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 I₁ (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 (1976a) 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 Kᵣ 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 O₂ (Hill et al., 1990).

Complete inhibition of nitrogenase activity in *K. pneumoniae* occurs during exposure to a DOC greater than 0.55 μM-O₂ (Hill et al., 1984). Earlier work by Hill (1976a) suggested that, in *K. pneumoniae*, the complete inhibition of nitrogenase activity by O₂ was reversible upon the removal of O₂. This implied the operation of a protective mechanism against O₂ damage to nitrogenase in *K. pneumoniae* similar to that in *Azotobacter*. However, since in these experiments the DOC within the assay system was not measured nor maintained, Hill’s evidence was not conclusive. To extend investigations into the reversibility of O₂ 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 μM-O₂; Hill et al., 1984). The requirement to measure DOCs over a range greatly in excess of 0.55 μM-O₂ excluded the use of the O₂-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 μM. Therefore a galvanic electrode with a functional range of 1.5–1200 μM-O₂ was used. In samples from chemostat cultures similar to those used by Hill (1976a), we confirm that, as in *Azotobacter*, inhibition of nitrogenase activity by O₂ 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 O₂. We also show that, in *K. pneumoniae*, the system donating electrons to nitrogenase in *vivo* is not more O₂-sensitive than nitrogenase itself.

**Methods**

*Organism and culture.* Klebsiella pneumoniae strain M5a1 was maintained on nutrient agar slopes in air at 20 °C and subcultured monthly. The chemostat apparatus and the procedures for establishing and maintaining anaerobic glucose-limited N₂-fixing and O₂-limited N₂-fixing chemostat populations have been described previously (Hill, 1976a). The medium was that of Hill (1976a) except that nitritotriacetic acid (300 μM), which is not a nitrogen source for *K. pneumoniae*, was included as an iron chelator, and p-aminobenzoic acid and biotin were omitted (Hill, 1976b). The steady states were grown at D = 0.1 h⁻¹ and at 30 °C. The biomasses for the steady-state anaerobic glucose-limited and O₂-limited populations were 230 ± 10 and 300 ± 10 μg protein ml⁻¹ respectively (means ± SD). The anaerobic specific C₃H₄O₃-reducing activity in samples from either culture was 62 ± 2.0 mmol C₃H₄O₃ (mg protein)⁻¹ min⁻¹ when estimated in flasks, as described previously (Hill, 1976a), 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 μM) to O₂ saturation (1200 μM). Maintenance of the desired DOC (±5%) was achieved by the continual correction of stirrer speed.

The double-walled glass reaction vessel (approximately 86 ml), fitted with a ground-glass cone carrying a threaded plastic top. The O₂ 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 Unicorn) 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 °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 O₂ electrode was used for the anaerobic control treatment (see Fig. 1).

The sterilizable galvanic O₂ 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 N₂.

The O₂ electrode was calibrated in 20 ml of spent medium which was recovered by passing a chemostat culture sample through a cellulose acetate membrane filter (045 μM pore size, Sartorius Instruments). A range of DOCs was obtained by sparging the stirred medium with the appropriate gas mixtures (N₂ + air or O₂). A linear response by the electrode was obtained from 1.5 to 1200 μM-O₂. 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 O₂ electrode; D, amplifier; E, indicator/controller; F, feedback thyristor controller; G, magnetic stirrers; H, multi-turn potentiometer.](image-url)
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 \(^{14}C\)-labelled amino acids into trichloroacetic-acid-precipitable polypeptides was used to measure protein synthesis. Samples (20 ml) of a glucose-limited anaerobic N\(_2\)-fixing population were transferred in N\(_2\)-flushed syringes to two N\(_2\)-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, C\(_2\)H\(_4\) (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 C\(_2\)H\(_4\) and C\(_2\)H\(_6\) by gas chromatography (Hill et al., 1984). The rate of protein synthesis was measured by estimating the incorporation of \(^{14}C\)-labelled amino acids into polypeptides after injecting the culture sample (1 ml) into a N\(_2\)-flushed capped (Subaseal closure) polycarbonate tube (10 ml, Nunc), containing 0.1 ml of 1.3 mm-\(^{14}C\)-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 μl) and disrupted by heating for 5 min in a boiling water bath (Cannon, 1980). Aliquots (5 μl) 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 μg ml\(^{-1}\)) used by Hill (1976a), the incorporation of \(^{14}C\)-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 μg ml\(^{-1}\)), erythromycin (50, 500 μg ml\(^{-1}\)) and tetracycline (20-200 μg 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 C\(_2\)H\(_4\)-reducing activity by about 20%. However, tetracycline immediately and completely inhibited protein synthesis. Concentrations greater than 100 μg ml\(^{-1}\) also decreased the specific C\(_2\)H\(_4\)-reducing activity (see Table 1). The lowest concentration of tetracycline which immediately and completely inhibited protein synthesis was 20 μg ml\(^{-1}\) (see Table 1); therefore the concentration routinely used to prevent de novo synthesis of nitrogenase in all experiments described below was 40 μg ml\(^{-1}\).

