Site-directed mutagenesis of the *Klebsiella pneumoniae* nitrogenase

Effects of modifying conserved cysteine residues in the α - and β -subunits

Helen M. KENT, Ioannis IOANNIDIS, Carol GORMAL, Barry E. SMITH and Martin BUCK*

A.F.R.C. Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, U.K.

The five conserved cysteine residues present in the α -subunit and the three conserved cysteine residues present in the β -subunit of nitrogenase component 1 were individually changed to alanine. Mutations in the α -subunit at positions 63, 89, 155 and 275 and in the β -subunit at positions 69, 94 and 152 all resulted in a loss of diazotrophic growth and component 1 activity and loss of the normal e.p.r. signal of the component 1 protein. Component 2 activity was retained. Replacement of cysteine-184 in the α -subunit with alanine greatly diminished, but did not eliminate, diazotrophic growth and component 1 activity. Substitution of serine for cysteine at position 152 in the β -subunit, in contrast with the substitution of alanine at this position, resulted in the formation of active component 1. Replacement of the non-conserved cysteine-112 in the β -subunit with alanine did not greatly perturb diazotrophic growth or the activity of component 1. Extracts prepared from a mutant, with cysteine-275 of the α -subunit replaced by alanine, complemented extracts of a mutant unable to synthesize the iron–molybdenum cofactor of nitrogenase, indicating that the alanine-275 substitution increases the availability of cofactor. Furthermore extracts of this mutant exhibited an e.p.r. signal similar to that of extracted iron–molybdenum cofactor. These data suggest a role for cysteine-275 as a ligand to the cofactor.

INTRODUCTION

The reduction of N_2 by symbiotic and free-living bacteria is catalysed by the metalloenzyme nitrogenase. The molybdenum-containing nitrogenase of the free-living *Klebsiella pneumoniae* consists of two separable protein components (Lowe *et al.*, 1985; Smith *et al.*, 1988). An iron protein (component 2, encoded by the *nifH* gene) serves to donate electrons to a molybdenum–iron protein (component 1, encoded by the *nifD* and *nifK* genes). Component 1 contains the site at which N_2 is bound and reduced.

Current evidence indicates that the metal atoms associated with the molybdenum–iron protein of nitrogenase are present as two distinct prosthetic groups, namely four [4Fe-4S]-type clusters, designated P clusters, and two iron–molybdenum cofactor (FeMoco) centres with a stoichiometry of $MoFe_6S_8S_{4-10}$. The latter are associated with a characteristic e.p.r. signal with g -values near 4.3, 3.7 and 2.01 and can be extracted intact from the protein (Shah & Brill, 1977). The total iron, sulphur and molybdenum complement of component 1 is 33 ± 3 Fe atoms, 30 ± 3 S^{2-} groups and 2.0 ± 0.1 Mo atoms per molecule (Smith *et al.*, 1988). It is probable that FeMoco is the site at which N_2 binds and is reduced (Hawkes *et al.*, 1984). However, in the absence of a three-dimensional structure for FeMoco or component 1 little direct information is available concerning the individual properties of the metal centres with respect to their structures, exact functions or spatial arrangements. Extrusion experiments indicate that the P clusters are liganded at least in part by cysteine residues

(Kurtz *et al.*, 1979). However, the spectroscopic properties of the P clusters (Zimmermann *et al.*, 1978; Smith *et al.*, 1980; McLean *et al.*, 1987; Hagen *et al.*, 1987) are not typical of the well-characterized [4Fe-4S] centres found in ferredoxins, raising the possibility of additional non-thiol ligands or an atypical structure for the iron–sulphur centres, two possibilities that may not be mutually exclusive. The reaction of extracted FeMoco with 1 mol of thiolate per g-atom of Mo suggests that cysteine residues could be involved in liganding FeMoco in the protein (Burgess *et al.*, 1980). Non-thiol ligands may also be involved in binding to FeMoco. Evidence in favour of this comes from the requirement for reagents with amide function to extract FeMoco from component 1 (Shah & Brill, 1977; Walters *et al.*, 1986), and is supported by studies of the interaction of purified FeMoco with amide groups and electron-spin-echo experiments with component 1 protein (Thomann *et al.*, 1987).

