Kinetics of MgATP-dependent Iron Chelation from the Fe-Protein of the Azotobacter vinelandii Nitrogenase Complex

EVIDENCE FOR TWO STATES*

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(Received for publication, November 2, 1988)

Chelation of Fe from the Fe-protein component (Av2) of Azotobacter vinelandii nitrogenase has been investigated. The chelation, which requires MgATP binding by Av2, is best described as a two-exponential process. The rates for the two phases differed by ~10-fold and increased as the concentration of MgATP was increased. The rates for both phases were 50% of maximum at approximately 1.5 mM MgATP. At MgATP concentrations >100 mM, the more rapid phase represented ~25% of the total Fe chelated from Av2. However, below 1000 mM MgATP, the proportion of the faster phase decreased until at 20 μM MgATP, only a single phase could be detected.

The properties of Av2 were studied at various stages of Fe chelation. The partially chelated protein was isolated from the reaction by gel filtration and was subjected to a second MgATP-dependent Fe chelation. Material isolated after the completion of the first phase regained biphasic kinetics in subsequent chelation reactions. However, if MgATP was present during the isolation of Av2, then only a single phase was observed in the subsequent chelation studies. In addition, the enzymatic activity of Av2 decreased concomitantly with total Fe chelation.

To account for these observations, a model is presented in which Av2 exists in two conformers. Fe chelation is proposed to occur from either conformer but only when two MgATP are bound. Both conformers bind MgATP with the same affinity but are distinguished by a 10-fold difference in chelation rate. The two conformers are in equilibrium and can interconvert only in the absence of MgATP. That is, MgATP binding prevents the conversion of the two conformational states.

Biological reduction of dinitrogen to ammonia is catalyzed by the nitrogenase complex which is composed of the Fe-protein and the MoFe-protein. The MoFe-protein contains the substrate binding and reduction site(s) while the Fe-protein serves as the unique electron donor for the reaction. For every electron transferred to the MoFe-protein, two ATP are bound and hydrolyzed by the Fe-protein (1, 2). Because the Fe-protein is a single electron donor, multiple cycles of electron transfer and ATP hydrolysis are needed for the reduction of nitrogenase substrates (3). Each electron transfer appears to involve a cycle of association/dissociation of the protein complex (4, 5).

The Fe-protein has a single 4Fe:4S cluster which bridges the two identical subunits of the protein. The four cysteinyl ligands for the Fe:S center are residues 97 and 132 from each subunit (residue numbering is based upon the protein sequence of Av2) (6). Although the exact mechanistic role of ATP hydrolysis in substrate reduction is not known, MgATP binding to the Fe-protein induces a conformational change that alters the properties of the Fe center. For example, the midpoint potential, E½, decreases ~100 mV (7, 8), the EPR spectrum becomes more axial (9), and the magnetic circular dichroism spectrum changes (10).

One of the most striking effects of MgATP binding by the Fe-protein is the change in the reactivity of the Fe:S cluster with chelators. Walker and Mortenson (11, 12) and Ljones and Burrus (13) found that the Fe center was rapidly removed by chelators in the presence of MgATP but not in the presence of MgADP or in buffer alone. Furthermore, MgADP inhibited the MgATP-induced Fe chelation. The specificity and MgATP dependence of the reaction have been exploited to investigate other properties of the enzyme. For example, the amount of MgATP-dependent Fe chelation is proportional to the enzyme activity and can be used to estimate the fraction of active Fe:S center present (13). Also, using iodoacetic acid to label cysteinyl residues exposed during the Fe chelation reaction, the putative cluster ligands were identified (6). Finally, the nucleotide-binding constants have been estimated from the initial rate of chelation as a function of MgATP concentration (12–15).

The time course for Fe chelation from reduced Fe-protein was described by previous workers as kinetically complex yet was not further characterized (11, 12). Furthermore, Fe chelation from oxidized Av2 occurs in two sequential steps with the formation of a discrete 2Fe:2S intermediate (16). Thus, a detailed analysis of the kinetics of Fe chelation from reduced Av2 also might reveal new properties of the protein and its Fe:S center. The time course of Fe chelation might provide a basis for comparing the reduced and oxidized states of Av2. Furthermore, in order to use the rate of Fe chelation as a measure of protein conformational change induced by nucleotide binding or by other agents, the relationship of initial rates to the full time course of Fe removal should be established. Finally, Fe chelation may prove a useful tool to probe subtle conformational differences between mutant forms of the protein.

* This work was supported, in part, by the Science and Education Administration, United States Department of Agriculture Grant 82-CRCA-1-1119, and National Institutes of Health, Grant GM 34321. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Av2, the Fe protein from Azotobacter vinelandii; Av1, the Mo-Fe protein from A. vinelandii; AdPPNP, 5'-adenyl-imidodiphosphate (AMP-PNP).
In this paper, we describe the kinetics for the full time course of Fe chelation from reduced Av2. As we observed for oxidized Av2, the removal of Fe is biphasic. However, unlike the sequential chelation of Fe from oxidized Av2, the Fe appeared to be removed, without intermediates, from two different forms of reduced Av2. The two forms, which interconvert in the absence of nucleotide, had a 10-fold difference in rate of Fe chelation.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, creatine phosphate, creatine kinase, and 2,2'-bipyridyl were obtained from Sigma. MgATP solutions were prepared by dissolving MgCl₂ and ATP in 50 mM Tris-HCl, pH 8.0 followed by titrating the solution to pH 8.0 with NaOH. The concentration of ATP solutions was determined spectrophotometrically using ε₃₂₅₀nm = 1.53 × 10⁵ M⁻¹ cm⁻¹. ATP, creatine phosphate, and creatine kinase solutions were prepared fresh daily. MgATP concentrations were determined from the known concentrations of Mg and ATP and the stability constant, 5.01 × 10⁻³ M⁻¹ (17). For the concentrations of Mg and ATP used in these experiments, MgATP need not be considered (17).

