Soluble CuA-binding Domain from the *Paracoccus* Cytochrome c Oxidase*

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In cytochrome c oxidase the C-terminal part of subunit II is outside the membrane and contains a copper center called CuA. We have expressed this domain of the *Paracoccus denitrificans* oxidase in a soluble form. Data obtained by quantitative copper-to-protein measurements, electrospray mass spectrometry, and electron paramagnetic resonance spectroscopy show that the center contains two copper atoms probably in a mixed valence configuration. Its absorbance spectrum is similar to that of the copper center A in nitrous oxide reductase. The EPR spectrum suggests that the center in the soluble protein is closely related to the native CuA site in the cytochrome oxidase complex. However, it seems likely that the copper center in the soluble domain is more exposed to the aqueous milieu than in the intact complex because its absorbance and EPR spectra are sensitive to pH. At alkaline pH one of the copper ligands in the site acquires type-2 character, indicating that it may be coordinately bound to a new ligand. The pK of this reversible change is about 8.2. The CuA-binding fragment is able to oxidize cytochrome c.

We have recently studied an engineered CuA-like center built into the Cyoa subunit of the Escherichia coli cytochrome bo quinol oxidase complex. These studies on the isolated soluble protein fragment and on its mutants have shown that this purple copper center is indeed binuclear. The main ligands of the two coppers appear to be 2 cysteines, 2 histidines, and a methionine (7). Here we continue this study with a homologous domain isolated from the soluble cytochrome c oxidase. It has been expressed from the ctaC gene of *Paracoccus denitrificans*. The results obtained with this native CuA-binding domain confirm the binuclear nature of the site.

EXPERIMENTAL PROCEDURES

Expression of the Subunit II Fragment—The region of ctaC coding for amino acid residues 128–290 (14) was amplified by polymerase chain reaction. The primers introduced the upstream NcoI and downstream HindIII restriction sites to the DNA fragment. The NcoI site affects the residue following N-terminal methionine which is valine in our construct but leucine in the native CtaC sequence (Tables I and II (14, 15)). The polymerase chain reaction fragment was inserted into a modified pET3d vector described in (4); the resulting plasmid was called pET.PD1. This construct was used to express the subunit II domain in *E. coli* BL21(DE3) cells. Protein purification, freshly streaked LB plates containing ampicillin (0.1 mg/ml) were used to inoculate 100-ml cultures in 250-ml Erlenmeyer bottles. After shaking (200 revolutions/min) for 3 h at 37 °C, these cultures were used to inoculate 1-liter cultures in twelve 2-liter Erlenmeyer bottles. The cells were grown 3-4 h to the optical density of 0.6 at 600 nm, and the expression was harvested with isopropyl-thio-galactoside (0.2 mM). The cells were washed 2.5× after induction, washed with 20 mM Tris (pH 8.2), and stored at −80 °C.

Protein Refolding and Purification—About 15 g of *E. coli* cells (the yield from 6 liters of bacterial culture) were suspended in 40 ml of our "standard Tris buffer": 20 mM Tris (pH 8.2) containing proteolytic inhibitors phenylmethylsulfonyl fluoride (0.15 mM) and benzamidine (5 mM), and broken with a French press at 4 °C. The viscosity of the suspension was reduced by adding deoxyribonuclease (2 mg) and MgSO₄ (1 mM). The suspension was centrifuged for 40 min at 40,000 revolutions/min in a Beckman Ti-45 rotor. Almost all of the supernatant was therefore discarded, and the pellet was resuspended into 50 ml of standard Tris buffer containing 3% (w/v) Triton X-100. After incubation for 2 h on ice, the suspension was briefly centrifuged to separate the inclusion body pellet from the solubilized membrane proteins. The pellet was washed with 50 ml of standard Tris buffer containing 1% of Triton X-100, and stored at −20 °C.