Comparison of the amino acid sequences of component 1 proteins from a variety of diazotrophs reveals that there are five conserved cysteine residues in the α -subunit (encoded by the *nifD* gene) and three conserved cysteine residues in the β -subunit (encoded by the *nifK* gene) (Ioannidis & Buck, 1987; Holland *et al.*, 1987; Wang *et al.*, 1988). Since component 1 possesses an $\alpha_2\beta_2$ structure, a total of 16 conserved cysteine residues are present as potential ligands to the P centres and to FeMoco, insufficient to provide four ligands to each P centre and one to each FeMoco. The demonstration that tyrosine, serine, proline and other residues can act as ligands to iron–sulphur centres (Evans & Leigh, 1988)

Abbreviation used: FeMoco, iron–molybdenum cofactor of nitrogenase.

* To whom correspondence should be addressed.

raises the possibility that these residues, in addition to cysteine residues, may ligand the prosthetic groups in component 1.

In order to attempt to identify ligands to the prosthetic groups of component 1 and to elucidate their role in nitrogenase function, we have systematically altered by directed mutagenesis the amino acid sequence of component 1 of *K. pneumoniae* nitrogenase, substituting alanine for cysteine at both conserved and non-conserved positions. Alanine substitution is expected to eliminate any metal-cluster-liganding property. The properties of 11 altered nitrogenases are described. Results demonstrate that each of the conserved cysteine residues present in the α - and β -subunits is required for maximal nitrogenase activity and diazotrophic growth. Both zero-growth and slow-growth phenotypes were observed. One mutation appears to influence the interaction of FeMoco with the α -subunit. Some of our preliminary data have been reported elsewhere (Smith *et al.*, 1988). Studies in which the conserved cysteine residues of component 1 of *Azotobacter vinelandii* nitrogenase were replaced by serine have also recently been reported (Brigle *et al.*, 1988; Dean *et al.*, 1988).

MATERIALS AND METHODS

Plasmids and bacterial strains

The *K. pneumoniae nifHDK* genes were expressed from the low-copy-number plasmid pWF23, which contains a 6.4 kb *EcoRI* fragment spanning these genes (Ioannidis & Buck, 1987; see Fig. 1). Derivatives of this plasmid bearing mutations in the *nifD* or *nifK* genes were constructed by ligating appropriate restriction fragments (Fig. 1) after oligonucleotide-directed mutagenesis to generate the changes in the *nifD* and *nifK* coding sequences. Oligonucleotide-directed mutagenesis was conducted on single-stranded DNA template derived from fragments of *nifD* and *nifK* genes ligated into M13mp18 and M13mp19, by employing methods described previously (Buck *et al.*, 1987). Specifically, 0.9 kb *Asp718-BamHI* (*nifH'D'*) or 1.5 kb *BamHI-HindIII* (*nifD'K'*) fragments were cloned (see Fig. 1). The *nifHDK* plasmids were maintained in *K. pneumoniae* UNF2392 (*nifH2231::Tn7*, *RecA56*, *srl300::Tn10*, *hsdRI*) (Filler *et al.*, 1986).

Growth conditions and media

For the growth of bacteria harbouring *nifHDK* plasmids carbenicillin and ampicillin were added to all growth media at a final concentration of 200 μ g/ml each. Diazotrophic growth was assessed by plating bacteria, cultured on fresh selection media, for single colonies on NFDM (Cannon *et al.*, 1974) plates and incubating the plates anaerobically at 29 °C for 4–6 days. In all cases where growth was detected on NFDM plates, the plating efficiency observed could not be accounted for by reversion of the plasmid-borne mutation. For the preparation of de-repressed cells, bacteria (1 litre) were grown to an EEL instrument reading of 0.5–0.6 at 540 nm overnight at 30 °C in NFDM supplemented with 1 mg of $(\text{NH}_4)_2\text{SO}_4$ /ml. These cells were harvested by centrifugation, washed with NFDM and resuspended in 1 litre of NFDM supplemented with 100 μ g of aspartic acid/ml. During de-repression (22–30 h) cultures were sparged with N_2 and gently shaken.

Pulse-labelling of cells

Bacteria were de-repressed in 4 ml of NFDM supplemented with 100 μ g of aspartic acid/ml for 5 h, after which 1 ml was removed and 5 μ Ci of [^{35}S]methionine was added to it. Labelled cells were harvested after 10 min, washed and resuspended in Laemmli (1970) sample loading buffer. ^{35}S -labelled polypeptides were analysed by polyacrylamide-gel electrophoresis followed by autoradiography.

