Oxygen inhibition of nitrogenase activity in *Klebsiella pneumoniae*

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A purpose-built oxystat has been used to study reversible inhibition of nitrogenase by O₂ in the facultative anaerobe *Klebsiella pneumoniae*. C₂H₂-reducing activity in samples from either an anaerobic glucose-limited or an O₂-limited diazotrophic chemostat culture was completely inhibited by exposure to a dissolved O₂ concentration (DOC) of 1.5 µM or above. Subsequently, under anaerobic conditions, C₂H₂-reducing activity returned in the absence of *de novo* protein synthesis. The amount of activity returning never reached 100% of the initial anaerobic activity before O₂ treatment. The degree of reversibility was inversely proportional to the log of DOC during exposure and was decreased by increasing the time of exposure to O₂ (about 60% reversibility occurred after a 20 min exposure to 6 µM-O₂). The failure to obtain complete recovery of activity was apparently not due to inactivation of the very O₂-sensitive pyruvate–flavodoxin oxidoreductase (*nifJ* product) which provides electrons for nitrogenase activity *in vivo*. Samples from the O₂-limited culture behaved similarly to those limited by glucose. Thus, ‘training’ of the organism to use O₂ during growth does not influence the tolerance of nitrogenase to O₂. Since the behaviour towards O₂ reported here for *K. pneumoniae* differs from that known to occur in *Azotobacter*, the mechanism of protection of nitrogenase from O₂ damage may differ in these organisms.

Introduction

Nitrogenase, the enzyme responsible for the reduction of N₂ to NH₃, comprises two redox proteins, both of which are irreversibly damaged by O₂ (Robson & Postgate, 1980). Therefore nitrogenase can only function under anaerobic conditions. In obligately aerobic *Azotobacter* spp. O₂ is excluded from the active site of nitrogen fixation by protective processes. These processes include respiratory and conformational protection which were first characterized in *A. chroococcum* (Drozd & Postgate, 1970; Robson & Postgate, 1980). Respiratory activity in *Azotobacter* spp. prevents the access of O₂ to the O₂-sensitive site of nitrogenase. The respiratory rate in *Azotobacter* spp. can adjust to a change in the rate of O₂ supply, thus maintaining protection of the O₂-sensitive sites. An increase in the supply of O₂ to *Azotobacter* spp. during growth causes an increase in the respiratory rate and in the optimum dissolved O₂ concentration (DOC) for nitrogenase activity. An increase in the O₂ supply also

causes an increase in the *d*-type cytochrome oxidase content, which is essential for aerotolerant nitrogenase activity in *A. vinelandii* (Kelly *et al.*, 1990).

However, when the level of O₂ supplied to a culture of *Azotobacter* spp. is greater than that consumed via respiration, nitrogenase activity is inhibited. The inhibition arises from the binding of the two O₂-sensitive proteins of nitrogenase to the Fe–S protein II (called the Shethna or protective protein) to form an O₂-tolerant complex (Scherings *et al.*, 1977). The inhibition is reversible, provided that the subsequent O₂ supplied to the culture is lowered to a level which can be consumed via respiration (Drozd & Postgate, 1970). The degree of reversibility of inhibition is not influenced by either the DOC causing inhibition or the duration of exposure to O₂ (Dingler & Oelze, 1985).

Hill (1976*a*) reported a tolerance of nitrogenase activity to O₂ in the anaerobically grown facultative anaerobe *Klebsiella pneumoniae*. This tolerance increased when the organism was grown in the presence of O₂. Additionally Hill *et al.* (1984) and Kavanagh & Hill (1990) reported an optimum DOC for nitrogenase activity in *K. pneumoniae* of 0.03 µM, which is near the apparent *K_m* for O₂ (0.02 µM) of the *d*-type cytochrome oxidase (the only oxidase detected during anaerobic or

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Abbreviation: DOC, dissolved oxygen concentration.

micro-aerobic nitrogen fixation in *K. pneumoniae*; Smith *et al.*, 1990). The functions of the *d*-type cytochrome oxidase in *K. pneumoniae* are to support energy-requiring processes and to protect anaerobic processes from O_2 (Hill *et al.*, 1990).

