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Expression, purification and characterization of the soluble Cu_A domain of cytochrome c oxidase of *Paracoccus versutus*

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Abstract **The key subunit Ⅱ of cytochrome c oxidase (CcO) contains a soluble binuclear copper center (Cu_A) domain. The Cu_A domain of *Paracoccus versutus* was cloned, expressed, purified and characterized. The gene encoding the Cu_A domain in pET11d vector was expressed in *E. coli* BL21 (DE3). The results showed that the Cu_A domain was expressed mostly in inclusion bodies and the Cu_A domain protein synthesized in *E. coli* cells represents approximately 10 percent of the total cellular proteins. Dissolved in urea, dialyzed and recombined with Cu^+/Cu^{2+} and purified by the Q-sepharose fast flow anion-exchange column and Sephadex G-75 gel filtration column, the soluble purple-colored protein, which shows a single band in electrophoresis, was obtained. The UV-visible absorption spectrum of Cu_A domain showed that there are intense band at 478 nm and a shoulder peak at 530 nm, and two weak bands at 360 and 806 nm respectively, which can be assigned to the charge transfer and the interactions of obitals of Cu—S and Cu—Cu in the mixed-valence binuclear metal center ($Cu_2S_2R_2$). The far-UV CD spectrum indicated that this domain is predominantly in β-sheet structure. The fluorescence spectra showed that its maximal excitation wavelength and maximal emission wavelength are at 280 and 345 nm, respectively.**

Keywords: cytochrome coxidase, Cu_A domain protein, gene expression, purification, spectra.

Cytochrome c oxidase (CcO) is the terminal enzyme of the electron transport chain in the inner mitochondria membrane of eukaryotes and in the cytoplasmic membrane of many aerobic bacteria. It transfers electrons from cytochrome c to oxygen molecule and reduces the dioxygen to water. Electron transfer is coupled to the proton pumping function. Concomitant with this electron transfer, CcO simultaneously pumps protons from the matrix side to the cytosol side of the membrane. In this way, an electrochemical gradient is generated for the synthesis of ATP. The equation of the overall reaction catalyzed by the enzyme is as follows:

$$4\ \text{cyt c}\ (Fe^{2+}) + 8\ H^+_{(inside)} + O_2 = 4\ \text{cyt c}\ (Fe^{3+}) + 4\ H^+_{(outside)} + 2\ H_2O$$

Cytochrome c oxidase is a complex molecule consisting of multiple subunits. Many researches[1—3] have shown that the catalytic core subunit Ⅰ of CcO contains the metal center structure of heme a and heme a_3-Cu_B, and the catalytic core subunit Ⅱ of CcO contains a soluble domain including a binuclear Cu_A center, which is the primary electron acceptor from cytochrome c. From Cu_A the electron is transferred to heme a and then to heme a_3-Cu_B binuclear center, it is here that oxygen molecule is reduced to water. The Cu_A center is a highly delocalized mixed-valence [$Cu^{+1.5}$—$Cu^{+1.5}$] binuclear copper complex, the two copper ions are bridged by two cysteine thiolates, which is a novel chemical structure in biological systems. Recent epidemic investigations have shown that there is an obvious relativity between CcO and mitochondrial diseases, e.g. CcO is abnormal in most patients with Alzheimer's disease[4,5]. Probably, it is attributed to the mutations of amino acids and improper assemblies of CcO. To explore the molecular mechanism for electron transfer of CcO and investigate the effects of mutations of amino acids on the structure, the properties and functions of CcO, as well as to provide the evidence for elucidating the molecular mechanism of mitochondrial diseases, the soluble binuclear copper center (Cu_A) domain of CcO from *Paracoccus versutus* was cloned, expressed, purified and characterized by using protein engineering techniques and biochemical methods.

1 Experimental

(ⅰ) Instruments, materials and reagents. Jasco J-715 Spectropolarimeter, HP8453 Diode Array Spectrophotometer (USA), and Cary Eclipse Fluorescence Spectrophotometer were used.

