The Role of Methionine 156 in Cross-subunit Nucleotide Interactions in the Iron Protein of Nitrogenase*

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A variant Fe protein has been created at the completely conserved residue methionine 156 by changing it to cysteine. The Azotobacter vinelandii strain expressing M156C is unable to grow under nitrogen-fixing conditions, and the purified protein cannot support substrate reduction in vitro. This mutation has an effect on the Fe protein’s ability to undergo the MgATP-induced conformational change as evidenced by the fact that M156C is chelated in the presence of MgATP with a lower observed rate than wild-type. While the electron paramagnetic resonance spectra of this protein are similar to those of the wild-type Fe protein, the circular dichroism spectrum is markedly different in the presence of MgATP, showing that the conformation adopted by M156C following nucleotide binding is different from the wild-type conformation. Although competition activity and chelation assays show that this Fe protein can still form a complex with the MoFe protein, this altered conformation only supports MgATP hydrolysis at 1% the rate of wild-type Fe protein. A model based on x-ray crystallographic information is presented to explain the importance of Met-156 in stabilization of the correct conformation of the Fe protein via critical interactions of the residue with Asp-43 and nucleotide in the other subunit.

The nitrogenase enzyme catalyzes the reduction of atmospheric dinitrogen to ammonia and thus plays a critical role in the global nitrogen cycle. This enzyme is found in a large number of organisms, ranging from symbiotic and free-living bacteria to cyanobacteria, and has been extensively studied in the soil bacterium Azotobacter vinelandii (1, 2). The nitrogenase enzyme consists of two oxygen-labile metalloproteins. The first is the molybdenum-iron (MoFe) protein, a Mr ~ 240,000 αβ2 heterotetramer encoded by the nifD and nifK genes. This protein has been crystallographically characterized by itself (3) and in a complex with the second nitrogenase protein (4). Additionally, the x-ray crystallographic structure of this protein from the obligate anaerobe Clostridium pasteurianum has also been solved (5). The resulting crystal structures have confirmed the existence of two different and interesting types of metal clusters. The first of these is called the iron-molybdenum cofactor (FeMo cofactor). This cofactor is a [Mo-7Fe-9S-homocitrate] cluster (3, 6) and is thought to be the site of substrate reduction (1). The second cluster is a [8Fe-7S] cluster called the P-cluster that appears to accept electrons as they are transferred to the MoFe protein (3, 6, 7).

The second protein in the nitrogenase enzyme is the iron (Fe) protein. This protein is a Mr ~ 60,000 αβ homodimeric protein and is encoded by the nifH gene. It has also been crystallographically characterized by itself (8) and in a complex with the MoFe protein (4). These crystal structures show that the Fe protein contains a single [4Fe-4S] cluster bound by two cysteinyl sulfurs from each identical subunit. The Fe protein is the only known reductant that is able to transfer electrons to the MoFe protein in such a way that the MoFe protein is then able to use them for substrate reduction.

Each Fe protein subunit binds a single molecule of MgATP at a Walker A consensus sequence (9, 10), approximately 19 Å from the [4Fe-4S] cluster (4, 8). This binding event triggers a conformational change that is required for the formation of a productive complex with the MoFe protein (11, 12). The conformational change is believed to be initiated by the breaking of a salt bridge between Lys-15 and Asp-125 by the MgATP γ-phosphate (13, 14). The subsequent movement of Asp-125 then communicates the nucleotide binding event to the [4Fe-4S] cluster through the movement of the polypeptide backbone connecting residue Asp-125 to the [4Fe-4S] cluster ligand Cys-132. This sequence of residues connecting Asp-125 to Cys-132 has been named switch II based on homology to other nucleotide binding proteins. While the residues in the Walker A sequence and the switch II sequence have been well characterized (11, 13–17), the proper function of these two regions of the protein is not the only requirement for the conformational change that occurs upon MgATP binding. We have previously identified a conserved region of the Fe protein that has been shown to play an important role in the protein’s ability to undergo the proper MgATP-induced conformational change (12, 18, 19). This region, which extends from residues 144 to 173, contains an α-helix designated helix ö5 (4, 8). Changes made at Ala-157, near the subunit interface, dramatically affect the protein’s ability to undergo the MgATP-induced conformational change. To better understand the role of this region in the MgATP-induced conformational change, a mutant Fe protein in which cysteine is introduced at the neighboring, completely conserved, Met-156 has been constructed, purified, and characterized.

EXPERIMENTAL PROCEDURES

All chemicals and reagents were obtained from Fisher, Baxter Scientific, or Sigma unless otherwise noted.