Complete inhibition of nitrogenase activity in *K. pneumoniae* occurs during exposure to a DOC greater than $0.55 \mu M-O_2$ (Hill *et al.*, 1984). Earlier work by Hill (1976*a*) suggested that, in *K. pneumoniae*, the complete inhibition of nitrogenase activity by O_2 was reversible upon the removal of O_2 . This implied the operation of a protective mechanism against O_2 damage to nitrogenase in *K. pneumoniae* similar to that in *Azotobacter*. However, since in these experiments the DOC within the assay system was neither measured nor maintained, Hill's evidence was not conclusive. To extend investigations into the reversibility of O_2 inhibition of nitrogenase activity in *K. pneumoniae*, particularly with regard to similarities of the protective mechanism to that in *Azotobacter*, we have developed an oxystat designed to maintain DOCs which are inhibitory to nitrogenase activity ($> 0.55 \mu M O_2$; Hill *et al.*, 1984). The requirement to measure DOCs over a range greatly in excess of $0.55 \mu M-O_2$ excluded the use of the O_2 -sensing systems employing leghaemoglobin (Bergersen & Turner, 1979) or photobacteria (Kavanagh & Hill, 1990) since these systems are only functional within a DOC range of 0.003 – $1 \mu M$. Therefore a galvanic electrode with a functional range of 1.5 – $1200 \mu M-O_2$ was used. In samples from chemostat cultures similar to those used by Hill (1976*a*), we confirm that, as in *Azotobacter*, inhibition of nitrogenase activity by O_2 is reversible. But, unlike the situation in *Azotobacter*, the amount of activity returning is never 100% and is dependent upon the DOC causing inhibition and the duration of exposure to O_2 . We also show that, in *K. pneumoniae*, the system donating electrons to nitrogenase *in vivo* is not more O_2 -sensitive than nitrogenase itself.

Methods

Organism and culture. *Klebsiella pneumoniae* strain M5a1 was maintained on nutrient agar slopes in air at $20^\circ C$ and subcultured monthly. The chemostat apparatus and the procedures for establishing and maintaining anaerobic glucose-limited N_2 -fixing and O_2 -limited N_2 -fixing chemostat populations have been described previously (Hill, 1976*a*). The medium was that of Hill (1976*a*) except that nitrilotriacetic acid ($300 \mu M$), which is not a nitrogen source for *K. pneumoniae*, was included as an iron chelator, and *p*-aminobenzoic acid and biotin were omitted (Hill, 1976*b*). The steady states were grown at $D = 0.1 h^{-1}$ and at $30^\circ C$. The biomasses for the steady-state anaerobic glucose-limited and O_2 -limited populations were 230 ± 10 and $300 \pm 10 \mu g$ protein ml^{-1} respectively (means $\pm$ SD). The anaerobic specific C_2H_2 -reducing activity in samples from either culture was 62 ± 2.0 nmol C_2H_4 (mg protein) $^{-1}$ min $^{-1}$ when estimated in flasks, as described previously (Hill, 1976*a*), or during the first anaerobic phase of an oxystat experiment (Fig. 2).

The oxystat. The oxystat (Fig. 1) was designed to maintain predetermined values of DOC in samples of liquid culture taken from a chemostat. These values ranged from the limit of stable detection of DOC by the oxygen electrode (typically $1.5 \mu M$) to O_2 saturation ($1200 \mu M$). Maintenance of the desired DOC ($\pm 5\%$) was achieved by the continual correction of stirrer speed.

The double-walled glass reaction vessel (approximately 86 ml), fitted with a ground-glass socket (SRB34, Quickfit) was closed by a ground-glass cone carrying a threaded plastic top. The O_2 electrode and two narrow steel tubes (1 mm internal diameter) were passed through the opening in the plastic top and were held in position with quick-setting silicon rubber (Dow Corning), which filled the inside of the cone. An injection head (1 mm internal diameter, Pye Unicam) was mounted on top of one tube, which provided the injection port (A in Fig. 1), and a venting non-return valve (Fisons) was mounted on top of the other (B in Fig. 1). The vessel carried a magnetic flea, and was maintained at $30^\circ C$ by pumping water from a thermostatically controlled water bath between the two glass walls of the vessel. An identical vessel fitted with a dummy O_2 electrode was used for the anaerobic control treatment (see Fig. 1).

The sterilizable galvanic O_2 electrode (C in Fig. 1) was based on the design of Johnson *et al.* (1964). The cathode and anode were contained in a bent Pyrex glass tube to ensure that the membrane-covered tip was always immersed within the liquid contained in the reaction vessel whilst not obstructing the magnetic flea. The surface of the electrolyte was continually flushed with a slow stream of N_2 .

The O_2 electrode was calibrated in 20 ml of spent medium which was recovered by passing a chemostat culture sample through a cellulose acetate membrane filter ($0.45 \mu m$ pore size, Sartorius Instruments). A range of DOCs was obtained by sparging the stirred medium with the appropriate gas mixtures (N_2 + air or O_2). A linear response by the electrode was obtained from 1.5 to $1200 \mu M-O_2$. At the end of each experiment the calibration was checked.

Fig. 1. Apparatus for the exposure of bacterial suspensions to anaerobic conditions or to maintained DOC. The DOC was measured and maintained within a culture in the vessel labelled 1 whilst an anaerobic culture was run simultaneously in the vessel labelled 2. The components of the oxystat, control vessel and dissolved oxygen control apparatus are indicated as follows: A, injection port for the introduction of liquids and gases; B, venting valve; C, membrane-covered O_2 electrode; D, amplifier; E, indicator/controller; F, feedback thyristor controller; G, magnetic stirrers; H, multi-turn potentiometer.

