Advances in Studies on Nitrogenase Crystallography

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Abstract: The function of biomacromolecule is dependent on its space structure. X-ray diffraction analysis is generally an important way to obtain structural information of biomacromolecules. Here, the main advances in the growth and X-ray diffraction analysis of nitrogenase crystals are briefly introduced and reviewed. At last, the challenge and prospect of nitrogenase crystallography are discussed.

Key words: nitrogenase; crystal growth; crystallographic structural analysis

Biological nitrogen fixation is performed by nitrogenase, which catalyzes the reduction of atmospheric N\textsubscript{2} to NH\textsubscript{3} (Milagros et al., 1999). Mo-containing nitrogenase is composed of two separable components designated the component I (MoFe protein) and the component II (Fe protein). Fe protein is a 60 000 Da dimer of identical y subunits connected through a single [4Fe-4S] cluster. It serves as an electron donor for MoFe protein in a reaction somehow coupled to MgATP hydrolysis. MoFe protein is a 220 000 - 240 000 Da tetramer of αβ2γ. It is often viewed as being composed of two identical halves that do not communicate with each other. Each half has one α subunit and one FeMoco, one P-cluster and one binding site for Fe protein (Kim and Rees, 1992; Peters et al., 1997; Schindelin et al., 1997).

Biosynthesis of MoFe protein (Av1) and Fe protein (Av2) in Azotobacter vinelandii Lipmann requires at least 15 different nif gene products (Nif). NifD, NifK and NifH have been implicated in the in vivo synthesis of α, β subunits of MoFe protein and γ subunit of Fe protein, respectively. NifZ has been implicated in the in vivo synthesis of P-cluster. If nifZ is deleted, Av1 synthesized in vivo is one of FeMoco-containing, P-cluster-changed form of Av1 (Zhong et al., 1996; Huang et al., 1999). And NifN, NifE, NifB, NifH, NifQ and NifV have been strongly implicated in the in vivo synthesis of FeMoco (Brigle et al., 1987; Paustian et al., 1989; Roll et al., 1995). If any of the later six genes is deleted or mutated, Av1 synthesized in vivo is one of P-cluster-containing, FeMoco-deficient form of Av1. The conditions for their full activation in vitro are not the same (Shah et al., 1977; Paustian et al., 1989; Tal et al., 1991; Zhao et al., 2003). It indicates that there is a structural difference between FeMoco-deficient Av1. However, the structural differences between the FeMoco-deficient or P-cluster-changed Av1 from mutants and Av1 from wild-type strain have not well been known.

Besides Mo-containing nitrogenase, there are other two genetically distinct nitrogenase systems in bacteria: a V-containing nitrogenase and an “iron only” nitrogenase lacking both Mo and V (Hales et al., 1990; Müller et al., 1992). Biosynthesis of their nitrogenases requires vnf and anf gene products, respectively. They are also composed of two separable components designated the component I (VFe protein and FeFe protein, respectively) and the component II (Fe protein). The three component I proteins have similar P-clusters and different FeMco in which M is Mo, V and Fe, respectively. But their structural differences have not well been known.

It was shown by a measurement using the perturbed angular correlation’s that the \textsuperscript{99}Mo had gone to the define place of Mo in FeMoco after an incubation of a partial metallocluster-deficient Av1 with a reconstituent solution containing \textsuperscript{99}Mo (Dong et al., 1996). And the partial metallocluster-deficient Av1 was activated by a reconstituent solution containing either Mn or Cr, leading to a suggestion that there could be nitrogenases MnFe protein and CrFe protein (Huang et al., 1994; 1995). The suggestion was supported by the later results that the MnFe protein and CrFe protein have been partially purified from a mutant UW3 of A. vinelandii grown on the Mn-, Cr-containing medium, respectively (Huang et al., 2001; 2002). Of course, more structural and functional evidence should be obtained to demonstrate their existence.

The function of biomacromolecule is dependent on its
space structure. A final elucidation of nitrogenase function is also dependent on its space structure. X-ray diffraction analysis is generally an important way to obtain structural information of biomacromolecules. However, the growth of crystals suitable for the analysis is usually very difficult, and often became a main hindrance for crystallography (Drenth et al., 1987). Fortunately, many crystals of nitrogenase component proteins have been obtained and analyzed by X-ray diffraction after a lot of efforts were made in the past more than 20 years.

