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Crystal Growth and Characterization of Residual Bacterioferritin in Partially Purified Nitrogenase CrFe Protein Solution from a Mutant UW3 of *Azotobacter vinelandii*

ZHAO Jian-Feng[1], LIU He-Li[2], ZHOU Hui-Na[1], WANG Zhi-Ping[3], ZHAO Ying[1], BIAN Shao-Min[1], LI Shu-Xing[2], BI Ru-Chang[2], HUANG Ju-Fu[1*]

(1. *Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, The Chinese Academy of Sciences*, Beijing 100093, China;
2. *Institute of Biophysics, The Chinese Academy of Sciences*, Beijing 100101, China;
3. *Institute of Nuclear Agriculture, Zhejiang University*, Hangzhou 310029, China)

Abstract: While attempting to obtain large crystals of nitrogenase CrFe protein, brown crystals and brick red crystals were simultaneously or independently obtained from CrFe protein preparation, which was partially purified from a mutant UW3 of *Azotobacter vinelandii* Lipmann grown on Mo-, ammonia-free but Cr-containing medium. SDS-PAGE and anoxic native-PAGE analysis consistently showed that the protein of the brown crystal was mainly composed of subunits (~60 kD) similar to those of Av1 (MoFe protein), while the protein of the brick red crystal was composed of ~20 kD subunits. And only the larger subunits rather than the smaller ones were detectable by Western blot to the antibody of Av1. Comparing with the large subunits, the amount of the small subunits in the partially purified CrFe protein solution was much smaller, indicating that the protein composed of the smaller subunits was one of contamination proteins for CrFe protein. Detection by 3, 5-diaminobenzoic acid of native-PAGE gels showed that the proteins forming the brick red crystal and the brown crystal were two kinds of iron-containing proteins with different electrophoretic mobility on the gel. The analysis of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) proved that the protein forming the brick red crystal was bacterioferritin of *A. vinelandii* (AvBF). X-ray diffraction to 2.34 Å resolution showed that the crystal belonged to space group H3, with unit-cell parameters a = 124.965 Å, b=124.965 Å and c = 287.406 Å. The detailed structural analysis published in the near future has confirmed that the brick red crystal is that of 24-meric bacterioferritin.

Key words: crystal growth and characterization; bacterioferritin; partially purified CrFe protein solution; mutant strain UW3 of *Azotobacter vinelandii*

The biological reduction of molecular nitrogen (N_2) is one of the most fundamental processes in nature. This process is catalyzed by nitrogenase which consists of two protein components (components Ⅰ and Ⅱ). During the separation of nitrogenase from *Azotobacter vinelandii*, a nitrogen-fixing bacterium, some important proteins including bacterioferritin had been purified and characterized. Like the bacterioferritins isolated from *Escherichia coli* and *Pseudomonas aeruginosa*, etc., bacterioferritin from *A. vinelandii* (AvBF) was shown to be a ferritin containing haem in addition to the non-heme iron core (Andrews *et al.*, 1991; Wai *et al.*, 1995). It was said that AvBF is a haem-containing multisubunit protein that performs the same functions of iron storage and iron detoxification as animal ferritins being studied more extensively (Stiefel and Watt, 1979). And now it is proposed that the ferritins' dual functions of storing iron and detoxification of iron or protection against oxygen are likely to be bacterioferritins' primary function (Carrondo, 2003). As a bacterioferritin, AvBF's significant difference from other ferritins, such as the presence of 12-heme groups and structurally disordered phospho-hydroxy mineral core, made great sense to elucidate how it functions. It is obvious that X-ray diffraction of AvBF crystal would help us to understand its structure-function relationship. However, its crystal structure has not been reported for a long time, though its preliminary X-ray crystallographic studies were described to show that the AvBF crystals belong to the cubic system, space group I432, with cell dimension 230 Å (Zhao *et al.*, 1984).

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* Author for correspondence. E-mail: <jfhuang@ibcas.ac.cn>.

