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Stopped-flow spectrophotometric and resonance Raman analyses of aldoxime dehydratase involved in carbon–nitrogen triple bond synthesis

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Abstract On stopped-flow analysis of aliphatic aldoxime dehydratase (OxdA), a novel hemoprotein, a spectrum derived from a reaction intermediate was detected on mixing ferrous OxdA with butyraldoxime; it gradually changed into that of ferrous OxdA with an isosbestic point at 421 nm. The spectral change on the addition of butyraldoxime to the ferrous H320A mutant showed the formation of a substrate-coordinated mutant, the absorption spectrum of which closely resembled that of the above intermediate. These observations and the resonance Raman investigation revealed that the substrate actually binds to the heme in OxdA, forming a hexa-coordinate low-spin heme.

Keywords: Heme; Reaction intermediate; Stopped-flow spectrophotometry; Resonance Raman spectroscopy; Nitrile; Dehydration

1. Introduction

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] [1–4] and isonitriles [R–N≡C] [5–7]. The microbial degradation of nitriles proceeds through two different enzymatic pathways [8,9]: (i) nitrilase catalyzes the hydrolysis of nitriles into acids [R–C(=O)–OH] and ammonia [10–12] and (ii) nitrile hydratase (NHase) catalyzes the hydration of nitriles to amides [R–C(=O)–NH_2] [13–16], which are subsequently hydrolyzed to acids and ammonia by amidase [17–19]. These enzymes have received much attention in applied fields [8,20] as well as academic ones [12,21]. One of the fruits of our application-oriented nitrile studies is the current industrial production of acrylamide and nicotinamide using the NHase of *Rhodococcus rhodochrous* J1 [1,9]. On the other hand, the NHase of *Pseudomonas chlororaphis* B23 [22], which was previously used as a catalyst for acrylamide manufacture [8,20,23], is now used for the production of 5-cyanovaleramide, a herbicide intermediate, at the industrial level [24].

Very recently, we discovered a gene (*oxdA*) [25] encoding an aldoxime dehydratase upstream of the NHase and amidase genes in *P. chlororaphis* B23 [26]. OxdA catalyzes the dehydration of aliphatic aldoximes [R–CH=N–OH] to the corresponding nitriles [R–C≡N], and thus we named it aliphatic aldoxime dehydratase (OxdA); it has been approved as a new enzyme by NC-IUBMB: EC 4.99.1.5. [27]. The OxdA reaction is surprising, particularly in the formation of the carbon–nitrogen triple bond and the efficient dehydration of a substrate in the presence of water [25–29]. The enzymatic conversion of an aldoxime to a nitrile is not only academically interesting but also expected to be applicable to the practical production of nitriles because it occurs under mild conditions [30].

OxdA contains protoheme IX as the prosthetic group [25]. The enzymatic reaction proceeds when the heme iron is in the ferrous state, but not in the ferric state. Resonance Raman (RR) investigation revealed that proximal ligand of the heme was a histidine residue and that positively charged or proton-donating residues were present in the distal heme pocket [31]. The combination of site-directed mutagenesis and spectroscopic measurements for OxdA demonstrated that His299 is the proximal ligand and that His320 is in the distal pocket, playing a crucial role in the reaction [32]. We initially found that the addition of butyraldoxime to ferrous OxdA caused a drastic change in the absorption spectrum of OxdA [25]. Thus, the heme of OxdA has been assumed to interact with a substrate in the reaction. However, no structural information on reaction intermediates has been obtained. Thus, we examined a reaction intermediate of OxdA in detail, using stopped-flow rapid scan and RR techniques, to shed new light on the OxdA reaction mechanism.

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Abbreviations: OxdA, aliphatic aldoxime dehydratase; RR, resonance Raman; 6c-ls, hexa-coordinate low-spin; NHase, nitrile hydratase; H320A, H320A mutant OxdA; OS-I, OxdA–substrate complex I

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2. Materials and methods

2.1. Preparation of recombinant OxdA and the H320A mutant

Recombinant OxdA and the H320A mutant (we will use the term "H320A" to refer to the H320A mutant OxdA in this paper) were overexpressed and purified according to the procedure described previously [31,32].

2.2. Spectral measurements

For all spectral measurements, sodium dithionite was used at the concentration of 10 mM in 100 mM potassium phosphate buffer (pH 7.0).

The stopped-flow rapid scan measurements were carried out using a RSP-601 stopped-flow spectrophotometer (Unisoku, Osaka). About 10 μM (subunit conc.) OxdA and various concentrations of butyraldoxime (Tokyo Kasei Kogyo, Tokyo) were put into separate reservoirs. Both solutions were thoroughly treated with bubbling N_2 gas and then sodium dithionite was put into the reservoirs. For measurements, equal volumes of the solutions were mixed and introduced into the optical cell, and absorption measurements were started. The dead time was 5 ms, which is the time for the sample solution to flow to the optical cell. The temperature of each sample was controlled with a thermostat (RM6, Lauda).

