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Aluminum Fluoride Inhibition of Nitrogenase: Stabilization of a Nucleotide·Fe-Protein·MoFe-Protein Complex†

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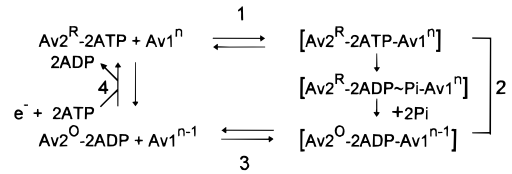
ABSTRACT: Coupling of ATP hydrolysis to electron transfer in nitrogenase has properties similar to nucleotide-dependent switch proteins. Aluminum fluoride, a powerful inhibitor of some switch proteins, is a progressive, slowly reversible ($t_{1/2}$ for reversal >21 h) inhibitor of nitrogenase that requires both component proteins (Fe-protein and MoFe-protein) and nucleotide (either ATP or ADP). The pseudo first-order inhibition is dependent on the aluminum fluoride species, AlF_4, and is linear with [Al] concentration (nonsaturating) at a pH optimum near 7.1−7.3. The inhibitor appears to react with the transient complex of the two component proteins and nucleotide. Although ADP can support the AlF inhibition, the rate of inhibition is more than 30-fold greater with ATP, which suggests the reactive conformation more closely resembles ATP hydrolysis. Conditions that increase enzymic turnover (protein concentration and component ratio) also increase the rate of inhibition, while ionic strength which slows enzymic activity spares the inhibition.. The inhibited protein was isolated by gel filtration chromatography and found to be an AlF_4-ADP-Fe-protein·MoFe-protein complex with the ratio of 2:1 that is consistent with two active sites per MoFe-protein $\alpha_2\beta_2$ tetramer. Hence, inhibition by AlF_4 is the stabilization of a complex that no longer hydrolyzes ATP or reduces substrates. We propose that AlF-ADP is tightly bound only in Fe-protein conformations obtained in the complex with MoFe-protein. Ligands (including Arg-46) at the base of a flexible flap on the Fe-protein could immobilize MoFe-protein−Fe-protein interface, thereby preventing dissociation of the complex.

An essential feature of the nitrogenase mechanism is the formation of a complex between the two metalloprotein components, the Fe-protein and the MoFe-protein[1] (Scheme 1) [see Orme-Johnson (1985) and Howard and Rees (1994), for a review of nitrogenase literature]. In this transient intermediate, two ATP are hydrolyzed and one electron is transferred (Hageman et al., 1980; Thorneley & Lowe, 1985). Only after multiple cycles of electron transfer are substrates such as dinitrogen reduced. Although Fe-protein is the ATP binding component, nucleotide hydrolysis is dependent on the association of the two proteins. These observations raise several fundamental questions including: what is the role of ATP hydrolysis in electron transfer and how does the MoFe-protein induce nucleotide hydrolysis by the Fe-protein?

Scheme 1. ATP-Dependent Electron Transfer Cycle of Nitrogenase[a]



[a]R and O, designate reduced and oxidized Fe-protein; n and $n-1$ designate increase in one electron in MoFe-protein.

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[1] Abbreviations: Av1, nitrogenase MoFe-protein component from *Azotobacter vinelandii*; Av2, nitrogenase Fe-protein component from *A. vinelandii*; AlF, the sum of all aluminum fluoride compounds present.

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Preliminary ideas about the nature of the ATP hydrolysis were based upon model building using the crystal structures of the individual components (Wolle et al., 1992b; Seefeldt et al., 1992; Howard, 1993; Howard and Rees, 1994). Because Fe-protein has a peptide fold and several conserved amino acid motifs similar to those in the G-protein super family of nucleotide-dependent switch proteins (Georgiadis et al., 1992; Wolle et al., 1992b), we proposed for nitrogenase a related nucleotide-dependent switch mechanism. Namely, we posited that ATP hydrolysis by Fe-protein causes a conformational change at the interface between the Fe-protein and the MoFe-protein. In this transient conformation the electron transfer path between donor and acceptor metal centers is opened. Subsequent expelling of the hydrolyzed phosphate would allow relaxation of the complex and closing of the electron transfer "gate". Toward the goal of spectroscopic and crystallographic studies on putative, short-lived conformational states of nitrogenase, the effects of ATP analogues on substrate reduction were investigated. We report here the isolation of an aluminum fluoride adduct of the ADP-Av2-Av1 complex which has the properties of a stabilized transition state or transient intermediate in nucleotide hydrolysis. After submitting this communication, we were made aware by one of the reviewers that a related study on nitrogenase with similar conclusions has now been published (Duyvis et al., 1996).

