Crystal structure of CO-bound cytochrome c oxidase determined by serial femtosecond X-ray crystallography at room temperature

Izumi Ishigami*, Nadia A. Zatsepin,Masahide Hikita,Chelsie E. Conrad, Garrett Nelson, Jesse D. Coe, Shibom Basu, Thomas D. Grant, Matthew H. Seaberg, Raymond G. Sierra, Mark S. Hunter, Petra Fromme, Raimund Fromme, Syun-Ru Yeh, and Denis L. Rousseau

*Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461; †Department of Physics, Arizona State University, Tempe, AZ 85287; Center for Applied Structural Discovery, The Biodesign Institute, Arizona State University, Tempe, AZ 85287; ‡School of Molecular Sciences, Arizona State University, Tempe, AZ 85287; Department of Structural Biology, Hauptman-Woodward Institute, State University of New York at Buffalo, Buffalo, NY 14203; and †SLAC National Accelerator Laboratory, Menlo Park, CA 94025

Edited by Harry B. Gray, California Institute of Technology, Pasadena, CA, and approved June 8, 2017 (received for review April 5, 2017)

Cytochrome c oxidase (CcO), the terminal enzyme in the electron transfer chain, translocates protons across the inner mitochondrial membrane by harnessing the free energy generated by the reduction of oxygen to water. Several redox-coupled proton translocation mechanisms have been proposed, but they lack confirmation, in part from the absence of reliable structural information due to radiation damage artifacts caused by the intense synchrotron radiation. Here we report the room temperature, neutral pH (6.8), damage-free structure of bovine CcO (bCcO) in the carbon monoxide (CO)-bound state at a resolution of 2.3 Å, obtained by serial femtosecond X-ray crystallography (SFX) with an X-ray free electron laser. As a comparison, an equivalent structure was obtained at a resolution of 1.95 Å, from data collected at a synchrotron light source. In the SFX structure, the CO is coordinated to the heme a3 iron atom, with a bent Fe–C–O angle of ~142°. In contrast, in the synchrotron structure, the Fe–CO bond is cleaved; CO relocates to a new site near CuA, which, in turn, moves closer to the heme a3 iron by ~0.38 Å. Structural comparison reveals that ligand binding to the heme a3 iron in the SFX structure is associated with an allosteric structural transition, involving partial unwinding of the helix-X between heme a and a3, thereby establishing a communication linkage between the two heme groups, setting the stage for proton translocation during the ensuing redox chemistry.

Bioenergetics | X-ray free electron laser | crystallography | cytochrome c oxidase | serial femtosecond crystallography

In the mammalian respiratory chain, cytochrome c oxidase (CcO), the final electron acceptor, catalyzes the four-electron reduction of oxygen to water and pumps protons across the inner mitochondrial membrane to establish an electrochemical proton gradient that is used to synthesize ATP. CcO contains four redox centers: two heme groups (heme a and heme a3) and two copper centers (CuA and CuB). Cytochrome c delivers electrons to the CuA center from which they transfer to heme a and then to the heme a3–CuB binuclear center (Fig. 1), where oxygen is reduced to water by using four protons (the substrate protons) from the matrix side of the membrane and four electrons delivered to CcO by cytochrome c. Associated with the oxygen reduction chemistry, four additional protons (the pumped protons) are translocated from the negative side to the positive side of the mitochondrial membrane. Whereas the mechanism of the O2 reduction is relatively well understood, the mechanism by which the redox energy is coupled to proton translocation remains unresolved in mammalian CcOs (1–4), although it is quite well-defined in bacterial CcOs (5, 6).

