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Growth of Large Single Crystals of Nitrogenase CrFe Protein and MnFe Protein

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Abstract: By using the liquid/liquid diffusion method at a suitable crystallization conditions, large single and dark brown crystals (the sides of the largest crystals were 0.20 mm × 0.20 mm × 0.07 mm and 0.18 mm × 0.18 mm × 0.05 mm, respectively) could be obtained from the solutions of nitrogenase CrFe protein and MnFe protein purified from a mutant UW_3 of *Azotobacter vinelandii* Lipmann grown in Cr- or Mn-containing but NH_3-free medium. The time of crystal formation, as well as the number, size, shape and quality of crystals obviously depended on the concentrations of PEG, $MgCl_2$ and NaCl. The liquid/liquid diffusion method seems to benefit CrFe protein and MnFe protein for the growth of large single crystals for X-ray diffraction analysis.

Key words: mutant UW_3 of *Azotobacter vinelandii*; nitrogenase CrFe protein and MnFe protein; growth of large single crystals

Three genetically distinct nitrogenase systems are composed of two separable proteins called component Ⅰ containing FeMco and P-cluster and component Ⅱ containing Fe_4S_4 cluster (Müller *et al*, 1992; Newton, 1992). M in the three FeM cofactors could be Mo, V or Fe, hence the three component Ⅰ proteins are called MoFe protein, VFe protein or FeFe protein. It is very important to elucidate the mechanism of nitrogen fixation by comparative studies on the structural and functional relationships between the Mo-containing protein and the last two proteins (Müller *et al*, 1992). From the cells of UW_3 strain grown on a Mo-free nitrogen-fixation medium containing Cr and Mn, respectively, nitrogenase CrFe protein and MnFe protein were partly purified and characterized (Huang *et al*, 2001; 2002). The studies on the relationship between structure and function of the two proteins would also help us to understand the mechanism of nitrogen fixation. The 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 from *A. vinelandii* in both the reduced state and the oxidized state (Kim and Rees, 1992; Peters *et al*, 1997), and nitrogenase complex of MoFe protein and Fe protein from *Azotobacter vinelandii* (Schindelln *et al*, 1997). Thus, it is necessary to grow large crystals of good quality of the two proteins for their structure analysis. However, the crystals obtained by the vapor diffusion method are not large enough for X-ray diffraction (Huang *et al*, 2001; Zhang *et al*, 2002). The present study is undertaken for the attempt to select the optimum methods and crystallization conditions to obtain the large crystals of high quality of nitrogenase CrFe protein and MnFe protein.

1 Materials and Methods

Growth of UW_3 strain of *Azotobacter vinelandii* Lipmann was carried out according to the method of Bishop *et al* (Müller *et al*, 1992; Newton, 1992) except the addition of 10 μmol/L Na_2CrO_4 or $MnSO_4$ (Huang *et al*, 2001; 2002). The CrFe protein and MnFe protein were obtained after chromatography twice on DEAE-52 columns, followed by chromatography on Sephacryl S-300 and Q-Sepharose columns (Huang *et al*, 2001; 2002). The proteins were in 25 mmol/L Tris buffer (pH 7.4) containing 1.7 mmol/L $Na_2S_2O_4$ (DT) and 0.25 mol/L NaCl.

The crystals were obtained by the liquid/liquid diffusion method at 20 ℃ in a small glass tube (about 0.10 – 0.15 cm × 5.0 cm). Fifteen μL precipitant solution and 15 μL protein solution were slowly added into the tube. Crystals of CrFe protein and MnFe protein were also cultured by vapor diffusion at 20 ℃ with both the hanging drop method and the sitting drop method (Mcpherson, 1983; Huang *et al*, 2000; 2001; Zhang *et al*, 2002). The precipitant solutions were composed of buffer (Tris or Hepes) and precipitant (PEG and Salts). The crystallization was first observed after incubation for 7 d. The precipitant solutions also contained 12.0 mmol/L DT and about 12% (V/V) glycerin which was used as a stabilizer of proteins.

All solutions used were rigorously degassed and saturated with Ar. All operations were performed under the

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atmosphere of Ar.