Mutant strain UW3 from *A. vinelandii* was unable to fix N_2 in the presence of Mo (Nif^-) but able to grow either under condition of Mo starvation or in a Mo-deficient medium containing Re or V (Bishop *et al.*, 1982). Two new nitrogenase components Ⅰ (CrFe protein and MnFe protein) have been partially purified from the mutant grown on Mo-deficient medium containing Cr and Mn, respectively (Huang *et al.*, 2001; 2002). While attempting to crystallize CrFe protein, large AvBF crystals were accidentally obtained. X-ray diffraction analysis shows that these crystals belong to space group H3, rather than space group I432 as reported before (Zhao *et al.*, 1984). The present studies focus on the growth and characterization of AvBF crystals as well as the identification of them from CrFe protein crystals.

1 Materials and Methods

1.1 Bacterial strains and growth conditions

The mutant strain UW3 of *Azotobacter vinelandii* Lipmann was kindly provided by Prof. Bishop. Its growth was carried out according to Bishop *et al.* (1982) except the addition of Na_2CrO_4 (Huang *et al.*, 2002). Precautions were taken to minimize contamination by Mo as Mo-deficient media were prepared. The chemicals of analytic grade and redistilled water were used. All solutions of inorganic chemicals were passed through a column with active carbon in order to decrease the residual Mo content in the chemicals (Schneider *et al.*, 1991).

1.2 Purification and Crystallization of nitrogenase CrFe protein

The purification of the protein was anaerobically performed according to Huang *et al.* (2002).

The crystals were obtained by the liquid/liquid diffusion method in a capillary. In a plexi-glass box fully filled with Ar, 15 μL precipitant solutions and the same volume of protein solution were carefully added into the capillary. The mouth of the capillary was greased. The capillaries were put into a large glass tube with a plastic stopper. The sealed tubes were put into a glass bottle with its tight cap, then stood for several months on the basement at 20 ℃.

All operations were performed under Ar atmosphere. All of the used solutions were rigorously degassed and filled with Ar before and after the addition of 0.3 mg/mL and 1.6 mg/mL DT for protein purification and crystallization, respectively.

Determination of protein concentration and anaerobic absorption spectrum were carried out by the method of Huang *et al.* (2001).

Anoxic native-PAGE, SDS-PAGE and Western blott were performed according to Shah and Brill (1973), Paustian *et al.* (1990) and Luo *et al.* (1995), respectively.

1.3 Mass spectrometry

The ~ 20 kD proteins from brick red crystals, in advance run on SDS-PAGE gel and developed with Coomassie brilliant blue staining, were excised, sequentially digested with trypsin in gel, and then applied to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Peptide mass fingerprints (PMFs) were acquired in the reflectron mode on a Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics) using an acceleration voltage of 20 kV. Monoisotopic peptide masses obtained from MALDI-TOF were queried against entries for NCBInr protein databases using a protein search program, Mascot (Matrix Science Ltd., London). The mass accuracy of 50 ppm in the parent ion mass and 0.1 Da in the product ion mass was set as search parameters.

1.4 Single crystal X-ray diffraction

The single crystal X-ray diffraction experiments were performed at the Beijing Synchrotron Radiation Facility (Beijing, China) beamline 3W1A at a wavelength of 0.980 1 Å using a MAR345 (MAR Research, Hamburg) image plate detector. No good cryocondition was obtained after lots of trials, most of which resulted in large mosaicities. As a result, all the data were collected at room temperature, leading to a relatively small mosaicity of 0.28°. A large crystal-to-detector distance 300 mm and a small oscillation 1.0° were adopted to reduce the overlap of reflections.

2 Results and Discussion

2.1 Formation of brick red crystals in partially purified CrFe protein solution

The precipitant composition is important for formation and subsequent growth of protein crystals. In order to obtain crystals of CrFe protein suitable for X-ray diffraction, the optimal composition of precipitant has been screened. The crystal number decreased and crystal size increased with both the decrease of PEG and NaCl concentrations under the same conditions (Table 1). Under the given conditions, brick red crystals and brown crystals were simultaneously observed in a capillary for crystallization of the partially purified CrFe protein solution (Fig.1). After precipitant was extensively optimized, there was only one large brick red crystal in a capillary incubated for 128 d. It is shown that the composition of the precipitant had indeed an important effect on the number, size and color of crystals formed from the partially purified CrFe protein solution.