Construction and Expression of the Variant A. vinelandii Strain—An A. vinelandii chromosomal fragment containing the nifH, nifD, and part of the nifK genes was cloned into the bacteriophage M13mp18. Site-directed mutagenesis was performed using the Mutagenic Mutagenesis Kit, version 2 from Bio-Rad. The mutagenesis oligonucleotide was purchased from Integrated DNA Technologies, Coralville, IA. The oligonucleotide was 24 bases long, was complementary to the region surrounding the Met 156 codon, and was degenerate, allowing the production of several Fe protein mutants at position 156. Following the mutagenesis procedure, single strand DNA sequencing (Sequenase II, U. S. Biochemical Corp.) was used to select bacteriophage that con-
tained mutated nifH genes. Double-stranded DNA was isolated from this phage using a Qiagen kit (Qiagen, Chatsworth, CA) and was used to transform two A. vinelandii strains, the wild-type Trans strain, and the ΔnifH strain DJ54 using a published method (20). Here, the chromosomal copy of the gene was replaced with the mutated gene through homologous recombination, allowing construction of variants in its native background, under the control of its native promoter. The recombination of the mutated nifH gene containing the Met to Cys mutation into the bacterial chromosome of the Trans strain resulted in a strain that was unable to grow under nitrogen-fixing conditions. All subsequent work was done on this Trans-derived strain.

**Culturing and Protein Purification**

The M156C A. vinelandii strain was grown in 180-liter batches in a 250-liter New Brunswick fermenter in Burk minimal medium under ammonium acetate-limiting derepressing conditions. The medium was made 2 mM in ammonium acetate, such that the ammonia was exhausted during mid-log phase. Cultures were then derepressed for an additional 3 h and harvested using a flow-through centrifugal harvester (Cepa, Germany). The cell paste was washed with 0.05 M Tris-HCl, pH 8.0, and was kept on dry ice until needed. The variant Fe protein was purified as described previously (21) with some slight modifications. In particular, a heat step was not performed, and the gel filtration column was AcA34 (ICF, Cédez, France). The purified Fe protein was analyzed by polyacrylamide gel electrophoresis, and its concentration was determined using the Biuret method (22).

**Spectroscopy**

For spectroscopic experiments, all samples were made in a Vacuum Atmospheres dry box under argon with less than 1 ppm oxygen. For EPR, the samples were 2 mM in Na$_2$S$_2$O$_4$. The spectra were recorded on a Bruker ESP 300 EZ spectrophotometer equipped with an Oxford Instruments ESR-9002 liquid helium continuous flow cryostat. For CD experiments, the [4Fe-4S] cluster was oxidized by passage over a specially prepared column as described previously (22). The column (1 × 10 cm) consists of, from top to bottom, 5 cm of indigo disulfonate dye (MC/B, Norwood, OH) bound to AG 1-X8 ion exchange resin (Bio-Rad) and 5-cm P6DG gel filtration resin (Bio-Rad). The protein was loaded onto the top of the column and was allowed to incubate longer than 15 min. It was then eluted with 0.05 M Tris-HCl, pH 8.0. The CD spectra were obtained using a Jasco J720 spectropolarimeter. Ultraviolet/visible spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer.

**Activity Assays**

Enzyme activity was determined by measuring hydrogen evolution under an argon atmosphere or ethylene evolution under a 10% acetylene/90% argon atmosphere. Both assays were performed by incubating the Fe and MoFe proteins together in the presence of a reaction mixture (19) and 20 mM sodium acetate, such that the ammonia was exhausted during mid-log phase. The M156C Fe protein was then purified anaerobically and, according to purification profiles and yields, was found to be present in approximately wild-type levels. When added to a standard nitrogenase assay for hydrogen evolution the wild-type Fe protein supported a rate of hydrogen evolution of 2224 nmol min$^{-1}$·mg protein$^{-1}$, but the M156C Fe protein was unable to support detectable levels of hydrogen evolution. Likewise, while the wild-type Fe protein supported acetylene reduction at a rate of 1612 nmol min$^{-1}$·mg protein$^{-1}$, the M156C Fe protein activity was undetectable. These data confirm that the Ni$^{2+}$ phenotype of the strain expressing M156C Fe protein is due to a defect in its electron transfer function.

