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High pressure refolding, purification, and crystallization of flavin reductase from *Sulfolobus tokodaii* strain 7

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ABSTRACT

Flavin reductase $HpaC_{St}$ catalyzes the reduction of free flavins using NADH or NADPH. High hydrostatic pressure was used for the solubilization and refolding of $HpaC_{St}$, which was expressed as inclusion bodies in *Escherichia coli* to achieve high yield in a flavin-free form. The refolded $HpaC_{St}$ was purified using Ni-affinity chromatography followed by a heat treatment, which gave a single band on SDS–PAGE. The purified refolded $HpaC_{St}$ did not contain FMN, unlike the same enzyme expressed as a soluble protein. After the addition of FMN to the protein solution, the refolded enzyme showed a higher activity than the enzyme expressed as the soluble protein. Crystals of the refolded enzyme were obtained by adding FMN, FAD, or riboflavin to the protein solution and without the addition of flavin compound.

Introduction

Heterologous protein expression is one of the most widely used technologies in life science researches, and often utilizes bacterial, yeast, insect or mammalian cells. Protein overexpression systems permit a high-level production of recombinant proteins, and some recombinant proteins form insoluble aggregates, known as inclusion bodies (IBs)[1], even after the optimization of protein expression conditions. IBs can be formed by misfolded intermediates [1] and/or properly folded polypeptides [2,3].

IBs are generally solubilized by using denaturating agents such as guanidine hydrochloride (GdnHCl) or urea, and then refolded by dilution, dialysis or gel filtration to obtain active proteins [4]. Refolding of IBs using denaturating agents is also applicable to the production of ligand-free proteins. The addition of redox reagents, detergents, amino acids (arginine, lysine, and glutamic acid), and sugars is effective to increase the recovery yield of refolded proteins, and high throughput screening kits containing these compounds are commercially available, *e.g.*, iFold 1–3 (Merck). However, the effects of these compounds are not fully understood, and there has been no refolding method that is universally applicable.

Recently, a new refolding technique using high hydrostatic pressure has been proposed by several groups [5–8]. High hydrostatic pressure has generally been shown to unfold native proteins above 400 MPa, but to dissociate aggregates and oligomers at 100–300 MPa [9–11]. A high pressure at 100–300 MPa disrupts both ionic and hydrophobic interactions, but does not affect hydrogen bonds. Therefore, high pressure refolding may be useful, since the high pressure induces protein dissociation without the need of a denaturating agent.

Flavin reductase $HpaC_{St}$ (ST0723) from the aerobic thermoacidophilic crenarchaeon *Sulfolobus tokodaii* strain 7 [12] catalyzes the reduction of free flavin compounds using NADH or NADPH. $HpaC_{St}$ is a member of the short-chain flavin reductase family, and two reaction mechanisms are proposed in this family of enzymes. One is a Ping Pong Bi Bi reaction mechanism in which NADH reduces the FAD cofactor and the cofactor transfers electrons to the FAD substrate [13]. The other is an ordered sequential mechanism in which NADH binds first and a substrate flavin compound binds second [14,15]. Previously, we reported the crystal structure of $HpaC_{St}$ expressed as the soluble form in recombinant *Escherichia coli* cells [16], which revealed that the enzyme existed as a homodimer, and each protomer contained an FMN molecule without the addition of FMN to the purified enzyme solution. On the other hand, the majority of $HpaC_{St}$ was expressed as IBs in the recombinant *E. coli* cells. The purpose of this study was to yield a greater amount of the native $HpaC_{St}$ and $HpaC_{St}$ in a flavin-free form by high pressure refolding and to obtain the crystals complexed with various flavin compounds to better understand the reaction mechanism of $HpaC_{St}$ in detail.

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[1] *Abbreviations used*: IB, inclusion body; GdnHCl, guanidine hydrochloride; IPTG, isopropyl β-D-thiogalactopyranoside; UV, ultraviolet; TCEP, tris(2-carboxyethyl) phosphine.

