Published in final edited form as:

Biochemistry. 2007 December 11; 46(49): 14058–14066. doi:10.1021/bi700446s.

Probing the MgATP-bound conformation of the nitrogenase Fe protein by solution small angle x-ray scattering

Ranjana Sarma, David W. Mulder, Eric Brecht, Robert K. Szilagyi, Lance C. Seefeldt[†], Hiro Tsuruta[†], and John W. Peters^{*}

Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717

[†]Stanford Synchrotron Radiation Laboratory, Stanford Linear Accelerator Center, Stanford University, MS 69, 2575 Sand Hill Road Menlo Park, CA 94025

[‡]Department of Chemistry and Biochemistry, Utah State University Logan, UT 84322

Abstract

The MgATP-bound conformation of the Fe protein of nitrogenase from *Azotobacter vinelandii* has been examined in solution by small angle x-ray scattering (SAXS) and compared to existing crystallographically characterized Fe protein conformations. The results of the analysis of the crystal structure of an Fe protein variant with a Switch II single amino acid deletion recently suggested that the MgATP-bound state of the Fe protein may exist in a conformation that involves a large-scale reorientation of the dimer subunits resulting in an overall elongated structure relative to the more globular structure of the MgADP-bound state. It was hypothesized that the Fe protein variant may be a conformational mimic of the MgATP-bound state of the native Fe protein based largely on the observation that the spectroscopic properties of the [4Fe-4S] cluster of the variant mimicked in part the spectroscopic signatures of the native nitrogenase Fe protein in the MgATP-bound state. In the present work, SAXS studies reveal that the large scale conformational differences between the native Fe protein and the variant observed by x-ray crystallography are also observed in solution. In addition, comparison of the SAXS curves of the Fe protein nucleotide-bound states to the nucleotide-free states indicates that the conformation of the MgATP-bound state in solution does not resemble the structure of the variant as initially proposed, but rather, at the resolution of this experiment, it resembles the structure of the nucleotide-free state. These results provide insights into the Fe protein conformations that define the role of MgATP in nitrogenase catalysis.

Introduction

Biological nitrogen fixation is a critical step in the global nitrogen cycle that is carried out exclusively by prokaryotes. The reduction of N₂ to two NH₃ molecules is catalyzed by the enzyme nitrogenase that exists in Mo, V, and Fe-only forms. Mo nitrogenase is the most extensively studied form and consists of two oxygen sensitive protein components termed the Fe protein and the MoFe protein (1). The Fe protein is a homodimer (~64 kDa) with a single [4Fe-4S] cluster bridging the two subunits and with each subunit possessing a single site for MgATP binding and hydrolysis. The MoFe protein, an $\alpha_2\beta_2$ heterotetramer (~230 kDa), has two types of complex iron-sulfur clusters termed the P-clusters (8Fe-7S) and the FeMo-cofactors (Mo-7Fe-9S-homocitrate), the latter serving as the sites for N₂ binding and reduction (2-4).

* Address correspondence to this author: VOICE 406-994-7211; FAX 406-994-7212; john.peters@chemistry.montana.edu.

During catalysis, the Fe protein and the MoFe protein associate with the transfer of a single electron from the Fe protein to the MoFe protein coupled to the hydrolysis of two MgATP molecules, one of each subunit of the Fe protein. Since eight electrons must be transferred to the MoFe protein for the reduction of one N₂ molecule to two NH₃ molecules and the reduction of two protons yielding H₂, multiple cycles of component protein interaction are required for the complete nitrogenase catalytic cycle. One of the more intriguing aspects of the nitrogenase mechanism is the involvement of MgATP. The coupling of MgATP hydrolysis to multiple-electron reduction reactions is unusual in biology. More common is the coupling of nucleoside triphosphate hydrolysis to a cellular process such as metabolic regulation (5-7), protein synthesis (8, 9), DNA repair (10, 11), or muscle contraction (12-14). Details of the role of MgATP binding and hydrolysis in nitrogenase catalysis remain unknown, although it is assumed to function to gate the unidirectional flow of electrons from the Fe protein towards the substrate (1). The ability to accumulate multiple electrons on the MoFe protein is necessary to complete the catalytic cycle since N₂ does not bind at the FeMo-cofactor until the enzyme is reduced by several electrons (2, 15-17).

The binding of nucleotides to the Fe protein is known to induce protein conformational changes as evidenced by changes in the spectroscopic properties of the [4Fe-4S] cluster (18-27). While these spectroscopic methods reflect changes in the electronic properties of the [4Fe-4S] cluster, they do not directly access changes to the overall structure of the Fe protein. To gain insights into nucleotide-dependent protein conformational states of the Fe protein, a number of x-ray crystal structures of Fe protein and Fe protein – MoFe protein complexes without and with bound nucleotides have been determined (28-35). These structures provide insights into the interactions of nucleotides with Fe protein, the role of the signal transduction pathways and switch regions, and intermolecular electron transfer between Fe and MoFe proteins. These studies have clearly shown that the Fe protein is capable of undergoing conformational changes manifested mainly as the rigid body reorientation of the two subunits, and based on these studies models for the conformational changes and the interaction that occur within the nitrogenase complex have been proposed. The structure of a key state in defining Fe protein nucleotide-dependent conformational change, the MgATP bound state, has not been elucidated.

