Protein crystal as a host to study small molecule reaction by time-resolved serial femtosecond crystallography

Capturing snapshots of dynamic changes in proteins and enzymes is essential for revealing their detailed mechanisms of action in real time. Time-resolved serial femtosecond crystallography (TR-SFX) is a powerful method for determining the real-space structures of dynamic changes in ultrashort intervals [1,2]. For example, the light-driven energy-harvesting mechanism of bacteriorhodopsin was revealed by conformational changes in 13 snapshot structures [2]. Most TR-SFX studies have used natural proteins and enzymes, whereas the use of small molecules is rare. Small molecule crystals diffract well but produce spots that are less numerous compared to biomacromolecules. This makes it challenging to determine the structural and conformational changes induced by SFX. Therefore, new methods, such as small-molecule SFX, are being developed; however, observing their dynamic structural changes is difficult [3,4].

In this context, the development of a new method for studying the dynamics of small molecules is highly important. To this end, we used a host-guest system to study metal complex reactions using light activation. A porous protein crystal was used as the host, and the metal center was fixed to the host as a guest [5]. This avoids the inherent difficulty in determining the structural changes in metal-complex reactions. Microcrystals of hen egg white lysozyme (HEWL) were used as a host and soaked in a photosensitive Mn-carbonyl complex to fix them into the porous solvent channels of the crystals (Fig. 1). The absorption and infrared spectra of the washed microcrystals confirmed the immobilization of the Mn-carbonyl on the crystal. The host-guest system was then applied to SFX at SACLA **BL2** (EH3 and 4b) to determine the structure under dark conditions (Fig. 2(a)). The results revealed the binding of the Mn-carbonyl complex at His15 to three carbon monoxide and two water molecules (Fig. 2(b)).

After confirming the structure of the Mn-carbonyl-bound HEWL, the microcrystals were subjected to TR-SFX at BL2 (EH3 and 4b) with 365 nm pump laser irradiation (Fig. 2(a)). The structures after 10 ns, 100 ns, and 1 μs of photoexcitation were determined (Fig. 2(c)). The difference in density features revealed that after 10 ns of light excitation, the axial CO started to release and was replaced by a water molecule (Fig. 2(c)-i). The process continued up to 1 μs. Subsequently, the 2nd CO ligand located at the equatorial position, was released. When the laser intensity was increased to 40 μJ at 1 μs delay time, the release of the 2nd CO ligand was prominent in the difference density map (Fig. 2(c)-iv). However, a further increase in the delay time to 17 ms hampered the precise assignment of the later-stage intermediates, possibly owing to the increased population of multiple intermediates. Therefore, our method is suitable for studying the early stages of the reactions. Although the CO release reaction from the Mn-carbonyl complex was previously investigated by transient spectroscopic

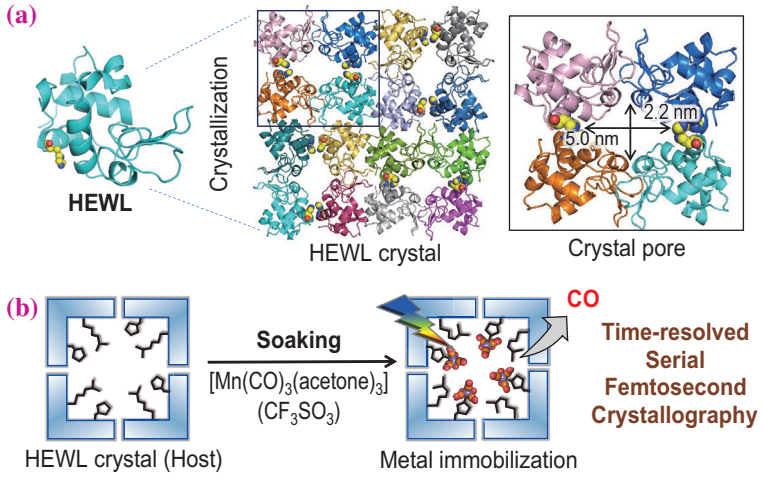

Fig. 1. Porous protein crystal as a host to immobilize small molecules. **(a)** Hen egg white lysozyme (HEWL) crystal and pore structure. **(b)** Schematic representing showing the immobilization of a Mn-carbonyl complex on the HEWL crystal host and light-induced CO release reaction study using the TR-SFX method. [5]

methods, real-time structure determination, particularly observing the release of 2nd CO, was not previously observed.

