Formation of a Tight 1:1 Complex of *Clostridium pasteurianum* Fe Protein−*Azotobacter vinelandii* MoFe Protein: Evidence for Long-Range Interactions between the Fe Protein Binding Sites during Catalytic Hydrogen Evolution†

Thomas A. Clarke, Silvana Maritano, and Robert R. Eady*

Nitrogen Fixation Laboratory, John Innes Centre, Colney, Norwich NR4 7UH, U.K.

Received February 8, 2000; Revised Manuscript Received June 6, 2000

ABSTRACT: It has been well documented that the combination of the MoFe protein of *Azotobacter vinelandii* nitrogenase (Av1) with the Fe protein (Cp2) from *Clostridium pasteurianum* nitrogenase produces an inactive, stable complex. However, we report that this heterologous nitrogenase has a low level of activity for H_2 evolution, with a specific activity of 12 nmol min^{-1} mg^{-1} of Av1. This activity does not arise from contaminating hydrogenase since it required the presence of both Cp2 and Av1 and showed saturation kinetics when increasing amounts of Cp2 were added to the assay. Incubation of the two proteins at a 4:1 Cp2:Av1 ratio in the absence of MgATP followed by analytical gel filtration showed, surprisingly, that the stoichiometry of the isolated complex was Av1·Cp2 instead of Av1·$(Cp2)_2$ as determined previously. The presence of MgATP in the elution buffer did not change the elution profile of the complex. The hydrodynamic radius of the isolated complex determined by dynamic light scattering was 5.93 ± 0.14 nm, intermediate between Av1 and a stable 2:1 nitrogenase complex, consistent with a 1:1 assignment for the Av1·Cp2 complex. When assayed with Av2, the isolated Av1·Cp2 complex showed full half-site reactivity with a specific activity of 750 nmol of C_2H_2 reduced min^{-1} mg^{-1} of Av1. The EPR spectrum of the isolated complex showed the Cp2 to be oxidized and the Av1 to retain the $S = {}^3/_2$ signal characteristic of FeMoco. In the presence of MgATP, under turnover conditions at a 2:1 ratio of Cp2:Av1, the [4Fe-4S] center of Cp2 was protected from the chelator 2,2′-bipyridyl. This is consistent with the formation of a tight 2:1 complex of Av1·$(Cp2)_2$ which is more stable than the homologous Cp nitrogenase. Assuming that the Lowe−Thorneley model for nitrogenase applies and that a rate-limiting dissociation of the complex is required for H_2 evolution, then with a rate of 0.032 s^{-1} the 1:1 complex is too stable to be involved in catalysis. The differences in the stability of the 2:1 and 1:1 complexes indicate cooperativity between the Fe protein binding sites of Av1, which structural data show to be separated by 105 Å. On the basis of these observations, we propose a model for nitrogenase catalysis in which the stable 1:1 complex formed between oxidized Fe protein and the one-electron-reduced MoFe protein plays an essential role. In this scheme, the two Fe protein binding sites of the MoFe protein alternately bind and release Fe protein in a shuttle mechanism associated with long-range conformational changes in the MoFe protein.

Nitrogenase, the enzyme system responsible for biological nitrogen fixation, catalyzes the reduction of dinitrogen to ammonia via the MgATP-dependent reaction:

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

Three related nitrogenase systems have been characterized, the first containing Fe and Mo, which is widespread and the most extensively studied (*1*, *2*), a second utilizing Fe and V, and a third based on Fe (see ref *3*). An unrelated superoxide-dependent Mo-containing system isolated from *Streptomyces thermoautotrophicus* has recently been described (*4*). The relative contribution that these systems make to global nitrogen cycling has yet to be assessed; however, it is likely that the conventional Mo-containing enzyme, which appears to be present in all nitrogen-fixing organisms other than *S. thermoautotrophicus*, is a major route for nitrogen fixation and is the enzyme studied in the present work. This Mo-containing enzyme is comprised of two separate component proteins: an Fe protein, responsible for MgATP-driven electron transfer, and a larger MoFe protein, which contains the catalytic site for nitrogen reduction. The electrons for the reaction are donated to the Fe protein from flavodoxin or ferredoxin in vivo and usually sodium dithionite in vitro (*1*−*3*). In addition to N_2, nitrogenase will reduce a number of alternative substrates including C_2H_2, and in the absence of a reducible substrate protons are reduced to H_2. Mo nitrogenases are a highly conserved enzyme family

† Financial support for this work was provided by the Biotechnology and Biological Sciences Research Council as part of the Competitive Strategic Grant to the John Innes Centre and a research studentship to T.A.C. and by the European Union for a Marie Curie fellowship (ERBFMBICT982909) to S.M.

* Address correspondence to this author: e-mail, robert.eady@bbsrc.ac.uk; tel, 44 1603 450728; fax, 44 1603 450018.

10.1021/bi0002939 CCC: $19.00 © 2000 American Chemical Society
Published on Web 08/18/2000

and have very similar physicochemical properties when isolated from a wide range of different nitrogen-fixing organisms (*5*). In many cases fully functional enzymes are formed by complementary components purified from different organisms, but the components of the nitrogenase system from *Clostridium pasteurianum* show a restricted degree of interchangeability with those of other organisms (*6*).

