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Expression, purification and molecular modelling of the Iro protein from *Acidithiobacillus ferrooxidans* Fe-1

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Abstract

The Iro protein was proposed to be involved in the iron respiratory electron transport chain in *Acidithiobacillus ferrooxidans*, it is a member of HiPIP family with the iron–sulfur cluster for electron transfer. The gene of Iro protein from *A. ferrooxidans* Fe-1 was cloned and then successfully expressed in *Escherichia coli*, finally purified by one-step affinity chromatography to homogeneity. The recombinant protein was observed to be dimer. The molecular mass of a monomer containing the $[Fe_4S_4]$ cluster was 6847.35 Da by MALDI-TOF-MS. The optical and EPR spectra results of the recombinant protein confirmed that the iron–sulfur cluster was correctly inserted into the active site of the protein. Molecular modelling for the protein revealed that Cys20, Cys23, Cys32 and Cys45 were in ligation with the iron–sulfur cluster, and Tyr10 was important for the stability of the $[Fe_4S_4]$ cluster. As we know, this is the first report of expression in *E. coli* of the Iro protein from *A. ferrooxidans* Fe-1.

Keywords: *Acidithiobacillus ferrooxidans*; Iro protein; Expression; Purification; His-tag; Molecular modelling

Acidithiobacillus ferrooxidans is one of the most studied bacteria that thrives in acidic mine drainage, it is a Gram-negative and chemolithotrophic bacterium, which obtains energy through the oxidation of ferrous ion to ferric ion with molecular oxygen as the terminal electron acceptor [1]. The first step of energy transduction in this organism is catalyzed by an unknown protein coupled to cytochrome c552 reduction. The unknown protein in the first step was proposed to be the Iro protein, but the mechanism for electron transfer of the Iro protein is not clear so far. In the second step, cytochrome c552 transfers electrons to molecular oxygen via cytochrome *c* oxidase [2,3]. It is reported that the rusticyanin, a blue copper protein, also participates in the Fe(II) oxidation pathway because it undergoes reduction during the Fe(II) oxidation [3].

The Iro protein is a member of the high redox potential iron sulfur proteins (HiPIPs),[1] which generally function in electron transport. The HiPIPs are usually isolated from the purple phototrophic bacteria, form a class of small proteins (6–10 kDa) containing a $[Fe_4S_4]$ cluster. The cluster undergoes one-electron reactions between $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^{3+}$. The HiPIPs have redox potentials ranging from approximately +50 to +450 mV [4]. Most HiPIPs characterized are monomeric except the *Rhodospirillum salinarum* iso-2 HiPIP, which appears to be a tetramer [5]. A number of molecular structures of HiPIP proteins from various sources have been resolved either by X-ray crystallography

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[1] *Abbreviations used:* C, Cys, cysteine; RUS, rusticyanin; *A. ferrooxidans*, *Acidithiobacillus ferrooxidans*; HiPIP, high redox potential iron–sulfur proteins; IPTG, isopropyl-D-thiogalactopyranoside; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RMSD, root mean square deviation; SDS, sodium dodecylsulfate; UV/vis, ultraviolet–visible spectroscopy. EPR, electronic paramagnetic resonance.

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or by NMR [6–11], and the mechanism for electron transfer was also studied.

The Iro protein has a molecular mass of 63 kDa, containing 18–20 atoms of non-haem iron and 6 atoms of inorganic sulfide [12], which was proposed to be a homo-multimer molecule with the smallest subunit of 6 kDa, but the number of subunit is still unclear because the crystal structure of the Iro protein is unavailable so far. The amino acid sequence of Iro protein was reported to consist of 90 amino acids, including a 37-residue signal sequence [13]. Another report revealed that this protein has a molecular mass of 24 kDa instead of 63 kDa, which was proposed to be a tetramer [14]. The Iro protein has not yet been extensively characterized mainly due to the lack of an efficient expression system for producing the protein in *E. coli*. The small size of the polypeptide and poor initiation of translation may result in difficulty in expression.

In this study, the gene of Iro protein was cloned and expressed in *E.coli*, finally purified by one-step affinity chromatography. The recombinant protein exhibited all the properties of native Iro protein and the iron–sulfur cluster was correctly incorporated into the active site cavity of the protein. As we know so far, this is the first report of expression in *E.coli* of the Iro protein from *A. ferrooxidans* Fe-1.

