Crystal Structure of a Conserved Hypothetical Protein MJ0927 from *Methanocaldococcus jannaschii* Reveals a Novel Quaternary Assembly in the Nif3 Family

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Received 30 May 2014; Revised 24 July 2014; Accepted 8 August 2014; Published 28 August 2014

Academic Editor: Cheng-Yang Huang

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A Nif3 family protein of *Methanocaldococcus jannaschii*, MJ0927, is highly conserved from bacteria to humans. Although several structures of bacterial Nif3 proteins are known, no structure representing archaeal Nif3 has yet been reported. The crystal structure of *Methanocaldococcus jannaschii* MJ0927 was determined at 2.47 Å resolution to understand the structural differences between the bacterial and archaeal Nif3 proteins. Intriguingly, MJ0927 is found to adopt an unusual assembly comprising a trimer of dimers that forms a cage-like architecture. Electrophoretic mobility-shift assays indicate that MJ0927 binds to both single-stranded and double-stranded DNA. Structural analysis of MJ0927 reveals a positively charged region that can potentially explain its DNA-binding capability. Taken together, these data suggest that MJ0927 adopts a novel quartenary architecture that could play various DNA-binding roles in *Methanocaldococcus jannaschii*.

1. Introduction

Given that numerous genomes of diverse organisms have been sequenced to date, it is not surprising that the number of hypothetical proteins with unknown functions has also steadily increased. Structures of hypothetical proteins can provide hints for deciphering their functions [1]. Since Nif3-like protein is widely distributed from bacteria to higher eukaryotes, this conserved hypothetical protein has been identified as a target from which the protein function can be inferred based upon a structural perspective [2].

The name of the Nif3-like superfamily was originated from the yeast Nif3, which was identified as an NGG1p-interacting protein in a yeast two-hybrid screen [3]. Sequence analysis of all reported Nif3-like proteins indicated that only the N- and C-terminal regions of the Nif3 proteins are highly conserved during evolution [4]. Studies of several eukaryotic Nif3 homologs have indicated that NIF3 family proteins are involved in transcriptional regulation [5]. For example, Akiyama et al. have demonstrated that murine Nif3 can interact with Trip5/CSN2 to function as transcriptional repressors [5]. Yet, the biological functions of Nif3 proteins in both prokaryotes and archaea kingdoms remain obscure.

To date, the tertiary structures of several bacterial Nif3 proteins have been determined by X-ray crystallography including those of SPI609 from *Streptococcus pneumonia* (2FYW), YqfO from *Bacillus cereus* (2GX8) [6], Ybgl from *Escherichia coli* (1NMP) [7], SA1388 from *Staphylococcus aureus* (2NYD) [8], and TTHA1606 from *Thermus thermophilus* HB8 (2YXB) [9]. These structures have all been observed to adopt a similar α/β structure, forming a toroidal hexamer. In most Nif3 structures, two metal ions were also
Table 1: Data collection statistics for the MJ0927 crystal. Values in parentheses are for the highest resolution shell. Data of C222₁ form has been used in previous publication [10].

