*MECHANISM OF THE ENZYMIC REDUCTION OF N_2: THE BINDING OF ADENOSINE 5'-TRIPHOSPHATE AND CYANIDE TO THE N_2-REDUCING SYSTEM**

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The *in vitro* reduction of N_2 is a complex process involving at least six different reactants: two proteins[1, 2] for which the names azoferredoxin (AzoFd) and molybdoferredoxin (MoFd) have been proposed,[3] an electron source, the electron acceptor, ATP,[4] and Mg^{2+}.[5-7] One of the goals of research in this area is to define the orderly and quantitative participation of these reactants leading to the reduction of the electron acceptor with concomitant breakdown of ATP to ADP and inorganic phosphate.[7]

The work described in this paper shows that (1) AzoFd reversibly binds both ATP, a reactant in N_2 reduction, and ADP, a specific inhibitor of N_2 reduction, and (2) MoFd reversibly binds cyanide, which is also reduced by the N_2-reducing system. It is suggested that the binding of ATP and of cyanide are partial reactions of the N_2-reducing system.

Methods.—Buffers: 2-Amino-2-(hydroxymethyl)-1,3-propanediol, dissolved in 0.5 equivalents of HCl, was used in cyanide-binding experiments at 50 mM (pH 8.1). Sodium piperazine-N,N'-bis (2-ethanesulfonic acid) (Calbiochem), dissolved in 0.4 equivalents of LiOH, was used in all other experiments at 50 mM (pH 6.6).

Biochemicals: Sodium salts of ATP, AMP, CDP, GDP, IDP, and UDP were purchased from Sigma. Trilithium ADP was purchased from Calbiochem. Uniformly labeled ^{14}C-ATP and ^{14}C-ADP were purchased from Schwarz BioResearch. $K^{14}CN$ was purchased from International Chemical and Nuclear Corporation.

Proteins: AzoFd (approximate purity 90%), prepared by a modification[8] of the method of Mortenson *et al.*,[3] was a gift from E. Moustafa. The molecular weight of 4×10^4 used in all binding calculations was estimated by Sephadex chromatography. MoFd, purified to a constant Mo/Fe/protein by a modification[9] of the method of Mortenson *et al.*,[3] was a gift from J. A. Morris. The molecular weight of 1.6×10^5 used in binding calculations was estimated by Sephadex chromatography.

Cyanide reduction: Cyanide is an excellent substrate for studies of the N_2-reducing system for several reasons: (1) it can be handled easily because it is a solid, (2) it is reduced by the N_2-reducing system to ammonia and methane,[10] which can be quantitated rapidly by gas chromatography, and (3) it can be readily obtained in a radioactive form, $K^{14}CN$. Cyanide solutions were prepared anaerobically and stored under hydrogen at $-20°$. The reduction was carried out at 22° in glass vials (8 ml vol) sealed with rubber serum stoppers and equipped with magnetic stirring bars. The gas phase in the vials was evacuated and replaced by hydrogen. AzoFd, MoFd, and the electron donor dithionite[11] were added anaerobically since they are extremely sensitive to oxygen. The reaction was started by the addition of ATP, and stopped when necessary by the addition of 0.05 ml of 10 *N* KOH. Unless indicated otherwise, the vials contained in 2.0 ml total volume the following buffered reaction mixture: 0.2 mg AzoFd, 0.8 mg MoFd, 4.0 mM KCN, 4.0 mM dithionite ($Na_2S_2O_4$), 2.0 mM ATP, and 2.0 mM $MgCl_2$. Gas samples were removed at appropriate time intervals and analyzed for methane by gas chromatography using a Porapak R column,[12] 1.8 mm diameter × 40 cm, at 22°.

