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A Labile Regulatory Copper Ion Lies Near the T1 Copper Site in the Multicopper Oxidase CueO*

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CueO, a multicopper oxidase, is part of the copper-regulatory *cue* operon in *Escherichia coli*, is expressed under conditions of copper stress and shows enhanced oxidase activity when additional copper is present. The 1.7-Å resolution structure of a crystal soaked in $CuCl_2$ reveals a Cu(II) ion bound to the protein 7.5 Å from the T1 copper site in a region rich in methionine residues. The trigonal bipyramidal coordination sphere is unusual, containing two methionine sulfur atoms, two aspartate carboxylate oxygen atoms, and a water molecule. Asp-439 both ligates the labile copper and hydrogen-bonds to His-443, which ligates the T1 copper. This arrangement may mediate electron transfer from substrates to the T1 copper. Mutation of residues bound to the labile copper results in loss of oxidase activity and of copper tolerance, confirming a regulatory role for this site. The methionine-rich portion of the protein, which is similar to that of other proteins involved in copper homeostasis, does not display additional copper binding. The type 3 copper atoms of the trinuclear cluster in the structure are bridged by a chloride ion that completes a square planar coordination sphere for the T2 copper atom but does not affect oxidase activity.

Copper is an essential cofactor for enzymes that generate cellular energy, detoxify oxygen-derived radicals, mobilize iron, coagulate blood, and cross-link connective tissue. However, free copper is toxic because of its role in generating reactive oxygen species that can damage lipids, proteins, and DNA and is therefore highly regulated in the cell and implicated in several diseases (1–4). In *Escherichia coli*, three systems for copper tolerance have been identified, the chromosomally encoded *cue* (for Cu efflux) and *cus* (for Cu sensing) systems (5), and the plasmid-encoded *pco* (for plasmid-borne copper resistance) (6). The *cue* system employs a cytosolic metalloregulatory protein, CueR, that induces expression of two proteins under conditions of copper stress, CopA and CueO. CopA is a copper efflux P-type ATPase that is located in the inner cell membrane (7), and CueO is a periplasmic multicopper oxidase that is stimulated by exogenous copper(II) (8–10). Disruption of the *CueO* gene renders cells more sensitive to copper (11), underlining its role in copper homeostasis. The *cus* system includes four proteins (CusCFBA) that are thought to allow copper transport directly from the periplasm across the outer membrane, and respond mainly to anaerobic copper stress (12). The *pco* system, like the *cue* system, contains a periplasmic multicopper oxidase (PcoA), a periplasmic copper binding protein (PcoC), and three other proteins (PcoBDE) (13).

Both the *cue* and *pco* systems rely on multicopper oxidases for copper tolerance; however, the functional role of these proteins remains unclear, as does the *in vivo* substrate. In general, multicopper oxidases couple four one-electron substrate oxidation steps to the four-electron reduction of dioxygen to water (14). CueO can oxidize a range of substrates *in vitro*, including catechols, siderophores, and Fe(II) (10), and this activity is greatly enhanced in the presence of excess (~100 μM) copper(II) ions (10, 15), suggesting that binding of a labile copper is important for enzymatic activity. We recently determined the crystal structure of CueO at 1.4-Å resolution (15) and showed that its structure is similar to that of other multicopper oxidases, such as ascorbate oxidase (16) and several laccases (17–19). As expected, CueO contains a T1 "blue copper" site, near the point at which the larger substrate is believed to bind, as well as a trinuclear copper center, where oxygen reduction occurs. However, CueO possesses a methionine-rich motif not found in these other proteins that could potentially be involved in copper binding. Portions of this motif (residues 356–371) are part of a helix lying near, and blocking access to, the T1 copper site (15); the remaining residues in the motif (380–402) are disordered in the structure.

Methionine-rich regions are found in numerous proteins involved in copper homeostasis, leading to the suggestion that such regions are involved in copper binding (recently reviewed in Refs. 20 and 21). Of these, Ctr1 (for copper transport protein 1) has been shown to require one or more MXXM motifs for activity in the yeast and human proteins (22), and CopA has been shown to bind seven extra copper ions (23). However, PcoC and the related periplasmic protein CopC bind only one copper ion (20, 21, 23). The recently determined PcoC crystal structure displayed an interface between methionine-rich regions in adjacent molecules in the crystal, leading the authors to suggest that the role of such regions may be in protein-protein recognition, possibly between PcoC and PcoA, rather than in copper binding (24).

