How Periplasmic Thioredoxin TlpA Reduces Bacterial Copper Chaperone Scol and Cytochrome Oxidase Subunit II (CoxB) Prior to Metallation**

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The atomic coordinates and structure factors (codes 4TXV and 4TXO) have been deposited in the Protein Data Bank (http://www.pdb.org/).

This study addresses a hitherto unresolved issue in the biogenesis of the aa₃-type cytochrome c oxidase (COX),2 a multi-meric integral membrane complex that performs the ultimate step in cellular respiration in many aerobic bacteria and in mitochondria (1–4). Being a heme- and copper-containing enzyme complex, the question of how its subunits plus heme and copper cofactors are assembled in the membrane has been answered only partially. Particularly intricate is the biogenesis of the membrane-anchored subunit II (CoxB) because it carries a binuclear Cu-Cu center (CubX) on a domain that faces the periplasmic side of the cytoplasmic membrane in Gram-negative bacteria or the intermembrane space in mitochondria (1, 2). Copper ions must therefore be delivered to these compartments for CubX assembly. The CubX center is the entry point of electrons coming from reduced cytochrome c, which are then guided to the membrane-integral subunit I via the low-spin heme A to the high-spin heme A₂-CuB center, the site of oxygen reduction (4, 5). The two copper ions in the oxidized form of the CubX center have an overall valence of +3 (+1.5/copper ion), and cytochrome c reduces them to +2 (+1/copper ion) (6).

Subunit II possesses a highly conserved amino acid sequence motif for copper binding (HX₃₆CXEXخمسXXHM) where the six ligands (underlined) are provided by the sulfur atoms of the methionine and the two cysteines, one imidazole nitrogen atom each of the two histidines, and the peptide bond carbonyl oxygen from the glutamate (1, 2).

When the CubX-binding domain of subunit II is secreted from the cytoplasm to the periplasm in the course of bacterial protein synthesis and membrane insertion, the two neighboring cysteine thiol (CXX₃₆C) in the aforementioned motif are prone to oxidation because the periplasm is an oxidizing environment (7, 8). Regardless of whether intramolecular disulfide bond formation occurs by air oxidation or is catalyzed by a DsbA-like dithiol oxidase (9, 10), such a constellation precludes complexation with copper ions. Enzyme-catalyzed re-reduction of such a disulfide bond (an off-pathway intermediate to CubX assembly) to the dithiol form would thus appear to be necessary for copper

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2 The abbreviations used are: COX, cytochrome oxidase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PSY, peptone-salts-extract medium; TEV, tobacco etch virus; TMPD, tetramethyl-p-phenylenediamine; Bis-Tris, 2-(bis[2-hydroxyethylamino]-2-hydroxyethyl)mpropane-1,3-diol; ESI, electrospray mass ionization; red, reduced; ox, oxidized.
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ion binding to subunit II. A strong candidate for a periplasmic CoxB reductase is the thioredoxin-like protein TlpA (11, 12), which has been discovered first in Bradyrhizobium japonicum, a member of the α-proteobacteria and one of the model organisms for studying bacterial and mitochondrial hemo- and metalloproteins (13–15). TlpA has a negative redox potential (E' = −256 mV), which predicts that it is a reductant in vivo (16, 17). The protein is anchored to the cytoplasmic membrane, with its thioredoxin domain facing the periplasm and, hence, ideally positioned to reduce membrane-associated substrates in this compartment. A tlpA knock-out mutant of B. japonicum is defective for cytochrome oxidase activity (11). In this work, we provide compelling functional and structural evidence that the oxidized CoxB protein is a substrate of the disulfide reductase TlpA and that CoxB reduction is an essential step in the biogenesis of active COX.

Incidentally, a CXC site such as in CoxB is also present in the B. japonicum ScoI protein (14), which is a homolog of the mitochondrial copper chaperone Sco1 (18, 19). The Cu2+-binding domain of the membrane-anchored ScoI and ScoI proteins faces the periplasm and intermembrane space, respectively, akin to CoxB. We had shown recently that TlpA serves as a reductant for ScoI (20). With few exceptions, the biogenesis of the CuA center in eukaryotic mitochondria and in prokaryotes depends on the presence of Sco1/ScoI (18, 19). One could argue, therefore, that the cytochrome oxidase deficiency in B. japonicum tlpA mutant cells is simply the consequence of a defect in forming a sufficient amount of intracellular Cu-ScoI complex for the synthesis of CuA on CoxB. As shown here, however, this explanation alone does not hold true because not only ScoI but also CoxB are direct substrates of TlpA. Furthermore, the requirement of ScoI for cytochrome oxidase activity, but not that of TlpA, can be bypassed by supplying high environmental copper concentrations. This strongly suggests that CuA can be assembled on CoxB only after the latter had been reduced by TlpA.

**EXPERIMENTAL PROCEDURES**

**Whole-cell Cytochrome Oxidase Test—**B. japonicum 110spc4 (WT) and mutants ΔtlpA (Bj3556 (17)), ΔscoI (Bj2575 (14)), coxA::Tn5 (COX132 (22)), and ΔcoxB (Bj3563 (14)) were grown aerobically at 30 °C in PSY medium (21) containing either 100 μg/ml spectinomycin (for wild type) or 100 μg/ml spectinomycin plus 100 μg/ml kanamycin (all other strains). To test for possible complementation of cytochrome oxidase deficiency, the medium was supplemented with 50 μM CuCl2. Cells from 2-ml culture volume were harvested by centrifugation and suspended in PSY to a final A600 of 25. Samples (5 μl) of these suspensions were spotted onto a filter paper soaked with saturated TMPD solution. Formation of indophenol blue upon the medium was supplemented with 50 mM H9262 32432 Journal of Biological Chemistry EXPERIMENTAL PROCEDURES

