Biochimica et Biophysica Acta, 527 (1978) 359—369

BBA 68606

NITROGENASE: PROPERTIES OF THE CATALYTICALLY INACTIVE COMPLEX BETWEEN THE *AZOTOBACTER VINELANDII* MoFe PROTEIN AND THE *CLOSTRIDIUM PASTEURIANUM* Fe PROTEIN

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(Received April 27th, 1978)
(Revised manuscript received July 21st, 1978)

Summary

The catalytically inactive complex generated by the combination of the *Azotobacter vinelandii* MoFe protein (Av1) and the *Clostridium pasteurianum* Fe protein (Cp2) inhibits N_2 reduction, C_2H_2 reduction, H^+ reduction and ATP hydrolysis catalyzed by the homologous nitrogenases. Kinetic data indicate that the inactive complex consists of two molecules of Cp2 to one molecule of Av1, with values for the inhibitor constant in the range of 1—10 nM. Inhibition of *C. pasteurianum* nitrogenase by Av1 produces a lag phase in acetylene reduction that increases with increasing Av1. The lag phase is found only at levels of Av1 sufficient to keep the ratio of Cp2 : Cp1 lower than 2. Gel filtration of a mixture of Av1 and Cp2 provides evidence for complex formation and indicates that each Av1 molecule binds more than one Cp2 molecule. The Av1-Cp2 complex binds two molecules of MgATP per molecule of Cp2. MgATP is not required for complex formation, but complex formation lowers the MgATP-Cp2 dissociation constant approx. 3-fold. Av1 protects the iron-sulfur center in Cp2 completely against the MgATP-induced reaction with chelators. This provides additional evidence for formation of the Av1-Cp2 complex and together with the results of the MgATP-binding studies suggests that the two binding sites for MgATP are some distance away from the iron-sulfur site on Cp2.

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Abbreviation: MES, 4-morpholineethansulfonic acid.

Introduction

Nitrogenase is the enzyme complex that catalyzes the reduction of N_2 to NH_4^+. Nitrogenase has been purified from a wide variety of organisms [1] and consists of two proteins; one containing molybdenum, non-heme iron, and acid-labile sulfur (the MoFe protein) and the other non-heme iron and acid-labile sulfur (the Fe protein). Both proteins are required for catalytic activity, as neither protein alone can perform any of the characteristic reactions of nitrogenase. Evidence of complex formation has been obtained from studies on the 'dilution effect' [2], sedimentation velocity [2,3], stopped-flow kinetic experiments [4], and activity titration curves [5—9]. Disagreement exists over the stoichiometry of the complex; complexes consisting of Fe protein : MoFe protein molar ratios of both 1 and 2 have been reported. Recently, the heterologous combination of the *Azotobacter vinelandii* MoFe protein (Av1 *) and *Clostridium pasteurianum* Fe protein (Cp2 *) was found to generate a tight-binding catalytically inactive complex [10]. The inactive complex consists of 2 Cp2 : 1 Av1. A 1 : 1 molar equivalent ratio of Av1 and Cp2 inhibited the homologous nitrogenase very weakly. This implied that although the MoFe protein has two binding sites for the Fe protein, a 1 : 1 molar equivalent ratio of MoFe protein to Fe protein produced near maximal activity. This could explain the difference in the values reported for the composition of the nitrogenase complex. In this report we describe some other properties of this complex.

Materials and Methods

Assay mixtures for the measurement of nitrogenase activity (C_2H_2 and N_2 reduction) were prepared as described previously [10]. Samples for ethylene formation were withdrawn from the reaction bottles, 5 and 10 min after initiation of the assay, in 1-ml plastic syringes fitted with 27-gauge needles. Assays were terminated after 15 min by adding 1 ml of a saturated K_2CO_3 solution. When reactions were run longer than 15 min, gas samples were taken every 5 min. Ethylene was analyzed on a Varian 600D gas chromatograph equipped with a flame ionization detector and a column of Porapak R, 150 cm long and 2 mm internal diameter, at 50°C with N_2 as the carrier gas. 0.5-ml gas samples were used for analysis. Ammonia production was determined by the method of Chaykin [11] after microdiffusion [12]. Assays of nitrogenase activity were carried out at 30°C unless otherwise stated. H_2 evolution was measured as described by Wang et al. [13]. Creatine release, as an index of ATP hydrolysis activity, was measured by the method of Eggleton et al. [14]. The gel equilibration method of Tso and Burris [15] was used to measure the binding of ATP to the nitrogenase proteins. The ATP-binding mixture contained 50 mg Sephadex G-50, 2 mg creatine phosphate, 0.05 mg creatine kinase, 0.25 mM $Na_2S_2O_4$,