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The atomic coordinates and structure factors (codes 1N68 (CueO+CuCl and 1PF3 (CueO M441L)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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TABLE I
X-ray data measurement and refinement statistics

Mutation	None + $CuCl_2$	M441L
Space Group	$P2_1$	$P2_1$
Cell Parameters		
a (Å)	50.00	50.57
b (Å)	90.94	91.29
c (Å)	53.81	53.97
β (°)	102.4	102.1
Temperature (K)	100	100
Resolution (Å)	26.3–1.7	1.0–1.5
Wavelength (Å)	1.378	0.90
Total/unique reflections	237,516/51,492	147,786/63,714
Completeness[a]	0.997/0.997	0.858[b]/0.936
Mean I/σ(I)[a]	11.6/3.0	20.2/2.9
R_{sym}[a]	0.075/0.256	0.066/0.180
R_{cryst}/R_{free}[c]	0.165/0.216	0.191/0.227
RMSD[e] bonds/angles (Å)	0.007/0.024	0.015/1.6[d]

[a] Overall/outer shell.
[b] Low completeness of overall data results from removal of reflections obscured by ice rings during integration.
[c] Test set contains 5% of reflections.
[d] Angle error in degrees.
[e] Root-mean-square deviation.

In the present work, we report the crystal structure of CueO bound to exogenous copper. We find a copper ion bound to the methionine-rich helix near the T1 center, ligated to residues required for copper tolerance. Mutations to the ligating residues yield reduced CueO oxidase activity *in vitro* and reduced copper tolerance *in vivo*.

EXPERIMENTAL PROCEDURES

Protein Expression and Crystallization—Expression and purification of wild-type and mutant forms of CueO from *E. coli* strain BL21 (Stratagene) using Strep-TagII affinity chromatography and crystallization by the hanging drop method, were carried out as previously described (9, 15). For data measurement, a crystal grown in 20% polyethylene glycol 4000, 0.2 M ammonium acetate, 0.1 M sodium acetate, pH 4.6, was soaked for 60 min in 10 mM $CuCl_2$ and 30% polyethylene glycol, transferred to 50% polyethylene glycol 4000, picked up in a small loop (Hampton), and flash-frozen in liquid nitrogen. Attempts to grow crystals in the presence of copper failed, and crystals that were placed in a solution made with $CuSO_4 \cdot 5H_2O$ dissolved. The crystal of the M441L mutant protein was grown by the same procedure.

X-ray Data Measurement and Structural Refinement—For the $CuCl_2$-soaked crystal, data were measured at Stanford Synchrotron Radiation Laboratory beam-line 9–2 and processed with D*TREK (25) (Table I). An anomalous difference Fourier electron density map calculated with phases from the CueO model with copper atoms deleted revealed the four expected copper sites, an additional highly occupied site near the T1 copper, and a partially occupied site on the surface of the protein. These atoms were added to the model and the structure refined with SHELX (26). Metal-ligand distances and angles were not restrained. Refinement cycles were interspersed with rebuilding using O (27). Final refinement included hydrogen atoms in calculated positions. Other programs of the CCP4 package (28) were used for structural analysis, and MolScript (29), Bobscript (30), and Raster3D (31) were used for figure preparation. Data for the M441L mutant were measured at the Advanced Photon Source, BioCARS, beam line 14-BMC. Data were processed with D*TREK and refined with REFMAC from the CCP4 suite (28).

Bacterial Strains and Growth Media—Strains used in this work are *E. coli* XLI blue, GR15 Δ*cueO* Δ*cusCFBA* (pASK-IBA3), GR15 (pCueO) (9), GR15 (pCueO M355L), GR15 (pCueO M441L), GR15 (pCueO D360A), and GR15 (pCueO D439A). *E. coli* cells were grown in Luria Bertani medium with antibiotics (100 μg/ml ampicillin and 15–20 μg/ml chloramphenicol) or with $CuCl_2$ added where appropriate.

Mutagenesis of cueO—Chromosomal DNA of *E. coli* strain W3110 was purified using Genomic-tips (Qiagen). Mutant *cueO* derivatives were generated by overlap extension-PCR from genomic DNA of *E. coli* W3110 with the Expand High Fidelity PCR system (Roche Applied Science) using the following primers: FW, 5′-GAAGAATTCATGCAACGTCGTGATTTCTTAAAAT-3′ with reverse primers M355L-U, 5′-TCGAGCATCGGGTCCA**G**AGAGAGTTGCAGCTTG-3′; D360A-U, 5′-CATCTGCATCCCCATCATA**G**CGAGCATCGGGTC-3′; D439A-U, 5′-GGATGCAGCATCATG**G**CGCCCACGCCAGAGATA-3′; M441L-U, 5′-TATGGAACGGATGCAGCA**G**CATGTCGCCCACGC-3′ yielding upper partial *cueO* genes and REV, 5′-GTCCTGCAGTACCGTAAACCCTAACATCATCCCC-3′ with forward primers M355L-D, 5′-CAAGCTGCAACTCTC**TC**TGGACCCGATGCTCGA-3′; D360A-D, 5′-GACCCGATGCTCG**C**TATGATGGGGATGCAGATG-3′; D439A-D, TATCTCTGGCGTGGGCG**C**CATGATGCTGCATCC-3′; M441L-D, GCGTGGGCGACATG**C**TGCTGCATCCGTTCCATA-3′ yielding lower partial *cueO* genes (restriction sites are underlined and base changes are bold). Cognate PCR fragments with introduced base pair changes were mixed, treated with *Dpn*I to digest the *dam*-methylated template DNA, purified, and used for a second round of PCR with primers FW and REV. The mutated full-length *cueO* genes were purified, cut, and cloned into plasmid pASK-IBA3 (IBA, Göttingen, Germany) *via* the *Eco*RI and *Pst*I restriction sites and transformed into *E. coli* XLI blue (Stratagene). The resulting plasmids expressed *cueO*-derivatives as C-terminal fusion proteins with the Strep-TagII epitope (SAWSHPNFEK), plus eight additional residues at the N terminus (GDRGPEF), which are removed along with the next 27 amino acids that comprise a signal sequence during export to the periplasm (15).

