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STRUCTURE OF MACROMOLECULAR COMPOUNDS

Recombinant Formate Dehydrogenase from *Arabidopsis thaliana*: Preparation, Crystal Growth in Microgravity, and Preliminary X-Ray Diffraction Study

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Abstract—Crystals of high-purity recombinant NAD^+-dependent formate dehydrogenase from the higher plant *Arabidopsis thaliana* (*Ara*FDH) were grown in microgravity in the Modul'-1 protein crystallization apparatus on the International Space Station. The space-grown crystals have larger sizes than those grown on Earth. X-ray diffraction data suitable for determining the three-dimensional structure were collected from the space-grown crystals to a resolution of 1.22 Å using an X-ray synchrotron source. The crystals belong to sp. gr. $P4_32_12$; the unit-cell parameters are $a = b = 107.865$ Å, $c = 71.180$ Å, $\alpha = \beta = \gamma = 90°$.

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INTRODUCTION

The growth of high-quality protein crystals suitable for determining the three-dimensional structures at high resolution is an important problem. The crystallization in microgravity is one efficient approach to improving the X-ray diffraction quality of crystals. The quality of the protein crystals depends significantly on the mass transfer to growing crystals, which occurs through convection or diffusion. In microgravity, where the convection is virtually absent, the diffusion transport dominates. A stable concentration gradient is established in the solution around the crystals so that the crystal growth occurs in the most favorable conditions at a low supersaturation. Due to the absence of sedimentation, crystals do not fall to the bottom of the vessel, which yields the uniform transfer of protein molecules to all growing faces and facilitates the formation of more isometric crystals in microgravity [1].

In 2006–2009 a series of experiments on the protein crystal growth in microgravity were carried out on the Russian Segment of the International Space Station (RS ISS) [2]. Crystals of several proteins (including genetically-engineered human insulin, carboxypeptidase, and sialylated insulin) were grown in the Modul'-1 protein crystallization apparatus constructed in the Special Design Bureau of the Shubnikov Institute of Crystallography of the Russian Academy of Sciences. These crystals had larger sizes and were of a higher X-ray diffraction quality than the earth-grown crystals of the corresponding proteins [2–4].

In this study we report the results of the crystallization of recombinant NAD^+-dependent formate dehydrogenase from *Arabidopsis thaliana* (*Ara*FDH) in the presence of sodium azide in the Modul'-1 protein crystallization apparatus on the RS ISS.

NAD^+-dependent formate dehydrogenases (EC 1.2.1.2, FDHs) catalyze the oxidation of the formate ion to carbon dioxide coupled with the reduction of NAD^+ to NADH. There are several types of this enzyme in nature, which differ in the subunit composition and the presence of different metal ions (molybdenum, tungsten), prosthetic groups, and even unusual amino acids (for example, selenocysteine in FDH from *E. coli*). The most structurally simple enzyme consists of two identical subunits, each composed of 360–400 amino acids. This type of FDH was found in different bacteria (methylotrophic, symbiotic nitrogen-fixing, pathogenic, deep-water, etc.), yeasts, microscopic fungi, and plants. Formate dehydrogenases from the bacteria *Pseudomonas* sp. 101 and *Moraxella* sp. C-1 and the yeast *Candida boidinii* have been characterized in most detail. The genes for these enzymes were cloned, and strains of *E. coli* for the hyperproduction of both wild and mutant recombinant enzymes were obtained [5–7].

In plants, the formate dehydrogenase activity was found for the first time in the early 1950s [8]. However, systematic studies of plant FDHs have not been performed because of the difficulties of isolating them. Plant FDHs attracted great attention in the early 1990s [9, 10] when it was shown that FDH found in

potato tuber mitochondria is a stress protein. Thus, the amount of FDH in mitochondria reached 9% of all mitochondrial proteins in the response of plants to stress [9].

Investigations of plant FDHs are of both theoretical and practical interest. A comparison of the amino-acid sequences of FDHs from different sources shows the closest homology between the active sites of plant FDHs and the active sites of related enzymes from bacteria [5, 7]. However, plant FDHs differ substantially from bacterial enzymes in their mechanism of catalysis [5, 11, 12]. From the practical point of view, the protein engineering of plant FDHs is also an important problem. In stressed plants, formate dehydrogenases provide cells with energy as a result of the NADH production via the oxidation of toxic formate. Plants with enhanced resistance to different stresses, such as drought, heat, cold, etc., can be designed by increasing the activity and stability of plant FDHs. It is very important to know the high-resolution three-dimensional structures for genetic-engineering experiments with proteins.

