The photoreduction of nitrogenase

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The photoreduction, without reductant dithionite, of N_2 to NH_3 or acetylene to ethylene catalysed by nitrogenase in the presence of Mg^{2+} ·ATP, eosin and NADH in the light has been established. There is an optimum NADH concentration for each particular eosin concentration. When the ratio of the iron protein component of nitrogenase from *Azotobacter vinelandii* (Av2)/the molybdenum–iron protein component of nitrogenase from *A. vinelandii* (Av1) is equal to 3 for 4×10^{-5} M eosin the optimum NADH concentration is 5×10^{-4} M. The rate of photoreduction (per one electron) of acetylene or N_2 under identical conditions was shown to be similar. The photoreductant-dependent ATPase

activity, in the presence of a given photochemical system in the light, was revealed. Eosin is shown to be the inhibitor of the coupling site. Concentrations of 8×10^{-6} – 1×10^{-4} M eosin do not inhibit the ATPase activity. The inhibition of substrate-reduction activity depends on the ratio of the nitrogenase components. Under conditions where the Av2/Av1 ratio is equal to 1 the rate of photochemical reduction is higher than in the presence of dithionite: the total electron flux through nitrogenase being increased 2.2-fold. We suggest that in this case the nitrogenase complex (1:1) works without dissociation.

INTRODUCTION

The enzyme nitrogenase (E.C. 1.18.6.1) from *Azotobacter vinelandii* consists of two protein components: a molybdenum–iron protein (Av1) (molecular mass 224 kDa) and an iron protein (Av2) (molecular mass 64 kDa). Nitrogenase, as a central enzyme of biological nitrogen fixation, catalyses the N_2 to NH_3 (or acetylene to ethylene) reduction at room temperature and atmospheric pressure (Smith et al., 1988). The key step of the nitrogenase reaction is the electron transfer from Av2, with a redox potential versus the normal hydrogen electrode at pH 7.0 (E_1^0) of -435 mV, to Av1, with E_1^0 values of 290 mV and -480 mV (Watt et al., 1980), against the potential gradient, with the formation of a so-called Av1 super-reduced state followed by the substrate reduction. This electron-transfer reaction is coupled with ATP hydrolysis (Thorneley and Lowe, 1982). Despite a great deal of research in this field some of the principal problems of the nitrogenase reaction have not been solved yet. The mechanism of coupling has remained unknown.

One of the most promising ways of gaining this information appears to be a photoreduction of nitrogenase over a microsecond range.

This paper presents results about the 'eosin-photosensitized' catalytic photoreduction of N_2 to NH_3 or acetylene to ethylene by nitrogenase from *A. vinelandii*. The properties of eosin as an inhibitor of the enzyme and a comparison of dithionite and NADH–eosin systems as reductants are examined.

MATERIALS AND METHODS

Materials

DEAE-cellulose DE-52 anion-exchange resin (Whatman, England), Sephadex G-150 (Pharmacia, Sweden), ATP, creatine phosphate, creatine kinase, eosin, Tris (Serva, F.R.G.) and NADH (Reanal, Hungary) were used. Other chemicals of analytical reagent grade were obtained from commercially available sources.

Purification of nitrogenase

Pure water and argon, crystalline Av1 and homogeneous Av2 from *A. vinelandii* OP were obtained as described (Syrtsova et al., 1983) with an additional DEAE-cellulose chromatography of Av2. Av1 and Av2 proteins had specific activities of 2000–2500 and 2000 nmol of acetylene reduced/min per mg of protein respectively at 303 K under standard conditions (Syrtsova et al., 1983). All purification procedures were carried out in an argon atmosphere.