A comprehensive scheme for the reduction of N_2 and the formation of H_2 by the homologous *Klebsiella pneumoniae* nitrogenase system has been developed (*7*−*10*). In this scheme the Fe protein functions as an MgATP-dependent one-electron reductant that binds to the MoFe protein and, following the transfer of each electron, dissociates in the rate-limiting step of nitrogenase turnover. Further cycles of electron transfer require the rereduction of $Kp2_{ox}$·$(MgADP)_2$ by dithionite and the replacement of MgADP by MgATP before formation of a new electron-transfer complex. A minimum of two such cycles are required for the reduction of protons to H_2. This general scheme has been shown to apply to the nitrogenase system of *Azotobacter vinelandii* (*11*). In addition, a scheme for the reduction of acetylene (C_2H_2) to form ethylene (C_2H_4), a standard method of determining nitrogenase activity, has been proposed (*12*, *13*). Although the reduction of C_2H_2 to C_2H_4 is also a two-electron reduction, the scheme proposes that this reaction requires that three electrons are transferred to the MoFe protein. Consequently, three rounds of complex association and dissociation are required before release of C_2H_4 occurs. In this mechanism, Kp1[1] with C_2H_2 bound is further reduced beyond the $2e^-$ state and is then irreversibly committed to form C_2H_4.

The X-ray crystallographic structures of the two component proteins and the putative transition state AlF_4^-·ADP complex of *A. vinelandii* nitrogenase have been determined (*14*−*17*). The Fe protein is a γ_2 64 000 Da protein with a single [4Fe-4S] center bridged between two identical subunits, each subunit having a binding site for one MgATP molecule (*16*, *17*). Both MgADP and MgATP bind to the free Fe protein (*2*), but there are conflicting reports about whether MgATP binds to the free Fe protein or after complex formation with the MoFe protein during turnover of the enzyme complex (*11*, *18*). Irrespective of the order of binding, MgATP hydrolysis appears to be essential for dissociation of the complex after electron transfer (*19*).

The MoFe proteins are 226 000 Da $\alpha_2\beta_2$ tetramers that contain two metallocenters in each $\alpha\beta$ dimeric subunit. A unique Fe_8-S_7 cluster, known as the P cluster,[2] is positioned at the interface between the $\alpha\beta$ subunits, and an iron−molybdenum−cofactor center (Fe_7S_9Mo-homocitrate), known as FeMoco, is located within the α subunit (*14*). It is generally accepted that the P cluster functions as an intermediate for electron transfer between the [4Fe-4S] cluster and FeMoco, the site of substrate binding and reduction (*2*, *14*, *17*).

During the past few years investigation of the structures of stabilized complexes formed by nitrogenase components, and of the reactions they catalyze, has utilized mutated forms of the Fe protein. Such mutated proteins are either locked in the ATP-bound conformation or form tight complexes with the MoFe protein (*20*, *21*). Other studies have utilized the stable transition-state analogues formed with BeF_3^- or AlF_4^- and MgADP (*15*, *22*−*24*). Tight complexes are also formed between some heterologous nitrogenases formed by component proteins purified from different nitrogen-fixing organisms, in particular, the Fe protein component of *C. pasteurianum* (Cp2) and the MoFe protein of *A. vinelandii* (Av1) or *K. pneumoniae* (Kp1) (*6*, *25*, *26*). Stopped-flow kinetic studies of such complexes have shown that, in the presence of MgATP, Cp2 rapidly transfers an electron to Kp1 (*27*) with a rate constant of 250 s^{-1} or, in the case of Av1, 100 s^{-1} (*18*, *28*). In the former case, an active nitrogenase is formed, but although the time course is linear for H_2 evolution, a 10 min lag for C_2H_2 reduction (*27*) and a 30 min lag for N_2 reduction (*29*) are observed before the time course becomes linear. The reasons for the existence of these long lag periods are not clear, but it has been proposed that a once-only activation step is required. In the case of the Cp2·Av1 system, although MgATP hydrolysis and electron transfer from Cp2 to Av1 occur, no reduction of substrates has been reported (*18*).

A surprising feature of all the tight complexes which have been investigated is the observation that although the EPR and optical properties of the Fe protein indicate that it is oxidized, consistent with electron transfer to the MoFe protein having occurred, neither the P clusters nor the FeMoco centers of the MoFe protein appear to change oxidation state. In the case of the AlF_4·ADP·Kp complex this has been rationalized by the finding that H_2 is evolved during the formation of the tight complex of Kp1·$[Kp2_{ox}$·$(AlF_4$·$ADP)_2]_2$ (*30*). In this paper the potential catalytic properties of the heterologous nitrogenase formed by the components Av1 and Cp2 are reexamined. The significance of these observations and the insight that they give to the role of Fe protein and its interaction with the MoFe protein in nitrogenase catalysis are discussed.

EXPERIMENTAL PROCEDURES

Nitrogenase Preparation. The nitrogenase component protein Av1 was prepared as described previously (*31*), and Cp2 was prepared as described in ref *32* with minor modifications. The specific activity as determined by C_2H_2 reduction of Av1 was 2000 nmol min^{-1} mg^{-1}, and the specific activity of the Cp2 samples ranged between 1200 and 1400 nmol min^{-1} mg^{-1}. The molybdenum content of Av1 was 1.9 Mo atoms mol^{-1}, as determined by Southern Analytical Ltd. (Brighton BN1 9PY, U.K.), using inductively coupled plasma emission analysis after acid hydrolysis.

Hydrogen Evolution. Assays were carried out in 8 mL glass vials containing 1 mL of assay mixture. Assay vials were sealed with Suba Seals (W. M. Freedman, Barnsley, U.K.) and flushed with argon. The assay mixture contained 100 mM Hepes, pH 7.4, 20 mM ATP, 50 mM $MgCl_2$, 20

[1] The nitrogenase metalloproteins are abbreviated according to the genus and species of the diazotrophs from which they were isolated. For example, the Fe protein from *Klebsiella pneumoniae* is Kp2, the *Azotobacter vinelandii* Fe protein is Av2, and the *Clostridium pasteurianum* protein is Cp2; the MoFe protein from *K. pneumoniae* is Kp1 etc.

