J. Biochem. 2022;171(1):13–15 https://doi.org/10.1093/jb/mvab118

JB THE JOURNAL OF BIOCHEMISTRY

JB Special Issue - Commentary

Crystallographic studies of cytochrome c and cytochrome c oxidase

Received 30 September 2021; accepted 19 October 2021; published online 26 October 2021

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I started on crystallographic studies of cytochrome c (Cyt.c) in the later 1960s at the Institute for Protein Research, Osaka University. The institute successfully built the structural model of ferro-Cyt.c by the multiple heavy atom replacement method in the early 1970s. In the early 1990s, crystals of cytochrome c oxidase (CcO) from bovine heart were obtained by using polyethylene glycol 4000 (Sigma) as the precipitant. We reported the first structure of a mammalian membrane protein at 2.8 Å resolution in 1995. High-resolution crystallography of CcO is in progress to understand the coupling mechanism of O_2 reduction and proton pumping. We determined the structure of the mammalian Cyt.c–CcO complex at 2.0 Å resolution and identified the 'soft and specific' interaction between Cyt.c and CcO, which affected high-efficiency inter-molecular electron transfer.

In the early 1960s, a research group at the Institute for Protein Research, Osaka University, led by Masao Kakudo initiated crystallographic studies of Cyt.c in order to understand the electron-transport mechanism of the respiratory system. I started on crystallographic studies of Cyt.c as a graduate student in the institute. Crystal structure analysis of bonito Cyt.c was performed in the infancy of protein crystallography in Japan. Because many protein crystals were initially required to establish x-ray experimental procedures, purification and crystallization of the protein were performed on a large scale. Crystals of Cyt.c for structure analysis were successfully prepared from bonito heart (*1*). A total of 3 g of protein was purified from 20 kg of frozen bonito hearts (*2*). The protein was crystallized by adding salt (salting-out) as follows. Ammonium sulphate powder was added to 10 ml of Cyt.c solution (7%, w/w) in a test tube until high turbidity was brought about. The turbid solution was left to stand at room temperature, leading to the development of crystalline nuclei in the amorphous precipitate that grew to large crystals in 1–2 weeks.

A computer-controlled four-circle diffractometer was developed to acquire diffraction intensity data for the protein crystals. Initially, various types of diffraction experiment were tried for structure analysis by the multiple heavy atom replacement method. Tamaichi Ashida developed computing programs for protein crystallography (*2*). It was hard to prepare heavy atom derivative crystals of Cyt.c because its solvent content of 37% in volume was too low to preserve isomorphism with the native crystal. Three ($K_3UO_2F_5$, K_2PtCl_4 and K_2HgI_4) were found to be useful for the preparation of heavy atom derivatives (*2*). The Hg-derivative and Pt-derivative crystals provided data with resolution lower than 6.0 and 4.0 Å, respectively, because of poor isomorphism. Ultimately, after obtaining $(CH_3)_2SnCl_2$ and K_2IrCl_6 derivatives, the crystal structure analysis of bonito ferro-Cyt.c was performed at 2.3 Å resolution ((*3*, *4*) PDB ID: 1CYC). The overall features of ferro-Cyt.c were very similar to those of horse ferri-Cyt.c (*5*), despite the various differences in physiological and chemical properties in the oxidized and the reduced states (*2*). Several basic residues on the molecular surface of Cyt.c were proposed to interact with CcO by chemical modification experiments (*6*). When ferro-Cyt.c was oxidized by O_2 in the crystalline state, difference Fourier synthesis indicated a structural alteration suggesting a modification in surface charge between the oxidized and reduced states (*7*).

In 1974, I talked with Shinya Yoshikawa, who was engaged in biochemical research on CcO, about conducting crystallographic studies of a mammalian CcO, but the purity of CcO obtained from bovine heart was insufficient for crystallization in those days. In 1980, however, he observed sparkle micro crystals during concentration of the protein. These crystals yielded x-ray diffractions with resolution as high as 8 Å (*8*). Square plate crystals were obtained by using polyethylene glycol 4000 (Sigma) as the precipitant. The crystals diffracted x-rays up to 2.6 Å resolution and belong to the orthorhombic space group (*9*). The resolution of the orthorhombic crystal has been improved up to 1.3 Å today. We reported the first structure of a mammalian membrane protein at 2.8 Å resolution in 1995 (*9*). The whole structures of 13 subunits containing Cu_A, heme *a*, heme a_3 and Cu_B were clearly assigned in the electron density map ((*9*, *10*) PDB ID: 5B1A). Crystal structure analyses of bovine CcO crystals in various reaction states have demonstrated that the enzyme regulates its exact functions by accurate structural changes at the sub-angstrom level (*11–19*).

The enzyme catalyses the reduction of O_2 in six distinct steps, which enable electron transfer to be coupled with proton pumping. Based on our structural studies, we have proposed a proton pumping mechanism, which involves a proton-conducting pathway (H-pathway) comprising a water channel and a hydrogen-bond network located between the negative side (N-side) and the positive side (P-side) surfaces of CcO (Fig. 1). A large water cluster including an Mg^{2+} ion linked to the H-pathway accepts protons from the water channel and supplies protons to the

