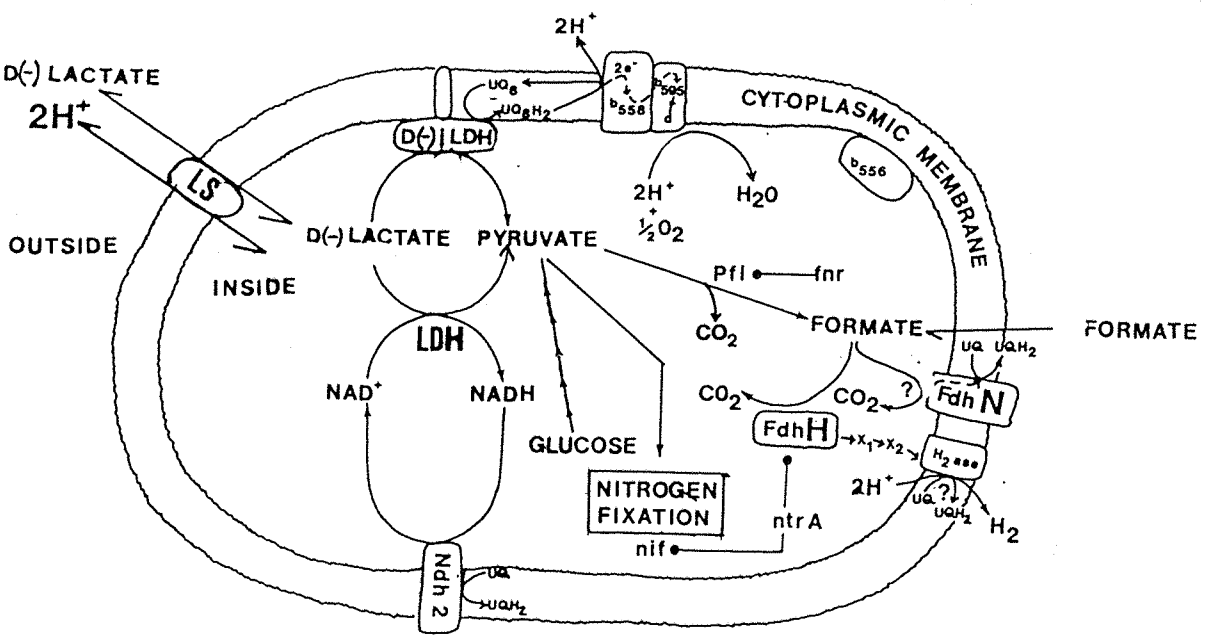
OXYGEN, RESPIRATION AND NITROGEN FIXATION IN THE FACULTATIVE ANAEROBE *KLEBSIELLA PNEUMONIAE*

A thesis submitted to the University of Southampton for the degree of Doctor of Philosophy.



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ABSTRACT

FACULTY OF SCIENCE
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OXYGEN, RESPIRATION AND NITROGEN FIXATION IN THE FACULTATIVE ANAEROBE *KLEBSIELLA PNEUMONIAE*

Biological nitrogen fixation is an oxygen-sensitive energy intensive process; because of the potential benefits of aerobic respiration for ATP production, nitrogen-fixing micro-organisms have adapted to various degrees to cope with and utilize oxygen during N_2 fixation.

Klebsiella pneumoniae has been the main 'work horse' for research into nitrogen fixation for many years. Although a facultative anaerobe, it too can utilize O_2 during N_2 fixation. The mechanism whereby O_2 regulates expression of the *nif* genes is not known. In the present work, the hypothesis that haem plays a role in this mechanism is tested by studying the oxygen regulation of nitrogenase synthesis in a Nif^+ *E. coli* strain deficient in haem synthesis. The O_2 regulatory mechanism is shown to be independent of haem status.

There is good evidence that the respiratory chain of *K. pneumoniae* has an important physiological role during nitrogen fixation yet little is known about it or its regulation under N_2-fixing conditions. A question of particular interest was the nature of the oxidase(s) responsible for respiration at the very low oxygen concentrations that allow *nif* gene expression. The cytochrome *d* oxidase complex was purified from *K. pneumoniae* and its O_2 affinity determined with leghaemoglobin (Km'21nM). This was consistent with the observed sensitivity to O_2 of *nif* gene expression; the oxidase was judged to be kinetically competent to support oxidative phosphorylation at low O_2 levels. In support of this, *E. coli* cytochrome *d* minus (Cyd^-) strains carrying the Nif^+ plasmid pRD1 failed to support microaerobic nitrogenase activity under conditions where ATP and reductant must have been generated as a result of respiration via cytochrome *d*. A model is proposed to describe how the oxidation of formate plus lactate supports O_2-dependent nitrogenase activity in Cyd^+ strains.

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LIST OF ABBREVIATIONS

5-ALA, 5-Amino laevulinic acid

CO, Carbon monoxide

COAS-H, Coenzyme A

D(-)iLDH, D(-) lactate ubiquinone reductase NAD^+ independent

DNA, Deoxyribonucleic acid

E^o, Standard mid point redox potential at pH7.0

Em, Mid point redox potential

EDTA, Ethylene diamine tetracetic acid

FeMoCo, Iron molybdenum cofactor

FF, Fast flow

FPLC, Fast protein liquid chromatography

K, Kilo daltons

Kb, Kilo base

LDH, NAD^+-linked lactate dehydrogenase

MM, Minimal medium

mV, Multi volts

MOPS, N-Morpholinopropane sulphonic acid

N, Any nucleotide

NA, Nutrient agar

NB, Nutrient broth

NFDM, Nitrogen deficient medium

ORF, Open reading frame

(ox), Oxidized

Q, Ubiquinone

Q_1H_2, Ubiquinol-1

R, Any purine nucleotide

(red), Reduced

RNA, Ribonucleic acid

SEM, Standard error of the mean

SDS-PAGE, Sodium dodecylsulphate polyacrylamide gel electrophoresis

Tris, (Hydroxymethyl) aminomethane

Y, Any pyrimidine nucleotide

To my family

CHAPTER 1

INTRODUCTION

1.1 Why study biological nitrogen fixation?

Nitrogen is a key element in the chemistry of life in proteins, nucleic acids and other biomolecules. Although the atmospheric form of nitrogen (dinitrogen) is virtually inexhaustible, most plants are limited in their growth by an inadequate supply of fixed nitrogen. In nearly all agricultural areas where sunlight or water supply do not limit crop growth, biological productivity is determined by the availability of inorganic nitrogen in the soil (Postgate, 1982). For this reason, some 30-40 per cent of the world's population is dependent on chemical N-fertilizers for its food. With a world population set to double by the middle of the next century, this can be seen as an approaching 'nitrogen crisis'. Increasing use of nitrogenous fertilizers is not necessarily the whole answer; it is often not an economic alternative in the third world while in the developed world the environmental impact of intensive farming methods is being questioned, particularly in the light of nitrate pollution of food and water supplies.

Dinitrogen is chemically so stable that only a relatively small number of microorganisms can convert dinitrogen into ammonia. No higher organisms have developed this ability although several participate indirectly by forming symbiotic associations with dinitrogen-fixing bacteria (Sprent, 1979; Haaker & Veeger, 1984). Industrially, dinitrogen reduction is an energy-intensive process requiring high temperatures and pressures. Biological nitrogen fixation also requires a

high energy input but takes place at physiological temperatures and pressures in a reaction catalysed by the enzyme nitrogenase:

(Eqn 1)

1) $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$

In the long term, genetically-improved biological systems might make substantial inroads into the demand for N-fertilizers and lead to increased crop productivity, particularly in the third world. Merrick and Dixon (1984), in their article "Why don't plants fix nitrogen?", concluded that "the effective co-existence of photosynthesis and nitrogen fixation in the cyanobacteria suggests that there is potential for the construction of diazotrophic crop plants". However, they also concluded that it was impossible to put a timescale on such a genetic engineering project.

A second, possibly more immediate approach is to extend our knowledge of the *Rhizobium*/legume symbiosis to the point where manipulation of the plant symbiotic genes and development of new symbioses are a feasible alternative.

There are however a number of important physiological constraints operating on nitrogen-fixing microorganisms which stem from the physicochemical properties of nitrogenase. These are directly relevant to the genetic engineering of nitrogen fixation in plants, since only when they are understood will we be in a position to give evolution a helping hand! These constraints include:

i) Nitrogenase requires large amounts of chemical energy derived from ATP hydrolysis.

ii) The microorganism must generate a strong reductant for dinitrogen reduction, which might otherwise have been used for catabolic or anabolic metabolism.

iii) Nitrogenase is irreversibly damaged by high levels of molecular oxygen and its synthesis is inhibited by oxygen.

iv) Nitrogenase has a low turnover number and high concentrations (5-30% of cell protein; 300 μM) are required *in vivo*; a great deal of energy has to be invested in enzyme synthesis.

Despite these constraints, the ability to fix nitrogen is scattered amongst obligate anaerobes, facultative anaerobes and obligate aerobes (Table 1.1). This thesis concerns the mechanism by which the facultative anaerobe *Klebsiella pneumoniae* is able to carry out microaerobic nitrogen fixation despite the constraints listed in i), ii) and iii) above.

Table 1.1 Selected genera of nitrogen-fixing organisms and their modes of nitrogen fixation

Genus or Species	Mode of Life	Mode of Nitrogen Fixation
1. *Azotobacter* *Berjerinckia*	obligate aerobic	obligate aerobic
2. *Azospirillum* *Xanthobacter flavus* *Rhizobium, Frankia*	obligate aerobic	only at low oxygen tensions (1-3 μM oxygen)
3. *Klebsiella pneumoniae* *Chromatium*	facultative anaerobic	only at very low oxygen tensions (30-100nM or anaerobically oxygen)
4. *Clostridium pasteurianum* *Desulphovibrio*	obligate anaerobic	obligate anaerobic

Genetic studies in the free-living nitrogen-fixing bacterium *Klebsiella pneumoniae (K. oxytoca)*, have made rapid progress, owing partly to its similarity to *Escherichia coli* and also because in this organism the *nif* genes are conveniently clustered as a single regulon close to the histidine operon, rather than scattered either on the chromosome or on megaplasmids as in other diazotrophs (Dixon, 1988).

Fig. 1.1 Map of the *nif* gene cluster of *K. pneumoniae*

The roles of the gene products are indicated above each gene. The horizontal arrows indicate the extent and direction of each transcript, black dots represent the location of *nif* promoters (from Dixon, 1988).

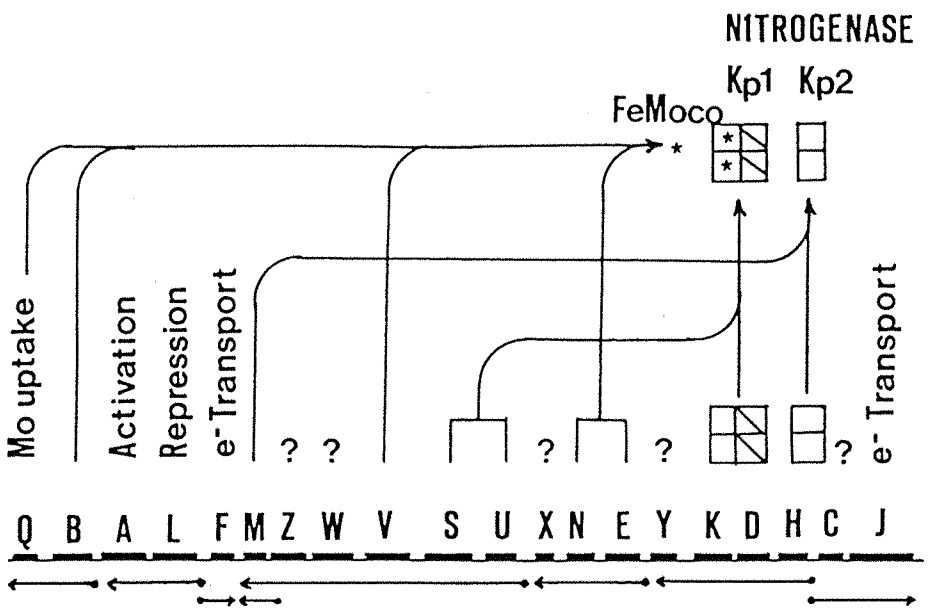
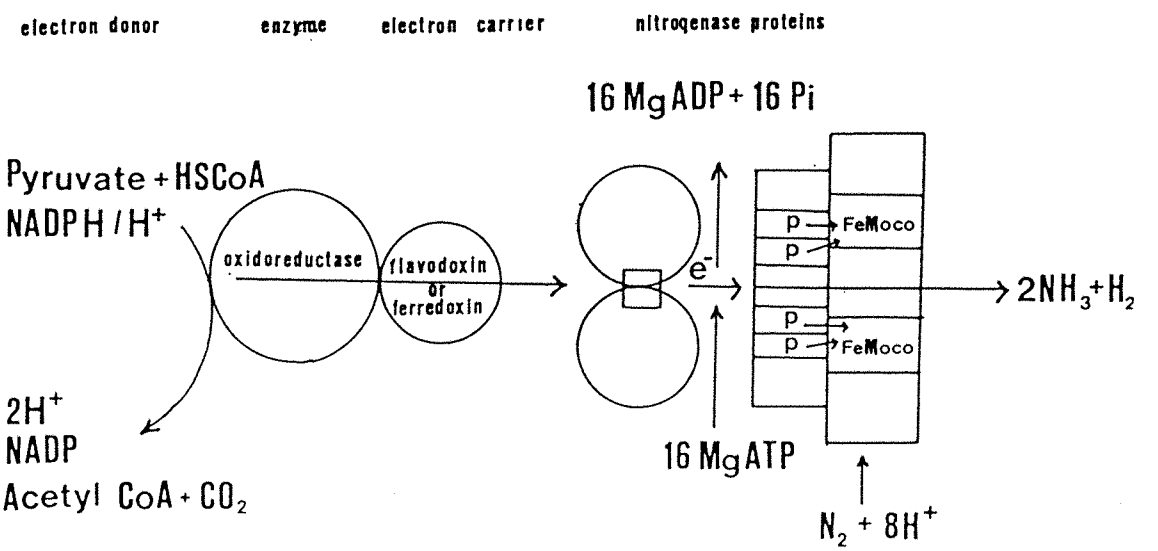


Fig. 1.2 Proteins involved in biological nitrogen fixation
(Modified from Bothe, 1984)

P denotes Fe/S P. clusters (see text).



Biochemical studies of purified nitrogenase from many organisms have shown them to have very similar properties, and genetic comparisons of the *nif* structural gene sequences show them to be remarkably highly conserved. More recently, an 'alternative' nitrogenase has been discovered containing vanadium (V) rather than molybdenum (Mo) at the N_2 binding site (Eady *et al.*, 1988).

1.1a Nitrogenase of *Klebsiella pneumoniae*

The well-characterized Mo-nitrogenase of *K. pneumoniae* consists of two oxygen sensitive redox proteins, one of which contains molybdenum and iron (Mo-Fe protein) and the other which contains only Fe (Fe protein) (Table 1.2). Some eight to nine of the *nif* genes, of which there are at least 20, are required for the synthesis of functional nitrogenase (Fig. 1.1, Table 1.3). *nifK* and *nifD* encode the polypeptides of the MoFe protein, and $\alpha_2\beta_2$ tetramer with subunits of 56K and 60K respectively. The active tetramer (Kp1) contains two MoFe cofactor centres; this extremely oxygen-sensitive cofactor can be extracted from the holoenzyme and used to activate the nitrogenase from *nif B* mutant strains. In this way, FeMoco (iron molybdenum cofactor) biosynthesis and insertion have been shown to require the products of at least four genes, *nifB*, *N*, *E* and *V* (Table 1.3). Under conditions of molybdenum deficiency, a molybdenum sequestering system encoded by *nifQ* is also required. The stoichiometry of FeMoco is variable (Mo, Fe6-8, S4-9), the structure being unknown; it does not contain any amino acid residues. Recently, FeMoco biosynthesis from Mo and ATP using the above gene products has been described *in vitro* (Shah *et al.*, 1986). The $NifV^-$ phenotype is particularly interesting. Mutations in the *nifV* gene result in the formation of a MoFe protein with an altered substrate specificity; N_2 is a relatively poor substrate, while C_2H_2 is an effective substrate. This phenotype was transferred with FeMoco when the latter was extracted from $NifV^-$ strains and used to

activate the aponitrogenase from a *nif B* mutant (Hawkes, McLean & Smith, 1984). This strongly suggests that FeMoco forms part of the substrate-binding and reducing site of nitrogenase. The *nifH* gene (structural gene for the Fe protein) is required for the biosynthesis of active FeMoco (Filler *et al.*, 1986). About 50 per cent of the iron in the MoFe protein can be extracted as four [4Fe-4S] clusters. The properties of these so-called "P" clusters are unusual and their precise role in dinitrogen reduction is unclear, but presumably involves electron donation to the FeMoco centres (Fig. 1.2).

All Fe proteins are dimers of 57-72k; the subunit is encoded by the *nifH* gene in *K. pneumoniae*. The amino acid sequence from nine bacterial sources has been determined; all show considerable similarity (Eady, 1986), with five invariant cysteine residues and three potential nucleotide-binding regions (Robson, 1984). The redox centre is a [4Fe-4S] cluster. Only one other *nif* gene product, NifM is required for biosynthesis of active Fe protein. The Fe proteins have an $E^{o'}$ in the range -240 to -393mV. A negative shift in the Em occurs when Fe protein binds two molecules of MgATP. The Fe protein then acts as a highly specific one electron donor to MoFe protein during enzyme turnover. All the Fe proteins are extremely oxygen sensitive with half lives for activity of about 45 seconds (Table 1.2).

Table 1.2 Properties of pure nitrogenase proteins

	MoFe protein	Fe protein
1. molecular weight	200-220 000	55-65 000
2. subunit composition	$\alpha_2\beta_2$	2 (identical)
3. Mo g atoms/mol	2	0
4. Fe g atoms/mol	24-36	4
5. Acid labile sulfur g atom/mol	20-36	4
6. oxygen sensitivity of nitrogenase (t 1/2)		
Klebsiella pneumoniae	10 min	<0.5 min
Azotobacter chroococcum	10 min	<0.5 min

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Table 1.3 Functions of the *K. pneumoniae nif* genes

nif gene	Gene product Molec wt/KD	Phenotype in strains containing lesion	Function of gene product *in vivo*
A	60	Nif- Dixon, (1984)	DNA binding protein Nif specific activator
L	50	Nif++ fast derepression with low N or O_2	antiactivator in response to N and O_2 Hill (1981, 1987)
B	52	Nif-	
N	?	inactive	FeMoCo biosynthesis
E	?	FeMoCo Shah & Brill (1977)	and insertion
V	40?	Altered substrate specificity Hawkes *et al.* (1984)	Late step in FeMoCo maturation. Hoover *et al.* (1985)
H	35	Nif- inactive/unstable FeMoCo	Fe protein structural gene. Dixon (1977) required for active FeMoCo. Filler (1986)
D	56	Nif-	MoFe protein struct
K	60	Roberts *et al* (1978)	-ural genes (Kp1)
M	28	Nif- defective Fe protein Roberts *et al.* (1978)	Maturation of [4Fe- 4S] cluster in Fe protein McLean *et al.* (1986)
J	120	Nif$^-$ *in vivo* not *in vitro* Hill & Kavanagh (1980)	Pyruvate flavodoxin oxidoreoductase Shah *et al.* (1983) e^- trans to flavodoxin
F	18	as above Hill & Kavanagh (1980)	flavodoxin, e-trans to Fe protein Deistung *et al.* (1985, 1986)

1.1b Enzymology

In addition to active nitrogenase components (MoFe protein and Fe protein) and a strong reducing agent (Eh<-400mV) to donate electrons to nitrogenase, MgATP and an anaerobic environment are required for activity. In addition to reducing N_2 to NH_3 and H^+ to H_2, nitrogenase will also reduce C_2H_2, azide, HCN and isocyanides and a number of other small molecules which have a triple bond. The reduction of acetylene to ethylene measured by gas chromatography provides a simple and sensitive assay for nitrogenase *in vitro* and *in vivo*. In the absence of any added reducible substrate, protons are reduced and H_2 is evolved. The reduction of all substrates except protons is inhibited by CO, and H_2 specifically and competitively inhibits the reduction of N_2. The Fe protein accepts electrons from a low potential donor, *in vivo*, mediated by the *nifF* product a flavodoxin, *in vitro*, usually sodium dithionite. The flavodoxin is maintained in the reduced state *in vivo* at the expense of intracellular pyruvate (Fig. 1.2) by the enzyme pyruvate: flavodoxin oxidoreoductase, the *nifJ* gene product.

A number of nitrogenase mechanisms have been proposed, by far the most comprehensive being that proposed by Thorneley & Lowe (1985). Their model consists of a computer simulation of eight sequential one-electron reductions of MoFe protein with side reactions. It is able to simulate all available experimental data from stopped flow and rapid quench experiments. A brief description of the simpler elements follows together with the important physiological conclusions.

In the absence of MoFe protein, the Fe protein cannot hydrolyse MgATP. Fe protein binds two molecules of MgATP which lowers the potential of the electrons at the [4Fe-4S] centre and allows complex formation between reduced Fe protein and oxidized MoFe protein (Fig. 1.3a). Reduction

Fig. 1.3a The Fe protein oxidation-reduction cycle

One electron is transferred from Kp2 to Kp1 with concomitant hydrolysis of 2 MgATP, the complex dissociates in the rate limiting step of the scheme (from Thorneley & Lowe, 1985).

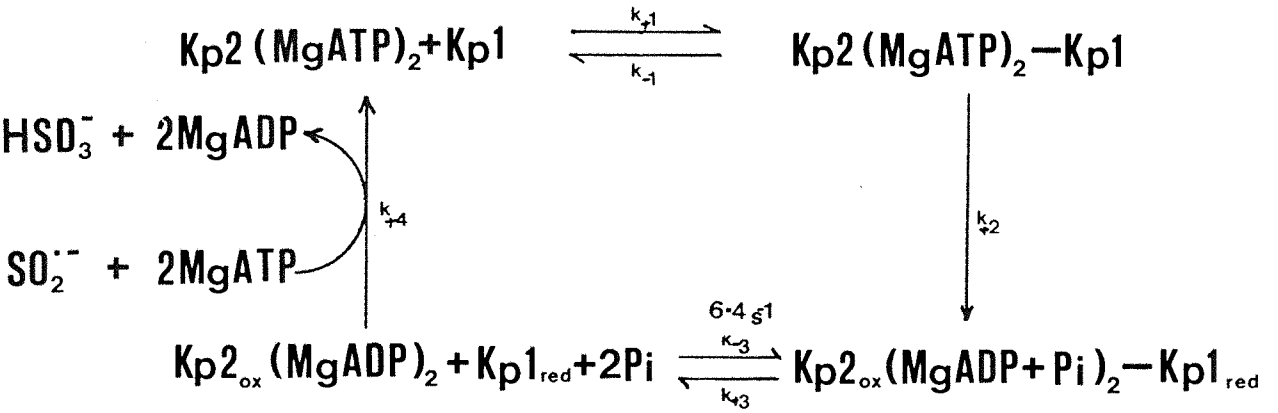
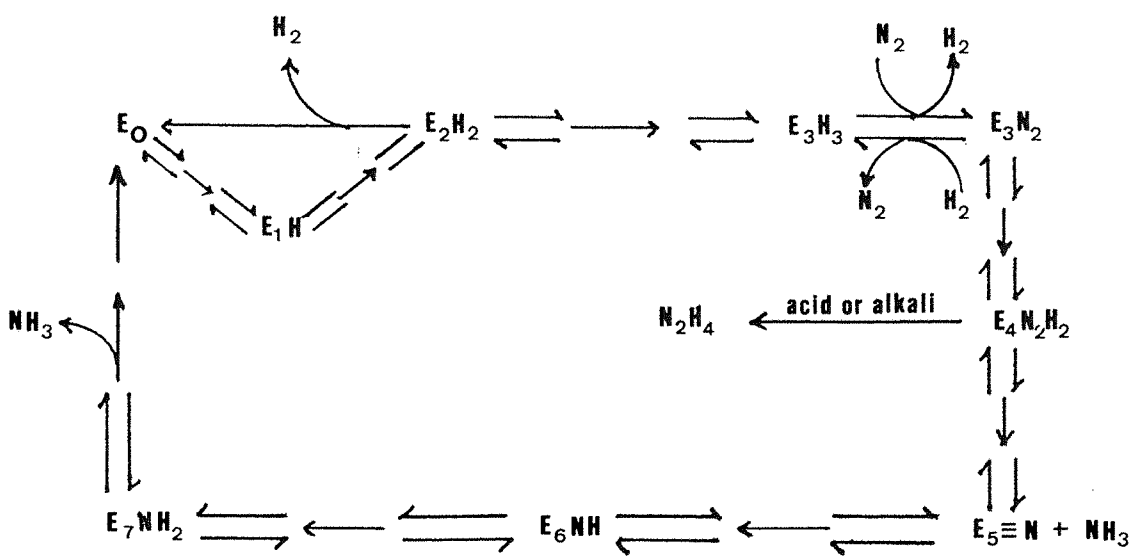


Fig. 1.3b Cycle for the turnover of MoFe protein during reduction of N_2

E_0 to E_7 represent different states of a half molecule of MoFe protein after sequential $1e^-$ reductions. There is evidence for the dinitrogen hydride intermediate at E_4 and for NH_3 release at E_5 or E_6. However, formation of nitride $E_5 \equiv N$ and its reduction are conjectural (from Thorneley & Lowe, 1985).



(one-electron) of the MoFe protein is made irreversible by the hydrolysis of MgATP. Within the MoFe protein, electrons are transferred to N_2 and H^+. The MoFe protein-Fe protein complex then dissociates in the rate-determining step in a cycle which is completed by reduction of the Fe protein and the replacement of MgADP by MgATP. The cycle then starts again. To account for the limiting stoichiometry identified *in vitro* (see Eqn 1), eight successive single electron transfers from Fe protein must occur. Under optimum conditions of Fe protein excess, only one mole of H_2 is produced as a result of N_2 binding by H_2 displacement. In the model, the MoFe protein has two active centres acting independently; the scheme shown in Fig. 1.3b is for a half-molecule of the MoFe protein showing the sequence of one electron reduction steps (for a full analysis, see Thorneley & Lowe, 1985). Conditions which minimise H^+ over N_2 reduction include: An excess of Fe protein over MoFe protein (2.5:1), a high *in vivo* enzyme concentration (300 μM) and slow dissociation of the Fe protein-MoFe protein complex after electron transfer, resulting in very slow enzyme turnover (6.4 S^{-1}). For reviews see, Thorneley and Lowe (1985); Haaker and Veeger (1984) and Postgate (1982).

1.2 The *nif* gene cluster of *Klebsiella pneumoniae*

The closely-linked *nif* genes span 24kb of the *K. pneumoniae* genome and comprise eight transcriptional units (Fig. 1.1). Initial mapping resulted in the identification of some 17 *nif* genes, but recently three additional open reading frames (ORFs) have been found, ORFC located between *nifH* and *nifJ* (Shah *et al.*, 1981) and ORFZ and ORFW located between *nifM* and *nifV* (Paul & Merrick, 1987). The functions of the *nif* gene products are shown in Table 1.3. For reviews, see Dixon (1984; 1988).

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1.2a Regulation of nitrogenase synthesis by fixed nitrogen and oxygen

Synthesis of nitrogenase in *K. pneumoniae* occurs under anaerobic, nitrogen-limiting conditions and repression of transcription is observed in strains grown on ammonia or in the presence of oxygen (Eady *et al.*, 1978). This is to be expected since nitrogenase is irreversibly inactivated by oxygen and nitrogen fixation is too energy intensive to be permitted under conditions of nitrogen sufficiency. Regulation of *nif* is at the transcriptional level; although effects on mRNA stability and protein turnover have been reported, they are quantitatively less significant.

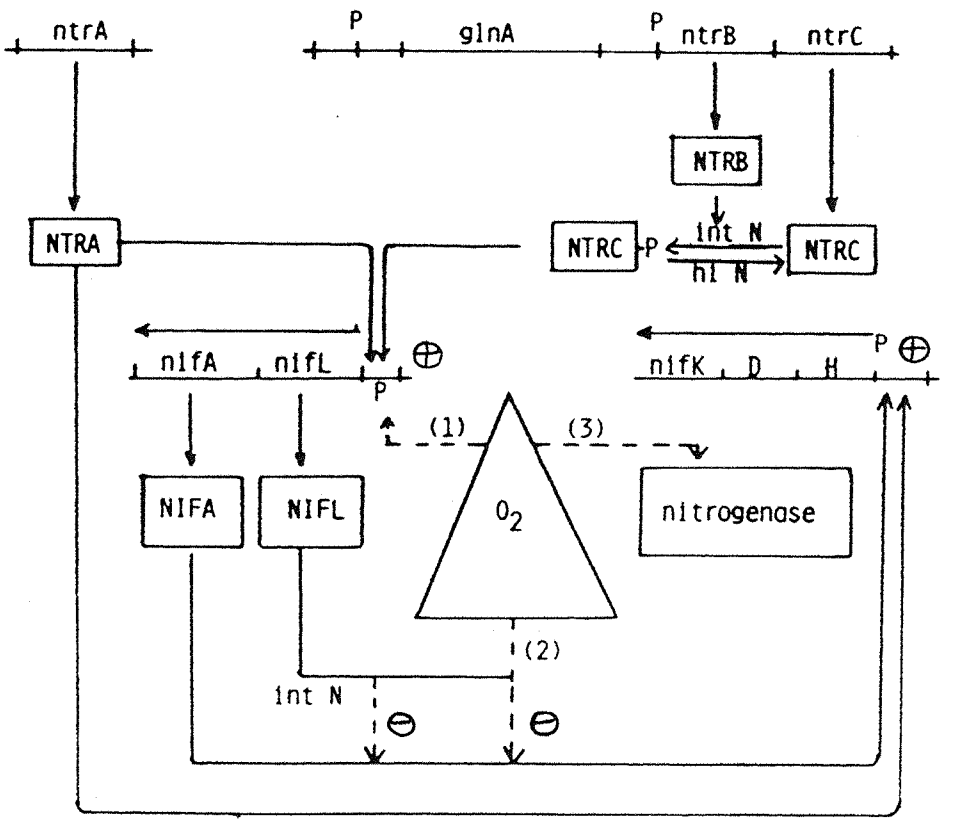
1.2ai Nitrogen regulation

The current model for the *nif* regulatory cascade is shown in Fig. 1.4. The central regulators of transcription are the products of the genes *nifA* and *nifL* (Fig. 1.1, Table 1.3). *NifA* encodes an activator of transcription, essential for the expression of each *nif* promoter. The *nifL* product is believed to be a negative regulator which responds to oxygen status, certain amino acids (as N-source) and low levels of ammonium ion. The main evidence for this is that non-polar deletions in *nifL* allow transcription to occur at intermediate levels of fixed nitrogen or dissolved oxygen that would normally be repressive (Hill, 1981). Unlike conventional repressors of transcription, the *nifL* product does not bind DNA and has no regulatory effect in the absence of the *nifA* protein. Its mode of action is thought to be direct inactivation of the *nifA* product in response to low levels of fixed nitrogen or oxygen (see Hill (1988), for a summary of the evidence).

At higher levels of fixed nitrogen, transcription from the *nifLA* promoter is itself repressed. This additional level of regulation is mediated by the *ntr* (nitrogen control) system (Merrick, 1987; 1988). This controls expression of several genes involved in the utilization of

Fig. 1.4 The current model for *nif* regulation in *K. pneumoniae*

The two-tier regulation by high (hi) and intermediate (int) levels of fixed nitrogen (N) are shown together with the three apparent interactions of oxygen. (1), 60 μM O_2 inhibits derepression of *nifLA*; (2) 100 nM O_2 inhibits derepression of the other nif operons via the antiactivator properties of the NifL protein; (3), 6 μM O_2 inhibits nitrogenase activity which through N starvation indirectly prevents nitrogenase synthesis (modified from Hill, 1988, with permission).



poor nitrogen sources, including arginine, proline and histidine as well as molecular nitrogen (Kushi *et al.*, 1986; Merrick *et al.*, 1987). The *ntr* system comprises three genes: *NtrB* and *ntrC*, which form a single operon like *nifLA* and the unlinked gene *ntrA*. The *ntrBC* operon lies immediately downstream from *glnA*, the gene for glutamine synthetase, the first enzyme in the pathway of ammonium assimilation used in conditions of nitrogen limitation. *NtrC* encodes a DNA-binding protein whose phosphorylation state is controlled by a kinase, the *ntrB* product, which modulates the activity of *NtrC*. *NtrB* can apparently 'sense' the nitrogen status of the cell; this involves a complex regulatory cascade mediated by the products of *glnB* (P*II* protein) and *glnD* (uridyl-transferase) (Bueno *et al.*, 1985). In the phosphorylated state, *NtrC* is able to activate the *glnA* p2 promoter (Fig. 1.4) and the *nifLA* promoter. Promoters subject to *ntr*-mediated activation, such as *nifLA* have the consensus sequence NTGGCRCR-N_4-TTGG$^{A}_{T}$, while other *nif* promoters activated by *NifA* have the consensus CTGGYAYR-N_4-TTGC$^{A}_{T}$, (where N = any nucleotide, R = Pyrimidine and Y = Purine) both extending from -26 to -10 relative to the transcription start site. Note that the element GG-N8-TTG is common to both (reviewed in Gussin *et al.*, 1986). In addition, full activation by *NtrC* and *NifA* at their respective target promoters requires the presence of an upstream enhancer sequence at least 100 nucleotides upstream from the transcription initiation site (Buck *et al.*, 1986; 1987). Biochemical evidence suggests that recognition of *NtrC* and *NifA* dependent promoters by RNA polymerase requires the product of the *ntrA* gene, an alternative sigma factor, replacing the rpo D-encoded σ^{70}, to allow recognition of *ntr* and *nif* promoters.

Nif expression is regulated at two levels, one general and sensitive to high fixed nitrogen levels, the *ntr* system, and one *nif* specific and sensitive to low levels of fixed nitrogen (for reviews see Dixon, 1984; 1988; Merrick, 1988).

1.1dii Oxygen regulation

Regulation of nitrogenase synthesis by oxygen is less well understood than regulation by fixed nitrogen because of the oxygen sensitivity of nitrogenase activity, difficulties in measuring very low oxygen tensions and the lack of techniques for selecting oxygen constitutive mutants (Hill, 1987). Many enzymes are said to be regulated by oxygen but the mechanisms of redox control or oxygen sensing are largely unknown (Cole, 1988a, b). Nitrogenase synthesis is extremely tightly regulated by oxygen. There are probably two reasons for this. Firstly, nitrogenase is irreversibly inactivated by oxygen (2 μM) and secondly, the low potential single electron transfers inherent in the nitrogenase mechanism may result in superoxide generation (Eqn 2) and production of toxic oxygen radicals.

2)—— $O_2 + e^- \rightarrow O_2$ $E^0 = -322$ mV. (Youngman, 1984).

Fig. 1.4 shows the three probable sites at which oxygen status affects *nif* expression (1), (2) and (3). Expression from the *nifL* promoter is insensitive to 6 μM oxygen, but is inhibited 50 per cent by 60 μM oxygen (1) (Dixon *et al.*, 1980). Using leghaemoglobin to monitor low dissolved oxygen concentrations showed that near the apparent Km of the principal terminal oxidase (100 nM oxygen) *nifH::lac* expression is 50 per cent inhibited (2) (Bergersen *et al.*, 1982). On the other hand, even lower levels (30 nM) can stimulate *nifH::lac* and *nifL::lac* expression probably owing to an increase in ATP levels as a result of oxidative phosphorylation (Hill *et al.*, 1984). A third site at which O_2 indirectly affects synthesis of nitrogenase is through its inhibitory effect on N_2 reduction, leading to N-starvation and switch-off of synthesis (Hill & Kavanagh, 1988; for review, see Hill, 1988).

1.3 Physiology

How is carbon catabolism coupled to nitrogen fixation in *K. pneumoniae*?

During anaerobic nitrogen-fixing growth in glucose-limited continuous culture, *K. pneumoniae* derives its energy from a mixed acid fermentation pathway (Fig. 1.5). The ATP requirement for nitrogen fixation has been estimated from the difference in molar growth yield per ATP (YATP) utilized between nitrogen fixing and ammonia assimilating cultures to be 29 moles ATP/mole N_2 fixed, assuming that maintenance energy requirements are the same in both (Hill, 1976a). This compares with the minimum ATP/N_2 ratio identified *in vitro* of 16 (Eqn 1).

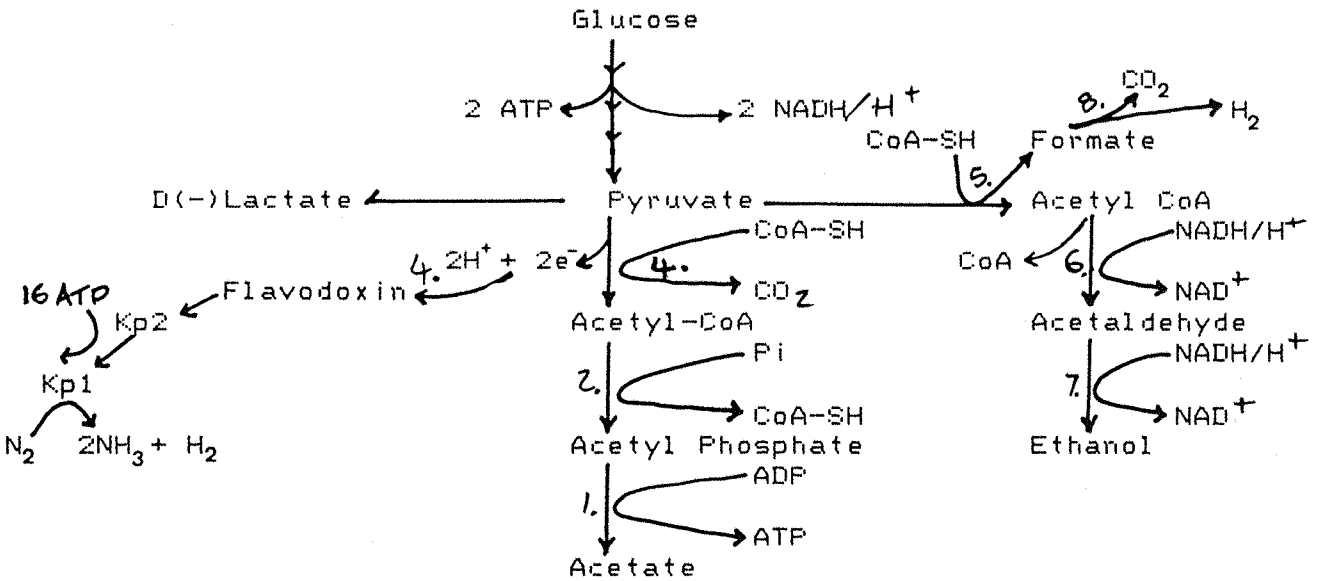
Only in *K. pneumoniae* is the electron transport pathway to nitrogenase genetically proven. Crude extracts of *K. pneumoniae* can couple the oxidation of pyruvate or formate to electron transport to nitrogenase (Yoch, 1974), whereas extracts from *nifF*$^-$ and *nifJ*$^-$ mutants cannot (Hill & Kavanagh, 1980). The *nifJ* gene product has been shown to be a pyruvate:flavodoxin oxidoreductase (Shah *et al.*, 1983). The enzyme catalyses a thioclastic reaction (Eqn 3) and is extremely sensitive to oxygen. The *nifF* product has been shown to be a flavodoxin which mediates rapid electron transport to the Fe protein (Deistung *et al.*, 1984).

3) —— Pyruvate + CoASH + 2 flavodoxin (ox) → acetyl CoA + CO_2 + 2 Flavodoxin (red) + $2H^+$

There is strong evidence that, like *E. coli*, *K. pneumoniae* is capable of conserving the energy in the thioester link of acetyl CoA (Eqn 3) as one molecule of ATP (see Fig. 1.5). *K. pneumoniae* can grow anaerobically with pyruvate as sole C-source with NH_4^+ or N_2 as nitrogen source (Hill, 1976a). Fermentation of glucose could yield a maximum of 3ATPs/glucose molecule. Since one mole of pyruvate can reduce two moles of flavodoxin

Fig. 1.5 Anaerobic catabolism of glucose during nitrogen fixation, a mixed acid fermentation

Enzymes include: (1), acetate kinase; (2), phosphotransacetylase; (3), pyruvate: ferridoxin oxidoreductase; (4), pyruvate flavodoxin oxidoreductase; (5), pyruvate formate lyase; (6), acetaldehyde dehydrogenase; (7), ethanol dehydrogenase; (8), formate hydrogen lyase.



Maximum yield 3 ATPs / Glucose consumed

and nitrogenase requires a minimum of 16 moles of ATP as against eight of flavodoxin; it is clear that synthesis of ATP rather than reductant limits nitrogenase activity under anaerobic conditions. When low O_2 concentrations were introduced into the gas supply of anaerobic glucose-limited N_2-fixing cultures, the molar growth yield for glucose and efficiency of N_2-fixation were increased by up to 82 per cent (Hill, 1976b). Although *K. pneumoniae* is often regarded as an anaerobic N_2-fixer, low concentrations of O_2 do improve the efficiency of carbon catabolism during diazotrophy, presumably by permitting oxidative phosphorylation. This is consistent with the stimulation of nitrogenase activity and synthesis by very low (30 nM) oxygen concentrations (Hill, 1976a, b; Hill *et al*, 1984). However, an obligate aerobic catabolism (ie microaerobic growth on succinate) cannot sustain N_2-fixing growth (Hill, S., unpublished results). Perhaps an obligate aerobic catabolism cannot provide adequate reducing power in the form of pyruvate for nitrogenase (see Section 4.2b). Obligate aerobic N_2-fixers such as *Azotobacter* do not possess a pyruvate:flavodoxin oxidoreductase (Gubler & Hennecke, 1986), but instead electron transport to nitrogenase is tightly coupled to aerobic respiration (Klugkist *et al.*, 1986) and requires an energized cytoplasmic membrane (Haaker *et al.*, 1974). The ultimate source of electrons is believed to be NADPH although the redox potential of the NADPH/$NADP^+$ couple (-330 mV) is not low enough for direct electron transport to nitrogenase, and reversed electron transport powered by proton-motive force has been proposed to have a role (Laane, 1979; 1980). Similar observations and suggestions have been made for the bacteroids of *Rhizobium* where a cluster of genes *fix ABCX* not found in *K. pneumoniae* (Gubler & Hennecke, 1986) are believed to code for proteins having a role in electron transport to nitrogenase.

Such fundamental differences in the mechanism of generating reducing power for nitrogenase in facultative anaerobes and obligate aerobes may be the main factor in determining whether the mode of diazotrophic growth is aerobic, microaerobic or anaerobic.

1.3a Mechanisms to protect nitrogenase from inactivation by oxygen; 'respiratory protection'

A wide range of devices has been developed by a variety of diazotrophs to protect their nitrogenase against damage by oxygen (see Bothe, 1985; Hill, 1988); these are listed in Table 1.4. Of these, only two 'respiratory protection' and perhaps 'conformational protection' are relevant in *Klebsiella pneumoniae*.

The concept of respiratory protection was originally developed in *Azotobacter* where the ability is most highly developed. It is based on the fact that the magnitude of the O_2 gradient between ambient concentration and the site of nitrogenase function is affected by the rate of O_2 uptake and resistance of the bacterial cell wall and membrane to oxygen diffusion. *Azotobacter* can adjust its O_2 demand to satisfy a very wide range of O_2 supply at the cost of increased inefficiency of N_2 fixation. It exhibits one of the highest rates of respiration of any known microorganism. The probable components contributing to a flexible respiratory activity in response to changes in O_2 supply are discussed below.

i) The respiratory chain

The key factor in 'respiratory protection' is the respiratory chain. Like many bacteria, the *Azotobacter* electron transport chain is branched at the dehydrogenase and oxidase ends. It probably contains two oxidases, a cytochrome *o* and a cytochrome *d* (Hofman *et al.*, 1980) through which the bulk of the electrons flux appears to pass (Fig. 1.6). The following explanation for the flexibility of respiratory activity of

Table 1.4 Devices that protect nitrogenase from damage by O_2 in N_2 fixing microorganisms

1. A high turnover of nitrogenase proteins.

2. Respiratory protection (*Azotobacter*, *Azospirillum*).

3. Protection by the oxyhydrogen reaction; H_2 evolution by nitrogenase and H_2 uptake coupled to respiration (*Azotobacter*), not proven.

4. Autoxidation of Fe protein at low O_2 concentration as a means of O_2 uptake (see 2 above) (*Azotobacter*) (Thorneley and Ashby, 1989).

5. Conformational protection (*Azotobacter*, possibly *K. pneumoniae*).

6. Formation of an air stable nitrogenase complex with a special Fe-S protein (Shethna protein) (*Azotobacter*).

7. Leghaemoglobin in the peribacteroid space buffered/facilitated O_2 transfer in combination with respiratory protection (*Rhizobium* species/legume symbioses).

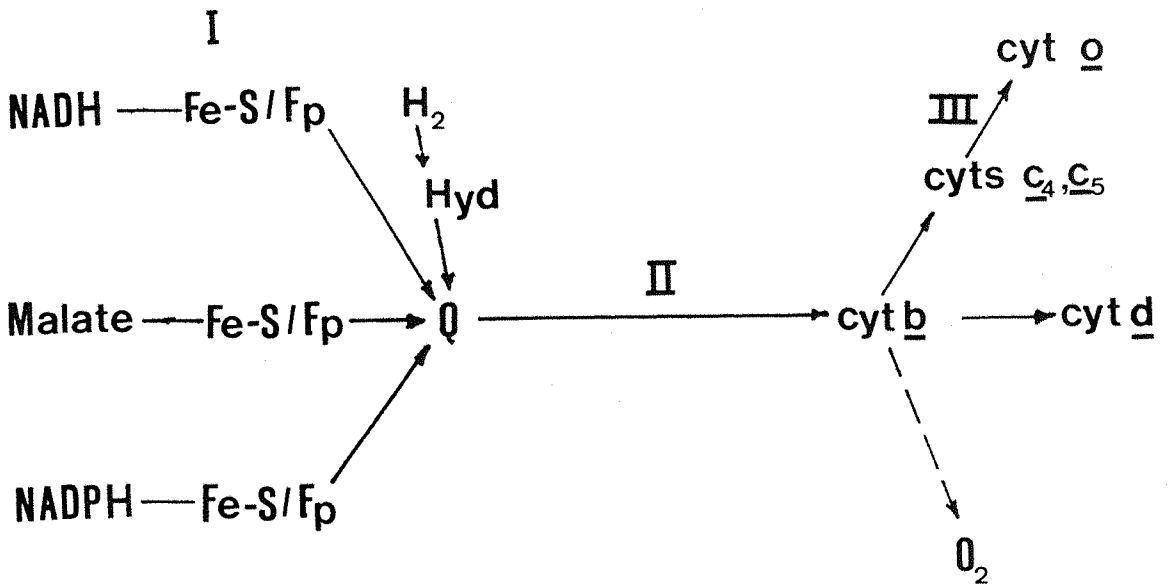
8. Compartmentation (nodules of legumes, vesides of actinomycetes (*Frankia*) and heterocysts of cyanobacteria).

9. Clustering of filaments and association with O_2 consuming bacteria (*Trichodesmium*, *Oscillatoria*).

10. Possession of superoxide dismutase, catalase and glutathione reductase.

Fig. 1.6 An interpretation of the respiratory chain of *Azotobacter vinelandii*

I, II and III are postulated coupling sites; Hyd, is a Ni containing uptake hydrogenase, modified from Haddock and Jones (1977). Note that cytochrome a_1 has been omitted because haem a_1 has not been detected in pyridine haemochrome spectra, and cannot be assigned on the basis of the 595 nm absorbance alone (see 4.2e). It is difficult to accept the validity of coupling site II in the absence of a cytochrome bc_1 complex and Q-cycle.



Azotobacter has been accepted over the years (see Haddock & Jones, 1977, for an account).

At low oxygen concentrations, electron transport from NADH to O_2 is tightly coupled to ATP synthesis, engaging three coupling sites (Fig. 1.6). This limb of the electron-transport chain terminates with a cytochrome *o* (Ackrell & Jones, 1971a, b) and presumably involves an as yet unidentified cytochrome bc_1 complex. At higher oxygen concentrations, cytochrome *d* is induced (Drozd & Postgate, 1970) and coupling sites I, II and III are bypassed, minimizing respiratory control and increasing the rate of electron flow. It must be emphasized that respiratory protection is not an instant response but an adaptive one, taking several minutes after a shift in oxygen supply.

In the facultative anaerobe *E. coli* cytochrome *d* is maximally expressed under acute O_2-limitation (Anraku & Gennis, 1987; Chapter 4, this work) and has a 'reputation' as a high affinity oxidase (K_S O_2 0.024 μM) (Rice & Hempfling, 1978). In *Azotobacter*, the situation appears to be reversed; cytochrome *d* is synthesised in response to excess oxygen and apparently has a low affinity for oxygen (K_S O_2 18 μM) while cytochrome *o* has the higher affinity for O_2 (K_S O_2 3.2 μM) (Hoffman *et al.*, 1979). Other workers (Jones, 1978; Jones *et al.*, 1978) reported that the pattern of cytochrome *d* expression was as described for other organisms; that is induction during acute O_2-limitation. These apparent differences are not easily rationalized and led Yates (1988) to speculate tht *Azotobacter* may have two cytochrome *d*s with differing O_2-affinities!

ii) Intermediary metabolism, the Fos^- mutants of *Azotobacter chroococcum*

An essential component of respiratory protection is the ability to generate increased reductant (NADH) in response to an increase in O_2 supply. This was illustrated by the work of Ramos and Robson (1985a, b) who isolated mutants of *Azotobacter chroococcum* unable to grow and fix N_2

under air. These mutants were called Fos$^-$ (Fixation on sugars$^-$); they showed low maximum respiration rates which were corrected by addition of carboxylic acids which led to an increased affinity for O_2. Some were shown to lack phosphoenol pyruvate carboxylase, while others were deficient in citrate synthase. Clearly mutations which affected the normal functioning of the TCA cycle, or catabolic metabolism in general, abolished N_2 fixation under air, because insufficient reductant for respiratory protection was generated.

1.3b Respiratory protection in *K. pneumoniae*?

