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Influence of Glycerol on Nitrogenase Reactions[1]

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Abstract—The influence of glycerol on the ATPase reaction of nitrogenase and reduction of the substrate (acetylene) is studied. Glycerol inhibits the ATPase nitrogenase reaction dependent on an electron donor. The reaction rate is halved at a glycerol concentration of 11% in the medium when the solution viscosity increases only 1.31 times. The electron donor–independent (decoupled) ATPase reaction of nitrogenase is inhibited to a lesser extent. The activation energies (E_a) of reactions studied in the presence of glycerol are determined. Despite the inhibition effect, glycerol in a concentration of 7.5% does not affect the E_a of acetylene reduction. The introduction of glycerol significantly decreases the E_a of the electron donor-dependent ATPase reaction. In the absence of glycerol, this reaction limits the nitrogenase reaction: $E_a = 14 \pm 1.4$ kcal/mol at temperatures higher than 21°C and $E_a = 50 \pm 10$ kcal/mol at temperatures below 21°C, which are close to the E_a of acetylene reduction. In the presence of 7.5% glycerol, the $E_a = 0.7 \pm 0.6$ kcal/mol at temperatures above 21°C and the $E_a = 2.4 \pm 0.6$ kcal/mol at temperatures below 21°C. This indicates that the reactions of substrate-binding and ATPase sites are decoupled in the presence of glycerol, and the step of substrate reduction becomes the limiting step of the nitrogenase reaction. Glycerol also has a noticeable effect on the E_a of the electron donor-independent ATPase reaction and the shape of the plot of $\log w$ vs. $1/T$ for this reaction. The data obtained indicate the specific interaction of glycerol with nitrogenase in the region of the ATPase site perhaps due to the distortion of the structure of hydrogen bonds, and this interaction changes the limiting step of the nitrogenase reaction.

INTRODUCTION

Nitrogenase (EC 1.18.6.1.) is an enzyme that catalyzes the reduction of N_2 to NH_3. The composition and structure of nitrogenases and the main steps of the nitrogenase reaction, which is a complicated enzymatic reduction process, have been indentified in the past decades. Nitrogenase from *Azotobacter vinelandii* is known to consist of two metalloproteins: a protein containing molybdenum and iron (Av1) and an iron-containing protein (Av2). The first protein is the heterotetramer of two types of $\alpha_2\beta_2$ subunits with an overall molecular weight of ~250 kD and contains two [8Fe-7S] P and two [Mo-7Fe-9S-homocitrate] clusters, which are the so-called FeMo cofactors (FeMoco), that is, the centers of activation and reduction of substrates [2]. The Av2 protein is the homodimer of two subunits with an overall molecular weight of ~64 kD [1] and it contains one [4Fe-4S] cluster symmetrically incorporated between two subunits. Both nitrogenase components (Av1 and Av2), MgATP, and an electron donor are needed for enzymatic activity. In addition to nitrogen, nitrogenase also reduces other compounds with a triple bond, for example, acetylene, cyanides, and others. At least two adenosine-5'-triphosphoric acid (ATP) molecules are hydrolyzed per each transferred electron. The hydrolyzed ATP molecules in the form of a complex with Mg^{2+} are bonded to Av2. Only the reduction of protons to dihydrogen occurs in an argon atmosphere. The overall stoichiometry of the nitrogenase-catalyzed reduction of nitrogen or acetylene can be described by the following schemes [3]:

$$N_2 + 8H^+ + 8\bar{e} + 16MgATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16P_i, \quad \text{(I)}$$

$$C_2H_2 + 2H^+ + 2\bar{e} + 4MgATP \longrightarrow C_2H_4 + 4MgADP + 4P_i, \quad \text{(II)}$$

where P_i is inorganic phosphate and ADP is adenosine-5'-diphosphoric acid.

The first step of the nitrogenase reaction is electron transfer from an external electron donor (dithionite or a photodonor *in vitro*, flavodoxin or ferredoxin 1 *in vivo*) to the Av2 $[4Fe\text{-}4S]^{2+}$ cluster. Then, in the process coupled with ATP hydrolysis, an electron from the $[4Fe\text{-}4S]^{1+}$ cluster in Av2 transfers to the P cluster in Av1 and further to FeMoco, reduced by dithionite ($FeMoco^N$) to form the so-called "super-reduced" FeMoco ($(*FeMoco)^{1-}$) [4]. The latter process does not occur without ATP. The half-reduction potential at pH 7.0 with respect to the hydrogen electrode (E_m) for the $[4Fe\text{-}4S]^{2+} \longleftrightarrow [4Fe\text{-}4S]^{1+}$ transition in the Av2 protein bonded to two MgATP molecules is equal to –0.43 V [5]. According to experimental data, E_m for the $FeMoco^N \longleftrightarrow (*FeMoco)^{1-}$ transition in Av1 ranges from –0.6 to –1 V [6]. Thus the electron transfer from Av2 to Av1 occurs against the potential gradient. Therefore, nitrogenase is categorized as an enzyme with

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energy coupling in which, according to the definition given by Blyumenfel'd [7], "one chemical transformation with a free energy decrease can provide the occurrence of another chemical transformation accompanied by an increase in the free energy." ATP hydrolysis is considered to liberate energy needed for electron transfer. However, the mechanism of energy coupling in nitrogenase remains unknown.