1 Crystallization of Nitrogenase

The growth of protein crystal is indeed a complex physical and chemical process. There are many factors affecting crystallization of proteins, such as temperature, purity and concentration of the protein, kind and concentration of precipitants and stabilizer, concentration and pH value of different buffer, gravitation, method for crystallization and technical bias, etc. (McPherson, 1976). Like some metalloenzymes, nitrogenase proteins are susceptible to O2 and this makes their crystallization more complicated and difficult.

The first method for crystallization of Av1 was put forward by Burns et al. (1970), then was modified by Shah et al. (1973) and was simplified by the 7th Laboratory, Institute of Botany, The Chinese Academy of Sciences (1973). But the crystals obtained in these laboratories were small needle-shape. The crystal is not suitable for X-ray diffraction analysis. Weininger et al. (1982) attempted to do X-ray diffraction analysis of MoFe protein crystals grown by using microdialysis method. But they did not succeed in analyzing the crystallographic structure of MoFe protein.

In 1992[2] both Kim and Rees, and Georgiadis et al. succeeded in growing the big crystals of Av1 and Av2 by the microcapillary batch method and in analyzing of these crystals by X-ray diffraction, respectively. Since then, the liquid/liquid diffusion method has been usually used for crystal growth of nitrogenase proteins (Table 1).

In the past 10 years, many factors affecting crystallization of nitrogenase proteins have been well studied in our laboratory, leading to formation of larger crystals of Av1, Av2, MnFe protein, CrFe protein and bacterioferritin from wild-type A. vinelandii. Some of them are being analyzed by X-ray diffraction (Table 1).

The precipitant solutions used in our laboratory contain PEG, Na2S2O3, DT and glycerin. The solutions could help to protect nitrogenase protein from denaturing. The protection of the solutions could come from the following factors: (1) excess DT which is able to reduce O2; (2) glycerin which is used to be a stabilizer for many proteins; and (3) viscosity of PEG and glycerin which obstructs O2 in diffusing to protein molecules (Zhao et al., 2003). PEG could absorb water from the environment around protein molecules, resulting in decreasing protein dissolusion, and could obviously decrease the dielectric constant of the medium, resulting in decreasing an effectively electrostatic shield between protein molecules (McPherson et al., 1976). So, PEG was usually used as a precipitate for protein crystallization. In fact, most of the big crystals with good quality in Table 1 were obtained only when PEG was used as one of the precipitants for crystallization of nitrogenase proteins. Like salts (MgCl2, NaCl, etc.), PEG has an optimum concentration for the crystallization. Only a few crystal nuclei are formed and subsequently grown to large crystals of good quality when the protein loses water at a suitable rate. The optimum concentration of a precipitant is changed with the protein kind, the other chemical concentrations and the method for crystallization.

Hepes or Tris is one kind of salt, but its basic role is to stabilize the pH value of the protein solution, since pH value is very important for the electric charge on protein and the stability of protein conformation (McPherson et al., 1976; Huang et al., 2001). Hence, pH value is an important factor affecting the size, number, shape and quality of crystals. The pH value of 8.1-8.4 is the optimum value for crystallization of Av1, Av2, Av1, MnFe protein and CrFe protein. Perhaps the optimum pH value is also dependent on protein kind and other conditions. For example, the crystals of Av1 used for X-ray diffraction analysis were formed at pH value of 9.5 (Schmid et al., 2002).

Excellent protein crystals are usually obtained under such a condition that the solution convection and wall effect are minimized. The microgravity on the spacecraft could decrease the convection and wall effect (Drenth et al., 1991). Up to date, the effect of the microgravity on the crystallization of nitrogenase has not been reported. Fortunately, both MnFe protein by the vapor diffusion in sitting drop method and CrFe protein by the liquid/liquid diffusion method were crystallized on the spacecraft (“Shenzhou” No. 3) in the space (Zhao et al., 2003). All formed crystals were single on the spacecraft, while twin crystals appeared on the ground under the same conditions. The size of the largest crystal grown in space from CrFe protein was 2-fold larger than that on the ground. But the size of the largest crystal grown in space from MnFe protein was not larger than that on the ground. The results show that the crystallization in space
either by the vapor diffusion method or by the liquid/liquid diffusion method might benefit the avoidance of twin crystal formation of the proteins. And the crystallization in space benefited the great diminution of crystal nuclei number and significant growth of CrFe protein crystals. The differences in the effect of microgravity on crystal number and size between the two proteins could be resulted from the diffusion method, or the protein kind, or other factors including the precipitant solutions, etc. It is reported that the liquid/liquid diffusion method benefits a gradually growth of both CrFe protein crystals and MnFe protein crystals (Zhang et al., 2002; Lu et al., 2003). It is indicated that the main factor affecting crystal growth is the diffusion method, rather than the kind of nitrogenase.

