Characterization of short-lived reaction intermediate in enzymatic nitrous oxide generation by XFEL crystallography and time-resolved spectroscopy

Nitrous oxide (N_2O) is a powerful greenhouse gas that is 310-times more potent than carbon dioxide, as well as a major ozone-depleting substance. The concentration of atmospheric N_2O has been increasing for more than 100 years, contributing to global warming [1]. The major anthropogenic cause of global N_2O emission is the microbial breakdown of nitrogen-based compounds contained in fertilizers for agriculture. A key biochemical process related to N_2O formation from fertilizers is microbial denitrification, a form of anaerobic respiration, in which nitrate (NO_3^-) is sequentially reduced to dinitrogen gas ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). In this process, nitric oxide reductase (NOR) catalyzes the formation of N_2O from nitric oxide (NO). Therefore, the elucidation of the catalytic mechanism of N_2O formation by NOR will help us resolve this aspect of global warming.

NOR isolated from the fungus *Fusarium oxysporum* (P450nor) is an enzyme involved in fungal denitrification. P450nor has a heme coordinated by a cysteine thiolate as an active site and catalyzes the reduction of NO to N_2O using a proton and electrons from NADH ($2NO + NADH + H^+ \rightarrow N_2O + NAD^+ + H_2O$) (Fig. 1). In addition to the global impact described above, the catalytic mechanism of P450nor has intrigued chemists since its first isolation in 1993 [2], because the P450nor-catalyzed reaction involves N–N bond formation and N–O bond cleavage. In the late 1990s, spectroscopic studies provided clues to the catalytic mechanism [3]. The binding of the first NO molecule to the resting ferric state produces the Fe^{3+}–NO form. Then, the Fe^{3+}–NO species is reduced with hydride (H^-) from NADH to form a short-lived reaction intermediate called intermediate I (*I*). Finally, the second NO molecule reacts with *I* to yield N_2O. Thus, knowing the chemical structure of *I* is crucial for understanding the mechanism of N_2O formation, i.e., N–N bond formation, by P450nor. However, despite extensive efforts over the decades by several approaches, including synthetic model and theoretical studies, the chemical structure of *I* remains to be solved, although four possible models, Fe^{2+}–NHO, Fe^{3+}–$NHO^{\bullet -}$, Fe^{3+}–$NHOH^{\bullet}$ and Fe^{4+}–$NHOH^-$, have been proposed. To establish the catalytic mechanism in P450nor, we need to obtain conclusive experimental information on the structure of *I*.

Most recently, we have developed time-resolved (TR) techniques using caged NO, which quantitatively releases NO, on the microsecond time scale upon UV irradiation to directly observe the catalytic reaction of P450nor and successfully characterized the Fe^{3+}-NO species by TR serial femtosecond crystallography (TR-SFX) at SACLA [4]. In this study, we utilized this technique of using caged NO to determine the coordination and electronic structures of the key reaction intermediate *I* in P450nor. Our work in this study is briefly summarized in Fig. 2. Our previous TR visible absorption measurement for the microcrystals of P450nor soaked in a solution containing NADH and caged NO showed that the formation of *I* in the microcrystal took ~5 s after UV light illumination, though it took only 1 ms in the solution state [4]. From this information, the microcrystals soaked with NADH and caged NO were scooped using a mesh

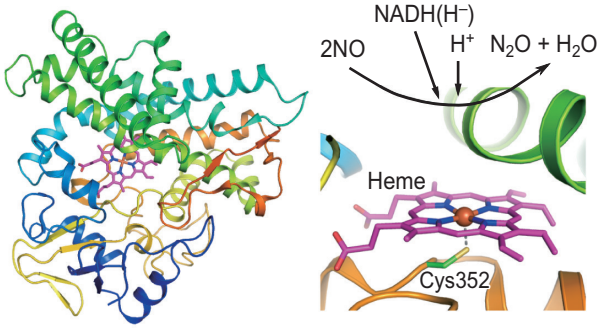

Fig. 1. Structure of P450nor. P450nor catalyzes the reduction of NO using NADH and proton.

