Soluble Cuₐ Domain of Cyanobacterial Cytochrome c Oxidase*

Received for publication, August 12, 2003, and in revised form, December 4, 2003
Published, JBC Papers in Press, December 12, 2003, DOI 10.1074/jbc.M308903200

Martina Paumann‡, Borjana Lubura‡, Günther Regelsberger§, Markus Feichtinger¶, Gunda Köllensberger§, Christa Jakopitsch§, Paul G. Furtmüller§, Günter A. Peschek§, and Christian Obinger‡‡

From the ‡Institute of Physical Chemistry, Molecular Bioenergetics Group, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria and the §Department of Chemistry, Division of Biochemistry, BOKU University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria

The genomes of several cyanobacteria show the existence of gene clusters encoding subunits I, II, and III of aa₃-type cytochrome c oxidase. The enzyme occurs on both plasma and thylakoid membranes of these oxygen phototrophic prokaryotes. Here we report the expression and purification of a truncated subunit II copper A (Cuₐ) domain (i.e. the electron entry and donor binding site) of cytochrome c oxidase from the cyanobacterium Synechocystis PCC 6803 in high yield. The water-soluble purple redox-active bimetallic center displays a relatively low standard reduction potential of 216 mV. Its absorption spectrum at pH 7 is similar to that of other soluble fragments from aa₃-type oxidases, but the insensitivity of both absorbance and circular dichroism spectra to pH suggests that it is less exposed to the aqueous milieu compared with other Cuₐ domains. Oxidation of horse heart cytochrome c by the bimetallic center follows monophasic kinetics. At pH 7 and low ionic strength the bimolecular rate constant is ($2.1 \pm 0.3 \times 10^{4}$ s⁻¹ M⁻¹), and the rates decrease upon the increase of ionic strength. Sequence alignment and modeling of cyanobacterial Cuₐ domains show several peculiarities such as: (i) a large insertion located between the second transmembrane region and the putative hydrophobic cytochrome c docking site, (ii) the lack of acidic residues shown to be important in the interaction between cytochrome c and Paracoccus Cuₐ domain, and (iii) an extended C terminus similar to Escherichia coli ubisqualin oxidase.

Cyanobacteria have evolved as the most ancient, oxygenic, plant-type photosynthetic organisms (1, 2). Since they were the first to produce O₂, they also may have been among the first to sense it and also to utilize it. Gradual modification of pre-existing photosynthetic electron transport and enzyme systems could have changed a photosynthetic into a respiratory chain (conversion hypothesis) (3). By elaborating mechanisms for aerobic respiration, cyanobacteria have uniquely accommodated both oxygenic photosynthesis and aerobic respiration within a single prokaryotic cell (4). The thylakoid membrane (ICM) is utilized for both photosynthetic and respiratory electron transport, while the cytoplasmic membrane (CM) only contains a respiratory electron transport chain but no photosynthetic reaction centers. In the thylakoid membrane, the photosynthetic and respiratory electron transport chains share the plastoquinone pool (Fig. 7), the cytochrome b₆f complex and extrinsic soluble electron carriers (5).

Spectroscopic (6), inhibitor (7), and EPR studies (8), indicated that cyanobacterial respiratory chains contain an aa₃-type cytochrome c oxidase. Finally, a gene cluster, encoding subunits II, I, and III of the aa₃-type cytochrome c oxidase has been cloned from Synechocystis PCC 6803 (9). A gene cluster encoding these genes in the same order has also been cloned from Synechococcus vulcanus (10). Sequencing of the entire genome of Synechocystis PCC 6803 (11) confirmed the existence of this set of genes encoding the aa₃-type cytochrome oxidase (CcO), but detailed analysis showed the occurrence of two additional sets of genes for terminal respiratory oxidases, interpreted as cytochrome bo- and bd-type quinol oxidases (12). Unfortunately, no functional quinol oxidase activity could so far be detected in any cyanobacterium subjected to whatever growth conditions (13). These heme-copper oxidases are members of the superfamily of terminal oxidases, which are redox driven proton pumps that couple the reduction of molecular oxygen to vectorial translocation of protons across the membrane (14). The superfamily is defined by a high sequence similarity within the largest subunit (subunit I) and a binuclear active site consisting of a high-spin heme (heme a₃) and a closely associated copper ion (Cuₐ). In addition to the binuclear center, CcO has a low-spin heme (heme a) within subunit I and another copper center (Cuₐ), which is located in the extrinsic domain of subunit II (15). Electrons donated from cytochrome c enter the oxidase complex via a conserved tryptophan in subunit II (16–18). Analysis of site-directed mutants has confirmed the classical view that the Cuₐ center, composed of two electronically coupled, mixed-valence Cu²⁺/Cu³⁺ (oxidized state) copper ions, is the acceptor site (19). By contrast, quinol oxidases, which use quinols instead of cytochromes c as electron donor (20), have lost the Cuₐ in the course of evolution (21).

In contrast to the wealth of knowledge on the mammalian and bacterial terminal oxidases, amazingly little is known about the respiration of cyanobacteria and most experiments

* This work was supported by the Austrian Science Fund (FWF Project P13069-CHE). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Chemistry, Division of Biochemistry, Metallprotein Research Group, BOKU, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria. Fax: 43-1-36006-6059; E-mail: christian.obinger@boku.ac.at.

1 The abbreviations used are: ICM, intracytoplasmic (thylakoid) membrane; CM, cytoplasmic (plasma) membrane; CcO, cytochrome c oxidase; SUII, subunit II; PCC, Pasteur Culture Collection; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ICP-SFMS, inductively coupled plasma sector field mass spectrometry; CD, circular dichroism; TMPD, N,N,N′,N′-tetramethyl-1,4-phenylenediamine; CHES, 2-cyclohexylamino)ethane-sulfonic acid.