The gene encoding the soluble Cu_A domain of cytochrome c oxidase from *Paracoccus versutus* was from the laboratory of Professor Gerard W. Canters, Leiden University, The Netherlands. *E. coli* BL21 (DE3) cells were reserved in our laboratory. Restriction endonucleases *Nco* Ⅰ and *Bam*H Ⅰ were purchased from New England BioLabs. Tryptone and Yeast Extract were purchased from Unipath Ltd., England. Isopropylthio-β-D-galactoside (IPTG) and Sephadex G-75 were obtained from Sigma. Dithiothreitol (DTT) was obtained from Shanghai Sangon Biotechnology Company. Q-Sepharose Fast Flow was purchased from Pharmacia Biotech. All other reagents were of analytical or biochemical quality.

(ⅱ) Gene cloning of Cu_A domain. The extraction and purification of plasmid, the sequencing of the gene, the preparation of the competent cells and fresh transformants were manipulated according to the general methods of molecular biology[6].

(ⅲ) Expression and purification of Cu_A domain[7]. The gene encoding the soluble Cu_A domain of *Paracoccus*

versutus was inserted into pET11d vector, the restriction sites were *Nco* Ⅰ and *Bam*H Ⅰ. The recombinant plasmid ($pETCu_A$) was transformed into *E. coli* BL21 (DE3) cells and the single colonies containing the $pETCu_A$ were screened with LB solid culture plate containing 100 μg/mL ampicillin. A small amount of LB liquid culture medium containing 100 μg/mL ampicillin was inoculated with a single colony from the freshly streaked plate of BL21 (DE3) cells containing the $pETCu_A$ plasmid. After incubation for 3 h at 37℃ with shaking at 200 r/m, these cultures were used to inoculate a large amount of LB liquid culture medium containing 100 μg/mL ampicillin. The LB cultures were incubated at 37℃ and shaked at 200 r/m, typically for about 3—4 h, until the optical density reached 0.4—0.6 at 600 nm, and the expression was induced with a final concentration of 0.2 mmol/L IPTG. The cells were harvested 2.5 h after induction, washed with 20 mmol/L Tris-HCl (pH 8.2) buffer.

The collected *E. coli* cells were suspended in the "standard buffer": 20 mmol/L Tris-HCl (pH 8.2) containing proteolytic inhibitors of 0.15 mmol/L phenylmethylsulfonyl fluoride and 5 mmol/L benzamidine. Then the lysozyme and DNase were added, and the mixture was stirred and broken by ultrasonication on ice. The suspension was centrifuged at 15000 r/min for 40 min at 4℃. The supernatant was discarded, and the pellet was re-suspended into the "standard buffer" containing 3% (*w*/*v*) Triton X-100. After incubation for 2 h on ice, the suspension was centrifuged to separate the inclusion body pellet from the solubilized membrane proteins. The pellet was washed with the "standard buffer" containing 1% (*w*/*v*) Triton X-100.

The pellet obtained above was dissolved in 6 mol/L urea, 20 mmol/L Tris-HCl (pH 8.2) solution. The dissolved protein was first dialyzed for 4 h against 2 mol/L urea, 0.5 mmol/L DTT, 20 mmol/L Tris-HCl (pH 8.2) buffer, then for 4 h against 0.5 mmol/L DTT, 20 mmol/L Tris-HCl (pH 8.2), and overnight against 200 μmol/L CuCl, 20 mmol/L Tris-HCl (pH 8.2). The final dialysis was done against 200 μmol/L $CuCl_2$, 20 mmol/L Tris-HCl (pH 8.2) for 4 h. Dialysis was carried out at 4℃. The supernatant of dialysis, which has a distinct purple color, was loaded onto a Q-Sepharose fast flow anion-exchange column that had been equilibrated with 20 mmol/L Tris-HCl (pH 8.2) buffer containing 200 μmol/L $CuCl_2$, and eluted with 0—1 mol/L NaCl linear gradient in 20 mmol/L Tris-HCl (pH 8.2) buffer containing 200 μmol/L $CuCl_2$. Fractions containing the purple colored Cu_A domain protein were pooled. After concentration in an ultrafilter, the target protein was loaded onto a Sephadex G-75 gel filtration equilibrated with 20 mmol/L Bis-Tris-HCl (pH 7.0) buffer containing 50 μmol/L $CuCl_2$, and the fractions of Cu_A domain protein were collected. All the columns were run at 4℃.