Two regions of MgATP binding to Av2 (one per each identical subunit) are at a distance of 15 Å from the metalloclusters involved in the electron transfer [8]. Therefore, MgATP is assumed to affect the [4Fe-4S] cluster in Av2 by distant conformational changes [5]. This assumption is confirmed by a series of publications. It is known that the MgATP-affected [4Fe-4S] cluster becomes more accessible to chelating reagents [9]. The ESR signal with 1/2 spin changes symmetry from rhombic to axial [10]. The redox potential shifts by ~120 mV toward negative values [11]. The NMR studies have shown that changes in the properties of the [4Fe-4S] cluster result from changes in the protein environment near the cluster rather than from changes in the Fe–Fe and Fe–S distances or the orientations of the ligands of the [4Fe-4S] cluster [12]. As shown by the circular dichroism method, MgATP and MgADP induce different conformational changes in the Fe protein structure [13]. Conformational changes in nitrogenase were also found upon formation of the Av1 · Av2 nitrogenase complex and assumed to facilitate electron transfer [14].

ATP hydrolysis can be suggested to induce conformational changes in the protein structure. They change the microenvironment of the iron–sulfur clusters and their E_m, creating a favorable free energy profile for distant electron transfer from Av2 to the P cluster and further to FeMoco in Av1.

It is very important for correct quantitative analysis of electron transfer reactions to take into account changes in the nitrogenase conformation during its functioning and the direct influence of these changes on the potential of the iron–sulfur clusters and, finally, on the electron transfer between the Av1 and Av2 clusters of nitrogenase.

In the nitrogenase reaction accompanied by slow conformational rearrangements, whose characteristic time is comparable with or longer than the time for electron transfer between the nitrogenase clusters, electron transfer reaction can occur in the adiabatic regime or "gated reaction" regime. In this case, electron transfer is independent of the distance between the transfer centers and determined by the time for the protein structure "alignment" during electron transfer [15, 16]. The possibility of this reaction regime can be judged by measuring the rate of relaxation rearrangements in the protein structure using the temperature, solvent viscosity, or specific action on the functional groups of the active site.

The purpose of this work is to study the influence of glycerol on the nitrogenase reactions and their activation energies. The results of this study provide information on the steps of energy coupling in nitrogenase, and the limiting step of the reaction is revealed in the presence and absence of glycerol. Data obtained will be used for a more detailed description of electron transfer in nitrogenase in the framework of the Marcus theory.

EXPERIMENTAL

Materials. DEAE cellulose (Whatman, Great Britain); G-150 Sephadex (Pharmacia, Sweden); ATP, glycerol, and Tris buffer (Serva, Germany); and sodium dithionite (Merck, Germany) were used. Water and argon were purified, the crystalline Av1 and homogeneous Av2 proteins were isolated from the *Azotobacter vinelandii OP* cells, and their specific activities (specific rates of the enzymatic reaction, w) were determined as described previously [17]. The specific activities of the Av1 and Av2 proteins determined in a tenfold excess of complementary protein were 2000–2500 and 2000 nmol of acetylene reduced per 1 mg of protein for 1 min at 30°C.

Protein concentration was determined by the biuret method and from the Mo and Fe content. The Mo and Fe content was determined by atomic absorption on an AAS I spectrometer (Carl Zeiss, Germany) with an HGA-74 graphite attachment (Perkin-Elmer, USA).

Specific rates (w) of nitrogenase reactions: the rates of substrate reduction or ATP hydrolysis (enzymatic activity) were determined by the initial linear region of the kinetic curve of product accumulation and expressed as the amount of products formed per protein amount per unit time. The concentrations of all nonprotein components of the medium were several times higher than their Michaelis constants to provide the maximum rate of the enzymatic reaction under the chosen experimental conditions.

Reduction rate of acetylene to ethylene was determined in an argon–acetylene (11 : 2, vol/vol) atmosphere in serum bottles with a rubber compacting stopper. The reaction system (2 ml) contained Av1 (2.75×10^{-3} mmol), Av2 (6×10^{-3} mmol), ATP (10 mmol), $MgCl_2$ (20 mmol), $Na_2S_2O_4$ (28 mmol), and Tris buffer (45 mmol, pH 7.2). After nitrogenase was added, incubation was carried out at 30°C for 30 s with continuous stirring. The reaction was stopped by the addition of trichloroacetic acid to a concentration of 5%, and the amount of ethylene formed was determined by chromatography of the gas phase on an LKhM-8 MD chromatograph.

