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氧敏感蛋白空间晶体生长的研究

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摘要： 为适应固氮酶蛋白等厌氧蛋白质空间晶体生长的要求，应在地面用简易而适用的厌氧加样装置以优化这类蛋白的结晶条件。用塑料袋或简易箱代替固氮酶实验室常用的笨重厌氧箱，获得了缺失 *nifZ* 固氮菌（*Azotobacter vinelandii* Lipmann）突变种的 MoFe 蛋白和含锰固氮培养基中生长的 UW_3 的 MnFe 蛋白晶体。并在远离固氮酶实验室的地方，使用由小食品塑料袋和急救用的氩气袋组成的更轻便的厌氧装置，用坐滴法也能使这两种蛋白结晶出来。结果表明，利用上述简易厌氧装置有望达到以上 2 种厌氧蛋白空间晶体生长的要求。

关键词： 氧敏感蛋白；空间晶体生长

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Studies on Crystalline Growth of O_2-susceptible Proteins in Space

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Abstract: In order to meet the requirement for crystalline growth of O_2-susceptible proteins in space, crystallization conditions on the earth was optimized for the proteins using a simple and suitable device for anaerobic addition of the protein samples. Nitrogenase is susceptible to O_2. Δ*nifZ* MoFe protein from a *nifZ* deleted strain and MnFe protein from mutant strain UW_3 grown on a medium containing Mn were crystallized at the first time in the world using an anaerobic device equipped with plastic bags or using a small simplified box, as a replacement for the cumbersome dry box. And the proteins could be also crystallized far from laboratory by sitting-drop method using a much lighter device. It was equipped with a smaller plastic food bag and a first-aid bag filled with Ar, as a substitute for the cumbersome dry box and the Ar cylinder, respectively. The results showed that the device could meet the requirement for studies on crystal growth of the above anaerobic proteins in space.

Key words: O_2-susceptible proteins; crystalline growth in space

In nature, a lot of metal-containing enzymes and proteins play an important role in the activity of life. Thus, great attention has been paid to the relation between their structure and function. Some enzymes and proteins are susceptible to O_2. For example, two component proteins of nitrogenase, component 1 (MFe protein, M = Mo, V, Fe or others) and component 2 (Fe protein), are very susceptible to O_2[1－4]. Studies on the relation between structure and function of nitrogenase component 1 proteins containing different M or MoFe proteins from mutant strains with *nif* genes deleted would help us to understand the mechanism of nitrogen fixation[4]. X-ray diffraction analysis is generally an important way to obtain structural informations of biological macromolecule, but the growth of crystals suitable for X-ray diffraction analysis usually is very difficult, and often becomes a main hindrance for crystallography[5]. Thus, an unique technique had to be used for the growth of big and high grade crystals of proteins[5]. There are a lot of factors affecting crystallization process related to the size and quality of crystals of proteins. Excellent crystals of proteins are usually obtained under such conditions as the convection of solution and wall effect are minimized. A tiny gravitation on the spacecraft could be of benefit to the decrease of the convection and wall effect[6]. However, travel on the spacecraft is expensive. Therefore, it is necessary to

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optimize crystallization condition on the ground. Even though the experiment performed on the ground was successful, the addition of the O_2-susceptible samples should be performed on the ground, followed by starting the crystallization process by an astronaut after spacecraft was launching into the sky. Because the dry box usually used in our nitrogenase laboratory is too big and too heavy, it is a great trouble to have the box at the base. Therefore, it is an important precondition to design a simpler device for anaerobic performance of crystallization of the O_2-susceptible proteins.

1 Materials and Methods

Growth of the mutant strain with *nifZ* deleted and UW_3 of *Azotobacter vinelandii* Lipmann, purification and characterization of their nitrogenase component 1 were carried out according to the method of Bishop *et al*[2], Burgess *et al*[3], and Zhong *et al*[7], respectively; except that the mutant UW_3 was grown in a medium containing 10 μmol/L $MnSO_4$.

Crystallization of nitrogenase component 1 from mutant strains was performed according to the method of Drenth *et al*[6] and Huang *et al*[8] with the exception, that is, a first-aid bag filled with Ar was substituted for the Ar cylinder and a small plastic bag or a simple box was substituted for the dry box (anaerobic box).

A rectangular device for crystallization used in the sitting-drop method was made of polycarbonic ester[9]. It was a type of vapor diffusion chambers. Each chamber was composed of one internal cell and two external pools. Five chambers of this type were combined as one block on a pedestal. The whole device included a pedestal and a cover board (Fig. 1). The equilibrium solution for crystallization was anaerobically added into the cells and pools, followed by the anaerobic addition of protein solutions into the cells. Then, the pedestal was air-tightly covered over with the cover board.