(ⅳ) Characterization of the Cu_A domain. SDS-PAGE was carried out by using the method of ref. [6], and the gel was stained in a solution containing 0.25% Coomassie Brilliant Blue R250. UV-visible absorption spectrum was measured in a solution containing 50 μmol/L Cu_A domain protein in 20 mmol/L Tris-HCl buffer (pH 7.5). Circular dichroism was employed in a solution containing 16 μmol/L Cu_A domain protein in 20 mmol/L Tris-HCl buffer (pH 7.5), purged with N_2 at a flow rate of 5 L/min. The path-length of the sample cuvette was 1 mm. The scan speed was 100 nm/min, the resolution was 0.2 nm, and the response time was 0.25 s. Fluorescence spectra were measured in a solution containing 1 μmol/L Cu_A domain protein in 20 mmol/L Tris-HCl buffer (pH 7.5). The slits of both excitation and emission were 5 nm. Excitation spectrum was recorded from 250 nm to 320 nm when the emission wavelength was fixed at 345 nm. Emission spectrum was recorded from 290 nm to 500 nm when the excitation wavelength was fixed at 280 nm.

2 Results and discussion

(ⅰ) The gene cloning and expression of the Cu_A domain. The nucleotide sequence of the gene encoding the soluble Cu_A domain of cytochrome c oxidase of *Paracoccus versutus* was measured. The gene is composed of 462 bp nucleotides, including the start codon ATG. It encodes the amino acids from 128 to 280 in subunit Ⅱ of CcO. SDS-PAGE analysis (fig. 1) showed that molecular weight of the soluble Cu_A domain protein is about 17500 u, this is consistent with the predicted molecular weight of 17248.6 u. The gene encoding the Cu_A domain, which was inserted into a pET11d vector by using its restriction sites *Nco* Ⅰ and *Bam*H Ⅰ, was transformed into *E. coli* BL21 (DE3), and they were incubated, screened and expressed in LB culture medium containing ampicillin. The expression conditions were optimized and it was found that a key factor for the gene expression was that only the fresh transformants could be expressed efficiently. A range of concentration of IPTG from 0 to 1 mmol/L was used to test the induction efficiency. It was shown that there was also a low expression in the absence of IPTG (see lane 1 in fig. 1), and the expression did not increased with the increasing concentration of IPTG after IPTG concentration reached 0.2 mmol/L. From lane 2 in fig. 1 of SDS-PAGE it is known that the Cu_A domain protein synthesized in *E. coli* cells represents approximately 10 percent of the total cellular protein. From lanes 3 and 4 in fig. 1 of SDS-PAGE, it was shown that there was only a small amount of the target protein in the supernatant of the whole-cell lysate, but most of the expected expression product existed in the precipitate, i.e. the Cu_A domain protein was expressed mostly in inclusion bodies in such a condition.

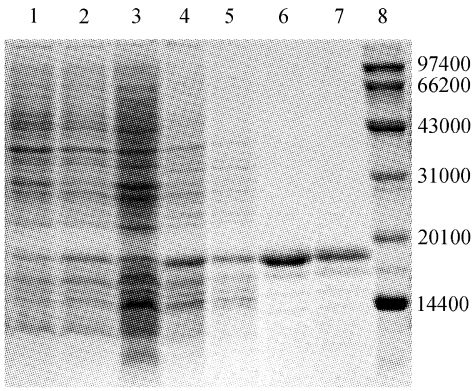

Fig. 1. SDS-PAGE analysis of polypeptide composition of the preparations after different purification steps. Lane 1, The whole-cell lysate of *E. coli* BL21(DE3) carrying pET11d. Lane 2, The whole-cell lysate of *E. coli* BL21(DE3) carrying pET11d, 0.2 mmol/L IPTG induction. Lane 3, Supernatant of the whole-cell lysate. Lane 4, The pellet of the whole-cell lysate. Lane 5, The purified inclusion bodies. Lane 6, Preparation after chromatography on Q-Sepharose. Lane 7, Preparation after chromatography on Sephadex G-75. Lane 8, Molecular weight markers of protein.