|                     | Se-Met Nif3 (P₂₁ form) | Nif3 (C222₁ form) |
|---------------------|------------------------|-------------------|
| **Data Collection** |                        |                   |
| Wavelength (Å)      | 0.9792                 | 0.9793            |
| Peak                |                        |                   |
| Edge                | 0.9793                 | 0.9641            |
| Remote              | 1.0000                 |                   |
| Space group         | P₂₁                    | C222₁             |
| Unit Cell (Å)       | 95.60, 77.21, 131.85   | 81.21, 172.94, 147.42 |
| β                   | 105.36                 |                   |
| Resolution range (Å)| 30–2.8 (2.90–2.80)     | 30–2.7 (2.80–2.68) |
|                    | 30–2.75 (2.85–2.75)    | 30–2.47 (2.56–2.47) |
| Total observations  | 340792 (34238)         | 189181 (18903)    |
| Unique reflections  | 45486 (4505)           | 51206 (5109)      |
| Completeness (%)    | 99.8 (99.8)            | 99.7 (99.9)       |
| I/σ (I)            | 23.1 (4.4)             | 16.1 (2.8)        |
| Rmerge (%)         | 5.9 (3.3)              | 5.1 (3.3)         |
| **Refinement**      |                        |                   |
| Resolution range (Å)| 30–2.68 (2.73–2.68)   | 30–2.47 (2.53–2.47) |
| Reflections (F > 0σ_F) | 51170 (2370)     | 37404 (2689)      |
| Rwork (%)          | 18.8 (26.0)            | 18.1 (24.1)       |
| Rfree (%)          | 25.7 (33.8)            | 23.6 (35.8)       |
| RMS deviations     |                        |                   |
| Bond lengths (Å)    | 0.008                  | 0.007             |
| Bond angles (°)     | 1.13                   | 1.08              |
| Average B-factors (Å²) | 58.0                 | 49.0              |
| Protein atoms       | 54.2                   | 45.6              |
| Water molecules     |                        |                   |
| Model content       |                        |                   |
| Protein residues    | 1464                   | 732               |
| Waters              | 381                    | 192               |

found to stably exist within the cavity of the hexameric toroid, although the functional roles of these metal ions are still unclear.

To understand the structural differences between archaeal and bacterial Nif3 proteins, MJ0927 was selected as a target for structure determination. The crystal structure of MJ0927 determined at 2.47 Å revealed that MJ0927 folds into two interlinked α/β domains. Six protomers of MJ0927 are found to form a novel hollow cage-like hexamer, in contrast to the toroid-shaped hexamers formed by other members of the Nif3 family. Additionally, we found that MJ0927 is a DNA-binding protein exhibiting the ability to bind to both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). These data indicate that MJ0927 is a new member of the Nif3 family.

2. Materials and Methods

2.1. Cloning, Expression, and Purification. The gene cloning, protein expression, purification, crystallization, and diffraction for the native MJ0927 have been previously reported [10]. For expression of selenomethionyl (Se-Met) labeled MJ0927, the E. coli BL21(DE3) host cells were cultured in M9 medium supplemented with 40 μg mL⁻¹ Se-Met at 37°C and induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM when the cell density reached an OD₆₀₀ of 0.6. Purification of selenomethionyl MJ0927 was performed using similar protocols as established for the native protein.

2.2. Crystallization, Data Collection, and Structure Determination. Purified selenomethionyl MJ0927 was concentrated to approximately 30 mg mL⁻¹ for crystallization. Crystals were grown at 4°C using the sitting-drop vapor diffusion method by mixing 1 μL of protein solution with 1 μL of reservoir solution containing 5% PEG3350, 0.1 M sodium acetate, pH 5.0, 0.3 M sodium formate, 0.1 M ammonium sulfate, and 3% poly-γ-glutamic acid polymer (PGA-LM). Crystals were flash-cooled in the mother liquor supplemented with 25% glycerol as a cryoprotectant. X-ray diffraction data were collected using the BL13BI beamline at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. A three-wavelength MAD data set was collected to solve the protein phases. Intensity data were scaled and reduced using the HKL-2000 program [11]. The Se-Met-labeled MJ0927 crystals were found to adopt space group P₂₁. The MAD dataset was used for solving the structure and an ab initio model was built by using the Autosol and Autobuild Wizards.
in the Phenix package [12], respectively. Twelve selenium sites were identified and the initial phases calculated from these sites were further improved by density modification. The resulting electron density map was readily interpretable and was used to build most of the MJ0927 structure, which was then refined by iterative manual model building in the Coot [13] and Phenix refinement module [14]. Finally, 244 residues out of a total of 249 amino acids could be clearly identified in the model of Se-Met MJ0927.