The experimentally observed intermediate structures were further verified using quantum mechanical/molecular mechanical studies. The observed intermediates followed the lowest-energy paths, in which axial CO was released first, followed the release of equatorial CO. However, the release of 3rd CO or subsequent changes was difficult, as the release of $Mn(CO)(wat)_4$ was found to be more energetically favorable. This explains why we did not observe intermediates in the later stages of the reaction.

Overall, the study demonstrated the potential and scope of applying TR-SFX to small molecule reactions using protein crystals as hosts. Our method avoids the challenges in the structure determination of small molecules. This study opens new possibilities for investigating the chemical reaction mechanisms of small molecules such as metal complexes or organic molecules. In addition, this study will be valuable for the development of artificial metalloenzymes.

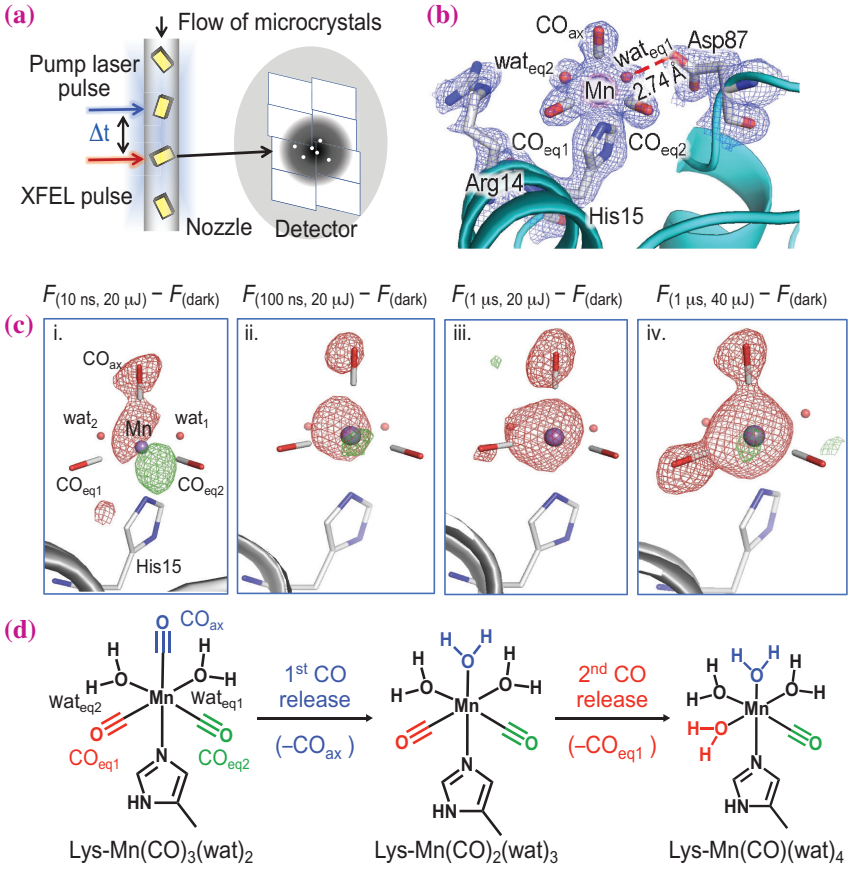

Fig. 2. Porous protein crystal as a host to study small molecule reaction. **(a)** A typical experimental setup to determine time-resolved structures by serial crystallography. **(b)** The dark structure of Mn-carbonyl is bound in the lysozyme crystal. **(c)** Observation of the CO release reaction through time-resolved structures at various delay times after photoexcitation. The difference $|F_o|_{light} - |F_o|_{dark}$ maps at ±3.2 σ (green/red) show the changes in the structures at various delay times after light excitation. **(d)** Schematics of the CO-releasing reaction steps for Mn-carbonyl, based on experimental results. [5]

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