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缺失 nifZ 的棕色固氮菌突变种钼铁蛋白的晶体生长研究

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摘要: 在一定的结晶条件下 ,缺失 *nifZ* 的棕色固氮菌 (*Azotobacter vinelandii* Lipmann) 突变种钼铁蛋白将从溶液中结晶出深棕色的短斜四棱柱晶体。PEG 8000、$MgCl_2$、NaCl、Tris 的浓度及缓冲液的 pH 对该蛋白的结晶及晶体生长影响的系统研究表明 ,浓度和 pH 较低时 ,不出晶体 ; 随着浓度逐渐提高及 pH 高于 8.0 ,在一周内出现大量小晶体 ;再提高浓度 ,便延长结晶时间但出质好、体积大而数少的晶体 ;然后晶体随浓度的提高而又变小、变多、甚至晶质变差 ,直至不再出晶体。影响晶体生长的各因子的最适浓度随其他条件的改变而有所不同。当缓冲液的 pH 为 8.2 而 PEG 8000、$MgCl_2$、NaCl、Tris 和蛋白质的浓度分别为 1.86 %、300 mmol/L、400 mmol/L、53 mmol/L 和 4.64 g/L ,在一滴悬滴液中只有一颗较大的优质晶体 (最大两边线均为 0.16 mm) 。

关键词: 棕色固氮菌 ; 缺失 *nifZ* 的突变种 ; MoFe 蛋白 ; 晶体生长
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Studies on Crystalline Growth of MoFe Protein from a nifZ Deleted Strain of Azotobacter vinelandii

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Abstract : Under a given condition of crystallization , dark brown short rhombohedron crystals could be obtained from Δ*nifZ* MoFe protein purified from a *nifZ* deleted mutant strain of *Azotobacter vinelandii* Lipmann. Systematic studies on the effect of concentrations of PEG 8000 , $MgCl_2$, NaCl , Tris and buffer pH on the crystallization and crystal growth of the protein showed that the protein could not be crystallized in lower concentrations of the chemicals and lower buffer pH. A large amount of smaller crystals of the protein appeared in a week with gradual increasing in the chemical concentrations and pH ≥8.0. When the chemical concentrations were further increased , the time for crystallization was increased and a few high grade crystals of larger size were formed. If the concentrations of the chemicals were continuously increased , many crystals with smaller size , and , sometimes of poor quality appeared again and eventually ceased to produce any crystals. The optimal concentration for each of the above mentioned chemicals varies with other variable factors. Only one bigger crystal (both of the longest two sides : 0.16 mm) could be obtained in a hanging drop of protein sample when the concentrations of PEG 8000 , $MgCl_2$, NaCl , Tris and protein were kept at 1.86 % , 300 mmol/L , 400 mmol/L , 53 mmol/L and 4.64 g/L , respectively , with Tris buffer pH 8.2.

Key words : *Azotobacter vinelandii* ; *nifZ* deletion mutant ; MoFe protein ; crystalline growth

FeMoco and P-cluster in molybdenum-iron (MoFe) protein of nitrogenase plays a very important role in the structure and function of the protein[1]. The biosynthesis of metalloclusters in nitrogen-fixation bacteria *in vivo* are controlled by many *nif* genes[2]. Thus , deletion of the relative genes in the bacteria would lead to synthesize new MoFe proteins , in which the metalloclusters are either deficient or changed in structure. Earlier studies showed the P-cluster in the Δ*nifZ* MoFe protein from *nifZ* deleted mutant strain of *Azotobacter vinelandii* was obviously changed , but not the FeMoco , indicating that the lower activity and instability of the protein in structure were

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mainly related to the changes in P-cluster[3-5]. Up to date, the studies on the relationship between structure and function of the protein has been a subject of importance and interest for understanding the mechanism of nitrogen fixation of nitrogenase. It is necessary to perform X-ray diffraction analysis of the protein crystal for better access of this relationship. However, the protein crystals obtained were not big enough[4,5] and the growth of crystal suitable for X-ray diffraction was usually very difficult; Thence, it has become a main hindrances for proteins crystallography[6]. Therefore, it is urgent and important to screen the most suitable conditions for growth of single larger crystal of high quality. The present study is undertaken for the attempt to select the most suitable condition for crystalline growth of the Δ*nifZ* MoFe protein which is not only susceptible to O_2, but also has a drawback in structure.