Although a true MgATP bound state of the Fe protein has not been determined, we have recently characterized the x-ray crystal structure of a variant of the Fe protein with a single deletion in the switch region that connects the nucleotide binding site to the [4Fe-4S] cluster (33, 34). The crystal structure of the L127Δ Fe protein shows a structure strikingly different from the previously determined structures, characterized by a large rigid-body reorientation of the two subunits. Spectroscopic and biochemical studies on this variant protein have revealed similarities in the spectroscopic properties of the nucleotide-free form of the L127Δ Fe protein and the MgATP-bound state of Fe protein (36). The L127Δ Fe protein is not capable of nitrogen reduction, but it can form a stable complex with the MoFe protein in the absence of nucleotides that resembles the native Fe protein – MoFe protein complex stabilized by MgADP-tetrafluoroaluminate (37, 38). Although the characterization of the [4Fe-4S] cluster of this variant provides support that the L127Δ Fe protein somehow mimics several aspects of the MgATP-bound native Fe protein (36), the relationship between the variant and the MgATP-bound state from the perspective of protein structure has not yet been examined.

Recent structural work in which a number of nitrogenase complexes stabilized in the presence of nucleotides and nucleotide analogs favor a different model in which Fe protein conformational changes were much less pronounced (32). From the results of this work, it was proposed that the cycle of complex association, coupled nucleotide hydrolysis and electron transfer, and complex dissociation involved the binding of the different states of the

Fe protein to the MoFe protein at different sites. In the present manuscript, small angle solution x-ray scattering studies have been implemented to analyze the structure of the Fe protein in nucleotide bound conformations to critically evaluate our hypotheses with respect to nucleotide-dependent conformational states and to determine the relationship between the structure of the authentic MgATP-bound form and the previously crystallographically characterized conformations.

Experimental Procedures

Protein Purification

Both the native Fe protein and the L127Δ Fe protein of nitrogenase from *Azotobacter vinelandii* were purified as described previously (18). The proteins were buffer exchanged into 20% glycerol buffer at pH 7.5 with 0.5M NaCl by Sephacryl-200 gel filtration chromatography. The native Fe protein was concentrated to 33 mg/mL and the L127Δ Fe protein was concentrated to 100 mg/mL using an Amicon™ concentration apparatus under Ar pressure and stored in liquid nitrogen.

Sample Preparation

All samples for the SAXS experiments were prepared in a glove box (Vacuum Atmospheres, Hawthorne, MA) operating under a N₂ atmosphere at less than 1 ppm of oxygen. All buffers, which contained 20% glycerol and 5 mM dithionite, were degassed under vacuum in sealed Wheaton™ vials in an atmosphere of 100% N₂. In order to insure the integrity of MgADP- and MgATP-bound samples, nucleotide stock solutions (100 mM) were prepared no more than 15 minutes prior to data collection. To ensure nucleotide saturation, three different nucleotide concentrations were initially used, specifically 1 mM ATP/ADP + 2 mM MgCl₂, 5 mM ATP/ADP + 10 mM MgCl₂, and 10 mM ATP/ADP + 20 mM MgCl₂. There were no discernable differences observed at the different concentrations and a final nucleotide concentration of 5 mM (a 20-25-fold molar excess) was used throughout the experiment to insure saturation.

Data Collection

SAXS data were collected at Beam Line 4-2 (39) at the Stanford Synchrotron Radiation Laboratories (SSRL) on five different occasions. On the first two occasions, conventional flat-window cells with thin mica windows were used. For data collections in which all of the data presented herein were recorded, a continuous flow cell incorporating a thin wall x-ray capillary was used. The flow cell was attached to a computer controlled syringe dispenser (Hamilton 500 series, 250 μL syringe) via oxygen impermeable tubing, and the samples were injected into the flow cell system from sealed vials under mildly positive N₂ pressure to maintain anaerobic conditions. The sample volume for each run was 80 μL and the dispenser was set in a continuous loop to allow the sample to flow 30 μL in the forward and reverse direction (4 μL/sec) relative to the x-ray beam position. The continuous flow of the sample evenly distributed 20 x-ray exposures, each lasting 10 seconds, over the entire 80 μL sample aliquot. This data collection strategy proved to be highly effective and the effects of x-ray radiation damage and protein aggregation were eliminated. In order to maintain anaerobic conditions throughout the experiments, the flow cell was flushed with anoxic buffer between each run. The capillary cell was maintained at 20 °C throughout the measurements. The detector pixels were calibrated to the momentum transfer Q , which equals $4\pi\sin(\theta)/\lambda$ (θ is one half of the scattering angle and λ is the wavelength) using the (100) reflection from a cholesterol myristate powder sample. Fe protein data were collected at an incident beam energy of 8700 eV using a MARCCD165 detector (MarUSA, Evanston, IL) at a distance of 1 meter from the sample cell.

Calibration

Lysozyme was used as a calibration standard for experimental setup and measured intensities as described previously (40). Reagent grade lysozyme (Fischer Scientific) was used to make up solutions (10 mM ammonium acetate, pH 5.0, 150mM NaCl) at 4 different concentrations of 2.5 mg/mL, 5mg/mL, 10mg/mL, and 20 mg/mL. Each of the samples was run in triplicate at 8950 eV. The experimental scattering curves were compared to the theoretical scattering curve generated using the crystal structure 193L.pdb (41) in CRY SOL (42).