Photoreduction

The lamp power supply (DRSh-500) equipped with a water light filter ($l = 15$ cm) was used. The light intensity, as measured by means of thermoelement RTN-20S, was 4×10^{17} quantum/cm² per s in the range 440–600 nm. The photoreduction was performed in a 15 ml serum bottle (with a rubber stopper) installed in a glass thermostat which was placed on a magnetic stirrer. The rate of photoreduction was measured in medium (2 ml) containing nitrogenase components, ATP, $MgCl_2$, eosin, and NADH solutions in 40 mM Tris buffer, at pH 7.2. The gas phase was a mixture of N_2 (66.6 kPa) (including 92–95% $^{15}N_2$) and argon (26.6 kPa) or argon (80 kPa) and acetylene (13.3 kPa). The samples were incubated at 303 K for 2 min; the reaction was started by simultaneous addition of nitrogenase and illumination (the blind was removed), and was terminated by simultaneous removal of illumination (blind was inserted) and addition of 0.2 ml of 50% (v/v) trichloroacetic acid by means of a syringe (or 0.6 ml of 0.5 M EDTA for H_2 analysis). This was followed by mixing at 303 K for 2 min before analysis of the gas phase. Aliquots of the gas phase were analysed for the presence of ethylene or H_2 by gas chromatography, with separation of ethylene and acetylene on a column of Porapak N (0.3 cm $\times$ 40 cm) at 303 K, or of H_2 on columns fitted with molecular sieves 0.5 nm (5 Å) (0.4 cm $\times$ 100 cm).

The NH_3 which was formed was collected for analysis from the reaction medium by a micro-diffusion method. Micro-diffusion was carried out in side-arm flasks sealed with a gas-tight stopper

by a method similar to that of Bulen et al. (1965). Trapping solution (1 ml of 0.1 M H_2SO_4) was added into the side-arm; 5 ml of reaction medium was mixed with 1 ml of a solution of 0.5 mg of ^{14}N carrier in the form of ammonium sulphate in the main compartment of the flask. Then 35 ml of saturated K_2CO_3 was added to the main compartment, the flask was quickly closed and incubated for 20 h at room temperature. Then the H_2SO_4 solution, with NH_3 diffused from the side-arm, was placed in one bulb of a Rittenberg tube and oxidized with alkaline hypobromide to N_2 under vacuum. The N_2 formed was analysed by m.s. The percentage abundance of ^{15}N was calculated from the formula:

$$\% ^{15}\text{N} = \frac{100}{2R+1} \quad R = \frac{N_{28}}{N_{29}}$$

where N_{28} and N_{29} are the values of mass peaks 28 and 29 (i.e. $^{14}\text{N}_2$ and $^{14}\text{N}^{15}\text{N}$).

When analysing $^{15}\text{N}_2$ gas with a mass peak of 30 the following formula was used:

$$\% ^{15}\text{N} = \frac{N_{29} + 2N_{30}}{2[N_{28} + N_{29} + N_{30}]} \times 100$$

The quantity of ^{15}N in the test solution was calculated as

$$^{15}\text{N} \text{ (mg)} = \frac{C(a-0.37)}{b-a}$$

where 0.37 is the natural content of ^{15}N in air; C , the quantity in terms of milligrams of nitrogen as carrier; a , the content (%) of ^{15}N in the test solution, and b , the content (%) of ^{15}N in the gas phase.

All values given in the tables and figures are mean values from three experiments. All tests on activity determination were carried out under a linear range of reaction rates. The error is 10%.

ATPase activity

This was measured by analysis of P_i formed. To determine the ATPase activity the ATP-generating system (creatine phosphokinase and creatine phosphate) was excluded from the test system and the ATP content was doubled. Aliquots (0.4 ml) of reaction mixture were taken by syringe after definite times of incubation and frozen in liquid nitrogen to terminate the reaction. To measure the P_i content, the frozen aliquots were melted in tubes at 277 K and 5.6 ml of 11.5% (v/v) trichloroacetic acid were added. The denaturated protein was separated by filtration and the P_i content in the filtrate was determined by the method of Taussky and Shorr (1953).

Protein concentration

Protein concentration was determined by the biuret method and by analysis of Mo and Fe.

Mo and Fe analysis

The Mo and Fe contents were analysed using an atomic-absorption spectrometer AAS I (Carl Zeiss) with a graphite set HGA-74 (Perkin-Elmer).