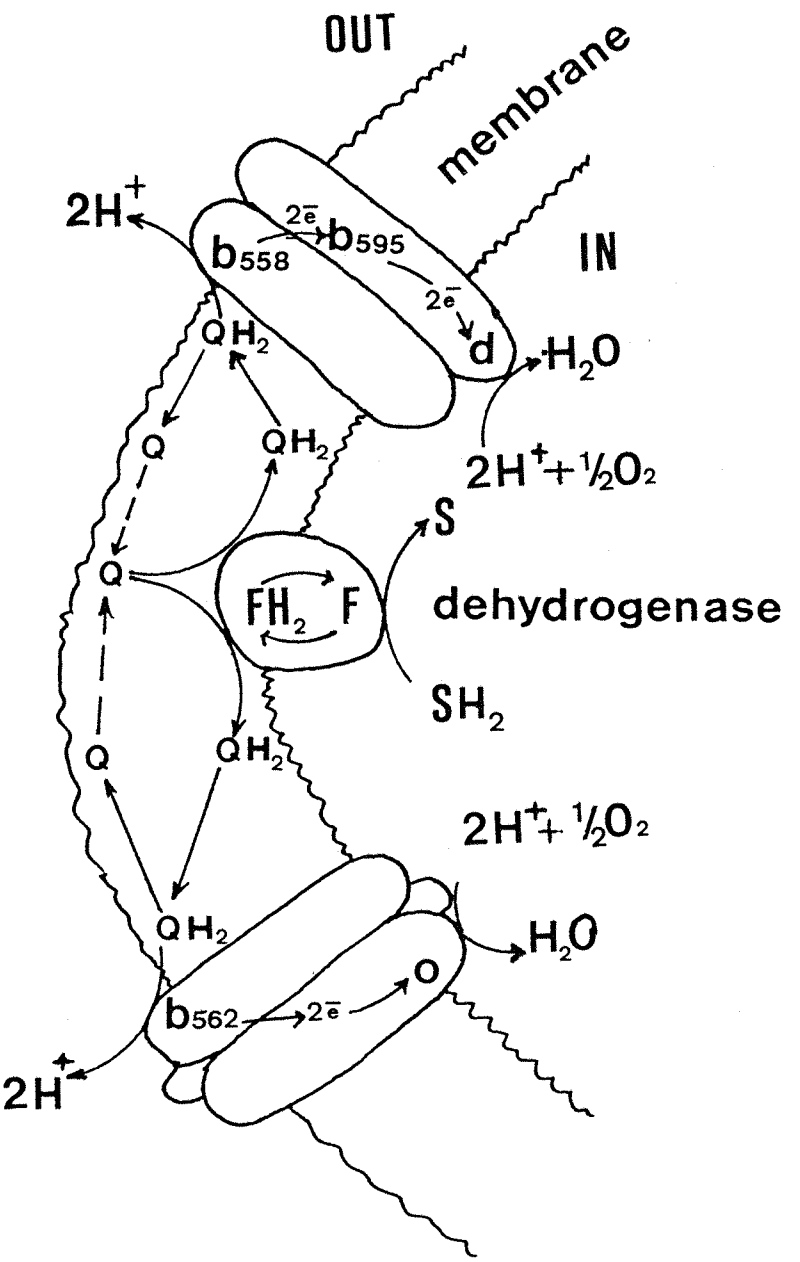
The role of respiration

Respiratory protection need not be confined to obligate aerobic nitrogen fixers. Facultative anaerobes grown anaerobically on glucose (under fermentative conditions) do express components of a respiratory chain; *K. aerogenes* contain cytochrome *b* and cytochrome *d* (Harrison, 1972); *E. coli* also contains low levels of these cytochromes (Jones, 1977) and gene fusions have shown that transcription of the *cyd* operon (cytochrome *d*) remains at a low constitutive level during anaerobiosis (Georgiou *et al.*, 1988a). However, none of these measurements has been done with *K. pneumoniae* with reference to N_2-fixing conditions. Samples from an anaerobic glucose-limited N_2-fixing chemostat culture of *K. pneumoniae* are capable of substantial O_2 uptake (200-300 nmol O_2 min^{-1} mg^{-1}). Hill (1976b) also showed that nitrogenase activities measured in samples from O_2-limited cultures were more tolerant of low atmospheric O_2 concentrations than in samples from anaerobic cultures, suggesting a degree of respiratory protection.

In *K. pneumoniae*, the sensitivity to inhibition by ambient O_2 of *nif* gene expression is greater than the sensitivity to inhibition of nitrogenase activity (Section 1.1dii). The main role of respiration may be to create and maintain an O_2 environment compatible with *nif* gene

Fig. 1.7 The aerobic respiratory chain of *E. coli*

A model for the organisation of the components of the aerobic respiratory chain in the cytoplasmic membrane of *E. coli*. A series of dehydrogenases reduce ubiquinone-8 (Q); the product ubiquinol-8 (QH_2) diffuses within the bilayer and can be oxidized by whichever oxidase is present, O-type oxidase (O) and cytochrome d oxidase (d). Either oxidase can serve as a coupling site generating a proton motive force by separating the two half-reactions on opposite sides of the membrane. F denotes FAD and S denotes substrate (modified from Anraku & Gennis, 1987).



expression and to supplement ATP production by oxidative phosphorylation (Section 1.2e). Presumably this must be achieved under conditions of oxygen which still allow sufficient fermentative metabolism for the production of pyruvate and low potential electrons for nitrogenase (Section 1.2e).

1.2 The aerobic respiratory chain of *E. coli* and cytochrome *d* oxidase complex

K. pneumoniae and *E. coli* are both members of the *Enterobacteriaceae* family and are hence closely related. Since very little information on the cytochromes and respiratory chain of *K. pneumoniae* is available, and none at all is available for N_2-fixing conditions (Harrison, 1972), it will be useful to consider the respiratory chain of *E. coli*.

E. coli is a facultative anaerobe whose respiratory chain composition varies with carbon source, the availability of oxygen, or with the replacement of O_2 by alternative electron acceptors (Ingledew & Poole, 1984); I shall consider here ony the aerobic respiratory chain.

In common with many bacteria, the aerobic respiratory chain of *E. coli* is branched, containing dehydrogenases which reduce ubiquinone on the inner side of the cytoplasmic membrane, and two terminal oxidases, each of which oxidizes ubiquinol, reducing molecular oxygen to water (Fig. 1.7). The low affinity cytochrome *o* is the principal oxidase under aerobic conditions and the high affinity cytochrome *d* oxidase (encoded by the *cyd* genes) predominates under microaerobic or O_2-limited conditions. Mutants of *E. coli* that lack either oxidase have no growth defects under the various laboratory conditions tested (Green & Gennis, 1983; Au *et al.*, 1985), implying a possible redundancy of terminal oxidases. One of the most outstanding problems is that of a physiological role for these oxidases. The higher affinity of cytochrome *d* oxidase for oxygen (assumed to be 24 nM) compared with that of O-type oxidase (200 nM) may

Table 1.5 Enzymatic properties of the two terminal oxidases of *E. coli*

Substrate	Cytochrome *o* complex	Cytochrome *d* complex
Substrate (Km/μM)		
Ubiquinol-1	48	230
Menadiol	38	1.7
O_2 †	2.9	0.38
O_2 #	0.2	0.024
Inhibitor (mM)[a]		
KCN	0.01	2
NaN_3	15	400
H_2O_2	300	120
HQNO	0.002	0.007
$ZnSO_4$ †	0.001	0.060

Data collected from Anraku and Gennis (1987); † Kita *et al.* (1984); #, Rice and Hempfling (1978) (assumed value from whole cell studies); (a), concentration required for 50% inhibition of ubiquinol-1 oxidase activity.

explain why cytochrome *d* is induced under oxygen deficient conditions (Rice & Hempfling, 1978); this is not however a *raison d'ètre*. It may be that facultative anaerobes growing fermentatively can do so more efficiently at low oxygen concentrations. It is interesting to note that some obligate anaerobes contain cytochrome *d* where its high affinity for O_2 may endow the cell with a mechanism for the 'respiratory protection' of O_2-labile enzymes (Haddock & Jones, 1977).

Table 1.5 shows the enzymatic properties of the two terminal oxidases of *E. coli*. Special attention is drawn to the difference in sensitivity of the two oxidase types to inhibition by NaN_3 and Zn^{2+} since this forms the basis of a screening test for the Cyd^- phenotype (Chapter 5).

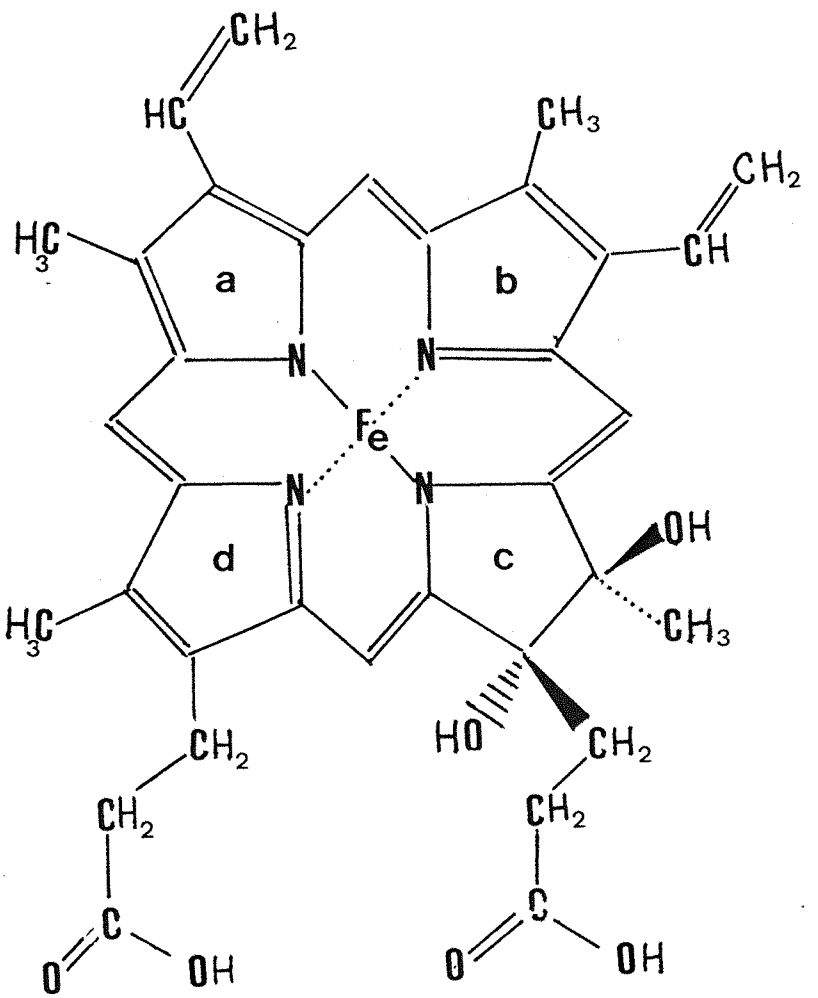
The cytochrome *d* complex has been characterized by a number of workers and found to contain two polypeptide subunits (Miller & Gennis, 1983) and four haem groups (Lorence *et al.*, 1986). Subunit I (Mr58K) and subunit 2 (Mr43K) are both encoded at the *cyd* locus mapping at min 16.5. The DNA

sequence of *cydA* (1) and *cydB* (2) have been determined (Green *et al.*, 1988) and regulation of expression has been shown to be transcriptionally controlled. Transcriptional activation is greatest under O_2-deficient conditions during growth on lactate or succinate and there is a low basal level of activation during anaerobic growth on glucose. The activation under O_2-deficient conditions is independent of the *fnr* gene product, cyclic AMP or cyclic AMP-binding protein (Georgiou *et al.*, 1988a). The deduced amino acid sequences indicate that both subunits are highly hydrophobic each having seven to eight transmembrane spanning helices. Gene fusions have identified four cytoplasmic facing regions within subunit 2, consistent with its hydropathy profile (Georgiou *et al.*, 1988b). Subunit 2 contains at least one *d* type haem which is actually a chlorin and is unique to the enzyme (Timkovitch *et al.*, 1985) (Fig. 1.8). This is believed to be the O_2-binding site. It has a characteristic absorbance peak in the reduced minus oxidized difference spectrum at 628 nm (see Chapter 4). Subunit 2 also contains a high spin protohaem, designated cytochrome b_{595} which has a role in the reduction of O_2 bound to haem *d* (Poole & Williams, 1987). Subunit I is a low spin b-type cytochrome responsible for quinol binding and oxidation (Lorence *et al.*, 1988).

Fig. 1.7 shows the functional organisation of the respiratory chain and the coupling sites for oxidative phosphorylation. Dehydrogenases located on the inner surface of the cytoplasmic membrane reduce ubiquinone-8 (UQ-8) in the bilayer. This serves as a branch point providing electrons to either oxidase. It has been proposed that, since the quinol oxidation site is near the periplasm, two protons are released when quinol is oxidized (Anraku & Gennis, 1987). The electrons are transferred back through the membrane via cytochrome b_{595} to reduce O_2

Fig. 1.8 The structure proposed for heme *d*

Heme *d* (Chlorin) prosthetic group in the cytochrome *d* complex. The chlorin binds O_2 and is presumably located on the inside surface of the cytoplasmic membrane. In principle, it can be synthesized from protohaem IX. One of the pyrrole rings is reduced, defining it as a chlorin, and two hydroxyl groups have been added (Timkovitch *et al.*, 1985; Anraku & Gennis, 1987).



bound to haem *d* where protons and electrons are consumed. The net reaction is the electrogenic transfer of one H^+ across the membrane/electron. The theoretical H^+/o ratio for this would be 2, and experimental values of 1.7 have been obtained in support of this model (Miller & Gennis, 1985). The *E. coli* respiratory chain is much less efficient than the mitochondrial chain, where three coupling sites I, III and IV operate in the oxidation of NADH.

1.5 Aims and present work

The mechanism whereby oxygen regulates the expression of the *nif* genes is not known, although evidence suggests that the action of the *nif* specific activator NifA is antagonized by NifL in response to oxygen (1.2aii). How oxygen status is communicated to NifL is not known. Chapter 3 tests the possibility that there is a role for haem, either bound to NifL or via its role in the respiratory chain in the oxygen-sensing mechanism.

There is good evidence that the respiratory chain of *K. pneumoniae* has an important physiological role in nitrogen fixation (1.2aii, 1.3, 1.3b); yet little is known about this respiratory chain or its regulation under N_2-fixing conditions. A question of particular interest is the nature of the oxidase(s) responsible for respiration at the very low oxygen concentrations that allow *nif* gene expression (1.2aii) and which support more efficient nitrogen fixation than occurs under anaerobic conditions (1.3, 1.3b). Chapter 4 reports the identity and properties of this oxidase (cytochrome *d*) and compares it with the available information on the *E. coli* enzyme. In the light of its measured O_2 affinity, it is proposed that the d-type oxidase fulfills the roles stated above.

Chapter 5 provides direct evidence for the involvement of cytochrome *d* in microaerobic nitrogen fixation by showing that strains deficient in cytochrome *d* cannot support microaerobic nitrogenase activity under

conditions where the ATP and reductant must be generated as a result of respiration via cytochrome *d*. A model outlining the respiratory chain expressed under N_2-fixing conditions and its involvement in O_2-dependent nitrogenase activity is proposed.

CHAPTER 2

MATERIAL AND METHODS

Materials and methods are divided into three sections, according to the chapter that they cover. Tables containing details of strains used will be found in the relevant results chapters.

Chapter 3

2.1a) Bacterial strains and growth conditions

The bacterial strains used in this work are listed in Table 3.1 and were always grown and incubated at 30°C.

Nutrient broth (NB) and nutrient agar (NA) were from Oxoid. Minimal medium (MM) and nitrogen-free medium (NFDM) containing glucose (1% w/v) have been described by Cannon (1980). For growth of A1002 and A1004a, MM and NFDM were supplemented with isoleucine, valine, methionine and cysteine (all at 40 μg/ml) and vitamin-free casamino acids (200 μg/ml) (Difco). 5-Aminolaevulinic acid (5-ALA) (30 μM) (from Aldrich) was added as required. Strain JC5466 (pRD1) was grown in MM supplemented with tryptophan (20 μg/ml), carbenicillin (200 μg/ml) and kanamycin (30 μg/ml).

2.1b) Bacterial matings

The donor strain JC5466 (pRD1) and the recipient strains A1002 and A1004a were grown anaerobically for 18 h in MM supplemented as described, harvested and washed once in saline phosphate (containing 100 mM-NaCl and 50 mM-phosphate buffer, pH 7.4). Matings were carried out by spotting 50 μl of the donor mixed with recipient onto the surface of a selective agar plate (MM supplemented with isoleucine, valine, methionine, carbenicillin, kanamycin and 5-ALA (concentrations above). After three days, two transconjugants were purified on selective media and checked for maintenance of the Haem-phenotype and for the presence of the Nif^+ phenotype by acetylene-reduction assays.

2.1c) Inoculum cultures of strains A1004a(pRD1) and A1002(pRD1)

These were grown anaerobically in NFDM supplemented as described except that the casamino acid concentration was increased to 500 μg/ml and 5-ALA was omitted; carbenicillin and kanamycin were included to maintain plasmid selection. Flasks (50 ml), containing 20 ml medium were inoculated with a single colony from a selective plate, sparged for 20 min with sterile nitrogen and capped with a Suba seal. Growth was for 18-40 h with shaking (50 oscillations/min). For A1004a(pRD1), this procedure gave acceptably low numbers of revertants and, because of the absence of 5-ALA, it provided cultures lacking haem (see Scott & Poole, 1987).

2.1d) Acetylene-reduction assays

A nif^+ phenotype was confirmed for all transconjugants by growth and subsequent assay for C_2H_2-reduction after N-limited growth in supplemented-NFDM containing carbenicillin, kanamycin and 5-ALA (50 μM as required), as described by Cannon (1980). Quantitative measurements of C_2H_2 reduction were performed under anaerobic conditions as described in Section 2.3c.

2.1e) Growth curves

Strain A1004a(pRD1) was grown in 20 ml of supplemented-NFDM in 100 ml Klett flasks anaerobically or aerobically in the presence or absence of 5-ALA (60 μM). Aerobic cultures were shaken (50 oscillations/min) under air; anaerobic cultures were sparged for 20 min with sterile nitrogen before capping with a Suba seal. Optical density measurements were recorded by using a Klett- Summersen photoelectric colorimeter. At the end of the experiment 10% C_2H_2 was introduced into the gas phase and the specific C_2H_2 reducing activity was determined.

2.1f) Detection of revertants

Two methods of detecting $Haem^+$ revertants were used: 1) ability to grow aerobically on NA or MM in which succinate (50 mM) replaced glucose

Rank-oxygen electrode at 30°C in a 2 ml reaction mixture containing phosphate buffer (50 mM, pH 7.4), NADH (0.5 mM) and D(-)lactate (20 mM). These substrates were chosen to give the greatest rate of oxygen consumption by these membranes, the rate of oxygen consumption being used as a guide to the oxidase activity.

2.1i) Immunoblot analysis

SDS-PAGE was performed as described by Laemmli (1970) in 10 per cent polyacrylamide gels. Protein was transferred to nitrocellulose membrane (Schleider and Schüll BA 83, 0.22 μm) overnight at 5V/cm (0.32 amp) using the bicarbonate blotting system of Dunn (1986). Blots were developed with rabbit antiserum raised to *Klebsiella pneumoniae* nitrogenase (Rennie *et al.*, 1978), and sheep anti-rabbit conjugated peroxidase (from Serotec). Immunologically-reactive bands were stained as described in Towbin *et al.* (1979) except that diaminobenzidine dihydrochloride (Sigma) instead of o-dianisidine was used.

2.1j) Pulse-labelling of derepressed cultures following exposure to oxygen

Strain A1004a(pRD1) (10 ml) was inoculated into 100 ml of supplemented-NFDM containing carbenicillin and kanamycin. Cultures were sparged with a slow stream of 1% CO_2 in nitrogen for 18 h until the C_2H_2-reducing activity was more than 10 nmol C_2H_4 produced/min/mg bacterial protein (early exponential phase). The number of $Haem^+$ revertants was always less than 2×10^{-6}.

Portions of culture (40 ml) were transferred in nitrogen-flushed syringes to two anaerobic reaction vessels supplied with nitrogen and mounted on magnetic stirrers. One vessel was equipped with a lead/silver oxygen electrode connected to an oxystat as described by Hill *et al.* (1981). Both vessels were isolated from the gas supply and 10% C_2H_4 was introduced into the gas phase. A small amount of air was introduced into

the vessel with the oxygen electrode (oxystat culture) and the stirring speed automatically adjusted to maintain a low oxygen concentration (1.4-2.6 μM oxygen) for the duration of the experiment. The control culture was maintained under anaerobic conditions. Samples (3-3.5 ml) of culture were removed from the reaction vessels as required and 2 ml of the sample was injected into an argon-flushed conical polycarbonate tube capped with a Suba seal containing 10 μCi ^{14}C-labelled amino acids (Amersham International). The tube was then incubated for 5 min with rapid shaking. Incorporation of labelled amino acids was stopped by addition of 100 μl of unlabelled casamino acids (10 mg/ml). Subsequent estimation of total polypeptide synthesis, measured as incorporation of radioactivity into trichloracetic acid-precipitable material, and of *nif* polypeptide synthesis by autoradiography of SDS-PAGE gels were performed as described by Eady *et al.* (1978) and Cannon (1980). Autoradiograms were scanned on a Chromoscan gel scanner. The *nif* gene products were identified by comparison with other pulse-labelling experiments in which the *nif* products were expressed from a multicopy plasmid during derepression (Cannon *et al.*, 1985) and therefore accounted for 90-100 per cent of total protein synthesis. In experiments where derepression had already occurred, the *nif* products accounted for only 20-30 per cent of total protein synthesis.

2.1k) Total bacterial protein and membrane protein

This was measured by the bicinchoninic acid assay (Smith *et al.*, 1985; Redinburgh & Turley, 1986). Culture samples (0.5-5.0 ml) were harvested by filtration or centrifugation, washed once in saline phosphate and suspended in 200 μl of 50 mM Tris/HCl, pH 7.4. Various volumes of bacterial suspension (or membrane particles) were then diluted with an equal volume of 1% SDS in 1.8% EDTA, and boiled for 5 min. The boiled suspensions (10 μl) were placed in wells of a 96 well microtitre plate

(Dynaech M129A; washed with Decon followed by acid between uses). BCA working reagent (200 μl) (Pierce Chemical Company, Illinois) was added to each well and the plates incubated for 1 h at 50°C. Absorbance measurements were made on a MR700 microplate reader (Dynatech, West Sussex, UK) with a 570 nm test filter and 450 nm reference. A standard curve was prepared with bovine serum albumin (0-2 mg/ml).

Chapter 4

2.2a) Growth of bacteria in continuous culture and preparation of membranes for determination of cytochrome and oxidase content

K. pneumoniae (oxytoca) (NCIB 12204) was maintained as described for *E. coli* by Smith *et al.* (1988).

The growth medium contained Na_2HPO_4 (73 mM); KH_2PO_4 (18 mM); nitriloacetic acid (300 μM) and the following (final concentration in mg l^{-1}): $MgSO_4.7H_2O$ (60); $MnSO_4.4H_2O$ (4.4); Na_2SO_4 (345); $CaCl_2.2H_2O$ (340); $Na_2MoO_4.2H_2O$ (7.6) and ferric citrate $5H_2O$ (49). The medium also contained 50 ml/litre of the trace element solution of Poole *et al.* (1979) (but omitting the cations listed above). The carbon sources were glucose (0.5-2%), glycerol (1%) or sodium succinate (50 mM). The nitrogen source was ammonium sulphate (6 mM), tryptone (0.7-1.0 gl^{-1}) or 'white spot' nitrogen gas (BOC). All anaerobic cultures were sparged with a stream (100 ml min^{-1}) of 'white spot' nitrogen. When necessary, the oxygen transfer rate was adjusted by diluting inflowing air with nitrogen gas or by altering the stirrer speed.

The bacteria were grown at 30°C in an 800 ml working volume chemostat. The pH was controlled to 6.7±0.2 by automatic addition of 2M-NaOH or 1M-HCl. The dissolved oxygen concentration in the culture was measured with a lead/silver oxygen electrode (L. H. Engineering Ltd.)

calibrated at 0% and 20% of air saturation. The lowest recordable oxygen concentration was about 1 μM.

Bacteria were considered to be in steady state conditions when the cell density was constant and there was no measurable limiting nutrient. The time allowed for a new steady state to be established after a change in growth conditions was six culture volume replacements. Culture samples were tested for purity and for *nif* phenotype by plating on minimal agar medium plus appropriate carbon source, on nutrient agar and on nitrogen-free solid medium (NFDM) (incubated anaerobically) as described by Cannon (1980). A given nutrient was considered to be growth-limiting when a decrease in its concentration led to a proportional decrease in cell density, when it was not detectable in the culture, and when an increase in concentration of non-limiting nutrients (nitrogen, oxygen or carbon) led to no change in cell density.

Bacteria for preparation of membranes were collected from 'overspill' culture (10 l) (at 4°C) over a few days. Bacterial suspensions were concentrated (five-fold) using a Pellicon membrane system (Millipore) and then harvested, and membrane fractions prepared exactly as described by Smith *et al.* (1988) (2.1 h).

The same medium as that described above was used for growth on a large scale for preparation of the *d*-type oxidase except that glucose was replaced by 0.1% glycerol plus 50 mM-sodium succinate, the tryptone concentration was increased to 4 gl^{-1}, ammonium sulphate (20 mM) and yeast extract (1 gl^{-1}) added and the phosphate concentration doubled (to 192 mM). Bacteria were grown in a 20 l fermenter at 30°C and harvested well into the stationary phase (at four days). The culture was sparged with a 4 l air per min with an agitation rate of 150 rpm. After visible growth had occurred, dissolved oxygen was not detectable.

2.2b) Spectrophotometric characterisation of haems, cytochromes and oxidases

All spectra were recorded on a Shimadzu UV-3000 dual beam/dual wavelength spectrophotometer fitted with an attachment for measurements at 77K when required. A spectral bandwidth of 2 nm, a lightpath of 10 mm (or 2 mm, when stated) and a scan speed of 100 nm/min were used. For characterisation of the haems present in purified oxidase or in membrane preparations and whole bacteria, the alkaline pyridine haemochrome method of Fuhrop and Smith (1975) was used (2.1g). Immediately after production of the haemochrome, the dithionite-reduced minus hydrogen peroxide-oxidised difference spectrum was recorded. For determination of cytochromes in membranes, samples in 20 mM-tris/HCl (pH 8.0) containing 1 mM-EDTA were oxidised by air (cytochrome *d*) or by addition of a crystal of ammonium persulphate or potassium ferricyanide. They were reduced by addition of sodium dithionite or appropriate respiratory substrate (20 mM). The extinction coefficients for reduced-minus-oxidised spectra were those used for determination of cytochromes in *E. coli*: total cytochrome *b*, 17.5 cm^{-1} mM^{-1} (558-575 nm) (Kita *et al.*, 1984a); cytochrome *d*, 7.4 cm^{-1} mM^{-1} (628-607 nm) (Lorence *et al.*, 1986). Determination of cytochrome *o* was from (reduced + CO)-minus-(reduced) spectra recorded after sparging with CO for 2 min. Extinction coefficients were those used for studies of *E. coli* cytochrome *o*: 145 cm^{-1} mM^{-1} (416-430 nm) (Kita *et al.*, 1984a).

2.2c) Purification of the cytochrome *d* oxidase complex

The method was based on that of Miller and Gennis (1983), the main difference being the replacement of chromatography on hydroxylapatite with anion exchange chromatography on Pharmacia Mono-Q. Membranes from 30 g wet weight of bacteria were suspended in 20 mM-tris/HCl (pH 8.0) containing 1 mM-EDTA and 0.5 M-KCl and centrifuged at 150,000 g for 90

min. They were resuspended in the same buffer (lacking KCl) and sodium deoxycholate added to give a final concentration of 0.2% and final protein concentration of 5 mg/ml. The suspension was stirred for 15 min on ice, the membranes sedimented (as above), and suspended in 75 mM-potassium phosphate (pH 6.3), containing 150 mM-KCl and 1 mM-EDTA (buffer A). The oxidase was solubilised from the membranes by addition of 120 mM-Zwittergent 3-12 (Calbiochem) to give a final detergent concentration of 60 mM and membrane protein concentration of 12 mg/ml. The mixture was stirred on ice for 30 min and centrifuged at 150,000 g for 2 h. Chromatography of the supernatant on DEAE-Sepharose FF (Pharmacia) was done essentially as described by Miller and Gennis (1983). Pooled active fractions were concentrated about three-fold by ultrafiltration (Amicon YM-50 membrane) to give a solution of 1 mg protein/ml, which was desalted on a Pharmacia PD10 column equilibrated with buffer A containing 4 mM-Zwittergent 3-12 and chromatographed on a 1 ml Mono Q column equilibrated in the same buffer, using a 33 ml linear salt gradient (150-40 mM). Peak fractions were concentrated to 1 mg protein/ml and glycerol added to a final concentration of 10% before storage in liquid nitrogen.

2.2d) Analytical gel filtration

This was done on a Pharmacia Superose-12 column equilibrated with buffer A containing 4 mM-Zwittergent and run at a flow rate of 0.3 ml/min.

2.2e) Determination of cytochrome *d* during purification

This was based on the oxidation of 2,3,5,6-tetramethyl *p*-phenylenediamine dihydrochloride (TMPD). This was dissolved (1 mg/ml) in 20 mM-tris/HCl (pH 7.9) containing 0.05% Triton X-100. Samples of column fractions (10 μl) were mixed with 200 μl of fresh TMPD reagent in wells of microtitre plates and absorbance at 630 nm recorded within 2 min using a microtitre plate reader (Dynatech).

2.2f) Protein determination and amino acid composition

Protein was measured by the bicinchoninic acid method adapted for use on microtitre plates as described by Smith *et al.* (1988) (2.1k). For determination of its amino acid composition 100 μg of purified oxidase was precipitated from solution with an equal volume of ice cold acetone, washed twice in acetone, suspended in 100 μl of Analar water (BDH) and transferred to a hydrolysis tube. HCl plus phenol (0.5 ml of 5M HCl containing 0.1% phenol) were added and the tube evacuated before hydrolysis at 110°C for 24 h. Amino acids were determined on a Perkin Elmer amino acid analyser.

2.2g) SDS-Polyacrylamide gel electrophoresis and assay for nif polypeptides

SDS-PAGE was done as described by Laemmli (1970) except that samples contained a final concentration of 4% SDS and were not heated unless specifically stated. Gels were stained with Coomassie brilliant blue or with silver stain (Bio-rad). *Nif* polypeptides were determined by immunoblot analysis as described by Smith *et al.* (1988) (2.1i).

2.2h) Determination of oxidase activity in the oxygen electrode

Oxygen consumption was measured in a Rank-oxygen electrode at 30°C in a 1 ml reaction mixture containing 50 mM-tris/HCl (pH 7.0 or 7.9), bovine serum albumin (1 mg/ml) and 0.05% Tween 20 (Sigma) or Triton X-100 (BDH). Electron donors were either 110 μM-ubiquinol-1 (half its Km concentration) (a gift from Hoffman La Roche), 1 mM-duroquinol (Sigma), or 1.0 mM-TMPD (Sigma). Dithiothreitol (8 mM) was used to maintain the quinols in the reduced state, and 5.0 mM-ascorbate to keep TMPD in its reduced state. Assays using duroquinol were done at pH 7.9.

2.2i) Determination of the oxygen affinity of the cytochrome *d* oxidase

This was measured by following the deoxygenation of leghaemoglobin. Ferric azide leghaemoglobin was the kind gift of F. J. Bergerson (CSIRO, Canberra, Australia); it was converted for use to the ferrous oxygenated form by the method of Bergersen and Turner (1980). A glass cuvette contained 50 μM-oxyleghaemoglobin, 0.05% Tween 20, 55 μM-ubiquinol-1 and 8 mM-dithiothreitol in 50 mM-sodium phosphate (pH 7.4), to give a total volume of 1.9 ml completely filling the cuvette which was sealed with a Suba seal so as to exclude all air. The spectrum of ferrous oxyleghaemoglobin was recorded in the dual wavelength mode between 500 nm and 600 nm (reference wavelength, 587 nm) using a buffer baseline. Purified oxidase (5 μg) was added and the change in fractional saturation of oxyleghaemoglobin against time was recorded at 575 nm (against a reference of 561 nm). The free dissolved oxygen concentration and total oxygen in the cuvette were calculated at 6 s intervals during deoxygenation by the method of Bergersen and Turner (1980) using the published binding constant of ferrous leghaemoglobin for oxygen of 43.5 nM. Steady state kinetic data were fitted to the Michaelis-Menten equation by computer-aided iterative regression (Biodata Handling with Microcomputers by R. B. Barlow, Elsevier-Biosoft).

2.2j) Reconstitution of cytochrome *d* oxidase complex with phospholipid

Phospholipid stocks (Sigma) were stored in chloroform:methanol (2:1) at -20°C. Samples were dried under nitrogen and solubilised by sonication until clear in 100 mM-Tris/HCl (pH 7.9) containing 50 mM-sodium cholate (recrystallized from methanol) to give a final concentration of 5-20 mg phospholipid/ml. Total bacterial phospholipid was prepared by solvent extraction of 50 g wet weight *K. pneumoniae* by the method of Bligh and Dyer (1959) as applied by Ames (1968). This was solubilised in the

same way as the pure phospholipids. Equal volumes of purified oxidase (1.1 mg/ml) and solubilised phospholipid were incubated together for 1 h at room temperature and a 10 μl sample of the incubation mixture diluted 100-fold into the assay mixture used for measurement of activity in the oxygen electrode as described above (the neutral detergent was omitted from the reaction mixture).

2.2k) Rabbit antisera to cytochrome *d* complex

The two subunits of cytochrome *d* oxidase complex were separated by preparative SDS-PAGE, bands were visualised with cold KCl (200 mM) and excised from the gel and packed into 10 ml pipettes. Using a rod gel apparatus, the two subunits were electroeluted at 100 volts for 18 h into a dialysis sack containing electrophoresis buffer (Laemmli, 1970). The protein was precipitated with three volumes of ice-cold acetone and resuspended to 100 μg ml^{-1} in sterile saline phosphate. Two New Zealand white rabbits were injected with 400 μg of subunit II or 400 μg of oxidase complex, in complete adjuvant (Sigma). Rabbits were boosted at monthly intervals with the same dose of antigen in incomplete adjuvant and bled after three months. Immunoblot analysis was as described in Section 2.1i.

Chapter 5

2.3a) Bacterial strains

The bacterial strains used in this work are listed in Table 5.2 and described in the legend. General growth conditions and maintenance of strains were as previously described.

2.3b) Continuous culture of *Klebsiella pneumoniae*

This was as described previously for Chapter 4 (Section 2.2a). *K. pneumoniae* (wild-type was grown in glucose-limited anaerobic conditions in a 450 ml working volume chemostat, with glucose (0.5% w/v) as sole C-source and N_2 as sole nitrogen source ($D=0.12hr^{-1}$).

2.3c) Measurement of whole cell acetylene reduction

Conical flasks (35 ml) containing 50 µl 20% w/v glucose were capped with Suba-seal closures and flushed with argon. Flasks were injected with 4 ml acetylene pC_2H_2 0.12 atm, equilibrated at 30°C for 5 min and the positive pressure released. Assays were started by injecting 2 ml of anaerobic culture and the flask subjected to vigorous shaking (100 cycles/min). The pC_2H_2 in flasks after equilibration and injection of culture was 0.06 atm. The Km for C_2H_2 reduction was 0.007 atm. For multiple assays, samples were injected at 30 s intervals and gas samples withdrawn every 5 min. Gas samples (0.5 ml) were analysed by a Pye Unican 204 gas chromatograph fitted with a 45 cm porapak N-column with N_2 as carrier gas. Ethylene and where necessary acetylene were measured with reference to external standards. The precise culture volume injected was obtained by difference after weighing each flask before and at the end of the assay and applying a density correction. The gas volume was obtained in a similar manner by filling the flask with water. Sampling error was assumed to have a normal distribution about the calculated regression line, from which activity (min^{-1} ml^{-1}) was obtained as the slope.

2.3d) Harvesting and washing bacteria anaerobically

Samples of anaerobic bacterial culture (20 ml) from either batch or continuous culture were harvested in Suba-seal capped argon-flushed plastic universals at 5000 x g for 15 min at 4°C. The pellets were resuspended in 5 ml of saline phosphate which had been sparged with argon for 30 min. Samples were continually flushed with a slow stream of argon and all syringes and needles were flushed with argon before use. Culture (filtrates) supernatants were maintained under argon and stored at -20°C.

2.3e) O_2-consumption measurements

The rate of O_2 consumption of anaerobically washed bacteria was determined in a Rank O_2-electrode at 30°C in 2 ml of saline phosphate pH

7.4. Potential fermentation products and substrates were dissolved in 100 mM sodium phosphate pH 7.4 and the final concentrations used were those shown in Fig. 5.3a, b.

2.3f) Formate plus lactate dependent microaerobic acetylene-reduction assays

These were done as described in Section 2.3c. Sodium formate and sodium D/L lactate (Sigma) solutions (40 mM) were prepared in 100 mM sodium phosphate pH 7.4, 0.5 ml of each was added to the acetylene-reduction assay flasks in place of glucose. Air (2 ml) was injected into the anaerobic gas phase before injection of acetylene and equilibration. The oxygen concentration in the gas phase was checked by injecting 0.5 ml gas samples, with an argon-flushed syringe into a katherometer equipped with a thermal conductivity detector.

2.3g) Bacterial matings

These were done exactly as described in Section 2.1b except that MM was unsupplemented and the selective medium for transconjugants was MM containing carbenicillin (200 μg/ml) and Kanamycin (30 μg/ml).

2.3h) Batch cultures of *E. coli* Cyd^- strains

The strains in Table 5.2 were maintained and grown for inocula either on NA or, of Nif^+ transconjugants, on minimal glucose medium supplemented as above (Section 2.3g). Anaerobic glucose-limited growth was achieved after 18-24 h of slow bubbling with CO_2 (1% v/v) in O_2-free N_2 at 30°C in 100-200 ml of modified NFDM medium. Modified NFDM contained 0.25% w/v glucose, twice the usual phosphate concentration, 5% v/v nutrient broth (derived from the inoculation culture), the trace element solution of Poole *et al.* (1979) and vitamin-free casamino acids (400 μg/ml). The concentration of fixed N in this medium did not repress *nif*, but was not adequate to yield a glucose-deficient culture at the end of growth.

Exhaustion of glucose was detected with Clinistix reagent strips (Miles Laboratories Ltd., Slough).

2.3i) Detection of the Cyd^- phenotype

Cyd^- strains were checked for reversion to Cyd^+ by plating on freshly-prepared nutrient agar containing $ZnSO_4$ (100 μM) and NaN_3 (100 μM), Cyd^- strains failed to grow while Cyd^+ strains did, owing to the expression of the Zn^{2+} and $azide^-$ insensitive oxidase cytochrome *d* (see Section 1.4, Table 1.5). Zn^{2+} alone was sufficient to inhibit the growth of RP56, while G0103 required azide as well for complete inhibition of growth (see Table 5.2).

2.3j) Determination of the D(-) lactate concentration in culture supernatants

D(-) lactate: ubiquinone reductase, otherwise known as NAD^+ independent lactate dehydrogenase (D(-)iLDH) (Garvie, 1980), was purified from *K. pneumoniae* membranes by a modification of the method of Kohn and Kaback (1973) (see Appendix 1) and used in a dye-linked assay system to measure D(-) lactate concentration in culture supernatants. The 1.0 ml assay contained 360 μM 2,6 dichlorophenol indophenol (Sigma) as acceptor (Km^{DCPIP} = 33 μM), two units of D(-)iLDH, 1 mg BSA ml^{-1} and 50 mM Tris HCl pH 8.0. The assay was initiated by the addition of D(-) lactate or a dilution of clture supernatant (Km^{lac} = 220 μM). The decrease in absorption at 600 nm was followed and the initial rate calculated using the extinction coefficient of Peel (1984) (21 cm^{-1} mM^{-1}). A calibration curve of rate versus D(-) lactate concentration was constructed by iterative fit to the Michaelis-Menten equation. The rates supported by dilutions of culture supernatants were used to obtain the D(-) lactate concentration by interpolation from the fitted curve.

CHAPTER 3

THE ROLE OF HAEMOPROTEIN IN THE CONTROL OF ENTERIC NITROGENASE SYNTHESIS BY OXYGEN

3.1 INTRODUCTION

Nitrogen fixation in the facultative anaerobe *Klebsiella pneumoniae* occurs only in anaerobic or microaerobic conditions (at less than 30 nM oxygen) (Hill *et al.*, 1984). At about 6μM oxygen (or higher) nitrogenase is inactivated and transcription from all the *nif* operons except for *nifLA* (the regulatory operon) is inhibited (Dixon *et al.*, 1980; Hill *et al.*, 1981; Merrick *et al.*, 1982; Cannon *et al.*, 1985). At concentrations of oxygen above 60 μM, expression from the *nifLA* promoter is also inhibited. The mechanism whereby oxygen regulates the expression of the *nif* genes is not known although the evidence suggests that in response to low concentrations of O_2, NifL antagonises the action of NifA, the *nif* specific transcriptional activator (Hill *et al.*, 1981; Merrick *et al.*, 1982; Filser *et al.*, 1983; Dixon *et al.*, 1980; Buchanon-Wollaston & Cannon, 1984; Drummond & Kennedy, 1984, see 1.1d2). The mechanism by which the oxygen status of the bacteria is communicated to the NifL product remains a matter for speculation, although it has been shown to be independent of the *fnr* gene product (Hill, 1985).

The sequence of NifL has recently been determined (Kim *et al.*, 1986; Drummond & Wootton, 1987). Drummond and Wootton's analysis of it has revealed a -Cys-X-X-Cys- sequence that is homologous with redox sensitive-sites in disulphide reductases such as thioredoxin, and homologous with metal-binding sites in redox centres of rubredoxins and cupredoxins. Drummond and Wootton (1987) also found a similarity between the amino acid sequences flanking the Cys-containing site and regions of

cytochrome *c* in which the two cysteines are involved in covalent bonding of haem; the amino acids responsible for the fifth and sixth ligands to the iron of the haem in cytochrome *c* (histidine and methionine) are however missing. Drummond and Wootton (1987) suggested that although NifL is not a cytochrome *sensu stricto*, a bound haem might be the basis of the redox sensitivity of NifL.

A more likely alternative involvement of haem protein in oxygen regulation is by way of a specific, oxygen-binding haem protein which, by analogy with other such proteins, would require a histidine (or cysteine) fifth ligand to the iron in the haem. A second alternative involvement of a haem protein would be as an intermediate component of an electron transport chain which might interact with NifL. Such an interaction is consistent with the observation that expression from the *nifH* promoter (the structural operon) is inhibited by 50 per cent at a dissolved O_2 concentration near the apparent Km (100 nM) of the principal terminal oxidase in *K. pneumoniae* (Bergersen *et al.*, 1982).

This chapter reports the results of experiments using an *E. coli* mutant defective in the synthesis of haem, at the first committed step in porphyrin biosynthesis. Strain A1004a (Table 3.1) contains a single lesion (UV-induced) in 5-aminolaevulinic acid (5-ALA) synthetase. The strain is unable to grow on non-fermentable C-sources without 5-ALA, but is able to grow anaerobically and aerobically with glucose as C-source (Haddock and Schairer, 1973) (subject to the growth medium). In order to limit the number of possible mechanisms to consider in explaining the role of oxygen and NifL in regulating nitrogenase synthesis, a plasmid carrying the *K. pneumoniae nif* genes (pRD1, Table 3.1) was introduced into the *Hem* A^- strain and its ability to regulate nitrogenase synthesis in response to low O_2 concentrations tested.

The importance of the stringent response for initiating *nif* derepression is also discussed and preliminary evidence presented that nitrogenase may have a physiological role in regulating its own synthesis under very N-starved conditions, through the effect of inhibitors on its own N_2-fixing activity.

3.2 RESULTS AND DISCUSSION

a) Anaerobic expression of nitrogenase in a Hem A^- *E. coli* (pRD1) transconjugate

The Nif^+ plasmid pRD_1 was used to construct transconjugants of the Hem A^- mutant *E. coli* A1004a and of the wild type Hem^+ *E. coli* A1002 (Table 3.1). Bacterial matings were performed as described in methods and the recipients and transconjugants were tested for C_2H_2-reducing activity in a standard assay procedure (Cannon, 1980). The recipient strains did not reduce C_2H_2 (data not shown; no naturally diazotrophic *E. coli* strain has yet been discovered). Transconjugates of both the Hem A^- and wild-type strains reduced C_2H_2, but the activity in the mutant transconjugant was much greater than in that of the wild-type (Table 3.2).

Anaerobic growth, as well as C_2H_2 reduction, of the wild-type transconjugate A1002 (pRD*1*) was always poor compared with that of the Hem A^- mutant A1004a (pRD*1*) or with any other *E. coli* strain carrying pRD_1. This difference in growth properties was due to an apparent greater sensitivity to the kanamycin needed to retain pRD*1*, suggesting that the two strains are not isogenic as previously thought. Since the addition of 5-ALA restored haem biosynthesis and membrane oxidase activity (Haddock, 1973; Haddock & Schairer, 1973) in the Hem A^- mutant (Table 3.2), the behaviour of cultures of this strain grown with 5-ALA was considered to be equivalent to that of a wild-type (Hem^+) phenotype.

Table 3.2 Effect of the presence of 5-aminolaevulinate (5-ALA) on the haem content and nitrogenase activity of *E. Coli* and of mutant lacking 5-ALA synthetase

Growth of bacteria and measurements of haem, oxidase activity and nitrogenase activity were as described in Methods, except that C_2H_2 reduction was measured in strain A1004a(pRD1) after 18 h growth from a 4-8% inoculum, and in strain A1002(pRD1) after 36 h growth from an 8-16% inoculum. The opitcal density of the cultures of the latter strain were about half those of the cultures of strain A1004a (pRD1). The reversion frequence to $Haem^+$ was less than 2×10^{-7}. nd, not determined.

	Growth without 5-ALA		Growth with 5-ALA	
	A1002 ($HemA^+$)	A1004a($HemA^-$)	A1002($HemA^+$)	A1004a($HemA^-$)
Strains without *nif*				
Haem *b* (pmol haem *b* / mg bacterial protein)	300	<<10	nd	1280
NADH plus D(-) Lactate oxidase activity (nmol oxygen/min/mg membrane protein)	93	1	nd	35
Strains carrying *nif* plasmid				
Acetylene reduction (nmol C_2H_4 min/mg protein)	3.2-3.7	16-21	2.8-3.6	48-50

Table 3.1 *E. coli* strains and plasmids

All *E. coli* strains used were derived from the K12 strain.

Strain or plasmid	Genotype	Source/reference
A1002	*mel*, *ilv*, *lac*I, *met*E	NCIB 11825 (Poole, 1979)
A1004a	*mel*, *ilv*, *lac*I, *met*E, *haem*A	Haddock (1973a, b)
JC5466	*trp*, *his*, *rec*A56, *rps*E	Dixon *et al.* (1977)
	Phenotype	
pRD1	Km^R, Cb^R, Tc^R, His^+, Nif^+	Dixon *et al.* (1976)

Table 3.2 shows that supplementation of anaerobic cultures of the Hem A^- transconjugate with 5-ALA resulted in a two- to three-fold increase in C_2H_2-reducing activity. The presence of nitrogenase was confirmed by an immunoblotting procedure (Fig. 2). Under anaerobic conditions haem is not absolutely required for nitrogenase synthesis or for the process of electron transport to the enzyme in *E. coli*. The rate of anaerobic growth was improved when haem biosynthesis was permitted (Fig. 3.1). This point is worth further discussion.

The biosynthesis of cysteine requires the reduction of sulphite to sulphide (Murphey *et al.*, 1974) which is catalysed by a sirohaem containing reductase, hence cysteine and methionine were made available in growth and derepression media. There is no other known role for a haemoprotein during fermentative metabolism under strictly anaerobic conditions, hence the increased growth rate in cultures of the hem A^- mutant supplemented with 5-ALA is difficult to explain.

3.2b) Does oxygen Inhibit nitrogenase synthesis in the Hem A^- *E. coli* (pRD,) transconjugate?

As expected, aerobic growth of the Hem A^- transconjugate was extremely poor unless 5-ALA was added (Fig. 3.1). The slow growth was probably due

to the lack of hydroperoxidases and of respiratory cytochromes. C_2H_2 was not reduced by these cultures. In neither the presence nor absence of added 5-ALA were the polypeptides of nitrogenase detected by the immunoblotting procedure at the end of aerobic growth (Fig. 3.2). The concentration of fixed N in the medium was that allowing anaerobic derepression of nitrogenase (as for Fig. 3.1).

Nitrogenase synthesis in wild-type *K. pneumoniae* is inhibited by 2μM dissolved oxygen (Hill *et al.*, 1981; Bergersen *et al.*, 1982), this was the lowest concentration of O_2 which can be reliably monitored using a conventional O_2-electrode. Fig. 3.3 shows the effect on the anaerobic growth of A1004a(pRD$_1$) without 5-ALA, of 2μM oxygen. The levels of nitrogenase polypeptides rapidly decreased (Fig. 3.2) and the C_2H_2-reducing activity [14 nmol C_2H_4 produced min^{-1}(mg protein)$^{-1}$ in sample (g) Fig. 3.3] dropped to zero (in sample (h), Figs. 3.2 and 3.3). Since the nitrogenase polypeptides were unstable under this aerobic condition, the effect of O_2 could not be monitored by using the accumulation of nitrogenase as a measure of synthesis. A more direct approach to measuring nitrogenase synthesis after exposure to low O_2 levels was adopted.

In *K. pneumoniae* the rate of nitrogenase polypeptide synthesis has been measured by a pulse-labelling technique in cultures exposed to a constant dissolved O_2 concentration (6μM). This has been used to distinguish mutants (NifL$^-$) that are defective in O_2 regulation of nitrogenase, from the wild type (Hill *et al.*, 1981; Cannon *et al.*, 1985). Therefore rates of nitrogenase polypeptide synthesis were measured in A1004a(pRD$_1$), by pulse-labelling in cultures exposed to anaerobiosis and to a dissolved O_2 concentration of 2μM in an oxystat. When the culture was grown with added 5-ALA (to restore haem biosynthesis), this concentration of O_2 inhibited synthesis of the *nifH*

Fig. 3.1 Effect of 5-ALA (60 μM) on anaerobic (○, ○) and aerobic (□, □) growth in strain A1004a(pRD$_1$)

Cultures were grown in supplemented -NFDM as described in Methods either without (○, □) or with (●, ■) 5-ALA. The nitrogenase content, assessed by immunoblot analysis in samples labelled (a), (b), (c) and (d), is shown in Fig. 3.2.