The determination of X-ray structures of various derivatives of bovine CcO (bCcO) has been very helpful in understanding many of the features of the enzyme as summarized in a recent review (3). However, the structures obtained with conventional synchrotron light sources were determined from protein crystals exposed to a high X-ray flux and high levels of cryoprotectants at cryogenic temperatures. It has been shown in CcO (7, 8) as well as other proteins and enzymes (9, 10) that the high-intensity synchrotron X-ray beam has many deleterious effects including reduction, ligand dissociation, and damage (11, 12). These deleterious effects cause irreversible changes in the protein structure leading to erroneous structural determinations, especially in protein systems containing electron-rich heavy metals (13). In oxidized forms of CcO, it has been shown that X-ray reduction of the metal centers occurs even with very short exposures to the X-ray beam (7, 14). Deleterious radiation-induced artifacts are especially evident in the structure of the CO-bound derivatives (bCcO-CO) obtained at low temperature by synchrotron radiation in which the X-ray beam has been shown to photodissociate the CO from the heme a3 iron atom (8). Hence, it is a challenging task to obtain damage-free structures of CcO that show its native structure.

To overcome these problems, we have used serial femtosecond X-ray crystallography (SFX), to determine the structure

Significance

A fundamental bioenergetics question is how the redox energy from oxygen reduction in cytochrome c oxidase (CcO) is coupled to proton translocation. Although crystallography has revealed many properties of the enzyme, the coupling mechanism remains elusive, in part due to artifacts caused by X-ray damage from synchrotron radiation. We determined the structure of CO-bound bovine CcO by serial femtosecond X-ray crystallography (SFX) with an X-ray free electron laser, outrunning radiation damage. The SFX structure is compared with the structure obtained with synchrotron radiation in which the Fe–CO bond was cleaved. Our results reveal that ligand binding to heme a3 triggers an allosteric structural transition that may play a critical role in proton translocation in the CcO family of enzymes.

Author contributions: J.I., M.H., P.F., S.-R.Y., and D.L.R. designed research; J.I., M.H., C.E.C., G.N., J.D.C., M.H.S., R.G.S., M.S.H., and R.F. performed research; S.-R.Y. contributed new analytic tools; N.A.Z., S.B., T.D.G., R.F., and D.L.R. analyzed data; and S.-R.Y. and D.L.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 5W97 and 5WAO).

1Present address: Institute of Materials Structure Science, Synchrotron Radiation Science Division II, Tsukuba, Ibaraki 305-0801, Japan.

2Present address: Center for Cancer Research, National Cancer Institute, Advanced Technology Research Facility, Frederick, MD 21702-1201.

3To whom correspondence should be addressed. Email: denisrousseau@einstein.yu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1705628114/-/DCSupplemental.
The calculated unbiased F$_o$–F$_c$ difference map, based on the refinement performed without the CO ligand, shows strong positive density the Cu$_{Cu}$–heme $a_1$ binuclear center (Fig. 2A). In the refined structure with CO modeled in (Fig. 2B), the Fe–CO and Fe–His bond lengths are 1.89 and 1.98 Å, respectively, and the Fe–C–O moiety adopts a bent conformation with an angle of ~142° (Fig. 2C). Electronically, the Fe–C–O favors a linear geometry that is perpendicular to the heme plane. However, the close proximity of Cu$_{Cu}$ to the heme $a_1$ iron atom in bC$_{CO}$ forces the iron-bound CO to move away from Cu$_{Cu}$ leading to the bent Fe–C–O conformation, possibly associated with an Fe–CO bond tilted from the heme normal, which cannot be differentiated by the current data.

**Structure of bCO–CO Obtained by Synchrotron Radiation.** As a comparison with the room temperature SFX structure, we obtained the bC$_{CO}$–CO structure with synchrotron radiation at 100 K at the Advanced Light Source (ALS) of the Lawrence Berkeley National Laboratory (beamline 8.2.1). The data collection strategy and analysis are described in SI Appendix, Table S3. The structure was determined to 1.95 Å and unit cell parameters of $a$, $b$, $c$ = 177.92, 182.55, 208.45 Å, respectively, and $\alpha = \beta = \gamma = 90°$ were obtained, similar to those previously reported for the CO-bound enzyme, where data were measured at cryogenic temperatures (178.09, 182.83, 206.93 Å, $\alpha = \beta = \gamma = 90°$) [Protein Data Bank (PDB): 3AG2] with a synchrotron source (SPring-8) (8). They are also comparable to those determined by SFX discussed above, with the exception that the b unit cell axis is 182.55 versus 189.8 Å, which possibly reflects the difference in the data acquisition temperatures.