This paper is available on line at http://www.jbc.org

10293
with inhibitors and electron donors were performed on intact cells or isolated membranes. The interaction with different cytochromes and plastocyanin was investigated and both cytochrome c₆ and plastocyanin were proposed as potential electron donors for the cyanobacterial CcO (22). This is interesting because cytochrome c₆ and plastocyanin have the same function in photosynthetic electron transport (Fig. 7) (29) and replaced each other depending on the growth conditions (24). Topographical models of the transmembrane subunits I, II, and III of the Synechocystis PCC 6803 cytochrome c oxidase (9, 25, 26) suggest that the C-terminal Cc₆ domain of subunit II is located in the periplasmic and thylakoid luminal spaces (Fig. 7). How the oxidase fulfills its function as electron acceptor from either a heme donor (i.e. cytochrome c₆) or a type I copper protein (i.e. plastocyanin) in different cellular environments is unknown. An answer to these questions needs the use of a purified cyanobacterial CcO. However, due to its exceedingly low concentration in both CM and ICM (<1 μmol/mg membrane protein) (27), only minute amounts of cyanobacterial CcO could be isolated and purified (28) and so far its heterologous expression was not successful.2 Here, we report for the first time (i) the cloning and successful expression in E. coli of the soluble C-terminal domain of subunit II of CcO from the cyanobacterium Synechocystis PCC 6803, (ii) insertion of Cu ions forming a typical redox active Cc₆ domain, (iii) its spectral properties in the pH range of 5.5–10.8, (iv) its electrochemical properties as well as (v) its direct reaction with horse heart cytochrome c at pH 7 and the effect of ionic strength on the reaction rates. Sequence and structural analysis demonstrate several peculiarities of the cyanobacterial Cc₆ domain, which are discussed with respect to its role in cyanobacterial respiration.

EXPERIMENTAL PROCEDURES

Materials—Standard chemicals and biochemicals were obtained from Sigma Chemical Co. at the highest grade available. Dynazyme EXT DNA polymerase was from Finnzyme and DNA ligase from MBI Fermentas; NdeI, BglII, and BamHI restriction enzymes were purchased from New England Biolabs, alkaline phosphatase was from Roche Diagnostics; GFX PCR DNA and Gel Band Purification Kit, Q Sepharose Fast Flow, DEAE Sepharose Fast Flow, and Superdex 75 HR were purchased from Amersham Biosciences; the Centriprep YM-10 concentrators were from Amicon.

Cloning and Heterologous Overexpression of the CcO SUII Soluble Domain—DNA and protein sequences of CcO SUII were based on the sequence provided by Cyanobase, the genome data base for Synechocystis sp. PCC 6803 (www.kazusa.or.jp/cyano/Synechocystis). Primers were designed for the expression of the periplasmic domain of the ctaC gene coding for the amino acid residues 137–332 of CcO SUII, excluding the two predicted transmembrane regions (26). The synthetic oligonucleotide primers were purchased from genXpress (Maria Worth, Austria). Primer 1 (5‘-GGG AAT TCC ATA TGG GAC ACA TGG GGA GCA TGG G-3‘) introduced an NdeI restriction site, an ATG start codon and removed a naturally occurring NdeI site. With primer 2 (5‘-GGGA AGA CCTTCA GAG TAG GAC ACA TGG GGA GCA TTG G-3‘) introduced an NdeI restriction site, an ATG start codon and a TGA termination codon were introduced and a naturally occurring BglII site was removed. A cell suspension of Synechocystis grown in BG11 medium (29) for 3 weeks at 35 °C in a shaker under illumination was used as a template for PCR, which was carried out under the following conditions: 95 °C for 10 min (hot start); 30 cycles of 94 °C for 40 s, 52 °C for 30 s, 72 °C for 60 s; followed by a final step of 72 °C for 10 min. A single PCR product of the expected size (614 bp) was obtained and purified using the GFX PCR DNA and Gel Band Purification Kit. The PCR product was digested with the restriction enzymes NdeI and BglII and cloned into the NdeI- and BamHI-digested, alkaline phosphatase-treated expression vector pET-3a (30). The insert was sequenced by the dideoxy chain termination method (31).

Competent E. coli BL21(DE3)pLysS was transformed by electroporation (Gene Pulser, Bio-Rad). Positive clones carrying the recombinant plasmid were detected by PCR and grown overnight in LB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol on an orbital shaker at 180 rpm and 37 °C. M9SB medium containing the same antibiotics was inoculated with the overnight culture in a 1:100 ratio and grown at 37 °C at 180 rpm to an OD₆₀₀ of 1.3. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. At this point 200 μM CuSO₄ was added as well. 4 h after induction cells were harvested by centrifugation (5000 × g, 10 min, room temperature) and stored at −80 °C. The cell paste (obtained from 1 liter of E. coli culture) was thawed and resuspended on ice in 40 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, 5 μM pepstatin, and 0.5 mM dithiothreitol). The lysis buffer was sonicated on ice (three times 40 s) and (to break down genomic DNA to reduce the viscosity of the suspension). The cell paste was removed by centrifugation (27,000 × g, 20 min, 4 °C). The soluble domain of CcO SUII was purified from the supernatant by column chromatography in three steps.

Protein Purification and Reconstitution of the Cc₆ Center—The supernatant was loaded on a Q Sepharose Fast Flow column (2.6 × 22 cm), which was equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.5 mM dithiothreitol. The column was washed with 150 ml of buffer, and proteins were eluted by the stepwise increase of NaCl in the buffer described above: 100 ml NaCl (100 mM), 100 ml NaCl (200 mM), respectively, followed by 250 ml NaCl (300 mM). Fractions were tested for Cu(His)₂₉ (final concentration 1.5 mM). After 1.5 h of incubation and stirring at 4 °C the color of the solution had changed from greenish blue to purple and no further color development could be observed. The protein solution was then loaded on a DEAE Sepharose Fast Flow column (2.6 × 12 cm), which was equilibrated with 50 mM phosphate buffer (pH 6.5). After washing the column with 150 ml of buffer, proteins were eluted by the stepwise increase of NaCl in the same buffer system. The Cu₆ domain-containing Cu₆ domain buffer: 100 mM NaCl (70 mM), 100 mM NaCl (100 mM), and 100 mM NaCl (130 mM), respectively, followed by 250 ml NaCl (150 mM). Purple-colored fractions were pooled and concentrated to a volume of 300 μl using Centriprep concentrators. Portions of 100 μl were loaded on a Superdex 75 HR 10/300 FPLC column, which was equilibrated with 67 mM phosphate buffer (pH 7.5) and 20 ml of 50 mM acetate buffer (pH 4.6). The Cu₆ site was reconstituted by the addition of Cu(His)₂₉ (final concentration 1.5 mM). After 1.5 h of incubation and stirring at 4 °C the protein was concentrated as described above and stored at −80 °C. All steps aside from the Superdex 75 column were carried out at 4 °C.

Determination of Mass and Cu/S Ratio—MALDI-TOF-MS was carried out on a DYNANO MALDI-TOF-MS (Thermo BioAnalysis, Santa Fe, New Mexico) with a sinapinic acid matrix. Spectra were recorded in the reflector mode using both standard and low-mass-range (setting 0.1) and calibrated externally using lysozyme. The protein sample was mixed with a 1% solution of the matrix in 70% acetonitrile, air-dried, and inserted into the mass spectrometer to acquire a spectrum.

