

Crystal Growth of Nitrogenase CrFe Protein and MnFe Protein in Space

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Abstract: Nitrogenase CrFe protein and MnFe protein were purified from a mutant strain UW3 of *Azotobacter vinelandii* Lipmann grown on a medium containing Cr and Mn, respectively. In order to meet the requirement for crystal growth of O₂-susceptible proteins including nitrogenase in space, crystallization conditions were optimized for the proteins using a simple and suitable device, as a replacement for the cumbersome anaerobic box (dry box), for anaerobic addition of the protein samples. In all used precipitant and protein solutions added in the simplified plexi glass box, CrFe protein and MnFe protein could be crystallized on the spacecraft in one week by the liquid/liquid diffusion method and vapor diffusion by the sitting drop method, respectively. All formed crystals were single on the spacecraft, but under the same condition twin crystals appeared on the ground. 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 difference in crystal growth in space between CrFe protein and MnFe protein could be resulted from the crystallization method, rather than the kind of protein.

Key words: mutant UW3 of *Azotobacter vinelandii*; O₂-susceptible proteins; nitrogenase CrFe protein and MnFe protein; crystal growth in space

Three genetically distinct nitrogenase systems are composed of two separable proteins called component containing FeMco and P-cluster and component containing Fe₄S₄ cluster (Kim and Rees, 1992; Müller *et al.*, 1992; Newton, 1992). Different M in these FeM cofactors is Mo, V and Fe, respectively, hence the three component proteins are called MoFe protein, VFe protein and FeFe protein, respectively. These proteins are obviously different from one another in substrate-reduction activity and spectroscopic characteristics. It is very important for elucidation the mechanism of nitrogen fixation to comparatively study on the structural and functional relationships among the three proteins (Müller *et al.*, 1992). Nitrogenase CrFe protein and MnFe protein have been purified, partly characterized and crystallized on the ground (Huang *et al.*, 2001; 2002; Lü *et al.*, 2003). Further studies on structure and function of the two proteins are of great importance in both theory and practice (Huang *et al.*, 2001; 2002). Elucidation of the mechanism of nitrogen fixation has been greatly advanced by X-ray diffraction analysis of MoFe protein (OP

Av1) from wild-type strain OP and *nifB* MoFe protein (*nifB*-Av1) from a *nifB* deleted mutant strain DJ1143 (Newton, 1992; Peters *et al.*, 1997; Schindelln *et al.*, 1997; Schmid *et al.*, 2002). Thus, it is urgent to grow big crystals with good quality of CrFe protein and MnFe protein for X-ray diffraction analysis. Excellent crystals of proteins are usually obtained under such a condition that the convection of solution and wall effect are minimized. The microgravity on the spacecraft could be beneficial to decrease the convection and wall effect (Drenth *et al.*, 1991). The O₂-susceptible samples should be anaerobically added on the ground, followed by starting the crystallization process by an astronaut or controllable machinery after spacecraft was launched into the sky (Dong *et al.*, 2001). Up to date, the effect of the microgravity on the crystallization of nitrogenase proteins has not been reported. Therefore, it is an important requisite to examine the simplest device for anaerobic performance of crystallization of the O₂-susceptible proteins, to optimize crystallization condition on the spacecraft and to study crystallization characteristics of nitrogenase in space.

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1 Materials and Methods

Growth of UW3 strain of *Azotobacter vinelandii* Lipmann, purification of CrFe protein and MnFe protein from the strain and determination of their protein concentration, substrate-reducing activity, metal contents and SDS-PAGE analysis were described elsewhere (Huang *et al.*, 2001; 2002).

Crystallization by vapor diffusion in the hanging drop method was performed by the method of Huang *et al.* (2001).

Crystallization by vapor diffusion in the sitting drop method was performed by the method of Fu *et al.* (1999). After addition of precipitant and protein solution in a simplified box filled with N₂ (Dong *et al.*, 2001), the pedestal was air-tightly covered over with the cover board in such a way that each channel did not keep vapor from diffusing.

