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Crystallization of Nitrogenase MoFe Protein from a Mutant *nif*E Deleted Strain of *Azotobacter vinelandii*

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Abstract: Under a suitable condition of crystallization, dark brown short rhombohedron crystals could be obtained from FeMoco-deficient MoFe protein (Δ*nif*E Av1) purified from a *nif*E deleted mutant DJ35 of *Azotobacter vinelandii* Lipmann grown in NH_3-limited medium. The number, size and quality of crystals were significantly affected by either the concentration of precipitants and buffer or diffusion method. The longest sides of the largest crystal of Δ*nif*E Av1 protein, which was obtained by vapor diffusion in the hanging drop method, were 0.12 and 0.13 mm, respectively.

Key words: mutant DJ35 of *Azotobacter vinelandii*; nitrogenase Δ*nif*E Av1; crystallization

Nitrogenase is a two-component metalloenzyme that catalyzes the MgATP-dependent reduction of N_2. For the Mo-dependent nitrogenase of *Azotobacter vinelandii*, Fe protein (Av2, *Nif*H) is a 60 000 molecular weight dimer of identical γ subunits connected through a single (4Fe-4S) cluster. It serves as an electron donor for the MoFe protein (Av1) in a reaction somehow coupled to MgATP hydrolysis. Av1 is often viewed as being composed of two identical halves that do not communicate with each other. Each half has one α subunit (*Nif*D) and β subunit (*Nif*K), one ($MoFe_7S_9$ homocitrate) cluster designated FeMoco, one (8Fe-7S) cluster designated P-cluster, and one binding site for Av2. FeMoco is connected to the protein by only two ligands, a Cys and a His, located within the α subunit. The P-clusters appear to be involved in electron transfer from Av2 to FeMoco which serves as the site of substrate binding and reduction (Burgess *et al*, 2001; Kim *et al*, 2001). Biosynthesis of both Av1 and Av2 is a complex process, requiring at least 15 different gene products. Six of them have been strongly implicated in the *in vivo* synthesis of FeMoco: *nif*N, *nif*E, *nif*B, *nif*H, *nif*Q and *nif*V (Roll *et al*, 1995). If any of the genes is deleted or mutated, Av1 synthesized *in vivo* is one of P-cluster-containing, FeMoco-deficient form (Apo-Av1) of Av1. FeMoco has been isolated and used to activate the Apo-Av1. For example, both *nif*B$^-$ Av1 from UW45 (a *nif*B point-mutation strain) and Δ*nif*E Av1 from DJ35 (a *nif*E deleted mutant) are a FeMoco-deficient Av1 and are able to be activated by FeMoco isolated from Av1 of wild-type *A. vinelandii* (Shah *et al*, 1977; Paustian *et al*, 1989; Tal *et al*, 1991; Zhao *et al*, 2003). The crystalline structure shows that the FeMoco occupies a cavity formed between domains αⅠ, αⅡ, and αⅢ, which is empty in Δ*nif*B Av1 (Schmid *et al*, 2002). Only minor differences exist in the β subunit and domains αⅠ and αⅡ, whereas domain αⅢ undergoes major structural rearrangements. A funnel formed in the α subunit, which is revealed to be predominantly positively charged, is of sufficient size to accommodate insertion of the negatively charged cofactor. The results are important for elucidating the mechanism of insertion by FeMoco and its chemical models. The Apo-Av1 from different gene-deletion mutants are somewhat different in both the structure and the characteristic (Tal *et al*, 1991). Hence, comparison of their crystalline structures is also important for understanding the mechanism of nitrogen fixation. This present study is undertaken for the attempt to select the optimum conditions for crystallization of nitrogenase Δ*nif*E Av1.

1 Materials and Methods

*nif*E-deletion mutant strain DJ35 of *Azotobacter vinelandii* Lipmann was kindly provided by Prof. Burgess in Department of Molecular Biology and Biochemistry, University of California. The construction and growth of DJ35 were reported by Brigle *et al* (1987).

Purification, determination of activity and protein concentration, and SDS-PAGE analysis of Δ*nif*E Av1 from DJ35 were carried out according to the method of Zhao *et al* (2003).