To facilitate the preparation of 400 mM stock solutions of 2,2'-bipyridyl, the suspension of the chelator in 50 mM Tris-HCl, pH 8.0, was warmed until the crystals melted and then was mixed vigorously. Because 2,2'-bipyridyl solutions are unstable for periods greater than a few minutes, they were prepared weekly. The chelator concentration was determined using ε₃₃₀nm = 1.51 × 10⁴ M⁻¹ cm⁻¹ in 50 mM HCl (18). The ε₅₂₀nm for the Fe(2,2'-bipyridyl) was 8.4 × 10⁴ M⁻¹ cm⁻¹ (11). For the o-bathophenanthroline disulfonate Fe complex, ε₅₃⁵nm = 2.21 × 10⁵ M⁻¹ cm⁻¹ was used (13).

The manipulations of enzymes and reagents were carried out under an Ar atmosphere on a Schlenk manifold or in a glove box. Hamilton gas tight syringes and stainless steel cannules were used for solution transfers. Ar was purified by passing the gas over heated BASF catalyst.

**Enzyme Purification and Characterization**—Avl and Av2 were purified by a modification of the method of Burgess et al. (15). The average specific activity of eight preparations of Av2 used in our experiments was 2770 ± 240 (mean ± S.D., n = 8) mol H₂ formed min⁻¹ mg⁻¹. Protein concentrations were determined by amino acid analyses. Upon treatment of Av2 with o-bathophenanthroline disulfonate Fe complex, ε₅₃⁵nm = 2.21 × 10⁵ M⁻¹ cm⁻¹ was used (13).

The manipulations of enzymes and reagents were carried out under an Ar atmosphere on a Schlenk manifold or in a glove box. Hamilton gas tight syringes and stainless steel cannules were used for solution transfers. Ar was purified by passing the gas over heated BASF catalyst.

**Enzyme Assay**—Nitrogenase catalyzed H₂ formation was determined in serum vials (13.4 ml volume) with 1.0 ml of assay mixture containing 50 mM Tris-HCl, pH 8.0, 6 mM ATP, 6 mM MgCl₂, 6 mM creatine phosphate, 0.1 mg of creatine kinase, and 15 mM Na₂S₂O₄. Prior to the addition of Na₂S₂O₄, the vials were deaerated by nine alternating cycles of evacuation and filling with Ar. The assay was initiated by addition of Av2 to vials containing Avl at 30 °C.

**Kinetic Analysis of Fe Chelation**—Absorbance data were collected in a Beckman model DU-8S spectrophotometer equipped with a thermostat-controlled, six-position cell changer. The data were stored in a North Star computer via the RS232 port on the spectrophotometer. Because analysis of observations taken at a constant time interval weights too heavily later stages of a reaction, a subset of the data points was selected using a constant absorbance change or the time point of the full reaction time course. A data weighting scheme based on a particular model was considered inappropriate.

The selected data were analyzed by nonlinear regression programs described by Bevington for the Marquardt algorithm (20). The programs have been adapted for North Star FBASIC by Dr. Eric Eccleston of our laboratory. All data sets were fit to equations consisting of 1, 2, and 3 exponential terms, with the absorbance value at infinite time (Ainf) permitted to vary as an adjustable parameter. The calculated Ainf value deviated by less than 3% from the experimentally determined value, but optimum convergence of the experimental results with the exponential equations required this additional degree of freedom.

The statistical parameter, chi-squared (χ²), was calculated for the fit of the data to equations 1-3 (see below). To determine which equation best fit the data, the χ² were compared as ratios. A ratio >20 was taken to indicate a preferential fit of the data to the equation with the smaller χ².

**RESULTS**

**Time Course of Fe Chelation**—A number of investigators have shown that the chelation of Fe from Av2 requires MgATP; with chelator alone or with MgADP, only small quantities of Fe not associated with catalytically active Fe-S centers are removed (6, 11–15). At saturating concentrations of MgATP and high concentrations of chelator, the reaction is complete in less than 10 min. However, if the reaction is slowed by reducing the chelator concentration, the full time course of the Fe chelation can be measured and is found to be kinetically complex. In Fig. 1A is shown the absorbance change with time for one concentration of reagents. When the absorbance is plotted as a logarithmic function versus time, the complexity of the kinetics becomes apparent; namely, there is a significant deviation from linear behavior at intermediate and late time points (see Fig. 1B).

In order to obtain a quantitative measure of the curvature, the data were analyzed by a nonlinear least squares procedure (see "Experimental Procedures"). The data were fit to Equations 1, 2, and 3 having one, two and three exponential terms, respectively.

\[
\begin{align*}
\text{Abs}_i &= P \exp(-k_a t) + \text{Abs}_{\text{inf}} \\
\text{Abs}_i &= P \exp(-k_a t) + P \exp(-k_b t) + \text{Abs}_{\text{inf}} \\
\text{Abs}_i &= P \exp(-k_a t) + P \exp(-k_b t) + P \exp(-k_c t) + \text{Abs}_{\text{inf}}
\end{align*}
\]

2 Nonlinear least squares procedure used for rate constants: kₐ, kₐ, kₐ refer to the observed rate constants experimentally measured, rate constants for Fe chelation obtained by curve fitting the data to Equations 1–3; k values with numerical subscripts refer to specific rate constants defined by Schemes 1–3.
Detection of both exponential terms. The more rapid rate constant, $k_2$, has a pre-exponential term ($P_1$) that represents $\sim 25\%$ of the total absorbance change. The remaining $75\%$ of the absorbance change is associated with the pre-exponential term ($P_2$) of the slower phase, $k_3$ (Equation 2). Thus, the Fe chelation occurs as a "burst" in absorbance followed by a slower development of the remaining absorbance. In subsequent figures, the contribution of the first phase to the chelation kinetics will be expressed as $\%P_1$, where $\%P_1 = (100 \times P_1)/(P_1 + P_2)$.