* The terminology of Thorneley and Eady [22] is employed; the MoFe protein is designated as 1 and the Fe protein as 2, the genus and species are designated by two letters, e.g. Av = *Azotobacter vinelandii* and Cp = *Clostridium pasteurianum*.

20 mM MES-KOH buffer, pH 6.6, [^{14}C]ATP as indicated, and $MgCl_2$ 1 mM in excess of the ATP concentration, in a total volume of 0.60 ml. Binding mixtures were incubated at 25°C for 10 min on a rotating mixer. The stock ATP (8[^{14}C]ATP, 4 sodium salt; purchased from New England Nuclear) had a specific radioactivity of 20 μCi/ml (0.26 mg ATP/ml in ethanol/water, 1 : 1, v/v). Samples (50 μl) were withdrawn from the supernatant in the vials in which ATP binding was assayed and were placed in 16 ml of Aquasol (New England Nuclear) and counted for 10 min in a Beckman model LS-100C liquid scintillation counter. Anaerobic gel filtration and calibration of the Sephadex G-200 column (2.5 cm diameter X 79 cm) were performed as described by Tso [16]. Elution profiles were monitored at 280 nm with an ISCO Model UA-5 absorbance monitor. The column effluent was concentrated under argon in a 50 ml Dialfo ultrafiltration cell with a PM-10 membrane (Amicon Corp.). Analytical polyacrylamide gel electrophoresis was performed by the method of Davis [17]. EPR spectra were recorded on a Varian E-line spectrometer at 13 K. The μmolar equivalent (μM equiv.) concentration, as defined previously [10] is the μmolar concentration of each component protein corrected for the specific activity of that component, assuming the specific activity of completely pure, active nitrogenase components is 3000 nmol C_2H_4 formed/min per mg protein. Protein assays were performed by the microbiuret [18] or the method of Lowry et al. [19] with bovine serum albumin as the standard. Chelation of the iron in the Fe protein was measured with the method described by Walker and Mortenson [20] modified to employ bathophenanthroline disulfonate instead of α,α'-dipyridyl [21].

Results and Discussion

Substrate inhibition

A. vinelandii nitrogenase inhibited by *C. pasteurianum* Fe protein (Cp2) produces linear rates of acetylene reduction, but *C. pasteurianum* nitrogenase inhibited by *A. vinelandii* MoFe protein (Av1) produces linear rates of acetylene reduction only after a lag phase [10]. The duration of the lag phase increases with increasing concentration of Av1 [10]. Time courses of the activity of *C. pasteurianum* nitrogenase inhibited by Av1 do not exhibit a lag phase as measured by N_2 reduction, H_2 evolution or ATP hydrolysis (data not shown). No lag phase was observed during Cp2 inhibition of *A. vinelandii* nitrogenase when N_2, C_2H_2 or H^+ were the substrates or when ATP hydrolysis was measured. The lag phase was observed only when the acetylene reduction activity of *C. pasteurianum* nitrogenase was followed during its inhibition by Av1.

Time-course measurements are performed routinely by withdrawing gas samples at 5, 10 and 15 min. Linear rates of acetylene reduction are established within 5 min after ethylene production can be detected; this is true with all levels of added inhibitor proteins and under all experimental conditions tried. Lag phases that extended for as long as 20 min also appeared to require less than 5 min after the onset of activity to change to linear acetylene reduction rates; however, the low activity in these assays decreased their accuracy.

