Electron transfer coupled with ATP hydrolysis in nitrogenase

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Nitrogenase, which is not a membrane protein *in vivo*, performs energy coupling: the transfer of an electron coupled with ATP hydrolysis from one protein component of nitrogenase, Fe protein (Av2), to another its protein component, MoFe protein (Av1), to form the so-called "super-reduced state" of the active site responsible for the reduction of the substrates, FeMo cofactor (FeMoco) containing Fe, Mo, S, and homocitrate. The review discusses recent publications on studying the electron transfer coupled with ATP hydrolysis in nitrogenase and evaluates a possible value of the redox potential of the super-reduced FeMoco.

Key words: nitrogenase, Fe protein, MoFe protein, FeMoco, nitrogen fixation.

Nitrogenase (EC 1.18.6.1) is the enzyme that catalyzes the reduction of N_2 to NH_3 and consists of two metalloproteids: molybdenum—iron-containing protein (MoFe protein) and iron-containing protein (Fe protein), which manifest the enzyme activity only in a complex. The MoFe protein and Fe protein from *Azotobacter vinelandii* are briefly named Av1 and Av2, respectively (those from *Klepsiella pneumoniae* are Kp1 and Kp2, *etc.*)*.

To manifest the enzyme activity, nitrogenase needs both nitrogenase components, MoFe and Fe proteins, MgATP, and an electron donor. In addition to nitrogen, nitrogenase also reduces other compounds with the triple bond: acetylene, cyanide, and others. Two electrons are needed for the reduction of acetylene to ethylene or two protons to dihydrogen, and eight electrons are needed for the reduction of nitrogen because one hydrogen molecule is always formed per nitrogen molecule. Only the reduction of protons to dihydrogen occurs in an argon atmosphere. At least two ATP molecules are hydrolyzed per each transferred electron, and they are bound to the Fe protein as a complex with Mg^{2+}. The general stoichiometry of nitrogen reduction catalyzed by nitrogenase can be described by the following scheme[1]:

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

The composition and structure of Av1 and Av2 have been established in recent years.[2,3] The structure of Av1 with a resolution of 2 Å provided data on the structure of Fe—S clusters in Av1.[3] More complete data on the redox potentials of the Fe—S clusters of Av2 and Av1 have been obtained.[4—8] Alternative electron donors for nitrogenase have been obtained: photodonors eosin—NADH[9] and 4′,5′-dibromofluorescein (DBF)—NADH.[10] The kinetics of the transfer of one and two electrons in nitrogenase with the photodonor was studied.[11] The ability of the photodonors[10—13] and the natural electron donor flavodoxin[14] to reduce Av2 bound to Av1 was found. The array of these data allows us to discuss the specific features of electron transfer in nitrogenase and to distinguish the problems to be solved for revealing the mechanism of nitrogen reduction.

The Av1 protein is a heterotetramer of two types of subunits $\alpha_2\beta_2$ with a total molecular weight of ~250 kDa. Nitrogenase contains three types of metal—sulfur clusters. The first stage of the enzymatic reaction is the transfer of an electron from the external electron donor (dithionite or photodonor *in vitro*, flavodoxin or ferredoxin 1 *in vivo*) to Av2, which is a homodimer of two subunits with a total molecular weight of ~64 kDa.[2] Then the transfer of an electron from Av2 to Av1 occurs, which is coupled with ATP hydrolysis. Av1 contains two P clusters with the [8Fe—7S] composition and two [Mo—7Fe—9S—homocitrate] clusters, the so-called FeMo cofactors (FeMoco), which are the region of substrate activation and reduction.[3]

Av2 contains one [4Fe—4S] cluster symmetrically incorporated between two subunits. In the presence of an excess of dithionite, the [4Fe—4S] cluster exists in the oxidation state 1+. The ESR spectrum reveals a mixture of spin states S = 1/2 and S = 3/2. The half-reduction potential E_m for the $[4Fe—4S]^{2+} \rightleftharpoons [4Fe—4S]^{1+}$ transition in Av2 equals −0.29 V.[4] Flavodoxin from *Azotobacter vinelandii* has the E_m potential equal to −0.515 V for the reversible transition between the semi-

* Abbreviations used: Av1 and Av2 are molybdenum—iron-containing and iron-containing components, respectively, of nitrogenase from *Azotobacter vinelandii*; E_m is the half-reduction potential at pH 7.0 *vs.* hydrogen electrode; P cluster is [8Fe—7S] cluster in Av1; FeMoco is molybdenum-, iron-, and homocitrate-containing cofactor in Av1; P^N and $FeMoco^N$ are P and FeMoco clusters reduced by dithionite, respectively; $(*FeMoco)^{1-}$ is FeMoco in the "super-reduced" (by one electron) state; DBF is 4′,5′-dibromofluorescein; ATP is adenosine-5′-triphosphate; ADP is adenosine-5′-diphosphate; P_i is phosphate; NADH is reduced β-nicotinamide adenine dinucleotide; and K_m is the Michaelis—Menten constant.