**RESULTS AND DISCUSSION**

**Fe Protein Residue Met-156 Is Essential for Substrate Reduction by Nitrogenase**

Met-156 is a completely conserved residue in the Fe protein of nitrogenase that is located at the base of helix a5 (4, 8). As shown in Fig. 1A, in the absence of nucleotides, this residue protrudes into the interface between the two subunits of the Fe protein and is far removed from the [4Fe-4S] cluster. Here we have constructed a M156C variant Fe protein and expressed the protein in its native background in A. vinelandii. In contrast to the wild-type A. vinelandii strain, this strain was unable to grow under N$_2$-fixing conditions. The Fe protein has three defined roles in N$_2$ fixation: it is required for the initial biosynthesis of FeMo cofactor (22–29); it is required for the insertion of preformed FeMo cofactor into FeMo cofactor-deficient MoFe proteins (30); and it is the only protein that can serve as an electron donor to the MoFe protein in support of substrate reduction (1). The first two functions can be monitored by EPR spectroscopy, because the FeMo cofactor center of wild-type holo-MoFe protein exhibits a characteristic S = 3/2 EPR signal (31) that can easily be observed in cell-free extracts. The EPR spectra of crude extracts of the strain expressing M156C Fe protein clearly show that the M156C strain is able to participate both in the biosynthesis and insertion of the FeMo cofactor (data not shown), indicating that the defect must be in the electron transfer function of the M156C Fe protein.

The M156C Fe protein was then purified aerobically and, according to purification profiles and yields, was found to be present in approximately wild-type levels. When added to a standard nitrogenase assay for hydrogen evolution the wild-type Fe protein supported a rate of hydrogen evolution of 2224 nmol min$^{-1}$·mg protein$^{-1}$, but the M156C Fe protein was unable to support detectable levels of hydrogen evolution. Likewise, while the wild-type Fe protein supported acetylene reduction at a rate of 1612 nmol min$^{-1}$·mg protein$^{-1}$, the M156C Fe protein activity was undetectable. These data confirm that the Ni$^{2+}$ phenotype of the strain expressing M156C Fe protein is due to a defect in its electron transfer function.

**The M156C Fe Protein Has Altered Reactivity with Nucleotides**

In the first step of a normal nitrogenase reaction the Fe protein binds two molecules of MgATP and undergoes a conformational change that is critical for the next step in the reaction, productive binding to the MoFe protein (11, 12). One method that has been used extensively to characterize the MgATP-induced conformational change is iron chelation by compounds such as a,a′-dipyridyl (25, 26). In the absence of nucleotides, in the presence of MgADP, the wild-type Fe protein’s [4Fe-4S]$^+$ cluster is almost entirely protected from chelation by a,a′-dipyridyl. This is also true for the M156C Fe protein (data not shown). Upon addition of MgATP, the reactivity of the chelator for the iron in the [4Fe-4S]$^+$ cluster of the wild-type Fe protein is greatly increased, such that the iron is entirely removed from the Fe protein’s cluster (26).
compares the behavior of the wild-type and M156C Fe proteins in a standard nitrogenase chelation assay. Clearly both proteins are able to bind MgATP and undergo a conformational change that makes the Fe atoms of the cluster accessible to the chelating reagent. This chelation reaction has complex biphasic kinetics (26) and was therefore analyzed by fitting a double exponential curve to the data as described under “Experimental Procedures.” The data from four experiments provided average wild-type values for $k_a$ and $k_b$ of $6.3 \times 10^{-2} \pm 4.1 \times 10^{-3}$ s$^{-1}$ and $9.7 \times 10^{-3} \pm 4.5 \times 10^{-4}$ s$^{-1}$, respectively. In contrast to this, the values associated with the M156C Fe protein were lower, $4.9 \times 10^{-2} \pm 4.1 \times 10^{-3}$ s$^{-1}$ (78% wild-type) and $5.2 \times 10^{-3} \pm 6.1 \times 10^{-5}$ s$^{-1}$ (54% wild-type) for $k_a$ and $k_b$, respectively. These decreased rates could be caused by a slower conformational change or by the protein ending up in a different conformation with the Fe atoms less accessible to the chelator, or both. We then proceeded to examine the M156C Fe protein using spectroscopic methods to determine whether it adopts the correct conformation upon MgATP binding or whether it adopts another conformation.

The Conformation of the M156C-MgATP Complex Is Different from the Wild-type

EPR spectroscopy can be used to characterize the Fe protein in the [4Fe-4S]$^{2+}$ oxidation state (31), and the change that occurs upon MgATP binding by monitoring the characteristic $S = 1/2$ signal that changes from rhombic in the nucleotide-free conformation to axial in the MgATP-bound conformation (32). The spectra of the wild-type and M156C Fe proteins in the presence of 2 mM Na$_2$S$_2$O$_4$ and in the absence and presence of MgATP are indistinguishable from each other (data not shown). These data suggest that the M156C Fe protein has a similar conformation to that of the wild-type Fe protein in the absence of nucleotide and that it undergoes a similar conformational change upon MgATP binding.