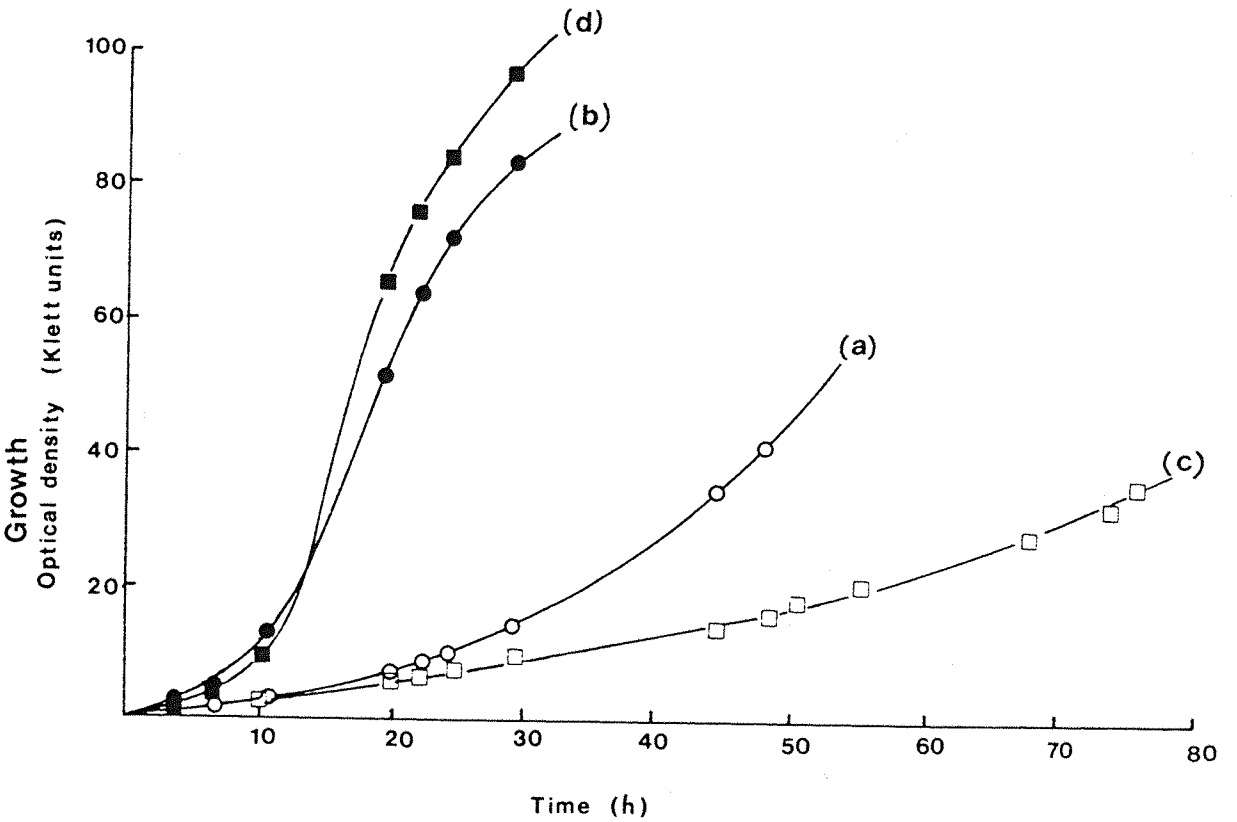


Fig. 3.2 Effect of oxygen on nitrogenase content in A1004a(pRD$_1$) growing without 5-ALA

Two cultures were grown anaerobically in 200 ml of supplemented-NFDM sparged with 1% CO_2 in N_2 (100 ml.min^{-1}). During mid-exponential growth, indicated by the vertical dotted line, air was introduced into the gas supply of one culture (open symbols) to maintain 2-6 μM dissolved O_2 for the subsequent 30 h of incubation. There was negligible O_2 uptake. Samples (10 ml) taken before (g) and after the introduction of air (h, i, j and k) were assayed for nitrogenase content by immunoblot analysis (Fig. 3), and for anaerobic C_2H_2-reducing activity. The latter was 14 nmole C_2H_4 produced.min^{-1}.mg protein^{-1} at (g) and zero at (h).

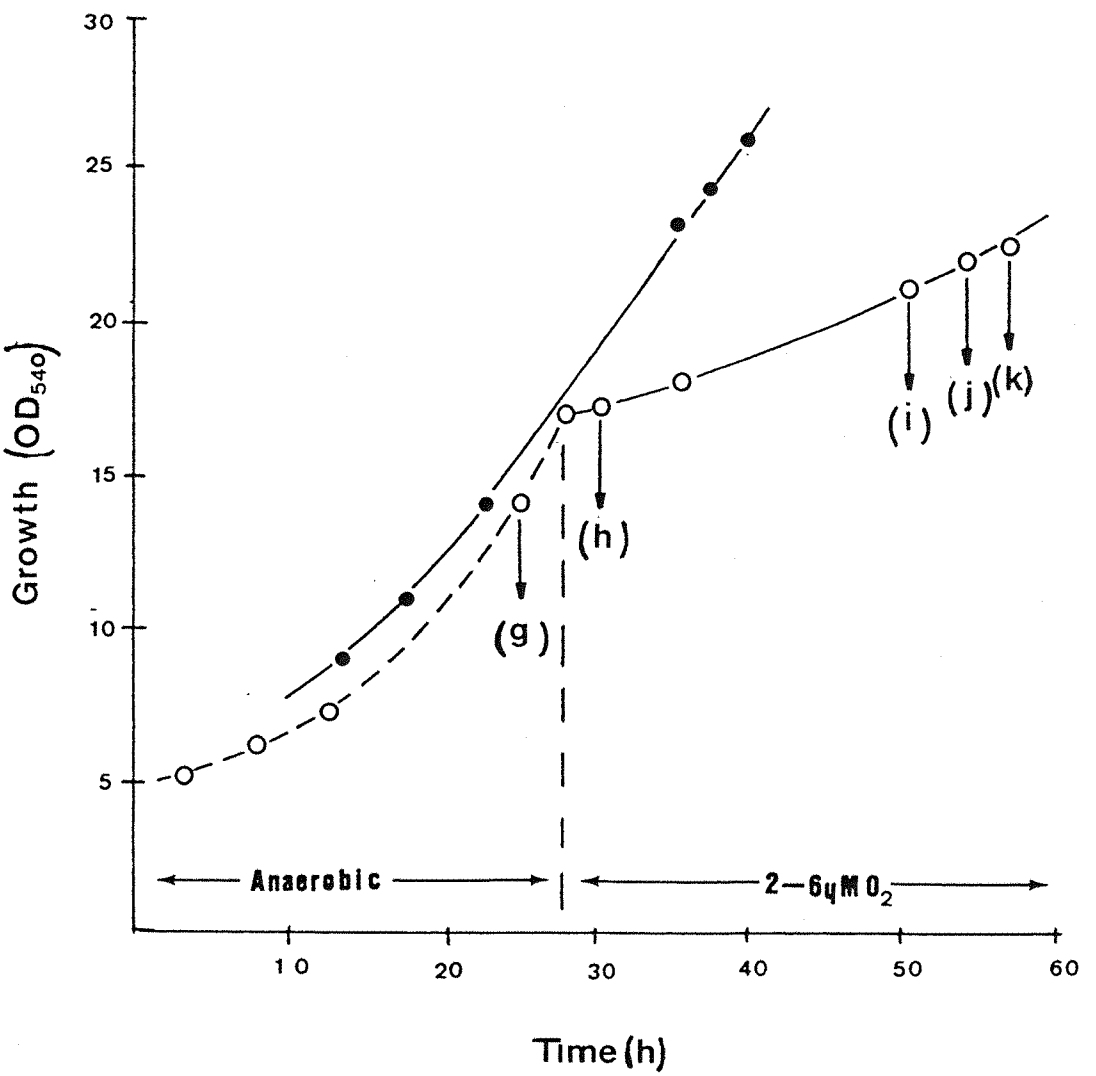
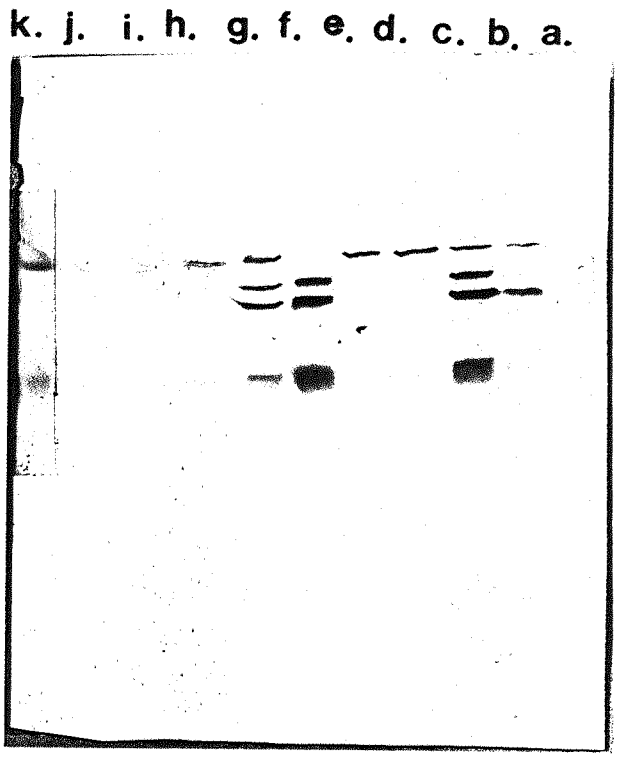


Fig. 3.3 Immunoblot analysis of nitrogenase levels in samples of A1004a(pRD$_1$)

Samples from growth depicted in Fig. 3.1 (samples a, b, c and d) and in Fig. 3.2 (samples g, h, i, j and k) were treated and analysed as described in Methods. Tracks contained 10 μg protein from anaerobic growth (a), anaerobic growth with 5-ALA (b), aerobic growth (c), aerobic growth with 5-ALA (d), anaerobic growth (g) and anaerobic growth followed by 2.5 h (h) and 22.5 h (i) treatment with 2μM-O_2. Bands labelled K, D and H indicate the polypeptides of nitrogenase and CA indicates a Gram negative common antigenic polypeptide (personal communication Dr. D. Nunn).



and *nifK* polypeptides within 10 min (*nifD* polypeptide was not sufficiently resolved to draw any conclusions) (Fig. 3.4a). The level of oxygen chosen (2μM) had no significant effect on the rate of overall protein synthesis (Fig. 3.5a). When 5-ALA was omitted (to prevent haem biosynthesis), the same response to oxygen was seen (Fig. 3.4b). The synthesis of *nifJ* polypeptide (pyruvate: Flavodoxin oxidoreductase), which is inhibited by O_2 (6μM) in *K. pneumoniae* wild-type but not in oxygen constitutive NifL$^-$ mutants (Hill *et al.*, 1981; Cannon *et al.*, 1985) was inhibited by 2μM-O_2 in A1004a(pRD1) both in the presence or absence of added 5-ALA (Fig. 3.4a, b). Thus the inability of strain A1004a(pRD1) to make haem does not alter the sensitivity to O_2 of the mechanism regulating the synthesis of nitrogenase and *nifJ* polypeptide.

During exposure to 2μM-O_2 the synthesis of no other polypeptides besides *nifH*, *K* and *J* was completely inhibited, although the synthesis of two polypeptides (8, 11 in Fig. 3.4a, b) were markedly enhanced regardless of whether or not the cultures were supplemented with 5-ALA (Fig. 3.6). As expected this level of O_2 completely inhibited C_2H_2-reducing activity (Fig. 3.6).

Synthesis of nitrogenase ceased after 90 min in both anaerobic control cultures (Fig. 3.4c) and C_2H_2 reduction in these cultures became non-linear (Fig. 3.6) suggesting inhibition of activity, or a reduction in nitrogenase levels (nett degradation). After 90 min the same two polypeptides (8, 11) which were induced in response to 2μM-O_2 were also induced (to higher levels) in the anaerobic control cultures (data not shown). Prolonged exposure to C_2H_2 (2 hrs) does not normally inhibit activity in the presence of an adequate supply of reductant, however C_2H_2 does compete strongly with N_2 as a substrate for nitrogenase therefore cutting the supply of fixed -N from nitrogen fixation for amino acid biosynthesis. The cultures used in this experiment probably became more N-starved with increasing exposure to C_2H_2.

Fig. 3.4a, b Effect of O_2 on *nif* polypeptide synthesis in A1004a(pRD,)

Cultures were supplemented (Hem^+) (a) or unsupplemented (Hem^-) (b) with 5-ALA, and exposed to either anaerobiosis (——) or 2± 0.6 (SEM), n=16)μM-O_2 (—) as described in Methods. Samples were removed at 10, 40, 70 and 100 min and pulse-labelled with ^{14}C-labelled amino acids for the measurement of the rate of total polypeptide synthesis and of *nif* polypeptide synthesis (see Methods). The microdensitometer traces shown are of autoradiographs of SDS-PAGE of extract prepared from the samples removed after an exposure of 40 min. J, D, K and H show the position of the *nif* polypeptides and 8 and (11) show two unidentified polypeptides, (5) shows the position of EF-Tu.

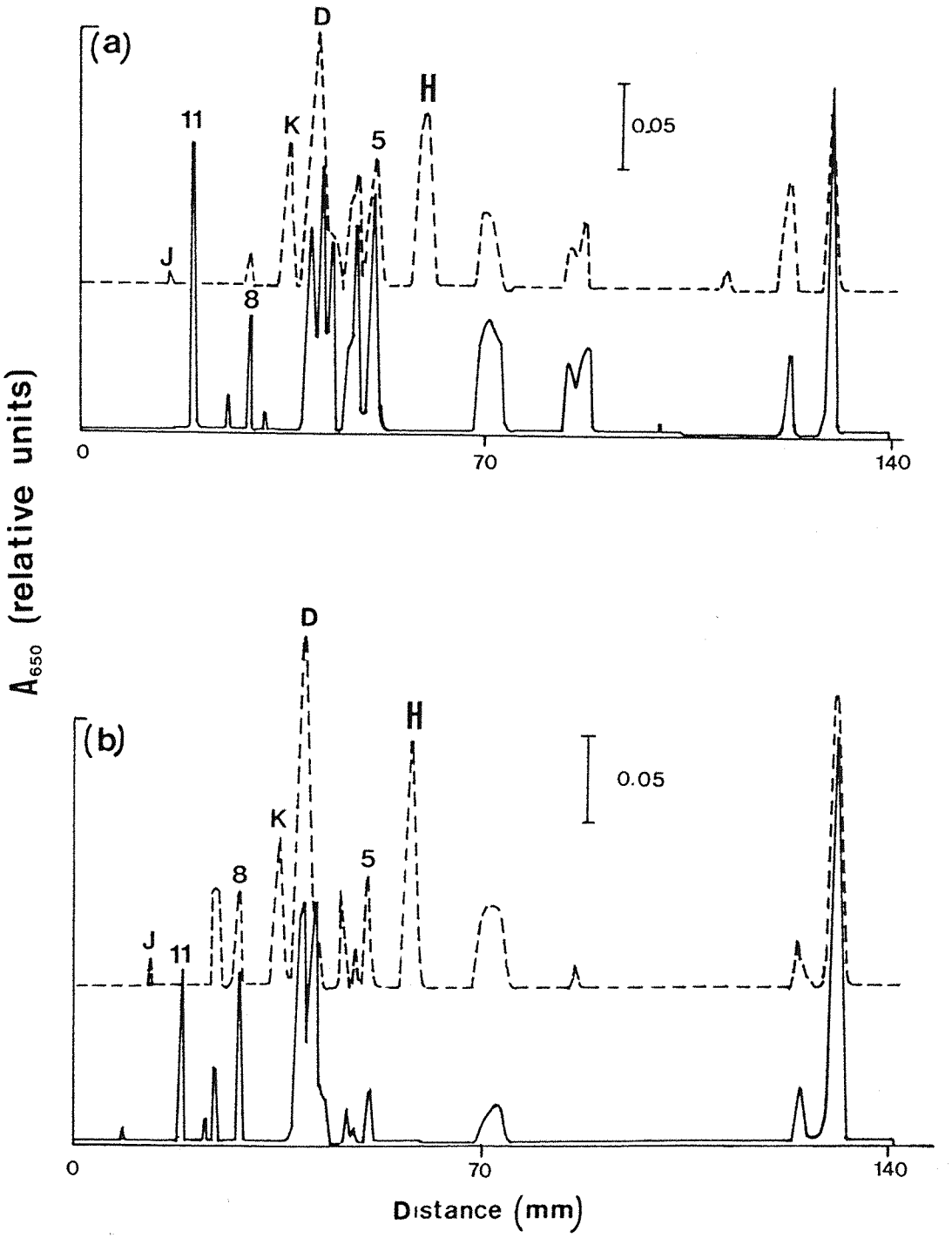


Fig. 3.4c Synthesis of NifH and NifK in the Haem$^+$ anaerobic control culture

The peaks from the microdensitometer trace shown in Fig. 3.4a were integrated for each of the time points at which samples were pulse-labelled. Rates of NifH (●) and NifK (○) polypeptide synthesis were expressed as a proportion of the rate of total protein synthesis.

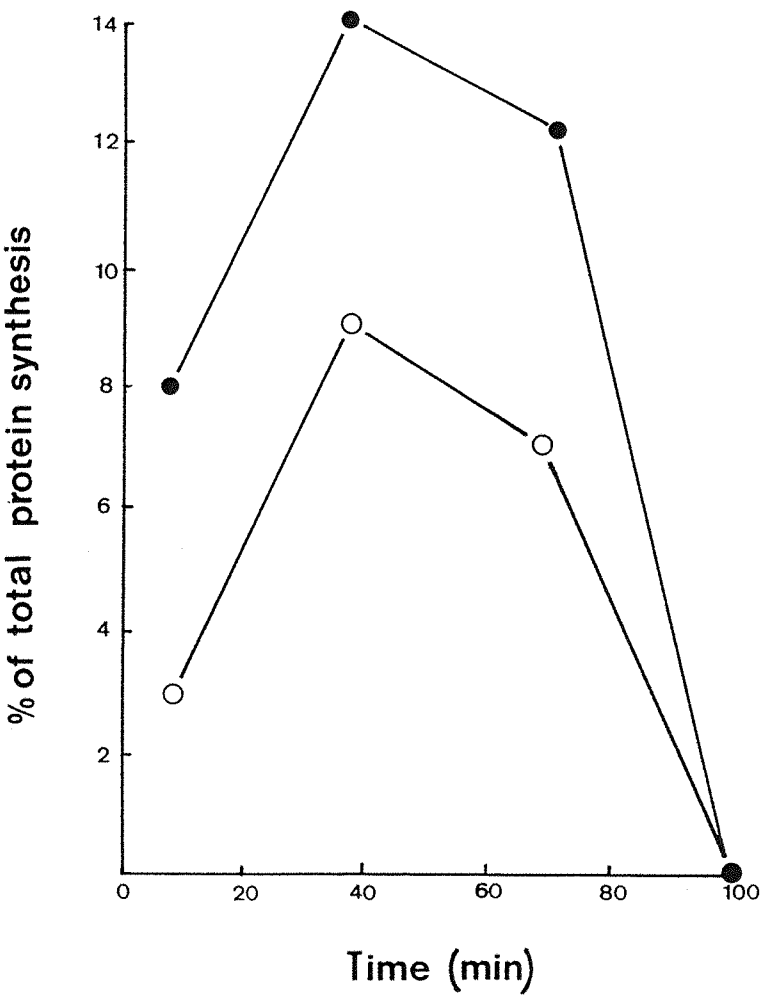


Fig. 3.5a, b Effect of O_2 on the rate of overall protein synthesis

Cultures were supplemented (Haem$^+$) (a) or unsupplemented (Haem$^-$) (b) with 5-ALA, and exposed to either anaerobis (---) or 2±0.6μM-O_2(—). ^{14}C labelled amino acid incorporation into TCA precipitable material was used to measure the overall rate of protein synthesis.

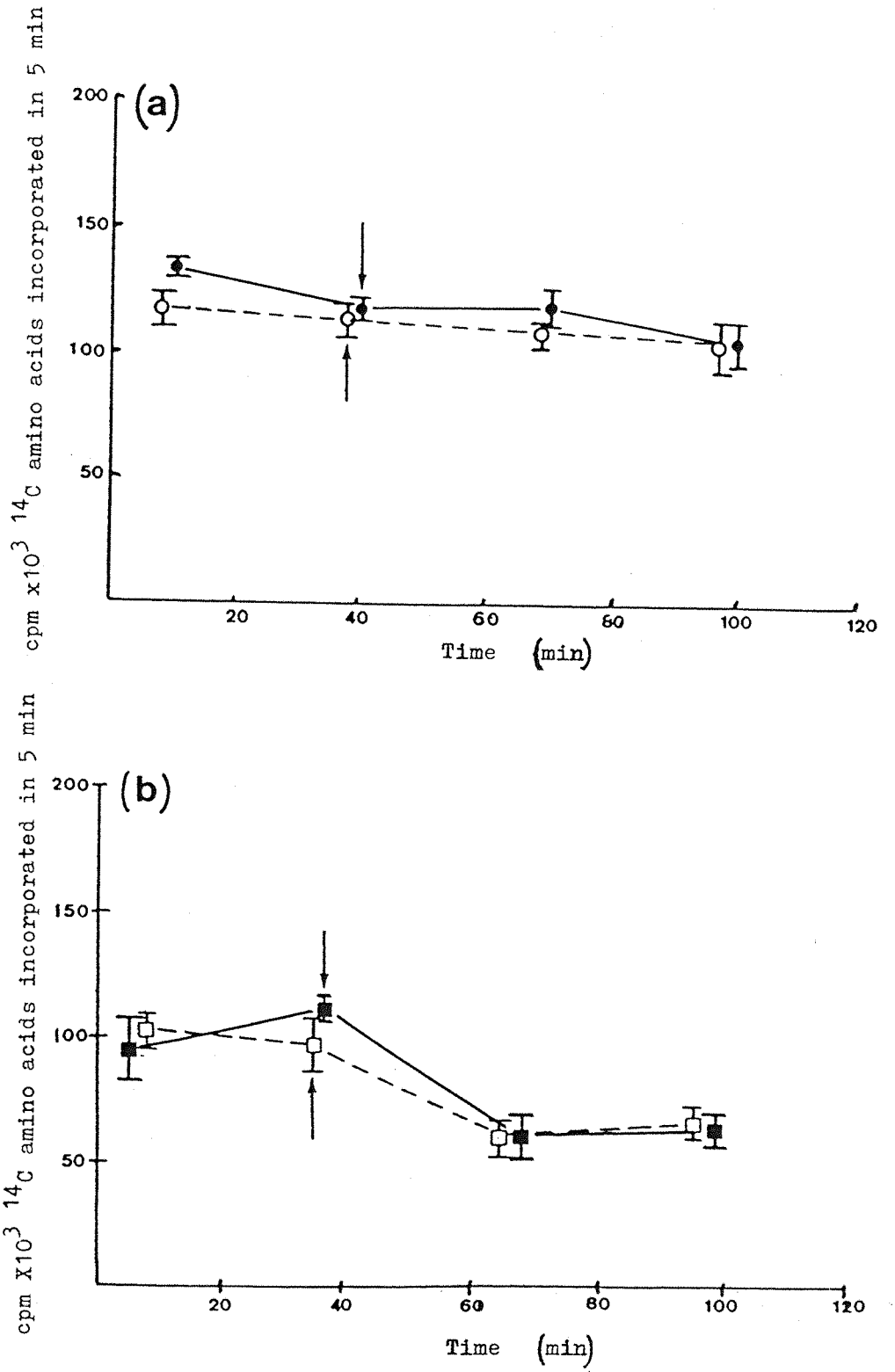
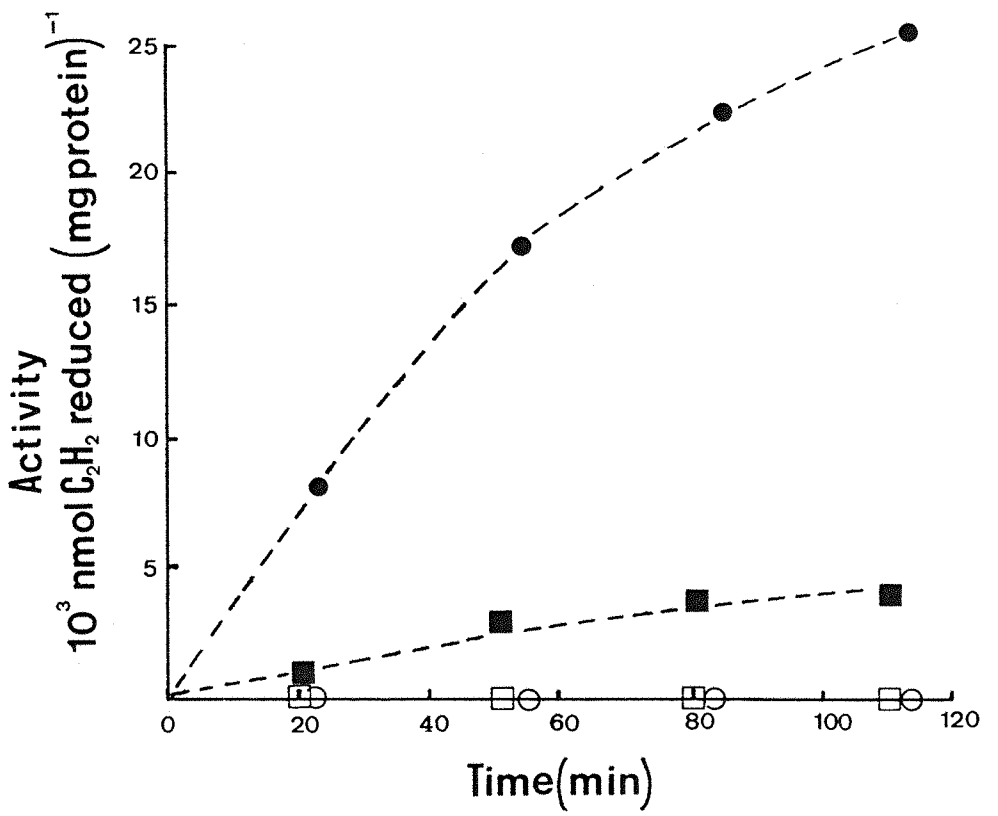


Fig. 3.6 Effect of oxygen on nitrogenase activity

This was measured *in situ* as C_2H_2-reduction in parallel with pulse-labelling experiments as described in Fig. 3.4a, b. ●, anaerobic, haem$^+$; ○, aerobic, haem$^+$; ■, anaerobic, haem$^-$; □, aerobic, haem$^-$.



An attempt was made to establish the identity of some of the other polypeptides detected in the pulse labelling procedure, particularly those enhanced in response to O_2 exposure and potentially N-starvation (8, 11 in Fig. 3.4).

Using information on the relative abundance and molecular weight of *E. coli* polypeptides, the most abundant protein in pulse labels of this type would be expected to be EF-Tu (5 in Fig. 3.4a, b) (Smith and Neidhardt, 1983). A candidate for the identity of polypeptide (8) was stringent factor (the *rel* A product).

3.3 Summary and further discussion

Two separate sets of conclusions were reached in this work. The first relates to the initial aims and concerns the involvement of haem in the O_2-regulation of nitrogenase synthesis; the second to a physiological role for nitrogenase activity in regulating nitrogenase synthesis under very N-starved conditions.

Nitrogenase was derepressed in anaerobic cultures of A1004a(pRD_1) unsupplemented with 5-ALA showing that there is no obligate requirement for haem for *nif* expression and electron transport to nitrogenase. Anaerobic growth without haem biosynthesis was poor compared with when haem biosynthesis was allowed, suggesting that there may be an as yet undescribed role for a cytochrome or haemoprotein during anaerobic fermentative growth.

Oxygen regulation of *nif* expression mediated by the *nifL* product was not lost when bacteria were deprived of haem. It is unlikely that traces of haem, undetected in these experiments satisfied the presumptive requirement of the *nifL* product, but not that for the respiratory chain, particularly since the *nifL* product is made in relatively large amounts (Cannon *et al.*, 1985). Hence the suggestion of Drummond and Wootton (1987) that the *nifL* product might contain a bound haem as the basis of

its 'redox sensitivity' is not supported. The lack of haem proteins renders the constitutively expressed respiratory chain unable to reduce O_2 to H_2O; the redox status of the components of the chain are therefore 'locked' in the reduced state regardless of the presence or absence of O_2. Hence the direct involvement of a functional respiratory chain in the mechanism of O_2-regulation of *nif* expression is also not supported. Similar experiments with hem A^- mutants have subsequently been used to show that the anaerobic regulator *fnr* does not interact with a haemoprotein or the respiratory chain (G. Unden, personal communication, 1988). Like *fnr* it is not known whether a small signal moleclule is required to modulate the activity of NifL. Possibilities for a mechanism of O_2 (redox) sensing could reside in alternative modes of reduction of O_2 or its dismutation. Whether there is a role for nitrogenase itself, or for a component of the electron transport chain to nitrogenase in generating the signal for the prevention of *nif* expression mediated by the *nifA* product remains to be seen, but possible candidates for the transducing protein (*nifL* product or a *nifAL* protein complex) have not yet been excluded.

Following transfer from growth on NH_4^+ to growth on N_2, anaerobic cultures of *K. pneumoniae* show the stringent response characteristic of N-starved organisms (Nair & Eady, 1984). Stringent control is well studied in *E. coli* (Gallant, 1979), in which deprivation of a required amino acid inhibits rRNA synthesis, increases the rate of protein turnover and arrests biosynthetic metabolism (Galland & Lazzarine, 1976). These changes are associated with an expansion in nucleotide polyphosphate pools catalysed by 'stringent factor' an enzyme located on the 50s ribosome, which is activated in response to uncharged $tRNA_s$ occupying the aminoacyl site of the translating ribosome. On N-starvation, N_2 fixing bacteria respond with changes in guanosine polyphosphate levels (Kleiner &

Phillips, 1981) and a decrease in intracellular amino acid levels (Kleiner, 1979). Under these conditions the *nif* genes are derepressed! Stringent control has been implicated in regulation of *nif* expression in *K. pneumoniae* because relaxed mutants (RelA$^-$), unable to synthesise guanosine polyphosphates, are also unable to derepress nitrogenase (Reisenberg *et al.*, 1982). Nair and Eady (1984) concluded that the *relA* product (stringent factor) was important for allowing *nif* expression under N-deficiency. Bacteria are effectively N-starved before *nif* is derepressed. No nett protein synthesis can occur and synthesis of nitrogenase must be at the expense of turnover of N reserves. These are in part mobilised by guanosine polyphosphate synthesis which activates proteases involved in protein turnover (Bridger & Paranchych, 1979). In the pulse labelling experiments described in Fig. 3.4a, b, c, nitrogenase had already been derepressed and its inhibition by O_2 or C_2H_2 probably resulted in increased N-starvation, possibly strengthening the stringent response. Derepression was temporarily stimulated before ceasing completely (Fig. 3.4c) presumably because of critical N-starvation and exhaustion of intracellular reserves. Hill and Kavanagh (1988) have subsequently shown that ongoing N_2 fixation can enhance *nif* gene expression but, on the other hand, that *nif* expression can also be curtailed by N-starvation. Whether this mechanism involves 'stringent factor' or not remains to be seen, but it is interesting to speculate that polypeptide (8) (in Fig. 3.4a, b) which incorporates label under these conditions is the 'stringent factor'.

CHAPTER 4

THE PURIFICATION, CHARACTERISATION AND ROLE OF THE CYTOCHROME *d* TERMINAL OXIDASE COMPLEX OF *KLEBSIELLA PNEUMONIAE* DURING NITROGEN FIXATION

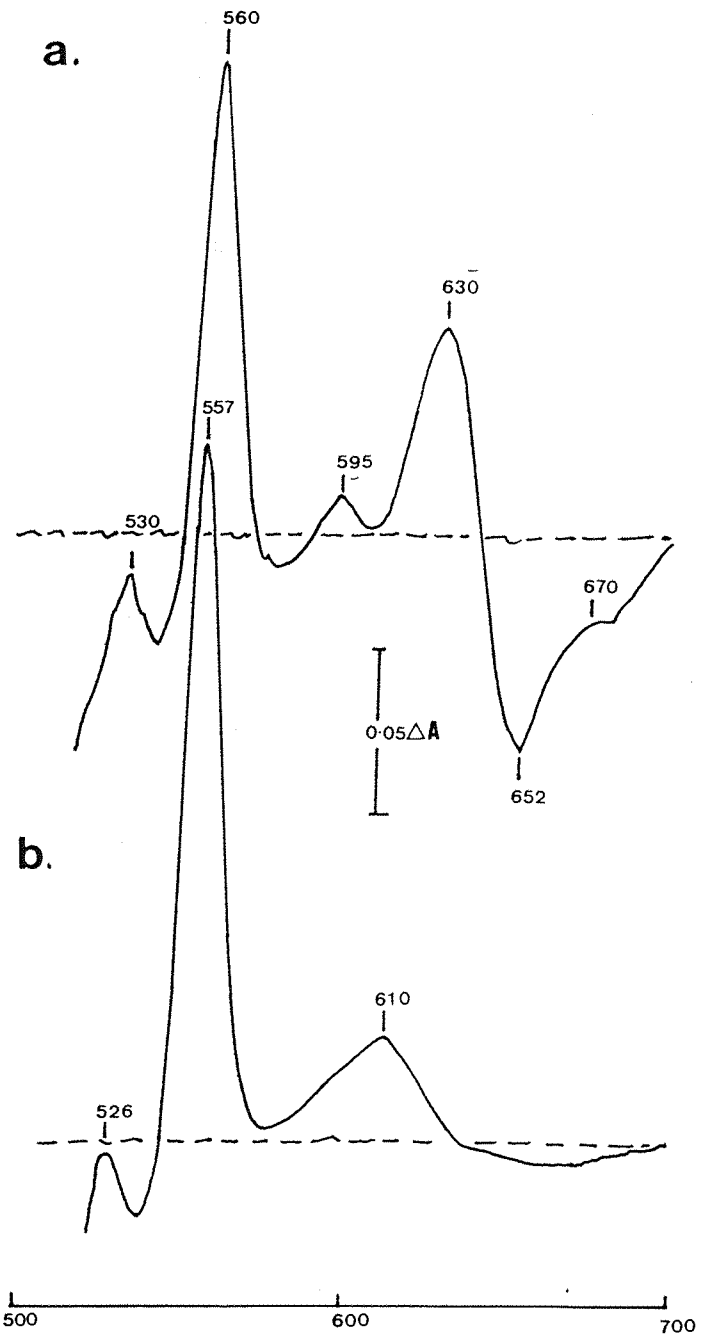
4.1 INTRODUCTION

Nitrogen fixation in the facultative anaerobe *Klebsiella pneumoniae* occurs only in anaerobic or microaerobic conditions. Low oxygen concentrations (optimum about 30 nM) enhance nitrogenase synthesis, activity and efficiency (moles nitrogen fixed per mole of carbon substrate consumed) compared with that occurring during anaerobiosis (Hill, 1976, 1988; Hill *et al.*, 1984). This is presumably due to provision of ATP for nitrogenase activity by way of oxygen-dependent respiration. Higher oxygen concentrations inhibit both synthesis and activity of nitrogenase, with 50 per cent inhibition of *nifH::lac* expression occurring at about 100 nM oxygen which is close to the estimated Ks of the dominant terminal oxidase (about 80 nM, estimated from oxygen consumption measurements with whole bacteria) (Bergersen & Turner, 1979; Bergersen *et al.*, 1982; Hill *et al.*, 1984; 1.3aii, 1.3b). This raises the important question of the nature of the oxidase(s) responsible for respiration at the very low oxygen (microaerobic) concentrations that support more efficient nitrogen fixation, and which is involved in creating conditions suitable for derepression of nitrogenase synthesis.

Very little information is available on the cytochromes of *Klebsiella pneumoniae* and none at all is available for nitrogen-fixing conditions (Harrison, 1972) but, because *K. pneumoniae* is related to *E. coli*, it might be expected that their cytochrome complements would be similar. *E. coli* has two terminal oxidases, an *o*-type oxidase and a cytochrome *d* oxidase (Anraku & Gennis, 1987; Ingledew & Poole, 1984; 1.4). They are similar in oxidising ubiquinol but they differ in their oxygen affinity

Fig. 4.1a, b Absorption spectra of the pyridine haemochrome of membranes from bacteria grown on succinate with tryptone as nitrogen source in oxygen-limited, microaerobic conditions

The protein concentration was 17 mg per ml. a) Dithionite-reduced minus H_2O_2 oxidized difference spectrum; b) Dithionite-reduced minus H_2O_2 oxidized difference spectrum after extraction of haem into alkaline pyridine.



and in the regulation of their synthesis. The cytochrome *o* complex has a lower affinity for oxygen (Km = 200 nM) and is predominant during growth with high oxygen levels, whereas cytochrome *d* has a higher affinity for oxygen (Km = 24 nM) and is predominant at lower oxygen concentrations (Kita *et al.*, 1984a, b; Rice & Hempfling, 1978). Observations using antibodies to cytochrome *d* of *E. coli* suggested that the membranes of *K. pneumoniae* are similar to those of *E. coli* in containing a *d*-type oxidase (Kranz & Gennis, 1985).

This chapter shows that the sole oxidase detectable during microaerobic nitrogen fixation is a *d*-type oxidase. This has been purified and characterised, and its exceptionally high affinity for oxygen (measured with leghaemoglobin) shown to be compatible with a critical role in supporting microaerobic nitrogen fixation. It is the first example of a high affinity oxidase to be isolated from a natural diazotroph, and is only the second class III oxidase to be studied in any detail (see Anraku & Gennis, 1987).

4.2 RESULTS AND DISCUSSION

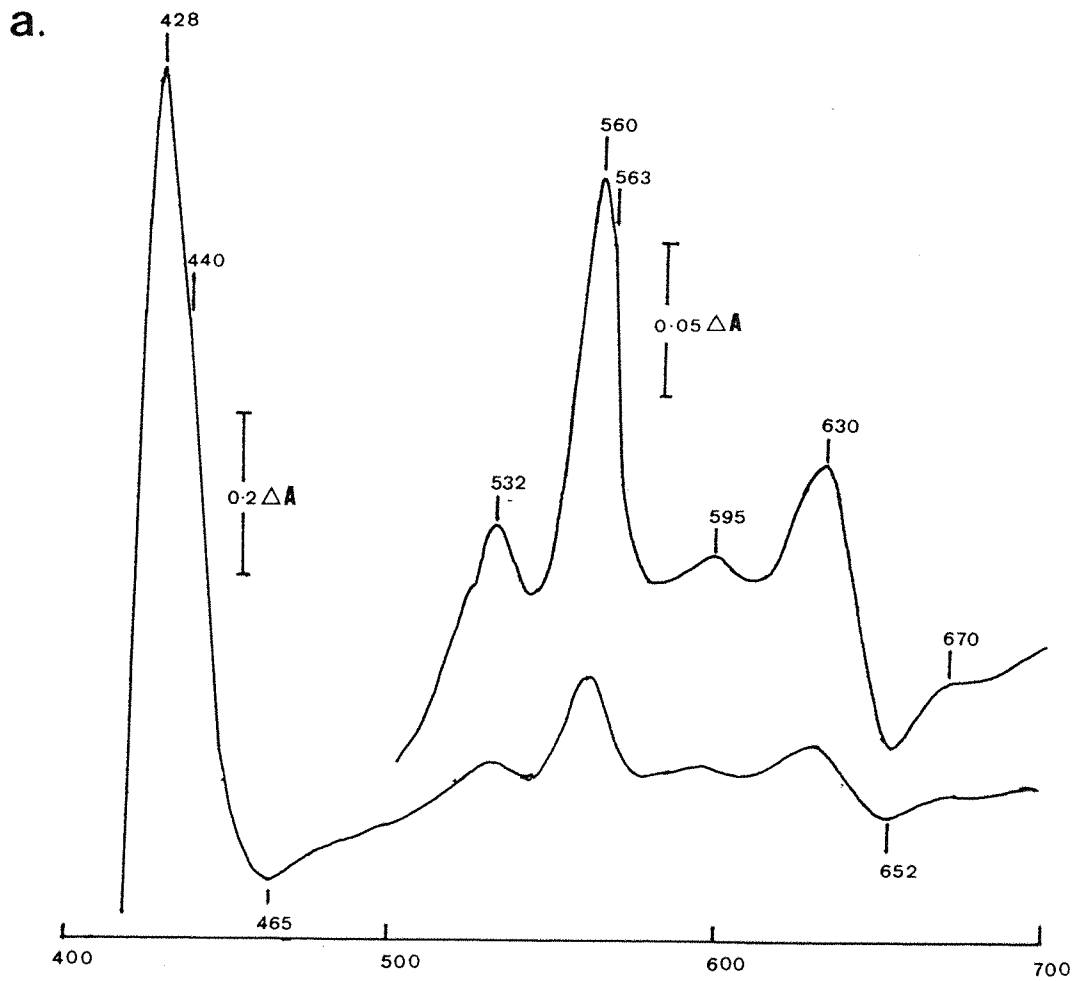
a) The cytochromes of *K. pneumoniae*

Spectra of pyridine haemochromes prepared from membrane fractions (Fig. 4.1a, b) showed peaks characteristic of haem *b* (557 nm) and haem *d* (Chlorin) (Barrett, 1965). No haem *c* or haem *a* was detected. Very low concentrations of cytochrome *b* were detected in soluble fractions, but these were not investigated further.

The spectra presented in Figs. 4.2 - 4.4 confirm that the only cytochromes in membranes of *K. pneumoniae* were *b*- and *d*-type cytochromes. Fig. 4.2a shows the spectra of membranes of bacteria grown in microaerobic conditions (oxygen-limited) with succinate, and tryptone as the nitrogen source. In these conditions *b*-type cytochromes (absorbing at 557-562 nm) and cytochrome *d* (peak at 630 nm) were

Fig. 4.2a, b, c Absorption spectra of membranes from bacteria grown on succinate with tryptone as nitrogen source in oxygen-limited, microaerobic conditions.

The protein concentration was 11.3 mg per ml. a) Dithionite-reduced minus persulphate-oxidised difference spectrum; b) (Reduced plus CO) minus reduced difference spectrum (after sparging with CO for 30s); c) after sparging with CO for 2 min.



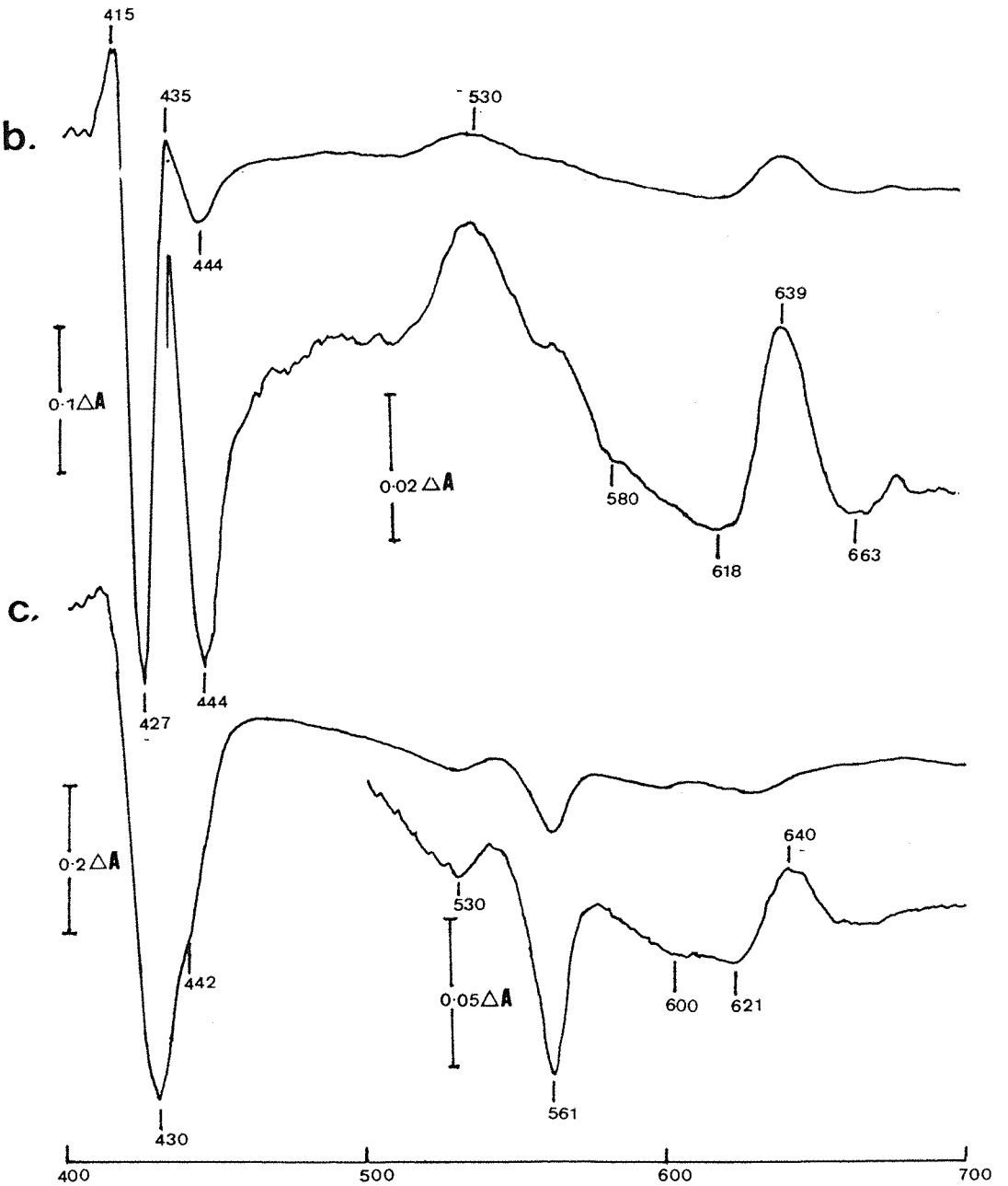
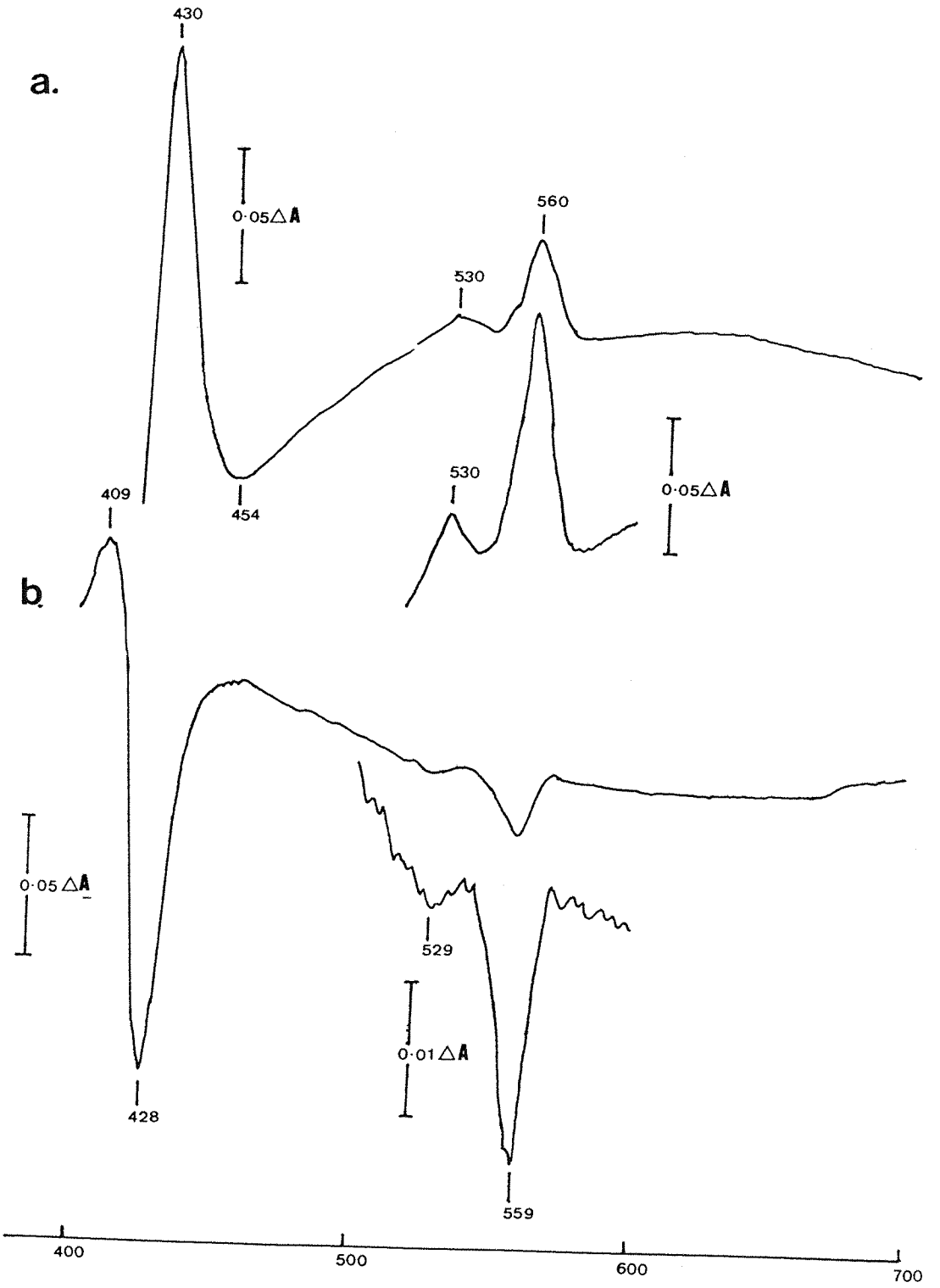

b.
c.
415
435
530
444
639
0·1△A
0·02△A
580
618
663
427
444
0·2△A
530
640
442
0·05△A
600
621
561
430
400
500
600
700

Fig. 4.3a, b <u>Absorption spectra of membranes from bacteria grown on glycerol with tryptone as nitrogen source in nitrogen-limited conditions (oxygen concentration, 10-20 uM)</u>

The protein concentration was 6.2 mg per ml. a) Dithionite-reduced minus persulphate-oxidised difference spectrum; b) (Reduced plus CO) minus reduced difference spectrum after sparging with CO for 2 min.



present. In the absence of any haem *a* the peak at 595 nm almost certainly corresponds to the cytochrome b_{595} component of the cytochrome *d* oxidase complex of *E. coli* (Lorence *et al.*, 1986; Poole, 1988; 1.4). Figs. 4.2b and 4.2c show that some of the *b*-type cytochrome (troughs at about 430 nm and 560 nm) and the cytochrome *d* (trough at about 620 nm) were able to react with CO, indicating the presence of some cytochrome *o* oxidase (an oxidase containing haem *b*).