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quinone and hydroquinone forms of flavodoxin[15] and can reversibly reduce the $[4Fe—4S]^{1+}$ cluster Av2 by one electron to the $[4Fe—4S]^{0}$ state in which all iron ions exist in the ferro form. The E_m potential for the $[4Fe—4S]^{1+} \rightleftharpoons [4Fe—4S]^{0}$ transition in Av2 is equal to −0.46 V.[4]

In the presence of dithionite, the MoFe protein gives only one ESR signal (S = 3/2) characteristic of FeMoco reduced by one electron ($FeMoco^N$). The $FeMoco^N \rightleftharpoons FeMoco_{ox}$ transition to occur requires the potential E_m −0.042 V.[5] In the process coupled with ATP hydrolysis, Av2 transfers at least one electron to $FeMoco^N$ to form the so-called "super-reduced" FeMoco[6]: $(*FeMoco)^{1-}$. The ESR signal (S = 3/2) disappears. According to experimental data, E_m for the $FeMoco^N \rightleftharpoons (*FeMoco)^{1-}$ transition in Av1 is −0.6 to −1 V.[16]

Unlike FeMoco, the P cluster is diamagnetic in the presence of dithionite. According to data in Ref. 7, this state corresponds to the cluster of ferro-iron P^N, which can reversibly be oxidized to the paramagnetic states P^{1+}, P^{2+}, and P^{3+}. The E_m potentials for the transitions $P^N \rightleftharpoons P^{2+}$, $P^N \rightleftharpoons P^{1+}$, $P^{1+} \rightleftharpoons P^{2+}$, and $P^{2+} \rightleftharpoons P^{3+}$ are equal to −0.307, −0.309, −0.309, and +0.09 V, respectively.[8] The ESR spectrum of the P^{2+} cluster exhibits the signal characteristic of S = 3. The ESR spectrum of the P^{1+} cluster contains the signals of the spin states S = 5/2 and S = 1/2. Published data favor the participation of the P cluster in the electron transfer inside Av1. The P clusters are oxidized when nitrogenase reduces N_2.[17] The authors[18] concluded that the intramolecular electron transfer in nitrogenase is not an elementary reaction but includes at least two steps: the transfer of an electron from Av2 to the unidentified region in Av1 (it is assumed that to the P cluster) followed by the reduction of FeMoco. This conclusion was based on the observation that, in the presence of NaCl, during the transfer of an electron from Av2 to Av1, changes in the absorption of Av1 occur yet before the transfer of an electron to FeMoco.[18]

An electron is transferred in nitrogenase to considerable distances.[19] In this process, the conformation changes, which occur during the Fe protein binding with MgATP or MgADP and were detected by various methods,[4] can play a substantial role. According to X-ray structural analysis data, the MgATP-binding regions exist in Av2 at a distance of approximately 15 Å from the [4Fe—4S] cluster and from the region of binding with Av1.

The crystal structures of the Av2 complex and Av1 stabilized by the nitrogenase inhibitor, aluminum fluoride (AlF_4^-) in a combination with ADP, have recently[20] been determined. This complex differs from a native complex by two Av2 molecules (unlike one molecule as in the native complex) per Av1 molecule. In this complex, the P cluster is localized at equal distances of 14 Å from the [4Fe—4S] cluster in Av2 and from FeMoco. The most substantial structural changes during complex formation occurred in Av2. The [4Fe—4S] cluster shifted by approximately 4 Å toward the protein surface in Av2. The conformational changes (the turn of each monomer of Av2 by 13 Å towards the surface of the subunits) occurred, and the nitrogenase complex became more compact, *i.e.*, gained the "closed" conformation. Such changes in a complex can facilitate the electron transfer.[20]