Circular dichroism (CD) spectroscopy in the visible region can be used to examine the environment of [Fe-S] clusters in proteins. For the Fe protein, weak but measurable CD is observed for the [4Fe-4S]$^{2+}$ oxidation state of the protein that changes upon binding of either MgATP or MgADP (33, 34). MgADP binding is known to induce a less dramatic conformational change in the Fe protein than does MgATP (1, 18). Fig. 3 shows the CD spectra of both the wild-type and M156C Fe proteins in the absence of nucleotide (top panel), in the presence of MgADP (middle panel), and in the presence of MgATP (bottom panel). The line shape of the wild-type and M156C Fe protein spectra is similar in the absence of nucleotide or in the presence of MgADP, suggesting that their conformations are very similar. In sharp contrast, the spectrum of the wild-type Fe protein in the presence of MgATP is strikingly different from that of the M156C Fe protein in the presence of MgATP. Thus, in the region of the [4Fe-4S]$^{2+}$ cluster, the conformation...
of the M156C Fe protein is somehow different from that of the wild-type protein.

We have considered the possibility that the differences observed in the EPR and CD spectra are entirely dependent on the redox state of the protein. If this were the case, we would conclude, based on the spectroscopic evidence, that the M156C Fe protein adopts the normal conformation in the [4Fe-4S]$^{1+}$ state, but adopts an abnormal conformation when oxidized to the [4Fe-4S]$^{2+}$ state. However, our experimental results suggest that this is not the case. Our chelation experiments, in which the Fe protein is in the [4Fe-4S]$^{1+}$ state, indicate that the M156C Fe protein adopts a conformation similar enough to the wild-type protein's conformation to allow iron chelation, but different enough to affect the observed rates of chelation. These different rates suggest an altered structure around the [4Fe-4S] cluster. As discussed below, the ability of the reduced M156C Fe protein to form a complex with the MoFe protein suggests a conformation that is similar overall to the wild-type protein, while the variant's inability to support MgATP hydrolysis and substrate reduction suggests conformational differences. These results, all of which are obtained from reduced Fe proteins, combined with the CD results indicate that the structure of the M156C Fe protein in either oxidation state is similar to, but not identical with, the wild-type structure.

The Role of Methionine 156 in A. vinelandii Fe Protein

The M156C Fe Protein Forms a Complex with the MoFe Protein

Taken together the above data show that the M156C Fe protein binds MgATP and undergoes a conformational change but that the final product is somehow different in the region of the [4Fe-4S] cluster. The next step in nitrogenase turnover for the wild-type protein is the formation of a complex between the Fe protein and the MoFe protein. We examined the interactions between these proteins using two techniques. The first technique is a modification of the standard chelation experiment in which MoFe protein is included in the cuvette. As discussed below, the ability of the reduced M156C Fe protein to form a complex with the MoFe protein suggests a conformation that is similar overall to the wild-type protein, while the variant's inability to support MgATP hydrolysis and substrate reduction suggests conformational differences. These results, all of which are obtained from reduced Fe proteins, combined with the CD results indicate that the structure of the M156C Fe protein in either oxidation state is similar to, but not identical with, the wild-type structure.

The M156C Fe Protein Is Blocked for MgATP Hydrolysis

The Fe protein alone does not hydrolyze MgATP. Instead, complex formation with the MoFe protein appears to bring this result by showing that the [4Fe-4S] cluster is covered up by the surface of the MoFe protein when the two proteins are in a complex. This prevents the chelator from gaining access to the [4Fe-4S] cluster, thus inhibiting chelation. Fig. 4 shows the results of a chelation experiment performed in the presence of the MoFe protein. It is immediately obvious that this mutant Fe protein does receive protection from chelation in the presence of the MoFe protein. Thus, by this criterion, the two proteins are able to form a complex.

The second technique we used was designed to answer the question: can the inactive M156C Fe protein compete with wild-type Fe protein in an activity assay? Fig. 5 shows the results of an experiment in which increasing amounts of either wild-type or M156C Fe protein were added to an activity assay with a fixed amount of wild-type Fe protein present. If the M156C Fe protein could not form a complex with the MoFe protein then the activity would not be affected by its presence. In contrast, Fig. 5 shows that the M156C Fe protein is able to compete effectively with the wild-type Fe protein in an activity assay, confirming the result obtained from the chelation protection assay. Because it has been known for several years that the MgATP conformation of the Fe protein is a prerequisite for formation of a productive complex (11, 12), these data also show that the global conformation obtained by the M156C Fe protein following MgATP binding must be very similar, in the MoFe protein binding regions, to that of the wild-type Fe protein-MgATP complex.

The MgATP-dependent chelation of purified wild-type and M156C by α,α’-dipyridyl. The reaction was performed as indicated under “Experimental Procedures.” The observed rates for the wild-type and M156C Fe proteins are listed in the text.