The *apo* forms of bacterial and plant formate dehydrogenases are in the open conformation, whereas the *holo* form (formate dehydrogenase in the complex with azide and the cofactor) is in the closed conformation. It should be noted that the binding of the cofactor is insufficient for the transformation of the enzyme into the closed form [13]. Crystals of FDH from *Arabidopsis thaliana* have been grown, and the three-dimensional structures of the *apo* form (open conformation) and the *holo* form of FDH in the complex with azide and NAD^+ have been established [14]. However, it remains unclear whether the presence of azide alone is sufficient for the transformation from the open to the closed conformation. In the FDH–NAD^+–azide ternary complex, azide mimics the substrate in the transition state of the reaction.

In this study, crystals of *Ara*FDH were grown from a solution of highly purified preparations of recombinant FDH from *Arabidopsis thaliana* in the presence of sodium azide both on earth and in microgravity. The crystals grown in microgravity had a larger size and were of a better quality than the earth-grown crystals. The X-ray diffraction data collected from the space-grown crystals were suitable for determining the structure of *Ara*FDH at higher resolution (1.22 Å) than for the earth-grown crystals.

EXPERIMENTAL

Biomass Production of Recombinant E. Coli Cells with FDH from A. thaliana

The plasmid of total cDNA of the gene *arafdh* was kindly supplied by Professor J. Markwell (University of Nebraska–Lincoln, United States). To express *Ara*FDH in *E. coli* cells from the gene *arafdh* by the polymerase chain reaction, the nucleotide sequence encoding the signaling peptide was removed [11]. The DNA fragment encoding only the mature enzyme was cloned into the plasmid *p*ET21a. The resulting plasmid *p1Ara*FDH was used to transform the BL21-CodonPlus(DE3) *E. coli* cells. To obtain a seed culture, a single colony was taken from a Petri dish and incubated in 20 mL of the 2YT medium (16 g/L bactotryptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) at 37°C for 6 h at 150 rpm in the presence of 150 μg/mL ampicillin. The inoculant was transferred into the fresh 2YT medium (200 mL) in a 1-l flask and cultured at 30°C for 3 h at 100 rpm. Then lactose was added to the flask at a concentration of 20 g/L, the cultivation temperature was decreased to 25°C, and then the cultivation was performed for 12–16 h. The cells were precipitated by centrifugation at 5000 g for 20 min at 4°C. The precipitate was resuspended in a 0.1-M phosphate buffer, pH 7.5, containing 10 mM EDTA (20 g of the cells per 90 mL of the buffer).

*Purification of Ara*FDH

To disrupt the cells, 44 mL of the suspension (two 22-mL portions) were frozen and thawed twice, and each portion was twice treated with ultrasound for 3 min with continuous ice cooling. The precipitate was removed by centrifugation at 20 000 g for 20 min. Solid ammonium sulfate was added with continuous stirring to the combined supernatant to 35% saturation. The resulting solution was kept overnight at 4°C. The precipitate of impurity proteins was removed by centrifugation at 20 000 g for 20 min. The enzyme solution was applied to a column packed with Phenyl-Sepharose Fast Flow (50 mL, Pharmacia Biotech) equilibrated with 35% saturated sulfate ammonium in a 0.1-M phosphate buffer, pH 7.0, containing 10 mM EDTA. The column was washed with 300 mL of the same solution. The enzyme was eluted with a linear descending concentration gradient of ammonium sulfate from 35 to 0%. The fractions containing active *Ara*FDH were combined and concentrated to 5 mL in an Amicon cell through an Amicon PM-10 membrane. The resulting enzyme solution was applied to a 2.5 × 100-cm column packed with 500 mL of Sephacryl S-200 (Pharmacia Biotech) equilibrated with a 0.1-M phosphate buffer, pH 7.0, containing 10 mM EDTA. The enzyme was eluted from the column at a rate of 25 mL/h. The elution of the enzyme was monitored with a Uvicord SII stopped-flow spectrophotometer at 278 nm, and 5-mL fractions were collected. The enzyme activity and the protein concentration in the fractions were determined. The fractions with a specific activity of 6.5 units per mg of the protein fractions were taken and combined. The yield of the purified enzyme was 1360 activity units (72% of the initial activity). According to the results of analytical SDS-polyacrylamide gel electrophoresis, the purity of the enzyme preparations was no lower than 95%.