Immunoblotting—To detect CueO, strains expressing CueO-derivatives were grown in Luria Bertani broth at 37 °C to A_{600} = 0.5. The inducer anhydrotetracycline (200 μg/l) was added and CueO-expression was carried out for 3 h at 30° C. Cells were harvested, aliquots separated on SDS-PAGE, and blotted onto a polyvinylidene difluoride membrane. CueO-derivatives were detected by their Strep-TagII-epitopes with a streptavidin-horseradish peroxidase conjugate, as described previously (13).

Functional Assays—The model substrate 2,6-dimethoxyphenol (DMP)[1] (Aldrich) was used to assess catalytic activity by monitoring the change in absorption at 468 nm in the presence of CueO, as described previously (9). CueO activity was also measured as oxygen uptake in presence of external $CuCl_2$ using DMP as an artificial electron donor. The change in oxygen concentration was monitored by an oxygraph instrument (Hansatech, Cambridge UK), as described previously (32). The assay mixture contained 0.1 M Tris buffer (pH adjusted with glacial acetic acid to specified values), 2 mM DMP, variable concentrations of $CuCl_2$, and 250 μM O_2 (estimated from Table values) at room temperature. Purified CueO protein was added to reach a concentration in the range of 25–100 μg/ml. The reaction was initiated by the addition of $CuCl_2$ (iron content < 0.005%; Mallinckrodt AR) and the initial rates were determined. Kinetic values for V_{max} and K_m were estimated using Lineweaver-Burk analyses and varying copper concentration between 0.14 and 20 mM. Water was millipore-purified and solutions were freshly prepared before each experiment.

Mass Spectroscopy—Mass spectral analyses were performed on a Thermoquest LCQ and a Bruker Reflex III MALDI-TOF instrument. Samples measured by MALDI-TOF were mixed with a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 70:30 H_2O/acetonitrile containing 0.1% of TFA, in such a way that the total amount of protein deposited was 1–2 pmol. In general, a laser attenuation of 80% was used. Electrospray ionization experiments were performed by direct injection of a ~10 μM solution of the protein in MeOH/H_2O, 1:1, containing 2% AcOH. All protein samples analyzed were in 20 mM Tris, pH 7.5, but diluted accordingly.

RESULTS

Structural Studies. Location of Labile Copper—Optimal CueO activity occurs only in the presence of excess Cu(II) ions. To uncover the molecular basis for this, diffraction data were measured from crystals that had been soaked in $CuCl_2$ and flash-frozen (see "Experimental Procedures" for details). Diffraction data were measured from a frozen crystal using x-rays with λ = 1.378 Å to maximize anomalous scattering by the copper atoms. The anomalous difference Fourier electron density map, using phases calculated from a CueO model with copper atoms removed, displayed six strong peaks, five of which are shown in Fig. 1. Four of the strong peaks (28–30σ) coincide with the previously defined T1 and trinuclear copper positions, whereas a fifth strong site (18σ) is located near the T1 copper position and is consistent with a fully occupied copper atom, referred to below as the labile or regulatory copper. A weaker

[1] The abbreviations used are: DMP, 2,6-dimethoxyphenol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

