Pre-steady-state Kinetics of Nitrogenase from Azotobacter vinelandii

EVIDENCE FOR AN ATP-INDUCED CONFORMATIONAL CHANGE OF THE NITROGENASE COMPLEX AS PART OF THE REACTION MECHANISM*

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Martina G. Duyvis, Hans Wassink, and Huub Haaker‡

From the Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

The pre-steady-state electron transfer reactions of nitrogenase from Azotobacter vinelandii have been studied by stopped-flow spectrophotometry.

With reduced nitrogenase proteins after the initial absorbance increase at 430 nm (which is associated with electron transfer from the Fe protein to the MoFe protein and is complete in 50 ms) the absorbance decreases, which, dependent on the ratio [Av2]/[Av1], is followed by an increase of the absorbance. The mixing of reductant-free nitrogenase proteins with MgATP leads after 20 ms to a decrease of the absorbance, which could be fitted (from 0.05 to 1 s) with a single exponential decay with a rate constant \( k_{\text{obs}} = 6.6 \pm 0.8 \text{ s}^{-1} \). This reaction of nitrogenase was measured at different wavelengths. The data indicate the formation of a species with a blue shift of the absorbance of metal-sulfur clusters of nitrogenase from 430 to 360 nm.

The absorbance decrease at 430 nm observed (after 50 ms) in the case of the reduced nitrogenase proteins could only be simulated well if, after the initial electron transfer from the Fe protein to the MoFe protein and before dissociation of the nitrogenase complex, an additional reaction was assumed. The rate constant of this reaction was of the same order as the rate constant of the MgATP-dependent pre-steady-state proton production by nitrogenase from A. vinelandii: \( k_{\text{obs}} = 14 \pm 4 \text{ s}^{-1} \) with reduced nitrogenase proteins and \( k_{\text{obs}} = 6 \pm 2 \text{ s}^{-1} \) with dithionite-free nitrogenase proteins (Duyvis, M. G., Wassink, H., and Haaker, H. (1994) Eur. J. Biochem. 225, 881–890).

It is proposed that in the presence and absence of reductant, the observed absorbance decrease at 430 nm of nitrogenase is caused by a change of the conformation of the nitrogenase complex, as a consequence of hydrolysis of MgATP.

Biological nitrogen fixation, which is the reduction of nitrogen to ammonia, is catalyzed by the enzyme system nitrogenase (EC 1.18.6.1). The molybdenum-containing nitrogenase of Azotobacter vinelandii consists of two metalloproteins, which cooperate in catalysis: the MoFe protein (Av1) and the Fe protein (Av2).

The MoFe protein is an \( \alpha_2\beta_2 \) tetramer and contains two types of metal-sulfur clusters: the FeMo cofactor (FeMoco), which contains Fe, S, Mo, and homocitrate, and the P-cluster, which contains Fe and S. FeMoco is generally considered to be the site of substrate reduction. Recent studies have indicated that electrons flow from the Fe protein via the P-cluster to FeMoco (2). For both clusters, structural models have been proposed by Kim and Rees (3), based on crystallographic analysis of the MoFe protein, but there is some discussion about the structure of the P-clusters (4, 5).

The other nitrogenase protein, the Fe protein (Av2), consists of two identical subunits sharing a single [4Fe-4S] cluster and contains two binding sites for MgATP and MgADP. The crystallographic structure of this protein has been determined by Georgiadis et al. (6). A remarkable similarity between the nucleotide binding sites of the Fe protein and of the molecular switch protein Ha-Ras p21 was found (6). The binding of MgATP or MgADP to the Fe protein leads to a decrease of the redox potential, changes in the EPR spectrum, and changes of the conformation of the Fe protein (1).