Detection of binding by gel filtration: An anaerobic column of 2.4 cm diameter was packed with 6.0 gm of Sephadex G-50, then washed with at least one bed volume of deaerated equilibrating buffer prior to the application and elution of the protein sample at

constant rate (60 ml/hr). $K^{14}CN$, ^{14}C-ATP, or ^{14}C-ADP were added to the equilibrating buffer at the low concentration of 0.1 mM since a high signal (bound substrate or inhibitor) to noise (background of free substrate or inhibitor) ratio was necessary for a quantitative estimation of substrate or inhibitor binding by the test proteins. $MgCl_2$ (0.5 mM) and dithionite (4.0 mM) were also present in the experiments testing ATP and AMP for binding to AzoFd in competition with ADP. The effluent was collected in 1.0-ml samples and analyzed for protein by the biuret method, and for radioactivity, which was measured at 22° in a liquid scintillation counter (Beckman model LS-150) by mixing 0.5 ml of the column effluent with 7.0 ml of a scintillation fluid containing 5.0 gm of 2,5-diphenyloxazole and 50 gm of naphthalene per liter of 1,4-dioxane.

Results.—ADP inhibition of cyanide reduction: ADP has been shown to inhibit reductant-dependent ATP hydrolysis,[5] acetylene reduction,[6] ATP-dependent H_2 evolution,[7] and N_2 reduction,[7] all of which are catalytic activities of the N_2-reducing system. The inhibition was not a result of ADP decreasing the amount of Mg^{2+} available,[5, 7] for example by forming a (Mg·ADP) complex. Increasing concentrations of ADP affect the rate of cyanide reduction (Fig. 1 or ATP-dependent H_2 evolution[7] in a similar manner. When the data from the experiment on the inhibition of cyanide reduction by ADP (Fig. 1) were plotted as log $(v/(v_0 - v))$ vs. log ADP,[13] the slope of the resulting curve (Fig. 2) indicated that the inhibition required the participation of three ADP molecules per cyanide-reducing unit. The specific inhibition of the N_2-reducing activity *in vitro* by an end product in ammonia synthesis, ADP (Table 1), suggests that the inhibition operates *in vivo* as an energy-conserving device. When the supply of energy in the cell becomes limiting, the resulting ADP build-up will decelerate the breakdown of ATP by the N_2-reducing system.

Binding of cyanide to MoFd: Earlier attempts to define the role of MoFd and AzoFd in N_2 reduction[14–16] were unsuccessful[5, 7, 17] because the techniques used[5]

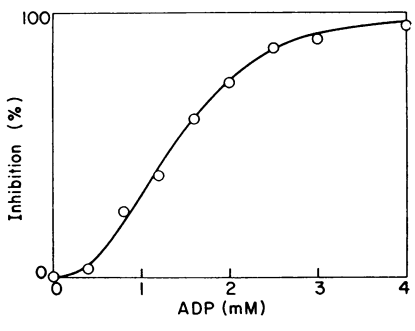


FIG. 1.—ADP inhibition of cyanide reduction. Cyanide reduction was assayed as described in *Methods*, in the presence of increasing concentrations of ADP. One hundred per cent activity corresponds to an initial rate of 60 mμmoles methane produced/min.

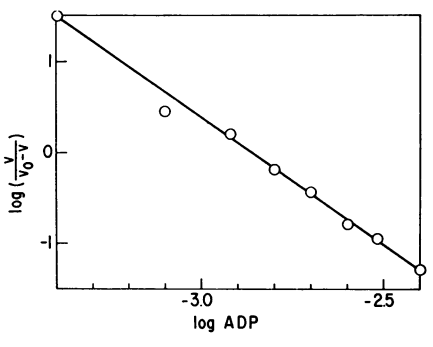


FIG. 2.—ADP inhibition of cyanide reduction. Initial rates of cyanide reduction in the presence (v) or absence (v_0) of ADP were taken from Fig. 1. The concentration of ADP is in moles/liter. The slope of the curve, $n = 2.8$, corresponds approximately to the number of ADP molecules required to inactivate the cyanide-reducing unit. The number of ATP molecules required to activate the acetylene-reducing unit is 2 or 3, depending on whether ADP is absent or present.[6]