1 Materials and Methods

Growth of the *nifZ* deleted mutant strain of *Azotobacter vinelandii*, purification and determination of the nitrogenase were carried out according to the method of Zhong *et al*[3] and Wang *et al*[4].

Determination of protein concentration, activity and metal content of the Δ*nifZ* MoFe protein were carried out, respectively, according to the method described previously[4,5].

Experimentation on crystalline growth of the Δ*nifZ* MoFe protein was performed according to the methods of Wang *et al*[4], Huang *et al*[5] and Drenth *et al*[7]. Unless described elsewhere, different crystalline solutions were prepared by addition of a given volume of protein precipitants and salts. PEG 8000, $MgCl_2$ and NaCl were dissolved in 83 mmol/L Tris buffer (about pH 8.2) containing 11% glycerin. By using the hanging drop method, the volumes of the protein, internal and external crystalline solutions added were 3, 3 and 1 000 μL, respectively. The samples should stand for 5-7 days or longer at 20 ℃ or 4 ℃. All of the solutions used were rigorously degassed and filled with Ar, and contained 2.1 g/L $Na_2S_2O_4$. All operations were done under atmosphere of Ar.

2 Results and Discussion

The Δ*nifZ* MoFe protein being homogeneous as determined by Coomassie staining of SDS gels here reported was the same as that reported in earlier paper[5]. From judgement of its substrate-reduction activity, circular dichroism (CD) spectrum, metals content, FeMoco extraction and PAGE etc., it was shown that the structure of P-cluster in the protein was changed[4,5]. Many experiments of crystal growth have evidenced the crystalline solution composed of PEG 8000, $MgCl_2$ and NaCl was the best one among tree crystalline solutions described earlier[4] in terms of quality, size of the crystal and probability.

2.1 Relations of the crystalline growth of Δnif Z MoFe protein to Tris concentration and its pH

2.1.1 Tris buffer pH As reported earlier[4], the pH of the buffer used for crystallization of the Δ*nifZ* MoFe protein was higher than 8.0, which is the same as that of OP MoFe protein[1], but different from that for the needle micro crystals of the OP MoFe protein which was pH 7.4[8]. When the Tris concentration was increased and NaCl was diluted to about 40 mmol/L[8], the Δ*nifZ* MoFe protein was difficult, if not at all, to be crystallized at pH ≤8.0. Even if the solution was originally buffered at pH between 8.1 and 8.2, there still existed a gradual reduction of the frequency and quality of crystallization after a period of time at a temperature of 4 ℃ as the pH decreased to 7.9. However, both the probability and quality of crystallization were restored by readjusting the same buffer pH to the optimal pH of 8.2. The size, number and quality of crystals were critically related to the pH value. Although the optimal pH was about 8.2, yet the Δ*nifZ* MoFe protein could be crystallized in the solutions with pH from 8.1 to 9.0 (Table 1).

Table 1 Effect of pH on crystalline growth of the Δ*nifZ* MoFe protein

pH of buffer[1)]	Crystal		
	Size	Number	Quality
9.0	Small	L. A[2)]	Worse
8.7	Small	Many	Poor
8.5	Big	10-20	Good
8.3	Big	10-20	Good
8.2	Bigger	6-13	Good
8.1	Big	10-20	Good

1) The final concentrations of Tris, NaCl, $MgCl_2$, glycerin, PEG 8000 and protein were 52.6, 322.6 and 315.3 mmol/L, 5.55%, 1.86% and 4.64 g/L, respectively. 2) Large amount.

2.1.2 Tris concentration in the buffer Like the MoFe protein from *Azotobacter vinelandii* (OP)[1], the Δ*nifZ* MoFe protein, could be crystallized when the Tris concentration was 53 mmol/L (pH ≥8.0) under the condition of suitable concentration of precipitant and salts. In order to obtain the best condition for the crystalline growth of the protein and to enhance the stability of pH in the system, an attempt was made to increase the Tris concentration. Unfortunately, result showed that the crystalline nuclei of the protein increased and the size of the crystal

decreased as the Tris concentration was increased (Table 2). It should be stressed that the effect of the Tris concentration on the crystallization was significant although the crystallization process in the two drops were not exactly the same because of the slight difference in operation.