MATERIALS AND METHODS

Av1 and Av2 were isolated under rigorous anaerobic conditions and were characterized by gel electrophoresis, amino acid analysis, and iron analysis as previously described (Wolle et al., 1992a). The specific activities were Av1 >1800 nmol of acetylene reduced min^{-1} mg^{-1} and Av2 > 2200 nmol of acetylene reduced min^{-1} mg^{-1} at pH 8.0.

High-resolution, analytical gel filtration chromatography was performed using a 1.0 × 28 cm column of Superdex 200 (Pharmacia) connected to anaerobic fluid pumps and in line with a 500 μL anaerobic sample injection loop. The elution was monitored by observing the absorbance at 420 nm. The data were collected and processed with the Beckman System Gold software. The column buffer was pH 7.3, 100 mM MOPS containing 100 mM NaCl and 2 mM sodium dithionite. The elution volume vs molecular weight was calibrated by multiple chromatography with proteins of known size: these were aldolase, serum albumin, catalase, ferritin, carboxypeptidase A, β-galactosidase, and thyroglobulin.

Acetylene was used as substrate for nitrogenase activity measurements. Standard assays contained 5 mM $MgCl_2$, 50 mM Tris/MOPS buffer, 15 mM creatine phosphate, 0.1 mg/mL creatine kinase, and 15 mM sodium dithionite. ATP, aluminum chloride, and sodium fluoride concentrations, time of incubation, and pH were varied as part of the experimental protocol. Individual conditions are given in the figure legends. Final reaction volume was 1.0 mL assay solution in 8.9 mL serum vials with an argon atmosphere containing 1.0 mL of acetylene gas. Assay temperature was 30 °C. At specified times, the reaction was stopped by adding glacial acetic acid and the ethylene in the gas phase was quantified by gas chromatography. The efficiency (coupling) of ATP hydrolysis to substrate reduction was calculated from simultaneously measuring substrate reduction and ATP equivalents used by increase in creatine (Ennor, 1957).

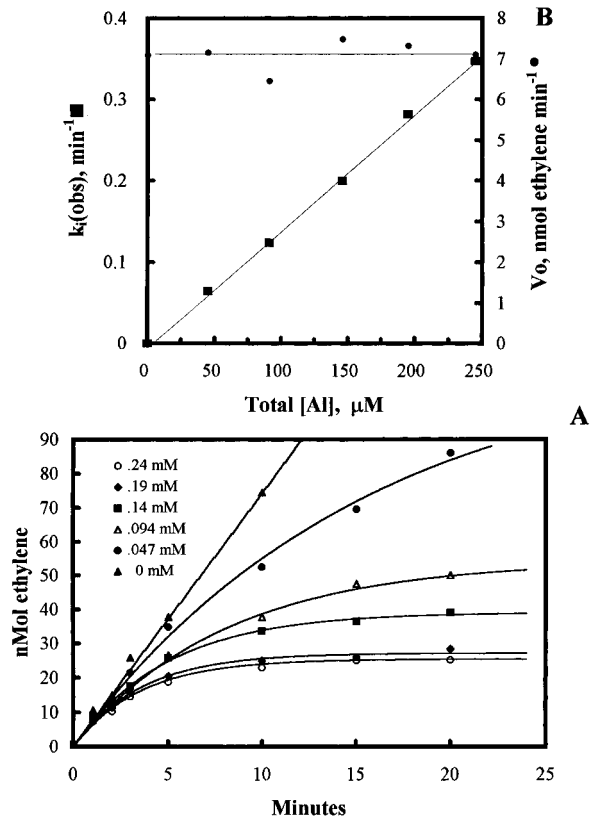

FIGURE 1: Inhibition of nitrogenase by aluminum fluoride at pH 7.3. (A) Av1 and Av2 were added to standard assay vials containing aluminum chloride and 5 mM NaF. Assays were quenched at the indicated times, and the ethylene in the gas phase was quantified. Solid lines are the nonlinear least-squares fit of the data using the equation given in the text. (B) Plot of reaction parameters obtained for the time course in A.