Potential for artifacts arising from Fe fluorescence or anomalous scattering effects from the [4Fe-4S] cluster in the Fe protein was probed by measuring scattering curves of buffer solutions containing different concentrations of Fe standards. Scattering curves were collected for ferrous ammonium sulfate at 0.1mM, 0.3mM, and 0.7mM in buffer solution at three different energies, 7050 eV, 7150 eV (near the Fe edge) and 8950 eV (remote relative to the Fe edge).

Data Analysis

The two dimensional images from the detector were azimuthally integrated, scaled for beam intensities, frame-averaged and background-subtracted with *MarParse* (39) to obtain the scattering curves. In order to assess systematic error in the data, each experimental run was carried out at least in duplicate. In addition, scattering curves of each corresponding buffer and nucleotide solution were subtracted from the individual protein scattering curves to eliminate any scattering contributions of the buffer solution. Radii of gyration (R_g) values were obtained from the Guinier plots, using the first 20-22 intensity points beyond the beam stop in the Q range $\sim 0.02 - 0.06 \text{ \AA}^{-1}$. Theoretical curves were generated with CRY SOL (42) from the crystal structure of the nucleotide-free native Fe protein (2NIP) (30), and the crystal structure of the nucleotide-free L127 Δ Fe protein (1RW4) (33). The electron pair distance distribution function, $P(r)$, was calculated using GNOM (43). In these calculations, scattering intensity points in the Q range $\sim 0.02 - 0.30 \text{ \AA}^{-1}$ were used. The maximum distance (D_{max}) values of 70 and 80 $\text{\AA}$ were assumed for the native and L127 Δ Fe protein calculations, respectively, based on the dimensions obtained from the corresponding crystal structures. These D_{max} values were verified to be valid in solution by running GNOM with varying D_{max} values. The best results were obtained at D_{max} 72 $\text{\AA}$ (native) and 82 $\text{\AA}$ (L127 Δ).

Ab initio shape reconstructions of the Fe protein in the nucleotide-free state and the L127 Δ Fe protein were calculated with DAMMIN (44). In this particular work, all DAMMIN calculations were constrained to two-fold symmetry and were run ten times to check for reproducibility of model construction. The ten separate, but similar, 3D structure models obtained by DAMMIN for each condition were spatially aligned and also analyzed for spatial discrepancy by SUPCOMB (45) which was run as a subprocess of DAMAVER (46). In this analysis dissimilar models are rejected and a most probable model representing the most populated volume among the ten models is given.

Results and Discussion

Several structures of the nitrogenase Fe protein have been determined and their characterization has revealed that the protein can exist in several different conformations (28-32). The main differences among these conformations are manifested in different orientations of the individual subunits with respect to one another. This indicates that during catalysis the Fe protein undergoes conformational changes upon nucleotide binding, complex association, and dissociation, which tune the spectroscopic properties of the Fe

protein's [4Fe-4S] cluster, modulate the distance between intermolecular redox partners (Fe protein [4Fe-4S] cluster and MoFe protein P cluster), and promote MgATP hydrolysis.

The overall differences in the dimensions of the Fe protein in different conformations from crystal structures, shown in figure 1, indicate that the Fe protein can exist in molecular shapes that range from a more globular protein, as observed for the nucleotide-free native Fe protein ($60 \text{ \AA} \times 47 \text{ \AA}$), to a more open or elongated shape, as observed for the L127 Δ Fe protein ($77 \text{ \AA} \times 40 \text{ \AA}$). The dramatic conformational difference observed between the crystal structures of native and L127 Δ Fe protein was not anticipated. There are limited interactions between the subunits in the structure of the L127 Δ Fe protein, suggesting a potentially higher degree of flexibility of the structure in comparison to the more globular compact conformations of the native nucleotide-free Fe protein. The limited interactions between the subunits of the L127 Δ Fe protein coupled with the large differences observed between the native structure and the variant structure make it necessary to investigate whether the structure observed predominates in solution or whether the crystalline lattice itself is stabilizing a local energy minimum of an overall conformationally dynamic L127 Δ Fe protein.

The structure of the L127 Δ Fe protein was probed using SAXS to examine if the structure observed by crystallography was present as a predominant structure in solution. The scattering curves of globular compact proteins are noticeably different from the curves of the proteins having elongated or ellipsoid shapes. The extent of the differences between the conformation of the L127 Δ Fe protein and the native Fe protein are large enough to result in discernable differences between the respective scattering curves. Scattering curves generated from the radial scattering intensities can be used to evaluate the radius of gyration (R_g) of a protein which is related to the protein's molecular mass and overall shape. The radius of gyration of a macromolecule is defined as the root mean square distance of the collection of electrons from their common centre of gravity. Theoretical scattering curves for the crystal structures and their R_g values for two conformations of the Fe protein are shown in figure 1C. The structure of the more elongated L127 Δ Fe protein has a higher anticipated radius of gyration because the elongated shape has a longer largest dimension. In addition to the larger R_g value, clear differences exist in the overall shape of the theoretical curves, especially in the Q range $<0.15 \text{ \AA}^{-1}$. The scattering data in this Q range is more sensitive to subtle conformational differences than R_g alone would indicate and can be exploited to distinguish the Fe protein conformational states.