**Protein Production and Purification—**For production of CoxBPD and its variants CoxBPD C229S and CoxBPD C233S in cytoplasmic inclusion bodies, Escherichia coli strain BL21(DE3) transformed with pET11a-coxBPD, PET11a-coxBPD C229S, or PET11a-coxBPD C233S, respectively, was used. The purification procedure for CoxBPD, CoxBPD C229S, and CoxBPD C233S was identical. E. coli BL21(DE3) transformed with pET11a-coxBPD was grown at 37 °C in 2YT medium (Tryptone, 16 g/liter; yeast extract, 10 g/liter; NaCl, 5 g/liter) containing ampicillin (100 μg/ml) until an A600 nm of 0.5 had been reached. CoxBPD expression was induced by the addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside, and cells were further grown at 30 °C for 4 h. Cells were harvested by centrifugation, suspended in 100 mM Tris-HCl, pH 8.0, 1 mM EDTA (3 ml/g wet cells), mixed with DNase I (50 μg/ml final concentration), and lysed with a Microfluidizer M-110L (Microfluidics, Westwood, MA). After the addition of 0.5 volumes of 60 mM EDTA-NaOH, pH 7.0, 1.5 mM NaCl, 6% (v/v) Triton X-100, the lysate was stirred at 4 °C for 1 h. The inclusion bodies were harvested by centrifugation (30 min, 48,000 × g, 4 °C) and washed five times at 4 °C with 100 mM Tris-HCl, pH 8.0, 20 mM EDTA to remove the Triton X-100. The inclusion bodies were solubilized in 100 mM Tris-HCl, pH 8.0, 6 mM guanidinium chloride, 1 mM EDTA, 100 mM DTT (20 ml/gram of inclusion body) at room temperature under stirring for 2 h. Insoluble material was removed by centrifugation, and CoxBPD was refolded from the supernatant by rapid dilution with 50 volumes of 20 mM Tris-HCl, 0.5 mM arginine-HCl, 1 mM EDTA, 10 mM DTT (pH 8.0) at room temperature. Refolded, reduced CoxBPD was concentrated to ~1.5 mg/ml and dialyzed against 20 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, pH 8.5 (4 °C). Precipitated protein was removed by centrifugation, and the supernatant was applied at 4 °C to a Resource Q column (GE Healthcare, Glattbrugg, Switzerland) equilibrated with the same buffer. The flow-through, containing reduced CoxBPD, was concentrated and subjected to gel filtration on Superdex 75 (GE Healthcare) in 20 mM Tris-HCl, 1 mM EDTA, 0.5 mM DTT (pH 8.0) at room temperature. The fractions containing pure, reduced CoxBPD (as judged after Coomasie Blue-stained SDS-PAGE) were stored at −20 °C until further use. The final yield of purified, reduced CoxBPD was 60 mg/100 ml of bacterial culture. The identity of the protein (including the N-terminal methionine) was verified by ESI mass spectrometry (calculated mass: 15,578.0 Da; found: 15,578.0 Da).

For the use of CoxBPD C233S in crystallization trials, a different expression construct was made that carried an N-terminal His6 tag. E. coli strain BL21(DE3) harboring pProExHTa-His6-coxBPD C233S was grown and induced with isopropyl-β-D-1-thiogalactopyranoside as described above for CoxBPD. In addition, the N-terminal methionine was removed by treatment with endonuclease Arginine-HCl, 1 mM EDTA, 10 mM DTT (pH 8.0) at room temperature. The fractions containing pure, reduced CoxBPD, as judged after Coomasie Blue-stained SDS-PAGE) were stored at −20 °C until further use. The final yield of purified, reduced CoxBPD, was 60 mg/100 ml of bacterial culture. The identity of the protein (including the N-terminal methionine) was verified by ESI mass spectrometry (calculated mass: 15,578.0 Da; found: 15,578.0 Da).

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µg/ml final concentration) were added, and cells were disrupted with a Microfluidizer. The lysate was centrifuged, and the pellet was mixed with 2 volumes of buffer A (20 mM Tris-HCl, 0.5 mM NaCl, 6 mM guanidinium chloride, 2 mM β-mercaptoethanol, pH 8.0) and stirred at 4 °C for 1.5 h. Insoluble material was removed by centrifugation, the supernatant was mixed with imidazole/HC1, pH 8 (final concentration: 10 mM) and loaded onto nickel-nitriirolactate-agarose (Qiagen, Switzerland) equilibrated with buffer A. The resin was washed extensively with buffer A containing 20 mM imidazole/HC1. His$_{6}$-tagged CoxBPD C233S was eluted with buffer A containing 200 mM imidazole/HC1, and protein fractions were refolded by dilution with 100 volumes of 20 mM Tris-HCl, 0.5 mM NaCl, 0.5 mM arginine- HC1, 5 mM β-mercaptoethanol, pH 8.0, and incubation for 1 h at room temperature. Refolded, His$_{6}$-tagged CoxBPD C233S was concentrated to 1.5 mg/ml (96 µM), and aggregates were removed by centrifugation. The N-terminal His$_{6}$ tag was then cleaved off by incubation (18 h, 4 °C) with TEV protease (7 µM) in 20 mM Tris-HCl, 0.3 mM NaCl, 5 mM β-mercaptoethanol, pH 8.5. The uncleaved protein and the cleaved tag were removed by binding to a nickel-nitriirolactate column as described above. The cleaved protein in the flow-through was concentrated to ∼1.5 mg/ml and subjected to gel filtration on Superdex 75. The final yield of purified CoxB$^{C233S}$ was 4.5 mg/liter of bacterial culture, and its mass was verified by ESI mass spectrometry (calculated mass: 17,396.9 Da; found: 17,397.0 Da).

TlpA$_{50}$, used for the disulfide exchange equilibrium with CoxB$_{PD}$, and TlpA$_{50}$ C110S, used for crystallization, were produced and purified as reported recently (20). TEV protease was used in excess, with initial concentrations of 5, 10, or 15 µM. The kinetic profiles were fitted globally with Berkeley Madonna (version 8.3.18), with a fixed value of 7.1 for the ratio between the forward ($k_{+}$) and reverse ($k_{-}$) reactions based on the determined equilibrium constant ($K_{eq} = k_{+}/k_{-}$).

Stop-flow Fluorescence Kinetics of Disulfide Exchange between TlpA and CoxB—The kinetics of the reduction of CoxB$_{PD}$ by TlpA$_{50}$ were recorded at 25 °C in 50 mM sodium phosphate, 0.1 mM EDTA, pH 7. The reactions were initiated with stop-flow mixing (1:1) in a SX20 stop-flow instrument (Applied Photophysics, Leatherhead, UK) and followed via the decrease in tryptophan fluorescence of TlpA$_{50}$ at 320 nm upon oxidation (excitation at 280 nm) for 20 min followed by gel filtration on a Hi-Prep 26/10 column as described above in 50 mM sodium phosphate, 0.1 mM EDTA, pH 7. Complete oxidation of CoxB$_{PD}$ red by DTNB was verified by reversed-phase HPLC (see above).

Disulfide-exchange Equilibrium between TlpA and CoxB—CoxB$_{PD}$ ox and TlpA$_{50}$ red were obtained after reduction with excess DTT (10 mM for CoxB$_{PD}$ and 5 mM for TlpA$_{50}$) for ∼30 min at room temperature in 20 mM Tris-HCl, 0.3 mM NaCl, 0.5 mM EDTA, pH 8 (CoxB$_{PD}$) or 10 mM Tris-HCl, pH 8 (TlpA$_{50}$) followed by centrifugation of CoxB$_{PD}$ at 100,000 × g for 30 min at 4 °C and removal of excess DTT from both proteins by gel filtration on Hi-Prep 26/10 Sephadex G25 columns (GE Healthcare). CoxB$_{PD}$ ox was obtained after incubation of CoxB$_{PD}$ Reduced (10–20 µM) with a 1.5-fold molar excess of DTNB in 50 mM sodium phosphate, 0.1 mM EDTA, pH 7, at room temperature for 20 min followed by gel filtration on a Hi-Prep 26/10 column as described above in 50 mM sodium phosphate, 0.1 mM EDTA, pH 7. Complete oxidation of CoxB$_{PD}$ red by DTNB was verified by reversed-phase HPLC (see above).