2 Results

Like other proteins, the crystal growth of MnFe protein and CrFe protein is indeed a complex physical and chemical process, and depends on many factors, such as temperature, purity and concentration of the protein, kind and concentration of precipitants, stabilizer and buffer in crystallization solutions, and pH value of the solution, etc (Mcpherson, 1983; Fu *et al*, 1999; Huang *et al*, 2000; 2001; Zhang *et al*, 2002). Although most of these factors had been relatively fully studied before, the longest side of their biggest crystals obtained by the vapor diffusion method was still less than 0.16 mm (Huang *et al*, 2001; Zhang *et al*, 2002). Thus, it is urgent and important to select the suitable conditions for crystallization and to extensively optimize the precipitant conditions in order to obtain larger single crystals of high quality.

2.1 Optimization of crystallization condition

2.1.1 Crystallization solution system The large crystals of MoFe proteins, which were purified from either *Azotobacter vinelandii* or *Clostridium pasteurianum*, were obtained in the following precipitant solutions composed of either: (1) PEG 4000, Na_2MoO_4 and NaCl in Tris buffer; (2) PEG 4000, $MgCl_2$ and NaCl in Tris buffer; (3) PEG 4000, $MgCl_2$ and CsCl in Tris buffer; (4) PEG 6000 and $MgCl_2$ in Tris buffer; (5) PEG 8000 and Na_2MoO_4 in Tris buffer (Weininger and Mortenson, 1982; Kim and Rees, 1992). In the past 3 years, the precipitant solution system composed of PEG 8000, $MgCl_2$ and NaCl was used for crystallization of CrFe and MnFe protein by the vapor diffusion method in our laboratory (Huang *et al*, 2001; Zhang *et al*, 2002). But the crystals obtained were not large enough for X-ray diffraction analysis. In order to obtain larger crystals of the two proteins, the precipitant solutions used for crystallization of MoFe protein should be experienced. By using the liquid/liquid diffusion method in the solutions composed of PEG 4000 and Na_2MoO_4, of which the concentrations were around the suitable ranges for growth of the large crystals of MoFe proteins (Kim and Rees, 1992), some small poor crystals were formed from the solution of MnFe protein, but none from the solution of CrFe protein (Tables 1 and 2). And in the solutions composed of PEG 6000 and $MgCl_2$ of which the concentrations were around the suitable ranges for growing the large crystals of MoFe proteins (Weininger and Mortenson, 1982), a large amount of small-sized crystals were formed from both MnFe protein and CrFe proteins (Table 3). Similar results were also obtained with other precipitant solutions suitable for MoFe proteins (Weininger and Mortenson, 1982; Kim and Rees, 1992). It turned out that in comparison with the

Table 1 Effect of PEG 4000 concentration on the crystallization of CrFe protein and MnFe protein by liquid /liquid diffusion method

PEG %[1] (W/V)	CrFe protein[2]		MnFe protein[2]	
	Crystal number	Crystal size	Crystal number	Crystal size
11.90	0	/	Some[3]	Small[4]
15.06	0	/	Some[3]	Small[4]
17.98	0	/	Some[3]	Small[4]

1), concentration of PEG was per cent content (W/V) and concentrations of Na_2MoO_4 and NaCl were 200.74 mmol/L and 139.66 mmol/L, respectively; 2), concentrations of CrFe protein and MnFe protein were 9.74 mg/mL and 16.24 mg/mL, respectively; 3), number of crystals was ≥ 40; 4), the longest side of crystal was < 0.05 mm.

Table 2 Effect of Na_2MoO_4 concentration on the crystallization of CrFe protein and MnFe protein by liquid/liquid diffusion method

Na_2MoO_4 (mmol/L[1])	CrFe protein[2]		MnFe protein[2]		
	Crystal number	Crystal size	Crystal number	Crystal size	Crystal quality
100.40	0	/	≥100	Small[3]	Poor[4]
200.74	0	/	≥100	Small[3]	Poor[4]
401.48	0	/	≥100	Small[3]	Poor[4]

1), concentrations of PEG 4000 and NaCl were 17.98% (W / V) and 139.66 mmol/L, respectively; 2) and 3), the same as those in Table 1; 4), crystal in the poor quality.