Aluminum and fluoride in buffered aqueous solution form compounds of the general composition $AlF_x(OH)_y$ (Martin, 1988). The mole fraction of each compound was calculated using the reported formation constants (Martin, 1988) and is expressed as a function of the total aluminum or fluoride concentration. In the absence of fluoride, insoluble $Al(OH)_3$ dominates at neutral pH. To avoid precipitation of aluminum, $AlCl_3$ was added as a 5 mM solution to buffered NaF in assay mixtures. All reaction concentrations of aluminum were calculated to be below the solubility product for $Al(OH)_3$.

RESULTS AND DISCUSSION

AlF with GDP or ADP has been found to mimic the triphosphate and thereby inhibit some nucleotide-dependent switch proteins (Chabre, 1990). In preliminary nitrogenase assays, using the standard, single 8 min incubation time, only a small decrease in activity was detected in the presence of AlF. However, the potency of inhibition became apparent when the full time course was monitored (Figure 1A). Initially, substrate reduction was minimally affected, yet by 20 min the activity had nearly ceased and the inhibition was complete. In control experiments, no inhibition was observed if fluoride or aluminum was omitted or if either nitrogenase component was incubated individually with AlF and MgATP before diluting into the complete assay. The nonlinear, time-dependent activity loss is characteristic of "slow" or "pro-

gressive" inhibitors of the form

$$E + S \rightleftarrows [ES] \xrightarrow{k_{cat}} E + P$$

$$[I] \updownarrow$$

$$[EI] \overset{k_i}{\rightleftarrows} EI^*$$

where the product formed with time is given by[2]

$$P = v_0(1 - e^{-k_i(obs)t})/k_i(obs)$$

for k_i(obs), the observed inhibitor rate constant, and v_o, the initial rate of product formation (Morrison & Walsh, 1988). The solid lines in Figure 1A are the nonlinear least-squares fit of the data at different total aluminum concentrations. Because the fluoride concentration is in large excess in this experiment, all AlF species increase in proportion to the added aluminum. The plots (Figure 1B) of the rate parameters from curve fitting vs aluminum concentration indicate AlF binds weakly, if at all, prior to the inactivation step. That is, AlF does not exhibit competitive inhibition with substrate reduction. For example, v_o is independent of the aluminum concentration while the inhibition rate constant, k_i(obs), is linear in this concentration range.[3] Likewise, incubation of the enzyme with AlF prior to initiating substrate reduction with ATP, had no effect on v_o or the time for onset of "slow" inhibition. These results indicate AlF inhibition is a second order reaction and the k_i(obs) is given by

$$k_i(obs) = k_i[AlF]$$

The nature of the AlF species that leads to inactivation was investigated by varying the pH and fluoride concentration. A full time course of activity was determined for each condition, and the kinetic parameters were obtained by curve fitting. The initial rate for substrate reduction, v_o, was not altered by the inhibitor (data not show) which confirmed the apparent lack of AlF binding previously inferred from the data in Figure 1B. In the pH range 5−8 and with millimolar fluoride, three AlF species, AlF_3, AlF_4, and AlF_3OH, are calculated to account for most of the aluminum. The mole fractions of each AlF species and the experimental, k_i(obs), are plotted in Figure 2. In both data sets, the rate of inactivation most closely follows the concentration of AlF_4 indicating the latter is the most likely reactive species.

In addition to aluminum and fluoride, Mg, ATP, and both protein components must be present for inhibition, indicating that enzymic turnover is an essential condition. Thus, factors that alter the enzymic turnover rate should also affect the rate of inhibition. For example, because the formation of the MoFe-protein·Fe-protein complex is a mass action equilibrium (step 1, Scheme 1), nitrogenase activity is proportional to both the component ratio and total protein concentration. As shown in Figure 3, the rate of AlF inhibition (k_i(obs)) increased as a function of component ratio and total protein concentration and was proportional to the level of overall enzymic activity. Most importantly, the apparent time of onset of inhibition decreased as the overall enzymic activity increased. This would be expected if AlF were reacting with an intermediate. However, as observed for the other conditions, the initial rate of substrate reduction was unaltered by AlF.

