 © 2009 The Chemical Society of Japan

Modification of Spectroscopic Properties and Catalytic Activity of *Escherichia coli* CueO by Mutations of Methionine 510, the Axial Ligand to the Type I Cu

Shinji Kurose,[1] Kunishige Kataoka,*[1] Naoya Shinohara,[1] Yuko Miura,[2] Maiko Tsutsumi,[2] Seiya Tsujimura,[2] Kenji Kano,[2] and Takeshi Sakurai[1]

[1]Graduate School of Natural Science and Technology, Kanazawa University, Kakuma, Kanazawa 920-1192

[2]Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502

Received November 4, 2008; E-mail: kataoka@cacheibm.s.kanazawa-u.ac.jp

Replacement of Met510, the axial ligand to the type I Cu in a cuprous oxidase CueO, with Leu afforded the three-coordinated type I Cu, while Gln, Ala, and Thr mutations led to the replacement of the thioether ligand with oxygen ligands (amide carbonyl group and water), and characteristic properties of absorption, circular dichroism, and electron paramagnetic resonance spectra of a variety of Met510 mutants were correlated with the changes in redox potential and enzyme activities.

Escherichia coli CueO (copper efflux oxidase) is a unique multicopper oxidase in terms of its molecular architecture to carry a methionine-rich helical region, beneath which the substrate-binding site for Cu^+ ion is located.[1–3] Because of the presence of this methionine-rich helical region, the access of organic substrates to the catalytic site is inhibited, and CueO functions as the sole known cuprous oxidase. Nevertheless, CueO has the same catalytic Cu centers as other multicopper oxidases: a type I Cu to function as the entry of electron and a trinuclear Cu center comprised of a type II Cu and a pair of type III Cu atoms to bind and reduce dioxygen to two water molecules.[4,5] The type I Cu is responsible for the charge-transfer bands, His(N) $\rightarrow Cu^{2+}$ at ca. 440 nm, $Cys(S^-)_\sigma \rightarrow Cu^{2+}$ at ca. 520 nm, and $Cys(S^-)_\pi \rightarrow Cu^{2+}$ at 610 nm, and the d–d transition bands at >700 nm. The type III Cu bridged with a hydroxide ion affords an intense charge-transfer band, $OH^- \rightarrow Cu^{2+}$ at ca. 330 nm, while the type II Cu does not give a conspicuous band in the visible region. The type I Cu and the type II Cu give rise to the characteristic signals in the electron paramagnetic resonance (EPR) spectra, while the type III Cu atoms are EPR-silent because of the strong antiferromagnetic interaction.

In order to study function and reaction mechanisms and apply to biofuel cells, we have performed point mutations and engineering of CueO.[3,6] In the present study, we carried out mutations of Met510 axially coordinating the type I Cu aimed at obtaining a variety of type I Cu centers (Figure 1 shows the sequence alignment around the type I Cu center in multicopper oxidases, nitrite reductases containing a type I Cu and a type II Cu, and blue copper proteins containing a type I Cu). We replaced Met510 with Leu to obtain the three-coordinated type I Cu center found in fungal laccases with more positive redox potentials.[4] In line with this, we replaced Met510 with Ala and Thr to study the effect of bulkiness and polarity of the side chain on redox potential, although these axial ligands have not been found in naturally occurring type I Cu centers. In addition, we replaced Met510 with Gln in order to obtain a type I Cu center with a more negative redox potential found in a class of blue copper proteins, phytocyanin such as stellacyanin and mavicyanin, and the Met to Gln mutants of nitrite reductase and blue copper proteins, azurin and pseudoazurin.[7–9] Recently, we have performed analogous mutations at Met467 in a multicopper oxidase, *Myrothecium verrucaria* bilirubin oxidase, but the compensatory binding of the distal Asn459, the unconserved residue in other multicopper oxidases (Figure 1), did not allow us to obtain systematic data in spite of the double mutations to avoid the binding of this amino acid.[10–12]

Experimental

With a QuikChange kit (Stratagene) the genes for Met510Leu, Met510Gln, Met510Ala, and Met510Thr with a 6xHis-tag at the C-terminal were synthesized by using the template plasmid pUCCueO′, in which the *Nco*I site was introduced at Ala375 as the silent mutation site (GCG → GCC), and the designed primers as shown in Chart 1.