Crystals of native MJ0927 in the C2221 space group were obtained using the reservoir solution containing 0.1 M (NH₄)₂SO₄, 0.3 M sodium formate, 0.1 M sodium acetate, 3% PGA-LM, and 20% MPD. The X-ray diffraction data of native MJ0927 were collected using the BL13C1 beamline at the NSRRC, Taiwan. The crystal structure was solved by the molecular replacement program Phaser [15] using the MJ0927 coordinates in P2₁ form as the template. Three molecules of MJ0927 are present in each asymmetric unit. Subsequent refinement of the coordinates and individual B factors were carried out in the Phenix refinement module. Noncrystallographic symmetry restraints were included only in the initial stages of refinement. The R values were refined to 18.1% and 23.6% for the Rwork and Rfree, respectively. The detailed crystallographic statistics were summarized in Table 1 [10]. Both coordinate sets have been deposited in the Protein Data Bank under the entries of 4IWG (C2221 form) and 4IWM (P2₁ form).

2.3. Electrophoretic Mobility-Shift Assay (EMSA). Two types of DNA were used for the DNA-binding experiments: a single-stranded 37-bp oligonucleotide and a double-stranded 37-mer oligonucleotide (5’-ATGTGAATCAGTATGGTT-ACTATCTGCTGAAGGAAAT-3’ and 5’-ATTTCTCTTC-AGCAGATGAAAACTACTGATTACAT-3’). These DNA were purchased from MDBio Inc. (Taiwan) and labeled by reaction with T4 polynucleotide kinase in the presence of [γ-32P]ATP. The purified His-tag-free MJ0927 was incubated with 25 nM ssDNA or dsDNA for 60 min, in a 10 μL solution containing 50 mM Tris-Cl, pH 8.0, 100 mM NaCl, 5% glycerol, 100 μM bovine serum albumin, and 2 mM Tris(2-carboxyethyl)phosphine at 37°C. The samples were then loaded onto a native gel of 5% nondenaturing polyacrylamide in 0.5x TB buffer (45 mM Tris-Cl, pH 8.0, and 45 mM boric acid). The DNA and protein-DNA complexes were separated by electrophoresis. Gels were exposed to phosphor storage screens and analyzed on a phosphorimager (Typhoon 9200, GE Healthcare).

3. Results and Discussion

Two crystal forms of MJ0927 were obtained, one in the P2₁ and the other in the C2221 space groups. The Se-Met labeled P2₁ crystal structure was solved at 2.8 Å resolution by using the multiwavelength anomalous diffraction (MAD) method. The refined structure was subsequently used as the template to solve the native C2221 structure by a molecular replacement approach. The final model was refined to 2.47 Å resolution with good Rwork and Rfree values. Residues 6–249 of the polypeptide chain were well defined in the electron-density maps, excluding the 5 N-terminal end residues which were invisible. A ribbon representation of a single MJ0927 monomer is shown in Figure 1(a). The P2₁ crystal form contains six molecules in each asymmetric unit, forming a hexameric spheroid with a 32-symmetry (Figure 1(b)). The six independent molecules in the asymmetric unit superpose well with pairwise root-mean-square deviations (rmsd) ranging from 0.222 to 0.310 Å. The C222₁ crystal form contains a single copy of a trimer in each asymmetric unit. No significant structural differences of the individual molecules were observed between the P2₁ and C222₁ crystal forms.

The overall structure of the MJ0927 monomer resembles a typical SCOP-classified NIF3-like fold [16] and is composed of 11 β-strands (β1–β11), nine α-helices (α1–α9), and one 310-helix. This protein appears to fold into two interlinked α/β Nif3 domains, each consisting of two α-helix layers sandwiching a single β-sheet (Figure 1(a)). The first domain (D1) contains a 5-stranded mixed β-sheet flanked by two α-helices and three α-helices on either side. The second domain (D2) is characterized by a central mixed β-sheet comprised of six β-strands with a pair of α-helices on both sides.