Elemental analysis of Cu and S in the investigated sample was performed by inductively coupled plasma sector field mass spectrometry (ICP-SPS; Finnigan MAT, Bremen, Germany). Measurements were carried out by means of a Finnigan MAT ELEMENT (Finnigan MAT, Bremen, Germany) high resolution ICP-SPS. This instrument is equipped with a double focusing mass separator consisting of a magnetic and an electric sector field of reversed Nie-Johnson design and has predefined resolution settings (key definition) of 400 (low), 4000 (medium) and 9500 (high). As a prerequisite for complete elimination of spectral interferences, the determination of Cu and S was conducted using the medium mass resolution setting. As introduction system, the microconcentric nebulizer (PFA, Elemental Scientific Inc., Omaha, NE) in combination with a cooled nebulizer spray chamber (setting 0.1) and calibrated externally using laurylparaffin. The protein sample was mixed with a 1% solution of the matrix in 70% acetonitrile, air-dried, and inserted into the mass spectrometer to acquire a spectrum.

2 S. Ferguson-Miller, C. Hiser, M. Paumann, G. A. Peschek, and C. Obinger, unpublished data.
gent grade hydrochloric acid (Merck, Darmstadt, Germany) was additionally cleaned by double subboiling distillation. Calibration standards for Cu and In were prepared by diluting 1000 µg g⁻¹ single element ICP-MS standard solutions (Merck, Darmstadt, Germany). Indium was used for internal standardization. For sulfur quantification a cyanide standard solution was prepared (Merck) with the sulfur concentration of 105.6 µg g⁻¹.

Prior to ICP-SFMS analysis the protein samples were digested following an open vessel treatment with HCl: 200 µl of 6 N HCl were added to 0.01 g of protein in buffer. After heating this mixture in PFA vessels (100 °C) for several hours, the solution was diluted to a final weight of 2 g by subboiled water. In order to exclude elemental sulfur losses by this preparation procedure two samples were spiked with 0.010 and 0.015 g cyanide solution for sulfur quantification. Copper concentration was quantified by external calibration. A dedicated software (GUM-workbench software, Metoddata G.m.b.H., Grenzach-Wyhlen, Germany) was employed for the calculations based on the numerical method of differentiation.

**UV-Vis Spectroscopy and Circular Dichroism Spectropolarimetry—** Steady-state spectrophotometric measurements were made on a diode-array spectrophotometer Spectord S10 (Zeiss) and a Hitachi Model U-3000 spectrophotometer, respectively. The protein concentration was calculated from the known amino acid composition and absorption at 280 nm according to Pace et al. (32), whereas the concentration of the binuclear copper center was calculated by using the extinction coefficient of 3800 M⁻¹ cm⁻¹ at 535 nm (this work).

Circular dichroism studies were carried out using a JASCO J-600 spectropolarimeter. Far-UV (200–300 nm) experiments were carried out using protein concentrations of 20 µM in 20 mM phosphate buffer, pH 7.0 to 11, and the hydropathy threshold for transmembrane helices was at 1.6.

**Sequence Analysis—** The kinetics of oxidation of ferrous horse heart cytochrome c (Sigma type IV, used without further purification) by the recombinant Cu₄A domain was followed in the single mixing mode at either 550 nm (Δεₒₒ) for Cu and 610 nm for In were prepared by diluting 1000 µl of 6 N HCl to 0.010 g of protein in buffer. After heating this mixture in PFA vessels (100 °C) for several hours, the solution was diluted to a final weight of 2 g by subboiled water. In order to exclude elemental sulfur losses by this preparation procedure two samples were spiked with 0.010 and 0.015 g cyanide solution for sulfur quantification. Copper concentration was quantified by external calibration. A dedicated software (GUM-workbench software, Metoddata G.m.b.H., Grenzach-Wyhlen, Germany) was employed for the calculations based on the numerical method of differentiation.

**Second-order rate constants were calculated from the slope of the line**

Equation 1:

\[
K' = \frac{[Cu₄A(ox)][TMPD(ox)]}{[Cu₄A(red)][TMPD(ox)]}
\]

**RESULTS AND DISCUSSION**

**Sequence Analysis, Secondary, and Tertiary Structure Prediction—** Synechocystis PCC 6803 cytochrome aa₃-type cytochrome c oxidase (CoO) consists of 3 subunits encoded by the *cta* operon. Fig. 1 illustrates the amino acid sequences of six cyanobacterial CoO subunits II in comparison to the corresponding sequences of subunit II of cytochrome c oxidase from *P. denitrificans*, bovine heart and *Rhodobacter sphaeroides* O subunits II in comparison to the corresponding sequences of subunit II of cytochrome c oxidase from *P. denitrificans*, bovine heart and *Rhodobacter sphaeroides* O subunits II. ClustalX version 1.81 (www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) (46) and DIALIGN-T-COFFEE (www.ch.embnet.org/software/Tcoffee.html) (44) and ClustalX version 1.81 (www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) (46). The three-dimensional model of subunit II of CoO from the cyanobacterium *Synechocystis* was constructed using SWISS-Model and the SWISS-PdbViewer (see www.expasy.ch/spdbv/). Model building was based on the crystal structure of CoO from *Paracoccus denitrificans* (47).