One time-course experiment involving Av1 inhibition of *C. pasteurianum* nitrogenase (0.4 μM equiv. Cp1, 1.1 μM equiv. Cp2 and 0.34 μM equiv. Av1

assayed at 30-s intervals) showed a lag phase of ≈140 s before linear rates of acetylene reduction were achieved (15.2 nmol C_2H_4 formed/min). Ethylene formation was first detected at 60 s (0.9 nmol C_2H_4) and creatine release at 30 s. Although variable, the measured creatine values were significantly above the control values at zero time. The rate of release of creatine was relatively constant during the 15-min experiment. Because ATP hydrolysis was occurring in the absence of acetylene reduction, one can infer either that ATP hydrolysis was uncoupled from substrate reduction or that ATP-dependent hydrogen evolution was occurring at a significant rate. Time-courses of hydrogen evolution by *C. pasteurianum* nitrogenase in the presence of 1 atm of N_2 were linear, both in the absence and presence of inhibitory Av1.

Inhibition of ATP hydrolysis

ATP hydrolysis by the homologous nitrogenases was inhibited by the Av1-Cp2 complex. Fig. 1A shows that ATP hydrolysis by *A. vinelandii* nitrogenase decreased with increasing Cp2 concentration, but leveled off near the combining ratio and remained relatively constant upon the addition of more Cp2.

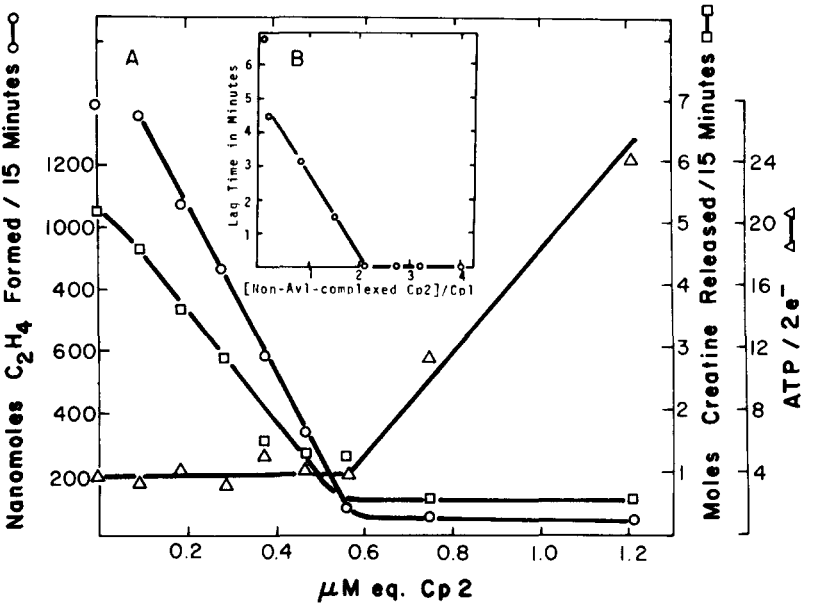

Fig. 1. (A) Inhibition of C_2H_2 reduction and ATP hydrolysis during Cp2 inhibition of *A. vinelandii* nitrogenase. All assays contained 0.22 μM equiv. of Av2, 0.68 μM equiv. of Av2 and Cp2 as indicated. ○———○, acetylene reduction; □———□, creatine release and △———△, ATP/$2e^-$. Acetylene reduction and creatine released were determined on the same assay vial. The combining ratio in this experiment was determined to be 2.7 Cp2/1 Av1. The specific activities were 2136 nmol C_2H_4 formed/min per mg protein for Av1, 1600 for Av2 and 1046 for Cp2. (B) Ratio of (non-Av1-complexed Cp2)/Cp1 vs. the lag time of C_2H_2 reduction during Av1 inhibition of *C. pasteurianum* nitrogenase. All assays contained 0.34 μM equiv. Cp1, 1.38 μM equiv. Cp2 and the following amounts of Av1 (right to left on the abscissa): 0, 0.11, 0.22, 0.33, 0.44, 0.55 and 0.66 μM equiv. The experimentally determined combining ratio for these components was 2.2 Cp2/1 Av1. The amount of non-Av1-complexed Cp2 was determined for each level of Av1 by assuming 1.0 μM equiv. of Av1 prevents 2.2 μM equiv. Cp2 from forming a homologous complex with Cp1. The ratios of the (total Cp2 minus Av1-complexed-Cp2)/Cp1 or (non-Av1-complexed Cp2)/Cp1 were plotted vs. the lag time observed for each addition of Av1. The specific activities were 1197 nmol C_2H_4 formed/min per mg protein for Cp1, 1960 for Cp2 and 2136 for Av1.