Materials and methods

Materials

Acidithiobacillus ferrooxidans Fe-1 was isolated in our laboratory, which was identified to be identical with previous report by genomic DNA sequence analysis [13]. A HiTrap chelating metal affinity column was purchased from GE healthcare LTD. HB101 competent cells, *E. coli* strain BL21(DE3) competent cells came from Invitrogen Life Technologies. The Plasmid Mini kit, a gel extraction kit and synthesized oligonucleotides were obtained from Sangon Company of Shanghai. *Taq* DNA polymerase, T4 DNA ligase and restriction enzymes came from MBI Fermentas of Germany. All other reagents were of research grade or better and were obtained from commercial sources.

Cloning of the Iro protein gene from A. ferrooxidans Fe-1

Genomic DNA from *A. ferrooxidans* Fe-1 was prepared using the EZ-10 spin column genomic DNA isolation kit from Bio Basic Inc., according to the manufacturer's instructions for bacterial DNA extraction. This genomic DNA was used as a template for PCR. The gene was amplified by PCR using primers that were designed to add six continuous histidine codons to the 5′-primer. The sequence of the forward primer was 5′- CGCGCGGATCCAGGAGGAATTTAAAATGAGAGGATCGCATCACCATCACCATCACGGAAGCATGCCTAAGGCAGCGGTGCAATATC-3′, containing a *Bam*HI site (GGATCC), a ribosome binding site (AGGAGGA), codons for the amino acid sequence MRGSHHHHHH (start codon and hexahistag), and codons for amino acids 2–11 of mature Iro protein. The sequence of the reverse primer was 5′-CTGCAGGTCGACTCATGCTGACTTAGGAACAAAGGCTACACA-3′, containing a SalI site (GTCGAC), a stop anticodon (TCA), and anticodons for the last six amino acids of mature Iro protein. PCR amplification was performed using *Taq* DNA polymerase, and samples were subjected to 25 cycles of 45 s of denaturation at 95 °C, 1 min of annealing at 55 °C, and 2 min of elongation at 72 °C in a Mastercycler Personal of Eppendorf Model made in Germany. The amplification products were analyzed by electrophoresis on a 0.9% agarose gel and stained with ethidium bromide. The resulting PCR product was gel purified, double digested and ligated into a pLM1 [15] expression vector resulting in the pLM1::IRO plasmid. The constructed pLM1::IRO plasmid was transformed into HB101 competent cells for screening purposes. The positive colony with a gene insert in the plasmid was identified by single restriction digestion of the plasmid with *Bam*HI, double restriction digestion of the plasmid with *Bam*HI and SalI, followed with agarose gel analysis. The identified positive colony was grown in LB medium containing ampicillin (50 mg/L), and the plasmid pLM1::IRO was isolated from harvested bacteria cells using a plasmid extraction kit. The isolated pLM1::IRO plasmid was then transformed into *E. coli* strain BL21(DE3) competent cells for expression purposes. DNA sequencing of the cloned Iro protein gene was performed and the inserted gene sequence was identified as previously reported without any mutation.

Expression of soluble recombinant protein of Iro protein from A. ferrooxidans Fe-1 in E. coli

We use the following procedure for expression of soluble Iro protein. Normally, the *E. coli* strain BL21(DE3) cells with pLM1::IRO plasmid was grown at 37 °C in 500 ml of TB medium containing ampicillin (100 mg/L) to an OD_{600} of 0.6. At this point, the cells were incubated at room temperature with the addition of 0.5 mM IPTG overnight with shaking at 180 rpm. The cells were harvested by centrifugation and the cell pellet was washed with an equal volume of sterile water. The cells were again harvested by centrifugation, suspended in start buffer (20 mM potassium phosphate, pH 7.4, 0.5 M NaCl), incubated with 5 mg lysozyme at room temperature for half an hour, and then stored at −80 °C for purification.

Purification of the Iro protein from A. ferrooxidans Fe-1

The cells were lysed by sonication four times for 30 s each time using a 150-W Autotune Series High Intensity Ultrasonic sonicator equipped with a 8 mm-diameter tip. The insoluble debris was removed by centrifugation and the clear supernatant was used for protein purification. The Hi-Trap column was first equilibrated with 0.1 M nickel sulfate to charge the column with nickel ions followed by 5 column volumes of MiliQ water to remove unbound nickel