ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by F. F. NORD

Edited by ALTON MEISTER

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INTERACTIONS OF DINITROGENASE AND DINITROGENASE REDUCTASE

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I. Introduction

Nitrogenase is a term that has been applied for many years to the enzyme system capable of fixing N_2, that is, reducing N_2 to 2 NH_3. When studies of the system were confined to intact organisms, it was presumed that nitrogenase was a single protein that conformed to the usual pattern of known enzymes. With the development of methods for preparing cell-free nitrogenase (1), efforts were made to purify the enzyme and to define its properties and mode of action more completely. It soon was shown (2) that the nitrogenase system consisted of two proteins rather than one and that these proteins could be separated (3). One of the proteins contained Mo and Fe, and the other was an Fe protein.

The nomenclature of the nitrogenase system has been varied and confused. We will retain the term nitrogenase to designate the complex of the two proteins and the term nitrogenase system for the complete system

including the two proteins plus reductant and MgATP. The MoFe protein will be called dinitrogenase* and the Fe protein dinitrogenase reductase.* The rationale for the nomenclature will be clarified by the discussion, but basically it rests on the observation that dinitrogenase binds and reduces N_2, hence it should be considered as the active enzyme in N_2 fixation. The reason two proteins must be present is that only the Fe protein appears able to reduce dinitrogenase, hence the name dinitrogenase reductase. The nomenclature seems rational, as it is based on the function of the nitrogenase components.

Although active cell-free preparations had been obtained from time to time (4), the first consistent cell-free preparations were recovered from the anaerobic bacterium *Clostridium pasteurianum* (1). Nitrogenase in a crude extract from dried cells was supported by pyruvate as a source of energy and reductant. The pyruvate was metabolized rapidly with the production of H_2, CO_2, and acetyl phosphate. Attempts to purify the enzyme led to the demonstration that two proteins were present and necessary (3). When it became apparent that the nitrogenase system worked only when the two protein components were together, it was natural to assume that they combined and functioned as a unit. Although initial observations had indicated that ATP was inhibitory to nitrogenase, it soon was shown (5) that MgATP in fact was an obligatory substrate for the system. It also was shown that ferredoxin and flavodoxin were common reductants for the nitrogenase system.

Nitrogenase functioned effectively in crude, unseparated extracts when supplied a suitable source of energy. When two proteins were separated, neither showed any activity. It was necessary to recombine them, and the mixture was commonly designated the nitrogenase complex, although no evidence for a persistent complex existed.

When separation techniques were perfected, it was apparent that the two proteins from *C. pasteurianum* were eluted from gel filtration chromatographic columns as symmetrical peaks, an indication that any complex between them must be a loose complex. Despite this evidence and the demonstration (6) that a system reconstructed from previously separated components regained full activity when supplied substrate and cofactors,

*Synonyms in the literature for dinitrogenase are MoFe protein, protein I, component I, molybdoferredoxin, azofermo, and for dinitrogenase reductase they are Fe protein, protein II, component II, azoferredoxin and azofer. For a shorthand designation we will use Av1 and Av2 to indicate *Azotobacter vinelandii* dinitrogenase and dinitrogenase reductase, respectively; Cp1 and Cp2 will be used for *Clostridium pasteurianum* components.

the concept of a relatively tight complex persisted. This was reflected in the position held by Bulen and LeComte (7) that the nitrogenase system should be studied as a crude preparation lodged on cellular fragments. This presumably was more representative of the naturally occurring nitrogenase system than the purified and reconstructed systems with some 20-fold greater specific activity.

Although the school of workers who employed reconstituted systems produced no evidence that nitrogenase components formed a persistent complex, they habitually referred to the nitrogenase cor plex. The persistent nature of the complex was seldom stated, but it often was implied in interpretation of results.