During nitrogenase catalysis, the Fe protein transfers electrons to the MoFe protein in a MgATP-dependent reaction. The generally accepted kinetic description of the mechanism of action of nitrogenase was developed by Lowe and Thorneley (7, 8). The model consists of two coupled cycles, the Fe protein cycle and the MoFe protein cycle. In the Fe protein cycle, the association of the reduced Fe protein and the MoFe protein to the nitrogenase complex is followed by the transfer of a single electron from the Fe protein to the MoFe protein, with concomitant hydrolysis of MgATP. After this, the nitrogenase complex dissociates; this is the rate-limiting step of the cycle (7). The Fe protein is reduced by the present reductant (flavodoxin or ferredoxin in vivo; sodium dithionite in vitro) and MgADP is replaced by MgATP. For the complete reduction of \( N_2 \) to 2NH_3 and \( H_2 \) (which is an inevitable side product of the nitrogenase reaction), eight electrons are needed. The sequence of eight Fe protein cycles, during which the MoFe protein is stepwise reduced, constitutes the MoFe protein cycle. In this scheme, one of the two independently functioning halves of the MoFe protein, which has been subjected to \( n \) Fe protein cycles and is consequently reduced by \( n \) electrons, is written as \( E_n \) (8). The rate constants of the reactions that constitute the Fe protein...
cycle are assumed to be independent of the level of reduction of the MoFe protein (9).

The presence of MgATP is an absolute requirement for electron transfer from the Fe protein to the MoFe protein. For MgATP hydrolysis by nitrogenase, on the other hand, it is not necessary that electron transfer take place: nitrogenase hydrolyzes MgATP even when no reduc tant is present and the Fe protein is oxidized (10).

The precise mechanism of action of MgATP hydrolysis in nitrogenase catalysis is not yet known. If the similarity between the Fe protein of nitrogenase and the molecular switch proteins is more than a structural one, it could be expected that nucleotide binding and hydrolysis are used by nitrogenase to switch between different conformations (1). Recently we (11) and Renner and Howard (12) have independently demonstrated that MgADP plus aluminum fluoride inhibits nitrogenase by stabilizing a complex between both nitrogenase proteins, suggesting a mode of action similar to that observed for molecular switch proteins (11–13). However, limited data are available that show that conformational changes occur in the nitrogenase complex during turnover. Mensink and Haaker (14) concluded from their experiments on the temperature dependence of the extent and rate of MgATP-induced electron transfer that electron transfer occurs via a highly disordered transition state, which indicates a major conformational change. It was proposed that the binding of MgATP to the nitrogenase complex causes a change of the conformation of the complex that triggers electron transfer (14, 15).

The transfer of the first electron from the Fe protein to the MoFe protein is accompanied by a fast increase of the absorbance at 430 nm, due to oxidation of the Fe protein. Following this absorbance increase, smaller absorbance changes are observed (2, 16), probably caused by subsequent redox changes of the MoFe protein. Lowe et al. (16) were able to simulate the absorbance changes that occur during the first 0.6 s of the reaction of the nitrogenase of Klebsiella pneumoniae.

In this paper, the absorbance changes associated with pre-steady-state electron transfer reactions by nitrogenase of A. vinelandii are presented. The shape of these curves differs significantly from the shape of the curves published for the reaction of K. pneumoniae nitrogenase; as a consequence, the absorbance changes cannot be simulated using the Lowe-Thorneley model (16). To describe the absorbance changes adequately, a reaction associated with an absorbance decrease must be assumed prior to dissociation of the nitrogenase complex. We present evidence that this reaction is a change of the conformation of the nitrogenase complex from the MgATP-bound to the MgADP-bound conformation.

**EXPERIMENTAL PROCEDURES**

**Cell Growth and Isolation and Preparation of Nitrogenase—**A. vinelandii ATCC strain 478 was grown and the nitrogenase component proteins were purified and assayed as described elsewhere (15). Specific activities of Av1 and Av2 were at least 8 and 2 mol\(^-1\) mol\(^-1\) of ethylene produced, respectively. Av1 contained 1.8 ± 0.2 mol of Mo/mol of Av1; the iron content of the Fe protein was 3.6 ± 0.3 mol of Fe/mol of Av2. Dithionite-free Av1 was prepared by running Av1 over a Bio-Gel P-2D column, which was equilibrated with 10 mM MgCl\(_2\) and 200 mM NaCl in 50 mM Tes/NaOH, pH 7.4. All buffers were saturated with argon. ATP (special quality) was obtained from Boehringer Mannheim.

