Biochem. J. (1991) 277, 457–464 (Printed in Great Britain)

Altered nitrogenase MoFe proteins from *Azotobacter vinelandii*

Analysis of MoFe proteins having amino acid substitutions for the conserved cysteine residues within the β-subunit

Harold D. MAY,* Dennis R. DEAN†‡ and William E. NEWTON*‡

*Department of Biochemistry and †Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, U.S.A.

The regions surrounding the three strictly conserved cysteine residues (positions 70, 95 and 153) in the β-subunit of the *Azotobacter vinelandii* nitrogenase MoFe protein have been proposed to provide P-cluster environments [Dean, Setterquist, Brigle, Scott, Laird & Newton (1990) Mol. Microbiol. **4**, 1505–1512]. In the present study, each of these cysteine residues was individually substituted by either serine or alanine by site-directed mutagenesis of the *nifK* gene, which encodes the MoFe protein β-subunit. A mutant strain for which the codon for Cys-153 is removed was also isolated. Significant structural or functional roles are indicated for the cysteine residues at positions 70 and 95, where substitution by either serine or alanine eliminates diazotrophic growth of the resulting strains and abolishes or markedly decreases both MoFe-protein acetylene-reduction activity and the intensity of the whole-cell $S = \frac{3}{2}$ e.p.r. signal. Changes introduced at position 153 have various effects on the functional properties of the enzyme. The strains produced either by deletion of the Cys-153 residue or its substitution by serine exhibit only a moderate decrease in diazotrophic growth and MoFe-protein activity and no loss of the whole-cell e.p.r.-signal intensity. In contrast, substitution by alanine eliminates diazotrophic growth and very markedly decreases both MoFe-protein activity and e.p.r.-signal intensity. These results are interpreted in terms of a metallocluster-driven protein rearrangement. After purification of the altered MoFe protein, in which serine replaces Cys-153, an investigation of its catalytic and spectroscopic properties confirms that neither the FeMo cofactor, i.e. the substrate-reduction site, nor the component–protein interaction site has been affected. Instead, these data indicate a disruption in electron transfer within the MoFe protein, which is consistent with a role for this residue (and region) at the P clusters.

INTRODUCTION

Molybdenum-dependent nitrogenase occurs only in prokaryotic organisms and it is responsible for catalysing the reduction of N_2 gas to NH_3 (for a review see Smith, 1990). This two-component enzyme (Bulen & LeComte, 1966) also catalyses the reduction of a variety of other small-molecule substrates, most notably acetylene to yield only ethylene (Dilworth, 1966; Schöllhorn & Burris, 1967). In the absence of added substrate, protons are reduced to H_2 gas (Burns & Bulen, 1965), the only catalysed reaction that is insensitive to the presence of CO (Hardy *et al.*, 1965; Bulen *et al.*, 1965). The smaller of the two protein components is the Fe protein (or component II), a homodimer (encoded by *nifH*) of approx. 63 kDa that has a single [4Fe-4S] cluster symmetrically bridged between its two subunits (Hausinger & Howard, 1983; Howard *et al.*, 1989; Georgiadis *et al.*, 1990). The larger component is the MoFe protein (or component I), which is a $\alpha_2\beta_2$ tetramer (encoded by *nifD* and *nifK*) of approx. 230 kDa. It contains two Mo atoms and about 30 Fe atoms and 30 S^{2-} groups per molecule. The Fe and S^{2-} contained within the MoFe protein appear to be equally distributed between metalloclusters of two distinct types (Shah & Brill, 1977; Zimmermann *et al.*, 1978). One of these cluster types, the P clusters, can be extruded as about four [4Fe-4S] clusters (Kurtz *et al.*, 1979), although their structure within the MoFe protein is unknown (Hagen *et al.*, 1987; Bolin *et al.*, 1990). The remaining Fe and S^{2-}, together with both Mo atoms, constitute two identical FeMo cofactors (Shah & Brill, 1977). There is considerable evidence to show that the MoFe protein contains the substrate-reducing sites (Orme-Johnson *et al.*, 1972; Smith *et al.*, 1973; Mortenson *et al.*, 1973), which are embodied in the FeMo cofactors (Hawkes *et al.*, 1984; Scott *et al.*, 1990). Enzymic turnover is effected under anaerobic conditions by a sequence of associations–dissociations of the two component proteins (Hageman & Burris, 1980; Lowe & Thorneley, 1984) during which the Fe protein acts as a one-electron donor to the MoFe protein and two molecules of MgATP are hydrolysed per electron transfer.

The properties of the P clusters and FeMo cofactors have been investigated by a variety of biophysical techniques. So far, these techniques have been unsuccessful in producing a complete structural or functional definition of either type of prosthetic group. Recently, a complementary approach, using site-directed mutagenesis, has been initiated (Brigle *et al.*, 1987*a*; Dean *et al.*, 1988, 1990*a*,*b*; Kent *et al.*, 1989, 1990; Scott *et al.*, 1990) to determine both the distribution of these prosthetic groups among the two subunit types and their individual catalytic responsibilities. Our strategy has involved formulation of a model (Dean *et al.*, 1990*a*) in which potential metallocluster domains located within the MoFe protein are tentatively assigned. This model is now being tested by placement of known amino acid substitutions for targeted key residues within the MoFe protein, followed by determination of the resulting catalytic and spectroscopic consequences (see, e.g., Dean *et al.*, 1990*a*). Salient features of our working model include: (i) regions within the MoFe protein encompassing the α-subunit histidine residue 195 and the cysteine residue 275 (numbers refer to the *Azotobacter vinelandii* sequence; see Brigle *et al.*, 1985) provide FeMo-cofactor domains; (ii) P-cluster domains are equally distributed among the α- and β-subunits of the MoFe protein;

‡ To either of whom correspondence should be addressed.

Table 1. Mutations introduced, Nif phenotypes and specific activities of the nitrogenase component proteins in crude extracts of wild-type and mutant strains of *A. vinelandii*

Specific activities are expressed as nmol of ethylene produced/min per mg of total extract protein in the presence of saturating concentrations of the complementary protein. Δ represents deletion of the Cys-153 residue.