In experiments on the determination of the ATPase activity (ATP hydrolysis rate), the incubation system was the same, but argon was used as a gas phase. After a 30 s incubation, a sample was taken via a syringe from the reaction system and frozen in liquid nitrogen to stop the reaction. For the measurement of

the content of inorganic phosphate, tubes with the samples were placed in a water bath at 4°C, and the samples were allowed to melt. Aliquots (0.4 ml) were taken, and 11.5% trichloroacetic acid (5.6 ml) was introduced into each tube at 4°C. Denatured protein was separated by filtration, and the content of phosphate was determined in the filtrate by the Taussky–Shorr procedure [18]. Dithionite was added to the reaction system when the donor-independent ATPase activity was measured. In each series of experiments (with or without dithionite), the initial content of phosphate in the reactants was determined in reference experiments using nitrogenase inactivated by incubation in air for 5 min. Reference experiments were carried out as those described for nitrogenase. The amount of phosphate in a reference sample was at most 10% of the phosphate content in experimental samples. The amount of phosphate formed due to the ATP hydrolysis for 30 s with the background subtracted was used for the calculation of ATPase activity.

When studying the temperature dependence of nitrogenase reactions, the reaction system without nitrogenase and a solution of nitrogenase were heated to a required temperature before mixing.

Specific rates of nitrogenase reactions are presented in nanomoles of the reaction product (ethylene or phosphate) formed per 1 mg of Av1 for 1 min. The mean-square error is 5%.

Activation energies were calculated from the $\log w - 1/T$ plots for the nitrogenase reactions. In each figure presented in the work, experimental points are averaged over three parallel experiments. For the E_a values obtained from the series of three $\log w - 1/T$ plots equal to 5–50 kcal/mol, the mean-square error of measurement is 10%, and for the E_a values lower than 5 kcal/mol the error is ±0.6 kcal/mol.

RESULTS AND DISCUSSION

The influence of glycerol on the nitrogenase reactions and their activation energies were studied in detail. It is known that glycerol is a cryoprotector; it strongly changes the solution viscosity and thus retards the rate of diffusion interactions of the substrate with enzyme or large conformational rearrangements inside the protein [19, 20]. In addition, glycerol can specifically interact with the surface amino acid residue of a protein, changing the interaction of protein with water molecules and thus distorting the structure of hydrogen bonds for the whole protein globule [21]. These hydrogen bonds are important for protein functioning. In turn, changes and distortions can prevent protons from entering the active site of an enzyme. Glycerol is a polydentate ligand and can interact with metal ions in metalloenzyme. Although it is difficult to distinguish the mechanisms of glycerol action (this could be a task of future work), the diffusion interactions, conformational changes, or proton transfer are the limiting steps of the complicated enzymatic reactions and, hence, glycerol should affect the E_a of this reaction. Thus, glycerol can be used to identify the limiting step of an enzymatic reaction. Since the metal-containing clusters of the Av1 protein are inside the protein globule [1], their interaction with glycerol is improbable. Molecules of compounds with a triple bond, which can be nitrogenase substrates, are known to be smaller than the glycerol molecule [5]. The [4Fe-4S] cluster in Av2 is coordinatively saturated and cannot interact with glycerol.

As in the case of other ATPases [22], the plots of $\log w$ vs. $1/T$ for the nitrogenase reaction are characterized by a break at 21–22°C [23]. The E_a values of substrate reduction by nitrogenase from *A. vinelandii* calculated from these plots are 50.2 kcal/mol ($T < 22$°C) and 15 kcal/mol ($T > 22$°C) [24]. The $\log w$–$1/T$ plots for all reactions of substrate reduction by nitrogenase are similar in shape [24]. Therefore, it is reasonable to assume that ATP hydrolysis is the limiting step of the reaction. The break in the plot of $\log w$ vs. $1/T$ can be explained by conformational changes in nitrogenase [24] or a change in the limiting step of the reaction at the break point [25]. However, a reason for these changes remains unclear.

To determine the enzymatic activity of nitrogenase, we studied the kinetics of acetylene reduction. The reaction rate was determined from the linear region of kinetic curves (Fig. 1). The linear dependence of the amount of the product substance on time was observed for 30 s with or without glycerol. The enzymatic activity of nitrogenase was also studied in ATP hydrolysis [26], where the plot of the amount of the product (P_i) vs. reaction time is also linear for 30 s regardless of the presence of glycerol.

Glycerol inhibits acetylene reduction (Fig. 2). When the glycerol concentration in the medium is 11%, the ratio of the reaction rate in the presence of glycerol to the reaction rate without glycerol is 0.5 at 30°C (Fig. 2). In this case, the solution viscosity increases only 1.31 times [27]. Therefore, inhibition cannot be explained by a change in the viscosity of the solution. In this case, the inhibition effect of glycerol can be attributed to its influence on the protein globule of nitrogenase, for example, to a change in the structure of hydrogen bonds near the active site. Our studies show that the influence of glycerol on acetylene reduction by nitrogenase is determined by glycerol effect on the ATPase activity of nitrogenase.

