 mM ATP	0.07
3	AzoFd (2.35 mg), 5 mM CP, 10 mM ATP	0.01
4	AzoFd, MoFd, 5 mM CP, 10 mM ATP	0.45
5	AzoFd, MoFd, 5 mM CP	0.09
6	AzoFd, MoFd, 5 mM CP, 10 mM ADP	0.05

[a] The mixtures were incubated in 0.05 M Tris-hydrochloride (pH 7) containing 5 mM $MgCl_2$ and 0.75 mM dithionite. CP, Carbamyl phosphate.

[b] The amount of CP bound was expressed on the basis of MoFd tetramer, except for experiment three, where it was expressed on the basis of AzoFd dimer.

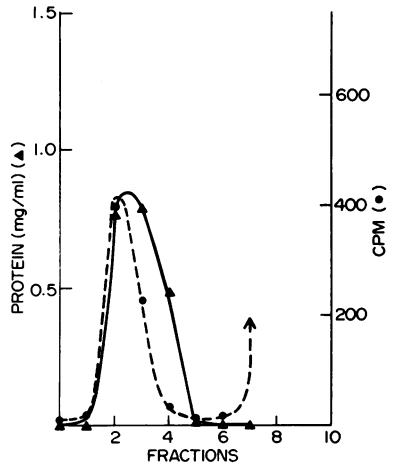

FIG. 3. *Binding of carbamyl phosphate (5 mM) to nitrogenase; 2.35 mg of AzoFd and 2.05 mg of MoFd were incubated with 5 mM $MgCl_2$, 10 mM ATP, 50 μliters of [^{14}C]carbamyl phosphate, and 0.05 M Tris-hydrochloride (pH 7) containing 0.75 mM $Na_2S_2O_4$ in a total volume of 1 ml. Anaerobic condition was maintained by keeping the incubation mixture under an atmosphere of N_2. After 1 min of incubation, 0.5 ml of the mixture was put on a Sephadex G-100 column (0.6 by 38 cm) equilibrated with 0.05 M Tris-hydrochloride (pH 7) containing 1.5 mM $Na_2S_2O_4$. Fractions (1 ml) were collected anaerobically. Samples were removed from each fraction for protein and radioactivity determinations. Unbound [^{14}C]carbamyl phosphate was eluted between fractions 7 and 10, and its radioactivity (indicated by the dash line and arrow) was much too high to be included within the scale of the figure.*

carbamyl phosphate to nitrogenase. In other words, operating nitrogenase is required for optimal binding. These observations show that binding is specific for MoFd and/or the complex. Additional experiments in which proteins such as bovine serum albumin were examined for binding of [^{14}C]carbamyl phosphate showed no binding.

A plot of the amount of carbamyl phosphate bound versus its increasing concentration was a segment of a rectangular hyperbola (Fig. 4). The curve approached 1 mol bound at concentrations of carbamyl phosphate of about 15 mM. The same data, presented in a reciprocal plot, are shown in the insert of Fig. 4. From this plot, the following values were deduced for n and K respectively, 2 and 5×10^{-5} M (see figure legend for the identification of terms n and K). Values of $n = 1.7$ and $K = 7 \times 10^{-5}$ M were obtained in a Scatchard plot of the same data. It is interesting to note that the K values are in the same order of magnitude as the endogenous level of carbamyl phosphate in the N_2-fixing culture shown in Table 5. It should also be pointed out that, under N_2-fixing conditions, nitrogenase is at least 50% repressed by the "NH_3" it produces (4).

DISCUSSION

The addition of 5 mM carbamyl phosphate to an in vitro acetylene reduction measurement catalyzed by nitrogenase caused a maximal decrease in activity of 50%. As reported previously (17), higher concentrations of carbamyl phosphate did not cause further inhibition. When 1 mM carbamyl phosphate was added to a N_2-fixing culture, not only was the nitrogenase activity inhibited, but its synthesis was also repressed. It was observed, moreover, that car-