Salemme (1972) pointed out that the liquid/liquid method utilizes free diffusion between protein solution and precipitant solution to attain the conditions of protein supersaturation requisite for the nucleation and subsequent growth of large single crystals. Protein and precipitant solutions are layered over each other, and allowed to diffuse to equilibrium. The sharp interface is helpful to decrease the diffusion rate, leading to the slow formation of nuclei and crystal growth of a few large crystals. Perhaps the microgravity in space could be helpful to further decrease the diffusion rate. However, using the sitting drop method or hanging drop method, the liquid/liquid diffusion always exists after the vapor diffusion method, the liquid/liquid diffusion always exists after the vapor diffusion method or hanging drop method, etc. It is reported that the liquid/liquid diffusion method might benefit the avoidance of twin crystal formation of the proteins. And the crystallization in space could be helpful to further decrease the diffusion rate.

<table>
<thead>
<tr>
<th>Component (strain)</th>
<th>Name(1)</th>
<th>Crystalline method(2)</th>
<th>Main precipitant (pH)</th>
<th>( \AA ) (3)</th>
<th>Author</th>
</tr>
</thead>
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<td>I (Wild type)</td>
<td>Cp1</td>
<td>-</td>
<td>PEG6K, MgCl₂ (7.5)</td>
<td>2.4</td>
<td>Weininger et al. (1982)</td>
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<td>Cp1</td>
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<td>Kim et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>Cp1</td>
<td>MB</td>
<td>PEG4K, MgCl₂/CsCl (8.0)</td>
<td>3.0</td>
<td>Kim et al. (1993)</td>
</tr>
<tr>
<td></td>
<td>Kp1</td>
<td>MB</td>
<td>PEG6K, MgCl₂ (8.0)</td>
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</tr>
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<td>-</td>
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<td>3.0</td>
<td>Weininger et al. (1982)</td>
</tr>
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<td>Peters et al. (1997)</td>
</tr>
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<td>Av1</td>
<td>VS</td>
<td>PEG6K, MgCl₂, NaCl (8.2)</td>
<td>-</td>
<td>Huang et al. (1998)</td>
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<td>MB</td>
<td>PEG4K, NaCl (8.0)</td>
<td>1.16</td>
<td>Einsle et al. (2002)</td>
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<tr>
<td>I (Mutant)</td>
<td>( \Delta ) n ZIP Av1</td>
<td>VH</td>
<td>PEG6K, MgCl₂, NaCl (8.2)</td>
<td>-</td>
<td>Huang et al. (2000)</td>
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<tr>
<td></td>
<td>( \alpha )-Gln(19)-Av1</td>
<td>MB</td>
<td>PEG400, Na₂MoO₄ (8.0)</td>
<td>2.5</td>
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<td></td>
<td>( \Delta ) n IE Av1</td>
<td>VH, VS &amp; MB</td>
<td>PEG6K, MgCl₂, NaCl (8.2)</td>
<td>-</td>
<td>Zhao et al. (2003)</td>
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<td>( \Delta ) n IH Av1</td>
<td>VH, VS &amp; MB</td>
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<td>Bian et al. (2003)</td>
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<td>( \Delta ) n IB Av1</td>
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<td>PEG6K, CHES (9.5)</td>
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<td>NifB Av1</td>
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<td>PEG6K, MgCl₂, NaCl (8.2)</td>
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<td>Mayer et al. (2002)</td>
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<td>PEG6K, MgCl₂, NaCl (8.2)</td>
<td>-</td>
<td>Huang et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>CrFe pro.</td>
<td>VH &amp; MB</td>
<td>PEG6K, MgCl₂, NaCl (8.2)</td>
<td>-</td>
<td>Lù et al. (2003)</td>
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<td>PEG4K, CaCl₂ (7.5)</td>
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<td>PEG4K, Na₂MoO₄ (8.0)</td>
<td>1.93</td>
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<tr>
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<td>Av2</td>
<td>Dialysis</td>
<td>2-methyl-2,4-pentanediol (7.8)</td>
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<td>Rees et al. (1983)</td>
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<tr>
<td></td>
<td>Av2</td>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>Georgiadis et al. (1990)</td>
</tr>
<tr>
<td></td>
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<td>MB</td>
<td>PEG4K, Na₂MoO₄, NaCl (8.3)</td>
<td>4.0</td>
<td>Georgiadis et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>Av2</td>
<td>MB</td>
<td>PEG4K, Na₂MoO₄ (8.0)</td>
<td>2.2</td>
<td>Schlessman et al. (1998)</td>
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<tr>
<td></td>
<td>Av2</td>
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<td>PEG4K, NaAc, Glycerol (8.5)</td>
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<td>PEG4K, NaCl, Glycerol (8.0)</td>
<td>2.25</td>
<td>Srop et al. (2001)</td>
</tr>
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<td>( \Delta ) Av2</td>
<td>MB</td>
<td>PEG4K, NaAc (8.5)</td>
<td>2.4</td>
<td>Jiang et al. (2000)</td>
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<tr>
<td>Complex</td>
<td>Av 2-ADP·ALF₁⁺-Av 1</td>
<td>MB</td>
<td>PEG8K, CaCodylate, MgCl₂ (6.5)</td>
<td>3.0</td>
<td>Schindelin et al. (1997)</td>
</tr>
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<td></td>
<td>( \Delta ) Av 2-Av 1</td>
<td>MB</td>
<td>PEG4K, Tris, NaoAc</td>
<td>2.2</td>
<td>Chiu et al. (2001)</td>
</tr>
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<td></td>
<td>Av 2-Av1</td>
<td>MB</td>
<td>PEG6K, MPD, NaCl (8.0)</td>
<td>3.2</td>
<td>Schmid et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Av 2-ADP·ALF₁⁺-Av 1</td>
<td>MB</td>
<td>PEG8K, MgCl₂, NaCl, AlCl₃, NaF (6.5)</td>
<td>2.3</td>
<td>Schmid et al. (2002)</td>
</tr>
</tbody>
</table>