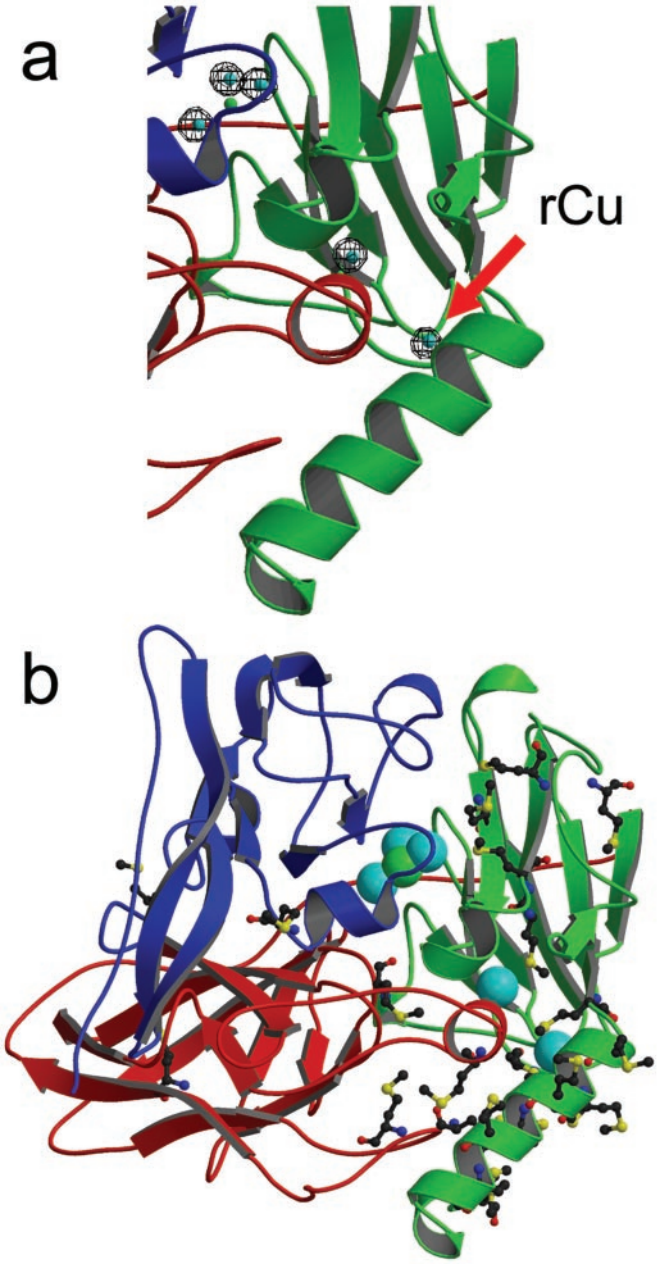

FIG. 1. **Location of copper ions and methionine residues in CueO.** *a*, ribbon drawing highlighting the positions (*cyan spheres*) and anomalous difference electron density peaks (contoured at 10σ) for the five highly occupied copper atoms. The newly discovered regulatory copper site (rCu) is indicated by an *arrow*; the partially occupied sixth copper site is not visible in this view. *b*, ribbon drawing highlighting the three related CueO domains (domain 1 in *blue*, 2 in *red*, 3 in *green*), the trinuclear copper center between domains 1 and 3 (copper in *cyan*, chlorine in *green*), the T1 and labile copper ions in domain 3, and the location of methionine residues (*ball-and-stick representation*). The labile copper lies near the T1 copper site and is tucked under one end of the methionine-rich helix (residues 356–371). Residues 380–402 are disordered in the crystal and are therefore missing from the figure.

peak (11σ) is apparently the result of a partially occupied copper-binding site on the surface of the protein that is unlikely to be of functional importance.

In the refined structure, the labile copper atom lies 7.5 Å from the T1 copper and is buried under the methionine-rich helix (Fig. 1). Coordination is through the SD atoms of Met-355 (2.2 Å) and Met-441 (2.5 Å), the OD1 atoms of Asp-360 (2.4 Å) and Asp-439 (2.1 Å), and a water molecule, HOH 2301 (3.1 Å), in a distorted trigonal bipyramidal coordination geometry (Fig. 2). Met-355 and HOH 2301 occupy axial positions, and Met-441, Asp-360, and Asp-439 are equatorial ligands. Interestingly, the labile copper and the T1 copper are directly linked through a hydrogen-bond from OD2 of Asp-439 to NE2 of His-443. As noted above, these two residues ligate the labile and T1 copper atoms, respectively, and the hydrogen-bond between them may provide a pathway for electron transfer between the two copper atoms. The largest structural change is for the side chain of Asp-439 with the ligating atom OD1 moving 2 Å, and CB moving 1.75 Å. The overall temperature factors for the labile copper and coordinating residues are somewhat higher than those for the other copper centers, averaging about 33 Å^2 *versus* 22 Å^2 for the entire protein (25 Å^2 for side chain atoms), but the positions for these atoms are clearly defined in the final electron density maps. In addition, there is no residual electron density in the former position of the Asp-439 side chain.