[2] Abbreviations: FeMoco, [1Mo-7Fe-9S-homocitrate] cofactor of nitrogenase; P cluster [8Fe-7S] cluster of nitrogenase; $Av1_{red}$·$Cp2_{ox}$, the 1:1 complex of oxidized Cp2 and Av1 reduced by one electron; $Av1_{(red)2}$·$(Cp2_{ox})_2$, the 2:1 complex of oxidized Cp2 and Av1 reduced by two electrons.

mM sodium dithionite, 50 mM creatine phosphate, and 0.1 mg of creatine kinase. Assays were started with the addition of 49 μg of Av1 (0.21 μM) and 14−475 μg of Cp2 (0.21−7.5 μM), incubated for 30 min at 30 °C, and then stopped with 0.1 mL of 30% w/v trichloroacetic acid. The amount of H_2 evolved was measured as described in ref *33*.

Fe Chelation from the [4Fe-4S] Cluster of Cp2 Using 2,2′-Bipyridyl. The reaction was carried out in a cuvette under an atmosphere of argon. A final volume of 2 mL contained 50 mM Tris-HCl buffer, pH 8.0, 4 mM $MgCl_2$, 6.25 mM 2,2′-bipyridyl, 1.3 mM sodium dithionite, 6 mM creatine phosphate, 0.125 mg mL^{-1} creatine kinase, and either 6.8 μM Cp2 alone or 6.8 μM Cp2 with 3.4 μM Av1 or Cp1. To initiate the reaction, ATP was added by syringe addition to give a final concentration of 3.9 mM. The increase in absorbance at 520 nm due to the formation of the Fe^{II} chelate complex was monitored.

Analytical FPLC Gel Filtration of Complexes. Samples for gel filtration were prepared by a short (∼3 min) incubation of Cp2 and Av1 at a molar ratio of 4:1 either in buffer containing 50 mM Hepes buffer, pH 7.4, and 2 mM sodium dithionite or in a reaction mixture lacking creatine kinase. Samples were run on an analytical HR 10/30 Superdex 200 column equilibrated with 50 mM Tris-HCl buffer, pH 7.4, and 2 mM dithionite. The flow rate was 0.2 mL min^{-1}, and the absorbance was monitored at 405 nm. In some experiments, as indicated, the equilibration and elution buffer contained 10 mM $MgCl_2$ and 5 mM ATP.

The elution profiles of the heterologous complex and Av1 were analyzed quantitatively to determine the relative proportions of Cp2 bound to Av1. The relative extinction coefficients used were 1 for Av1 and 0.18 for $Cp2_{ox}$; thus an Av1·Cp2 complex would have an extinction coefficient of 1.18, and an Av1·$(Cp2)_2$ complex would have an extinction coefficient of 1.36. These relative extinction coefficients are those for *K. pneumoniae* nitrogenase components (*33*), but given the similarity of the chromophores present in Cp2 and Av1 to those of Kp2 and Kp1, it can be assumed that the relative extinction coefficients will be applicable.

Dynamic Light Scattering. Dynamic light scattering on all samples was performed using a Dynapro-MSTC instrument (Protein Solutions, Inc., Charlottesville, VA) equipped with a 40 mW, 843 nm solid-state laser. Samples were diluted with 50 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, and 2 mM sodium dithionite to a final concentration between 0.2 and 2 mg mL^{-1} before filtration through a 0.01 μm filter. Samples were filtered before filling the 12 μL quartz flow cell. Between 20 and 30 readings were taken for each concentration of sample. The data were analyzed and the hydrodynamic radii determined using Dynamics version 5.19.06, the software package supplied with the machine.

RESULTS AND DISCUSSION

MgATP-Dependent H_2 Evolution by Av1Cp2 Nitrogenase. The original description of the properties of the heterologous nitrogenase formed by Cp2 and Av1 (*25*) reported that it was incapable of reducing N_2 or C_2H_2 (*6*, *26*). Subsequently, the inhibitory effect of Cp2 on the activity of the homologous Av nitrogenase in the presence of excess Av2 was shown to be a consequence of the formation of a tight nondissociating complex between Cp2 and Av1 (*6*). The formation of this tight complex was previously proposed to account for the reported lack of activity.

It was recently reported that electron transfer from Cp2 to Av1 occurs both in the presence and, unexpectedly, in the absence of nucleotides. In addition, the [4Fe-4S] center of Cp2 remains oxidized in the presence of the reductant sodium dithionite (*18*). Pre-steady-state stopped-flow studies by these authors showed that electron transfer from Cp2 to Av1 was fastest in the presence of MgATP and occurred with an apparent first-order rate constant of 100 s^{-1}. This rate is comparable to those of the homologous Cp (*27*) and Av nitrogenases (*20*, *34*) where the rate-limiting step in nitrogenase turnover with dithionite as electron donor is considered to be dissociation of the Fe·MoFe protein complex following electron transfer (*35*).

Some 24 years ago one of us demonstrated that, in the analogous nitrogenase formed between Cp2 and Kp1, the time courses for N_2 or C_2H_2 reduction were not linear and a slow time-dependent change in substrate specificity occurred (*27*). The recent report of Chan et al. (*18*), that electron transfer occurred in the Cp2·Av1 complex, prompted us to investigate the potential hydrogenase activity of this nitrogenase system to determine whether the reported inactivity arose from a protracted lag period before substrate reduction occurred.

When assayed for 30 min under an atmosphere of argon, under the standard conditions for nitrogenase assay, we readily detected low levels of H_2 evolution from mixtures of Av1 and Cp2. No H_2 was evolved from either protein incubated alone, indicating that this activity was a property of the combined proteins and did not arise from contaminating hydrogenase activity. This is an important finding, since C_2H_2 reduction assays at a Cp2:Av1 molar ratio of 2:1 or 26:1 confirmed the original report (*6*) that C_2H_2 was not a substrate, since we did not detect any C_2H_4 even after an overnight incubation. Our detection of H_2 evolution contrasts with an earlier report (*26*) that the addition of Cp2 to an assay of Av nitrogenase caused complete inhibition of the reduction of all substrates, including protons. However, since their assays were only incubated for a reaction time of 10 min, the low rate of H_2 evolution we observe may have not been recognized as significant.