The rate of ATP hydrolysis remained at approx. 12% of the uninhibited rate when the combining ratio was reached (2.7 Cp2/1 Av1). This final rate of ATP hydrolysis varied between 10 and 40%, but in most experiments the rate was near 40% of the uninhibited control. The rate of substrate reduction after the combining ratio had been reached was 10% or less of the uninhibited rate, so in the presence of Cp2 in excess of the combining ratio, ATP hydrolysis was largely uncoupled from substrate reduction. The ATP/$2e^-$ ratio remains fairly constant until the combining ratio is reached, whereupon the ATP/$2e^-$ ratio rises rapidly to over 100 with increasing Cp2. This inhibition pattern was observed for both Av1 and Cp2 as inhibitors, and when N_2, C_2H_2 and H^+ were used as substrates.

The combining ratio of the Av1-Cp2 complex

The combining ratios of the Av1-Cp2 complex were determined from velocity vs. inhibitor plots and reciprocal velocity vs. inhibitor plots [23,24]. Regardless of substrate used, combining ratios of Cp2/Av1 for both inhibition of *C. pasteurianum* nitrogenase by Av1 and inhibition of *A. vinelandii* nitrogenase by Cp2 range between 2 and 3 (usually between 2.4 and 2.7). This range of combining ratios was found for all preparations of Av1 and Cp2 used; specific activities of Av1 have varied between 700 and 2100 nmol C_2H_4 formed/min per mg Av1 and specific activities of Cp2 have varied between 550 and 2200 nmol C_2H_4 formed/min per mg Cp2. The observed ratio may reflect the assumptions made about the maximum specific activity of the nitrogenase components. Whereas specific activities for Cp2 of 3000 nmol C_2H_4 formed/min per mg Cp2 have been obtained [5], the highest specific activities for Av1, which can be crystallized, approach only 2500 nmol C_2H_4 formed/min per mg Av1. If the combining ratios are based on this lower value for the maximal specific activity of Av1, the adjusted combining ratios are 2.0—2.3. However, the combining ratios reported in this communication are based on a maximum specific activity for Av1 of 3000 nmol C_2H_4 formed/min per mg Av1.

The data for H_2 evolution, acetylene reduction and N_2 reduction all indicate that a 1 : 1 ratio of Cp2 to Av1 inhibits the homologous nitrogenase very weakly or not at all. A 2 : 1 ratio of Cp2 to Av1 inhibits very strongly. No interaction is seen between the two Fe protein binding sites of the MoFe protein. If one of the two binding sites on Av1 for Av2 is blocked by Cp2, substrate reduction is not inhibited, implying that only one binding site on Av1 for Av2 is required for a normal rate of substrate reduction and ATP hydrolysis. Thus, a 1 : 1 complex of Av1 : Av2 gives near normal activity, even though Av1 can bind two Av2. This may help explain the discrepancies among reports on the homologous complex ratios. The discrepancies also could reflect species differences in the dissociation constants for the two Fe protein binding sites [10].

Inhibition constant of the Av1-Cp2 complex

The inhibition constant of the Av1-Cp2 complex was calculated from reciprocal velocity vs. inhibitor plots [25]. Comparison of the velocity vs. inhibitor and reciprocal velocity vs. inhibitor plots with the theoretical plots published by Morrison [25] indicate that the K_i (inhibition constant) for the

Av1-Cp2 complex should be at least 100 times less than the K_m for either homologous nitrogenase. Determinations of K_i values from hydrogen evolution data, for both Cp2 and Av1 as inhibitors, give values between 0.001 and 0.011 μM. Values as low as 0.004 μM have been obtained for Cp2 inhibition of C_2H_2 reduction by *A. vinelandii* nitrogenase. Inhibition constants have been calculated from N_2 reduction results, but the observed values of K_i have been quite variable because the ammonia assay is not sensitive enough to accurately measure low levels of activity. The most reliable K_i values from N_2 reduction are between 0.020 and 0.030 μM.