The current generated by the O₂ electrode was attenuated with a variable resistance (0–1 k Ω) in order to improve the signal-to-noise ratio required for the control of DOC. The electrode output (typically 10 μ A for air-saturated water at 30 °C), was amplified by a multi-range O₂ meter (model FL3, Western Biological; D in Fig. 1), which also displayed the DOC in analogue form and transformed the signal into a mV (0–100) output. This variable mV output was recorded on a multi-range chart recorder (model S35-922/150, Griffin Nesco) and was fed to a Clearspan indicator controller (model P130L, Kent Industrial Measurements; E in Fig. 1). The controller compares a preset voltage, related to the maintained DOC required, with the incoming voltage, and the difference activates relay circuits. Their rate of response to yield the 0–10 mA output was set to minimize hunting. This setting is dependent upon the relationship between the maintained DOC, the O₂ concentration of the gas phase, and the O₂ demand of the culture. The resultant variable output (0–10 mA) was fed into a AC thyristor controller (model MAC240-10-8, United Automation; F in Fig. 1) which in turn processed the incoming signal to provide the current to drive a magnetic stirrer (Rank Bros.; G in Fig. 1) at a speed sufficient to maintain the required DOC. The original single-turn potentiometer incorporated in the stirrer speed control was replaced by a potentiometer (250 Ω 10 turn; H in Fig. 1) which allowed fine manual control.

Supplies of N₂, O₂ and air were carried in hard nylon tubing (3.2 mm external diameter, 1.6 mm internal diameter, Phase Separation). Their fluxes were measured by either rotameters (Rotameter) or meterates (Meterates) and were modified by precision gas-flow controllers (Negretti & Zambra Aviation). The gases were passed through H₂O contained in dreschel bottles (250 ml) before they were introduced into the reaction vessel via stainless steel hypodermic needles that pierced the sampling port.

Inhibition of protein synthesis by antibiotics. In the earlier work of Hill (1976a) chloramphenicol was assumed to inhibit protein synthesis because it had prevented the derepression of nitrogenase activity in *K. pneumoniae* (Tubb & Postgate, 1973). Chloramphenicol and several other antibiotics were tested for their ability to inhibit *de novo* protein synthesis without inhibiting nitrogenase activity. The incorporation of ¹⁴C-labelled amino acids into trichloroacetic-acid-precipitable polypeptides was used to measure protein synthesis. Samples (20 ml) of a glucose-limited anaerobic N₂-fixing population were transferred in N₂-flushed syringes to two N₂-sparged reaction vessels of the design described above for the anaerobic control treatment. Both vessels were maintained at 30 °C and contained 60 mM-glucose (final concentration). One vessel also contained the antibiotic to be tested. Immediately after the injection of the culture, C₂H₂ (10 ml) was injected into both vessels, and a sample (1 ml) of the culture was removed from each vessel initially and at the end of the incubation period to measure the rate of protein synthesis. Gas samples (0.5 ml) were taken at 5 min intervals during incubation for analysis of C₂H₂ and C₂H₄ by gas chromatography (Hill *et al.*, 1984). The rate of protein synthesis was measured by estimating the incorporation of ¹⁴C-labelled amino acids into polypeptides after injecting the culture sample (1 ml) into a N₂-flushed capped (Subaseal closure) polycarbonate tube (10 ml, Nunc), containing 0.1 ml of 1.3 mM-¹⁴C-labelled Casamino acids (9.25 kBq in 0.1 ml; Amersham). After 10 min agitation at 30 °C the assay was terminated by injecting 0.05 ml vitamin-free Casamino acids (10 mg ml⁻¹; Difco). The organisms were harvested by centrifugation (15 min at 5000 g) and washed once in saline phosphate buffer (Cannon, 1980), containing vitamin-free Casamino acids (1 mg ml⁻¹). The organisms were then resuspended in Laemmli double-strength sample buffer (40 μ l) and disrupted by heating for 5 min in a boiling water bath (Cannon, 1980). Aliquots (5 μ l) of the supernatant obtained following centrifugation for 15 min at 5000 g were spotted onto Whatman no. 1 paper and the total polypeptide synthesis was measured

as the incorporation of radioactivity into trichloroacetic-acid-precipitable material as described by Cannon *et al.* (1985). At the concentration of chloramphenicol (100 μ g ml⁻¹) used by Hill (1976a), the incorporation of ¹⁴C-labelled amino acids was inhibited by only 59% within 30 s, and by 90% after 30 min. Increasing the concentration of chloramphenicol fivefold had little effect (Table 1). Therefore five other antibiotics, kanamycin, gentamicin, streptomycin (50, 75 μ g ml⁻¹), erythromycin (50, 500 μ g ml⁻¹) and tetracycline (20–200 μ g ml⁻¹) were tested under the conditions described for chloramphenicol. Erythromycin and kanamycin failed to inhibit protein synthesis. Gentamicin inhibited protein synthesis but, like streptomycin, also inhibited the specific C₂H₂-reducing activity by about 20%. However, tetracycline immediately and completely inhibited protein synthesis. Concentrations greater than 100 μ g ml⁻¹ also decreased the specific C₂H₂-reducing activity (see Table 1). The lowest concentration of tetracycline which immediately and completely inhibited protein synthesis was 20 μ g ml⁻¹ (see Table 1); therefore the concentration routinely used to prevent *de novo* synthesis of nitrogenase in all experiments described below was 40 μ g ml⁻¹.

