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Crystalline Growth of Nitrogenase CrFe Protein

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Abstract: Under a suitable condition of crystallization, dark brown rhombohedron crystals (the lengths of the longest two diagonals were 0.25 and 0.12 mm, respectively) could be obtained from nitrogenase CrFe protein purified from a mutant UW_3 of *Azotobacter vinelandii* Lipmann grown in Cr-containing but NH_3-free medium. The possibility of crystallization, as well as the number, size and quality of crystals obviously depended on the concentrations of PEG 8000, $MgCl_2$, NaCl, Tris and Hepes buffer, and methods of crystallization. The optimum concentrations of the chemicals for crystallization of CrFe protein were slightly different from those for crystallization of MnFe protein from UW_3 grown in Mn and Δ*nifZ* MoFe protein from a *nifZ* deleted strain of *A. vinelandii*. The crystal seemed to be formed from CrFe protein.

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

Three genetically distinct nitrogenase systems are composed of two separable proteins called component Ⅰ which contains FeMco and P-cluster and component Ⅱ which contains Fe_4S_4 cluster[1,2]. Different M in cofactors is Mo, V and Fe, respectively. In these systems, hence the three component Ⅰ proteins are called MoFe protein, VFe protein and FeFe protein, respectively. A body of studies show that these proteins are obviously different from one another in substrate-reduction activity and spectroscopic characters. 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 much less active proteins[2]. Nitrogenase CrFe protein or MnFe protein has been purified from UW_3 strain grown on a Mo-free nitrogen-fixation medium containing Cr or Mn, and has been partly characterized[3,4]. The studies on the relationship between structure and function of the two proteins would also help us to understand the mechanism of nitrogen fixation. And Cr, especially Cr (Ⅵ), is an important pollutant inducing distortion and cancer[5]. This strain could concentrate Cr (Ⅵ), leading to harnessing its pollution[4]. The further studies on structure and function of CrFe protein are of great importance in both theory and practice. Elucidation of the mechanism of nitrogen fixation has been greatly advanced by X-ray diffraction analysis of MoFe protein from *Azotobacter vinelandii* in both the reduced state[6] and the oxidized state[7], and of nitrogenase complex of MoFe protein and Fe protein from *A. vinelandii*[8]. Thus, it is necessary to grow big crystals with good quality of CrFe protein for X-ray diffraction analysis. However, the growth of crystal suitable for X-ray diffraction analysis is usually very difficult and become a hindrance for protein crystallographic research[9]. The study on crystalline processing of bio-macromolecules is indeed a specialized scientific technique that requires relative knowledge and experience[9], so, it is often called "art", other than just "science". Nitrogenase proteins are O_2-susceptible bio-macromolecules, which make the growth of their crystals more difficult. Up to date, aside from the big crystals of MoFe protein and Mo-containing nitrogenase complex[6-8], small crystals of MnFe protein[10] and Δ*nifZ*-MoFe protein[11], there has been no report on the growth of big crystals of other nitrogenase component Ⅰ. The present study is undertaken for the attempt to select the optimum conditions for crystalline growth with big size and high quality of nitrogenase CrFe 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*[12] except the addition of 10 μmol/L Na_2CrO_4[4]. The CrFe protein was obtained after chromatography twice on DEAE 52 columns, followed by chromatography on Sephacryl S-300 and Q-Sepharose columns[3,4]. The protein was in 25 mmol/L Tris buffer (pH 7.4) containing 1.7 mmol/L $Na_2S_2O_4$ (DT) and 0.25 or 0.50 mol/L NaCl. Determination of protein concentration, substrate-reducing activity and SDS-PAGE analysis of CrFe protein were performed according to the methods of Huang *et al*[3,4].

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Crystals of CrFe protein were obtained by vapor diffusion at 20 ℃ using the hanging drop method or the sitting drop method[10,11,13]. The volumes of the protein internal and external crystalline solutions added were 3, 3 and 1 000 μL, respectively, for the hanging drop method, and 15, 15 and 2 × 300 μL, respectively, for the sitting drop method. The crystals could also be formed by the liquid diffusion method at 20 ℃ in a small glass tube (about 0.1 cm × 5.0 cm), into which 15 μL of crystalline solution was added, then followed by addition of 15 μL protein solution. The crystalline solutions were composed of the buffer (Tris or Hepes), precipitant (PEG 8000) and salts (NaCl and $MgCl_2$). The crystalline solutions also contained 12.0 mmol/L DT and about 12% (V/V) glycerin which was used as a stabilizer of proteins. The used solutions were rigorously degassed and filled with Ar. All the operation was performed under an atmosphere of Ar.