**RESULTS AND DISCUSSION**

**Sequence Analysis, Secondary, and Tertiary Structure Prediction—** Synechocystis PCC 6803 cytochrome aa₃-type cytochrome c oxidase (CoO) consists of 3 subunits encoded by the *cta* operon. Fig. 1 illustrates the amino acid sequences of six cyanobacterial CoO subunits II in comparison to the corresponding sequences of subunit II of cytochrome c oxidase from *P. denitrificans*, bovine heart and *Rhodobacter sphaeroides* CoO as well as subunit II of ubiquinol oxidase from *E. coli* aligned by the program T-COFFEE. Similar results were obtained when the programs DIALIGN and ClustalX (using “identity matrix” as protein weight matrix) were applied. The T-COFFEE program was used as inputs. The input sequence was analyzed by applying the stored rules for various sequence features of known protein sorting signals. Finally, the possibility for the input protein to be localized at each candidate site was reported.
oxidase (51) are known and clearly show two transmembrane helices. A hydropathy plot (not shown) indicates that the primary sequence of subunit II of Synechocystis comprises two membrane spanning regions at the N terminus. The program PSORT (for Gram-negative bacteria), which predicts the presence of signal sequences by both McGeoch’s (52) and von Heijne’s (53) method suggests the presence of a signal sequence at the N terminus and a possible cleavage site between Ser20 and Leu21. In P. denitrificans (Fig. 1) the signal sequence (residue 1–28) is cleaved off after translocation and the three-dimensional structure of mature P. denitrificans subunit II comprises an N-terminal loop (residues 29–53), two transmembrane helices including their connection (residues 54–132) and a C-terminal globular domain (residues 133–280). Using the program MEMSAT of PSIPRED for topology analysis of subunit II of Synechocystis oxidase, it was predicted that residues 47–68 and 91–109 form the transmembrane helix segments, and that residues 69–90 are located on the cytoplasmic side of the membrane, whereas both the N-terminal loop (residues 21–46) and the soluble CuA containing domain (residues 110–332) are positioned on the other side of the membrane. As Fig. 1 clearly shows very similar results were obtained for CcO subunits II of five other cyanobacterial species, namely Synechococcus PCC 7002, Nostoc PCC 7120, T. elongatus BP-1, Synechococcus WH 8102, and P. marinus MIT 9313 using the same program. Since immunocytochemical localization and cytochrome c oxidase activities clearly demonstrated that cyanobacterial CcO is localized in both CM and ICM (27, 54–55), in cyanobacteria the CuA containing domain of CcO is most likely positioned in the periplasm or thylakoid lumen. Thus, the pathways of electron transfer on the outside of the plasma membrane and within the thylakoid appear to be different (see also Fig. 7).

**Fig. 1.** Amino acid sequence alignment of subunit II of cytochrome c oxidase from the cyanobacteria Synechocystis PCC 6803, Synechococcus PCC 7002, Nostoc PCC 7120, T. elongatus BP-1, Synechococcus WH 8102, and P. marinus MIT 9313. Comparison with sequences of subunit II of cytochrome c oxidase with known three-dimensional structure. The alignment was produced with the program T-COFFEE. Fully conserved residues are highlighted in red, highly conserved residues are highlighted in yellow, *#* mark gray-colored copper ligands, underlined are known and potential N-terminal signal peptides as well as C-terminal propeptides. Transmembrane regions and putative transmembrane regions are printed in blue. The cytoplasmic loop between the two transmembrane regions are printed in green. Amino acids at the putative hydrophobic electron entry site are printed in orange. The arrow marks the beginning of the soluble domain of recombinant Synechocystis subunit II. Abbreviations: Par.den., P. denitrificans; Rh.sph., R. sphaeroides; Bovine heart, cytochrome c oxidase from bovine heart; Prochloro.mar.MIT 9313, P. marinus MIT 9313; Thermosyn.elong.BP-1, T. elongatus BP-1; E. coli Ubiq.Ox, ubiquinol oxidase from E. coli.
contains a specific insertion located after the second transmembrane region (Pro^{120}\text{--}Gly^{166}), which is not found in \textit{P. denitrificans}, bovine heart and \textit{R. sphaeroides} CuO subunit II, nor in \textit{E. coli} ubiquinol oxidase subunit II, whereas other cyanobacteria show similar insertions (see Figs. 1 and 3). A peculiarity of the insertion Pro^{120}\text{--}Gly^{166} in \textit{Synechocystis} CuO SUII is its abundance of methionines and histidines (\textsuperscript{121}Met-Ala-His-Asn-His-Met-Gly-His-Met-Gly-Ser-Met-Gly-Asn-Met-Val-Ala-Met\textsuperscript{148}), which does not seem to represent the cytochrome c binding site but might be another metal-binding site. Furthermore, cyanobacterial CuO subunits II lack a region immediately after the potential cytochrome c binding site of \textit{P. denitrificans} (\textsuperscript{161}Ala\textsuperscript{161}\text{--}Ala\textsuperscript{185} \textit{P. denitrificans} numbering).

With PSIPRED one less \(\beta\)-sheet and two less \(\alpha\)-helices are predicted at the N terminus of the soluble domain as well as two more \(\alpha\)-helices at the \textit{Synechocystis}-specific extended C terminus (Ser\textsuperscript{206}\text{--}Leu\textsuperscript{232}; line 1 in Fig. 2A), which is not found in \textit{Paracoccus} CuO but is similar to ubiquinol oxidase from \textit{E. coli} (Fig. 1). As to be seen in Fig. 1 not all the subunits II of cyanobacterial CuOs have an extended C terminus.

Both predictions suggest a \(\beta\)-barrel in the core of the soluble domain of \textit{Synechocystis} CuO SUII (Fig. 2B) similar to the soluble portion of subunit II of \textit{P. denitrificans} (47) and bovine heart CuO (49) and to class I copper proteins such as plasto-
Cyanobacterial Cuₐ Domain

Fig. 3. Overlay of the three-dimensional structure of P. denitrificans CeO subunit II and the model of the soluble Cuₐ domain (see Fig. 2) of the corresponding Synechocystis protein (gray). The regions where the cyanobacterial model differs from the P. denitrificans CeO subunit II structure are depicted in black. Copper atoms are shown in light gray.

cyanin or azurin (47). In detail, the model of the Synechocystis Cuₐ domain suggests β-sheets at Gly¹³⁷–Met¹³⁹, Met¹⁴²–Asn¹⁴⁴, Ala¹⁴⁷–Val¹⁵⁴, Leu¹⁵⁶–Ser¹⁶², Ala¹⁹⁷–Ile¹⁹⁹, Val²⁰³–Met²⁰⁷, His²¹⁴–Phe²¹⁶, Gln²²⁸–Val²²⁷, Ser²³³–Phe²³⁷, Gly²⁴¹–Val²⁴⁷, and Ser²⁶²–His²⁶⁷.

Recombinant Cuₐ Domain of Synechocystis Cytochrome c Oxidase—The N terminus of the ctaC gene was truncated in order to obtain a water-soluble gene product (starting at the position indicated by the arrow in Fig. 1). Amino acid residues 137–332 of CeO SUII were expressed, excluding the two predicted transmembrane regions (26). The recombinant protein was expressed in soluble form and was successfully separated from other cytosolic proteins by several chromatographic steps as described under “Experimental Procedures” and demonstrated in the inset to Fig. 4. Production of the soluble cyanobacterial Cuₐ domain in E. coli contrasts other purification protocols that describe main formation of inclusion bodies followed by different refolding procedures (57–59). The cyanobacterial protein loaded on the Q Sepharose Fast Flow column was colorless. Spectroscopically no purple copper could be found in this fraction. After pooling the relevant fractions, Cu(His)₂ was added and the Cuₐ site was reconstituted successfully. The characteristic purple fraction was further purified by DEAE Sepharose Fast Flow and Superdex 75 HR chromatography (inset to Fig. 4). Comparison with the gel filtration elution profile of standard proteins proved the cyanobacterial Cuₐ-binding domain to be a monomer with an apparent molecular mass of about 21 kDa, which is consistent with SDS-PAGE analysis. The theoretical isoelectric point of the Cuₐ domain is 4.3.