As already mentioned, the ATP hydrolysis in nitrogenase is coupled with electron transfer. This implies that this process mainly occurs in the presence of an external electron donor (for example, dithionite or a photodonor *in vitro*, flavodoxin or ferredoxin *in vivo*) during electron transfer from Av2 to Av1. However, low ATPase activity is observed in the absence of an electron donor. The electron donor-independent MgATP hydrolysis of nitrogenase occurs when Av2 is oxidized

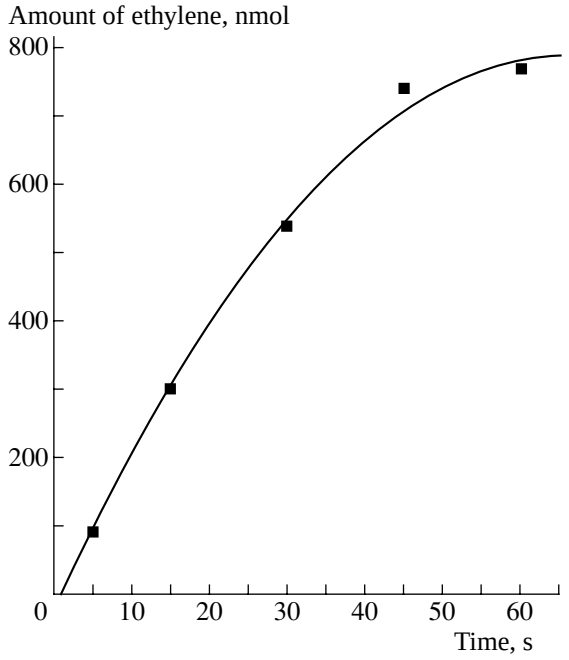

Fig. 1. Kinetic curve of acetylene reduction in the presence of ATP without the ATP-regenerating system at 30°C. An acetylene–argon mixture is the gas phase.

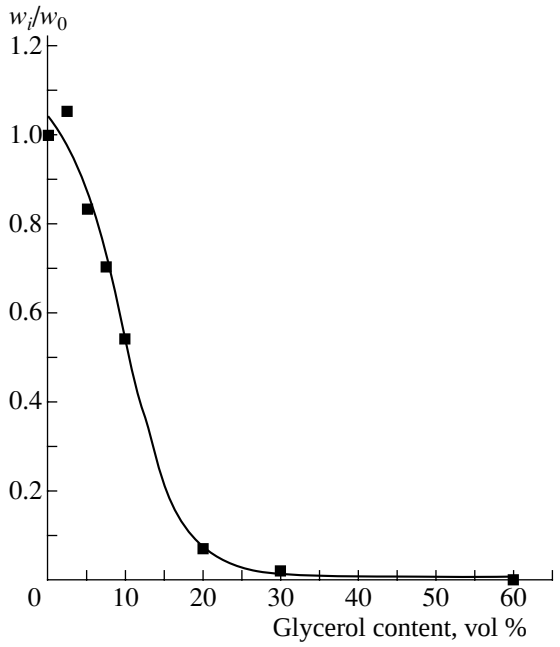

Fig. 2. A plot of the ratio of the rates of acetylene reduction by nitrogenase at 30°C in the (w_i) presence and (w_0) absence of glycerol vs. glycerol content.

[28] or under conditions that are not optimal for electron transfer (at certain values of temperature, pH, and ratio of protein components [5]). The rate constants of competitive inhibition by MgADP for the donor-dependent and donor-independent ATPase activities of nitrogenase are the same. This indicates that these reactions occur at the same active site [5]. In this work, we used an Av2 : Av1 ratio equal to 2.2 : 1 to monitor both the donor-dependent and donor-independent ATPase activities in parallel. The overall ATPase activity (ATP hydrolysis rate) in the presence of dithionite and the donor-independent ATPase activity (in the absence of dithionite) were determined from experimental data. The donor-dependent ATPase activity was determined by the difference between the first and second activities. Glycerol inhibits the dependent activity to a greater extent than the donor-independent ATPase activity. The plots of the reaction rates in the presence and absence of glycerol vs. glycerol content are shown in Fig. 3. The glycerol inhibition of the donor-dependent ATPase reaction is parallel to its inhibition of acetylene reduction (Fig. 2). Based on these data, we can suggest that glycerol inhibits acetylene reduction due to inhibition of the donor-dependent ATPase reaction. This conclusion was confirmed by the study of the glycerol influence on the E_a of the nitrogenase reactions.

A new class of inhibitors of nitrogenase (for example, pentachlorophenol [26]) affecting the donor-dependent ATPase activity but not affecting the donor-independent ATPase activity has been discovered previously. These inhibitors were named inhibitors of the coupling site in nitrogenase. Pentachlorophenol decreased the donor-dependent ATPase activity and inhibited the reduction of a substrate to the same extent. Glycerol can also be categorized as such an inhibitor because it decreases the donor-dependent ATPase activity to a greater extent.