Strain	Codon altered	Codon change	Substitution	Nif phenotype	Specific activity: MoFe protein	Specific activity: Fe protein
Wild-type	None	None	None	+	51.7	19.9
DJ243	*nifK*70	TGC→GCC	Cys→Ala	−	0.5	24.3
DJ84	*nifK*70	TGC→TCC	Cys→Ser	−	0.1	14.3
DJ120	*nifK*95	TGC→TCC	Cys→Ser	−	0.2	24.7
DJ252	*nifK*153	TGC→GCC	Cys→Ala	−	1.4	33.0
DJ122	*nifK*153	TGC→TCC	Cys→Ser	+	24.8	25.5
DJ219	*nifK*153	TGC→Δ	Cys removed	+	18.6	26.3

(iii) regions surrounding the strictly conserved cysteine residues 62, 88 and 154 in the α-subunit and 70, 95 and 153 in the β-subunit provide P-cluster environments.

We and others have previously reported results that support the assignment of the α-subunit His-195 region (Scott *et al.*, 1990) and Cys-275 region (Dean *et al.*, 1990*b*; Kent *et al.*, 1989, 1990) as providing FeMo-cofactor domains. We have also described experiments designed to probe the proposed P-cluster environments in the α-subunit (Dean *et al.*, 1990*a*). Our reported results on the *A. vinelandii* MoFe protein and those of others from complementary studies on the *Klebsiella pneumoniae* MoFe protein are in full agreement. Here, we describe the biochemical and e.p.r. spectroscopic analyses of nitrogenase from six different mutant strains of *A. vinelandii*, each of which contains an altered MoFe-protein β-subunit changed at one of the three conserved cysteine residue positions.

MATERIALS AND METHODS

Strain construction

Methods for site-directed mutagenesis, gene replacement and the isolation of mutant strains were performed as described or cited previously (Brigle *et al.*, 1987*a*). The six mutant strains analysed in the present study are listed in Table 1. The isolation of strain DJ122, having the β-subunit residue Cys-153 substituted by serine, was previously reported (Dean *et al.*, 1988). MoFe proteins having an alteration within the MoFe-protein β-subunit primary sequence are designated by the mutated gene (*nifK*), the amino acid position altered and the single-letter code for the normal and substituting amino acid. For example, an altered MoFe protein having the β-subunit Cys-153 position substituted by serine is designated K153C→S (also see Table 1).

Growth conditions and media

Wild-type and mutant strains were grown at 30 °C in a modified Burk medium (Strandberg & Wilson, 1968). This medium contained 0.01 mM-Na_2MoO_4 to ensure that only Mo-dependent nitrogenase was under study. When required, a fixed-nitrogen source of filter-sterilized urea was added to a final concentration of 20 mM. Diazotrophic growth characteristics of the strains were determined by inoculating 50 ml of nitrogen-free Burk medium in a 250 ml side-arm flask to 10–20 Klett units and monitoring growth with a Klett–Summerson colorimeter fitted with a no. 54 filter. For purification and kinetic studies, strains DJ122 and DJ219 were grown with N_2 as the sole source of nitrogen; all others were grown on urea-supplemented media and de-repressed for nitrogenase synthesis. De-repression of nitrogenase and preparation of crude extract were performed as described previously (Jacobson *et al.*, 1989).

Nitrogenase assays

The MoFe-protein and Fe-protein activities of each crude extract were assayed for acetylene reduction in the presence of an optimal concentration of its purified complementary protein as reported previously (Brigle *et al.*, 1987*b*), except that 50 μl portions of the extract were titrated to maximal activity. The complementary proteins were purified from *A. vinelandii* strain OP as described previously (Burgess *et al.*, 1980) and had specific activities of 1040 (Fe protein) and 1608 (MoFe protein) nmol of ethylene formed/min per mg. For the native and altered MoFe proteins purified in this work, assays were performed as described previously (Wherland *et al.*, 1981), except that NH_3 was determined with an *o*-phthalaldehyde/mercaptoethanol reagent at pH 7 (Corbin, 1984) and the dithionite concentration was adjusted as required. The amount of MoFe protein added was calculated from its Mo content, assuming two Mo atoms per molecule. Acetylene-reduction assays were performed under a 10% acetylene/90% argon atmosphere and ethylene production was quantified by gas chromatography on a Poropak N column. When required, CO (10%) was added by gas-tight syringe to the appropriate assay vial during the preincubation period. Each assay contained a total of 1 mg of nitrogenase proteins in 1 ml to avoid complications introduced by both high and low protein concentrations. Protein concentrations were determined by the biuret method (Gornall *et al.*, 1948). Mo and Fe contents were determined as described previously (Burgess *et al.*, 1980).

E.p.r. spectroscopy

De-repressed whole cells were prepared for e.p.r. spectroscopy as described earlier (Scott *et al.*, 1990). The spectra of these samples (at 50 mW) and of purified MoFe proteins (at 5 mW) were recorded on a Bruker ER300 instrument at 9.46 GHz and at 8 K maintained by liquid-helium boil-off.

Gel electrophoresis

For SDS/PAGE, samples and gels were prepared according to the Laemmli (1970) procedure. The gels used were 12% polyacrylamide (1.35% cross-linker) with a 4% stacking gel. Electrophoresis was performed at 20 mA/gel in a Hoefer Mighty Small apparatus (Hoefer, San Francisco, CA, U.S.A.) and the gels were stained with Coomassie Blue (R-250). Two-dimensional gel electrophoresis as described by O'Farrell (1975) was used to

examine the accumulation of nitrogenase component proteins in crude extracts.