The theoretical mass of the truncated apoprotein (which has lost the N-terminal methionine, 131 Da) is 20,988 Da. Adding copper (2 × 63.5 Da) gives a theoretical mass of the holoprotein of 21,115 Da, which fits well with the prominent mass peak at 21,094 Da in the MALDI-TOF mass analysis (Fig. 4).

Spectral Characterization—The pure cyanobacterial Cuₐ domain has a characteristic purple color indicating its oxidized state. At pH 7.0 it exhibits two strong absorbance maxima at 482 nm and 535 nm, respectively. It was suggested that these two absorption bands arise from an interaction between the two Cu ions (60). Two additional maxima are present at 359 nm and 785 nm (Fig. 5). The extinction coefficients (obtained from the slope of the plot of absorbance of peak maximum versus Cuₐ domain concentration) at pH 7.0 were determined to be 27900 M⁻¹ cm⁻¹ (280 nm), 1580 M⁻¹ cm⁻¹ (359 nm), 2820 M⁻¹ cm⁻¹ (482 nm), 3080 M⁻¹ cm⁻¹ (535 nm), and 1840 M⁻¹ cm⁻¹ (785 nm). At 550 nm (α-peak of reduced horse heart cytochrome c) the extinction coefficient is 2860 M⁻¹ cm⁻¹. In intact cytochrome c oxidase only the flat peak at 785 nm can be seen because the other absorbances are covered by the hemes. The energies and extinction coefficients of the transitions of the cyanobacterial Cuₐ domain are similar to that found for other Cuₐ domains (57–59), however small differences in the position and relative intensity of these bands suggest some differences in the copper environment.

The spectral changes over a wide pH range were investigated. Fig. 5A shows extreme spectral states at pH 5.5, 7.0, and 10.8 and Fig. 5B demonstrates the pH dependence of the extinction coefficients at selected wavelengths. Between pH 5.5 and 8 neither the position nor the intensity of the peak maximum change significantly. This is in contrast to other Cuₐ domains, which show a more pronounced pH dependence between pH 6 and 8. For P. denitrificans Cuₐ domain a peak close to 8.2 was determined which was interpreted as a change in the ligation of the copper center due to solvent exposition. By contrast the copper site in intact P. denitrificans cytochrome c oxidase complex is not sensitive to alkaline pH, suggesting that the sensitivity is an artifact of the isolated domain. Even lower P/K values are determined in other Cuₐ domains (59). In case of the cyanobacterial protein the absorption maxima did not change in the pH range 5.5–8 but they all shifted to lower wavelengths at higher pH values (pH 10: 349 nm, 476 nm, 534 nm, 780 nm). This is a difference between the cyanobacterial protein and that of P. denitrificans (49, 53), since in the latter the weak absorbance at 360 nm shifted to both higher wavelength and intensity at basic conditions. The relative insensitivity of the cyanobacterial isolated purple center to pH suggests that it is less exposed to the aqueous milieu than the corresponding Paracoccus protein. At pH ≥ 10 the typical signature of the purple copper center of the cyanobacterial enzyme deteriorates.

The CD spectrum of the protein in the far-UV region was almost independent of pH (not shown), indicating that the overall secondary structure of the protein remains unchanged. Also in the near-UV around 280 nm no definite changes in the pH range 5.5–10.8 were observed (not shown) in contrast to observations with the P. denitrificans domain (61). This could indicate a more compact structure of the cyanobacterial protein.

Inductively coupled plasma sector field mass spectrometry of
the purified CuA-domain allowed the determination of a molar S/Cu ratio. A molar S/Cu ratio of 5.7 ± 0.9 (expanded uncertainty) was found. The uncertainty was calculated according to the ISO/GUM guide (62) using the uncertainty propagation procedure. Taken into account the known number of cysteines (two) and methionines (eleven) in the recombinant truncated Synechocystis protein this value suggests an occupancy of the binuclear center of more than 90% of the recombinant protein (as is supported by the UV-vis and MALDI-TOF data). This was a good precondition to investigate its redox activity and its direct reaction with cytochrome c.

Redox Properties—The CuA binding domain can be reduced with dithionite, ascorbate, reduced horse heart cytochrome c, and TMPD (N,N,N',N'-tetramethyl-1,4-phenylenediamine) resulting in the loss of its purple color.

The reaction between TMPD and the oxidized binuclear center is monophasic. The bimolecular rate constant was determined to be (4.0 ± 0.4) × 10^5 M⁻¹ s⁻¹ at pH 7.0. An excess of TMPD was necessary to reduce the copper protein and an equilibrium between the redox couples CuA (ox)/CuA(red) and TMPD(ox)/TMPD(red) was established and allowed to assign the reduction potential of the binuclear center by spectroscopic determination of the concentration of the involved species at equilibrium (34). Experiments with different TMPD concentrations were performed in order to achieve equilibria with various CuA (ox)/CuA (red) ratios (Table I). Using the known standard reduction potential of TMPD(ox)/TMPD(red) of 266 mV (35) allowed the determination of the unknown standard value for the CuA (ox)/CuA (red) couple, since under equilibrium conditions both redox couples have the same reduction potential. The determined midpoint potential of the cyanobacterial protein is lower, (216 ± 2) mV at pH 7.0, compared with the values published for the corresponding proteins from P. denitrificans (240 mV) (57), Thermus thermophilus (250 mV) (58), and Sulfolobus acidocaldarius (237 mV) (59).

Cytochrome c Oxidation—Reduced horse heart cytochrome c is oxidized when it is mixed with the oxidized CuA-binding domain. From the spectroscopically determined equilibrium concentrations an equilibrium constant of about 0.16 could be estimated (not shown) fitting well with the positive reaction Gibbs energy of 4.25 kJ/mol (see below), which corresponds to an equilibrium constant of 0.18. The kinetics of oxidation of cytochrome c and reduction of CuA was monitored by following the decrease of absorbance at 550 and 785 nm, respectively. A fast and simultaneous monophasic decrease of absorbance at both wavelengths was observed (Fig. 6, A and B). Due to the weak amplitude at 785 nm, only the oxidation of cytochrome c was followed in determination of actual rate constants.