Nitrogenase assays

Whole-cell acetylene-reduction assays were conducted as described previously (Buck *et al.*, 1985). Crude extracts from 1 litre of de-repressed cultures were prepared as described previously (Filler *et al.*, 1986) and stored in liquid N_2 . Component 1 and component 2 were each assayed with an optimal concentration of the complementary component as described previously (Eady *et al.*, 1972). The specific activities of components 1 and 2 used were 1420 and 1200 units/mg respectively (1 unit of activity is defined as 1 nmol of C_2H_2 reduced/min).

Complementation of *nifB* mutant crude extracts

Cell-free extracts of de-repressed *K. pneumoniae* Kp5058 (Cannon *et al.*, 1977; Hawkes & Smith, 1983), a *nifB* mutant unable to synthesize FeMoco, were incubated with those of the various *nifD* and *nifK* mutants at 30 °C for 1 h and then assayed for component 1 activity (Filler *et al.*, 1986).

Complementation of extracts of the *nifD* and *nifK* mutants with FeMoco

A 200 μ l portion of each extract was incubated for 30 min at 30 °C with 10 μ l of 200 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and 10 μ l of purified FeMoco (Smith, 1980), capable of producing an activity of 28 700 units/ml of FeMoco when saturated with an extract from a *nifB* mutant. Then 50 μ l portions were withdrawn and assayed from component 1 activity.

E.p.r. experiments

Extracts were concentrated until the component 2 activity was 1250 units/ml, by using a Minicon B15 concentrator (Amicon) inside an anaerobic glovebox (Faircrest Engineering, Croydon, Surrey, U.K.) operating at less than 1 p.p.m. O_2 . The extracts were then placed in 3 mm-internal-diam. quartz e.p.r. tubes and the spectra were measured with a Brüker ER200D-SRC instrument updated with an ERO23M signal channel, and ERO32 field controller and an ESP1600 computer control system. Sample temperature was controlled by using an Oxford Instruments ESR9 liquid-He cooling system.

RESULTS

Single amino acid substitutions in *nifD*-gene and *nifK*-gene products

Each of the 11 substitutions in *nifDK* listed in Table 1 was generated by directed mutagenesis. The appropriate restriction fragment (Fig. 1) spanning the site of the mutation was in each case sequenced in its entirety, confirming that only the desired base changes were present. During the course of repeatedly resequencing these regions the following differences to published *nifD* and *nifK* gene sequences were noted,

Table 1. Mutations introduced into the α - and β -subunits of *K. pneumoniae* nitrogenase component 1

Plasmid	Gene and amino acid position mutated	Codon change	Substitution
pWF23 (wild-type)	None	None	None
pII1	<i>nifD</i> 63	TGC to GCC	Ala for Cys
pHK12	<i>nifD</i> 89	TGC to GCC	Ala for Cys
pHK10	<i>nifD</i> 155	TGC to GCC	Ala for Cys
pHK16	<i>nifD</i> 155	TGC to AGC	Ser for Cys
pII2	<i>nifD</i> 184	TGC to GCC	Ala for Cys
pII3	<i>nifD</i> 275	TGC to GCC	Ala for Cys
pII4	<i>nifK</i> 69	TGC to GCC	Ala for Cys
pHK13	<i>nifK</i> 94	TGC to GCC	Ala for Cys
pHK14	<i>nifK</i> 112	TGC to GCC	Ala for Cys
pHK11	<i>nifK</i> 152	TGC to GCC	Ala for Cys
pHK15	<i>nifK</i> 152	TGC to AGC	Ser for Cys

with nucleotide 1 referring to the A of the ATG *nifD* or *nifK* initiation codon: *nifD* gene 33-C $\rightarrow$ G, 46-C $\rightarrow$ G, 115-A $\rightarrow$ G, 121-A $\rightarrow$ G, 260-C $\rightarrow$ T, 261-T $\rightarrow$ C, 287-A $\rightarrow$ G, 498-C $\rightarrow$ G (differing from the sequence reported by Scott *et al.*, 1981), 705-T $\rightarrow$ C, 1110-G $\rightarrow$ C, 1113-C $\rightarrow$ G and 1329-T $\rightarrow$ G (differing from the sequence reported by Ioannidis & Buck, 1987), and *nifK* gene 394-T $\rightarrow$ C (differing from the sequence reported by Holland *et al.*, 1987). These sequence corrections change the predicted amino acid sequences of the *nifD*-gene product (as reported by Scott *et al.*, 1981) at the following positions: 16-Gln $\rightarrow$ Glu, 39-Lys $\rightarrow$ Glu, 41-Lys $\rightarrow$ Glu, 87-Ala $\rightarrow$ Val and 96-Glu $\rightarrow$ Gly. The corrections to sequence at positions 705, 1110, 1113 and 1329 in the *nifD* gene and position 394 in the *nifK* gene have also been noted independently (Steinbauer *et al.*, 1988).