Purification of MoFe protein from strain DJ122

The altered MoFe protein from DJ122 cells and native MoFe protein from wild-type cells were purified in parallel to provide a comparative analysis at each stage of the protocol. Crude extracts, prepared as described above, were heated in a water bath at 56 °C for 5 min, cooled and then centrifuged at 47000 *g* for 1 h. The resulting supernatants were then fractionated by chromatography on a Pharmacia f.p.l.c. system (Piscataway, NJ, U.S.A.) with monitoring at 405 nm. All columns were made anaerobic by equilibration with degassed 25 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-$Na_2S_2O_4$ before use and all operations were carried out anaerobically. Fractionation was accomplished with a 0.15–0.5 M-NaCl gradient run at 4 ml/min for 26 min on a Mono Q HR 10/10 anion-exchange column. Both altered and native MoFe proteins were eluted at 0.2 M-salt and were collected. Similar behaviour was observed when larger volumes (containing 500 mg of total protein) were chromatographed anaerobically on a 2.5 cm × 14 cm DEAE-Sepharose (Pharmacia) column.

The Mono Q fraction containing the partially purified MoFe protein was then made 0.5 M in $(NH_4)_2SO_4$ and applied to a Pharmacia phenyl-Superose HR 5/5 hydrophobic interaction column, the equilibration buffer of which included 0.5 M-$(NH_4)_2SO_4$. An $(NH_4)_2SO_4$ gradient (0.5–0.0 M run at 0.5 ml/min for 30 min) eluted wild-type MoFe protein at 0.33 M, and the altered protein was eluted at 0.29 M. The collected fraction was then dialysed against 25 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-$Na_2S_2O_4$ within an anaerobic enclosure (Vacuum Atmospheres Corp., Hawthorne, CA, U.S.A.), concentrated by pressure dialysis in a Spectra/Por S25-10 cell fitted with a type C membrane (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) and stored in liquid N_2.

The larger amounts of both wild-type and altered (K153C→S) MoFe protein obtained by DEAE-Sepharose chromatography were further purified by using crystallization techniques described previously (Burgess *et al.*, 1980).

RESULTS

Diazotrophic growth and nitrogenase activities of crude extracts

Separate substitution by alanine or serine within the MoFe-protein β-subunit at the Cys-70 position to give the strains DJ243 (K70C→A) and DJ84 (K70C→S) respectively or substitution by serine at the Cys-95 position to give strain DJ120 (K95C→S) eliminated the diazotrophic-growth capacity of the respective mutants. In contrast, the diazotrophic-growth capability of cells with alterations at the Cys-153 residue position in the β-subunit varied depending on the substitution. Substitution by alanine at this position to give DJ252 (K153C→A) eliminated diazotrophic growth whereas substitution by serine to give DJ122 (K153C→S) had a minimal effect. DJ219, a mutant strain having the Cys-153 position deleted (K153→Δ), also remained capable of diazotrophic growth. These results are summarized in Table 1. The same diazotrophic-growth phenotypes found for *A. vinelandii* mutant strains having the MoFe-protein β-subunit Cys-153 residue substituted either by serine (Dean *et al.*, 1988) or by alanine (the present study) were also reported independently for equivalent mutant strains of *K. pneumoniae* (Kent *et al.*, 1989).

Fe-protein and MoFe-protein acetylene-reduction activities present in nitrogenase-de-repressed extracts of all the mutant strains described in this study are shown in Table 1. Effective de-repression of nitrogenase synthesis in each of these strains is evidenced by their Fe-protein acetylene-reduction activity (Table 1) and by their accumulation of MoFe-protein subunits as determined by two-dimensional gel-electrophoretic analyses (results not shown; see, e.g., Brigle *et al.*, 1987*a*). Thus the lack of MoFe-protein activity in crude extracts of strains DJ84 (K70C→S) and DJ120 (K95C→S) must arise from a defect in nitrogenase catalysis rather than from a decrease in the accumulation of nitrogenase component proteins. Both strains DJ243 (K70C→A) and DJ252 (K153C→A) exhibited very low, but detectable, levels of MoFe-protein activity. These activities were surprising because both strains exhibit a strictly Nif^- phenotype and the equivalent *K. pneumoniae* strains were reported to have no such activity (Kent *et al.*, 1989). The MoFe-protein activities present in strains DJ122 (K153C→S) and DJ219 (K153C→Δ) were 48% and 36% of wild-type activity, and these values correlate with their slower diazotrophic growth rates (approx. 4–5 h doubling times compared with approx. 3 h for wild-type). A *K. pneumoniae* mutant strain equivalent to DJ122 exhibited 27% of the wild-type MoFe-protein activity (Kent *et al.*, 1989).

E.p.r. studies on whole cells

Under reducing conditions, the FeMo-cofactor centres of the native MoFe protein exhibit a biologically unique $S = \frac{3}{2}$ e.p.r. signal having characteristic *g* values near 4.3, 3.7 and 2.01. The *g* values near 4 are also easily discernible in de-repressed whole cells of wild-type *A. vinelandii* or in its crude extracts (Davis *et al.*, 1972). No $S = \frac{3}{2}$ e.p.r. signal was observed in whole cells of strain DJ84 (K70C→S) or strain DJ120 (K95C→S). This result parallels the observed lack of both diazotrophic growth and MoFe-protein activity in these strains. In contrast, whole cells of strains DJ122 (K153C→S), DJ219 (K153C→Δ), DJ243 (K70C→A) and DJ252 (K153C→A) all exhibited an $S = \frac{3}{2}$ e.p.r. signal, which was identical in lineshape, width and *g* values with that of the native MoFe protein. However, the intensity of these whole-cell signals did not directly correlate with the MoFe-protein activities present in their corresponding crude extracts. The $S = \frac{3}{2}$ e.p.r. signal intensity present in the Nif^+ strains, DJ219 (K153C→Δ) and DJ122 (K153C→S), was approximately equal to that present in wild-type cells, even though their catalytic activity was about 40–50% of wild-type. DJ252 (K153C→A) and DJ243 (K70C→A) cells exhibited an $S = \frac{3}{2}$ e.p.r. signal at about 25% and 2% the intensity of wild-type signal, and their corresponding MoFe-protein activities were only about 3% and 1% respectively of wild-type. The results with strains DJ252 (K153C→A) and DJ243 (K70C→A) contrast with the equivalent *K. pneumoniae* mutant strains (Kent *et al.*, 1989), which were reported to lack both an $S = \frac{3}{2}$ e.p.r. signal and MoFe-protein activity.