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Materials and methods

Expression and inclusion body preparation of flavin reductase $HpaC_{St}$

The aerobic thermoacidophilic crenarchaeon *S. tokodaii* strain 7 was kindly provided by Dr. Toshihisa Ohshima (The University of Tokushima, Japan) and its genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen). The gene of $HpaC_{St}$ protein (st0723) was amplified from the genomic DNA of *S. tokodaii* strain 7 by PCR with the forward primer (5′-GGGAATTCCATATGGCTGAAG TTATCAAAAGTATAATGAGGAAATTTCCT-3′) with a *Nde*I restriction site (underlined) and the reverse primer (5′-CGCGGATCCCTACTA-CAAGCTAGAGAGTTTATAGTATTTTCT-3′) with a *Bam*HI restriction site (underlined), and cloned into the *Nde*I/*Bam*HI site of pET-28a(+) (Novagen) for the expression of N-terminally His-tagged $HpaC_{St}$ protein. *E. coli* Rosetta(DE3) (Novagen) transformed with this plasmid was grown in 10 mL of LB medium containing 30 μg/ml kanamycin at 37 °C overnight. The pre-culture was added to 1.5 L of LB medium in a 5 L shaking flask, and the cells were cultured at 37 °C. When the optical density at 600 nm reached at 0.6, the protein expression was induced by adding IPTG (isopropyl β-D-thiogalactopyranoside) to a final concentration of 1 mM, and the culture was continued at 20 °C for 12 h. The cells (approximately 4 g wet weight) were harvested by centrifugation at 4500 *g* at 4 °C for 5 min and suspended in 70 ml ice-cold water. The suspension was sonicated (Branson 250-D; amplitude 60%) on ice for 7 min, and subjected to a centrifugation at 40,000 *g* at 4 °C for 30 min. The pellet was resuspended in wash buffer A (50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM TCEP, 0.5 mM EDTA, 5% (v/v) glycerol, and 125 mM NDSB-201), and centrifuged at 8000 *g* at 4 °C for 15 min. The pellet was resuspended in wash buffer B (50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM TCEP, 0.5 mM EDTA, and 5% (v/v) glycerol) and subjected to a centrifugation at 8000 *g* at 4 °C for 15 min. The wash procedure using wash buffer B was repeated twice. The pellet was stored at −80 °C until use.

High hydrostatic pressure refolding

The refolding buffer conditions were examined using HiPER-Fold Starter kit (Barofold) (Table 1). The pellet was suspended in water, and the protein concentration of the pellet was measured by a BCA protein assay (Thermo Scientific). Each refolding buffer (10×) was added to prepare a refolding test sample (500 μl) whose protein concentration was 0.5 mg/ml. Refolding was performed in a high pressure vessel (Barofold). High pressure (200 MPa) was applied at room temperature for 16 h, and the samples were depressurized 25 MPa every 5 min until 0.1 MPa was reached. After the centrifugation at 15,000 *g* at 25 °C for 10 min, the supernatants were analyzed by SDS–PAGE.

The large-scale refolding was carried out using a 39 ml tube (Beckman) designed for ultracentrifugation. The pressurization/depressurization procedures were the same as those for the initial screening. The depressurized sample was centrifuged at 15,000 *g* at 25 °C for 10 min, and the supernatant was dialyzed against Sol A (25 mM Tricine–HCl (pH 8.5), 400 mM NaCl, and 5 mM imidazole). The dialyzed protein was loaded onto a 3 ml Ni Sepharose 6 Fast Flow column (GE Healthcare). After a wash step with Sol B (25 mM Tricine–HCl (pH 8.5), 400 mM NaCl, and 50 mM imidazole), the protein was eluted with Sol C (25 mM Tricine–HCl (pH 8.5), 400 mM NaCl, and 200 mM imidazole). The protein solution was heated at 80 °C for 20 min and centrifuged at 15,000 *g* at 25 °C for 15 min. The supernatant was dialyzed against 15 mM Tricine–HCl (pH 8.5) at 25 °C. The protein concentration was determined by the Bradford method [17] using bovine serum albumin as the standard protein.