For rate determination the concentration of the CuA domain was constant (2 μM), whereas the cytochrome c concentration varied between 5 and 30 μM (Fig. 6C). In addition the effect of ionic strength for the reaction was studied. At pH 7 and 5 mM phosphate buffer, the apparent bimolecular rate constant between cytochrome c and the cyanobacterial CuA domain was determined to be (2.1 ± 0.3) × 10^4 M⁻¹ s⁻¹. The rates decreased at higher salt concentrations. In Fig. 6D the logarithms of k app are plotted against the square root of ionic strength. The decrease of the rates in this Bronsted plot indicates that electrostatic interactions are important for the reaction between horse heart cytochrome c and the cyanobacterial CuA domain.

Using the Bronsted law (63) log k app = log k o + 2BzAzB/V, the number of charges on the protein interfaces that control the interaction between the two proteins can be estimated: k app is the bimolecular rate constant at ionic strength I, k o is the bimolecular rate constant at I = 0 (i.e. 4.4 × 10^6 M⁻¹ s⁻¹), and B is a temperature-dependent term, whose value at 25 °C is about 0.6. The slope of the plot (the zA/zB product with zA and zB representing the electron transfer-sensitive charges on the protein surfaces) is 1.9, which indicates that about one effective charge of opposite sign on each protein interface interacts in the reaction between Synechocystis CuA domain and horse heart cytochrome c. A similar effect was observed in the reaction between horse heart cytochrome c and the Paracoccus protein. The corresponding rate constant of 3 × 10^5 M⁻¹ s⁻¹ (57) is about ten times higher than that of the cyanobacterial domain and the slope of the presented Bronsted plot was about
published cytochrome c oxidase structures (47, 49, 50) this Trp is near the putative cytochrome c binding site and molecular modeling placed the indole ring of this Trp within a distance of 4–5 Å from the exposed heme edge of cytochrome c (65) which supports its role as the site of electron entry. The corresponding residue in the aligned cyanoabacterial CrO SUII is a tyrosine (Tyr175 in Synechocystis CrO subunit II), with the exception of Synechococcus WH 8102, where interestingly a phenylalanine is found (Fig. 1). This putative electron entry in cyanoabacterial CrO SUII is part of a conserved sequence rich in aromatic amino acids ((Tyr/Phe)-Ala-(Trp/Phe)-(Ile/Leu)-Phe-(Thr/His)-Tyr). Similar to Paracoccus CrO this region could contribute to a hydrophobic loop, which is thought to participate in cytochrome c binding and facilitates rapid electron transfer (16, 47).

At horse heart cytochrome c a strong positive surface potential is evident, while several acidic residues have been suggested to participate in docking on a negatively charged patch on subunit II of Paracoccus or bovine heart CrO. It has been shown for these CrO that the closer a charged side chain is to this hydrophobic region, the more important it is to binding affinity (65). At the interface between subunit II and cytochrome c, the most influential horse heart cytochrome c residues, Lys13 and Lys72, are most probably paired with Asp163 and Asp706 (Paracoccus numbering, see Fig. 1). Both residues have been shown to be involved in both horse heart cytochrome c and bacterial cytochrome c552 binding by the Paracoccus enzyme (66). In cyanoabacterial CrO SUII only the latter amino acid is conserved (Asp211 in Synechocystis; Fig. 1), because Asp163 (Paracoccus numbering) is part of a region not present in cyanobacteria. Further interactions between lysines on horse heart cytochrome c and Glu154 and Asp187 in Paracoccus CrO have been proposed (66), but interestingly the corresponding amino acids in cyanobacteria are not acidic (Fig. 1).

Despite these discrepancies, Synechocystis CuA domain has a relatively low pI-value of 4.3 and thus a negatively charged surface. How the Synechocystis-specific insertion (Pro120–Gly166 in Synechocystis CrO SUII contains 4 Asp and 2 Glu) and the extended C terminus contribute to the protein structure and surface is unknown. The model based on the Paracoccus structure placed the insertion at the opposite side of the electron entry site (Fig. 3) and lacks the C-terminal 47 amino acids (Ser286–Leu332), since they are not present in Paracoccus CrO. Modeling based on the structure of ubiquinol oxidase from E. coli did not give reliable results (not shown). If and how the C terminus influences and modulates the donor binding cannot be answered at present.

Summary—Cyanobacteria are unique in possessing cytochrome c oxidase in two distinct membranes, namely CM and IC, and so the extrinsic CuA domain of CrO is located in two distinct environments (Fig. 7), namely the periplasmic space and the thylakoid lumen. The here described fully redox-active recombinant soluble domain of subunit II of Synechocystis CrO now enables the study of interaction with potential endogenous electron donors. Several differences with so far studied cytochrome c oxidases have been detected, namely a long insertion immediately after the second transmembrane helix, an extended C terminus (similar to ubiquinol oxidase from E. coli), differences in the distribution of acidic amino acids (and thus in the putative donor binding site), insensitivity to pH changes and a relatively low reduction potential of 216 mV at pH 7.0. The geometry of the binuclear copper center including the nature of the ligands seems to be identical to other CrOs and a tyrosine most probably serves as electron entry site. The CuA domain is an acidic protein, as are the two in vitro electron donor candidates cytochrome c552 and plastocyanin. The pH val-

---

**Fig. 5.** A, absorbance spectra of the Synechocystis CuA domain (100 μM) at pH 5.5, 7, and 10.8, respectively. Conditions: 50 mM phosphate buffer (pH 5.5, 7) and carbonate buffer (pH 10.8), respectively. B, variation of extinction coefficients (M⁻¹ cm⁻¹) at selected wavelengths with pH. Conditions: 100 μM CuA domain, 50 mM buffer with overlapping pH values (phosphate buffer, pH 5.5–8, Tris/HCl, pH 8–9, CHES, pH 9–10, carbonate buffer, pH 10–10.8).

-3.8 (calculated from Table I in Ref. 57). This indicates that the interaction between the cyanobacterial CuA domain and horse heart cytochrome c is less affected by electrostatic interactions. These results are in agreement with differences in the surface properties of subunit II suggested by sequence analysis (see below). The less positive reduction potential (216 mV) of the cyanobacterial copper protein compared with the Paracoccus protein (240 mV) could be responsible for the observed lower rate constant of the reaction between horse heart cytochrome c and the cyanobacterial electron acceptor. Calculating with a reduction potential of horse heart cytochrome c of 260 mV (64), gives a more positive reaction Gibbs energy ΔG° of 4.25 kJ/mol for the Synechocystis CuA domain compared with 1.93 kJ/mol for the Paracoccus CuA domain.