**Stopped-flow Spectrophotometry**—Stopped-flow spectrophotometry was performed with a HI-TECH SF-51 stopped-flow spectrophotometer (Salisbury, Wills, United Kingdom) equipped with an anaerobic kit and a data acquisition and analysis system. If not indicated otherwise, the absorbance changes were measured at 430 nm. The reaction temperature was 20.0 ± 0.1 °C. The mixing ratio was 1:1. For measurement of electron transfer, one syringe of the stopped-flow apparatus contained Av1 and Av2 (concentrations as indicated), and the other syringe contained 10 mM ATP; both syringes contained 10 mM MgCl\(_2\), 4 mM sodium dithionite, and NaCl (concentration as indicated) in 50 mM Tes/NaOH, final pH 7.4. In case of the dithionite-free experiments, one syringe contained dithionite-free Av1 and oxidized Av2 (concentrations as indicated), and the other syringe contained 10 mM ATP; both syringes contained 100 mM NaCl and 10 mM MgCl\(_2\) in 50 mM Tes/NaOH, final pH 7.4.

**Simulations**—The simulations of the absorbance changes associated with electron transfer were performed with the computer program KINSIM, written and distributed by Barshop et al. (17).

**RESULTS**

**Absorbance Changes of A. vinelandii Nitrogenase during Electron Transfer**—Fig. 1 shows the absorbance changes (at 430 nm) that are observed when consecutive electron transfer steps from the Fe protein to the MoFe protein take place and the influence of the ratio \([\text{Av2}] / [\text{Av1}]\) on these absorbance changes. The absorbance increase immediately after the mixing of the nitrogenase proteins with MgATP, caused by the transfer of one electron from the reduced Fe protein to the MoFe protein, is complete within 100 ms at all \([\text{Av2}] / [\text{Av1}]\) ratios (at 20 °C). The maximum absorbance increase is reached when \([\text{Av2}] / [\text{Av1}] = 8\) (Av1 is an \((\alpha\beta)_2\) dimer; each Av1 binds two molecules of Av2); \(k_{\text{obs}}\) does not depend on the ratio of the nitrogenase proteins \((k_{\text{obs}} = 68.3 ± 11.4 \text{ s}^{-1})\). The absorbance increase at \([\text{Av2}] / [\text{Av1}] = 9\) can be fitted to a single exponential with \(\Delta A_{430} = 0.119\) and rate constant \(k_{\text{obs}} = 70.5 \text{ s}^{-1}\). The presence of 100 mM NaCl in the reaction mixture, necessary to prevent precipitation of Av1 at low \([\text{Av2}] / [\text{Av1}]\) ratios, lowers the observed rate constant of electron transfer (in this case from \(-100 \text{ s}^{-1}\) to \(-70 \text{ s}^{-1}\)) and increases the \([\text{Av2}] / [\text{Av1}]\) ratio at which the maximum absorbance is reached. After 100 ms, the absorbance decreases, which, dependent on the ratio \([\text{Av2}] / [\text{Av1}]\), is followed by an increase of the absorbance (see Fig. 1). These absorbance changes are slower and less pronounced when NaCl is present in the reaction mixture, as can be seen by comparing the stopped-flow traces in Fig. 1 (in the presence of 100 mM NaCl) and the stopped-flow trace in Fig. 2 (obtained in the absence of salt).

Lowe et al. (16) simulated the absorbance changes observed in the first 0.6 s after the mixing of the nitrogenase proteins from K. pneumoniae with MgATP by ascribing the absorbance changes after the initial absorbance increase to the redox changes of Kp1 described in the MoFe protein cycle (8). Their simulation used the rate constants for the Fe protein cycle and the MoFe protein cycle given by Lowe and Thorneley in (8). Different absorption coefficients were attached to the subsequent redox changes of Kp1 from state E\(_0\) to E\(_4\). As noticed by the authors, their simulation slightly but reproducibly deviates
Pre-steady-state Kinetics of Nitrogenase