(ii) Isolation and purification of Cu_A domain. When the expression strain was fermented on a large scale, about 8.2 g *E. coli* BL21 (DE3) cells in wet weight was obtained per 4 liters of LB culture medium. After the cells were broken and the suspension was centrifuged, the obtained pellet, consisting of the inclusion bodies and membrane proteins, was solubilized by surfacant Triton X-100 and then centrifuged and washed to discard the solubilized membrane proteins, and finally the pure inclusion bodies precipitates were obtained. From lane 5 in fig. 1 of SDS-PAGE, it is indicated clearly that most membrane proteins had been removed and there were hardly the other proteins in the pure inclusion bodies. In this state, the Cu_A domain protein presented itself in the apo-form. Therefore, it needed further treatment for getting the holo-form. The pure inclusion bodies were dissolved in urea and dialyzed against different buffers containing DTT and Cu^+ and Cu^{2+}. Cysteines in the protein were reduced to free Cys-SH form in the presence of DTT. Furthermore, with the addition of Cu^+ and Cu^{2+}, the apo-Cu_A domain protein could be recombined and renatured to the holo-form, and it could be observed that the protein solution had become distinct purple. The protein was purified by using the Q-sepharose fast flow anion-exchange column, and the purple colored Cu_A domain protein was eluted at 0.45 mol/L NaCl when eluted with 0—1 mol/L NaCl linear gradient. Finally, the purified protein which was more than 95% pure was obtained by the Sephadex G-75 gel filtration column (see lane 7 in fig. 1).

(iii) Characterization of Cu_A Domain. Fig. 2 is the UV-visible absorption spectrum of Cu_A domain protein in 20 mmol/L Tris-HCl buffer at pH 7.5. It shows that there is an intense band at 478 nm and a shoulder peak at 530 nm, and two weak bands at 360 and 806 nm, respectively. Only the weak peak of Cu_A domain protein corresponding to 806 nm can be seen in the intact CcO complex, but other peaks cannot be observed, because the intense absorbance of heme a and heme a_3 effectively mask the optical spectra of Cu_A. The UV-visible absorption spectrum of Cu_A domain is different from that of blue copper protein which is dominated by a ligand-to-metal charge transfer (LMCT) band near 600 nm. However, it is very similar to that of the Cu_A domain in the N_2O reductase[8] and in CcO of *Paracoccus denitrificans*[7]. Cu_A is a binuclear center with a mixed-valence state [$Cu^{+1.5}$—$Cu^{+1.5}$], the two copper ions are bridged by the two cysteine thiolates, and the copper ions and the Cys sulphurs lie in one plane. The ligands for each Cu^{2+} form a distorted tetrahedron. These absorption bands can be assigned to the interactions of charge transfer and their obitals of Cu—S and Cu—Cu in the mixed-valence binuclear metal center ($Cu_2S_2R_2$)[9]. Many studies have shown that it is a novel chemical structure in biological system, and it is different from that known in blue copper protein[1, 2, 9].

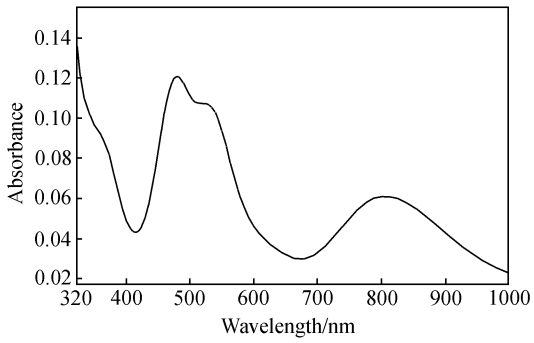

Fig. 2. UV-visible absorbance spectrum of Cu_A domain of *Paracoccus versutus*. The spectrum was obtained from a solution containing 50 μmol/L protein in 20 mmol/L Tris-HCl buffer at pH 7.5.