Dependence of the acetylene reduction lag phase on the Cp2/Cp1 component ratio

The lag phase of acetylene reduction with Av1 as inhibitor of *C. pasteurianum* nitrogenase depends on the ratio (non-Av1-complexed Cp2)/Cp1 (Fig. 1B). *C. pasteurianum* nitrogenase was inhibited by various levels of Av1 and the lag times and combining ratio determined (2.2 Cp2/1 Av1). The non-Av1-complexed Cp2 was calculated at each addition of Av1 by assuming each μM equiv. of Av1 complexes with 2.2 μM equiv. of Cp2 and, in effect, eliminates these 2.2 μM equiv. of Cp2 from forming a homologous complex with Cp1. The ratio of the remaining Cp2 and the Cp1 were plotted versus the lag time observed for each addition of Av1. Lag phase resulted only when the ratio of (non-Av1-complexed Cp2)/Cp1 was less than 2. A lag phase is not induced with homologous nitrogenase components when the ratio of the Fe protein to the MoFe protein is less than 2. Ratios of Cp2/Cp1 between 0.17 and 0.0012 show a variable lag phase of 1—1.5 min. This lag phase does not increase significantly with decreasing Cp2/Cp1 ratios. Thus, the lag phase caused by Av1 inhibition of *C. pasteurianum* nitrogenase at ratios of Cp2/Cp1 between 0.2 and 2 appears different from that caused by ratios of Cp2/Cp1 below 0.20.

Gel filtration studies

An elution profile from anaerobic chromatography of Av1 on Sephadex G-200 is shown in Fig. 2. Av1 was applied to the column, the elution profile was recorded, and the Av1 protein peak (Peak B) was collected and concentrated under argon. Next, a mixture of Av1 (same concentration as Peak B) and Cp2 (4-fold in excess over the calculated combining ratio of Av1-Cp2) was applied to the column and the elution profile was recorded and the protein peaks (Peaks A and C) were collected and concentrated. The elution profiles (Fig. 2) revealed that the first peak of the Av1-Cp2 sample (Peak A) emerged at a smaller elution volume (V_e) than the peak of the Av1-only sample indicating a higher molecular weight for the complex in Peak A.

Anaerobic analytical polyacrylamide gel electrophoresis of fractions obtained from Peak A showed two protein bands corresponding to Av1 and Cp2; fractions from Peak B gave only a single protein band corresponding to Av1. Addition of Cp2 to a sample of the Av1 protein from Peak B before electrophoresis produced the identical band pattern as found with fractions from Peak A. The EPR spectrum of these fractions from Peak A revealed the presence of both MoFe protein and Fe protein signals (data not shown). This spectrum was very similar to the spectrum of a 2 : 1 mixture of Cp2 and Av1.

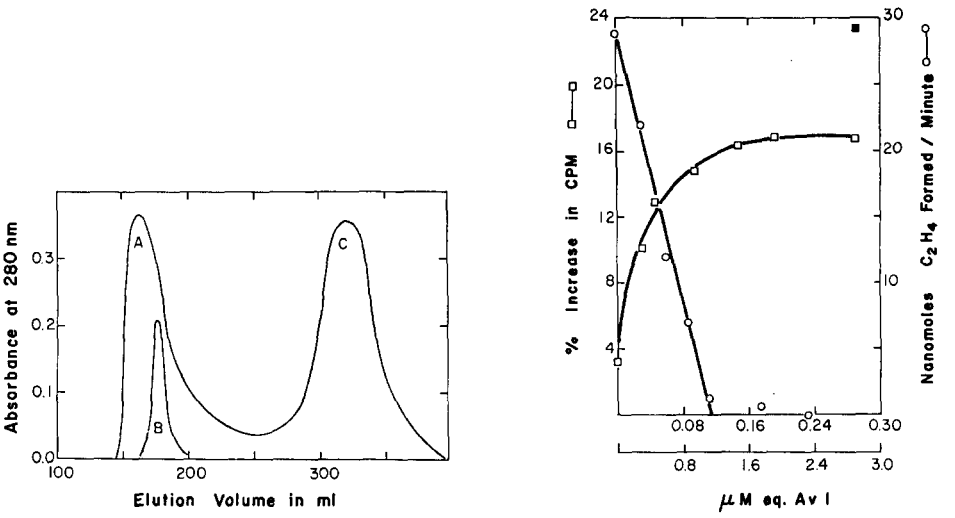

Fig. 2. Elution profile of Av1 and the Av1-Cp2 complex on Sephadex G-200. The same concentration of Av1 was applied to the column in each experiment. The Av1 protein sample (B) contained 7 μM equiv. Av1 and the Av1-Cp2 sample (A) contained 7 μM equiv. Av1 and 63 μM equiv. of Cp2. Both samples were applied to the column in the same volume. The increased absorbance of the Av1-Cp2 peak (A) relative to the Av1 peak (B) is due to the presence of the Cp2; peak C is from Cp2 alone. The Sephadex G-200 column was 2.5 cm diameter × 79 cm and was maintained at 12°C. Absorbance at 280 nm was recorded continuously on an Isco Model UA-5 absorbance monitor. Peaks A, B and C were collected separately, concentrated, assayed by acetylene-reducing activity and by polyacrylamide gel electrophoresis as described in Materials and Methods.