Information on the function of dinitrogenase and dinitrogenase reductase emerged clearly with the application of EPR techniques almost simultaneously in three laboratories (8-10). Both dinitrogenase and dinitrogenase reductase have characteristic EPR spectra, and the changes in the signals induced by MgATP and by oxidation and reduction have been very helpful in defining the path of electron transfer in the systems.

It was established that when MgATP was added to dinitrogenase reductase at pH 8, the EPR spectrum of the protein was altered; MgATP does not change the EPR signal from dinitrogenase (8). This information suggested that dinitrogenase reductase rather than dinitrogenase bound MgATP, and other techniques (11,12) showed that this was in fact true. The binding of MgATP is accompanied by a lowering of the oxidation-reduction potential of dinitrogenase reductase from around -300 to around -400 mv.

The EPR signal of dinitrogenase is altered drastically as it goes from the oxidized state, in which form it is isolated under anaerobic conditions, to the reduced (sometimes described as the superreduced) state. When reductant is exhausted, the dinitrogenase returns to the oxidized state as evidenced by reappearance of the characteristic EPR signal (8). Putting these observations together supported the scheme of electron transfer indicated in Figure 1.

As indicated in Figure 1, MgATP binds specifically to dinitrogenase reductase in the reduced (12) or oxidized state (13). The dinitrogenase reductase then is reduced by ferredoxin or flavodoxin or by $Na_2S_2O_4$ and acquires the ability to reduce dinitrogenase. The dinitrogenase reductase serves as a one-electron carrier to dinitrogenase. The two components of nitrogenase combine momentarily to transfer an electron, and MgATP hydrolysis accompanies this electron transfer. The best evidence suggests that 2 MgATP are hydrolyzed with each electron transferred. As all re-

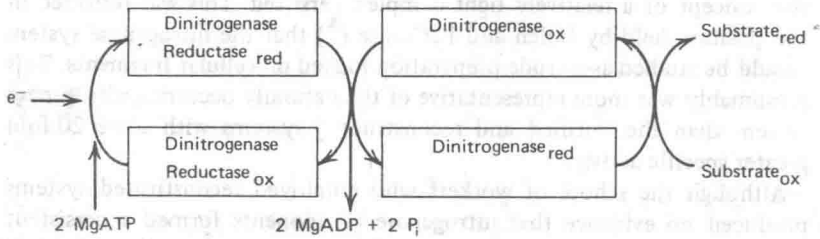

Fig. 1. Electron transfer in the nitrogenase system.

actions of nitrogenase require the transfer of at least two electrons, it is evident that dinitrogenase reductase must react more than once with each dinitrogenase molecule to charge it with two or more electrons. The charging process appears to be at random, and there is no evidence that electrons can be passed among molecules of dinitrogenase.

Once charged, the dinitrogenase can bind and reduce substrates without the direct involvement of dinitrogenase reductase. It is because of the ability of free dinitrogenase to bind and reduce substrates that we believe it should be designated as the active enzymatic unit of the nitrogenase system by referring to it as dinitrogenase.

The EPR and other studies established that the electron transfer was from ferredoxin (or other reductant) to dinitrogenase reductase (which specifically bound two MgATP per molecule) to dinitrogenase to substrate. Nothing in this scheme required that the dinitrogenase reductase and dinitrogenase form a persistent complex during electron transfer. Nevertheless, common reference to the nitrogenase complex implied that the two proteins stayed together for some time. The literature even produced the term "Azotophore" to designate the complex as isolated on a particle.