Table 2 Effect of Tris concentration on crystalline growth of the Δ*nif Z* MoFe protein

Tris concentration (mmol/L)	Crystal			
	Drop 1[1)]		Drop 2[1)]	
	Size	Number	Size	Number
53.5	Small	1	Bigger	3
73.2	Small	23	Small	25
108.1	Smallest	L.A[2)]	Smallest	Lot of

1) The final concentration of NaCl, $MgCl_2$ and glycerin were 320.4, 152.7 mmol/L and 5.20 %, respectively, buffer pH 8.3, the others were the same as those in Table 1; 2) L.A, large amount.

Table 3 Effect of concentration of PEG 8000 on crystalline growth of the Δ*nif Z* MoFe protein

Final concentration[1)] (%)	1.60	1.70	1.85	1.91	2.00	2.20	2.40	2.61	2.81
Crystal size	/	Small	Bigger	Small	Higher		Smaller		
Crystal number	No[2)]	Many	Some	Many	Higher		More		

1) The final concentrations of Tris, NaCl, $MgCl_2$, glycerin and protein were 53.5, 320.3, 145.6 mmol/L, 4.6 % and 4.64 g/L, respectively; 2) No crystal.

2.2 Relationship between the crystallization of the Δnif Z protein and the concentration of PEG 8000

As shown in Table 3, the concentration of PEG had an important effect on both the possibility of crystallization and the crystal size of the protein. Under the same concentration of $MgCl_2$, NaCl, glycerin, protein, Tris and pH of the buffer, the protein could not be crystallized when the PEG concentration was ≤1.60 %, but then a few bigger crystals appeared when the PEG concentration was ≥1.70 %. However, further increase of PEG concentration to ≥1.90 % could gradually increase the crystal number and decrease the size. It was indicated that around 1.85 % was the optimal PEG concentration for the formation of a few bigger crystals. Although the results were slightly different from the previous study[4] that the lowest and highest PEG concentration caused the appearance of larger crystals, yet in the present study the most suitable range of PEG concentration for the formation of the biggest crystals was between 1.8 % - 1.9 %.

PEG is a precipitant of protein and could absorb water from the environment around the protein molecules, resulting in decreasing protein dissolution. Crystal nuclei could not be formed in the PEG solution below a critical concentration, whereas only the largest crystals were formed at the optimal PEG concentration, in which the formation of small number of the crystal nuclei was very slow. So that, it could provide the nuclei with their possibility to grow to a few large crystals. On the contrary, when the PEG concentration was too high, the formation of the crystal nuclei was accelerated, leading to the appearance of large number of small-sized crystals. With further increase of PEG concentration cessation of crystallization occurred consequent upon protein precipitation prior to the formation of crystal nuclei.

2.3 Salt concentration related to the crystallization of the Δnif Z MoFe protein

2.3.1 $MgCl_2$ The concentration of $MgCl_2$ could significantly affect the crystallization process of the Δ*nif Z* MoFe protein (Table 4). The protein was not crystallized in too low or too high concentrations of $MgCl_2$. Although the protein could be crystallized in a given range of its concentration (75 - 350 mmol/L), yet the number, size and quality of the crystals obtained were closely concentration dependent, that is: when the concentration was lower, the crystals were large in number, smaller in size and poorer in quality; when the concentration was somewhat increased, the crystals were in small number, bigger in size and better in quality, a few big and high grade crystals were observed at concentration of 350 mmol/L and the protein precipitation was also less (Fig. 1). When the concentration was further increased large number and smaller size of the crystals again occurred and finally no crystal could be observed when the concentration reached 400 mmol/L or higher.

