Observation of enzymatic reactions by time-resolved X-ray crystallography using photosensitive caged substrate

Enzymes can catalyze various kinds of chemical reactions with high efficiency and selectivity under mild conditions. The elucidation of the mechanism of the catalytic reactions of enzymes is therefore crucial for understanding how nature designs active site structures for efficient catalytic reactions. The combination of X-ray structural analysis of the static state and time-resolved spectroscopic analysis of dynamic information is an invaluable strategy to reveal the molecular mechanism of enzyme-catalyzed reactions. However, a method for the direct observation of enzyme structures during their catalytic reactions can be a much more powerful tool for completely understanding the mechanism of enzymatic reactions. X-ray free electron lasers (XFELs) have provided a new method for time-resolved (TR) protein X-ray crystallography, which has great potential for observation of enzyme structures along with catalytic reactions. An experimental technique using XFELs, serial femtosecond crystallography (SFX), in which diffraction images are collected from a continuous flow of microcrystals with random orientation, opened a new avenue for TR crystallography [1]. The SFX technique was combined with pump-probe TR crystallography, in which photoirradiation is utilized as a reaction trigger. This TR-SFX technique has already led to major achievements in the analysis of structural dynamics in several photosensitive proteins such as photosystem II [2] and bacteriorhodopsin [3]. Thus, we consider that a system for the initiation of enzymatic reactions by photoirradiation will assist the TR-SFX characterization for enzymatic reactions.

In this project, we utilized a photosensitive caged compound as a reaction trigger in a TR-SFX experiment and applied this system to the structural characterization of the intermediate in a reaction catalyzed by nitric oxide reductase isolated from the fungus *Fusarium oxysporum* (P450nor). P450nor has a heme that is ligated by cysteine thiolate as an active site and catalyzes the reduction of nitric oxide (NO) to nitrous oxide (N_2O) ($2NO+NADH+H^+ \rightarrow N_2O+NAD^++H_2O$). Since N_2O is a powerful greenhouse gas as well as a major ozone-depleting substance, the mechanism of N_2O generation from NO has intrigued researchers. On the basis of spectroscopic studies, the reaction mechanism was proposed as shown in Fig. 1 [4]. The enzyme is in the ferric state with a water molecule as the sixth ligand in the resting state. The binding of the first NO molecule induces the dissociation of the water ligand, producing a ferric NO-bound form as the initial reaction intermediate. Then, the ferric NO-bound species is reduced with hydride (H^-) from NADH to form the second intermediate called intermediate *I* (*I*). Although *I* is in a two-electron reduced state, the protonation state is still under debate. Finally, a second NO molecule electrophilically attacks *I* to generate N_2O. To completely understand the NO reduction mechanism, study of the TR structure is highly desirable.

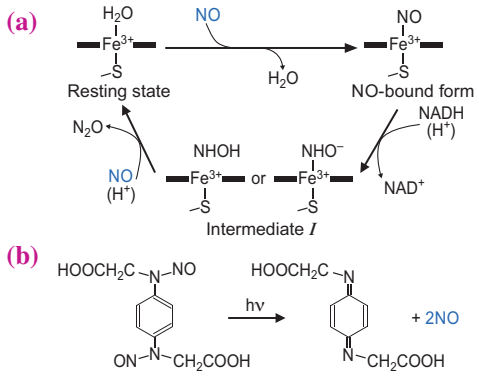

Fig. 1. **(a)** Proposed mechanism of NO reduction reaction catalyzed by P450nor. The protonation state in intermediate *I* is still under debate. **(b)** Chemical structure of caged NO used in this project. In the current TR experiments, photolyzed NO from caged NO initiates the P450nor-catalyzed NO reduction.

For the initiation of the P450nor-catalyzed reaction in TR-SFX experiments, we used caged NO (N,N'-bis(carboxymethyl)-N,N'-dinitroso-p-phenylenediamine sodium salt), which quantitatively releases NO on the microsecond time scale upon UV irradiation (Fig. 1). Prior to the TR-SFX experiments, we tested whether the P450nor reaction system with caged NO works or not by TR spectroscopic analysis. Using P450nor microcrystals soaked into a solution containing NADH and caged NO, we measured the changes in TR visible absorption induced by UV irradiation (Fig. 2) [5]. A positive difference peak at 437 nm was observed 20 ms after UV irradiation, indicating that NO released from caged NO reacts with the resting enzyme to form the ferric NO-bound species. Another positive peak at 450 nm was detected with the concomitant decay of the peak at 437 nm in the second time scale. These spectral changes indicate the formation of *I* upon the reduction of the ferric NO-bound form with NADH. Furthermore, the N-N stretching band of N_2O at 2,228 cm^{-1} was observed after UV irradiation of the sample by microscopic infrared spectroscopy at SPring-8 **BL43IR**, showing that P450nor catalyzes NO reduction to yield N_2O even in the crystalline state. These spectroscopic data demonstrate that P450nor produces N_2O through the formation of a ferric NO-bound form and *I* in the crystalline state, as observed

in the solution sample. However, the rate of *I* formation was two orders of magnitude lower in the crystalline sample than in the solution (Fig. 2). It is plausible that the slow accommodation of NADH into the active site of P450nor in the crystal is due to the packing effect. Thus, kinetic analysis of the crystalline sample by TR spectroscopy is important to determine the measurement points in the TR-SFX experiment.