The plasmids containing the mutated site were digested with *Nco*I and *Bam*HI, and exchanged with the corresponding gene fragments (about 450 bp) of pUCCueO′ to give pUCCueO-M510L/Q/A/T. *E. coli* Origami (DE3)/pLac I (Novagen) was transformed with the mutant plasmids. Cultivation of the transformants and purification of the mutant proteins were carried out as described previously.[3] Protein concentration was determined by using the BCA (bicinchoninate) protein assay reagent (Pierce) and from the absorption intensity at 280 nm.

The total copper content in each CueO mutant was determined by atomic absorption spectroscopy on a Varian SpectrAA-50 spectrometer. Absorption spectra were measured on a JASCO V-560 spectrometer for the wavelength region 240–900 nm and circular dichroism (CD) spectra on a JASCO J-500C spectropolarimeter for the wavelength regions 300–800 nm (photomultiplier detector) and 700–1000 nm (Pb-S cell detector). X-band EPR spectra were measured on a JEOL JES-RE1X spectrometer at 77 K.

Published on the web April 11, 2009; doi:10.1246/bcsj.82.504

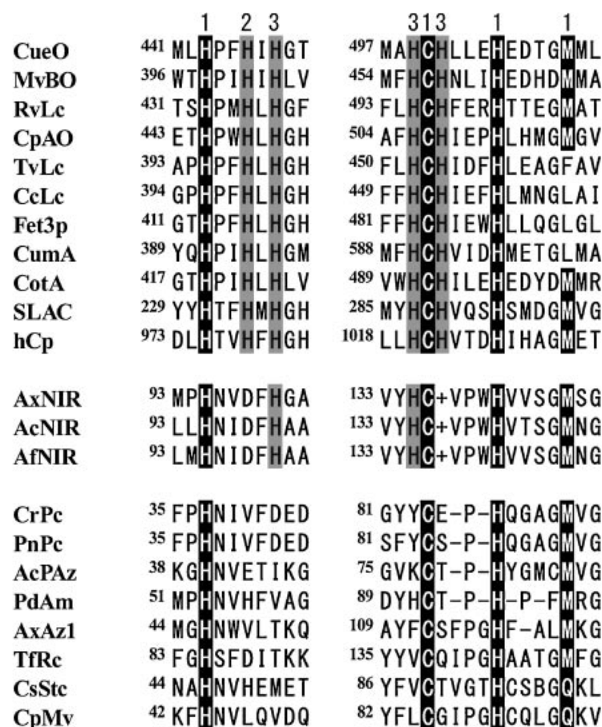

Figure 1. Amino acid sequence alignment around the type I Cu binding site of multicopper oxidase, nitrite reductase, and blue copper protein. 1, 2, and 3 represent type I Cu ligand, type II Cu ligand, and type III Cu ligand, respectively. Plus represents the five amino acids as spacers. MvBO, *Myrothecium verrucaria* bilirubin oxidase; RvLc, *Rhus vernicifera* laccase; CpAO, *Cucurbita pepo* ascorbate oxidase; TvLc, *Trametes versicolor* laccase; CcLc, *Coprinus cinerius* laccase; SLAC, small laccase from *Streptomyces coelicolor*; hCp, human ceruloplasmin; AxNIR, *Alcaligenes xylosoxidans* nitrite reductase; AcNIR, *Achromobacter cycloclastes* nitrite reductase; AfNIR, *Alcaligenes faecalis* nitrite reductase; CrPC, *Chlamydomonas reinhardtii* plastocyanin; PnPc, *Populus nigra* plastocyanin, AcPAz, *Achromobacter cycloclastes* pseudoazurin; PdAm, *Paracoccus denitrificans* amicyanin; TfRc, *Thiobacillus ferroxidans* rusticyanin; CsStc, *Cucumis sativus* stellacyanin; CpMv, *Cucurbita pepo* mavicyanin.