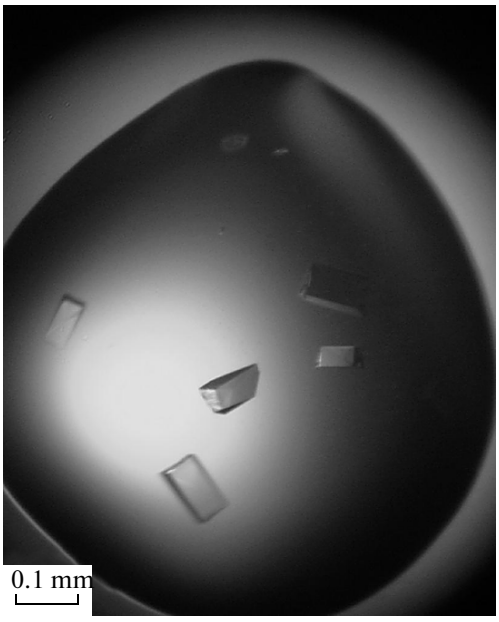

Fig. 1. Crystals of the *Ara*FDH complex with azide grown by the hanging-drop vapor-diffusion method.

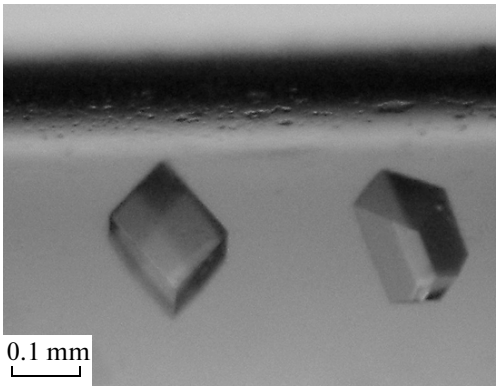

Fig. 2. Crystals of the *Ara*FDH complex with azide grown in capillaries.

The enzyme, which was stored without any loss of activity at 4°C in a 0.1 M phosphate buffer, pH 7.0, containing 10 mM EDTA, was used for crystal growth experiments.

*Crystallization of Ara*FDH *in the Presence of Sodium Azide*

Crystallization by the hanging-drop vapor-diffusion method. The preliminary search for the conditions of crystal growth on earth was carried out by the hanging-drop vapor-diffusion method based on the conditions proposed in [14]. Crystals were grown from solutions of the enzyme in a 0.1-M potassium phosphate buffer, pH 7.0, containing 0.01 M EDTA and 0.01 M sodium azide with the use of 1.9–2.3 M ammonium sulfate in a 0.1-M Bis-Tris buffer, pH 5.6, as the precipitant. The largest crystals were obtained at a protein concentration of 5.8 mg/mL with the use of 2.1 M ammonium sulfate (Fig. 1).

Crystallization in small-volume capillaries by the free-interface-diffusion technique. A 10-μL protein solution with a protein concentration of 10–16 mg/mL was placed onto a siliconized glass cover slide near a drop of a reservoir solution of the same volume. The protein solution, followed by the reservoir solution, was successively sucked into the capillary by placing the tip of the capillary into these drops. Then both ends of the capillary were sealed with picein; 3.6–4.0 M ammonium sulfate in a 0.1-M Bis-Tris buffer, pH 5.6, was used as the precipitant. The largest crystals (up to 0.10 mm) grew at a protein concentration of 14 mg/mL with the use of 3.8 M ammonium sulfate. Cubic and prismatic crystals grown in the capillaries are shown in Fig. 2.

Protein crystallization by the free-interface-diffusion technique in the Modul'-1 protein crystallization apparatus on earth and in microgravity. The Modul'-1 crystallization apparatus was prepared and loaded as described in [2]. The crystallization conditions for microgravity are given in Table 1. Under the same conditions, the control experiment in the Modul'-1 apparatus was performed on earth. The crystals of *Ara*FDH grown in microgravity in the Modul'-1 crystallization apparatus are shown in Figs. 3a and 3b.