The mutated *nifD* and *nifK* gene sequences were introduced into the low-copy-number plasmid pJEL101 (Løve-Larsen *et al.*, 1984) by ligating appropriate *nifHDK* gene fragments into *EcoRI*-restricted vector DNA (Fig. 1 and Table 1). In these constructs *nifHDK* gene is

expressed from the *nifH*-gene promoter, the low copy number of the plasmids preventing unbalanced *nif*-gene expression (through titration of the positive control protein NifA), which would occur with high-copy-number *nifHDK* plasmids (Reidel *et al.*, 1983). Expression of chromosomal *nifHDK* genes in *K. pneumoniae* UNF2392 was prevented by the transposon in the *nifH* gene, thus ensuring that only plasmid-borne *nifHDK* gene sequences gave rise to the component 1 and component 2 polypeptides.

Biosynthesis of mutant NifD and NifK polypeptides

Each mutant plasmid-borne *nifD* and *nifK* gene (Table 1) was assessed for its ability to express mutant NifD and NifK polypeptides. Pulse-labelled cells were analysed for their polypeptides by SDS/polyacrylamide-gel electrophoresis (Fig. 2). In all cases (including the 155-Cys $\rightarrow$ Ser substitution in NifD polypeptide; results not shown) the mutant *nifD* and *nifK* genes directed the synthesis of polypeptides of the expected molecular mass and abundance under de-repressing conditions (Fig. 2). Therefore phenotypes associated with the various mutants (see below) cannot be simply attributed to gross instability or loss of the mutant polypeptides.

Diazotrophic growth and whole-cell acetylene reduction assays

Results of these assays are summarized in Table 2. Substituting alanine for cysteine at positions 63, 89, 155 and 275 in the α -subunit and at positions 69, 94 and 152 in the β -subunit resulted in barely detectable growth under N₂, with the cells forming pin-point colonies on plates. In contrast, substituting alanine for cysteine at position 184 in the α -subunit permitted slow diazotrophic growth, and small colonies formed after 4 days.

It has been reported that replacing the conserved cysteine at position 153 with serine in the *A. vinelandii* β -subunit (the equivalent residue being cysteine-152 in the *K. pneumoniae* β -subunit) permits diazotrophic growth (Dean *et al.*, 1988). Our data show that the presence of serine but not alanine in place of cysteine at position 152 in the *K. pneumoniae* β -subunit permits good diazotrophic growth. In contrast, substituting serine for cysteine at position 154 (equivalent to 155 in the

Fig. 1. Five conserved cysteine residues encoded by *nifD* and three conserved cysteine residues encoded by *nifK* shown on the 6.4 kb *EcoRI* (E) restriction fragment cloned into pJEL101

Restriction sites used in the mutagenesis experiments are *Asp*718 (A), *Bam*HI (B) and *Hind*III (H). The transcription of *nifHDK* gene from the N₂-regulated *nifH*-gene promoter is indicated by the arrow above the restriction sites.

Fig. 2. Gel electrophoresis of pulse-labelled proteins from *K. pneumoniae* nitrogenase component 1 with mutated and wild-type α - and β -subunits