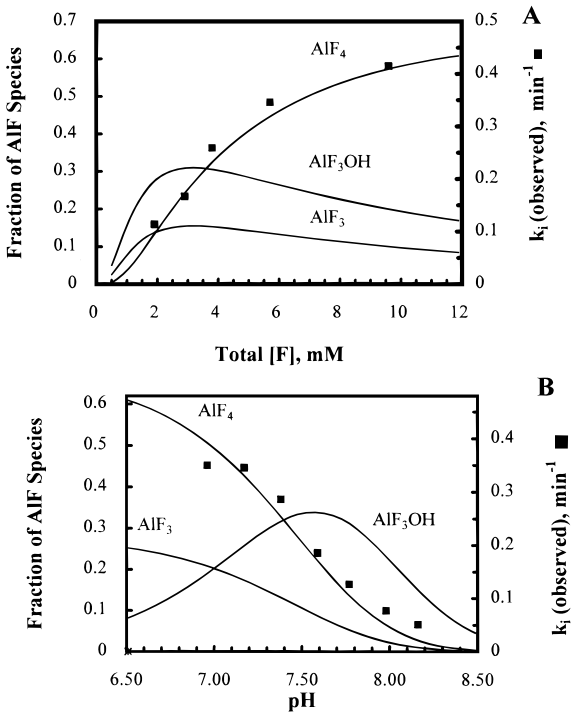

FIGURE 2: AlF species leading to inhibition of nitrogenase. Av1 and Av2 were added to standard assay vials and time course was evaluated as in Figure 1. Solid lines are the calculated mole fraction for each AlF species. (A) pH 7.3, [NaF] concentration varied at constant 194 μM aluminum chloride. (B) pH of assays adjusted with constant 240 μM aluminum chloride and 5 mM NaF.

A second condition that alters nitrogenase activity by affecting formation of the MoFe-protein·Fe-protein complex is ionic strength (Burns et al., 1985; Deits and Howard, 1990). Under turnover conditions, salts are competitive with either component (competitive inhibition at step 1, Scheme 1). Hence, inhibition of complex formation should "spare" quasiirreversible AlF inhibition. To investigate this hypothesis, the nitrogenase components were incubated with AlF, a MgATP generating system, and various concentrations of NaCl. Samples were withdrawn and diluted 50-fold (to an AlF concentration below the threshold for inhibition), and the residual activity was determined. As shown in Figure 4, NaCl had a significant "sparing" effect on AlF inhibition. The concentration for 50% sparing (ca. 150 mM) was identical to that for 50% reversible salt inhibition under the same conditions.

Because in some nucleotide-dependent switch proteins AlF, in combination with GDP or ADP, mimics the triphosphate, we considered nitrogenase intermediates that bind or exchange nucleotides as potential sites for AlF inhibition. In experiments not shown here, AlF did not alter the K_m for ATP nor change the efficiency of ATP coupling. At high Av2 ratio, 2.1−2.3 ATP were hydrolyzed per electron transferred to substrate throughout the inhibition time course such that ATP hydrolysis ceased when substrate reduction was fully inhibited. That is, the inhibited enzyme does not turn over ATP. Nevertheless, if AlF replaces a phosphate

[2] This equation can be written to include a slow reversal of EI* to accommodate noncovalent inhibition and includes an expression for product formation at steady state (Morrison & Walsh, 1988). Curve fitting using the more general expression indicated that the steady-state rate for the AlF inhibited nitrogenase is negligible and can be omitted from the initial rate analysis.

[3] AlF binding and nonlinear aluminum dependence cannot be excluded at higher, impractical aluminum concentrations.