2.3.2 NaCl The same as $MgCl_2$, the concentration of NaCl could obviously affect the crystalline process of the protein when the concentration of $MgCl_2$ was 145.0 or 300.1 mmol/L and the other requirements for the crystallization were the same (Table 5). When the concentration of NaCl was lower, the crystals were in large number and smaller size. Then, with the increase in the concentration of NaCl, the number of crystals was decreased, their size increased and their quality improved. If it was higher, not only the crystals were in small number, bigger size and better quality, but also the amount of protein precipitate was decreased. But the time for crystalline formation and growth were prolonged (Fig. 2). In one hanging drop there was only one crystal which was the biggest one (Fig. 2, B). If the NaCl concentration was again further increased, then, the opposite situation appeared with the crystals being in large number and small size again, and finally no crystallization when the concentration if reached

Table 4 Effect of concentration of $MgCl_2$ on crystalline growth of the $\Delta nifZ$ MoFe protein

Exp. 1 (322.8 mmol/L[1])				Exp. 2 (320.2 mmol/L[1])			
Final concentration (mmol/L)[2]	Crystal			Final concentration (mmol/L)[2]	Crystal		
	Size	Number	Quality		Size	Number	Quality
25.2	No[3]			200.5	Smallest	10	Poor
50.4	No[3]			249.6	Small	10 - 20	Good
75.6	Small	7 - 8	Poor	300.1	Bigger	10 - 20	Good
100.9	Bigger	20 - 30	Poor	325.3	Bigger	2 - 7	Good
126.1	Bigger	20 - 30	Poor	350.5	Bigger	2	Good
				399.7	No[3]		

1) The final concentration of NaCl; 2) The final concentration of Tris, glycerin, PEG 8000 and protein were 53.4 mmol/L, 4.82 %, 1.85 % and 4.64 g/L respectively; 3) See Table 3.

Table 5 Effect of NaCl concentration on crystalline growth of the $\Delta nifZ$ MoFe protein

Final concentration of NaCl[1] (mmol/L)	Crystal					
	Exp. 1 (145.0 mmol/L[2])			Exp. 2 (300.1 mmol/L[2])		
	Size	Number	Quality	Size	Number	Quality
250.0	Smaller	L.A[4]	Good	Small	Some	Poor
300.3	Small	Some	Good	Big	10	Poor
350.3	Small	Some	Good	Bigger	4	Good
400.5	Smallest	Lot of	Good	Bigger	1[5]	Good
450.7	Smallest	Lot of	Good	Big	Some[6]	Good
500.9	No[3]			No[3]		

1) The final concentration of Tris, glycerin, PEG 8000 and protein were the same as those listed indicated in Table 2; 2) The final concentration of $MgCl_2$; 3) See Table 3; 4) See Table 1; The crystal not observed in the first week, then, only one crystal was observed in 5) and some crystals in 6) 3 months later.

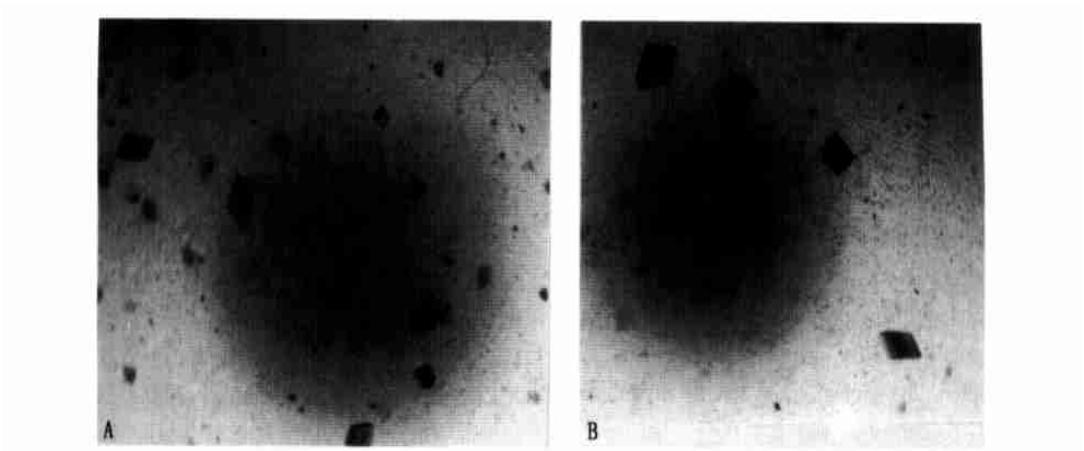

Fig. 1. The crystals of Δnif MoFe protein obtained by using 300.1 (A) and 349.2 (B) mmol/L $MgCl_2$ ($\times 150$)*.
Besides NaCl (400.5 mmol/L), other crystallization conditions were described in Table 2. Crystals were observed 1 week later.