CD Spectra and Thermal Unfolding Transitions of CoxB$_{PD}$—CD spectra of CoxB$_{PD}$ (0.2 mg/ml) in 10 mM sodium phosphate, pH 7.0, were recorded at 25 °C with a J-715 spectropolarimeter (Jasco, Easton, MD; 0.1-cm path length). Thermal unfolding transitions of oxidized and reduced CoxB$_{PD}$ in 10 mM sodium phosphate, pH 7.0 (supplemented with 5 mM DTT in the case of reduced CoxB$_{PD}$) were followed via the increase in the far-UV CD signal at 218 nm upon unfolding (heating rate: 1 K/min) and fitted and normalized according to a reversible thermal unfolding equilibrium (Equation 3)

$$S = \{(y_{y} + m_{y} \times T) \times (y_{u} + m_{u} \times T) \times e^{\Delta H_{f}(RT/\ln(T-T_{m}/T_{m}))}/(1 + e^{\Delta H_{f}(RT/\ln(T-T_{m}/T_{m}))}) \}$$

where $S$ represents the observed CD signal at 218 nm, $y_{y}$ and $y_{u}$ are the $y$ axis intercepts at zero $K$, and $m_{y}$ and $m_{u}$ are the slopes of the pre- and post-transition baselines, respectively, $T$ is the
temperature, \( T_m \) is the melting temperature, and \( \Delta H_m \) is the enthalpy change of unfolding at \( T_m \).

In Vitro Reconstitution of the CuA Center in CoxBPD—The CuA center in CoxBPD was reconstituted via the reaction described from the recently described CuA reconstitution in subunit II of the \( ba_3 \)-type cytochrome oxidase from \( T. thermophilus \) (6).

\[ 2 \text{CoxB}_{25} + 3 \text{Cu}^{2+} \rightarrow \text{CoxB}_{25} \cdot \text{Cu}^{2+} + \text{CoxB}_{25} + 2 \text{H}^+ \]

\[ \text{REACTION 1} \]

Reduced CoxB\(_{25}\) (50 \( \mu \)M) was incubated with CuCl\(_2\) (500 \( \mu \)M) in 50 mM Bis-Tris-HCl, pH 7.0, overnight at room temperature. Excess CuCl\(_2\) was removed by gel filtration in 50 mM Bis-Tris-HCl, pH 7.0, the solution was concentrated to \( \sim 150 \mu \)M protein, and precipitated material was removed by ultracentrifugation. The measured absorbance spectrum with its maxima at 367, 479, and 813 nm confirmed the formation of the CuA center.

Preparation and Crystallization of the TlpA-ScoI Mixed Disulfide Complex—Purified TlpA\(_{C110S}\) and ScoI\(_{C74S}\) were incubated with excess DTT (2 mM) in 10 mM Tris-HCl, pH 8 (TlpA\(_{C1105}\)) or 50 mM succinic acid-NaOH, 0.2 mM EDTA, 0.1 M NaCl, 1 mM DTT, pH 5.3 (ScoI\(_{C74S}\)) at room temperature for 30 min followed by the removal of DTT by gel filtration on Sephadex G-25 (GE Healthcare) in 10 mM Tris-HCl, pH 8. TlpA\(_{C1105}\) (40 \( \mu \)M) was activated by incubation with a 20-fold molar excess of DTT in 10 mM Tris-HCl, pH 8, for 20 min. The resulting TlpA\(_{C1105}\)-TNB derivative was again purified by gel filtration and incubated with an equimolar amount (50 \( \mu \)M each) of ScoI\(_{C74S}\) overnight at room temperature. After gel filtration on HiLoad Superdex 75 16/60 column (GE Healthcare) in 20 mM Tris-HCl, 0.3 M NaCl, pH 8.0 (at 4 °C), fractions containing the pure TlpA\(_{C1105}\)-ScoI\(_{C74S}\) mixed disulfide complex were buffer-exchanged to 10 mM Tris-HCl, pH 8, and concentrated to 10 mg/ml. Fragile crystals in the form of clusters were grown with the sitting-drop vapor diffusion technique by mixing 0.75 \( \mu \)l of the TlpA-ScoI complex (10 mg/ml in 10 mM Tris-HCl, pH 8) with 0.75 \( \mu \)l of precipitant solution (final concentration: 3% glycerol, 0.2 M NaSCN, and 16% (w/v) PEG 3350) and equilibration over 100 \( \mu \)l of well solution (3% glycerol, 0.2 M NaSCN, 28% (w/v) PEG 3350) at 4 °C. Crystals were cryo-protected in precipitant solution containing 25% ethylene glycol and flash-frozen in liquid nitrogen.

Preparation and Crystallization of the TlpA-CoxB Mixed Disulfide Complex—The TlpA\(_{C1105}\)-CoxB\(_{PD}^{C233S}\) complex was generated as described above for TlpA\(_{C1105}\)-ScoI\(_{C74S}\). The final reaction that led to the mixed disulfide complex was performed with equimolar concentrations (15 \( \mu \)M each) of CoxB\(_{PD}^{C233S}\) and TlpA\(_{C1105}\)-TNB in 10 mM Tris-HCl, pH 8 (1 h at room temperature). The TlpA\(_{C1105}\)-CoxB\(_{PD}^{C233S}\) complex was purified by gel filtration on HiLoad Superdex 75 16/60 as described above and finally concentrated to 12 mg/ml in 10 mM Tris-HCl, pH 8.0. ESI mass spectrometry and SDS-PAGE analysis revealed that seven C-terminal residues in CoxB\(_{PD}^{C233S}\) (residues 273–279) had been cleaved off by an unknown protease (calculated mass of the cleaved mixed disulfide complex: 36,284.5 Da; found: 36,287.5 Da). The TlpA\(_{C1105}\)-CoxB\(_{PD}^{C233S}\) stock solution (3 \( \mu \)l) was mixed with 1 \( \mu \)l of precipitant solution (25% (w/v) PEG 2000 monomethyl ether, 0.8 M formic acid-NaOH, 0.1 M sodium cacodylate, pH 6.5). Rod-like crystals grew at 4 °C within 13 weeks (sitting-drop vapor diffusion). Crystals were cryo-protected in precipitant solution containing 15% glycerol and flash-frozen in liquid nitrogen.

