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固氮酶锰铁蛋白的晶体生长

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摘要： 从以 Mn 代钼的固氮培养基中固氮生长的固氮菌（*Azotobacter vinelandii* Lipmann）突变种 UW_3 中分离纯化的 MnFe 蛋白，在一定的结晶条件下，可从溶液中析出深棕色的短斜四棱柱晶体。Tris 和 Hepes 缓冲液、NaCl、$MgCl_2$ 和 PEG 的浓度及结晶方法等，对该蛋白的出晶率、晶核数目、晶体大小和质量均有明显的影响，PEG 浓度的改变还可使该蛋白晶体的晶型发生变化。MnFe 蛋白结晶所需的上述化合物的最适浓度与缺失 *nifZ* 固氮菌突变种 Δ*nifZ* MoFe 蛋白结晶所需的最适浓度有所不同。SDS 凝胶电泳表明，晶体溶解后的蛋白与结晶前的 MnFe 蛋白基本相同。结果表明，该晶体为 MnFe 蛋白的晶体。

关键词： 固氮菌突变种 UW_3；含锰培养基；固氮酶锰铁蛋白；晶体生长
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Growth of the Crystals of Nitrogenase MnFe Protein

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Abstract: Under a suitable condition of crystallization, dark brown short rhombohedron crystals could be obtained from nitrogenase MnFe protein purified from a mutant UW_3 of *Azotobacter vinelandii* Lipmann grown in Mn-containing but Mo- and NH_3-free medium. The possibility of crystallization, and number, size and quality of crystals were obviously dependent on concentrations of NaCl, $MgCl_2$, PEG 8000, Tris and Hepes buffer and on methods for crystallization. PEG concentration affected on the shape of the crystals. The optimal concentrations of the chemicals for crystallization of MnFe protein were slightly different from those for crystallization of Δ*nifZ* MoFe protein from a *nifZ* deleted strain of *Azotobacter vinelandii*. SDS-PAGE showed that the protein from the dissolved crystals was almost the same as MnFe protein before crystallization, indicating that the crystal was formed from MnFe protein.

Key words: mutant UW_3 of *Azotobacter vinelandii*; Mn-containing medium; nitrogenase MnFe protein; crystalline growth

It is well known that three genetically distinct nitrogenase systems exist in bacteria, the "classical" Mo-containing nitrogenase, a vanadium-containing one and an "iron only" nitrogenase lacking both Mo and V[1,2]. All of them are composed of two separable proteins called component Ⅰ and Ⅱ. There are P-cluster and different M cofactors in component protein Ⅰ M is Mo, V or Fe in these systems, hence they are called MoFe protein, VFe protein and FeFe protein, respectively. These proteins are obviously different from each other in substrate-reducting activity and spectroscopic properties. More attention has been paid to the comparative studies on the relationships between the structures and functions between the most active Mo-containing system and the last two much less active systems. These studies are of great importance for the elucidation of the mechanism of nitrogen fixation[2].

Nitrogenase MnFe protein was purified from UW_3 strain grown on a Mo-free nitrogen-fixation medium containing Mn, and its structure and function had been partly characterized[3]. The studies on the relationship between structure and function of the Mo-Fe protein would also help us to understand the mechanism of nitrogen fixation. For this, it is necessary to grow big crystals with good quality for X-ray diffraction analysis.

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Table 1 Different effect of Tris concentration on the crystallization of Δ*nifZ* MoFe protein and MnFe protein*

Tris[1] (mmol/L)	Crystals					
	MnFe (16.72 mg/mL)			Δ*nifZ* MoFe (9.28 mg/mL)		
	Number[2]	Size	Quality	Number[2]	Size	Quality
53	0	–	–	11 – 16	Middle and small	Good
43	3 – 6	Middle	Good	⩾40	Middle and small	Poor
33	15 – 30	Middle and small	Good	13 – ⩾40	Middle and small	Poor

* Crystallization by the hanging drop method. 1) The concentrations of glycerin, PEG 8000, NaCl and $MgCl_2$ in the solution were 11.57%, 4.00%, 149.75 and 599.05 mmol/L, respectively, and the concentrations of NaCl in Δ*nifZ* MoFe and MnFe protein solution were 500 and 250 mmol/L, respectively; 2) Crystal numbers in two drops of solution under the same condition.