Crystallization of Δ*nif*E Av1 by vapor diffusion in the hanging drop method was carried out by the method of Mcpherson *et al* (1983) and Dong *et al* (2001). Its crystallization by the liquid/liquid diffusion method was performed by the method of Zhang *et al* (2002). Fifteen μL of crystalline solution and the same volume of protein solution were added into a small glass tube (about 0.1 cm × 5.0 cm). Unless described elsewhere, all prepared

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samples for crystallization stood for 7 d at about 20 ℃.

2 Results and Discussion

Crystallization of protein is affected by many factors, such as temperature, purity and concentration of the protein, kind and concentration of precipitants, stabilizer and buffer in precipitant solutions, pH of the solution, and method for crystallization (Mcpherson *et al*, 1983). In order to obtain big crystals, the main factors should be optimized.

2.1 Effect of precipitant solution on the crystallization

2.1.1 Hepes concentration Hepes or Tris is one kind of salts, but its basic role is 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 (Mcpherson *et al*, 1983). Hepes concentration could affect the number, size and quality of crystals formed (Table 1). With the increase of Hepes concentration under the condition in Table 1, the crystal number increased and the crystal size decreased. A few big crystals were usually formed at 61 mmol/L Hepes.

2.1.2 PEG 8000 concentration As shown in Table 2, the concentration of PEG 8000 had an important effect on the number, size and quality of crystals. Like nitrogenase CrFe protein and MnFe protein (Huang *et al*, 2001; Zhang *et al*, 2002), there was the optimal range of PEG concentration for the crystallization of Δ*nif*E Av1. The crystals of the protein could not form or only a large number of small-sized formed when the PEG concentration was out of the range. At the condition in Tables 1 and 2, the optimal PEG concentration for the crystallization of Δ*nif*E Av1 is about 5.50%.

PEG could absorb water from the environment around protein molecules, resulting in decreasing protein dissolution, and could obviously decrease the dielectric constant of the medium, resulting in decreasing an effectively electrostatic shield between protein molecules (Mcpherson *et al*, 1983). Only a few crystal nuclei were formed and subsequently grown to large crystals with good quality when the protein lost water at a suitable rate (Fig. 1). However, when the PEG concentration was too low (≤4.0%), the crystal nuclei were not able to form; when the PEG concentration was too high, the formation

Table 1 Effect of Hepes concentration on crystallization of △*nif*E Av1 by vapor diffusion in the hanging drop method

Precipitant[1]		Crystals			
PEG (%)	Hepes (mmol/L)	Drop 1[2]		Drop 2[2]	
		Number	Size	Number	Size
5.00	50.69	0	/	0	/
	61.01	1	Big	0	/
	71.18	2	Small[3]	0	/
	81.35	2/2	Middle/small	12	Small
5.50	50.69	2	Big	1	Big
	61.01	3	Biggest	3/3	Big/twin
	71.18	> 40	Small	> 40	Small
	81.35	12	Small	29	Small

1), the concentration of glycerin, NaCl and $MgCl_2$ in the precipitant solution (pH 8.2 – 8.3) are 11.57% (V/V), 490.12 mmol/L and 598.95 mmol/L, respectively, and the concentration of protein in 25 mmol/L Tris buffer (pH 7.4) containing 250 mmol/L NaCl is 11.48 mg/mL; 2), crystals form in two drops of solution under the same condition after incubation for 7 d at 20 ℃; 3), crystals of bad quality.

Table 2 Effect of PEG 8000 concentration on crystallization of Δ*nif*E Av1 by vapor diffusion in the hanging drop method

Precipitant[1]		Crystals			
Hepes (mmol/L)	PEG (%)	Drop 1[2]		Drop 2[2]	
		Number	Size	Number	Size
50.84	3.79	0	/	0	/
	4.00	0	/	0	/
	4.43	0	/	0	/
	5.00	1	Small	2/4	Big/small
	5.50	9	Big	3/2/4	Big/twin/small
	6.00	0	/	0	/
61.01	3.79	0	/	0	/
	4.00	0	/	0	/
	4.43	1	Middle	0	/
	5.00	1	Middle	0	/
	5.50	2	Big	0	/
	6.00	40	Small	50 – 60	Small

1), the same as in Table 1; 2), crystals formed in two drops of solution under the same condition after incubation for 9 d at 20 ℃.