At the Pullman International Symposium on Nitrogen Fixation in 1974, Burris and Orme-Johnson (14) quoted the statement of Ljones (15), "A possible mechanism for the oxidation of excess Fe protein involves dissociation of the active complex between the two proteins after each cycle of electron transfer is completed. . . . It is not known whether the two nitrogenase proteins in the steady state remain together in a complex during repeated catalytic cycles or shuttle between associated and dissociated forms, but if the presented mechanism is correct, the comparison of rates in the steady state and during oxidation of excess Fe protein suggests that formation and dissociation of the active complex of the two proteins are as fast as or faster than electron transfer. . . . Interaction among the

components can be considered as an enzyme-substrate reaction with Mo-Fe protein being the enzyme and reduced Fe protein the substrate. Formation of a complex between oxidized Fe protein and Mo-Fe protein is then analogous to product inhibition." Burris and Orme-Johnson (14) also described experiments in which dinitrogenase was reduced enzymatically, was rapidly separated from dinitrogenase reductase on a DEAE cellulose column, and then was exposed immediately to C_2H_2. Although small amounts of C_2H_4 were formed, the experiments were not definitive because traces of dinitrogenase reductase remained in the separated dinitrogenase, and because ubiquitous H^+ always was present to serve as a substrate during the separation and before C_2H_2 could be added.

Based on the conviction that the two proteins of nitrogenase associated and dissociated with each electron transfer, we suggested (16,17) that the proteins be designated nitrogenase and nitrogenase reductase, but such terminology was not acceptable to the International Commission on Biochemical Nomenclature. (We now prefer the designations dinitrogenase and dinitrogenase reductase so that the term nitrogenase can be retained in its accepted sense as the combination of the two proteins.) Subsequently (18), data on the tight-binding complex between nitrogenase proteins have been analyzed in terms of dinitrogenase reductase binding to and serving as the substrate for dinitrogenase. Hageman and Burris (17) used the terminology nitrogenase and nitrogenase reductase and presented experimental evidence indicating that in fact the two proteins do associate and dissociate at each turn of the catalytic cycle. Although this concept is not accepted universally, it appears to have gathered support recently, while support for the concept of a persistent nitrogenase complex has decreased.

II. Dinitrogenase and Its Association with Dinitrogenase Reductase

A. HOMOLOGY AMONG NITROGENASE COMPONENTS

The complete nitrogenase system is required for substrate-reducing activity (19,20) since neither dinitrogenase nor dinitrogenase reductase has activity by itself. This absolute requirement for both proteins prompted the question whether the component proteins from different sources could be mixed to generate an active hybrid complex.

Detroy et al. (21) interchanged the component proteins from *Clostridium pasteurianum, Azotobacter vinelandii, Klebsiella pneumoniae,* and *Bacillus polymyxa* in the 16 possible combinations of the two proteins. Four crosses recombined homologous components. Six heterologous

crosses yielded active hybrids and six were inactive. The crosses between proteins from closely related organisms actively reduced N_2, whereas crosses formed between more diverse organisms did not. These data implied that the nitrogenase protein components from organisms with similar evolutionary development could form an active hybrid complex with a complementary heterologous protein. Organisms more remote in evolutionary development synthesized proteins structurally distinct and unable to form an active nitrogenase complex.

Dahlen et al. (22) confirmed that recombined fractions from different organisms are capable of forming a catalytically active system and that these combinations apparently are restricted to organisms with similar physiological properties. They crossed components and tested their activity with the alternative substrates acetylene, azide, and cyanide. They demonstrated that N_2 reduction and alternative substrate reduction were performed by the same enzyme system.

Further reports on cross reactions followed promptly. Kelly (23) extended these studies to include ATP-hydrolyzing activity, and the range of organisms was expanded to include the photosynthetic bacteria (24,25), the microaerophilic bacteria (25), the blue-green algae (24), and the symbiont from soybean root nodules (26). The early use of cross reactions to investigate the interactions of the components was of limited value because pure, homogeneous proteins of high activity were not available. The primary applications of cross reactions were (*a*) to demonstrate the relatedness of the component proteins from different sources, and (*b*) to measure the activity of components obtained in low yield, by supplementing them with a complementary, heterologous component that could be obtained in greater quantity.