1 Materials and Methods

Growth of UW_3 strain of *Azotobacter vinelandii* Lipmann, purification, determination of protein concentration and SDS-PAEG analysis of MnFe protein were carried out according to the method of Huang *et al*[3] and Bishop *et al*[4].

Crystallization and characterization of the crystals of MnFe protein were performed, according to the methods of Drenth *et al*[5], Fu *et al*[6], Huang *et al*[7,8] and Dong *et al*[9].

2 Results and Discussion

There are many factors affecting crystallization of proteins, such as temperature, purity and concentration of the protein, kind and concentration of precipitants, stabilizer and buffer in crystalline solutions, pH of the solution, method for crystallization and technical bias, etc[7]. Therefore, it is necessary to optimize the above conditions in order to obtain big crystals of good quality.

2.1 Effect of crystalline solution on the growth of crystals of MnFe protein

2.1.1 Tris buffer As shown in Table 1, the process of crystallization of MnFe protein was siginificantly affected by Tris concentration, and the optimal concentration (43 mmol/L) of the crystallization for this protein was slightly lower than that (53 mmol/L) for Δ*nifZ* MoFe protein.

2.1.2 Hepes buffer In comparison with Tris buffer, Hepes buffer was more beneficial to increase the possibility of crystallization of MnFe protein and to decrease the number of crystal nuclei under a given condition (Table 2). For the crystallization of Δ*nifZ* MoFe protein there was no such difference between Tris buffer and Hepes buffer under the same condition[7].

The role of buffer is basically 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.

2.1.3 PEG 8000 As shown in Table 3, the concentration of PEG 8000 had an important effect on the possibility of crystallization, the number of crystal nuclei and the size and quality of crystals. Like Δ*nifZ* MoFe protein[7], there was the best suitable range of PEG concentration for the crystallization of MnFe protein. The crystals of the protein could not be formed or only small crystals were formed when the PEG concentration was out of this range.

Table 2 Effect of Hepes and Tris buffer on crystallization of MnFe protein*

PEG[1] (%)	Crystals			
	Hepes (81 mmol/L)		Tris (81 mmol/L)	
	Number[2]	Size	Number[2]	Size
1.86	13 – 24	Middle/Small	0	–
2.00	4 – 12	Middle/Small	11 – 16	Middle/Small

* and 2), same as in Table 1; 1), final concentration of protein, glycerine, NaCl and $MgCl_2$ in the solution: 8.36 mg/mL, 5.79%, 203 and 301 mmol/L, respectively.

Table 3 Effect of PEG 8000 concentration on the crystallization of MnFe protein*

Exp. 1 (145 mmol/L[1])			Exp. 2 (300 mmol/L[1])		
PEG[2] (%)	Crystal		PEG[2] (%)	Crystal	
	Number	Size		Number	Size
1.50	Many	Small	2.00	0	–
1.72	Many	Small	2.26	0	–
1.93	Many	Small	2.50	1	Small
2.22	3	Bigger	2.76	15	Big
2.76	1	Bigger	3.00	20 – 30	Small
3.00	1	Small	3.22	50 – 100	Smaller

*, same as in Table 1; 1), final concentration of $MgCl_2$; 2), final concentration of protein, glycerin and NaCl: 3.61 mg/mL, 5.55% and 200 mmol/L, respectively.

The optimal PEG concentration for the crystallization of this MnFe protein was slightly higher than that for Δ*nifZ* MoFe protein[7]. Bigger crystals could be obtained at about 2.2% PEG and 300 mmol/L $MgCl_2$.

The concentration of PEG affected the crystal shape of MnFe protein under given condition (Fig. 1). The slant of the short rhombohedron crystals increased with the increase of the PEG concentration from 1.86% to 2.29% and 3.00%.