A

Fig. 2. Simulation of the absorbance changes associated with electron transfer from the Fe protein to the MoFe protein. ---, stopped-flow trace (ΔAexp) obtained after the mixing of the nitrogenase proteins with MgATP; ——, simulation of the absorbance changes; -----, Eo, one of two independently functioning halves of MoFe protein, reduced by n electron equivalents. Concentrations after mixing: 7.7 μM Av1; 46.2 μM Av2; 4 mM Na₂S₂O₄. The simulation uses rate constants determined for A. vinelandii nitrogenase (see Table I). A, simulation of the absorbance changes and the appearance of species Eo, using the model and the absorbance coefficients according to Lowe et al. (16). B, simulation of the absorbance changes and the appearance of species Eo, using the model as in panel A with an additional reaction (associated with an absorbance decrease) before dissociation of the nitrogenase complex (see Table II, simulation 3).

from the stopped-flow trace between the first 30 and 200 ms. It was suggested that these small deviations are the result of intramolecular reactions (such as electron transfer between the metal-sulfur clusters of Kp1 or from clusters to bound protons) at the Eo level of reduction of Kp1.

The stopped-flow traces (20 °C) obtained after the mixing of the A. vinelandii nitrogenase proteins with MgATP (Figs. 1 and 2) have a much more pronounced shape than the curves obtained with the nitrogenase proteins from K. pneumoniae (23 °C) (16). For A. vinelandii nitrogenase, the absorbance clearly decreases after the initial increase of the absorbance, whereas for the published K. pneumoniae ratios (ΔKp2/ΔKp1 = 8/1 and 1/1), this is not the case (16).

We tried to simulate the stopped-flow trace obtained after the mixing of Av1 and Av2 with MgATP in the absence of salt by using the same model and absorption coefficients and rate constants for the MoFe protein cycle as Lowe et al. (16). In addition, we used the rate constants of the Fe protein cycle as determined for the A. vinelandii proteins at 20 °C (see Table I). The absorption coefficients used for species E1, E2, E3, and E4 (relative to Eo) are given in Table II, simulation 1. It is obvious from Fig. 2A that the absorbance decrease observed in the stopped-flow trace is absent in the simulation. It was possible, however, to simulate the stopped-flow trace by allowing other absorption coefficients for the different redox states of Av1, but to obtain a good simulation, some of these absorbance coefficients had to be extremely large (see Table II, simulation 2). Therefore, we did not consider this to be the right solution to the problem.

To acquire an adequate simulation of the stopped-flow trace, an additional reaction (with an absorbance decrease) with a rate constant of about 1 s⁻¹ had to be included in the model of the reaction mechanism; this reaction must take place before dissociation of the nitrogenase complex. With this addition to the model and small changes of the absorbance coefficients of the different redox states of the MoFe protein (see Table II, simulation 3), a good fit of the stopped-flow trace and the simulated absorbance changes was obtained (see Fig. 2B). The stopped-flow traces obtained at different [Av2]/[Av1] ratios could also be simulated with this adjusted model (data not shown). The rate constant of the added reaction is of the same order as the MgATP-dependent pre-steady-state proton production: kobs = 14 ± 4 s⁻¹ (reduced nitrogenase proteins) (18). To be able to simulate their data of pre-steady-state phosphate release caused by the reaction of the nitrogenase proteins from K. pneumoniae with MgATP (23 °C), Lowe et al. (19) assumed a kinetic scheme in which first an electron is transferred (kobs = 176 s⁻¹), followed by on-enzyme MgATP hydrolysis (k = 50 s⁻¹), after which phosphate is released (kobs = 22 s⁻¹), and finally the nitrogenase complex dissociates (k = 6.4 s⁻¹). We also added the on-enzyme MgATP hydrolysis step to our kinetic scheme, with a rate constant k = 40 s⁻¹ (20 °C), after electron transfer; with this addition and slightly altered absorbance coefficients (see Table II, simulation 4), also a good simulation of the stopped-flow data was obtained (data not shown).

However, it is not absolutely necessary to add this step to the kinetic scheme because it does not clearly improve the simulation.

Absorbance Changes of Dithionite-free Nitrogenase Proteins—The hypothesis that MgATP hydrolysis induces an absorbance decrease at 430 nm in the nitrogenase complex was tested with dithionite-free nitrogenase proteins. After the mixing of dithionite-free nitrogenase proteins (oxidized Fe protein, MoFe protein with FeMoco and P-cluster dithionite-reduced (see “Experimental Procedures”) with MgATP, after a delay of about 20 ms, a decrease of the absorbance at 430 nm was observed. An example of the obtained stopped-flow trace is given in Fig. 3. The absorbance decrease could be fitted from 0.05 to 1 s, to a single exponential: ΔA430 = 0.011·exp(−6.23·t). The absorbance decrease increases with the component protein ratio and saturates at [Av2]/[Av1] > 10 with ΔA430 = 0.020 ([Av1] = 2.7 μM). The observed rate constant of the absorbance decrease is independent of the ratio [Av2]/[Av1] (data not shown).