The exposure of crystals at 100 K to synchrotron radiation, at a total exposure of less than 25 MGy, led to CO dissociation from the heme $a_1$ iron, as well as its movement toward Cu$_{Cu}$ (Fig. 2D–F). We define this photoprodust as bC$_{CO}$–CO$^*$. The CO occupies a position in which the Cu–C and Cu–O distances are similar, as shown in Fig. 2F. The Cu$_{Cu}$–Fe distance decreased from 5.30 Å, when the CO was coordinated to the heme $a_1$ iron atom, to 4.92 Å. This change in distance involves primarily movement of Cu$_{Cu}$; the position of the iron atom changes less than that of Cu$_{Cu}$, which changes by ~0.28 Å. This change is a result of the Cu moving from its nearly planar position, defined
by the three histidine ligands in the CO-bound structure, to an out-of-plane position in the bCcO–CO* structure.

**Structural Changes in Helix-X.** Apart from the differences in the CO position and the Cu₈-heme α₃ distances, the overall structure of the CO-bound and CO-photodissociated proteins were quite similar, except for a section of helix-X. Helix-X is a critical structural element of bCcO as it lies between heme α and heme α₃ and contains their axial ligands (H378 and H376, respectively) (Fig. 1). In bCcO–CO*, the helical structure of this part of the sequence is stabilized by a series of H-bonding interactions between backbone N-H groups and C=O groups located three or four residues earlier along the sequence. In the SFX-based undamaged bCcO–CO structure, the [380–384] amino acid fragment is partially unwind due to the disruption of the H-bonding interactions as illustrated in Fig. 3. Specifically, the H bonds between the peptide carbonyls of S382/M383 and their partner residues observed in bCcO–CO* are not present, due to a large reorientation of the carbonyls. Similarly, the H bond between the carbonyl of Y379 and the amine group of G384 is not present due to reorientation of the amine group. It is important to note that the [380–385] amino acid fragment constitutes one of the most flexible regions of the bCcO structures, in particular the S382 residue, as evident in the B-factor plots shown in *SI Appendix*, Figs. S3 and S4. The partial unwinding of the α-helical structure of the [380–385] fragment culminates in large movement of the Ca and peptide carbonyl group of S382 by as much as 1.5 and 2.8 Å, respectively (Fig. 3A). We define the synchrotron structure with the intact H-bonding interactions as a closed structure and the SFX structure with the disrupted H-bonding interactions as an open structure.

How does ligand binding to heme α₃ trigger the partial unwinding of the helix-X? One might expect that this modulation is a consequence of a change in the position of the helix-X histidine, H376, which is coordinated to heme α₃, where the exogenous ligand binds. Despite the large differences in the backbone between residues 380 and 384, there are only minimal differences (Fig. 3A) between residues 376–379, the region that contains the heme axial His ligands (376 and 378). This demonstrates that it is not the histidine movement due to the presence or absence of a ligand on heme α₃ that causes the structural differences. Instead, the differences originate in the interaction of the C pyrrole ring of heme α₃ with residue V380, one of the most common heme face contact residues (23). V380 is the only residue that is in contact with heme α₃ face (Fig. 4) and is part of the region of the helix-X backbone that changes. Upon release of the CO, a heme α₃ distortion brings about the change in position of V380 (*SI Appendix*, Fig. S5).