(1) Av1, Kp1 and Cp1 are MoFe protein from Azotobacter vinelandii, Klebsiella pneumoniae and Clostridium pasteurianum, respectively, and Av2, Kp2 and Cp2 are Fe protein from the strains, respectively. MnFe protein and CrFe protein are component 1 purified from a mutant UW3 strain of A. vinelandii that grew on a Mo-free nitrogen-fixation medium containing Mn and Cr, respectively. (2) MD, microdialysis; MB, microcapillary batch method; VH, the vapor diffusion using hanging drop method; VS, the vapor diffusion using sitting drop method. (3) X-ray diffraction at \( \AA \) resolution, the data published was in the corresponding paper of references.
addition of the sample and the sharp interface is hardly formed, leading to increase of the diffusion rate. It is easy to form a larger amount of small crystals.

Most of biomacromolecule crystallization processes belong not only to indeed studies in scientific field, but also to technological studies with a half of experience. It needs a given techniques and relative knowledge, and sometimes needs to use unique technique. Therefore, the growth of crystals suitable for X-ray diffraction is still a main hindrance for protein crystallographic research although the knowledge and techniques of crystallography have been greatly improved.

2  Crystalline Structural Analysis of Nitrogenase

Georgiadis et al. (1992), Kim and Rees (1992) reported the first crystal structures of Av2 and Av1. It is viewed as a fieldstone in the studies on nitrogenase structure. From then on, 10 MoFe proteins, 7 Fe proteins and 5 nitrogenase complexes have been crystallographically determined and put into PDB (Protein Data Bank).

2.1  Nitrogenase Fe protein

Fe protein (NifH) has three different roles in the nitrogenase enzyme system. Apart from serving as the physiological electron donor to Av1, NifH is involved in FeMoco biosynthesis and in maturation of the FeMoco-deficient Av1 (Rangaraj et al., 1999). Binding of Fe protein with ATP plays a key role in electron transfer. The crystallographic structure of Av2 demonstrated that a single [4Fe-4S]-Cys₄ clusters bridged symmetrically between two identical subunits. Although Av2 and Cp2 share only 69% amino acid sequence identity, their subunit folds and dimer arrangements are very similar to one another (Schlessman et al., 1998). Av2 and Cp2 possess the common core elements of nucleotide-binding proteins. The elements consist of: (1) a predominantly parallel β-sheet flanked by α-helices; (2) a phosphate-binding loop (P-loop), or Walker A motif; and (3) two switch regions switch I and switch II.