FIG. 2. MgATP-dependent chelation of purified wild-type and M156C by α,α’-dipyridyl. The reaction was performed as indicated under “Experimental Procedures.” The observed rates for the wild-type and M156C Fe proteins are listed in the text.

FIG. 3. Circular dichroism spectra of oxidized wild-type (thin lines) and M156C (thick lines) in the absence of nucleotide (upper panel), in the presence of 5 mM MgADP (middle panel), and in the presence of 5 mM MgATP (upper panel). Protein concentrations were determined using the Biiuret method (22).
residues within the Fe protein into the correct orientation to catalyze MgATP hydrolysis (1, 2, 4, 35). As shown above, the M156C Fe protein binds MgATP, undergoes a conformational change, and binds to the MoFe protein. Once that complex is formed, however, the rate of MgATP hydrolysis is negligible compared with that of the wild-type at 86 nmol·min⁻¹·mg⁻¹ and 7770 nmol·min⁻¹·mg⁻¹ Fe protein, for the M156C and wild-type Fe proteins, respectively. Thus the conformational difference observed by CD appears to prevent the correct orientation of groups involved in MgATP hydrolysis within the M156C Fe protein.

The possibility that the system is blocked at a step subsequent to MgATP hydrolysis, such as phosphate or MgADP release seems unlikely. Phosphate release has been shown to occur prior to complex dissociation (36). Thus, if there were a problem with phosphate release, we would expect to see the formation of a tight complex. If a tight complex were forming between the M156C Fe protein and the MoFe protein, we would expect to see a much greater degree of protection from chelation than the intermediate protection we observed (Fig. 4). Furthermore, we would expect to see a greater degree of inhibition when the M156C Fe protein is titrated into an activity assay (Fig. 5). Finally, no abnormal peaks were observed during gel filtration chromatography while the M156C Fe protein was being purified that would suggest the presence of a tight complex with the MoFe protein. Thus, the M156C Fe protein does not form a tight complex with the MoFe protein. This line of reasoning does not rule out blockage at the MgADP release step. However, the CD data suggest that the M156C Fe protein is in the normal conformation with MgADP bound. Small angle x-ray scattering experiments have shown that the conformation of the MgADP-bound form of the Fe protein is similar to the MgADP-free form of the protein that is shown in Fig. 1A. In this conformation, Met-156 is not near the nucleotide binding site, and therefore, there is no reason to expect that it would influence the release of MgADP.

**Structural Considerations**

**Met-156 Is Unlikely to Be Important for the Initial Binding of MgATP or for the MgADP Conformation of the Protein—As** shown in Fig. 1A, in the absence of nucleotides Met-156 protrudes into the interface between the two Fe protein subunits. Modeling of the smaller M156C residue in this position indicates that the mutation should not affect either the local or global conformation of the protein. Indeed the *in vivo* stability of the protein and the chelation, EPR, and CD data presented here are all consistent with the conformation of the M156C Fe protein being the same as the conformation of the wild-type protein in the absence of nucleotides.

Comparisons of the sequences and structures of a family of proteins that have energy transduction mechanisms involving switching between conformational states upon nucleotide binding or hydrolysis (e.g. H-Rasp²¹, recA, myosin, Fe protein) reveals two highly conserved regions that are involved in nucleotide binding (37). One region is the P-loop or Walker A motif that in the Fe protein contains residues 9–16. The second region, which corresponds to the switch II region in the G-protein family, extends from residues 125 to 132 in the Fe protein. In the absence of nucleotide in the wild-type Fe protein, there are no contacts between the Met-156 side chain and either of the known nucleotide binding regions of the Fe protein.¹ If, as now seems likely, the nucleotides bind to the Fe protein initially in the ras-like mode (4), then there is no reason to think that substitution of Met-156 by the smaller residue cysteine should interfere with the initial binding of nucleotides.

As will be considered in detail, the binding of MgATP causes a large global conformational change that results in the two subunits coming together and the radius of gyration of the

¹ It should be noted that the original structure of the iron protein (8) showed partial occupancy of one subunit of the iron protein with ADP that copurified with the iron protein. The ADP lacked Mg²⁺ and was in an unusual cross-subunit mode in which there were van der Waals contacts between the ADP-ribose C8 and the δ sulfur of Met-156. Based on the new complex structure, it seems likely at this time that the binding mode for the adventitious ADP is not relevant to the mechanism (4).
protein contracting on average by 2 Å (4, 18). MgADP binding also causes a conformational change in the Fe protein. Although the structure of the MgADP form is not available, x-ray scattering experiments and other data show that this conformational change is much less dramatic and does not involve a global contraction of the protein (1, 18). It is therefore unlikely to involve the coming together of the two subunits. If that is the case, then substitution of Met-156 by a cysteine should not influence the MgADP conformational change which is consistent with the CD data shown in Fig. 3.