Fig. 3. Saturation curve of ATP binding to the Av1-Cp2 complex. The ATP binding assays contained 50 μM [^{14}C]ATP, 1.05 mM $MgCl_2$ and 2.71 μM equiv. Cp2 (Cp1 does not affect the ATP binding results). The C_2H_2 reduction assays contained 0.271 μM equiv. Cp2 and 0.170 μM equiv. Cp1. Both ATP binding assays and acetylene reduction assays were performed at 25°C. The Av1 scale from 0 to 0.30 μM equiv. refers to acetylene reduction assays. The Av1 scale from 0 to 3.0 μM equiv. refers to ATP binding assays. □——□, percent increase in cpm; ○——○, nmol C_2H_4 formed/min; ■, ATP binding data representing the value found with 50% more Cp2 than in the assay at 2.85 μM equiv. Av1 and 2.71 μM equiv. Cp2 to insure an excess of unbound ATP was present. The specific activities were 1150 nmol C_2H_4 formed/min per mg protein for Cp1, 1760 for Cp2 and 1878 for Av1.

This indicates that Av1-Cp2 forms a tight complex in the absence of ATP, and the low dissociation constant allows the recovery of the complex even by the slow process of gel filtration; the Av1-Cp2 complex eluted 10 h after the sample was applied. Fig. 3 shows that the elution profile for the Av1-Cp2 complex (Peak A) is asymmetrical and trails back to Peak C; the absorbance does return to the baseline after Peak C has emerged from the column. These features indicate that some dissociation is occurring during the course of the experiment, but the dissociation constant is very low. Calibration of the Sephadex G-200 column indicated a molecular weight of 216 000 for Av1 and 300 000—350 000 for the Av1-Cp2 complex. The calibration curve for elution volumes vs. the logarithm of the molecular weights was not linear in the region of the Av1-Cp2 elution volume, so the molecular weight determined was inaccurate. This transport technique does not establish the stoichiometry of the complex, only the lower limit, but the data do indicate that one Av1 binds more than one Cp2. The last peak (Peak C) of the Av1-Cp2 sample indicated a molecular weight of 60 000, corresponding to Cp2.

The specific activity, as determined by acetylene reduction by the Av2-Cp2 complex (Peak A), was only 30% as high as that of the Av1 only sample (Peak B) when each was assayed in the presence of saturating Av2. The 70% inhibition of Av1 in the Av1-Cp2 complex, as calculated from several velocity vs. inhibitor plots, indicates a ratio of Cp2/Av1 of 1.6—1.9. The composition cannot be determined accurately by these calculations, but again the data indicate more than one Cp2 is bound per Av1.

Binding of MgATP to the Av1-Cp2 complex

The effect of Av1 on the binding of MgATP by Cp2 was determined with gel equilibration technique [15]. Fig. 3 shows that the ATP binding increases with additions of Av1 at constant Cp2 until the combining ratio (2.4 Cp2/1 Av1) was reached. Additions of Av1 beyond the combining ratio do not further increase ATP binding. The increase in radioactivity outside the gel was not affected by mixing Av1 and Cp2 before addition to the assay or by allowing Cp2 to react with MgATP before the addition of Av1. Cp1 does not affect the ATP binding of Cp2 or of the Av1-Cp2 complex.