A rectangular device for crystallization used in the liquid/liquid diffusion method was also made of polycarbonic ester. Each chamber was composed of one small round hole and one piston by which the hole was divided into two. Five chambers of this type were combined as one block on a pedestal. The whole device included a pedestal and a cover board. In the same box filled with N₂, more than 1/2 volume of the hole was fully filled with about 100 μ L precipitant solution, followed by turning the piston in such a way that the added solution could not come into contact with the protein solution added later on the upside. The hole on the upside was washed twice with an anaerobic buffer and entirely dried with filter paper, then followed by fully filling with about 100 μ L protein solution. Then the pedestal was air-tightly covered over with the cover board.

All prepared samples for crystallization stood for 3 d at 15 °C. After the spacecraft was launched into the sky, the vapor diffusion and liquid/liquid diffusion were started by moving the cover board for the sitting drop method to open

the vapor passageway for vapor diffusion and by tuning piston to open the liquid passageway for the liquid/liquid diffusion method, respectively. The samples stood for 7 d at about 20 °C in space.

All the solutions were rigorously degassed and filled with Ar. Proteins were dissolved in 0.2 - 0.5 mol/L NaCl and 25 mmol/L Tris buffer (pH 7.4).

2 Results

2.1 Special requirement for crystallization in space

2.1.1 Anaerobic addition of samples Since the two component proteins of all nitrogenase are O₂-susceptible proteins, the protein and precipitant solutions for crystallization have to be added in a dry box filled with Ar or N₂. When the samples were added in a simplified plexi glass box filled with Ar or N₂, large crystals of MnFe protein could be formed from 4 precipitant solutions by vapor diffusion in the hanging drop method (Table 1). However, the protein was not easy to be crystallized from the solutions added under atmosphere of air. It was shown that the box could basically meet the requirement for the anaerobic addition of nitrogenase proteins. Perhaps, the reason why a few crystals were crystallized from some drops aerobically added was that the precipitant solution could protect some of the protein molecules against O₂.

After OP Av1 (0.15 mL) stood in a small tube filled with Ar for 48 h at room temperature, the protein was almost precipitated and inactivated. But under the same condition, the same Av1 (0.05 mL) mixed with 0.10 mL precipitant solution (Table 1, precipitant 1) retained its 75% activity. This indicates that the precipitant solution could help to protect nitrogenase protein from denature. The protection of the solution 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) high viscosity of

Table 1 Effect of the anaerobic addition of samples on the crystallization of MnFe protein by vapor diffusion in the hanging drop method

Precipitant ⁽¹⁾	Crystals ⁽²⁾							
	Air ⁽³⁾				Air ⁽⁴⁾			
	Drop 1 ⁽⁵⁾		Drop 2 ⁽⁵⁾		Drop 1 ⁽⁵⁾		Drop 2 ⁽⁵⁾	
	Number	Size	Number	Size	Number	Size	Number	Size
1	2 / 7	Middle/small	16	Small	0	/	2	Small
2	1	Small	2	Small	0	/	0	/
3	1	Small	2	Small	0	/	0	/
4	26	Small	23	Small	2	Small	2	Small

(1), concentration of MgCl₂, DT, Hepes (pH 8.2), glycerin and protein (dissolved in 250 mmol/L NaCl in 25 mmol/L Tris buffer, pH 7.4) were 598.95 mmol/L, 9.24 mmol/L, 80.94 mmol/L, 11.57% and 19.06 g/L, respectively; and concentration of PEG 8000 and NaCl in precipitant solutions were 4.43% and 192.56 mmol/L in the solution 1, respectively; 5.00% and 490.14 mmol/L in the solution 2, respectively; 4.43% and 245.07 mmol/L in the solution 3, respectively; 4.71% and 245.07 mmol/L in the solution 4, respectively; (2), incubation for 7 d at 20 °C on the ground; (3) and (4), the protein and precipitant solutions were added under an atmosphere of Ar and air, respectively; (5), crystals in two drops of solution under the same condition.