The MJ0927 hexamer can be described as two stacked trimers, consisting of monomers A-B-C and D-E-F, which are related by a two-fold symmetry (Figure 1(b)). The intratramer

| Type of contacts | Monomer A Residue | Monomer D Residue |
|-----------------|------------------|------------------|
| Salt bridges | Arg79[O] | Asn29[ND2] |
|                | Phe81[O] | Gly35[N] |
|                | Ile78[O] | Ile78[N] |
|                | Lys75[O] | Arg79[NH1] |
|                | Gly27[O] | Arg79[NH1] |
|                | Gly27[O] | Arg79[NH2] |
|                | Leu32[O] | Asn80[ND2] |
|                | Asn29[OD1] | Asn80[ND2] |
|                | Gln33[O] | Phe81[N] |
|                | Gly35[O] | Thr82[OG1] |
| Hydrogen bonds | Asp96[OD1] | Tyr86[OH] |
|                | Asp36[OD1] | Lys87[NZ] |
|                | Asn29[ND2] | Arg79[O] |
|                | Gly35[N] | Phe81[O] |
|                | Ile78[N] | Ile78[O] |
|                | Arg79[NH1] | Lys75[O] |
|                | Arg79[NH1] | Gly27[O] |
|                | Arg79[NH2] | Gly27[O] |
|                | Asn80[ND2] | Asn29[OD1] |
|                | Phe81[N] | Gln33[O] |
|                | Tyr86[OH] | Asp96[OD1] |
Figure 1: (a) Stereo view of MJ0927 monomer. The protein is rainbow-colored from blue at the N-terminus to red at the C-terminus. (b) The top and side views of the MJ0927 hexamer. The monomers A–F are colored in green, blue, cyan, red, yellow, and magenta, respectively. (c) The top and side views of the TTHA1606 hexamer. Each of the six monomers is in a different color.
Figure 2: (a) Stereo view of the MJ0927 dimer. (b) A stereo top-view of the MJ0927 trimer along its 3-fold axis.

The interface comprises two major regions, one consisting of a β-strand–helix interaction between β-strand β5 and helices α7 and α8 derived from one adjacent subunit, while the other is formed by helices α7 and α8, and β-strand β5 derived from another subunit (Figure 2(b)). The surface area of each subunit buried in the intratramer interface is 1110 Å², representing ~9.1% of the surface area of each subunit. There are five direct hydrogen bonds and six salt bridges and extensive hydrophobic interactions between the monomers across the intratramer interface (Table 3). Contacts between molecules A and D, B and E, or C and F occur through the interdimer interactions. The surface area of each subunit buried in the interdimer interface is 1207 Å², which constitutes ~10% of each subunit surface area. A total of 21 direct hydrogen bonds and four salt bridges are observed across the interdimer interface of each subunit (Table 2), with significant hydrophobic interactions formed by the helix α3, the β-strand β1, and the loop region Lα3–β3 (Figure 2(a)). The hexamer is stabilized by a combination of interdimer and intratramer interactions. These results are consistent with the previous gel filtration experiment showing that MJ0927 exists as a hexamer in solution [10].

Since MJ0927 is a thermophilic archaeal Nif3 protein, it is not surprising that the quaternary structure of MJ0927 differs from other bacterial Nif3 proteins with regard to the stabilizing structural elements, particularly the structural arrangement at the intratramer interfaces. As seen in Figure 2(b), the intratramer interfaces are tightly packed between the second domains of MJ0927 and the three subunits are arranged in a tail-to-tail manner. To date, trimer contacts in these solved bacterial Nif3 structures are only

Table 3: Interacting surfaces in MJ0927 and interactions at the intratramer interface.

| Type of contacts | Monomer A Residue | Monomer B Residue | Monomer C Residue |
|-----------------|------------------|------------------|------------------|
| Salt bridges    | Asp22            | His204           |                  |
|                 | Lys192           | Glu191           |                  |
|                 | Lys127           | Asp194           |                  |
| Hydrogen bonds  | Tyr188[OH]       | Glu191[OE1]      |                  |
|                 | Lys127[NZ]       | Ala193[O]        |                  |
|                 | Lys110[N]        | Glu211[OE1]      |                  |
|                 | Glu191[OE1]      | Lys110[N]        |                  |
|                 |                  |                  |                  |

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found in the SA1388 and YqO [6, 8], which use PII-like domains that appear to cap the openings on either side of the central channel in the toroidal rings. Therefore, the trimer organization of MJ0927 is a unique structural feature that likely contributes to its increased structural stability and which has never been observed in other Nif3 proteins. In addition, a sequence alignment further revealed that the Nif3 members in the Methanotorris, Methanocaldococcus, and Methanococcus species likely exhibit similar hexameric spherical architectures, as residues involved in the hexamer formation are found to be highly conserved (Figure 3). Although the biological function of MJ0927 remains unclear, this sequence analysis suggests that the hexameric spherical structure and its overall shape are preserved during evolution and thus may have functional significance.