Activities of the mutants for oxidizing 2,2′-azinodi-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were colorimetrically determined from the absorption changes at 420 nm in acetate buffer, pH 5.5.[3] One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute based on the molar extinction coefficients of the one-electron oxidized product (radical cation, $ABTS^{+\bullet}$), 36.0 $mM^{-1}\,cm^{-1}$ ($M = mol\,dm^{-3}$).[13]

Results and Discussion

All Met510 mutants of CueO were purified to homogeneity to give a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (53 kDa, not shown). Met510Leu, Met510Gln, Met510Ala, and Met510Thr contained 3.6, 4.0, 4.0, and 3.5 copper ions per protein molecule, respectively (Experimental error, ca. 10%). We also tried to prepare Met510Phe containing another type I Cu center found in fungal laccases (Figure 1), but its copper content was 2.6 in spite of the changes in cultivation conditions and dialysis against Cu^{2+} or Cu^{+}. While such reduced copper content has been reported in the mutants for copper ligand of bilirubin oxidase, CotA and laccase,[14–16] we excluded data on Met510Phe without a multiplying factor.

M510L(+), 5′-GATACGGGGCTGATGTTAGGG-3′

M510L(−), 5′-CCCTAACATCAGCCCCGTATC -3′

M510Q(+), 5′-GATACGGGGCAGATGTTAGGG-3′

M510Q(−), 5′-CCCTAACATCTGCCCCGTATC -3′

M510A(+), 5′-GATACGGGGGCTATGTTAGGG-3′

M510A(−), 5′-CCCTAACATAGCCCCCGTATC -3′

M510T(+), 5′-GATACGGGGACGATGTTAGGG-3′

M510T(−), 5′-CCCTAACATCGTCCCCGTATC -3′

Chart 1.

Met510Leu/Gln/Ala/Thr gave a strong absorption band derived from the charge-transfer, $Cys(S^{-})_{\pi} \rightarrow Cu^{2+}$ at 610–613 nm (Figure 2A top: black line for the recombinant CueO (rCueO), blue line for Met510Leu, green line for Met510Gln, red line for Met510Ala, and orange line for Met510Thr). The type I Cu had been fully oxidized as ascertained by the fact that the absorption intensities of the 610–613 nm band of rCueO and mutants did not increase upon reaction with an oxidizing reagent, hexacyanoferrate(III). Therefore, Met510 mutants have a decreased extinction coefficient, $\varepsilon = 3900$–$4700\,M^{-1}\,cm^{-1}$ from that of rCueO, $\varepsilon = 6300\,M^{-1}\,cm^{-1}$. All Met510 mutants showed a strong absorption band at ca. 330 nm, $\varepsilon = 4800$–$6200\,M^{-1}\,cm^{-1}$ comparable to that of rCueO, $\varepsilon \approx 5000\,M^{-1}\,cm^{-1}$, indicating that the electronic state of the trinuclear Cu center was not disturbed by the mutations at the remote site, ca. 13 Å (Å $= 10^{-10}$ m) apart. The d–d band originating in the type I Cu of Met510Leu was observable at ca. 780 nm as a shoulder similarly to rCueO. On the other hand, those of Met510Gln/Ala/Thr shifted to ca. 800 nm and their intensities were considerably reduced (The broadened features at 700–900 nm were not due to shift in baseline, but were intrinsic to the mutants). Analogous spectral features in the d–d band of the type I Cu have been also reported for the axial ligand mutants of blue copper proteins, bilirubin oxidase, and nitrite reductase.[7,8,12] The spectral features of Met510Ala and Met510Thr were unexpectedly analogous to those of Met510Gln, suggesting that an O atom occupied the vacant axial position (vide infra).

In the CD spectra of rCueO and Met510Leu (Figure 2A bottom) the positively-signed bands coming from the charge-transfers, $Cys(S^{-})_{\pi} \rightarrow Cu^{2+}$ and $Cys(S^{-})_{\sigma} \rightarrow Cu^{2+}$ were observed at 580 and ca. 500 nm (shoulder), respectively. On the other hand, the CD spectral features of Met510Gln were