In order to provide the driving force required for electron transfer, the redox potentials of Fe protein have to be changed. The [4Fe-4S] cluster contributes largely in the process. Mutagenesis studies have revealed that the conserved Phe, at position 135 in Av2 plays an important role in defining several biochemical and biophysical properties of the [4Fe-4S] cluster (Ryle et al., 1996). The crystallographic structure of the Trp-substituted Av2 shows that the substitution did not obviously change Fe protein conformation, indicating that the changes in the properties of the cluster could be only resulted from change in its local environment (Jang et al., 2000). The cluster is generally thought to undergo a one-electron redox cycle between the [4Fe-4S]³⁺ and the [4Fe-4S]⁴⁺. Watt et al. (1994) presented the evidence for further reduction to the [4Fe-4S]⁵⁺. And Strop et al. (2001) obtained the first crystallographic view of an all-ferrous [4Fe-4S]⁴⁺ cluster. It was suggested that the solvent accessibility of the all-ferrous Av2 cluster might play a role in its ability of formation of the oxidation states. It is generally believed that Fe protein serves as a one-electron donor and binds two ATPs during inter-protein electron transfer to yield an overall ATP/e⁻ ratio of 2. If the all-ferrous protein can function mechanistically as a two-electron donor, its high efficiency of energy utilization would be very intriguing.

2.2  Nitrogenase MoFe protein

Biochemical and biophysical studies have indicated that the FeMoco and P-cluster in MoFe protein play an important role in substrate reduction and electron/proton transfer. More attention has been paid to their structure and function. Since the two clusters have been neither chemically synthesized nor crystallized from solutions extracted from MoFe protein because of their novel structures and unique physical and chemical properties (Howard et al., 1996; Einsle et al., 2002), their structure and fine composition were controverted before X-ray diffraction analysis of Av1 (Kim and Rees, 1992).

The crystallographic structure of Av1 at 2.7Å resolution showed that the α- and β-subunits in the α₂β₂ tetramer had similar polypeptide folds. The subunits consist of three domains (designated α I, II, III and β I, II, III, respectively). The FeMoco is completely buried within the α-subunit, whereas the P-cluster occurs at the interface between α- and β-subunits. In this model of Av1, each center consists of two bridged clusters: the FeMoco has 4Fe:3S and 1Mo:3Fe:3S clusters bridged by three non-protein ligands and the P-cluster contains two 4Fe:4S clusters bridged by two cysteine thiol ligands. FeMoco containing Mo/Fe/S as well as homocitrate in the proportions 1:7:9:1 is connected to the protein through the side chains of only two residues bound to Fe and Mo sites located at the opposite ends of the cluster. The structures of MoFe protein from different sources are basically identical. The Cp1 crystallographic structure at 3.0Å resolution also showed that FeMoco has 4Fe:3S and 1Mo:3Fe:3S clusters and the P-cluster contained two 4Fe:4S clusters (Kim et al., 1993). NiFV Kp1 from a nifV-mutation mutant with FeMoco containing citrate instead of homocitrate can reduce C₂H₂ and H⁺, but its N₂ reduction activity is only ~7% that of the wild type. Ten years ago, it was not clear whether citrate
took the place of homocitrate. The crystallographic structure of Ni\textsuperscript{IV} Kp1 showed that citrate occupied the site of homocitrate in half of the protein, and water molecules occupied the site of homocitrate in the remainder (Mayer et al., 2002). Citrate differs from homocitrate by only a \(-\text{CH}_2\), but the difference results in a significant functional change of FeMoCo. It indicates that the structure of FeMoCo is a highly unique one, in which the homocitrate indeed plays an important role in reduction of N\textsubscript{2}.

In the cell, the FeMoCo is synthesized as a separate, but protein-associated entity. It is subsequently inserted into a P-cluster-containing, FeMoCo-deficient form of the MoFe protein. The crystallographic structure of \(\Delta nifB\) Av1 shows that like Av1, the \(\alpha\) and \(\beta\) subunits of \(\Delta nifB\) Av1 also consist of three domains each. Compared with Av1, except the \(\alpha\) \(\beta\) \(\gamma\) , the rest remains essentially unchanged. The residue rearrangements occurring in domain \(\alpha\) \(\beta\) \(\gamma\) create a positively charged funnel that is of sufficient size to accommodate insertion of the negatively charged FeMoCo. The result may help to explain a special requirement of Av2 and MgATP for \(\Delta nifH\) Av1 to be activated by FeMoCo. It is possible to suggest that the insertion funnel and accommodation site for FeMoCo in \(\Delta nifH\) Av1 are different from those of some FeMoCo-deficient Av1, leading to obstruction of FeMoCo insertion. Only after the residue rearrangements were changed with Av2 and MgATP, they are suitable for insertion and accommodation of FeMoCo.