RESULTS

The photoreduction of N_2 and acetylene in the nitrogenase system

The photoreduction of N_2 to NH_3 or acetylene to ethylene in the absence of dithionite has been observed. Figure 1 shows the dependence of eosin-photosensitized N_2 reduction on duration of illumination. The yield of NH_3 increases linearly with increasing illumination duration for about 20 s and then slows down. Similar kinetic behaviour for reduction of acetylene was observed. Figure 2 shows the dependence of acetylene photoreduction on eosin concentration. The photoreduction rate increases linearly with an increase in the eosin concentration from 1×10^{-5} to 3×10^{-5} M at a concentration of 5×10^{-4} M NADH. The rate was linear for about 30 s.

The lag which has been observed previously (Druzhinin et al., 1989) is absent. We consider that the lag arose due to diffusion of ethylene formed in the solution, because in that study the reaction was not stopped when the gas samples were taken for analysis. In the present study the reaction was stopped before gas samples were removed for analysis, and the reaction medium was mixed for 2 min at 303 K to allow full diffusion of the gas products formed.

The existence of a lag of several seconds would come to light when the method of performing photochemical experiments over a millisecond range is used.

The dependence of eosin-photosensitized acetylene reduction in the nitrogenase system on NADH concentration under conditions where the ratio of Av2:Av1 is equal to 3 is shown in Table 1. The tests were conducted over the linear range of reaction rate. In the case of 10^{-5} M NADH and 4×10^{-5} M eosin the reaction was linear for only 10 s. There is an optimum NADH concentration for each particular eosin concentration. Thus for 4×10^{-5} M eosin the maximum activity is reached at 5×10^{-5} M NADH and there is no further change until the NADH concentration reaches 2×10^{-2} M, when activity starts to

Figure 1 Eosin-photosensitized catalytic reduction of N_2 in the nitrogenase system depending on illumination duration (t)

The system composition was (μmol in ml): Av1, 5.4×10^{-3} ; Av2, 1.6×10^{-2} ; eosin, 4×10^{-2} ; NADH, 0.5; ATP, 10; MgCl_2 , 5; Tris, 40, pH 7.2, at 303 K. The lamp power supply was DRSh-500 ($\lambda > 350$ nm).

Figure 2 Time courses of eosin-photosensitized acetylene reduction by nitrogenase in the presence of different eosin concentrations

The system composition was (μmol in ml): Av1, 5.4×10^{-3} ; Av2, 1.7×10^{-2} ; eosin 6×10^{-2} (●), 4×10^{-2} (○), 3×10^{-2} (○), 2×10^{-2} (▽) and 1×10^{-2} (▲); the other components and the lamp power supply are the same as in Figure 1. Expts. were performed at 303 K. The error is $\pm 10\%$.

Table 1 The dependence on NADH concentration of eosin-photosensitized acetylene reduction in the nitrogenase system

The assay system composition was (μmol /ml): Av1, 5.4×10^{-3} ; Av2, 1.7×10^{-2} ; ATP, 10; MgCl_2 , 5; Tris, 40, pH 7.2 at 303 K. The lamp power supply is DRSh-500 ($\lambda > 350$ nm). The duration of illumination was 20 s in Expts. 2–15 and 10 s in Expt. 1. In the absence of nitrogenase in the light, or with nitrogenase in the dark ethylene was not formed.

Expt.	Eosin concn. ($10^5 \times \text{M}$)	NADH concn. ($10^4 \times \text{M}$)	Ethylene formed (nmol)
1	4	0.1	60 ± 6
2	4	1	205 ± 20
3	4	5	270 ± 25
4	4	10	270 ± 25
5	4	20	300 ± 30
6	4	100	280 ± 30
7	4	200	310 ± 30
8	4	400	160 ± 15
9	8	100	380 ± 35
10	8	5	430 ± 40
11	10	20	440 ± 40
12	12	200	515 ± 50
13	20	400	300 ± 30
14	60	200	390 ± 40
15	100	400	80 ± 8

become inhibited. The photochemical activity can be increased if the eosin concentration is $> 4 \times 10^{-5}$ M if the NADH concentration is $\geq 5 \times 10^{-4}$ M (Table 1). An eosin concentration $> 1.2 \times 10^{-4}$ M is not effective, due to the probable effect of eosin inhibition (cf. Figure 3).

