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Nature of Oxygen Inhibition of Nitrogenase from *Azotobacter vinelandii*

(kinetics/oxygen/uncompetitive inhibition)

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ABSTRACT **The reduction of nitrogen, acetylene, azide, and cyanide at various oxygen concentrations by nitrogenase from *Azotobacter vinelandii* was measured with a well-defined system. Oxygen inhibited the reduction of each substrate uncompetitively. The inhibition constants (K_i) were 0.014, 0.023, 0.008, and 0.003 atm of oxygen for reduction of nitrogen, acetylene, azide, and cyanide, respectively. The system used included ATP-generating components, subcellular particles from *A. vinelandii* with high nitrogenase specific activity, and illuminated spinach chloroplasts plus carriers to supply electrons. Oxygen did not affect the photochemical electron donating system, but it did inhibit nitrogenase-dependent ATP hydrolysis.**

The effects of O_2 on biological N_2 fixation have been investigated mainly with intact free-living aerobic bacteria such as *Azotobacter vinelandii* and *Azotobacter chroococcum* or with detached root nodules of higher plants. Parker and Scutt (1, 2) described the O_2 inhibition of N_2 fixation by *A. vinelandii* as competitive. They suggested that O_2 and N_2 might compete as alternative acceptors of respiratory electrons, and regarded N_2 fixation as a form of respiration. Bond (3) reported competitive inhibition by O_2 of N_2 fixation in detached nonleguminous root nodules. Bergersen (4) also reported that O_2 is a competitive inhibitor of N_2 fixation in detached soybean nodules. However, secondary effects of O_2 on the metabolic activities of intact organisms may complicate interpretation of such results. Dilworth (5), for example, reported that O_2 inhibits pyruvate dehydrogenase.

A well-defined cell-free system was needed for the study of O_2 inhibition of N_2 fixation. Such a system required nitrogenase, an ATP-generating system, and an electron donating system. Highly purified nitrogenase is irreversibly inactivated by O_2, and hence is not suitable. Likewise, the two most widely used electron-donating systems for cell-free N_2 fixation ($Na_2S_2O_4$ and H_2 plus clostridial ferredoxin and hydrogenase) are unstable in O_2, and are thus unsuitable. We chose to study O_2 effects on subcellular particles from *A. vinelandii;* these particles have high nitrogenase specific activity, and they are not irreversibly inactivated by O_2 (6).

The photochemical electron-donating system as described by Benemann *et al.* (7) was used; this system transports electrons from ascorbate to azotobacter flavoprotein through dichlorophenolindophenol and illuminated spinach chloroplasts. The reduced flavoprotein, which is not readily oxidized by O_2 (7, 8), can donate electrons to nitrogenase.

MATERIALS AND METHODS

Source of Chemicals. Ascorbic acid was obtained from Merck and Co., 2,6-dichlorophenolindophenol, ATP, creatine kinase, and tris(hydroxymethyl)aminoethane from Sigma Chemical Co., sodium azide from Fisher Scientific Co., sodium cyanide and calcium carbide (used to generate acetylene) from Allied Chemical Co., and N_2, O_2, and A from National Cylinder Gas Co.. The disodium salt of creatine phosphate was prepared by the method of Ennor and Stocken as modified by Peansky, Kuby, and Lardy (9).

Preparation of Nitrogenase, Chloroplasts, and Flavoprotein. *A. vinelandii* strain OP was grown on a modified Burk's medium (10). After washing with 0.025 M Tris·HCl buffer at pH 7.3, the cells were suspended in the same buffer at a ratio of 2 ml buffer to 1 g of cell paste. The cells were disintegrated by passage through a 40-ml Aminco French pressure cell at a pressure of about 16,000 psi. Whole cells and cell debris were removed by centrifugation at 16,000 × *g* for 30 min. The subcellular particles containing active nitrogenase were obtained from the supernatant by successive centrifugation at 105,000 × *g* for 1 hr (sedimented material discarded) and at 145,000 × *g* for 5 hr (sedimented particles saved). These particles were resuspended in 0.025 M Tris·HCl buffer (pH 7.3) to a protein concentration of 75 mg/ml and were stored at −20°.

Chloroplasts were isolated from spinach by the method of Yamashita and Butler (11). Photosystem II was destroyed by heating the chloroplasts at 55° for 5 min (12). The heated chloroplast suspension was diluted to 3 mg chlorophyll per ml and stored at −20°.

Flavoprotein from *A. vinelandii* was recovered by butanol extraction and purified by a method similar to that of Shethna *et al.* (13); it was concentrated to 0.2 μmol/ml and stored at −20°. The concentration of oxidized flavoprotein was determined spectrophotometrically at 450 nm, based on a millimolar extinction coefficient of 12.2 (8).