2 Results and Discussion

The crystal growth of protein is indeed a complex physical and chemical process[9]. In order to obtain big crystals of high quality, it is necessary to optimize the factors affecting crystallization, such as the temperature, purity and concentration of the protein, the kind and concentration of precipitants, salts, stabilizer and buffer in the crystalline solutions, pH of the solution, the method for crystallization and technical bias, etc[9-11].

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

2.1.1 Buffer It was shown earlier that the optimum pH value was 8.1 – 8.4 for MnFe protein[10] and *ΔnifZ* MoFe protein[11]. As shown in Tables 1, 2, the crystalline process of CrFe protein is significantly affected by the kind and concentration of buffer in the indicated pH range of 8.1 – 8.4. CrFe protein could be crystallized either in Tris or Hepes buffer, but forming different sizes and numbers of crystals in different buffer concentrations. The crystals were less in number, bigger in size and better in quality when the concentrations of Tris and Hepes were 61, 81 and 61 mmol/L, respectively.

The buffer could not only stabilize the pH value of the protein solution, but also change the electric charge on protein and protein conformation[9-11], leading to different results of crystallization.

2.1.2 PEG 8000 Like the MnFe protein and *ΔnifZ* MoFe protein[10,11], 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. There was the most suitable range of PEG concentration for the crystallization of the protein. The crystals of the protein could not be formed or only a lot of smaller crystals were formed when the PEG concentration was out of the range of 2.20% – 3.00% (Tables 1 – 3). A lot of results showed that the optimum concentration of PEG for crystallization of the protein was slightly higher than that for *ΔnifZ* MoFe protein[11], even though the optimum concentrations shown in Table 3 were not the same when the batches and concentrations of the proteins were different. The bigger crystals shown in Fig. 1A are usually formed when the PEG concentration is 2.20% – 2.40% in presence of 300 mmol/L $MgCl_2$.

2.1.3 $MgCl_2$ The concentration of $MgCl_2$ significantly affected crystallization of CrFe protein, and the number and size of crystals (Tables 4, 7). Two big, one middle and one small crystals appeared simultaneously in a drop of crystalline solution when the concentration of $MgCl_2$ was 149.77 mmol/L (Fig. 1A), and a great number of small-sized crystals and twin crystals appeared when the concentration of $MgCl_2$ was greatly increased. The most suitable concentration for the crystallization of CrFe protein was about 250 mmol/L in Tris buffer, which was slightly

Table 1 Effect of Tris concentration on crystallization of CrFe protein by vapor diffusion in the hanging drop method

PEG[1)] (%)	Concentration of Tris buffer (mmol/L)					
	41		61		81	
	Crystal		Crystal		Crystal	
	Number	Size	Number	Size	Number	Size
2.25	≥100	Smaller	≥40	Middle	20 – 25[2)]	Middle
2.50	≥40	Middle/small	≥40	Middle/small	≥40	Middle/small

1) Final concentrations of protein, NaCl and $MgCl_2$ were 8.10 mg/mL, 204.85 mmol/L and 299.53 mmol/L, respectively; 2) Crystal numbers in two drops of solution under the same condition.

Table 2 Effect of Hepes concentration on crystallization of CrFe protein by vapor diffusion in the hanging drop method

PEG[1)] (%)	Final concentration of Hepes buffer (mmol/L)							
	51		61		71		81	
	Crystal		Crystal		Crystal		Crystal	
	Number	Size	Number	Size	Number	Size	Number	Size
2.22	0	–	9 – 27[2)]	Middle	30 – 40[2)]	Middle[3)]	≥40	Smaller[3)]
2.38	20 – 30[2)]	Middle[3)]	13 – 20[2)]	Middle	≥60	Middle[3)]	≥40	Smaller[3)]

1) Final concentrations of protein, Tris, NaCl and $MgCl_2$ were 4.87 mg/mL, 12.50 mmol/L, 245.07 mmol/L and 299.48 mmol/L, respectively; 2) The same as in Table 1; 3) Bad crystal in the quality.

lower than that for *ΔnifZ* MoFe protein[11]. Although the optimum concentration for crystallization of CrFe protein was similar to that for MnFe protein, the range of the concentration for crystallization of CrFe protein was larger than that for MnFe protein[10].