The reaction of the dithionite-free nitrogenase proteins with MgATP was measured at different wavelengths. Between 320 and 400 nm, the absorbance increases with a maximum at 360 nm, whereas between 400 and 750 nm, the absorbance decreases with a minimum around 430 nm (see Fig. 3, inset). This indicates that MgATP hydrolysis during the Fe protein cycle induces a blue shift of the absorbance spectrum of the nitrogenase complex; some absorbance of metal-sulfur clusters around 430 nm shifts to 360 nm.
TABLE I

Rate constants of the Fe protein cycle, determined for nitrogenase from *Azotobacter vinelandii*, as used in the simulations

| Rate constant | Value         | Reaction                        | Reference |
|---------------|---------------|---------------------------------|-----------|
| $k_{-1}$      | $5 \times 10^4$ M$^{-1}$·s$^{-1}$ | Association active complex      | 26        |
| $k_1$         | 15 s$^{-1}$   | Electron transfer                | 26        |
| $k_{-2}$      | 100 s$^{-1}$  | Dissociation of complex          | This study|
| $k_3$         | 3.3 s$^{-1}$  |                                 | This study|
| $k_{-4}$      | $2.3 \times 10^6$ M$^{-1}$·s$^{-1}$ | Reduction Av2ox (MgADP)$_2$     | This study|
| $k_4$         | 5.6 s$^{-1}$  |                                 |           |

TABLE II

Absorbance coefficients ($\Delta e_{430}$) used for simulation of the absorbance changes at 430 nm observed after mixing of the nitrogenase proteins from *A. vinelandii* with MgATP

The absorbance coefficients for the appearance of species $[Av2^{\text{ox}}(\text{ATP})_2 Av1^{\text{†}}]$ and $[Av2^{\text{red}}(\text{ATP})_2 Av1^{\text{†}}]_{\text{ATP}}$ and $[Av2^{\text{ox}}(\text{ADP})_2 Av1^{\text{†}}]_{\text{ADP}}$; the absorbance coefficients for the appearance of species $E_1$ are relative to $E_0$. All absorbance coefficients ($\Delta e_{430}$) are in cm$^{-1}$·M$^{-1}$. It was assumed that 62% of all Av2 present was active with respect to electron transfer and 38% was inactive (12, 27). In simulation 3, an extra reaction ($k = 14$ s$^{-1}$) is added to the model used in simulations 1 and 2; this reaction is accompanied with an absorbance change at 430 nm, defined by the absorbance coefficient of species $[Av2^{\text{ox}}(\text{ATP})_2 Av1^{\text{†}}]_{\text{ATP}}$. In simulation 4, an extra delay ($k = 40$ s$^{-1}$) is added to the model of simulation 3; this delay is not accompanied with an absorbance change at 430 nm.

| Species                | 1                     | 2                     | 3                     | 4                     |
|------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| $[Av2^{\text{ox}}(\text{ATP})_2 Av1^{\text{†}}]_{\text{ATP}}$ | 5.0                   | 5.0                   | 5.9                   | 5.5                   |
| $[Av2^{\text{ox}}(\text{ADP})_2 Av1^{\text{†}}]_{\text{ADP}}$ | 0.0                   | 0.0                   | -0.70                 | -0.60                 |
| $E_1$                  | 0.0                   | 10.0                  | -0.25                 | 0.3                   |
| $E_2$                  | -2.2                  | 60.0                  | -1.0                  | 0.4                   |
| $E_3$                  | -2.2                  | -55.0                 | 6.0                   | 4.5                   |

$^{a}$Av1$, one of two independently functioning halves of the MoFe protein; Av1$^{\text{†}}$, MoFe protein with super-reduced FeMoco; Av2$^{\text{ox}}$ and Av2$^{\text{red}}$, oxidized and reduced Fe protein, respectively; $[Av2^{\text{ATP}}]_2 Av1^{\text{ATP}}$ and $[Av2^{\text{ADP}}]_2 Av1^{\text{ADP}}$, nitrogen complex in the MgATP-bound and MgADP-bound conformation, respectively.