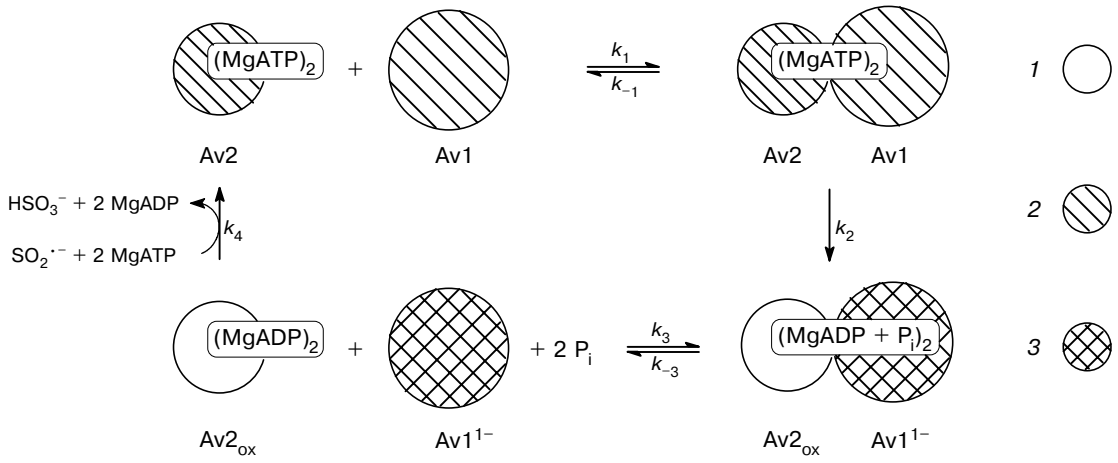
It has been known long ago that when MgATP or MgADP is bound to Av2 its redox potential changes from −0.29 to −0.43 V and the protein undergoes conformational changes.[4] The Av2 protein has two regions of MgATP binding (one per each identical subunit) in the oxidized and reduced states. It has been shown[21] that region of MgATP binding in Av2 contains the sequence of amino acids homological to those described for other ATPases.

The P cluster exhibited the properties that can play a substantial role in its functioning.[3] Considerable distinctions in the P^N and P_{ox} structures were revealed. In each of these states, the P cluster is the [8Fe—7S] cluster. In the oxidized state it can be presented as a single cluster consisting of two [4Fe—S] and [4Fe—3S] clusters connected by the bridging S(1) sulfur. The P cluster undergoes the redox-dependent structural rearrangement, which can be coupled with the transfer of an electron or a proton to FeMoco. The interconversion between the oxidation states of the P cluster was observed and included the migration of two Fe atoms and replacement of their coordination with the protein by the coordination with the central sulfur atom S(1). The oxidation of the P cluster is accompanied by the coordination of Ser188(β) and the amide nitrogen of Cys88(α) with the Fe atoms of the P cluster. Since both these ligands are protonated in the free state and can be deprotonated in the bound state, the authors[3] assumed that during the two-electron oxidation of the P cluster two protons can be liberated and the replacement of the ligands in the P cluster can result in the coupled transfer of electrons and protons from the P cluster to FeMoco. This assumptions are favored by the data,[8] which show that the electron transfer and oxidation of P^{1+} to P^{2+} are also accompanied by the coupled proton transfer at physiological pH values.

The helix sequence of amino acids binding the ligand of the P cluster of Cys62(α) with Val70(α) in the region of FeMoco has been revealed.[22] It is assumed that this helix is the potential way for the transfer of an electron directly from the P cluster to FeMoco. The reduction center of the FeMoco substrates is a cluster of the new type containing both Mo and Fe. In this cluster each Fe—S—Fe group binds two 4Fe—3S and 1Mo—3Fe—3S subclusters as bridges and forms two 4Fe—4S faces. The Val70(α) residue is localized at a short distance from one of these faces.[22]

It has been considered up to recent time that the Fe protein is the one-electron donor for the MoFe protein. The results of studying the kinetics of the transfer of an

Scheme 1



Note. The smaller sphere is Av2, and that of a larger size is Av1 in the oxidized (*1*), reduced (*2*), and super-reduced (*3*) states. $k_1 = 4.4 \cdot 10^6$ mol^{-1} L s^{-1}, $k_{-1} = 6.4$ s^{-1}, $k_2 = 200$ s^{-1}, $k_3 = 4.4 \cdot 10^6$ mol^{-1} L s^{-1}, $k_{-3} = 6.4$ s^{-1}, and $k_4 = 3.0 \cdot 10^6$ mol^{-1} L s^{-1}.

electron from the Fe protein to the MoFe protein with the artificial electron donor, dithionite,[23] are presented in Scheme 1.