Protein Conformation—There are no significant structural changes in the protein upon copper binding. The coordinating side chains reorient to accommodate copper ligation, which is associated with small shifts in nearby backbone positions. The overall root-mean-square deviation in Cα atom positions between the structures with and without added copper is 0.18 Å; the largest shift (0.84 Å) was for the ligating residue Asp-439. It is possible that crystal lattice contacts could mask larger changes in this region upon binding copper, given that the structure was obtained *via* soaking of existing crystals with $CuCl_2$; however, this seems unlikely because of the sparse number of contacts in the region. Furthermore, the crystals have oxidase activity.

As noted, the labile copper ion is buried under the methionine-rich helix (residues 356–371), which, as in the absence of the labile copper, contains several non-copper-ligating methionine residues with poorly ordered side chains. The disorder is particularly evident in the methionine SD and CE atoms, where very large temperature factors are displayed. Amino acids 380–402 are not visible in the present electron density maps, as was also true in the absence of the labile copper. We have confirmed, by MALDI mass spectroscopic analysis of the frozen, copper-soaked crystals as prepared for data collection, that the protein is intact and not cleaved in this region. Possibly, the 22 unobserved amino acids become ordered in the presence of substrate or are involved in as-yet-unknown protein-protein interactions. A sixth partially occupied copper atom is found on the surface of the protein away from the active site region (15 Å from the T2 copper) and bound to His-488, Asp-132, and HOH 2001.

Trinuclear Copper Cluster—In the initial report of the crystal structure of CueO, we noted an unusual trinuclear center geometry containing a linear copper-oxygen-copper geometry (15). The copper atoms of the trinuclear center lie in nearly identical positions in the previous and present structures; however, in the present structure, modeling of the bridging atom as a single oxygen resulted in a low temperature factor (3 Å^2) and a 5σ residual electron density peak, indicating the need for more electrons at this site. The density is clearly spherical, and modeling as dioxygen, as was done in the structure of laccase from *Melanocarpus albomyces* (19), resulted in an unsatisfactory refinement. Modeling the atom as chlorine (10 mM $CuCl_2$ was added to the crystal), refined well, yielding copper-to-chlorine distances of 2.3 and 2.5 Å (unrestrained), similar temperature factors for copper and chlorine atoms (~20 Å^2), and no residual electron density. The modeled geometry is appropriate for a copper-chlorine-copper moiety. Furthermore, this chlorine is 3 Å from the T2 copper and completes a square planar coordination sphere for that copper ion.

We have reinvestigated the identity of the bridging atom in

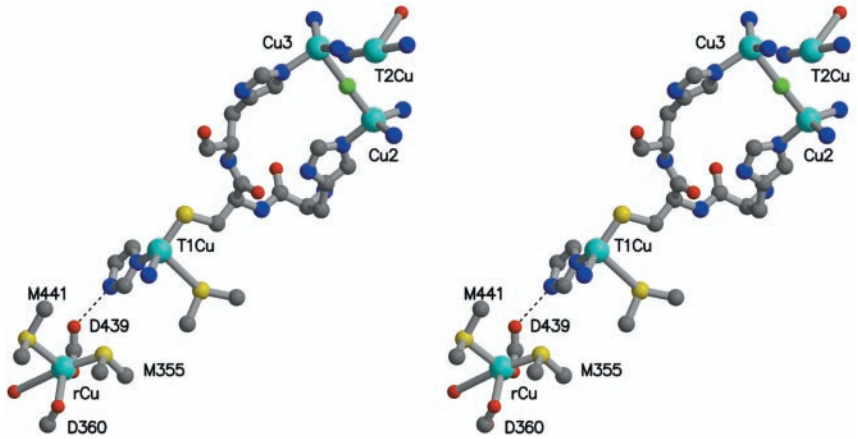

FIG. 2. **Stereo view of the coordination spheres.** Copper atoms are shown in *cyan*, oxygen in *red*, nitrogen in *blue*, carbon in *gray*, and chloride in *green*. The residues around the T1 and trinuclear copper centers (T2Cu, Cu2, and Cu3) are unchanged in the new complex and ligated through His-443, Cys-500, His-505, and Met-510 (T1Cu); His-101, His-446, and a water molecule (T2Cu), histidines 103, 141, and 501 (Cu2); and histidines 143, 448, and 499 (Cu3). The atom bridging Cu2 and Cu3 in the trinuclear center is a chloride ion (see text). The regulatory copper (rCu) is located near the T1 site and ligated to Met-355 and Asp-360 of the methionine-rich region, as well as Asp-439, Met-441, and a water molecule. The hydrogen-bond between OD2 of Asp-439 and NE2 of His-443 is indicated with a *dashed line*.

the original structure and both oxygen and chlorine refine satisfactorily in this position with acceptable temperature factors; the oxygen temperature factor was somewhat, but not dramatically, low (14 $Å^2$), and the chlorine temperature factor was high (30 $Å^2$) compared with the average of the copper atoms of the trinuclear cluster (14, 22, and 30 $Å^2$). Because the geometry of the trinuclear cluster we observed is that expected for a bridging chloride ion, we now believe that the structure previously reported contains a bridging chlorine atom in the trinuclear cluster.