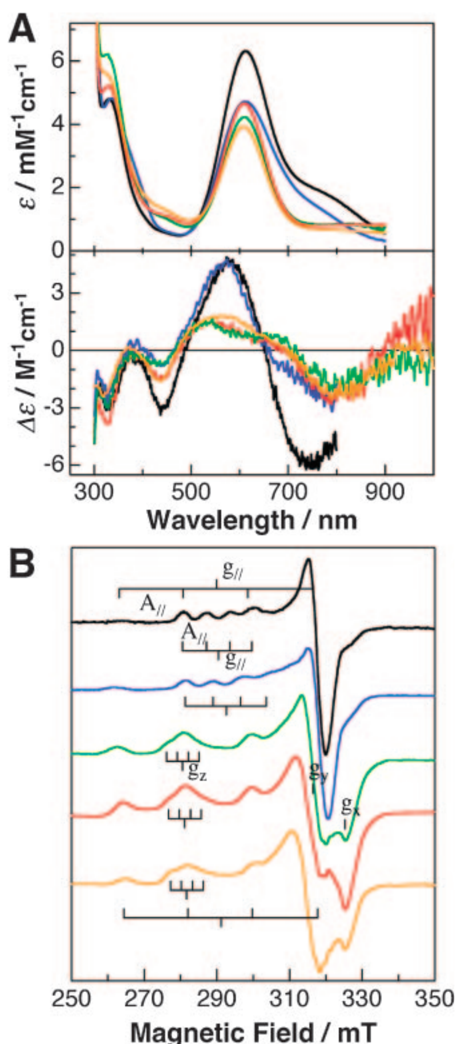

Figure 2. Absorption (A, top), CD (A, bottom), and EPR (B) spectra of rCueO and Met510 mutants, Met510Leu/Gln/Ala/Thr. Spectra of rCueO, Met510Leu, Met510Gln, Met510Ala, and Met510Thr are figured with black, blue, green, red, and orange lines, respectively. Absorption and CD spectra were measured at room temperature using a 1 cm path-length quartz cell for ca. 100 µM proteins in 0.1 M phosphate buffer, pH 7. The units of the ordinate are based on protein molecule. EPR spectra were measured at 77 K, frequency 9.2 GHz, microwave power 4 mW, modulation 1 mT at 100 kHz, filter 0.3 s, sweep time 4 or 8 min, and amplitude 400.

modified due to the replacement of the thioether ligand with the amide oxygen in the corresponding region.[17] Similar to the case of the absorption spectrum, Met510Ala and Met510Thr gave analogous CD spectra to that of Met510Gln, supporting that an O atom occupied the vacant axial position. The intensity of the CD band coming from the d–d transitions of Met510Leu was reduced to almost half of that of rCueO, being accompanied by a shift in wavelength from 745 to ca. 790 nm. The intensities of the CD band derived from the d–d transitions of Met510Gln/Ala/Thr were also prominently reduced with a red-shift of ca. 50 nm in wavelength. Although the noise level of these CD spectra of Met510Ala in the region 800–1000 nm is considerably high, it is apparent that the d–d band is involved in the transition of the d_{z^2} orbital at >1000 nm for Met510Gln/Ala and may also be for Met510Thr.[16] The CD band at 330 nm due to the charge-transfer, $OH^- \rightarrow Cu^{2+}$ of Met510Leu/Gln/Ala/Thr was as intense as that of rCueO (Figure 2A bottom).

Both the type I Cu and type II Cu signals were observed in the EPR spectra of the Met510 mutants (Figure 2B and Table 1). The spin Hamiltonian parameters of Met510Leu were $g_{//} = 2.22$ and $A_{//} = 8.4 \times 10^{-3}\,cm^{-1}$ for the type I Cu, and $g_{//} = 2.24$ and $A_{//} = 20 \times 10^{-3}\,cm^{-1}$ for the type II Cu. While the type I Cu became three-coordinated by the mutation, the EPR signal remained axial with a slight modification in the parameters from those of rCueO, $g_{//} = 2.24$ and $A_{//} = 6.7 \times 10^{-3}\,cm^{-1}$. The parameters of the type II Cu were unchanged after the mutation. On the contrary, Met510Gln/Ala/Thr afforded the rhombic type I Cu signal typical of the phytocyanin-like type I Cu center: $g_z = 2.31$–2.34, $g_y = 2.06$–2.07, $g_x = 2.00$, $A_z = 3.5$–$3.6 \times 10^{-3}\,cm^{-1}$.[7–9,11,17,19] The total number of the EPR detectable Cu^{2+} was 2.0 per protein molecule within the experimental error, ca. 10%, for all mutants.