Purification of the altered MoFe protein from DJ122

Partial purification of the altered MoFe protein from crude extracts prepared from wild-type or strain-DJ122 (K153C→S) cells was achieved by a heat step, followed by either f.p.l.c. over Mono Q or conventional chromatography over DEAE-Sepharose. Either of the latter procedures resulted in complete recovery of MoFe-protein activity, which was devoid of Fe-protein activity. Similar levels of purification were achieved for both the wild-type and strain-DJ122 (K153C→S) MoFe proteins as judged by MoFe-protein activity and PAGE criteria (see Fig. 1). At this stage of purification, the wild-type MoFe protein had a specific activity of 640 nmol of ethylene formed/min per mg compared with 197 nmol/min per mg for the K153C→S protein, and both proteins had a metal content of 28 ± 4 Fe atoms per Mo

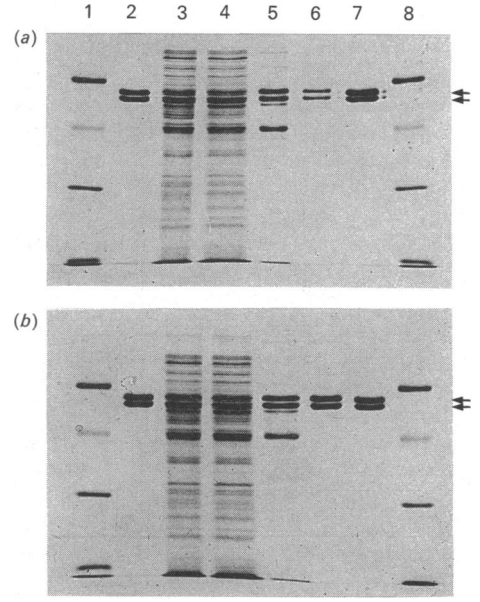

Fig. 1. SDS/PAGE of the nitrogenase MoFe proteins from (*a*) wild-type and (*b*) DJ122 strains of *A. vinelandii* during purification

Cells were grown and de-repressed and crude extracts prepared as described in the Materials and methods section. Lanes 1 and 8, molecular-mass standards (BSA, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; RNAase, 13.7 kDa); lanes 2 and 7, 2.0 μg of crystallized native MoFe protein; lane 3, 20 μg of extract protein; lane 4, 20 μg of 56 °C-treated extract protein; lane 5, 7.5 μg of MoFe-protein fraction purified by f.p.l.c. Mono Q anion-exchange chromatography; lane 6, 3.0 μg of MoFe-protein fraction after Mono Q and phenyl-Superose chromatography. Arrows identify the α- and β-subunits of the MoFe protein.

atom. Further parallel purification of the wild-type and K153C→S MoFe proteins, by either crystallization (of the DEAE-Sepharose fractions) or hydrophobic-interaction chromatography (of the Mono Q fractions), produced material having specific activities of 1443 and 618 nmol/min per mg respectively with an average Fe/Mo ratio of 14±2:1. As was found in crude extracts, the purified K153C→S protein has about 50% of the wild-type activity. The yield of the K153C→S protein on crystallization, however, was much lower than the yield off the phenyl-Superose column (8% versus 70%).

Table 2 lists the maximum specific activities with respect to N_2 fixation, H_2 evolution under argon and acetylene reduction for the wild-type and K153C→S MoFe proteins obtained at various stages of their purification. If these activities are expressed on the basis of ng-atoms of Mo present rather than on mg of MoFe protein present in the assay, in order to minimize any differences in purity among different batches of the same protein as well as among native and altered protein samples, the altered protein displays about 60% of the native MoFe-protein catalytic capacity.

The e.p.r. spectrum of the purified K153C→S MoFe protein was identical with the spectrum of the native MoFe protein in the $g = 4$ region, but its $g = 2$ component was relatively broader and slightly more intense (results not shown). This effect probably results from a lower purity of the altered protein sample. On a Mo basis, the e.p.r. spectral intensities (measured as the relative heights of the $g = 3.7$ feature) of the native and K153C→S MoFe proteins were comparable.

Comparison of the substrate-reduction characteristics of wild-type and K153C→S MoFe proteins

Substrate-reduction data were obtained in parallel for the native and altered proteins to detect any change in substrate-reduction specificity, electron flux, inhibitor (CO) susceptibility or electron-allocation pattern under an N_2 atmosphere caused by the introduced substitution. In the presence of saturating Fe protein, the electron flux through either the wild-type or K153C→S MoFe protein is constant and independent of the substrate (N_2, H^+ or acetylene) being reduced (see Table 2). Under 100% N_2, both the native and altered proteins divert approx. 30% of the flux to H_2 evolution. Under 10% acetylene, ethylene is the only hydrocarbon product. When 10% CO is

Table 2. Maximum specific activities of MoFe proteins purified from wild-type and DJ122 strains of *A. vinelandii*

Specific activities are expressed in terms of nmol of product/min per mg under the atmospheres indicated and in the presence of saturating concentrations of purified native *A. vinelandii* Fe protein. Total $2e^-$ under N_2 represents all products formed on the basis of electron pairs ($1.5 \times NH_3$ plus H_2) to allow a direct comparison among substrates. MQ, PS and DEAE represent purification by Mono Q, phenyl-Superose and DEAE-Sepharose chromatography respectively; cryst. represents purification by crystallization. N.D., not determined. Numbers in parentheses are ng-atoms of Mo per mg of protein in the samples.