Bacteria were grown under repressing (+NH₄⁺) or de-repressing (−NH₄⁺) conditions. The positions of the *nifH*-gene-, *nifD*-gene- and *nifK*-gene-encoded polypeptides and the size of the molecular-mass markers are indicated. Lane a, *K. pneumoniae* M5a1, −NH₄⁺ (wild-type); lane b, *K. pneumoniae* M5a1, +NH₄⁺ (wild-type); lane c, *K. pneumoniae* UNF2392, −NH₄⁺ (chromosomal *nifH*::Tn7); lane d, *K. pneumoniae* UNF2392 (pJEL101), −NH₄⁺ (vector plasmid); lane e, *K. pneumoniae* UNF2392 (pWF19), −NH₄⁺ (*nifH* plasmid); lane f, *K. pneumoniae* UNF2392 (pWF23), −NH₄⁺ (*nifHDK* plasmid); lane g, *K. pneumoniae* UNF2392 (pWF23), +NH₄⁺ (*nifHDK* plasmid); lane h, 63DCys → Ala (pII1), −NH₄⁺; lane i, 89DCys → Ala (pHK12), −NH₄⁺; lane j, 155DCys → Ala (pHK10), −NH₄⁺; lane k, 184DCys → Ala (pII2), −NH₄⁺; lane l, 275DCys → Ala (pII3), −NH₄⁺; lane m, 69KCys → Ala (pII4), −NH₄⁺; lane n, 94KCys → Ala (pHK13), −NH₄⁺; lane o, 112KCys → Ala (pHK14), −NH₄⁺; lane p, 152KCys → Ala (pHK11), −NH₄⁺; lane q, 152KCys → Ser (pHK15), −NH₄⁺.

Table 2. Influence of mutations in the *nifD* and *nifK* genes of *K. pneumoniae* upon diazotrophic growth and whole-cell acetylene-reduction activity

Bacteria harbouring mutant and wild-type α - and β -subunits of nitrogenase component 1 were assessed for their ability to form colonies when grown with N₂ as sole nitrogen source and to reduce acetylene as a measure of nitrogenase activity in whole cells. Typical values for 100% acetylene reduction were 30 nmol of C₂H₄ formed/min per A₆₀₀ unit of cells.

Plasmid and mutation	Growth on N ₂	Whole-cell C ₂ H ₂ reduction (%)
pWF23 (wild-type)	++++	100
pWF19 (<i>nifDK</i> Δ)	—	0
pII1 (<i>nifD</i> 63Cys → Ala)	—	< 1
pHK12 (<i>nifD</i> 89 Cys → Ala)	—	1
pHK10 (<i>nifD</i> 155 Cys → Ala)	—	< 1
pHK16 (<i>nifD</i> 155 Cys → Ser)	—	< 1
pII2 (<i>nifD</i> 184 Cys → Ala)	+	15
pII3 (<i>nifD</i> 275 Cys → Ala)	—	1
pII4 (<i>nifK</i> 69 Cys → Ala)	—	< 1
pHK13 (<i>nifK</i> 94 Cys → Ala)	—	< 1
pHK14 (<i>nifK</i> 112 Cys → Ala)	++++	100
pHK11 (<i>nifK</i> 152 Cys → Ala)	—	< 1
pHK15 (<i>nifK</i> 152 Cys → Ser)	++	25

K. pneumoniae α -subunit) in the α -subunit of the *A. vinelandii* nitrogenase resulted in severe loss of diazotrophic growth (Brigle *et al.*, 1988; Dean *et al.*, 1988), and in *K. pneumoniae* neither serine nor alanine at

position 155 in the α -subunit is sufficient for diazotrophic growth (Table 2).

For each of the mutants examined whole-cell acetylene-reduction assays closely paralleled the patterns of growth observed under N₂, indicating a similar decreased activity towards N₂ and acetylene as substrates. Replacement of a non-conserved cysteine residue by alanine (cysteine-112 in the β -subunit) had little effect upon diazotrophic growth and acetylene reduction.

Component 1 and component 2 activities in cell-free extracts

The mutations introduced into the α - and β -subunits of nitrogenase in many cases resulted in a severe loss of capability for diazotrophic growth and acetylene-reducing activity (Table 2). The *nifH*-, *nifD*- and *nifK*-encoded polypeptides were in all cases detected in the mutants (Fig. 2), and the above experiments did not define which nitrogenase component was defective. Therefore we sought to measure the activity of component 2 and to assay for component 1 activity in crude extracts of the mutants.

In all cases where mutations in the α - or β -subunits of nitrogenase resulted in the loss of nitrogenase activity (Table 2) it was necessary to de-repress for substantially longer than with the wild-type (or with mutant nitrogenases that retained activity) to obtain extracts with significant component 2 activity. The data given in Table 3 are from extracts prepared from cells grown for times that produced optimal component 2 activities under the de-repressing conditions examined. Clearly in all cases significant component 2 activity was present in the crude extracts, whereas component 1 activity in eight of the mutants was absent. These eight mutants failed to grow diazotrophically (Table 2). The cysteine-to-alanine

Table 3. Specific activities of the component proteins in crude extracts of mutant and wild-type *K. pneumoniae* nitrogenases

Extracts were prepared at the indicated times after resuspension of washed cells in NFDm without NH_4^+ . Specific activities were determined as nmol of C_2H_2 reduced/min per mg of extract: component 1 in the presence of saturating concentrations of component 2, and component 2 in the presence of optimum concentrations of component 1.