The rate-limiting stage is the dissociation of nitrogenase to components after the intramolecular transfer of an electron from Av2 to Av1 ($k_{-3} = 6.4$ s^{-1} at 23 °C). The electron donor is the $SO_2^{\bullet -}$ radical anion formed upon the dissociation of dithionite $S_2O_4^{2-} \rightleftharpoons 2\ SO_2^{\bullet -}$ ($K = 1.5 \cdot 10^{-9}$ mol L^{-1}; the dissociation rate constant is equal to 1.7 s^{-1}). It has been established that dithionite cannot reduce the Fe protein when it is in a complex with the MoFe protein. In this case, dissociation determines the rate of the nitrogenase reaction and, moreover, it is necessary for the Fe protein to be reduced again after the electron transfer. Since the dissociation of nitrogenase "disguises" the stages of the nitrogenase reaction, a certain mechanism of reactions occurred during nitrogen reduction was not established. As we mentioned previously,[24] in our opinion, dissociation in the presence of dithionite is, in fact, an artifact. The data are available that when the concentration of dithionite is higher than $2 \cdot 10^{-3}$ mol L^{-1} nitrogenase is dissociated to the components.[25] Therefore, K_m for dithionite in the reaction considered is equal to $9 \cdot 10^{-3}$ mol L^{-1}.[26] This concentration of dithionite is necessary for the dissociation of nitrogenase. It is known that NaCl in the concentration higher than 0.09 mol L^{-1} results in the forced dissociation nitrogenase to the components.[27] Dithionite, probably, has the same effect.

New recent data change substantially the concept of the electron transfer in nitrogenase. The found property of dithionite as an electron donor for nitrogenase differs it from the natural donor flavodoxin. Unlike dithionite, flavodoxin can transfer one more electron to Av2 reduced by one electron.[4] Therefore, it is assumed that, under natural conditions, two electrons can transfer from Av2 to Av1, and the mechanism of electron transfer can differs from that in the case of dithionite.[4] In fact, unlike dithionite, flavodoxin reduces both Av2 bound to Av1 and free Av2 in a solution. The apparent rate constants of these reactions are 400 s^{-1} and >1000 s^{-1}, respectively.[14]

It is likely that photodonors act as flavodoxin. In the case of the photodonors, eosin or DBF, in the presence of NADH, the reducing agent for nitrogenase is the photodonor radical anion with E_m for the transition photodonor $\rightleftharpoons$ photodonor radical anion equal to −0.58 V.[28] The close E_m values of flavodoxin and the photodonor indicate that these donors can reduce Av2 to Av2^{2-} by the successive transfer of two electrons. The stages of the transfer of the first and second electrons in nitrogenase were detected by kinetic laser spectroscopy using the DBF—NADH photodonor in the presence of nitrogen[11] (Scheme 2).

Two electrons are transferred from Av2 to Av1 successively one after another without the limiting stage between these reactions as in the case of dithionite (Fig. 1).[11] It has been established[29] that, in the case of the photochemical eosin—NADH system and dithionite

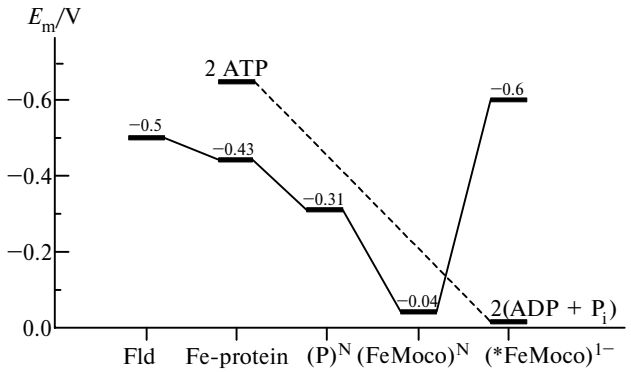
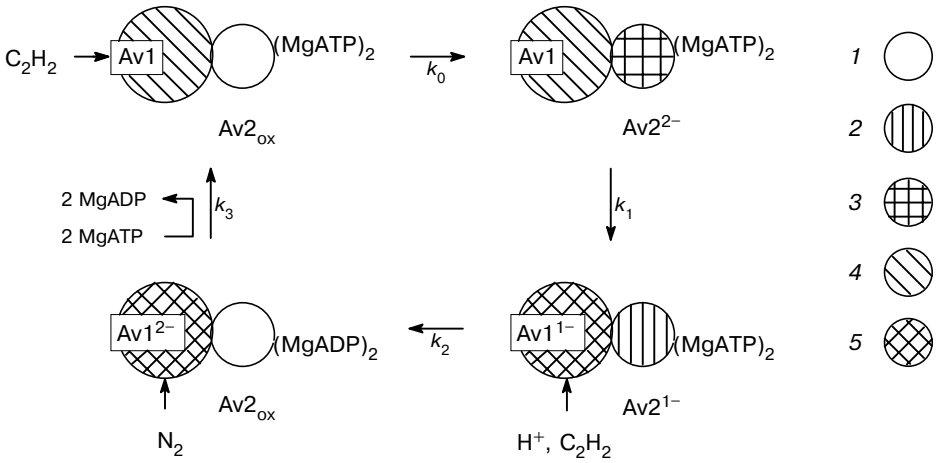


Fig. 1. Energy profile of the transfer of one electron in nitrogenase coupled with the hydrolysis of two MgATP molecules. Fld is flavodoxin.