X-ray data collection and characterization of crystals. The X-ray diffraction data were collected from one of the space-grown crystals to 1.22 Å resolution at the European Molecular Biology Laboratory (EMBL Outstation, Hamburg, DESY synchrotron, beamline X13) at 100 K. The X-ray data were processed with the use of the DENZO and SCALEPACK program packages [15]. The crystals belong to sp. gr. $P4_32_12$; the unit-cell parameters are $a = b = 107.865$ Å, $c = 71.180$ Å, $\alpha = \beta = \gamma = 90°$. The X-ray diffraction data collection statistics are presented in Table 2. The Matthews coefficient [16] was 2.32 and the solvent content was 47%.

RESULTS AND DISCUSSION

For experiments on the protein crystal growth in microgravity, the Modul'-1 protein crystallization apparatus was designed in the Special Design Bureau of the Shubnikov Institute of Crystallography, Russian Academy of Sciences. In this apparatus the crystal growth occurs by the free-interface diffusion. This is the method of choice in experiments performed in microgravity, because it prevents or reduces the Marangoni convection.

Active recombinant formate dehydrogenase from *Arabidopsis thaliana* for crystallization experiments was expressed with the use of the hyperproducing strain of *E. coli* [11]. The procedures developed for the cultivation and purification allowed the production of

Table 1. Crystallization of the complex of *Ara*FDH with azide in microgravity in the Modul'-1 protein crystallization apparatus

Cell	Composition of the protein solution	Composition of the reservoir solution	Result
1	18 mg/mL, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.9 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Amorphous precipitate
2	18 mg/mL, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.8 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Cubic crystals with dimensions $0.2 \times 0.2 \times 0.2$ mm. Prismatic crystals with dimensions $0.5 \times 0.5 \times 1.0$ mm
3	15 mg/mL, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.9 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Crystals: cubic with dimensions $0.2 \times 0.2 \times 0.2$ mm and prismatic with dimensions $0.3 \times 0.3 \times 0.6$ mm
4	16 mg/mL, 0.01 M ADPR, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.9 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Amorphous precipitate
5	18 mg/mL, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.9 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Amorphous precipitate
6	18 mg/mL, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.8 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Cubic crystals with dimensions $0.4 \times 0.4 \times 0.4$ mm
7	15 mg/mL, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.8 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Crystals: cubic with dimensions $0.2 \times 0.2 \times 0.2$ mm and rectangular-prismatic with dimensions $0.1 \times 0.1 \times 0.3$ mm
8	13.5 mg/ml, 0.01 M ADPR, 0.6 M ammonium sulfate, 0.01 M EDTA, 0.01 M sodium azide in 0.1 M potassium phosphate buffer, pH 7.0	3.9 M ammonium sulfate in 0.1 M Bis-Tris buffer, pH 5.6	Amorphous precipitate

FDH from *Arabidopsis thaliana* in large amounts (up to 1 g of the target protein per liter of the medium) and in the homogeneous state, which made it possible to systematically characterize the enzyme (including the experiments on the crystal growth and the determination of the three-dimensional structure). Crystals of the *apo* and *holo* forms of *Ara*FDH have been grown before, and the three-dimensional structures of the enzyme have been established at resolutions of 1.7 and 2.0 Å, respectively [14].

The conditions for the crystal growth on earth in the presence of azide ions were found by the hanging-drop vapor-diffusion method. Like in the previous study [14], a solution of sulfate ammonium in a 0.1 M Bis-Tris buffer, pH 5.6, was used as the precipitant. Protein solutions in a potassium phosphate buffer contained sodium azide and EDTA. The crystals grown in the drops reached the size of 0.10 mm (Fig. 1). These conditions were used as the initial conditions for the crystal growth on earth in capillaries by the free-interface-diffusion technique. However, no crystals grew in capillaries under the conditions used in the hanging-drop vapor-diffusion method. The conditions for the crystal growth in capillaries were optimized by varying both the protein and precipitant concentrations. Crystallization by the free-interface-diffusion method generally requires higher concentrations of both the protein and the precipitant compared to those used in the vapor-diffusion method, because the volume of the solution after both components are mixed in the former method remains constant during the experiments. By contrast, in the vapor-diffusion method, the volume of the drops composed generally of equal volumes of protein and reservoir solutions decreases until the concentration of the precipitant in the drop becomes equal to that in the reservoir solution. In our experiments, the protein concentration in capillaries was increased by a factor of more than two and the concentration of sulfate ammonium was increased to 3.6–4.0 M. Crystals appeared within 7–9 days and reached the largest size (approximately 0.2 mm) after 3–4 weeks.