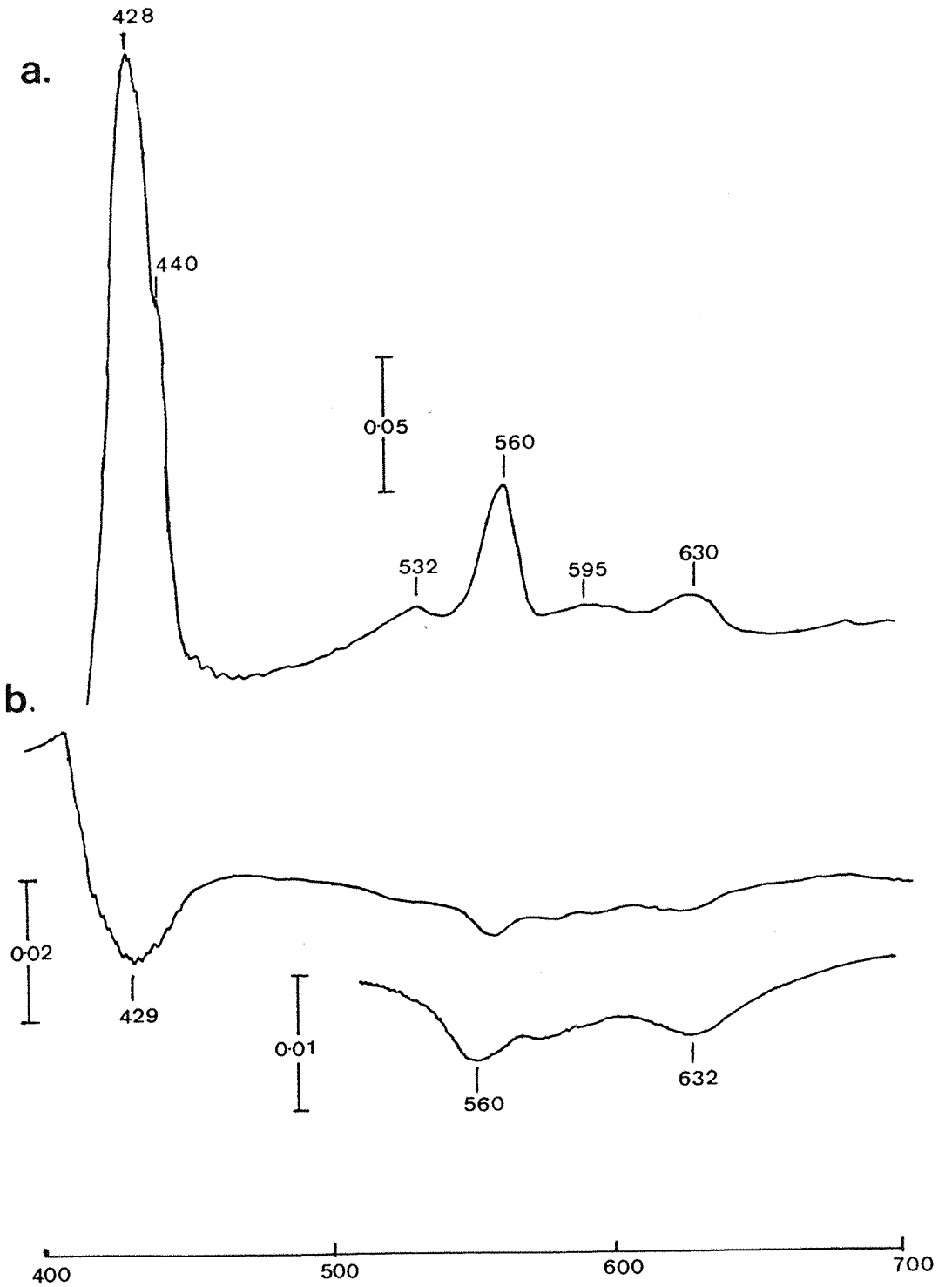
The reaction of the various cytochromes with CO was time / concentration dependent, cytochrome *o* reacting most rapidly (Fig. 4.2b). The reaction of cytochrome *d* oxidase with CO is more complex. In *E. coli* two separate components of the oxidase, cytochrome b_{595} and cytochrome *d* both react with CO and in certain regions of the spectrum (580-620 nm) their signals overlap (Lorence, 1985). This gives rise to the complex spectra seen in Fig. 4.2b, c. These spectra were very similar to those obtained with purified cytochrome *d* complex (see later), except that the trough due to cytochrome *o* at 427 nm (Fig. 4.2b) was absent from the spectrum of the pure complex (Fig. 4.10b).

The spectra shown in Fig. 4.3a, b demonstrate that cytochrome *d* was absent during growth in aerobic conditions (oxygen-excess) on glycerol with tryptone as the limiting nutrient. In these growth conditions, the only CO-reactive species was a *b*-type cytochrome shown by a trough in the CO difference spectrum at 428 nm and 559 nm, presumably cytochrome *o*. This confirms earlier observations with *E. coli* where cytochrome *o* predominates when cells are grown at high O_2 levels in batch cultures (Kranz *et al.*, 1984) and continuous culture (Rice & Hempfling, 1978).

Fig 4.4 shows the spectra of membranes from bacteria grown on glucose with dinitrogen as nitrogen source and oxygen as the growth-limiting nutrient, which indicate that in these microaerobic conditions cytochromes *b* and *d* were present. The spectrum shown in Fig. 4.4b is not

Fig. 4.4a, b **Absorption spectra of membranes from nitrogen-fixing bacteria grown on glucose in oxygen-limited, microaerobic conditions (oxygen concentration less than 1 μM)**

The protein concentration was 18 mg ml^{-1}. a) Dithionite-reduced minus persulphate-oxidised difference spectrum; b) (Reduced plus CO)-minus-(reduced) difference spectrum after sparging with CO for 2 min.



characteristic of cytochrome *o*, but is probably due to the CO reactivity of some of the haem *b* of the *d*-type oxidase, as shown in spectra of the pure protein (Figs. 4.9 and 4.10). If the spectrum were assumed to be due to the presence of cytochrome *o* then its content would be 6 nmol (mg protein)$^{-1}$, which is about 10 per cent of the cytochrome *d* present and less than 2 per cent of the cytochrome *o* present in any other growth conditions.

These results indicate that *K. pneumoniae* is very similar to *E. coli* in having two potential oxidases, cytochrome *o* and cytochrome *d*, the relative amounts being determined by the growth conditions.

Table 4.1 summarises the results of a broader survey of the effect of growth conditions on synthesis of the cytochromes of *K. pneumoniae*. Cytochromes were quantified from spectra using *E. coli* extinction coefficients. It was evident that in all anaerobic conditions the major potential oxidase was cytochrome *d* oxidase; this was regardless of the nitrogen source (dinitrogen, ammonia or tryptone). It is possible that cytochrome *o* was repressed by glucose as is the case in *E. coli* for respiratory enzymes subject to catabolite repression such as succinate dehydrogenase (Guest, 1981); however there is no direct evidence for this at present. By contrast cytochrome *d* synthesis was completely repressed in the presence of excess oxygen (10 μM-O_2) as shown previously with the oxidase of *Klebsiella aerogenes* (Moss, 1956; Harrison, 1972) and of *E. coli* (Ingledew & Poole, 1984). In the present work with *K. pneumoniae*, both oxidases were only present together during respiratory growth in oxygen-limited conditions with glycerol or succinate, when the total cytochrome content was also at a maximum.

Table 4.1 The effect of growth conditions on synthesis of cytochromes in continuous culture

K. pneumoniae was grown as described in methods with a dilution rate (D) of 0.15 h^{-1}. The glucose concentration was 0.5% except for growth conditions 2 and 11, when it was 2%. The concentration of ammonium chloride in 3 and 7 was 6 mM. The concentration of fumarate in 4 was 30 mM. The concentration of tryptone was 0.5 gl^{-1} in condition 5, 0.7 gl^{-1} in 9 and 11, and 1 gl^{-1} in 8 and 10.

Cytochrome concentrations in membranes were measured from difference spectra of the sort illustrated in Figs. 4.2-4.4; they are expressed as pmol/mg membrane protein. The values presented for cytochrome *b* are total values including the cytochrome *o*. Although the results presented here are from single steady state cultures, each growth state was repeated at least once and the results were always within 10% of those recorded here. The lowest concentration of the cytochromes that could be determined with confidence was 10 pmol/mg membrane protein. In microaerobic conditions (oxygen-limited) the oxygen concentration was below the limit of detection by the oxygen electrode (1 μM). In aerobic conditions the oxygen concentrations were 10-200 μM. All cultures grown with dinitrogen as sole nitrogen source were shown to contain *nif* polypeptides as detected by immunoassay. In addition to these cultures, microaerobic cultures with glycerol as carbon source and tryptone as nitrogen source also had low levels of *nif* polypeptides; this is indicated by +/- in the Table. n.d., less than 10 pmol cytochrome (mg membrane protein)$^{-1}$.

	Carbon source	Oxygen status	*nif* expression	Growth Limitation	Nitrogen source	Cytochrome concn. (pmol/mg protein) Cyt b	Cyt *o*	Cyt *d*
1.	Glucose	anaerobic	+	carbon	dinitrogen	120	nd	60
2.	Glucose	anaerobic	+	nitrogen	dinotrogen	110	nd	40
3.	Glucose	anaerobic	-	carbon	ammonia	100	nd	40
4.	Glucose + fumarate	anaerobic	+	carbon	dinitrogen	160	nd	70
5.	Glucose	anaerobic	-	carbon	tryptone	110	nd	50
6.	Glucose	microaerobic	+	oxygen	dinitrogen	130	nd	60
7.	Glucose	microaerobic	-	oxygen	ammonia	150	nd	60
8.	Glycerol	microaerobic	+	oxygen	tryptone	560	180	170
9.	Succinate	microaerobic	-	oxygen	tryptone	640	430	390
10.	Glycerol	aerobic	-	nitrogen	tryptone	360	220	nd
11.	Succinate	aerobic	-	nitrogen	tryptone	500	290	nd

The pattern of oxidase expression described above was confirmed using the difference between cytochrome *d* and cytochrome *o* sensitivity to inhibition by CN^- as an indicator of oxidase type in the membrane (see 1.4, Table 1.5).

The NADH oxidase activity of membranes from growth states 1-7 (Table 4.1) were insensitive to inhibition by KCN (I_{50} = 1 mM); in *E. coli* a characteristic of cytochrome *d* (Anraku & Gennis, 1987). In contrast, membranes which contained only cytochrome *o* (10, 11) were extremely sensitive to inhibition to KCN (I_{50} = 50 μM) while those which contained both oxidases (8, 9) showed a mixed inhibition (data not shown).

The only oxidase detected during nitrogen fixation was the *d*-type oxidase. This was true for all nutrient limitations tested (nitrogen, carbon or oxygen) in which the main energy source for growth was fermentation (anaerobic) or fermentation with microaerobic respiration. Expression of cytochrome *d* was increased seven-fold during microaerobic growth with succinate, a phenomenon utilised in the isolation of the enzyme (see later).

These results identify the oxidase responsible for the O_2 uptake activity reported in N_2 fixing *K. pneumoniae* by Bergersen and Turner (1979) and suggest that the nitrogen fixation occurring under microaerobic conditions may be supported by way of a respiratory chain involving cytochrome *d* oxidase. In order to investigate this possibility further, the cytochrome *d* oxidase complex was purified and characterised with respect to its oxygen affinity.

4.2b) Nitrogenase expression during growth with an aerobic metabolism

It was shown in the last section that all N_2 fixing cultures expressed cytochrome *d*. The nitrogenase levels of bacteria in cultures 1-11 (Table 4.1) were measured to ascertain whether the reverse statement was true;

i.e. do all cultures which express cytochrome *d* (and are N-limited) express *nif*. Nitrogenase levels were measured by immunoblot analysis and by ELISA which utilised a monoclonal antibody specific for Kp1. Nitrogenase expression is shown by a (+) or (-) in Table 4.1. Nitrogenase accounted for 10 per cent of the soluble protein in growth states (1, 2 and 4); 2.5 per cent in (6) and 0.5 per cent in (8). Surprisingly, although nitrogenase was expressed at a low level during microaerobic growth with glycerol (8), it was not expressed under the same conditions when succinate replaced glycerol as carbon source. Since *K. pneumoniae* can (unlike *E. coli*) ferment glycerol (Johnson & Levine, 1985), the requirement for a fermentative, rather than an obligate aerobic metabolism for *nif* expression cannot be ruled out.

4.2c) Purification of cytochrome *d* oxidase

When grown on succinate under oxygen-deficient conditions, the cytochrome *d* complex constituted about 10 per cent of the membrane protein. This was extracted and purified as described in Methods (2.2c). Bacterial membranes were isolated and washed with buffer containing 0.5 M KCl, this removed about 20 per cent of the total cytochrome *b* while leaving all the cytochrome *d* in the membranes. Extraction of these washed membranes with 0.2 per cent (w/v) deoxycholate removed little cytochrome *d* but increased its specific content by 1.5 fold. This step was particularly useful because it led to selective release of D-lactate dehydrogenase and cytochrome b-556 (see Appendix 1), both of which could be readily purified by other techniques. Solubilisation of the cytochrome *d* from the resulting enriched membranes was then achieved with the zwitterionic sulphobetaine detergent Zwittergent 3-12 as described by Miller and Gennis (1983), whose procedure forms the basis of this purification method. The results of a representative purification are summarised in Table 4.2. The elution

Fig. 4.5a Ion exchange chromatography of cytochrome *d* oxidase complex on DEAE-Sepharose FF column

Bacterial membranes were solubilised in 2.5 ml buffer B containing 60 mM Zwittergent 3-12 and 78 ml adsorbed to a 2.5*15 cm column of DEAE-Sepharose FF. The column was developed with a linear gradient from 150 to 300 mM KCl in 800 ml of buffer B containing 6 mM zwittergent 3-12. Cytochrome *d* oxidase activity was measured in column fractions using the instant oxidase test described in Methods (2.2e). ⊢——⊣ = pooled fractions.

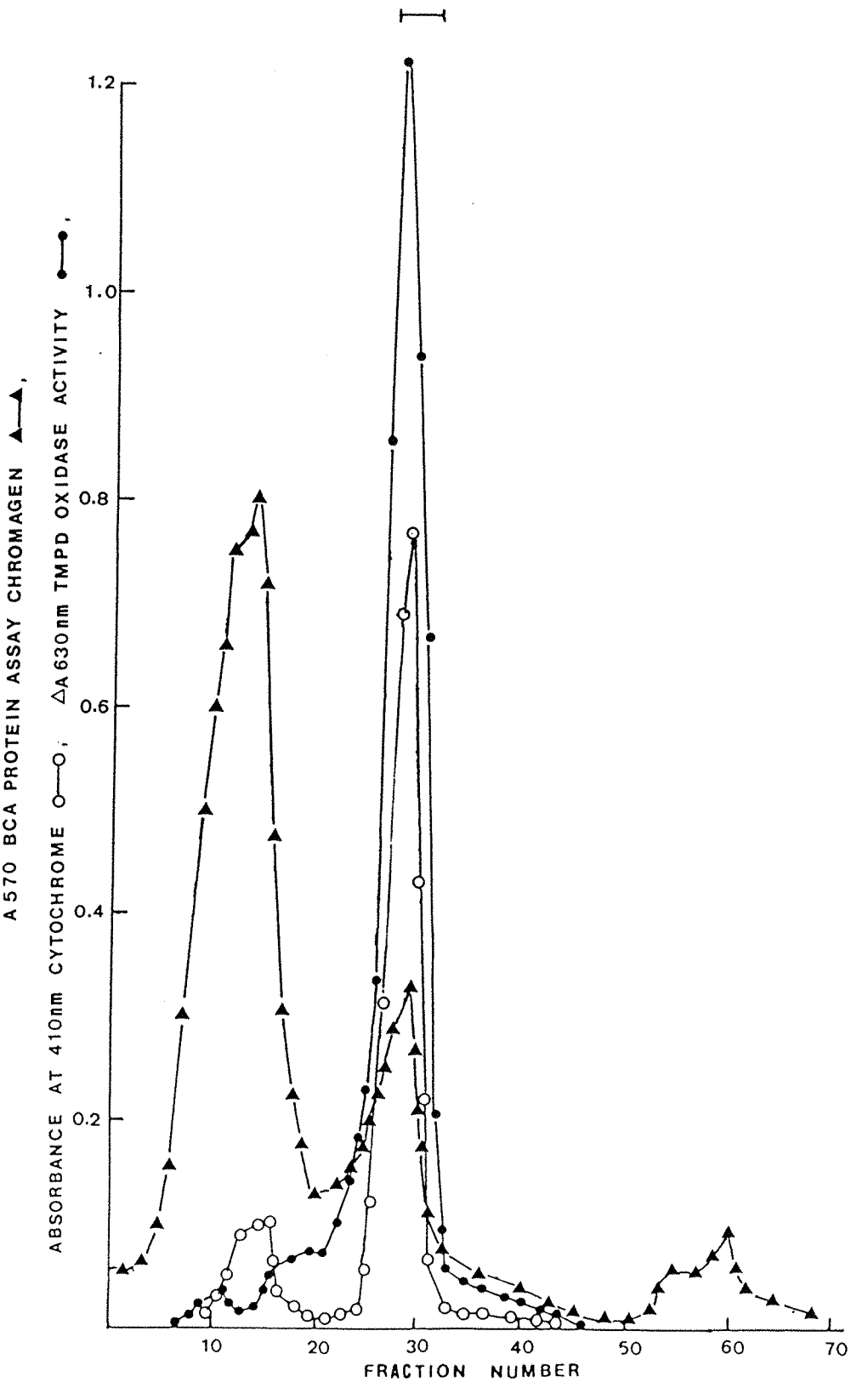
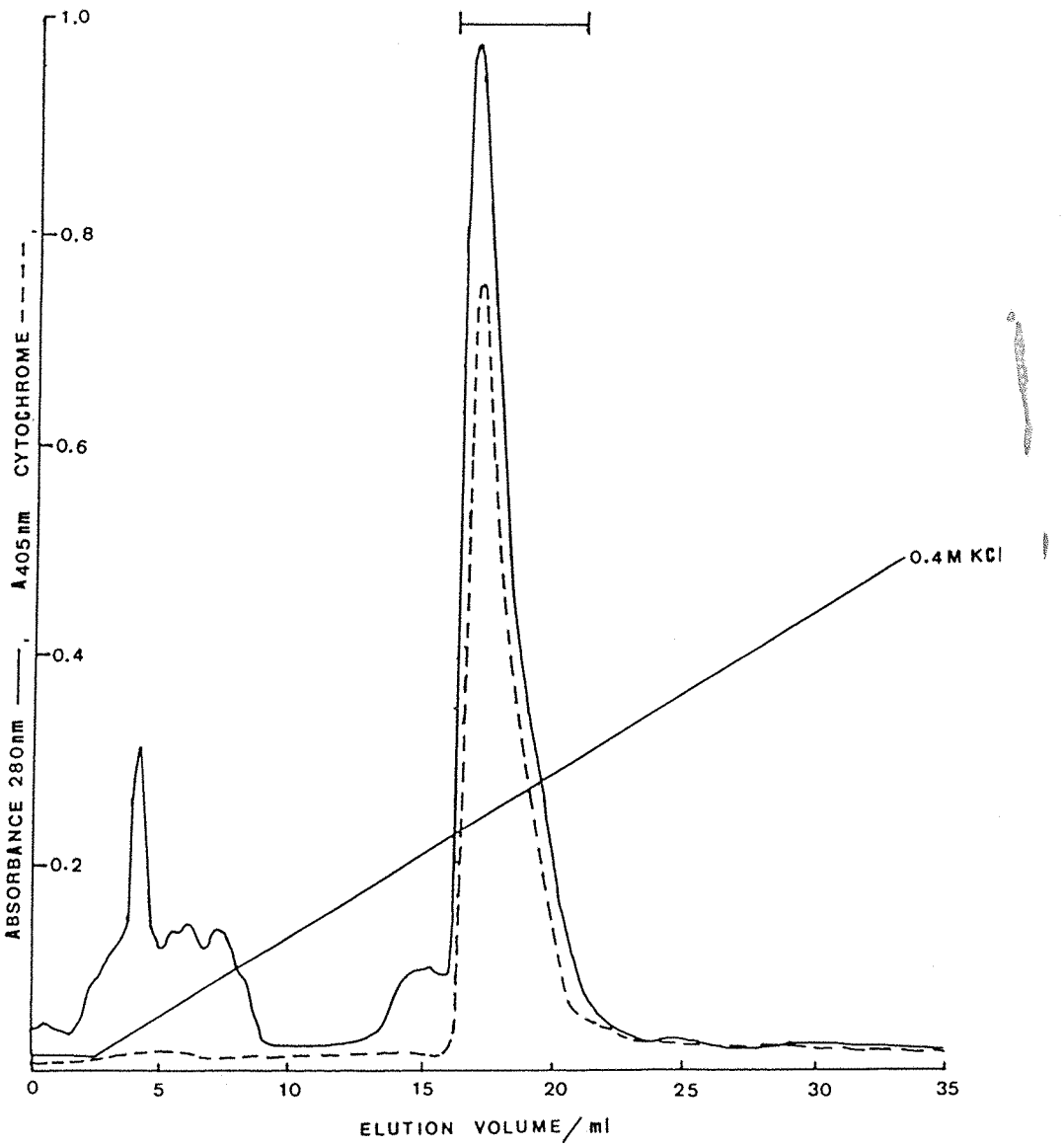


Fig. 4.5b Rechromatography of cytochrome *d* on Fplc Mono Q

Pooled fractions from the DEAE-column were desalted and applied to a 1 ml Mono Q column (HR 5/5) which was developed with a linear KCl gradient as described in methods. |——| = pooled fractions.



profile after ion exchange chromatography of the solubilised membrane extract on DEAE-sepharose FF is shown in Fig. 4.5a. Two haem containing peaks were resolved. The first and smaller contained a b-type cytochrome (b_{556}) which had no oxidase activity (TMPD or Ubiquinol). The second and larger had a high specific oxygen uptake activity and high haem *b* and haem *d* content. Fractions from this peak were pooled and concentrated before chromatography on MONO Q (Fig. 4.5b).

Table 4.2 Purification of the cytochrome *d* oxidase complex

The method is described in the Methods section (2.2c). The cytochrome *b*-558 component was determined from the reduced minus oxidised difference spectrum, using the extinction coefficient of 22 mM^{-1} cm^{-1} (560-580 nm) (Green *et al.*, 1986); the cytochrome *d* component was determined from the reduced minus oxygenated difference spectrum, using the extinction coefficient of 7.4 $mM^{-1}cm^{-1}$ (628-607 nm) (Lorence *et al.*, 1986). The values given for the membranes are prior to detergent washing. The puriication factor presented is based on specific cytochrome *d* content. When based on specific oxidase activity measured in 0.05% Triton X-100 using ubiquinol-1 as substrate, the purification factor was 5.6 overall, presumably due to loss of some activity during purification.

Purification step	Protein (mg)	Cytochrome content (nmol/mg) Cyt *b*-558	Cyt *d*	Abs_{412nm}/Abs_{280}	Purification	Yield (%)
Membranes	638	1.7	2.0	-	1	100
Solubilised membranes	224	3.7	4.8	0.12	2.5	87
DEAE-Sepharose	27	10.4	13.3	0.54	6.8	29
Mono-Q	6	15.6	19.3	0.92	9.9	9

The overall yield was about 10 per cent and the increase in purity about 10-fold on the basis of specific cytochrome *d* content. The complex could not be purified further by any methods attempted and the oxidase eluted as a single symmetrical peak during FPLC gel filtration on Superose-12 (Fig. 4.5c). On the basis of this, and of SDS-PAGE, and

spectral analysis ($Abs_{412\ nm}/Abs_{280\ nm}$ = 0.92), it was concluded that the oxidase complex was at least 95 per cent pure.

4.2d) The relative molecular mass of components of the purified oxidase complex

SDS-PAGE showed that the complex consisted of two polypeptides, designated I and II (Fig. 4.6a and b). The larger subunit (I) had an apparent Mr of 52K. As found with the enzyme from *E. coli* (Miller & Gennis, 1983), the apparent Mr of subunit II showed a dependence on the gel density during electrophoresis. To resolve this problem, the Mr was measured from gels with a range (7.5-15%) of acrylamide concentrations and an averaged Mr determined by Ferguson analysis (Fig. 4.7a, b) (Hendrich & Smith, 1978) This gave Mr values for the two subunits of 52K and 36K.

More recently, the molecular weight of the two subunits from *E. coli* has been deduced from the DNA sequence of the cloned *cyd* operon, to be 58K and 43K (Green *et al.*, 1988). The aggregation state of the dissociated subunits was very temperature-sensitive, 2 min. heating (at 70°C) during sample preparation for SDS-PAGE resulting in complete aggregation of subunit I and its retention at the gel boundary. Similar temperature-sensitive aggregation has been reported for the γ subunit, (Nar I, another hydrophobic cytochrome) of membrane-bound nitrate reductase from *E. coli* (De Moss, unpublished observation). Boiling samples for 2 min. prevented either subunit from entering the gel. Often a larger polypeptide was seen (Mr 80K) as a faint band. This was absent when the subunits were separated by preparative SDS-PAGE and then run separately. If the subunits were mixed, this band was once more seen, suggesting that it was due to reassociation of the subunits during electrophoresis.

Fig. 4.5c Analytical gel filtration of cytochrome *d* oxidase complex

Oxidase protein (0.5 mg) was loaded in 0.5 ml onto a fplc superox 12 column and eluted with buffer *B* containing 4 mM zwittergent 3-12 at a flow rate of 0.5 ml/min.

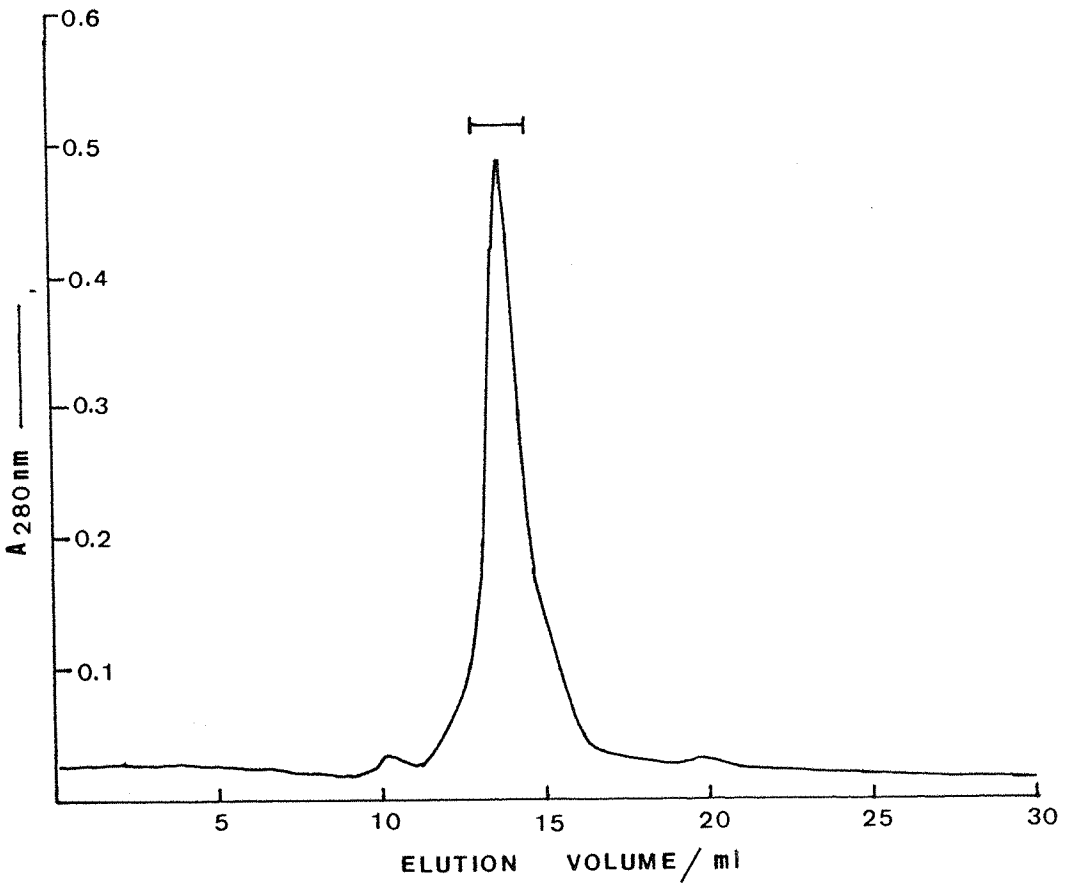


Fig. 4.6a, b, c SDS-PAGE of the purified cytochrome *d* complex

The 12% acrylamide gels below show samples from a representative purification of cytochrome *d* oxidase: Track 1) 23 μg bacterial membrane protein; 2) 10 μg zwittergent 3-12 membrane extract; 3) 5 μg oxidase DEAE peak; 4) 5 μg oxidase Mono Q peak; 5) 2 μg oxidase superose-12 peak. a) Detection with silver stain; b) staining with coomassie blue; c) β-mercaptoethanol omitted during sample preparation, staining for haem by the method of Thomas *et al.* (1976).

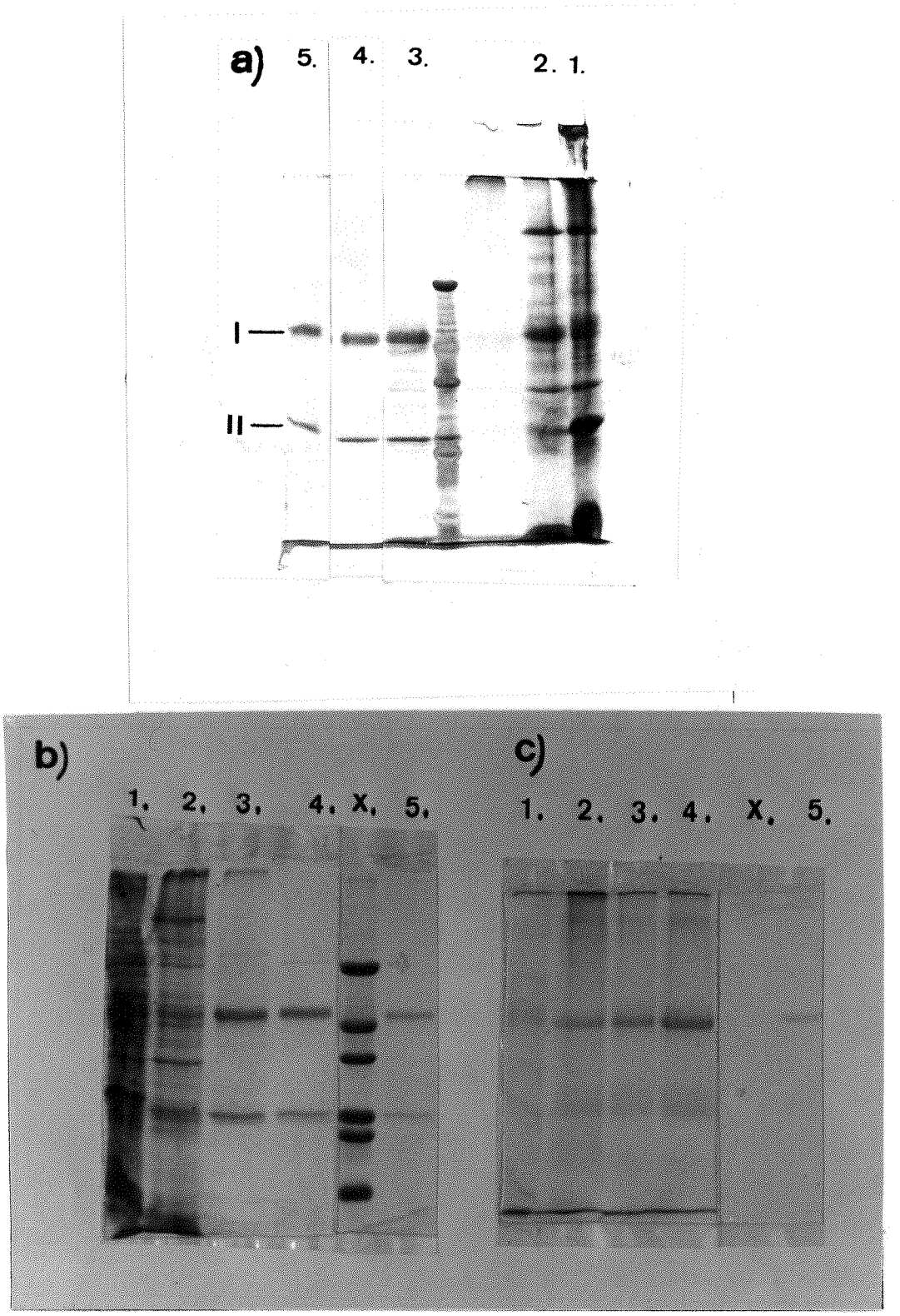
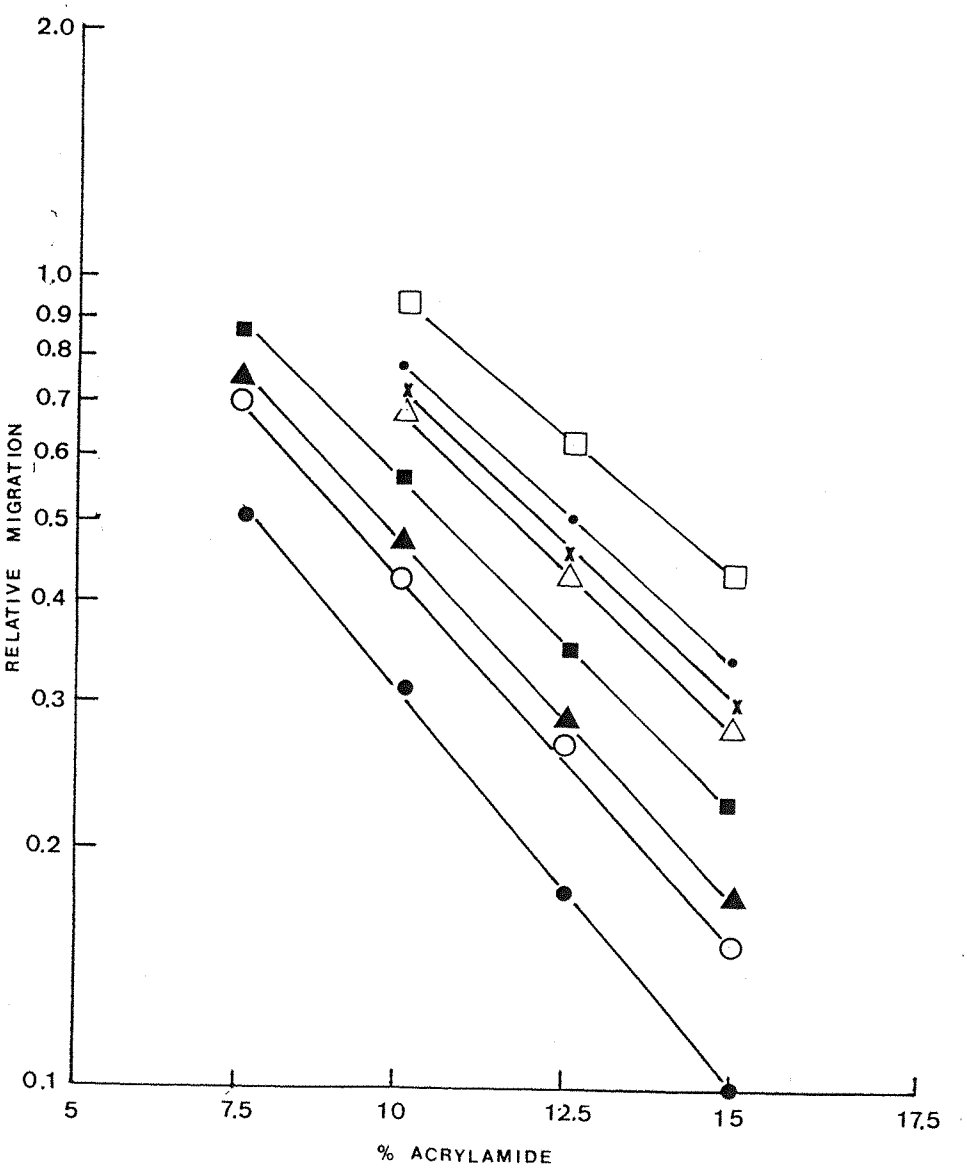


Fig. 4.7a, b <u>Ferguson plot analysis of the apparent molecular weights of the two subunits of the cytochrome *d* oxidase complex</u>

(a) Shows the dependence of the relative mobility (Rf) on the percentage acrylamide. The protein standards with Mr ( ) were: (●) BSA, (66K); (▲) Ovalbumin, (45K); (■) Glyceraldehyde 3P DH, (36K); (X) Carbonic anhydrase, (29K); (•) Trypsinogen, (24K); (□) Trypsin inhibitor, (20K); Subunit I, (52K); Subunit II, (36K).

The slopes of the lines in (a) were plotted against the molecular weight of the standard proteins to give the calibration curve in (b).

a.



b.

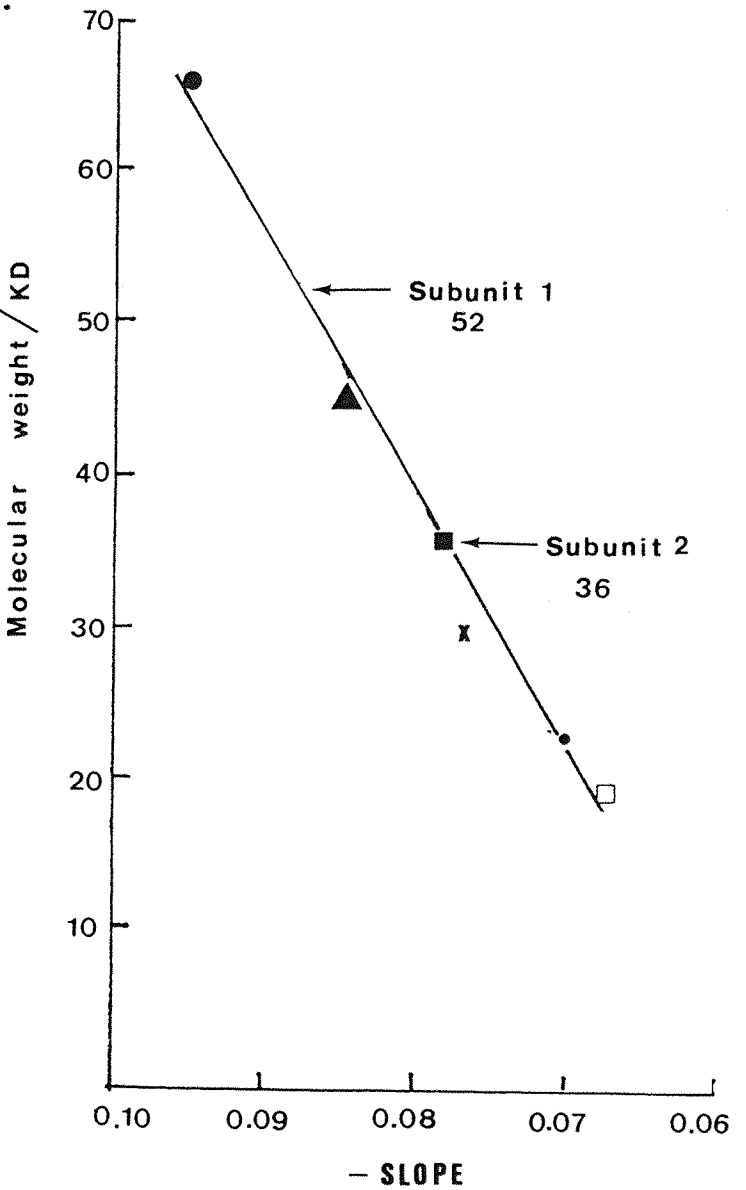

70
60
50
40
30
20
10
Molecular weight / KD
Subunit 1
52
Subunit 2
36
0.10
0.09
0.08
0.07
0.06
– SLOPE

Fig. 4.8 Determination of the apparent molecular weight of the oxidase complex by gel filtration

The standard proteins were chromatographed under the same conditions as the oxidase complex in Fig. 4.5c, they were: (O) B-Amylase, (200K); (□) ADH, (150K); (Δ) BSA, (67K); (O) Ovalbumin, (45K); (□) cytochrome c, (12.5K). The void volume was determined with blue dextran and the total column volume with tyrosine.

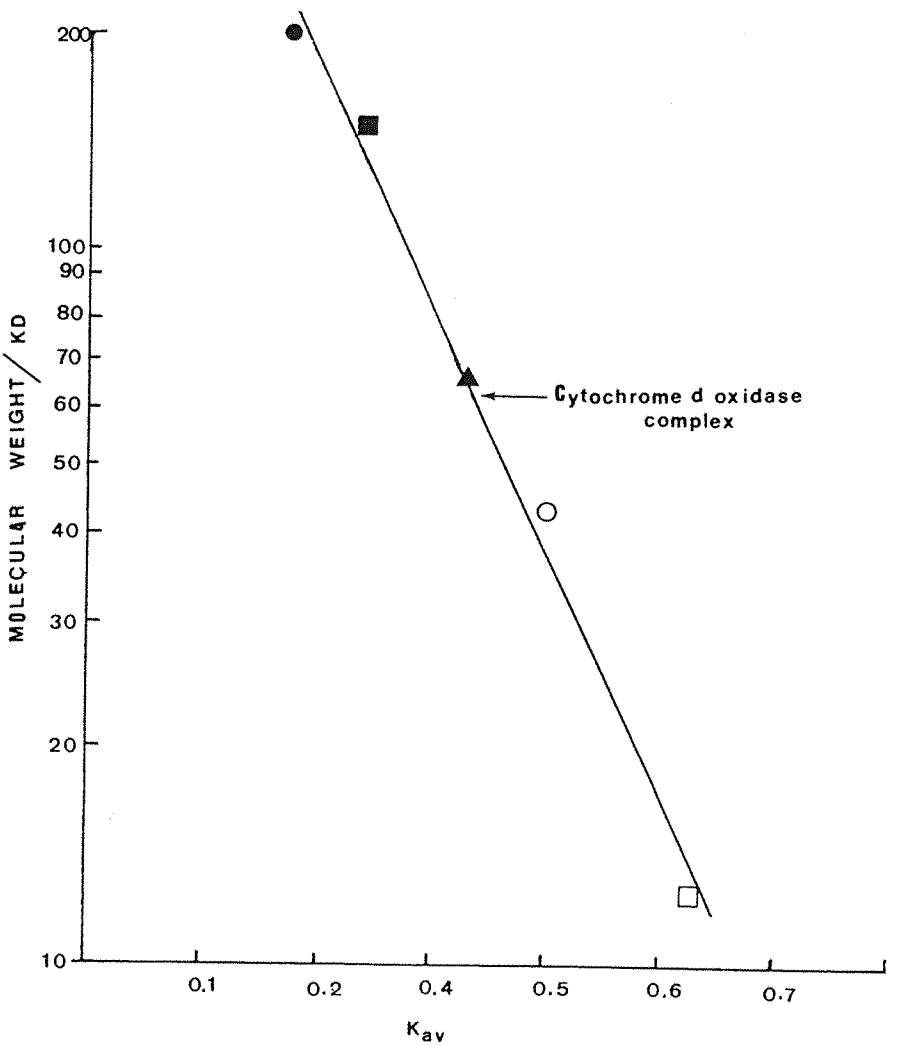


Fig. 4.6c shows that, providing SDS-PAGE was done under oxidising conditions, subunits I and II could be stained for peroxidase activity, suggesting that both subunits were able to retain haem even under denaturing conditions. Note that subunit I stained much more intensively than subunit II.

During gel filtration on Superose-12 in 4 mM zwittergent, the cytochrome *d* complex eluted as a single peak corresponding to an apparent Mr of about 70K (Fig. 4.8), suggesting that the oxidase as isolated was a heterodimer with subunits of 52K and 36K. When this was repeated in 10 mM -deoxycholate, the oxidase was in a much more highly aggregated state eluting as a single broad peak with an apparent Mr of 240K.

The active form of the *E. coli* enzyme has recently been shown to be a heterodimer (Miller *et al.*, 1988).

4.2e) Cytochrome composition and absorption spectra of the purified oxidase complex

Fig. 4.9 shows the UV-visible absorption spectrum of the air-oxidised (as isolated) form of the enzyme. The absorption at 646 nm is a feature not normally encountered in the spectra of oxidised cytochromes. In *E. coli* it has been suggested that cytochrome *d* can form a room temperature stable intermediate with O_2 in a way that is entirely analogous to haemoglobin (Poole *et al.*, 1983). This is the species responsible for the absorption peak at 650 nm. This can be shown to be true for *K. pneumoniae* cytochrome *d*. By first reducing a sample of oxidase in a sealed cuvette with sufficient substrate (Q_1H_2) to use up all the oxygen and then reoxidising the enzyme with a trace of H_2O_2, the true oxidised spectrum rather than the 'oxygenated' spectrum was generated (Fig. 4.9). In Fig. 4.10 the dithionite reduced form of cytochrome d characterised by a peak at 630 nm, reacted with CO resulting in a shift to 637 nm. Incubation of the oxygenated form of the enzyme with CO gave a similar

Fig. 4.9 Absorption spectra (room temperature) of the air-oxidized form of the cytochrome *d* complex

The sample contained 65 μg oxidase protein/ml in 75 mM-potassium phosphate (pH 6.3) containing 150 mM KCl, 1 mM EDTA and 4 mM zwittergent. Spectra are as follows: ——, air oxidized; - - -, air oxidized after sparging with CO for 30s; ·····, spectrum after the removal of all the oxygen from a sealed 1 ml cuvette by addition of 55 μM Ubiquinol-1 and 16 mM dithiothreitol followed by oxidation with a trace of hydrogen peroxide (6 μl of 2% v/v H_2O_2).

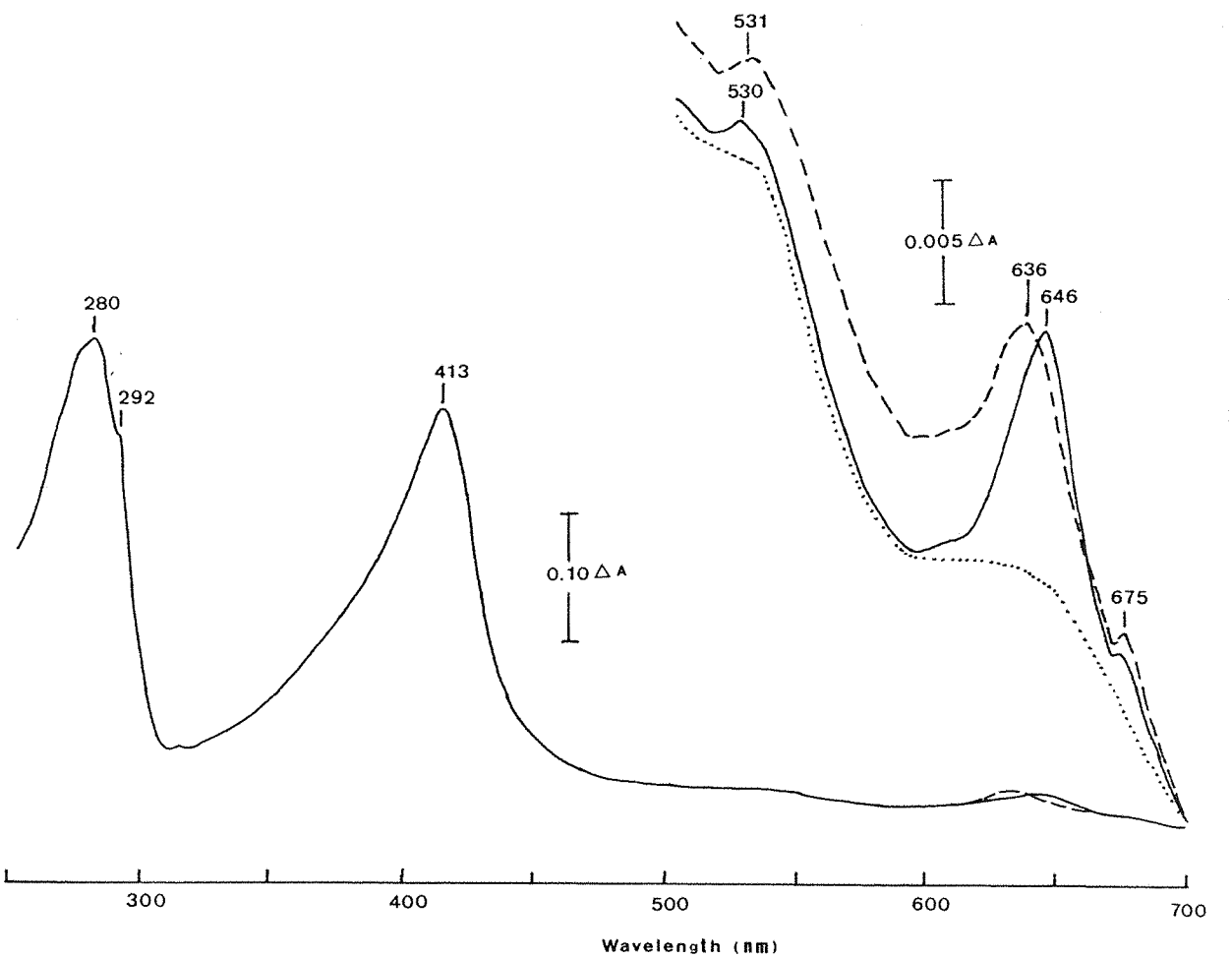


Fig. 4.10 Absorption spectra (at room temperature) of dithionite-reduced cytochrome *d* oxidase

The conditions were as described in Fig. 4.9 spectra: ——, dithionite-reduced oxidase; - - -, dithionite-reduced oxidase after sparging with CO for 30s.