No significant absorbance change was observed when the dithionite-free nitrogenase proteins were mixed with MgADP nor when either oxidized Av2 or dithionite-free Av1 was mixed with MgATP. The absorbance decrease observed after the mixing of the dithionite-free nitrogenase complex with MgATP is therefore a property of the nitrogenase complex, not of the individual nitrogenase proteins. No electrons are transferred from the Fe protein to the MoFe protein because the Fe protein is oxidized. EPR measurements indicated that the FeMoco $S = 3/2$ signal did not decrease significantly during the first 2 s. No signals associated with oxidized P-clusters, like those reported by Peercy et al. (20) or Tittsworth and Hales (21), were observed, even after a prolonged incubation (23 s). It is therefore clear that the absorbance decrease is caused by a reaction other than electron transfer. The rate of the absorbance decrease (after 50 ms) is of the same order as the rate observed for the MgATP-dependent pre-steady-state proton production for dithionite-free nitrogenase proteins: $k_{\text{obs}} = 6 \pm 2$ s$^{-1}$ (18). This suggests that the absorbance decrease at 430 nm is caused by the same reaction that is causing the MgATP-dependent pre-steady-state proton production.

**DISCUSSION**

In our previous article, we suggested that the binding of MgATP to the nitrogenase complex induces electron transfer from the Fe protein to the MoFe protein. After electron transfer, MgATP is hydrolyzed and the conformation of the nitrogenase complex changes; this change is accompanied by the production of protons (18). On the basis of the data and simulations presented here, we suggest that this conformational change of the nitrogenase complex causes a shift of the absorbance maximum from 430 nm to 360 nm, resulting in a decrease of the absorbance at 430 nm. In this hypothesis, a conformational change of the nitrogenase complex could alter the environment of the metal-sulfur clusters of nitrogenase, causing an absorbance change, as observed for various types of chromophores in proteins (22, 23).

The absence of a pronounced absorbance decrease in the *K. pneumoniae* nitrogenase stopped-flow traces (16), in contrast to the traces obtained for *A. vinelandii* nitrogenase, might be caused by a different rate constant of the proposed conformational change of the nitrogenase complex, assuming that such a conformational change exists for *K. pneumoniae* nitrogenase. The absorbance decrease is no longer visible in our simulation if a lower value for the rate constant is chosen (4 s$^{-1}$) or if a much higher value is chosen (100 s$^{-1}$) (data not shown). In the first case, the change of the conformation coincides with the dissociation of the nitrogenase complex ($k_{\text{obs}} \approx 3$ s$^{-1}$), and in the other case, the absorbance decrease becomes a contribution to the (lowered) initial absorbance increase, caused by electron transfer.

Lowe et al. (16) only observed an absorbance decrease after

![Absorbance changes after the mixing of the dithionite-free nitrogenase proteins with MgATP](image-url)
Reduced nitrogenase proteins

\[
\begin{align*}
&\text{Av}_2^{\text{red}} \text{Av}_1^1 \\
&+ \text{ATP} \\
&\rightarrow \text{Av}_2^{\text{red}}(\text{ATP})_2 \text{Av}_1^1 \\
&\Delta e^*: k_{\text{obs}} = 113 \text{ s}^{-1} \\
&\Delta A_{430} = 4.85 \text{ mM}^{-1} \cdot \text{cm}^{-1}
\end{align*}
\]

