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# Capturing reaction intermediates of enzymes by time-resolved XFEL crystallography

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Time-resolved (TR) crystallography using X-ray free electron lasers (XFELs) is being established and increasingly applied to proteins for visualizing their structural dynamics as "molecular movies". At SACLA, we have applied this technique to study the dynamics of two respiratory enzymes, bovine cytochrome *c* oxidase (CcO) and fungal NO reductase (P450nor). CcO is the terminal oxidase of cell respiration that catalyzes the reduction of O2 (substrate) to H2O at the heme-cupper site, coupled with proton pumping across the mitochondrial inner membrane. In this study, with the pump-probe scheme, we tracked the structural dynamics of CcO following photo-dissociation of CO (photolabile inhibitor) from the heme, which mimics the reverse process of substrate binding. As a result, we successfully observed gate opening processes in the proton-pump pathway, coupled with CO dissociation in the μs time domain. Next, we turned our focus to observing enzymatic reactions induced by substrate binding. The pump-probe technique with photosensitive caged substrates, which can release substrates by light illumination, may be useful for this purpose. To test this methodology, we performed TR crystallography with a caged substrate, using P450nor as a simple model enzyme. P450nor is an enzyme that catalyzes the reduction of NO (substrate) to N2O at the heme site in the anaerobic respiration in the fungal mitochondrion. To track the NO reduction reaction, we used caged NO that can release NO by UV illumination with a quantum yield of ~1.4. Although the crystal packing affects the reaction rate, we first captured the P450nor-NO complex (initial intermediate) at 20 ms after the caged-NO photolysis. Observation of the subsequent NO reduction reaction of this enzyme is underway.

**Primary author:** KUBO, Minoru (RIKEN SPring-8 Center)

**Presenter:** KUBO, Minoru (RIKEN SPring-8 Center)

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