To quantitate the heme α₃ distortion, we used normal-coordinate structure decomposition (NSD) (24) to analyze the heme structures. In the SFX structure of bCcO–CO the composite out-of-plane distortion is 0.603 Å, whereas in the bCcO–CO* structure it is 0.866 Å, with the largest changes occurring in the domains (from 0.428 to 0.670 Å) and saddling (from 0.256 to 0.517 Å) coordinates. A full description of the NSD results and descriptions of the domain and saddling structural distortions are presented in *SI Appendix*, *Discussion*. The heme distortion results in a 0.5 Å movement of the C2 and C3 atoms of the C pyrrole ring of heme α₃, as well as a change in the orientation of the vinyl group attached to it (see the expanded view in Fig. 4 and *SI Appendix*, Fig. S5). Together these conformational changes induce the movement of V380, triggering the partial unwinding of the helix-X structure. Thus, V380 initiates the change in the helix-X structure and transmits the heme α₃ state to the peptide backbone, regulating the open and closed structures.

**Fig. 3.** Allosteric structural transition in the Helix-X fragment induced by ligand binding to heme α₃. (A) Superimposed structures of helix-X in bCcO–CO and bCcO–CO* obtained by SFX (green) and by synchrotron radiation (coral), respectively. The arrows designate the [380–384] peptide backbone movements (in Å) induced by ligand binding to heme α₃. In the comparison of the two structures, the Ca carbon atoms of S382 and M383 differ by 1.5 Å, and the backbone carbonyl groups differ by 2.8 Å. Comparison of the alpha-helix hydrogen bonding in (B) the bCcO–CO structure obtained by SFX and (C) the bCcO–CO* structure obtained by synchrotron radiation. In Cα, in which the CO ligand was dissociated from the heme α₃ iron atom, the normal α-helical H-bonding is established, whereas in bCcO, the SFX structure, the α-helical H-bonding patterns of the carbonyl groups of Y379, S382, and M383 are disrupted.

**Fig. 4.** Helix-X mediated allosteric structural transition induced by ligand binding to heme α₃. Superimposed structures of bCcO–CO and bCcO–CO* obtained by SFX (green) and synchrotron radiation (coral), respectively, showing the ligand binding induced conformational change to the heme α₃, in particular the C-pyrrole ring, and its propagation to heme α via the 380–384 residue segment of the helix-X. The farnesyl OH group of heme α is highlighted by the blue background. The expanded view shows the interaction region between V380 and the C-pyrrole ring of heme α₃. The red arrows identify the distance change of the C2 atom (0.5 Å) due to the increased heme distortion in the photodissociated state.
steric constraints owing to its sp² electronic configuration, which favors a 120° Fe–O–O angle. The absence of steric crowding would also suggest a shorter Fe–Cu₃ distance for O₂ compared with CO. In accord with this, in the reduced bCcO–NO complex, in which Fe–N–O adopts an electronically favored bent geometry, the Fe–Cu₄ distance (4.92 Å) is short (8). On the other hand, our synchrotron data revealed that CO dissociation from the heme iron leads to the movement of Cu₃ towards the heme α₃ ion. The apparent facile change in position of Cu₃B, as a function of the ligand coordination on the heme α₃ iron atom, suggests that during the catalytic cycle, Cu₃ is able to move in response to the change in the ligation states of the heme α₃ thereby modulating the reactivity of the binuclear center. As suggested recently, a shortening of the Fe–Cu₃ distance could play an important role in ligand stabilization (27).

A structure of the CO-bound derivative of bCcO was reported by Muramoto et al. (8) in which the crystals were placed in a capillary and measured at room temperature (280 K) by synchrotron radiation. In that structure, CO remained bonded to heme α₃ but the orientation of the CO was reported (8) to be essentially perpendicular with an Fe–C–O angle of 164–168°, in sharp contrast to the 142° we obtained in the SFX measurements reported here. Because the measurements of Muramoto et al. (8) were made with synchrotron radiation, radiation damage may have affected the final structure. In addition, CO photodissociation by the synchrotron X-ray beam would be expected, so the structure may represent a photostationary state, in which the CO was continually being dissociated and rebound to the heme α₃ iron atom.