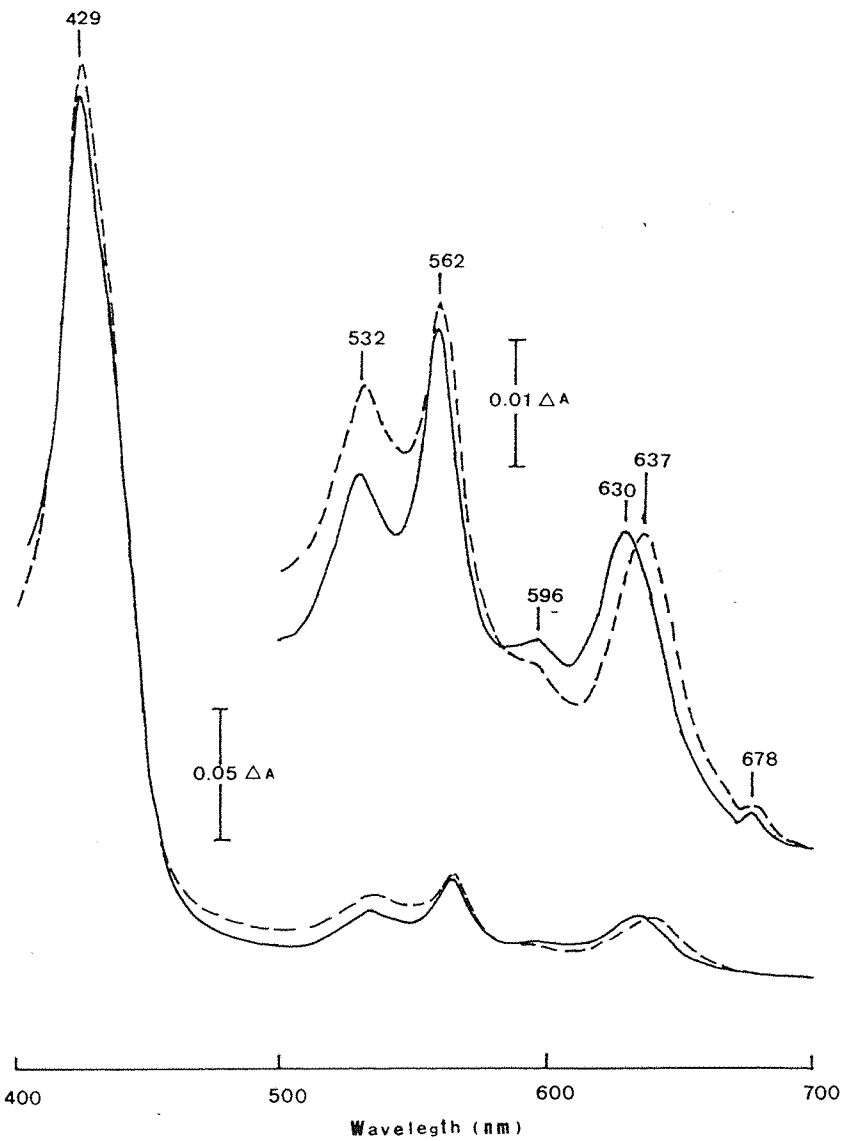


Fig. 4.11a, b Room temperature difference spectra of the cytochrome *d* complex

The conditions were as described in Fig. 4.9. a) Dithionite-reduced minus air-oxidized (oxygenated) difference spectrum; b) (dithionite reduced plus CO) minus dithionite reduced difference spectrum; ——, CO was sparged for 10s; — - -, CO was sparged for 30s.

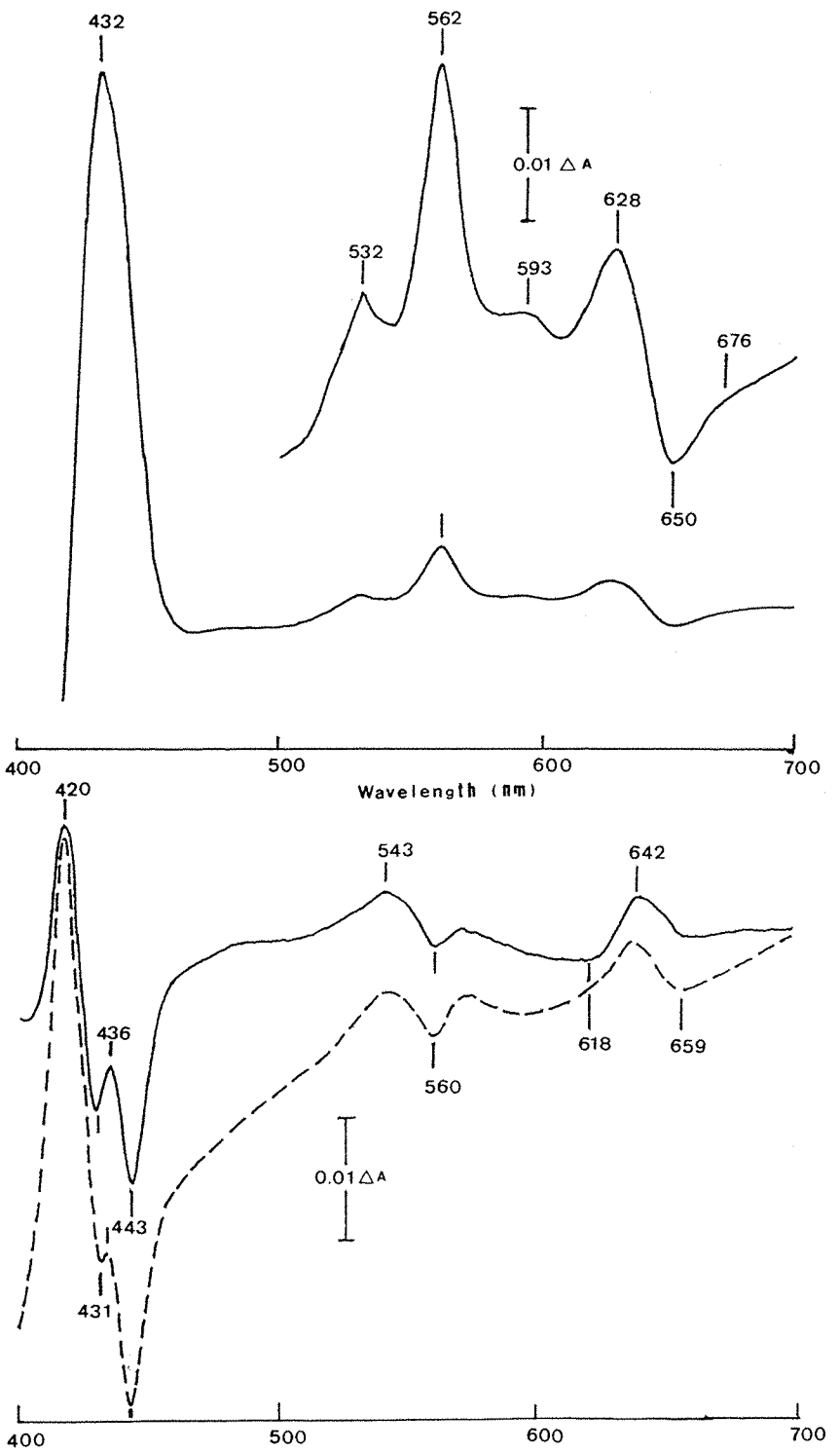
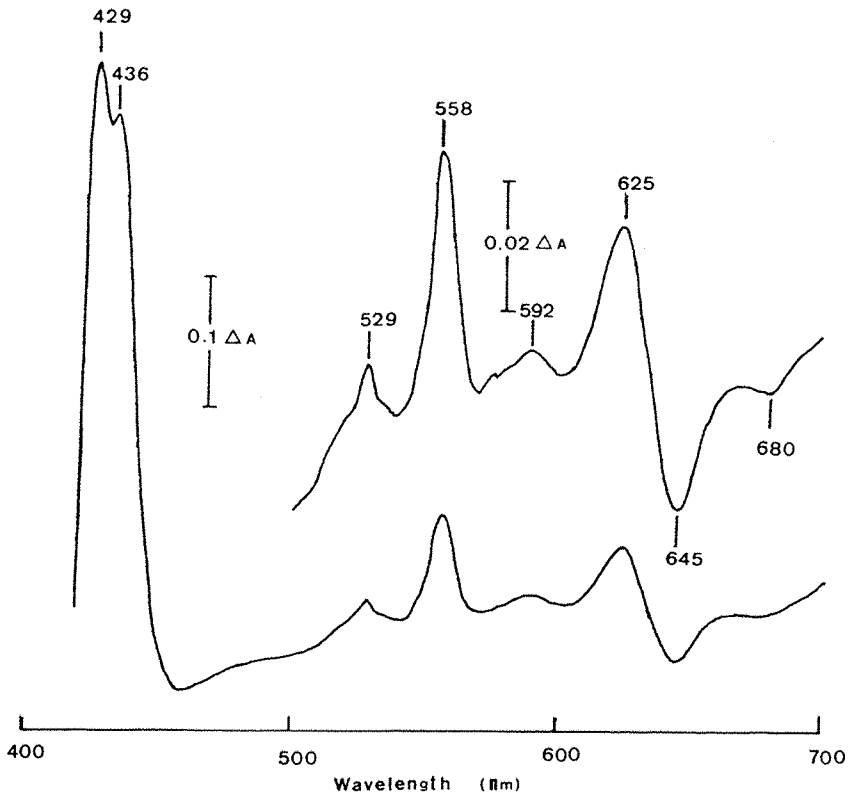
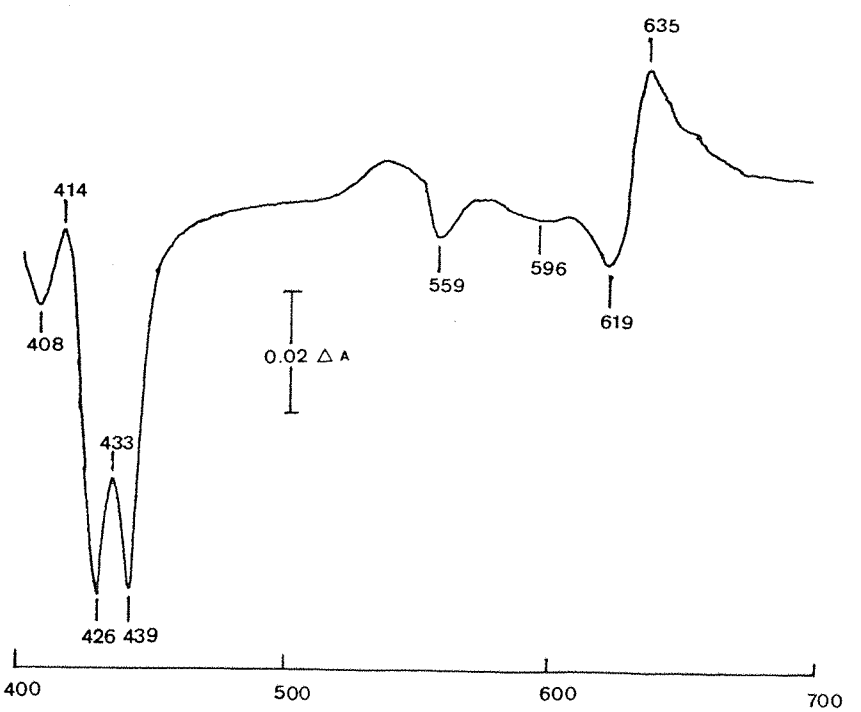


Fig. 4.12a, b Low temperature difference spectra (MK) of the cytochrome *d* complex

The conditions were as described in Fig. 4.9 except that the oxidase concentration was doubled (130 μg per ml) and the light path was 2 mm. (a) Dithionite-reduced minus air-oxidised (oxygenated) difference spectrum; (b) (dithionite-reduced plus CO) minus dithionite-reduced difference spectrum (CO was sparged for 30s before freezing).





peak at 636 nm (Fig. 4.9), suggesting that CO was able to displace bound O_2. *K. pneumoniae* cytochrome *d* oxidase can form a room temperature stable O_2 intermediate like the *E. coli* enzyme. By analogy with other O_2 binding haemoproteins, it is likely that cytochrome *d* must be in the ferrous (Fe^{2+}) state to bind O_2.

The absorption spectra shown in Figs. 4.11a, b and 4.12.a, b confirm that the purified cytochrome *d* oxidase complex is almost identical to that of *E. coli* (Miller & Gennis, 1983; Kita *et al.*, 1984b).

The reduced minus oxygenated difference spectrum at room temperature (Fig. 4.11a) showed absorbances characteristic of cytochrome *b* at 562 nm and cytochrome *d* at 628 nm. The smaller peak at 593-595 nm has proved 'historically' more controversial because of its similarity to the recognised absorption maxima of cytochromes a_1 (Poole, 1983). In the case of *E. coli* the true identity of the 595 nm band has been explained by Koland *et al.* (1984) and Lorence *et al.* (1986), who showed that this feature is the α peak of a high spin haem b centre, designated cytochrome b_{595}. More recently a specific role for cytochrome b_{595}, in the reduction of oxygen bound to cytochrome *d* has been proposed (Poole & Williams, 1987). The *K. pneumoniae* oxidase contained the 595 nm band and no cytochrome a_1 was detected by spectra of the pyridine haemochrome. The complex contained twice as much haem b (28.3 nmol/mg) as could be accounted for by the low spin b_{558} component alone (13.6 nmol/mg) [using the extinction coefficient recommended by Lorence *et al.* (1986) for the quantification of b_{558} in the presence of b_{595}]. The haem d content was 19.3 nmol/mg using the extinction coefficient of Lorence *et al.* (1986) but was only 15.2 nmol/mg using the extinction coefficient of Kita *et al.* (1984). Cytochrome b_{558}, cytochrome b_{595} and cytochrome *d* are present in the complex in the ratio of 1.1:1.0:(1.4-1.1). The higher ratio of cytochrome *d* (1.4) is similar to that determined for the *E. coli* enzyme by

Lorence *et al.* (1986) who concluded that there are two moles of cytochrome *d* per mole of oxidase complex. More recent estimates based on e.p.r. measurements indicate one mole each of cytochrome b_{595} and cytochrome *d* per mole of the *E. coli* oxidase (Rothery & Ingledew, 1989). The data for the *Klebsiella* oxidase also favour one mole of cytochrome *d* per mole of complex.

The low temperature spectrum (Fig. 4.12a) shows that the peak associated with cytochrome b_{558} was not split and so probably represents a single component. The Soret peak was split showing a peak due to the low spin b_{558} at 429 nm and a shoulder at 436 nm probably due to the high spin b_{595}, since cytochrome *d* is not thought to contribute to absorbance in the Soret region (Rothery *et al.*, 1987). Both these components reacted with CO (Fig. 4.11b) although the reaction of b_{558} was partial and was probably due to partial denaturation.

4.2f) Amino acid composition of the oxidase complex and similarity to *E. coli* cytochrome *d* complex

The amino acid compositions of the *Klebsiella* and *E. coli* cytochrome *d* complexes were very similar (Table 4.3). The polarity percentage (mole % of polar amino acids) was extremely low (30%); this is characteristic of intrinsic membrane proteins (Capaldi & Vanderkooi, 1970).

The homology between *E. coli* and *K. pneumoniae* cytochrome *d* suggested by the similar amino acid composition was confirmed immunologically. Rabbit antisera were raised against the whole oxidase complex and against subunit II. The anti complex antiserum reacted with a polypeptide on Western blots of SDS extracts of *E. coli* membranes that comigrated with subunit I of the *Klebsiella* oxidase. The pattern of regulation of cytochrome *d* synthesis described in section 4.2a, based on spectroscopic measurements was confirmed at the protein level by Western blot

Table 4.3 Comparison of the rleative abundance of amino acid residues in cytochrome *d* oxidase complex from *E. coli* (Miller & Gennis, 1983) and *K. pneumoniae* (this work)

Amino acid residue	mole % *E. coli*	*K. pneumoniae*
Asx	8.2	7.8
Thr	6.2	4.2
Ser	4.9	3.2
Glx	7.2	7.6
Gly	9.3	11.7
*Ala	11.5	10.6
*Val	8.8	8.4
*Met	3.5	4.4
*Pro	4.1	4.0
*Ile	7.1	5.5
*Leu	12.8	13.0
*Tyr	3.4	3.7
*Phe	6.9	8.0
His	1.5	2.3
Lys	4.1	2.8
Arg	1.4	1.6
% Hydrophobic	43.0	43.7
% Asx + Glx	15.4	15.4
% Basic	7.0	7.4

* = potentially hydrophobic residue

analysis. Unfortunately, subunit II failed to transfer efficiently during blotting as noted previously by Kranz and Gennis (1985).

4.2g) Substrate specificity and kinetic studies with the oxidase complex

Table 4.4 presents the steady state kinetic parameters of the purified oxidase complex which demonstrate that the preparation is an active quinol oxidase. The pH optimum with ubiquinol-1 as substrate was 7.9 (±0.1). The specific activity was about 30 per cent of that of the complex isolated from *E. coli* (Miller & Gennis, 1983). The turnover number (Kcat) of the oxidase (in terms of cytochrome *d* content) was 400 s^{-1} at pH 7.9 with ubiquinol-1 as substrate and with 0.05% Triton X-100 in the reaction mixture. This compares with an estimated turnover number *in vivo* (for a range of growth states) of 300-700 s^{-1}. The natural substrate for the oxidase is likely to be ubiquinol-8, the predominant form of quinone in enteric bacteria. A yellow neutral lipid spectroscopically identical with ubiquinones was isolated from the neutral lipid fraction of *K. pneumoniae* by the method of Collins (1985). Using ubiquinol-1 as substrate the Ki for KCN was 0.33 mM; this compares with the value of 2 mM-KCN required for 50 per cent inhibition of the *E. coli* enzyme (Anraku & Gennis, 1987).

Table 4.4 Steady state kinetic parameters of the cytochrome *d* oxidase complex

All reaction mixtures contained 0.05% Triton X-100 and all rates were corrected for any auto-oxidation of substrates. n.d., not determined.

Substrate	pH	Km value (mM)	Vmax (μmol O_2/min/mg protein)
Ubiquinol-1	7.0	0.22	50
Ubiquinol-1	7.9	0.21	117
Duroquinol	7.0	0.19	10
Duroquinol	7.9	nd	18
TMPD	7.0	2.8	32

4.2h) The requirement for activity of the oxidase for detergent or neutral lipid

The oxidase was similar to mitochondrial cytochrome aa_3 in that it was largely inactive after isolation and purification when assayed in the absence of phospholipid or neutral detergents (Table 4.5). There was no activity when the charged detergent (cholate) or the zwitterionic detergent (Zwittergent), that were used in the solubilisation and purification of the enzyme, replaced the neutral detergents Tween or Triton, as shown in preliminary studies of the *E. coli* oxidase (Lorence *et al.*, 1986). Exchanging the Zwittergent with cholate by gel filtration prior to assay had no effect on the measured rates. The optimum concentration of Tween 20 in the assay mixture was about 0.05 per cent. Above 0.2 per cent this detergent was inhibitory. The results in Table 4 also show that the neutral detergent was not required when the oxidase was first reconstituted with phospholipid prior to assay. The highest activity was obtained with phospholipids extracted from *K. pneumoniae*, but rates were not much lower when phosphatidyl glycerol or phosphatidyl ethanolamine was used; these are the main phospholipids in membranes of *E. coli*, and probably also of *K. pneumoniae* (Ames, 1968).

4.2i) The affinity of pure cytochrome *d* oxidase for oxygen

Estimates of the oxygen affinity of the cytochrome *d* of other bacteria have been published but these have been based on measurements with oxygen electrodes which are not suitable for accurate determinations at low oxygen concentrations (Hill, 1988). The purified cytochrome *d* oxidase from *K. pneumoniae* was able to catalyse the removal of oxygen from a solution containing soyabean oxyleghaemoglobin (Ks, 43.5 nM) resulting in its complete deoxygenation. The rate of the later phase of deoxygenation was used to calculate the steady state oxygen consumption kinetics at concentrations of free oxygen below 200 nM (Fig. 4.13).

Table 4.5 The dependence of cytochrome *d* oxidase activity on detergents or phospholipids

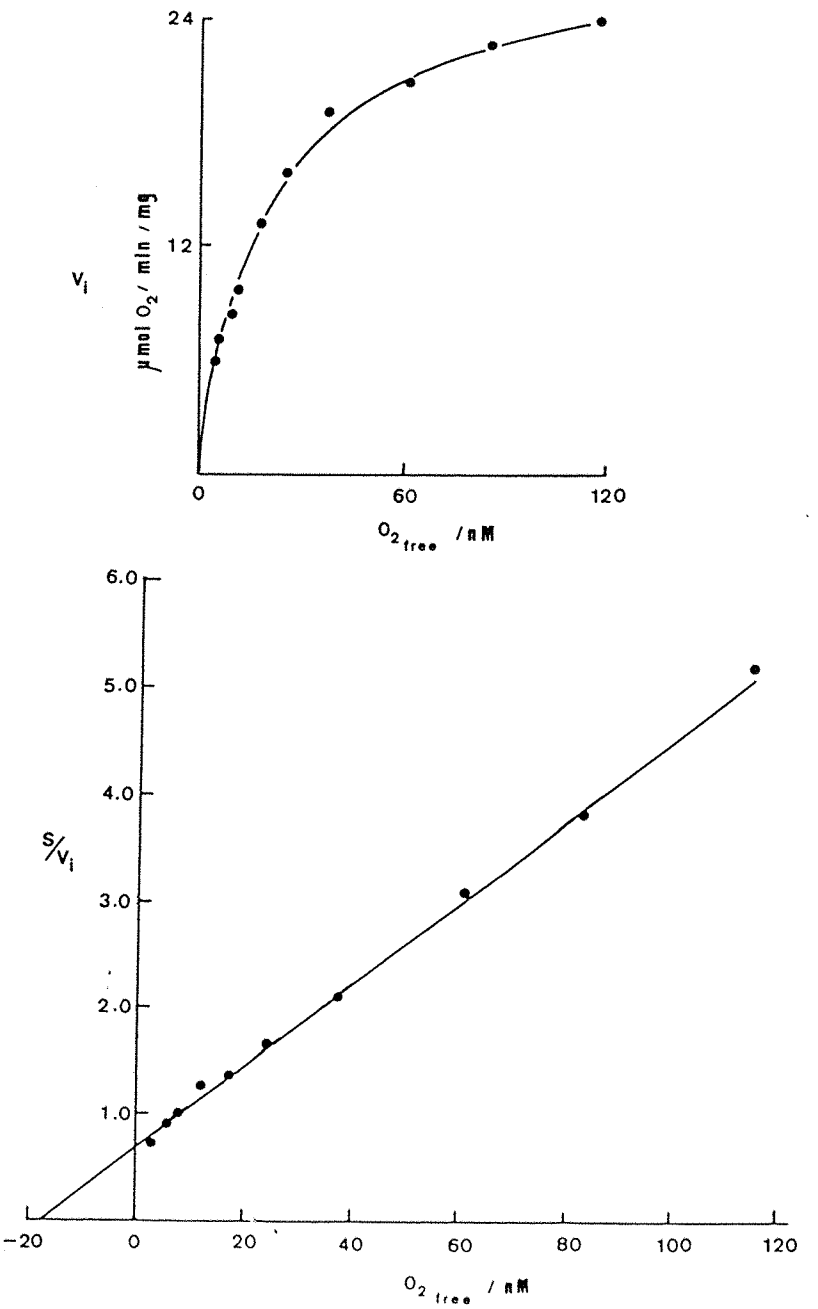
Activity was measured in the standard oxygen electrode assay system with duroquinol (1 mM) as substrate using oxidase isolated in Zwittergent. The values in parentheses are for oxidase which was first transferred to 5 mM cholate by passage down a PD10 gel filtration column. To measure the effect of phospholipid on activity of the oxidase equal volumes of purified oxidase were mixed with phospholipid (previously solubilised in Tris buffer (pH 7.9) containing 50 mM cholate as described in Methods. The final concentrations of phospholipid in the 'reconstitution' mixtures were as follows (mg ml^{-1}): phosphatidyl choline (10.0), phosphatidyl glycerol (9.5), phosphatidyl ethanolamine (5.0) and total phospholipid from *K. pneumoniae* (5.0). Activity of the oxidase was then determined by using 0.01 ml of this mixture in a 1 ml reaction mixture in an oxygen electrode assay. All the 'reconstitution' assays recorded below therefore contain 0.5 mM cholate. For the control recorded in the Table, cholate was omitted from the 'reconsntitution' mixture. The recorded values are averages of three assays, all of which gave values within 5% of those recorded. Although no systematic examination of the effect of phospholipid concentration was undertaken, doubling of the phospholipid concentrations did not increase the activities above the values recorded below.

It should be noted that the incubation period in Tris buffer, which was part of the 'reconstitution' process, led to a decrease to 48% of the specific activity of the oxidase (assayed in the presence of Tween 20); the activities recorded after 'reconstitution' should before be compared with a control value of 5.7 μmol/min/mg.

Activity in standard assay			Activity after 'reconstitution'	
Detergent	Specific activity (μmol O_2/min/mg)		Phospholipid	Specific activity (μmol O_2/min/mg)
None	0.02	(0.02)	None	0.70
Triton X-100 (0.05%)	4.93	(5.22)	Phosphatidyl choline	1.04
Tween 20 (0.05%)	11.94	(13.00)	Phosphatidyl glycerol	7.46
Cholate (0.05%)	0.07	(0.14)	Phosphatidyl ethanolamine	7.02
Zwittergent (0.5%)	0.02	(0.02)	Total phospholipid from *K. pneumoniae*	8.50
Cholate (0.05%) plus Tween 20 (0.05%)	7.31			

Fig. 4.13 The oxygen affinity of cytochrome *d* oxidase

The deoxygenation of soya bean leghaemoglobin (50 μM) was followed over 2 min in a sealed cuvette containing 5 μg of oxidase, ubiquinol (55 μM) and 0.05% Tween 20 at pH 7.4 as described in Methods (2.2i). (a) Computer-aided iterative fit to the Michaelis Menten equation, Km^{O_2} = 20.6 ± 1.6 nM, $Vmax^{O_2}$ = 26.5 ± 0.6 μmol^{O_2}/min/mg, demonstrating the simple hyperbolic curve that describes the reaction of the oxidase with oxygen; (b) least squares fit to the Hanes plot, Km^{O_2} = 20.0 nM $Ymax^{O_2}$ = 26.2 μol O_2/min/mg.



These results demonstrate that the oxidase has a single high affinity site for binding oxygen with an apparent Km' value of 20 nM. This value is much lower than that published for any other purified bacterial oxidase and, in particular, it is much lower than that measured for the purified cytochrome *d* oxidase of *E. coli* (380 nM) (Kita *et al.*, 1984b). However, the value for the *E. coli* protein was obtained from measurements with an oxygen electrode and hence is likely to be subject to error for measurements at low oxygen concentrations. The value assigned to cytochrome *d* oxidase in whole cells of *E. coli* (24 nM) (Rice & Hempfling, 1978) was closer to the value measured for the pure oxidase from *K. pneumoniae* in the present work. It is important to appreciate that the value measured is an apparent Km^{O_2} and not an absolute binding constant. No systematic attempt was made to study the effect of reductant concentration (UQ_1-H_2) on the apparent Km^{O_2}, although preliminary findings suggested that the apparent Km^{O_2} would decrease with increasing concentration of UQ_1-H_2. The binding constant for O_2 is therefore less than 20 nM.

As stated in the Introduction, it has been previously shown that 50 per cent inhibition of synthesis of nitrogenase occurred at about 100 nM oxygen which was close to the Ks of the dominant terminal oxidase; this was estimated from oxygen consumption measurements with whole bacteria using the same system for oxygen determination as used in the present work for measurement of the Km value for the purified oxidase (Bergersen & Turner, 1979; Bergersen *et al.*, 1982; Hill *et al.*, 1984). The estimated value using whole bacteria was about 80 nM. The apparent discrepancy between this value and that for the oxidase purified in the present work (20 nM) must presumably be due to gradients of oxygen between

the growth medium and the site of binding of oxygen to the oxidase, for which there is experimental evidence (Bergersen & Trichant, 1985).

4.3 SUMMARY AND DISCUSSION

The results described above confirm that *K. pneumoniae* contains a *d*-type oxidase very similar to that previously described in *E. coli* (Miller & Gennis, 1983; Lorence *et al.*, 1986; Miller *et al.*, 1988; Kita *et al.*, 1984b; Finlayson & Ingledew, 1985). The three cytochrome components, b_{558}, b_{595} and *d* which form the most abundant part of the respiratory chain present in the membranes of O_2 deficient *K. pneumoniae* co-purified as a single complex. These components defied further separation by conventional chromatography. The complex oxidised ubiquinol after activation with neutral detergent or phospholipid with turnover numbers sufficient to account for the respiration rate in whole bacteria. The complex was a heterodimer in the detergent system used to purify it and contained polypeptides with apparent molecular weights of 52K and 30-36K designated I and II. The complex was highly hydrophobic as judged by amino acid composition, consistent with an intrinsic membrane protein. The relative abundance of amino acid residues in the *K. pneumoniae* oxidase was very similar to that of the *E. coli* oxidase, indicative of a high degree of homology between the two proteins; this was confirmed immunologically.

The subunit sizes of the cytochrome *d* oxidase complex from *E. coli* have been reported with some variability; 57K and 43K (Miller & Gennis, 1983); 51K and 26K (Kita *et al.*, 1984), and more recently 70K and 43K (Finlayson & Ingledew, 1985). The aggregation state of subunit I in SDS was particularly temperature-sensitive; this can lead to its selective disappearance on SDS-PAGE if the sample is heated. Together with the presence of the undissociated oxidase under some conditions, this can lead to confusion in assigning the subunit molecular weights correctly. This

may explain the high molecular weight reported for subunit I by Finlayson and Ingledew (1985).

Like the *E. coli* cytochrome *d* oxidase the *K. pneumoniae* enzyme can form a stable oxygenated intermediate (Poole *et al.*, 1983; Koland *et al.*, 1984) strongly suggesting that cytochrome *d* is the oxygen-binding site. Analysis of the steady state oxygen consumption kinetics of the purified *Klebsiella* oxidase using oxyleghaemoglobin to measure dissolved oxygen concentrations below 200 nM were consistent with a single high affinity oxygen-binding site with an apparent Km' of 20 nM. In the light of this, it would seem reasonable that the oxidase should contain one mole of cytochrome *d* per mole of complex as suggested by the spectroscopic data. The Km for oxygen of the *E. coli* enzyme has been reported as 24 nM (Rice & Hempfling, 1978) and 0.23-0.38 μM (Kita *et al.*, 1984).

In *K. pneumoniae*, Bergersen *et al.* (1982) showed that very low oxygen concentrations inhibited derepression of both *nifH::lac* and pRD1 *nif*; 50 per cent inhibition of derepression occurred at 100 nM-O_2. The measurement of the affinity of *Klebsiella* cytochrome *d* oxidase for oxygen by a method which does not suffer from the problems associated with conventional O_2- electrodes, showed that the oxidase was competent to create and maintain O_2 concentrations compatible with *nif* expression. In addition the oxidase provides a means of oxidative phosphorylation at the very low O_2- levels (30 nM) that have been shown to enhance nitrogen fixation in *K. pneumoniae* (Hill *et al.*, 1984) (see Conclusions).

4.4 CONCLUSIONS

In *E. coli* no specific role for cytochrome *d* oxidase or cytochrome *o* has been proposed; mutants lacking either enzyme being able to grow normally under laboratory conditions (Au *et al.*, 1985; Green & Gennis, 1983). Georgiou *et al.* (1988) have shown that the regulation of synthesis of cytochrome *d* in *E. coli* is at the level of transcription, the

cyd operon being transcriptionally inactive at high oxygen levels. They concluded that there was no obvious need for the oxidase, except perhaps as an oxygen scavenger for which there has been no demonstrated need in *E. coli*.

The main feature that distinguishes *K. pneumoniae* from *E. coli* is that *K. pneumoniae* is able to fix nitrogen. In this organism the cytochrome *d* oxidase was always produced in nitrogen-fixing conditions, when it was the sole oxidase. Its most important property in this context is its extremely high affinity for molecular oxygen, the highest affinity for any oxidase previously described (Km', 20 nM). It may be that the exceptionally high affinity for O_2 is a property unique to the *Klebsiella* *d*-type oxidase. It is proposed that this ensures that it is able to lower the free oxygen in solution so that nitrogenase synthesis may be derepressed and so that it may be protected from the inhibitory effects of oxygen (respiratory protection). The affinity of the alternative oxidase, cytochrome *o* (Km, 200 nM), measured in whole cells of *E. coli*, is about 10 per cent of that of cytochrome *d* and hence usually unable to maintain the low oxygen concentration essential for derepression of nitrogenase (less than about 100 nM). It is also proposed that the cytochrome *d* oxidase is able to function as the terminal oxidase in an energy-generating electron transport chain in order to support an enhanced efficiency of nitrogen fixation at low oxygen concentrations. The presence of this oxidase after anaerobic growth on glucose presumably explains why, after glucose depletion, nitrogen fixation only occurs in the presence of added oxygen (Hill, 1976). Presumably the glucose fermentation products are providing electrons for this electron transport to oxygen by way of the cytochrome *d* oxidase described in this work. This hypothesis is tested using *cyd* mutants in Chapter 5.

It is clear that this proposed role for cytochrome *d* oxidase is not directly relevant to its function in *E. coli* as it does not fix nitrogen. There may be some as yet unidentified oxygen-sensitive enzymes whose activities provide some benefit to the organism during microaerobic growth. A candidate during growth on fermentable C-sources might be the formate H_2-lyase complex.

CHAPTER 5

A ROLE FOR CYTOCHROME *d* OXIDASE IN N_2 FIXATION BY ENTERIC BACTERIA

5.1 INTRODUCTION

In diazotrophs, a high affinity terminal oxidase has been implicated in N_2 fixation as a means of generating ATP at low O_2 concentrations (Appleby, 1984; Ramos & Robson, 1985). However, in none of them has this requirement been established; this is due mainly to the lack of defined oxidase mutations in diazotrophs, particularly those affecting terminal oxidases with high O_2-affinity.

In *Azotobacter vinelandii*, for which the concept of 'respiratory protection' was originally proposed (Dalton and Postgate, 1969), several oxidase mutants have been described. TMPD oxidase mutant Av-11 (Hoffman *et al.*, 1979) appears to be blocked between cytochrome C_4 and C_5; while a second class of mutant Av-90 (Hoffman *et al.*, 1980) lacked the spectral properties of c-type cytochromes (Fig. 1.6). Both mutants were reported to grow normally under N_2-fixing conditions, calling into question the importance of cytochrome *o* and providing indirect evidence that cytochrome *d* (the only other oxidase) was responsible for the respiration which protects and supports N_2-fixation. Subsequently McInerney *et al.* (1982) showed that Av-11 utilised O_2 less efficiently during O_2-limitation, although it was still capable of sufficient respiratory protection for N_2-fixing growth.

A 'shotgun' approach using transposon Tn5 mutagenesis in *Bradyrhizobium japonicum* has produced mutants which are both Nif^- and Nod^- due to the loss of the bc_1 complex. This prevented electron flow to the high affinity branch of the respiratory chain, but not to cytochrome *o*, which still permitted aerobic growth at high ambient oxygen concentrations

which were incompatible with N_2-fixation (H. Hennicke, personal communication).

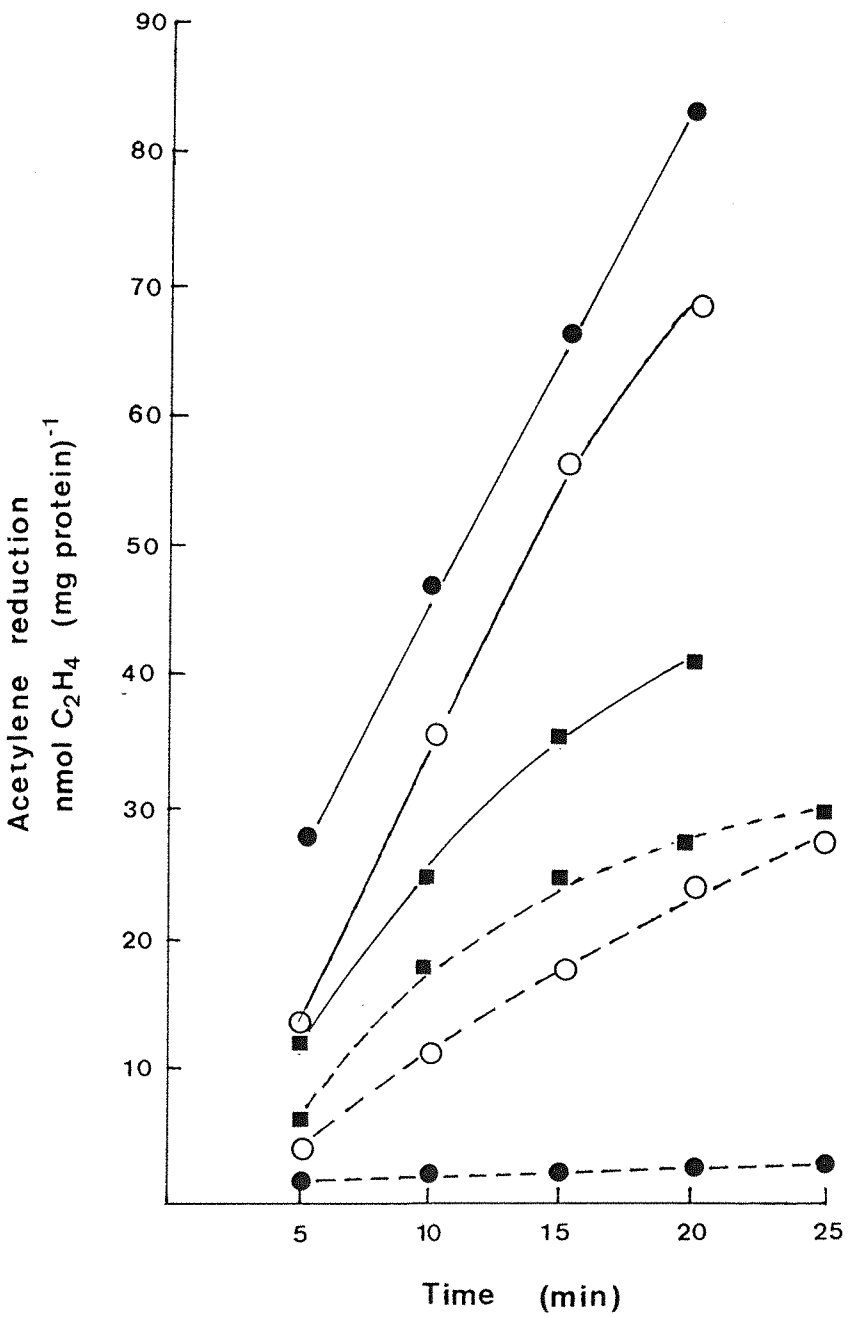
In the enteric N_2 fixer *K. pneumoniae*, O_2-dependent nitrogenase activity was greatest near the apparent Km of the cytochrome *d* oxidase complex (30 nM) (Hill, 1976b; Hill *et al.*, 1984; Chapter 4, this work) which according to spectroscopic measurements was the only oxidase expressed during nitrogen fixation. Hill (1976b) showed that, when small amounts of oxygen were introduced into the gas supply of an anaerobic glucose-limited N_2-fixing chemostat culture, the molar growth yield for glucose and the efficiency of N_2-fixation were increased up to 82 per cent. This led to the suggestion that a proportion of the ATP required for N_2 fixation and growth was generated by oxidative phosphorylation; leading to the production of more oxidized fermentation products.

The aim of this chapter is to provide direct evidence that cytochrome *d* oxidase has a physiological role in microaerobic nitrogen fixation.

The expression and regulation of *K. pneumoniae nif* genes in an *E. coli* background is the same as in the parental background. Therefore well characterised mutants of *E. coli* lacking cytochrome *d* oxidase were used in experiments to establish a physiological role. The first step was to set up an assay to test for the dependence of nitrogenase activity on respiration via cytochrome *d*. Hill (1976b) had shown that, in the absence of glucose, low oxygen concentrations were required for nitrogenase activity in samples from an anaerobic glucose-limited chemostat culture, implying that fermentation products were supplying electrons for nitrogenase activity and respiration. The first part of this work covers the identification of the 'active' fermentation products, and the standardisation of an assay which measures O_2-dependent nitrogenase activity in *K. pneumoniae*. The second part describes an experiment in which this assay was used to compare the O_2-dependent

Fig. 5.1 The effect of O_2 concentration in the gas phase on acetylene-reducing activity

Acetylene-reducing activity was measured in bacterial samples (2 ml) withdrawn from an anaerobic glucose-limited continuous culture in 35 ml flasks as described in Methods. The effect of O_2 concentration in the presence (————) and absence (- - - -) of added glucose (0.5% w/v) was determined with three different oxygen concentrations in the gas phase; anaerobic (<0.01% v/v) (●); 0.6% v/v oxygen (○) and 1.8% v/v oxygen (■). The agitation rate was constant at 90 cycles/min.



nitrogenase activities of *E. coli* cytochrome *d* minus (*Cyd*$^-$) and *Cyd*$^+$ strains carrying the Nif$^+$ plasmid pRD1. The results support the proposal that cytochrome *d* has an important physiological role in N_2-fixation in enteric bacteria. A model is proposed to explain how the identified fermentation products could supply both reductant in the form of pyruvate and ATP for N_2-fixation, via a respiratory chain terminated by cytochrome *d* oxidase.

5.2 RESULTS

5.2a) The requirement for oxygen for nitrogenase activity

K. pneumoniae was grown in anaerobic glucose-limited continuous culture with N_2 as sole nitrogen source. Samples were removed and nitrogenase activity was measured in flask assays as described in Methods. Fig. 5.1 shows the effect of varying the oxygen concentration in the gas phase on nitrogenase activity, with or without added glucose. The results were similar to those reported previously by Hill (1976b).

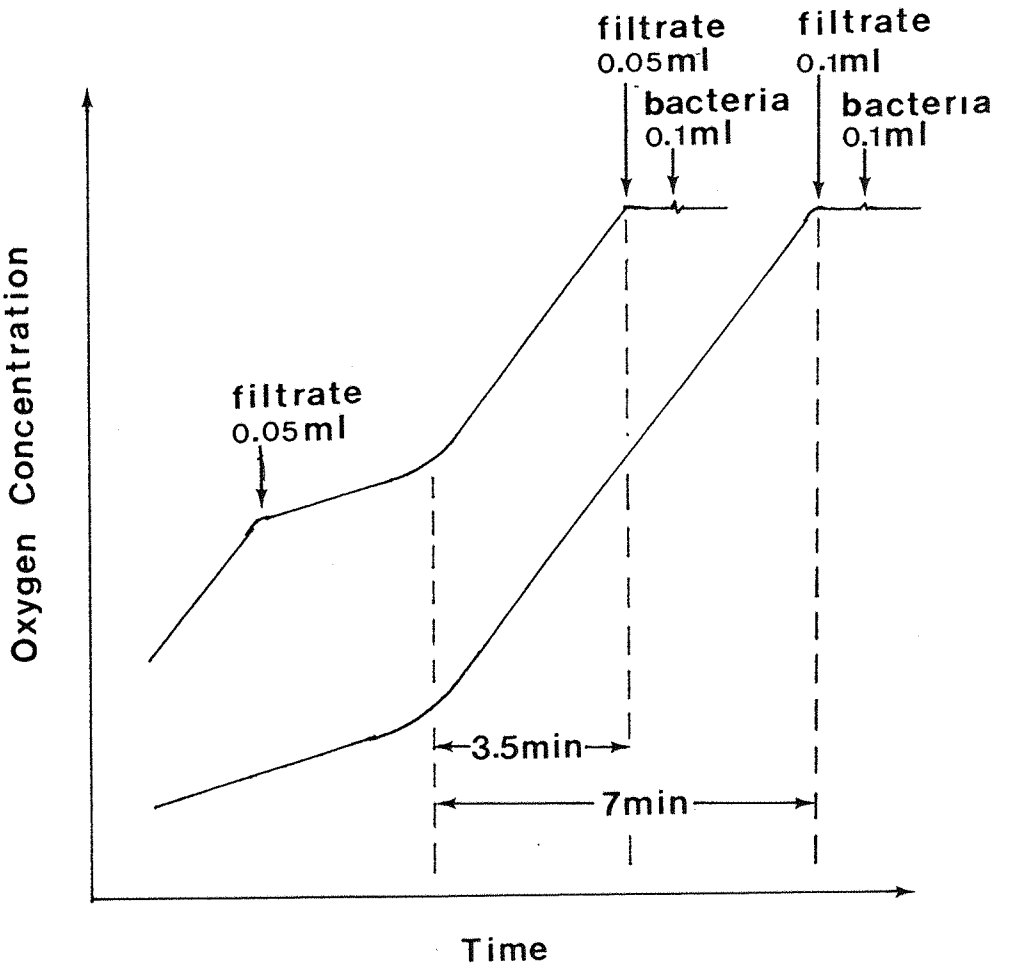
In the presence of glucose, O_2 at concentrations above 0.6% v/v inhibited activity. In complete contrast, in samples not supplemented with glucose, activity increased with increasing oxygen concentration. The maximum activity measured with 0.6% v/v oxygen in the gas phase was 37% of that in anaerobic samples containing added glucose. Presumably the glucose fermentation products provided electrons for nitrogenase activity and respiration via cytochrome *d* oxidase (see 5.1). An oxygen concentration of 1.2% v/v was chosen for further assays designed to show oxygen-dependent nitrogenase activity.

5.2b) The identity of the reductant(s) which support microaerobic nitrogenase activity

The mixed acid fermentation pathway used by *K. pneumoniae* during anaerobic metabolism of glucose was reviewed in Chapter 1. The obvious candidates for the source of reductant in glucose-limited chemostat

Fig. 5.2 Oxygen electrode trace showing the biphasic oxygen consumption by anaerobically-grown bacteria supported by culture filtrate

Bacteria were harvested from an anaerobic glucose-limited continuous culture and washed twice (Methods, 2...). O_2 consumption stimulated by addition of various amounts of culture filtrate was followed in a Rank O_2 electrode in a 2 ml reaction volume.



samples, which supported oxygen-dependent nitrogenase activity were the end products of anaerobic fermentation. Under glucose-limited conditions, these include (in order of abundance): acetate, ethanol, carbon dioxide, H_2, formate and lactate (Table 5.4). During N-limited growth on glucose, more lactate is excreted and 2,3-butanediol and succinate are produced. (Teixeria de Mattos & Tempest, 1983; 1984; Hill, 1976a).

Fig. 5.2 shows an O_2-electrode trace in which O_2 consumption by washed bacteria from an anaerobic glucose-limited chemostat was stimulated by addition of 'culture filtrate' from the same growth state. O_2 consumption was biphasic, with a fast rate and a slow rate, suggesting that a mixture of at least two substrates were supporting O_2 consumption. The fast rate was of relatively short duration, while the slow rate was of much longer duration. These results are shown quantitatively in Fig. 5.3a. The duration of the 'fast rate' was proportional to the amount of filtrate present; the initial rate was also dependent on the amount present. In contrast, the slow rate was unaffected by the amount of filtrate added. These results suggest that filtrates contain small amounts of a rapidly-oxidized substrate and much larger amounts of a slowly-oxidized substrate.

Fig. 5.3b shows the rates obtained when O_2 consumption was stimulated by the addition of a potential fermentation product. Formate was the only substrate tested whose oxidation rate was rapid enough to account for the fast rate; similarly succinate could account for the slow rate. Lactate, ethanol and acetate were not oxidized significantly. Pyruvate, although not a fermentation product under these conditions (Teixeira de Mattos & Tempost, 1983), was oxidized at a rate which increased rapidly with time. Mixtures of formate and pyruvate gave an O_2 consumption rate which approached that obtained with glucose alone (the growth substrate).

The biphasic oxygen consumption kinetics supported by culture filtrates could be accounted for if these contained 0.5-1.0 mM formate and between 2 and 4 mM succinate.

Table 5.1 shows the results of an experiment in which bacteria from an anaerobic glucose-limited chemostat culture were harvested and washed under strictly anaerobic conditions and tested for their ability to reduce acetylene microaerobically and anaerobically when incubated with mixtures of four known fermentation products of *K. pneumoniae*. As shown previously (Fig. 5.1), the maximum acetylene-reducing activity was measured with glucose anaerobically. Oxygen-dependent nitrogenase activity was supported by a mixture of fermentation products; formate, lactate, succinate and ethanol. Neither formate nor lactate was able to support microaerobic nitrogenase activity separately, but a mixture of formate plus lactate was effective (Table 5.1 and Fig. 5.4). The rates measured with either formate plus lactate or with culture filtrates were 31% of the rate measured anaerobically with glucose. In Fig. 5.4, O_2-dependent acetylene reduction supported by filtrate became non-linear after 25 min, this was probably due to formate depletion. The idea that the rate of supply of reductant rather than O_2 was limiting in any of these microaerobic nitrogenase assays was supported by the observation that oxyleghaemoglobin, which increases O_2 availability at low free O_2 concentrations (Appleby, 1984), had no effect on the measured rates (Table 5.1).

The only non-fermentable substrates which supported microaerobic nitrogenase activity in *K. pneumoniae* were a mixture of lactate plus formate. A model to explain this finding is discussed in Section 5.3 and Fig. 5.6

Fig. 5.3a, b Comparison of the rates of bacterial oxygen consumption measured with culture filtrate or potential fermentation products as substrates

Oxygen uptake rates were measured using the same procedure as for Fig. 5.2. The rates shown in the histograms were measured with the following reductants.

Fig. 5.3a
a) No addition
b) 0.5% w/v glucose
c) 0.2 ml culture filtrate
d) (c) after 14 min
e) 0.1 ml culture filtrate
f) (e) after 7 min
g) 0.05 ml culture filtrate
h) (g) after 3.5 min

Fig. 5.3b
i) 10 mM formate
j) 1 mM formate
k) 10 mM succinate
l) 10 mM D/L lactate
m) 0.1% v/v ethanol
n) 10 mM pyruvate
o) (n) after 5 min
p) 10 mM pyruvate + formate
q) 10 mM formate + succinate

(Time/min) = duration of oxygen consumption at the rate shown (see Fig. 5.2).