Mutagenesis and CueO Activity—To investigate whether the labile copper atom contributes to CueO activity, four mutants were constructed (M355L, D360A, D439A, and M441L), each of which has one ligand removed from the copper coordination sphere. The UV-visible absorption spectra are the same for the wild-type protein and the M355L, D360A, and D439A proteins, confirming that these mutations have not affected copper incorporation into the nearby T1 copper site. However, for the M441L protein, the absorbance at 610 nm, relative to that at 280 nm, shows about 50% less intensity, suggesting that the mutation has affected copper incorporation into the T1 copper site. X-ray diffraction data for the M441L mutant protein has been measured and the structure analyzed. The M441L polypeptide is identical with that of the wild-type protein (Protein Data Bank code 1KV7) except for the replacement of the side chain at the mutation site. However, a large negative peak (-10σ) appears centered at the T1 copper site. Setting the occupancy of the T1 copper to one half results in the disappearance of this negative peak, indicating that the T1 copper is about half depleted, in agreement with the spectroscopic results.

None of the mutant CueO proteins were as effective as wild-type CueO in protecting cells against copper-induced cell death; two of them, M355L and D360A, are completely inactive (Fig. 3). Oxidase activity of the purified mutant proteins is also decreased by the mutations (Table II). Under the conditions used (pH 6.8, 200 mM Tris-acetate buffer), all mutant CueO proteins show significantly lower oxidase activity; one, M355L, shows essentially no activity. Thus, disruption of the labile copper-binding site has led to a reduced copper oxidase specific activity *in vitro* and loss of the copper tolerance phenotype *in vivo*. D439A is the most active of the four mutant proteins, in agreement with the results of the copper tolerance experiments.

To assess whether the mutations led to proteins with altered stability, we examined oxidase activity after incubating at elevated temperatures. Wild-type CueO and the four mutant proteins were subjected to 10-min incubations, ±2 mM $CuCl_2$, at 30, 40, 50, 60 and 70 °C, followed by measurement of their activities at 25 °C. Wild-type CueO is reasonably thermostabile, displaying 67% of its activity after 70 °C incubation in the absence of added copper and 91% in the presence of excess copper. Thus, binding of the labile copper has a small stabilizing affect on the protein. Mutants M355L, M441L, and D439A

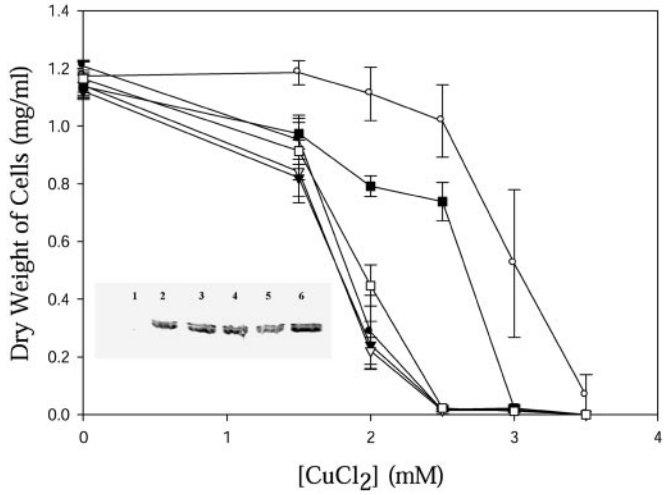

FIG. 3. **Growth of *E. coli* strains with mutant CueO proteins.** Overnight cultures were diluted 1:500 into fresh Luria Bertani medium with indicated concentrations of $CuCl_2$. Cell growth was monitored as milligrams per milliliter dry weight after 16 h of incubation at 37 °C with shaking for *E. coli* strain GR15 (Δ*cueO* Δ*cusA*) with pCueO (○), pCueO M355L (▼), pCueO D360A (▽), pCueO D439A (■), pCueO M441L (□), or pASK-IBA3 (●). Shown are the averages of three independent experiments. *Inset*, immunoblot against Strep-TagII-labeled CueO mutants from crude extracts of the different *cueO*-derivatives, indicating a high level of expression for the mutant proteins The lanes contain GR15 (Δ*cueO* Δ*cusA*) with pASK-IBA3 (*lane 1*), pCueO (*lane 2*), pCueO M355L (*lane 3*) pCueO D360A, (*lane 4*), pCueO D439A (*lane 5*), and pCueO M441L (*lane 6*).