**Allosteric Structural Transition.** The data reported here show that ligand binding to the heme α₃ iron is associated with partial unwinding of the helical structure in helix-X generating an open conformation. In both the SFX bCcO–CO structure (open) and the synchrotron-based bCcO–CO⁺ structure (closed), all four of the metal redox centers are reduced. However, in the former structure, CO is coordinated to heme α₃ whereas in the latter, heme α₃ is ligand-free in a five-coordinate high spin configuration, i.e.,

\[
(Cu^{1+}, Fe^{2+}, Cu^{1+}, Fe^{2+} - CO) \text{ (open)}
\]

\[
\to (Cu^{1+}, Fe^{2+}, Cu^{1+} \cdots CO, Fe^{2+}) \text{ (closed}).
\]

In Eq. 1, φ represents photodissociation by the synchrotron radiation beam, and the ⋯ represents the observation that the CO is close to, but not coordinated to, CuB. Thus, the removal of ligand coordination of the iron atom on heme α₃ is sufficient to induce the change from the open to the closed conformation and establish the α-helical H-bonding.

Structural differences in helix-X, in various derivatives of bCcO (28), and in its bacterial analogs (29), have been reported previously (SI Appendix, Table S2). Recently, new high-resolution structures of reduced and oxidized bCcO obtained with synchrotron radiation at 50 K were reported (30). To reduce the effects of radiation damage each frame was taken with a 10-s exposure and the crystals were translated by 100 μ after each round of 10 shots. For the measurements, 16 oxidized and 8 reduced crystals were used. In their reduced and oxidized structures, helix-X adopted closed and open conformations, respectively, similar to those defined in this work (SI Appendix, Figs. S6 and S7), although in bCcO–CO⁺ the helix does not relax all of the way back to the same position as in the fully reduced enzyme, likely due to restrictions imposed by the low-temperature measurements. Inspection of other reported structures of bCcO, revealed that when the ferrous heme α₃ iron is in an exogenous ligand-free state, helix-X adopts a closed conformation; conversely, when the heme α₃ iron is coordinated by an exogenous ligand, in either the oxidized or reduced state, helix-X adopts an open conformation. It is notable that the reduced bCcO–NO complex has alternate structures (PDB: 3AG3) (8), with helix-X in both the open and the closed structures, possibly due to partial photodissociation of the NO by the synchrotron radiation. (See SI Appendix, SI Text and Table S2, for a discussion of the helix-X conformations in other derivatives.)

The series of structural changes in the helix-X region induced by ligand binding to heme α₃ (red arrows) are summarized in Fig. 5. Ligand binding to heme α₃ (indicated by a in Fig. 5) leads to a change in the heme distortion (indicated by b in Fig. 5). The change in the heme structure triggers the movement of V380 (indicated by c in Fig. 5), which induces a partial unwinding in helix-X (indicated by d in Fig. 5), causing a reorientation of S382 (indicated by e in Fig. 5). This poises S382 to interact with the OH of the farnesyl side chain, but it is over 8 Å away when heme a is reduced. However, during the catalytic reaction, heme a alternates between an oxidized and reduced state (gray arrows). When heme a becomes oxidized (indicated by f in Fig. 5) the OH of the farnesyl side chain rotates by 160° (indicated by g in Fig. 5) to be within ~3 Å of S382 in the ligand-bound (open) state (SI Appendix, Fig. S8). The allosteric structural transition establishes a dynamic communication pathway between the two hemes, offering structural insights into previously proposed proton translocation mechanisms in bCcO (1, 4, 28, 31).

In proton translocation studies of bovine and bacterial CcOs, three proton channels have been postulated (SI Appendix, Fig. S9). The K and D channels connect the negative side of the membrane to the vicinity of the heme α₃–Cu₃ binuclear center (32–35), whereas the H channel passes from the negative side of the membrane via the heme a center to the positive side (31, 36, 37). Because the catalytic sites of the heme–copper oxidases from eukaryotic and prokaryotic species share the same structural architecture, it has been proposed that the proton pumping mechanism is conserved across species (38, 39). However, this general concept has been questioned by the lack of agreement between those mechanisms shown to be operative in bacterial CcOs (5, 6) and those proposed for bCcO (1, 4, 28, 31). Illustrative of the distinct properties of the different species is the finding that although a structural change in helix-X, similar to that in bCcO, is seen in *Rhodobacter sphaeroides* CcO (RsCcO)
(29), the OH group of the heme α farnesyl side chain does not undergo the 160° rotation upon the change in the redox state of heme α as it does in bCcO. Thus, what functional role, if any, the conformation change in helix-X plays in bacterial CcOs remains to be determined.