Table 1 Effect of PEG 8000 and NaCl concentrations on crystallization of proteins in the partially purified CrFe protein solution by liquid/liquid diffusion method

	Precipitant		Crystals	
Solution(1)	NaCl (mmol/L)	PEG (%)	Number	Size(2)
#1	987.20	7.00	40	Small
#2	987.20	6.00	4/2	Large/middle
#3	987.20	5.57	2/1	Large/middle
#4	1 073.04	5.57	1(3)	Large
#5	1 158.89	5.57	3/10	Middle/small
#6	987.20	4.71	3(4)	Large

(1), concentrations of $MgCl_2$, glycerin, Hepes buffer (pH 8.2) in the precipitant and the protein in 25 mmol/L Tris buffer (pH 7.4) containing 250 mmol/L NaCl were 599.75 mmol/L, 11.57 % (V/V), 74.57 mmol/L and 6.53 mg/mL, respectively; (2), the longest side of large, middle and small crystal was >0.20 mm, 0.05−0.20 mm and <0.05 mm, respectively; (3), brick red in color; the others were brown or brick red; (4), incubation time was 163 d; the others were 128 d.

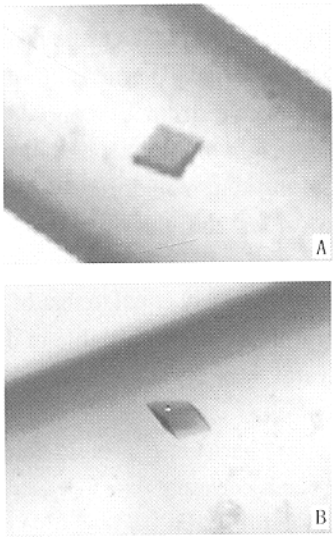

Fig.1. Brick red crystal (**A**) and brown crystals (**B**) formed in the partially purified CrFe protein solution incubated with the solution #4 and the solution #6 of Table 1 for 128 d and 163 d, respectively. The crystal diagonals in A are 0.28 mm and 0.20 mm, respectively; and both of longer sizes for the crystal in B are 0.16 mm.

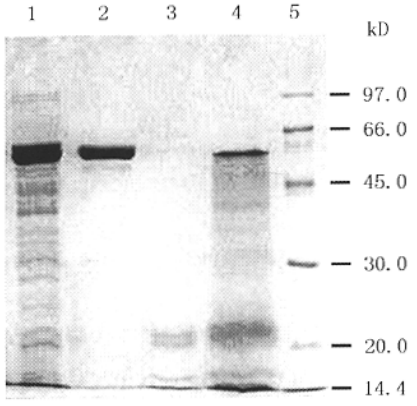

Fig.2. SDS-PAGE (12%) of partially purified CrFe protein solution before and after crystallization. 1, partially purified CrFe protein solution before crystallization (5.17 μg); 2, crystalline Av1 (4.79 μg); 3, crystals picked out, most of which were brick red; 4, crystals picked out, most of which are brown; 5, marker (low molecular standard).

2.2 Characterization of brick red crystals

In order to clarify the reason about different colors of crystals, the subunit composition of the crystal proteins was analyzed. The brown and brick red crystals were picked out, then washed and dissolved under atmosphere of Ar with 25 mmol/L Tris-HCl buffer containing 0.3 mg/L DT, respectively. After centrifugation, the supernatants were analyzed by SDS-PAGE. Both brown crystals and the brick red crystals had one band at ~14 kD and two bands at ~20 kD, but only the former had bands (~60 kD) similar to those of Av1 (Fig.2). On SDS-PAGE gel of the partially purified CrFe protein solution before crystallization, the two main bands were indeed at ~60 kD position even though there were several bands with low molecular weights. And the two main bands were detectable by Western blotting analysis of SDS gel, while other bands with lower molecular weight were not detectable (Fig.3). Like crystalline Av1, a main band at the same position as that of Av1 on the anoxic native gel of the partially purified protein could be detected as an iron-containing protein (Fig.4). This indicates that CrFe protein in the partially purified CrFe protein solution seemed to be an iron-containing protein composed of the similar subunits to those of Av1. As reported earlier (Huang *et al.*, 2002), the partially purified CrFe protein was shown to contain Fe and Cr (atom ratio of Fe to Cr was 11.60) and to have ~70% of C_2H_2- and H^+-reduction activity of Av1. Therefore, it is reasonable to consider that the protein should be a nitrogenase component Ⅰ composed of the ~60 kD subunits. The smaller subunits of the brown crystal shown in lane 4 of Fig.2 could be one of the following subunits. (1) Subunits forming the brick red crystal. Some

brick red crystals could be mixed with the brown ones because it was rather difficult to exactly differentiate the crystals' kind. (2) The possible third subunit of CrFe protein. It has been reported that Apo-Av1 from some mutants could be composed of α, β and γ subunits which could be decomposed to fractions with molecular weights of ~20 kD and ~14 kD (Homer *et al.*,1995). Even though the final identification of the subunits is on the way, it is reasonable to suppose that the brown crystal was from CrFe protein and the brick red crystal from another protein.