PEG and glycerin which obstructs O₂ in diffusing to protein molecules in the internal solution.

2.1.2 The suitable condition for crystallization The samples added on the ground have to stand for at least 2 - 3 d before the spacecraft is launched into the sky. By vapor diffusion in the sitting drop method, the liquid/liquid diffusion between the protein and precipitant solutions starts immediately while an addition of the two solutions is performed. It is possible to form a few crystals or crystal nuclei before the spacecraft was launched into the sky (Table 2). Therefore, it is necessary to select the crystallization conditions in which crystal nuclei could not be formed before the vapor diffusion started, and large single crystals of good quality could be formed after the diffusion started in the sky. In general, the conditions in which 10 - 30 crystals of good quality could be formed had to be selected in the preliminary experiment. But in the following experiments, the conditions in which a few high quality crystals could be formed should be selected in order to obtain a few large single crystals with high quality. When the special device described in "Materials and Methods" was used for crystallization by the liquid/liquid diffusion method, the liquid/liquid diffusion between the protein and precipitant solutions did not start before the liquid passageway was opened. A body of experiments showed that unlike the crystalliza-

tion by the vapor diffusion method, CrFe protein could hardly be crystallized by the liquid/liquid diffusion method before the diffusion started. Therefore, it is necessary to select the conditions in which a few big and high grade single crystals are formed.

2.2 Crystallization of nitrogenase proteins in space

2.2.1 Nitrogenase MnFe protein Under the conditions in Table 3, a body of twin crystals were formed from MnFe protein on the ground by vapor diffusion in the sitting drop method, but these twin crystals did not appear when crystallized on the spacecraft "Shenzhou" No.3 by the same method. In comparison with crystals grown on the ground, crystals grown in space were not larger. The result was similar to that obtained on the spacecraft "Shenzhou" No.2 by the same method in a sample solution containing 16.71 g/L MnFe protein, 5.51% PEG 8000, 598.95 mmol/L MgCl₂, 490.14 mmol/L NaCl, 11.57% glycerin and 80.94 mmol/L. The largest size of the crystals grown in space and on the ground were 0.16 mm × 0.16 mm × 0.07 mm and 0.16 mm × 0.16 mm × 0.05 mm, respectively. In the two experiments, not only the protein batches and concentration were different, but also precipitant solutions had somewhat difference from one another. It seems that the crystallization of MnFe protein on the spacecraft by the sitting drop method benefits possibly an avoidance of twin crystals,

Table 2 Crystallization of MnFe protein by vapor diffusion in the sitting drop method before the vapor diffusion on the crystallization

Precipitant ⁽¹⁾	Crystals ⁽²⁾			
	MnFe 1 ⁽³⁾		MnFe 2 ⁽⁴⁾	
	Before diffusion ⁽⁵⁾	After diffusion ⁽⁶⁾	Before diffusion ⁽⁵⁾	After diffusion ⁽⁶⁾
1	No	Yes	No	Yes
2	Yes	Yes	Yes	Yes ⁽⁷⁾
3	No	Yes	No	Yes ⁽⁸⁾

(1), concentration of DT, glycerin, PEG 8000 and NaCl were 9.24 mmol/L, 11.57%, 5.57% and 987.20 mmol/L, respectively; and concentration of Tris buffer (pH 8.2), Hepes buffer (pH 8.2) and MgCl₂ were 75.20 mmol/L, 0 mmol/L and 303.99 mmol/L in precipitant solution 1, respectively; 0 mmol/L, 74.57 mmol/L and 599.75 mmol/L in precipitant solution 2, respectively; 0 mmol/L, 74.57 mmol/L and 308.44 mmol/L in precipitant solution 3, respectively; (2), the same as in Table 1; (3) and (4), the two batches of proteins of which concentrations were 16.23 g/L and 9.62 g/L, and were dissolved in 250 mmol/L and 200 mmol/L NaCl in 25 mmol/L Tris buffer, pH 7.4, respectively; (5) and (6), incubation for 2 d at 15 °C and for another 5 d at 20 °C, respectively; (7), >30 crystals with good quality; (8), >30 crystals with bad quality.