Table 2. X-ray data-collection statistics for crystals of *Ara*FDH

Resolution, Å	1.22 (1.23–1.22)*
Sp. gr.	$P4_32_12$
Unit-cell parameters, Å, deg	$a = b = 107.87$, $c = 71.18$, $\alpha = \beta = \gamma = 90$
Mosaicity, deg	0.7
Total number of reflections	15153970
Number of independent reflections	123669
Wavelength, Å	0.96
Completeness of the data set, %	99.4 (98.7)
Rsym, %	6.1 (44.4)
$I/\sigma(I)$	14.2 (3.4)

* The data for the last resolution shell are given in parentheses.

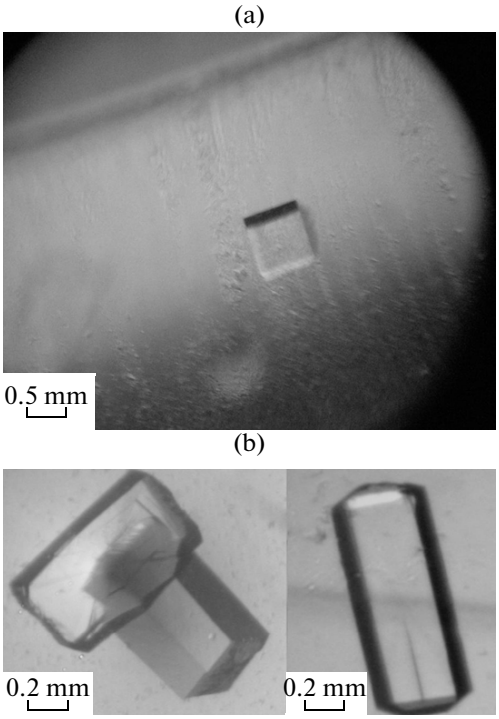

Fig. 3. Crystals of the *Ara*FDH complex with azide grown in the Modul'-1 protein crystallization apparatus on the International Space Station during the ISS-17 space flight; (a) a cubic crystal in a cell of the crystallization apparatus; (b) prismatic crystals after withdrawing from the cell of the crystallization apparatus.

The crystals of *Ara*FDH were grown on the RS ISS during the ISS-17 space flight (August–October, 2008). To find the conditions for the crystal growth in microgravity in the Modul'-1 protein crystallization apparatus, the protein and the precipitant concentrations were varied in a narrow range optimal for the crystal growth in capillaries (Table 1). The difference in the conditions of the crystal growth is attributed, in particular, to the presence of an air interlayer in the capillaries and, consequently, to the presence of the solution–air interface. The interface facilitates the nucleation, whereas the cells of the Modul'-1 apparatus are completely filled with solutions.

After the space flight, the cells of the Modul'-1 apparatus and the cells of the control crystallization apparatus remaining on earth were opened and the contents were transferred to Petri dishes. Space-grown crystals were found in four out of eight cells (Table 1). Inspection with a light microscope showed that the crystals are cubic or prismatic in shape. The average crystal size of the cubic form was $0.3 \times 0.3 \times 0.3$ mm, and some crystals were as large as $0.4 \times 0.4 \times 0.4$ mm (Fig. 3a). Prismatic crystals found in cell 2 had an average size of $0.7 \times 0.3 \times 0.3$ mm, and some crystals were as large as $1.0 \times 0.4 \times 0.4$ mm (Fig. 3b). Such large crystals were never grown on earth. The cells of the control crystallization apparatus on earth contained mainly amorphous precipitates, and the crystals found in the cells were smaller than 0.05 mm.

The X-ray diffraction data from the space-grown crystals were obtained to 1.2–1.3 Å resolution using a synchrotron radiation source (DESY synchrotron, Hamburg, beamline X13), whereas the resolution for the earth-grown crystals was lower than 1.7 Å. The X-ray diffraction set collected from the space-grown crystals is suitable for determining the three-dimensional structure of *Ara*FDH at a resolution of no lower than 1.3 Å.

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