Table 3 Effect of PEG 6000 concentration on the crystallization of CrFe protein and MnFe protein by liquid/liquid diffusion method

$MgCl_2$ (mmol/L)[1]	PEG % (W/V)	CrFe protein[2]		MnFe protein[2]	
		Crystal number	Crystal size	Crystal number	Crystal size
301.10	5.02	≥100	Small[3]	≥100	Smaller
	10.04	0	/	0	/
	15.06	≥100	Small[3]	0	/
498.98	5.02	≥40	Small[3]	≥100	Small[3]
	10.04	0	/	≥100	Small[3]
	15.06	≥40	Small[3]	≥100	Small[3]
679.64	15.06	0	/	≥100	Small[3]

1), concentrations of PEG 6000 and NaCl were 17.98% (W/V) and 139.66 mmol/L, respectively; 2) and 3), the same as those in Table 1.

PEG 8000/$MgCl_2$ system (Huang *et al*, 2001; Zhang *et al*, 2002), the suitable systems for MoFe protein were not better than the original solution for CrFe protein and MnFe protein. Therefore, it is more urgent to further optimize the compositions of the original solution consisting of PEG 8000, $MgCl_2$ and NaCl, rather than to change the precipitant systems.

2.1.2 Optimum compositions of the precipitant solution composed of PEG 8000, $MgCl_2$ and NaCl

PEG 8000 Like crystallization by the vapor diffusion methods (Huang *et al*, 2001; Zhang *et al*, 2002), the concentration of PEG 8000 had an important effect on the number of crystal nuclei, the size and quality of crystals and time of crystal formation when MnFe and CrFe proteins were crystallized by the liquid/liquid diffusion method. The two proteins were also slightly different in the time of crystal formation, size of crystals and the most suitable PEG concentration (Table 4 to Table 6). In the presence of 599 mmol/L $MgCl_2$ and 986 mmol/L NaCl, the PEG concentrations at which the biggest crystals of CrFe protein and MnFe protein formed were usually about 5.00% and 7.00% (Figs.1,2).

Table 4 Effect of PEG 8000 concentration and incubation time on the crystal growth of CrFe protein and MnFe protein by the liquid/liquid diffusion method

Protein[1]	PEG 8K %[2] (W/V)	Incubation time				
		One week		> 150 days		> 210 days
		Crystal number	Crystal size	Crystal number	Crystal size	Size of the biggest one
CrFe	4.71	0	/	> 10	Big	0.16 mm × 0.16 mm × 0.06 mm
	5.00	0	/	2	Big	0.20 mm × 0.20 mm × 0.07 mm
	6.00	> 40	Middle[3]	> 40	Bigger	0.12 mm × 0.12 mm × 0.05 mm
	7.00	> 40	Small[4]	> 40	Small[4]/bigger	0.12 mm × 0.10 mm × 0.10 mm
MnFe	4.71	0	/	0	/	/
	5.57	4	Small[4]	4	Small[4]	Nd[5]
	7.00	> 10	Middle[3]	> 10	Bigger	0.08 mm × 0.07 mm × 0.07 mm

1) and 4), the same as those in Table 1; 2), concentrations of Tris, Hepes, NaCl and $MgCl_2$ were 25.00 mmol/L, 74.60 mmol/L, 985.86 mmol/L and 598.95 mmol/L, respectively; 3), the longest side of crystal was ≥0.05 mm; 5), not determined.

Table 5 Effect of $MgCl_2$ concentration and incubation time on the crystal growth of CrFe protein and MnFe protein by the liquid/liquid diffusion method

Protein[1]	$MgCl_2$ (mmol/L)[2]	Incubation time			
		One week		> 150 days	
		Crystal number	Crystal size	Crystal number	Crystal size
CrFe	308.46	0	/	0	/
	445.24	0	/	> 20	Middle[3]
	596.95	> 20	Middle[3]	> 20	Middle[3]
MnFe	308.46	> 20	Middle[3]	> 20	Middle[3]
	445.24	0	/	0	/
	596.95	4	Small[4]	4	Small[4]
	793.98	0	/	0	/
	856.80	> 100	Small[4]	> 100	Small[4]

1), 3) and 4), the same as those in Table 1 and Table 4; 2), concentrations of PEG 8000, Tris, Hepes and NaCl were 5.572% (W/V) and 25.00 mmol/L, 74.60 mmol/L and 985.86 mmol/L, respectively.