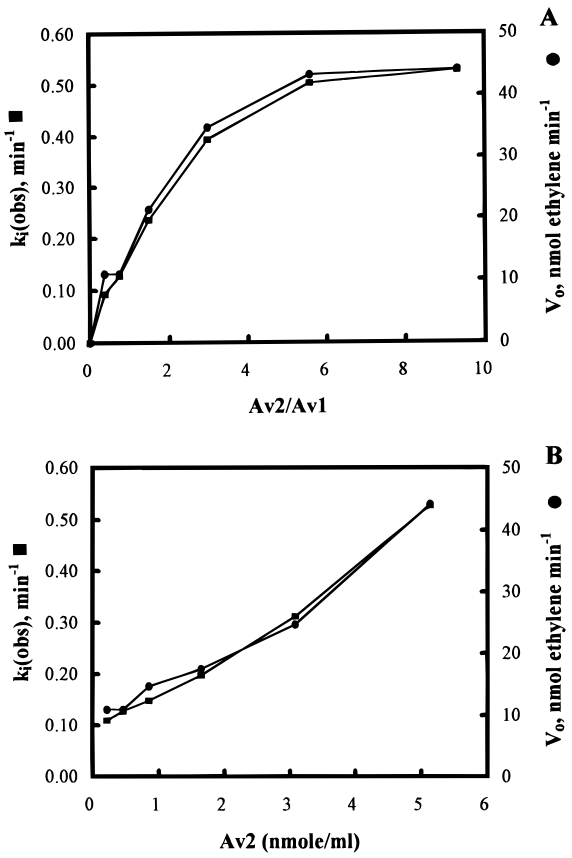

FIGURE 3: AlF inhibition of nitrogenase at 194 μM aluminum chloride and 8 mM NaF at pH 7.3. Time course obtained and evaluated as in Figure 1. (A) Ratio of Av2/Av1 varied by adjusting Av2 concentration at constant Av1 (0.53 μM active sites) (B) Ratio Av2/Av1 constant with total protein concentration varied. Data are reported in terms of Av2 concentration.

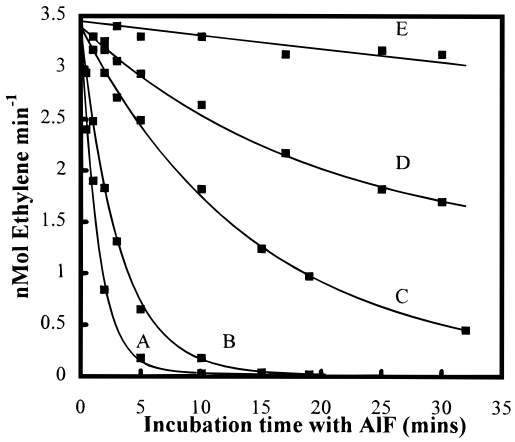

FIGURE 4: Effect of NaCl on AlF inhibition of nitrogenase. Av1 and Av2 were incubated with 194 μM aluminum chloride, 6 mM NaF, 1.5 mM MgATP, and a 15 mM creatine phosphate regeneration system at pH 7.3. At the indicated times, samples were diluted 50-fold into standard 8 min acetylene reduction assays (no added salt). (A) 75 mM NaCl; (B) 175 mM NaCl; (C) 275 mM NaCl; (D) 375 mM NaCl; (E) 475 mM NaCl. Solid lines are the least-squares fit of the data to a first order exponential decay.

during ATP turnover, then, by microscopic reversibility, AlF plus MgADP should also be inhibitory. When AlF, MgADP, and *both* components (pseudoenzymic turnover conditions) are incubated, the enzyme is progressively inactivated as determined in subsequent standard assays (Figure 5). Neither component alone was inhibited by MgADP plus AlF.[4] Under the identical conditions, the rate of inactivation with MgATP

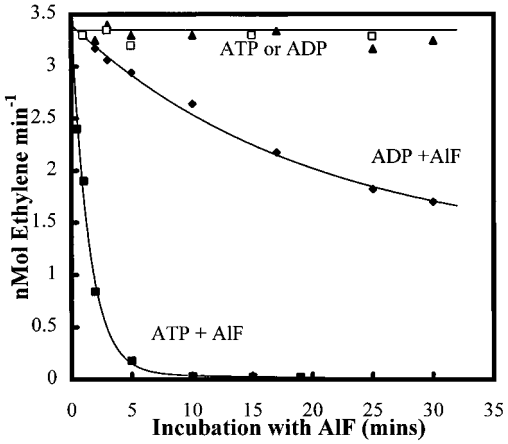

FIGURE 5: Nucleotide requirement for MgADP or MgATP in AlF inhibition of nitrogenase. Av1 and Av2 were incubated with 194 μM aluminum chloride, 6 mM NaF, and either 1.5 mM MgATP, and a 15 mM creatine phosphate regeneration system *or* 1.5 mM MgADP without a regenerating system at pH 7.3. At the indicated times, samples were diluted 50-fold into standard 8 min acetylene reduction assays. Solid lines are the least-squares fit of the data to a first-order exponential decay. Control experiments are enzyme incubations without AlF.

was >30 times that with MgADP, a strong indication that the AlF-trapped conformation originates directly from ATP hydrolysis. Because AlF inhibition with MgATP is much faster and is carried out in the presence of an ATP regenerator, it is unlikely that inhibition with ATP is from the accumulation of free ADP during turnover.