The first set of experimental runs, for both the native and L127 Δ Fe proteins, were carried out at three different protein concentrations, 5 mg/mL, 10 mg/mL, and 20 mg/mL. A decrease in the R_g values for both the native (5 mg/mL – $25.7 \text{ \AA}$, 10 mg/mL – $22.7 \text{ \AA}$, and 20 mg/mL – $21.3 \text{ \AA}$) and the L127 Δ Fe protein (5 mg/mL – $28.1 \text{ \AA}$ and 20 mg/mL – $26.5 \text{ \AA}$) were reproducibly observed indicative of some interparticle interference. Subsequent measurements were conducted at a concentration of 5 mg/mL to limit interference since high quality data with good signal could be obtained.

The experimental scattering curves of the solution structures of the nucleotide-free states of the native Fe protein and L127 Δ Fe protein differences are shown in figure 2A. The curves are consistent with the salient features of the theoretical curves for the two proteins based on the known structures of the proteins obtained by crystallography (Figure 1C), including the key distinguishing differences observed in the Q range of $0.1 - 0.2 \text{ \AA}^{-1}$. Additionally, the R_g values obtained from the Guinier plots (Figure 2A inset) for the native Fe protein ($25.2 \pm 0.1 \text{ \AA}$) and the L127 Δ Fe protein ($28.1 \pm 0.1 \text{ \AA}$) are near the simulated values of 25.0 and 27.1 (Figure 1C). The R_g value for the L127 Δ is nearly $\sim 1 \text{ \AA}$ larger than the simulated spectra but clearly the differences in comparing the native and L127 Δ Fe protein are consistent with the

observation that the native Fe protein exists in a more globular state and the L127 Fe protein is more elongated in structure, however the lack of a precise fit to the simulated curve may suggest that the crystalline lattice in L127 Δ Fe protein may be imposing some structural constraints on the conformation. In order to validate if the anticipated molecular shape and of the native Fe protein and the L127 Δ Fe protein is consistent with the observed scattering curves, the scattering data were analyzed by the indirect Fourier transform method using GNOM. This analysis evaluates the electron pair distance distribution function of a protein molecule in solution and also gives an estimate of the R_g values. The R_g values obtained by GNOM are similar as those obtained using the Guinier plots (Figure 2B). The peak of the pair distribution function of the L127 Δ Fe protein occurs at a longer distance than the peak for the native Fe protein, clearly supporting that the L127 Δ Fe protein in solution is predominantly in the elongated structural state as observed by crystallography.

The scattering curves can be further exploited to calculate shape reconstructions which can be compared directly to the existing crystal structures. The most probable low resolution structural models of the native Fe protein and the L127 Δ Fe protein computed from the experimental scattering data using DAMMIN are shown in figure 2C and D. The average normalized spatial discrepancy (NSD) values over all independent models was 0.55 (± 0.03) for the native Fe protein and 0.63 (± 0.01) for the L127 Δ Fe protein. One model for the L127 Δ Fe protein was excluded by DAMAVER due to a slightly higher NSD value. The range of NSD values in all cases indicates satisfactory similarity among nine or ten individually computed models from which the most probable models were computed by DAMAVER. As indicated in figure 2 the shape reconstructions generated from the experimental curves are consistent with the overall globular state of the native Fe protein versus the more elongated shape for the L127 Δ Fe protein observed by x-ray crystallography (Figure 1).

Although the salient differences of the scattering profiles in comparing the native Fe protein and L127 Δ Fe protein can be rationalized in the context of the differences in the simulated scattering curves, a close comparison of the simulated and experimental scattering curves of both proteins (Figure 3), however, reveals significant differences at the higher scattering angles ($>0.15 \text{ \AA}^{-1}$). The differences in the experimental data are reproducible and could be result of slight conformation variation or conformation dynamics in solution in comparison to the crystal structure. The potential for the discrepancy to be associated with artifacts resulting from the experimental setup was addressed using the protein lysozyme as a calibration standard. Under the experimental setup used in this study the experimental scattering data for lysozyme was consistent with the simulated data indicating that no artifacts were introduced at the level of the data collection apparatus or experimental design.

Since the Fe protein contains a [4Fe-4S] cluster, the potential for artifacts occurring as a result of Fe fluorescence or anomalous scattering effects was also addressed by examining the scattering curves of Fe standards at different incidence energies. Scattering curves of ferrous ammonium sulfate solutions at 0.1mM, 0.3mM, 0.7mM at three different incident energies (7050eV, 7150eV, and 8950 eV) near and remote relative to the Fe absorption edge (7113eV) were examined to address the potential influence of the presence of Fe in the Fe protein on the experimental scattering data. These control experiments revealed a small contribution of Fe in the signal intensities of scattering curves and a small dependence on incident energies. However the degree of the differences observed was orders of magnitude less than the intensity differences observed at the higher angles in our data indicating that the [4Fe-4S] cluster of the Fe protein is unlikely to be the source of the discrepancy between the Fe protein experimental and simulated scattering curves.

Having established that the difference between solution structures of the L127Δ Fe protein and the native Fe protein can be clearly distinguished using SAXS, we used this experimental method as a relative benchmark to ask the question whether the different nucleotide bound states resemble the conformations previously described. These studies are very important for the characterization of the MgATP-bound state since it has not yet been possible to obtain a structure of a MgATP-bound state of the native Fe protein crystallographically. For the MgADP-bound state of the native Fe protein, the structure has been crystallographically determined and the overall structure at a low resolution approximately resembles the conformation observed for the native Fe protein and in essence serves as an internal control for this analysis. Previous SAXS experiments probing the nucleotide-bound states of the Fe protein reported R_g values for the nucleotide-bound conformations very similar to those observed for the nucleotide-free native Fe protein (47) (48). In the current study, the effect of nucleotide binding to both the native and L127Δ Fe protein are examined in parallel to directly assess whether the L127Δ Fe protein is a mimic of the MgATP state as suggested previously (33).