	Specific activity				
	100% N_2			100% Ar	10% C_2H_2
Sample	NH_3	H_2	Total $2e^-$	H_2	C_2H_4
MQ					
Wild-type (5.2)	334.4	270.4	771.9	717.1	693.5
K153C→S (2.4)	100.5	50.0	200.8	225.6	191.3
MQ/PS					
Wild-type (5.8)	687.9	490.7	1522.5	1519.0	1432.6
K153C→S (4.2)	300.3	204.5	655.0	N.D.	604.7
DEAE					
Wild-type (3.2)	327.4	177.6	668.6	724.5	591.8
K153C→S (2.0)	96.6	58.4	203.3	243.8	202.3
DEAE/cryst.					
Wild-type (5.5)	789.3	562.1	1746.0	1635.7	1454.1
K153C→S (3.9)	367.8	213.3	765.0	748.8	632.2

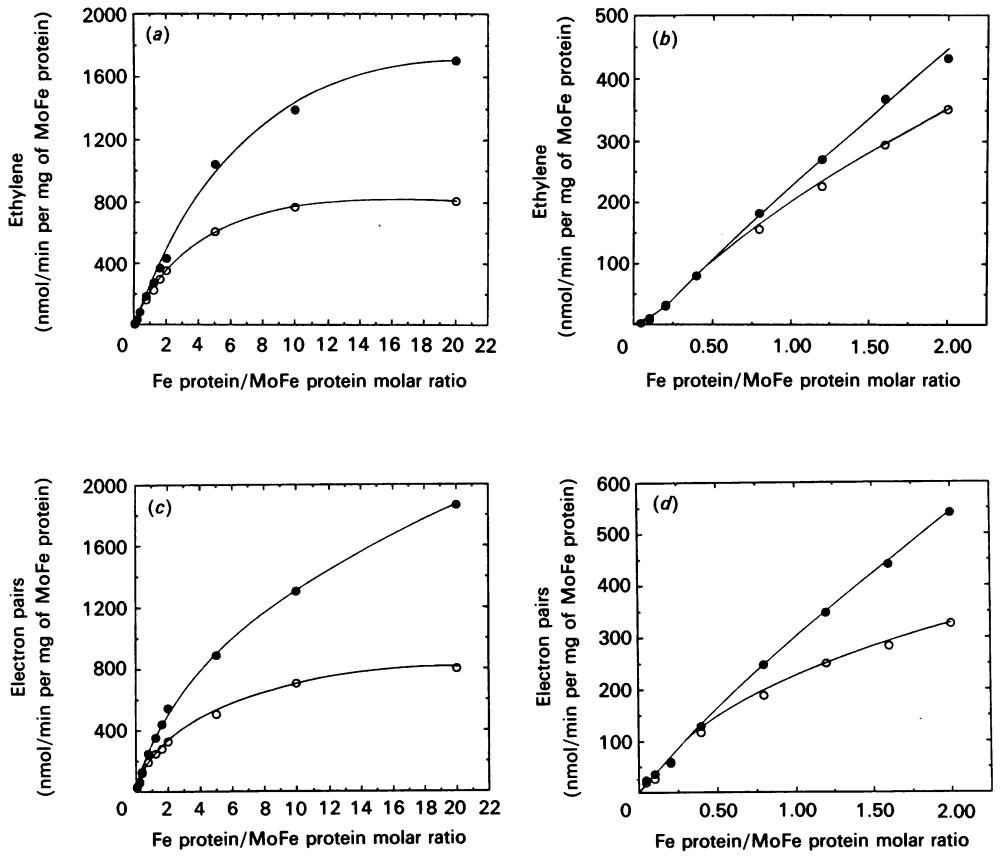

Fig. 2. Product formation by wild-type and K153C→S nitrogenase under atmospheres of either 10% acetylene (*a* and *b*) or 100% N_2 (*c* and *d*) as a function of the component-protein ratio

Partially purified (DEAE-Sepharose fractions) wild-type (●) and K153C→S (○) MoFe proteins were assayed as described in the Materials and methods section with various molar ratios of purified native *A. vinelandii* Fe protein added. Specific activity in nmol of product/min per mg of MoFe protein is plotted against Fe protein/MoFe protein molar ratio in the ranges 0.05:1 to 20:1 (for *a* and *c*) and 0.05:1 to 2.0:1 (for *b* and *d*). Nitrogen fixation is expressed as electron pairs, i.e., $2e^-$, which is calculated as ($1.5 \times NH_3$ plus H_2).

present (results not shown), the flux through either MoFe protein remains effectively unchanged and the only observable product is H_2 under any of the three atmospheres.

The only difference noted among the two MoFe proteins was in electron flux, which was first investigated by changing the Fe protein/MoFe protein molar ratio (Hageman & Burris, 1980) over the range of 0.05:1 to 80:1. For 10% acetylene (Figs. 2*a* and 2*b*), the rate of ethylene production (and therefore flux) is virtually the same for the wild-type and K153C→S MoFe proteins up to a Fe protein/MoFe protein molar ratio of about 1:1 (170 nmol of ethylene formed/min per mg). Above this ratio, the rates diverge significantly to a different maximum for each MoFe protein (1976 versus 837 nmol of ethylene formed/min per mg for the wild-type and K153C→S MoFe proteins respectively). Both systems saturate at about the same molar ratio of 20:1. Under 100% N_2, the same trends and comparable rates are observed for NH_3 and H_2 production by both MoFe proteins (Figs. 2*c* and 2*d*). The observed rate for both MoFe proteins at a molar ratio of 0.8:1 was 218, whereas maximum rates at a ratio of 20:1 were 1867 and 803 nmol of electron pairs/min per mg of MoFe protein for wild-type and K153C→S MoFe proteins respectively. The electron distribution to H_2 and NH_3 changes identically with changing flux for both the native and altered MoFe proteins; H_2 accounts for more than 50% of the flux at ratios of less than 0.4:1 and for about 27% at ratios greater than 5:1.

The responses of the native and altered MoFe proteins to changes in electron flux were also investigated by holding the Fe protein/MoFe protein molar ratio constant at about 1:1 and varying the dithionite concentration. When electron flux was controlled in this way, the specific activities of the native and K153C→S MoFe proteins responded in parallel. At dithionite concentrations of 0.25 mM, 0.50 mM and 1.00 mM, the specific activities for both proteins were 49±3, 81±6 and 111±2 nmol of H_2/min per mg respectively.