Reductant-free nitrogenase proteins

\[
\begin{align*}
&\text{Av}_2^{\text{red}} \text{Av}_1^1 \\
&+ \text{ATP} \\
&\rightarrow \text{Av}_2^{\text{red}}(\text{ATP})_2 \text{Av}_1^1 \\
&\Delta H^*: k_{\text{obs}} = 6 \text{ s}^{-1} \\
&\Delta A_{430} = 3.70 \text{ mM}^{-1} \cdot \text{cm}^{-1}
\end{align*}
\]

\[
\begin{align*}
&[\text{Av}_2^{\text{red}}(\text{ATP})_2 \text{Av}_1^1(\text{ATP})] \\
&\Delta H^* : k_{\text{obs}} = 14 \text{ s}^{-1} \\
&\Delta A_{430} =
\end{align*}
\]

\[
\begin{align*}
&[\text{Av}_2^{\text{red}}(\text{ATP})_2 \text{Av}_1^1]_{\text{ADP}} + 2\text{P} \\
&\rightarrow [\text{Av}_2^{\text{red}}(\text{ATP})_2 \text{Av}_1^1]_{\text{ADP}} + 2\text{P} \\
&\Delta H^* : k_{\text{obs}} = 3.3 \text{ s}^{-1}
\end{align*}
\]

\[
\begin{align*}
&\text{Av}_2^{\text{red}}(\text{ATP})_2 + \text{Av}_1^1 \\
&\Delta H^* : k_{\text{obs}} = 3.3 \text{ s}^{-1}
\end{align*}
\]

SCHEME 1. Pre-steady-state reactions of nitrogenase with MgATP. All rate constants were measured for A. vinelandii nitrogenase. For the abbreviations used, see Table II. \(\Delta e^*\), electron transfer from the Fe protein to the MoFe protein was observed; \(\Delta H^*\), proton production was observed; \(\Delta A_{430}\) and \(\Delta A_{250}\) (in \(\text{mm} \cdot \text{cm}^{-1}\) s\(^{-1}\)), an absorbance change at 430 nm was observed.

The mixing of the nitrogenase proteins with MgATP under a \(\text{C}_2\text{H}_2\) atmosphere. This decrease in the absorbance was not followed by an increase. Similar results were obtained with the nitrogenase proteins from A. vinelandii under a \(\text{C}_2\text{H}_2\) atmosphere (data not shown), but this absorbance decrease started only after 200 ms and happened at a much lower rate than the absorbance decrease presented in this paper. Kp1 with \(\text{C}_2\text{H}_2\) bound cannot be reduced further than the \(E_3\) state (24), and no absorbance increase is observed after the absorbance decrease (16). This shows that the reduction of Kp1 from the \(E_3\) state to the \(E_4\) state in the absence of \(\text{C}_2\text{H}_2\) causes an increase of the absorbance at 430 nm (Table II, simulation 1).

The stopped-flow traces obtained with A. vinelandii nitrogenase could not be simulated with the model of Lowe et al. (16); the kinetic scheme had to be adjusted as discussed under “Results.” Also, the absorption coefficients of the different \(E_n\) states were slightly altered (Table II, simulations 1 and 3). One should not attach too much importance to the value of these absorption coefficients; what absorbance changes really accompany the reduction of the MoFe protein to each of the subsequent \(E_n\) states are not known. We realize that the absorbance changes of the Fe protein, caused by oxidation or reduction, also contribute considerably to the stopped-flow traces. It was observed that the rate of reduction of oxidized \(\text{Av}_2\) after dissociation of the nitrogenase complex has a large influence on the appearance of the stopped-flow traces (data not shown).

On the basis of the described experiments, we suggest the following sequence of events after the mixing of the nitrogenase proteins with MgATP (see Scheme 1). Binding of MgATP to the nitrogenase complex is fast and induces a conformational change of the complex, which, if the Fe protein is reduced, allows rapid electron transfer from the Fe protein to the MoFe protein (\(k_{\text{obs}} \sim 100 \text{ s}^{-1}\) in the absence of salt). In the altered conformation, MgATP is hydrolyzed and a second conformational change of the nitrogenase complex (to the MgADP-bound conformation) takes place, which changes the environment of the metal-sulfur clusters and causes a blue shift of the absorbance maximum at 430 nm (\(k_{\text{obs}} \sim 14 \text{ s}^{-1}\)). We propose that this reaction is also associated with proton production (18) and P release (19) Hereafter, the nitrogenase complex dissociates (\(k_{\text{obs}} \sim 3 \text{ s}^{-1}\); this rate constant was calculated from the rate of turnover under optimal conditions at 20 °C (18), assuming that the dissociation of the nitrogenase complex is the rate-limiting step of the catalytic cycle (7)).

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