The pellet obtained above was dissolved in 40 ml of 6 M urea, 20 mM Tris (pH 8.2). The dissolved protein was first dialyzed for 4 h against 4 M urea, 20 mM Tris (pH 8.2). The dissolved protein was then dialyzed against 20 mM Tris (pH 8.2), 0.5 mM dithiothreitol (DTT), and overnight against 20 mM Tris, 200 μM CuCl₂. The final dialysis was done against 20 mM Tris (pH 8.2), 200 μM CuCl₂ for 4 h. Dialysis was carried out at 4 °C.

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1 The abbreviations used are: DTT, dithiothreitol; BisTris, bis(2-hydroxyethyl)mimnotriis(hydroxyethyl)methane; MES, 2-(N-morpholino)ethanesulfonic acid.
Expression of a Soluble CuA Domain

The sequence of the C-terminal domain of subunit II

The amino acid sequence of subunit II is taken from Ref. 14. N-terminal residues methionine and valine which are added to the native sequence, are shown in bold. C-terminal extension of the protein (15) is indicated with small letters. The copper-binding ligands (7) are underlined. Residue numbers are on the right.

| MVFRS | QEMPNDPDLV IKAIGHQYW YSEYPNPGVA FDALMLEKEA LDAGYSEDE | 180 |
|       | YLLATDNPV VPVGKVVLVQ VTATDVHAW TIPFAVQKD APVRIAQQLW | 230 |
|       | FSVQEYVYP GQCSLGCIN HAYMPIVVKA VSEQYEAWL AGAEEKFAA | 280 |
|       | dasdylpasp vklasae | 297 |

*Table I*
The primers used in plasmid constructions

The NcoI and HindIII restriction sites are underlined, and the beginning and the end of the reading frame are shown.

*Table II*

| Primer | (128) |
|        | MVFRS | QEMP |
|        | 118   | 5'-GCCGCCATGGTGTTCCGAGGATGCCG-3' |
|        |       | (280) |
|        | AKEEFAA |  |
|        | 155   | 3'-CGCTTTCTCCTTAAGCGGCGGATTTTCGAGGGC-5' |

*Table III*

Copper/protein ratios

| pH | Cu/protein | EPR-detectable Cu |
|----|------------|-------------------|
| 7.0 | 1.9 | 53 |
| 9.0 | 1.9 | 57 |

**Fig. 1. Purification of the subunit II fragment.** Polypeptide composition of the preparations after different purification steps are shown. Lane 1, total protein extract of E. coli expressing the subunit II fragment; lane 2, purified inclusion bodies; lane 3, preparation after chromatography on Q-Sepharose; lane 4, preparation after the final Superdex 75 column. Molecular mass scale (kDa) is shown on the left side.

in a 3.5-kDa cutoff tubing. A part of the protein precipitates during dialysis. This was removed by centrifugation for 40 min at 45,000 revolutions/min in a Beckman Ti-60 rotor.

The supernatant was applied to a Q-Sepharose fast flow anion-exchange column (20 ml bed volume) equilibrated with 20 mM Tris (pH 8.2) containing 200 μM CuCl₂. The flow rate was 6 ml/min, and a linear NaCl gradient from 0 to 1 M was developed during 120 min. The peak fractions containing the subunit II fragment, which elutes at 45-50 ml, were saved, concentrated in Microsep concentrators (Filtron Co., MA) to approximately 1 ml and loaded to a Superdex 75 HiLoad gel filtration column (120 ml, Pharmacia LKB Biotechnology Inc.) which had been equilibrated with 20 mM BisTris (pH 7.0) containing 200 mM NaCl. The flow rate was 1.0 ml/min. The subunit II fragment elutes at 65-70 ml. All the columns were run with a Pharmacia fast protein liquid chromatography instrument at room temperature.