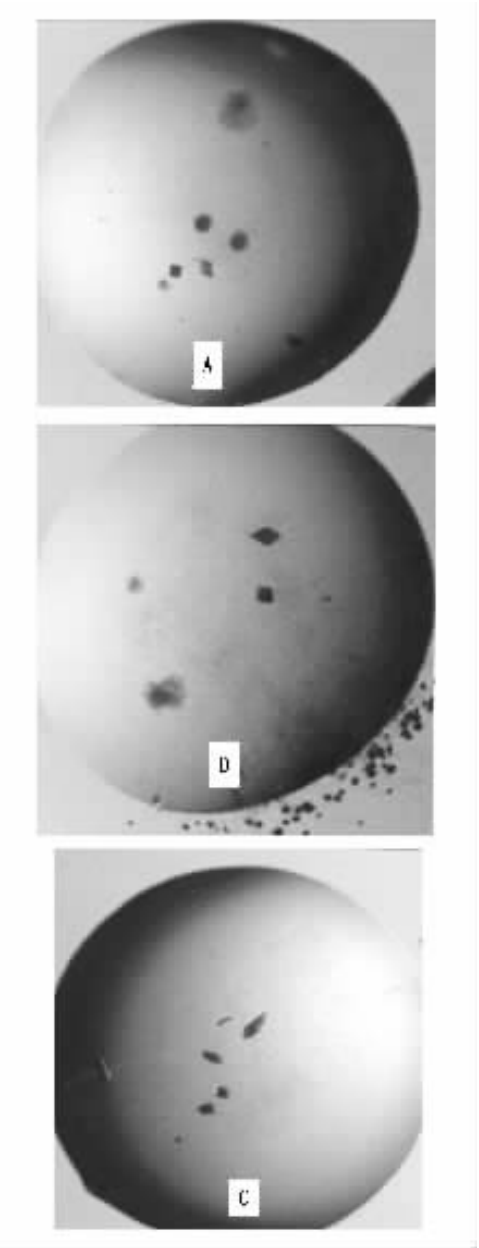

Fig. 1. The crystal shapes of MnFe protein in the presence of 1.86% (A), 2.29% (B) and 3.00% (C) of PEG 8 000 (×40). Final concentration of protein, glycerin, Tris, $MgCl_2$ and NaCl: 8.36 mg/mL, 5.79%, 52.96, 299.53 and 130.00 mmol/L, respectively.

PEG could absorb water from the environment around the protein molecules, resuting in decreasing protein dissolution[7]. Only a few crystal nuclei were formed and slowly grown to larger crystals with good quality when the protein lost water at a suitable rate (Fig. 1B). However, when the PEG concentration was too high, the formation of the crystal nuclei was accelerated with accelerating loss of water from the protein, leading to the following possibilities: 1) the size and quality of crystals were reduced and the number of crystals was increased, 2) the crystal shape was changed due to changes of the arrangement of protein molecules on the nuclei (Fig. 1C), and 3) the protein precipitation prior to the formation of crystal nuclei.

2.1.4 $MgCl_2$ The concentration of $MgCl_2$ significantly affected crystallization of the protein, and the number and size of crystals (Table 4). Its optimal concentration (about 250 mmol/L) for the crystallization of MnFe protein was slightly lower than that for Δ*nifZ* MoFe protein in Tris buffer[7]. But bigger protein crystals could form at 300 mmol/L of $MgCl_2$ when the final concentration of NaCl and PEG were 373 mmol/L and 2.215%, respectively.

Table 4 Effect of $MgCl_2$ concentration on crystallization of MnFe proteins from different preparations*

$MgCl_2$ (mmol/L)	Crystals			
	Protein solution 1[1)]		Protein solution 2[2)]	
	Number	Size	Number	Size
101			0	–
145			0	–
150	21	Middle and small		
200	16	Middle and small	2/1	Big/Small
250	3	Middle	1	Middle
300	0	–	0	–
320			0	–

*, see Table 1; 1), final concentration of protein, glycerin, PEG and NaCl: 8.36 mg/mL, 5.69%, 2.00% and 318.07 mmol/L, respectively; 2), final concentration of protein, glycerin, PEG, and NaCl: 3.61 mg/mL, 5.69%, 1.86% and 200.00 mmol/L, respectively.