Table 3 Effect of PEG 8000 concentration on crystallization of CrFe protein from different batches by vapor diffusion in the hanging drop method

PEG (%)	Crystal			
	Exp. 1[1)]		Exp. 2[2)]	
	Number	Size	Number	Size
1.86	0	–		
1.89			3	Smaller
2.00	≥10	Small		
2.11			≥100	Smaller
2.22			≥60	Smaller
2.26	4	Middle/small	7	Middle/small
2.38			3	Middle/small
2.50			22	Middle/small
2.75			≥40	Middle/small
3.00	9	Big/middle		
3.50	≥100	Smaller		
4.00	≥100	Smaller		

1) Final concentrations of protein, Tris, NaCl and $MgCl_2$ were 8.10 mg/mL, 52.96 mmol/L, 204.85 mmol/L and 299.53 mmol/L, respectively; 2) Final concentrations of protein, Tris, Hepes, NaCl and $MgCl_2$ were 4.87 mg/mL, 12.50 mmol/L, 40.68 mmol/L, 245.54 mmol/L and 299.48 mmol/L, respectively.

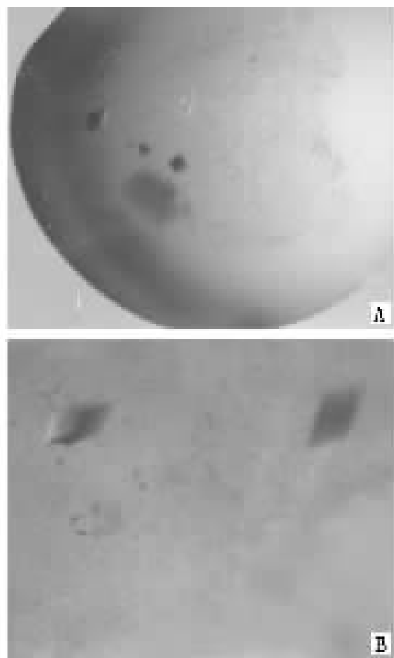

Fig.1. Crystals of CrFe protein (16.20 mg/mL) formed by (A) vapor diffusion in the hanging drop method (×40) and (B) vapor diffusion in the sitting drop method (×200). Final concentration of PEG 8000, $MgCl_2$, NaCl and Tris were 2.22%, 149.77 mmol/L, 198.07 mmol/L, 52.96 mmol/L in A and 2.00%, 202.03 mmol/L, 298.39 mmol/L, 52.96 mmol/L in B, respectively.

Table 4 Effect of $MgCl_2$ concentration on crystallization of CrFe protein by vapor diffusion in the hanging drop method

$MgCl_2$[1)]	Crystal			
	Exp. 1[2)]		Exp. 2[3)]	
	Number	Size	Number	Size
144.91	≥40	Small	≥100	Middle/small
256.60	10	Big/middle	15	Middle/small
297.22	19	Middle/small	15	Middle/small

1) Final concentrations of protein, Tris and NaCl were 8.10 mg/mL, 53.13 mmol/L and 204.87 mmol/L, respectively; 2) and 3) Final concentrations of PEG 8000 were 2.00% and 2.22%, respectively.

2.1.4 NaCl Under the condition shown in Table 5, the concentration of NaCl, at which the crystal nuclei was less, was about 200 mmol/L. Unlike *ΔnifZ* MoFe protein[11], the concentration of NaCl did not significantly affect the number of crystals (Table 6).

Table 5 Effect of NaCl concentration on crystallization of CrFe protein by vapor diffusion in the hanging drop method

NaCl[1)]	Crystal			
	Drop 1[2)]		Drop 2[2)]	
	Number	Size	Number	Size
130.00	21	Middle/small	23	Middle/small
204.87	3	Middle/small	15	Middle/small
252.51	17	Middle/small	25	Middle/small
320.57	≥40	Middle/small	≥40	Middle/small

1) Final concentrations of protein, PEG 8000, Tris and $MgCl_2$ were 8.10 mg/mL, 2.22%, 53.13 mmol/L and 297.23 mmol/L, respectively; 2) The same as in Table 1.

Table 6 Comparison of the effect on crystallization of CrFe protein between the sitting drop method and hanging drop method

NaCl[1)]	Crystal			
	Sitting drop method		Hanging drop method	
	Number	Size	Number	Size
125.00	7	Big/middle	≥40	Middle/small
202.02	11	Big/middle	≥40	Middle/small

1) Final concentrations of protein, PEG 8000, Tris and $MgCl_2$ were 8.10 mg/mL, 1.86%, 53.13 mmol/L and 298.48 mmol/L, respectively.