The number of ATP molecules bound per Cp2 protein molecule (N) and their dissociation constants (K_D) were determined for both Cp2 and the Av1-Cp2 complex (Fig. 4). Cp2 binds 1.7 ATP molecules per molecule of Cp2 (N = 1.7) with a dissociation constant (K_D) of 53 μM. Tso and Burris [15] reported a K_D of 17 μM and N = 1.7. The Av1-Cp2 complex data were calculated in two ways: (a) the concentration of the complex was considered to be equal to one-half the concentration of Cp2 (3.68 μM equiv. of complex) and; (b) the Av1-Cp2 complex concentration (3.07 μM equiv. of complex) was calculated on the basis of a complex of 2.4 Cp2/1 Av1, the experimentally determined combining ratio. The two methods gave: (a) N = 3.6 and K_D = 15 μM and; (b) N =

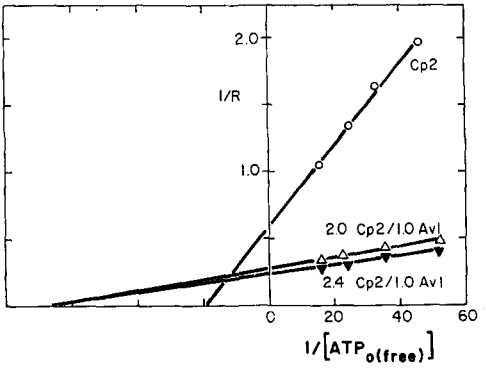

Fig. 4. Determination of the number of ATP molecules bound per Cp2 and per Av1-Cp2 complex and the ATP dissociation constants. R is the amount of ATP bound per protein molecule under experimental conditions. ($ATP_{0(free)}$) is the free ligand concentration outside the gel. Cp2 assays (○———○) contained 7.36 μM equiv. of Cp2. Av1-Cp2 assays contained 3.68 or 3.07 μM equiv. of Av1-Cp2 (see text for details). The concentrations of ATP in the assay vials were 25, 35, 50 and 75 μM. N, the number of ATP molecules bound per Cp2, was 1.7 and K_D, the dissociation constant, was 53 μM. For Av1-Cp2 equivalent to 3.68 μM equiv. (△———△), N = 3.6 and K_D = 15 μM. For Av1-Cp2 equivalent to 3.07 μM equiv. (▼———▼), N = 4.3 and K_D = 14 μM. The specific activities were 1878 nmol C_2H_4 formed/min per mg protein for Av1 and 2210 for Cp2.

4.3 and $K_D = 14\,\mu M$, respectively. A complex of 2 Cp2/1 Av1 binds 4 ATP molecules; each Cp2 binds 2 ATP's. Complex formation between Av1 and Cp2 does not block the ATP binding sites on Cp2 but does cause the ATP to bind more tightly. Premixing of the two components or adding them separately did not affect the extent of ATP binding. A conformational change induced by Av1 upon complex formation could account for the decrease in the dissociation constant.

Inhibition by Av1 of the MgATP-induced reaction between Cp2 and iron chelators

The binding of MgATP to the Fe protein of nitrogenase is believed to induce a conformational change in the protein [1]. One of several lines of experimental evidence for such an effect was provided by the observation of Walker and Mortenson [20] that the binding of MgATP to Cp2 dramatically increases the accessibility of the Fe-S cluster on Cp2 to reaction with the iron(II)-chelator α,α'-dipyridyl. We have recently confirmed and extended these observations, using the water-soluble iron(II)-chelator bathophenanthroline disulfonate, which offers advantages of high sensitivity and high rates of reaction [21].

The reaction between Cp2 and α,α'-dipyridyl in the presence of MgATP is partially inhibited by Cp1 [20], but interpretation of this observation is complicated by the onset of electron transfer and ATP hydrolysis. Use of Av1 instead of Cp1 should alleviate this problem. The data in Table I demonstrate that Av1 will completely inhibit the MgATP-dependent reaction between Cp2 and bathophenanthroline disulfonate. These data furnish additional evidence in support of a tight complex between Cp2 and Av1.

Conclusions

The time-courses, velocity vs. inhibitor plots and reciprocal velocity vs. inhibitor plots for both Av1 inhibition of *C. pasteurianum* nitrogenase and

TABLE I

EFFECT OF Av1 ON THE MgATP-DEPENDENT REACTION BETWEEN Cp2 AND BATHOPHENANTHROLINE DISULFONATE

Rates at 25°C of iron (II)-bathophenanthroline disulfonate complex formation were calculated from absorbance changes at 535 nm. Reaction mixture of 1.00 ml were injected into cuvettes that were sealed with rubber serum stoppers and had been evacuated and flushed with N_2. The reaction mixture was: 20 mM Tris-HCl, pH 7.4; 0.1 mM bathophenanthroline disulfonate; 2 mM $MgSO_4$; 1 mM ATP; 5 mM $Na_2S_2O_4$; Av1 and Cp2 as indicated; ATP was added last.