Table 3 Effect of $MgCl_2$ concentration on crystallization of Δ*nif*E Av1 by vapor diffusion in the hanging drop method

Precipitant[1)]		Crystals			
Hepes (mmol/L)	$MgCl_2$ (mmol/L)	Drop 1[2)]		Drop 2[2)]	
		Number	Size	Number	Size
61.01	275.99	7/1	Small/small twin	12	Small
	399.30	> 40	Small	> 40	Small
	499.12	1/3	Middle/small	4	small
	598.95	0	/	0	/
	698.77	0	/	0	/
81.35	275.99	6/5	Middle/small twin	> 40	Small
	399.30	5/3	Small/small twin	10	Small twin
	499.12	> 40	Small	> 40	Small and smaller
	598.95	6	Small twin	2	Small
	698.77	0	/	0	/

1), the concentration of glycerin, PEG 8000, NaCl and the protein are 11.57% (V/V), 4.30%, 245.06 mmol/L and 11.48 mg/mL, respectively. The protein solution contains 25 mmol/L Tris (pH 7.4); 2), the same as in Table 1.

Fig.1. The only two crystals (0.13 mm × 0.12 mm and 0.13 mm × 0.10 mm, respectively) of Δ*nif*E Av1 form in one hanging drop. The concentration of Hepes and PEG 8000 are 61.01 mmol/L and 5.50%, respectively. The other crystallization conditions are described in Table 1.

of the crystal nuclei was accelerated with accelerating water loss from the protein, leading to the formation of a large number of small-sized crystals.

2.1.3 $MgCl_2$ concentration $MgCl_2$ had also a significant effect on protein crystallization. The number and size of crystals depended on the $MgCl_2$ concentration (Table 3). The optimal concentration of $MgCl_2$ was apparently relative to that of Hepes. It decreased from about 499 mmol/L to the range of 276 – 399 mmol/L when Hepes concentration increased from 61 mmol/L to 81 mmol/L.

2.1.4 NaCl concentration NaCl had also an effect on protein crystallization by using the hanging drop method. When NaCl concentration increased from 35 mmol/L to 490 mmol/L, the crystal number decreased and the crystal size increased; but a large number of small crystals formed when the concentration further increased (Table 4). The optimal concentration of NaCl for crystallization by the hanging drop method was in a range of 350 – 490 mmol/L, which was slightly lower than that by liquid /liquid diffusion method. This shows that the optimal concentration somewhat depended on the crystallization method.

2.2 Effect of protein batch on crystallization

In general, the size and quality of protein crystals are affected by their purity and batch (Mcpherson *et al*, 1983). As shown earlier by the analysis of SDS-PAGE, Δ*nif*E Av1 of the 1st batch were somewhat purer than that of the 2nd batch (Zhao *et al*, 2003). Even though Δ*nif*E Av1 of the two batches could be crystallized, the crystals from the 1st batch were slightly less in number and larger in size than those from the 2nd batch (Table 5). It is consistent with the reported results that MnFe protein and CrFe protein could be crystallized from their partially purified samples and the high purity of protein could benefit the formation of a few large crystals of protein (Huang *et al*, 2001; Zhang *et al*, 2002).

2.3 Effect of diffusion method on crystallization

Owing to less needed protein, faster diffusion speed and convenience for observation, the hanging drop method is usually adopted to optimize the crystallization condition for the liquid/liquid diffusion method. Δ*nif*E Av1 was crystallized by both the liquid/liquid diffusion method and the hanging drop method in the same conditions (Table 4). However, unlike crystallization by vapor diffusion in the hanging drop method, the effect of NaCl concentration on the crystal number was not easy to be observed when using the liquid/liquid diffusion method. Although the sample volume in each tube with the liquid/liquid diffusion method was 5-fold of that in each hanging drop, their nucleus numbers were independent on the volume and less 5-fold in the most treatments. It keeps more protein being used for a subsequent growth, leading to forming a few large crystals after incubation for a long time. In the vapor diffusion method, the diffusion process includes the liquid/liquid diffusion between solutions and the vapor diffusion between the internal cell and external pool. In the liquid/liquid diffusion method, there is only a diffusion process at the interface between protein and precipitant solutions at the beginning, then followed by diffusing to equilibrium. 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 *et al*, 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.