**Spectroscopy—Optical spectra** were recorded with a Perkin Elmer Lambda 2 Spectrophotometer at room temperature. Electrospray mass spectra were recorded with a Sciex API III instrument as described in Ref. 7. Approtein samples for mass spectra were produced by incubating the subunit II fragment with 20 mM EDTA for 24 h. The protein samples were desalted by gel filtration on Sephadex G-25 columns (PD-10, Pharmacia) equilibrated with distilled water. 0.05% formic acid was added to the sample just before spraying into the mass spectrometer. EPR spectra were recorded with a Bruker ER 200D-SRC X-band spectrometer equipped with a standard TE102 rectangular cavity and an Oxford Instruments ESR-9 helium flow cryostat. Temperatures above 100 K were obtained with a nitrogen gas flow system. Quantifications of the EPR spectra were performed under non-saturating conditions as described earlier (16). For EPR, the samples were exchanged to buffers with desired pH by gel filtration on PDlO columns.

**Fig. 2. Crystals of the CuA-binding domain.** The crystals were grown by vapor diffusion in hanging drops as described under "Experimental Procedures." Bundles of needle-like crystals formed in 2-3 days. The crystals are purple (not shown). The scale bar is 0.1 mm.

**Miscellaneous—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate** was carried out using the buffer system of Laemmli (17). Analytical ultracentrifugation was kindly carried out with a Beckman XLA Optima centrifuge by Ariel Lustig (Biocenter, University of Basel). Copper was determined according to Ref. 18. The free copper was removed from the samples by gel filtration on PD10 columns.
Expression of a Soluble CuA Domain

Fig. 3. Electrospray mass spectra. The samples were prepared as described under "Experimental Procedures." A, the apoprotein form of the subunit II fragment. B, The Cu-binding protein. The original mass spectra has been deconvoluted as described in Ref. 21.

Table IV

| Sample | Observed mass | Calculated mass | Mass difference |
|--------|---------------|-----------------|-----------------|
| Apoprotein | 17,146 | 17,146 | 0 |
| Purple protein | 17,272 | 17,272 | 126 |
| A' | 17,142 | 17,142 | 0 |
| B' | 17,142 | 17,142 | 0 |

*The two forms (a and b) of the apoprotein correspond to the full-length sequence (Table I) and to the protein which has lost its N-terminal methionine. The molecular masses of these proteins with added copper (a' and b') are shown above. The mass differences have been calculated for each of the two forms in the presence and absence of copper (i.e. a'-a and b'-b).*

RESULTS

Expression of the CuA-binding Domain—Several constructs were made in order to express the CuA-binding domain from subunit II of the P. denitrificans cytochrome c oxidase in E. coli. The highest amount of protein was expressed with a construct which has its N terminus immediately after the second predicted transmembrane helix (20) and its C terminus 17 residues before ctaC translation stop codon (Tables I and II). These last 17 amino acids make a C-terminal extension of the precursor which is removed post-translationally (15). The protein was expressed at a high level in the cytoplasm of E. coli, but it was mostly in inclusion bodies (Fig. 1). Only 10–20% of the fragment was found in soluble fraction. We could not find spectroscopically any purple copper in this minor soluble fraction after purification and addition of copper. This indicates that the soluble form subunit II fragment may be modified or incorrectly folded in the bacterial cytoplasm.

In contrast, we were able to produce a homogeneous protein preparation with a characteristic purple copper spectrum by refolding the denatured protein of inclusion bodies by the removal of urea (see "Experimental Procedures"). It appeared to be necessary to refold the protein in the presence of DTT in order to obtain a preparation which binds CuA quantitatively. It is also necessary to introduce the copper after the refolding of the protein. The addition of copper during the folding process led to the formation of a colorless product.

The renatured protein was purified using two chromatographic steps: first anion-exchange on a Q-Sepharose column and then gel filtration on a Superdex 75 column (Fig. 1). In the anion-exchange chromatography, the subunit II fragment was separated from β-lactamase, the other major protein in the inclusion bodies (upper band in lane 2 in Fig. 1). The protein was more than 95% pure after chromatography on Q-Sepharose, and the purification was finalized with the gel filtration step. The hydrodynamic molecular mass of the subunit II fragment was measured to be 17 kDa by sedimentation equilibrium. This agrees with the calculated molecular weight (Table IV) and shows that the protein is a monomer.