Since highly purified, active component proteins have become available, cross reactions have been used mainly to corroborate experimental results obtained first with homologous components. Thorneley et al. (27) showed that the complementary proteins from *Klebsiella pneumoniae* and *Azotobacter chroococcum* formed a complex in the absence of MgATP and sodium dithionite that could be analyzed by sedimentation velocity. A 1:1 complex was observed between *A. chroococcum* dinitrogenase + *K. pneumoniae* dinitrogenase reductase and *K. pneumoniae* dinitrogenase + *A. chroococcum* dinitrogenase reductase. These complexes dissociated in the presence of sodium dithionite, a response similar to that of the homologous complexes (27,28).

Smith et al. (29) investigated the heterologous combination of the dinitrogenase from *K. pneumoniae* and dinitrogenase reductase from *C.*

pasteurianum. Stopped-flow spectrophotometric studies revealed that electron transfer from *C. pasteurianum* dinitrogenase reductase to *K. pneumoniae* dinitrogenase occurred at the same rate as with the homologous proteins from *K. pneumoniae*. The electron transfer reaction displayed the same dependence on ATP concentration, but ATP hydrolysis was uncoupled from substrate reduction.

Recently, Emerich and Burris (30) reported a survey of the cross reactions between dinitrogenase and dinitrogenase reductase from eight different organisms. The survey was performed with highly purified proteins, and the proteins were titrated against each other to give the maximal activity possible under a specific set of assay conditions (Table I). Of the 64 possible combinations of protein components, eight are homologous combinations. Of the 56 possible nonhomologous crosses, 55 were tested and 45 produced measurable substrate reduction. Among these active heterologous crosses, activity ranged from 100% down to 1%. When the nitrogenase activity was low, the preparations frequently hydrolyzed considerable amounts of ATP, that is, the ATP hydrolysis was poorly coupled to the substrate-reducing reaction. This indicates that ATP hydrolysis and substrate reduction are not coupled in an obligatory manner.

Of the combinations that yielded no measurable activity, at least four generated inactive complexes that inhibited all the characteristic activities of the active complex (18, Section II.C). So the similarity between nitrogenase protein components extends even to those combinations of components that have no detectable substrate-reducing activity. Thus 49 of the 55 heterologous crosses assayed formed active or inactive complexes; the components clearly were able to bind to one another.

The high percentage of cross reactivity implies that nitrogenase proteins have originated from the same evolutionary stem and have retained many common properties during evolutionary development. The major divergence has been observed with *C. pasteurianum* protein components. When 0% activity was recorded, it always involved a cross with one of the proteins from *C. pasteurianum*. *C. pasteurianum* gave the lowest number of active crosses, whereas *K. pneumoniae* and *B. polymyxa* gave active crosses with components from all other organisms.

B. THE QUESTION OF ASSOCIATION–DISSOCIATION

The nature of the active complex is manifest in the association–dissociation behavior of dinitrogenase and dinitrogenase reductase. The rate of association between component proteins is rapid, less than 10 msec (10,31,32), but the dissociation rate has not been measured. There are con-

TABLE I

Cross Reactions among Nitrogenase Components from Various Organisms[a]

Av = *Azotobacter vinelandii*, Kp = *Klebsiella pneumoniae*, Rr = *Rhodospirillum rubrum*, Sl = *Spirillum lipoferum*, Cv = *Chromatium vinosum*, Rj = *Rhizobium japonicum*, Bp = *Bacillus polymyxa*, and Cp = *Clostridium pasteurianum*.