Scheme 2



Note. The smaller sphere is Av2 in the oxidized (*1*) and reduced by one (*2*) or by two electrons (*3*) states. The larger sphere is Av1 in the reduced (*4*) and "super-reduced" (*5*) states. Sort arrows show the reduction state of Av1 when the substrate is bound to it. Constants $k_0 = 200\ s^{-1}$, $k_1 = 158\ s^{-1}$, $k_2 = 54\ s^{-1}$, and $k_3 = 18\ s^{-1}$.

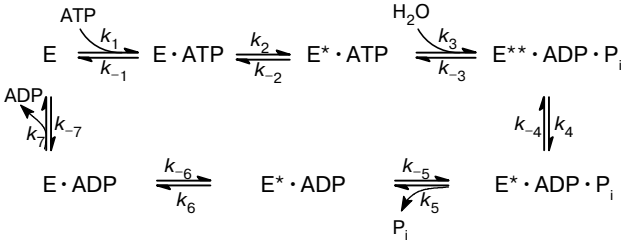
concentrations not higher than $4 \cdot 10^{-4}$ mol L^{-1}, the Av1 · Av2 (1 : 1) nitrogenase complex functions. The slow process ($0.1\ s^{-1} < k < 2.0 \cdot 10^2\ s^{-1}$ at 20 °C) of nitrogenase dissociation to the Av1 and Av2 components was not observed. It has been found that the reduction of Av2 in a complex with Av1 has the same efficiency as that in the free state in a solution[10—13]: the rate constant of a second order for the reduction of Av2 in the nitrogenase composition (in a complex with Av1) with the eosin—NADH photodonor is equal to $1.12 \cdot 10^7$ $mol^{-1}\ L\ s^{-1}$, and that with the DBF—NADH photodonor is $1.2 \cdot 10^8$ $mol^{-1}\ L\ s^{-1}$.

The main specific feature should be taken into account when considering results of studies of the kinetics of electron transfer in nitrogenase: the latter is an enzyme that performs energy coupling in which "one chemical transformation with a decrease in the free energy can provide the occurrence of another chemical transformation accompanied by an increase in the free energy." [30] Despite various biological systems of energy transformation (the system of oxidative phosphorylation, the actin—myosin complex, nitrogenase) have different structures and perform different biological functions, the main regularities of ATP hydrolysis in H^+-ATPase, myosin, and nitrogenase are similar.[31] These ATPases hydrolyze ATP at the phosphoanhydride O—P_γ bond. They catalyze direct and intermediate [^{18}O] exchange (see further). In a medium containing ^{18}O-labeled P_i or water, the isotope exchange of oxygen occurs, which can be reduced to the enrichment (in the case of labeled water and nonlabeled P_i) or depletion (in the case of labeled P_i nonlabeled water) of P_i in the ^{18}O isotope. For standard hydrolysis, only one oxygen atom in the molecule of eliminating P_i is replaced by ^{18}O. During the hydrolysis of ATP by ATPases, ^{18}O (2—4 ^{18}O atoms) is additionally inserted into the eliminating P_i. The ^{18}O-exchange reaction that occurs at the intermediate stage of ATP hydrolysis was named "intermediate ^{18}O exchange." ATPases also catalyze the "direct ^{18}O exchange" or "exchange with the medium," which occurs with free P_i or after its elimination from ATP or with P_i added to the medium in the absence of ATP (see review[31]). The stage of the hydrolytic cleavage of ATP is reversible. As a rule, the stage determining the rate of MgATP hydrolysis is the stage of MgADP liberation to the medium. These ATPases have at least two regions of MgATP binding, between which the cooperative interaction appears. The polarity of the environment of the catalytic center changes at the "coupling stage" (see below): the hydrophilic-hydrophobic transition is observed due to the change in the local conformation. The ATPases considered have similar sets of functional groups in the active site, *etc*. The authors of the mentioned review cite the works in studying the interaction of MgATP and MgADP with nitrogenase.

The most completely studied[32] sequence of stages of ATP hydrolysis by myosin (E) is presented in Scheme 3.