**Biphasic Fe Chelation as an Intrinsic Property of Av2**—A series of control experiments were undertaken to demonstrate that the observed kinetic complexity of Fe chelation is a property of Av2 and not a consequence of the experimental conditions or protein purification methods. A potential source of the kinetic complexity could be the generation of an inhibitor during the chelation reaction. For example, MgADP, the product of nitrogenase turnover, is a potent inhibitor of the MgADP-regenerating system was included in all our experiments. The capacity of the regenerating system was verified by adding Av1 (up to 1 molar %) to Fe chelation reaction mixtures. Even this relatively large amount of Av1 had no effect on the kinetics of the Fe chelation reaction as long as the complete MgATP regeneration system was present (Fig. 2).

Other potential inhibitors, such as the products of the Fe
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Fig. 2. Effect of ATP-regenerating system and full time course of Fe chelation from Av2. Reaction mixture contained 4.0 mM 2,2'-bipyridyl, 11.3 nM Av2, 2.0 mM creatine phosphate, and 1.0 mM ATP (other reaction components as described under "Experimental Procedures"). Reactions were initiated by addition of ATP at time 0. (i) 0.1 mg of creatine phosphokinase; (ii) 0.5 nM Av1; (iii) 0.5 nM Av1 and 0.1 mg of creatine phosphokinase.

Table I

| [Avn] (uM) | 1.25 | 3.1 | 6.4 | 24.0 | 60 |
|------------|------|-----|-----|-----|----|
| %P1        | 11.2 | 19.1| 27.5| 60  |    |

TABLE II

| Time sample | %Fe removed from protein (observed) | %Fe removed from protein (calculated, 2 steps) | %Fe removed from protein (calculated, 1 step) |
|-------------|-----------------------------------|-----------------------------------------------|---------------------------------------------|
| min         |                                   |                                               |                                             |
| 0           | 0.50                              | 0.108                                         | 0.108                                       |
| 20          | 0.50                              | 0.012                                         | 0.012                                       |
| 30          | 0.50                              | 24.0                                          | 24.0                                        |
| 60          | 0.50                              | 60                                            | 60                                          |

* [Av2] = 39.5 nM, [2,2'-bipyridyl] = 3.0 mM, [ATP] = 0.5 mM. Reaction was initiated with ATP, and quenched at the indicated time by addition of 10 mM ADP. In control experiments, it was shown that MgADP immediately stopped the absorption change due to chelation of Fe from Av2. The reaction mixture was transferred anaerobically to a 1.0 x 10-cm Sphadex G-25 column equilibrated with deoxygenated 0.05 M Tris, pH 8.0, containing 3 mM Na2SO4, 0.5-mL fractions were collected. The Fe content of the protein and salt peaks were determined. The observed data represents the percentage of the Fe removed from the protein at the indicated times. An identical reaction mixture was monitored spectrophotometrically, and the reaction time course was fit to Equation 2.

Expected percentage of Fe removed from Av2 calculated from the kinetic parameters in footnote a assuming two steps of Fe chelation.

Expected percentage of Fe removed from Av2 assuming no Fe was chelated in the initial rapid phase of a sequential process. Based upon this assumption, a differential equation was derived to calculate the theoretical values.

Chelation reaction (apoAv2, Fe-2,2'-bipyridyl complex, or sulfide), had no effect on the reaction time course (data not shown). Likewise, successive Fe chelation experiments performed without removing the products of the previous reaction had biphasic kinetics identical to the initial reaction. In addition, Av2 purified by an alternate method omitting the heat step exhibited the same chelation kinetics. Finally, a biphasic reaction time course with a %P1 of ~25% was also observed with the chelator o-bathophenanthroline disulfonate (data not shown). Thus, the two-exponential character of the Fe chelation time course appears to be an intrinsic property of Av2.

Competence of MgAdPNNP to Induce Fe Chelation—The results above do not exclude the possibility that Fe chelation may be a result of MgATP hydrolysis and enzymic turnover of Av2. To investigate this possibility, the nonhydrolyzable MgATP analog, MgAdPNNP, was used in place of MgATP. Not only was MgAdPNNP competent to induce complete Fe chelation, but also the kinetics of the reaction were best described by Equation 2 (Table I). The slower rates observed with MgAdPNNP probably are due to Av2 not being saturated with the analog. Thus, hydrolysis of MgATP is not a necessary condition for Fe release nor does it produce the observed biphasic kinetics.

Both Kinetic Phases Represent Fully Chelated Fe—The work of Cowart et al. (21) indicates that Fe is chelated from transferrin in a biphasic process. For transferrin, the first phase was shown to be a spectral change due to the formation of a mixed ligand complex of chelator and protein; the removal of Fe occurred only in the second phase. We examined the possibility that the early kinetic phase in our experiments also simply represented a spectral change. Fe chelation experiments were performed in which the amount of protein-bound Fe and of chelated Fe was determined by gel filtration. The results are shown in Table II. Even at one min the Fe chelated from Av2 was that expected if both kinetic phases involved the chelation and release of the Fe from the protein.