The Fe protein was incubated with a molar excess of either MgATP or MgADP for five to ten minutes prior to data collection. The significant molar excess of MgATP and the short time of incubation prior to data collection insured that the Fe protein would be predominantly in the MgATP-bound form, with minimal hydrolysis to MgADP. The scattering curves for the native Fe protein (5 mg/mL) in the absence of nucleotides and in the presence of 5 mM MgADP or MgATP (~20-25 fold excess) are shown in figure 4A. The scattering curves reveal that in the presence of either MgADP or MgATP, the structure of the Fe protein at the low resolution of the scattering experiment resembles the native state and does not undergo a large scale conformational change to resemble the L127Δ Fe protein structure. The estimations of the radius of gyration are very similar (~25.0) to that obtained for the native Fe protein, and the key discriminating features of the scattering profile observed in the Q range $0.1-0.2 \text{ \AA}^{-1}$ resemble the native Fe protein more than the L127Δ Fe protein. This is consistent with the previously characterized crystallographic structure of the Fe protein with bound MgADP which was observed to exist in the same overall conformation as the nucleotide-free Fe protein. These results clearly indicate that the MgATP-bound state in solution does not exist in the elongated structure observed for the L127Δ Fe protein. Thus, in overall shape, the L127Δ Fe protein does not appear to faithfully mimic the conformation of the MgATP-bound state.

Although the addition of nucleotides does not result in the large scale conformational change proposed in our previous work, comparison of the scattering curves of native and nucleotide bound states indicate subtle differences (Figure 4A). Since the R_g and shape of the scattering curves are nearly the same then we can conclude the structures of the native and nucleotide bound states must be very similar, however, the subtle differences suggest that the states behave differently in solution which may be a result of slight variability of the structures or dynamics in solution that cannot be revealed at the resolution of the current study.

Although the L127Δ Fe protein is essentially inactive, it is competent in MgATP and MgADP binding, therefore we have done a parallel study examining the effects on SAXS curves in the presence of saturating concentrations of MgADP and MgATP (Figure 4B). The differences in the scattering curves of the L127Δ Fe protein in comparing the nucleotide-free and nucleotide-bound forms are more pronounced than those observed for the native Fe protein. In addition, the R_g values of the L127Δ Fe protein are decreased by ~1-2 Å upon addition of nucleotides to more closely approximate the R_g values of the nucleotide-free Fe protein obtained by simulation. This suggests that the L127Δ Fe protein, in the presence of MgADP or MgATP, undergoes a conformational change that is likely manifested in movement of the subunits toward a more globular structure or toward a conformation that

more closely approximates the structure the L127Δ Fe protein observed crystallographically. However, since the R_g values are approximately midway between the R_g values observed for the native and L127Δ Fe protein by simulation one might anticipate a structure in which the subunits of the dimer are reoriented to an approximation intermediary between the structures of the native and nucleotide-free Fe protein. The variance in observed values is consistent with a larger degree of conformational flexibility in the L127Δ Fe protein dimer in comparison to the native Fe protein that is likely to be a result of the relatively small number of intersubunit interactions in the L127Δ Fe protein. Recently the structure of the L127Δ Fe protein with bound MgATP was determined (34). This structure was generated by soaking crystals of the nucleotide-free state of the L127Δ Fe protein with MgATP just prior to cryo-cooling for data collection. The structure of the MgATP bound state did not differ in overall conformation or the relationship between the two subunits of the dimer when compared to the nucleotide-free form of the L127Δ Fe protein. The observation that these two structures have the same overall shape crystallographically but clearly differing shapes in solution strongly suggest that the limited intersubunit contacts allow for a more flexible structure and multiple states distinct from the native Fe protein structure can be observed and thus the crystalline lattice in this case may be stabilizing a local minimum energy structure. The apparent conformational flexibility of the L127Δ Fe protein notwithstanding, the overall conclusion of the current work clearly indicates that the more elongated structure of the L127Δ Fe protein observed by crystallography predominates in solution and that this state is not a faithful mimic of the MgATP state is well supported.

Summary and Conclusions

In the present study, SAXS was used to probe and analyze the structure of the nitrogenase Fe protein in defined states in solution. The results described herein established that the conformation that the more elongated L127Δ Fe protein observed in the crystal structure predominates in solution. Using the crystal structures of the native Fe protein, the MgADP-bound state of the native Fe protein, and the L127Δ Fe protein as benchmarks, it is concluded that the structure of the L127Δ Fe protein observed crystallographically either in the presence or the absence of bound nucleotides does not closely resemble the MgATP-bound conformation of the Fe protein in solution.