TABLE II
Kinetic parameters for the reaction of CueO with DMP at pH 6.5

Protein	V_{max}	K_m Cu^{2+}
	μmolO_2/mg protein/min	*mM*
Wild type	3.1 ± 0.15	0.163 ± 0.03
D360A	0.87 ± 0.08	1.6 ± 0.08
D439A	1.03 ± 0.09	0.59 ± 0.03
M355L[a]	0.086 ± 0.02	82. ± 20
M441L	0.38 ± 0.06	0.27 ± 0.03

[a] The kinetic values did not vary appreciably as a function of protein concentration, except for M355L, in which the low activity introduced greater uncertainty, and estimates of K_m varied between 73 and 90 mM. Numbers in parentheses are estimated errors in the measurement.

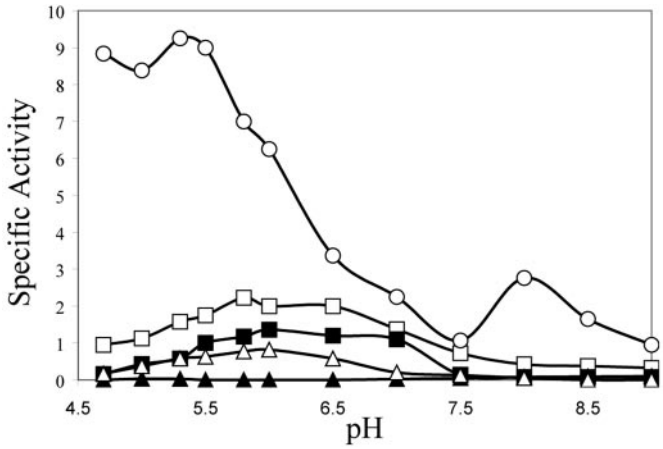

FIG. 4. **pH dependence of oxidation of DMP by CueO.** Purified CueO proteins were assayed for phenol oxidase activity in the pH range of 4.8–9.0. The reaction mixtures contained (in 2 ml): 0.1 M Tris acetate buffer (constant Tris concentration with varying amount of acetic acid), 250 μM O_2, 2 mM DMP, and 1 mM $CuCl_2$ and protein in the range of 50 to 200 μg. The reaction was started by the addition of $CuCl_2$, and the initial reaction rates were determined by measuring oxygen consumption using an oxygen electrode. The data represent the average of three measurements that varied by approximately ±5%. ○, CueO; ▲, M355L; △, D360A; ■, D439A; □, M441L.

retained 93, 60, and 63% of their original activity, respectively, after incubation at 70 °C in the presence of excess copper, and thus display stabilities similar to those of wild-type CueO but without the added stabilization through labile copper binding (the value for M355L is less precise because of the very low inherent activity in this protein). Mutant D360A was found to be more thermolabile and displayed only 32% activity after incubation. In the physiological temperature range of 25 to 37 °C, only small losses in activity were observed for any of the proteins; thus, we conclude that the altered kinetics of the mutants is predominately caused by loss of binding at the labile copper site.

The pH dependence of the CueO reaction with oxygen was measured for the wild-type enzyme and the four mutants. The initial reaction rates were determined in the pH range of 4.8–9.0 (Fig. 4). The wild-type enzyme has a broad activity peak between pH 5 and 6 and a second activity peak at pH 8 that was not observed for the mutants. The pH dependence of the reaction as monitored by oxygen consumption is different from that reported previously for the colorimetric determination of DMP oxidation. Oxygen consumption is directly linked to enzyme turnover and is therefore likely to provide a more reliable rate estimate than the indirect measurement of rates through a colorimetric assay based upon the polymerization of DMP oxidation products.

In both *in vitro* and *in vivo* measures of CueO activity, the mutants located on the methionated helix, M355L and D360A, are the most impaired; M355L is almost inactive. The mutants of the copper-binding residues nearest the trinuclear center, D439A and M441L, retain considerably more activity, despite the M441L mutant apparently having a somewhat depleted T1 copper site.

Effect of Chloride on Oxidase Activity—To investigate whether the bridging chloride ion affected enzyme activity, oxygen consumption was measured in the presence and absence of chloride. CueO apo-protein was isolated under chloride-free conditions in Tris·SO_4 buffer. Oxygen consumption was measured as described above in Tris·SO_4 buffer, pH 8.0, using 2 mM DMP and 22.5 μg/ml copper-free apo-protein. The reaction was initiated by the addition of 1 mM $CuSO_4$ or $CuCl_2$. No lag phase was observed in oxygen consumption, indicating that reconstitution occurs quickly on the time scale of this experiment. A rate of 2.9 μM O_2/mg protein/min was observed for the $CuSO_4$ reconstituted protein, comparable with that seen for the enzyme reconstituted with chloride (3.3 μM O_2/mg of protein/min).