The influence of glycerol on the E_a of the nitrogenase reactions was studied to determine the limiting step of the nitrogenase reaction. The activation energies were determined from the temperature dependence of the reaction rates (w) at a constant concentration of the reactants [29] using the plots of $\log w$ vs. $1/T$ by the formula $E_a = 2.303R\Delta \log w / \Delta (1/T)$, where R is the universal gas constant equal to 1.987 cal deg^{-1} mol, T is the absolute temperature, and $\Delta \log w / \Delta(1/T)$ was determined from the plot.

In the presence of glycerol, the temperature plot of acetylene reduction (Fig. 4) remained the same as that without glycerol [24] with a break in the plot of $\log w$ vs. $1/T$ for this reaction at 21°C. The activation energies are presented in Table 1. Thus, the partial inhibition of the donor-dependent ATPase reaction and subsequent acetylene reduction had no effect on the E_a of acetylene reduction. This fact was explained by the study of the E_a of ATP hydrolysis by nitrogenase.

In the study of the temperature relation of the rate of ATP hydrolysis by nitrogenase, we compared in parallel experiments the electron donor (dithionite)–dependent and donor-independent ATPase activities determined as the amount of inorganic phosphate formed

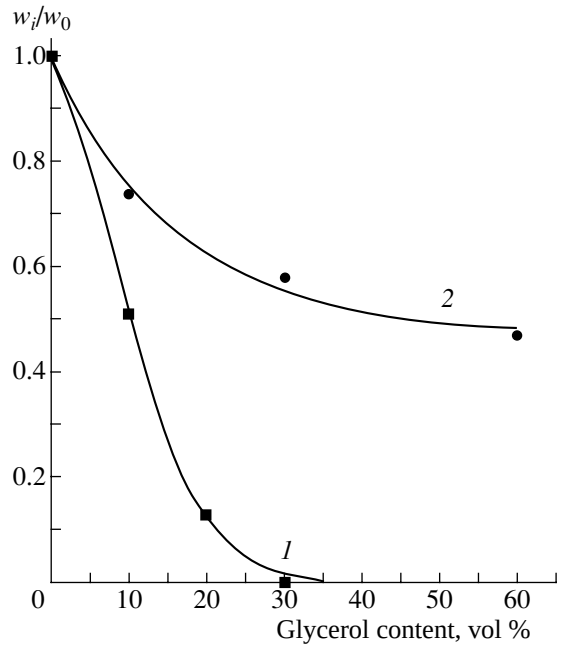

Fig. 3. Plots of the ratio of the rates of the ATPase reaction vs. glycerol content at 30°C: (*1*) the difference in the presence and absence of dithionite and (*2*) in the absence of dithionite. Argon is the gas phase.

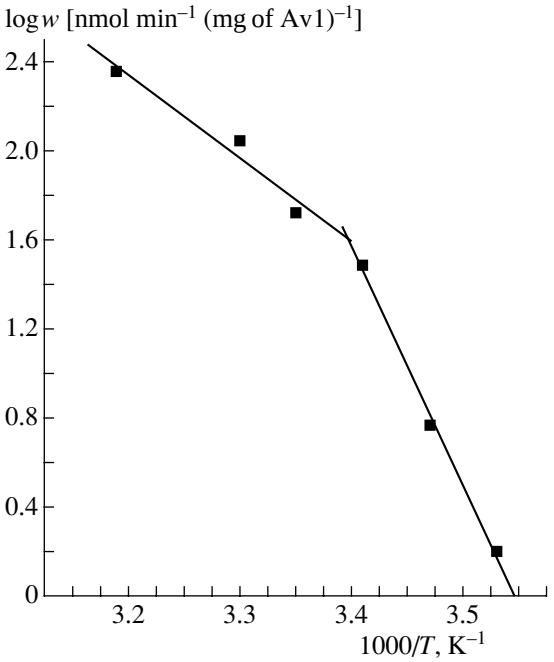

Fig. 4. A plot of $\log w$ vs. $1/T$ of acetylene reduction by nitrogenase in the presence of 7.5% glycerol.

(P_i) (Table 2). It was found that at temperatures < 21°C the ATPase activity of nitrogenase becomes decoupled to a great extent, independent of an electron donor.

At temperatures > 21°C, in the absence of glycerol, for the donor-dependent ATPase activity (reaction in the presence of dithionite), the E_a = 14 kcal /mol (Table 1), which is close to the E_a value of acetylene reduction in this temperature interval. Below 21°C, in the absence of glycerol, for the donor-dependent ATPase activity, the E_a can only be calculated by two points (15 and 20°C) with an error of 20% (Table 2). According to several experiments, the E_a is equal to 50 kcal/mol, which is also close to the E_a value of acetylene reduction. Thus, we conclude that for the donor-dependent ATPase reaction, in the absence of glycerol, the plot of $\log w$ vs. $1/T$ has the same character as that for substrate reduction: both the break at 21°C and the E_a values above and below this temperature are retained (Fig. 5). Thus, in the absence of glycerol, the electron donor–dependent ATPase nitrogenase reaction is responsible for the activation parameters. At temperatures > 21°C, the donor-independent ATPase activity remains unchanged when the temperature changes (the E_a value of this reaction becomes close to zero (Table 1)), and