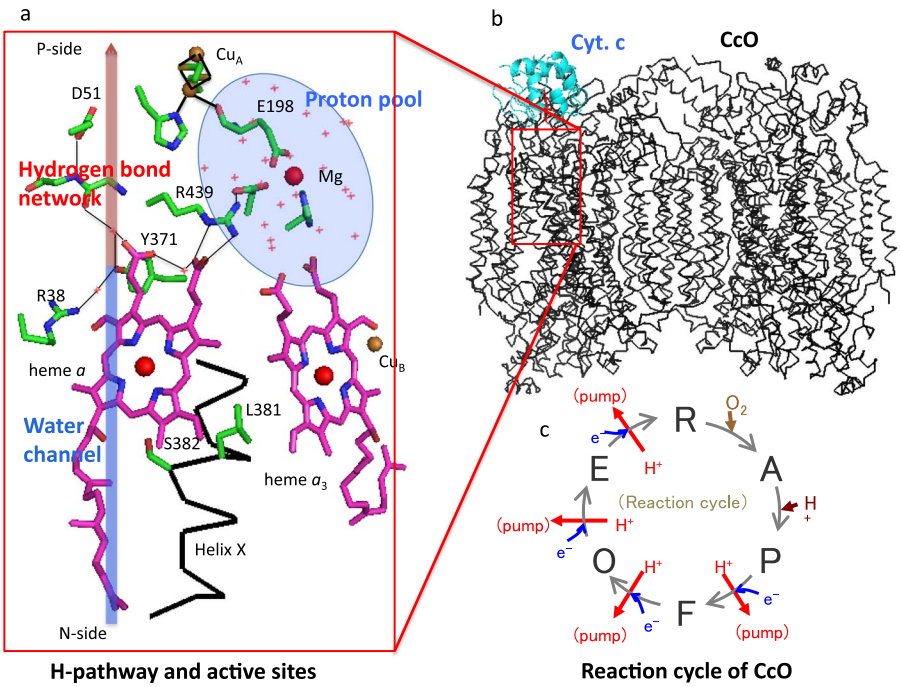

Fig. 1. Structure and function of CcO. (a) The H-pathway, comprising a water channel (blue line) and a hydrogen-bond network (red arrow), is the pathway for proton pumping. An electron from Cyt.c is accepted at Cu_A and transferred to heme a_3 via heme a. Protons stored in the proton pool (highlighted in light blue) are obtained from the N-side of CcO via the water channel. (b) Structure of the Cyt.c-CcO complex. CcO is shown as a black Cα trace, Cyt.c as a light blue ribbon drawing and heme as a red stick model. (c) The reaction cycle of CcO comprises six steps. In the R-state the proton pool obtains four protons via the water channel. A proton is then transferred from the proton pool to the P-side of CcO via the hydrogen-bond network upon electron transfer at each step of the reaction step from the P- to F-, F- to O-, O- to E- and E- to R-states.

hydrogen-bond network; thus, the water cluster functions as a pool of protons. In total, four protons are accumulated in the proton pool, via the water channel of the H-pathway, from the N-side surface of CcO in the R-state. When an O_2 molecule is transferred to Cu_B in the O_2 reduction centre in the R-state, the vinyl group of heme a_3 shifts by 0.3 Å. This small translational shift of the vinyl group effects a conformational change of the residues around Ser 382 in the helix X of subunit I to close the gate of the water channel of the H-pathway. When the O_2 molecule moves from Cu_B to the Fe_{a3} site to generate the A-state of the enzyme, closure of gate is completed. The A-state is then converted to the P-state by accepting a proton. As soon as the enzyme is converted to the P-state, electron transfer from Cu_A to Fe_{a3} via Fe_a takes place to affect proton transfer from the proton pool to the P-side surface via the hydrogen-bond network to generate the F-state. The same coupling of electron transfer and proton transfer observed during conversion from the P-state to the F-state is repeated during conversion from the F- to O-, O- to E- and E- to R-states. Electron migration from Cu_A to heme a_3 via heme a changes the electronic states of the Cu ion of Cu_A and Fe ion of heme a. Structural changes of Asp51, the water cluster, Arg438 of subunit I and Glu 198 of subunit II coordinating to Mg^{2+}, affected by the redox states of Cu and Fe_a ions, promote proton transfer from the proton pool to the P-side of the enzyme. We are working on crystallographic studies to elucidate precise alterations of structures affected by the electron migration.

Kyoko Shinzawa-Itoh, Satoru Shimada and others of University of Hyogo obtained rectangular plate crystals of the Cyt.c–CcO complex diffracting x-ray at 2.0 Å resolution in 2015. We determined the structure of the mammalian Cyt.c–CcO complex and identified the electron transfer pathway from Cyt.c to Cu_A of CcO (***20***) PDB ID: 5IY5). The specific interaction between Cyt.c and CcO is stabilized by only a few electrostatic interactions between side chains located within a small contact surface area. Between the two proteins are three layers of water molecules with a long inter-molecular span; the middle water layer lies between the other two layers without a significant direct interaction with either protein. These features of the docking interface represent the first known example of a new class of protein–protein interaction, which we have termed as ‘soft and specific’ interaction. This interaction is likely to contribute to the rapid association and dissociation of the Cyt.c–CcO complex, which is needed for efficient reduction of CcO.

The mammalian CcO regulates its exact functions by accurate structural changes. Cyt.c has positively charged region on the molecular surface. The ‘soft and specific’ interaction between the basic residues of Cyt.c and the acidic residues of CcO promotes efficient reduction of CcO. I am grateful to the permanent employment system in 1970th, which enabled me to conduct the long-term research on CcO and Cyt.c.

Funding

This work was a part of PDB50 activity supported by grants from the Database Integration Coordination Program from the National Bioscience Database Center (NBDC)-JST (Japan Science and Technology Agency).

Conflict of Interest

None declared.

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