Procedures for exposing samples to controlled DOC and to anaerobiosis for the measurement of nitrogenase (C\(_2\)H\(_4\)-reducing) activity during and after O\(_2\) treatment. Equal aliquots of culture (20 or 30 ml, the larger volume was used for the preparation of leflaxin) were transferred in N\(_2\)-flushed syringes from the chemostat and injected into the oxystat and control vessels (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 μg ml\(^{-1}\) and 60 mm respectively, and were flushed with N\(_2\) (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 N\(_2\) flowing (400 ml min\(^{-1}\)) through the culture. Upon removal of the N\(_2\) supply, 10 ml C\(_2\)H\(_4\) was injected into both vessels (B in Fig. 2). Due to the high solubility of C\(_2\)H\(_4\) in the stirred culture samples this injection did not activate the venting valve. Over the following 20 min, at approximately 5 min intervals, 0.5 ml N\(_2\) was injected into the gas phase, and 0.5 ml of a gas sample was removed for analysis of C\(_2\)H\(_4\) and C\(_2\)H\(_6\). Both vessels were then sparged for 7 min with N\(_2\) (400 ml min\(^{-1}\)) to drive out C\(_2\)H\(_4\) and C\(_2\)H\(_6\) (C to D in Fig. 2). The stirrers were turned off and the N\(_2\) supply to the oxystat vessel was replaced by an air or O\(_2\) supply (E in Fig. 2). This supply was maintained for a predetermined time (estimated by trial and error) in order to provide sufficient O\(_2\) 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 C\(_2\)H\(_4\) 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 O\(_2\) exposure, the cultures were sparged with N\(_2\) (400 ml min\(^{-1}\)) until the DOC had returned to zero (G to H in Fig. 2), when 10 ml of C\(_2\)H\(_4\) was injected into each vessel, and samples were taken for analysis of C\(_2\)H\(_4\) and C\(_2\)H\(_6\) at 10 min intervals over the following 60 min. The effect of O\(_2\) 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 C\(_2\)H\(_4\)-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% C\(_2\)H\(_4\) 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 N\(_2\)-flushed syringes (at the time indicated between G and H in Fig. 2) and were then injected into N\(_2\)-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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Antibiotic concn (μg mL⁻¹)</th>
<th>Percentage incorporation* of ¹⁴C-labelled amino acids following exposure to antibiotic for:</th>
<th>Specific activity [nmol C₂H₄ · mg protein⁻¹ · min⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 0.5 min</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0</td>
<td>100 (4 ± 3)</td>
<td>67.4 ± 20</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>64 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>41 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>13 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>8 (1)</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0</td>
<td>100 (1)</td>
<td>61.8 ± 20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.6 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.3 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.2 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.2 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.2 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0.2 (1)</td>
<td></td>
</tr>
</tbody>
</table>

ND, Not determined.

*Percentage incorporation into polypeptides = [(β emission with antibiotic)/β emission of control] × 100, where β emission of control (100%) = 110 000 ± 1000 c.p.m. (mean ± SD). Figures in parentheses indicate the number of different assays.

ND, Not determined.

Subaseal closures. The organisms were harvested by centrifugation (3000 g for 15 min at 4 °C) and resuspended in 1 ml N₂-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% YO/O₂/O₂. The bottles were shaken for 1 h at 30 °C, and 0.5 ml gas samples, which were replaced by Ar, were analysed for C₂H₂ and C₂H₄ at 15 min intervals. The ATP-dithionite-dependent specific C₂H₄-reducing activity in the Triton-treated sample taken from the control vessel was 45 ± 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 C₂H₄-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 O₂-limited chemostats (Hill, 1976a) and, consistent with this earlier work, the biomass was about 25% higher in the latter culture (see Methods). The anaerobic glucose-supported C₂H₂-reducing activity in samples from either culture was linear over 2 h in the presence of tetracycline (40 µg ml⁻¹) (Fig. 2) and the specific activity of both cultures was similar (see Methods). No anaerobic C₂H₂-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 C₂H₂ reduction occurred (data not shown).