**The Cytochrome c Interaction Domain and Electron Entry on the Cyanobacterial CuA Domain**—Efficient electron transfer between cytochrome c and the CuA domain requires both rapid complex formation and rapid product dissociation. During complex formation transient interactions occur and paths for intermolecular electron transfer have to be active. Electron transfer between the donor and the binuclear acceptor side has been thought to be strongly dependent on the presence of a tryptophan (e.g. Trp149 in P. denitrificans, see Fig. 1), which when mutated, abolishes electron transfer (14–16). In the so far...
ues of cytochrome c₆ and plastocyanin are 4.94 (11) and 5.6 (67), respectively. Both cytochrome c₆ and plastocyanin are electron donors to Photosystem I in the thylakoid and, unlike the cationic mitochondrial cytochrome c (Fig. 7), it was shown that CM and ICM preparations react best with cytochrome c₆ at high ionic strength (27), while it reacts effectively with Photosystem I at low ionic strength. It is known that in the thylakoid lumen ionic strength may rise as H⁺/H₂O₁, Mg²⁺/H₂O₁, and Cl⁻ accumulate during photochemical activity. And it is also known that oxidase activity is induced in the plasma membrane by growth under high ionic strength conditions. So it was hypothesized that the ionic strength can act as a "switch" for the electron transfer pathways in both plasma membrane and thylakoids (27). The newly available recombinant electron acceptor of a cyanobacterial CcO for the first time allows the study of actual protein interactions and electron transfer reac-

### Table I

Parameters of the redox equilibrium and standard reduction potential \( E^\circ \) of the soluble binuclear copper center of subunit II of cytochrome c oxidase from Synechocystis dependent on the initial TMPD concentration

| TMPD(red) initial concentration | Cu₄(ox) | Cu₄(red) | TMPD(ox) | TMPD(red) | \( K' \) | \( E^\circ \) |
|--------------------------------|---------|---------|----------|-----------|--------|---------|
| 5 \( \mu \)M                  | 1.09    | 0.91    | 0.91     | 4.09      | 0.1858 | 223     |
| 10 \( \mu \)M                 | 0.95    | 1.05    | 1.05     | 8.95      | 0.1297 | 214     |
| 20 \( \mu \)M                 | 0.69    | 1.31    | 1.31     | 18.69     | 0.1331 | 214     |
| 30 \( \mu \)M                 | 0.52    | 1.48    | 1.48     | 28.52     | 0.1477 | 217     |
| 40 \( \mu \)M                 | 0.49    | 1.51    | 1.51     | 38.49     | 0.1209 | 212     |

**Fig. 6. Reaction of cyanobacterial Cu₄ domain with horse heart cytochrome c.** A and B, typical time courses of the absorbance changes in the reaction between 50 \( \mu \)M reduced cytochrome c and 10 \( \mu \)M oxidized Cu₄ domain at pH 7.0 (20 mM phosphate buffer). Oxidation of cytochrome c was followed at 550 nm (A), reduction of the copper domain was followed at 785 nm (B). C, plot of \( k_{obs} \) values against concentration of reduced cytochrome c. Reaction conditions: 2 \( \mu \)M oxidized copper domain, 5–30 \( \mu \)M reduced horse heart cytochrome c, 20 mM phosphate buffer (pH 7.0), reaction was followed at 550 nm; D, dependence of \( k_{app} \) on ionic strength. Conditions as in C with the exception of variation of ionic strength from 5–300 mM.
tions to understand the physiological role of an aa₃-oxidase in oxygenic phototrophic bacteria.