Mutation	Crude-extract specific activity (nmol/min per mg)		Time of de-repression (h)
	Component 1	Component 2	
None (pWF23)	158	56	23
<i>nifDK</i> Δ (pWF19)	1.0	17	24
<i>nifD</i> 63Cys $\rightarrow$ Ala (pII1)	0.3	27	27
<i>nifD</i> 89Cys $\rightarrow$ Ala (pHK12)	0.3	37	27
<i>nifD</i> 155Cys $\rightarrow$ Ala (pHK10)	0.07	16	30
<i>nifD</i> 155Cys $\rightarrow$ Ser (pHK16)	0	8.0	28
<i>nifD</i> 184Cys $\rightarrow$ Ala (pII2)	6	70	28
<i>nifD</i> 275Cys $\rightarrow$ Ala (pII3)	0	33	29
<i>nifK</i> 69Cys $\rightarrow$ Ala (pII4)	0	57	29
<i>nifK</i> 94Cys $\rightarrow$ Ala (pHK13)	0.02	26	25.5
<i>nifK</i> 112Cys $\rightarrow$ Ala (pHK14)	63	57	25
<i>nifK</i> 152Cys $\rightarrow$ Ala (pHK11)	0.05	16	30
<i>nifK</i> 152Cys $\rightarrow$ Ser (pHK15)	42	40	23

mutation at position 184 in the α -subunit resulted in a significant diminution of component 1 activity, correlating with its diminished diazotrophic growth and whole-cell acetylene-reducing activity (Table 2). The presence of serine but not alanine at position 152 in the β -subunit resulted in the formation of component 1 with considerable activity, again correlating with good diazotrophic growth and whole-cell acetylene-reducing activity. Replacement of the non-conserved cysteine-112 in the β -subunit by alanine also resulted in the formation of component 1 with high activity.

Complementation of crude extracts from a *nifB* mutant

Two types of prosthetic groups are potentially liganded by the conserved cysteine residues of component 1 that we have replaced with alanine. These are the iron-sulphur P centres and FeMoco. Component 1 protein prepared from a *nifB* mutant can be activated by the addition of purified FeMoco, thus providing an assay for available FeMoco (Hawkes & Smith, 1983). Lesions in the *nifB* gene prevent the biosynthesis of FeMoco. Therefore the ability of mutant extracts to complement extracts prepared from a *nifB* mutant for component 1 activity may provide a means of distinguishing which amino acid substitutions influence the interaction of FeMoco with the α - and β -subunits. Complementation assays of this type were conducted with extracts prepared from mutants unable to form active component 1 (Table 3). A low but significant activity was generated with all extracts examined (Table 4), and we attribute this to the presence of FeMoco synthesized but not bound to component 1 polypeptides, as previously observed (Filler *et al.*, 1986). However, extracts prepared from the *nifD* cysteine-275-to-alanine mutant produced about 10-fold higher complemented activity than obtained with any of the other mutant extracts tested (Table 4). Therefore replacement of cysteine-275 in NifD polypeptide by alanine appears to make significantly more FeMoco available to the component 1 polypeptides from *nifB*⁻ cells.

Table 4. Complementation of *nifB* mutant extracts with extracts prepared from *K. pneumoniae* with mutated nitrogenase component 1 α - and β -subunits

Complemented *nifB* mutant component 1 activity was measured with saturating concentrations of component 2. Specific activities of added extracts with mutated α - and β -subunits are given in Table 3, and were subtracted from those obtained after incubation with the *nifB* mutant extract. Active extracts (pWF23 and *nifD* Ala-184) were always inhibited by addition of the *nifB* extract.