2.1.5 NaCl The crystal number was dependent on the concentration of NaCl (Table 5). In certain range (200 to 350 mmol/L), the number of crystals decreased with the increase of NaCl concentration. However, in comparison with for Δ*nifZ* MoFe protein[7], the effect on the size of the crystals was smaller for MnFe protein.

Table 5 Effect of NaCl concentration on the crystallization of MnFe protein*

NaCl[1)] (mmol/L)	Crystals			
	Drop 1		Drop 2	
	Number	Size	Number	Size
205	15	Middle/Small	30	Middle/Small
249	12	Middle/Small	20	Middle/Small
300	10	Middle/Small	> 40	Middle/Small
351	5	Middle/Small	15	Middle/Small

*, see Table 1; 1), final concentration of protein, glycerin, PEG and $MgCl_2$: 8.36 mg/mL, 5.69%, 2.25% and 299.53 mmol/L, respectively.

2.2 Comparison of hanging drop with sitting drop method for the crystallization of MnFe protein

When MnFe protein was crystallized by the methods described previously[9], it was found that the methods had affected both the possibility of crystallization, and the number, size and quality of crystals (Table 6). Although the volume of protein in each cell of the sitting drop method was 5 folds of that of the hanging drop method,

Table 6 Crystallization of MnFe protein by hanging drop method* and sitting drop method

PEG %	Crystals					
	Hanging drop method*				Sitting drop method[4)]	
	Drop 1		Drop 2			
	Number	Size	Number	Size	Number	Size
1.86[1)]	0	–	0	–	12	Small
1.86[2)]	2/1	Middle small	1/1	Middle/Small	2/11	Middle/Small
2.22[3)]	2/3/7	Bigger/Middle/Small	1/2	Big/Middle	7	Bigger
2.38[3)]	8	Middle	8	Middle	10/2	Middle /Small
2,50[3)]	2/4/7	Bigger/Middle/Small	2	Middle	More	Small

*, the same as in Table 1; 1), final concentration of protein, glycerin, Hepes, Tris and NaCl: 9.53 mg/mL, 5.69%, 40.47, 12.50, 373 mmol/L, respectively; and final concentration of $MgCl_2$: 153.00, 199.69 and 300.25 mmol/L in 1), 2) and 3), respectively; Quality of crystals in 4) was better than that in *.

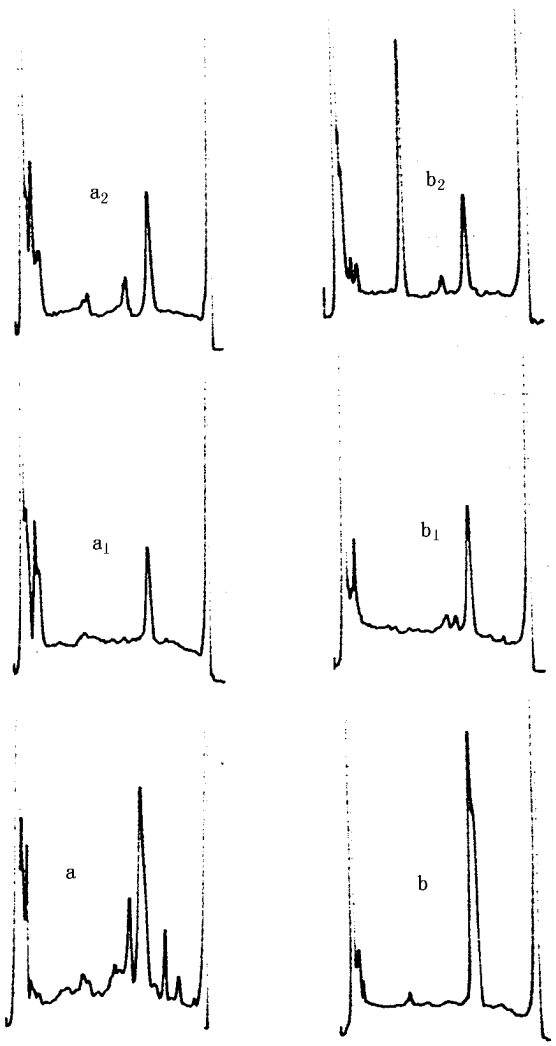

Fig.2. Electrophoresis patterns for SDS-PAGE of crystalline MnFe protein by CS-910 Dual-Wavelength TLC Scanner at 595 nm.
a, MnFe protein before crystallization (5.9 μg); a_1 and a_2, the first and second dissolved solution from crystalline drops with crystals by 0.3 mol/L NaCl, respectively; b, MoFe protein from *Azotobacter vinelandii* OP strain (4.8 μg); b_1 and b_2, the first and second dissolved solution from drops without crystals by 0.3 mol/L NaCl, respectively.

neither number nor size of crystals was completely proportional to the solution volume.