Absorption, CD, and EPR spectra of Met510Ala and Met510Thr indicated that the axial site of the type I Cu was not vacant but was occupied by an O atom. In the case of Met510Gln, the origin of the O atom is the amide carbonyl group in the side chain of Gln. On the other hand, the origin of the O atom in Met510Ala and Met510Thr is presumed to be a water molecule, which might become accessible to the type I Cu site by the substitutions of amino acids with less bulky side chains. The occupation of a water molecule in the open axial site of the type I Cu has been reported for Met98Ala of amicyanin and Met121Asp and the deleted mutant from Met121 to Lys128 at the C-terminal end (End-121) of azurin, although the mutations of azurin also induced a shortening of the distance from the carbonyl O atom of Gly45 to the type I Cu, 2.97 to 2.74 Å leading to an approximately trigonal-bipyramidal geometry.[20,21] These approaches of the carbonyl O atom of Gly45 to the type I Cu produced a four-coordinated Cu site in the Met121Ala mutant of azurin without allowing the occupation of a water molecule in the open axial site.[22] On the other hand, a recent crystallographic study on the CotA mutant at the distal Ile494 to Ala showed the coordination of a water molecule at the type I Cu site leading to a five-coordinated Cu site with an approximately trigonal-bipyramidal geometry.[19] The axial site of the type I Cu center in Met144Ala of nitrite reductase was vacant, and accordingly three-coordinated.[23,24] In contrast, the hydroxy group of Thr182 was directed to the mutated type I Cu center of the so-called green nitrite reductase with a distance of 3.4–3.6 Å (protein data bank (PDB) code 1MZZ), giving the spectra typical of the type I Cu center with an axial symmetry.[25,26] In the case of the Met469 mutants of bilirubin oxidase, the distal Asn459 compensatory occupied the vacant site.[12] However, the amino acid sequence (Figure 1) and the X-ray crystal structure of CueO indicate that neither potential compensatory amino acid such as Asn and Gln nor peptide backbone is present within the coordination sphere of the type I Cu center.[2] The diversity of the modifications on the type I Cu center demonstrates that position and rigidity of the axial ligands have a profound influence in the determination of the geometry and spectroscopic properties.

Table 1. Kinetic Parameters for the Oxidation of ABTS, EPR Spectral Data, and Redox Potentials of rCueO and Met510 Mutants

Enzyme	Kinetic parameters[a)]		Type I Cu signal in EPR		Redox potential[b)]
	K_m/mM	k_{cat}/s^{-1}	$g_{//}$/cm^{-1}	$A_{//}$/10^{-3} cm^{-1}	E_m/V vs. SHE
rCueO	6.5 ± 0.3	1.2 ± 0.11	2.24	6.7	0.50
Met510Leu	3.7 ± 0.2	6.1 ± 0.11	2.22	8.4	(0.54)[c)]
Met510Gln	7.1 ± 0.9	0.036 ± 0.002	2.34[d)]	(3.6)[d)]	0.37
Met510Ala	6.0 ± 0.6	0.035 ± 0.002	2.31[d)]	(3.5)[d)]	(0.38)[c)]
Met510Thr	5.9 ± 0.5	0.16 ± 0.006	2.32[d)]	(3.5)[d)]	(0.47)[c)]

a) One unit of activity is defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute in 0.1 M acetate buffer (pH 5.5). b) Midpoint potential at pH 5 determined by cyclic voltammetry using a carbon–gel electrode[18] and unpublished data. c) Midpoint potential estimated from voltammetric responses in the presence of O_2. d) g_z and A_z values are indicated. g_y and g_x values are 2.06–2.07 and 2.00, respectively.

We have determined the redox potential of the type I Cu in rCueO to be 0.50 V (vs. SHE (standard hydrogen electrode)) at pH 5 (0.44 V at pH 7) by electrochemical study using a carbon–gel electrode.[18] The value of Met510Leu is shifted toward positive potential by 40 mV, whereas those of Met510Gln, Met510Ala, and Met510Thr toward negative potentials by ca. 130, 120, and 30 mV, respectively (Detailed electrochemical data will be published elsewhere). When the axial site of the type I Cu center in mutants was vacant as in fungal laccases,[4,5] the redox potential was shifted to positive potential: the redox potentials of the type I Cu in Met121Ala of azurin and Met144Ala of nitrite reductase were shifted from 310 to 373 mV and 240 to 314 mV, respectively.[21,22] On the other hand, when an O atom coordinated the axial site in place of an S atom, the redox potential was shifted to negative potential: the redox potentials of the type I Cu in Met121Asp and End-121 of azurin changed from 346 mV to 327 and 205 mV, respectively.[19] The type I Cu center in stellacyanin, which has the coordination of the amide O atom, shows the most negative redox potential, 184 mV, among the type I Cu centers.[27] The slight shift in the redox potential of Met510Thr toward negative potential by ca. 30 mV might be a result of an integrated effect of the coordination of an O atom and hydrophobicity caused by the side chain of the Thr residue. Supporting this, the redox potential of the Thr mutant of nitrite reductase, in which the hydroxy group of Thr182 was simply oriented to the type I Cu (vide supra), changed from 247 to 354 mV.[25]

