Crystallographic studies of cytochrome c and cytochrome c oxidase

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

Tomitake Tsukihara*

1Graduate School of Life Science, University of Hyogo, 3–2–1 Koto, Kamigori-cho, Akoh-gun, Hyogo 678–1297, Japan
2Institute for Protein Research, Osaka University, 3–2 Yamadaoka, Suita, Osaka 565–0871, Japan

*Tomitake Tsukihara, Graduate School of Life Science, University of Hyogo, 3–2–1 Koto, Kamigori-cho, Akoh-gun, Hyogo 678–1297, Japan. Tel.: 072-621-0017, Fax: 072-621-0017, email: tsuki@protein.osaka-u.ac.jp

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 O₂ 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 (K₃UO₂F₅, K₂PtCl₄ and K₂HgI₄) 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 (CH₃)₂SnCl₂ and K₂IrCl₆ 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 O₂ 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 diffractions 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 Cu₄, heme α, heme α₃ and Cu₈ 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 subangstrom level (11–19).

The enzyme catalyses the reduction of O₂ 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 a Mg²⁺ ion linked to the H-pathway accepts protons from the water channel and supplies protons to the
Fig. 1. Structure and function ofCcO. (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 CuA 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 $C$ 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.

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).
REFERENCES

1. Takano, T., Sugihara, A., Ando, O., Ashida, T., Kakudo, M., and Okunuki, K. (1968) Two kinds of cytochrome c from heart muscle of bonito possibly due to the age. J. Biochem. 63, 808–810

2. Ashida, T., Ueki, T., Tsukihara, T., Sugihara, A., Takano, T., and Kakudo, M. (1971) The crystal structure of bonito katsuo ferrocytochrome c at 4.0 Å resolution. J. Biochem. 70, 913–924

3. Ashida, T., Tanaka, N., Yamane, T., Tsukihara, T., and Kakudo, M. (1973) The crystal structure of bonito (katsu) ferrocytochrome c at 2.3 Å resolution. J. Biochem. 73, 463–465

4. Tanaka, N., Yamane, T., Tsukihara, T., Ashida, T., and Kakudo, M. (1977) The crystal structure of ferrocytochrome c I. General features of the horse and bonito proteins at 2.8 Å resolution. J. Biochem. 73, 147–162

5. Dickerson, R.E., Takano, T., Eisenberg, D., Kallil, O.B., Samson, L., Cooper, A., and Margoliash, E. (1971) Ferrocytochrome c. Science 193, 1136–1144

6. Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E. (1978) Definition of cytochromes c binding domain by chemical modification. III. Kinetics of reaction of carboxyldinitrophenyl cytochrome c with cytochrome c oxidase. J. Biol. Chem. 253, 149–159

7. Tsukihara, T., Yamane, T., Tanaka, N., Ashida, T., and Kakudo, M. (1973) Oxidation of ferrocytochrome c in crystalline state—structural change and anion binding. J. Biochem. 73, 1163–1167

8. Yoshikawa, H., Shimada, A., Hatano, K., Tadehara, H., Yano, N., Shinzawa-Itoh, K., Muramoto, K., and Yoshikawa, S. (1995) Structure of metal sites of oxidized bovine heart mitochondrial membrane: composition and x-ray diffraction studies. Proc. Natl. Acad. Sci. USA 88, 1354–1358

9. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) Structure of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 Å resolution. Science 269, 1069–1074

10. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å. Science 272, 1136–1144

11. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, J., Libeu, C.P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) Redox-coupled crystal structural change in bovine heart cytochrome c oxidase. Science 280, 1723–1729

12. Tsukihara, T., Shimokata, K., Katayama, Y., Shinada, H., Muramoto, K., Aoyama, H., Mochizuki, M., Shinzawa-Itoh, K., Yamashita, E., Yao, M., Ishimura, Y., and Yoshikawa, S. (2003) The low-spin heme of cytochrome c oxidase as the driving element of the proton-pumping process. Proc. Natl. Acad. Sci. USA 100, 15303–15309