REFERENCES
1. Nitschke, W., and Rutherford, A. W. (1991) Trends Biochem. Sci. 16, 241–245
2. Blankenship, R. E. (2001) Trends Biochem. Sci. 6, 4–6
3. Broda, E., and Peschek, G. A. (1979) J. Theor. Biol. 81, 201–212
4. Peschek, G. A. (1986) Biochim. Biophys. Acta 875, 27–32
5. Peschek G. A. (1996) Biochem. Soc Trans. 24, 729–733
6. Peschek, G. A., Schmetterer, G., Lauritsch, G., Nitschmann, W. A., Kielzl, P. F., and Muchl, R. (1982) Arch. Microbiol. 131, 261–265
7. Wastyn, M., Achata, T., Treka, M., and Peschek, G. A. (1987) Biochem. Biophys. Res. Commun. 130, 102–111
8. Fry I. V., and Peschek, G. A. (1988) Methods Enzymol. 176, 450–459
9. Alge, D., and Peschek, G. A. (1993) Biochem. Biophys. Res. Commun. 191, 9–17
10. Sone, N., Tano, H., and Ishizuka, M. (1993) Biochim. Biophys. Acta 1188, 130–138
11. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirokawa, S., Suzuki, T., Sato, M., Nakazaki, N., Nishino, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) DNA Res. 3, 109–136
12. Howitt, C. A., and Vermaas, W. F. J. (1986) Biochemistry 25, 17944–17951
13. Fromwald, S., Zeder, R., Wastyn, M., Lübben, M., and Peschek, G. A. (1999) Arch. Biochem. Biophys. 367, 122–128
14. Wijkstra M. (1989) Nature 383, 776–778
15. Abramson, J., Svensson-Ek, M., Byrne, B., and Iwata, S. (2001) Biochim. Biophys. Acta 1544, 1–9
16. Witt, H., Malatesta, F., Nicolletti, F., Brunori, M., and Ludwig, B. (1998) J. Biol. Chem. 273, 5132–5136
17. Zhen, Y., Hoganson, C. W., Babcock, G. T., and Ferguson-Miller, S. (1999) J. Biol. Chem. 274, 38032–38041
18. Wang, K., Zhou, Y., Sadusski, R., Grinell, S., Gerern, L., Ferguson-Miller, S., Durham, B., and Millet, F. (1999) J. Biol. Chem. 274, 38042–38050
19. Malatesta, F., Nicolletti, F., Zickermann, V., Ludwig, B., and Brunori, M. (1998) FEBS Lett. 434, 322–324
20. Calhoun, M. W., Thomas J. W., and Gennis, R. B. (1994) Trends Biochem. Sci. 19, 325–330
21. Castresana, J., Lübben, M., Saraste, M., and Higgs, D. G. (1994) EMBO J. 13, 2516–2525
22. Lockau, W. (1995) Arch. Microbiol. 168, 336–340
23. Navarro, J. A., Hervas, M., and De La Rosa, M. A. (1997) J. Biol. Inorg. Chem. 2, 11–12
24. Sandmann, G., and Börger, P. (1980) Plant Sci. Lett. 17, 417–424
25. Alge, D., Schmetterer, G., and Peschek, G. A. (1994) Gene 138, 127–132
26. Alge, D., and Peschek, G. A. (1993) Biochem. Mol. Biol. Int. 29, 511–525
27. Nicholls, P., Oster, G., and Niederhauser, H., and Peschek, G. A. (1992) Biochim. Biophys. Acta 1095, 184–190
28. Heffer, U., Scherer, S., and Böger, P. (1988) Biochim. Biophys. Acta 934, 186–190
29. Stanier, R. Y., Kunizawa, R., Mandel, M., and Cohen-Bazire, G. (1974) Bacteriol. Rev. 35, 171–205
30. Studier, W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1979) Methods Enzymol. 165, 60–89
31. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
32. Pace, C. N., Vagias, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
33. Bani, L., and Assfalg, M. (2001) in Handbook of Metalloproteins (Messerschmidt, A., Huber, R., Poulos, T., and Wieghardt, K., eds) Vol. 1, pp. 35–43, Wiley, New York
34. Sorensen, D., Seefeldt, L. C., and Parker, V. D. (2000) Anal. Biochem. 287, 118–125
35. Dutton, L. (1978) Methods Enzymol. 54, 411–435
36. Hennig, L. (1999) BioTechniques 26, 1170–1172
37. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
38. Jones, D. T. (1999) J. Mol. Biol. 292, 195–202
39. Jones, D. T. (1999) J. Mol. Biol. 287, 797–815
40. McGuffin, L. J., and Jones, D. T. (2003) Bioinformatics 19, 874–881
41. Jones, D. T. (1998) FEBS Lett. 423, 281–285
42. Jones, D. T., Taylorm, W. O., and Thornton, J. M. (1994) Biochemistry 33, 3038–3049
43. Nakai, K., and Horton, P. (1999) Trends Biochem. Sci. 24, 34–36
44. Notredame, C., Higgins, D., and Heringa, J. (2000) J. Mol. Biol. 302, 265–217
45. Morgenstern, B. (1999) Bioinformatics 15, 211–218
46. Jeanpoulou, F., Thompson, J. D., Gouy, M., Higgins, D. G., and Gibson, T. J. (1998) Trends Biochem. Sci. 23, 403–405
47. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature 376, 660–669
48. Lassmann, T., and Sønnhammer, E. L. L. (2002) FEBS Lett. 529, 126–130

Fig. 7. Scheme of bioenergetic membrane functions in a cyanobacterium (5). PSI and PSII, photosystems I and II; PQ, plastoquinone; FNR, ferredoxin-NADP reductase; c₆, soluble cytochrome c₆; PC, plastocyanin; DH₉/₂, either bacteria-like nonproton-translocating one-subunit NADH dehydrogenase, or mitochondria-like multisubunit NADH dehydrogenase; Cyt b₆f, cytochrome b₆f complex; Cyt aa₃, cytochrome c oxidase; Cu₆, binuclear copper center of subunit II of cytochrome oxidase; F-type, F-type H⁺-translocating ATPases.
49. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) Science 272, 1136–1144
50. Svensson-Ek, M., Abramson, J., Larsson, G., Tornroth, S., Brzezinski, P., and Iwata, S. (2002) J. Mol. Biol. 321, 329–339
51. Abramson, J., Riistama, S., Larsson, G., Jasaitis, A., Svensson-Ek, M., Laakkonen, L., Puustinen, A., Iwata, S., and Wikström, M. (2000) Nat. Struct. Biol. 7, 910–917
52. McGeoch, D. J. (1985) Virus Research 3, 271–276
53. von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690
54. Peschek, G. A., Obinger, C., Fromwald, S., and Bergmann, B. (1994) FEMS Microbiol. Lett. 124, 431–438
55. Peschek, G. A., Obinger, C., Sherman, D. M., and Sherman, L. A. (1994) Biochim. Biophys. Acta 1187, 369–372
56. Wilmanns, M., Lappalainen, P., Kelly, M., Sauer-Eriksson, E., and Saraste, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11949–11951
57. Lappalainen, P., Aasa, R., and Malmström, B. G. (1993) J. Biol. Chem. 268, 26416–26421
58. Slutter, C. E., Sanders, D., Wittung, P., Malmström, B. G., Aasa, R., Richards, J. H., Gray, H. B., and Fee, J. A. (1996) Biochemistry 35, 3387–3395
59. Komorowski, L., Anemüller, S., and Schäfer, G. (2001) J. Bioenerg. Biomembr. 33, 27–34
60. Larsson, S., Kallebring, B., Wittung, P., and Malmström, B. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7167–7171
61. Gupta, S., Warne, A., Saraste, M., and Mazumdar, S. (2001) Biochemistry 40, 6180–6189
62. Guide to the Expression of Uncertainty in Measurement (1995) International Organization for Standardization, ISO/GUM, Geneva, Switzerland
63. Bronsted, J. N., and La Mer, V. K. (1924) J. Am. Chem. Soc. 46, 555–573
64. Pettigrew, G. W., and Moore, G. R. (1987) Cytochromes c: Biological Aspects, Springer Verlag, Berlin
65. Roberts, V. A., and Pique, M. E. (1999) J. Biol. Chem. 274, 38051–38060
66. Drosou, V., Reincke, B., Schneider, M., and Ludwig, B. (2002) Biochemistry 41, 10629–10634
67. Romero, A., De la Cerda, B., Varela, P. F., Navarro, J. A., Hervas, M., and De la Rosa, M. (1998) J. Mol. Biol. 275, 327–336
Soluble CuA Domain of Cyanobacterial Cytochrome c Oxidase
Martina Paumann, Borjana Lubura, Günther Regelsberger, Markus Feichtinger, Gunda Köllensberger, Christa Jakopitsch, Paul G. Furtmüller, Günter A. Peschek and Christian Obinger

J. Biol. Chem. 2004, 279:10293-10303.
doi: 10.1074/jbc.M308903200 originally published online December 12, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308903200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 11 of which can be accessed free at http://www.jbc.org/content/279/11/10293.full.html#ref-list-1