Nitrogenase Synthesis in *Klebsiella pneumoniae*: Comparison of Ammonium and Oxygen Regulation

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Rates of nitrogenase synthesis by *Klebsiella pneumoniae* were measured by pulse-labelling organisms with a mixture of ^{14}C -labelled amino acids followed by sodium dodecyl sulphate gel electrophoresis and autoradiography. Populations from an NH_4^+ -repressed, SO_4^{2-} -limited chemostat ($0.46 \text{ mg dry wt ml}^{-1}$), when released from NH_4^+ repression, simultaneously synthesized detectable quantities of the three nitrogenase polypeptides 45 min before acetylene-reducing activity was observed. Exposure of populations synthesizing nitrogenase to air or NH_4^+ ($200 \mu\text{g N ml}^{-1}$) repressed synthesis of both component proteins simultaneously, the rate initially decreasing by half in 11 to 12 min; in the presence of NH_4^+ a second slower phase with an approximate half-life of 30 min was observed. With 5% O_2 in N_2 the half-lives for the decreases in the rates of synthesis were 30 min for the Fe protein and 33 min for the Mo-Fe protein. Oxygen also repressed nitrogenase in a glutamine synthetase constitutive derivative of *K. pneumoniae* (strain sk24) which escapes NH_4^+ repression. Regulation of nitrogenase by O_2 may therefore be independent of glutamine synthetase.

INTRODUCTION

Nitrogen fixation by the facultative anaerobe *Klebsiella pneumoniae* occurs only under anaerobic conditions (Pengra & Wilson, 1958) or at very low dissolved O_2 tensions (Klucas, 1972; Hill, 1975, 1976*a, b*). Chemostat populations fix N_2 at dissolved O_2 tensions between 2 and 10 mmHg but not at 15 mmHg, and Klucas (1972) suggested that a high respiratory rate at low dissolved O_2 tension as reported by Harrison & Loveless (1971) and Harrison & Pirt (1967) decreased the dissolved O_2 tension to a permissive level for nitrogenase synthesis. The inability of *K. pneumoniae* to fix N_2 under aerobic conditions must in part be due to inactivation of its O_2 -sensitive nitrogenase, but O_2 , like NH_4^+ , may also repress nitrogenase synthesis as claimed by St John, Shah & Brill (1974).

In this paper we report a study of O_2 as a regulator of nitrogenase synthesis in *K. pneumoniae* and present evidence, using a mutant in which nitrogenase synthesis is NH_4^+ -constitutive, that regulation by O_2 differs mechanistically from regulation by NH_4^+ .

METHODS

Organism and culture. The *K. pneumoniae* strains used in this work were the wild-type strain m5a1, in which nitrogenase synthesis is repressed by NH_4^+ , and strain sk-24 (Shanmugam & Valentine, 1975), a double mutant lacking glutamate synthase activity in which both nitrogenase and glutamine synthetase activities are derepressed in the presence of NH_4^+ . The strains were maintained at 20°C in air on 2% (w/v) nutrient agar slopes and subcultured monthly. For strain sk-24, approx. 0.1% (w/v) glutamine was included in the agar.

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The medium and conditions used for the growth of strain m5a1 in SO_4^{2-} -limited continuous culture were those of Tubb & Postgate (1973), except that the glucose concentration was decreased to 10 g l^{-1} and NH_4Cl was included at a concentration (0.25 g l^{-1}) just sufficient to repress nitrogenase synthesis. The chemostat was operated at a dilution rate of 0.1 h^{-1} which gave a population of $0.46 \text{ mg dry wt ml}^{-1}$. The presence of NH_4^+ in the supernatant from the culture vessel was determined by the method of Chaney & Marbach (1962).

Batch cultures of strain sk-24 were grown on the same medium as used for chemostat culture, except that the Na_2SO_4 concentration was increased 10-fold, and L-glutamine ($100 \mu\text{g ml}^{-1}$) and $(\text{NH}_4)_2\text{SO}_4$ (1 mg ml^{-1}) were included. Cultures were established by inoculating 100 ml of the sterilized medium in 250 ml conical flasks with about 0.2 ml of an 18 h nutrient broth culture and grown under N_2 at 30°C on a rotary shaker; they were harvested after about 40 h.