Table 1. Activation energies (E_a, kcal/mol) of the nitrogenase reactions

Nitrogenase reaction	Glycerol content, %	Temperature of reaction mixture	
		below 21°C	above 21°C
Acetylene reduction	7.5	51 ± 5	15 ± 1.5
Electron donor–dependent ATPase activity	0	50 ± 10	14 ± 1.4
	7.5	2.4 ± 0.6	0.7 ± 0.6
Electron donor-independent ATPase activity	0	12 ± 1.2	0*
	7.5	5.3 ± 0.5	5.3 ± 0.5

Note: The errors take into account the mean-square measurement error determined for the series of three experiments.
* Since the reaction rate is temperature-independent in this case, E_a is close to zero with an accuracy of 0.6 kcal/mol.

Table 2. Amounts of the product (P_i) in the ATPase nitrogenase reaction in the presence and absence of dithionite

T, °C	P_i, nmol min^{-1} (mg of Av1)$^{-1}$	
	$+Na_2S_2O_4$	$–Na_2S_2O_4$
10	430 ± 20	430 ± 20
15	680 ± 30	630 ± 30
20	900 ± 45	720 ± 35
25	1240 ± 60	890 ± 45
30	1470 ± 70	890 ± 45
35	1730 ± 85	890 ± 45
40	1870 ± 95	890 ± 45

Note: The reaction system contains the components indicated in the experimental section. Argon is the gas phase.

at temperatures below 21°C the E_a = 12 kcal/mol. Therefore, when the ATPase reaction depends on an electron donor and is coupled with electron transfer from Av2 to Av1, an activation barrier appears and can presumably be related to the inclusion of a new step into the sequence of steps of the complicated enzymatic reaction of ATP hydrolysis. This can be a step associated with the conformational change or proton transfer, etc. The low or zero E_a values indicate the complicated character of the reaction. The simplest way to explain this is as follows. All steps of ATP hydrolysis are known to be reversible (see review [31]). When E_a for any inverse reaction preceding the apparent value increases, it can be lower than zero, equal to zero, or negative, since the total E_a is equal to the sum of the E_a of particular steps. At a low E_a (0–2.5 kcal/mol), taking into account the mean-square error of w determination (5%), the error of E_a calculation is 0.6 kcal/mol.

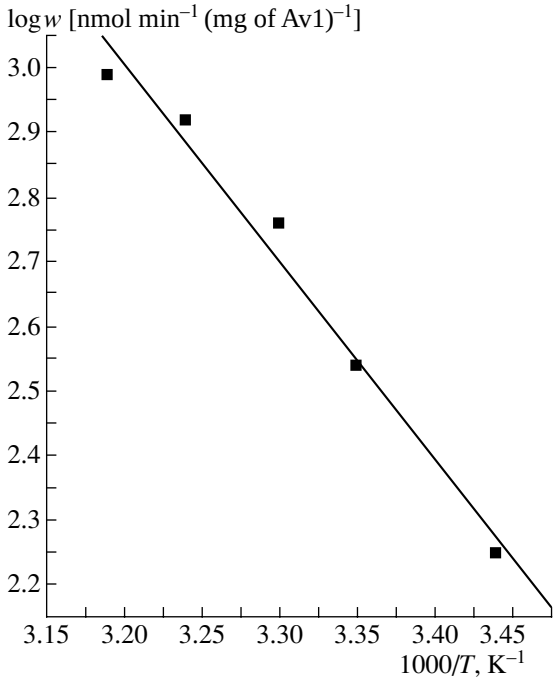

Fig. 5. A plot of $\log w$ vs. $1/T$ of the electron donor–dependent ATPase reaction. The reaction mixture contains no glycerol. Argon is the gas phase.

Note that the ATPase activity was measured in this work by the accumulation of inorganic phosphate P_i. The ATPase reaction of nitrogenase, as well as the reactions of other ATPases, consists of several steps [30, 31]. The region of MgATP binding in Av2 contains the same sequence of amino acids as other ATPases [32]. As discussed previously [30], the limiting step of the ATPase reaction, as well as the nitrogenase turnover, can be a change in the conformation before the liberation of the MgATP hydrolysis products: MgADP or P_i (as in the case of other ATPases). However, so far for nitrogenase there are no clear data which of the steps is slower: liberation of MgADP or P_i. These reactions are both slow. The P_i liberation after ATP hydrolysis is characterized by a reaction rate constant of 22 s^{-1} [33]. It is unknown whether this reaction is preceded by the limiting step of the conformation change. The rate of MgADP separation from the nitrogenase molecule is low even in the step of the first electron transfer: the first MgADP molecule is separated from nitrogenase with $k_{app} < 0.2$ s^{-1}, and the second molecule separates with $k_{app} > 0.6$ s^{-1} [34]. Data on various ATPases provide evidence that the rate of MgADP elimination can increase in the presence of MgATP (see review [31]). For most ATPases, the conformation of ATPase in a complex with MgADP changes slowly to the initial conformation without MgATP followed by the fast step of MgADP escape to a solution or substitution by MgATP. Electron transfer is synchronous with one of the steps of the ATPase reaction preceding the step of liberation of the products of ATP hydrolysis to the medium [30]. When dithionite is used as an electron donor, the limiting step of the nitrogenase reaction is characterized by a rate constant equal to 6.4 s^{-1} at 23°C [35]. Thorneley and Lowe [35] believe that the nitrogenase reaction is limited by the dissociation of nitrogenase to the protein components after electron transfer from Av2 to Av1. The authors of [30] presented arguments that the nitrogenase reaction is limited by a change in the conformation before the liberation of the products of ATP hydrolysis to the medium. In the presence of a photodonor, nitrogenase does not dissociate [30]. Thus, the nature of the limiting step of the nitrogenase reaction remains disputable.