General Procedure for Study of O_2 Inhibition. The assay mixture in a final volume of 1.5 ml contained the following components in μmoles: ATP, 7.5; creatine phosphate, 50; $MgCl_2$, 15; flavoprotein, 0.06; ascorbate, 20; dichlorophenolindophenol, 0.10; and Tris·HCl (pH 7.3), 37.5; plus nitrogenase, 7.5 mg protein; creatine kinase, 0.2 mg protein; and chloroplasts, 600 μg chlorophyll.

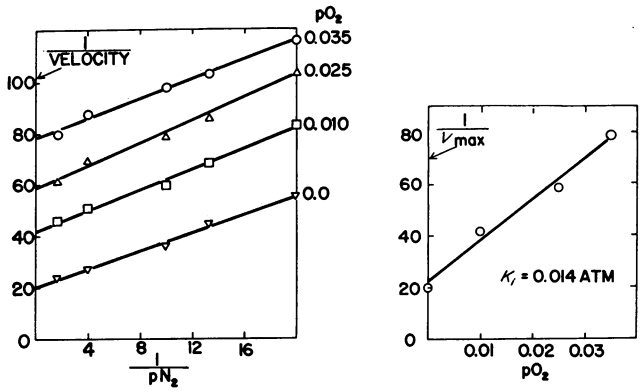


FIG. 1 (*left*). Double reciprocal plot of velocity of N_2 fixation against pN_2 at different pO_2. Both pN_2 and pO_2 are expressed in atm. The velocity is expressed as μmoles of ammonia formed per min. The reaction mixtures were incubated for 15 min.

FIG. 2 (*right*). Plot of reciprocals of maximum reaction velocities ($1/V$), obtained from y-axis intercepts in Fig. 1, against pO_2. The intercept of this line with the y-axis divided by the slope of this line yields K_i.

The assay mixture was placed in 21-ml serum bottles (nominal size 15 ml) capped with serum stoppers. The bottles were evacuated and flushed five times with purified A. N_2, acetylene, azide, or cyanide was added at various concentrations as indicated in the legends to the figures. The reaction bottles, after addition of these substrates, were incubated in the light (1500 cd) at 30° for 5 min. During this incubation, an appropriate amount of O_2 was injected into each bottle to produce the desired pO_2. The reactions were initiated by addition of nitrogenase, and the reaction mixtures were incubated for 15 or 20 min depending on the nature of the substrate supplied.

The reactions were stopped by addition of 0.5 ml or 0.1 ml of 25% Cl_3CCOOH when acetylene or cyanide, respectively, was the substrate. The amount of ethylene formed from acetylene reduction, or methane formed from cyanide reduction, was determined by gas chromatography with a flame ionization detector. The column, 1/8 in diameter and 5 ft long, was packed with Porapak R. When N_2 or azide was the substrate, the reaction was stopped by addition of 2 ml of saturated K_2CO_3. Ammonia was collected by microdiffusion (14), and its quantity was determined by the indophenol method (15).

Effect of O_2 on Rate of Flavoprotein Reduction. Flavoprotein in the standard assay mixture (0.06 μmol flavoprotein; nitrogenase and substrate omitted) was exposed to light under a pO_2 of 0.0 or 0.075 atm for varied periods. The reaction bottles were darkened immediately after the incubation period. We removed chloroplasts from the reaction mixtures by centrifuging them anaerobically and in the dark. The amount of flavoprotein reduced was measured by the decrease in absorbance at 450 nm.

Effect of O_2 on Nitrogenase-Dependent ATP Hydrolysis. Nitrogenase-dependent ATP hydrolysis was measured under standard assay conditions at various oxygen concentrations. The amount of ATP hydrolyzed was expressed as creatine released. Under the conditions of the experiment, the equilibrium of the creatine kinase reaction kept the adenine nucleotide in the form of ATP (16), so that creatine released was equivalent to ATP utilized. Creatine was measured by the method of Eggleton *et al.* (17).

RESULTS

O_2 Inhibition of N_2 Fixation. Fig. 1 indicates that O_2 inhibited N_2 fixation uncompetitively. Each point in this plot, and the subsequent plots for reduction of other substrates, represents an average of two determinations. The lines were fitted by the method of least squares.

The kinetic equation for the reciprocal formulation of uncompetitive inhibition is as follows:

$$\frac{1}{v} = \frac{1}{V}\frac{K}{[S]} + \frac{1}{V}\left(1 + \frac{I}{K_i}\right)$$

where v is the initial velocity at an initial substrate concentration of S, V is the maximum velocity, and K is the Michaelis constant; I is the concentration of inhibitor and K_i, is the dissociation constant of the enzyme–inhibitor complex.