We carried out the TR-SFX experiment using a lipidic cubic-phase injector with a hydroxyethyl cellulose matrix at SACLA **BL3**. The current system for TR-SFX allows us to carry out measurements for a time window of up to 20 ms. Therefore, we focused on the characterization of the formation of the ferric NO-bound species, the initial reaction intermediate, in the TR-SFX experiment on the basis of the kinetic information. Figure 3 shows the structures of P450nor before caged NO photolysis and 20 ms after caged NO photolysis, both of which were determined at a resolution of 2.1 Å at ambient temperature [5]. In the structure at 20 ms, there is a clear positive electron density at the heme distal coordination sphere in the F_o(20 ms) – F_o(without UV irradiation) difference electron density map, which is assignable to the NO ligand. The NO coordination geometry in the NO-bound structure determined by TR-SFX shows a slightly bent Fe-N-O structure (158°). In the case of the NO-bound structure determined at SPring-8 **BL26B2**, the orientation of NO is more bent (147°) (Fig. 3). Since the reduction of the ferric NO-bound form generally induces the conformational change of the Fe-NO moiety to a bent conformation, the structure determined at SPring-8 could be partially reduced by X-ray irradiation, but the structure determined by TR-SFX is the ferric NO-bound form without any X-ray reduction. Thus, we can conclude that the method using a photosensitive caged compound with pump-probe TR-SFX is a powerful tool for dynamic structural analyses of enzymes during their catalytic reactions. Various caged compounds including caged ATP and photoinduced electron donors are available, allowing us to further elucidate the dynamic aspects of proteins based on time-resolved structural analysis.

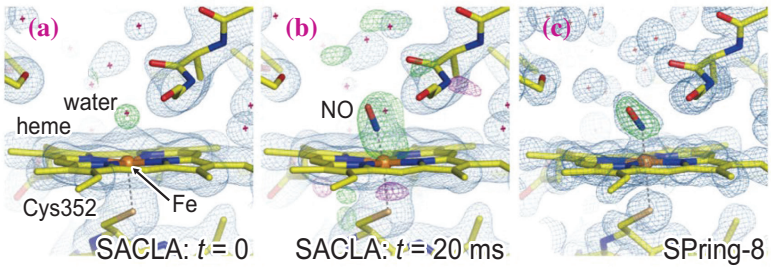

Fig. 3. Active site structure of P450nor determined at SACLA and SPring-8. **(a)** The structure determined by TR-SFX before UV irradiation at ambient temperature. A water molecule coordinates to a heme iron. **(b)** Structure determined by TR-SFX 20 ms after caged NO photolysis at ambient temperature. Green mesh represents the F_o(20 ms) – F_o(without UV irradiation) difference electron density map, which indicates the presence of a NO ligand at the sixth coordination sphere of the heme. **(c)** NO-bound structure determined by synchrotron crystallography at 100 K. The NO-bound form was prepared by soaking NO gas into P450nor crystal in the absence of NADH. Green mesh represents the F_o – F_c electron density map.

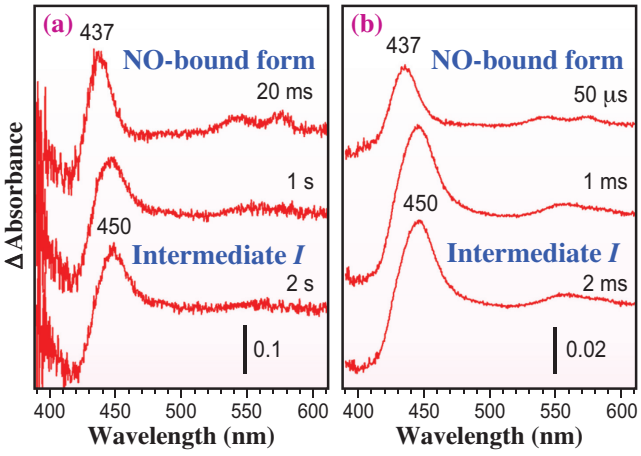

Fig. 2. TR visible absorption difference spectra of P450nor after caged NO photolysis in the crystalline state **(a)** and solution state **(b)**. The difference spectra were obtained by subtracting the spectrum recorded before UV irradiation to induce the caged NO photolysis. The spectra showed that NO released from caged NO upon UV irradiation reacts with ferric P450nor to form a ferric NO-bound state, which is followed by the formation of intermediate *I* in both crystalline and solution samples.

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