X-ray Crystallography and Structure Determination—The crystal structure of the TlpA\(_{C1105}\)-ScoI\(_{C74S}\) mixed disulfide was solved by molecular replacement with PHASER (25), using the crystal structure of the soluble domain of \( B. japonicum \) TlpA (Protein Data Bank (PDB) entry 1jfu) and the crystal structure of \( B. subtilis \) Sco1 (PDB entry 1x zo) as search models. The asymmetric unit of the TlpA-ScoI crystal structure contains four complexes, where chains A and B form complex 1, chains C and D form complex 2, chains E and F form complex 3, and chains G and H form complex 4. For the analysis of the structure, mostly complex 2 (chains C+D) was employed. The structure of the TlpA\(_{C1105}\)-CoxB\(_{PD}^{C233S}\) complex was also solved by molecular replacement with PHASER (25), using the structures of \( B. japonicum \) TlpA (PDB entry 1jfu) and subunit II of cytochrome \( c \) oxidase from \( R. sphaeroides \) (PDB entry 1m56), in which the N-terminal membrane helices (residues 1–130), residues 151–191, the C-terminal helix (residues 270–289), and the loop (residues 250–261) containing the two copper-binding cysteines are absent. PHENIX (26) and Refmac (27) were used for refinement and model validation. COOT (28) was used for manual model building. Secondary structure elements were assigned with DSSP (29). T-Coffee (30) was used for multiple sequence alignments. The Pdb2pqr web server (31) and APBS (32) were employed for analysis of electrostatics, PyMOL (The PyMOL Molecular Graphics System, Version 1.6.0.0, Schrödinger, LLC) was employed for generation of structural figures, and ALINE (33) was employed for producing figures of sequence alignments. Interface analysis of the structures was performed with EPPIC (34). PISA (35) was used to localize hydrogen bonds and salt bridges in the dimer interfaces.

RESULTS

Excess Environmental Cu\(^{2+}\) Rescues Cytochrome Oxidase Activity in a scoI but Not in a tlpA Deletion Mutant of \( B. japonicum \) — \( B. japonicum \) is a plant nodule bacterium found in the root nodules of soybean and other legumes. It is the host of \( B. japonicum \) and is capable of fixing atmospheric nitrogen. The \( B. japonicum \) genome contains a single copy of the \( tlpA \) gene, which encodes a thioredoxin-like protein that is involved in the assembly of the \( ba_3 \)-type cytochrome oxidase. The \( tlpA \) gene is essential for the growth of \( B. japonicum \) on solid media.

In this study, we investigated the role of \( tlpA \) in the assembly of the \( ba_3 \)-type cytochrome oxidase in \( B. japonicum \). To this end, we used a \( tlpA \) knockout strain of \( B. japonicum \) and compared its growth and respiratory properties to those of the wild-type strain. The results showed that the \( tlpA \) knockout strain had a reduced respiratory capacity and was unable to grow on solid media. However, when the growth medium was supplemented with Cu\(^{2+}\), the \( tlpA \) knockout strain was able to grow and show normal respiratory activity.

The results of this study suggest that \( tlpA \) is involved in the assembly of the \( ba_3 \)-type cytochrome oxidase in \( B. japonicum \) and that Cu\(^{2+}\) plays a role in this process. The mechanism by which \( tlpA \) affects the assembly of the cytochrome oxidase is not yet known. Further studies are needed to elucidate the role of \( tlpA \) in the assembly of the \( ba_3 \)-type cytochrome oxidase and the function of Cu\(^{2+}\) in this process.
**TlpA Is a Specific Reductant of CoxB**—The possible target for TlpA-catalyzed thiol-disulfide exchange is the cysteine pair Cys^{229}/Cys^{233} in the periplasmic domain of CoxB. This domain (CoxB_{PD}) was expressed in *Escherichia coli* in the form of cytoplasmic inclusion bodies and purified after *in vitro* refolding under reducing conditions (Fig. 2). The dithiol of Cys^{229}/Cys^{233} in CoxB_{PD} could be quantitatively converted to the disulfide form by oxidation with DTNB. Both reduced and oxidized CoxB_{PD} showed almost identical, cooperative thermal unfolding transitions, arguing for an intact tertiary structure (Fig. 2, B and C). This was further confirmed by the successful *in vitro* reconstitution of the Cu_{A} center in reduced CoxB_{PD} by the addition

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**FIGURE 1.** Whole-cell cytchrome oxidase activity is rescued in *B. japonicum* scoI but not tlpA mutant by excess Cu^{2+} in the growth medium. The respective *B. japonicum* mutants were grown aerobically in PSY medium with or without 50 μM CuCl_{2} and spotted onto a filter paper soaked with the redox indicator TMPD. Active COX in cells such as the wild type (WT) converted TMPD to indophenol blue. Mutants of subunits I (coxA) and II (coxB) of COX served as negative controls. – Cu, without copper supplement; + Cu, with copper supplement.

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**FIGURE 2.** Characterization of CoxB_{PD}, the recombinant, periplasmic domain of CoxB (residues 128–265) from *B. japonicum*. A, ESI mass spectrum of purified, reduced CoxB_{PD} (calculated mass: 15,578.0 Da; found: 15,578.0 Da). Inset: Coomassie Blue-stained SDS-polyacrylamide gel of purified CoxB_{PD} (B-PD); St.: molecular mass standard. a. u., arbitrary units. B, far-UV CD spectra of oxidized (blue) and reduced (red) CoxB_{PD} at pH 7.0 and 25 °C. C, both oxidized and reduced CoxB_{PD} show cooperative thermal unfolding transitions, demonstrating the presence of intact tertiary structure. Thermal unfolding transitions of oxidized (blue) and reduced (red) CoxB_{PD} in 10 mM NaH_{2}PO_{4}-NaOH, pH 7.0, at a heating rate of 1 K/min were followed via the increase in the far-UV CD signal at 218 nm upon unfolding, tentatively fitted according to a reversible thermal unfolding equilibrium (solid lines), and normalized. Apparent melting temperatures of 317.5 ± 0.2 and 318.5 ± 0.2 K were obtained for CoxB_{PDox} and CoxB_{PDred}, respectively. D, UV/VIS spectrum of Cu_{A}-CoxB_{PD} after *in vitro* reconstitution of the Cu_{A} center, showing Cu_{A}-specific absorbance maxima at 367, 479, and 813 nm.
of Cu\(^{2+}\) ions (Fig. 2D) (6). Oxidized CoxB\(_{PD}\) failed to assemble the Cu\(_A\) center.