Based on structural and mutagenesis studies of bCcO (31), it has been proposed that the H channel is the major pathway for proton translocation in bCcO, and the translocation is gated by D51, heme α, and the S382 residue/farnesyl side chain OH pair (3). Thus, repositioning of S382 during the allosteric transition plays a critical role in proposed H channel mechanisms (1, 3, 4). Contrary to the H channel mechanisms in the bovine enzyme, extensive mutagenesis, theoretical, and structural studies in bacterial CcOs have led to the conclusion that the major proton translocation pathway is the D channel (5, 40, 41). (See SI Appendix, SI Text, for additional information on the H and D channel mechanisms.) To interrogate the effect of S425 in RscCcO (equivalent to S382 in bCcO) on proton translocation in the D pathway, Brzezinski and coworkers (42) mutated S425 to alanine and measured its proton translocation efficiency during the O2 reaction cycle as a function of pH. They found that structural changes in S425 propagate to the D pathway, thereby modulating proton transfer rates (42). These data suggest that the S382-linked allosteric structural transition may play a role in proton translocation in both mammalian and bacterial oxidases.

Ligand Reactivity. It is well established that in solution, following CO photodissociation from the heme α3 iron atom, the CO first binds to CuB; it subsequently dissociates from CuB and exits the protein matrix without geminately rebinding to the heme α3 iron atom (43). This has been postulated as evidence for conformational changes at the binuclear center thereby hindering the CO from rebinding to the heme α3 iron atom (43). However, our current data do not support this hypothesis, because no structural changes in the binuclear center that can limit the rebinding rate are identifiable in the structure of bCcO-CO*.

To better understand the ligand binding/dissociation reactions of bCcO, it is necessary to consider the differences between CO and O2 binding based on the binding reaction scheme shown in Fig. 6. Inset, and the corresponding free energy curves. This reaction coordinate is analogous to those derived for the myoglobin reaction with ligands (44). In bCcO, for a ligand to bind the heme α3 iron, it has to first migrate into the protein matrix by overcoming the outer barrier (OB) to form the encounter complex (labeled in gray). Subsequently, it binds to CuB by crossing inner barrier 1 (IB-1) and then moves from CuB to the heme α3 iron by crossing inner barrier 2 (IB-2). The initial migration into the protein is expected to be similar to CO (red curve) and O2 (gray curve), represented by their overlap in the outer barrier. Because the bimolecular binding of O2 to heme α3 is over 103 times faster than that of CO (45), the inner barriers must be lower for O2 than for CO. For the CO reaction, IB-2, and possibly also IB-1, is higher compared with the O2 reaction because in essentially all heme proteins the barrier for binding to the ferrous iron atom is much higher for CO than for O2 (44, 46). For example, owing to this barrier, in myoglobin the binding rate is 35 times slower for CO than for O2. Frauenfelder and Wolynes (47) have shown that this difference in the CO and O2 binding rates in heme proteins is a result of the spin transitions required for the binding of CO to a heme iron atom, which does not occur for O2 binding.

Consistent with the hypothesized energy curves shown in Fig. 6, Woodruff and coworkers were able to determine that the on rate of CO from CuB to the heme α3 iron is 1,030 s−1 (43), at least 35 times slower than the on rate of O2 from CuB (48). In any case, the energy curve associated with the CO reaction indicates that once CO is photodissociated from the heme α3 iron in bCcO, it could either rebind to the heme iron by crossing the IB-2 or escape out of the protein by crossing IB-1 and OB. Which route it takes depends on the branching ratio R (R = kIB-2/kIB-1), in which kIB-1 and kIB-2 represent the rate constants for crossing inner barrier 1 and inner barrier 2, respectively. The low branching ratio for CO, due to the higher IB-2 than IB-1 barrier, accounts for the observed low geminate CO recombination yield.