The proposal that the L127Δ Fe protein might mimic the conformation of the MgATP-bound state of the Fe protein was based on the results of a wealth of biochemical and spectroscopic experiments that indicate clear parallels between the biochemical and spectroscopic properties of the L127Δ Fe protein and the native Fe protein in the presence of bound MgATP. In addition, it was shown that the L127Δ Fe protein was capable of undergoing a conformational change in the presence of the MoFe protein to form a stable complex that closely resembles the Fe protein – MoFe protein complex stabilized in the presence of MgADP and tetrafluoroaluminate (37). Since the overall solution structures of the L127Δ Fe protein and the MgATP-bound state of the Fe protein, as determined by SAXS, do not appear to closely resemble each other, these results suggest that similar complexes can therefore be formed via at least two different pathways of Fe protein conformational change triggered by the MoFe protein. Also, apparently similar spectroscopic and biochemical properties, including similarities in EPR spectral features and metal chelation properties, can be obtained by markedly different protein conformations. This indicates the need to further dissect the factors that influence the spectroscopic and electronic structure properties of the [4Fe-4S] cluster of the Fe protein.

Acknowledgments

This work was supported by National Institutes of Health Grants GM069938 (JWP) and GM59087 (LCS). Portions of this research were carried out at the Stanford Synchrotron Radiation Laboratory, a national user facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Department of Energy, Office of Biological and Environmental Research and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences.

References

1. Peters JW, Szilagyi RK. Exploring new frontiers of nitrogenase structure and mechanism. *Curr Opin Chem Biol*. 2006; 10:101–108. [PubMed: 16510305]
2. Howard JB, Rees DC. How many metals does it take to fix N₂? A mechanistic overview of biological nitrogen fixation. *Proc Natl Acad Sci USA*. 2006; 103:17088–17093. [PubMed: 17088547]
3. Barney BM, Lee HI, Dos Santos PC, Hoffman BM, Dean DR, Seefeldt LC. Breaking the N₂ triple bond: insights into the nitrogenase mechanism. *Dalton Trans*. 2006:2277–2284. [PubMed: 16688314]
4. Dos Santos PC, Igarashi RY, Lee HI, Hoffman BM, Seefeldt LC, Dean DR. Substrate interactions with the nitrogenase active site. *Acc Chem Res*. 2005; 38:208–214. [PubMed: 15766240]
5. Sprang SR. G protein mechanisms: insights from structural analysis. *Annu Rev Biochem*. 1997; 66:639–678. [PubMed: 9242920]
6. Sunahara RK, Tesmer JJ, Gilman AG, Sprang SR. Crystal structure of the adenylyl cyclase activator G_sα. *Science*. 1997; 278:1943–1947. [PubMed: 9395396]
7. Sondek J, Lambright DG, Noel JP, Hamm HE, Sigler PB. GTPase mechanism of Gproteins from the 1.7-Å crystal structure of transducin α-GDP-AIF-4. *Nature*. 1994; 372:276–279. [PubMed: 7969474]
8. Kjeldgaard M, Nyborg J. Refined structure of elongation factor EF-Tu from *Escherichia coli*. *J Mol Biol*. 1992; 223:721–742. [PubMed: 1542116]
9. Jurnak F. Structure of the GDP domain of EF-Tu and location of the amino acids homologous to *ras* oncogene proteins. *Science*. 1985; 230:32–36. [PubMed: 3898365]
10. Story RM, Steitz TA. Structure of the recA protein-ADP complex. *Nature*. 1992; 355:374–376. [PubMed: 1731253]
11. Story RM, Weber IT, Steitz TA. The structure of the *E. coli* recA protein monomer and polymer. *Nature*. 1992; 355:318–325. [PubMed: 1731246]
12. Rayment I. The structural basis of the myosin ATPase activity. *J Biol Chem*. 1996; 271:15850–15853. [PubMed: 8663496]
13. Rayment I, Holden HM, Whittaker M, Yohn CB, Lorenz M, Holmes KC, Milligan RA. Structure of the actin-myosin complex and its implications for muscle contraction. *Science*. 1993; 261:58–65. [PubMed: 8316858]
14. Rayment I, Rypniewski WR, Schmidt-Base K, Smith R, Tomchick DR, Benning MM, Winkelmann DA, Wesenberg G, Holden HM. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science*. 1993; 261:50–58. [PubMed: 8316857]
15. Lowe DJ, Thorneley RN. The mechanism of *Klebsiella pneumoniae* nitrogenase action. The determination of rate constants required for the simulation of the kinetics of N₂ reduction and H₂ evolution. *Biochem J*. 1984; 224:895–901. [PubMed: 6395863]
16. Thorneley RN, Lowe DJ. The mechanism of *Klebsiella pneumoniae* nitrogenase action. Pre-steady-state kinetics of an enzyme-bound intermediate in N₂ reduction and of NH₃ formation. *Biochem J*. 1984; 224:887–894. [PubMed: 6395862]
17. Barney BM, Laryukhin M, Igarashi RY, Lee HI, Dos Santos PC, Yang TC, Hoffman BM, Dean DR, Seefeldt LC. Trapping a hydrazine reduction intermediate on the nitrogenase active site. *Biochemistry*. 2005; 44:8030–8037. [PubMed: 15924422]