**Met-156 Is Critical after the Conformational Change Has Occurred in Orientation of the MgATP in the Other Subunit**—A unique feature of the Fe protein when compared with other nucleotide binding proteins is that it is a dimer of two identical subunits. When two MgATP molecules bind, one in each subunit, a global conformational change occurs that affects a single [4Fe-4S] cluster bridged between the two subunits. This conformational change not only involves the P-loop and the switch II regions that are conserved among nucleotide binding proteins, but it also involves a third region that is unique to the Fe protein (12). This third region contains helix α5 located at the subunit interface, which extends from residues 151–176 and includes Met-156. Many of the residues in this region, including Met-156, are completely conserved in the more than 20 sequences that are available (12).

Our earlier mutagenesis studies have shown that Ala-157 is critical for the initial stages of the MgATP-induced conformational change (12), which is now known to involve a substantial movement of helix α5 (4). Ala-157 is not critical for any subsequent reaction (19). In the initial stages of the conformational change the two subunits are apart and Ala-157 is responding to the MgATP that is binding to the same subunit. As shown in Fig. 1A and discussed above, even though Met-156 is adjacent to residue 157, its side chain protrudes into the cleft between the two subunits. As a result this residue is unlikely to be important until after the conformational change has occurred to the point that the two subunits have come together, explaining why the M156C does undergo the conformational change while the A157S Fe protein does not.

**Fig. 7. A model of the position of the magnesium (Mg) atom as it is found in the complexed Fe protein relative to those found in other MgADP-AlF4− or MgGDP-AlF4−-containing protein structures.** The Mg2+ from the Fe protein is represented as a yellow sphere and is about 1.7–2.1 Å away from the Mg2+ found in the other nucleotide structures. The figure was created by superimposing the aluminum atom, phosphorus atoms, and the connecting oxygen atoms of the AlF4− or MgGDP-AlF4− group and the β- and α-phosphates of nucleotides as they are found in two forms of the rat brain protein G-I (blue and green) (41, 45), truncated myosin head (red) (44), and the Fe-MoFe complex structure (yellow) (4).

**A Model for the Role of Met-156**

The chelation, EPR, and MoFe protein binding data presented above show that the global conformation adopted by the M156C Fe protein following MgATP binding must be very similar to that of the wild-type Fe protein. The change in the CD (Fig. 3) and the inability to hydrolyze MgATP are therefore
Fe protein is coordinated to the nucleotide only through the atoms associated with these nucleotides is coordinated tightly with both the γ-phosphate (or AlF$_4^-$) and β-phosphate. The sole exception is casein kinase, which has an interesting γ–α coordination (46).

As shown in Fig. 7, the γ carbon atom of Met-156 makes contact with one of the oxygen atoms of the α-phosphate of MgADP-AlF$_4^-$ at a distance of 3.0 Å and comes close to the ribose ring. The M156C mutation would replace this γ carbon with a larger more polar sulfur residue. This small change is unlikely to have a major effect, however, because the nucleotide in that region is already tightly packed by interactions with residues in its own subunit presumably before Met-156 enters from the other subunit. In addition, the removal of the δ-sulfur and ε-carbon atoms of Met-156 should give the γ atom room to move out of the way to avoid bumping the nucleotide and it is difficult to see how these direct interactions with the nucleotide could translate to changes in the environment of the [4Fe-4S] cluster.

These issues led us to examine the possibility that the effects of the M156C mutation were caused by indirect interactions that were somehow transmitted to the switch II region that connects Asp-125 to Cys-132, a ligand to the [4Fe-4S] cluster. Indeed a D129E Fe protein that had been characterized previously (16) has many properties in common with our M156C mutation in that it undergoes the conformational change, ends up in a somewhat different conformation based on spectroscopy, binds to the MoFe protein, but fails to hydrolyze MgATP or carry out subsequent reactions. As shown in Fig. 6, Asp-125 is the switch II residue closest to Met-156, and in the complex structure it is oriented around Mg$^{2+}$. Our closer examination of this residue led us to the interesting observation shown in Fig. 7 that the Mg$^{2+}$ atom in the Fe protein structure is in an unusual location relative to its expected location. The crystal structures of several other nucleotide binding proteins have been solved, some in the presence of nucleotide triphosphate (40), some in the presence of nonhydrolyzable nucleotide analogues (41–43), and some in the presence of the transition state analogue MgADP-AlF$_4^-$ (41, 44, 45), as is the case with the Fe protein:MoFe protein complex (4). In all but one case, the Mg$^{2+}$ atom associated with these nucleotides is coordinated tightly with both the γ-phosphate (or AlF$_4^-$) and β-phosphate. The sole exception is casein kinase, which has an interesting γ–α coordination (46).