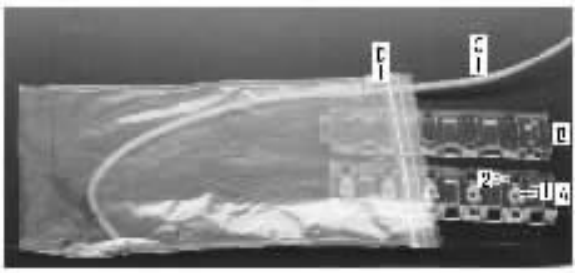

Fig. 1. A simple device for crystallization of O_2-susceptible proteins in the sitting-drop method.
A, pedestal with 5 crystallization chambers (1) and 10 reservoirs (2); B, cover board; C, plastic tube connecting to a first-aid bag filled with Ar or a Ar cylinder; D, small plastic bag (22 cm × 16 cm).

The device for the hanging-drop method was a plastic plate (10.2 cm × 10.2 cm × 2.2 cm) composed of 16 reservoirs (external pools) with 16 cover slides. 2 – 3 μL and 1 000 μL of equilibrium solution were anaerobically added on the slides and into the pool, respectively, followed by anaerobic addition of protein (two to three μL) to the equilibrium solution on the slides. The cover slides were turned over and put air-tight against the reservoir with greased edges.

The microsyringes used for injection of both the protein and the equilibrium solutions were washed with DT ($Na_2S_2O_4$) stock solution (20.0 mg/mL in 0.02 mol/L NaOH and degassed). All solutions were thoroughly degassed and filled with Ar, and all the bottles containing solutions should maintain a positive pressure and anaerobic condition with Ar. The final concentration of DT was 2.1 and 0.3 mg/mL in the equilibrium solutions and protein solutions, respectively.

2 Results

2.1 Crystallization in a small plastic food bag by the sitting-drop method

As shown in Fig. 1, a pedestal with 5 cells and 10 pools on it (Fig. 1, A) and cover board (Fig. 1, B), were connected with a plastic tube (Fig. 1, C) to a first-aid bag filled with Ar. They were put into a small plastic food bag (22 cm × 16 cm) washing with Ar, then the mouth of the bag was closed. To replace the air by Ar the bag was swollen with Ar, then was pressed for 5 – 6 times. As 80% – 90% air in the bag could be replaced by each press, it was reasonable to estimate that the residual air in the bag was decreased to about 1×10^{-4} – 1×10^{-6} by volume. The content of the residual air in the bag met the requirement for the anaerobic experiment. Then, the equilibrium and protein solutions for crystallization were injected into pools and cells, respectively, by means of microsyringe needles passing through the plastic bag. Finally, the device with samples were incubated at 20 ℃ for one week after the pedestal was air-tightly covered over with the cover board.

By using the simple device as shown in Fig. 1, crystals could be formed not only from nitrogenase MoFe protein and bacterioferritin of *Azotobacter vinelandii* (OP) in our laboratory[10], but also from Δ*nifZ* MoFe protein (Fig. 2) and MnFe protein (purified from mutant strain UW_3 grown on a Mn-containing medium) in either the Institute of Biophysics or the Institute of Shanghai Technological Physics. Even though the crystals of Δ*nifZ* MoFe protein were smaller than those formed by the hanging-drop method (Fig. 3), they were similar to each other in

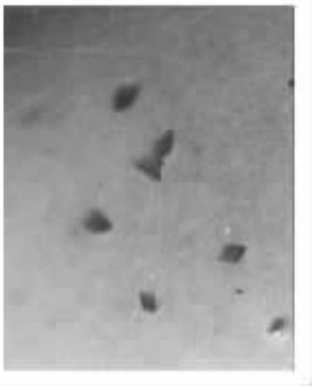

Fig.2. Crystals of Δ*nifZ* MoFe protein formed by the sitting drop method in the small plastic bag (×150).
Concentration of protein, PEG 8000, NaCl and $MgCl_2$ is 4.57 mg/mL, 1.90%, 323 mmol/L and 152 mmol/L, respectively.

Fig. 3. Crystals of Δ*nifZ* MoFe protein by the hanging-drop method in the plastic bag (×60).
Concentration of protein, PEG 8000, NaCl and $MgCl_2$ is 3.92 mg/mL, 2.65%, 320 mmol/L and 145 mmol/L, respectively.

color and shape. It was indicated that the simple and portable device was very suitable for carrying out the anaerobic experiments at any place.

2.2 Crystallization in a plastic bag by the hanging-drop method

By using the hanging-drop method, one to three drops of protein samples (2 – 4 μL/drop) and the equilibrium solutions (2 – 4 μL/drop) could be added on each small cover slides, that is, 16 – 48 different samples or different treatments could be performed on one plate. The hanging-drop method is superior to the sitting-drop method in performing more treatments with less protein samples. Therefore, it was usually used in the experiments.

However, in the hanging-drop method the plastic bag described in Fig.1 was too small and had to be replaced by a bigger one, and the first-aid bag replaced by an Ar cylinder. To fully deplete the air, the bag with device in it was swollen with Ar and then pressed for 5 – 6 times. The equilibrium and protein solutions were added on 4 slides and into relevant pools, respectively. Then, the pools were covered over with the turned over slides. The same procedure was repeated until all 16 slides were completed. By this way the brown crystals of Δ*nifZ* MoFe protein could be formed (Fig.3) after being incubated for several days at 20 ℃.