Table 6 Effect of NaCl concentration and incubation time on the crystal growth of CrFe protein and MnFe protein by the liquid/liquid diffusion method

Protein[1]	NaCl (mmol/L)[2]	Incubation time				
		One week		> 150 d		> 210 d
		Crystal number	Crystal size	Crystal number	Crystal size	Size of the biggest one
CrFe	858.44	4	Small[3]	4	Small[3]	Nd[4]
	987.20	> 20	Middle[3]	> 20	Middle[3]	Nd[4]
	1 073.04	> 10	Middle[4]	> 10	Big	Nd[4]
	1 158.90	4	Small[3]	4	Small[3]	Nd[4]
MnFe	858.44	/	/	0	/	/
	987.20	4	Small	4	Small[3]	Nd[4]
	1 073.04	0	/	1	Big	0.18 mm × 0.18 mm × 0.05 mm
	1 158.90	0	/	1/1	Big/Small	0.16 mm × 0.12 mm × 0.05 mm

1) and 3), the same as those in Table 1; 2), concentrations of PEG 8000, Tris, Hepes, $MgCl_2$ were 5.572 (W/V), 25.00 mmol/L, 74.60 mmol/L and 598.95 mmol/L, respectively; 4), not determined.

Table 7 Effect of crystalline methods on the crystallization of MnFe protein

PEG 8K %[1]	Sitting drop[2]		Liquid diffusion[2]	
	Crystal number	Crystal size	Crystal number	Crystal size
4.71	0	/	4	Small[3]
5.00	0	/	> 60	Small[3]
5.57	3	Small[3]	> 60	Small[3]
6.00	> 20	Middle[4]	> 60	Small[3]
7.00	> 40	Small[3]	> 60	Small[3]

1), concentrations of protein, PEG 8K, Tris, Hepes, $MgCl_2$ and NaCl were 16.24 mg/mL, 5.572% (W/V) and 25.00 mmol/L, 74.60 mmol/L, 599.75 mmol/L and 987.20 mmol/L, respectively; 2), incubation for one week; 3) and 4), the same as those in Table 1 and Table 4.

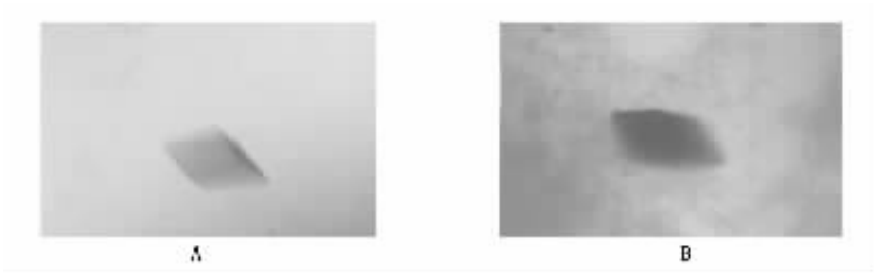

Fig.1. Crystals of CrFe protein observed after incubation for 210 d in the crystalline solution containing 4.71% (W/V) PEG 8000 (A, 0.16 × 0.16 × 0.06 mm) and 5.00% (W/V) PEG 8000 (B, 0.20 × 0.20 × 0.07 mm).
Other conditions for crystallization were the same as that in Table 4.

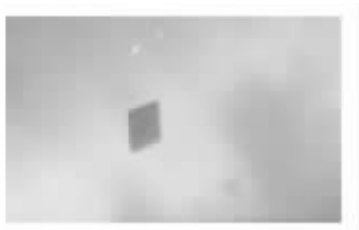

Fig.2. Crystals of MnFe protein observed after incubation for 210 d in the crystalline solution containing 3.50% (W/V) PEG 8000 (0.08 × 0.07 × 0.07 mm).
Other conditions for crystallization were the same as that in Table 4.