Expression of nitrogenase activity. Samples of strain m5a1 from a fully-repressed SO_4^{2-} -limited continuous culture were collected anaerobically under N_2 , centrifuged at 4000 g for 10 min, and the pellet was resuspended at 30°C in derepression medium (half the original volume) previously sparged with N_2 for 15 min. This medium was of the same composition as the growth medium in the chemostat except that it contained no NH_4^+ and the SO_4^{2-} concentration was increased 10-fold so as to be non-limiting. Derepressing cultures ($520 \mu\text{g protein ml}^{-1}$) were incubated at 30°C in acid-washed tubes ($15 \times 1.5 \text{ cm}$) capped with Suba seals and bubbled with humidified N_2 (80 ml min^{-1}). Gas was passed into the culture through a hypodermic needle immersed in the medium, a shorter needle in the gas headspace allowing gas exit. Samples for measurement of nitrogenase activity or for radioactive labelling were removed at intervals with syringes.

To investigate the effect of NH_4^+ or O_2 on nitrogenase synthesis, cultures were derepressed for 4 to 5 h after which time nitrogenase formed a substantial proportion of the protein being synthesized. The cultures, derived from the same chemostat populations, were then exposed to O_2 or NH_4^+ and the rate of synthesis of nitrogenase was measured by pulse-labelling with a mixture of ^{14}C -labelled amino acids as described below.

Radioactive labelling. Samples (2 ml) were removed from the derepression vessel and injected into Ar-filled acid-washed test tubes incubated at 30°C , sealed with Suba seals and containing a mixture of ^{14}C -labelled amino acids (CFB 104; The Radiochemical Centre, Amersham) to give a final concentration of $2.5 \mu\text{Ci ml}^{-1}$. Uptake of radioactive material was stopped after 4 min by addition of unlabelled Casamino acids (Difco) to a final concentration of 1 mg ml^{-1} . The organisms were harvested by centrifuging at 4000 g for 10 min, washed in 4 ml of saline phosphate [$0.85 \text{ } (\text{w/v}) \text{ NaCl}$ in $75 \text{ mM-phosphate buffer pH } 7.2$] containing Casamino acids (5 mg ml^{-1}), collected by centrifugation and frozen by immersing the centrifuge tubes in liquid nitrogen. At this stage the labelled organisms could be stored at -20°C .

Electrophoresis and autoradiography. The pelleted organisms were resuspended in 0.25 ml of Ortec gel sample buffer (see below) containing $2 \text{ } (\text{w/v})$ sodium dodecyl sulphate (SDS) and $5 \text{ } (\text{w/v})$ β -mercaptoethanol, then heated in a boiling water bath for 5 to 10 min. To measure radioactivity incorporated into acid-insoluble material, $10 \mu\text{l}$ of the extract was applied to a 2 cm square of Whatman 3MM paper which was then immersed in ice-cold trichloroacetic acid ($5 \text{ } (\text{w/v})$) for 15 min. The paper was rinsed for 5 min in ethanol and then in acetone and dried. The dried paper was placed in a vial with 6 ml of scintillation fluid (NE23; Nuclear Enterprises, Beenham, Berkshire) and the radioactivity was measured in a scintillation counter (Nuclear Enterprises 8310).

Volumes of SDS extract containing approximately 100000 c.p.m. were applied to 8 mm-wide sample wells in 1.5 mm-thick polyacrylamide slab gels. The discontinuous gel buffer system used was that described in the Ortec manual (Ortec 4200 electrophoresis system, Ortec, Oak Ridge, Tennessee, U.S.A.) except that $0.1 \text{ } (\text{v/v})$ SDS was included in both the gel and electrode buffers, and the latter also contained 2 mM-EDTA . In this work the electrode buffer contained SDS (Koch-Light, batch no. 40134) to ensure resolution of both subunits of Kp1 (Kennedy *et al.*, 1976). A maximum current of 40 mA was used during electrophoresis.