Binuclear Nature of Copper Center—The results of colorimetric copper measurements (Table III) show that the mon-
Expression of a Soluble CuA Domain

Fig. 4. Absorbance spectra of CuA domain. Purified protein samples were passed through small gel filtration columns equilibrated with 40 mM BisTris-propane buffer at different pH values. All samples were 100 μM. The absorbance scale is given by a bar. The locations of spectral maxima at pH 6-10 (from top to bottom) are shown.

Table V

Spectral properties of CuA site in the soluble domain at pH 7.0

| nm  | m.M⁻¹ cm⁻¹ |
|-----|------------|
| 363 | 1.2        |
| 420 | 0.8        |
| 480 | 3.0        |
| 808 | 1.6        |

omeric protein binds almost exactly two coppers. We also carried out electrospray mass spectroscopy to determine the number of copper atoms bound in the COII domain. The mass spectrometric data indicate that the apoprotein has two different molecular species with a mass difference of 131 daltons (Fig. 3A). The mass of the larger apoprotein fragment is 17,146 daltons which is equivalent to the calculated molecular mass without a formyl group in the N-terminal methionine. The 17,014 dalton mass of the smaller species probably corresponds to the protein which has lost the N-terminal methionine (131 daltons). Both protein species can apparently bind copper (Fig. 3B); note, however, that the smaller form with two bound copper atoms (17,142 daltons) cannot be resolved from the larger apoprotein (17,146 daltons). The mass differences between each apoprotein species and their copper saturated forms are 126 and 128 daltons (Fig. 3 and Table IV). This result indicates that there are indeed two coppers (127 daltons) bound to this fragment. Only about 50% of this copper is visible in EPR which is consistent with the proposed mixed valence structure (Table III).

Spectroscopical Properties of the CuA Center—The CuA-binding domain has a characteristic purple color. At pH 7.0 it has a strong absorbance maximum at 480 nm with a shoulder at 530 nm. Two additional maxima are present at 363 and 808 nm (Fig. 4). Only the latter flat peak can be seen in the intact cytochrome oxidase complex. However, the absorbance spectrum is similar to the spectra of the center A in N₂O reductase (22-24) and of the engineered CuA-like center in purple CyoA (4). The extinction coefficients for these maxima are given in Table V.

The EPR spectrum of the Cu-binding domain (Fig. 5) at the neutral or acidic pH is similar to the spectrum of the native CuA site in cytochrome oxidase. The g values and hyperfine coupling constants are the same as those of the intact oxidase and the engineered purple CyoA (see Refs. 2, 4, 9).

The Isolated CuA Center Is Exposed to Solvent—The absorbance spectrum of the CuA domain is dependent on pH. At pH 6-7 the strongest absorbance is in the 450-550 nm region, whereas the absorbance around 360 nm is weak. With increasing pH the absorbance in the 360 nm region increases and is shifted to 370 nm. Simultaneously, there is a decrease of
The optical spectrum of CuA cannot be recorded in the intact oxidase complex because it is mostly covered by the absorbance of hemes. However, magnetic circular dichroic spectrum of the center in cytochrome oxidase has suggested which is close to 8.2 (Fig. 6). The absorbance changes caused by pH are reversible.

Also the EPR spectrum of the CuA domain changes at alkaline pH (Fig. 5). The CuA spectrum becomes merged to another signal which is similar to the spectrum of a type 2 copper center. The EPR spectrum of the samples at low pH begins to broaden at temperatures above 130 K which is typical of the EPR behavior of CuA center in cytochrome c oxidase (25). This broadening is not seen for the type 2 signal at pH 9. No change in the EPR spectrum of the CuA site in the intact Paracoccus cytochrome c oxidase could be detected when the pH was increased to 9. The monomer binds two coppers at pH 7 and 9 (Table III), and in both cases only half of the copper is visible in EPR.

The sensitivity of the isolated purple center to pH suggests that it is more exposed to the aqueous milieu than the site in the intact oxidase complex. This could also have an effect on the redox potential of the center. We have not been able to measure the midpoint potential accurately because the titration cannot be carried out reversibly.