18. Ryle MJ, Lanzilotta WN, Seefeldt LC. Elucidating the mechanism of nucleotide-dependent changes in the redox potential of the [4Fe-4S] cluster in nitrogenase iron protein: the role of phenylalanine 135. *Biochemistry*. 1996; 35:9424–9434. [PubMed: 8755721]
19. Lanzilotta WN, Holz RC, Seefeldt LC. Proton NMR investigation of the [4Fe-4S]¹⁺ cluster environment of nitrogenase iron protein from *Azotobacter vinelandii*M: defining nucleotide-induced conformational changes. *Biochemistry*. 1995; 34:15646–15653. [PubMed: 7495793]
20. Deits TL, Howard JB. Kinetics of MgATP-dependent iron chelation from the Fe-protein of the *Azotobacter vinelandii* nitrogenase complex. Evidence for two states. *J Biol Chem*. 1989; 264:6619–6628. [PubMed: 2785107]
21. Howard JB, Rees DC. Nitrogenase: a nucleotide-dependent molecular switch. *Annu Rev Biochem*. 1994; 63:235–264. [PubMed: 7979238]
22. Lindahl PA, Day EP, Kent TA, Orme-Johnson WH, Munck E. Mossbauer, EPR, and magnetization studies of the *Azotobacter vinelandii* Fe protein. Evidence for a [4Fe-4S]¹⁺ cluster with spin S = 3/2. *J Biol Chem*. 1985; 260:11160–11173. [PubMed: 2993304]
23. Lindahl PA, Gorelick NJ, Munck E, Orme-Johnson WH. EPR and Mossbauer studies of nucleotide-bound nitrogenase iron protein from *Azotobacter vinelandii*. *J Biol Chem*. 1987; 262:14945–14953. [PubMed: 2822707]
24. Ljones T, Burris RH. Nitrogenase: the reaction between the Fe protein and bathophenanthrolinedisulfonate as a probe for interactions with MgATP. *Biochemistry*. 1978; 17:1866–1872. [PubMed: 656366]
25. Lanzilotta WN, Fisher K, Seefeldt LC. Evidence for electron transfer-dependent formation of a nitrogenase iron protein-molybdenum-iron protein tight complex. The role of aspartate 39. *J Biol Chem*. 1997; 272:4157–4165. [PubMed: 9020128]
26. Walker GA, Mortenson LE. Effect of magnesium adenosine 5'-triphosphate on the accessibility of the iron of clostridial azoferredoxin, a component of nitrogenase. *Biochemistry*. 1974; 13:2382–2388. [PubMed: 4364777]
27. Zumft WG, Mortenson LE, Palmer G. Electron-paramagnetic-resonance studies on nitrogenase. Investigation of the oxidation-reduction behaviour of azoferredoxin and molybdoferredoxin with potentiometric and rapid-freeze techniques. *Eur J Biochem*. 1974; 46:525–535. [PubMed: 4368670]
28. Georgiadis MM, Komiya H, Chakrabarti P, Woo D, Kornuc JJ, Rees DC. Crystallographic structure of the nitrogenase iron protein from *Azotobacter vinelandii*. *Science*. 1992; 257:1653–1659. [PubMed: 1529353]
29. Schindelin H, Kisker C, Schlessman JL, Howard JB, Rees DC. Structure of ADP x AIF₄⁻-stabilized nitrogenase complex and its implications for signal transduction. *Nature*. 1997; 387:370–376. [PubMed: 9163420]
30. Schlessman JL, Woo D, Joshua-Tor L, Howard JB, Rees DC. Conformational variability in structures of the nitrogenase iron proteins from *Azotobacter vinelandii* and *Clostridium pasteurianum*. *J Mol Biol*. 1998; 280:669–685. [PubMed: 9677296]
31. Jang SB, Seefeldt LC, Peters JW. Insights into nucleotide signal transduction in nitrogenase: structure of an iron protein with MgADP bound. *Biochemistry*. 2000; 39:14745–14752. [PubMed: 11101289]
32. Tezcan FA, Kaiser JT, Mustafi D, Walton MY, Howard JB, Rees DC. Nitrogenase complexes: multiple docking sites for a nucleotide switch protein. *Science*. 2005; 309:1377–1380. [PubMed: 16123301]
33. Sen S, Igarashi R, Smith A, Johnson MK, Seefeldt LC, Peters JW. A conformational mimic of the MgATP-bound “on state” of the nitrogenase iron protein. *Biochemistry*. 2004; 43:1787–1797. [PubMed: 14967020]
34. Sen S, Krishnakumar A, McClead J, Johnson MK, Seefeldt LC, Szilagyi RK, Peters JW. Insights into the role of nucleotide-dependent conformational change in nitrogenase catalysis: Structural characterization of the nitrogenase Fe protein Leu127 deletion variant with bound MgATP. *J Inorg Biochem*. 2006; 100:1041–1052. [PubMed: 16616373]