$MgCl_2$ When CrFe protein and MnFe protein were crystallized by the liquid/liquid diffusion method, the time of the crystal formation and the number and size of their crystals significantly depended on the concentration of $MgCl_2$ (Table 5). The suitable concentrations for crystallization of the two proteins were slightly different, and the crystal of CrFe protein was usually slightly larger in size than that of MnFe protein.

NaCl When CrFe protein and MnFe protein were crystallized by the liquid/liquid diffusion method, the concentration of NaCl in the precipitant solutions significantly affected not only the time of the formation of crystal, the number and the size of crystals, but also the crystal shape (length ratio of sides). Under the condition shown in Table 6, the crystals of the two proteins could continue to grow only in the presence of about 1 073 mmol/L NaCl. And the length ratio of the longer two sides of the crystal of the MnFe protein decreased from 1.00 (Fig. 3A) to 0.75 (Fig. 3B) with the increase of NaCl concentration from 1 073 mmol/L to 1 159 mmol/L.

2.2 Optimization of crystallization method

In comparison with the vapor diffusion method, the crystals of MnFe protein obtained with liquid/liquid diffusion method in one week were usually not less in number and not larger in size (Table 7), but this method could facilitate the gradual growth of a few large crystals from the nuclei created at the initial interface (Zhang *et al*, 2002). A few large crystals shown in Fig. 1 to Fig. 3 were obtained with the liquid/liquid diffusion method other than the vapor diffusion method after incubation for a long time.

The crystals of CrFe protein in Fig. 1B and MnFe protein in Fig. 3A are the largest crystals obtained in our laboratory. They are much larger than those obtained from the same batches of the two proteins under similar crystallization conditions by vapor diffusion in either hanging drop method or sitting drop method in the past three years (Huang *et al*, 2001; Zhang *et al*, 2002).

3 Discussion

In our laboratory, several nitrogenase component Ⅰ proteins of *A. vinelandii* and its mutants, such as, CrFe protein (Zhang *et al*, 2002), MnFe protein (Huang *et al*, 2001), △*nifZ*-MoFe protein (Huang *et al*, 2000), *nifB*-MoFe protein, △*nifE*-MoFe protein and △*nifH*-MoFe protein (the last three crystals will be published later), have been successfully crystallized by the vapor diffusion method. However, up to date, the crystal of △*nifZ*-MoFe protein is the largest one of which the longest side length was ≤ 0.16 mm (Huang *et al*, 2000) under a lot of crystallization conditions, including the protein concentration and batch (Huang *et al*, 2000). The crystals shown in Fig. 1B and Fig. 3A obtained by the liquid/liquid diffusion method were significantly larger than those obtained before. This indicated that the liquid/liquid diffusion method could induce the crystallization process of CrFe and MnFe protein to benefit the formation of large crystals. Salemme (1972) pointed

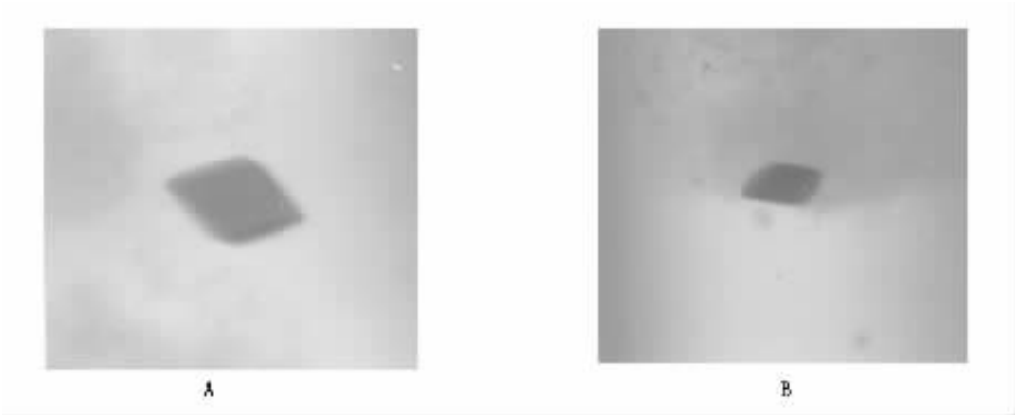

Fig.3. Crystals of MnFe protein observed after incubation for 210 d in the crystallization solution containing NaCl of 1 074 mmol/L (A, 0.18 × 0.18 × 0.05 mm) and 1 159 mmol/L (B, 0.16 × 0.12 × 0.05 mm).
Other conditions for crystallization were the same as those in Table 6.