As shown in Fig. 7 in sharp contrast, the Mg$^{2+}$ atom in the Fe protein is coordinated to the nucleotide only through the AlF$_4^-$, it is 1.7–2.1 Å removed from its location in other proteins and at its closest point is 3.6 Å away from the β-phosphate. This is not an artifact of the use of the transition state analogue because the structures shown in Fig. 7 are all from MgADP-AlF$_4^-$ or Mg-GDP-AlF$_4^-$ complexes. Rather it appears that the Fe protein was designed to have this unusual location for the Mg$^{2+}$, because as shown in Fig. 8, the atom is held in place by a several completely conserved residues that are positioned to draw the Mg$^{2+}$ away from a typical β,γ coordination. The critical residues include Ser-16 at 2.4 Å, Asp-125 at 3.8 Å, Asp-39 at 2.9 Å, and Asp-43 at 3.7 Å. In addition to its apparent electrostatic interactions with the Mg$^{2+}$ atom, Asp-125 is hydrogen-bonded to Ser-16 (4). Thus, the position of Asp-125 seems strongly dependent upon the position of the Mg$^{2+}$ atom. Should this pocket around the Mg$^{2+}$ atom be disturbed, it seems likely that this would affect this atom’s position. As discussed above and demonstrated elsewhere (13) the position of Asp-125 is important for the Fe protein to adopt the correct conformation upon MgATP binding. Given the close proximity of Met-156 to Asp-43, one of the pocket residues, we propose that the role of the conserved residue Met-156 is to restrict the movement of Asp-43 and stabilize this pocket as it protrudes into the opposite subunit following the initial conformational change. Replacement of Met-156 with cysteine results in the removal of the last two atoms of the methionyl side chain. This mutation would give Asp-43 more freedom to move, causing a disturbance of the pocket around the Mg$^{2+}$ atom and thus resulting in the reorientation of the pocket residues, especially Asp-125, due to movement of the Mg$^{2+}$ atom. Like other mutations that affect the switch II pathway, the movement of Asp-125 could easily cause subtle alterations in the correct conformation of the Fe protein, resulting in a protein that has an altered conformation around the [4Fe-4S] cluster (Fig. 3) and is unable to hydrolyze MgATP. As discussed above, Met-156 protrudes into the area between the two subunits and only has interactions with other residues following the binding of MgATP and the subsequent conformational change in which the two subunits are drawn together. Thus, we propose that Met-156 acts to stabilize the pocket containing Mg$^{2+}$ following the initial conformational change and that it does not participate in nucleotide binding or the initial conformational change.

**Fig. 8.** A stereo image of the pocket of negatively charged residues around the Mg$^{2+}$ atom. The distances of the relevant atoms from the Mg$^{2+}$ atom are 2.4 Å for the Ser-16 O–γ, 2.9 Å for the Asp-39 O–δ, 3.7 Å for the Asp-43 O–δ, and 3.8 and 3.9 Å for the two Asp-125 O–δ atoms.
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REFERENCES