In all cases of the successive complicated enzymatic reaction of ATP hydrolysis (Scheme 3), the storage of energy as a strained protein conformation occurs at stages 1—3. The maximum changes in the standard free energies ΔF° occur at the stage of the addition of ATP to myosin (−51.8 kJ mol^{-1}), and the minimum changes occur at stage 3 (−5.4 to −11.7 kJ mol^{-1}). Thus, the stage of the O—P bond cleavage is reversible. The elimination of the products (ADP, P_i) requires energy expenditure (+21.3 kJ mol^{-1}).[33] The rate-limiting stage is the isolation of the ATP hydrolysis products into the medium: sometimes P_i but, in most cases, as in myosin in the presence of actin, this is the reaction of the two-stage elimination of MgADP (Scheme 3, stages 6 and 7). The conformation of the MgADP-containing enzyme is

Scheme 3



Note. The conformationally changed states of myosin are marked by asterisk. Equilibrium constants: $K_1 = 4.5 \cdot 10^3$ mol^{-1} L, $K_2 \geq 2 \cdot 10^4$, $K_3 = 9.0$, $K_5 = 7.3 \cdot 10^{-3}$ mol L^{-1}, $K_6 = 3.5 \cdot 10^{-3}$, $K_7 = 2.7 \cdot 10^{-4}$ mol L^{-1}. $K_{tot} = 7.5 \cdot 10^5$ mol L^{-1}. Rate constants of direct reactions (s^{-1}): $k_2 = 400$, $k_3 = 160$, $k_4 = 0.06$ (in the presence of actin, 20), $k_6 = 1.4$.

slowly transformed into the conformation corresponding to the initial enzyme followed by the stage of the fast liberation of MgADP to the medium (stage 7). Energy transformation in myosin is based on the revolution of conformational changes accompanying ATP binding and cleavage.[32] This process occurs at the so-called "coupling" stage 4 (Scheme 3). In the systems of energy transformation in a complex with MgATP and MgADP, the active site is shielded from the medium, and the ATP hydrolysis, which occurs at several stages, is coupled with reactions at stage 4 (they are not shown in Scheme 3) to which the coupling is directed: electron transfer in nitrogenase, mechanical work in actomyosin, *etc.* In nitrogenase this fruitful "work" of the ATP functioning should be the formation of $(*FeMoco)^{1-}$. This can be one or several elementary steps at which FeMoco in Av1 is prepared for the accepting of an electron from Av2. Above we presented the data that the reduction of Av2 is accompanied by substantial conformational rearrangements,[2] and the redox potential of Av2 changes when Av2 binds with MgATP or MgADP.[3] Perhaps, processes associated with protonation,[34,35] due to which the acceptor properties of FeMoco can enhance, occur. In these processes, MgADP should remain "bound" on the enzyme for the time necessary for the formation of $(*FeMoco)^{1-}$. Indeed, it is known[36] that the rate of MgADP elimination from the nitrogenase molecule even at the stage of transfer of the first electron is low: the first MgADP molecule separates from nitrogenase with $k_{eff} \leq 0.2$ s^{-1}, and the second molecule does with $k_{eff} \geq 0.6$ s^{-1}. It is known from data on various ATPases that the rate of MgADP elimination can increase in the presence of MgATP.[31] It is most likely that, in the presence of MgATP, the rate constant of the separation of MgADP from nitrogenase becomes equal to the rate-limiting stage. After $(*FeMoco)^{1-}$ was formed, the conformation of nitrogenase have to become the same as in the initial state.

It has been established in 1995 that P_i is liberated after the intramolecular electron transfer during ATP hydrolysis by nitrogenase.[37] In this connection, the fact of energy coupling in nitrogenase was called into question.[37] However, the reactions considered must occur actually in this sequence if we take into account that in the systems of energy coupling, to which nitrogenase belongs, the ATP hydrolysis products, ADP and P_i, are liberated to the solution after the "coupling" stage 4 (Scheme 3) at which, in the case of nitrogenase, the intramolecular electron transfer should occur.

The energy profile of the transfer of one electron in nitrogenase is presented in Fig. 1. It is seen that the electron transition occurs against the redox potential due to the use of the energy of ATP hydrolysis, as discussed above, to form the "super-reduced" FeMoco: $(*FeMoco)^{1-}$. The E_m values are presented for the process of the one-electron reduction of Av2 (in a complex with MgATP) and the P and FeMoco clusters in Av1 with dithionite. The $(*FeMoco)^{1-}$ potential can be estimated. Taking into account that two ATP molecules are hydrolyzed per each transferred electron and ΔF° of the hydrolysis of one ATP molecule is -30.5 kJ mol^{-1} at 25 °C (pH 7.0), we can estimate that for the transfer of one electron E_m for the $FeMoco^N \rightleftharpoons (*FeMoco)^{1-}$ transition in Av1 can reach -0.67 V (-0.042 V + (-0.63 V)). In biological systems of energy transformation, the efficiency of ATP hydrolysis can reach 85%.[31] Assume that in nitrogenase the efficiency of ATP hydrolysis is not lower than 85%, then for the transition indicated E_m is ca.-0.6 V. If after the transfer of the first electron from Av2 to Av1 the electron is not further transferred to the substrate,[11] the subsequent transfer of the second electron in the process coupled with the hydrolysis of two MgATP molecules gives the "super-reduced" state of FeMoco: $(*FeMoco)^{2-}$. The transfer of the second electron is not shown in Fig. 1. Published data on the E_m value for the $(*FeMoco)^{1-} \rightleftharpoons (*FeMoco)^{2-}$ transition are lacking. According to the calculation as that for $(*FeMoco)^{1-}$, we can obtain $E_m \approx -1$ V for the latter transition.