Extract added	Complemented activity (nmol of C_2H_4 formed/min per mg of Kp5058 extract)
None	0
UNF 2392 (background strain)	0 (Filler <i>et al.</i> , 1986)
pWF23 (wild-type)	-10.7
<i>nifD</i> (Ala-63)	0.26
<i>nifD</i> (Ala-89)	0.45
<i>nifD</i> (Ala-155)	0.20
<i>nifD</i> (Ala-184)	-0.15
<i>nifD</i> (Ala-275)	3.5
<i>nifK</i> (Ala-69)	0.36
<i>nifK</i> (Ala-94)	0.36
<i>nifK</i> (Ala-152)	0.40

Complementation of mutant extracts with FeMoco

We considered the possibility that in some extracts component 1 activity may have been lost because FeMoco was weakly bound and therefore readily degraded. Component 1 activity might be restored by the addition of purified FeMoco to extracts of the mutants. However, incubation of extracts with FeMoco (see the Materials and methods section) did not result in any significant increase in component 1 activity (results not shown).

Fig. 3. E.p.r. spectra of extracts from mutants with (a) cysteine-152 in the β -subunit replaced by alanine, (b) cysteine-152 in the β -subunit replaced by serine or (c) cysteine-275 in the α -subunit replaced by alanine

Samples were prepared as described in the Materials and methods section. Spectra were recorded at 12K at 9.4348 GHz with field modulation of 27.77G at 100 kHz and a microwave power of 103 mW. g -values are indicated on the stick diagrams.

E.p.r. studies on extracts of mutants

The FeMoco centres in dithionite-reduced component 1 exhibit a characteristic e.p.r. signal, with g -values near 4.3, 3.7 and 2.01 (Lowe *et al.*, 1985). Although many cell components can contribute signals that obscure the $g = 2$ region, the higher g -values are readily distinguishable in whole cells or crude extract preparations (Davis *et al.*, 1972). Examination of all extracts from mutants for this signal was undertaken (see the Materials and methods section). Only extracts from cells capable of diazotrophic growth, i.e. from the mutants with cysteine-112 replaced by alanine in NifK polypeptide, cysteine-152 replaced by serine in NifK polypeptide and cysteine-184 replaced by alanine in NifD polypeptide exhibited the normal component 1 signal. For example a strong signal was observed when cysteine-152 in the β -subunit was replaced with serine (Fig. 3b) but not when it was replaced with alanine (Fig. 3a). The presence of alanine rather than cysteine at position 184 in the α -subunit resulted in a relatively weak signal, correlating with the slow diazotrophic growth of this mutant and its low component 1 activity (see Tables 2 and 3). A broader signal, with g -values near 4.5 and 3.5, was observed in extracts from the mutant with cysteine-275 replaced by alanine in the NifD polypeptide (Fig. 3c). This signal may reasonably be associated with the loosely bound FeMoco in these extracts. When FeMoco is extracted into *N*-methylformamide it exhibits a broader e.p.r. spectrum, with g -values near 4.6 and 3.4. This spectrum is sharpened by addition of thiophenol to become more like the protein spectrum (Rawlings *et al.*, 1978). In extracts (Fig. 3c) the lower-field component of the observed signal is overlaid by other signals. These were observed with variable intensities in all extracts and are tentatively assigned to cytochrome-containing membranous material.

DISCUSSION

Interspecies sequence comparisons have revealed that the α -subunit of nitrogenase has five and the β -subunit three cysteine residues that are always conserved (Ioannidis & Buck, 1987; Holland *et al.*, 1987; Wang *et al.*, 1988). The retention of nitrogenase activity by a mutant in which a non-conserved cysteine residue (cysteine-112 in the β -subunit) was replaced with alanine adds credence to the notion that conservation of primary sequence is likely to reflect regions of important functionality. Our results clearly demonstrate that the conserved cysteine residues are important but not necessarily essential for the formation of active component 1. For example cysteine-184 in the α -subunit can be replaced by alanine without eliminating diazotrophic growth and component 1 activity, whereas similar replacements of cysteine-63, -89, -155 and -275 in the α -subunit and cysteine-69 and -94 in the β -subunit do; cysteine-152 in this subunit can be replaced by serine but not alanine. Clearly the conserved cysteine residues do not all have equivalent functionality.

In addition to their component 1 activity phenotypes, all mutants tested that were deficient in nitrogenase activity showed different de-repression characteristics. In general a longer period of de-repression was required to detect component 2 activity if component 1 activity was low or lacking. The mutations introduced into the α - and β -subunits do not appear to result in their rapid degradation or in the loss of the capacity to form active component 2. It is possible that the contribution of combined nitrogen from N_2 reduction catalysed by nitrogenase promotes nitrogenase biosynthesis under the de-repressing conditions used. This could reflect the synthesis or activity of a regulatory gene product or a more global limitation on macromolecular biosynthesis.