Procedures for exposing samples to controlled DOC and to anaerobiosis for the measurement of nitrogenase (C₂H₂-reducing) activity during and after O₂ treatment. Equal aliquots of culture (20 or 30 ml; the larger volume was used for the preparation of leaky cells) were removed in N₂-flushed syringes from the chemostat and injected into the oxystat and control vessels (at A in Fig. 2). Both vessels contained tetracycline and, when required, glucose in 1.1 ml of distilled water to give final concentrations of 40 μ g ml⁻¹ and 60 mM respectively, and were flushed with N₂ (200 ml min⁻¹) for 10 min prior to the injection of the culture sample. The stirrers were switched on and sparging was continued for 5 min with the N₂ flowing (400 ml min⁻¹) through the culture. Upon removal of the N₂ supply, 10 ml C₂H₂ was injected into both vessels (B in Fig. 2). Due to the high solubility of C₂H₂ in the stirred culture sample this injection did not activate the venting valve. Over the following 20 min, at approximately 5 min intervals, 0.5 ml N₂ was injected into the gas phase, and 0.5 ml of a gas sample was removed for analysis of C₂H₄ and C₂H₂. Both vessels were then sparged for 7 min with N₂ (400 ml min⁻¹) to drive out C₂H₄ and C₂H₂ (C to D in Fig. 2). The stirrers were turned off and the N₂ supply to the oxystat vessel was replaced by an air or O₂ supply (E in Fig. 2). This supply was maintained for a predetermined time (estimated by trial and error) in order to provide sufficient O₂ to maintain the desired DOC (Fig. 2). After the removal of the gas supplies, the stirrer and the oxystat control mechanism were switched on. As soon as the desired DOC had been reached, 10 ml C₂H₂ was injected into each vessel (F in Fig. 2) and 0.5 ml gas samples were taken for analysis at approximately 5 min intervals. After O₂ exposure, the cultures were sparged with N₂ (400 ml min⁻¹) until the DOC had returned to zero (G to H in Fig. 2), when 10 ml of C₂H₂ was injected into each vessel, and samples were taken for analysis of C₂H₄ and C₂H₂ at 10 min intervals over the following 60 min. The effect of O₂ treatment in the absence of glucose was investigated by injecting 60 mM-glucose into both vessels (H in Fig. 2) following the exposure of the culture sample to the desired DOC. No C₂H₂-reducing activity was detected in either vessel until glucose was added. The anaerobic specific activity of glucose-limited chemostat samples in the presence of glucose was measured both in the control vessel and in 7 ml assay bottles. The bottles, capped with a Subaseal closure, were shaken for 1 h at 30 °C and contained in 1 ml culture sample, 60 mM-glucose and 10% C₂H₂ in Ar. The specific activities were the same in both vessels. The concentration of glucose remaining following all experiments was at least 30 mM.

Preparing and assaying leaky cells. Samples of 20 ml were removed from the oxystat and from the control vessels in N₂-flushed syringes (at the time indicated between G and H in Fig. 2) and were then injected into N₂-flushed plastic universal bottles which were capped with

Table 1. *Effect of antibiotics on anaerobic protein synthesis and the specific nitrogenase activity in K. pneumoniae*

Antibiotic	Antibiotic concn ($\mu\text{g ml}^{-1}$)	Percentage incorporation* of ^{14}C -labelled amino acids following exposure to antibiotic for:		Specific activity [$\text{nmol C}_2\text{H}_4 (\text{mg protein})^{-1} \text{min}^{-1}$]
		< 0.5 min	30 min	
Chloramphenicol	0	100.0 $\pm$ 3.0	74.0 $\pm$ 5.0 (3)	61.8 $\pm$ 2.0
	50	64.0	12.4 (1)	61.8 $\pm$ 2.0
	100	41.0	9.9 (1)	61.8 $\pm$ 2.0
	200	13.2	7.2 (1)	ND
	500	8.2	6.6 (1)	ND
Tetracycline	0	100.0 $\pm$ 2.0	76.0 $\pm$ 2.0 (5)	61.8 $\pm$ 2.0
	20	0.6 $\pm$ 0.3	0.5 $\pm$ 0.2 (2)	61.8 $\pm$ 2.0
	40	0.2 $\pm$ 0.1	0.2 $\pm$ 0.05 (3)	61.8 $\pm$ 2.0
	50	0.2 $\pm$ 0.05	0.2 $\pm$ 0.05 (3)	61.0 $\pm$ 2.0
	100	0.2 $\pm$ 0.1	0.2 $\pm$ 0.05 (2)	61.0 $\pm$ 2.0
	150	0.2 $\pm$ 0.1	0.2 $\pm$ 0.06 (2)	57.0 $\pm$ 2.0
	200	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1 (2)	51.0 $\pm$ 3.0

ND, Not determined.