13. Shinzawa-Itoh, K., Aoyama, H., Muramoto, K., Terada, H., Kurauchi, T., Tadahara, Y., Yamasaki, A., Sugimura, T., Kurono, S., Tsujimoto, K., Mizushima, T., Yamashita, E., Tsukihara, T., and Yoshikawa, S. (2007) Structures and physiological roles of all the integral lipids of bovine heart cytochrome c oxidase. EMBO J. 26, 1713–1725

14. Aoyama, H., Muramoto, K., Shinzawa-Itoh, K., Hirata, K., Yamashita, E., Tsukihara, T., Ogura, T., and Yoshikawa, S. (2009) A peroxide bridge between Fe and Cu ions in the O2 reduction site of fully oxidized cytochrome c oxidase could suppress the proton pump. Proc. Natl. Acad. Sci. USA 106, 2165–2169

15. Hirata, K., Shinzawa-Itoh, K., Yano, N., Takemura, S., Kato, K., Hatanaka, M., Muramoto, K., Kawahara, T., Tsukihara, T., Yamashita, E., Tono, K., Ueno, G., Hikima, T., Murakami, H., Inubushi, Y., Yabashi, M., Ishikawa, T., Yamamoto, M., Ogura, T., Sugimoto, H., Shen, J.R., Yoshikawa, S., and Ayo, H. (2014) Determination of damage-free crystal structure of an X-ray-sensitive protein using an XFEL. Nat. Methods 11, 734–736

16. Yano, N., Muramoto, K., Shimada, A., Takemura, S., Baba, J., Fujisawa, H., Mochizuki, M., Shinzawa-Itoh, K., Yamashita, E., Tsukihara, T., and Yoshikawa, S. (2016) Cytochrome c oxidase collects four pumping proton equivalents in each catalytic cycle. J. Biol. Chem. 291, 23882–23894

17. Shimada, A., Hatanou, K., Tadahara, H., Yano, N., Shinzawa-Itoh, K., Yamashita, E., Muramoto, K., Tsukihara, T., and Yoshikawa, S. (2018) X-ray structural analyses of azidobound cytochrome c oxidases reveal that the H-pathway is critically important for the proton-pumping activity. J. Biol. Chem. 293, 14868–14879

18. Shimada, A., Etoh, Y., Kito-Fujisawa, R., Sasaki, A., Shinzawa-Itoh, K., Hiromoto, T., Yamashita, E., Muramoto, K., Tsukihara, T., and Yoshikawa, S. (2020) X-ray structures of catalytic intermediates of cytochrome c oxidase provide insights into its O2 activation and unidirectional proton-pump mechanisms. J. Biol. Chem. 295, 5818–5833

19. Shimada, A., Kubo, M., Baba, S., Yamashita, K., Hirata, K., Ueno, G., Noma, T., Kimura, T., Shinzawa-Itoh, K., Baba, J., Hatanou, K., Eto, Y., Miyamoto, A., Murakami, H., Kumakura, T., Owada, S., Tono, T., Yabashi, M., Yamaguchi, Y., Yanagisawa, S., Sakaguchi, M., Ogura, T., Komiya, R., Yan, J., Yamashita, E., Yamamoto, M., Ayo, H., Yoshikawa, S., and Tsukihara, T. (2017) A nanosecond time-resolved XFEL analysis of structural changes associated with CO release from cytochrome c oxidase. Proc. Natl. Acad. Sci. USA 114, 1603042

20. Shimada, S., Shinzawa-Itoh, K., Baba, J., Aoe, S., Shimada, A., Yamashita, E., Kang, J., Tanen, M., Yoshikawa, S., and Tsukihara, T. (2017) Complex structure of cytochrome c-cytochrome c oxidase reveals a novel protein-protein interaction mode. EMBO J. 36, 291–300