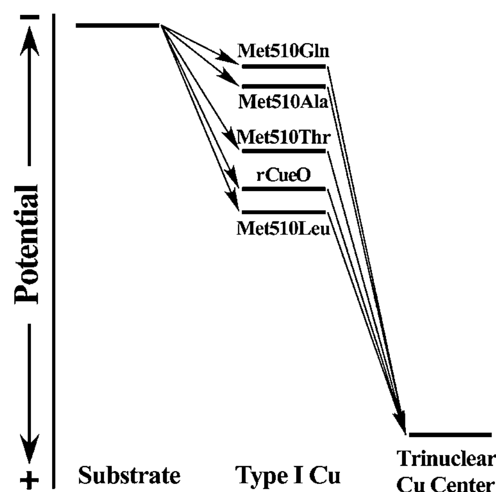

Figure 3. Scheme of the redox potential of the Met510 mutants of CueO to show how the electron transfers to and from the type I Cu center are modified. The potential of substrate is arbitrarily positioned, while the relative difference in the redox potentials of the Cu centers are taken into consideration in preparing the scheme based on Ref. 18 and unpublished data.

The K_m and k_{cat} values for the oxidation of ABTS by the Met510 mutants are tabulated in Table 1 together with the redox potential and the spin Hamiltonian parameters of the type I Cu. Met510Leu exhibited higher activities than rCueO presumably because the shift in the redox potential toward positive potential by ca. 40 mV favored the electron transfer between substrates and the type I Cu, although the intramolecular electron transfer between the type I Cu and the trinuclear Cu center would have become slightly unfavorable. Accordingly, it appears that the former thermodynamic driving force of electron transfer is superior to the latter in the case of Met510Leu. An analogous conclusion has been also derived from a flash-photolysis study.[2] In contrast, the shift in redox potential of Met510Gln/Ala/Thr by 30–130 mV toward negative potentials favored the electron transfer between the type I Cu and the trinuclear Cu center, while the electron transfer between substrates and the type I Cu became unfavorable. The order of enzyme activity, Met510Leu > rCueO > Met510Thr > Met510Ala ≈ Met510Gln, coincides with the order of the redox potential of type I Cu, indicating that the electron transfer between substrate and the type I Cu is a dominating process to determine the overall enzyme activity of CueO.

Met510, the axial ligand to the type I Cu in CueO, was substituted with Leu, Ala, Thr, and Gln. The type I Cu center in Met510Leu showed an axial symmetry similar to the type I Cu center in fungal laccases, and its redox potential shifted toward positive potential promoting the oxidizing activity of ABTS. On the other hand, the type I Cu center in Met510Gln/Ala/Thr showed a rhombic symmetry due to the coordination of an O atom, and their redox potentials were shifted to negative potentials leading to the drastic decreases in enzyme activities. Figure 3 schematically shows how the mutations at Met510 affect the electron transfer to and from the type I Cu and overall enzyme activities. This is the first example of the four axial

variants of the type I Cu center (thioether, amide oxygen, H_2O, and vacancy) prepared for multicopper oxidase, in correlation with their spectroscopic properties and enzyme activities.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 19350081 to T.S.) and NEDO. The authors also thank Toyota Motor Corporation and Mandom Corporation for financial support.