Under our conditions, using a 20:1 ratio of Cp2:Av1, no lag in the formation of H_2 was detected; the time course was linear for up to 60 min and was not inhibited by 30% v/v C_2H_2. This lack of inhibition provides further evidence that the activity is due to nitrogenase rather than to a low level of contaminating hydrogenase since C_2H_2 is a potent inhibitor of hydrogenase activity (*36*). The reduction of H^+, but not C_2H_2, is characteristic of heterologous nitrogenases under conditions of low electron flux, a finding consistent with more reduced species of the MoFe protein being involved in C_2H_2 reduction (*7*, *37*). The simulations of the Lowe−Thorneley model for C_2H_2 reduction (*12*) assign the three-electron-reduced form of the MoFe protein as that responsible for the formation of C_2H_4. Assuming this scheme to apply in the present case, then the Cp2Av1 nitrogenase appears not to achieve this level of reduction. The low rate of H_2 evolution we observe may arise from slow cycling of the first steps of the Lowe−Thorneley model or from repetition of the proposed once-only activation of MoFe protein, which

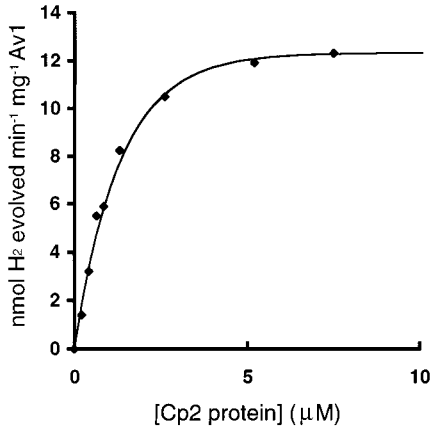

FIGURE 1: Effect of [Cp2] on the activity of Av1. H_2 evolution assays containing 46 μg of Av1 were initiated by addition of increasing amounts of Cp2 and incubated for 30 min before stopping with 30% trichloroacetic acid. The amount of H_2 formed was measured by gas chromatography as described in ref *32*. The saturation level of Av1 is at a Cp2:Kp1 molar ratio of approximately 25:1. The rate constant of the reaction is approximately 0.034 s^{-1}. The line is not a fit but shows the trend for activity increase as [Cp2] is increased.

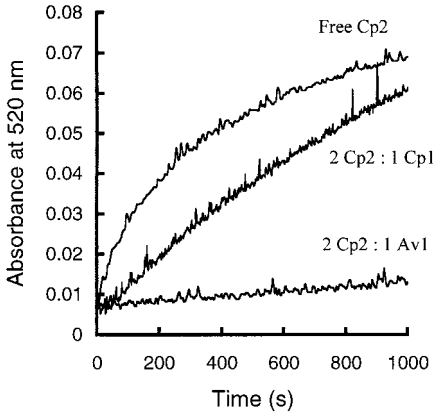

FIGURE 2: Rate of Fe chelation of the [4Fe-4S] cluster of Cp2 in the presence and absence of Cp1 and Av1. Mixtures containing either 6.8 μM Cp2 or 6.8 μM Cp2 with 3.4 μM Cp1 or Av1 were prepared as described in Experimental Procedures, and the change in absorbance caused by the formation of the Fe chelate complex was read at 520 nm.

results in H_2 evolution normally stoichiometric with Mo content (*37*).

Effect of Component Ratio on Hydrogen Evolution. Figure 1 shows the effect on the rate of H_2 evolution of the addition of increasing amounts of Cp2 to assays containing a fixed amount of Av1. The enzyme activity increases hyperbolically and saturates at a Cp2:Av1 molar ratio of 25:1 to give a limiting specific activity of 12 nmol of H_2 evolved min^{-1} mg^{-1} of Av1. This activity is low, being approximately 0.5% of that measured for Av1 when assayed in the homologous Av system.

The shape of the MoFe protein activity titration curve (Figure 1) is typical of those obtained with homologous nitrogenases, which have been simulated by Thorneley and Lowe (*10*). Thus it is reasonable to assume that their scheme is generally applicable to catalysis by heterologous nitrogenases. From the maximum rate of H_2 evolution measured at a saturating level of Cp2, a limit of $\sim$0.032 s^{-1} can be put on the rate of the dissociation of the complex following electron transfer. This value contrasts with those of 6.4 s^{-1} for Kp nitrogenase (*35*) and 10 s^{-1} for Av nitrogenase (*34*). Consequently, the low rate of H_2 evolution we observe is consistent with the slower dissociation of a complex of the two proteins following the initial rapid electron-transfer reaction, when compared with homologous nitrogenases. The slow turnover rate of the Cp2Av1 nitrogenase is unlikely to arise from a slow rate of nucleotide exchange or phosphate release from the complex, since it has been shown that the ATPase activity of the complex is high (*28*). Thus dissociation of the complex does not appear to be required for the catalysis of MgATP hydrolysis, but it seems likely that dissociation of the two proteins is a prerequisite for enzyme turnover leading to substrate reduction.