Thus, our results with Av2 are different from those with transferrin.

Dependence of Fe Chelation on the Concentration of Av2—The interaction between Av2 molecules was considered as a potential mechanism for generating the observed complex time course of Fe chelation. The effect of varying the concentration of Av2 was studied in a series of Fe chelation experiments, and the results are presented in Fig. 3. While there are changes in the apparent rate constants, %P1 and the ratio of $k_a$ to $k_b$ remain unaltered. That is, the biphasic nature of Fe chelation is not a consequence of protein concentration.

Loss of Enzymatic Activity with Fe Chelation—The Fe:S cluster of Av2 is essential to its catalytic activity. We examined the relationship between Fe chelation and the catalytic activity of Av2 by removing samples of Av2 from an Fe chelation reaction mixture and assaying for Av2 catalytic activity. The results are shown in Fig. 4. Despite the higher level of uncertainty inherent in the methods for activity

Control experiments showed no effect of 2,2'-bipyridyl in the nitrogenase assay under these conditions.
The rate of Fe chelation from Av2 is dependent upon the Fe chelation mixture. Kinetic analysis of spectrophotometric data; control experiment was performed in which ATP was omitted from the reaction mixture. A duplicate Fe chelation experiment was monitored in the spectrophotometer. A control experiment was performed in which ATP was omitted from Fe chelation mixture. Kinetic analysis of spectrophotometric data; $k_a = 0.104 \text{ min}^{-1}$, $k_b = 0.009 \text{ min}^{-1}$, $\%P1 = 16.6$. Kinetic analysis of activity data; $k_a = 0.088 \text{ min}^{-1}$, $k_b = 0.012 \text{ min}^{-1}$, $\%P1 = 13.8$.

**FIG. 4.** Loss of nitrogenase activity during the course of Fe chelation from Av2. The Fe chelation experiment contained 0.6 mM ATP, 4.0 mM 2,2'-bipyridyl, and 10 $\mu$M Av2. At timed intervals, 0.3-ml samples of the reaction mixture were transferred anaerobically to assay vials (see “Experimental Procedures”) containing Av1 in an amount optimal for the expression of Av2 activity. Assays were run for 8 min and analyzed for Fe chelation. In order to obtain measurable chelation rates over the range of MgATP concentrations to be studied, the concentration of 2,2'-bipyridyl was varied (see legend to Fig. 6). For those MgATP concentrations where the 2,2'-bipyridyl concentration was changed, a reaction was run at both the higher and lower chelator concentrations. Once $k_a$ and $k_b$ were corrected measurements, the time course of activity loss also appears to be biphasic. Furthermore, the kinetic parameters for activity loss and Fe chelation are in reasonable agreement. Most importantly, partial enzyme activity persisted after the first phase of Fe chelation and was not completely lost until all Fe was chelated.

**Effect of 2,2'-Bipyridyl Concentration on Fe Chelation**—In part, the rate of Fe chelation from Av2 is dependent upon the concentration of chelator (Fig. 5). For all concentrations of 2,2'-bipyridyl studied, the reaction was biphasic. Both rate constants ($k_a$ and $k_b$) were second order in chelator concentration above ~1 mM chelator (Fig. 5A) and not a consequence of the experimental protocol. Most importantly, partial enzyme activity persisted after the first phase of Fe chelation and was not completely lost until all Fe was chelated.

**FIG. 5.** 2,2'-Bipyridyl concentration dependence of Fe chelation from Av2. Reactions were carried out with 10 $\mu$M Av2 and 1.0 mM ATP.

**FIG. 6.** ATP concentration dependence of Fe chelation from Av2. Reactions carried out with 10 $\mu$M Av2. MgCl$_2$ concentration was equimolar to that of ATP at ATP concentrations above 1 mM and was 1.0 mM for all ATP concentrations below 1.0 mM. MgATP concentration was varied in three steps (2.4 mM, 4.8 mM, and 9.6 mM) as the concentration of ATP was reduced. At each concentration of ATP where 2,2'-bipyridyl concentration was changed, a reaction was run for both chelator concentrations. The data were corrected for the second order dependence of $k_a$ and $k_b$ on 2,2'-bipyridyl concentration (see Fig. 5) and are expressed here for 2.4 mM chelator. Solid lines represent theoretical curves generated from Equation 4 using $k_b = 0.6 \text{ min}^{-1}$, $k_a = 0.06 \text{ min}^{-1}$, $k_1 = 0.02 \text{ min}^{-1}$, $k_2 = 0.0063 \text{ min}^{-1}$, $D = B = 1.0$, and $K_i = K_i' = 0.62 \text{ mM}$ (see "Discussion").
rected for the second order dependence upon 2,2'-bipyridyl concentration (see Fig. 5), the two reactions were found to have identical kinetic parameters. The results were normalized to a single chelator concentration 2.4 mM and are presented in Fig. 6.

The rate constants $k_a$ and $k_b$ had a similar sigmoidal dependence on the MgATP concentration and reached maximum values above 2-3 mM MgATP. The most striking effect of MgATP concentration was the change in $\%P_1$ (see Fig. 6C). Even though the individual rate constants increased 4-5-fold above $\sim 75 \mu M$ MgATP, $\%P_1$ remained constant. As the concentration of MgATP was decreased below 75 mM, $\%P_1$ decreased concomitantly. This shift to a first order process at low MgATP is evident in the semilogarithmic plot of Fig. 7 where the second phase is no longer discernible, c.f. the results in Fig. 1 for a higher MgATP concentration. Curve fitting analysis confirmed the presence of only one exponential process. Note that $\sim 95\%$ of the Fe was chelated in the single, slow phase observed at low MgATP concentration. Thus, all Fe is accessible to chelation at all MgATP concentrations by either the apparent biphasic or monophasic process.