Table 1
Buffers used for refolding of $HpaC_{St}$.

Condition No.	Buffer
1	50 mM Acetate (pH 4), 5 mM TCEP
2	50 mM Acetate (pH 5), 5 mM TCEP
3	50 mM MES (pH 6), 5 mM TCEP
4	50 mM TES (pH 7), 5 mM TCEP
5	50 mM TAPS (pH 8), 5 mM TCEP
6	50 mM CHES (pH 9), 5 mM TCEP
7	50 mM CAPS (pH 10), 5 mM TCEP
8	50 mM Acetate (pH 4), 5 mM TCEP, 500 mM Arginine
9	50 mM Acetate (pH 5), 5 mM TCEP, 500 mM Arginine
10	50 mM MES (pH 6), 5 mM TCEP, 500 mM Arginine
11	50 mM TES (pH 7), 5 mM TCEP, 500 mM Arginine
12	50 mM TAPS (pH 8), 5 mM TCEP, 500 mM Arginine
13	50 mM CHES (pH 9), 5 mM TCEP, 500 mM Arginine
14	50 mM CAPS (pH 10), 5 mM TCEP, 500 mM Arginine
15	50 mM TAPS (pH 8), 5 mM TCEP, 1.5% (w/v) n-Octyl-β-D-glycopyranoside
16	50 mM TAPS (pH 8), 5 mM TCEP, 0.02% (w/v) Brij 35
17	50 mM TAPS (pH 8), 3.3 mM reduced glutathione, 1.65 mM oxidized glutathione
18	50 mM CHES (pH 9), 3.3 mM reduced glutathione, 1.65 mM oxidized glutathione
19	50 mM TAPS (pH 8), 3.3 mM reduced glutathione, 1.65 mM oxidized glutathione, 500 mM Arginine
20	50 mM CHES (pH 9), 3.3 mM reduced glutathione, 1.65 mM oxidized glutathione, 500 mM Arginine

Size exclusion chromatography

For the oligomeric state analysis, the supernatant obtained by centrifugation after the high-pressure treatment and the purified refolded sample of $HpaC_{St}$ were loaded onto a Superdex 75 10/300 GL column (GE Healthcare) pre-equilibrated with 50 mM CAPS (pH 10), 0.2 M NaCl and 1 mM TCEP. For the quaternary structure analysis of $HpaC_{St}$ in the presence or absence of FMN, the purified refolded $HpaC_{St}$ was injected onto a Superdex 75 10/300 GL column pre-equilibrated with 50 mM Tris–HCl (pH 8.5) and 0.2 M NaCl with or without the addition of FMN to the enzyme solution. The FMN-bound $HpaC_{St}$ was prepared by adding FMN at an FMN-to-$HpaC_{St}$ molar ratio of 5:1 and incubating at room temperature for 15 min. The column was eluted at a flow rate of 0.5 ml/min at 25 °C. Protein peaks were detected by UV absorbance at 280 nm.

Spectral analysis

Absorption spectra were recorded at 25 °C in the UV–VIS absorbance mode on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

CD measurements

Far-ultraviolet (UV) CD spectra were measured at 25 °C using a Jasco J-720 spectropolarimeter, which was set for a 200–260 nm wavelength range, 0.2 nm step resolution, 0.5 s response time, 1 nm bandwidth, and eight scans per sample. Samples of 5 μM refolded or solubly expressed $HpaC_{St}$ dissolved in 10 mM Tricine–HCl (pH 8.5) and 60 μM FMN were analyzed.

Flavin reductase assay

Flavin reductase assay was performed in a reaction mixture containing 6 μg/ml protein, 50 mM MOPS–KOH (pH 7.0), 200 μM NADH, and 30 μM FMN. Enzyme activity was determined by measuring the decrease in absorbance of NADH at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}) using a Beckman DU-7400 spectrophotometer.