TABLE 1. *The specificity of the inhibition of cyanide reduction by ADP.*

Additions	Activity (%)
None	100
ADP (4.0 mM)	6
CDP (4.7 mM)	91
GDP (4.1 mM)	86
IDP (4.3 mM)	94
UDP (5.2 mM)	95
AMP (4.0 mM) + $Na_4P_2O_7$ (4.0 mM)	92
K_2HPO_4 (4.0 mM) + Na_2HAsO_4 (4.0 mM)	94

Cyanide reduction was assayed (as described in *Methods*) in the presence of various nucleoside phosphates at the concentrations shown. One hundred per cent activity corresponded to an initial rate of 60 mμmoles methane produced/min.

were incapable of detecting reversible associations between proteins and substrates. This led us to adapt for use under anaerobic conditions a simple method capable of detecting reversible as well as irreversible associations between two chemical species of different molecular weights.[18] The method consists of first equilibrating a Sephadex G-50 column with buffer containing a substrate of the N_2-reducing system (cyanide or ATP), or an inhibitor (ADP), and any other necessary reactant (e.g. $MgCl_2$). Next the test protein is passed through the column. If the protein has an affinity for the substrate (or inhibitor), one should be able to detect an accumulation of substrate (or inhibitor) above the equilibrium level and coincident with the protein peak. In the absence of any interaction, the equilibrium concentration of the substrate (or inhibitor), constant throughout the column, should not be disturbed by passage of the protein band. When MoFd and AzoFd were tested in this manner, the elution pattern of protein and radioactivity showed binding of $^{14}CN^-$ to MoFd (Fig. 3), but not to AzoFd (Fig. 4). The binding was not enhanced by the addition of other reac-

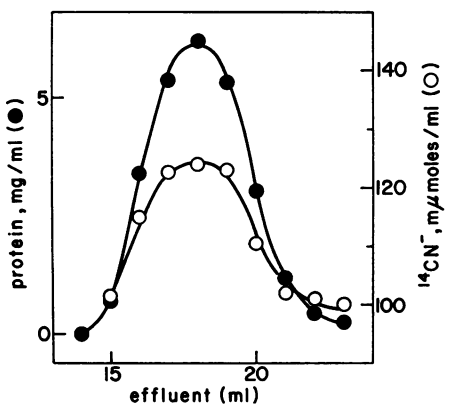


FIG. 3.—Binding of cyanide by MoFd. The equilibrating buffer for the Sephadex column (see *Methods*) contained 0.1 mM $^{14}CN^-$ (at this concentration of cyanide, the ratio of cyanide bound per MoFd was 0.6).

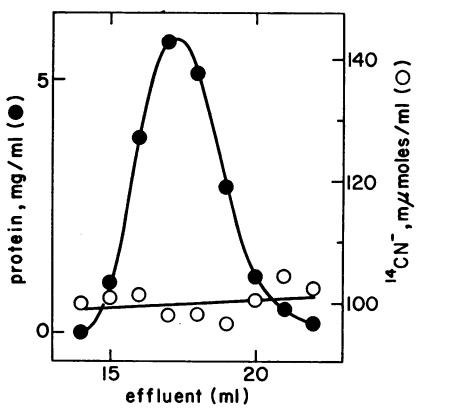


FIG. 4.—Lack of binding of cyanide by AzoFd. The equilibrating buffer for the Sephadex column (see *Methods*) contained 0.1 mM $^{14}CN^-$. Identical results were obtained when 4.0 mM dithionite, 2.0 mM ATP, and 2.0 mM $MgCl_2$ were also added to the buffer.

tants in N_2 reduction, singly or in any combination (the binding of cyanide by MoFd in the presence of AzoFd, ATP, Mg^{2+}, and dithionite was not tested since cyanide is converted to methane and ammonia under these conditions). The binding was reversible since no radioactivity could be detected with MoFd which had been incubated with $^{14}CN^-$ and then passed through Sephadex G-50 to separate unbound $^{14}CN^-$. Efforts to detect N_2 binding by MoFd, using anaerobic spectrophotometric techniques, are in progress, and will be reported elsewhere.[9]

Binding of ATP and ADP to AzoFd: Since ATP and ADP are chemically similar, it is reasonable to expect that they might bind to the same site on the N_2-reducing system. This site must be on AzoFd since AzoFd binds both ATP (Fig. 5) and ADP (Fig. 6), whereas MoFd binds neither. Like the binding of