The Inverse Correlation of the Fe–CO and C–O Vibrational Modes. In CO-bound heme proteins, there is a well-established inverse correlation between the frequencies of the Fe–CO and the C–O stretching modes. However, the frequencies of the modes in CcO–CO fail to fall on the inverse correlation line; instead, they lie above the line as shown in SI Appendix, Fig. S10 (49). It was shown by Yu and coworkers (26) that distortion of the CO, from its typical linear and perpendicular orientation, by distal pocket constrictions, can result in deviations from the correlation curve, in which the data points lie above the curve, possibly due to a reduction in the effective mass of the Fe–C–O moiety due to its bent conformation, thereby modifying the frequencies of the stretching vibrations. The structural data we report here reveal that the Fe–C–O moiety of bCcO-CO is bent due to the steric interaction with CuB; hence, they offer a molecular explanation for the deviation of the CcO data from the inverse correlation line. It is notable that in some studies of CcO–CO, a second set of data falling on the inverse correlation line were also observed (49), indicating that in a certain population of the enzyme molecules, CuB has moved away from the heme α3 iron atom, allowing the CO to bind in a linear fashion.

Materials and Methods

Isolation and purification of bCcO from bovine hearts was carried out by standard procedures (21, 22). To generate highly purified bCcO from the isolated enzyme, crystals were formed and harvested. These crystals were redissolved and used as the source for subsequent crystal growth. Large crystals were grown and then crushed to form seed stock to grow microcrystals (~20 x 20 x 4 μm) for the SFX measurements and large crystals (~300 x 300 x 60 μm) for the synchrotron radiation measurements. All crystals were grown in phosphate buffer at pH 6.8 with variable amounts of detergent (decylmaltoside) and PEG-4000, which were optimized for each preparation. For the SFX measurements the crystals were reduced with a minimal amount of dithionite and exposed to CO to form the CO derivative, which was monitored by microscopic spectroscopy. The crystal suspensions were kept at 4 °C throughout and used within a few hours of their preparation. For the synchrotron measurements, the crystals were soaked in ethylene glycol by a stepwise procedure to reach 45% in a CO-saturated buffer. The crystals were immediately frozen and stored under liquid nitrogen for the X-ray measurements.
The SFX experiments were carried out at the LCLS at SLAC National Accelerator Laboratory. A gas dynamic virtual nozzle delivered micrometer-sized gas-phase droplets loaded with a liquid jet, to the pulsed XFEL beam at a flow rate of ~10 μL/min. The data were collected at 9.5 keV energy, a duration of 40 fs, and a repetition rate of 120 Hz. The data were sorted, indexed, and merged with the Cheetah (17) and CrystFEL (18) programs and refined with the programs in the Collaborative Computational Project No. 4 (CCP4) suite. The synchrotron X-ray diffraction measurements were carried out at beamline 8.2.1 at ALS with premerz-HClo crystals of bCCo-CO at 100 K. The data were collected at 1.2 keV, and the full flux was 6 × 10^{11} photons per s. For premerging of the data, XDS software (50) was used; scaling was done with Aimless, molecular replacement was done with PhaserMR, and refinement was done with Refmac5 from the CCP4 program suite (51). Additional methods and materials details are available in SI Appendix.

ACKNOWLEDGMENTS. The SFX experiments were carried out at the LCLS at the SLAC National Accelerator Laboratory. LCLS is an Office of Science User Facility operated for the US Department of Energy Office of Science by Stanford University. Use of the LCLS, SLAC National Accelerator Laboratory, is supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract DE-AC02-76SF00515. Parts of the sample delivery system used at LCLS for this research were funded by NIH Grant P41GM103393, formerly P41RR01209. This work was supported by National Science Foundation (NSF) Science and Technology Center Award 1231306, CHE-1404929 (to D.L.R.), and Division of Biological Infrastructure, Advances in Biological Informatics (ABI) Grant 1565180 (to N.A.Z.) and National Institutes of Health (NIH) Awards GM098799 (to D.L.R.), GM115773 (to S.-R.Y), and R01GM105583 (to P.F.). We also acknowledge support from the BionodesCenter for Applied Structural Discovery at Arizona State University.