After electrophoresis, the gel was dried in a slab gel drier (Pharmacia GSD-14) and exposed to a Kodirex X-ray film for 45 to 90 h. The films were scanned with a Fisons Vitatron microdensitometer and the peaks were measured by cutting out and weighing to obtain relative values of radioactive intensity. Under these conditions the intensity of the bands obtained from microdensitometer tracings was proportional to the number of counts applied to the gel. It is estimated that this method will detect the three nitrogenase polypeptides if present at a minimum of about $2 \text{ } (\text{v/v})$ each of total proteins synthesized.

Enzyme assays. Nitrogenase activity of *K. pneumoniae* was measured essentially as described by Hill (1976b), except that 1 ml of culture was assayed in an 8 ml serum bottle. Glutamine synthetase [L-glutamate: ammonia ligase (ADP-forming); EC 6.3.1.2] was measured in intact organisms as described by Bender *et al.* (1977).

Fig. 1. Microdensitometer traces of autoradiographs of SDS gels of extracts prepared from *K. pneumoniae* M5a1 grown under conditions where nitrogenase synthesis was repressed by NH_4^+ (—) or after 6 h derepression (----). The gels were loaded with approximately 100000 c.p.m. of incorporated acid-insoluble ^{14}C and after electrophoresis were exposed to X-ray film for 45 h. The autoradiographs were scanned at 2 cm min $^{-1}$ using a Fisons Vitatron densitometer with minimum damping.

RESULTS

Autoradiographs of SDS gels of protein synthesized by *K. pneumoniae* in a pulse-labelling experiment under conditions where nitrogenase was either repressed or derepressed, showed clear differences. The Mo-Fe protein of *K. pneumoniae* nitrogenase (Kp1) comprises two types of subunits of approx. mol. wt 50000 and 60000, present in equal numbers (Eady *et al.*, 1972). Under the appropriate conditions of SDS electrophoresis these subunits can be separated, and have been shown to be different polypeptides (Kennedy *et al.*, 1976). In this paper, the 50000 and 60000 mol. wt subunits will be referred to as the α and β subunits of Kp1 respectively. The Fe protein of nitrogenase (Kp2) has a single type of subunit of mol. wt 37000 (Eady *et al.*, 1972). Figure 1 shows typical microdensitometer scans of gels from organisms under conditions where nitrogenase was repressed by NH_4^+ and from organisms during derepression. The labelling pattern was sufficiently different to enable the three nitrogenase polypeptides to be easily identified by comparison with the migration of highly-purified nitrogenase proteins. The identity and significance of the few other bands which changed in intensity is not known.

Derepression of nitrogenase

When washed organisms from a chemostat culture were resuspended in NH_4^+ -free medium and bubbled with N_2 , acetylene reduction was first detected after about 2 h. The enzymic activity increased rapidly and showed a linear increase with time from about 3 to 5 h (Fig. 2). At intervals during derepression, samples were removed from the culture and pulse-labelled with ^{14}C -labelled amino acids as described in Methods. Autoradiography of the SDS gels showed nitrogenase polypeptides to be present 1 h after the start of derepression, and then to increase as a proportion of the total radioactivity incorporated into protein for the next 2 h. After this time the rate of synthesis increased only very slowly, whereas the rate of acetylene reduction was increasing rapidly (Fig. 2). The nitrogenase polypeptides constituted a substantial proportion of the proteins synthesized: usually 30%, with a range of 12 to 40%. The reasons for this variation are not clear since the organisms originated from

Fig. 2. Derepression of nitrogenase activity in *K. pneumoniae* m5a1. At zero time organisms were washed free of NH_4^+ , and rates of nitrogenase synthesis and nitrogenase activity were measured at various times as described in Methods: ●, acetylene reducing activity; □, α subunit of Kp1; ○, β subunit of Kp1; △, subunit of Kp2; ▲, acetylene reducing activity in the presence of NH_4^+ ($200 \mu\text{g N ml}^{-1}$ added at zero time) or when 5% (v/v) O_2 in N_2 was bubbled through the culture.