The dependence on Cp2 concentration for the activity titration of Av1 suggests that this association is much weaker than that involved in the formation of a primary 2:1 complex. The requirement for Fe protein in excess of 2:1 for maximum MoFe protein activity has been rationalized in the Lowe−Thorneley scheme in the following way. For preparations of Fe protein which are fully active, maximum activity is expected to occur at a 2:1 ratio of nitrogenase components, provided that the rate of rereduction of Fe $protein_{ox}$·MgADP is fast when compared with the rate of complex formation of this species with MoFe protein to form an abortive complex (*10*). In the present case, the effect of free Cp2 is predominantly on a complex of $Av1_{red}$·$Cp2_{ox}$. The rate of dissociation of this complex is slow, so that at a 2:1 ratio the free [$Cp2_{ox}$] would be low and would not recomplex with Av1 to any significant extent. The implication of these results is that the enzymatic activity of the system is reductant-limited during the reactions leading to H_2 evolution. The shape of the activity titration curve (Figure 1) makes it unlikely that the observed activity involves reduction of Cp2 in the complex by dithionite as a 20-fold excess of Cp2 is required for maximum activity. Thus, a potential role for the excess Cp2 in our experiments is that of an electron donor to the complex, as has been suggested to occur with the homologous Av nitrogenase (*37*). However, a rate-limiting dissociation of the Av1·Cp2 complex is required for product release as in the Lowe−Thorneley scheme.

Stability of the Av1·Cp2 Complex during Turnover. If our assignment of the low catalytic activity of the Av1Cp2 nitrogenase as arising from the slow dissociation of the proteins as described above is correct, then this heterologous complex should be more long-lived under turnover conditions than that formed by homologous nitrogenase components. It has been shown that under assay conditions, in the presence of MgATP, the presence of homologous MoFe protein partially protects the Fe in the [4Fe-4S] center of the Fe protein from chelation, an effect attributed to the cluster being less accessible when the nitrogenase complex is formed (*39*−*41*). This protection is essentially complete for those nitrogenase systems where mutated forms of the Fe protein form tight complexes with MoFe protein, and this property has been exploited to gain insight into the stability of nitrogenase complexes under turnover conditions.

We used this method to compare the stability of the 2:1 complex formed between Cp2 and Av1 under our assay conditions. Figure 2 shows the iron chelation of free Cp2 and of preincubated mixtures of Cp1:Cp2 and Av1:Cp2 at a molar ratio of 1:2. An increase in absorbance over 1000 s in

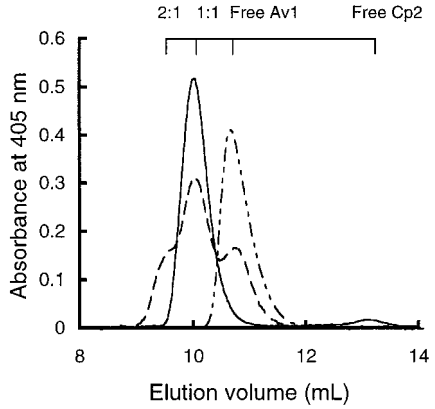

FIGURE 3: FPLC gel filtration traces of incubation mixtures containing Av1, a mixture of Av1:Cp2 at a 1:4 molar ratio, and a 1:1 equilibrium mixture of Kp1:Kp2 with MgADP and BeF_3^-. Incubation mixtures were run on an analytical Superdex 200 column (HR 10/30), equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM sodium dithionite, under nitrogen and eluted at a flow rate of 0.2 mL min^{-1}. The absorbance was measured at 405 nm.

the sample of Cp2 after addition of ATP is consistent with the iron being chelated from the Cp2 as MgATP binds and causes a conformational change which exposes the [4Fe-4S] center of the protein. The rate of chelation is lower in the presence of Cp1 and is almost totally inhibited in the mixture containing the Av1·Cp2 complex. This can be accounted for by the formation of a tight complex between Cp2 and Av1 that protects the [4Fe-4S] cluster from chelation by 2,2′-bipyridyl. The almost complete protection conferred on Cp2 indicates that, under turnover conditions, both of the Fe protein binding sites on Av1 are occupied by Cp2 and that the dissociation rate is markedly lower that in the homologous Cp nitrogenase system. It also shows that our Cp2 preparations are fully competent in complex formation. The formation of a 2:1 complex under assay conditions is consistent with the inhibition of Av nitrogenase activity by Cp2 (*25*, *26*), and the reported kinetic analysis was consistent with a finite dissociation constant for this complex.

Isolation and Composition of the Av1·Cp2 Complex. It has previously been shown that the Av1·Cp2 complex is sufficiently stable to be isolated by gel filtration using Sephadex G-200. However, the authors were not able to accurately determine the M_r of the complex owing to technical limitations, although a combining ratio of 1.6 to 1.9 was determined (*26*). We have recently reported conditions for analytical gel filtration, using the improved chromatography material now available, which demonstrated the importance of the flow rate at which the column is developed on the resolution of equilibrium mixtures containing the 2:1 and 1:1 complexes of Kp nitrogenase (*24*). Using this methodology, we have reinvestigated the nature of the complex formed by Av1 and Cp2 and, surprisingly, find the elution properties of the complex to be those of a 1:1 molar complex.

The two component proteins were mixed at a molar ratio of 4:1 Cp2 (40 μM) and Av1 (10 μM) before chromatography on an analytical HR 10/30 Superdex 200 column. The elution profile (Figure 3) showed a major peak eluted at a retention volume of 10.8 mL and a minor peak at 13.5 mL. When Av1 and Cp2 were passed through the same column separately under the same conditions, Av1 was eluted at 11.7 mL and Cp2 at 13.5 mL. These data are consistent with the earlier report that a species with a higher M_r than Av1 was sufficiently stable to be isolated by this method (*26*). However, when the $MgADPBeF_3^-$-containing equilibrium mixture of Kp1 and the complexes Kp1·Kp2 and Kp1·$(Kp2)_2$ were used to calibrate the column, the Av1·Cp2 elution volume corresponded to that of a 1:1 complex (Figure 3). In our experiments there is no indication that higher ordered complexes are present, since the profile is smooth on the leading edge and essentially the absorbance is at zero at a volume corresponding to a 2:1 complex. The presence of MgATP in the elution buffer had no significant effect on the elution profile of the Av1·Cp2 complex, consistent with the earlier report (*26*), indicating that the ATP-bound conformation of Cp2 is not required for complex formation.