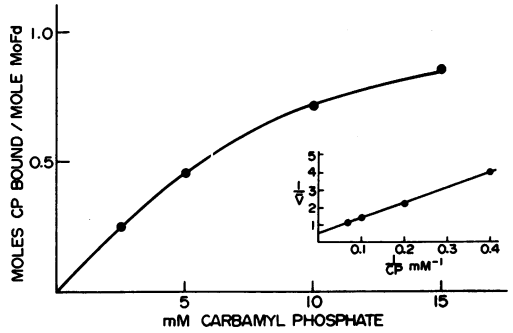

FIG. 4. *A plot of the amount of carbamyl phosphate bound to nitrogenase versus increase of ligand concentration. Each experiment was performed under identical conditions except for the amount of [^{14}C]carbamyl phosphate added. The experimental condition was described in the legend of Fig. 3. The inset is a reciprocal plot of the data presented in Fig. 4. V = average number of moles of carbamyl phosphate bound per mole of MoFd. The intercept on the ordinate axis is l/n; the slope is l/Kn, where K = dissociation constant of the bound complex, n = maximal number of binding sites.*

TABLE 5. *Endogenous levels of carbamyl phosphate in N_2-fixing culture and NH_3-growing culture*[a]

Growth condition	Endogenous CP (μM)	
	Range	Avg
N_2	57.6–71.7	64.7
12 mM NH_3	97.6–109.5	103.5

[a] N_2-fixing and NH_3-growing cells (500 ml culture) were harvested at mid-log phase and washed as described in Materials and Methods. Calculations of cell volume was based on the volume of a cylinder with the following dimensions: diameter, 1 μm; and length, 3 μm. Cell counts were determined by counting a 10 × dilution of the cultures in a Petroff-Hauser counter. CP, Carbamyl phosphate.

bamyl phosphate had a differential effect on the various reactions catalyzed by nitrogenase. It specifically inhibited substrate reduction (other than H^+) without affecting the concomitant ATP hydrolysis and electron flow of at least part of the nitrogenase system, i.e., H^+ reduction was not affected. Carbamyl phosphate can be a potentially significant tool to the elucidation of the mechanism of nitrogenase because of its differential effect on nitrogenase activities and because unlike CO it is not a gas.

Results from the binding experiments indicated that carbamyl phosphate was bound optimally to nitrogenase only in the presence of the operating nitrogenase system, i.e., in the presence of AzoFd, MoFd, Mg^{2+}, and ATP. The lack of binding of carbamyl phosphate to AzoFd alone and the results from the ATP hydrolysis experiment seem to rule out AzoFd as the binding site. The results suggest that carbamyl phosphate binds at a site or sites located on MoFd. The results also suggest that a conformational change of MoFd induced by AzoFd in the presence of reductant, Mg^{2+}, and ATP is essential to either create or expose the binding site(s). An alternative explanation for the requirement of both AzoFd and MoFd is that the binding sites are formed when AzoFd and MoFd complex and that a site or sites on both are required. The present data are insufficient to discriminate between these two possibilities.

As suggested from the kinetics of binding, there can be two carbamyl phosphate-binding sites. This could mean that each MoFd dimer binds one carbamyl phosphate. The present models for the mechanism of nitrogenase do not indicate whether the MoFd dimer or the tetramer is the functional unit. To explain the in vitro result that only 50% inhibition was observed at saturating levels of carbamyl phosphate, it can be postulated that carbamyl phosphate causes a decelerated rate of formation of the active MoFd-AzoFd complex. A maximal deceleration of 50% is reached at 5 mM inhibitor. The equilibrium between the AzoFd-MoFd complex and the individual components could be shifted to favor dissociation of the complex. Another possible explanation of the carbamyl phosphate inhibition is that the structure of the complex is changed such that it has decreased catalytic activity.

The K value obtained from the binding experiments is the overall dissociation constant for the two sites. It does not indicate the intrinsic dissociation constant for each individual site. The observation that this K value is very close to the endogenous level of carbamyl phosphate in N_2-fixing cells suggests that the nitrogenase in such a culture is partially repressed. This conclusion agrees with the previous finding by our laboratory that partial (70% or more) repression of nitrogenase exists in a normal N_2-fixing culture (4). The endogenous carbamyl phosphate in an NH_3-grown culture is approximately twice that of a N_2-fixing culture. Since nitrogenase is not synthesized in a culture growing in the presence of excess NH_3, the control by carbamyl phosphate would have to be a very sensitive one which can respond to small fluctuations in its concentration.

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