Fig. 3 (a). Effect of NH_4^+ on nitrogenase synthesis in *K. pneumoniae* m5a1. A derepressed culture was exposed to ammonium acetate ($200 \mu\text{g N ml}^{-1}$) and samples were removed at intervals for measurement of nitrogenase synthesis: □, α subunit of Kp1; ○, β subunit of Kp1; △, subunit of Kp2. (b) Semi-logarithmic plot of the same data. In both (a) and (b), rates of synthesis of nitrogenase polypeptides are expressed as a percentage of the initial proportion of radioactivity incorporated into nitrogenase.

a continuous culture which remained in a steady state. The correlation between acetylene-reducing activity and intensity of the nitrogenase bands was therefore rather variable.

Effect of ammonium ions on nitrogenase synthesis

When ammonium acetate ($200 \mu\text{g N ml}^{-1}$) was added to a comparable sample from the chemostat, nitrogenase remained repressed and no acetylene reduction (Fig. 2) or synthesis of nitrogenase polypeptides was observed. The addition of NH_4^+ ($200 \mu\text{g N ml}^{-1}$) to a derepressing culture of *K. pneumoniae* resulted in an immediate decrease in the intensity of nitrogenase bands on autoradiograms (Fig. 3a). A semi-logarithmic plot of these data (Fig. 3b) gave an approximate half-life of 11 min for the initial decay of rate of synthesis of both Kp1 and Kp2, which was followed by a decreased rate of decay at longer times, with an approximate half-life of 30 min.

Fig. 4. Effect of O_2 on pre-formed nitrogenase and on acetylene reducing activity. Cultures of *K. pneumoniae* m5a1 were bubbled with 5% (v/v) O_2 in N_2 (open symbols) or air (closed symbols) in the presence of chloramphenicol (1 mg ml^{-1}), and nitrogenase was monitored by autoradiography of SDS gels of extracts: $\square$, $\blacksquare$, α subunit of Kp1; $\circ$, $\bullet$, β subunit of Kp1; $\triangle$, $\blacktriangle$, subunit of Kp2; ∇ , $\blacktriangledown$, acetylene reducing activity. When acetylene reduction was to be measured, chloramphenicol was omitted and nitrogenase activity was determined as described in Methods.

Fig. 5(a). Effect of O_2 on nitrogenase synthesis in *K. pneumoniae* m5a1. Nitrogenase synthesis was measured by autoradiography of SDS gels of extracts prepared from derepressed organisms exposed to 5% (v/v) O_2 in N_2 (open symbols) or air (closed symbols) for various periods: $\square$, $\blacksquare$, α subunit of Kp1; $\circ$, $\bullet$, β subunit of Kp1; $\triangle$, $\blacktriangle$, subunit of Kp2. (b) Semi-logarithmic plot of the same data. In both (a) and (b) rates of synthesis of nitrogenase polypeptides are expressed as a percentage of the initial proportion of radioactivity incorporated into nitrogenase.

Effect of oxygen on pre-formed nitrogenase

As a preliminary to studying O_2 as a repressor, it was necessary to determine the stability of pre-formed nitrogenase to O_2 . Bacteria were therefore removed from the chemostat to the derepression medium and after 4 h the organisms were pulse-labelled with a mixture of ^{14}C -labelled amino acids and then exposed to air or 5% (v/v) O_2 in N_2 in the presence of chloramphenicol (1 mg ml^{-1}). Samples were removed at intervals for autoradiography of SDS gels. The nitrogenase bands were unaffected by 5% O_2 over 30 min and only a small decrease in their intensity occurred during the next 1.5 h (Fig. 4). When the cultures were bubbled with air, the intensity of the nitrogenase bands immediately but slowly decreased. Kp1 showed a greater sensitivity towards air than did Kp2, which was only slowly degraded.

Table 1. *Effect of NH_4^+ or oxygen on nitrogenase synthesis by the NH_4^+ -constitutive mutant *K. pneumoniae* SK-24*

Batch cultures of SK-24 were centrifuged and the organisms were resuspended in derepression medium supplemented with glutamine ($100 \mu\text{g ml}^{-1}$). Three 10 ml cultures were incubated under the conditions indicated below. After 6 h the rate of acetylene reduction was measured. Samples were also removed and pulse-labelled with a mixture of ^{14}C -labelled amino acids for measurement of nitrogenase synthesis as described in Methods.