* Percentage incorporation into polypeptides = $[(\beta \text{ emission with antibiotic})/(\beta \text{ emission of control})] \times 100$, where β emission of control (100%) = 110000 $\pm$ 1000 c.p.m. (mean $\pm$ SD). Figures in parentheses indicate the number of different assays.

Subaseal closures. The organisms were harvested by centrifugation (3000 *g* for 15 min at 4 °C) and resuspended in 1 ml N_2 -sparged 25 mM-HEPES pH 7.6 containing 20 mM-NaCl, 100 μg dithiothreitol and 2% (v/v) Triton X-100. The mixture was incubated with occasional shaking for 1 h at room temperature. Duplicate 0.2 ml samples of the mixture were then injected into 7 ml assay bottles capped with Subaseal closures and containing 33 mM-Tris/HCl pH 8.0, 5.3 mM-ATP, 13.3 mM-MgCl₂, 26.7 mM-creatine phosphate, 50 μg creatine kinase and 26.4 mM-sodium dithionite in a final volume of 1.0 ml under 10% C_2H_2 in Ar. The bottles were shaken for 1 h at 30 °C, and 0.5 ml gas samples, which were replaced by Ar, were analysed for C_2H_2 and C_2H_4 at 15 min intervals. The ATP-dithionite-dependent specific C_2H_2 -reducing activity in the Triton-treated sample taken from the control vessel was 45 $\pm$ 1.6% of the glucose-supported specific activity in a similar sample before the Triton treatment (see Table 3). The use of 1% or 4% in place of 2% Triton resulted in a lowering of the ATP-dithionite-dependent C_2H_2 -reducing activity, whereas shortening (30 min) or lengthening (4 h) the time of treatment with 2% Triton was without effect.

Results

Inhibition of nitrogenase activity by O₂

Diazotrophic populations were grown either in anaerobic glucose-limited or in O_2 -limited chemostats (Hill, 1976*a*) and, consistent with this earlier work, the biomass was about 25% higher in the latter culture (see Methods). The anaerobic glucose-supported C_2H_2 -reducing activity in samples from either culture was linear over 2 h in the presence of tetracycline (40 $\mu\text{g ml}^{-1}$) (Fig. 2) and the specific activity of both cultures was similar (see Methods). No anaerobic C_2H_2 -reducing activity was detected in the absence of glucose (data not shown). When glucose-supplemented samples from either culture

were exposed to a DOC of 1.5 μM no C_2H_2 reduction occurred (data not shown).

Reversibility of the O₂ inhibition of nitrogenase activity

The complete inhibition by O_2 of nitrogenase activity in glucose-supplemented samples from an anaerobic glucose-limited chemostat was partially reversible in the presence of 100 μg chloramphenicol ml^{-1} (Hill, 1976*a*). However, we found that under our assay conditions chloramphenicol only partially inhibited protein synthesis, whereas 40 μg tetracycline ml^{-1} was completely effective without influencing nitrogenase activity (see Methods). Therefore tetracycline was used in the experiments described below.

Samples from the glucose-limited N_2 -fixing chemostat were introduced into the oxystat and control vessel, both of which contained glucose and tetracycline. After the initial anaerobic C_2H_2 reducing-activity had been measured in both vessels the sample in the oxystat vessel was exposed to a maintained DOC of 6 μM for 20 min (Fig. 2). The control sample was exposed to N_2 . When the O_2 -treated sample was returned to anaerobic conditions, activity recovered without a lag to 58% of that in the anaerobic control (Figs 2 and 3). Therefore we conclude that in the absence of *de novo* protein synthesis, the inhibition of nitrogenase activity by O_2 is reversible.

The addition of 60 mM-glucose to samples from the anaerobic glucose-limited chemostat culture increased the rate of O_2 uptake 1.6-fold (Hill, 1976*a*). Thus O_2 exposure in the absence rather than in the presence of