Regulation of the Kinetic Heterogeneity of Fe Chelation by MgATP—The results presented in the section above indicate that the apparent number of kinetic phases as well as the rate of Fe chelation are controlled by the MgATP concentration. This raises the question as to whether the observed two phases at higher ATP concentrations indeed represent two subpopulations of Fe. Equation 2 predicts that at late time points the remaining Fe should be composed of a single, kinetically homogeneous subpopulation. To test this hypothesis, an Fe chelation reaction was conducted at a MgATP concentration (1 mM) sufficient to exhibit maximal biphasic kinetics. After $\sim 4$ half-lives of the first phase, the chelation rate was substantially accelerated by the inclusion of additional MgATP, yet the progress curve remained a single exponential process. As predicted from the results of previous experiments (Fig. 6), the rate of chelation obtained a maximal value at $\sim 2-3$ mM MgATP. Thus, we conclude that when the first phase is complete, the second becomes as a homogeneous population of molecules. Furthermore, the addition of MgATP is not, in itself, sufficient to induce biphasic reaction kinetics. Comparable results are obtained when the reaction is accelerated at late time points with 2,2'-bipyridyl (data not shown).

The results shown in Fig. 8 suggest that a kinetically homogeneous form of Av2 exists in the second phase of Fe chelation. Isolation of the protein at this point should provide material for further study to determine the properties of at least one of the Fe subpopulations. The Av2 remaining at late time points in the chelation reaction was isolated by ion exchange or gel filtration chromatography. When the reisolated protein was subjected to a further Fe chelation experiment, the time course of the reaction was again biphasic, with kinetic properties of native Av2. Thus, the isolation process apparently was sufficient to restore kinetic heterogeneity to Av2. That is, removal of a reaction component might have permitted regeneration of the two Fe subpopulations. To identify which, if any, component was involved, the protein from the Fe chelation reaction was isolated by gel filtration on columns equilibrated with either 2,2'-bipyridyl or MgATP. The reisolated Av2 was subjected to a second Fe chelation experiment and the kinetic pattern determined. For protein isolated in the presence of 2,2'-bipyridyl, a biphasic time course indistinguishable from native Av2 was observed. In contrast, Av2 isolated in the presence of MgATP had essentially a single exponential time course (Table III). Thus, not only is MgATP required for Fe chelation from Av2 but it appears to affect the conversion of different species of Fe which, in turn, are responsible for the biphasic kinetics of chelation.

In the experiments above, the initial MgATP concentration was sufficiently high that the Fe chelation was biphasic and the reaction behaved as though it contained two Fe populations. In contrast, when the concentration of MgATP is below $\sim 75 \mu M$, the reaction appears to be a single exponential process throughout (see Fig. 8). This raises new, corollary questions: at low MgATP, does the reaction contain a single population of Fe and can the reaction be made biphasic if it was initially monophasic?

To investigate these questions, the Fe chelation reaction was performed using the appropriate low MgATP concentration to produce a single exponential time course. After $\sim 60\%$ of the Fe was chelated, the MgATP concentration was increased to 0.2 mM. As shown in Fig. 9, the reaction was accelerated and immediately became biphasic. Although the two new phases accounted for only the last 40% of the Fe to be chelated, they had the same $\%P_1$ and ratio of $k_a$ to $k_b$ as observed for reactions which were biphasic throughout. Furthermore, the rates for the two phases were identical to those obtained for a reaction initially at the higher MgATP concentration. These experiments demonstrate the unique ability of MgATP to induce the expression of kinetic heterogeneity in Av2. In addition, we conclude that heterogeneity among Fe
Effect of isolation conditions on the chelation kinetics of Av2

Data for $k_1/k_0$ and $\%P_1$ are presented as the numerical mean of experiments with the standard deviation in parentheses. Reactions were initiated with $[\text{Av2}] = 40 \mu M$, $2,2'-$bipyridyl $= 3.0 \, mM$, and $[\text{ATP}] = 0.5 \, mM$. After 3-4 half-lives of the rapid reaction phase, as determined in duplicate spectrophotometric studies, the reaction mixture was chromatographed. For gel filtration experiments, the reaction mixture was applied anaerobically to a $1 \times 10$-cm Sephadex G-25 column equilibrated with $25 \, mM$ Tris-Cl, $pH$ 8.0, containing $3.0 \, mM \text{Na}_2\text{S}_2\text{O}_4$, with the indicated additional compounds. For DEAE-chromatography, the reaction mixture was applied to a $0.7 \times 4$-cm DEAE-Sepharose column equilibrated with $25 \, mM$ Tris-Cl, $pH$ 8.0, containing $3.0 \, mM \text{Na}_2\text{S}_2\text{O}_4$. The protein peak was eluted with $0.5 \, mM$ NaCl in the same buffer. In both gel filtration and DEAE procedures, the protein peak was collected directly into an anaerobic spectrophotometer cuvette and subjected to a second round of Fe chelation.