Unlike the earlier results, the crystallographic structures of Cp1, Av1 and Kp1 show that the P-cluster is a \([8\text{Fe}-7\text{S}]\) cluster, other than \([8\text{Fe}-8\text{S}]\) cluster (Bolin et al., 1993; Peters et al., 1997; Mayer et al., 1999). Crystallography analysis of Av1 with defined oxidation states has shown that only P-cluster undergoes a redox-dependent structural rearrangement (Peters et al., 1997; Drennan et al., 2003). This structural rearrangement involves the exchange of two Fe atom ligands from the shared S atom (in the reduced state) to the polypeptide (in the oxidized state). It is shown that P-cluster, other than FeMoCo, participates in electron transfer. It indicates that the analysis of the redox states of the P-cluster is also important for insights into the mechanism of nitrogenase. Crystallographic analysis has also identified three forms of the P-cluster, which were corresponding to the \(P^\text{N}\), \(P^{\text{OX}}\), \(P^{\text{em-OX}}\) states, respectively (Mayer et al., 1999). The result is consistent with the results obtained from an extensive studies on spectrosopes including EPR, CD, MCD and Mössbauer of MoFe protein. The studies on spectrosopes have shown that P-cluster could exist in the following oxidized states: the all-ferrous state (\(P^\text{N}\)), the two-electron oxidized state (\(P^{\text{OX}}\)) and the one-electron oxidized state (\(P^{\text{em-OX}}\)). Thus, the role for this cluster in coupling electron and proton transfer in nitrogenase could be demonstrated.

With the improvement of growth of large single crystal and technique of X-ray diffraction, structural information at high resolution was obtained, leading to having much accuracy structure of the proteins. The crystallographic structure of Av1 at 1.16Å resolution not only demonstrated the \([8\text{Fe}-7\text{S}]\) structure of P-cluster, but also showed that N is most likely the previously unrecognized atom of FeMoCo (Einsle et al., 2002). This new advance provides either an opportunity or a challenge for scientists to think again about how N\textsubscript{2} inserts and reduce in FeMoCo.

### 2.3 Nitrogenase complex

At the protein level, the basic mechanism of nitrogenase involves the following: (1) formation of a complex between the reduced Fe-protein with two bound ATP molecules and MoFe-protein; (2) electron transfer between the two proteins coupled to the hydrolysis of ATP; (3) dissociation of the Fe-protein accompanied by reduction and exchange of ATP for ADP; (4) repetition of this cycle until sufficient numbers of electrons (and protons) have been accumulated so that available substrates can be reduced (Rees et al., 2000). It is in the transient complex between the Fe protein and the MoFe protein that electron transfer occurs, ultimately resulting in substrate reduction at a remote site (FeMoCo) in the MoFe protein. Hence, it was speculated that nucleotide hydrolysis might serve as a regulator of conformational switching leading to electron transfer.

Jang et al. (2000) obtained the Fe protein bonding MgADP state and determined its crystallographic structure. The results show that switchs I and II underwent significant structural changes after Fe protein bound nucleotide. This result provided an opportunity to differentiate the individual contributions arising from nucleotide binding, nitrogenase complex formation, and complex-dependent nucleotide hydrolysis in the nitrogenase enzyme system.