Culture conditions	Nitrogenase activity [nmol C_2H_2 reduced min^{-1} (mg protein) $^{-1}$]	Rate of nitrogenase synthesis (% of total proteins)		
		Kp1		Kp2
		α subunit	β subunit	
N_2 plus NH_4^+ ($200 \mu\text{g N ml}^{-1}$)	10.9	5	5.6	9.4
N_2	11	7	5.9	8.5
Air	0	0	0	0

Bubbling with either air or 5% O_2 resulted in an immediate decrease in nitrogenase activity of intact organisms as measured by the acetylene-reduction assay (Fig. 4).

Effect of oxygen on nitrogenase synthesis

When 5% O_2 was bubbled through a derepressing population of *K. pneumoniae*, there was a lag of about 12 min before an effect on the rate of synthesis of Kp2 was observed (Fig. 5a). The decrease in synthesis rate of both subunits of Kp1 appeared to be parallel and was initiated earlier than the decrease in the rate of Kp2 synthesis. A semi-logarithmic plot of the data (Fig. 5b) gave approximate half-lives of 33 min for Kp1 synthesis and 30 min for Kp2 synthesis. When the culture was bubbled with air, no lag was observed before the rate of nitrogenase synthesis decreased. The rate of decrease was more rapid than that observed with 5% O_2 : the half-life was about 12 min and no synthesis of Kp1 subunits was detected after 30 min. In contrast, synthesis of Kp2 continued at a decreased rate up to 180 min.

As the effect of O_2 in decreasing the intensity of the nitrogenase bands occurred more rapidly than could be accounted for by the degradation of O_2 -damaged proteins (compare Fig. 4 and Fig. 5), O_2 must have repressed nitrogenase synthesis.

*Effect of oxygen and NH_4^+ on an NH_4^+ -constitutive mutant of *K. pneumoniae**

Glutamine synthetase mediates repression of nitrogenase by NH_4^+ in *K. pneumoniae* (Tubb, 1974; Streicher *et al.*, 1974). *Klebsiella pneumoniae* strain SK-24 lacks glutamate synthase activity and synthesis of glutamine synthetase and nitrogenase occurs at derepressed levels in the presence of NH_4^+ . If repression by O_2 is also mediated by glutamine synthetase this strain would be expected to escape O_2 repression. The effects of N_2 , N_2 plus NH_4^+ and 20% O_2 in N_2 (air) on nitrogenase synthesis and activity are shown in Table 1. After 6 h in derepression medium supplemented with glutamine ($100 \mu\text{g ml}^{-1}$), the rates of nitrogenase activity and synthesis were similar in organisms under N_2 or N_2 plus NH_4^+ , but organisms exposed to 20% O_2 during this time had no nitrogenase activity or nitrogenase synthesizing ability. In cultures exposed to O_2 , nitrogenase activity was undetectable after 45 min. Figure 6 shows typical autoradiographs of extracts of these organisms which indicate that no synthesis of the nitrogenase proteins occurred in organisms which had been exposed to O_2 .

Fig. 6. Autoradiograms showing the effects of NH₄⁺ and O₂ on nitrogenase synthesis in *K. pneumoniae* sk-24. Cultures of sk-24 were grown in derepression medium supplemented with glutamine (100 µg ml⁻¹). After 6 h samples were removed and pulse-labelled with a mixture of ¹⁴C-labelled amino acids and then subjected to SDS electrophoresis. Cultures were grown under (a) N₂ plus ammonium acetate (200 µg N ml⁻¹); (b) N₂; (c) air.

Effect of oxygen on glutamine synthetase activity

The average adenylation level of glutamine synthetase in intact *K. pneumoniae* m5a1 was measured by the method of Bender *et al.* (1977). In this assay hexadecyltrimethylammonium bromide is used to prevent changes in adenylation occurring during sample manipulation. The specific activities of glutamine synthetase in samples from NH₄⁺-grown and N₂-fixing cultures sparged with N₂ were 72 and 221 nmol γ-glutamyl hydroxamate min⁻¹ (mg protein)⁻¹ respectively. When the sparging gas was changed to air the total activity in both cultures increased by approximately 25% within 45 min, but in neither case did the ratio of biosynthetically active (nonadenylylated) enzyme to total enzyme (0.7 in both cultures) change significantly.