Table 1: Hydrodynamic Properties of the Isolated Av1·Cp2 Complex Compared with Av1 and 1:2 Complexes of Nitrogenase Components

sample	proposed stoichiometry	hydrodynamic radius (nm)
Av1	isolated Av1	5.32 ± 0.16
Av1·Cp2 complex	1:1 Av1:Cp2	5.93 ± 0.14
Kp1·Kp2 ($MgADP·BeF_3^-$)$_2$	1:2 Kp1:Kp2	6.71 ± 0.30

Quantification of the areas under the elution peak profiles of Av1 and the Av1·Cp2 complex, using the extinction coefficients assigned in the Experimental Procedures section, gave a ratio of 1:1.22 for the areas of Av1·Av1·Cp2 complex. This value is essentially that predicted for a 1:1 Av1·Cp2 complex (1.18) since the presence of an additional Cp2 in a potential Av1·$(Cp2)_2$ complex would have resulted in an increase of the area under the peak of the complex to 1.36.

To confirm the stoichiometry of the complex, the three peaks that eluted at different volumes on gel filtration were collected and subject to analysis by dynamic light scattering. Table 1 shows the hydrodynamic radii obtained for the three species with elution volumes of 9.6, 10.0, and 10.7 mL. There is a clear difference between the hydrodynamic radii of these species. Isolated Av1 is the lowest, and the BeF_3^-Kp1·$(Kp2)_2$ complex is the largest. The hydrodynamic radius of the complex formed by Av1 and Cp2 is intermediate between the two values, consistent with the formation of a 1:1 complex between Av1 and Cp2, as indicated by the gel filtration data.

EPR Spectra of the Isolated Av1·Cp2 Complex. The EPR spectrum of the isolated 1:1 complex showed only the $S = {}^3/_2$ features characteristic of the dithionite-reduced FeMoco centers of Av1 and the absence of the $S = {}^1/_2$ EPR signal characteristic of Cp2 (Figure 4). This feature was also not detected when the spectrum was measured at 18 K and 10 mW, optimum conditions for the detection of this species. This indicates that the Fe protein in the complex is oxidized, as reported for an unresolved mixture of Av1Cp1 (*18*). Since this complex was formed in the absence of MgATP, this finding provides further evidence for modulation of the redox potential driven by complex formation, in addition to those which may be associated with nucleotide binding (see ref *42*). No change in line width of the FeMoco signal nor signals attributable to oxidized P clusters were observed for the isolated complex. These findings differ from the original report on the complex isolated by gel filtration on Sephadex S-200 where an $S = {}^1/_2$ signal of reduced Cp2 was present (*26*). In the latter case it seems likely that the excess Cp2

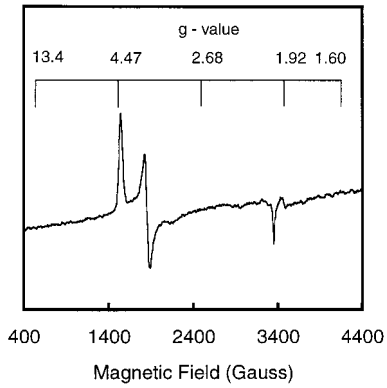

FIGURE 4: EPR spectrum of the isolated Av1·Cp2 complex in 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM sodium dithionite. The Av1·Cp2 complex was isolated using the analytical Superdex 200 column (HR 10/30). The spectrum was recorded at 10 K, 9.41 GHz, and 100 mW microwave power.

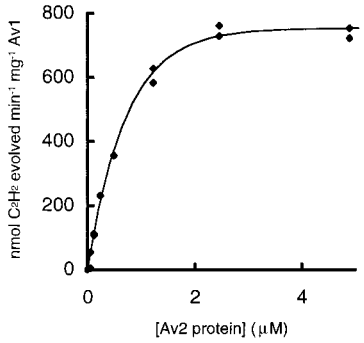

FIGURE 5: Effect of Av2 concentration on the nitrogenase activity of the isolated Av1·Cp2 complex. Standard C_2H_2 reduction assays containing 68 μg of the Av1·Cp2 complex were initiated by the addition of increasing amounts of Av2 and incubated for 10 min before the amount of C_2H_4 produced was measured by gas chromatography. The saturation level of the Av2·Av1 complex is approximately 17:1.

present in the incubation mixture may not have completely resolved from the complex, and the signal arose from this species. It has only recently been shown that the flow rate at which gel filtration columns are developed has a marked effect on the separation of free nitrogenase components from nitrogenase complexes (*24*).