Either the sitting drop method or the hanging drop method is a type of vapor diffusion, but both the route of the diffusion and the ratio of solution volumes in the internal cell to that in the external cell were different from each other. These difference could affect on the rate of water loss from the environment around protein, resulting in a change of the crystallization process.

The results above indicated that: 1) the optimal concentration for each of the chemicals above varied with other variable factors; 2) it is possible to control the number, size and quality of crystals to certain extent by changing both the factors and the methods of crystallization. In general, the crystals are able to grow with less crystal nuclei. Therefore, it is possible to obtain bigger crystals of good quality when the crystallization condition is optimized.

Although both MnFe protein and Δ*nifZ* MoFe protein[7] could be crystallized in the solutions containing the above chemicals and buffers, the optimal condition for their crystallization was similar, but not completely the same. Perhaps, why the crystals from the two proteins were dark brown in colour, was that the two proteins contained a large amount of iron.

2.3 Analysis of the crystalline protein by SDS-PAGE

Solutions from the crystalline drops with and without crystals were collected separately in a flow of Ar. The collected solutions were centrifuged to remove the supernatant solutions. The pellets were washed twice with 0.6 mL and 0.3 mL of an anaerobic Tris buffer and centrifuged. The washed pellets were resuspended twice in 0.1 mL and then 0.2 mL of an anarobic buffer containing 0.30 mol/L NaCl, then by vibrated and centrifuged. The supernatant solutions from the pellets with and without crystals were labelled as a_1, a_2 and b_1, b_2, respectively. Although either a_1, a_2 or b_1, b_2 on gels of SDS-PAGE had a main band, they were slightly different from each other in both the height of the band and the height ratio of the main band to small bands (Fig. 2). Unlike electrophoresis pattern for SDS-PAGE of the crystalline MoFe protein from *Azotobacter vinelandii* OP (Fig. 2b) and Δ*nifZ*

MoFe protein[7] which were composed of α and β subunits represented by the two bands, the MnFe protein had only one band, whose position was similar to that of the α-subunit[3], and was contaminated by other proteins of lower molecular mass. This indicated that MnFe protein was partially purified and composed of the same subunits or two kinds of subunits of similar molecular mass. The amount of the subunits in b_2 was smaller than that in b_1, and was much more than that of the contamination protein. The amount of the protein dissolved from the drops without crystals was obviously more than that from Δ*nifZ* MoFe protein[8]. The reasons could be that: 1) The used concentration of NaCl was 0.3 mol/L, other than 0.25 mol/L[8]; 2) Δ*nifZ* MoFe protein was deficient in structure, leading to the formation of an undissolvable protein. In comparison with b_1 and b_2, either a_1 or a_2 contained much less contamination proteins. The amount of protein in a_2 was more than that in a_1, but the protein in a_2 was less pure than in a_1. The reason could be that when the pellet was first treated with 0.3 mol/L NaCl, the concentration of NaCl was slightly decreased by the residual buffer without NaCl (used for washing the pellet), leading to incomplete dissolution of crystals in the pellet; but when the pellet was treated again with 0.3 mol/L NaCl, the NaCl concentration should increase, leading to better dissolution of both MnFe protein and the contamination proteins.

Although the protein used in Fig. 2 was the least pure one of the three batches (concentration: 7.22, 17.22 and 19.06 mg/mL, respectively), the crystals formed from three proteins were similar in the shape and colour. This means that the crystals above could be one of MnFe proteins. However, the final conclusion could be made only after X-ray diffraction analysis of the protein crystal.

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