out that the liquid/liquid method utilizes free diffusion between a protein solution and a 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 in small crystallization tubes, and allowed to diffuse to equilibrium. At oneset 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 (Salemme, 1972). This transient protein supersaturation induces the formation of nuclei which serve 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 nuclei created at the initial interface (Salemme, 1972). The sharp interface is helpful to decrease the diffusion rate, leading to the slow formation of nuclei and growth of a few large crystals. It is not difficult to form such a sharp interface between the solutions by means of decreasing of tube diameter and careful addition of the solutions. Perhaps, it is the reason why all of the large crystals of nitrogenase component Ⅰ proteins were obtained by the crystallization method (Weininger and Mortenson, 1982; Kim and Rees, 1992).

PEG, NaCl and $MgCl_2$, as precipitants for proteins (Mcpherson, 1983; Huang *et al*, 2000; 2001; Zhang *et al*, 2002), could change the electric charge on protein and absorb water molecules from the environment around the protein molecules, resulting in a decrease of protein dissolution (Mcpherson, 1983; Huang *et al*, 2000; 2001; Zhang *et al*, 2002). Therefore, their concentrations have an important effect on the rate and degree of water loss, and the electric charge on protein, resulting in change of time of the nucleus formation, as well as the number, size, shape and quality of crystals. The large crystals appeared only when fewer crystal nuclei formed slowly. The optimum salt concentrations for crystallization and crystal growth of the proteins were affected by the change of concentrations of other chemicals and batches of the protein when crystallization was performed by the liquid/liquid diffusion method. And the most suitable concentration of either PEG or $MgCl_2$ or NaCl for the formation of big crystals of these two proteins was slightly dependent on the crystallization method.

Since protein supersaturation is requisite for the nucleation and subsequent growth of large single crystals and the time for attaining supersaturation is shortened with the increase of the protein concentration, the protein concentration could affect the crystallization. Therefore, the suitable conditions for crystallization could be changed with the change of the protein concentration. In general, there is a suitable concentration range for the crystallization of each protein. In our experiments with different concentrations (6 – 20 mg/mL) of CrFe protein or MnFe protein, the effect of protein concentration on their crystallization was seemly less than that of the protein properties and the crystallization condition.

The CrFe protein and MnFe protein were purified from the same strain which grew on the media containing different metals. Thus, they are different from each other in both metal and subunit composition (Huang *et al*, 2001; 2002). Therefore, it is reasonable to think that the differences could result in the differences in their crystallization conditions, such as, the optimum concentrations of PEG, NaCl and $MgCl_2$.

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(Managing editor: HE Ping)

固氮酶铬铁蛋白和锰铁蛋白大单晶的培养

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摘要： 用液/液扩散法，从分别含 Cr 和 Mn 的无氨培养基中生长的固氮菌(*Azotobacter vinelandii* Lipmann)突变种 UW_3 中纯化出的 CrFe 蛋白和 MnFe 蛋白，在合适的结晶条件下生长出深棕色大单晶(最大晶体的尺寸分别为 0.20 mm × 0.20 mm × 0.07 mm 和 0.18 mm × 0.18 mm × 0.05 mm)。PEG、$MgCl_2$ 和 NaCl 的浓度对这两种蛋白的出晶时间、晶核数目、晶体大小和形状都有明显影响。结果表明，用此结晶法有利于 CrFe 蛋白和 MnFe 蛋白生长出可供 X-射线衍射分析的大单晶。

关键词： 棕色固氮菌突变种 UW_3；固氮酶铬铁蛋白和锰铁蛋白；大单晶生长

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