Table III

| Resolution conditions | $k_1/k_0$ | $\%P_1$ | $X_1/X_2$ | No. of experiments |
|-----------------------|-----------|---------|------------|-------------------|
| Starting material     | 9.6 (0.4) | 19 (2)  | 38         | 3                 |
| DEAE-chromatography   | 9.4 (0.9) | 17 (2)  | 59         | 4                 |
| Gel filtration ($pH 8.0$) | 9.1 (0.5) | 15 (2)  | 90         | 3                 |
| Gel filtration (+ 3 mM ATP) | 10.4 (0.9) | 15 (2)  | 64         | 4                 |
| Gel filtration (+ 3 mM ATP) | 4.2 (4)  | $<5$ (3) | 4          | 3                 |

Fig. 9. Effect of increased concentrations of MgATP at late time points in Fe chelation from Av2. A, Fe chelation was initiated in two cuvettes containing 10 $\mu M$ Av2, 20.0 $\mu M$ 2,2'-bipyridyl, and 10 $\mu M$ ATP. At the time indicated by the arrow, ATP (final concentration of 0.2 $\mu M$) was added to one cuvette; the other was left unchanged. B, expanded time scale for rapid Fe chelation following addition of ATP (data from A).

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DISCUSSION

Model for Fe Chelation—Two limiting models described by Equation 2 can be considered for the biphasic Fe chelation from Av2. Fe could be chelated sequentially in two partial reactions (Scheme 1), or the Fe could be removed by parallel reactions from two different sub-populations of Av2 (Scheme 2). An additional possibility, that the first phase represents formation of a mixed ligand complex of chelator and protein, has been excluded by the gel filtration experiments (Table II). Although Schemes 1 and 2 are kinetically equivalent, their specific rate laws are considerably different and can be distinguished by experimental conditions.

$$\text{Av2}(4\text{Fe}) \xrightarrow{k_1} \text{Av2}(x\text{Fe}) \xrightarrow{k_2} \text{apoAv2}$$

**Scheme 1**

$$\text{Av2} (4\text{Fe}) \xrightarrow{k_1} \text{apoAv2}$$

$$\text{Av2'} (4\text{Fe}) \xrightarrow{k_2} \text{apoAv2}$$

**Scheme 2**

However, neither of these mechanisms is sufficient to account for the experimental results. For example, the sequential model (Scheme 1) posits the formation of an intermediate with fewer than the original four Fe. Yet, the enzymatic activity decreased simultaneously with removal of all four Fe, not with just the first phase (Fig. 4). This would require the unlikely condition that Av2 with fewer than four Fe be catalytically active. In addition, Av2, reisolated after the first phase of Fe chelation, exhibited the same biphasic Fe chelation behavior as the original protein (Table III). For Scheme 1 to account for either of these results, the original Fe center would have to be reconstituted during the subsequent manipulation of the protein. Although other Fe:S proteins are known to reform active Fe centers from fragmented clusters, such reconstitution has not been found in the presence of chelator as is our condition (22, 23).

Likewise, the parallel model of Scheme 2 is not consistent with all the experimental results. Because both rate constants for Fe chelation have the same dependence on MgATP (see Fig. 6), Scheme 2 is inadequate to explain the change in number of phases as the concentration of MgATP is altered. As for Scheme 1, it is difficult to rationalize by Scheme 2 how the two phases of Fe chelation could be re-established after exhaustion of the first phase and isolation of the protein (Table III).

To overcome the limitations in Schemes 1 and 2, we have developed Scheme 3 as a minimal model consistent with our chelation data. Scheme 3 is a modification of Scheme 2 with
the significant addition that the two subpopulations of protein (Av2a and Av2b) are allowed to interconvert but only in the absence of MgATP. One of these forms (arbitrarily Av2a) is approximately 10-fold more reactive toward Fe chelation, i.e. $k_1$ is 10 times $k_2$. As in the other schemes, chelation of Fe can occur only when two MgATP are bound. To allow for possible co-operativity in nucleotide binding, interaction constants $B$ and $D$ have been included.

Scheme 3 appears to qualitatively account for several of the enigmas unexplained by Schemes 1 and 2. At low concentrations of MgATP, a high proportion of the Av2 molecules are in the nucleotide free form and the conversion of Av2a and Av2b is more rapid than chelation. Thus, the reaction is from a pool of components in rapid equilibrium and the observed rate is a weighted average of the two pathways. The result is that the reaction appears monophasic and slow (see Fig. 7).

As the concentration of MgATP is increased, the rate of conversion between the nucleotide-free forms is less than $k_2$, which allows the two pathways to be expressed as biphasic kinetics. Over these intermediate MgATP concentrations, a high proportion of the Av2 molecules are in the nucleotide free form and the conversion of Av2a and Av2b is more rapid than chelation. Thus, the reaction is from a pool of components in rapid equilibrium and the observed rate is a weighted average of the two pathways. The result is that the reaction appears monophasic and slow (see Fig. 7).

The striking feature of the model is the requirement for the MgATP binding and dissociation to occur only when two MgATP are bound. Thus, the reaction is from a pool of components in rapid equilibrium and the observed rate is a weighted average of the two pathways. The result is that the reaction appears monophasic and slow (see Fig. 7).

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Finally, when conversion of Av2a is blocked by the higher MgATP concentrations, the more reactive Av2a will be depleted in the rapid initial phase leaving a kinetically homogeneous material, Av2b (see Fig. 8). It is this latter material which, if isolated in the presence of MgATP, retains a single kinetic phase in subsequent chelation reactions yet can re-equilibrate to the two forms in the absence of nucleotides (see Table III).