Assuming a function of TlpA as a CoxB reductase, we predicted (i) that the cysteine pair Cys\(^{229}/\)Cys\(^{233}\) in CoxB\(_{PD}\) is less reducing than the active-site cysteine pair of TlpA (Cys\(^{107}/\)Cys\(^{110}\)), and (ii) that reduction of CoxB by TlpA would have to proceed fast, i.e., with a rate constant above the threshold (~10\(^{-3}\) M\(^{-1}\) s\(^{-1}\)) for physiological disulfide-exchange reactions between thioredoxin-like oxidoreductases and their substrates (36). We first determined the equilibrium constant (\(K_{eq}\)) of the disulfide-exchange reaction at pH 7.0 and 25 °C between TlpA and CoxB at pH 7.0 and 25 °C. A, determination of the equilibrium constant (\(K_{eq}\)) of the reduction of CoxB by TlpA. Reduced TlpA (TlpA\(_{Sa}\)) and oxidized CoxB (CoxB\(_{PD}\)) were mixed at initial concentrations (in \(\mu\)M) of 5 and 10, 5 and 5, or 10 and 5, respectively, and incubated for 18 h. The reactions were quenched with formic acid. All four redox species were separated by reversed-phase HPLC, detected via absorbance at 220 nm, and quantified by peak integration. The equilibrium constants deduced from the individual HPLC runs proved to be identical within experimental error, demonstrating that the disulfide-exchange equilibrium had been attained. AU, absorbance unit. B, stopped-flow fluorescence kinetics of the reduction of CoxB by TlpA. TlpA\(_{Sa}\) (1 \(\mu\)M) was mixed with a 5-, 10-, or 15-fold excess of CoxB\(_{PD}\), and the reaction was followed via the decrease in tryptophan fluorescence upon oxidation of TlpA. Data were fitted globally (solid lines) with Berkeley Madonna\(^TM\) according to the indicated disulfide-exchange equilibrium, with a fixed ratio of 7.1 between the second-order rate constants of the forward and reverse reactions (\(k_2\) and \(k_-2\), respectively), and normalized. The estimated error of the rate constants is about 20%.
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constants, deduced from the individual HPLC runs after peak integration, proved to be independent of the initial CoxBPD: TlpAS ratio within experimental error, demonstrating that equilibria had been attained. The deduced $K_{eq}$ value of 7.1 ± 0.6 translates into a redox potential ($E^\circ_0$) of $-231 \pm 1$ mV for CoxBPD. This shows that reduction of CoxBPD by TlpAS is thermodynamically favorable. The equilibrium is, however, far less on the side of oxidized TlpAS as compared with the reduction of ScoI (residues 30–196) by TlpAS, which has an equilibrium constant of 1740 (20).

Next, the kinetics of the attainment of the disulfide-exchange equilibrium between CoxBPD and TlpAS were followed after stopped-flow mixing of reduced TlpAS with a 5-, 10-, or 15-fold excess of oxidized CoxBPD, where we made use of the strong decrease in the tryptophan fluorescence of TlpAS upon oxidation (16, 20). The obtained rate constants of $8.4 \times 10^4$ and $1.2 \times 10^4$ M$^{-1}$ s$^{-1}$ for the forward and reverse reaction, respectively, demonstrate rapid disulfide exchange between both proteins (Fig. 3B). The rate constant of CoxBPD reduction is thus very similar to that of the reduction of ScoI by TlpAS (9.4 × 10$^4$ M$^{-1}$ s$^{-1}$) (20) and a strong hint that both CoxB and ScoI are physiologically substrates of the periplasmic reductase TlpA.

**Trapping and Crystallizing the TlpA-ScoI and TlpA-CoxB Heterodisulfides**—Disulfide exchange between thioredoxins and their substrates occurs via mixed disulfide intermediates, in which the more N-terminal, active-site cysteine of a thioredoxin forms the intermolecular disulfide with the substrate protein (37). In accordance with this reaction scheme, the active-site thiol of Cys$^{107}$ in TlpA forms a transient mixed disulfide with Cys$^{78}$ of ScoI (20). A mixed disulfide is kinetically unstable because it is rapidly attacked by a neighboring cysteine thiol. Therefore, a stable mixed disulfide between TlpAS and ScoI$\varsigma$ was prepared by using the single-cysteine variants TlpAS$^{C110S}$ and ScoI$\varsigma$C74S. Activation of TlpAS$^{C110S}$ with DTNB and subsequent reaction with ScoI$\varsigma$C74S yielded the TlpAS$^{C110S}$-ScoI$\varsigma$C74S complex.

To determine the cysteine residue in CoxBPD, which forms the mixed disulfide intermediate with TlpA, the single-cysteine variants CoxBPD$^{C229S}$ and CoxBPD$^{C233S}$ were tested for their reactivity with DTNB-activated TlpAS$^{C110S}$ in a similar way as described previously with ScoI (20). The result was that predominantly Cys$^{229}$ of CoxB reacted with Cys$^{107}$ of TlpA. Based on this finding, a stable TlpAS$^{C110S}$-CoxBPD$^{C233S}$ mixed disulfide complex was prepared as described above for the TlpAS$^{C110S}$-ScoI$\varsigma$C74S complex. The two purified mixed disulfide complexes were crystallized, and the x-ray structures of TlpAS$^{C110S}$-ScoI$\varsigma$C74S and TlpAS$^{C110S}$-CoxBPD$^{C233S}$ were solved at 2.2 and 2.0 Å resolution, respectively (Fig. 4; Table 1).

**The Folds of TlpA, ScoI, and CoxB in the Context of Mixed Disulfide Complexes**—In both mixed disulfide complexes, the structure of TlpAS$^{C110S}$ is identical to that determined previously for wild-type TlpAS, which shows a characteristic thioredoxin-like fold with a central β-sheet flanked by five α-helices and the active-site cysteine pair at the N-terminal end of helix α2 (12) (Figs. 4 and 5). In the TlpAS$^{C110S}$-ScoI$\varsigma$C74S complex, ScoI also displays a thioredoxin-like fold, composed of nine β-strands and six α-helices with a β-hairpin extension between α-helix 4 and β-strand 8, which is characteristic of Sco-like proteins (38). Unlike the active-site cysteines in bona fide thioredoxins, however, the Cu$^{2+}$-ion-liganding cysteine pair Cys$^{74}$/Cys$^{78}$ is located at a different position in the thioredoxin fold, namely in the loop segment (residues 71–80) between strand β3 and helix α1 (Figs. 4A and 5). Given that physiological disulfide-exchange reactions between thiol-disulfide oxidoreductases with a thioredoxin fold have not been observed and are kinetically restricted (7, 36), the reaction between TlpA and ScoI is unusual. This interaction is probably due to the different location of the Cys$^{74}$/Cys$^{78}$ cysteine pair in ScoI, which might facilitate the accessibility of the Cys$^{74}$-Cys$^{78}$ disulfide bond in oxidized ScoI for rapid, nucleophilic attack by Cys$^{107}$ of TlpA.

Apart from two exceptions (see below), CoxBPD$^{C233S}$ in the mixed disulfide complex with TlpAS$^{C110S}$ adopts essentially the basic fold that had been observed in the structure of the periplasmic CuA-center-containing domain of subunit Cox2 in the aa$_3$-type cytochrome oxidases from Paracoccus denitrificans (1, 39), bovine heart mitochondria (2, 40), and R. sphaeroides (41). Specifically, CoxBPD$^{C233S}$ adopts an extended Greek key-like five-stranded β-sheet (Greek key plus one additional, antiparallel strand), a three-stranded β-sheet, a β-hairpin, and a C-terminal α-helix (Figs. 4B and 5). The intermolecular disulfide bond in TlpAS$^{C110S}$-CoxBPD$^{C233S}$ was photoreduced during x-ray data collection (Fig. 4B, lower right inset), with a final refined distance of 2.9 Å between the two S atoms (which compares with an ideal distance of 2.05 Å). A proper disulfide between Cys$^{107}$ (TlpA) and Cys$^{229}$ (CoxBPD), however, can be modeled upon a slight conformational change of both cysteine side chains without changing the overall geometry of the interface.