The experiments on Fe-protein/MoFe-protein-molar-ratio-dependence can also be used to investigate whether the Fe protein–MoFe protein interaction has been affected by the introduced substitution. When the data are recalculated as Fe-protein specific activity versus Fe protein/MoFe protein molar ratios (results not shown), similar patterns of inhibition of Fe-protein activity (Eady *et al.*, 1972) result with both wild-type and K153C→S MoFe proteins under either N_2 or a 10% acetylene atmosphere. For all four data sets, the average maximum specific activity calculated for the Fe protein is 939 nmol of product/min per mg of protein. The molar ratio at which maximum Fe-protein specific activity is achieved is about 0.4:1 and 0.8:1 for the K153C→S and wild-type MoFe proteins respectively.

DISCUSSION

It is generally accepted that P clusters serve as intermediate electron carriers or as electron-storage units within the MoFe

protein. Requirements for extrusion of the P clusters from the MoFe protein indicate that they are bound within the MoFe protein mainly through cysteine mercaptide ligands. We have previously proposed that the conserved MoFe-protein β-subunit cysteine residues 70, 95 and 153 are likely candidates to serve as direct P-cluster ligands, as are the analogously conserved cysteine residues 62, 88 and 154 found within the α-subunit (Dean *et al.*, 1990*a*). If this hypothesis is correct and P clusters are involved in the intramolecular delivery of electrons to the substrate-reduction site, substitutions placed at any of these positions should either eliminate or alter electron transfer internal to the MoFe protein and therefore impact upon nitrogenase catalysis. Substitution by alanine or serine for either the MoFe-protein β-subunit Cys-70 or Cys-95 residue eliminates diazotrophic growth of the respective mutant strains and markedly decreases or abolishes MoFe-protein activity and the e.p.r.-signal intensity. These residues therefore do appear to have important structural or functional roles within the MoFe protein.

Interpretation of the results obtained with mutant strains having the MoFe-protein β-subunit Cys-153 residue either deleted or replaced by serine or alanine is more complicated because differential effects occur depending upon the specific alteration. Substitution of the Cys-153 residue by serine does not cause a severe decrease in diazotrophic growth although it does affect MoFe-protein specific activity. This result indicates either that the β-subunit Cys-153 residue does not provide an essential P-cluster ligand or that some other suitable ligand arrangement is formed in its absence. One possibility is that a functional replacement could occur at the same residue position. Namely, serine might serve as a functional replacement for the β-subunit Cys-153 residue. Another possibility is that a functional replacement could arise from a changed folding pattern within the altered polypeptide such that a new ligand arrangement is formed. In the case of MoFe protein from strain DJ219, where the β-subunit Cys-153 residue is removed, the polypeptide must seek a new ligand arrangement if Cys-153 normally provides an essential P-cluster ligand. Such a metallocluster-driven protein re-arrangement has been reported for studies involving site-directed substitution of the known cysteine ligands of the [4Fe-4S] cluster of ferredoxin I from *A. vinelandii* (Martin *et al.*, 1990). In contrast with the above results, substitution of Cys-153 by alanine apparently prevents the repositioning of a functionally competent replacement residue. Thus substitution by alanine at the Cys-153 position must place constraints on the folding of the β-subunit such that juxtapositioning of a suitable replacement ligand is not favoured.

If substitution of the β-subunit Cys-153 residue by serine results in a new P-cluster ligand arrangement, some change in the functional properties of the P clusters is expected. As shown in Table 2, the K153C→S MoFe-protein specific activity maximum is about 50% of that of the wild-type MoFe protein. This decrease in specific activity cannot be explained by either inherent instability of the altered protein or the absence of P clusters because comparable yields of protein containing similar Fe contents are obtained when altered and native MoFe proteins are purified in parallel. The decrease in electron flux through the altered MoFe protein, which is reflected by its lower maximum specific activity, is therefore due to (i) an alteration in the intramolecular delivery of electrons to the substrate-reduction site, (ii) the actual substrate-reduction site within the K153C→S MoFe protein being compromised, or (ii) disruption of specific component–protein interactions. To distinguish among these possibilities, we compared the catalytic and spectroscopic properties of wild-type MoFe protein and K153C→S MoFe protein that had been purified in parallel. Results of these experiments are discussed below.

Previous studies have demonstrated that amino acid substitutions placed within either of the proposed FeMo-cofactor-binding domains located within the MoFe-protein α-subunit can result in changes in its catalytic and spectroscopic properties (Scott *et al.*, 1990; Dean *et al.*, 1990*b*). Such alterations may include (i) changes in the linewidth, lineshape and g values of the characteristic $S = \frac{3}{2}$ e.p.r. signal, (ii) the ability to catalyse the four-electron reduction of acetylene to yield ethane as an additional product (wild-type Mo-dependent nitrogenase catalyses the reduction of acetylene by two electrons to yield ethylene only), (iii) changes in the relative distribution of electron flux to different substrates, and (iv) CO inhibition of nitrogenase-catalysed proton reduction (nitrogenase-catalysed H_2 evolution is normally insensitive to CO). The K153C→S MoFe protein could not be distinguished from the wild-type MoFe protein on the basis of e.p.r. spectroscopy, relative distribution of electron flux to different substrates or susceptibility to the inhibitor CO. Taken together, these results indicate that the FeMo-cofactor sites have not been changed in the altered K153C→S MoFe protein and therefore cannot be responsible for the lowered maximum specific activity observed for the altered MoFe protein.

The alternative explanation is that the K153C→S MoFe protein is defective in its capacity to deliver electrons to the substrate-reduction site. Such a defect could arise from an alteration either in the interaction of the component proteins (i.e. in the delivery of electrons from the Fe protein to the MoFe protein) or in the delivery of electrons within the MoFe protein to the substrate-reduction site. These possibilities may be distinguished by the system's response as the electron flux is varied. Electron flux through nitrogenase can be controlled by manipulating one of several parameters, e.g. ATP concentration, dithionite concentration or the component-protein ratio. Variation in any of these factors produces the same result in terms of changing the flux (Hageman & Burris, 1980).