Source of dinitrogenase reductase	Source of dinitrogenase: Av	Kp	Rr	Sl	Cv	Rj	Bp	Cp
Av	+	100% 1,2	⩾67% 1	⩾25% 1	⩾30% 1	=100%* 1	⩾61% 1	0 1,2
Kp	⩾93% 1,2	+	⩾4% 1	⩾40% 1	⩾23% 1	100%* 1	⩾57% 1	⩾8% 1,2
Rr	⩾91% 2	⩾98% 2	+	⩾26% 2	⩾77% 2	⩾50%* 2	⩾47% 2	0 1,2
Sl	⩾60%+ 2	=100%+ 2	~100% 2	+	⩾60%+ 2	⩾5% 2	⩾4% 1	0 2
Cv	=100%++ 2	⩾27%++ 2	⩾50%++ 2	⩾5%++ 2	+	N.D.	⩾1% 2	0 2
Rj	⩾88% 2	⩾88% 2	⩾85% 2	⩾24% 2	⩾28% 2	+	⩾71% 2	0 2
Bp	19% 1	⩾66% 1	⩾28% 1	⩾18%** 1	⩾5% 1	~100%* 1	+	⩾38% 1
Cp	0 1,2	⩾17% 1	0 1	0 1	0 1	0 1	⩾34% 1	+

[a]Abbreviations are as follows: + , homologous cross reaction; N.D., not determined; 1, activity (expressed as % activity in all cases) of this cross reaction was determined by titrating a constant amount of dinitrogenase with varying amounts of dinitrogenase reductase (the activity of this cross is compared to the homologous nitrogenase system of the dinitrogenase); 2, activity of this cross reaction was determined by titrating a constant amount of dinitrogenase reductase with varying amounts of dinitrogenase (the activity of this cross is compared to the homologous nitrogenase system of the dinitrogenase reductase); *, activity compared with the Rj1 × Av2 cross reaction; **, activity compared with the Sl1 × Av2 cross reaction; +, activity compared with the Kp1 × Sl2 cross reaction; ++, activity compared with the Av1 × Cv2 cross reaction.

flicting reports on the lifetime of the active complex. Thorneley (31) has reported evidence for the existence of a tight complex between the components from *K. pneumoniae*, but Hageman and Burris (17,33) have presented substantial kinetic evidence for a transient complex functioning between components of *A. vinelandii* nitrogenase. Further evidence con-

cerning the lifetime and composition of the catalytically active complex will be presented in Section III.C.

1. Dilution Effects

The question of the association-dissociation behavior of dinitrogenase-dinitrogenase reductase was first examined in reports on the dilution effect (27,34-44). The dilution effect refers to the disproportionately low specific activity found when the ratio of dinitrogenase to dinitrogenase reductase is kept constant but their total concentration is decreased. The nature of the dilution effect was not addressed until Sorger (42) and Shah et al. (43) reported that the effect could be overcome by additions of dinitrogenase or dinitrogenase reductase. Sorger (42) indicated that additions of dinitrogenase reductase to partially purified extracts of *A. vinelandii* restored the activity; dinitrogenase additions slightly enhanced the activity. Shah et al. (43) presented evidence that the dilution effect observed in *A. vinelandii* extracts could be overcome by adding either dinitrogenase or dinitrogenase reductase. The enhancement by dinitrogenase was concentration dependent, as an excess of dinitrogenase produced inhibition. Yates (41) found that additions of proteins other than dinitrogenase or dinitrogenase reductase also eliminated the dilution effect, and concluded that these various proteins prevented dissociation of the catalytic complex.

Thorneley et al. (27) analyzed the dilution effect in terms of dissociation of an active 1:1 dinitrogenase:dinitrogenase reductase complex. Their data, obtained with the component proteins from *K. pneumoniae, A. chroococcum,* and the heterologous crosses between these two organisms, agreed well with the theoretical results predicted from their model. The kinetic studies of Silverstein and Bulen (44) showed that dinitrogenase and dinitrogenase reductase were in dynamic equilibrium between associated components and nonassociated components. Dinitrogenase and dinitrogenase reductase are freely dissociable from the associated complex, and the degree of association in a dynamic equilibrium is concentration dependent. Therefore, the dilution effect is evident below a critical concentration of the component proteins, determined by the association constant, because the level of associated components decreases more rapidly than the total protein concentration.