The influence of glycerol on the activation parameters of the nitrogenase reactions was studied at a 7.5% concentration of glycerol in the sample. The solution viscosity changed insignificantly [27]. In the presence

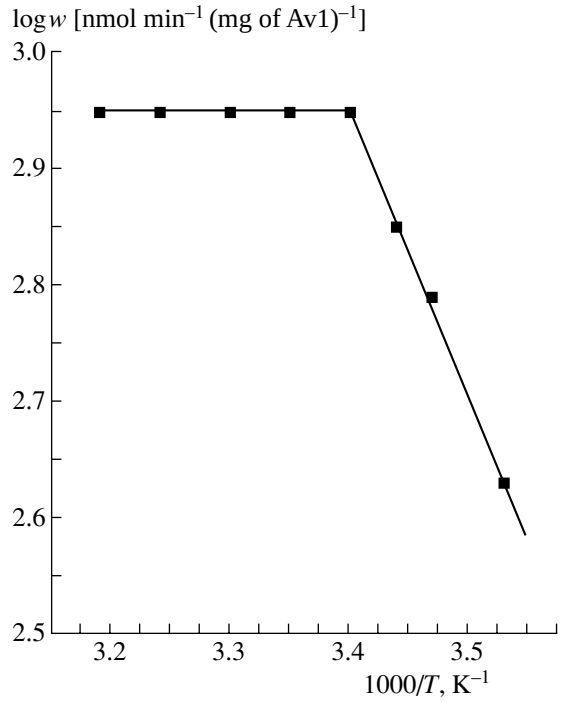

Fig. 6. A plot of $\log w$ vs. $1/T$ of the electron donor-independent ATPase reaction. The reaction mixture contains no glycerol or dithionite. Argon is the gas phase.

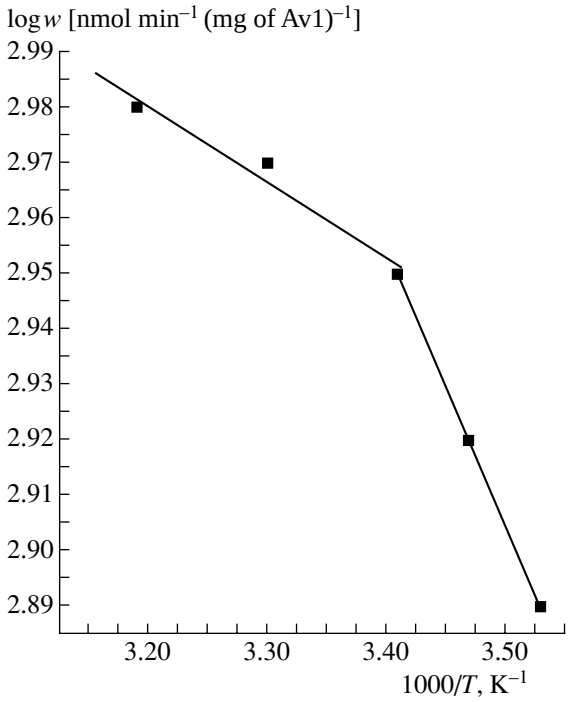

Fig. 7. A plot of $\log w$ vs. $1/T$ of the electron donor-dependent ATPase reaction in the presence of 7.5% glycerol. Argon is the gas phase.

of 7.5% glycerol, the $\log w$–$1/T$ plot for the electron donor–dependent ATPase reaction has a break point at 21°C (Fig. 7). However, the E_a of this reaction changes substantially. At temperatures higher than 21°C, the E_a is only 0.7 kcal/mol, and at $T < 21$°C, the $E_a = 2.4$ kcal/mol (Table 1). The considerable change in the E_a (~20 times) indicates a substantial change in the reaction mechanism. In the absence of glycerol, the rates of acetylene reduction and ATP hydrolysis change similarly with temperature, whereas in the presence of glycerol the reduction of acetylene and the ATP reaction at different temperatures are inhibited to different extents: acetylene reduction is retarded more substantially with a temperature decrease than the ATPase reaction. The reaction of substrate reduction becomes slower (limiting). Perhaps, in this case, the reaction is limited by some step between the ATP hydrolysis and acetylene reduction: electron or proton transfer. Under the same conditions, for the donor-independent ATPase reaction, the break on the $\log w$–$1/T$ plot disappears in the whole temperature interval from 10 to 40°C, and the E_a = 5.3 kcal/mol (Fig. 8, Table 1).