From the equation, the slopes of all the lines in Fig. 1 should be equal. Although the observed values of the slopes are not all equal (1.74, 2.02, 2.14, and 1.83 for the lines at pO_2 of 0, 0.01, 0.025, and 0.035 atm, respectively), they are within the experimental error of this rather complex assay system. We calculated the K_i graphically by plotting the vertical intercepts of Fig. 1 against the pO_2, as illustrated in Fig. 2. The K_i for N_2 fixation was 0.014 atm of O_2.

O_2 Inhibition of Acetylene Reduction. O_2 was an uncompetitive inhibitor of acetylene reduction as indicated by Fig. 3. The differences in the slopes of the lines are small. The K_i value for acetylene reduction was 0.023 atm of O_2.

O_2 Inhibition of Azide Reduction. The reduction of azide by nitrogenase was inhibited uncompetitively by O_2 (Fig. 4). The K_i value was 0.008 atm of O_2.

O_2 Inhibition of Cyanide Reduction. The study of the O_2 inhibition of cyanide reduction was difficult, because cyanide acted both as a substrate and as an inhibitor of nitrogenase.

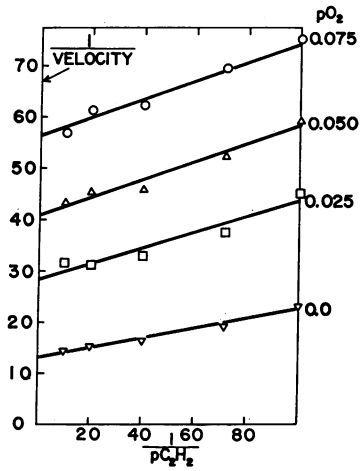


FIG. 3. Double reciprocal plot of velocity of C_2H_2 reduction against pC_2H_2 at different pO_2. The velocity is expressed as μmoles of C_2H_4 produced per min. The incubation period was 20 min.

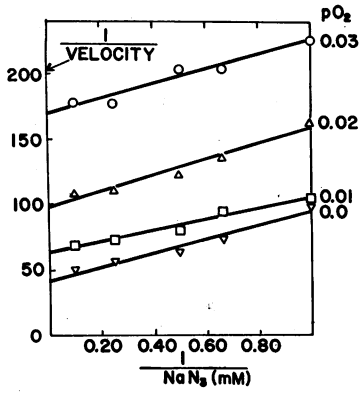


FIG. 4. Double reciprocal plot of velocity of azide reduction against concentrations of azide (mM) at different pO_2. The velocity is expressed as μmoles of ammonia formed per min. Reaction mixtures were incubated for 20 min.

The rate of cyanide reduction was much lower than the rate of reduction of the other three substrates. Fig. 5, nevertheless, indicates clearly that O_2 inhibited the reduction of cyanide uncompetitively. The K_i was 0.003 atm of O_2.

Effect of O_2 on the Rate of Flavoprotein Reduction. We tested the effect of O_2 on the photochemical electron transport system to determine whether the observed effects of O_2 were on electron transport rather than on nitrogenase *per se*. Inhibition by O_2 on any component of the photochemical system would decrease the rate of flavoprotein reduction. A lowered concentration of reduced flavoprotein would decrease the rate of substrate reduction.

O_2 apparently does not affect the reduction of flavoprotein by the photochemical system (Fig. 6). The initial rates of flavoprotein reduction were somewhat faster in 0.075 atm of O_2 than in the absence of O_2. In each case, the reduction ceased after 3 min of incubation.

O_2 Inhibition of Nitrogenase-Dependent ATP Hydrolysis. Inhibition by O_2 of ATP hydrolysis could in turn inhibit substrate reduction. O_2 does inhibit ATP hydrolysis (Fig. 7); the plot of the reciprocals of maximum velocities of ATP hydrolysis against pO_2 is linear and indicates a K_i of 0.025 atm O_2.

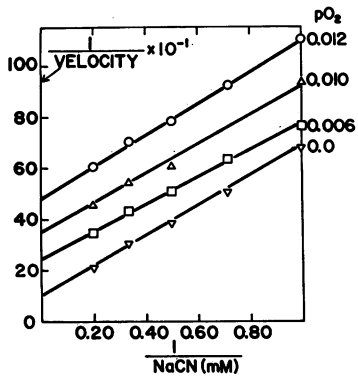
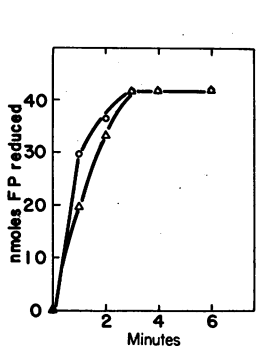


FIG. 5. Double reciprocal plot of velocity of cyanide reduction against concentrations of cyanide (mM) at different pO_2. The velocity is expressed as μmoles of methane formed per min.. The incubation period was 30 min.