Fig. 2. Time course of a typical experiment illustrating the partial reversibility of the complete O₂ inhibition of nitrogenase activity. Culture samples were introduced under N₂ into the oxystat and control vessels (at A: see Methods), both of which contained tetracycline and glucose. C₂H₂ was injected at B and C₂H₂-reducing activity was monitored in both vessels (○). The cultures were sparged with N₂ (between C and D). Air was introduced to the oxystat at E to achieve a DOC of 6 μM, followed by the injection of C₂H₂ to both vessels (F). C₂H₂-reducing activity was monitored in the oxystat (●) and control (○) vessels for 20 min, then both vessels were sparged with N₂ (between G and H). C₂H₂ was injected into both vessels at H and C₂H₂-reducing activity was measured over a further 1 h in the oxystat (▲) and control (○) vessels. To provide sufficient O₂ to maintain a DOC of 6 μM for 20 min in a glucose-supplemented 20 ml sample from the anaerobic glucose-limited population with a potential Q_{O_2} of 299 ± 40 nmol (mg protein)⁻¹ min⁻¹, the 66 ml head space of the oxystat vessel was flushed for 2 min with air flowing at 50 ml min⁻¹.

glucose might lead to a lower return of activity after the removal of O₂. Samples from the glucose-limited population were exposed for 20 min to a maintained DOC in the absence or in the presence of glucose. The subsequent levels of activity returning after the removal of O₂ were the same. This experiment was performed with three different DOCs (6, 48 or 96 μM), and in each case the presence of glucose during O₂ exposure made no difference to the level of nitrogenase activity returning (Table 2). Thus, a moderate change in the rate of O₂ consumption during O₂ treatment apparently does not affect the mechanism protecting nitrogenase activity from irreversible O₂ damage.

To determine whether the DOC during O₂ exposure influenced the level of nitrogenase activity returning after the removal of O₂, samples were exposed to a range of DOCs (1.5–1200 μM) for 20 min. The anaerobic activity after O₂ treatment, compared to that of the anaerobic control (the degree of reversible O₂ inhibition), never equalled 100%, even following exposure to the lowest DOC examined (1.5 μM) (Fig. 3). The percentage re-

Table 2. Influence of the duration of O₂ exposure, in the presence or absence of 60 mM-glucose, on the reversibility of nitrogenase activity

Data were derived from at least three experiments under each condition shown.

DOC (μM)	Duration of exposure (min)	Degree of reversibility (%)*	
		Glucose present	Glucose absent
6	15	ND	77.4
	20	58.0 ± 3.0	57.0 ± 3.0
	40	30.0 ± 1.0	ND
48	10	47.8 ± 2.0	51.0 ± 2.0
	20	32.0 ± 3.0	38.0 ± 1.0
96	15	30.8 ± 2.0	ND
	20	25.0 ± 2.0	26.0 ± 2.0

ND, Not determined.

*The activity of the anaerobic (glucose-supplemented) control, taken as 100%, was 61.8 ± 2.0 nmol C₂H₄ (mg protein)⁻¹ min⁻¹ (mean ± SD, *n* = 11).

Fig. 3. Relationship between log DOC and percentage reversibility of nitrogenase activity. C₂H₂-reducing activity was measured in culture samples exposed to either O₂ or N₂ for 20 min as described in Fig. 2 and Methods. Samples from the anaerobic glucose-limited culture were exposed to O₂ in the presence (○) or absence (□) of glucose. Samples from the O₂-limited culture were exposed to O₂ in the presence of glucose (▲). Samples from the anaerobic glucose-limited culture were exposed to O₂ in the absence of glucose and then assayed with dithionite and ATP (●) after treatment to make the cells leaky as described in Methods. Error bars show the greatest deviation for all experiments for a particular log DOC. The coordinate for the abscissa has been displaced to improve clarity. The precise DOCs used were 1.5, 6, 12, 24, 48, 96, 240, 480 and 1200 μM.

versibility decreased with increasing DOC. Over the range of DOCs examined there appeared to be a linear relationship between the percentage reversibility and log DOC (Fig. 3).

Hill (1976a) showed that a doubling of the time of exposure to air approximately halved the degree of apparent reversibility of O₂ inhibition of nitrogenase activity. The effect of varying the length of exposure (10, 15, 20 or 40 min) to a range of maintained DOCs (6, 48

Table 3. Degree of reversibility of nitrogenase activity in whole and leaky cells following O_2 treatment in the presence of 60 mM-glucose

O_2 treatment (μ M)	Specific activity* in whole cells with glucose	Specific activity* in leaky cells with dithionite-ATP	Relative activity of leaky cells compared to whole cells (%)	Degree of reversibility (%)†	
				Whole cells	Leaky cells
0	61.8 $\pm$ 2	27.0	43.7	NA	NA
12	31.5	14.0	44.4	51 $\pm$ 2.0	51.8 $\pm$ 2.0
240	10.5	4.9	46.6	17 $\pm$ 1.0	18.1 $\pm$ 1.0

NA, Not applicable.

* Specific activity = nmol C_2H_4 produced (mg protein) $^{-1}$ min $^{-1}$.† Results are means $\pm$ SD ($n = 3$).

or 96 μ M) was investigated. Consistent with Hill (1976a) we found that the degree of reversible O_2 inhibition was decreased in response to a longer exposure to O_2 . However, the percentage reversibility was apparently not proportional to the duration of O_2 exposure (Table 2).