DISCUSSION

We have discovered a fifth bound copper in CueO that is labile and ligated to two aspartates, two methionines, and a solvent molecule (Fig. 2). This copper ion is linked to the N-terminal-most methionine in the methionine-rich region, near the T1 copper site and the putative substrate-binding site (Fig. 1). Mutation of any of the four ligating residues leads to reduced or lost copper(II) stimulated activity *in vitro* (Table II) and reduced copper tolerance *in vivo* (Fig. 3). Taken together, these data confirm a regulatory role for the labile copper, and we have therefore designated this the regulatory copper site (rCu).

Regulatory Copper Ligation Geometry—Copper ions in proteins have been found bound to the peptide chain *via* histidine, cysteine, methionine, carboxylic acid (Asp, Glu), and N-terminal amide ligation, but histidine ligation is by far the most common (33). Ligation sites that do not include histidine are rare; only three examples have previously been described (24, 34, 35). Thus, the finding that methionine and aspartate residues coordinate the regulatory copper is surprising. The regulatory copper is probably in the Cu(II) oxidation state, given that carboxylic acid ligands are considered "hard" and do not bind to Cu(I) in this fashion. Furthermore, trigonal bipyramidal complexes of the sort found for rCu are well known for Cu(II), whereas Cu(I) prefers lower coordination numbers. The buried rCu site is also unlike those for the copper chaperone proteins, where the ligation site is on the protein surface and copper is ligated by cysteine residues (36).

The present structure also displays an unexpected bridging chloride ion at the trinuclear center, apparently resulting from the high chloride concentration in the crystal. This chloride bridge apparently does not affect catalysis, because we measure similar activity for CueO in the presence or absence of chloride. Furthermore, preliminary structural data with chloride-free protein indicate that a bridging oxygen occurs with a trinuclear center geometry similar to that found in ascorbate oxidase (16).

Functional Role for rCu—The major difference between the structure of CueO and that of the related proteins ascorbate oxidase and laccase is the methionine-rich insert located near the T1 copper site. In ascorbate oxidase and laccase, the T1 copper lies near the surface of the protein, whereas in CueO, the T1 site is buried under a methionine rich helix that limits access to substrate. The binding of rCu at this site, only 7.5 Å away and linked to the T1 copper through a hydrogen-bond, suggests that rCu may have a catalytic role in the protein. Certainly, binding of the copper at the rCu site alters activity, allowing catalysis, as previously described, but also interfering with direct reduction of the T1 copper by photoactivated deazariboflavin (15).

Interestingly, labile M(II) binding sites have been observed in ceruloplasmin, a human multicopper oxidase found in plasma with ferroxidase activity (37). Ceruloplasmin, in its normal state, contains six Cu atoms: the four copper atoms usually seen in multicopper oxidases and two additional copper ions in T1-like sites. Two more labile M(II) binding sites have been identified, one of which lies in domain 6 and is analogous to the labile site we find in CueO. Mutagenesis of two of the ligands to the domain 6 ceruloplasmin labile M(II) binding site reduces ferroxidase activity by half (38), suggesting that Fe(II) binding at the labile sites is a step in iron oxidation.

Although CueO also shows ferroxidase activity *in vitro*, it is unlikely that the mechanism proposed for iron oxidation in ceruloplasmin is operative in CueO. CueO shows only slight ferroxidase activity *in vitro* unless Cu(II) is present in solution and, presumably, bound at the rCu binding site. Furthermore, the ligand set of the labile Cu seems not to be favorable for Fe(II) binding.

The true substrate for CueO remains unknown; however, biochemical (10) and genetic[2] evidence is accumulating that suggests enterobactin, an iron siderophore, is the true substrate. Enterobactin oxidation possibly interferes with copper receptor mechanisms or leads to a copper-binding product.

Role of the Methionine-Rich Region—Two of the rCu-ligating residues, Met-355 and Asp-360, lie at the beginning of the methionine-rich helix-loop that covers the entrance to the T1 copper site. Mutation of these residues causes a bigger loss of CueO activity than mutation of the residues nearer the T1 copper. Insertion of the T1 copper into the protein seems not to be affected by these residues, unlike the M441L mutation. Aside from these residues involved in rCu ligation, none of the other nine methionines we see in the helix-loop region are altered in the present structure, although all of the side chains are disordered in both CueO structures. Furthermore, the remaining five methionines that lie in the disordered portion of the methionine rich region remain disordered. These results suggest that the methionine-rich region in CueO, and by extension in other proteins, is not present simply to bind copper. Other possible roles in CueO include involvement in substrate binding or in mediating protein-protein interactions, as has been suggested for PcoC (24).

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[2] G. Grass and C. Rensing, unpublished observations.

A Labile Regulatory Copper Ion Lies Near the T1 Copper Site in the Multicopper Oxidase CueO

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