Reversibility of the O₂ inhibition of nitrogenase activity

The complete inhibition by O₂ 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, 1976a). 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 N₂-fixing chemostat were introduced into the oxystat and control vessel, both of which contained glucose and tetracycline. After the initial anaerobic C₂H₂-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 N₂. When the O₂-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 O₂ is reversible.

The addition of 60 mM-glucose to samples from the anaerobic glucose-limited chemostat culture increased the rate of O₂ uptake 1.6-fold (Hill, 1976a). Thus O₂ exposure in the absence rather than in the presence of
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 exposure 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 reversibility 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

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

<table>
<thead>
<tr>
<th>DOC (μM)</th>
<th>Duration of exposure (min)</th>
<th>Degree of reversibility (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>15</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>58.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>30.0 ± 1.0</td>
</tr>
<tr>
<td>48</td>
<td>10</td>
<td>47.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>32.0 ± 3.0</td>
</tr>
<tr>
<td>96</td>
<td>15</td>
<td>30.8 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>25.0 ± 2.0</td>
</tr>
</tbody>
</table>

*ND, Not determined.

*The activity of the anaerobic (glucose-supplemented) control, taken as 100%, was 618 ± 2.0 nmol C₂H₄ (mg protein)⁻¹ min⁻¹ (mean ± SD, n = 11).
we found that the degree of reversible O₂ inhibition was proportional to the duration of O₂ exposure (Table 2).

ATP and reducing power. However, when sodium or 96 µM-O₂ decreased in response to a longer exposure to O₂, the treatment had inactivated the natural supplies of glucose-supported C₂H₄-reducing activity. This indicated to O₂ was greater in samples from O₂-limited than from not maintained, the return of activity following exposure but the percentage reversibility was apparently not proportional to the duration of O₂ exposure (Table 2).

In the earlier work (Hill, 1976a), where the DOC was not maintained, the return of activity following exposure to O₂ was greater in samples from O₂-limited than from glucose-limited nitrogen-fixing chemostats. Using maintained DOCS that completely inhibit nitrogenase activity, we compared percentage recovery of C₂H₄-reducing activity after exposure to O₂ in glucose-supplemented samples from the O₂-limited chemostat with those from the glucose-limited chemostat. Following treatments with 6, 24 and 48 µM-O₂ for 20 min, the subsequent percentage recovery of anaerobic C₂H₄-reducing activity was the same in both types of culture samples (Fig. 3).

Irreversible inhibition by O₂ of nitrogenase activity

To determine whether the electron donation system to nitrogenase in vivo is more sensitive to O₂ 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 C₂H₄ for the final measurement of anaerobic C₂H₄ reduction the contents of the control and oxygen-treatment vessels were removed by syringe and the C₂H₄-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 C₂H₄-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, C₂H₄-reducing activity was restored (Table 3). This activity was linear for 60 min and was 44 ± 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 µM-O₂ for 20 min. The degree of reversibility of O₂ 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 O₂ sensitive than nitrogenase itself.

Discussion

Our finding of complete inhibition of nitrogenase activity in samples exposed to a DOC of 1-5 µm is consistent with the results of Hill et al. (1984), who found, using leghaemoglobin as the O₂-sensor, almost complete inhibition by a DOC of 0.55 µm 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 C₂H₄-reducing activity in samples from glucose-grown batch cultures of K. pneumoniae (strain Kp1) was completely inhibited only when the DOC was > 5-2 µm. The reason for these differences in the apparent sensitivity of nitrogenase activity to O₂ may lie in the various strains or the growth conditions used.

To confirm the earlier observations (Hill, 1976a) that O₂ inhibition of nitrogenase activity in K. pneumoniae can be reversible and to investigate the mechanisms that prevent O₂-inactivation of nitrogenase activity we improved the methodology. Previously the DOC in assays for C₂H₄ 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 nitro-
genase, 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 con-
centration 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, 1976a). During O₂-limited
steady-state diazotrophic growth the biomass is pro-
tritional to the partial pressure of supplied O₂, so the
organisms are probably carrying out a mixed fermenta-
tive and oxidative catabolism (Hill, 1976a). Hill (1976a)
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 differ-
ence 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 aerobic Azo. 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 nitro-
genase 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 chromatog-
raphy (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 nitorgenase, 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 presence 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 conforma-
tional 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 oxido-
ductase), 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; Drummmond, 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 mM) or a high (240 mM) 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 nifF product is not more O₂ sensitive than the nitrogenase.

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References