Table 4 Effect of NaCl concentration and diffusion method on the crystallization of Δ*nif*E Av1

Precipitant[1]		Crystals			
Hepes (mmol/L)	NaCl (mmol/L)	Hanging drop method[2]		Liquid/liquid diffusion method[3]	
		Number	Size	Number	Size
50.84	35.01	> 60	Small	> 40	Small
	140.04	> 60	Small	> 40	Small
	245.06	> 40	Small	> 40	Small
	350.09	20	Small	> 40	Small
	490.12	9	Big	> 40	Middle and small
	595.09	13	Middle and small	> 40	Middle and small
61.01	35.01	> 40	Small	> 40	Small
	140.04	> 40	Small	> 40	Small
	245.06	1	Small	> 40	Small
	350.09	5/6	Big/middle	> 40	Small
	490.12	1	Big	> 40	Middle and small
	595.09	1	Small	> 40	Middle and small

1), the concentration of glycerin, PEG 8000 and $MgCl_2$ in the precipitant solution are 11.57%, 5.50% and 598.95 mmol/L, respectively and the concentration of protein in 25 mmol/L Tris buffer (pH 7.4) containing 250 mmol/L NaCl is 11.48 mg/mL; 2) and 3), incubation for 9 d and 19 d, respectively.

Table 5 The comparison of crystallization between different batches of Δ*nif*E Av1 by vapor diffusion in the hanging drop method

Protein batch	PEG[3] (%)	Crystals			
		Drop 1[4]		Drop 2[4]	
		Number	Size	Number	Size
1[1]	5.00	1	Small	2/4	Big/small
	5.50	9	Big	3/2/4	Big/twin/small
2[2]	5.00	23	Big and middle	27	Middle and small
	5.50	14	Big and middle	40	Middle and small

1) and 2), the proteins of batch 1 and batch 2 are dissolved in 25 mmol/L Tris buffer (pH 7.4) containing 250 mmol/L NaCl, which concentrations are 11.48 and 19.70 mg/mL, respectively; 3) and 4), the same as in Table 1.

3 Conclusion

As discussed above, protein crystallization is a complex process. Precipitant has the greatest effect on the process in all the factors. Besides, given the extreme oxygen sensitivity of Δ*nif*E Av1 protein, technical bias is also an important aspect. Under strictly anaerobic and optimized conditions, the largest obtained crystal of Δ*nif*E Av1 protein was 0.12 mm × 0.13 mm.

After modifying the optimal conditions for crystallization of MnFe protein and CrFe protein, their large crystals with good quality had been obtained by using the liquid/liquid diffusion method (Lü *et al*, 1983). It is reasonable to think that it is possible to get larger crystals with good quality by the liquid/liquid diffusion method. Further studies on this direction are under the way in our laboratory.

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棕色固氮菌缺失 *nif*E 的突变种固氮酶钼铁蛋白的结晶

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摘要: 从限氨固氮培养基中培养棕色固氮菌(*Azotobacter vinelandii* Lipmann)缺失 *nif*E 的突变种 DJ35 中,分离纯化得到缺失 FeMoco 的钼铁蛋白(Δ*nif*E Av1)。在一定条件下结晶得到深棕色短斜四棱柱晶体。结晶溶液中各组分的浓度以及结晶方法等对其晶核数目、晶体大小和质量有明显影响。目前用气相扩散的悬滴法所得的最大晶体的二维边长分别为 0.12 mm 和 0.13 mm。

关键词: 棕色固氮菌 DJ35 突变种;固氮酶 Δ*nif*E 钼铁蛋白;结晶

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