PEG, NaCl and $MgCl_2$, as precipitants for proteins[9] could not only change the electric charge on protein, but also adsorb water from the environment around the protein molecules, resulting in decreasing protein dissolution[9–11]. Therefore, their concentrations have an important effect on the rate and degree of water loss, and the electric charge on protein, resulting in a change of the rate of the nucleus formation, and the number, size and quality of crystals. In general, the nuclear number is increased when the rate of water loss is too high, leading to the appearance of a great number of small-sized and poor crystals, but the crystals can hardly form and grow when the degree of water loss is too low[11]. In our study, the optimum salt concentrations for the crystallization and crystal growth of the protein were influenced by the change of concentrations of other chemicals and batches of the protein.

2.2 Effect of crystalline methods on the growth of crystals of CrFe protein

Unlike MnFe protein and *ΔnifZ* MoFe protein[10,11], the crystals of CrFe protein formed by vapor diffusion in the sitting drop method were less in number and bigger in size than those by vapor diffusion in the hanging drop method (Table 6). Under the same conditions of the protein and the crystalline solutions, ten medium-sized crystals and a great number of small crystals appeared in two drops of solution of the hanging drop, respectively, and ten big and medium-sized crystals appeared in a cell of the sitting drop, the biggest one of which (the lengths of the longest two diagonals were 0.25 and 0.12 mm, respectively) is shown in Fig.1B. Although the sample volume in each cell with the sitting drop method was 5-fold of that in each hanging drop, their nucleus numbers were independent on the volume. In fact, although the two methods for crystallization are of a type of vapor diffusion, the route of diffusion and the ratio of solution volume in the internal cell or drop to that in the external cell were different from each other. These differences could affect the rate of water loss from the environment around protein, leading to the change of crystallization process[10].

The crystals of CrFe protein could also be formed by using liquid/liquid diffusion method (Table 7), and both the shape and colour of the crystals were similar to those formed by using vapor diffusion method.

The above crystals were dark brown in colour, which might be due to that the protein contained a large amount of iron[4].

Table 7 Comparison of the effect on crystallization of CrFe protein between the vapor diffusion method and the liquid/liquid diffusion method

$MgCl_2$ 1)	Crystal			
	Hanging drop method		Liquid/liquid diffusion method	
	Number	Size	Number	Size
138.00	0	–	0	–
199.65	≥100	Smaller	0	–
249.56	≥100	Smaller	0	–
299.48	≥100	Small/smaller	≥100	Smaller
349.39	5 – 20 2)	Middle/small	≥60	Smaller

1) Final concentrations of protein, PEG 8000, Tris, Hepes and NaCl were 4.87 mg/mL, 2.22%, 12.50 mmol/L, 30.52 mmol/L and 245.54 mmol/L, respectively; 2) The same as in Table 1.

3 Conclusion

The above results showed that each condition for optimal crystal growth of protein was affected not only by the change of the concentration of other chemicals and the protein batches, but also by the change of the method for crystallization[9-11]. And the number, size and quality of its crystals could be roughly controlled by these changes. It is, therefore, possible to obtain the big crystals of high quality by means of further selecting the optimum condition for crystallization of CrFe protein.

The CrFe proteins of the three batches used in this study are the same as those described earlier[4]. One of them was shown to be basically homogeneous from SDS-PAGE analysis[4]. The crystals formed from the three proteins were similar in shape and colour. And the characteristics of crystallization of CrFe protein was similar to, but not exactly, that of MnFe protein and *ΔnifZ* MoFe protein[10,11], indicating that the crystals could be of CrFe protein, although the final identification could only be made after X-ray diffraction analysis.

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固氮酶铬铁蛋白的晶体生长

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摘要: 在合适的结晶条件下,从含 Cr 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 蛋白可从溶液中析出深棕色斜四棱柱晶体,晶体最大的两条对角线长度分别可达 0.25 mm 和 0.12 mm。PEG 8000、$MgCl_2$、NaCl、Tris 和 Hepes 缓冲液的浓度及结晶方法等对该蛋白的出晶率、晶核数目、晶体大小和质量都有明显影响。CrFe 蛋白结晶所需的上述化合物的最适浓度与在 Mn 中生长的固氮菌突变种 UW_3 的 MnFe 蛋白和缺失 *nifZ* 固氮菌突变种的Δ*nifZ* MoFe 蛋白结晶所需的最适浓度有所不同。结果表明,该蛋白晶体可能为 CrFe 蛋白的晶体。

关键词: 固氮菌突变种 UW_3;含铬培养基;固氮酶铬铁蛋白;晶体生长

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