500 mmol/L.

It was shown from above that, in the presence of two different $MgCl_2$ concentrations, the regulation of NaCl affecting the crystalline growth possesses was similar to that of $MgCl_2$. In other words, the highest concentrations (450 - 500 mmol/L) of NaCl that block the formation appearance of the crystals was similar to each other in the two conditions of $MgCl_2$. When a higher $MgCl_2$ concentration was needed for the formation of larger crystals, the higher concentration of NaCl (400 mmol/L) was also required. This indicates that the two salts as well as PEG acted in coordination with each other in the crystallization of the protein. By the same token, when the concentration of NaCl and $MgCl_2$ was decreased to 320 mmol/L and 350 mmol/L, respectively, the bigger crystals of the protein could also be produced.

Crystallization and crystal growth of a protein is indeed a complex physical and chemical process. The gradual formation of crystals is the result of an absorption of the water molecules around the protein through a slow diffusion from the protein solution as the inner pool to the outer pool of the salt solution (the reservoir) due to a concentration gradient in which the concentration is 2 folds higher in the reservoir. In general, the slower diffusion allows more possibility for the formation of smaller crystal nuclei and larger crystals.

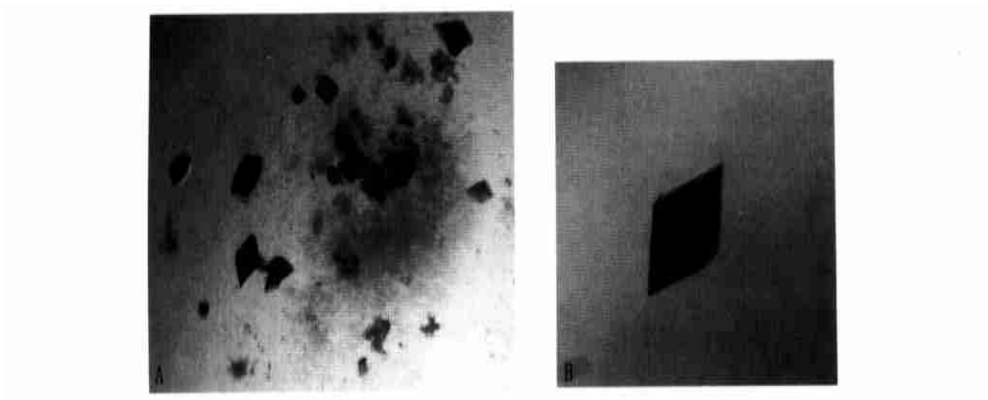

Fig. 2. The crystals of Δ*nif* MoFe protein obtained by using 300.3 mmol/L (A) and 400.5 mmol/L NaCl * (B).
Other crystallization conditions were described in Table 5 (Exp.2), pictures were made 3 months later. A. Crystals could be observed 1 week Later (×150); B. Crystal did not emerge 1 week later, but only one crystal (×320, both of longer sizes: 0.16 mm) in one drop consisted of 3 μL precipitant/salts and 3 μL protein was observed 3 months later (see note of 5) in Table 5).

Factors affecting the crystallization process, other than those mentioned above are temperature, protein concentration and stabilizer, technical bias, etc. The crystals of the Δ*nifZ* MoFe protein grown at 4 ℃ were no better than those at 20 ℃. The effect of the protein concentration on the crystal growth was significant, but it seemed not to be obvious if it was used with the range of its concentration in this experiment. But the addition of suitable glycerin was favorable to the stability of the protein, the decrease in precipitation and the large crystal growth. The technical error could also make the formation of crystals different both in number and size between the two drops of the protein solution even under the same conditions (Table 1, 2). Two technical biases existed: (1) the error of the very small sample volume which led to the significant change in the concentration. (2) In the hanging drop method, the addition of the tiny sample on the cover slide made it impossible to let the solution be differently mixed. Therefore, it is necessary to further optimize the requirements above in order to get the large crystals with better quality.

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