TlpA Displays Non-overlapping Surface Areas for Recognition of Different Substrates—The individual monomers in both mixed disulfide complexes are oriented such that their N termini are located on the same side of the heterodimers (Fig. 6A). Due to the presence of an N-terminal transmembrane domain in full-length CoxB and the N-terminal membrane anchors in wild-type TlpA and ScoI, the structures are fully consistent with a topologically suitable positioning on the periplasmic side of the cytoplasmic membrane for disulfide exchange between TlpA and ScoI or CoxB in vivo.

Remarkably, the solved structures of the two mixed disulfide complexes uncovered two separate TlpA interfaces for interaction with either ScoI or CoxB (Figs. 4 and 6), i.e., both interfaces overlap only in a comparatively small area. The interface area of TlpAS$^{C110S}$-CoxBPD$^{C233S}$ (997 Å$^2$) is more spacious than that of TlpAS$^{C110S}$-ScoI$\varsigma$C74S (775 Å$^2$). An upper-bound estimate for the shared overlap area is about 350 Å$^2$ (measured on the TlpA surface with the program EPPIC (34)). Herein, the TlpA residues Cys$^{107}$, Lys$^{171}$, and Met$^{179}$ are involved in similar types of interactions with both ScoI and CoxB (Table 2). Although Cys$^{107}$ forms the respective intermolecular disulfide bond, Lys$^{171}$ forms a salt bridge with Glu$^{84}$ of ScoI (Fig. 4A, lower right inset) or with the Cu$_n$-coordinating Glu$^{233}$ of CoxB (Fig. 4B, lower right inset), and Met$^{179}$ forms a two-fold backbone-
backbone interaction with Cys$^{229}$ of CoxB (Fig. 4B, lower right inset) or with Cys$^{78}$ of ScoI (Fig. 4A, left inset) where the backbone amide and backbone carbonyl of Met$^{179}$ of TlpA are in close proximity to the backbone carbonyl and backbone amide of Cys$^{229}$ of CoxB and Cys$^{78}$ of ScoI, respectively (Table 2).

All other interactions at the protein-protein interfaces are specific to the individual mixed disulfide complexes (Table 2;
for example, see Fig. 4A, lower right inset). The interface between Tlp\textsubscript{A}\textsubscript{1105} and Sco\textsubscript{L}\textsubscript{C74S} is characterized by a unique cluster of π-stacking interactions between four aromatic residues (Trp\textsubscript{106} and Phe\textsubscript{123} of Tlp\textsubscript{A} and Phe\textsubscript{83} and Phe\textsubscript{123} of Sco\textsubscript{L}). Trp\textsubscript{106} of Tlp\textsubscript{A} and Phe\textsubscript{123} of Sco\textsubscript{L} are conserved in Tlp\textsubscript{A} homologs and Sco\textsubscript{L} -like proteins, respectively conserved (Table 2). The backbone amides of these leucines (Trp\textsubscript{106} and Phe\textsubscript{123} of Tlp\textsubscript{A}, and Phe\textsubscript{83} and Phe\textsubscript{123} of Sco\textsubscript{L})\textsubscript{C74S} present detailed views of specific, polar interactions. In some of the views, the structures were rotated by the indicated angle. Residues are shown as sticks, whereas hydrogen bonds are shown as red dotted lines. Yellow sticks: sulfur atoms; red sticks: oxygen atoms; blue sticks: nitrogen atoms; yellow broken line in lower right inset of B: distance between Cys\textsubscript{229} of Cox\textsubscript{BPD}\textsubscript{C233S} and Cys\textsubscript{107} of Tlp\textsubscript{A}\textsubscript{1105}, indicating the position of the intermolecular disulfide that was lost due to radiation damage; N: N terminus of the respective polypeptide chain.

**DISCUSSION**

Because of the unique subunit and cofactor composition and the peculiar topology of cytochrome oxidase in the bacterial cytoplasmic membrane or mitochondrial inner membrane, the biogenesis of this enzyme is a very complex, multifactorial process. Although a substantial number of assembly factors for heme and copper insertion have so far been identified, our mechanistic understanding about how they assemble and act in concert is still incomplete. Results reported here add an important piece to the puzzle by showing how Tlp\textsubscript{A} prepares Sco\textsubscript{L} and cytochrome oxidase subunit II (Cox\textsubscript{B}) for copper ion insertion and formation of the Cu\textsubscript{A} center. The epistatic nature of Tlp\textsubscript{A} function became evident from the fact that the cytochrome oxidase defect in a B.\textit{japonicum} sco\textsubscript{L} mutant, but not in a tlp\textsubscript{A} mutant, could be corrected phenotypically by supplying excess

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**TABLE 1**

Statistics of crystallographic data collection and refinement

|  | Tlp\textsubscript{A}\textsubscript{1105}-Cox\textsubscript{BPD}\textsubscript{C233S} | Tlp\textsubscript{A}\textsubscript{1105}-Sco\textsubscript{L}\textsubscript{C74S} |
|---|---|---|
| No. of complexes per a.u. | 2 | 4 |
| No. of amino acids/complex | 184 (Tlp\textsubscript{A}\textsubscript{1105}) + 159 (Cox\textsubscript{BPD}\textsubscript{C233S}) | 184 (Tlp\textsubscript{A}\textsubscript{1105}) + 175 (Sco\textsubscript{L}\textsubscript{C74S}) |
| Processing | | |
| Space group | P2\textsubscript{1}, 2, 2 | P1 \_2, 1 |
| α, β, γ (°) | 74.83, 81.63, 109.37 | 60.05, 173.22, 66.69 |
| Resolution (Å) | 65.4–2.0 (2.1–2.0) | 50.0–2.2 (2.3–2.2) |
| R\textsubscript{free} (Å) | 4.0 | 4.4 |
| R-value (%), R-free (%), | 7.5 (84.8) | 15.8 (131.9) |
| Completeness (%) | 98.8 (97.7) | 98.9 (94.7) |
| Redundancy | 3.5 (3.5) | 7.5 (4.5) |
| Refinement | | |
| Resolution (Å) | 65.4–2.0 | 49.3–2.2 |
| No. of reflections (test set) | 301,996 (2304) | 68,109 (1363) |
| R\textsubscript{work}/R\textsubscript{free} (%) | 16.5/21.0 | 17.4/20.7 |
| Twin law | h, -k, -l | h, -k, -l |
| Twin fraction | 0.5 | 0.5 |
| No. of atoms | | |
| Protein | 4820 | 10,193 |
| Ligand/ion | 15 | 15 |
| Water | 444 | 590 |
| B-factors (Å\textsuperscript{2}) | 38.9 | 44.2 |
| Protein | 44.2 | 35.0 |
| Ligand/ion | 39.3 | 30.6 |
| Water | 3.9 | 0.5 |
| Ramachandran analysis\textsuperscript{d} | | |
| Favorable (%) | 96.8 | 96.0 |
| Allowed (%) | 2.9 | 3.8 |
| Outlier (%)\textsuperscript{d} | 0.3 | 0.2 |
| Bond length (Å) | 0.017 | 0.006 |
| Bond angles (°) | 1.832 | 1.080 |

\textsuperscript{a} Data were collected from a single crystal.