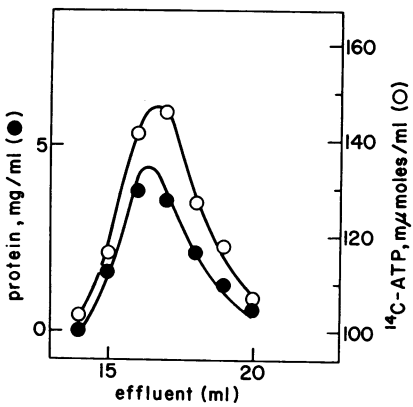


FIG. 5.—Binding of ATP to AzoFd. The equilibrating buffer for the Sephadex column (see *Methods*) contained 0.1 mM ^{14}C-ATP, and 0.5 mM $MgCl_2$. No binding could be detected in the absence of added $MgCl_2$.

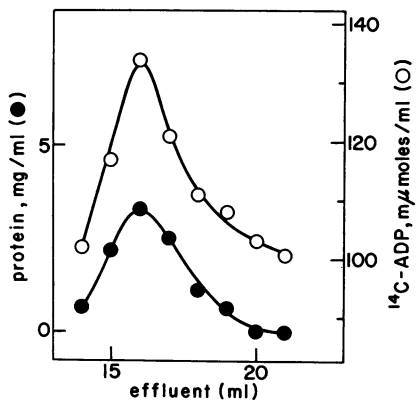


FIG. 6.—Binding of ADP to AzoFd. The equilibrating buffer for the Sephadex column (see *Methods*) contained 0.1 mM ^{14}C-ADP.

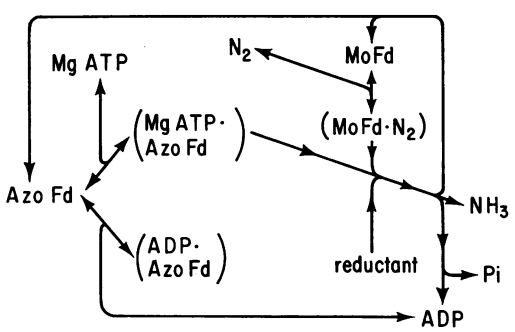
ATP, the binding of ADP by AzoFd was reversible, but unlike the binding of ATP, the binding of ADP did not require added Mg^{2+}. Calculations with the data available indicate that under the experimental conditions described, AzoFd binds both ATP and ADP to the same extent (0.4 ATP or ADP per AzoFd). The low ratio of ATP (or ADP) bound per AzoFd is a consequence of the necessity (see *Methods*) of using a low ATP concentration (5% of the optimal concentration for N_2 reduction[5]) in binding experiments. In addition, if both ATP and ADP bind at the same site, they should compete in binding to AzoFd. In fact, the amount of ^{14}C-ADP bound per AzoFd decreased to less than 15 per cent when the equilibrating buffer contained either 1.0 mM ^{12}C-ATP or 1.0 mM ^{12}C-ADP in addition to the 0.1 mM ^{14}C-ADP. In contrast, 1.0 mM AMP had no effect on the binding of 0.1 mM ADP, which suggests that AzoFd either does not bind AMP or does not bind AMP at the ADP (or ATP) site.

Binding of ADP to the N_2-reducing system: The binding of ATP by the N_2-

reducing system was not examined since any interpretation would be complicated by a simultaneous conversion of ATP to ADP and P_i. However, the binding of ADP by the N_2-reducing system can be shown (Fig. 7) since ADP is not metabolized by the system. When the binding of ADP by AzoFd was compared to the binding by the combination of AzoFd and MoFd (the N_2-reducing system), it was clear that MoFd did not affect the quantity of ADP bound per AzoFd although it retarded the dissociation of the (ADP·AzoFd) complex. This sluggish dissociation accounts for the ADP curve's being skewed toward the back of the protein curve (Fig. 7).