In sum, the kinetic evaluation points to AlF reacting, quasiirreversibly, with a form of the enzyme that is transiently activated by ATP hydrolysis, most likely the two-component complex (step 2, Scheme 1). To detect such a complex, a reaction mixture containing AlF inhibited nitrogenase was separated on an analytical column of Superdex S-200. The salient results are shown in Figure 6. Av1 or Av2 with or without MgATP/AlF had elution volumes for their individual molecular weights predicted by the column calibration. Likewise, only the individual components were detected in uninhibited (no AlF) reactions (Figure 6A). However, when the AlF-inhibited mixture was separated, there was a new higher molecular weight species of ca. 370 000 (Figure 6C). Integration of the protein peaks indicated that absorbance of the 370 000 molecular weight material was entirely accounted for by the decrease in the Av2 and Av1 peaks. The experiments in Figure 6 have identical protein concentrations (Av2 in ca. 15-fold molar excess) and clearly show complete conversion of Av1 to this new material. Figure 6B shows an intermediate time of reaction (ca. 2 min) where the activity indicated partial inhibition. For this reaction time, the elution time of the higher molecular weight peak was intermediate between Av1 and that observed for complete inhibition. This peak was broadened presumably as a mixture of Av1 and higher molecular weight species. With excess Av1 (data not shown), the Av2 peak disappeared, and a broad peak containing the excess Av1 and higher molecular weight material also was observed.

[4] A well documented property of free Fe-protein is a conformational change induced by binding MgATP that allows iron chelation from the metallocluster; MgADP inhibits the chelation reaction (Walker & Mortenson, 1973; Ljones & Burris, 1978; Deits & Howard, 1989). AlF plus MgADP was unable to induce the chelation state (Renner and Howard, unpublished results).

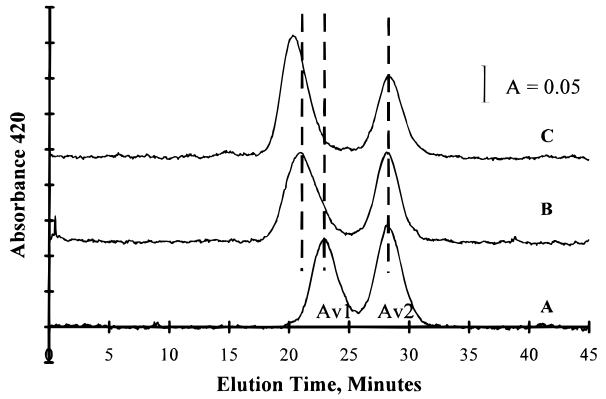

FIGURE 6: Separation of nitrogenase components by gel exclusion chromatography. Reaction mixtures of Av1 and Av2 were applied to a 1.0 cm × 28 cm column of Superdex S-200 equilibrated with pH 7.3 in 100 mM MOPS/Tris buffer containing 100 mM NaCl. (A) Nitrogenase assay reaction mixture after 10 min incubation, no AlF. (B) Nitrogenase assay reaction mixture after 2 min incubation with AlF. (C) Nitrogenase assay reaction mixture after 30 min incubation with AlF. Assay reaction was 5 mM Mg ATP and 15 mM creatine phosphate regenerating system at 30 °C. For B and C, 145 μM $AlCl_3$ and 6 mM NaF were included.