In the presence of creatine kinase, under conditions where the Av2/Av1 ratio is equal to 3, and at concentrations of 4×10^{-5} M eosin and 5×10^{-4} M NADH the photochemical activity did not change (cf. Figure 2), and rose with increase of the eosin concentration to 2×10^{-4} M at 5×10^{-4} M NADH. Such an effect

Figure 3 The dependence of the degree of inhibition of acetylene reduction by eosin in the darkness in the nitrogenase system on the Av2/Av1 ratio [(●), 2.8; (○), 1; (▲), 0.5]

The system composition was (μmol in ml): Av1, 6×10^{-3} ; Av2, 1.68×10^{-2} (●), 6×10^{-3} (○) and 3×10^{-3} (▲); ATP 10; MgCl_2 , 5; Tris, 40, pH 7.2; sodium dithionite, 28. Expts. were performed at 303 K. The reaction time was 30 s.

Table 2 Comparison of catalytic photoreduction of acetylene and N_2 in the nitrogenase system with eosin and NADH

The system composition (μmol /ml): Av1, 6.5×10^{-3} ; Av2, 2.6×10^{-2} ; eosin, 4×10^{-2} ; NADH, 0.5; ATP, 5; MgCl_2 , 5; Tris, 40, pH 7.2; creatine phosphate, 50; creatine kinase, 0.3 mg/ml. The ^{15}N content in air (background) is 0.37%. Experiments were conducted at 303 K. The lamp power supply is DRSh-500 ($\lambda > 350$ nm). The illumination duration was 20 s. For microdiffusion of NH_3 5 ml of reaction mixture was used.

Gas phase	^{15}N (%) in the probe (with background)		Product formed (nmol)	
	Light	Dark	NH_3	Ethylene
N_2 (94.8% ^{15}N)	1.22 ± 0.06	0.37 ± 0.02	150	—
Argon/ C_2H_2 (13:2)	—	—	—	520

is observed also for an Av2/Av1 ratio equal to 1: the optimum eosin concentration increased to 2×10^{-4} M and photochemical activity also increased. Therefore creatine kinase has decreased the inhibitory effect (cf. Figure 3), probably by binding of eosin. But on increasing eosin concentration to more than 2×10^{-4} M in the last case photochemical activity decreased, due to the inhibitory effect of eosin for Av2/Av1 ratio of 1 (cf. Figure 3).

The comparison of N_2 and acetylene photoreduction under the same conditions can be seen in Table 2. Under identical conditions the amount of ethylene formed is 3.47-fold higher than the amount of NH_3 . This value is in close agreement with the number of electrons needed for the enzymic reduction of N_2 (8) and acetylene (2) in the nitrogenase system. Therefore the rate (per one electron) of acetylene or N_2 photoreduction under identical conditions is similar. The yield of reaction products formed at NADH concentrations $< 5 \times 10^{-4}$ M was limited by the NADH concentration (Table 1).

In the presence of leucoeosin, generated under illumination, the acetylene reduction was not detected under the conditions of the nitrogenase reaction.

Table 3 Comparison of the degree of inhibition of acetylene- and N₂-reduction by eosin in the nitrogenase system

The system composition was ($\mu\text{mol/ml}$): Av1, 1×10^{-2} ; Av2, 5×10^{-3} ; eosin, 0.15; sodium dithionite, 28; ATP, 10; MgCl₂, 5; Tris, 40, pH 7.2. Controls were experiments performed without eosin. The ¹⁵N content in air (background) was 0.37%. Expts. were performed at 303 K. The reaction duration was 30 s. For microdiffusion of NH₃ 2 ml of reaction mixture was used.

Gas phase	¹⁵ N (%) in sample (with background)		Ethylene formed (nmol)		Degree of inhibition (%)
	Control	Expt.	Control	Expt.	
N ₂ (94.8% ¹⁵ N)	0.67 ± 0.01	0.49 ± 0.01	—	—	67 ± 6
Argon/C ₂ H ₂ (13:2)	—	—	97 ± 9	24 ± 2	75 ± 7

Table 4 Effect of eosin on the ATPase activity of nitrogenase in the presence of acetylene

The system composition was ($\mu\text{mol/ml}$): Av1, 1×10^{-2} ; Av2, 6×10^{-3} ; ATP, 10; MgCl₂, 5; Tris, 40, pH 7.2; sodium dithionite, 28. ATPase activity in the absence of dithionite with eosin or without it was equal to 27 ± 3 nmol of P_i/min per 1 mg of protein. Expts were performed at 303 K. The reaction duration was 30 s.