The Av1-Av2 complex stabilized with ADP \(\text{ALF}_4^-\) was crystallographically analyzed (Schindelin et al., 1997). The result shows that Av2 underwent substantial conformational changes and Av1 did not directly interact with the nucleotide. It is indicated that both Av1 and Av2 played a role in the stabilization of Av2-nucleotide intermediate in nucleotide hydrolysis. A conformational change in Av2 results in a modified binding surface that permits the \([4\text{Fe}-4\text{S}]\) cluster in Av2 to approach the Av1P-cluster ~4Å closer than possible with simple van der walls contact between
the individual protein components. Interactions in the complex have broad implications for signal and energy transduction mechanisms in the multiprotein complex. Another complex, EDC [-N-[3-(dimethylamino)propyl]-N'-
ethylcarbodiimide] cross-linked Av1-Av2, was also crystallographically analyzed (Schmid et al., 2002). The authors proposed that EDC cross-linking trapped a nucleotide-independent precomplex of the nitrogenase proteins driven by complementary electrostatic interactions. The precomplex subsequently rearranges in a nucleotide-dependent fashion to the electron transfer competent state observed in the ADP-ALF4 structure. This gives us dynamic insights into nitrogenase mechanism.

In the case of the Fe protein, Leu 127 and Asp 125-x_3-x_5-Gly 128 in the conserved sequence motif is part of the switch II region. The Av2 with a deletion of residue Leu127 (ΔAv2) can form a tight, inactive complex with the Av1 in the absence of nucleotide (denoted ΔAv2-Av1 complex) (Chiu et al., 2001). MgATP was bound to the crystal by soaking the crystals in MgATP solution. This demonstrates that dissociation of the nitrogenase complex is not required for nucleotide binding, whereas kinetic analysis has shown that dissociation of the Av1-Av2 complex is the rate-determining step. Consequently, the requirement and role for complex dissociation in the nitrogenase mechanism should be reconsidered. The differences in nucleotide binding between the L127 ΔAv2-Av1 and Av2-ADP-ALF4 Av1 complexes indicate that the mechanism of nucleotide hydrolysis by L127 ΔAv2 must be different from that proposed for the wild-type protein.

3 Prospects of Nitrogenase Crystallography

From the above discussion, it is not difficult to notice that more and more attention should be paid to the following crystallographic studies.

3.1 Nitrogenase-substrate complex

The X-ray diffraction analysis of MoFe protein and Fe protein crystals only provides important information about their “static” structures. In fact, the information is only a necessary base for fully understanding the mechanism of nitrogen fixation. The clear elucidation of the nitrogen fixation mechanism is only dependent on full knowledge about the exact “dynamic” changes in space structures of peptide chains and metalloclusters in two proteins during binding and reducing substrates. The crystallographic analysis of both Av1-Av2-ADP complex and Av1 with different redox states is able to provide some “dynamic” structural information. The crystallographic studies have demonstrated that some changes in conformation of enzyme molecule could appear after the enzyme binds substrate

McPherson et al., 1976]. It is reasonable to expect that the crystallographic analysis of nitrogenase-substrate complex should provide more and more information about the “dynamic” structural changes of nitrogenase. The information from the complex should be much more important than those from individual proteins. It has also been demonstrated that under some condition substrate-bound enzyme sometimes is easier crystallized [McPherson et al., 1976]. Therefore, it is undoubtedly worth to try crystallization of the nitrogenase-substrate complex when groping about conditions for crystallization of nitrogenase components.

3.2 Mutant nitrogenase

It is certain that the changes of the metalloclusters in MoFe protein and Fe protein could result in the changes in space structures of the peptide chains around the clusters when nitrogenase binds and reduces substrate. It is necessary for the exact elucidation of nitrogen fixation to obtain the information about the relation of the changes between the clusters and peptides. The crystallographic structures of AniB Av1 and NifV Kp1 show that it is very useful for knowledge of the structural changes to comparative study on crystallographic structures of nitrogenase from wild-type strain and different mutant strains.

3.3 Nitrogenases with the heterometal centers

From the bioinorganic point of view, it is of great importance to elucidate the influence of the heterometal center M (Mo, V, Fe) on the structure of related protein and/or the corresponding FeMco which may be present in different redox states, its electric structure, especially on the selectivity for substrate reduction (Müller et al., 1992). It is possible that the M in FeMco of MnFe protein and CrFe protein could be Mn and Cr, respectively (Huang et al., 2001; 2002). The existence of the proteins with different heterometaals thus provides the opportunity for better understanding of the N2 fixation mechanism by comparative biochemical and biophysical studies.

With improving techniques of both crystal growth and X-ray diffraction analysis, it is hopeful to perform the structural analysis of the three kinds of crystals mentioned above, resulting in some important advances or breakthrough of understanding mechanism of nitrogen fixation. As the crystal growth is still a main “hindrance”, a realization of the hopes is greatly dependent on the successful growth of large single crystals with good quality of the proteins mentioned above.
References:


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