\textsuperscript{b} Highest resolution shell is shown in parentheses.

\textsuperscript{c} Determined with PHENIX.

\textsuperscript{d} Two Ramachandran outliers at the border of the allowed region (Pro\textsubscript{76} of Sco\textsubscript{L}\textsubscript{C74S} (Tlp\textsubscript{A}\textsubscript{1105}), Sco\textsubscript{L}\textsubscript{C74S}, chains B and F), Pro\textsubscript{141} of Tlp\textsubscript{A}\textsubscript{1105} (Tlp\textsubscript{A}\textsubscript{1105}, Cox\textsubscript{BPD}\textsubscript{C233S}, chain A), and Ala\textsubscript{167} of Cox\textsubscript{BPD}\textsubscript{C233S} (Tlp\textsubscript{A}\textsubscript{1105}, Cox\textsubscript{BPD}\textsubscript{C233S}, chain B). 2\textit{F}_{o}-\textit{F}_{c} density, however, mandates this conformation of the residues.
Cu$^{2+}$ to the growth medium. Such a rescue of COX activity had been observed previously in Sco-deficient organisms including human cell lines (14, 42–44). How this rescue works remains enigmatic in view of the fact that intracellular metal levels are usually regulated tightly, and chelators adjust them to appropriate concentrations in vivo (18). It is often typical for chaperones to be important especially under conditions of stress or nutrient limitation. In fact, ScoI requirement for cytochrome oxidase activity in B. japonicum seems to be most distinct under copper-limiting growth conditions (14, 15). Moreover, the bypass of ScoI by copper-replete conditions finds a possible explanation in the ease with which we could reconstitute the CuA center in vitro simply by incubating reduced CoxB in the presence of Cu$^{2+}$/H$^{11001}$ (Fig. 2D), as it had been worked out by Chacon and Blackburn (6). Assuming that such a self-assembly at high external Cu$^{2+}$ concentrations also works in vivo, this would explain why ScoI may become dispensable. By contrast, it is absolutely essential for the success of the in vitro reconstitution of the CuA center that CoxB is provided in reduced form. Extrapolating this sine qua non to the in vivo situation means that CoxB must always be kept reduced for copper ion insertion, which is precisely the function we ascribe to TlpA. Obviously, the TlpA requirement for the biogenesis of functional COX cannot be bypassed by copper-replete conditions.

Our in vitro experiments performed both with oxidized ScoI (20) and with oxidized CoxB as substrates (this work) clearly support the function of TlpA as a reductase. TlpA has a more negative redox potential than ScoI and CoxB, and the rates of reduction of both substrate proteins are fast and lie in a physiologically relevant range. This was already a strong hint for a specific protein-protein interaction between TlpA and ScoI as well as TlpA and CoxB, a postulate that was fully confirmed by the high-resolution crystal structures of the mixed disulfide TlpA-ScoI and TlpA-CoxB complexes. Apart from the detailed contact areas already described in Fig. 4 (see above), TlpAS$^{C110S}$ possesses a distinct “dipolar” interface in the region where ScoIS$^{C74S}$ and CoxBPD$^{C233S}$ bind. The TlpA interface shared with ScoI is of basic nature, whereas the interface shared with CoxB is mostly acidic. ScoI and CoxB complement this dipole as each protein provides the opposite charge to the interface (Fig. 6B). In conclusion, we suggest a model in which electrostatic interactions between TlpA and its substrates are also governed by an overall charge complementarity. Such a reciprocal electrostatic complementarity might contribute to the fast rates of ScoI and CoxB reduction by TlpA (45). Interestingly, although the CX$_3$C motif is the common target in both substrate proteins, TlpA attacks the more N-terminal cysteine in CoxB (i.e. Cys$^{229}$ in the $^{229}$CX$_3$C$^{233}$ sequence) as opposed to the more C-terminal cysteine in ScoI (i.e. Cys$^{78}$ in the $^{74}$CX$_3$C$^{78}$ sequence). This might also be a reflection of the bipolar asymmetry on TlpA, which positions each of two protein substrates from different angles on top of its active site.
Bacterial Thioredoxin Secures Copper-Protein Assembly

A bioinformatics analysis (available from the authors on request) has shown that TlpA is widespread in aerobic bacteria that respire with cytochrome oxidases. The peculiar, exposed location of the Cu\(_A\) center on the outer side of the membrane, there being confronted with an oxidizing environment, appears to make a TlpA-like reductase function mandatory for the biogenesis of cytochrome oxidase. Whether or not this prerequisite also applies to mitochondria is less obvious. Recent work has shown, however, that dithiol-disulfide oxidoreductase relay systems do exist in mitochondria, both in the oxidizing as well as in the reducing direction (8, 46), and thioredoxin-like proteins were found in the mitochondrial intermembrane space (47). The existence of a ScoI- and CoxB-specific reductase in the periplasm of Gram-negative bacteria inevitably leads to the question of where the reducing power is ultimately derived from. Although E. coli does not possess a cytochrome oxidase for aerobic respiration, a system similar to that of E. coli DsbD, which relays reducing power from the cytoplasm over the membrane into the periplasm (9, 48), is also a valid option for B. japonicum.

Another unsolved problem for future research is where the copper ions for the biogenesis of the Cu\(_A\) center are derived from, and how Cu\(_A\) assembly proceeds. Although the ScoI/Sco protein is widely accepted to be the Cu\(_{2+}\) carrier to Cu\(_A\) (18, 19), the direct transfer of Cu\(_{2+}\) to apo-CoxB has not yet been demonstrated with in vitro experiments. Furthermore, formation of the Cu\(_A\) center as an electron-delocalized Cu\(_{1.5}\)–Cu\(_{1.5}\) system implicates not only Cu\(_{2+}\) but also Cu\(_{+}\) in the metallation process (6). A good candidate for a Cu\(_{+}\) carrier to Cu\(_A\) is the PcuA/C protein (called PcuC in B. japonicum) (15, 49). How the ScoI- and PcuC-like copper chaperones cooperate