In order to investigate Scheme 3 in quantitative detail, we have solved the relevant differential equations, assuming that

\[ \frac{d[Av2a(\text{ATP})]}{dt} = k_1[Av2a(\text{apo})] - k_2[Av2b(\text{apo})] \]

\[ \frac{d[Av2b(\text{apo})]}{dt} = k_2[Av2b(\text{apo})] - k_1[Av2a(\text{apo})] \]

These equations have a large number of variables which, we anticipate, might allow for multiple solutions. To fit the data, we have limited ourselves to those we consider potentially relevant to the nitrogenase enzymology. We have constrained the two rate law constants, $k_1$ and $k_2$, to the limiting, observed rates at saturating MgATP. $K_s$ and the co-operativity were considered to be the same for both conformers and limited to approximately 2 to 3. One set of values clearly best fit the experimental results (see Fig. 6). The striking feature of the model is the requirement for the very slow rate of conversion between Av2a and Av2b. However, other slow conformational changes have been observed in macromolecules such as the cis-trans isomerization, the binding of slow inhibitors, and in the complement protein family of C-3, C-4, and C-2-macroglobulin (24-26). In order for a faster rate of conversion between Av2a and Av2b to be accommodated by Scheme 3, a large negative cooperativity for MgATP binding and $\mu M$ dissociation constants would be required. We considered these parameters outside the acceptable limits of the previously reported binding constants (1, 2, 8).

Most importantly, Equation 4 appears adequate to describe the full time course of Fe chelation from Av2 in the presence of nucleotides and chelators. Our results appear to require two active conformers of Av2. It should be noted that there are other reports of multiple forms of nitrogenase Fe-protein. For example, recent low temperature Mossbauer, magnetic susceptibility, and EPR results on reduced Av2 have been interpreted as indicating the presence of Av2 molecules in two spin states, $S = 1/2$ and $S = 5/2$ (27-29). Because the Fe:S center is affected by the protein isomerization in all of these
phenomena, there is intrinsic interest in the properties of the conformations. Although a synthesis of the origin of the various multiple forms is not yet possible, it seems reasonable that there is some underlying correlation which is related to the unique property of the Fe-protein as the specific electron donor in nitrogenase.

Regardless of the physical interpretation of the differences in Fe:S cluster reactivity, the present study makes clear that the interaction of Av2 with MgATP is more complex than previously realized. That is, factors other than MgATP binding per se can dramatically affect the reactivity of the Fe:S cluster to chelators; these factors establish the reactivity difference between Av2a and Av2b. In addition, the ability of MgATP to inhibit equilibration between conformers of Av2 reveals a previously unnoticed allosteric effect in Av2. This effect should be borne in mind when correlation is made between structure and function. For example, MgATP may restrict protein flexibility and may bias the enzyme toward catalytically active conformations. Further, care must be taken that all Av2 molecules in a reaction comprise a single ensemble, or that the rate of protein isomerization is taken into consideration. Finally, the isomerization properties of Fe-protein may be altered by selective mutation. This study provides a basis to assess the effects of the mutations.

APPENDIX

For Scheme 3, we propose the presence of two conformers of Av2, Av2a, and Av2b, which equilibrate via k3 and k4. Fe chelation can occur only from those forms of Av2a and Av2b which have 2 MgATP bound. MgATP is designated as S in the equations below. Binding and dissociation of MgATP are assumed to be rapid compared to k1, k2, k3, and k4. The dissociation constants for MgATP with AvPa and Av2b are K2 and K3, respectively. Other abbreviations employed are B and D, which represent the interaction between the two MgATP molecules on Av2a and Av2b, respectively; [Av2] is the concentration of all Fe-containing Av2 molecules; [Av2a] and [Av2b] are the concentrations of the conformers; and m and n are the stoichiometries of Fe in Av2a and Av2b, respectively. The molar extinction coefficients of Av2a, Av2b, and the Fe(2,2'-bipyridyl)S complex at 520 nm are represented as EAv2a, EAv2b, and E(Fe(2,2'-bipyridyl)S), respectively.

Solution of Differential Equations for Scheme 3—Two differential equations can be written describing the net flux of Av2a and Av2b. From conservation of mass,

\[ \Sigma \text{Av2a} = \text{[Av2a]} + \text{[SAv2a]} + \text{[Av2aS]} + \text{[SAv2aS]} \]

Substituting,

\[ \Sigma \text{Av2a} = \text{[Av2a]} + \frac{2[S][\text{Av2a}]}{K_2^2} + \frac{[S]^2[\text{Av2a}]}{B(K_3)^2} \]

where

\[ W = 1 + \frac{2[S]}{K_2^2} + \frac{[S]^2}{B(K_3)^2} \]

The first differential equation for flux of Av2a is

\[ \frac{d(\Sigma \text{Av2a})}{dt} = k_3[\text{Av2b}] - k_3[\text{Av2a}] - k_1[\text{SAv2aS}] \]

Substituting,

\[ \frac{d([\text{Av2a}])}{dt} = \frac{k_3}{X} \frac{[\text{Av2b}]}{[\text{Av2a}]} - \frac{k_1}{X} \frac{[\text{SAv2aS}]}{[\text{Av2a}]} \]

where

\[ X = \frac{WB(K_3)^2}{S^2} \]

The analogous equations for Av2b are

\[ \Sigma \text{Av2b} = Y[\text{Av2b}] \]

where

\[ Y = 1 + \frac{2[S]}{K_1^2} + \frac{[S]^2}{D(K_4)^2} \]

The differential equation for the net flux of Av2b is

\[ \frac{d(\text{Av2b})}{dt} = \frac{k_3}{Y} \frac{[\text{Av2a}]}{[\text{Av2b}]} - \left( \frac{k_1}{Z} + \frac{k_1}{Y} \right) \frac{[\text{Av2b}]}{[\text{Av2a}]} \]

where

\[ Z = Y \frac{D(K_4)^2}{B} \]