DISCUSSION

The lag in expression of nitrogen fixation during derepression. Fleming & Haselkorn (1974), using a comparable radio-labelling technique, reported synthesis of nitrogenase components long before the appearance of enzymic activity during heterocyst development in *Nostoc muscorum*. The extreme sensitivity of the acetylene reduction assay for nitrogenase makes it unlikely that the lag is an artefact due to insensitive enzyme assay. Under conditions of derepression, supplies of ATP or of reducing equivalents, both necessary for nitrogenase function, would not be expected to limit nitrogenase activity. A possible explanation is that the lag represents a period in which a specific electron donor protein to nitrogenase, for example a flavodoxin, is synthesized. Under our conditions of electrophoresis, peptides of molecular weight 20000 and below would remain undetected. The lag is unlikely to be due to the dissociation of Kp1 and Kp2 at low protein concentrations since complex formation is rapid ($K > 10^7$ M⁻¹ s⁻¹; Thorneley, 1975) and tight ($K_{\text{complex}} = 3 \times 10^7$ M⁻¹; Thorneley, Eady & Yates, 1975). The possibility that it is due to the slow assembly of Kp1 or Kp2 from their respective subunits cannot be excluded since nothing is known about these processes.

The lag in repression of nitrogenase synthesis by oxygen. The lag observed before 5% O₂ has an effect on nitrogenase synthesis may be attributed to the time taken for the dissolved O₂ tension to reach a repressive level. Measurement of the rates of increase in dissolved O₂ indicates that this occurs relatively slowly at the gas flow rates and culture densities used in our experiments (S. Hill, personal communication). Further work will be required to correlate dissolved O₂ tension with the regulation of nitrogenase synthesis, but the dif-

ferential repressive effect of O₂ on Kp1 and Kp2 may indicate a greater sensitivity to dissolved O₂ of the processes leading to synthesis of Kp1.

Stability of nitrogenase proteins in vivo. Our results using chloramphenicol agree with the observations of St John *et al.* (1974) who demonstrated that on exposure of intact organisms to air, both the enzymic activity and the antigenic cross-reacting material of Kp1 decreased faster than those of Kp2. This contrasts with the O₂-sensitivity of the highly-purified nitrogenase components, since Kp2, with a half-life of 45 s in air, is at least 10 times more sensitive to inactivation than Kp1 (Eady *et al.*, 1972).

Kinetics and mechanisms of oxygen repression. The half-lives of the reduction in rates of nitrogenase synthesis under maximum O₂ or NH₄⁺ repression were similar (11 to 12 min) and the lag in the effect of 5% O₂ has been attributed above to slow diffusion of oxygen to the appropriate regulatory site. Nevertheless, this kinetic similarity may not reflect a similar mode of action at the molecular level, since our experiments with the NH₄⁺-constitutive strain show that O₂ represses nitrogenase synthesis despite the fact that glutamine synthetase activity remains little changed on exposure of the culture to O₂.

The experiments reported here cannot distinguish between effects of O₂ on transcription or on translation since only completed nitrogenase polypeptides are measured. Such effects might be distinguished by pulse-labelling experiments in which organisms are exposed to O₂ in the presence of differential inhibitors of transcription and translation. Loss of nitrogenase activity in cultures exposed to NH₄⁺ compared with loss of activity after exposure to chloramphenicol or rifampicin suggest that NH₄⁺ acts at the transcriptional level (Tubb & Postgate, 1973; Collmer & Lamborg, 1976). The *nif* gene cluster is located near the genes for histidine biosynthesis in *K. pneumoniae* and may include as many as three genes involved in regulation (*nifA*, *nifL* and *nifE*, see Kennedy & Dixon, 1977). It is conceivable that one of these gene products mediates repression of nitrogenase synthesis by O₂.

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