The estimation of E_m for the one-electron redox transition of FeMoco described in this work (-0.6 V) is likely correct. This conclusion is confirmed by published data.[38] In the case of Av2 specially changed by site-specific mutagenesis (deletion by *Leu*127 : L127Δ), E_m for the $[4Fe—4S]^{2+} \rightleftharpoons [4Fe—4S]^{1+}$ transition in the Av1 complex with Av2 becomes equal to -0.62 V.[38] One electron can be transferred from Av2 to Av1 without MgATP but with a lower rate than in the presence of MgATP. The second electron is not transferred without MgATP. In this case, the electron transfer cannot occur against the redox potential. Therefore, E_m for the $FeMoco^N \rightleftharpoons (*FeMoco)^{1-}$ transition cannot be lower than -0.6 V.

As follows from the presented material, despite considerable progress in studying the structure and redox properties of nitrogenase clusters, substantial problems remain in understanding of the mechanism of nitrogenase functioning. The main question: how does the

accumulation of eight electrons occur for nitrogen reduction on FeMoco and what is the role of the P clusters in this process? It is unclear whether P^N can accept electron(s) or not. Whether the capability of the P cluster of having 3 oxidation states (P^{1+}, P^{2+}, P^{3+}) allows the accumulation of electrons on it for subsequent nitrogen reduction? Why only one $P^{2+} \rightleftharpoons P^{1+}$ transition is pH-dependent[8]? Since the natural donor flavodoxin transfers two electrons to Av2, does this imply that electrons are transferred from Av2 to Av1 in pairs or successively one after another, as it has been shown when the photodonor was used[11]?