Substituting Equation 2 into the derivative of Equation 1 with respect to time and rearranging gives

\[ \frac{d([\text{Av2a}])}{dt} + \left( \frac{k_1}{X} + \frac{k_1}{Z} + \frac{k_1}{W} + \frac{k_1}{Y} \right) \frac{[\text{Av2a}]}{dt} = 0 \]

A solution to this differential equation is

\[ [\text{Av2a}], = C_1 \exp^{r_1 t} + C_2 \exp^{r_2 t} \]

where

\[ r_1 = \frac{-(G + H)}{2} \quad \text{and} \quad r_2 = \frac{-(G - H)}{2} \]

and

\[ G = \frac{k_1}{X} + \frac{k_2}{Z} + \frac{k_1}{W} + \frac{k_1}{Y} \]

and

\[ H = \left[ \frac{k_1}{X} + \frac{k_2}{Z} + \frac{k_1}{W} + \frac{k_1}{Y} + \frac{4k_3k_4}{WY} \right]^{1/2} \]

and C1 and C2 are constants established by the initial conditions.

Evaluation of C1 and C2—Assume that Av2a and Av2b are in equilibrium via k3 and k4 at zero time. Their respective initial concentrations, [Av2a], and [Av2b], are given by

\[ [\text{Av2a}], = \frac{k_3[\text{Av2}],}{(k_1 + k_3)} \]

and

\[ [\text{Av2b}], = \frac{k_3[\text{Av2}],}{(k_1 + k_3)} \]

where [Av2], is the initial concentration of all Av2 present. Furthermore, at t = 0, Equation 3 becomes

\[ [\text{Av2a}], = C_1 + C_2 \]

The derivative of Equation 3 is

\[ \frac{d([\text{Av2a}])}{dt} = r_1 C_1 + r_2 C_2 \]

Substituting Equation 1 at t = 0 and Equation 4 into Equation 5 gives

\[ C_1 = \left( \frac{1}{r_1 - r_2} \right) \left[ \frac{k_3[\text{Av2}],}{W} - \left( \frac{k_3}{W} + \frac{k_1}{X} \right) [\text{Av2}], \right] \]

Since, by equilibrium,

\[ k_1[\text{Av2}], = k_3[\text{Av2}], \]

the equation simplifies to

\[ C_1 = - \left( \frac{[\text{Av2}],}{(r_1 - r_2)} \right) \frac{k_1}{X} \]
By substituting back into Equation 4, 
\[ C_z = \frac{[Av2a]_0}{(r_1 - r_2)} \frac{k_z}{X + r_1} \]
Substituting the above values for \( C_1 \) and \( C_2 \) into Equation 3 gives the full differential equation 
\[ \frac{[Av2a]_0}{(r_1 - r_2)(k_0 + k_t)} \left[ \left( \frac{k_2}{X} + r_1 \right) \exp^{(y)} - \left( \frac{k_1}{X} + r_1 \right) \exp^{(y)} \right] \]
and 
\[ \frac{[Av2b]_0}{(r_1 - r_2)(k_0 + k_t)} \left[ \left( \frac{k_2}{Z} + r_2 \right) \exp^{(y)} - \left( \frac{k_2}{Z} + r_2 \right) \exp^{(y)} \right] \]

**Evaluation of Equations in Terms of Absorbance Change** — For the Fe chelation reaction from Av2 with 2,2'-bipyridyl, rates of reaction are determined from the net absorbance change at 520 nm and can be expressed, at time \( t \), as 
\[ \Delta Abs = \frac{m([Av2a] - [Av2a]_0) + n([Av2b] - [Av2b]_0)}{(r_1 - r_2)(k_0 + k_t)} \]

Substituting Equation 9 into Equation 8 yields 
\[ \Delta Abs = \frac{[Av2a]_0 (r_1 - r_2)(k_0 + k_t)}{(r_1 - r_2)(k_0 + k_t) + \epsilon_{Fe}(2,2'bp) \epsilon p} \]
Substituting Equations 6 and 7 into Equation 10 gives 
\[ \Delta Abs = \frac{\epsilon_{Fe}(2,2'bp) \epsilon p (m([Av2a] - [Av2a]_0) + n([Av2b] - [Av2b]_0))}{(r_1 - r_2)(k_0 + k_t) + \epsilon_{Fe}(2,2'bp) \epsilon p} \]

Equation 11 can be simplified for our purposes by setting \( m = n = 4 \) and setting the molar extinction coefficients for Av2a and Av2b equal to that of Av2 determined in bulk. The result is 
\[ \Delta Abs = \epsilon_{Fe}(2,2'bp) \epsilon p \frac{P_1 \exp^{(y)} + P_2 \exp^{(y)}}{(r_1 - r_2)(k_0 + k_t)} \]
where 
\[ P_1 = \frac{([Av2a]_0 (r_1 - r_2)(k_0 + k_t) \left[ \left( \frac{k_2}{X} + r_1 \right) + k_0 \left( \frac{k_2}{Z} + r_2 \right) \right]}{(r_1 - r_2)(k_0 + k_t)} \]
and 
\[ P_2 = \frac{([Av2b]_0 (r_1 - r_2)(k_0 + k_t) \left[ \left( \frac{k_2}{X} + r_1 \right) + k_0 \left( \frac{k_2}{Z} + r_2 \right) \right]}{(r_1 - r_2)(k_0 + k_t)} \]

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