In the earlier work (Hill, 1976a), where the DOC was not maintained, the return of activity following exposure to O_2 was greater in samples from O_2 -limited than from glucose-limited nitrogen-fixing chemostats. Using maintained DOCs that completely inhibit nitrogenase activity, we compared percentage recovery of C_2H_2 -reducing activity after exposure to O_2 in glucose-supplemented samples from the O_2 -limited chemostat with those from the glucose-limited chemostat. Following treatments with 6, 24 and 48 μ M- O_2 for 20 min, the subsequent percentage recovery of anaerobic C_2H_2 -reducing activity was the same in both types of culture samples (Fig. 3).

Irreversible inhibition by O_2 of nitrogenase activity

To determine whether the electron donation system to nitrogenase *in vivo* is more sensitive to O_2 than nitrogenase itself, it was necessary to bypass the natural electron donation system. This was achieved by making the cells leaky with Triton X-100, to enable sodium dithionite and ATP to enter in order to provide electrons for nitrogenase activity. Samples from the glucose-limited chemostat were exposed to a maintained DOC or anaerobiosis as shown in Fig. 2. Prior to the injection of C_2H_2 for the final measurement of anaerobic C_2H_2 reduction the contents of the control and oxygen-treatment vessels were removed by syringe and the C_2H_2 -reducing activity in whole and leaky cells was measured (see Methods). The treatment with Triton of samples removed from the anaerobic control vessel prevented glucose-supported C_2H_2 -reducing activity. This indicated that the treatment had inactivated the natural supplies of ATP and reducing power. However, when sodium dithionite and ATP were added to the leaky cells, C_2H_2 -reducing activity was restored (Table 3). This activity

was linear for 60 min and was 44 $\pm$ 1.6 % of the activity of untreated samples supplied with glucose (Table 3). When ATP was provided without sodium dithionite no activity was detected (data not shown), indicating that dithionite was providing electrons for nitrogenase activity in leaky cells.

The anaerobic activity in intact cells supplied with glucose and in leaky cells supplied with ATP and sodium dithionite was measured after samples from the glucose-limited cultures had been treated with either 12 or 240 μ M- O_2 for 20 min. The degree of reversibility of O_2 inhibition compared to the anaerobic control was similar in whole and leaky cells (Table 3). Therefore, we conclude that the electron donation system to nitrogenase is no more O_2 sensitive than nitrogenase itself.

Discussion

Our finding of complete inhibition of nitrogenase activity in samples exposed to a DOC of 1.5 μ M is consistent with the results of Hill *et al.* (1984), who found, using leghaemoglobin as the O_2 -sensor, almost complete inhibition by a DOC of 0.55 μ M in samples from sucrose-grown batch cultures of a derivative of strain M5a1 [UNF767(pRD1)] which carries a chromosomal *nifH::lac* fusion and the Nif^+ plasmid pRD1. However, Goldberg *et al.* (1987) reported that the C_2H_2 -reducing activity in samples from glucose-grown batch cultures of *K. pneumoniae* (strain Kp1) was completely inhibited only when the DOC was > 5.2 μ M. The reason for these differences in the apparent sensitivity of nitrogenase activity to O_2 may lie in the various strains or the growth conditions used.

To confirm the earlier observations (Hill, 1976a) that O_2 inhibition of nitrogenase activity in *K. pneumoniae* can be reversible and to investigate the mechanisms that prevent O_2 -inactivation of nitrogenase activity we improved the methodology. Previously the DOC in assays for C_2H_2 reduction was neither measured nor maintained, and the inclusion of chloramphenicol in these assays was

assumed to inhibit nitrogenase synthesis. In the present work a range of DOCs in culture samples during assays for acetylene reduction was achieved by using a purpose-built oxystat and we established that, by replacing chloramphenicol with tetracycline to inhibit protein synthesis in these assays, the rapid synthesis of nitrogenase, which can occur following O₂ removal (Cannon *et al.*, 1985), does not contribute to the return of C₂H₂-reducing activity after O₂ inhibition. Our results show that, after complete inhibition of nitrogenase activity by O₂, C₂H₂-reducing activity returns under anaerobiosis without a lag. However, the amount of activity returning was never 100%, even in culture samples exposed to the lowest DOC we could control (1.5 µM O₂).

Goldberg *et al.* (1987) also reported reversible O₂ inhibition of nitrogenase activity in *K. pneumoniae*. They observed that 80–90% of the initial anaerobic activity returned after 20 min exposure to either 1 or 5.3 µM-O₂. In contrast, we obtained only 69% of the initial activity following a 20 min exposure to 1.5 µM-O₂. This recovery dropped to 58% after a 20 min exposure to 6 µM-O₂. Goldberg *et al.* (1987) apparently did not prevent *de novo* protein synthesis in their assays. Thus in addition to the possibility that their strain differed from ours, the greater recovery of activity in their experiments may have been due to some resynthesis of nitrogenase. On the other hand, their O₂ treatments did not completely inhibit C₂H₂ reduction whereas ours did. Thus, the O₂ concentration *in vivo* may have been lower in their experiments than in ours. Hence, less damage to nitrogenase may have taken place, which could account for their greater recovery of activity.