Cp2 (μg protein)	Av1 (μg protein)	Rate (nmol Fe released/min)
70	0	0.93
0	130	0.06 *
70	130	0.28
70	260	0.03
70	390	0.00
140	260	0.66

* Identical rates in the presence or absence of ATP.

Cp2 inhibition of *A. vinelandii* nitrogenase, measured as C_2H_2 reduction, N_2 reduction and H_2 evolution are all similar except for Av1 inhibition of acetylene reduction by *C. pasteurianum* nitrogenase. In this case, a lag is exhibited before the rates of acetylene reduction become linear. The lag phase results from the interaction between homologous *C. pasteurianum* nitrogenase components in the presence of Av1, when the ratio of Cp2/Cp1 is less than 2. No lag phase in acetylene reduction is evident between homologous *C. pasteurianum* nitrogenase components, in the absence of Av1, at ratios of Cp2/Cp1 between 0.2 and 2. Lag phases have been observed by others [26,27] and are believed to result from changes in the catalytic electron allocation to the MoFe protein. Our results can be interpreted in an analogous way.

The plots of velocity vs. concentration of Av1 as inhibitor of *C. pasteurianum* nitrogenase show that acetylene reduction can be completely inhibited at the combining ratio of the heterologous components Av1 and Cp2. In all other cases, (a) time-courses of substrate reduction are linear at all levels of inhibitor, and no lag phase is observed; (b) in the velocity vs. inhibitor plots increasing inhibitor concentration rapidly decreases the velocity until the combining ratio is reached, then the plots break sharply and further increments of inhibitor only slightly decrease the velocity. At the combining ratio, the activity is reduced to 10% or less of the uninhibited rate.

The rate of ATP hydrolysis also was inhibited by formation of the Av1-Cp2 complex. ATP hydrolysis decreased with increasing inhibitor concentration until the combining ratio was reached, whereas the ATP/$2e^-$ ratio remained constant. Further additions of the inhibitor beyond the combining ratio did not markedly decrease ATP hydrolysis, but the ATP/$2e^-$ ratios rose sharply.

The Av1-Cp2 complex can be isolated by gel filtration. This shows that the Av1-Cp2 complex has a very low dissociation constant and that ATP is not required for complex formation. The molecular weight of the complex was estimated to be between 300 000 and 350 000, implying a ratio of Cp2/Av1 higher than 1.

ATP binds to Cp2 in the Av1-Cp2 complex with a 3-fold lower dissociation constant than for free Cp2. The number of ATP molecules bound per Cp2 is not affected by the presence of Av1; 4 ATP's are bound per complex of 2 Cp2/1 Av1 or 2 ATP's per Cp2. The lower dissociation constant for ATP in the Av1-Cp2 complex may result from a conformational change caused by complex formation.

Av1 protects the iron in Cp2 from chelation in the presence of MgATP, suggesting that Av1 upon binding to Cp2 covers the iron-sulfur site of Cp2 and prevents access for the chelator. On the other hand, the MgATP binding sites on Cp2 are accessible in the Av1-Cp2 complex, and the Cp2 in the complex undergoes a conformational change upon binding of MgATP, as evidenced by a change in the EPR spectrum (data not shown) similar to that observed upon binding of MgATP to free Cp2 [28]. Together, these observations indicate that the two binding sites for MgATP are located some distance from the iron-sulfur site on the Fe protein of nitrogenase. This suggests as a model, a complex with close contact between the Fe-S site on the Fe protein and an electron acceptor site on the MoFe protein, but with free access to the ATP sites retained.

Acknowledgements

We thank W.W. Cleland for helpful discussions, R.V. Hageman for the nitrogenase components from *A. vinelandii*, and W.H. Orme-Johnson for use of the EPR facilities. This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, by Public Health Service Grant AI-00848 from the National Institute of Allergy and Infectious Diseases, by the National Science Foundation Grant PCM 74-17604, and by National Institutes of Health Training Grant GM-00236 BCH from the National Institute of General Medical Sciences.

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