Thus, in this study we found the specific influence of low concentrations of glycerol on the nitrogenase reactions. It is known that glycerol is a good cryoprotector, favors the stabilization of biological structures and macromolecules at low temperatures, and retains their activity at concentrations of 60% and higher [21]. Our experiments show that at glycerol concentrations as low as 7.5%, the E_a of the donor-dependent ATPase reaction decreases significantly. The shape of the plots of $\log w$ vs. $1/T$ changes for both the donor-dependent and donor-independent ATPase reactions simultaneously with the decoupling of the electron donor-dependent ATPase reaction and substrate reduction.

The data obtained indicate that the glycerol effect is caused by neither a change in the solution viscosity nor by the diffusional mobility of substrates or protein as a whole. It is more probable that glycerol specifically interacts with the surface amino acid residues of the protein globule in the region of the ATPase site and thus affects the internal "chemical" protein structure, which also contains hydrogen bonds. When assuming that the coupling of ATP hydrolysis with electron transfer is associated with protein rearrangement or diffusional migration of a proton, then the total E_a value of the ATP hydrolysis should include the E_a of protein rearrangement (conformational changes) or the E_a of proton motion. In this case, decoupling due to the elimination of conformational changes or proton motion can change the E_a of ATP hydrolysis.

The results obtained make it possible to decouple the ATPase reaction and substrate reduction by rather slight actions on the protein globule, thus transforming substrate reduction into the limiting step. The elementary steps of electron transfer between the metal-containing clusters of nitrogenase possibly will be studied in the future.

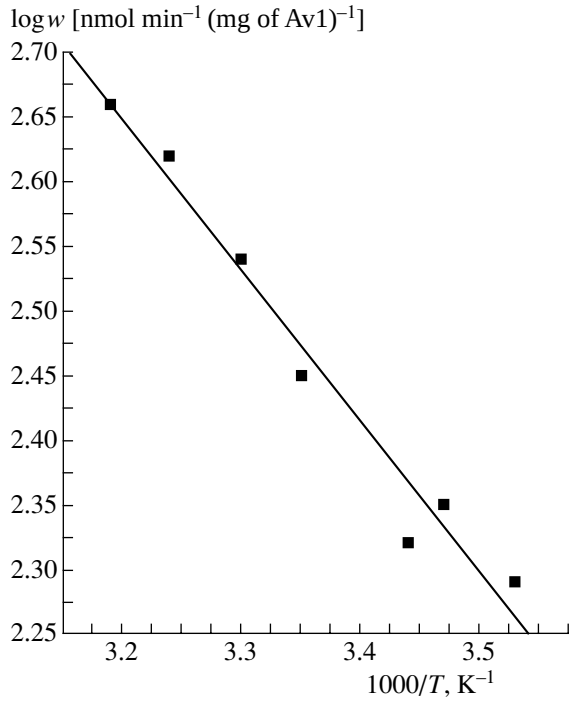

Fig. 8. A plot of log*w* vs. 1/*T* of the electron donor-independent ATPase reaction in the presence of 7.5% glycerol. The reaction mixture contains no dithionite. Argon is the gas phase.

CONCLUSIONS

(1) The study of the glycerol influence on the nitrogenase reactions has revealed its inhibition effect at low concentrations (7–11%) and established that at room temperature glycerol inhibits acetylene reduction and the electron donor-dependent ATPase reaction to the same extent. This effect is not related to a change in the solution viscosity because the reaction rate is halved when the solution viscosity increases by 30%.

(2) The plot of log*w* vs. 1/*T* for the reduction of the substrate (acetylene) in the presence of 7.5% glycerol is characterized by parameters analogous to those for this reaction in the absence of glycerol.

(3) In the absence of glycerol, the electron donor-dependent ATPase reaction in the temperature interval 10–40°C is characterized by activation parameters close to those of acetylene reduction. The electron donor-dependent ATP hydrolysis is the limiting step.

(4) In the presence of 7.5% glycerol, the E_a of the electron donor-dependent ATPase reaction sharply decreases.

(5) Above 21°C, the rate of the electron donor-independent ATPase reaction is temperature-independent.

(6) The different temperature plots of the rates of acetylene reduction and the electron donor-dependent ATPase reaction in the presence of 7.5% glycerol indicate that these reactions are decoupled by glycerol. Under these conditions, reduction of the substrate becomes the limiting step. This is a new effect, which will enable a future independent study of the ATP hydrolysis and elementary steps of electron transfer between the nitrogenase clusters.

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