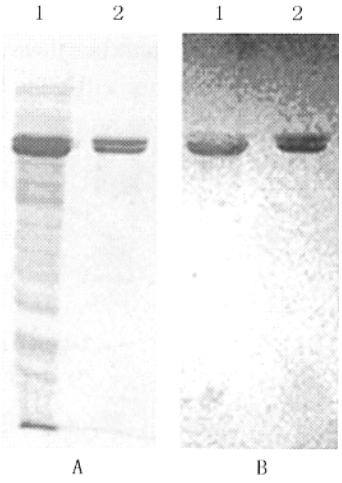

Fig.3. SDS gel (13%) of partially purified CrFe protein solution stained with Coomassie R-250 (**A**) and Western blot of SDS gel developed with antibody to Av1 (**B**). Lane 1, partially purified CrFe protein solution (5.5 μg in **A**, 11.0 μg in **B**); lane 2, crystalline Av1 (5.0 μg in **A**, 10.1 μg in **B**).

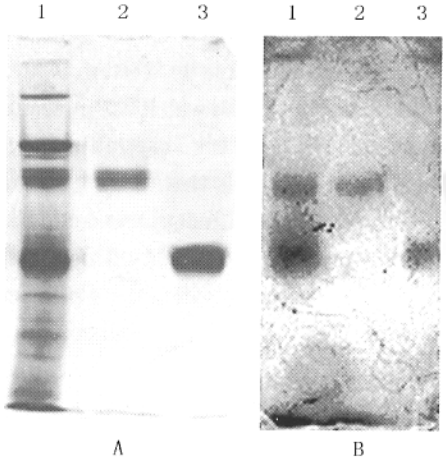

Fig.4. Anoxic native gel (6%) of partially purified CrFe protein solution stained with Coomassie R-250 (**A**) and 3,5-diaminobenzoic acid (**B**). Lane 1, partially purified CrFe protein solution (34.5 μg in **A**, 344.8 μg in **B**); lane 2, brick red crystals fromed from partially purified CrFe protein solution; lane 3, crystalline Av1 (50. 30 μg in **A**, 150.96 μg in **B**).

It is well shown in Fig.4 that there were only two iron-containing proteins including CrFe protein in the partially purified CrFe protein solution. CrFe protein migrated quicker than the other protein did on native gel. The protein of the brick red crystal was detected at the same position as that of the protein with smaller electrophoretic mobility, showing that the protein seemed to be the same as that of the brick red crystal. It is reasonable to consider that the brick red crystal was formed from an iron-containing protein, rather than CrFe protein. An absorption spectrum of the partially purified CrFe protein solution (in DT-containing solution) had three peaks at 416 nm, 526 nm and 556 nm, respectively (Fig.5). These absorption peaks were similar to those of AvBF from wild type strain of *A. vinelandii* (Li *et al.*, 1980). It was shown that a protein similar to AvBF indeed existed in the partially purified CrFe protein solution. As it is known, bacterioferritin is composed of 24 subunits with molecular weight of ~20 kD, half of which contains porphyrin, resulting in a small difference between the subunits (Andrews *et al.*, 2003). It is shown in Fig.2 that the molecular weights of the subunits composing the brick red crystal were approximately equal to that of bacteroferritin subunits. Therefore, it was reasonable to deduce that the brick red crystal was crystallized from the residual bacterioferritin.