1. Burgess, B. K., and Lowe, D. J. (1996) *Chem. Rev.* 96, 2963–3011
2. Howard, J. B., and Rees, D. C. (1996) *Chem. Rev.* 96, 2965–2982
3. Kim, J., and Rees, D. C. (1992) *Nature* 360, 555–560
4. Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B., and Rees, D. C. (1997) *Nature* 387, 370–376
5. Kim, J., Woo, D., and Rees, D. C. (1993) *Biochemistry* 32, 7104–7115
6. Chen, L., Gavini, N., Tsuruta, H., Eliezer, D., Burgess, B. K., Doniach, S., and Rees, D. C. (1998) *J. Biol. Chem.* 273, 1653–1659
7. Peters, J. W., Stowell, M. H. B., Soltes, S. M., Finnegyn, M. G., Johnson, M. K., and Rees, D. C. (1997) *Biochemistry* 36, 1181–1187
8. Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., and Rees, D. C. (1992) *Science* 257, 1432–1433
9. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J.* 1, 945–951
10. Robson, R. L. (1984) *FEMS Lett.* 173, 39–388
11. Seefeldt, L. C., Morgan, T. V., Dean, D. B., and Mortenson, L. E. (1992) *J. Biol. Chem.* 267, 6680–6688
12. Gavini, N., and Burgess, B. K. (1992) *J. Biol. Chem.* 267, 21179–21186
13. Wolfe, D., Dean, D. B., and Howard, J. B. (1992) *Science* 256, 992–995
14. Ryle, M. J., Lanzilotta, W. N., Mortenson, L. E., Watt, G. D., and Seefeldt, L. C. (1995) *J. Biol. Chem.* 270, 13112–13117
15. Seefeldt, L. C., and Mortenson, L. E. (1993) *Protein Sci.* 2, 91–102
16. Lanzilotta, W. N., Ryle, M. J., and Seefeldt, L. C. (1995) *Biochemistry* 34, 10713–10723
17. Ryle, M. J., and Seefeldt, L. C. (1996) *Biochemistry* 35, 4766–4775
18. Chen, L., Gavini, N., Tsuruta, H., Ehezer, D., Burgess, B. K., Deniach, S., and Hodgson, K. O. (1994) *J. Biol. Chem.* 269, 3289–3294
19. Bursey, E. H., and Burgess, B. K. (1998) *J. Biol. Chem.* 273, 16927–16934
20. Page, W. J., and von Tigerstrom, M. (1970) *J. Bacteriol.* 130, 1058–1061
21. Burgess, B. K., Jacobs, D. B., and Steifel, E. I. (1980) *J. Bacteriol.* 140, 236–245
22. Gonzales, A., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* 177, 751–766
23. Ashby, G. A., and Thornley, R. N. F. (1987) *Biochem J.* 246, 455–465
24. Wehr, C. T. (1990) in *Nucleic Acid Constituents by High Performance Liquid Chromatography*, Varian Associates Inc., Palo Alto, CA
25. Walker, G. A., and Mortenson, L. E. (1974) *Biochemistry* 13, 2382–2388
26. Deits, T. L., and Howard, J. B. (1989) *J. Biol. Chem.* 264, 6619–6628
27. Filler, W. A., Kemp, R. M., Ng, J. C., Hawkes, T. R., Dixon, R. A., and Smith, B. E. (1986) *Eur. J. Biochem.* 160, 371–377
28. Robinson, A. C., Burgess, B. K., and Dean, D. R. (1986) *J. Bacteriol.* 166, 180–186
29. Robinson, A. C., Dean, D. R., and Burgess, B. K. (1987) *J. Biol. Chem.* 262, 14327–14332
30. Robinson, A. C., Chun, T. W., Li, J. G., and Burgess, B. K. (1989) *J. Biol. Chem.* 264, 10088–10095
31. Palmer, G., Multani, J. S., Cretney, W. C., Zumft, W. G., and Mortenson, L. E. (1972) *Arch. Biochem. Biophys.* 153, 325–332
32. Zumft, W. G., Palmer, G., and Mortenson, L. E. (1973) *Biochem. Biophys. Acta* 292, 413–421
33. Stephens, P. J., McKenna, C. E., Smith, B. E., Nguyen, H. T., McKenna, M.-C., Thomson, A. J., Devlin, F., and Jones, J. B. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 2585–2589
34. Ryle, M. J., Lanzilotta, W. N., Seefeldt, L. C., Scarrow, R. C., and Jensen, G. M. (1990) *J. Biol. Chem.* 271, 1551–1557
35. Howard, J. B., and Rees, D. C. (1994) *Annu. Rev. Biochem.* 63, 235–264
36. Lowe, D. J., Ashby, G. A., Brune, M., Knights, H., Webb, M. R., and Thornley, R. N. F. (1995) in *Nitrogen Fixation: Fundamentals and Applications* (Tikhonovich, I. A., Provorov, N. A., Romonov, V. I., and Newton, W. E., eds), pp. 103–118, Kluwer Academic, Dordrecht, The Netherlands
37. Yoshida, M., and Amano, T. (1995) *FEBS Lett.* 359, 1–5
38. Duyvis, M. G., Wassink, H., and Haaker, H. (1996) *FEBS Lett.* 380, 233–236
39. Reiner, K. A., and Howard, J. B. (1996) *Biochemistry* 35, 5353–5358
40. Tari, L. W., Matte, A., Paganenth, U., Goldie, H., and Delbaere, L. T. J. (1990) *Nat. Struct. Biol.* 3, 355–363
41. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) *Science* 265, 1405–1412
42. Gulik, A. M., Bauer, C. B., Thoden, J. B., and Rayment, I. (1997) *Biochemistry* 36, 11619–11628
43. Raw, A. S., Coleman, D. E., Gilman, A. G., and Sprang, S. R. (1997) *Biochemistry* 36, 15660–15669
44. Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) *Biochemistry* 34, 8960–8972
45. Tesmer, J. J. G., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) *Biochemistry* 36, 2382–2388
46. Carmel, G., Leichus, B., Cheng, X., Patterson, S. D., Mirza, U., Chait, B. T., and Kuret, J. (1994) *J. Biol. Chem.* 269, 7304–7309