Eosin concn. (10 ⁴ × M)	Degree of inhibition of acetylene reduction (%)	Amount of P _i formed (nmol/min per mg of protein)	Degree of inhibition of ATPase activity (%)
—	—	700 ± 70	—
1	32 ± 3	700 ± 70	0
2.5	97 ± 10	645 ± 60	10

Inhibition of nitrogenase by eosin

Table 3 sums up the results on inhibition of N₂ and acetylene reduction by eosin in the presence of ATP and dithionite in darkness. Eosin inhibits both processes to the same extent.

Inhibition increases with reduction of the Av2/Av1 ratio (Figure 3).

For identification of the active centre, which eosin interacts with, the effect of eosin on ATPase activity was studied (Table 4). Eosin inhibits the ATPase activity to a markedly lower extent than the acetylene reduction. In the concentration range 8×10^{-6} – 1×10^{-4} M eosin does not inhibit ATPase activity at all. The slight inhibition of ATPase activity was detected only under an eosin concentration which is higher than that used for N₂ or acetylene photoreduction (Figures 1 and 2).

Photoreductant-dependent ATPase

Nitrogenase revealed in the light a photoreductant-dependent ATPase activity (Table 5). In the darkness without dithionite, or in the presence of the photochemical system NADH–eosin, reductant-independent ATPase activities are practically equal. Under the light, in the presence of the photochemical system, an increase of ATPase activity was detected. In this case the ATPase activity, with dithionite as a donor, is slightly above the activity of photoreductant-dependent ATPase. This is presumably because of the deficiency of photoreductant (Table 1).

Table 5 Effect of the photochemical system eosin–NADH on nitrogenase ATPase activity

The system composition was ($\mu\text{mol/ml}$): Av1, 6×10^{-3} ; Av2, 1.68×10^{-2} ; sodium dithionite, 28; ATP, 10; MgCl₂, 5; Tris, 40, pH 7.2; eosin, 6×10^{-2} ; NADH, 0.5. '+' indicates in the presence of dithionite and '–' in the absence of dithionite. Expts were performed at 303 K. The lamp power supply was DRSh-500 ($\lambda > 350$ nm). Duration of illumination was 30 s.

Reductant	Amount of P _i formed (nmol/min per mg of protein)
Sodium dithionite	
+	730 ± 70
–	80 ± 8
Photochemical system	
In the light	520 ± 50
In the darkness	90 ± 9

Figure 4 Acetylene reduction (△, ▲) and hydrogen evolution (○, ●) during light-dependent (○, △) and dithionite-dependent (●, ▲) nitrogenase reactions

The experiments (○, △) were carried out in the presence of 0.2 mM eosin and 0.5 mM NADH; in the other experiments (●, ▲) 28 mM dithionite was used. Both types of the reaction mixtures contained (μmol in ml): Av1, 5.4×10^{-3} ; Av2, 5.5×10^{-3} ; ATP, 5; MgCl₂, 5; Tris, 40, pH 7.2; creatine phosphate, 50; and creatine kinase, 0.3 mg/ml. Expts. were performed at 303 K. The lamp power supply is the same as used in Figure 1.

Comparison of the donor activities of dithionite and eosin–NADH in the light

For comparison an Av2/Av1 ratio equal to 1 was chosen. The tests were carried out in the presence of an ATP-generating system giving high activity. As is seen from Figure 4 the activity with the photochemical system NADH–eosin is markedly higher

than in the presence of dithionite without eosin. Both the acetylene-reducing (1.7-fold) and H_2 -evolving (2.4-fold) activities are increased. Therefore the total electron flux through nitrogenase increased 2.2-fold. In control tests without nitrogenase, or in the presence of EDTA with nitrogenase, both acetylene and H^+ were not reduced.