An additional consideration regarding the nature of the dilution effect is the dissociation of dinitrogenase and dinitrogenase reductase into their respective subunits at low protein concentration. Huang et al. (45) reported that the sedimentation coefficient of dinitrogenase from *C.*

pasteurianum decreased at protein concentrations below 5 mg/ml, and suggested that this indicated the dissociation of dinitrogenase into its subunits. Dinitrogenase from *A. chroococcum* dissociates into subunits at protein concentrations below 3 mg/ml (46). Dissociation of dinitrogenase into subunits may account in part for the dilution effect if the subunits are not catalytically active.

2. *Titration with Excess Dinitrogenase; Inhibition and Combining Ratio*

The ratio of dinitrogenase to dinitrogenase reductase in the active electron-transferring nitrogenase complex has been estimated most frequently by measuring activity after mixing or titrating various amounts of one protein component against a constant amount of the complementary component. Ratios of both 1:1 and 1:2 for the dinitrogenase:dinitrogenase reductase complex have been reported. The titration curves for N_2 reduction, C_2H_2 reduction, and H_2 evolution all have the same shape (47). Addition of a constant amount of dinitrogenase to various amounts of dinitrogenase reductase produces a hyperbolic saturation curve. Holding dinitrogenase reductase constant and varying the level of dinitrogenase yields a curve that increases with increasing dinitrogenase concentration to a maximum that is followed by decreasing activity (43,47,50). Excess dinitrogenase inhibits substrate reduction, but it apparently does not inhibit ATP hydrolysis (Sections III.A and III.B). Generally it has been believed that inhibition of activity of excess dinitrogenase results from formation of inactive or less active complexes between dinitrogenase and the active dinitrogenase-dinitrogenase reductase complex (see Section III.B). Ljones and Burris (51) proposed that at least two types of complexes could be formed: (*a*) at optimal ratios of dinitrogenase and dinitrogenase reductase, complexes are formed catalyzing ATP hydrolysis coupled to electron transfer, and (*b*) at high ratios of dinitrogenase to dinitrogenase reductase, complexes are formed that are relatively inefficient and catalyze uncoupled ATP hydrolysis. Stopped-flow kinetic analysis revealed the rate of electron transfer from dinitrogenase reductase to dinitrogenase was not inhibited by high ratios of dinitrogenase to dinitrogenase reductase (31). Thorneley et al. (52) observed that sodium dithionite reduced free, uncomplexed dinitrogenase reductase more rapidly than reduced dinitrogenase reductase is complexed and oxidized by dinitrogenase.

Hageman and Burris (33) reported that increasing the ratios of dinitrogenase to dinitrogenase reductase increases the K_m for dithionite. Thus

inhibition by excess dinitrogenase results in part from nonsaturation of the system with sodium dithionite at high dinitrogenase levels. The inhibition can be overcome by employing a better reductant (33). Hageman and Burris (33) have shown that at high concentrations of dinitrogenase relative to dinitrogenase reductase, dinitrogenase reductase transfers electrons randomly to dinitrogenase molecules. The nitrogenase complex dissociates after each electron transfer. When the steady-state level of reduced dinitrogenase decreases, substrate-reducing activity is reduced, but disproportionately.

3. *Ultracentrifugal Studies of Complex Formation*

Complex formation during catalysis has not been observed directly. Eady (28) and Thorneley et al. (27) cited evidence for complex formation between dinitrogenase and dinitrogenase reductase under conditions that did not support substrate reduction. Sedimentation coefficients of the component proteins were measured in an ultracentrifuge, both individually and mixed in various ratios, plus and minus ATP and sodium dithionite. Dinitrogenase:dinitrogenase reductase ratios of 1:1 yielded a single sedimentation peak with a sedimentation coefficient greater than either protein alone. Ratios of dinitrogenase reductase to dinitrogenase larger than 1:1 produced an extra peak corresponding to uncomplexed dinitrogenase reductase. Oxygen-inactivated proteins did not form a complex. Sodium dithionite, a functional *in vitro* reductant, prevented complex formation. The observed complexes were not catalytically functional and may not be relevant to the complexes present during enzyme turnover.