1. Ishigami I, Hikita M, Egawa T, Yeh SR, Rousseau DL (2015) Proton translocation in cytochrome c oxidase: Insights from proton exchange kinetics and vibrational spectroscopy. Biochim Biophys Acta 1847:98-107.
2. Yoshikawa S, Muramoto K, Shinzawa-Itoh K (2013) Proton-pumping mechanism of cytochrome C oxidase. Annu Rev Biochem 82:205-223.
3. Yoshikawa S, Shimada A (2015) Reaction mechanism of cytochrome c oxidase. Chem Rev 115:1936-1989.
4. Egawa T, Yeh SR, Rousseau DL (2013) Redox-controlled proton gating in bovine cytochrome c oxidase. PLoS One 8:e63669.
5. Kaila VR, Verkhoisky MJ, Wikström M (2010) Proton-coupled electron transfer in cytochrome oxidase. Chem Rev 110:7602-7081.
6. Wikström M, Sharma V, Kasprzak K, Hepler JR, Hummer G (2015) New perspectives on proton pumping in cellular respiration. Chem Rev 115:1916-2221.
7. Liu B, et al. (2009) Combined microspectrophotometric and crystallographic examination of chemically reduced and X-ray radiation-reduced forms of cytochrome bc oxidase from Thermus thermophilus. Structure of the reduced form of the enzyme. Biochemistry 48:8468-8478.
8. Muramoto K, et al. (2010) Bovine cytochrome c oxidase structures enable O2 reduction with minimization of reactive oxygens and provide a proton-pumping gate. Proc Natl Acad Sci USA 107:7740-7745.
9. Hersleth HP, et al. (2007) Crystallographic and spectroscopic studies of peroxide-derived myoglobin compound II and occurrence of protonated FeIV O. J Biol Chem 282:23372-23386.
10. Carugo O, Djinovic-Carugo K (2005) When X-rays modify the protein structure: Radiation damage at work. Trends Biochem Sci 30:213-219.
11. Sliz P, Harrison SC, Rosenbaum G (2003) How does radiation damage in protein crystals depend on X-ray dose? Structure 11:13-19.
12. Sanitsivil R, et al. (2011) Radiation damage in protein crystals is reduced with a micro-sized X-ray beam. Proc Natl Acad Sci USA 108:6127-6132.
13. Garman EF (2010) Radiation damage in macromolecular crystallography: What is it and why should we care? Acta Crystalogr D Biol Crystallogr 66:339-351.
14. Aoyama H, et al. (2009) A peroxide bridge between Fe and Cu ions in the O2 reduction site of fully oxidized cytochrome oxidase could suppress the proton pumping process. J Biol Chem 284:10265-10272.
15. Chapman NN, Cramer L, Timmehaus N (2014) Diffraction before destruction. Philos Trans R Soc Lond B Biol Sci 369:20130313.
16. Chapman NN, et al. (2011) Femtosecond X-ray protein nanocrystallization. Nature 470:70-77.
17. Barty A, et al. (2014) Cheetah: Software for high-throughput reduction and analysis of femtosecond X-ray diffraction data. J Appl Cryst 47:1118-1131.
18. White TA, et al. (2012) CrystFEL: A software suite for snapshot crystallography. J Appl Cryst 45:335-341.
19. White TA, et al. (2016) Recent developments in CrystFEL. J Appl Cryst 49:680-689.
20. Boutet S, et al. (2012) High-resolution protein structure determination by femtosecond crystallography. Science 337:362-364.
21. Yoshikawa S, Choc MG, O’Toole MC, Caughey WS (1977) An infrared study of CO stretching and Fe-C-O bending vibrations in sterically hindered carbonmonoxy myoglobin. Chem Rev 77:139-154.