Based on a comparison of protein amount with iron amount on the bands corresponding to bacterioferritin, CrFe protein and Av1 in Fig.4, it was impossible to estimate that the bacterioferritin contained much more iron atoms than CrFe protein or Av1 (30 irons per molecule) did. Hence, it could be speculated that the crystallized bacterioferritin was, to a large extent, a hollow tetracosamer without iron

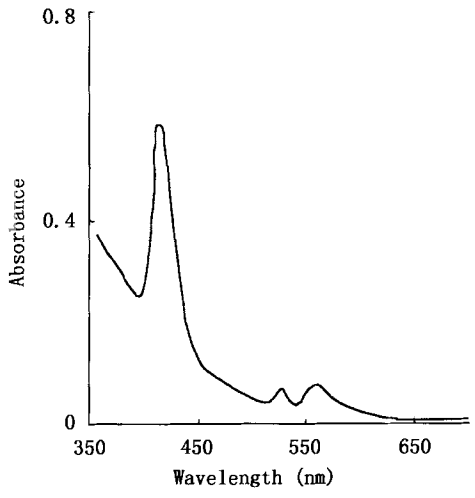

Fig.5. Absorption spectrum of partially purified CrFe protein solution. Concentration of protein and path of cell are 3.56 mg/mL and 4.98 mm, respectively.

core, which is subsequently confirmed by X-ray diffraction analysis (Liu *et al.*, 2004). The reason why the crystallized bacterioferritin is hollow needs further investigations.

2.3 Identification of bacterioferritin crystal

To investigate the protein composition of the crystals, we used MALDI-TOF MS to obtain sequence information of their subunits in SDS gel. The two bands of about 20 kD were cut and digested in-gel with trypsin, and mass spectra of the resulting peptides (peptide mass fingerprints) were acquired. The list of apparent peptide masses was then used to screen databases for correspondence to predict tryptic digests of known proteins. The analysis result of the larger subunit (Table 2) showed that 13 fragments matched 100% with the tryptic peptides from 18 207 Da bacterioferritin of *A. vinelandii* (AvBF), and the matched peptides accounted for 69% (107/156 amino acid residues) of total sequence. A similar conclusion was drawn from the analysis result of the smaller subunit, except that 1 538.54 Da peptide (positions 144−156 amino acids without modification) was not detected. It can be concluded that the protein compositions of two bands were identical, which was in accordance with the result of Grossman *et al.* (1992). Therefore, it was proved that protein composition of the crystals was AvBF.

The X-ray diffraction data integration and scaling were performed with the programs DENZO and SCALEPACK (Otwinowski *et al.*, 1997). As shown in Fig.6, the brick red crystal can diffract to 2.34 Å resolution. The crystals belong to space group H3, with unit-cell parameters a = 124.965 Å, b = 124.965 Å and c = 287.406 Å. A typical "432" point group symmetry was found by calculating the self-rotation function with MOLREP (Vagin *et al.*, 1997), confirming that the crystal protein used for diffraction was 24-meric bacterioferritin. The detailed structure determination and analysis will be introduced in a forthcoming publication (Liu *et al.*, 2004).

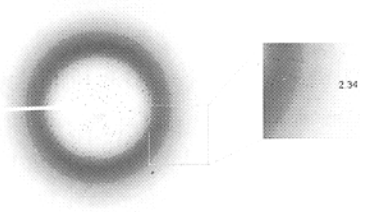

Fig.6. A diffraction pattern of brick red crystal and a zoomed-in region with a reflection at 2.34 Å.

3 Conclusions

From the partially purified CrFe protein solution, both the brown crystals and brick red crystals were observed simultaneously under given conditions. The analyses by many biochemical techniques have shown that the proteins forming the two crystals are nitrogenase CrFe protein and bacterioferritin, respectively. The determination by mass spectrometry and preliminary crystallographic analysis of the latter crystal further shows that the brick red crystal was that of bacterioferritin.

In the partially purified CrFe protein solution, CrFe protein and bacterioferritin were different in both protein kind and content. The former was the main protein composed of subunits similar to $\alpha_2\beta_2$ of Av1 while the latter was one of a contamination protein composed of 24 subunits similar to those of AvBF. However, it is unexpected that two different proteins could be simultaneously crystallized under some

Table 2 Correlation of the peptide masses of larger subunit as determined by MALDI-TOF analysis to calculated mass data of AvBF