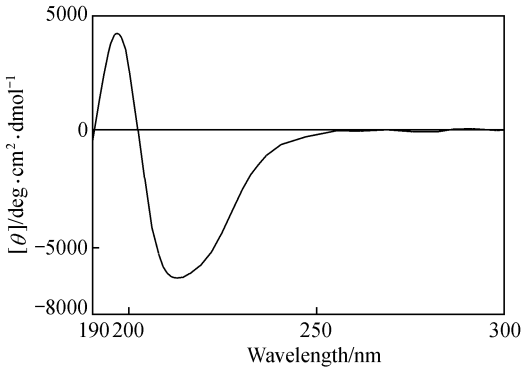

Fig. 3. Far-UV CD spectrum of the Cu_A domain of *Paracoccus versutus*. The spectrum was obtained from a solution containing 16 μmol/L protein in 20 mmol/L Tris-HCl buffer at pH 7.5.

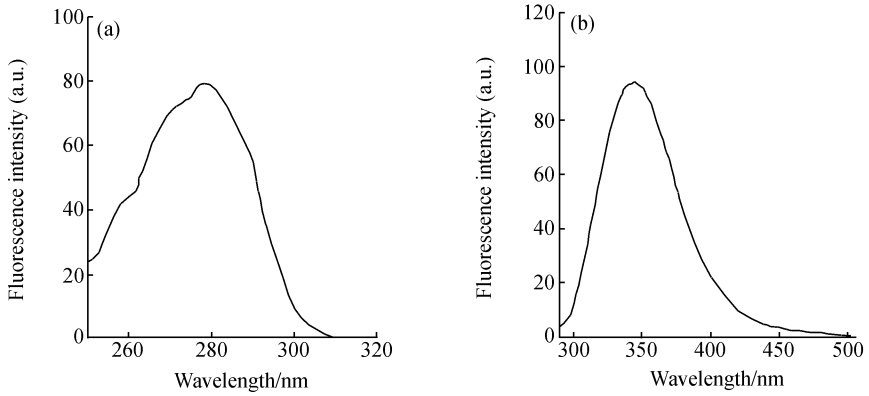

Fig. 4. Fluorescence spectra of the Cu_A domain from *Paracoccus versutus*. The spectra were obtained from a solution containing 1 μmol/L protein in 20 mmol/L Tris-HCl buffer at pH 7.5. (a) The excitation spectrum; (b) the emission spectrum.

Fig. 3 is the far-UV circular dichroism (CD) spectrum of Cu_A domain protein in 20 mmol/L Tris-HCl buffer at pH 7.5. It displays an obvious negative peak at 213 nm, which indicates that this protein is predominantly of β-sheet structure. The CD spectrum of Cu_A domain protein is similar to that of the water-soluble recombinant Cu_A-domain of the cytochrome ba_3 from *Thermus thermophilus*[10], and is in agreement with the X-ray structure reported for the *Paracoccus denitrificans* CcO molecule[1].

Fig. 4 is the fluorescence spectra of Cu_A domain protein in 20 mmol/L Tris-HCl buffer at pH 7.5. Fig. 4(a) is the excitation spectrum and fig. 4(b) is the emission spectrum. The maximal excitation wavelength is 280 nm, and there are three shoulder peaks at 258, 270 and 288 nm respectively, corresponding to its absorption spectrum. These bands are attributed to the phenylalanines (Phe), tyrosines (Tyr) and tryptophans (Trp) of the Cu_A domain protein, the band at 258 nm is contributed by Phe and others by Tyr and Trp. Excitation above 280 nm abolishes the fluorescence emission of Phe, and only exhibits the fluorescence emission of Trp in proteins due to Tyr-to-Trp energy transfer and quenching of nearby groups on the peptide chain. Trp emission is very sensitive to the microenvironment polarity, and its maximal emission wavelength is not identical in different proteins. The Cu_A domain protein has 5 Trp and 8 Tyr fluorophores, the maximal emission wavelength is 345 nm, which indicates that there is only a small exposure to the protein surface among these Trp residues since the free Trp emits at 351—361 nm, depending on the pH value[11]. Furthermore, the result is also in agreement with the X-ray structure reported for the *Paracoccus denitrificans* CcO molecule[1]. The fluorescence spectra of this protein had not been reported as far as we have known. The further study on the Cu_A domain is being undertaken.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant No. 39990600).

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(Received April 16, 2001)