Table 1: Properties of Isolated 370 000 Molecular Weight Complex[a]

(a) molecular weight	found by chromatography, 370 000 calculated (Av1 = 233 000, Av2 63 000); 359 000 for 2 Av2/1/Av1
(b) component ratio	Av2·Av1; 1.95
(c) nucleotide ratio	2.8−3.4 ADP/complex or 1.4−1.7 ADP/Av2
(d) fluoride ratio	3.6 ± 0.8 F^-/ADP
(e) $t_{1/2}$ dissociation	>21 h

[a] Except for the fluoride analysis, the data are the average for three different samples. The component ratio was determined by analytical SDS gel electrophoresis using individual components of known concentration as standards and by amino acid analysis. Nucleotide content was determined from the amount of tritium in the isolated complex and the specific activity of the [8-^{3}H]ATP used to generate the complex. The specific activity of the ATP in the initial reaction mixture was determined by quantitative nucleotide ion exchange chromatography and scintillation counting of a portion. Protein concentration was determined by amino acid analysis. Fluoride concentration (average five samples from one protein preparation) was determined with a fluoride specific electrode after acid precipitation of the proteins. The dissociation of the complex was estimated by regain of acetylene reduction activity and by integration of the elution profile from a chromatogram following incubation of the isolated complex for 18 h at room temperature. The complex generated by starting with ADP had identical gel chromatography elution properties and the same stoichiometry as the complex formed with ATP.

The 370 000 molecular weight material prepared using [8-^{3}H]ATP was isolated by gel chromatography and the composition determined. Some of the properties of the material are given in Table 1. By four criteria the 370 000 molecular weight material is a complex of Av1 and Av2: (1) Av2 and Av1 were found in a molar ratio of *ca.* 2 Av2/1/Av1 by gel electrophoresis in sodium dodecyl sulfate; (2) the absorbance of complex formed was equivalent to the combined decrease in absorbance of Av1 and Av2; (3) amino acid analysis of the high molecular weight material was consistent only with the ratio of 2 Av2/1/Av1, and (4) Edman degradation gave the expected three amino acid residues at each cycle consistent with an Av1·Av2 complex.

An important property of the complex is the tight binding of [^{3}H]adenosylnucleotide. By contrast, no nucleotide was associated with excess, free Av1 or Av2 in the presence of AlF. Even under turnover conditions, individual protein components do not form stable AlF-nucleotide complexes. When [γ-^{32}P]ATP was used in the inhibition reaction, no radioactivity was found in the complex, from which we conclude that the adenosylnucleotide form in the complex is ADP. Likewise, protein bound fluoride was found only in the complex and in a ratio of ca. 4 F^-/ADP. This is consistent with the formation of an AlF_4 adduct with the ADP-bound conformation of the Fe-protein·MoFe-protein complex.

The stoichiometry of the complex is best fit as 2.7−3.4 ADP/2/Av2/1/Av1. Because MoFe-protein is an $\alpha_2\beta_2$ tetramer having two active sites, the presence of two Av2 is consistent with both sites being occupied. Less clear is the interpretation of the intermediate ADP composition (1.4−1.7 instead of 2 ADP/Av2). While free Fe-protein has two nucleotide binding sites (one for each identical subunit), in the complex it appears there is less than full occupancy of two ADP sites, yet formation of the Av1·Av2 complex is complete. Hence, the stabilization of the complex may need only one of the two nucleotide sites to be occupied.

Several approaches were used to assess reversibility of the complex. Partial recovery (ca. 25%) of enzymic activity was found after incubating the isolated complex in a standard assay mix without AlF for 920 min at 21 °C. The fractional regain in activity correlated with the appearance of free Av1 and Av2 on gel filtration (figure not shown). After 21 h, approximately 45% of the Av2 originally part of the complex was free while the absorbance peak representing the higher molecular weight material was broadened and asymmetrical. The $t_{1/2}$ for dissociation of the complex was estimated to be >21 h in the absence of AlF, and likely, indefinitely stable in the presence of AlF.

How AlF might stabilize the ADP·Av2·Av1 complex can be considered in light of the recently reported structures of the trimeric G-protein, transducin (G_α), and myosin with various nucleotide analogues (Sondek et al., 1994; Fisher et al., 1995). Although AlF could stabilize the interface between Av1 and Av2 directly, more likely is the stabilization of the nucleotide binding site. In the high-resolution structure of G_α, Arg-178 and Gln-204 stabilize an octahedral array of the Al moiety containing four equatorial fluoride atoms, a bridging oxygen to the β-phosphate, and an axial water molecule. This has been described as a likely transition state for nucleotide hydrolysis. Arg-178 and Gln-204 are from opposite sides of the nucleotide binding region and, as ligands to the AlF, stabilize the nucleotide binding cleft. These interactions and other salt bridges appear to be unique in the AlF-GDP analogue structure.

