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Heterologous overproduction of 2[4Fe4S]- and [2Fe2S]-type clostridial ferredoxins and [2Fe2S]-type agrobacterial ferredoxin

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ABSTRACT

Ferredoxins are small, acidic proteins containing iron–sulfur clusters that are widespread in living organisms. They play key roles as electron carriers in various metabolic processes, including respiration, photosynthesis, fermentation, nitrogen fixation, carbon dioxide fixation, and hydrogen production. However, only several kinds of ferredoxins are commercially available now, greatly limiting the investigation of ferredoxin-related enzymes and metabolic processes. Here we describe the heterologous overproduction of 2[4Fe4S]- and [2Fe2S]-type clostridial ferredoxins and [2Fe2S]-type agrobacterial ferredoxin. Adding extra iron and sulfur sources to the medium in combination with using *Escherichia coli* C41(DE3) harboring pCodonplus and pRKISC plasmids as host greatly enhanced iron–sulfur cluster synthesis in the three ferredoxins. After induction for 12 h in terrific broth and purification by affinity chromatography and anion exchange chromatography, approximately 3.4 mg of streptavidin (Strep)-tagged and 3.7 mg of polyhistidine (His)-tagged clostridial 2[4Fe4S] ferredoxins were obtained from 1 l of culture. Excitingly, after induction for 24 h in terrific broth, around 40 mg of His-tagged clostridial [2Fe2S] and 23 mg of His-tagged agrobacterial [2Fe2S] ferredoxins were purified from 1 l of culture. The recombinant ferredoxins apparently exhibited identical properties and physiological function to native ferredoxins. No negative impact of two different affinity tags on ferredoxin activity was found. In conclusion, we successfully developed a convenient method for heterologous overproduction of the three kinds of ferredoxins with satisfactory yields and activities, which would be very helpful for the ferredoxin-related researches.

1. Introduction

Ferredoxins are a group of iron- and sulfur-containing acidic proteins of low molecular mass (normally 6–15 kDa, occasionally up to 25 kDa) that are widespread in living organisms [1]. The iron and sulfur atoms in the proteins are organized into iron–sulfur clusters of mainly the [2Fe2S] or [4Fe4S] ([3Fe4S]) types, which can accept or discharge electrons depending on the change in oxidation state (+2 or +3) of iron atoms. The redox potential of ferredoxin is usually below −200 mV and can be as low as −500 mV in obligate anaerobic microorganisms [2,3]; however, some exceptions exhibit a redox potential ranging from +150 to +450 mV [4,5]. Therefore, ferredoxins can function as electron carriers in various metabolic processes, including respiration, photosynthesis, fermentation, nitrogen fixation, carbon dioxide fixation, and hydrogen production [1,6–8]. Recently, a novel energy coupling mechanism, flavin-based electron bifurcation, was discovered to play a key role in energy conservation of anaerobic archaea and bacteria [3,9]. Ferredoxin is a substrate required in all reactions catalyzed by electron-bifurcating enzymes reported till date [2,10–16]. Despite of the ubiquitous occurrence of ferredoxins and their involvement in a wide variety of metabolic processes, only several kinds of ferredoxins are commercially available, greatly limiting the investigation of ferredoxin-related enzymes and metabolic processes.

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There are many publications reporting the methods for ferredoxin production from various organisms or from recombinant *Escherichia coli* harboring plasmids with different ferredoxin genes [17–25]. The methods described usually include the large-scale of cultivation of organisms and purification by several complicated chromatography steps, which are labor intensive and time consuming, especially in case of strictly anaerobic microorganisms. Heterologous production in *E. coli* is hindered by low expression level, protein degradation, insufficient iron–sulfur cluster assembly, and iron–sulfur cluster degradation [23–26]. In addition, most studies aimed to characterize the properties of the small proteins without considering the productivity. Another important point is that the yield after purification was relatively low because of the loss during the multiple steps of purification, no matter from the wild type organisms or from the recombinant *E. coli*, where most of the recombinant ferredoxins were not fused with a tag.

Although ferredoxins from different organisms exhibit different properties, some are functionally interchangeable *in vitro* with varying efficiency [2,10,12–15,21,27]. This would benefit ferredoxin-involving biochemical studies, if several typical ferredoxins can be easily yielded in high amounts. For example, 2[4Fe4S] type ferredoxin from *Clostridium pasteurianum* has been shown ideal activities with many enzymes from different anaerobic bacteria and archaea, and has been widely used in their assays, especially for the electron bifurcating/confurcating enzymes [2,10,12–16,21]. In this report, we attempted to heterologously express three ferredoxin genes encoding ferredoxins of either [2Fe2S] or 2[4Fe4S] types from *C. pasteurianum* DSM 525 and *Agrobacterium tumefaciens* S33 [28,29]. The target proteins were designed to be tagged with streptavidin (Strep) or polyhistidine (His) in order to simplify the purification procedure by affinity chromatography and to reduce the loss during the purification caused by multiple separation steps. Native *C. pasteurianum* DSM 525 2[4Fe4S] ferredoxin is composed of 55 amino acids (5.63 kDa) and has a very low redox potential ($E^{0'} = -410$ mV), even as low as −500 mV *in vivo* [2,3,12,27,30–32]. *C. pasteurianum* DSM 525 [2Fe2S] ferredoxin is a homodimer with a molecular mass of 23.1 kDa in its native state. Each subunit is composed of 102 amino acids, and its midpoint redox potential is around −300 mV [33–35]. The *A. tumefaciens* S33 [2Fe2S] ferredoxin is composed of 106 amino acids (11.4 kDa), and its midpoint redox potential was reported to be approximately −220 mV [18]. It has 46.7% identity at protein sequence to the well-known putidaredoxin from *Pseudomonas putida* (redox potential of −240 mV) [36,37].