We first chose to control electron flux by changing the component-protein ratio and to monitor the effects on catalysis under either an N_2 or a 10% acetylene atmosphere. At high Fe protein/MoFe protein molar ratios ($\geq 20:1$), electron flux through nitrogenase is greatest and the specific activity of MoFe protein is maximized (Wherland *et al.*, 1981). The Fe protein/MoFe protein molar ratio required to reach maximal NH_3 formation under 100% N_2 was found to be about 20:1 for both the altered and the wild-type MoFe proteins (see Fig. 2). As the component-protein ratio was decreased, i.e. the flux decreased, the difference between the two specific activities also decreased until the activities became equal at a molar ratio of about 1:1 and below. The same trend was observed for both H_2 evolution and NH_3 formation under N_2 and for ethylene formation. Under N_2 the changing flux affected electron distribution between NH_3 formation and H_2 evolution identically for the wild-type and the altered MoFe protein. These results indicate that interaction of the altered MoFe protein with the Fe protein is not significantly affected as a consequence of the amino acid substitution, otherwise a decrease in the altered MoFe-protein specific activity, relative to the wild-type MoFe-protein specific activity, should be apparent at all component/protein ratios.

This conclusion was tested by a second series of experiments in which electron flux was controlled via the dithionite concentration. In these experiments electron flux was measured by H_2 evolution under argon. To minimize the effect of the Fe protein/MoFe protein ratio on activity and to circumvent limitation of the assay by dithionite exhaustion, the component-protein ratio was kept constant at about 1:1. Under these conditions, the specific activity of both the native and K153C→S MoFe protein increased in parallel with increasing

dithionite concentration. These flux-related data are in agreement with those obtained by changing the component-protein ratio.

Further confirmation was forthcoming from determining the maximum specific activity of the wild-type Fe protein used in these assays. If indeed the introduced mutation has no effect on the Fe protein–MoFe protein interaction, the maximum specific activity of the Fe protein measured should be independent of the MoFe protein used. The maximum specific activity of the Fe protein determined in this way (939 nmol of product/min per mg) compares well with the specific activity of 1040 nmol/min per mg measured before the experiments.

Our interpretation of the results is that, under conditions of high electron flux, the maximum specific activity of the altered MoFe protein is limited by the intramolecular delivery of electrons to the substrate-reduction site. Thus, when electron flux through the system becomes limited in some other way, for example by lowering the Fe protein/MoFe protein molar ratio, the altered and wild-type MoFe proteins have nearly the same specific activity. This interpretation is in line with the hypothesis that the MoFe-protein β-subunit Cys-153 residue is associated with a P cluster and that P clusters are involved in mediating electron transfer to the substrate-reduction site.

The observation that it is possible to alter a proposed P-cluster environment with no apparent effect on the FeMo-cofactor environment, as in the K153C→S MoFe protein, is consistent with previous reports that these metal clusters are separate entities (Zimmermann *et al.*, 1978; Hawkes & Smith, 1983; Paustian *et al.*, 1990; Bolin *et al.*, 1990). However, on the basis of e.p.r. spectroscopic analyses, not all altered MoFe proteins that have an amino acid substitution placed within a proposed P-cluster domain appear to have a complete complement of FeMo cofactor. For example, the mutant strain of *A. vinelandii* that has the Cys-90 residue in the β-subunit substituted by serine exhibits no $S = \frac{3}{2}$ e.p.r. signal. Similar results have been reported for other mutant strains isolated from *A. vinelandii* (Dean *et al.*, 1990*a*) and *K. pneumoniae* (Kent *et al.*, 1990) that have amino acid substitutions placed within proposed MoFe-protein α-subunit P-cluster domains. Several different possibilities might explain these results: (i) elimination of P-cluster functionality could prevent insertion of the FeMo cofactor into the apo-MoFe protein; (ii) certain substitutions within proposed P-cluster domains could have global effects on the MoFe-protein structure such that the FeMo-cofactor-binding site is not accessible; or (iii) certain substitutions within proposed P-cluster domains could prevent the proper assembly of the MoFe-protein tetramer. Evidence that supports this last possibility has been reported (Kent *et al.*, 1990).

In summary, results reported here and previously (Dean *et al.*, 1988; Kent *et al.*, 1990; Dean *et al.*, 1990*a*) indicate that cysteine residues targeted as probable P-cluster ligands do have important roles in maintaining the catalytic integrity of the MoFe protein. Our success in purifying the MoFe protein from one such mutant strain, plus the recent progress concerning the elucidation of the three-dimensional structure of the MoFe protein from *Clostridium pasteurianum* (Bolin *et al.*, 1990), shows considerable promise for advancing a description of the organization and function of the P clusters within the MoFe protein.

We acknowledge support from the National Science Foundation (Grant DMB 85-10029) and the U.S. Department of Agriculture Competitive Research Grants Office (Grant 89-37120-4803). We thank Kevin Brigle for isolating the mutants, John Windle for assistance with e.p.r. spectroscopy and Mary Weiss for assistance with assays.