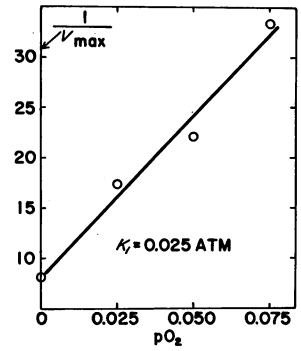


FIG. 6 (*left*). Time course of the photochemical reduction of *A. vinelandii* flavoprotein (FP) under different pO_2. The assay conditions and the method for measurement of the reduced flavoprotein are described under *Methods*. Δ——Δ, 0.0 atm O_2; O——O, 0.075 atm O_2.

FIG. 7 (*right*). Plot of reciprocals of maximum velocities (1/V) of ATP hydrolysis against pO_2. Velocity is expressed as μmoles of creatine released per min. The amount of creatine released was corrected for reductant-independent ATP hydrolysis.

DISCUSSION

O_2 uncompetitively inhibits the reduction of N_2, acetylene, azide, and cyanide by the cell-free nitrogenase system from *A. vinelandii*. This finding is contrary to the report by Parker and Scutt (2) that O_2 is a competitive inhibitor of N_2 fixation by intact *A. vinelandii* cells.

In the cell-free system, O_2 potentially could inhibit nitrogenase activity at four different sites: (*a*) on the photochemical electron-transport system, which would result in a decreased rate of flavoprotein reduction; (*b*) at the site where reduced flavoprotein donates its electrons to nitrogenase (we designate this as the electron-accepting site); (*c*) at the ATP-hydrolysis site; and (*d*) at the substrate binding site(s).

O_2 does not inhibit, in fact it enhances somewhat, the photochemical electron-transport system (Fig. 6), but it does inhibit nitrogenase-dependent ATP hydrolysis (Fig. 7). We are studying further the kinetics of ATP hydrolysis. The effect of O_2 on the electron-accepting site was not studied.

The K_i values observed for O_2 inhibition on the four substrates tested (Table 1) increase in the same order as the maximum velocities for reduction of these substrates. Although the significance of this observation is not immediately

TABLE 1. *Summary of kinetic parameters of nitrogenase reactions*

Substrate	K_i	V	V (per 2 e^-)
CN^-	0.003	0.010	0.030
N_3^-	0.008	0.023	0.023
N_2	0.014	0.025	0.075
C_2H_2	0.023	0.076	0.076
ATP	0.025	0.123	

K_i is expressed in atm of O_2. Maximum velocity (V) for cyanide reduction is expressed as μmoles of CH_4 formed per min; V of N_3^-, as μmoles of NH_3 formed per min; V of N_2, as μmoles of N_2 reduced per min; V of C_2H_2, as μmoles of C_2H_4 formed per min; and V of ATP, as μmoles of creatine released per min.

apparent, any hypothesis concerning the catalytic mechanism of nitrogenase must be compatible with these data. The maximum velocities of reduction of cyanide and azide relative to electron pairs required for their reduction (Table 1) are about a third the maximum velocities for N_2 and acetylene.

Hwang (18) reported that acetylene was a noncompetitive inhibitor of N_2 fixation and N_2, a noncompetitive inhibitor of acetylene reduction. Cyanide and azide each inhibited acetylene reduction or N_2 fixation noncompetitively, but cyanide, azide, and methyl isocyanide inhibited each other competitively. Hwang (18) suggested different binding sites or modified binding sites on nitrogenase for various substrates: a N_2-binding site (also binds H_2, N_2O, and NO), an acetylene-binding site, an azide-binding site (also binds cyanide and methylisocyanide), and a CO-binding site. The differences in K_i values for O_2 among various substrates support Hwang's suggestion that there are distinct differences in the binding sites on nitrogenase for specific substrates.

In the current study, O_2 inhibited the reduction of four substrates uncompetitively. This could indicate that once the substrate (*S*) is bound to the free enzyme (*E*), O_2 binds to a site on the enzyme–substrate complex (*ES*). Although the binding sites for the substrates may differ (18), the effect of O_2 binding onto the *ES* complexes is the same for all four substrates, as O_2 produces uncompetitive inhibition with each complex. The K_i values found would indicate that O_2 binds most tightly to the enzyme–cyanide complex, and progressively less tightly to the enzyme–azide, enzyme–N_2, and enzyme–acetylene complexes.

Although the uncompetitive inhibition can be explained by a binding of O_2 to an *ES* complex, it is more probable that O_2 inhibits the reduction of nitrogenase by the azotobacter flavoprotein, i.e., O_2 affects the electron-accepting site. If the reaction mechanism of nitrogenase is of the ping-pong type, in which the nitrogenase first is reduced and then the reduced nitrogenase donates electrons to the substrate, the inhibition of nitrogenase reduction by O_2 would cause uncompetitive inhibition against the substrate.

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