Table 3 Effect of a tiny gravitation on the spacecraft on crystallization of MnFe protein by the vapor diffusion in the sitting drop method

Precipitant ⁽¹⁾	Crystals			
	On the ground ⁽²⁾		In the space ⁽³⁾	
	Number	Longest side	Number	Longest side
1	~60	0.050 × 0.050 ⁽⁴⁾	>>100	0.013 × 0.013
2	~100	0.050 × 0.050 ⁽⁴⁾	>>100	0.025 × 0.025
3	>>100	0.038 × 0.038 ⁽⁴⁾	>100	0.020 × 0.020

(1), concentrations of glycerin, Tris buffer (pH 8.2), DT and protein (dissolved in 200 mmol/L NaCl in 25 mmol/L Tris buffer, pH 7.4) were 11.57%, 75.20 mmol/L, 9.24 mmol/L and 9.62 g/L, respectively; and concentrations of NaCl, MgCl₂ and PEG 8000 were 981.14 mmol/L, 303.99 mmol/L and 5.524% in the solution 1, respectively; and 858.43 mmol/L, 599.75 mmol/L and 5.571% in the solution 2, respectively; (2) and (3), after standing on the ground for 3 d at 15 °C, incubation for 7 d on the ground and in space, respectively; (4), there were a lot of twin crystals.

rather than a crystal growth.

2.2.2 Nitrogenase CrFe protein Like the crystallization of MnFe protein by the sitting drop method, a body of twin crystals were also formed from CrFe protein on the ground by the liquid/liquid diffusion method in the two precipitant solutions in Table 4. But these twin crystals did not appear when crystallized on the spacecraft "Shenzhou" No.3 by the same method. And in comparison with crystals grown on the ground, not only were the crystals in space larger, but also was the crystal number significantly diminished. The differences depended on the composition of the precipitant solutions. The crystals of CrFe protein grown in the space in the precipitant solution 3 were about 2-fold larger than those grown on the ground in the same solution (Fig.1, Table 4). It indicates that the crystallization in space by the liquid/liquid diffusion method might benefit not only the avoidance of forming twin crystals, but also the diminution of crystal nuclei and gradual growth of crystals.

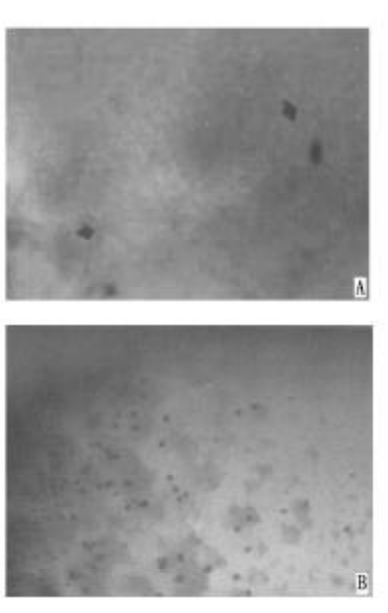


Fig.1. Crystals of CrFe protein grown on the spacecraft (A. 0.100 mm × 0.100 mm) and the ground (B. 0.050 mm × 0.050 mm) for 7 d by the liquid/liquid diffusion method. The protein and the precipitant were the same as in the solution 3 in Table 4.

3 Discussion

The simplified plexi glass box could meet requirement for an anaerobic addition of the O₂-susceptible protein. And nitrogenase protein is partially protected from O₂ by the used precipitant solutions.