35. Schmid B, Einsle O, Chiu HJ, Willing A, Yoshida M, Howard JB, Rees DC. Biochemical and structural characterization of the cross-linked complex of nitrogenase: comparison to the ADP-ALF₄⁻-stabilized structure. *Biochemistry*. 2002; 41:15557–15565. [PubMed: 12501184]
36. Ryle MJ, Seefeldt LC. Elucidation of a MgATP signal transduction pathway in the nitrogenase iron protein: formation of a conformation resembling the MgATP-bound state by protein engineering. *Biochemistry*. 1996; 35:4766–4775. [PubMed: 8664266]
37. Chiu H, Peters JW, Lanzilotta WN, Ryle MJ, Seefeldt LC, Howard JB, Rees DC. MgATP-Bound and nucleotide-free structures of a nitrogenase protein complex between the Leu 127 Delta-Fe-protein and the MoFe-protein. *Biochemistry*. 2001; 40:641–650. [PubMed: 11170380]
38. Lanzilotta WN, Fisher K, Seefeldt LC. Evidence for electron transfer from the nitrogenase iron protein to the molybdenum-iron protein without MgATP hydrolysis: characterization of a tight protein-protein complex. *Biochemistry*. 1996; 35:7188–7196. [PubMed: 8679547]
39. Smolksy IL, Liu P, Niebuhr M, Ito L, Weiss TM, Tsuruta H. Biological small-angle X-ray scattering facility at the Stanford Synchrotron Radiation Laboratory. *J Appl Cryst*. 2007
40. Priddy TS, MacDonald BA, Heller WT, Nadeau OW, Trehella J, Carlson GM. Ca²⁺-induced structural changes in phosphorylase kinase detected by small-angle X-ray scattering. *Protein Sci*. 2005; 14:1039–1048. [PubMed: 15741333]
41. Vaney MC, Maignan S, Ries-Kautt M, Ducruix A. High-resolution structure (1.33 Å) of a HEW lysozyme tetragonal crystal grown in the APCF apparatus. Data and structural comparison with a crystal grown under microgravity from SpaceHab-01 mission. *Acta Crystallogr D Biol Crystallogr*. 1996; 52:505–517. [PubMed: 15299672]
42. Svergun D, Barberato C, Koch M. CRY SOL - A program to evaluate x-ray solution scattering of biological macromolecules from atomic coordinates. *J Appl Cryst*. 1995; 28:768–773.
43. Svergun D. Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J Appl Cryst*. 1992; 25:495–503.
44. Svergun DI. Restoring low resolution structure of biological macromolecules from solution scattering using simulated annealing. *Biophys J*. 1999; 76:2879–2886. [PubMed: 10354416]
45. Kozin MB, Svergun DI. Automated matching of high- and low-resolution structural models. *J Appl Cryst*. 2001; 34:33–41.
46. Volkov VV, Svergun DI. Uniqueness of ab initio shape determination in small-angle scattering. *J Appl Cryst*. 2003; 36:860–864.
47. Chen L, Gavini N, Tsuruta H, Eliezer D, Burgess BK, Doniach S, Hodgson KO. MgATP-induced conformational changes in the iron protein from *Azotobacter vinelandii*, as studied by small-angle x-ray scattering. *J Biol Chem*. 1994; 269:3290–3294. [PubMed: 8106367]
48. Grossmann GJ, Hasnain SS, Yousafzai FK, Eady RR. Evidence for the selective population of FeMo cofactor sites in MoFe protein and its molecular recognition by the Fe Protein in transition state complex Analogues of Nitrogenase. *J Biol Chem*. 2001; 276:6582–6590. [PubMed: 11084023]

Figure 1.

Surface renderings and maximum dimensions of the nitrogenase Fe protein in different structural conformations including **A)** the native nucleotide free (2NIP.pdb) state in blue, **B)** L127Δ nucleotide free in red (1RW4.pdb). The two views of the Fe protein showing are separated by a ninety degree rotation about the horizontal axis. The bottom view displays the models viewed from the top side of the top view. **C)** Theoretical scattering curves obtained from CRYSOLE for the crystal structures of Fe protein in the conformations shown in **(A)** and **(B)** with the same coloring scheme. The nucleotide-free native state of the protein (2NIP.pdb) in blue and the nucleotide-free L127Δ Fe protein in red (1RW4.pdb).

Figure 2.

A) Experimental scattering curves of nucleotide free native Fe protein in blue and nucleotide-free L127 Δ Fe protein (5 mg/mL) represented in red and Guinier plots as inset (**A**), where intensities are artificially offset to aid comparison. **B)** Electron pair distribution function plots generated from the scattering curves in (**A**) with calculated R_g values for the nucleotide free forms of the native Fe protein (blue) and the L127 Δ Fe protein (red). *Ab initio* low resolution structural reconstruction from the scattering curves obtained using DAMMIN of the native Fe protein (blue) (**C**), nucleotide-free L127 Δ Fe protein (red) (**D**).

Figure 3.

A) Comparison of experimental scattering profile of 5 mg/mL Fe protein in blue and theoretical scattering curves of nucleotide-free native Fe protein (2NIP.pdb - brown). **B)** Comparison of experimental scattering profile of 5 mg/mL L127Δ variant Fe protein in red and theoretical scattering curves of nucleotide-free L127Δ variant Fe protein (1RW4.pdb - violet).

Figure 4.

A) Experimental scattering curves of nucleotide-free Fe protein (blue), along with nucleotide bound conformations of 5 mg/mL native Fe protein incubated with 5mM MgATP (magenta), MgADP (green circles) and Guinier plots for Fe protein with bound MgATP (magenta) and MgADP (green) as inset (**A**). **B)** Experimental scattering curves of nucleotide-free L127 Δ variant Fe protein (red), along with nucleotide bound conformations of 5 mg/mL L127 Δ variant Fe protein incubated with 5mM MgATP (gray), MgADP (aqua) and Guinier plots for Fe protein with bound MgATP (gray) and MgADP (aqua) as inset (**A**). **C) and (D)** Pair distribution function plots generated from the scattering curves (**A**) and (**B**), respectively, with calculated R_g values for the native Fe protein with MgATP (magenta) and MgADP (green) and calculated R_g values for the L127 Δ variant Fe protein with MgATP (gray) and MgADP (aqua).