In this study, we tested several *E. coli* hosts, sources of iron and sulfur, and induction procedures to develop a convenient way to overproduce these three kinds of ferredoxins. We successfully overexpressed and purified Strep- and His-tagged clostridial 2 [4Fe4S] and His-tagged clostridial and agrobacterial [2Fe2S] ferredoxins with satisfactory yields and activities, paving the way for the large-scale production of ferredoxins. This would benefit the ferredoxin-related researches.

2. Materials and methods

2.1. Biochemicals

Native 2[4Fe4S] ferredoxin and ferredoxin-dependent monomeric [FeFe]-hydrogenase were purified under anaerobic conditions from the cells of *C. pasteurianum* DSM 525 as described in Refs. [11,19,38]. The electron-bifurcating iron–sulfur flavoprotein complex NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase (NfnAB) was prepared by heterologous expression according to a previous report [14]. Spinach [2Fe2S] ferredoxin, $NADP^+$, NADPH, NAD^+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma–Aldrich Corp.

2.2. Strains and growth

E. coli C41(DE3) harboring pCodonPlus and pRKISC [25] was a gift from Dr. Yasuhiro Takahashi (Osaka University, Osaka, Japan) and was used as the host for the expression of ferredoxins in this study. The pRKISC plasmid contains the *E. coli isc* locus (iron–sulfur cluster) [39] and has been successfully used for the expression of iron–sulfur proteins [25,40].

C. pasteurianum DSM 525 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and was grown on glucose–ammonium medium [14].

A. tumefaciens S33, deposited at the China Center for Type Culture Collection (CCTCC; Wuhan, Hubei, China) under the accession number CCTCC M206131, was grown in nicotine medium plus 1 g l^{-1} glucose, 0.2 g l^{-1} ammonium sulfate, and 1 g l^{-1} yeast extract [28,29].

E. coli was aerobically cultivated at 37 °C in medium containing antibiotic(s) depending on the plasmids harbored, where carbenicillin (25 mg l^{-1}) was added for maintaining pET-51b(+) Ek/LIC; ampicillin (100 mg l^{-1}) was added for pET-Duet1; and chloramphenicol (25 mg l^{-1}) and tetracycline (10 mg l^{-1}) were added for *E. coli* C41(DE3) harboring pCodonplus and pRKISC. Lysogeny broth (LB) was used for routine cell propagation. Tryptone phosphate (TP) broth and terrific broth (TB) were the working media for gene expression in *E. coli* BL21(DE3) and *E. coli* C41(DE3), respectively.

For plasmid propagation, *E. coli* cells were inoculated into 5 ml LB and cultivated with a shaking speed of 200 revolutions per minute (rpm) overnight. Then, the cells were harvested by centrifugation at 6000 × *g* for 10 min for plasmid extraction.

2.3. Construction of recombinant plasmids for ferredoxin expression

C. pasteurianum DSM 525 2[4Fe4S] ferredoxin gene is 171 nucleotides long (including the stop codon), and it is located on a DNA fragment (nucleotides 169–339) with the accession number M11214 in the GenBank database [30]. *C. pasteurianum* DSM 525 [2Fe2S] ferredoxin gene is 309 nucleotides long with accession number as Z19005 in the European Molecular Biology Laboratory database [34]. *A. tumefaciens* S33 [2Fe2S] ferredoxin gene is complement to the region from 4127 to 4447 on the scaffold with the accession number as JFFS01001129 in Genbank database.

Ferredoxin genes were amplified by polymerase chain reaction (PCR) using KOD Hot Start DNA Polymerase (Merck KGaA, Darmstadt, Germany) or High-Fidelity FastPfu DNA Polymerase (ShineGene Molecular Bio-Technologies, Inc., Shanghai, China) with the primers listed in Table 1 using *C. pasteurianum* DSM 525 and *A. tumefaciens* S33 genomic DNA as templates. The genomic DNA of *C. pasteurianum* DSM 525 and *A. tumefaciens* S33 was extracted from the cells and purified using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA).

To construct a recombinant plasmid expressing Strep-tagged 2 [4Fe4S] ferredoxin from *C. pasteurianum* DSM 525, the primers Strep-fd4-Cp-s and Strep-fd4-Cp-a (Table 1) were designed for ligation with the pET-51b(+) Ek/LIC vector (Merck KGaA), which carries an N-terminal eight-amino acid Strep-Tag II coding sequence. After purification using a MinElute PCR Purification Kit (Qiagen, Hilden, Germany), the blunt PCR product was treated with T4 DNA Polymerase in the presence of dATP to generate specific vector-compatible overhangs. Next, it was annealed into the linear pET-51b(+)Ek/LIC with single-stranded complementary overhangs