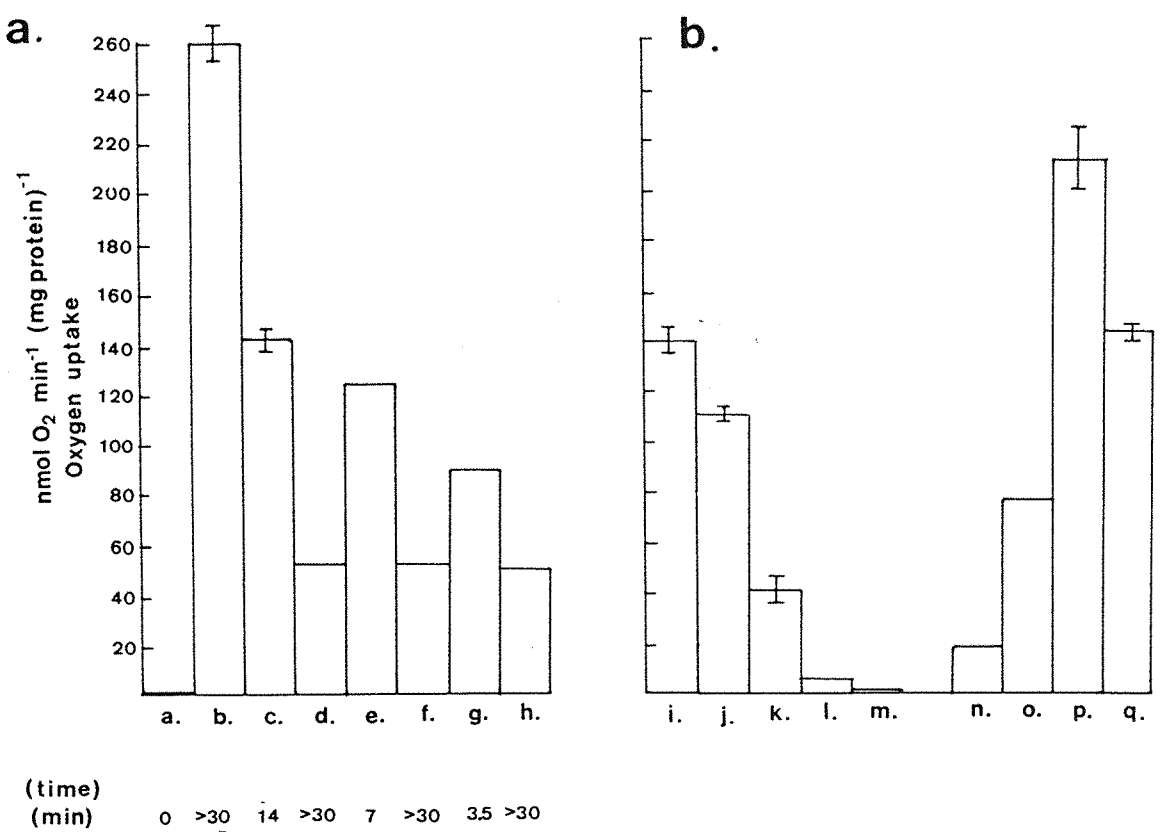


Fig. 5.4 Microaerobic acetylene reduction supported by a mixture of formate and D/L lactate

The procedure and conditions are the same as described for Table 5.1. The time course of acetylene reduction for some of the assays in Table 5.1 are shown: anaerobic with glucose (▲), microaerobic (1.2% v/v O_2) with lactate (△), microaerobic with formate (□), microaerobic with 20 mM (lactate plus formate) (○) and microaerobic with a 2-fold dilution of culture filtrate (●).

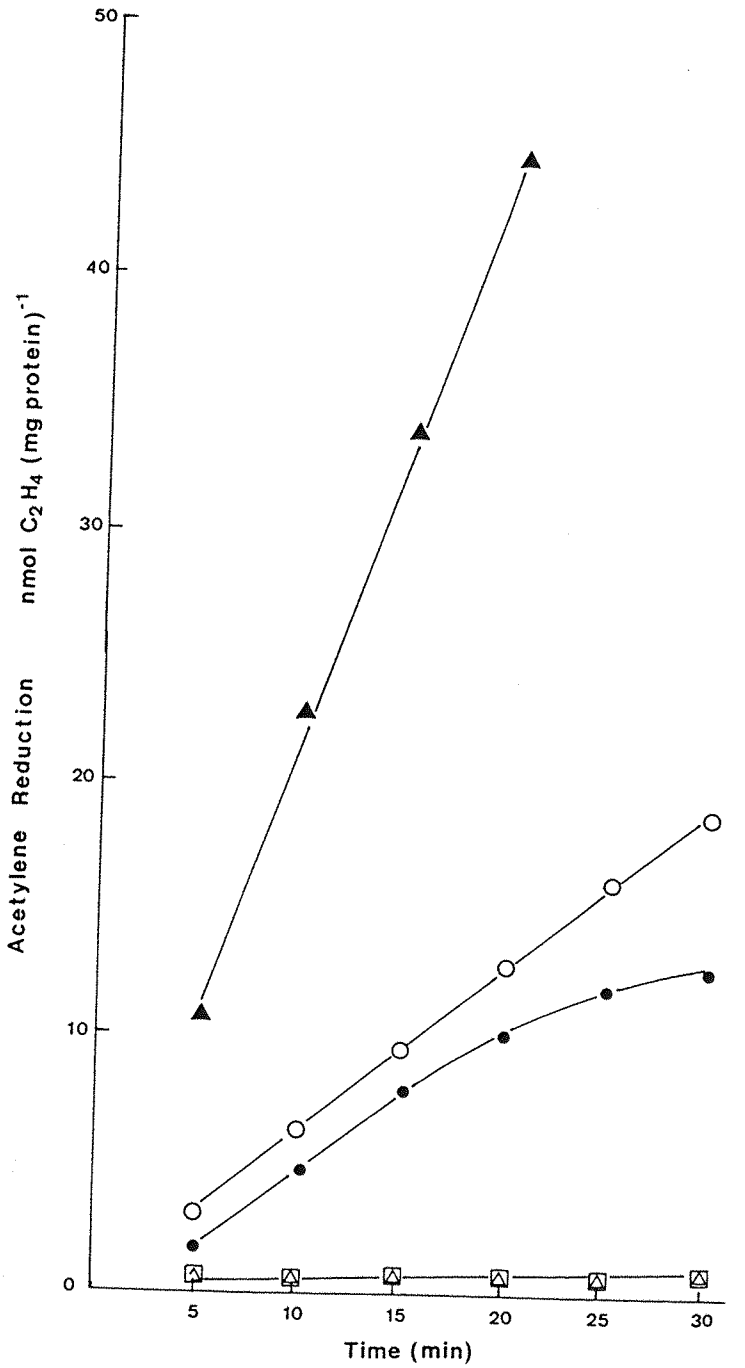


Table 5.1 The ability of fermentation products to support microaerobic acetylene reduction in *K. pneumoniae*

Substrate	Nitrogenase Activity % of control	
	Microaerobic	Anaerobic
Glucose (0.5%)	# 80%	100%
Culture filtrate (*2 dilution) (see Fig. 5.4)	25%	0
Culture filtrate + 100 μM oxyleghaemoglobin	27%	0
Formate, lactate succinate and ethanol	32%	0
Formate, lactate and succinate	32%	0
Formate and lactate	31%	0
Lactate	0.5%	0
Formate	0.9%	0
Pyruvate	0	26

Bacteria were grown in an anaerobic glucose-limited chemostat culture. They were harvested, washed twice anaerobically and acetylene-reducing activities measured in shaking flask assays as described in Methods (2.3c, f). The rates obtained when glucose was replaced with a fermentation product (20 mM) were measured and expressed as a percentage of the anaerobic glucose supported rate. Assays were either anaerobic under Argon or microaerobic, with 1.2% v/v oxygen in the gas phase. Culture filtrate refers to the supernatant obtained after harvesting bacteria. (#; rate was decreasing with time, to 10% after 30 minutes.)

Table 5.2 A summary of the properties of the strains used in this work

E. coli K12 strains	Genotype	Phenotype	Description
RP1	F-	Wild type $Zn\ Az^R$	Both oxidases functional.
RP56	F-, cyd C*	$Zn\ Az^S$	Cannot make haem *d*. Cytochrome *o* is the sole oxidase.
GO103	F-, rpsL, thi, gal, Δcyd1::Kan	$Zn\ Az^S$ Km	Gene encoding subunit I of cytochrome *d* deleted.
GR70N	F-, rpsL, thi, gal	$ZnAz^R$	Both oxidases functional.
K. pneumoniae M5a1		Wild type	Both oxidases functional.

* not proven

$ZnAz^S$ = unable to grow on aerobic NA containing 100 μM $2n^{2+}$ and Azide.

Strain GO103 was obtained courtesy of R. B. Gennis, University of Illinois, USA. It contained a 2.9 Kb deletion in the *cyd* operon spanning the coding region for subunits I and II and a Kanamycin cassette inserted 1.5 Kb upstream from the transcriptional start of the *cyd* genes (R. B. Gennis, personal communication, as yet unpublished).

Strain RP56 was obtained courtesy of R. K. Poole, Kings College, London. It was less well defined and contained a lesion (probably point mutation) mapping outside the *cyd* structural genes at min 18 on the *E. coli* chromosome (R. K. Poole, personal communication). It failed to make spectroscopically detectable cytochrome *d* and appeared to map at the same locus as *Cyd C* mutants which apparently fail to make haem *d* (Georgiou *et al.*, 1987).

Both strains were characterised independently by difference spectroscopy and sensitivity to inhibition of growth on nutrient agar containing Zn^{2+} and azide (see Methods) and shown to lack functional cytochrome *d* oxidase. In addition, immunoblot analysis using the antiserum raised to *Klebsiella* cytochrome *d* (Chapter 4) showed that RP56 did not synthesize cytochrome *d* apoprotein, presumably as a regulatory consequence of the mutation in *cyd* C, as previously reported by Georgiou *et al.* (1987).

5.2c) Evidence for a physiological role for cytochrome *d* during microaerobic nitrogen fixation

Cytochrome *d* oxidase has been shown to have a very high affinity for oxygen (Km' = 21 nM) and it is the only oxidase expressed under anaerobic nitrogen-fixing growth conditions (4.2a). Microaerobic acetylene reduction supported by lactate plus formate must therefore be dependent on a respiratory chain terminated by cytochrome *d* oxidase. The following experiment was designed to test this proposition by using a mutant lacking the oxidase.

The Nif^+ plasmid pRD1 was used to construct transconjugants of the two *E. coli* Cyd^- strains (cyd = cytochrome *d*) RP56 and G0103, and of a wild type (Cyd^+) *E. coli*, RP1 (Table 5.2). Normally such transconjugants are tested for a Nif^+ phenotype after N-limited growth, by a standard method (Cannon, 1980); this relies on respiration to create O_2-deficient conditions for nitrogenase synthesis and activity during the 18h of growth before measurement of acetylene reduction. All transconjugates were Nif^+, although Cyd^- strains only reduced acetylene when N_2 rather than air was initially present in the culture gas phase and when allowed to grow for 26 hours.

Table 5.3 shows the specific acetylene-reducing activity for each strain measured after growth and derepression under strictly anaerobic conditions. Growth and derepression of RP56(pRD1)(Cyd^-) was always inferior to that of the parental strain RP1(pRD1)(Cyd^+). However, because the mutagen used to create the lesion in RP56 was N-methyl N' nitro N- nitroso guanidine and in view of the growth differences under anaerobic fermentative conditions (Table 5.3), the isogenicity of the two strains cannot be assumed.

Table 5.3 Acetylene-reducing activities of *E. coli* cytochrome *d* mutant and wild type strains, carrying the Nif+ plasmid pRD1

Transconjugants	Growth (OD 540)	Acetylene-reducing activity nmol C_2H_4 min^{-1}(mg protein)$^{-1}$
RP1(pRD1)	26	48±2
RP56(pRD1)	16	17±2
GO103(pRD1)	34	46±3
K. pneumoniae M5a1	37	105±5

Cultures were grown in batch culture under strictly anaerobic, derepressing conditions in modified NFDM (see Methods). Culture samples were removed after 20 h growth and assayed for acetylene reduction under anaerobic, glucose-sufficient conditions. The final pH of cultures was always between 6.4 and 7.0.

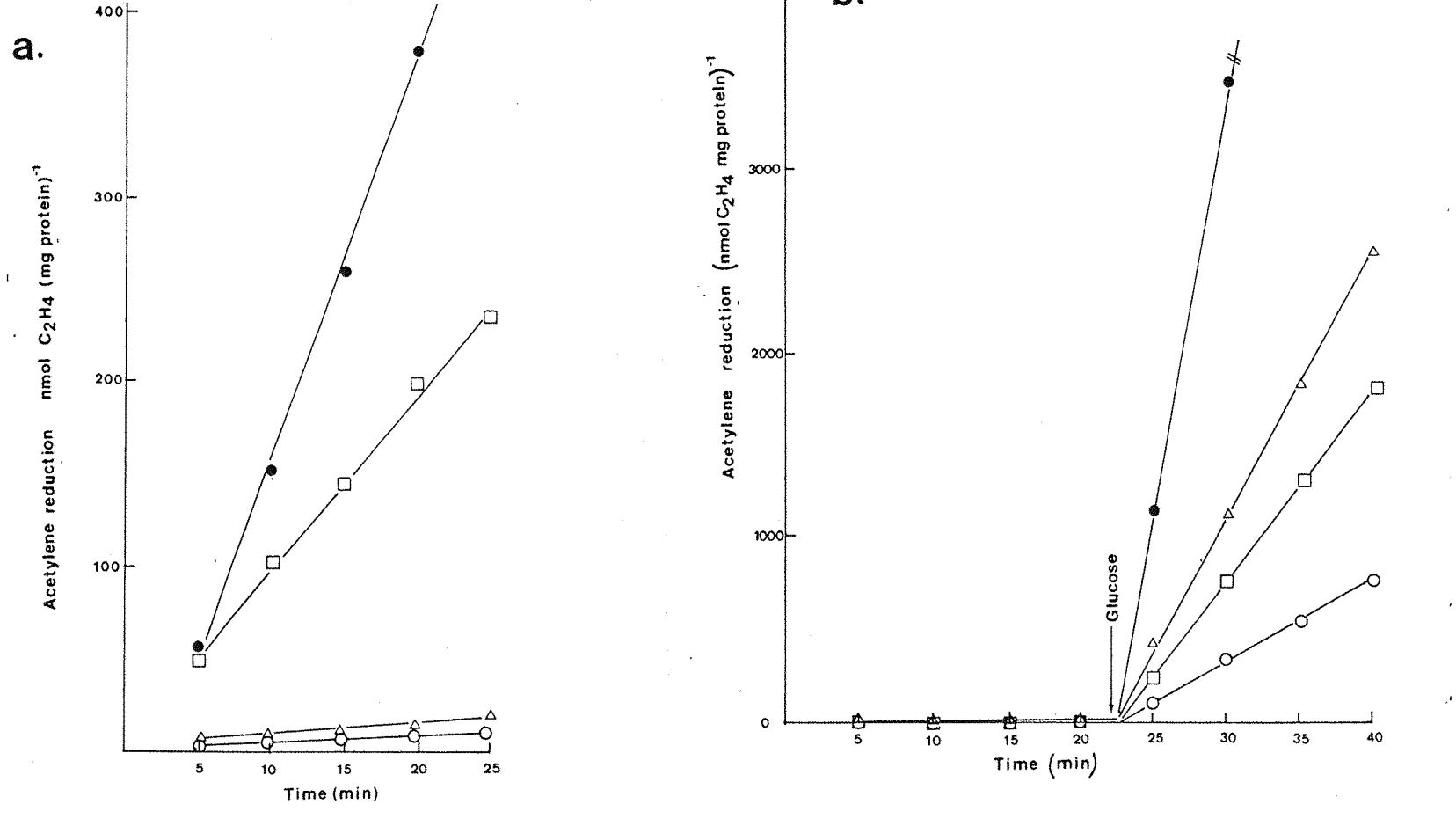
The second *Cyd*$^-$ strain, GO103(pRD1) showed the fastest growth of all. Unfortunately an isogenic *Cyd*$^+$ strain was not available when the transconjugants were constructed. It was assumed that the faster growth of GO103(pRD1) than RP1(pRD1) was a property of the strain rather than the mutation in cytochrome *d*. This was subsequently shown to be true by comparing the anaerobic growth of GO103 in glucose-minimal medium with that of its isogenic parent GR70N (data not shown).

The nitrogenase activities of all transconjugants were between 16-48% of that found with wild-type *K. pneumoniae*. During growth in selective-media (media containing carbenicillin and kanamycin), there was no evidence for the loss of either the Nif$^+$ plasmid (as judged by acetylene reduction) or the *cyd* mutation (as judged by the retention of zinc and azide sensitivity during growth on nutrient agar).

The *E. coli* transconjugants were tested for microaerobic oxygen-dependent acetylene reduction with formate plus lactate as substrate as described previously for *K. pneumoniae* (see Fig. 5.4; Table 5.1).

Fig. 5.5a, b Microaerobic acetylene reduction supported by formate and lactate in *E. coli Cyd*$^+$ *and Cyd*$^-$ strains carrying the Nif$^+$ plasmid pRD1

E. coli Cyd$^+$ *and Cyd*$^-$ strains carrying the Nif$^+$ plasmid pRD1 were grown in 200 ml of modified NFDM under N_2 for 18 hrs or until the specific acetylene-reducing activity of all cultures exceeded 10 nmol C_2H_2 reduced min^{-1}(mg bacterial protein)$^{-1}$. Bacteria (20 ml) were harvested and washed twice anaerobically (see Methods). Acetylene-reducing activities were measured in shaking flask assays containing a 20 mM mixture of formate plus D/L lactate in 1 ml of 100 mM sodium phosphate buffer pH7.4. Assays were either microaerobic (1.2% v/v O_2 in the gas phase) Fig. 5.5a, or anaerobic (under argon) Fig. 5.5b. Assays were started by the addition of 1 ml of resuspended bacteria. The following strains were used: (□), RP1(pRD$_1$), wild-type; (○), RP56), *Cyd* C$^-$; (△), G0103(pRD1), ▲△ cyd; (●), *K. pneumoniae* M5a1 wild-type. In the anaerobic control experiment in Fig. 5.5b, glucose (0.5% w.v) was added after 22 min.



The results in Fig. 5.5a show that only *Cyd*$^+$ strains (containing cytochrome *d*) were able to reduce acetylene with lactate plus formate in microaerobic conditions. All strains failed to reduce acetylene with lactate plus formate anaerobically (Fig. 5.5b), but were capable of reducing acetylene anaerobically after the addition of glucose.

Previously described experiments with *K. pneumoniae* (Fig. 5.4) used bacteria grown in continuous culture, where glucose was undetectable. The present experiments utilised washed bacteria grown in batch culture from which the glucose had not been exhausted. Probably as a result of this difference in growth regime, the microaerobic nitrogenase activities measured were not as high as previous, amounting to ony 10% of the rate obtained anaerobically with glucose (Fig. 5.5b).

These results strongly suggest that a functional respiratory chain terminating in cytochrome *d* is required to couple formate/lactate oxidation to nitrogen-fixation in enteric bacteria. This is further discussed in Section 5.4.

5.2d) Effect of the *Cyd*$^-$ mutation on the oxygen sensitivity of nitrogenase derepression in *E. coli* transconjugants

Initial attempts to obtain nitrogenase derepression in *Cyd*$^-$ strains in batch cultures sparging with 1% CO_2 in high purity N_2 always failed at high gas flow rates, whereas *Cyd*$^+$ strains derepressed normally. When gas flow rates were decreased to the minimum required to agitate the culture, then all strains derepressed normally to give the specific activities shown in Table 5.3. It may be that *Cyd*$^-$ strains were unable to scavenge successfully the low levels of oxygen in the culture due to oxygen contamination present in the gas supply, especially at high N_2 flow rates. No detailed experiments relating nitrogenase synthesis to a defined rate of O_2 supply were done, but this perliminary observation suggests that derepression of *Cyd*$^-$ strains may be abnormally oxygen-sensitive.

5.3 DISCUSSION

Like *E. coli*, *K. pneumoniae* is able to ferment glucose to a variety of products including acetate, ethanol, lactate, succinate and 2,3-butanediol. All arise from either phospho-*enol*-pyruvate or pyruvate, and under anaerobic conditions their syntheses maintain the redox balance.

During anaerobic fermentation in glucose-limited continuous culture, *K. pneumoniae* produces relatively small amounts of lactate and formate (Table 5.4; Hill, 1976). The precise amounts are dependent on the growth rate and nutritional limitation; for example, lactate production increases with dilution rate in glucose-limited culture, and increases dramatically when glucose is not growth-limiting (Teixeria de Mattos, 1983). The levels of lactate in culture filtrates from anaerobic N_2-fixing glucose-limited continuous cultures were calculated from the data of Hill (1976a) to be 1.0 mM. In the present work an independent estimate of 1.5 mM was made using purified *K. pneumoniae* D(-) lactate: ubiquinone reductase, coupled in a dye linked assay system (see Appendix 1 and Methods, 2.3j). Interestingly, Ugurbil *et al.* (1978) have shown by ^{13}C NMR that when O_2 was introduced into an anaerobic glucose-fermenting *E. coli* culture, lactate was the first fermentation product to be metabolised, followed much later by succinate and acetate. Unfortunately, formate could not be detected owing to the position of the ^{13}C label in the original glucose molecule. Estimates of formate reported in the literature make it one of the least abundant fermentation products (Table 5.4). Most formate is metabolised by the formate H_2-lyase system, but small amounts escape and are excreted, particularly at more alkaline pH values (Harrison and Pirt, 1967). Only 0.5-1.0 mM formate would be required to account for the duration of the 'fast phase' of O_2 consumption reported in Section 5.2b, in reasonable agreement with

the formate concentration calculated from the experimental data of Hill (1976a) or Teixeira de Mattos (1983) of 0.4 mM.

Table 5.4 Products of anaerobic glucose fermentation

Fermentation product	*E. coli* (#) (batch culture)		*K. pneumoniae* (*) (glucose-limited chemostat)	
	(%)	conc/mM	(%)	conc/mM
Lactic acid	44.6	24.8	1.0- 2.0	0.6- 1.1
Succinic acid	5.0	2.1	5.9-22.0	2.5- 9.4
Acetic acid	16.8	14.0	30.6-32.3	25.6-26.9
Formic acid	0.8	0.9	0.3- 0.4	0.3- 0.4
Carbon dioxide	12.4	13.5	-	-
Ethanol	16.1	17.5	20.4-23.0	22.0-25.0

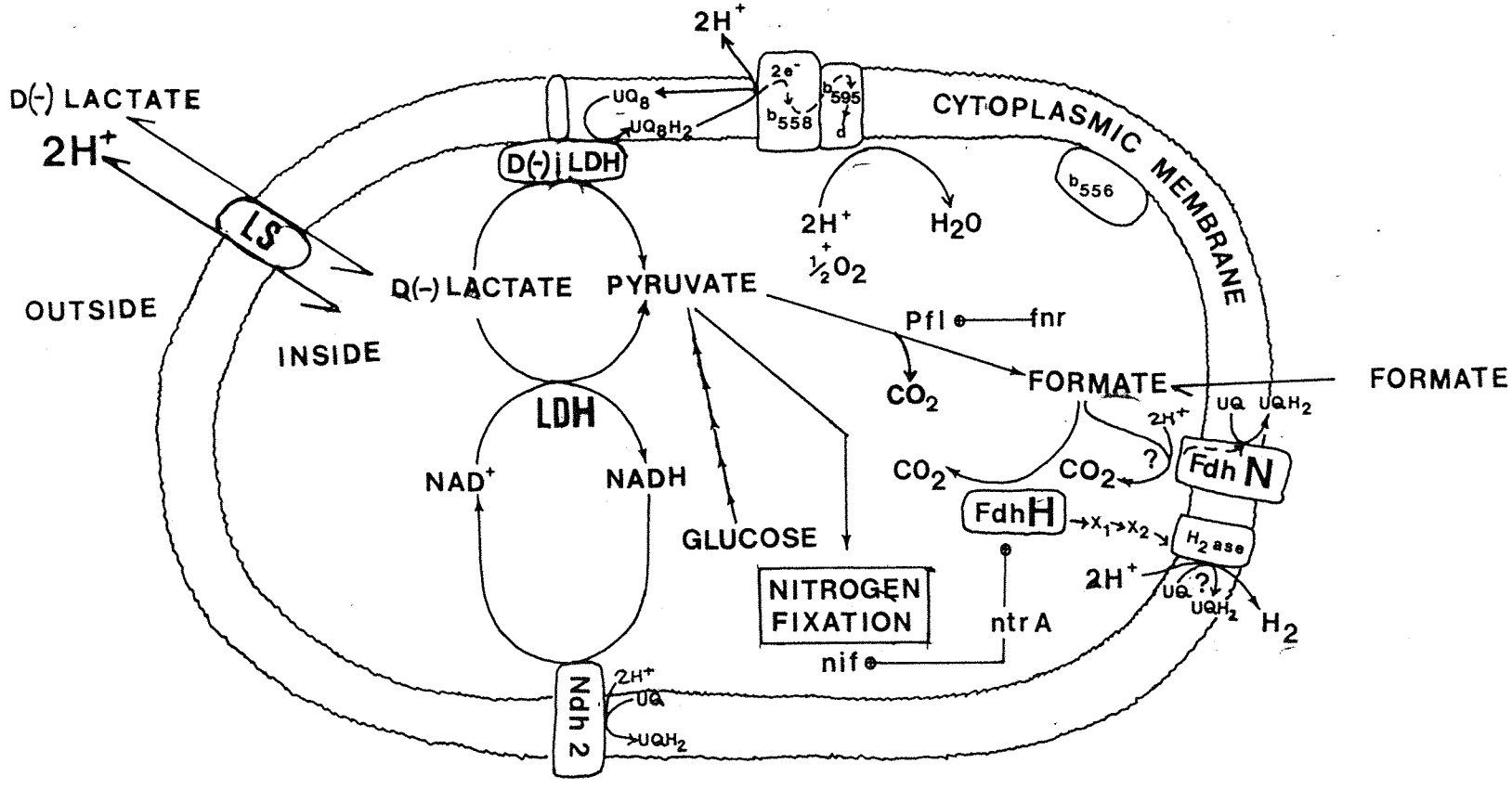
(%), percentage of glucose carbon fermented by weight. (#), calculated from the data of Doelle (1969). (*), calculated from the data of Hill (1976a). See also Teixeira de Mattos (1983). Note that much larger amounts of lactate are produced in batch cluture than in glucose-limited continuous culture.

In *K. pneumoniae* lactate plus formate were the only substrates which could support nitrogenase activity by stimulating respiration. The role of lactate is puzzling because washed bacteria failed to oxidize lactate at a significant rate (Section 5.2b). However, in *E. coli* there is considerable evidence that lactate uptake proceeds by a proton symport mechanism (depicted in Fig. 5.6) with a proton/lactate stoichiometry of 2/1 (Konings, 1985). This means that membrane translocation of lactate is electrogenic, with its intracellular accumulation being driven by proton motive force (Brink and Konings, 1980), hence de-energized bacteria will not take up lactate. In fact, during fermentation, lactate excretion has the potential to generate a proton motive force by the reverse of the same mechanism.

Could the oxidation of formate via a respiratory chain terminated by cytochrome *d* generate the proton motive force necessary to drive lactate uptake? This seems very likely. *E. coli* has two potential routes of formate oxidation. The aerobically expressed formate dehydrogenase

Fig. 5.6 A model to explain how lactate plus formate could supply both reductant in the form of pyruvate, and ATP for N_2-fixation via a respiratory chain terminated by cytochrome *d* oxidase

For evidence for the model see text. The components are identified as follows: LS, lactate symport system; LDH, NAD^+ linked lactate dehydrogenase; D(-)iLDH, D(-) lactate: Ubiquinone reductase (see Appendix 1); UQ_8H_2, $Ubiquinol_8$; Cyt*d*, cytochrome *d* oxidase complex (Chapter 4); Pfl, pyruvate formate lyase; Fdh_N, aerobic formate dehydrogenase; Fdh_H, anaerobic formate dehydrogenase of the formate hydrogen lyase system; X_1 and X_2, unidentified electron transfer components in F H_2-lyase system; H_2ase, hydrogenase (Hyd1); Ndh2, low affinity NADH dehydrogenase (Unemoto and Hayashi, 1988): *fnr* and *ntrA*, transcriptional activiation factors (see Chapter 1); b_{SS6}, mid-chain cytochrome *b* (see Appendix 1).



(Fdh_N) which transfers reducing equivalents to a quinone and terminal electron acceptor, and Fdh_H which transfers electrons to a hydrogenase (Hyd1) and is part of the formate-H_2 lyase system (Ballantine and Boxer, 1985; Stewart, 1988). *K. pneumoniae* has equivalent enzymes. Only the Fdh_H equivalent would be expected to be expressed under the anaerobic fermentative conditions reported in this chapter. Although whole bacteria from these conditions oxidize formate, aerobically prepared membrane particles do not, presumably becaude Fdh_H is only loosely membrane attached (Stewart, 1988) or is O_2 sensitive. The identity of the electrons carried between Fdh_H and the hydrogenase (X_1 and X_2 in Fig. 5.6) is not known; nor is it known whether they can pass electrons to an oxidase, but this would be the simplest explanation of the results described in this chapter. Proton stoichiometries for the formate to O_2 reaction catalysed by Fdh_N of $4H^+/2e$ have been measured, suggesting that the enzyme can pump protons (Jones, 1980a, b, 1979). Whether this is true of Fdh_H remains to be seen, but energy conservation for the oxidation of ubiquinol via cytochrome *d* alone is $2H^+/e$ (Anraku and Gennis, 1987; Section 1.4).

A model to explain the mechanism by which fermentation products could supply both reductant in the form of pyruvate, and ATP for N_2-fixation via a respiratory chain terminated by cytochrome *d* oxidase is shown in Fig. 5.6.

It is proposed that:

a) Lactate and formate are secreted during fermentation under anaerobiosis.

b) Glucose exhaustion occurs and nitrogenase activity ceases because ATP and pyruvate are no longer being made. (Pyruvate is the only source of low potential electrons for *K. pneumoniae* nitrogenase, see 1.3; Haaker & Klugkist, 1987.)

c) A shift from anaerobic to microaerobic conditions allows energization of the membrane by formate oxidation via a respiratory chain terminated by cytochrome *d*, and ongoing respiration maintains a low O_2 concentration in the vicinity of nitrogenase.

d) Lactate uptake takes place via the proton symport mechanism described by Konings (1985).

e) Under the more oxidizing conditions *in vivo* imposed by microaerobicity, the equilibrium between accumulated lactate and pyruvate should shift in the direction of pyruvate (see legend for the enzymes which could catalyse this reaction).

f) The accumulated pyruvate can supply additional formate for further respiration or more importantly low potential electrons to support nitrogenase activity.

REFERENCES

ACKRELL, B. A. C. & JONES, C. W. (1971a). The respiratory system of *Azotobacter vinelandii*. Properties of phosphorylating respiratory membranes. Eur. J. Biochem. 20, 22-28.

ACKRELL, B. A. C. & JONES, C. W. (1971b). The respiratory system of *Azotobacter vinelandii*. Oxygen effects. Eur. J. Biochem. 20, 29-35.

AMES, G. F. (1969). Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. J. Bacteriol. 95, 833-843.

ANRAKU, Y. & GENNIS, R. B. (1987). The aerobic respiratory chain of *Escherichia coli*. Trends Biochem. Sci. 12, 262-266.

APPELBY, C. A. (1984). Leghaemoglobin and *Rhizobium* respiration. Ann. Rev. Plant Physiol. 35, 443-478

APPLEBY, C. A. & BERGERSEN, F. J. (1980). Preparation and the experimental use of leghaemoglobin. In Methods for evaluating Biological Nitrogen Fixation. Ed. F. J. Bergersen, pp. 315-335. J. Chichester: Wiley & Son.

AU, D. C. T., LORENCE, R. M. & GENNIS, R. B. (1985). Isolation and characterisation of an *E. coli* mutant lacking the cytochrome *o* terminal oxidase. J. Bacteriol. 161, 123-127.

BALLANTINE, S. P. & BOXER, D. H. (1985). Properties of two hydrogenases from *E. coli*: Ch 5 of Microbial gas metabolism. Ed. M. K. Poole & C. S. Dow. Academic Press.

BARRETT, J. (1956). The prosthetic group of cytochroma *a*₂. Biochem. J. 64, 626-639.

BERGERSEN, F. J. & TURNER, G. L. (1979). Systems utilising oxygenated leghaemoglobin and myoglobin as sources of free dissolved oxygen at low concentrations for experiments with bacteria. Analytical Biochemistry 96, 165-174.

BERGERSEN, F. J., KENNEDY, C. & HILL, S. (1982). The influence of low oxygen concentrations on derepression of nitrogenase in *Klebsiella pneumoniae*. J. Gen. Microbiol. 128, 909-915.

BERGERSEN, F. J. & TRINCHANT, J. C. (1985). Flux of O_2 affects apparent optimal concentrations of O_2 in suspensions of bacteria respiring microaerobically. J. Theoretical Biol. 115, 93-102.

BLIGH, E. G. & DYER, W. J. (1959). A rapid method for total lipid extraction and purification. Canadian J. Biochem & Physiol. 37, 911-917.

BOTHE, H. (1985). The role of oxygen in biological nitrogen fixation. Periodicum Biologorum 87, 111-120.

BRIDGER, W. A. & PARANCHYCH, W. (1979). Guanosine tetraphosphate: coordinator of metabolic response to nitrogen starvation in bacteria. Trends Biochem. Sci. 4, 176-179.

BRINK, B. T. & KONINGS, W. N. (1980). Generation of an electrochemical proton gradient by lactate efflux in membrane vesicles of *E. coli*. Eur. J. Biochem. 111, 59-66.

BUCHANAN-WOLLASTON, V. & CANNON, F. C. (1984). Regulation of *nif* transcription in *Klebsiella pneumoniae*. In Advances of Nitrogen Fixation Research p. 732. Edited by C. Veeger and W. A. Newton. The Hague: Nijhoff-Junk.

BUCK, M., MILLER, S., DRUMMOND, M. & DIXON, R. A. (1986). Upstream activator sequences are present in the promoters of nitrogen fixation genes. Nature 320, 374-8.

BUCK, M., CANNON, W. & WOODCOCK, J. (1987). Transcriptional activation of the *K. pneumoniae* nitrogenase promoter may involve DNA loop formation. Mol. Microbiol. 1, 243-9.

BUENO, R., PATEL, G. & MAGASANIK, B. (1985) Role of *gln B* and *gln D* gene products in regulation of the gln ALG operons of *E. coli*. J. Bact. 164, 816-822.

CANNON, F. C. (1980). Genetic studies with diazotrophs. In Methods for Evaluating Biological Nitrogen Fixation pp. 367-413. Edited by F. J. Bergersen). Chichester: Wiley.

CANNON, M., HILL, S., KAVANAGH, E. & CANNON, F. C. (1985). A molecular genetic study of *nif* expression in *Klebsiella pneumoniae* at the level of transcription, translation and nitrogenase activity. Mol. Gen. Genet. 198, 198-206.

COLE, J. A. (1988a). Some new developments in anaerobic bacterial metabolism and its genetic regulation. Sci. Prog. 72, 239-58.

COLE, J. A. (1988b). Assimilatory and dissimilatory reduction of nitrate to ammonia. In The Nitrogen and Sulphur Cycles, pp 281-329. Eds. J. A. Cole & S. J. Ferguson. Symposium 42 Soc. Gen. Microbiol.

DALTON, H. & POSTGATE, J. R. (1969). Effect of oxygen on growth of *Azotobacter chroococum* in continuous culture. J. Gen. Microbiol. 54, 463-73.

DEISTUNG, J., CANNON, F. C., CANNON, M. C., HILL, S. & THORNELEY, R. N. F. (1985). Electron transfer to nitrogenase in *K. pneumoniae*. Biochem. J. 231, 745-753.

DEISTUNG, J. & THORNELEY, R. N. F. (1986). Electron transfer to nitrogenase. Biochem. J. 239, 69-75.

DIXON, R. A. (1984). The genetic complexity of nitrogen fixation. J. Gen. Microbiol. 130, 2745-2755.

DIXON, R. A. (1988). Genetic regulation of nitrogen fixation. In The Nitrogen and Sulphur Cycles pp 417-439. Soc. gen. microbiol. sym. 42. Eds. J. A. Cole & S. J. Ferguson.

DIXON, R. A., CANNON, F. & KONDOROSI, A. (1976). Construction of a P-plasmid carrying nitrogen fixation genes from *Klebsiella pneumoniae*. Nature 260, 268-271.

DIXON, R. A., KENNEDY, C., KONDOROSI, A., KRISHNAPILLAI, V. & MERRICK, M. (1977). Complementation analysis of *Klebsiella pneumoniae* mutants defective in nitrogen fixation. Mol. Gen. Genet. 157, 189-198.

DIXON, R. A., EADY, R. R., ESPIN, G., HILL, S., IACCARINO, M., KAHN, D. & MERRICK, M. (1980). Analysis of regulation of *Klebsiella pneumoniae* nitrogen fixation (*nif*) gene cluster with gene fusions. Nature 286, 128-132.

DOELLE, H. W. (1975). Bacterial Metabolism. Academic Press.

DROZD, J. W. & POSTGATE, J. R. (1970). Effect of oxygen on acetylene reduction, cytochrome content and respiratory activity of *Azotobacter chroococcum*. J. Gen. Micro. 63, 63-73.

DRUMMOND, M. H. & WOOTTON, J. C. (1987). Sequence of *nifL* from *Klebsiella pneumoniae*: mode of action and relationship to two families of regulatory proteins. Mol. Microbiol. 1, 37-44.

DUNN, S. D. (1986). Effects of modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins in western blots by monoclonal antibodies. Analytical Biochem. 157, 144-153.

EADY, R. R. (1986). Enzymology of freeliving diazotrophs. In Nitrogen Fixation. Eds. W. J. Broughton & A. Puhler. Vol. 4 Molecular Biology, pp. 1-49. Oxford: Clarendon Press.

EADY, R. R., ISSACK, R., KENNEDY, C., POSTGATE, J. R. & RATCLIFFE, H. (1978). Nitrogenase synthesis in *Klebisella pneumoniae*: comparison of ammonium and oxygen regulation. J. Gen. Microbiol. 104, 277-285.

EADY, R. R., ROBSON, R. L. & SMITH, B. E. (1988). Alternative and conventional nitrogenases. In The Nitrogen and Sulphur Cycles Symposium 42, pp. 364-382. Eds. J. A. Cole & S. J. Ferguson. Soc. Gen. Microbiol.

FILLER, W. A., KEMP, R. M., NG, J. C., HAWKES, T. R., DIXON, R. A. & SMITH, B. E. (1986). The *nif H* gene is required for the synthesis or stability of the iron-molybdeneum cofactor of nitrogenase from *K. pneumoniae*. Eur. J. Biochem. 160, 371-377.

FILSER, M., MERRICK, M. & CANNON, F. (1983). Cloning and characterisation of *nifLA* regulatory mutations from *Klebsiella pneumoniae*. Mol. Gen. Genet. 119, 485-491.

FINLAYSON, S. D. & INGLEDEW, W. J. (1985). Cytochrome *bd* of *Escherichia coli*: its isolation and study by EPR. Biochemical Soc. Trans. 13, 632-633.

FUHRHOP, J. H. & SMITH, K. M. (1975). Laboratory methods. In Porphyrins and Metalloporphyrins pp. 757-869. Edited by K. M. Smith. Amsterdam: Elsevier Pub. Co.

GALLANT, J. A. (1979). Stringent control in *E. coli*. Ann. Rev. Genet. 13, 393-415.

GALLANT, J. A. & LAZZARINI, R. A. (1976). The regulation of ribosomal RNA synthesis and degradation in bacteria. In Protein Synthesis: A series of advances pp. 309-359. Edited by E. McConkey. New York: Dekker.

GARVIE, E. I. (1980). Bacterial lactate dehydrogenases. Microbiol. Rev. 44, 106-139.

GEORGIOU, C. D., FANG, H., & GENNIS, R. B. (1987). Identification of the *cyd* C locus required for the expression of the function form of the cytochrome *d* terminal oxidase complex of *E. coli*. J. Bact. 169, 2107-12.

GEORGIOU, C. D., DUEWEKE, T. J. & GENNIS, R. B. (1988a). Regulation of expression of the cytochrome *d* terminal oxidase in *E. coli* is transcriptional. J. Bact. 170, 961-966.

GEORGIOU, C. D., DUEWEKE, T. J. & GENNIS, R. B. (1988b). β-Galactosidase gene fusions as probes for the cytoplasmic regions of subunits I and II of the membrane-bound cytochrome *d* terminal oxidase from *E. coli*. J. Biol. Chem. 263, 13130-13137.

GREEN, G. N & GENNIS, R. B. (1983). Isolation and characterisation of an *E. coli* mutant lacking cytochrome *d* terminal oxidase. J. Bact. 154, 1269-1275.

GREEN, G. N., FANG, H., LIN, R. J., NEWTON, G., MATHERS, M., GEORGIOU, C. D. & GENNIS, R. B. (1988). The nucleotide sequence of the cyt locus encoding the two subunits of the cytochrome *d* terminal oxidase of *Escherichia coli*. J. Biol. Chem. 263, 13138-13143.

GUBLER, M. & HENNECKE, H. (1986). *fix A, B* and *C* genes are essential for symbiotic and free-living, microaerobic nitrogen fixation. FEBS Lett. 200, 186-192.

GUSSIN, G. N., ROBSON, C. W. & AUSUBEL, F. W. (1986). Regulation of nitrogen fixation genes. Ann. Rev. Genet. 20, 567#591.

HAAKER, H., DE KOK, A. & VEEGER, C. (1974). Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*. Biochim. Biophys. Acta 357, 344-357.

HAAKER, H. & VEEGER, C. (1984). Enzymology of Nitrogen Fixation. Trends Biochem. Sci. 9, 188.

HAAKER, H. & KLUGKIST, J. (1987). The bioenergetics of electron transfer to nitrogenase. FEMS Microbiol. Rev. 46, 57-71.

HADDOCK, B. A. (1973). The reconstitution of oxidase activity in membranes derived from a 5-aminolevulinic acid-requiring mutant of *Escherichia coli*. Biochem. J. 136, 877-884.

HADDOCK, B. A. & SCHAIRER, H. U. (1973). Electron transport chains of *Escherichia coli* reconstitution of respiration in a 5-aminolevulinic acid-requiring mutant. Eur. J. Biochem. 35, 34-45.

HADDOCK, B. A. & JONES, C. W. (1977) Bacterial respiration. Bact. Rev. 41, 47-99.

HARRISON, D. E. F. (1972) Physiological effects of dissolved oxygen tension and redox potential on growing populations of micro-organisms. J. App. Chem. Biotech. 22, 417-440.

HARRISON, D. E. F. & PIRT, S. J. (1967). The influence of dissolved oxygen concentration on the respiration and glucose metabolism of *Klebsiella aerogenes*. J. Gen. Microbiol. 46, 193-211.

HAWKES, T. R., McLEAN, P. A. & SMITH, B. A. (1984). Nitrogenase from *nif V* mutants of *K. pneumoniae* contain an altered form of the iron molybdenum cofactor. Biochem. J. 217, 317-321.

HENDRICH, J. L. & SMITH, A. J. (1978). Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Archives of Biochemistry and Biophysics 1126, 155-164.

HILL, S. (1976a). Influence of atmospheric oxygen concentration on acetylene reduction and efficiency of nitrogen fixation in intact *Klebsiella pneumoniae*. J. Gen. Microbiol. 93, 335-345.

HILL, S. (1976b). The apparent ATP requirement for nitrogen fixation in growing *K. pneumoniae*. J. Gen. Microbiol. 95, 297-312.

HILL, S. (1985). Redox regulation of entneric *nif* expression is independent of the *fnr* gene product. FEMS Microbiol. Lett. 29, 5-9.

HILL, S. (1988). How is nitrogenase regulated by oxygen? FEMS Microbiol. Rev. 54, 111-130.

HILL, S. & KAVANAGH, E. P. (1980). Roles of *nif F* and *nif J* gene products in electron transport to nitrogenase in *K. pneumoniae*. J. Bacteriol. 141, 470-475.

HILL, S., KENNEDY, C., KAVANAGH, E., GOLDBERG, R. B. & HANAU, R. (1981). Nitrogen fixation gene (*nif L*) involved in the oxygen regulation of nitrogenase synthesis in *K. pneumoniae*. Nature 290, 424-426.

HILL, S., TURNER, G. L. & BERGERSEN, F. J. (1984a). Synthesis and activity of nitrogenase in *Klebsiella pneumoniae* exposed to low concentrations of oxygen. J. Gen. Microbiol. 130, 1061-1067.

HILL, S., TURNER, G. L. & BERGERSEN, F. J. (1984b). Synthesis and activity of nitrogenase in *K. pneumoniae* exposed to low oxygen concentrations. In Advances in Nitrogen Fixation Research, pp. 260-266. Eds. C. Veeger & W. E. Newton. The Hague: Nijhoff/Junk.

HILL, S. & KAVANAGH, E. (1988). Oxygen control and autoregulation of the *nif* structural operon in *Klebsiella pneumoniae* involves nitrogenase function. In Nitrogen Fixation: One hundred years after Gustav Fischer. Eds. H. Bothe, F. J. D. de Bruijn & W. E. Newton.

HOFFMAN, S., MORGAN, V. 7 DER VARTANIAN, D. V. (1979). Respiratory-chain characteristics of mutants of *Azotobacter vinelandii* negative to tetramethyl phenylenediamine oxidase. Eur. J. Biochem. 100, 19-27.

HOFMAN, S., IRWIN, R., CARREIRA, L. A., MORGAN, T., ENSLEY, B. & DER VARTANIAN, D. V. (1980). Studies of photochemical action spectra on N.N.N^1,N^1-Tetramethyl-p-phenylene-diamine-Oxidase negative mutants of *Azotobacter vinelandii*. Eur. J. Biochem. 105, 177-185.

HOOVER, T. R., SHAH, V. K., ROBERTS, G. & LUDDEN, P. W. (1985). *Nif V* dependent low molecular weight factor required for *in vitro* synthesis of iron molybdenum cofactor of nitrogenase. J. Bacteriol. 167, 999-1003.

HWANG, J. C., CHEN, C. H. & BURNS, R. H. (1973). Inhibition of nitrogenase catalysed reductions. Biochim. et Biophys. Acta 292, 256-270.

INGLEDEW, W. J. & POOLE, R. K (1984). The respiratory chains of *Escherichia coli* Microbiol. Rev. 48, 222-271.

JOHNSON, E. A. & LEVINE, R. L. (1985). Inactivation of glycerol dehydrogenase of *K. pneumoniae* and the role of divalent cations. J. Bact. 164, 479-483.

JONES, C. W. (1978). Microbial oxidative phosphorylation. Biochem. Soc. Trans. 6, 361-3.

JONES, C. W., BRICE, J. M. & EDWARDS, C. (1978). Bacterial cytochromes and oxidases and respiratory chain energy conservation. In Functions of Alternative Terminal Oxidases pp. 89-98. Eds H. Degn, D. Lloyd & G. C. Hill. Oxford: Pergamon Press.

JONES, R. W. (1979). Hydrogen-dependent proton translocation by membrane vesicles from *E. coli*. Biochem. Soc. Trans. 7, 1136-1137.

JONES, R. W. (1980a). Proton translocation by the membrane-bound formate dehydrogenase of *E. coli*. FEMS Microbiol. Lett. 8, 167-171.

JONES, R. W. (1980b). The role of membrane-bound hydrogenase in the energy-conserving oxidation of molecular hydrogen by *E. coli*. Biochem. J. 188, 345-350.

KENNEDY, C. & DRUMMOND, M. (1975). The use of cloned *nif* regulatory elements from *K. pneumoniae* to examine *nif* regulation in *Azotobacter vinelandii*. J. Gen. Microbiol. 131, 1787-1795.

KIM, Y-M., AHN, K-J., BEPPU, T. & UOZUMI, T. (1986). Nucleotide sequence of the *nifLA* operon of *Klebsiella oxytoca* NG13 and characterisation of the gene products. Mol. Gen. Genet. 205, 253-259.

KITA, K., KONISHI, K. & ANRAKU, Y. (1984a). Terminal oxidases of the *Escherichia coli* aerobic respiratory chain: purification and properties of cytochrome b_{562} O complex from cells in early exponential phase of aerobic growth. J. Biol. Chem. 259, 3368-3374.

KITA, K., KONISHI, K. & ANRAKU, Y. (1984b). Terminal oxidases of *Escherichia coli* respiratory chain. Purification and properties of the cytochrome *b*-558*d* complex from cells grown with limited oxygen, and evidence of a branched electron carrying system. J. Biol. Chem. 259, 3375-3381.

KLEINER, D. (1979). Regulation of ammonium uptake and metabolism by nitrogen-fixing bacteria III. *Clostrideum pasteurianum* Arch. Microbiol. 120, 263-270.

KLEINER, D. & PHILIPS, S. (1981). Relative levels of guanosine 5'-diphosphate, 3'-diphosphate (ppGpp) in some N_2 fixing bacteria during derepression and repression of nitrogenase. Arch. Microbiol. 128, 341-342.

KLUGKIST, J., VOORBERG, J., HAAKER, H. & VEEGER, C. (1986). Characterisation of three different flavodoxins from *Azotobacter vinelandii*. Eur. J. Biochem. 155, 33-40.

KOHN, L. D. & KABACK, H. R. (1973). Mechanisms of active transport in isolated bacterial membrane vesicles. J. Biol. Chem. 243, 7012-7017.

KONINGS, W. N. (1985). Generation of metabolic energy by end-product efflux. TIBS 10, 317-319.

KRANZ, R. G., BARASSI, C. J. & GENNIS, R. B. (1984). Immunological analysis of the heme proteins present in aerobically-grown *Escherichia coli*. J. Bacteriol. 158, 1191-1194.

KRANZ, G. R. & GENNIS, R. B. (1985). Immunological investigation of the distribution of cytochromes related to the two terminal oxidases of *Escherichia coli* in other Gram-negative bacteria. J. Bacteriol. 161, 709-713.

KUSHI, S., SEI, K. & KEENER, J. (1986). Nitrogen regulation in enteric bacteria. In Regulation of Gene Expression 25 Years On, pp. 139-154. Eds. I. Booty & G. F. Higgins. Cambridge University Press.

LAANE, C., KRONE, W., KONINGS, W. N., HAAKER, H. & VEEGER, C. (1979). The involvement of the membrane potential in nitrogen fixation by bacteroids of *Rhizobium leguminosarum*. FEBS Lett. 103, 328-332.