UV–Vis absorption spectra of ferrous H320A and the H320A–substrate complex were recorded with a Shimadzu UV-1700 PharmaSpec spectrophotometer. Ferrous H320A was prepared by adding sodium dithionite to purified H320A under anaerobic conditions. The H320A–substrate complex was prepared by adding butyraldoxime (final 5 mM) to ferrous H320A.

RR spectra were acquired as described previously [31], using 413.1 nm excitation. The laser power was 3 mW at the cell. Ferrous H320A and the H320A–substrate complex were prepared as described above.

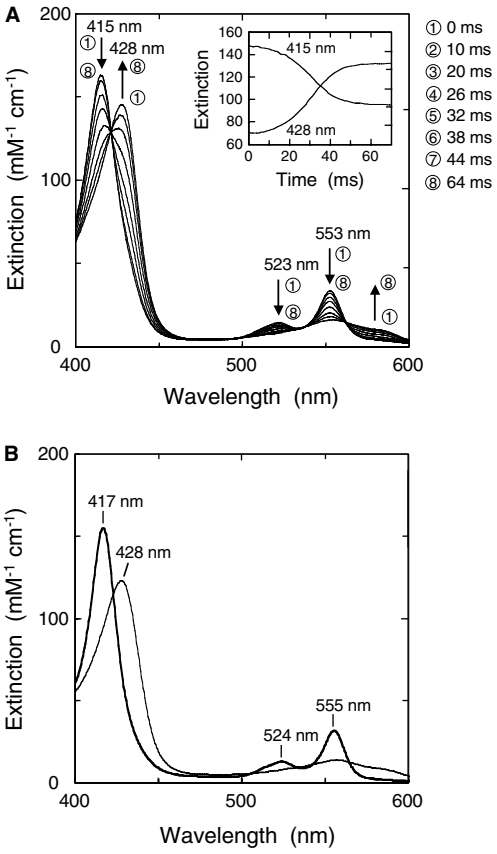

Fig. 1. (A) Absorption spectral change of OxdA at 0–70 ms (each value indicated in the figure is the time after the dead time of 5 ms) after mixing ferrous OxdA with butyraldoxime (final 500 μM) in the stopped-flow experiment at 25 °C. The spectra measured at 0, 10, 20, 26, 32, 38, 44, and 64 ms after the mixing are shown. The gate time, which refers to the amount of time that the gate of the detector in the stopped-flow apparatus is open for one scan, is 2 ms. Inset shows the time course of the absorbance change at 415 and 428 nm. (B) Absorption spectra of ferrous H320A before (thin line) and after (thick line) the addition of butyraldoxime measured under a static condition at room temperature. Extinction coefficients in both (A) and (B) are for the enzyme subunit.

3. Results and discussion

3.1. Stopped-flow rapid scan analysis of the OxdA reaction

We first examined the reaction of OxdA with butyraldoxime on the millisecond scale using the stopped-flow rapid scan technique. Immediately after mixing ferrous OxdA with butyraldoxime, we observed a spectrum derived from a new species (trace 1 of Fig. 1A). The spectrum showed an extremely sharp and intense Soret peak at 415 nm, which was blue-shifted from that of ferrous OxdA (428 nm), and well-resolved α and β peaks at 553 and 523 nm, respectively. The spectrum of this new species changed completely into that of ferrous OxdA at 64 ms (trace 8 of Fig. 1A) with the isosbestic point being at 421 nm, demonstrating that the new spectrum was derived from a reaction intermediate. The spectrum of the intermediate is slightly different from that previously observed by adding butyraldoxime into ferrous OxdA [25], probably because of difference in the purification procedures of the enzyme; e.g., the use of 2-mercaptoethanol, the resultant damage of the purified enzyme, and/or an extremely low content of the heme (0.69 mol of heme/mol of subunit) in the previous purification. Although we studied the reaction of OxdA with butyraldoxime at a much lower substrate concentration (~50 μM) and/or 5 °C, we could not detect any additional species prior to the intermediate nor the formation process of the intermediate, suggesting that the formation of the intermediate was very fast compared with the time scale of the stopped-flow measurement.

3.2. Kinetic analysis of the OxdA reaction

To kinetically examine the decay of the reaction intermediate, the time course of the absorption change at 415 nm was investigated with various amounts of butyraldoxime. When ferrous OxdA was mixed with a final 5 μM (almost equal to the concentration of the enzyme subunit) butyraldoxime, the decay of the absorption at 415 nm showed a single exponential feature (Fig. 2A). From the time course of the absorbance change, we estimated the rate constant for the decay of the reaction intermediate at 360 s^{-1}, this being consistent with the rate constant for the reaction turnover under a steady-state condition (313 s^{-1}), which was deduced from the specific activity (468 units/mg) previously reported [32].

On the other hand, when OxdA was mixed with an excess amount (~50 mM) of butyraldoxime, the decay of the absorption at 415 nm showed a sigmoidal feature (Fig. 2B); absorbance at 415 nm was maintained for a longer time with a higher concentration of aldoxime. This phenomenon suggests that the reaction intermediate is accumulated in the reaction mixture under the steady-state condition and that the rate-limiting step in the OxdA reaction is a conversion of the intermediate to the next species.