loop, and *I* was captured by rapid freezing 5 s after UV illumination. To avoid the radiation-induced redox change at the active site and precisely determine the structure of *I*, the X-ray diffraction data for the state of *I* free of radiation-induced damage were collected by fixed-target SFX at SACLA **BL3**. Compared with the Fe^{3+}–NO state in which the NO ligand adopts a slightly bent configuration (Fe–NO = 1.6 Å, N–O = 1.15 Å, Fe–N–O = 158°) [4], the FeNO unit is more bent in *I* (Fe–NO = 1.91 Å, N–O = 1.27 Å, Fe–N–O = 138°), as shown in Fig. 3. In addition to the crystal structure, the TR-infrared (TR-IR) measurement of the microcrystal sample showed that a NO stretching frequency (νNO) sensitive to the electronic structure was observed at 1298 cm^{-1} in *I* [5], which is distinct from νNO of 1853 cm^{-1} in the Fe^{3+}–NO state. Using such structural and spectroscopic information on *I* in the theoretical calculation, we can conclude that *I* is a singly protonated radical species, Fe^{3+}–$NHO^{\bullet-}$ [5]. Our finding of the radical character of *I* demonstrates that the radical coupling of *I* and the second NO molecule forms the N–N bond, as shown in Fig. 3. Thus, the recent progress of the time-resolved technique and XFEL crystallography has enabled us to answer a long-standing question regarding the mechanism of enzymatic N_2O formation.

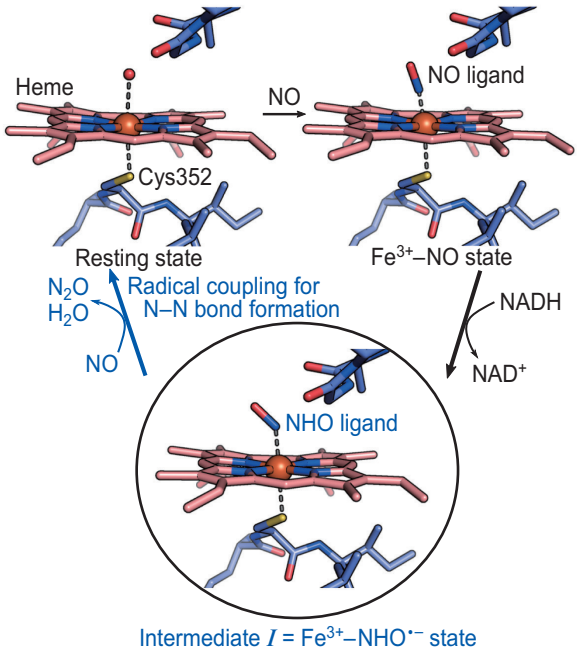

Fig. 3. Reaction mechanism of N_2O formation by P450nor. Current results reveal that *I* is Fe^{3+}–$NHO^{\bullet-}$. The radical character of *I* facilitates the formation of N–N bond via the radical coupling mechanism.

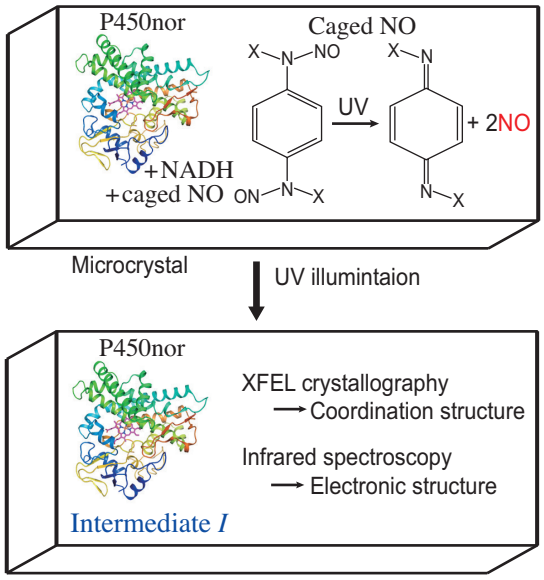

Fig. 2. Schematic representation of the method used in this work.

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