2.3 Crystallization in a simplified plexi-glass box

In order to further decrease risk of exposure of the proteins to air, the plastic bag was replaced by a small plexi-glass box. Two rubber gloves were fixed on both sides of the box. The back board was movable and could be tightened with screws. On the board there were some rubber stoppers with holes in it through which 5 small hard tubings were inserted. And an entrance for Ar, an exit for air and mouths of 3 balloons were connected with the tubings. While the box was washed with Ar, the balloons in the box were also fully filled with Ar so as to decrease the volume of gas exchange. After washing the box with Ar for more than 10 – 20 min, the equilibrium and protein solutions were added with microsyringe needles inserted into the stoppers on the back board. If the box was equipped with an Ar cylinder, it would be suitable for either the sitting- or the hanging-drop methods. Large and high grade crystals of Δ*nifZ* MoFe protein could be formed by using the box (Fig.4). It was reported that the brown crystals were really of this protein[8]; and the crystals were regularly changed in number, size and quality with the equilibrium solutions[11]. Brown crystals were formed from purified MnFe protein of the mutant strain UW_3 by using the same device (Fig. 5), and the size and number of the crystals were also dependant on the equilibrium solutions. As the final concentration of $MgCl_2$ in the

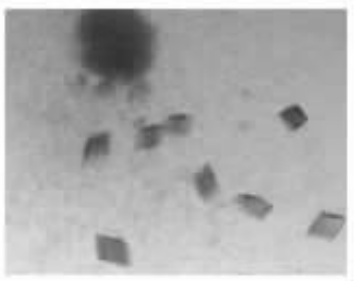

Fig.4. Crystals of Δ*nifZ* MoFe protein formed by the hanging-drop method in the simple box (×180).
Concentration of protein, PEG 8000, NaCl and $MgCl_2$ is 4.57 mg/mL, 1.85%, 320 mmol/L and 145 mmol/L, respectively.

Fig.5. Crystals of MnFe protein, which was purified from the mutant strain UW_3 grown on a containing-Mn medium, formed by the hanging-drop method in simple box (×180).
Concentration of protein, PEG 8000, NaCl and $MgCl_2$ is 3.61 mg/mL, 1.86%, 320 mmol/L and 200 mmol/L, respectively.

equilibrium solution was increased (150, 200, 250 and 300 mmol/L, respectively), the crystal size gradually increased and its number decreased (21, 16, 3 and 0, respectively). It was found that the optimal concentration of $MgCl_2$ was about 250 mmol/L under conditions shown in Fig.5. As UW_3 was able to grow in a Mo- and NH_3-free medium containing Mn, nitrogenase could be synthesized as the purified protein from its cells was able to reduce C_2H_2, H^+ and N_2, and contained Mn and Fe (with 10.4/1.0 of Fe/Mn ratio), this could be a protein of nitrogenase containing Fe and Mn (to be published elsewhere). Thus, like the crystal of Δ*nifZ* MoFe protein containing Fe and Mo crystals, they appeared to be brown in color (Fig.4 and Fig.5). But there were some tiny differences between them in optimal conditions for crystallization.

3 Discussion

From the above results, it was clear that the dry box and the Ar cylinder used for the anaerobic device could be successfully replaced by the small plastic bag and the first-aid bag filled with Ar, respectively. The content of Ar in the bag was enough for adding 5 samples on one pedestal even if the bag had been filled two months ago. The cheep and portable bags were able to satisfy the requirement for crystallization experiments with O_2-susceptible samples. Like in other two anaerobic devices, the crystals formed were gradually decomposed and finally disappeared after opening its cover; but the crystals could be maintained in the device for 3 – 4 months at 20 ℃ in good shape and sometimes could even continue growing. As the device for the sitting-drop method is decided to be used on the spacecraft, the small food bag and the first-aid bag filled with Ar have been chosen as the favorite devices for the anaerobic experiments at the base or any place far from a laboratory. The reason why the crystals formed by the sitting-drop method are somehow smaller than those formed by the hanging one is unknown, perhaps it is still not in the optimal condition for crystallization.

Although in the hanging-drop method the big plastic bag could also be replaced by the small food bag, but there are still drawbacks, that is, the risk of exposure to the air is increased and much agron is spent since the bag has to be opened several times. In this case Ar supplied by the first-aid bag was not enough. Therefore, Ar cylinder has to be used again. O_2 is not only toxic, but also it is an oxidant for MoFe protein. The metalloclusters of the protein and its other fractions had different susceptibilities to O_2. They will be oxidized, and irreversibly damaged by oxidation during exposing to O_2[12]. They could be protected against O_2 to some degree by DT in solution. Thus, in the present of DT and under strong Ar flow, it is possible to induce the protein crystallization in the device, with which studies on seeking for the optimal crystallization conditions could be carried out in the laboratory where the Ar supply was enough. Therefore, using very cheap and easily made device with the bag are still feasible choice.

The use of the simplified box spent much Ar than that of the small bag, so that the Ar cylinder had to be used. But in comparison with the big bag, the risk of exposure to O_2 was less during addition of the samples in the box. Therefore, the simplified box is also good in the anaerobic device for crystallization of O_2-susceptible proteins by the hanging-drop method.

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