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References

1. B. E. Smith, in *Nitrogen Fixation: Achievements and Objectives*, Eds. P. M. Gresshof, L. E. Roth, G. Stacey, and W. E. Newton, Chapman and Hall, New York—London, 1990, 3.
2. M. M. Georgiadis, H. Komiya, P. Chakrabarti, D. Woo, J. J. Kornuc, and D. C. Rees, *Science*, 1992, **257**, 1653.
3. J. W. Peters, M. H. B. Stowell, S. M. Soltis, M. G. Finnegan, M. K. Johnson, and D. C. Rees, *Biochemistry*, 1997, **36**, 1181.
4. B. K. Burgess and D. J. Lowe, *Chem. Rev.*, 1996, **96**, 2983.
5. A. J. Pierick, H. Wassink, H. Haaker, and W. R. Hagen, *Eur. J. Biochem.*, 1993, **212**, 51.
6. W. H. Orme-Johnson, W. H. Hamilton, T. L. Jones, M.-Y. W. Tso, R. H. Burris, R. H., V. K. Shah, and W. J. Brill, *Proc. Natl. Acad. Sci. USA*, 1972, **69**, 3142.
7. E. Münck, H. Rhodes, W. H. Orme-Johnson, L. C. Davis, W. J. Brill, and V. K. Shah, *Biochim. Biophys. Acta*, 1975, **400**, 32.
8. W. N. Lanzilotta, J. Christiansen, D. R. Dean, and L. C. Seefedt, *Biochemistry*, 1998, **37**, 11376.
9. S. Yu. Druzhinin, L. A. Syrtsova, A. M. Uzenskaya, and G. I. Likhtenshtein, *Biochem. J.*, 1993, **290**, 627.
10. S. Yu. Druzhinin, L. A. Syrtsova, N. N. Denisov, N. I. Shkondina, and V. Yu. Gak, *Biokhimiya*, 1998, **63**, 1164 [*Biochemistry (Moscow)*, 1998, **63**, 996 (Engl. Transl.)].
11. L. A. Syrtsova, V. A. Nadtochenko, N. N. Denisov, E. A. Timofeeva, N. I. Shkondina, and V. Yu. Gak, *Biokhimiya*, 2000, **65**, 1353 [*Biochemistry (Moscow)*, 2000, **65**, 1145 (Engl. Transl.)].
12. S. Yu. Druzhinin, L. A. Syrtsova, E. T. Rubtsova, and N. I. Shkondina, *Biokhimiya*, 1996, **61**, 480 [*Biochemistry (Moscow)*, 1996, **61**, 355 (Engl. Transl.)].
13. S. Yu. Druzhinin, L. A. Syrtsova, E. T. Rubtsova, and N. I. Shkondina, *Biokhimiya*, 1996, **61**, 2165 [*Biochemistry (Moscow)*, 1996, **61**, 1567 (Engl. Transl.)].
14. M. G. Duyvis, H. Wassink, and H. Haaker, *Biochemistry*, 1998, **37**, 17345.
15. G. D. Watt, *Anal. Biochem.*, 1979, **99**, 399.
16. W. E. Newton, F. A. Schultz, S. F. Gheller, S. Lough, J. W. McDonalds, S. D. Conradson, B. Hedman, and K. O. Hodgson, *Polyhedron*, 1986, **5**, 567.
17. D. J. Lowe, K. Fisher, and R. N. F. Thorneley, *Biochem. J.*, 1993, **292**, 93.
18. M. G. Duyvis, R. E. Mensink, H. Wassink, and H. Haaker, *Biochim. Biophys. Acta*, 1997, **1320**, 34.
19. L. C. Seefeldt, M. J. Ryle, J. M. Chan, and W. N. Lanzilotta, in *Biological Nitrogen Fixation for the 21-st Century, Proc. of the 11 Int. Congress on Nitrogen Fixation*, Paris, France, Eds. C. Elmerich, A. Kondorosi, and W. E. Newton, Kluwer Acad. Publ., Dordrecht, 1997, 39.
20. H. Schindelin, C. Kisker, J. L. Schlessman, J. B. Howard, and D. C. Rees, *Nature*, 1997, **387**, 370.
21. R. L. Robson, *FEBS Lett.*, 1984, **173**, 394.
22. J. Christiansen, V. L. Cash, L. C. Seefeldt, and D. R. Dean, *J. Biol. Chem.*, 2000, **275**, 11459.
23. R. N. F. Thorneley and D. J. Lowe, *Biochem. J.*, 1983, **215**, 393.
24. L. A. Syrtsova, V. A. Nadtochenko, and E. A. Timofeeva, *Biokhimiya*, 1998, **63**, 1176 [*Biochemistry (Moscow)*, 1998, **63** (Engl. Transl.)].
25. R. R. Eady, *Biochem. J.*, 1973, **135**, 531.
26. R. C. Burns, *Biochim. Biophys. Acta*, 1969, **171**, 253.
27. A. Burns, G. D. Watt, and Z. C. Wang, *Biochemistry*, 1985, **24**, 3932.
28. M. S. Chan and J. Bolton, *Solar Energy*, 1980, **24**, 561.
29. S. Yu. Druzhinin, L. A. Syrtsova, A. V. Khramov, A. P. Moravskii, and N. I. Shkondina, *Biokhimiya*, 1995, **60**, 1512 [*Biochemistry (Moscow)*, 1995, **60** (Engl. Transl.)].
30. L. A. Blyumenfel′d, *Biofizika*, 1976, **21**, 946 [*Biophysics*, 1976, **21** (Engl. Transl.)].
31. L. A. Syrtsova, in *Uspekhi biologicheskoi khimii* [*Progress in Biological Chemistry. Collection of Works*], Nauka, Moscow, 1989, 130 (in Russian).
32. C. R. Bagshaw, D. R. Trentham, R. G. Wolcott, and P. D. Boyer, *Proc. Natl. Acad. Sci. USA*, 1975, **72**, 2592.
33. R. G. Wolcott and P. D. Boyer, *J. Supramol. Struct.*, 1975, **3**, 154.
34. L. A. Syrtsova, Doct. (Chem.) Thesis, Institute of Chemical Physics in Chernogolovka, RAS, Chernogolovka, 1989, 325 pp. (in Russian).
35. L. A. Syrtsova, A. M. Uzenskaya, S. Yu. Druzhinin, A. V. Khramov, A. P. Moravskii, and G. I. Likhtenshtein, *Biofizika*, 1988, **33**, 391 [*Biophysics*, 1988, **33** (Engl. Transl.)].
36. J. Cordewener, A. Asbroek, H. Wassink, R. R. Eady, H. Haaker, and C. Veeger, *Europ. J. Biochem.*, 1987, **162**, 265.
37. D. J. Lowe, G. A. Ashby, M. Brune, H. Knights, M. R. Webb, and R. N. F. Thorneley, in *Nitrogen Fixation: Fundamentals and Applications, Proc. of the 10 Int. Congress on Nitrogen Fixation*, St. Petersburg, Russia, Eds. I. A. Tikhonovich, N. A. Provorov, V. I. Romanov, and W. E. Newton, Kluwer Acad. Publ., Dordrecht, 1995, 103.
38. W. N. Lanzilotta and L. C. Seefeldt, *Biochemistry*, 1997, **36**, 12976.

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