DISCUSSION

The phenomenon of charge photoseparation with participation of the triplet state of eosin (T) with an $E_{\frac{1}{2}}$ of -950 mV (Chibisov, 1981), was used for photoreduction of nitrogenase. In the presence of electron donor, NADH, the semi-reduced eosin (Rd) with an $E_{\frac{1}{2}}$ value of -580 mV and the colourless leucoeosin (L), with $E_{\frac{1}{2}}$ values of -700 and -1000 mV for the reaction $\text{eosin} + 2e \rightleftharpoons L$, and $E_{\frac{1}{2}}$ values of -170 and -400 mV for the reaction $L \rightleftharpoons Rd$ (Chan and Bolton, 1980), are formed.

It was of interest to determine the nature of the electron donor for the nitrogenase reduction. Kinetic laser spectroscopy was used (Druzhinin et al., 1989) for the detection of the intermediates of the eosin photochemical transformation (T, Rd, L). As was shown by the pulse-laser method, Rd was an electron donor for nitrogenase.

The main photochemical reactions in eosin photoreduction can be described by scheme 1:

where $E_{ox.}$ and $E_{red.}$ are nitrogenase in its oxidized or reduced forms respectively.

We have shown that L does not transfer electrons to nitrogenase; this is to be expected because the one-electron acceptor, Av2, can not be reduced by L because the redox potential of the system $L \rightleftharpoons Rd$ is not sufficient for the reduction of Av2.

Eosin is unlikely to be the inhibitor of the substrate reduction centre, because it does not contain triple bonds and has great dimension.

On the base of results obtained eosin is a decoupling reagent which does not effect the ATPase centre but inhibits substrate reduction, interrupting the electron transfer from Av2 to Av1 (Uzenskaya and Syrtsova, 1984).

Nitrogenase is an energy-coupling enzyme. Dithionite, the electron donor, increases the ATP hydrolysis rate in steady-state conditions (reductant-dependent ATPase). In a nitrogenase system using dithionite as a donor a minimum of two ATP molecules are hydrolysed for one electron transfer (Winter and Burris, 1976).

It was shown in Table 5 that nitrogenase revealed, in the light, photoreductant-dependent ATPase activity. The number of hydrolysed ATP molecules in the presence of photoreductant is twice the electron equivalents supplied by a given photochemical system.

As is seen from the above results eosin with NADH is an alternative reductant system for nitrogenase activity.

It was of interest to compare the donor systems: dithionite and eosin-NADH. Thorneley and Lowe (1983) showed that the rate

of dissociation of nitrogenase into its components after electron transfer from Av2 to Av1 was rate limiting with dithionite as the artificial electron donor. It is known that nitrogenase forms a complex of Av2 with Av1 in a 1:1 ratio. Thus Eady (1973) has shown that under ratios of Av1/Av2 varying from 1:1 to 1:7 (Av2 in excess) in the dithionite-free solution only the complex with stoichiometry 1:1 is formed. But maximum enzymic activity in the presence of dithionite is observed when Av2 is in excess of Av1 (for example, see Thorneley and Lowe, 1984). Such behaviour of the nitrogenase complex can be understood if the scheme of Thorneley and Lowe (1983) for the sequence of nitrogenase turnover reactions is taken into consideration. In this scheme dissociation of nitrogenase is the rate-limiting step.

The question may arise about the specific role of dithionite in this sequence of reactions. It is known that 2 mM dithionite both reversed and prevented the interaction between the two nitrogenase components (Eady, 1973).

Therefore we compared the donor capacities of two systems (dithionite and eosin-NADH), especially under an Av2/Av1 ratio equal to 1, when the 1:1 complex had to exist without dithionite according to the result of Eady (1973). We suggested that such a complex had to be more active with eosin-NADH because dissociation does not take place in this case, and the eosin can reduce Av2 in the complex. As can be seen from Figure 4 the photochemical reduction in this case is actually more effective than activity in the presence of dithionite. The total electron flux through nitrogenase increased 2.2-fold in the presence of eosin-NADH in the light.

Now we must work out the method for estimation of the stoichiometry of nitrogenase components in the nitrogenase complex under the turnover-reaction process and the method for estimation of the nitrogenase dissociation rate in the turnover process, in order to check our prediction. This work is in progress.

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