LAANE, C., KRONE, W., KONINGS, W. N., HAAKER, H. & VEEGER, C. (1980). Short-term effect of ammonium chloride on nitrogen fixation by *Azotobacter vinelandii* and by bacteroids of *Rhizobium leguminosarum*. Eur. J. Biochem. 103, 39-46.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

LORENCE, R. M., KOLAND, J. G. & GENNIS, R. B. (1986). Coulometric and spectroscopic analysis of the purified cytochrome *d* oxidase complex of *Escherichia coli*: evidence for the identification of 'cytochrome a^1' as cytochrome b_{595}. Biochemistry 25, 2314-2321.

LORENCE, R. M., CARTER, K., GENNIS, R. B., MATSUSHITA, K. & KABACH, H. (1988). Trypsin proteolysis of the cytochrome *d* complex of *E. coli* selectively inhibits ubiquinol oxidase activity while not affecting N,N,N',N'-Tetramethyl-p-phenylenediame activity. J. Biochem. 263, 5271-5276.

McINERNEY, M. J., HOLMES, S. K. & DER VARTANIAN, D. V. (1982). Effect of O_2 limitation on growth and respiration of the wild type and on ascorbate-tetramethyl-p-phenyline diamine oxidase-negative mutant strain of *Azotobacter vinelandii*. J. Bioenergetics & Biomembranes 14, 451-56.

McLEAN, H., HANSEN, LEMLEY, KOBLAN, ORME JOHNSON (1986). *K. pneumoniae nif M* gene product required for stabilisation and activation of the nitrogenase iron protein in *E. coli*. J. Biol. Chem. 261, 772-778.

MATSUSHITA, K. & KABACK, R. H. (1986). D(-) lactate oxidation and generation of the proton electrochemical gradient in membrane vesicles from *E. coli* GR19N and in proteoliposomes reconstituted with purified D(-) lactate dehydrogenase and cytochrome *o* oxidase. Biochem. 25, 2321-2327.

MERRICK, M. J. & DIXON, R. A. (1984). Why don't plants fix nitrogen? Trends in Biotech. 2, 162-165.

MERRICK, M. J. (1988). Regulation of nitrogen assimilation by bacteria. In The Nitrogen and Sulphur Cycles, pp. 331-361, Symposium 42. Eds. J. A. Cole & S. J. Ferguson. Soc. for Gen. Microbiol.

MERRICK, M. J., HILL, S., HENNECKE, H., HAHN, M., DIXON, R. & KENNEDY, C. (1982). Repressor properties of the *nifL* gene product in *Klebsiella pneumoniae*. Mol. Gen. Genet. 185, 75-81.

MERRICK, M. J., AUSTIN, S., BUCK, M., DIXON, R., DRUMMOND, M., HOLTEL, A. & MacFARLANE, S. (1987). Regulation of nitrogen assimilation in enteric bacteria. In Phosphate Metabolism in Micro-organisms. Eds. A. Torriani & S. Silver. Washington DC: ASM Press.

MILLER, M. J. & GENNIS, R. B. (1983). Purification and characterisation of the cytochrome *d* terminal oxidase complex from *Escherichia coli*. J. Biol. Chem. 248, 9159-9165.

MILLER, M. J. & GENNIS, R. B. (1985). J. Biol. Chem. 260, 14003-14008.

MILLER, M. J., HERMODSON, M. & GENNIS, R. B. (1988). The active form of the cytochrome *d* terminal oxidase complex of *Escherichia coli* is a heterodimer containing one copy of each of the two subunits. J. Biol. Chem. 263, 5235-5240.

MOSS, F. (1956). Adaptation of the cytochromes of *Aerobacter aerogenes* in response to environmental oxygen tension. Australian J. Exp. Biol. 34, 395-406.

MURPHY, M. J., SIEGEL, L. M., TOUE, S. R. & KARNIN, H. (1974). Sirohaem a new prosthetic group participating in six electron reduction reactions catalysed by both sulphite and nitrite reductases. Proc. Natl. Acad. Sci. USA 71, 612-616.

NAIR, B. M. & EADY, R. R. (1984). Nitrogenase synthesis in *Klebsiella pneumoniae*: Enhanced *nif* expression without accumulation of Guanosine 5'-Diphosphate 3'-Diphosphate. J. Gen. Microbiol. 130, 3663-3069.

PAUL, W. & MERRICK, M. J. (1987). The nucleotide sequence of the *nif* M gene of *K. pneumoniae* and identification of a new *nif* gene - *nif*2. Eur. J. Biochem.

PEEL, J. L. (1984). The use of electron acceptors and carriers. Chapter 1, pp. 367-413. Chichester: Wiley.

POOLE, R. K. (1988). Bacterial cytochrome oxidases. In Bacterial Energy Transduction pp. 231-291. Ed. C. Anthony. London: Academic Press.

POOLE, R. K., WARING, A. J. & CHANCE, B. (1979). The reaction of cytochrome *o* in *Escherichia coli* with oxygen low-temperature and spectral studies. Biochem. J. 184, 379-389.

POOLE, R. K. & CHANCE, B. (1981). The reaction of cytochrome *o* in *Escherichia coli* K12 with oxygen; evidence for a spectrally and kinetically distinct cytochrome *o* in cells from oxygen-limited cultures. J. Gen. Microbiol. 126, 277-287.

POOLE, R. K. & WILLIAMS, H. W. (1987). Proposal that the function of membrane bound a_1-like haemoprotein (cytochrome b_{595}) in *E. coli* is a direct electron donation to cytochrome *d*. FEBS Lett. 217, 49-52.

POSTGATE, J. R. (1982). The Fundamentals of Nitrogen Fixation. Cambridge University Press.

RAMOS, J. R. & ROBSON, R. L. (1985a). Isolation and properties of mutants of *Azotobacter chroococum* defective in aerobic N_2 fixation. J. Gen. Microbiol. 131, 1449-58.

RAMOS, J. R. & ROBSON, R. L. (1985b). Lesions in citrate synthase that affect aerobic nitrogen fixation by *Azotobacter chroococcum*. J. Bact. 162, 746-51.

REDINBAUGH, M. G. & TURLEY, R. B. (1986). Adaptation of the Bicinchoninic acid protein assay for use with microtitre plates and sucrose gradient fractions. Analytical Biochem. 153, 267-271.

REISENBERG, D., ERDEI, KONDOROSI, E. & KARI, C. (1982). Positive involvement of ppGpp in derepression of *nif* operon in *Klebsiella pneumoniae*. Mol. Gen. Genet. 185, 198-204.

RENNIE, R. J., FUNNEL, A. & SMITH, B. E. (1978). Immunochemistry of nitrogenase as a probe for the enzyme mechanism: Evidence for multiple forms and an $MgATP^{2-}$ binding site on the MoFe protein. FEBS Letters 91, 158-161.

RICE, C. W. & HEMPFLING, W. P. (1978). Oxygen-limited continuous culture and respiratory enzymes in *Escherichie coli*. J. Bacteriol. 134, 115-124.

ROBERTS, G. P., MacNEIL, T., MacNEIL, D. 7 BRILL, W. J. (1978). Regulation and characterisation of protein products coded by the *nif* genes of *K. pneumoniae*. J. Bacteriol. 136, 267-279.

ROBSON, R. L. (1984). Identification of possible adenine nucleotide binding sites in nitrogenase proteins by amino acid sequence comparison. FEBS Letts. 173, 394-398.

ROTHERY, R. A. & INGLEDEW, W. J. (1989). The cytochromes of anaerobically-grown *Escherichia coli*: an electron paramagnetic resonance study of the *in situ* cytochrome *bd* complex. Biochem. J. (in press).

SCOTT, R. I. & POOLE, R. K. (1987). Evidence for unequal segregation of cytochromes at cell division in *Escherichia coli*. FEMS Microbiol. Lett. 43, 1-4.

SHAH, V. K. & BRILL, W. J. (1977). Isolation of the iron molybdenum cofactor from nitrogenase. Proc. Natl. Acad. Sci. USA 74, 3249-3253.

SHAH, V. K., STACEY, G. & BRILL, W. J. (1983). Electron transport to nitrogenase: purification and characterisation of pyruvate:flavdoxin oxidoreductase, the *nifJ* product. J. Biol. Chem. 258, 12064-12068.

SHAH, V. K., IMPERIAL, J., VGALDE, R. A., LUNDDEN, P. W. & BRILL, W. J. (1986). *In vitro* synthesis of the iron molybdenum cofactor of nitrogenase. Proc. Natl. Acad. Sci. USA 83, 1636-1640.

SHEN, S-C., XUE, Z-t., KONG, Q-t & WU, Q-L. (1983). An open reading frame upstream from the *nif* H *gene of K. pneumoniae*. Nucleic Acids Res. 11, 4241-50.

SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J. & KLENK, D. C. (1985). Measurement of protein using bicinchoninic acid. Analytical Biochem. 150, 76-85.

SMITH, A., HILL, S. & ANTHONY, C. (1988). A haemoprotein is not involved in the control by oxygen of enteric nitrogenase synthesis. J. Gen. Microbiol. 134, 1499-1507.

SPRENT, J. I. (1979). The biology of nitrogen-fixing organisms. McGraw-Hill.

STEWART, V. (1988). Nitrate respiration in relation to facultative metabolism in Enterobacteria. Microbiol. Rev. 52, 190-232.

TEIXEIRA DE MATTOS, M. J. & TEMPEST, D. W. (1983). Metabolic and energetic aspects of the growth of *Klebsiella aerogenes* NCTC418 on glucose in anaerobic chemostat culture. Arch. Microbiol 134, 80-83.

TEIXEIRA DE MATTOS, M. J. STREEKSTRA, H. & TEMPEST, D. W. (1984). Metabolic uncoupling of substrate level phosphorylation in anaerobic glucose-limited chemostat cultures of *Klebsiella aerogenes* NCTC418. Arch. Microbiol. 139, 260-264.

THOMAS, P. E., RYAN, D. & LEVIN, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulphate polyacrylamide gels. Analytical Biochem. 75, 168-176.

THORNELEY, R. N. F. & LOWE, D. J. (1985). Kinetics and mechanism of the nitrogenase enzyme system. In Molybdenum Enzymes pp. 221-284. Ed. T. G. Spiro. New York: Wiley & Sons.

TIMKOVITCH, R., CORK, M. S., GENNIS, R. B. & JOHNSON, P. Y. (1985). J. Am. Chem. Soc. 107, 6069-6075.

TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

UGURBIL, K., BROWN, T. R., HOLLANDER, J. A., GLYNN, R. & SCHULMAN, R. G. (1978). High resolution ^{13}C NMR studies of glucose metabolism in *E. coli*. Proc. Natl. Acad. Sci. USA 75, 3742-3746.

UNEMOTO, T. & HAYASHI, M. (1988). Characterisation of the respiratory chain-linked NADH-Quinone reductase from *E. coli*. 5th European Bioenergetics Conference Reports, short reports, Vol. 5, p 151.

YATES, M. G. (1988). The role of oxygen and hydrogen in nitrogen fixation. In The Nitrogen and Sulphur Cycles, pp. 383-417. Eds. J. A. Cole & S. J. Ferguson. Symposium 42. Soc. Gen. Microbiol.

YOCH, D. C. (1974). Electron transport carriers involved in nitrogen fixation by the coliform *K. pneumoniae*. J. Gen. Microbiol. 83, 153-164.

YOUNGMAN, R. J. (1984). Oxygen activation: is the hydroxyl radical always relevant? Trends Biochem. Sci. 9, 280-283.

APPENDIX 1

Other components of the membrane bound aerobic respiratory chain of *K. pneumoniae* expressed during nitrogen fixation

This appendix is designed to describe briefly two other components present in the membranes of nitrogen fixing *K. pneumoniae* both of which are depicted in the model shown in Fig. 5.6 Section 5.3. No additional conclusions will be drawn, this appendix merely serves to satisfy the author's desire to account for his laboratory time!

A1) D(-) lactate: ubiquinone reductase

In Section 5.3, reference is made to the measurement of lactate concentration in culture supernatants using the enzyme D(-) lactate: Ubiquinone reductase (D(-)iLDH) purified from *K. pneumoniae* (see Section 2.3j). In the model depicted in Fig. 5.6 D(-)iLDH is proposed to have a role in lactate oxidation. Using the assay described in Section 2.3j, D(-)iLDH was found to be constitutively expressed in all the growth states described in Table 4.1. It was associated with the cytoplasmic membrane fraction, requiring low concentrations of deoxycholate (0.2% w/v) to release it (Section 4.2c), but was subsequently soluble in the absence of detergent. It was purified by ammonium sulphate fractionation (30-70% cut) followed by anion exchange chromatography on fplc Mono Q), eluting at 200 mMKCl. The pure complex was a flavoprotein absorbing at 450 nm with a shoulder at 480 nm. The 450 nm peak disappeared when the enzyme was reduced with excess D(-) lactate and reappeared on oxidation with a larger excess of ubiquinone-1. The enzyme was free of contaminating cytochrome and had an apparent Mr of 115K. The complex consisted of two polypeptides of apparent Mr 63K and 26K by SDS-PAGE. It was tested for its ability to oxidize a variety of potential substrates; it oxidized only D(-) lactate (Vmax = 23 μmol DCPIP reduced min^{-1} mg protein $^{-1}$) and

D/L hydroxybutyrate, at half the rate measured with D(-) lactate. Oxidation of lactate by membrane particles was sensitive to the quinone antagonist HQNO (I_{50} = 20 μM) and the purified enzyme could reduce ubiquinone-1 (Km^{UQ-1} = 4 μM) at a rate 80% of that obtained with DCPIP as electron acceptor (Km^{DCPIP} = 33 μM). The D(-)iLDH purified from *K. pneumoniae* was very similar to that reported from *E. coli* (Futai, 1973; Pratt, 1979; Kohn & Kaback, 1973). Two properties were notably different: The *K. pneumoniae* enzyme appeared to possess an extra small subunit and was able rapidly to reduce ubiquinone-1.

Table A.1

This table is supplementary to Table 4.1 in Chapter 4; it reports the measured D(-) lactate concentration in some of the growth states described in Table 4.1, by the D(-)iLDH method (Section 2.3j).

Growth state	D(-) lactate concentration /mM†
(1)	1.4-1.6
(6)	0.7-0.5
(8)	2.3-2.7
(9)	0

† range, two measurements only

A2) Cytochrome b_{556}

During anaerobic or microaerobic N_2-fixing growth the cytochrome *d* oxidase complex accounted for 50 per cent of the membrane-bound cytochrome *b* (see Table 4.1). Like D(-)iLDH a cytochrome *b* was released by treatment of the membranes with 0.2% w/v deoxycholate (Section 4.2c). Solubilized cytochrome *b* was brought to 20 per cent ammonium sulphate saturation and absorbed to a phenyl-sepharose column and eluted with a decreasing ammonium sulphate gradient. The cytochrome eluted at 2 per

cent saturation but required 0.1% w/v Triton X-100 for solubility. It was purified to a single polypeptide (Mr17K SDS-PAGE) by anion exchange chromatography on fplc MonoQ. It appeared to be oligomeric during gel filtration in the presence of 0.1% w/v Triton X-100 with an Mr of 100K. The α peak of the cytochrome absorbed at 556 nm (77K) and had a haem *b* content of 10.9 nmol/mg protein. The A412/280 ratio was 1.2 and the mid point redox potential at pH 7.0 was 5 mV.

No physiological role could be assigned to cytochrome b_{556} from *K. pneumoniae*; there are however strong similarities to the b_{556} isolated from *E. coli* by Kita *et al.* (1978). *E. coli* b_{556} has been shown to be the product of the *Sdh C* gene (Kita *et al.*, 1989) and is the membrane anchor subunit of the succinate dehydrogenase complex. Under some conditions, it can be isolated independently (Kita *et al.*, 1978; Kita *et al.*, 1989). One would not expect succinate dehydrogenase to be expressed under anaerobic conditions (Iuchi & Lin, 1988; Iuchi *et al.*, 1989). However fumarate reductase is expressed at a low level during anaerobic fermentation and is noted to have a remarkably similar subunit composition to succinate dehydrogenase, including a membrane anchor subunit which is a b-type cytochrome (Cole *et al.*, 1985).

References to Appendix 1

COLE, S. T., CONDON, C., LEMIRE, B. D. & WEINER, J. H. (1985). Biochimica et Biophysica Acta 811, 381-403.

FUTAI, M. (1973). Membrane bound D(-) lactate dehydrogenase from *E. coli*. Purification and properties. Biochem. 12, 2469-2475.

IUCHI, S., CAMERON, D. C. & LIN, E. C. C. (1989). Asecon second global regulator gene (*arc* B) mediating repression of enzymes in aerobic pathways of *E. coli*. J. Bact. 171, 868-873.

IUCHI, S. & LIN. E. C. C. (1988). *arc* A (dye), a global regulatory gene in *E. coli* mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. USA 84, 3901-3905.

KITA, K., VIBAT, R. T., MEINHARDT, S., GUEST, J. R. & GENNIS, R. B. (1989). One step purification from *E. coli* of complex II (Succinate: Ubiquinone oxidoreductase). Associated with succinate reducible cytochrome b_{556}. J. Biol. Chem. 264, 2672-2677.

KITA, K., YAMATO, I. & ANRAKU, Y. (1978). Purification and properties of cytochrome b_{556} in the respiratory chain of aerobically grown *E. coli* K12. J. Biol. Chem. 253, 8910-8915.

KOHN, L. D. & KABACK, R. H. (1973). Mechanisms of active transport in isolated bacterial membrane vesicles. J. Biol. Chem. 243, 7012-7017.

PRATT, E. A., FUNG, L. W. M., FLOWERS, J. A. & HO, C. (1979). Membrane-bound D(-) lactate dehydrogenase from *E. coli*. Purification and properties. Biochem. 18, 312-316.

APPENDIX 2

Publications which resulted from this work

HILL, S., VIOLLET, S., SMITH, A. T. & ANTHONY, C. (1989). Roles for enteric cytochrome d oxidase complex in N_2 fixation and microaerobiosis. J. Bact. (submitted).

SMITH, A., HILL. S. & ANTHONY, C. (1988). A haemoprotein is not involved in the control by oxygen of enteric nitrogenase synthesis. J. Gen. Micro. 134, 1499-1507.

SMITH, A., HILL, S. & ANTHONY, C. (1988). The purification and characterization of the cytochrome *d* terminal oxidase complex of *Klebsiella pneumoniae* and determination of its oxygen affinity: Proposal of a physiological role during microaerobic nitrogen fixation. Fifth European Bioenergetics Conference, Short reports. 5, 99.

SMITH, A., HILL, S. & ANTHONY, C. (1989). The purification, characterization and role of the cytochrome *d* terminal oxidase complex of *Klebsiella pneumoniae* during nitrogen fixation. J. Gen. Micro. (in press).

Figure legends

Fig. 1. Absorption spectra of membranes from bacteria grown on succinate with tryptone as nitrogen source in oxygen-limited, microaerobic conditions. The protein concentration was 11.3 mg per ml. a) Dithionite-reduced minus persulphate-oxidised difference spectrum; b) (Reduced plus CO) minus reduced difference spectrum (after sparging with CO for 30s); c) after sparging with CO for 2 min.

Fig. 2. Absorption spectra of membranes from bacteria grown on glycerol with tryptone as nitrogen source in nitrogen-limited conditions (oxygen concentration, 10-20uM). The protein concentration was 6.2 mg per ml. a) Dithionite-reduced minus persulphate-oxidised difference spectrum; b) (Reduced plus CO) minus reduced difference spectrum after sparging with CO for 2 min.

Fig. 3. Absorption spectra of membranes from nitrogen-fixing bacteria grown on glucose in oxygen-limited, microaerobic conditions (oxygen concentration less than 1 uM). The protein concentration was 18 mg ml^{-1}. a) Dithionite-reduced minus persulphate-oxidised difference spectrum; b) (Reduced plus CO)-minus-(reduced) difference spectrum after sparging with CO for 2 min.

Fig. 4. Absorption spectra (at room temperature) of dithionite-reduced cytochrome *d* oxidase. The sample contained 65ug per ml of purified oxidase in 75 mM-potassium phosphate (pH 6.3) containing 150 mM-KCl, 1 mM-EDTA and 4 mM-Zwittergent. ____, dithionite reduced oxidase; ----, dithionite-reduced oxidase after sparging CO for 30 s.

Fig. 5. Absorption spectra (room temperature) of the air-oxidised form of the cytochrome *d* complex. Conditions are as described in Fig. 4. Spectra are as follows: ____, air oxidised; -----, air oxidised after sparging with CO for 30

s; ....., spectrum after removal of all oxygen from sealed cuvette by addition of 5 mM ubiquinol-1 and 16 mM dithiothreitol followed by oxidation with a trace of hydrogen peroxide (6 ul of 2% H_2O_2).

Fig. 6. Low temperature difference spectra (77K) of the cytochrome *d* complex. Conditions are as described in Fig. 4 except that the protein concentration was doubled (130 ug per ml) and the light path was 2 mm. a) Dithionite-reduced minus air-oxidised (oxygenated) difference spectrum; b) (dithionite-reduced plus CO) minus dithionite-reduced difference spectrum (CO was sparged for 30 s before freezing).

Fig.7. The oxygen affinity of cytochrome *d* oxidase

The deoxygenation of soyabean leghaemoglobin (50 uM) was followed over 2 min in a sealed cuvette containing 5ug of oxidase, ubiquinol-1 (55 uM) and 0.05% Tween 20 at pH 7.4 as described in Methods. a) Computer-aided iterative fit to the Michaelis Menten equation of initial rates , demonstrating the simple hyperbolic curve that describes the reaction of the oxidase with oxygen; b) Hanes plot of the data presented in Fig. 7a.

Table 1. *The effect of growth conditions on synthesis of cytochromes in continuous culture*

K. pneumoniae was grown as described in methods with a dilution rate (D) of 0.15 h^{-1}. The glucose concentration was 0.5% except for growth conditions 2 and 11, when it was 2%. The concentration of ammonium chloride in 3 and 7 was 6 mM. The concentration of fumarate in 4 was 30 mM. The concentration of tryptone was 0.5 gl^{-1} in condition 5, 0.7 gl^{-1} in 9 and 11, and 1 gl^{-1} in 8 and 10.

Cytochrome concentrations in membranes were measured from difference spectra of the sort illustrated in Figs. 1-3; they are expressed as pmol/mg membrane protein. The values presented for cytochrome *b* are total values including the cytochrome *o*. Although the results presented here are from single steady state cultures, each growth state was repeated at least once and the results were always within 10 % of those recorded here. The lowest concentration of the cytochromes that could be determined with confidence was 10 pmol/mg membrane protein. In microaerobic conditions (oxygen-limited) the oxygen concentration was below the limit of detection by the oxygen electrode (1 uM). In aerobic conditions the oxygen concentrations were 10-200uM. All cultures grown with dinitrogen as sole nitrogen source were shown to contain *nif* polypeptides as detected by immunoassay. In addition to these cultures microaerobic cultures with glycerol as carbon source and tryptone as nitrogen source also had low levels (about 5% of nitrogen fixing cultures) of *nif* polypeptides; this is indicated by +/- in the Table. n.d., less than 10 pmol cytochrome (mg membrane protein)$^{-1}$.

Table 1.

	Carbon source	Oxygen status	*nif* expression	Growth limitation	Nitrogen source	Cytochrome concn. (pmol/mg protein) Cyt b	Cyt o	Cyt *d*
1.	Glucose	anaerobic	+	carbon	dinitrogen	120	nd	60
2.	Glucose	anaerobic	+	nitrogen	dinitrogen	110	nd	40
3.	Glucose	anaerobic	-	carbon	ammonia	100	nd	40
4.	Glucose + fumarate	anaerobic	-	carbon	dinitrogen	160	nd	70
5.	Glucose	anaerobic	-	carbon	tryptone	110	nd	50
6.	Glucose	microaerobic	+	oxygen	dinitrogen	130	nd	60
7.	Glucose	microaerobic	-	oxygen	ammonia	150	nd	60
8.	Glycerol	microaerobic	+/-	oxygen	tryptone	560	180	170
9.	Succinate	microaerobic	-	oxygen	tryptone	640	430	390
10.	Glycerol	aerobic	-	nitrogen	tryptone	360	220	nd
11.	Succinate	aerobic	-	nitrogen	tryptone	500	290	nd

Table 2. *Purification of the cytochrome d oxidase complex*

The method is is fully described in the methods section. The cytochrome *b*-558 component was determined from the reduced minus oxidsed difference spectrum, using the extinction coefficient of 22 mM^{-1} cm^{-1} (560-580nm) (Green *et al.*, 1986); the cytochrome *d* component was determined from the reduced minus oxygenated difference spectrum, using the extinction coefficient of 7.4 $mM^{-1}cm^{-1}$ (628-607nm) (Lorence *et al.*, 1986). The values given for the membranes are prior to detergent washing. The purification factor presented is based on specific cytochrome *d* content. When based on activities of the oxidase measured in 0.05% Triton X-100 using ubiquinol-1 as substrate, the purification factor was 5.6 overall, presumably due to loss of some activity during purification.

Purification step	Protein (mg)	Cytochrome content (nmol/mg)		Abs_{412nm}/Abs_{280}	Purification	Yield (%)
		Cyt *b*-558	Cyt *d*			
Membranes	638	1.7	2.0	-	1	100
Solubilised membranes	224	3.7	4.8	0.12	2.5	87
DEAE-Sepharose	27	10.4	13.3	0.54	6.8	29
Mono-Q	6	15.6	19.3	0.92	9.9	9

Table 3. *Steady state kinetic parameters of the cytochrome d oxidase complex*

All reaction mixtures contained 0.05% Triton X-100 and all rates were corrected for any auto-oxidation of substrates. n.d., not determined.

Substrate	pH	Km value (mM)	V_{max} (umol O_2/min/mg protein)
Ubiquinol-1	7.0	0.22	50
Ubiquinol-1	7.9	0.21	117
Duroquinol	7.0	0.19	10
Duroquinol	7.9	nd	18
TMPD	7.0	2.8	32

Table 1 *The dependence of cytochrome d oxidase activity on detergents or phospholipids*

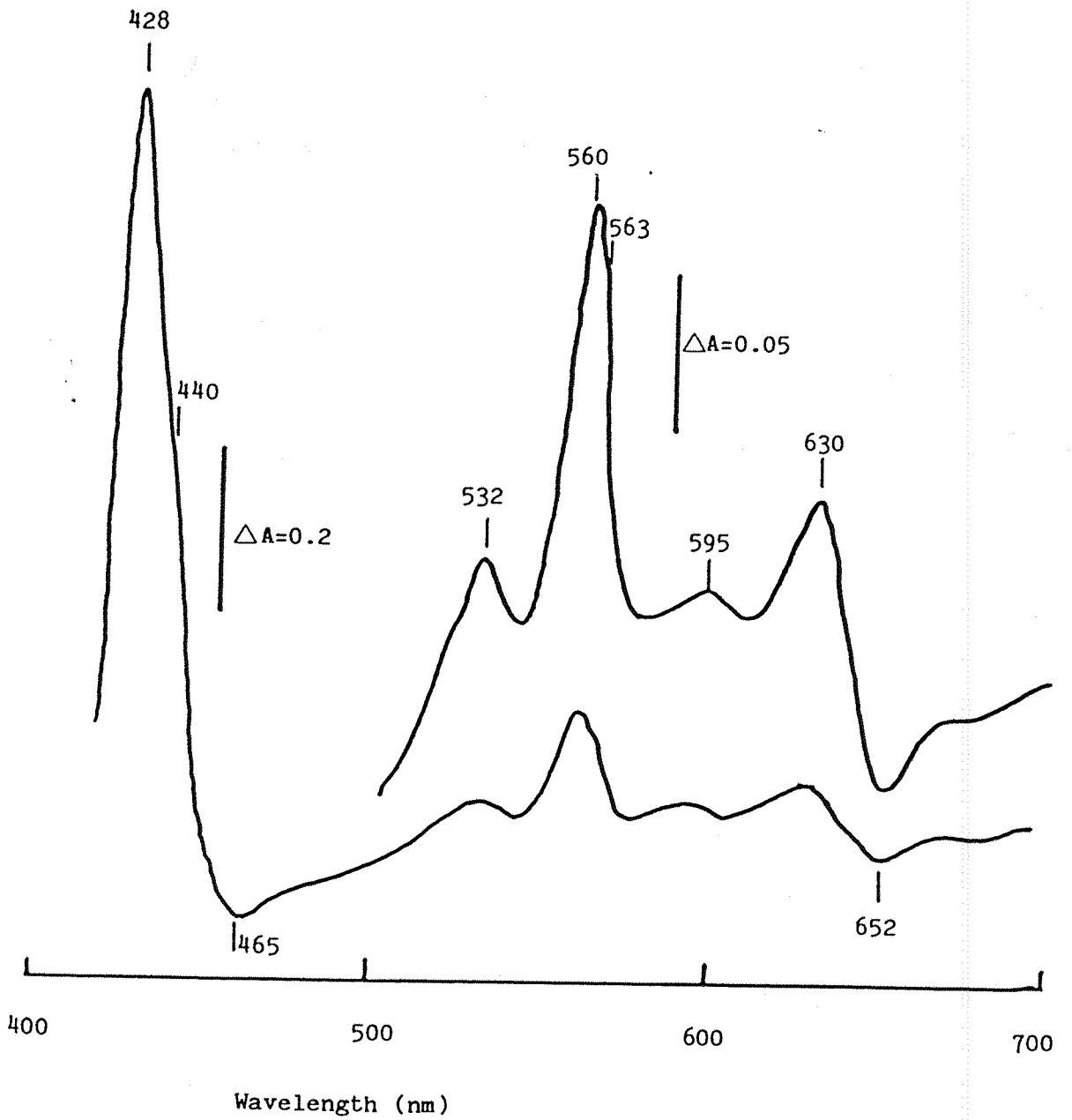
Activity was measured in the standard oxygen electrode assay system with duroquinol (1mM) as substrate using oxidase isolated in Zwittergent. The values in parentheses are for oxidase which was first transferred to 5mM cholate by passage down a PD10 gel filtration column. To measure the effect of phospholipid on activity of the oxidase equal volumes of purified oxidase were mixed with phospholipid (previously solubilised in Tris buffer (pH 7.9) containing 50mM cholate as described in Methods. The final concentrations of phospholipid in the 'reconstitution' mixtures were as follows (mg ml^{-1}): phosphatidyl choline (10), phosphatidyl glycerol (9.5), phosphatidyl ethanolamine (5.0) and total phospholipid from *K. pneumoniae* (5.0). Activity of the oxidase was then determined by using 0.01 ml of this mixture in a 1ml reaction mixture in an oxygen electrode. All the 'reconstitution' assays recorded below therefore contain 0.5 mM cholate. For the control recorded in the Table, cholate was ommitted from the 'reconstitution' mixture. The recorded values are averages of 3 assays, all of which gave values within 5% of those recorded. Although no systematic examination of the effect of phospholipid concentration was undertaken, doubling of the phospholipid concentrations did not increase the activities above the values recorded below.

It should be noted that the incubation period in Tris buffer, which was part of the 'reconstitution' process, led to a decrease to 48% of the specific activity of the oxidase (assayed in the presence of Tween 20); the activities recorded after 'reconstitution' should therefore be compared with a control value of 5.7 umol/min/mg.

Table 4 contd.

Activity in standard assay		Activity after 'reconstitution'	
Detergent	Specific activity (umol O_2/min/mg)	Phospholipid	Specific activity (umol O_2/min/mg)
None	0.02 (0.02)	None	0.70
Triton X-100 (0.05%)	4.93 (5.22)	Phosphatidyl choline	1.04
Tween 20 (0.05%)	11.94 (13.00)	Phosphatidyl glycerol	7.46
Cholate (0.05%)	0.07 (0.14)	Phosphatidyl ethanolamine	7.02
Zwittergent (0.5%)	0.02 (0.02)	Total phospholipid from *K. pneumoniae*	8.50
Cholate (0.05%) plus Tween 20 (0.05%)	7.31		

Figure 1a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Wavelength (nm)

Figure 1b,c

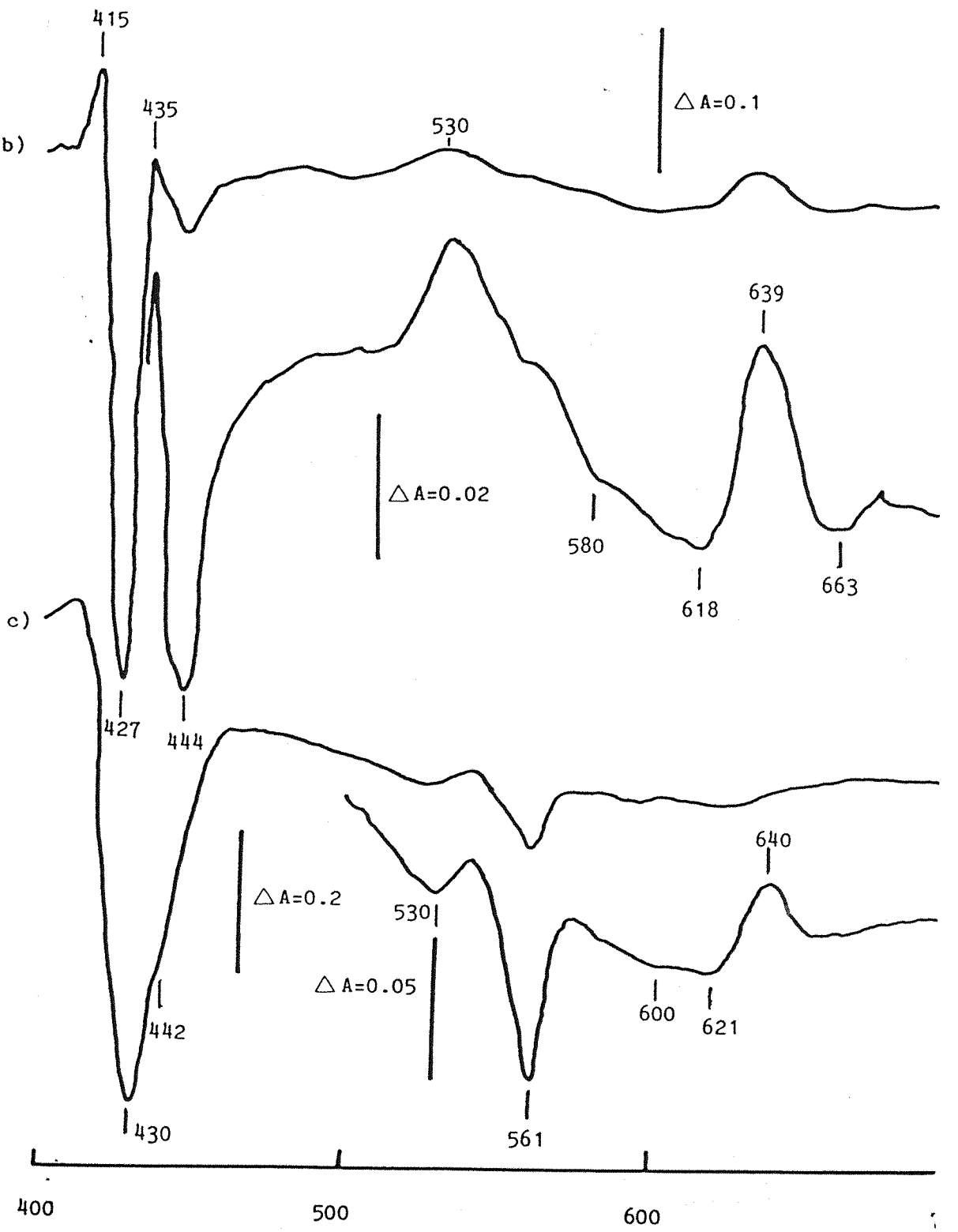
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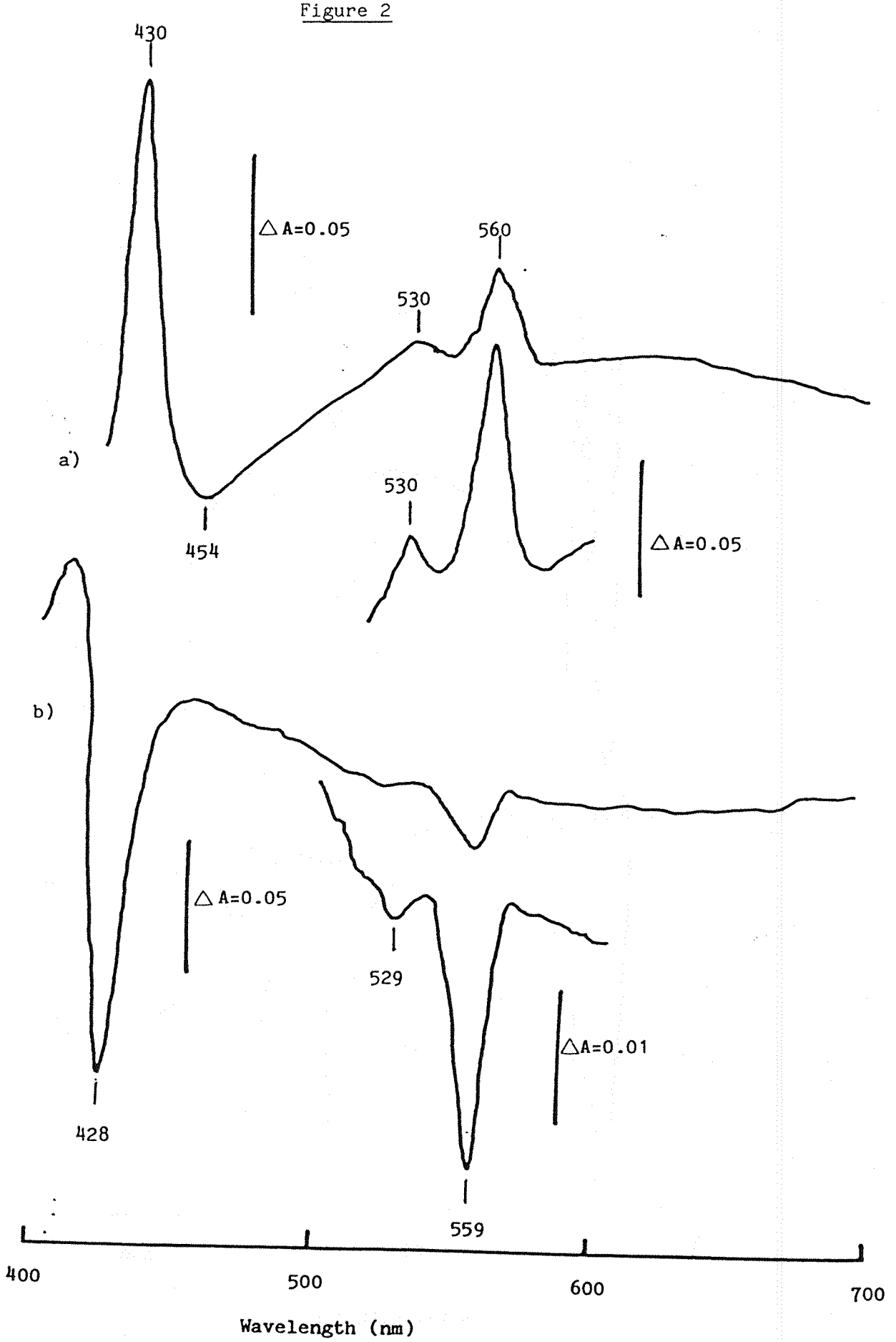
Figure 2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Wavelength (nm)

Figure 3

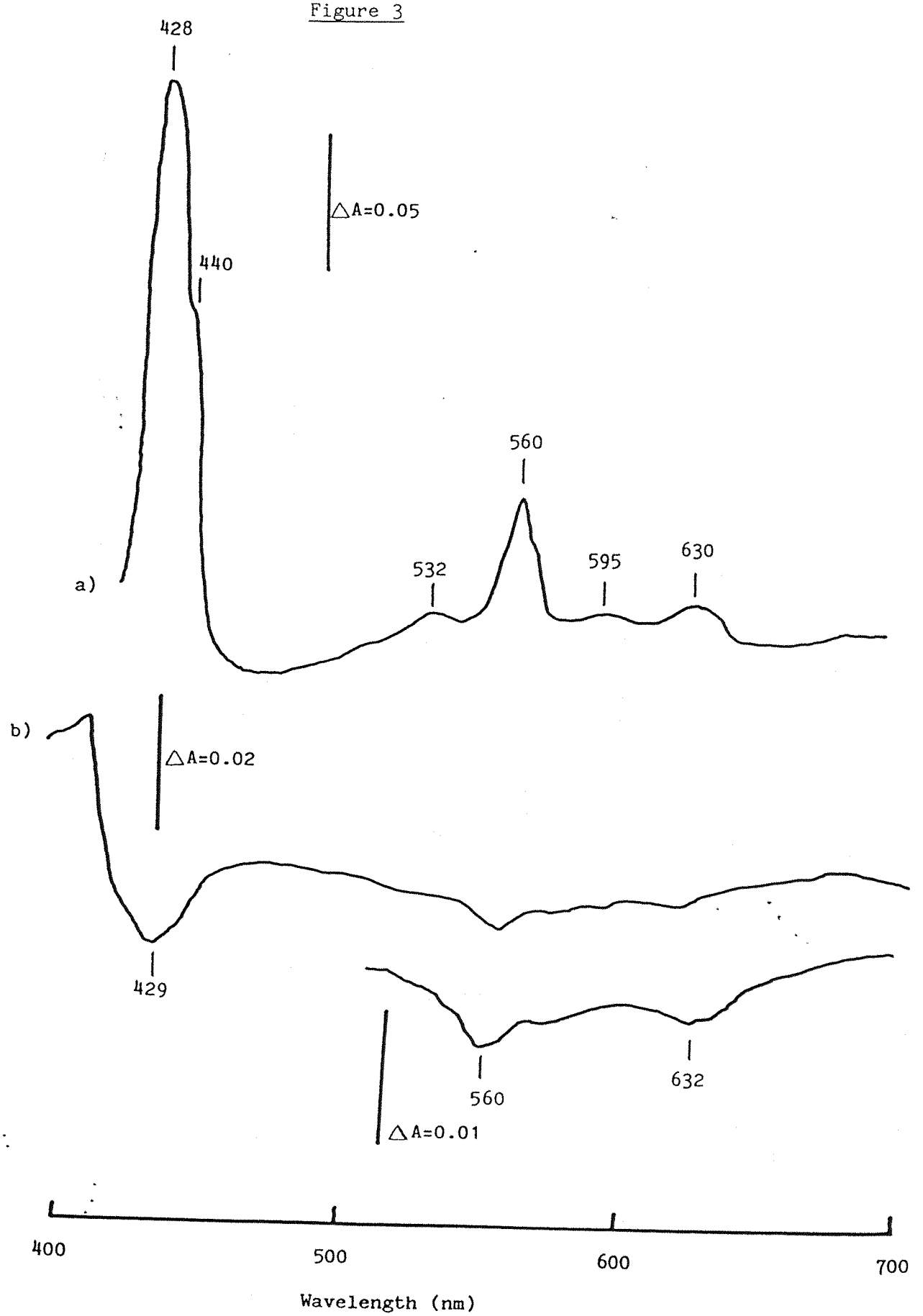
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Figure 4

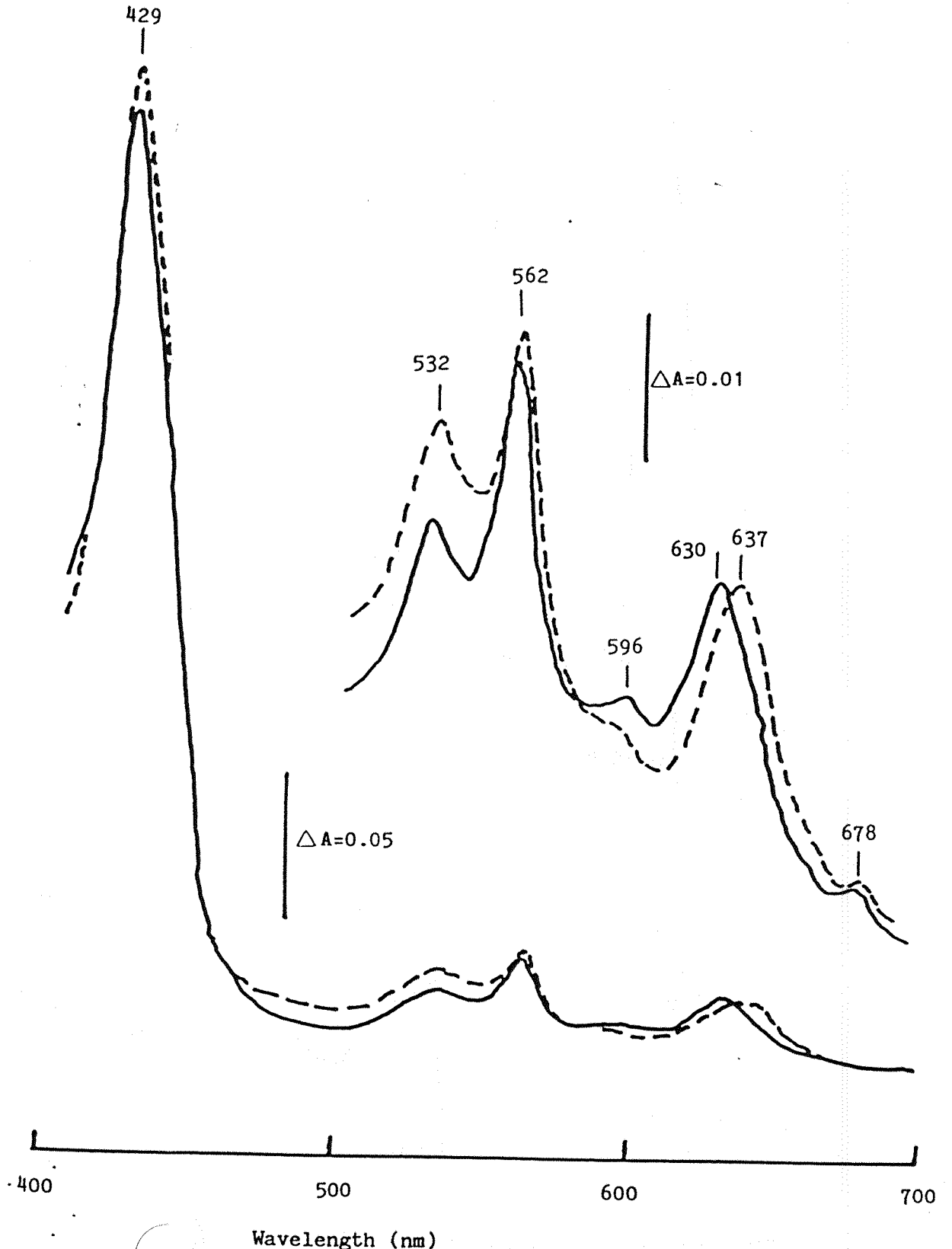
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Figure 5