Implications for Homologous Nitrogenase Turnover. The dissociation rate of the 1:1 $Av1_{red}$·$Cp2_{ox}$ complex, which is stable during the 60 min required for its isolation by gel filtration, is too slow for this species to be catalytically competent during turnover, since the rate-limiting step for H_2 evolution by Av1Cp2 nitrogenase has a rate constant of $\sim$0.032 s^{-1}. The stability of this 1:1 complex, as reflected in its behavior on gel filtration, is unaffected by the presence of MgATP; therefore, under assay conditions it cannot participate in a reaction mechanism involving its obligatory dissociation. If the species $Av1_{red}$·$Cp2_{ox}$ is stable during turnover, as our data and those of Chan et al. (*18*) indicate, then activity must arise from reactions involving the second Fe protein binding site. The activity we observe can be rationalized if the Fe protein binding sites on Av1 are no longer equivalent when only one Cp2 is tightly bound and if H_2 evolution occurs by a slow dissociative mechanism via the reactions of the Lowe−Thorneley cycle. For this to occur, the binding of a second Cp2 to the unoccupied Fe protein binding site of the Av1·Cp2 complex must trigger a global conformational change in Av1 across the $\alpha\beta$ subunit pairs, which result in the other binding site, located some $\sim$105 Å away (*15*), adopting a conformation which results in the ready dissociation of the bound Cp2. We propose the following sequence for protein complex formation: a model in which the two FeMoco sites function alternately in a shuttle mechanism with the $Av1_{red}$·$Cp2_{ox}$ species being considerably more stable than the 1:2 $Av1_{(red)2}$·$(Cp2_{ox})_2$ complex. In the initial stages of turnover, the binding of Cp2 to Av1 results in a rapid electron transfer to form the species $Av1_{red}$·$Cp2_{ox}$ which then binds a second Cp2, resulting in the transfer of a second electron, to form $Av1_{(red)2}$·$(Cp2_{ox})_2$. The chelation data (Figure 2) indicate that this latter complex is more stable than the homologous Cp nitrogenase complex. Subsequent electron transfer and proton reduction involve the prior rate-limiting dissociation of a single $Cp2_{ox}$, which is then reduced by dithionite, followed by reassociation of reduced Cp2 with the $Av1_{red}$·$Cp2_{ox}$ complex.

We favor a model in which the Fe protein binding sites of Av1 bind and release Cp2 alternately. Although the $Av1_{(red)2}$·$(Cp2_{ox})_2$ complex may be regarded as being symmetrical, there is an increasing body of experimental data indicating that the Fe protein undergoes a cascade of MgATP- and MgADP-induced conformational changes during the catalytic cycle of ATP hydrolysis and electron transfer, following binding to MoFe protein. Thus, potentially slow changes in conformation which may be required to facilitate dissociation would be expected to have occurred at the interface of Av1 and the Cp2 resident on Av1 for the longest time.

Inhibition studies of the homologous Av nitrogenase by Cp2 provide supporting evidence for such interaction between the Fe protein binding sites. It has been shown that the combining ratio of Cp2/Av1 resulting in >90% inhibition of substrate reduction by the homologous Av1Av2 nitrogenase lies between 2.4 and 2.7, *but at the 1:1 ratio only very weak inhibition is observed* (*25*). The authors concluded that a 1:1 complex of Av2 to Av1 has full activity under these conditions; i.e., the Cp2·Av1·Av2 complex is unimpaired with regard to catalytic activity. This finding is difficult to reconcile with the widely accepted scheme for nitrogenase function in which the FeMoco centers of the two halves of the $\alpha_2\beta_2$ tetramer of MoFe proteins function independently (see ref *2*), since under these circumstances such a complex should exhibit only 50% activity. Since marked inhibition occurs at ratios close to 2, these inhibition data are not consistent with preparations of Cp2 being a mixture with different affinities for Av1 but can be explained in the light of more recent findings, and the model proposed above, by the formation of a very tight Av1·$Cp2_{ox}$ complex which on interacting with a molecule of Av2 exhibits full activity.

To test this possibility the activity of the isolated 1:1 complex was measured in the presence of excess Av2. As the Av2 concentration was increased, normal saturation kinetics were observed (Figure 5). The specific activity at saturating levels of Av2 was 750 nmol of C_2H_2 reduced min^{-1} (mg of Av1)$^{-1}$, corresponding to the activity expected from a half-functional Av1; i.e., the Av1·Cp2 complex remains stable and full half-site reactivity is obtained. These results indicate that the isolated Av1·Cp2 complex persists when transferred to assay mixtures containing MgATP and the unoccupied site undergoes turnover. However, the

reactivity of the complex in the presence of Av2 contrasts with the inhibition data suggesting that the Cp2·Av1·Av2 complex has full activity (*25*). The reason for this difference is not clear at the present time.

The ratio of Cp2 to Av1 resulting in maximum activity was twice that required for the Av1·$Cp2_{ox}$ complex (compare Figures 1 and 5). This suggests that the concentration of Fe protein binding sites when Av1 was titrated with Fe protein is double that compared with the titration of the complex and is consistent with both sites functioning during H_2 evolution by the Av1·Cp2 complex, consistent with the mechanism proposed above.

Cooperativity between the Fe protein binding sites on MoFe protein has been observed during studies of the inhibition of Av nitrogenase by tight-binding mutated species of Av2 (*20*, *41*). In these experiments sigmoidal inhibition kinetics were observed. However, not all tight complexes show this behavior, and in the cases so far investigated cooperativity is only observed when catalytic activity is the measured parameter. For example, a physical study of the equilibrium between the 1:1 and 2:1 inhibited ADP·BeF_3 transition-state complexes of Kp nitrogenase components showed that the two binding sites for Kp2 on Kp1 were the same with one or both sites unoccupied (*24*). This contrasts with experiments in which the rate of formation of this complex (determined from inactivation of Kp1 containing one or both FeMoco binding sites occupied) where clear evidence of long-range conformational changes was obtained (*30*). These differences may indicate that the conformational states of MoFe protein responsible for these effects may only be significantly populated during enzyme turnover, but for long-lived complexes involving Cp2 such as the Av1·Cp2 reported here, they appear to predominate in the interaction with Fe protein.

ACKNOWLEDGMENT

We thank Prof. Barry Smith and Dr. David Lowe for stimulating and constructive discussions of the data presented here and for comments on the manuscript. We also thank Dr. Shirley Fairhurst for help with the EPR measurements and Gillian Ashby for purified Av nitrogenase.