References

1 C. Rensing, G. Grass, *FEMS Microbiol. Rev.* **2003**, *27*, 197.
2 S. A. Roberts, A. Weichsel, G. Grass, K. Thakali, J. T. Hazzard, G. Tollin, C. Rensing, W. R. Montfort, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2766.
3 K. Kataoka, H. Komori, Y. Ueki, Y. Konno, Y. Kamitaka, S. Kurose, S. Tsujimura, Y. Higuchi, K. Kano, D. Seo, T. Sakurai, *J. Mol. Biol.* **2007**, *373*, 141.
4 T. Sakurai, K. Kataoka, *Cell. Mol. Life Sci.* **2007**, *64*, 2642.
5 T. Sakurai, K. Kataoka, *Chem. Rec.* **2007**, *7*, 220.
6 Y. Ueki, M. Inoue, S. Kurose, K. Kataoka, T. Sakurai, *FEBS Lett.* **2006**, *580*, 4069.
7 M. A. Hough, J. F. Hall, L. D. Kanbi, S. S. Hasnain, *Acta Crystallogr., Sect. D* **2001**, *57*, 355.
8 K. Kataoka, K. Yamaguchi, S. Sakai, K. Takagi, S. Suzuki, *Biochem. Biophys. Res. Commun.* **2003**, *303*, 519.
9 K. Kataoka, A. Kondo, K. Yamaguchi, S. Suzuki, *J. Inorg. Biochem.* **2000**, *82*, 79.
10 A. Shimizu, T. Sasaki, J. H. Kwon, A. Odaka, T. Satoh, N. Sakurai, T. Sakurai, S. Yamaguchi, T. Samejima, *J. Biochem.* **1999**, *125*, 662.
11 K. Kataoka, R. Kitagawa, M. Inoue, D. Naruse, T. Sakurai, H.-W. Huang, *Biochemistry* **2005**, *44*, 7004.
12 K. Kataoka, K. Tsukamoto, R. Kitagawa, T. Ito, T. Sakurai, *Biochem. Biophys. Res. Commun.* **2008**, *371*, 416.
13 K. Otsuka, T. Sugihara, Y. Tsujino, T. Osakai, E. Tamiya, *Anal. Biochem.* **2007**, *370*, 98.
14 P. Durão, I. Bento, A. T. Fernandes, E. P. Melo, P. F. Lindley, L. O. Martins, *J. Biol. Inorg. Chem.* **2006**, *11*, 514.
15 A. Shimizu, J.-H. Kwon, T. Sasaki, T. Satoh, N. Sakurai, T. Sakurai, S. Yamaguchi, T. Samejima, *Biochemistry* **1999**, *38*, 3034.
16 A. E. Palmer, R. K. Szilagyi, J. R. Cherry, A. Jones, F. Xu, E. I. Solomon, *Inorg. Chem.* **2003**, *42*, 4006.
17 T. Sakurai, S. Suzuki, in *Multi-copper Oxidases*, ed. by A. Messerschmidt, World Scientific, Singapore, **1997**, pp. 225–250.
18 Y. Miura, S. Tsujimura, S. Kurose, Y. Kamitaka, K. Kataoka, T. Sakurai, K. Kano, *Fuel Cells* **2009**, *9*, 70.
19 P. Durão, Z. Chen, C. S. Silva, C. M. Soares, M. M. Pereira, S. Todorovic, P. Hildebrandt, I. Bento, P. F. Lindley, L. O. Martins, *Biochem. J.* **2008**, *412*, 339.
20 C. J. Carrell, J. K. Ma, W. E. Antholine, J. P. Hosler, F. S. Mathews, V. L. Davidson, *Biochemistry* **2007**, *46*, 1900.
21 L. M. Murphy, R. W. Strange, B. G. Karlsson, L. G. Lundberg, T. Pascher, B. Reinhammar, S. S. Hasnain, *Biochemistry* **1993**, *32*, 1965.
22 L.-C. Tsai, N. Bonander, K. Harata, G. Karlsson, T. Vänngård, V. Langer, L. Sjölin, *Acta Crystallogr., Sect. D* **1996**, *52*, 950.
23 M. J. Ellis, M. Prudêncio, F. E. Dodd, R. W. Strange, G. Sawers, R. R. Eady, S. S. Hasnain, *J. Mol. Biol.* **2002**, *316*, 51.
24 O. Farver, R. R. Eady, G. Sawers, M. Prudêncio, I. Pecht, *FEBS Lett.* **2004**, *561*, 173.
25 K. Olesen, A. Veselov, Y. Zhao, Y. Wang, B. Danner, C. P. Scholes, J. P. Shapleigh, *Biochemistry* **1998**, *37*, 6086.
26 L. Basumallick, R. K. Szilagyi, Y. Zhao, J. P. Shapleigh, C. P. Scholes, E. I. Solomon, *J. Am. Chem. Soc.* **2003**, *125*, 14784.
27 B. R. M. Reinhammar, *Biochim. Biophys. Acta* **1972**, *275*, 245.