Discussion.—Significant binding of ADP by the N_2-reducing system is predicted on the basis that (1) the system is responsible for at least 80 per cent of the ATP breakdown catalyzed by N_2-reducing clostridial extracts;[1] (2) this ATP breakdown is inhibited by ADP;[5] and (3) the system has the same number of binding sites (Fig. 2) and very nearly the same affinity for ATP and ADP (Figs. 5 and 6). It is therefore unlikely that a protein contaminant not involved in N_2 reduction could account for *all* of the ADP binding reported above since AzoFd bound the same amount of ADP, whether tested alone or as part of the N_2-reducing system. Furthermore, the fact that MoFd did not bind ADP and yet greatly affected the reversal of ADP binding by the AzoFd fraction (Fig. 7) suggests that a protein capable of interacting with MoFd, e.g. AzoFd, was responsible for the binding observed.

In summary, the use of available information on the binding reactions described above permits the construction of a more detailed picture of the paths of all but one of the reactants in the N_2-reducing system:



As shown in the diagram, AzoFd binds MgATP reversibly to form the potentially reactive complex (MgATP·AzoFd). The bond(s) between substrate and protein in this complex is presently under investigation. The Mg^{2+} requirement for N_2 reduction is explained by the inability of AzoFd to bind ATP in the absence of added Mg^{2+}. MoFd is responsible for the reversible binding of the electron acceptor (N_2 or CN^-) to form the complex (MoFd·N_2). Again, the nature of the bond(s) between substrate and protein is not known. The necessity of having two specific sites, one for the binding of MgATP and the other for the binding of the electron acceptor (N_2 or CN^-), cannot be the whole explanation for the requirement for two proteins in N_2 reduction since MoFd does not bind ATP and yet is required for the conversion of AzoFd-bound ATP to

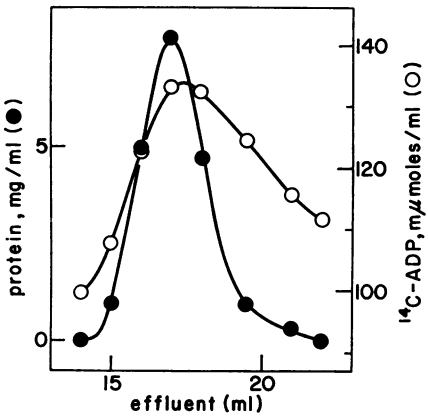


FIG. 7.—Binding of ADP to AzoFd plus MoFd. The equilibrating buffer for the Sephadex column (see *Methods*) contained 0.1 mM ^{14}C-ADP, 0.5 mM $MgCl_2$, and 4.0 mM dithionite. AzoFd and MoFd were combined in a 1.0:1.3 weight ratio. Identical results were obtained when AzoFd and MoFd were combined in a 1.0:4.0 weight ratio.

ADP and inorganic phosphate. The mechanism by which electrons are accepted by the N_2-reducing system and used to reduce N_2 is unknown, but the fact that no requirement for dithionite could be demonstrated for the binding of either Mg-ATP or cyanide suggests that the point of entry for the electrons may be at the level of (1) (MgATP·AzoFd), (2) (MoFd·N_2), or (3) the combination of these two complexes, rather than at the level of AzoFd or MoFd. One of the products of ATP metabolism by the N_2-reducing system, ADP, is capable of preventing N_2 reduction by acting as an inert ATP analog, i.e. reversibly forming the unreactive complex (ADP·AzoFd), thus effectively removing AzoFd which otherwise would react with MgATP. The lack of inhibition of cyanide reduction by several other nucleoside phosphates is probably a consequence of their inability to bind to the ATP site. This is supported by the fact that AMP, which was incapable of competing with ADP for the ATP binding site, is also inactive in inhibiting acetylene reduction,[6] ATP-dependent H_2 evolution,[7] reductant-dependent ATP hydrolysis,[7] and cyanide reduction (Table 1).

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Abbreviations: ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-phosphate; ADP, CDP, GDP, IDP, and UDP, 5′-diphosphates of adenosine, cytidine, guanosine, inosine, and uridine; P_i, inorganic orthophosphate.

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