REFERENCES

1. Howard, J. B., and Rees, D. C. (1996) *Chem. Rev. 96*, 2965−2982.
2. Burgess, B. K., and Lowe, D. J. (1996) *Chem. Rev. 96*, 2983−3011.
3. Eady, R. R. (1996) *Chem. Rev. 96*, 3013−3030.
4. Hofmann-Findeklee, C., Gadkari, D., and Meyer, O. (2000) in *Nitrogen Fixation: From Molecules to Crop Productivity* (Pedrosa, F. O., Hungria, M., Yates, M. G., and Newton, W. E., Eds.) pp 23−30, Kluwer Academic Publishers, Dordrecht.
5. Eady, R. R., and Smith, B. E. (1979) in *Dinitrogen Fixation* (Hardy, R. W. F., Bottomley, F., and Burns, R. C., Eds.) Vol. 1, pp 399−490, John Wiley & Sons, New York.
6. Emerich, D. W., and Burris, R. H. (1978) *J. Bacteriol. 134*, 936−943.
7. Lowe, D. J., and Thorneley, R. N. F. (1984) *Biochem. J. 224*, 877−886.
8. Thorneley, R. N. F., and Lowe, D. J. (1984) *Biochem J. 224*, 886−894.
9. Lowe, D. J., and Thorneley, R. N. F. (1984) *Biochem. J. 224*, 895−902.
10. Thorneley, R. N. F., and Lowe, D. J. (1984) *Biochem. J. 224*, 903−909.
11. Duyvis, M. G., Wassink, H., and Haaker, H. (1998) *Biochemistry 37*, 17345−17354.
12. Lowe, D. J., Fisher, K., and Thorneley, R. N. F. (1990) *Biochem. J. 272*, 621−625.
13. Ashby, G. A., Dilworth, M. J., and Thorneley, R. N. F. (1987) *Biochem. J. 247*, 547−554.
14. Peters, J. W., Stowell, M. H. B., Soltis, S. M., Finnegan, M. G., Johnson, M. K., and Rees, D. C. (1997) *Biochemistry 36*, 1181−1187.
15. Schindelin, H., Kisker, C., Shlessman, J. L., Howard, J. B., and Rees, D. C. (1997) *Nature 387*, 370−376.
16. Georgiadis, M. M., Komiya, H., Chakabarti, P., Woo, D., Kornuc, J. J., and Rees, D. C. (1992) *Science 257*, 1653−1659.
17. Schlessman, J. L., Woo, D., Joshua-Tor, L., Howard, J. B., and Rees, D. C. (1998) *J. Mol. Biol. 280*, 669−685.
18. Chan, J. M., Ryle, M. J., and Seefeldt, L. C. (1999) *J. Biol. Chem. 274*, 17593−17958.
19. Seefeldt, L. C., and Dean, D. R. (1997) *Acc. Chem. Res. 30*, 260−266.
20. Lanzilotta, W. N., Fisher, K., and Seefeldt, L. C. (1997) *J. Biol. Chem. 272*, 4157−4165.
21. Lanzilotta, W. N., and Seefeldt, L. C. (1996) *Biochemistry 35*, 16770−16776.
22. Duyvis, M. G., Wassink, H., and Haaker, H. (1996) *FEBS Lett. 380*, 233−236.
23. Renner, K. A., and Howard, J. B. (1996) *Biochemistry 35*, 5353−5358.
24. Clarke, T. A., Yousafzai, F. K. Y., and Eady, R. R. (1999) *Biochemistry 38*, 9906−9913.
25. Emerich, D. W., and Burris, R. H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4369−4373.
26. Emerich, D. W., Ljones, T., and Burris, R. H. (1978) *Biochim. Biophys. Acta 527*, 359−369.
27. Smith, B. E., Thorneley, R. N. F., Eady, R. R., and Mortenson, L. E. (1976) *Biochem. J. 157*, 439−447.
28. Larsen, C., Christensen, S., and Watt, G. D. (1995) *Arch. Biochem. Biophys. 323*, 215−222.
29. Smith, B. E., Eady, R. R., Thorneley, R. N. F., Yates, M. G., and Postgate, J. R. (1977) in *Recent developments in nitrogen fixation* (Newton, W. E., Postgate, J. R., and Rodreguez-Barruceco, Eds.) pp 191−203, Academic Press, New York.
30. Yousafzai, F. K. Y., and Eady, R. R. (1999) *Biochem. J. 339*, 511−515.
31. Mensink, R. E., Wassink, H., and Haarker, H. (1992) *Eur. J. Biochem.*, 289−294.
32. Nelson, M. J., Levy, M. A., and Orme-Johnson, W. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 147−150.
33. Eady, R. R., Smith, B. E., Cook, K. A., and Postgate, J. R. (1972) *Biochem. J. 133*, 655−675.
34. Duyvis, M. G., Wassink, H., and Haaker, H. (1994) *Eur. J. Biochem. 225*, 881−890.
35. Thorneley, R. N. F., and Lowe, D. J. (1983) *Biochem. J. 215*, 393−403.
36. Smith, L. A., Hill, S., and Yates, M. G. (1976) *Nature 262*, 209−210.
37. Liang, J., and Burris, R. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9446−9450.
38. Hageman, R. V., and Burris, R. H. (1978) *Biochemistry 17*, 4117−4124.
39. Deits, T. L., and Howard, J. B. (1989) *J. Biol. Chem. 264*, 6619−6628.
40. Bursey, E. H., and Burgess, B. K. (1998) *J. Biol. Chem. 273*, 29678−29685.
41. Lanzilotta, W. N., Ryle, M. J., and Seefeldt, L. C. (1995) *Biochemistry 34*, 10713−10723.
42. Seefeldt, L. C., Chan, J. M., Wu, W., and Huyett, J. E. (2000) in *Nitrogen Fixation: From Molecules to Crop Productivity* (Pedrosa, F. O., Hungria, M., Yates, M. G., and Newton, W. E., Eds.) pp 19−22, Kluwer Academic Publishers, Dordrecht.

BI0002939