Peptide	Experimental mass	Theoretical mass	ΔDa(1)	Position(2)	Peptide sequence	Modification
1	909.52	909.53	−0.01	77−84	(K)LLIGEHTK(E)	None
2	963.56	963.59	−0.03	6−13	(K)IVIQHLNK(I)	None
3	1 082.57	1 082.60	−0.03	93−102	(K)LEQAGLPDLK(A)	None
4	1 169.49	1 169.51	−0.02	31−39	(R)MYEDWGLEK(L)	None
5	1 185.47	1 185.50	−0.03	31−39	(R)MYEDWGLEK(L)	1-Met-ox(3)
6	1 416.57	1 416.60	−0.03	43−53	(K)HEYHESIDEMK(H)	None
7	1 432.54	1 432.59	−0.05	43−53	(K)HEYHESIDEMK(H)	1-Met-ox(3)
8	1 538.54	1 538.69	−0.15	144−156	(K)IGLENYLQSQMDE	None
9	1 554.57	1 554.69	−0.12	144−156	(K)IGLENYLQSQMDE	1-Met-ox(3)
10	1 631.69	1 631.72	−0.03	103−117	(K)AAIAYCESVGDYASR(E)	None
11	1 682.93	1 682.96	−0.03	62−76	(R)ILFLEGLPNLQELGK(L)	None
12	1 839.08	1 839.06	0.02	61−76	(K)RILFLEGLPNLQELGK(L)	None
13	1 984.08	1 984.09	−0.01	14−30	(K)ILGNELIAINQYFLHAR(M)	None

(1), ΔDa means mass experimented-mass calculated; (2), positions refer to the positions of amino acid residues in the polypeptide chain; (3), Met-ox refers to oxidation of methionine in the peptide sequence.

conditions. It indicates that a protein with low purity still has an opportunity to be crystallized under some conditions.

In general, protein solubility value is not unique but of several kinds, which makes a protein to form crystals with different shapes under several conditions (Mcpherson *et al.*, 1983). Crystal formation of two or more proteins in one solution depends mainly on protein properties and other factors. During diffusion of a protein into a precipitant, the local concentration of the protein and the precipitant in different position of a capillary are different from one another, leading to form crystals of different proteins with different shape and size. Therefore, it is not unexpected that CrFe protein and the residual bacterioferritin in the partially purified CrFe protein solution were simultaneously crystallized or only the latter was crystallized under a given condition. The crystallization of the residual bacterioferritin should be an example for crystallization of the minor contamination protein.

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棕色固氮菌突变种 UW3 部分提纯的固氮酶 CrFe 蛋白溶液中残存细菌铁蛋白的晶体生长及鉴定

赵剑峰[1] 刘合力[2] 周会娜[1] 汪志平[3] 赵 颖[1] 边少敏[1] 李树兴[2] 毕汝昌[2] 黄巨富[1*]

(1. 中国科学院植物研究所光合作用与环境分子生理学重点实验室，北京 100093;
2. 中国科学院生物物理研究所，北京 100101;
3. 浙江大学原子核农业科学研究所，杭州 310029)

摘要： 从无钼、无氨而含铬的固氮培养基中生长的棕色固氮菌(*Azotobacter vinelandii* Lipmann)突变种UW3中纯化得到了部分纯的CrFe蛋白。在试图培养CrFe蛋白大晶体时发现，棕色晶体和砖红色晶体可同时或单独出现。SDS-PAGE和厌氧天然PAGE皆表明，棕色晶体主要由与固氮酶钼铁蛋白(Av1)类似大小的亚基(~ 60 kD)组成，而砖红色晶体则由~20 kD亚基组成。免疫分析表明只有~ 60 kD的亚基可与固氮酶钼铁蛋白的抗体反应，而~ 20 kD亚基则无这种反应。在部分纯的CrFe蛋白溶液中，~ 20 kD的总蛋白含量远低于~ 60 kD蛋白的含量，表明由这种小亚基组成的蛋白只是CrFe蛋白溶液中的一种污染蛋白。用3,5-二氨基苯甲酸染色的天然电泳表明，形成砖红色和棕色晶体的蛋白是迁移率不同的两种含铁蛋白。质谱分析表明砖红色晶体蛋白为棕色固氮菌的细菌铁蛋白。分辨率为2.34 Å的X射线衍射结果也表明，砖红色晶体属于H3空间群，晶胞参数为a = 124.965Å, b= 124.965Å和c = 287.406 Å。即将发表的三维结构解析表明，此砖红色晶体确为24聚体的细菌铁蛋白。

关键词： 晶体生长及鉴定；细菌铁蛋白；部分纯铬铁蛋白；棕色固氮菌突变种

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* 通讯作者。 E-mail: <jfhuang@ibcas.ac.cn>。

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