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 Outline of Research Result

# Analysis of Reaction Mechanism of Aldoxime Dehydratase and Its Application to Production of Useful Compounds

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## Research aims

We have extensively studied the biological metabolism of toxic compounds (that have a triple bond between carbon and nitrogen) such as nitriles [R-C≡N] and isonitriles [R-N≡C]. Recently, we discovered a novel hemoprotein that catalyzes the dehydration of aliphatic aldoximes to the corresponding nitriles in *Pseudomonas chlororaphis* B23, which was previously used as a catalyst for acrylamide manufacture, and is now used for the industrial production of 5-cyanovaleramide. We named it aliphatic aldoxime dehydratase (OxdA); it has been approved as a new enzyme by NC-IUBMB: EC 4.99.1.5. (<http://www.chem.qmul.ac.uk/iubmb/enzyme/EC4/99/1/5.html>). The OxdA reaction is very unique and intriguing in the following: (i) dehydration of the substrate in an aqueous solution; (ii) utilization of the favorable hydrophobic environment of the heme pocket on catalysis of dehydration; and (iii) synthesis of a C–N triple bond, which is a highly toxic functional group. The OxdA reaction is not only academically interesting but also expected to be applicable to the practical production of nitriles because it occurs under mild conditions, in contrast with the chemical dehydration of aldoximes under harsh conditions. The reaction mechanism of OxdA has yet to be explored, because reaction intermediates in the catalytic cycle are not understood well. We previously reported that ferrous OxdA, containing a 5-coordinated high spin heme and His299 as its proximal ligand, is the reactive form of the enzyme, and that His320 in the distal heme pocket would play a crucial role in donating a proton to a substrate during aldoxime dehydration. We recently detected an initial reaction intermediate (named ‘OS-I’), the heme coordination structure of which was suggestive of Fe(II)–

N(OH)=CH–R, butyraldoxime being used as the substrate. However, information on the reaction intermediate of OxdA has been quite limited and, thus, the reaction mechanism of OxdA is not yet understood well.

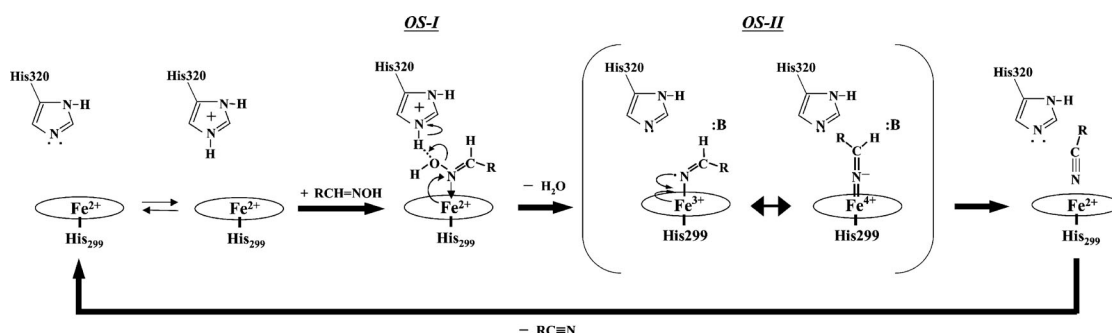
## Methods

Ferrous OxdA, which is an active form of OxdA, was prepared by adding sodium dithionite to purified OxdA under anaerobic conditions. The reaction product (butyronitrile) was determined with a gas chromatograph (GC-14BPF; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a glass column packed with Gaskuropack 56 (80/100% mesh; GL-Science, Tokyo, Japan).

The absorption spectrum was recorded with a Shimadzu UV-1700 PharmaSpec spectrophotometer (Kyoto, Japan). Resonance Raman spectra were obtained with excitation at 413.1 nm with a Kr<sup>+</sup> ion laser (Spectra Physics, model 2060).

## Results

We found that the addition of a large amount of butyraldoxime to a solution of ferrous OxdA with a low enzyme concentration yields a novel long-lived OxdA-substrate complex (named ‘OS-II’), whose UV-vis spectrum exhibits a Soret peak at 415.5 nm. A gradual decrease in the Soret peak of OS-II and a concomitant increase in the Soret peak of ferrous OxdA occurred 10 min after the addition of butyraldoxime, the spectrum of ferrous OxdA finally being observed. Although the reaction rate on synthesis of butyronitrile was reduced, the stoichiometric conversion of bu-



tyraldoxime to butyronitrile was confirmed by gas chromatography. In order to obtain a more detailed insight into OS-II, we have carried out resonance Raman (RR) spectroscopic studies on OS-II. Intriguingly, the frequency of the  $\nu_4$  band at  $1379\text{ cm}^{-1}$  of OS-II was even higher than that of ferric OxdA at  $1376\text{ cm}^{-1}$ . OS-II would be considered to contain a highly oxidized heme compared with that involved in ferric OxdA. We used [ $^{15}\text{N}$ ]butyraldoxime ( $\text{C}_3\text{H}_7\text{CH}=\text{}^{15}\text{NOH}$ ), and observed the butyraldoxime-isotope dependence of the RR spectra of OS-II. Intriguingly, the  $857\text{ cm}^{-1}$  vibrational frequency is much higher than the Fe–N single bond stretching frequency ( $500\text{--}600\text{ cm}^{-1}$ ) observed for the NO adducts of hemoproteins. These findings suggest that the bond order between the heme iron and the nitrogen atom of the substrate part in OS-II is more than one; an Fe=N double bond may be formed. We propose a possible mechanism for the creation of a carbon-nitrogen triple bond from aldoxime by OxdA.

### Conclusion

Herein, we demonstrated for the first time that aldoxime dehydratase, a unique heme-containing enzyme, forms a

novel reaction intermediate with a highly oxidized heme by directly binding a substrate with its heme iron.

### References

- 1) Oinuma, K-I., Hashimoto, Y., Konishi, K., Goda, M., Noguchi, T., Higashibata, H. & Kobayashi, M. "Novel aldoxime dehydratase involved in carbon-nitrogen triple bond synthesis of *Pseudomonas chlororaphis* B23: Sequencing, gene expression, purification and characterization" *J. Biol. Chem.*, **278**, 29600–29608 (2003)
- 2) Oinuma, K-I., Ohta, T., Konishi, K., Hashimoto, Y., Higashibata, H., Kitagawa, T., & Kobayashi, M. "Heme environment in aldoxime dehydratase involved in carbon-nitrogen triple bond synthesis" *FEBS Lett.*, **568**, 44–48 (2004)
- 3) Konishi, K., Ishida, K., Oinuma, K-I., Ohta, T., Hashimoto, Y., Higashibata, H., Kitagawa, T., & Kobayashi, M. "Identification of crucial histidines involved in carbon-nitrogen triple bond synthesis by aldoxime dehydratase" *J. Biol. Chem.*, **279**, 47619–47625 (2004)
- 4) Oinuma, K-I., Kumita, H., Ohta, T., Konishi, K., Hashimoto, Y., Higashibata, H., Kitagawa, T., Shiro, Y. & Kobayashi, M. "Stopped-flow spectrophotometric and resonance Raman analyses of aldoxime dehydratase involved in carbon-nitrogen triple bond synthesis" *FEBS Lett.*, **579**, 1394–1398 (2005)
- 5) Konishi, K., Ohta, T., Oinuma, K-I., Hashimoto, Y., Kitagawa, T. & Kobayashi, M. "Discovery of a reaction intermediate of aliphatic aldoxime dehydratase involving heme as an active center" *Proc. Natl. Acad. Sci. USA*, **103**, 564–568 (2006)