REFERENCES

Bolin, J. T., Ronco, A. E., Mortenson, L. E., Morgan, T. V., Williamson, M. & Xuong, N.-H. (1990) in Nitrogen Fixation: Achievements and Objectives (Gresshoff, P. M., Stacey, G., Roth, L. E. & Newton, W. E., eds.), pp. 117–124, Chapman and Hall, New York

Brigle, K. E., Newton, W. E. & Dean, D. R. (1985) Gene **37**, 37–44

Brigle, K. E., Setterquist, R. E., Dean, D. R., Cantwell, J. S., Weiss, M. C. & Newton, W. E. (1987*a*) Proc. Natl. Acad. Sci. U.S.A. **84**, 7066–7069

Brigle, K. E., Weiss, M. C., Newton, W. E. & Dean, D. R. (1987*b*) J. Bacteriol. **169**, 1547–1553

Bulen, W. A. & LeComte, J. R. (1966) Proc. Natl. Acad. Sci. U.S.A. **56**, 979–986

Bulen, W. A., Burns, R. C., LeComte, J. R. & Hinkson, J. (1965) in Non-Heme Iron Proteins: Role in Energy Conversion (San Pietro, A., ed.), pp. 261–274, Antioch Press, Yellow Springs

Burgess, B. K., Jacobs, D. J. & Stiefel, E. I. (1980) Biochim. Biophys. Acta **614**, 196–209

Burns, R. C. & Bulen, W. A. (1965) Biochim. Biophys. Acta **105**, 437–445

Corbin, J. L. (1984) Appl. Environ. Microbiol. **47**, 1027–1030

Davis, L. C., Shah, V. K., Brill, W. J. & Orme-Johnson, W. H. (1972) Biochim. Biophys. Acta **256**, 512–523

Dean, D. R., Brigle, K. E., May, H. D. & Newton, W. E. (1988) in Nitrogen Fixation: Hundred Years After (Bothe, H., DeBruijn, F. J. & Newton, W. E., eds.), pp. 107–113, Gustav Fischer, Stuttgart and New York

Dean, D. R., Setterquist, R. A., Brigle, K. E., Scott, D. J., Laird, N. F. & Newton, W. E. (1990*a*) Mol. Microbiol. **4**, 1505–1512

Dean, D. R., Scott, D. J. & Newton, W. E. (1990*b*) in Nitrogen Fixation: Achievements and Objectives (Gresshoff, P. M., Stacey, G., Roth, L. E. & Newton, W. E., eds.), pp. 95–102, Chapman and Hall, New York

Dilworth, M. J. (1966) Biochim. Biophys. Acta **127**, 285–294

Eady, R. R., Smith, B. E., Cook, K. A. & Postgate, J. R. (1972) Biochem. J. **128**, 655–675

Georgiadis, M. M., Chakrabarti, P. & Rees, D. C. (1990) in Nitrogen Fixation: Achievements and Objectives (Gresshoff, P. M., Stacey, G., Roth, L. E. & Newton, W. E., eds.), pp. 111–116, Chapman and Hall, New York

Gornall, A. G., Bardawall, C. J. & David, M. M. (1948) J. Biol. Chem. **177**, 751–766

Hageman, R. V. & Burris, R. H. (1980) Biochim. Biophys. Acta **591**, 63–75

Hagen, W. R., Wassink, H., Eady, R. R., Smith, B. E. & Haaker, H. (1987) Eur. J. Biochem. **169**, 457–465

Hardy, R. W. F., Knight, E., Jr. & D'Eustachio, A. J. (1965) Biochem. Biophys. Res. Commun. **20**, 539–544

Hausinger, R. P. & Howard, J. B. (1983) J. Biol. Chem. **258**, 13486–13492

Hawkes, T. R. & Smith, B. E. (1983) Biochem. J. **209**, 43–50

Hawkes, T. R., McLean, P. A. & Smith, B. E. (1984) Biochem. J. **217**, 317–321

Howard, J. B., Davis, R., Moldenhauer, B., Cash, V. L. & Dean, D. (1989) J. Biol. Chem. **264**, 11270–11274

Jacobson, M. R., Cash, V. L., Weiss, M. C., Laird, N. F., Newton, W. E. & Dean, D. R. (1989) Mol. Gen. Genet. **219**, 49–57

Kent, H. M., Ioannidis, I., Gormal, C., Smith, B. E. & Buck, M. (1989) Biochem. J. **264**, 257–264

Kent, H. M., Baines, M., Gormal, C., Smith, B. E. & Buck, M. (1990) Mol. Microbiol. **4**, 1497–1504

Kurtz, D. M., Jr., McMillan, R. S., Burgess, B. K., Mortenson, L. E. & Holm, R. H. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4986–4989

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Lowe, D. J. & Thorneley, R. N. F. (1984) Biochem. J. **224**, 877–886

Mortenson, L. E., Zumft, W. G. & Palmer, G. (1973) Biochim. Biophys. Acta **292**, 422–435

Martin, A., Burgess, B. K., Stout, C. D., Cash, V., Dean, D. R., Jensen, G. M. & Stephens, P. J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 598–602

O'Farrell, P. H. (1975) J. Biol. Chem. **250**, 4007–4021

Orme-Johnson, W. H., Hamilton, W. D., Jones, T. L., Tso, M.-Y., Burris, R. H., Shah, V. K. & Brill, W. J. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 3142–3145

Paustian, T. D., Shah, V. K. & Roberts, G. P. (1990) Biochemistry **29**, 3515–3522

Schöllhorn, R. & Burris, R. H. (1967) Proc. Natl. Acad. Sci. U.S.A. **58**, 213–216

Scott, D. J., May, H. D., Newton, W. E., Brigle, K. E. & Dean, D. R. (1990) Nature (London) **343**, 188–190
Shah, V. K. & Brill, W. J. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3249–3253
Smith, B. E. (1990) in Nitrogen Fixation: Achievements and Objectives (Gresshoff, P. M., Stacey, G., Roth, L. E. & Newton, W. E., eds.), pp. 3–13, Chapman and Hall, New York
Smith, B. E., Lowe, D. J. & Bray, R. C. (1973) Biochem. J. **135**, 331–341
Strandberg, G. W. & Wilson, P. W. (1968) Can. J. Microbiol. **14**, 25–31
Wherland, S., Burgess, B. K., Stiefel, E. I. & Newton, W. E. (1981) Biochemistry **20**, 5132–5140
Zimmermann, R., Münck, E., Brill, W. J., Shah, V. K., Henzl, M. T., Rawlings, J. & Orme-Johnson, W. H. (1978) Biochim Biophys. Acta **537**, 185–207

Received 14 September 1990/7 January 1991; accepted 16 January 1991