C. TIGHT-BINDING COMPLEXES*

Tight-binding, heterologous complexes have helped to reveal the nature of the active complex and the role of association-dissociation of the complex during catalysis. Several heterologous combinations of components forming tight-binding, inactive complexes have been reported (18). The tight-binding complex formed between dinitrogenase from *Azotobacter vinelandii* (Av1) and dinitrogenase reductase from *Clostridium pasteurianum* (Cp2) has been partially characterized (18).

Av1 inhibits N_2 reduction, C_2H_2 reduction, H^+ reduction, and ATP hydrolysis by the dinitrogenase-dinitrogenase reductase complex from *C. pasteurianum* (Cp1 + Cp2). In reciprocal fashion, Cp2 inhibits these char-

*In this section the following nomenclature is used: Av = *Azotobacter vinelandii*, Cp = *Clostridium pasteurianum*, 1 = dinitrogenase, and 2 = dinitrogenase reductase.

acteristic activities of Av1 + Av2 (Fig. 2). These inhibitions are specific as Av2 will not inhibit Cp1 + Cp2, and Cp1 will not inhibit Av1 + Av2. The inhibition requires active components, as neither oxygen-inactivated Av1 nor Cp2 nor cold-inactivated Cp2 can suppress the activity of the homologous components. All evidence indicates that Av1 and Cp2 bind together tightly to inhibit substrate reduction and ATP hydrolysis. Binding between heterologous components does not guarantee enzyme activity; apparently they must dissociate to be functional.

The inhibition patterns caused by Av1 and Cp2 are those expected of tight-binding inhibitors (53,54). Titration experiments and kinetic analyses show two molecules of Cp2 bind to one molecule of Av1. A 1:1 ratio of Cp2:Av1 is not inhibitory or is weakly inhibitory, whereas a 2:1 ratio is strongly inhibitory. Evidently, Av1 has two binding sites for Av2, but only one needs to be occupied to catalyze virtually full activity. The inhibition constants calculated from measurements with different substrates are in the range of 1-10 n*M*. The low dissociation constant for the Av1-Cp2 complex, the inhibition induced mostly by the second molecule of Cp2, and the rapidity of the interaction between Av1 and Cp2 indicate that these heterologous components interact as fast as homologous protein components.

The tight-binding between Av1 and Cp2 permits isolation of the inactive complex by gel filtration (55). This method illustrates the tight-binding nature of the complex, but does not establish the stoichiometry. It does

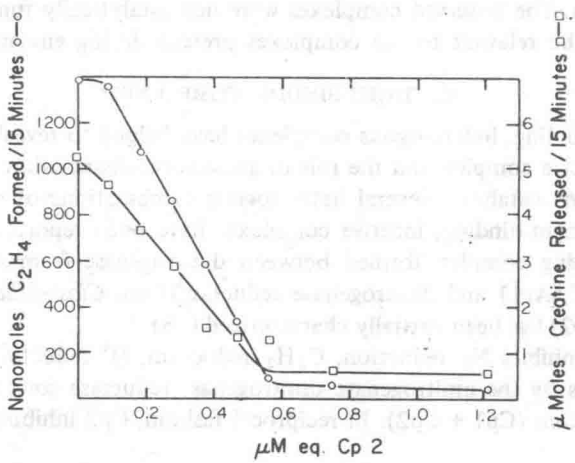

Fig. 2. Inhibition of C_2H_2 reduction and ATP hydrolysis during Cp2 inhibition of Av1 + Av2. All assays contained 0.22 μ*M* eq. of Av1 and 0.68 μ*M* eq. of Av2 and Cp2 as indicated.