Results from our e.p.r. studies show that FeMoco is not bound in its normal environment in any of the mutants that we have examined that fail to grow on N_2 . It seems improbable that all of the conserved cysteine residues that we have replaced with alanine directly ligand FeMoco. Furthermore we do not expect a gross perturbation in the structure of the polypeptide backbone resulting from substituting alanine (for example the cysteine-112-to-alanine mutation in the NifK polypeptide is relatively benign). Changes in the FeMoco environment resulting from the alanine substitutions could be due to the loss of P centres liganded by the cysteine residues and required of the integrity of component 1 and the binding of FeMoco.

Possible explanations for substitutions in either subunit influencing the environment of FeMoco include the binding of the P centres or FeMoco itself between subunits or that P centres must be present in the polypeptides before FeMoco is inserted. This last possibility would tend to support the 'all-or-none' hypothesis that was suggested in order to explain the observation that the ratio of iron species, representing both P centres and FeMoco, observed in the Mössbauer spectra of component 1 preparations appeared to be independent of the specific activity and iron content of the protein, i.e. that all preparations consisted of mixtures of molecules containing all, none or exactly half of the centres present in fully active protein (Smith *et al.*, 1980). Consistent with the above is our finding that simple addition of

FeMoco to extracts of the mutants did not restore activity.

The presence of a thiol (cysteine) or hydroxy (serine) group at position 152 in the β -subunit appears to be adequate for formation of active component 1, perhaps indicating that the thiolate ligand to a metal normally provided by cysteine-152 can be replaced by oxygen. It seems possible that the amino acid sequence adjacent to this position, which is rich in hydroxy groups, Ser-Thr-Thr, may contribute to the ability of serine-152 to replace cysteine-152 in the β -subunit. This could explain the lack of equivalence when cysteine-155 in the α -subunit is compared with cysteine-152 in the β -subunit (replacement of cysteine-152 by serine in the α -subunit does not produce active component 1); the environment of cysteine-155 is Gln-Ser-Glu. It also calls into question the degree of functional similarity between the *N*-terminal thirds of the α - and β -subunits inferred from their apparent homology, particularly with respect to the three conserved cysteine residues present in each of these regions (Thöny *et al.*, 1985; Holland *et al.*, 1987; Wang *et al.*, 1988).

The *nifB* complementation phenotype of the cysteine-275-to-alanine replacement in the α -subunit shows an increase in the availability of FeMoco in crude extracts as compared with the other mutants. This could result from a global change in protein structure resulting in FeMoco being loosely bound to the mutant polypeptide, or simply the loss of an essential ligand to FeMoco. Since the α - and β -polypeptides synthesized in the absence of FeMoco are activated by purified FeMoco and a gross distortion of the polypeptide structure resulting from substituting alanine for cysteine is not expected, we currently favour the notion that cysteine-275 is close to or ligands FeMoco. This suggestion is supported by the e.p.r. spectrum of extracts of the mutant with cysteine-275 replaced by alanine in the NifD polypeptide, which is similar to that of extracted FeMoco lacking thiol ligation. Residues around the 275 position have amide and carboxylate functions (asparagine-271, asparagine-280 and glutamic acid-287) and, on the basis of the requirement for solvents with amide function to extract FeMoco, may be N-ligands to FeMoco, further supporting the suggestion (Brigle *et al.*, 1985) that the position 275 region of the α -subunit is involved in an interaction with FeMoco. Directed mutagenesis of the homologous region of the *A. vinelandii nifE*-gene product influences FeMoco biosynthesis, again supporting the suggestion of an interaction of FeMoco with the position 275 region of the α -subunit (D. R. Dean, personal communication). The failure of added FeMoco to restore activity to extracts of the cysteine-275-to-alanine mutant may indicate that the cysteine-275 residue is essential for the activity of bound cofactor.

The replacement of cysteine by alanine at position 184 in the α -subunit did not eliminate component 1 function completely. It is possible that, since mutations in residues 186 and 195 in the α -subunit abolish assembly of the α - and β -subunits, this region of the α -subunit is involved in subunit assembly (Zamir & Govenzensky, 1988), and thus assembly and not solely the liganding of a prosthetic group may be influenced by the alanine substitution at position 184. Biochemical and further spectroscopic analysis of the nitrogenase proteins from the mutants should help to identify the precise nature of the bio-

chemical lesions resulting from the mutagenesis described in this paper.

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