Reaction with Cytochrome c—Fig. 7 shows that reduced cytochrome c is oxidized when it is mixed with oxidized CuA-binding fragment. The equilibrium constant for the redox reaction between the CuA-binding domain and cytochrome c is 0.7 as estimated from the titration data similar to those shown in the figure. This would correspond to a reduction potential of 240 mV for the CuA site in the soluble domain.

**DISCUSSION**

This study on a native CuA-binding domain confirms the earlier conclusion on the binuclear structure of the center which was reached by studying an engineered copper center (4, 7). The Paracoccus subunit II fragment has potential for further structural and functional investigations on the CuA site. It may also be useful for future experiments on the interaction and electron transfer between cytochrome c and the copper site. However, the isolation of the CuA site from the complex has altered the center. It has become pH sensitive and somewhat labile; this could be explained by a more open structure, which may allow water to enter. It seems to be difficult to oxidize and reduce it reversibly; this has hampered our efforts to measure the redox potential of the isolated CuA site.

The copper site in the intact cytochrome oxidase complex is not sensitive to the slightly alkaline pH. This sensitivity seems to be an artifact of the isolated domain. The phenomenon is, however, interesting. The EPR spectra at high pH suggest a change in the ligation of the copper center. The pK of this change (8.2) would fit to the deprotonation of a thiol group. A cysteine with a thiolate should be a stronger metal ligand than a cysteine with a protonated sulfhydryl group. Therefore, we think that another amino acid is responsible for the spectral change. One possibility is that a tyrosine residue is close to the copper center and coordinates to one of the coppers when it loses a proton and becomes a phenolate. Oxygen ligation would fit to the appearance of a type 2 copper center.

An aromatic residue in the vicinity of copper site could be involved in the electron transfer pathway from CuA to the other metal centers in cytochrome c oxidase. It has also been proposed that a tyrosine could be a ligand of the reduced CuA center (26).

The optical spectrum of CuA cannot be recorded in the intact oxidase complex because it is mostly covered by the absorbance of hemes. However, magnetic circular dichroic spectrum of the center in cytochrome oxidase has suggested

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**FIG. 6. Titrations of the absorbance changes.** Titrations at 480 and 370 nm are shown in the upper and lower panels, respectively. The data are taken from Fig. 4.

**FIG. 7. Reaction with cytochrome c.** 5 μM reduced horse heart cytochrome c was oxidized with 5, 10, and 15 μM CuA domain. The spectra were recorded 30 s after each addition. The same amount of oxidized CuA domain was also added to the reference cuvette. The extinction coefficient of reduced cytochrome c at 550 nm is an order of magnitude higher than that of the oxidized copper domain. The dotted line indicates the spectrum of the 5 μM cytochrome c oxidized with potassium ferricyanide in the 550 nm region. The absorbance scale is given on the left.

Absorbance at 450–550 and 810–820 nm (Fig. 4). An isosbestic point for the spectral change is present at 420 nm. Titration of the absorbance changes at 370 and 480 nm gives a pK for the spectral change is present at cytochrome c was oxidized with 5, 10, and 15 oxidized CuA domain was also added to the reference cuvette. The absorbance changes at 370 and 480 nm are shown in the upper and lower panels, respectively. The data are taken from Fig. 4. The extinction coefficient of reduced cytochrome c at 550 nm is an order of magnitude higher than that of the oxidized copper domain. The dotted line indicates the spectrum of the 5 μM cytochrome c oxidized with potassium ferricyanide in the 550 nm region. The absorbance scale is given on the left.

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Expression of a Soluble CuA Domain

that the optical spectrum has a strong signal in the 500 nm region (27). The spectrum at pH 6 and 7 (Fig. 4) has the same features as the optical spectra of nitrous oxide reductase and the engineered purple copper site in CyoA (4, 7, 22-24). They all have an absorbance maximum in the 480–540 region. However, the three spectra have also subtle differences which suggest that the geometry and ligation of binuclear sites are not exactly the same.

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