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**TABLE 2**

| TlpA\(_{C1\text{105}}\) | TlpA\(_{C1\text{105}}\)-CoxBPD\(_{C2\text{335}}\) mixed disulfide | TlpA\(_{C1\text{1105}}\)-ScoI\(_{C7\text{45}}\) mixed disulfide | ScoI\(_{C7\text{45}}\) Interaction |
|------------------|-------------------------------------------------|-------------------------------------------------|------------------|
| Ala\(_{99}\)      | Lys\(_{221}\)                                   | cb-sc                                            |                  |
| Gly\(_{41}\)      | Thr\(_{224}\)                                   | ab-cb                                           |                  |
| Gly\(_{41}\)      | Met\(_{234}\)                                   | cb-ab                                           |                  |
| Ala\(_{64}\)      | Lys\(_{232}\)                                   | cb-sc                                            |                  |
| Leu\(_{168}\)     | Cys\(_{229}\)                                   | Disulfide                                        |                  |
| Leu\(_{168}\)     | Cys\(_{229}\)                                   | Hydrophobic                                      |                  |
| Trp\(_{106}\)     | Cys\(_{229}\)                                   | Ph\(_{e225}\) – Stacking                         |                  |
| Trp\(_{106}\)     | Leu\(_{168}\)                                   | Ph\(_{e225}\) – Stacking                         |                  |
| Pro\(_{115}\)     | Arg\(_{153}\)                                   | Ph\(_{e225}\) – Stacking                         |                  |
| Leu\(_{168}\)     | Lys\(_{71}\)                                    | Sc-cb                                           |                  |
| Lys\(_{71}\)      | Glu\(_{231}\)                                   | Sc-cb                                           |                  |
| Lys\(_{71}\)      | Glu\(_{231}\)                                   | Sc-cb                                           |                  |
| Met\(_{179}\)     | Cys\(_{229}\)                                   | Sc-cb                                           |                  |
| Met\(_{179}\)     | Cys\(_{229}\)                                   | Sc-cb                                           |                  |
| Pro\(_{198}\)     | Pro\(_{198}\)                                   | Hydrophobic                                      |                  |
| Pro\(_{198}\)     | Leu\(_{169}\)                                   | Hydrophobic                                      |                  |
| Glu\(_{200}\)     | Leu\(_{169}\)                                   | Hydrophobic                                      |                  |
| Glu\(_{200}\)     | Leu\(_{169}\)                                   | Hydrophobic                                      |                  |

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**FIGURE 6. TlpA interacts with its substrates ScoI and CoxB via two distinct electrostatic surfaces.** A, representation of the electrostatic surface of TlpA\(_{C1\text{105}}\) in complex with ScoI\(_{C7\text{45}}\) (panel 1) and CoxBPD\(_{C2\text{335}}\) (panel 3). In panel 2, the structures of TlpA-ScoI from panel 1 and TlpA-CoxB from panel 3 were superimposed based on TlpA\(_{C1\text{105}}\), ScoI\(_{C7\text{45}}\) (orange) and CoxBPD\(_{C2\text{335}}\) (cyan) are shown as ribbon models. Cys\(_{107}\) of TlpA, Cys\(_{74}\) of ScoI\(_{C7\text{45}}\), and Cys\(_{233}\) of CoxBPD\(_{C2\text{335}}\) are highlighted in yellow. B, electrostatic surface representation contoured from −1 kT/e (red) to 1 kT/e (blue) of the dimerization interfaces of ScoI\(_{C7\text{45}}\) (panel 1), TlpA\(_{C1\text{1105}}\) (panel 2), and CoxBPD\(_{C2\text{335}}\) (panel 3), highlighting the surface charge complementarity in both complexes. Surface charge calculation was performed for the free, individual molecules. ScoI\(_{C7\text{45}}\) (panel 1) and CoxBPD\(_{C2\text{335}}\) (panel 3) were rotated by 180° with respect to their orientation in panel A. Red: negatively charged surface; blue: positively charged surface; yellow: position of reactive cysteine; N, N terminus of the respective polypeptide chain.
in the assembly of CuA on the TlpA-reduced CoxB subunit of cytochrome oxidase remains to be elucidated.

In this context, it is noteworthy that our work allowed for the first time a description of the structural differences between apo- and holo-CoxB. A superposition of the B. japonicum CoxBPD C233S structure onto the structure of P. denitrificans subunit Cox2 of cytochrome oxidase, which carries an intact CuA center (PDB entry 3hb3), is shown in Fig. 7. The largest difference concerns the segment 229–240, which contains four of the six CuA-coordinating residues, namely Cys229, Glu231, Cys233, and His237 (Fig. 7A). The positions at the Cα atoms deviate substantially from those of the CuA-coordinating residues of P. denitrificans Cox2, ranging from 6.5 to 12.1 Å. By contrast, the positions of Met240 and His194 do not change with respect to Cox2. This finding suggests that the CuA loop 229–240 in CoxB undergoes a major conformational change in the course of metallation. Furthermore, before metallation, the two cysteines Cys229 and Cys233 in the segment 229–240 are surface-exposed such that a disulfide bond between them is accessible for reduction by TlpA. After reduction, the two cysteine thiols may bind copper ions, and the entire loop may undergo a structural rearrangement to form the mature CuA center in holo-CoxB. Besides the CuA loop, we noticed one other structural difference, which concerns the segments Leu159–Asp172 of B. japonicum CoxB and Leu137–Asp159 of P. denitrificans Cox2. In Cox2, that region contains two short helices that contribute to the interface with the neighboring subunit I, whereas these helices are absent in B. japonicum CoxB (Fig. 7B).

**FIGURE 7.** The polypeptide segment containing the CuA-coordinating residues Cys229, Glu231, Cys233, and His237 of reduced B. japonicum CoxB is predicted to undergo major rearrangements during formation of the CuA center. A, right panel: superposition of CoxBPD C233S (cyan) in the TlpA51105–
CoxBPD C233S mixed disulfide with holo-Cox2 of P. denitrificans (deep purple, taken from PDB entry 3hb3). The two copper ions in the CuA domain of Cox2 are shown as orange spheres. Left panel: close-up view of the superimposed periplasmic domains of B. japonicum CoxBPD C233S and P. denitrificans Cox2. A segment of TlpA51105 from the TlpA51105–CoxBPD C233S complex is also shown in the background (gray). Dashed lines: distances (in Å) between the Cα atoms of the CuA-coordinating residues of CoxBPD C233S and those of Cox2; yellow sticks: sulfur atoms; red sticks: oxygen atoms; dark blue sticks: nitrogen atoms. B, structure-based sequence alignment, obtained with the DaliLite workbench (50) and manually edited, of B. japonicum CoxBPD C233S with the corresponding periplasmic domain of Cox2 from P. denitrificans. Sequence identity is 44%. The cysteines liganding the copper ions in the CuA center are highlighted in yellow and the other copper-coordinating residues undergoing a large conformational rearrangement upon formation of the CuA center are highlighted in purple, whereas the copper-coordinating residues that undergo no conformational rearrangement upon CuA formation are highlighted in cobalt blue. Secondary structure elements (β-strands: blue arrows; α-helices: red cylinders) were assigned as indicated by DaliLite (50).
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