A diazotrophic glucose-limited chemostat population of *K. pneumoniae* can be 'trained' to use O₂. This is achieved by gradually introducing O₂ so that the DOC remains below that detectable on a galvanic O₂ electrode (approximately 0.5 µM-O₂). An increase in O₂ supply resulting in detectable DOC indicates the subsequent washout of the culture (Hill, 1976*a*). During O₂-limited steady-state diazotrophic growth the biomass is proportional to the partial pressure of supplied O₂, so the organisms are probably carrying out a mixed fermentative and oxidative catabolism (Hill, 1976*a*). Hill (1976*a*) found that the percentage return of nitrogenase activity in samples from the O₂-limited population was greater than that in samples from the anaerobic glucose-limited population. In contrast, we found no difference when the DOC was maintained during O₂ treatment. The difference observed previously was probably due to the greater density of the O₂-limited population causing a lower DOC during treatment. Hence, the changes in physiology associated with the use of O₂ during diazotrophic growth apparently do not influence the process preventing O₂ damage to nitrogenase.

In the obligately aerobobic *Azotobacter* spp. the complete inhibition of nitrogenase activity by O₂ can be partially or fully reversible (Drozd & Postgate, 1970). The degree of reversibility is independent of the DOC and the duration of O₂ exposure but depends upon the DOC during growth (Dingler & Oelze, 1985). The degree of reversibility in *A. vinelandii* is greater in organisms grown at a lower compared to a higher DOC (Dingler & Oelze, 1985). On the other hand, we have shown that in *K. pneumoniae* the degree of reversibility is decreased upon an increase in either the DOC or the time of O₂ exposure, and the degree of reversibility is independent of whether O₂ is present during growth. These differences in behaviour of *A. vinelandii* and *K. pneumoniae* may reflect differences in the mechanisms protecting nitrogenase from O₂ damage. In *A. vinelandii* the reversible inhibition of nitrogenase activity by O₂ arises from the formation of an O₂-tolerant complex involving the nitrogenase proteins and the Fe-S protective protein (Haaker & Veeger, 1977), whereas in *K. pneumoniae* no such protective protein or complex has been identified by using techniques employing immunology or chromatography (Hochman *et al.*, 1987).

Goldberg *et al.* (1987) suggested that the reversibility of O₂ inhibition of nitrogenase activity in *K. pneumoniae* could simply occur by changes in the direction of electron flow either to nitrogenase, under anaerobiosis, or to the respiratory chain when O₂ is present. A potential candidate for the regulation of activity may be the post-translational modification of the *nif*-specific flavodoxin by the covalent attachment of coenzyme A (Thorneley *et al.*, 1992). In addition, the diversion of electron flow within nitrogenase may occur, as Thorneley & Ashby (1989) have shown that without loss of activity high concentrations of the Fe protein of *K. pneumoniae* nitrogenase can reduce O₂ to H₂O stoichiometrically. However, at concentration ratios of Fe protein to O₂ of less than 4 they observed irreversible damage, possibly as a result of the production of O₂⁻. The property of reversibility implies that O₂-sensitive sites remain anaerobic or O₂-limited during the inhibition of activity. Our results show that 17–18% of the initial anaerobic nitrogenase activity returned after 20 min exposure to air-saturated medium (240 µM-O₂) and activity was detected after 20 min exposure to O₂ saturation (1200 µM-O₂). The existence of an O₂ gradient of this magnitude between the medium and the O₂-sensitive sites *in vivo* suggests that protein-protein interactions or conformational changes may be involved in regulating nitrogenase activity and preventing damage.

Electrons for nitrogenase activity in *K. pneumoniae* are supplied from pyruvate by the action of the very O₂-sensitive *nifJ* product (pyruvate-flavodoxin oxidoreductase), and are then transferred to nitrogenase via the *nifF*

product, flavodoxin (Hill & Kavanagh, 1980; Nieva-Gomez *et al.*, 1980; Shah *et al.*, 1983; Deistung *et al.*, 1985; Drummond, 1986; Wahl & Orme-Johnson, 1987; Thorneley & Deistung, 1988). Therefore we investigated whether the failure to obtain full return of anaerobic nitrogenase activity following exposure to O₂ was due to impairment of the processes supplying electrons for nitrogenase activity rather than to damage of nitrogenase itself. Our experiments with leaky cells showed that after O₂ treatment with either a low (12 µM) or a high (240 µM) DOC the subsequent anaerobic activity of nitrogenase *in vivo* was apparently not limited by the electron supply from pyruvate. Therefore the failure to obtain full return of activity is due to O₂ damage of nitrogenase. These results also suggest that *in vivo* the *nifJ* product is not more O₂ sensitive than the nitrogenase.

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