A core of conserved structures and residues that approximate the positions of the comparable residues in the G-protein family compose the nucleotide binding site of myosin (Fisher et al., 1995). Likewise, in myosin, the nucleotide binding region connects two domains or the "jaws" of the actin binding site with substantial movement between halves of the nucleotide binding site when AlF-ADP is bound. The structural homology between G-proteins, myosin, and Av2 is compelling, and, remarkably, there is a counterpart to Arg-178 of G_α and Arg-238 of myosin, namely, Av2 Arg-46.[5]

[5] J. B. Howard, manuscript in preparation.

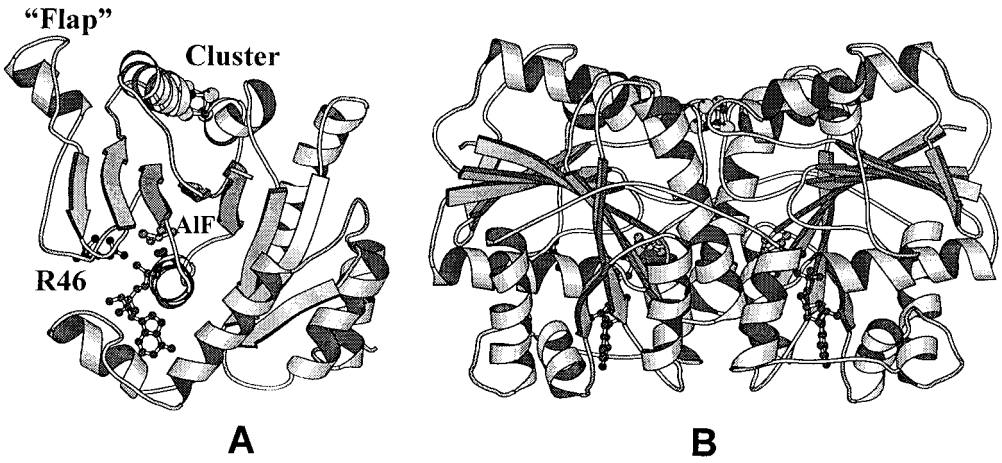

FIGURE 7: Ribbons diagram of the polypeptide fold of the Av2 dimer with ball and stick models of Fe·S cluster, Arg-46, and overlay of G_α GDP-AlF. View A is ca. 90° rotation toward the viewer around the molecular two-fold axis of view B. To simplify view A, the background subunit has been omitted. Prepared with the program MOLSCRIPT (Kraulis, 1991).

Figure 7 shows the Av2 structure with AlF_4 and MgGDP overlaid from the G_α-protein coordinates after aligning the "P-loop" and Asp 125 (a putative Mg ligand) with their counterparts in G_α-protein. In Av2, the putative ATP binding site is beneath the likely MoFe-protein binding face, and events in nucleotide hydrolysis must be transmitted by some mechanism of signal transduction to the surface (Georgiadis et al., 1992; Wolle et al., 1992b; Howard, 1993). The Av2 Arg-46 is at the base of a loop or flap connected to the core Av2 subunit fold. The top of the flap is part of a pocket of acidic residues that has been identified as a major interaction site with MoFe-protein (Willing and Howard, 1990; Howard, 1993; Peters et al., 1994). Hence, it is tempting to speculate that AlF may stabilize the ADP·Av2·Av1 complex by a series of interactions across the flap−core interface which in turn stabilizes interactions between Av1 and Av2 at the acidic pocket on the surface. Interestingly, the flap region in the crystal structure of Av2 is the least well defined and is somewhat disordered (Georgiadis et al., 1992). Hence, this region is mobile and flexible as would be expected for a binding interface with Av1. Although there are several nucleotide complexes that must exist during nitrogenase catalysis, AlF appears to stabilize the unique conformation only obtained as a consequence of ATP hydrolysis and only arises when both protein components are appropriately aligned. We suggest that this is the condition or conformation closest to that for electron transfer.

Analogues that resemble the transition state are potentially strong, quasi irreversible inhibitors and hence stabilize otherwise temporary macromolecular conformations. With the nitrogenase system, we have the potential to study the class of molecular switches that form protein−protein complexes during nucleotide hydrolysis. This has prospects for elucidating common themes in a wide range of other nucleotide-dependent proteins.

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