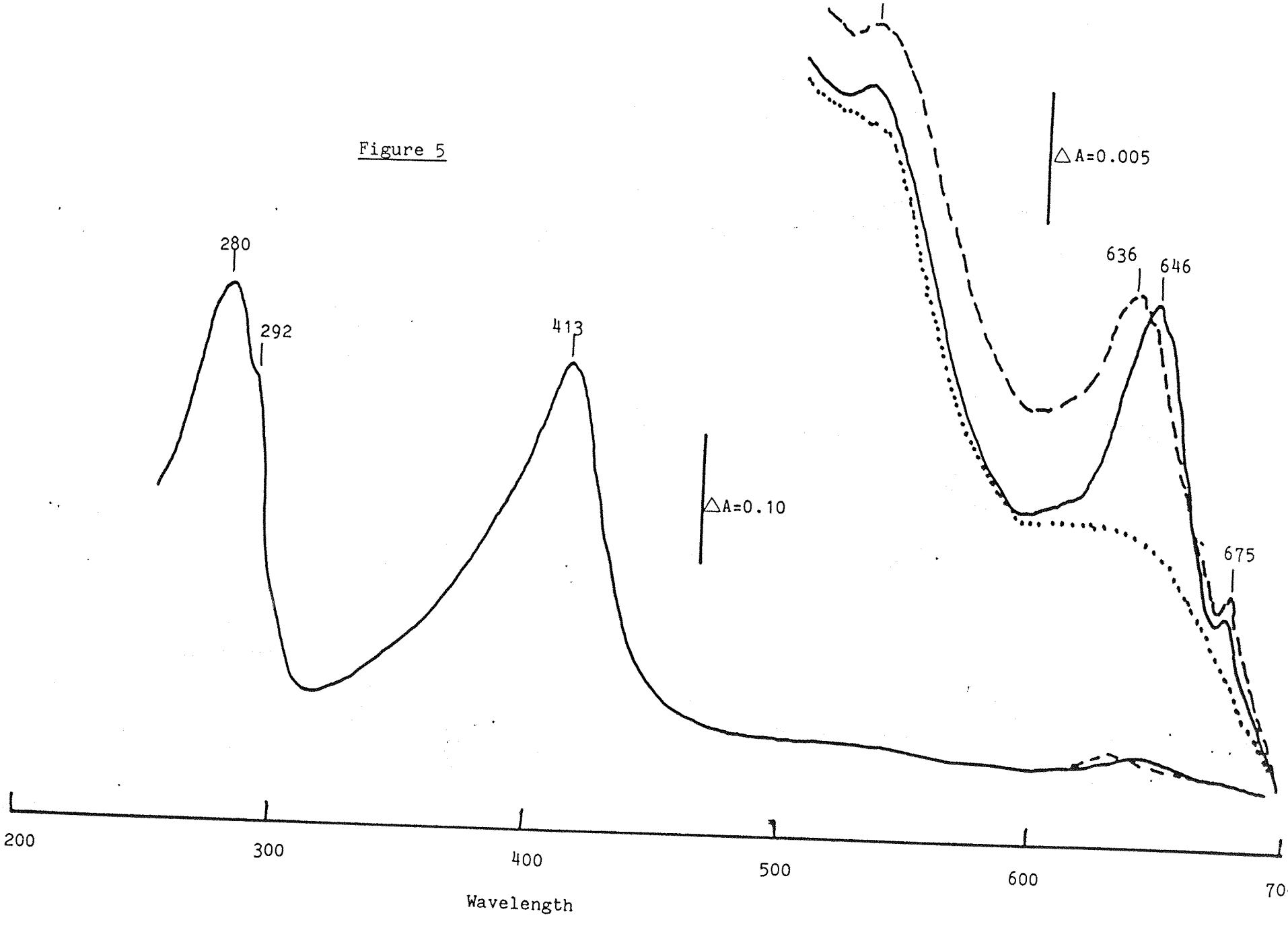
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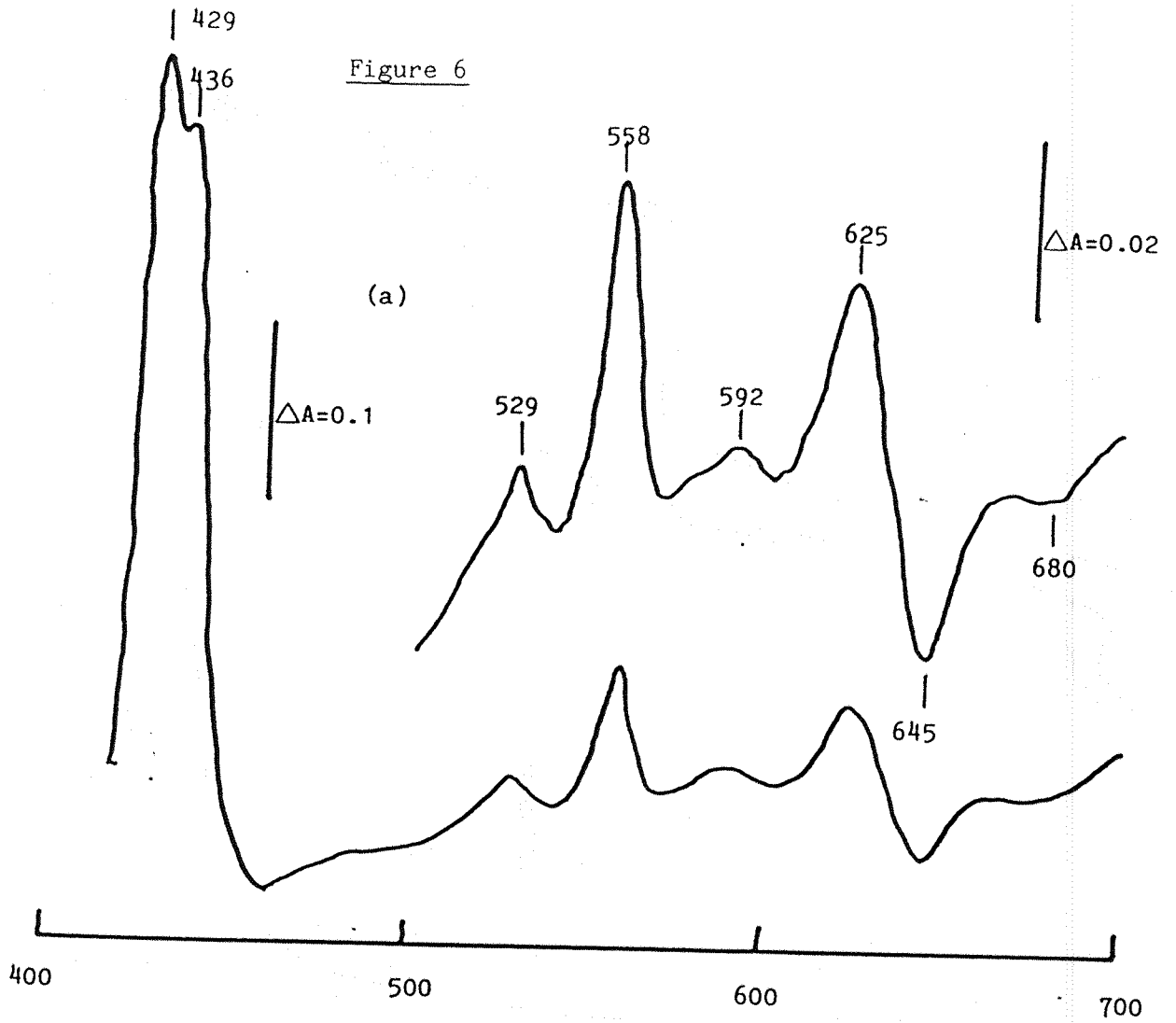
Figure 6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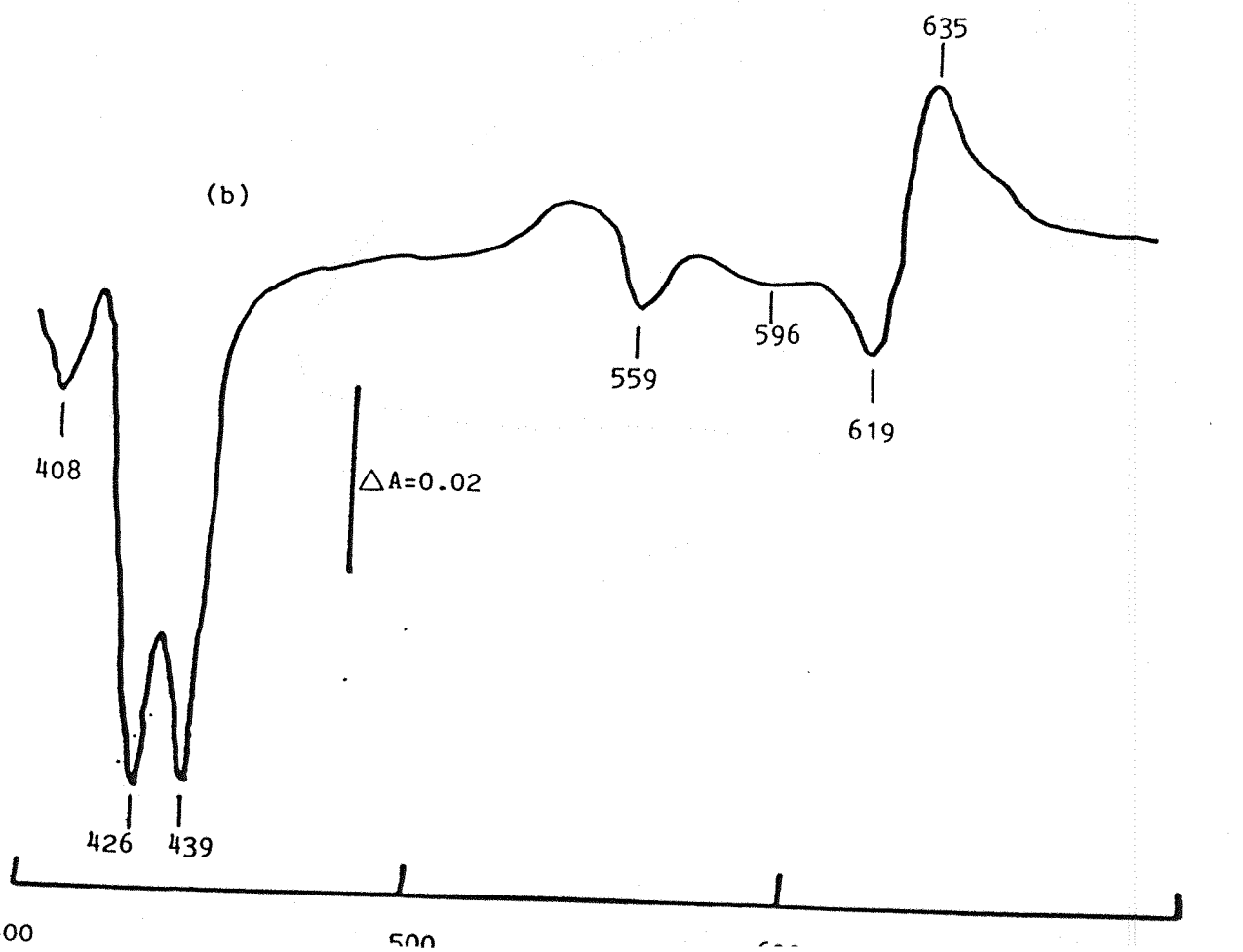
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(b)
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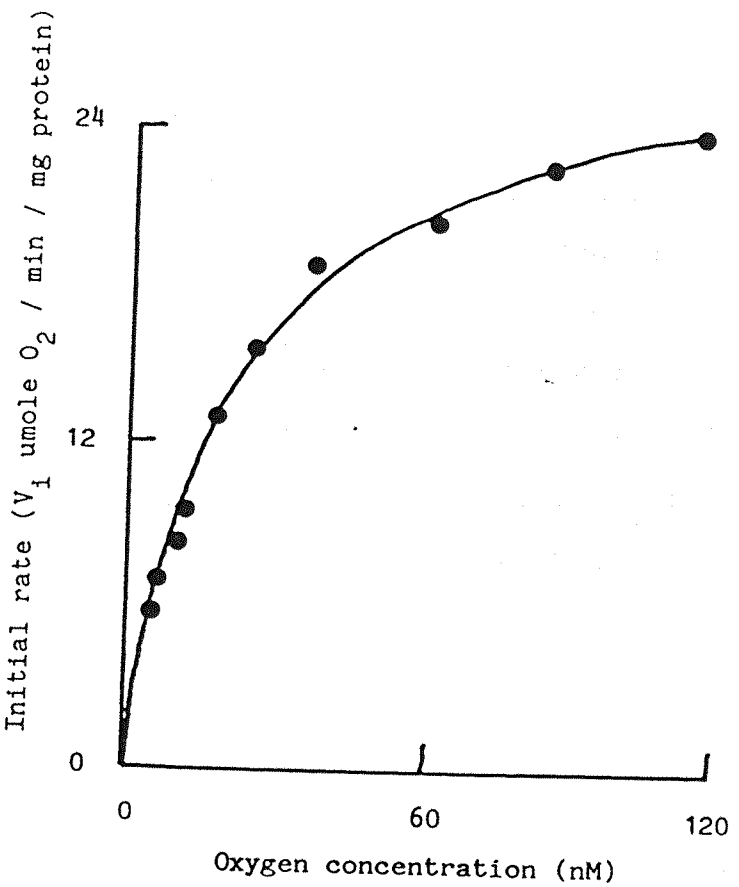
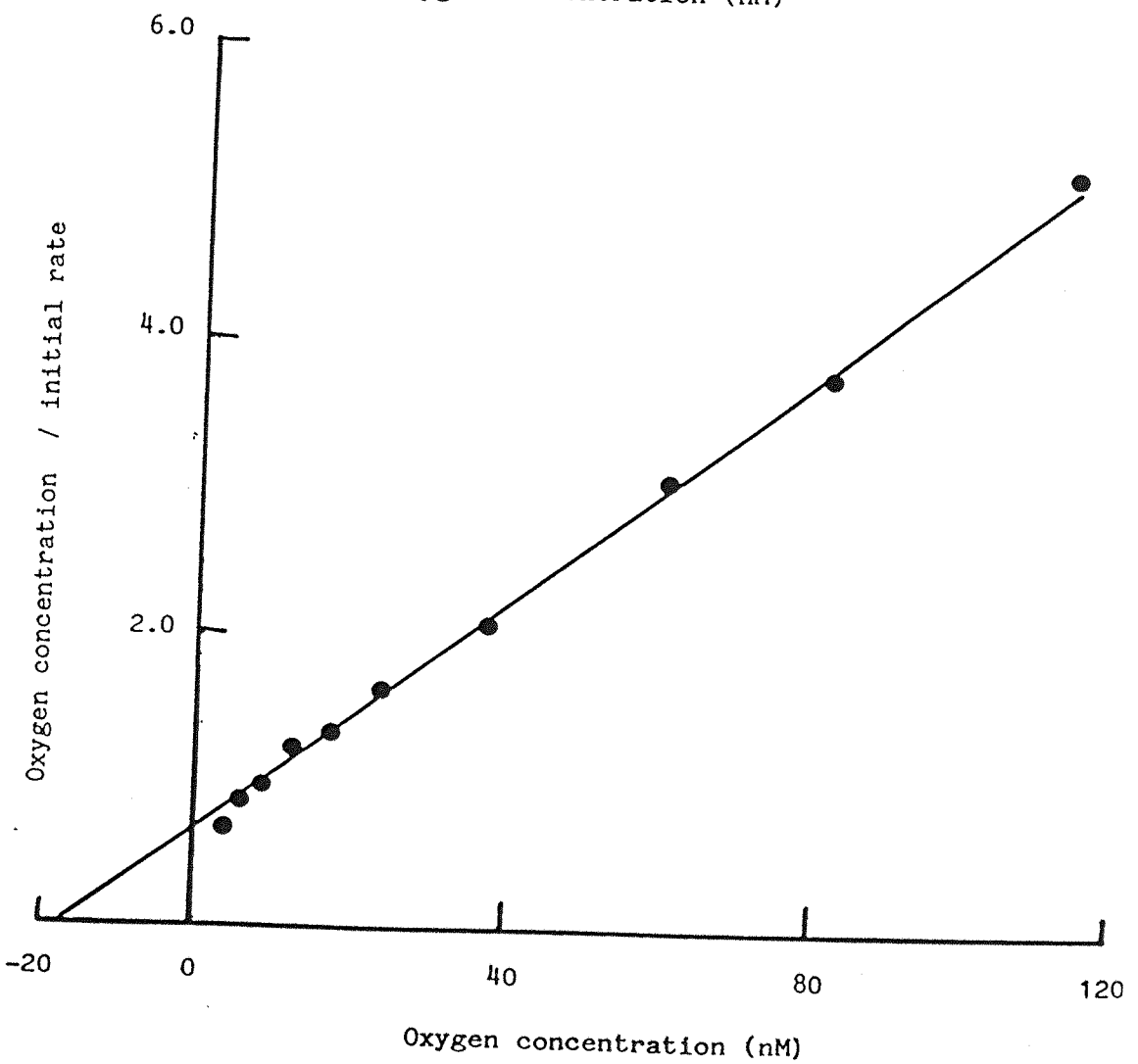
619
408
△A=0.02
426
439
400
500

Figure 7


Initial rate (V_i umole O_2 / min / mg protein)
24
12
0
0
60
120
Oxygen concentration (nM)


Oxygen concentration / initial rate
6.0
4.0
2.0
-20
0
40
80
120
Oxygen concentration (nM)

Roles for enteric cytochrome d oxidase complex in N_2 fixation and microaerobiosis

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Running title: Enteric cytochrome d oxidase in microaerobiosis

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Escherichia coli strains that lacked cytochrome d, the terminal oxidase with a high affinity for O_2, grew anaerobically as well as the wild-type, and were not impaired in the ability to evolve H_2 from either glucose or formate. The anaerobic synthesis and activity of nitrogenase in transconjugants of these strains carrying Klebsiella pneumoniae nif genes was also normal. However the behaviour towards O_2 of anaerobically-grown populations differed from that of the wild-type in the following ways: 1. the potential O_2 uptake was lower, 2. H_2 evolution, and, in the transconjugants, nitrogenase activity, when supported by fermentation, were more strongly inhibited by O_2, 3. Microaerobic O_2-dependent nitrogenase activity, which occurred in the absence of a fermentable carbon source in the wild-type was not detected in the mutants. These results show that the cytochrome d oxidase complex serves two functions to conserve energy under microaerobic conditions and to protect anaerobic processes from O_2 inhibition.

The biological reduction of N_2 to NH_4^+ is an O_2-sensitive and energy requiring process, which occurs in obligate anaerobes, facultative anaerobes and obligate aerobes. In aerobes respiration provides protection for nitrogenase from O_2 damage as well as ATP for nitrogenase activity (34,41). Oxidases with differing affinities for O_2 often terminate a branched respiratory chain in prokaryotes (1,28). In diazotrophs a terminal oxidase with high affinity

for O_2 is likely to be important for N_2 fixation so that ATP production is maintained at low O_2 concentrations. However, in none of them has this requirement been unequivocally established, although the following evidence supports this hypothesis: firstly, the biochemical and physiological analyses of microaerophilic N_2 fixation, such as that occurring in the legume symbiosis, shows that the O_2 concentration surrounding the bacteroids is very low (2,4,5). Secondly, the inferior growth yield of an ascorbate-TMPD oxidase-negative mutants of Azotobacter vinelandii during O_2-limited N_2-dependent growth, suggests that the higher affinity cytochrome a_1o branch of the respiratory chain is needed for energy conservation at low dissolved O_2 concentration (DOC) (25). Thirdly, the correction of O_2-sensitive mutants of Azotobacter chroococcum to aerotolerance by the provision of adequate TCA cycle intermediates suggests that a high electron pressure to maintain an adequate O_2 uptake at low DOC is required for nitrogen fixation in air (30,31,32).

In the facultative anaerobe Klebsiella pneumoniae the efficiency of N_2 fixation (mg N fixed.g glucose consumed^{-1}) is improved by providing small amounts of O_2 (16). The optimum DOC for nitrogenase activity is near the apparent K_m of the cytochrome d oxidase complex (25 nM O_2) (20, Smith, A., Hill, S., Anthony, C., submitted). The respiratory chain in K. pneumoniae (Smith, A., Hill, S., Anthony, A., submitted) appears to be similar to that of the better studied one in E. coli (1,28). In E. coli (13,27,33,39) and

in K. pneumoniae (Smith, A., Hill, S., Anthony, C., submitted) the low affinity cytochrome o oxidase is found under aerobic conditions, whereas the high affinity cytochrome d oxidase (encoded by cyd genes) predominates under microaerobic or anaerobic conditions. In K. pneumoniae the latter oxidase is present under conditions permitting nif expression (Smith, A., Hill, S., Anthony, C., submitted), i.e. under either anaerobiosis or microaerobiosis during N limited growth (18). Mutants of E. coli lacking either oxidase do not show growth defects under the various laboratory conditions tested (3,11), implying a possible redundancy in terminal oxidases (9). Experiments with cydA$^+$B$^+$ and cydA$^-$B$^-$ strains of E. coli and with transconjugants of these strains carrying the K. pneumoniae nif genes on a plasmid are reported here. They have revealed that the cytochrome d oxidase complex has two roles: to support energy-requiring processes under microaerobiosis, such as nitrogenase activity, and to protect anaerobic reactions from O_2 inhibition.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

E. coli strains J62-1 and UNF3501 were transduced with chloramphenicol resistant phage P1 grown on E. coli G0103, which carries a kanamycin resistance cassette close to the partially deleted cydAB locus (R.B. Gennis, personal

communication). Clones resistant to kanamycin (Km) (25 $\mu g.ml^{-1}$) were checked for failure to grow on freshly prepared nutrient agar (NA) containing $ZnSO_4$ (0.1 mM) and NaN_3 (0.1 mM) (NAAZ) which is a property of Cyd^- mutants (22; A.T. Smith, unpublished). Transformations of strains J62-1 and UNF3500 with pNG2 were performed by the method of (26). Transformants were selected by resistance to tetracycline (Tc) (5 $\mu g.ml^{-1}$) on NA, and were checked for growth on the NAAZ medium, and for the presence of the desired plasmid by PvuI and EcoRI restriction analysis (24). His^+ E. coli transconjugants were selected from matings with suitable K. pneumoniae strains carrying either pRD1 or pMF100 (6).

Growth conditions. Strains were maintained and grown for inocula either on NA or, for Nif^+ transconjugants, on minimal glucose medium (6) supplemented, when required, with tryptophan and proline (each at 25 $\mu g.ml^{-1}$). Where necessary Tc (5 $\mu g.ml^{-1}$) was added. Anaerobic glucose-limited growth was achieved after 18 to 24 h of bubbling (about 50 $ml.min^{-1}$) with CO_2 (1% v/v) in N_2 at 28°C in 15 to 50 ml of a modified NFDM medium (6) containing glucose (0.25% w/v), twice the usual phosphate concentration, the trace elements of Pope et al. (29), nutrient broth (5% v/v), vitamin-free casamino acids (800 $\mu g.ml^{-1}$), and, when required, tryptophan, proline or histidine (all at 25 $\mu g.ml^{-1}$). Exhaustion of glucose was detected by Clinistix (Miles Laboratories Ltd., Sl2 4LY, U.K.) and growth was estimated by measurements of optical density (540 nm) and protein concentration using the BSA

reagent (Pierce, CH1 4EF, U.K.) in microtitre plates (38).

Assays. H_2 evolution and C_2H_2 reduction assays were performed in suba-seal capped serum bottles (8 ml) containing the indicated substrates in 0.1 ml under Ar and, for the latter assays, 10 kPa of C_2H_2. The desired atmospheric O_2 was achieved by injecting a volume of air. The assay, performed at 30°C with shaking (136 strokes of 3.8 $cm.min^{-1}$), was started by injecting a sample of culture (1.0 ml), that had been collected anaerobically, and was terminated after either 30 or 60 min by injecting 0.1 ml of 30% (w/v) trichloroacetic acid. Gas samples (0.5 ml) were then taken for analysis by gas chromatography (20). For measurements of O_2 uptake a sample of culture (3 ml) was stirred under air in a Clark-type O_2 electrode chamber (Rank Bros., CB5 9DA, U.K.) at 30°C until the O_2 concentration had reached approximately 180 μM when air was excluded. O_2 consumption supported by endogenous fermentation products was allowed to proceed until the O_2 concentrations had reached approximately 150 μM when the indicated addition (in 0.3 ml) were made. Initial rates of O_2 uptake were calculated assuming that the O_2 concentration in air saturated saline phosphate buffer (6) was 240 μM.

RESULTS

Strain GO103 carries a Km^R cassette about 750 base pairs away from a <u>cydAB</u> deletion (R.B. Gennis, personal

communication). Because the phenotypes of this deletion mutation might be influenced by a variety of other characteristics associated with the respiratory chain two E. coli strains UNF3501 and J62-1 were transduced to Km^R with P_1 grown on GO103. These two strains were chosen because the Nif^+ plasmids could be retained by his prototrophy and in such transconjugants good levels of nitrogenase have previously been found. The transductants had received the cydAB deletion because, unlike $cydA^+B^+$ strains, they failed to grow on NAAZ medium (see Methods). Furthermore, the introduction of pNG2 (12), which carries the cydAB genes, restored the ability to grow on this medium.

Anaerobic growth, H_2 evolution and C_2H_2 reduction. The concentration of glucose and fixed N in the modified NFDM medium was adequate to yield good anaerobic glucose-limited growth after about 18 hours incubation of all the $cydA^-B^-$ and $cydA^+B^+$ strains listed in Tables 2 and 3. The yields, measured either by optical density (data not shown) or by estimates of protein content (Tables 2 and 3) were similar in wild-type and mutant strains.

The evolution of H_2, presumably via pyruvate formate-lyase and formate hydrogenlyase, in strains that did not carry nif genes was used as a measure of electron flux associated with fermentation. Samples of glucose-limited cultures showed only traces of H_2 evolution (data not shown). Addition of formate resulted in about twice the amount of H_2 evolution than did the addition of glucose. These activities

were similar in cydA$^+$B$^+$ and cydA$^-$B$^-$ strains (Table 2A and 2B).

C_2H_2 reduction by Nif$^+$ transconjugants was used as a measure of electron flux associated with nitrogenase activity or as a reporter of ATP production. Anaerobic samples of glucose-limited cultures showed very little C_2H_2 reduction (Table 3B). Addition of glucose enhanced the anaerobic C_2H_2-reducing activities to similar extents in cydA$^+$B$^+$ and cydA$^-$B$^-$ Nif$^+$ strains (Table 3A).

O_2-dependent C_2H_2 reduction. In the absence of added glucose, C_2H_2 reduction by the anaerobic glucose-limited cultures of cydA$^+$B$^+$ strains, UNF3501 (pRD1) and J62-1 (pMF100), was significantly improved by providing low levels of O_2 (Table 3B and Fig. 1), as had been found previously in K. pneumoniae (16). This O_2-dependent activity was increased in strain UNF3501 (pRD1) by providing fermentation products such as formate plus lactate (Table 3B). These products were chosen because together they supported microaerobic nitrogenase activity in washed cells of K. pneumoniae (A.T. Smith, unpublished). Here washing the E. coli Nif$^+$ transconjugants was avoided in case the cytochrome d oxidase complex was involved in protecting nitrogenase from O_2 damage (see below). The optimum atmospheric O_2 concentration (about 3kPa, Fig. 1) for activity was similar to that found previously for K. pneumoniae (16). Strikingly no O_2-dependent C_2H_2-reducing activity was found in cultures of cydA$^-$B$^-$ strains, UNF3502 (pRD1) and UNF3500 (pMF100), even when

fermentation products were added (Table 3B) or when the atmospheric O_2 concentration was varied (Fig. 1). The introduction of the recombinant plasmid pNG2, carrying the cydAB genes, to strain UNF3500 (pMF100) restored O_2-dependent C_2H_4-reducing activity (Table 3B). Therefore we conclude that the cytochrome d oxidase complex is required to support microaerobic nitrogenase activity in E. coli.

O_2 uptake rate. The failure of strains UNF3502 (pRD1) and UNF3500 (pMF100) to show O_2-dependent nitrogenase activity suggested that they had a defect in the ability to take up O_2. The O_2 uptake rates of the glucose-limited cultures (measured near 150 μM O_2 in a Rank O_2 electrode), are shown in Table 2C and 3C. O_2 consumption in the presence of added formate or glucose was slower in the $cydA^-B^-$ than in the $cydA^+B^+$ strains. Although glucose enhanced O_2 uptake in the $cydA^-B^-$ strains formate was consistently without effect. When pNG2 was introduced into $cydA^-B^-$ strain UNF3500 and UNF3500 (pMF100) the O_2 uptake rates were similar to those in the $cydA^+B^+$ strains J62-1 and J62-1 (pMF100) respectively (Tables 2C and 3C). The rates of O_2 uptake in $cydA^-B^-$ strains unlike those in $cydA^+B^+$ strains declined as the DOC decreased (data not shown). Microaerobic C_2H_2 reduction in K. pneumoniae is optimal at 30 nM O_2 (20) and does not occur at 5 μM O_2 (E. Kavanagh & S. Hill, unpublished observation), which is near the limit of sensitivity of the Rank O_2 electrode. Therefore in the microaerobic assays for C_2H_2 reduction (Table 3A and 3B) or H_2 evolution (Table 2A and 2B)

the rates of O_2 uptake in *cydA⁻B⁻* strains were probably much slower than those shown in Table 2C and 3C.

O_2 inhibition of electron transfer to nitrogenase and hydrogenase. In *K. pneumoniae* glucose-supported nitrogenase activity is not improved but is partially inhibited by providing low levels of O_2 (16). Diversion of electron flow away from nitrogenase to the respiratory chain could account for this inhibition (10). A similar pattern of inhibition by O_2 of glucose-supported nitrogenase activity occurred in *cydA⁺B⁺* strains UNF3501 (pRD1) and J62-1 (pMF100) (about 50% inhibition occurred under 1.3 kPa O_2) (Table 3A). On the other hand, this inhibition was much more marked in *cydA⁻B⁻* strains, UNF3502 (pRD1) and in UNF3500 (pMF100) (99% inhibition occur under 1.3 kPa O_2) (Table 3A). The introduction of pNG2 into strain UNF3500 (pMF100) gave rise to transformants which showed the same inhibition by O_2 of nitrogenase activity as had been found in the *cydA⁺B⁺* strain J62-1 (pMF100) (Table 3A).

The production of H_2 during glucose fermentation by *E. coli* involves at least one O_2-sensitive enzyme complex, pyruvate formate-lyase (23). In addition the enzyme complex formate hydrogenlyase (35,36) may be O_2 sensitive. Neither the formate dehydrogenase H nor hydrogenase III of this complex has been purified so their O_2 sensitivities are unknown. The H_2 evolution supported by either glucose or formate in samples of anaerobic glucose-limited cultures of the *cydA⁺B⁺* strain, J62-1 was inhibited by O_2 (about 50%

inhibition occurred under 0.8 kPa O_2) (Table 2A and 2). The inhibition by O_2 of H_2 evolution was much greater in the *cydA⁻B⁻* strain UNF3500 (95% inhibition occurred under 0.8 kPa O_2)(Table 2A and 2B). Also the level of inhibition in strain UNF3500 carrying pNG2 was similar to that in the *cydA⁺B⁺* strain J62-1 (Table 2A and 2B). The degree of inhibition by O_2 was similar whether the substrate was either glucose or formate. This indicates that, *in vivo*, pyruvate formate-lyase activity was not markedly more sensitive to O_2 than formate hydrogenlyase activity.

DISCUSSION

A specific role for the cytochrome d oxidase complex of *E. coli* has not so far been described. The phenotypes described here of strains carrying a deletion in the *cydAB* genes, strongly suggest that this oxidase is important in facultative anaerobiosis for exploiting microaerobic conditions. The deletion mutation was transduced into two *E. coli* strains. Both backgrounds gave similar results which suggests that the deletion was responsible for the phenotypes. Such mutants were considered to lack the cytochrome d oxidase complex because they failed, unlike *cydA⁺B⁺* strains, to grow aerobically in the presence of azide and zinc, a property which was corrected when the plasmid pNG2 was present *in trans*. The selection of transductants carrying the *cydAB* deletion utilized a Km^R cassette inserted ≈750 base pairs away from the deletion. Although the

phenotypes of these transductants were corrected by pNG2, this plasmid carries sufficient DNA to complement the cydAB deletion and also the location of the Km^R cassette (R.B. Gennis, personal communication). Therefore, the mutation responsible for these phenotypes could reside at either locus. However, in the following discussion it has been assumed that the cydAB deletion was responsible.

The cytochrome d oxidase complex is synthesised during anaerobic growth of E. coli (13,27,39), Klebsiella aerogenes (14) and of K. pneumoniae (Smith, A., Hill, S., Anthony, C., submitted). Thus the anaerobic glucose-limited culture of the $cydA^+B^+$ strains used in this work should have contained cytochrome d oxidase. A small peak at 630 nm, indicative of cytochrome d, was seen in the absorption spectra of crude extracts of anaerobic glucose-limited growth of the $cydA^+B^+$ srain UNF3501 (pRD1) (S. Hill, unpublished). The following evidence suggests that such cultures of the $cydA^-B^-$ strains did not contain cytochrome d oxidase. 1. The potential O_2 uptake rate was much lower than in $cydA^+B^+$ strain. 2. This defect was restored by the presence of pNG2 in trans. 3. Packed cells of anaerobic glucose-limited cultures of strains carrying pNG2 were greenish in colour, attributed to the cytochrome d oxidase (12) unlike those of the haploid strains. 4. No peak at 630 nM was seen in the absorption spectra of crude extracts of strain UNF3502 (pRD1) (S. Hill, unpublished).

Anaerobic glucose-limited cultures of $cydA^-B^-$ strains were capable of a slow consumption of O_2 (Table 2C and 3C).

Therefore the cytochrome o oxidase complex may have been synthesised under these growth conditions. Spectral evidence indicates that this oxidase is synthesised under anaerobic conditions in E. coli (13) and in K. aerogenes (14), although Smith, A., Hill, S., Anthony, C. (submitted) were unable to detect cytochrome o oxidase complex in anaerobically grown K. pneumoniae. Alternatively O_2 consumption by the samples of anaerobic cultures of cydA$^-$B$^-$ strains could occur by processes which normally transfer electron to acceptors other than O_2. The reduction of O_2 by the Fe protein of nitrogenase is such a reaction (40), but was not responsible in these experiments as O_2 uptake rates were not improved by the presence of the Nif$^+$ plasmid.

The apparent inhibition by O_2 of glucose-supported C_2H_2-reducing activity in K. pneumoniae (10) and in E. coli cydA$^+$B$^+$ carrying nif genes (Table 3A) could be accounted for by competition for the electrons arising from glucose oxidation between respiration and nitrogenase (10). Similarly, competition for electrons arising from either glucose or formate oxidation could explain the inhibition by O_2 of H_2 evolution in E. coli strain J62-1 lacking a Nif$^+$ plasmid (Table 2A and 2B)(D. Boxer personal communication). However in the cydA$^-$B$^-$ strains, in which electron flux to respiration was slower than in cydA$^+$B$^+$ strains, the inhibition by O_2 of C_2H_2 reduction (Table 3A) and of H_2 evolution (Table 2A and 2B) was not lower but higher than in the cydA$^+$B$^+$ strains. Therefore, at least in the cydA$^-$B$^-$ strains, O_2 was probably inhibiting nitrogenase and some

process associated with formate hydrogenlyase activity.

The DOC in the assays under a particular pO_2 was influenced by the rate of respiration alone, because the shaking rate and the protein content of the culture samples were kept constant. Because of the slower respiratory rate, the DOC in assays of the *cydA⁻B⁻* strains was higher than in those of the *cydA⁺B⁺* strains under the same conditions of a particular substrate addition and pO_2 value. Therefore in strains lacking the cytochrome d oxidase complex more O_2 was present to inhibit O_2-sensitive process. Hence O_2 uptake by the high affinity cytochrome d oxidase complex can provide protection to O_2 sensitive processes such as nitrogenase and formte hydrogenlyase. In aerobic diazotrophs such a function is referred to as the respiratory protection of nitrogenase (34,41).

C_2H_2 reduction by glucose-limited culture of *cydA⁺B⁺* Nif⁺ transconjugants (Table 3B, Fig. 1) like those of *K. pneumoniae* (16) was dependent upon the presence of low levels of O_2. The DOC achieved by the *cydA⁺B⁺* strain under 3 kPa O_2 (Fig. 1) was probably near 30 nM O_2 as this is the optimum DOC for nitogenase activity in *K. pneumoniae* (20). Further work, using either leghaemoglobin or photobacteria to measure such low levels of dissolved O_2 (see 18) is necessary to confirm the optimum concentration. The precise nature of the carbon substrate supporting this activity in *E. coli* has also to be elucidated. Lactate and formate are likely candidates as they are needed by *K. pneumoniae* (A. Smith, unpublished) and they enhanced activity in *E. coli* (Table 3B). Pyruvate,

the source of electrons for nitrogenase in K. pneumoniae (19,37), and presumably in E. coli Nif$^+$ transconjugants, could be generated by the action of lactate dehydrogenase. ATP to drive nitrogenase was probably provided by respiratory activity. Microaerobic respiration benefits other anabolic processes, besides N_2 fixation, because growth yields in anaerobic glucose-limited chemostats of E. coli (15) and K. pneumoniae (17) are increased by providing limiting O_2.

Microaerobic C_2H_2 reduction was not detected in cydA$^-$B$^-$ Nif$^+$ transconjugants, even when a range of low levels of O_2 were provided (Fig. 1), when either lactate plus formate (Table 3B) or pyruvate plus formate (S. Viollet unpublished) were added. Such inactivity indicates that cytochrome d oxidase complex is required to support nitrogenase activity in E. coli Nif$^+$ transconjugants under microaerobic conditions.

Our results strongly suggest that the high affinity cytochrome d oxidase complex in enteric bacteria serves two functions, to conserve energy under microaerobic conditions and to protect anaerobic process from O_2 inhibition. Such functions are essential for aerobic diazotrophy, not only in facultative anaerobes but also in obligate aerobic diazotrophs (34,41), and probably for the persistence of enteric bacteria in the gut where fluctuating supplies of carbon sources and O_2 are common (see 21).

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LITERATURE CITED

1. Anraku, Y. and Gennis, R.B. 1989. The aerobic respiratory chain of Escherichia coli. Trends Biochem. Sci. 12:262-266.

2. Appleby, C.A. 1984. Leghemoglobin and respiration. Ann. Rev. Plant Physiol. 35:443-478.

3. Au, D.C-T., Lorence, R.M. and Gennis, R.B. 1985. Isolation and characterization of a Escherichia coli mutant linking the cytochrome o terminal oxidase. J. Bacteriol. 161:123-127.

4. Bergersen, F.J. 1982. Root nodules of legumes: structure and function. John Wiley & Sons Ltd., Chichester.

5. Bergersen, F.J. 1984. Oxygen and the physiology of diazotrophic microorganisms, p. 171-180. In C. Veeger and W.E. Newton (ed.), Advances in nitrogen fixation research. Martinus Nijhoff/Dr. W. Junk, The Hague.

6. Cannon, F.C. 1984. Genetic studies in diazotrophs, p. 367-413. In F.J. Bergersen (ed.) Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Chichester.

7. Dixon, R.A., Cannon, F.C., and Kondorosi, A. 1976. Construction of a plasmid carrying nitrogen fixation genes from Klebsiella pneumoniae. Nature 260:268-271

8. Filser, M., Merrick, M. and Cannon, F. 1983. Cloning and characterization of nifLA regulatory mutations from Klebsiella pneumoniae. Mol. Gen. Genet. 191:485-591.

9. Georgiou, C.D., Dueweke, T.J. and Gennis, R.B. 1988.

Regulation of expression of the cytochrome d terminal oxidase in Escherichia coli is transcriptional. J. Bacteriol. 170:961-966.

10. Goldberg, L., Nadler, V., and Hochman, A. 1987. Mechaism of nitrogenase 'switch off' by oxygen. J. Bacteriol. 169:874-879.

11. Green, G.N. and Gennis, R.B. 1983. Isolation and characterization of an Escherichia coli mutant lacking cytochrome d terminal oxidase. J. Bacteriol. 154:1269-1275.

12. Green, G.N., Kranz, J.E. and Gennis, R.B. 1984. Cloning of cyd gene and locus coding for the cytochrome d complex of Escherichia coli. Gene 32:99-106.

13. Haddock, B.A., Downie, J.A. and Garland, P. 1976. Kinetic characterization of the membrane-bound cytochromes of Escherichia coli grown under a variety of conditions by using a stopped-flow dual-wave length spectrophotometer.

Biochem. J. 154:285-294.

14. Harrison, D.E.F. 1972. A study of the effect of growth conditions on chemostat-grown Klebsiella aerogenes and kinetic change of A_{500nm} absorption band. Biochim. Biophys. Acta 275:83-92.

15. Harrison, D.E.F. and Loveless, J.E. 1971. The effect of growth conditions on respiratory activity and growth efficiency in facultative anaerobes grown in chemostat culture. J. Gen. Microbiol. 68:35-43.

16. Hill, S. 1976. Influence of atmospheric oxygen concentration on acetylene reduction and efficiency of nitrogen fixation in intact Klebsiella pneumoniae. J. Gen. Microbiol. 93:335-345.

17. Hill, S. 1976. The apparent ATP requirement for nitrogen fixation in growing Klebsiella pneumoniae. J. Gen. Microbiol. 95: 297-312.

18. Hill, S. 1988. How is nitrogenase regulated by oxygen? FEMS Microbiol. Rev. 54: 111-130.

19. Hill, S. and Kavanagh, E. 1980. Roles of nifF and nifJ gene products in electron transport to nitrogenase in Klebsiella pneumoniae. J. Bacteriol. 141:470-475.

20. Hill, S., Turner, G.L. and Bergersen, F.J. 1984. Synthesis and activity of nitrogenase in Klebsiella pneumoniae exposed to low concentration of oxygen. J. Gen. Microbiol. 130:1061-1067.

21. Ingledew, W.J. and Poole, R.K. 1984. The respiratory chains of Escherichia coli. Microbiol. Rev. 48:222-271.

22. Kita, K., Konishi, K. and Anraku, Y. 1984. Terminal

oxidases of Escherichia coli aerobic respiratory chain ~~aerobic~~ purification and properties of cytochrome b_{558}-d complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. J. Biol. Chem. 259:3375-3381.

23. Knappe, J. 1987. Anaerobic dissimilation of pyruvate, p.151-155. In F.C. Neidhardt (ed.), Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology Vol.1. American Society for Microbiology, Washington, D.C.

24. Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. In Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

25. McInerney, M.J., Holmes, K.S. and Dervartanian, D.V. 1982. Effect of O_2 limitation on growth and respiration of the wild-type and an ascorbate-tetramethyl-p-phenylenediamine-oxidase-negative mutant strain of Azotobacter vinelandii. J. Bioenerg. Biomembr. 14:451-456.

26. Merrick, M.J., Gibbins, J.R. and Postgate, J.R. 1987. A rapid and efficient method for plasmid transformation of Klebsiella pneumoniae and Escherichia coli. J. Gen. Microbiol. 133:2053-2057.

27. Moss, F. 1952. The influence of oxygen tension on respiration and cytochrome a_2 formation of Escherichia coli. Aust. J. Exp. Biol. Med. Sci. 30:351-540.

28. Poole, R.K. 1988. Bacterial cytochrome oxidases, p.231-291. In C. Anthony (ed.) Bacterial Energy Transduction. Academic Press, London.

29. Poole, R.K., Waring, A.J. and Chance, B. 1979. The

reactions of cytochrome o in Escherichia coli with oxygen: low temperature and spectral studies. Biochem. J. 184:379-389.

30. Ramos, J. and Robson, R.L. 1985. Isolation and properties of mutants of Azotobacter chroococcum defective in aerobic nitrogen fixation. J. Gen. Microbiol. 131:1449-1458.

31. Ramos, J. and Robson, R.L. 1985. Lesions in citrate synthase that affect aerobic nitrogen fixation by Azotobacter chroococcum. J. Bacteriol. 162:746-751.

32. Ramos, J. and Robson, R.L. 1987. Cloning of the gene for phosphenolpyruvate carboxylase from Azotobacter chroococcum an enzyme important in aerobic N_2 fixation. Mol. Gen. Genet. 208:418-484.

33. Rice, C.W. and Hempfling, W.P. 1978. Oxygen-limited continuous culture and respiratory energy conservation in Escherichia coli. J. Bacteriol. 134:115-124.

34. Robson, R.L. and Postgate, J.R. 1980. Oxygen and hydrogen in biological nitrogen fixation. Ann. Rev. Microbiol. 34:183-207.

35. Sankar, P., Lee, J.H., and Shanmugam, K.T. 1988. Gene-product relationships of fhlA and fdv genes of Escherichia coli. J. Bacteriol. 170:5440-5445.

36. Sawers, R.G., Ballantine, S.P. and Boxer, D.H. 1985. Differential expression of hydrogenase isoenzymes in Escherichia coli: evidence for a third isoenzyme. J. Bacteriol. 164:1324-1331.

37. Shah, V.K., Stacey, G. and Brill, W.J. 1983. Electron transport to nitrogenase purification and characterization

of pyruvate: flavodoxin oxidoreductase, the nifJ gene product. J. Biol. Chem. 258:12064-12068.

38. Smith, A., Hill, S. and Anthony, C. 1988. A haemoprotein is not involved in the control by oxygen of enteric nitrogenase synthesis. J. Gen. Microbiol. 134:1499-1507.

39. Thomas, A.D., Doelle, H.W., Westwood, A.W. and Gordon, G.L. 1972. Effect of oxygen on several enzymes involved in the aerobic and anaerobic utilization of glucose in Escherichia coli. J. Bacteriol. 112:1099-1105.

40. Thorneley, R.N.F. and Ashby, G.A. 1989. Oxidation of nitrogenase iron protein by dioxygen without inactivation could contribute to high respiration rates of Azotobacter and facilitate nitrogen fixation in other aerobic environments. Biochem. J. 261:181-187.

41. Yates, M.G. 1988. The role of oxygen and hydrogen in nitrogen fixation, p.383-416. In J.A. Cole and S.J. Ferguson (ed.), The Nitrogen and Sulphur Cycles. Cambridge University Press, Cambridge, New York.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or derivation
E. coli		
G0103	F^-, *rpsL*, *thi*, *gal*, *Δcydab*, Km^R [a]	R.B. Gennis
J62-1	*pro*, *his*, *trp*, *nal*, *lac*	N. Datta
UNF3500	*pro*, *his*, *trp*, *nal*, *lac*, *Δcydab*, Km^R [a]	J62-1 x P1Cm^R grown on G0103
JC5466	*trp*, *his*, *rpsE*, *recA56*	N. Willetts
UNF3501	*trp*, *his*, *rpsE*	Rec^+ derivatives of JC5466 selected on methyl-methane-sulphonate
UNF3502	*trp*, *his*, *rpsE*, *Δcydab*, Km^R [a]	UNF3501 x P1Cm^R grown on G0103
Plasmids		
pRD1	Km^R, Cb^R, Tc^R, Gnd^+, His^+, Nif^+, $ShiA^+$ Tra^+, IncP	7
pMF100	Gnd^+, His^+, Nif^+, $ShiA^+$, Tra^+, IncP	8
pNG2	Tc^R, *cydA*$^+$*B*$^+$	12

[a]The Km^R cartridge is located at the *BglII* site 5′ to *ΔcydAB* (R.B. Gennis, personal communication).

Table 2. footnotes.

[a]Ability to grow on NAAZ medium.

[b]The standard deviations is shown in parenthesis.

[c]Activities of strain J62-1 (wt) were H_2 evolution (μmoles H_2.mg protein^{-1}.30 min^{-1}) supported by glucose 12.4(2.2) or by formate 28.5(7.6), and O_2 uptake (nmol O_2.mg protein^{-1}.min^{-1}) supported by endogenous substrate 73(31).

[d]not tested.

TABLE 3. Growth, nitrogenase (C_2H_2-reducing) activity and O_2 uptake rates in samples of anaerobic cultures of Cyd^+ and of Cyd^- *E. coli* Nif^+ transconjugants.

Strain	Cyd[a] phenotype	Growth (mg bacterial protein.ml culture^{-1})	(A) C_2H_2 reduction supported by glucose (13 mM) (% of wt under anaerobiosis)				(B) C_2H_2 reduction supported by fermentation products (% of wt with glucose under anaerobiosis)			(C) O_2 uptake (% of wt with no additions)		
			Atmospheric O_2 (kPa)				Atmospheric O_2 (kPa)			Additions		
			0	0.8	1.3	2.7	0	1.3	1.3[c]	none	glucose (13 mM)	formate (46 mM)
Transconjugants carrying pRD1												
UNF3501	+	0.14	100[d]	84	52(7)[b]	17	0.8(0.2)	4(4)	12(3)	100[e]	681	698(78)
UNF3502	-	0.14	79(18)	7(3)	1	<1	<0.1	<0.1	<0.1	91(20)	162	79(37)
Transconjugants carrying pMF100												
J62-1	+	0.11	100[d]	65	38(4)	7	0.1(0.1)	5(4)	5	100[e]	176(28)	79(29)
UNF3500	-	0.12	100(25)	1	<1	<1	0.4(0.5)	<0.1	<0.1	29(7)	66(4)	25(6)
Transconjugants carrying pMF100 transformed with pNE2												
J62-1	+	0.14	90(17)	47	33(7)	12	0.4(0.1)	6(2)	5	167(48)	337	85
UNF3500	-	0.13	108(17)	55	38(11)	12	0.2(0.1)	4(3)	3	126(9)	181(6)	99

Table 3. footnotes.

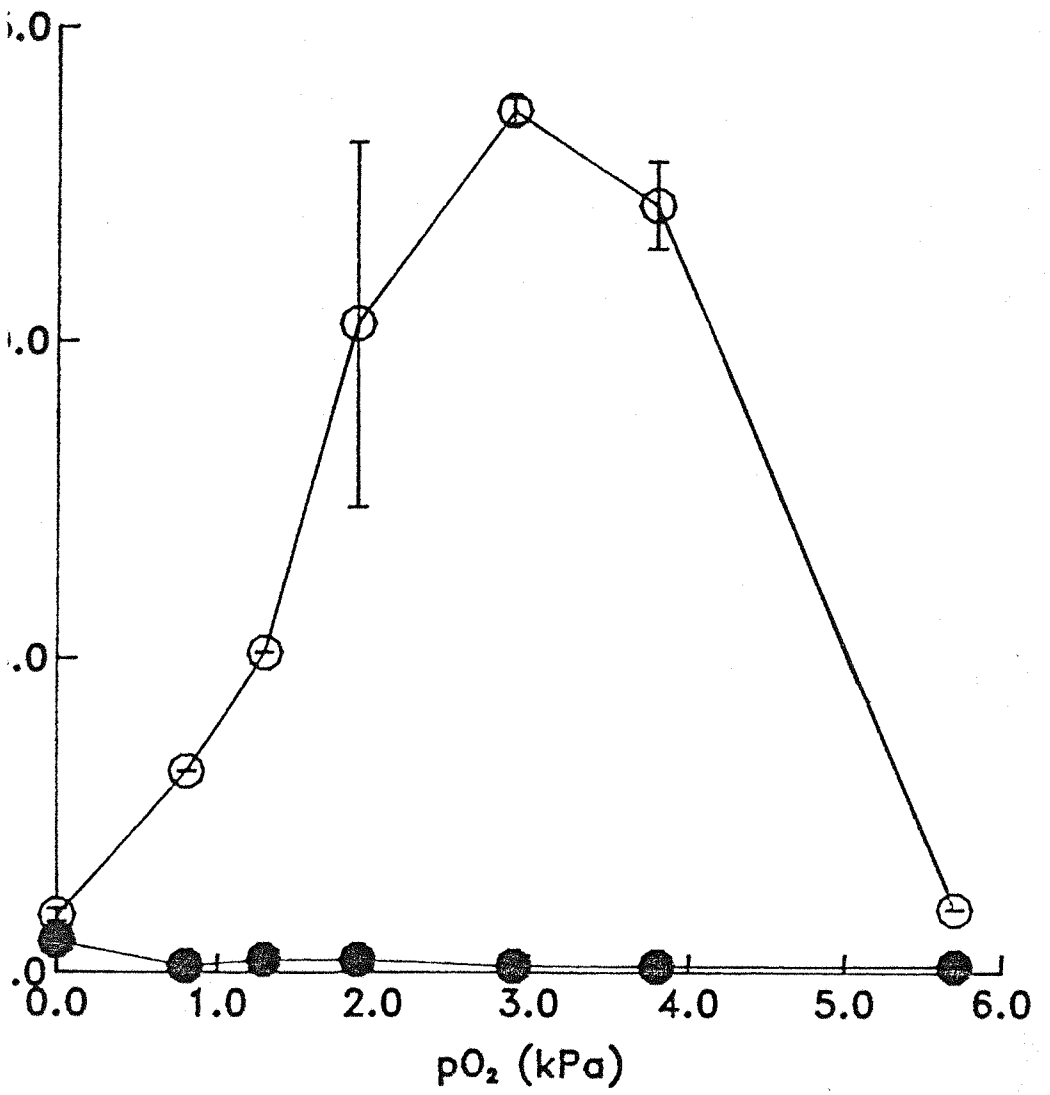
[a,b] See Table 2.

[c] Assays were supplemented with lactate (46 mM) and formate (46 mM).

[d] Activities (μmoles C_2H_4 produced.mg protein.h^{-1}) were 2.6(0.5) for UNF3501(pRD1) and 2.3(0.4) for J62-1(pMF100).

[e] O_2 uptake (nmoles O_2.mg protein.min^{-1}) were 20(3) for UNF3501(pRD1) and 89(12) for J62-1(pMF100).

FIG. 1. Influence of atmospheric pO_2 on microaerobic nitrogenase activity (C_2H_2 reduction) supported by fermentation products in *cydA*$^+$*B*$^+$ (○) and *cydA*$^-$*B*$^-$ (●) *E. coli* Nif$^+$ transconjugants. Strains UNF3501(pRD1) and UNF3502(pRD1) were assayed with lactate (46 mM) and formate (46 mM). The anaerobic glucose-supported activity of strain UNF3501(pRD1) (100%) is shown in Table 3.



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