Additionally, sequence analysis revealed that the highly conserved metal-binding motifs are located in the cavity between the D1 and D2 domains, which consist of three histidines, one glutamate, and one aspartate (Figure 3). In the three bacterial Nif3 protein structures (YbgI, YqO, and SA1388) [6–8], two divalent metal ions were found to occupy the metal-binding sites. However, no metal ion was observed in the metal-binding site of MJ0927 even though MJ0927 contains these conserved residues. The absence of endogenous metal ions in MJ0927 is similar to the structures reported for SP1609 and TTHA1606 [9], although the reason for this phenomenon is still unclear.

Since TTHA1606 has been shown to bind ssDNA [9], we are also interested to see if MJ0927 were capable of binding DNA. To assess the DNA-binding ability of MJ0927, purified proteins were incubated with ssDNA or dsDNA and the resulting complexes were separated by EMSA. Results of this analysis indicated that MJ0927 indeed is able to bind to
both ssDNA and dsDNA (Figure 4(a)). The dsDNA-binding property of MJ0927 was not observed in other Nif3 family proteins reported to date.

No Nif3-DNA complex structure has ever been reported yet. However, structural analyses of MJ0927 could provide some insights as to how the Nif3 protein binds to DNA. From the determined Nif3 structures reported to date, only those of MJ0927 and TTHA1606 were shown to bind ssDNA. Similar to TTHA1606, MJ0927 also possesses positively charged residues clustered on the helices α2 and α3 near the putative active site. Most of these residues share high sequence similarity between the MJ0927 and TTHA1606 Nif3 proteins, suggesting that the positively charged region may be involved in DNA binding (Figure 2(a)). Furthermore, the quaternary assembly of MJ0927 is significantly different to those previously solved bacterial Nif3 proteins. The bacterial Nif3s except for SA1388 and YqfO consist of three dimers forming toroidal ring quaternary structures, which possess an opened channel in the center of the hexamer (Figure 1(c)), whereas MJ0927 adopts an unusual assembly comprising a trimer of dimers that forms a cage-like architecture (Figure 1(b)). The architecture of MJ0927 leads to three large openings (Figure 4(b)). The diameter of the openings is approximately 33 Å, which is large enough to allow ssDNA or dsDNA entry. The sphere assembly of MJ0927 leads to larger openings than that of TTHA1606. Therefore, MJ0927 binds to both ssDNA and dsDNA, but TTHA1606 only binds to ssDNA.

### 4. Conclusions

MJ0927 is a member of the Nif3 family and is highly conserved among bacteria and humans. Here we describe the crystal structure of MJ0927, revealing an unusual hexameric assembly. Electrophoretic mobility-shift assays indicated that MJ0927 can bind to both ssDNA and dsDNA. The studies presented here clearly indicate that hexameric MJ0927 possesses ssDNA- and ds-DNA-binding properties and helps to further define its function. In a proposed follow-up study, we will try to determine the structure of MJ0927 in complex with DNA with the intent of identifying the DNA-binding residues of this protein to gain insight into its mechanism of action.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

### Authors’ Contribution

Sheng-Chia Chen and Chi-Hung Huang contributed equally to this work.

### Acknowledgments

The authors thank the National Synchrotron Radiation Research Center (NSRRC, Taiwan) for assistance during data collection. The authors are grateful to the staff of TCX-D900, Technology Commons, College of Life Science and Center for System Biology, NTU for help with the Art Robbins Instruments Phoenix protein crystallization robot. This work was supported by Grants from the National Science Council (NSC99-2313-B-241-001 and NSC100-2313-B-241-006 to Yeh
Chen) and by the Ministry of Education, Taiwan, ROC under the ATU plan.

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