The crystallization in the space either by the vapor diffusion method or by the liquid/liquid diffusion method might benefit the avoidance of twin crystal formation of both MnFe protein and CrFe protein. And the effect of microgravity on the crystal growth of CrFe protein by the liquid/liquid diffusion method is much larger than that of MnFe protein by the vapor diffusion method. The differences could be resulted from the diffusion method, or the protein kind, or other factors including the precipitant solutions, etc. It is reported earlier that the liquid/liquid diffusion method benefits a gradual growth of both CrFe protein crystals and MnFe protein crystals, leading to forming a few large crystals after incubation for a long time. After incubation for seven months, the largest crystal of CrFe protein and MnFe protein were 0.20 mm × 0.20 mm × 0.07 mm and 0.18 mm × 0.18 mm × 0.05 mm, respectively (Lü *et al.*, 2003). This indicates that the main factor that affects 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. At onset of interdiffusion of the protein and precipitant layers, the protein in the immediate vicinity of the interface is exposed to a transient supersaturating concentration of precipitant. This transient protein supersaturation induces the formation of nuclei which serves as crystal growth loci. At equilibrium the overall precipitant concentration in the total volume is substantially less than that required to spontaneously precipitate the protein, facilitating the gradual growth of a few large crystals from the

Table 4 Effect of a tiny gravitation on the spacecraft on crystallization of nitrogenase CrFe protein by the liquid/liquid diffusion method

Precipitant ⁽¹⁾	Crystals			
	On the ground ⁽²⁾		In the space ⁽³⁾	
	Number	Longest side	Number	Longest side
1	>>100 ⁽⁴⁾	0.050 × 0.050	>>100	0.063 × 0.063
2	>>100 ⁽⁴⁾	0.063 × 0.063	>100	0.075 × 0.063
3	>>100	0.050 × 0.050	4	0.100 × 0.100

(1), concentrations of glycerin, Tris buffer (pH 8.2), NaCl, DT and protein (dissolved in 250 mmol/L NaCl in 25 mmol/L Tris buffer, pH 7.4) were 11.57%, 75.20 mmol/L, 194.83 mmol/L, 9.24 mmol/L and 11.32 g/L, respectively; and concentration of PEG 6000 and MgCl₂ were 10.00% and 150.57 mmol/L in the solution 1, respectively; 10.00% and 249.49 mmol/L in the solution 2, respectively; 9.50% and 197.88 mmol/L in the solution 3, respectively; (2)-(4), the same as in Table 3.

nuclei created at the initial interface. The sharp interface is helpful to decrease the diffusion rate, leading to the slow formation of nuclei and 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, a liquid/liquid diffusion exists always after addition of the sample and the sharp interface is hardly formed, leading to the increase of the diffusion rate. It is easy to form a larger amount of small crystal.

As reported earlier, the larger crystals of the two proteins could be obtained by the liquid/liquid diffusion method after incubation for a long time (Lü *et al.*, 2002). Therefore, it is hopeful that some large single crystals of the two proteins would be obtained in the future in the space station.

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固氮酶CrFe蛋白和MnFe蛋白的空间晶体生长

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摘要: 从分别生长于含Mn和Cr培养基中的棕色固氮菌(*Azotobacter vinelandii* Lipmann) 突变种UW3分离纯化出MnFe和CrFe蛋白。为适应包括固氮酶在内的氧敏感蛋白的空间晶体生长的要求, 应用简易而适用的厌氧加样装置代替固氮酶实验室所用的笨重厌氧箱(dry box), 在地面进行厌氧加样。在充满氮气的简便有机玻璃箱内厌氧加样的所有样品中, 分别用液/液扩散法和汽相扩散的坐滴法都可在一周内使MnFe和CrFe蛋白在宇宙飞船上从溶液中结晶出来。在所用的数种蛋白沉淀剂中, 飞船上形成的所有晶体都为单晶, 而地面上在多数沉淀剂中都生成大量孪晶。在相同沉淀剂中用液/液扩散法, 飞船上生成CrFe蛋白的最大晶体比地面生成的最大晶体大1倍。而在相同沉淀剂中用汽相扩散的坐滴法, 飞船上生成的MnFe蛋白最大晶体却没有地面生成的最大晶体大。这种差异也许是由不同结晶方法而不是不同蛋白所引起的。

关键词: 棕色固氮菌(*Azotobacter vinelandii*) UW3; 氧敏感蛋白; 固氮酶MnFe蛋白和CrFe蛋白; 空间晶体生长

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