Regulation of Aldoxime Dehydratase Activity by Redox-dependent Change in the Coordination Structure of the Aldoxime-Heme Complex*

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Katsuaki Kobayashi‡, Shiro Yoshioka‡, Yasuo Kato§, Yasuhisa Asano§¶, and Shigetoshi Aono¶¶

From the ‡Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, 5-1 Higashiya, Myodaiji, Okazaki 444-8787, Japan and the ¶Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Kosugi, Toyama 939-0398, Japan

Phenylacetaldoxime dehydratase from Bacillus sp. strain OxB-1 (OxdB) catalyzes the dehydration of Z-phenylacetaldoxime (PAOx) to produce phenylacetonitrile. OxdB contains a protoporphyrin that works as the active center of the dehydration reaction. The enzymatic activity of ferrous OxdB was 1150-fold higher than that of ferric OxdB, indicating that the ferrous heme was the active state in OxdB catalysis. Although ferric OxdB was inactive, the substrate was bound to the ferric heme iron. Electron paramagnetic resonance spectroscopy revealed that the oxygen atom of PAOx was bound to the ferric heme, whereas PAOx was bound to the ferrous heme in OxdB via the nitrogen atom of PAOx. These results show a novel mechanism by which the activity of a heme enzyme is regulated; that is, the oxidation state of the heme controls the coordination structure of a substrate-heme complex, which regulates enzymatic activity. Rapid scanning spectroscopy using stopped-flow apparatus revealed that a reaction intermediate (the PAOx-ferrous OxdB complex) showed Soret, a, and g bands at 415, 555, and 524 nm, respectively. The formation of this intermediate complex was very fast, finishing within the dead time of the stopped-flow mixer (3 ms). Site-directed mutagenesis revealed that His-306 was the catalytic residue responsible for assisting the elimination of the hydrogen atom of PAOx. The pH dependence of OxdB activity suggested that another amino acid residue that assists the elimination of the PAOx hydrogen atom. His-306 in OxdB is identified as the catalytic residue responsible for assisting the elimination of the PAOx hydrogen atom.

Aldoxime dehydration is one of the most useful methods for nitrile synthesis. As the chemical dehydration of aldoximes requires harsh conditions, a biological process for aldoxime dehydration is required to establish an environmentally benign process. Recently, Asano and Kato discovered a novel heme-containing lyase aldoxime dehydratase (EC 4.2.1.29) from various aldoxime- or nitrile-degrading microorganisms through their studies on microbial aldoxime metabolism (1–4). Aldoxime dehydratase could be applicable for the enzymatic synthesis of nitriles from aldoximes (1–7).

Phenylacetaldoxime dehydratase from Bacillus sp. Oxd-1 (OxdB) catalyzes the dehydration reaction of Z-phenylacetaldoxime (PAOx) to produce phenylacetonitrile (1 eq) under mild conditions (2, 4). OxdB exists in a monomer of a 40-kDa polypeptide containing a protoheme (2). The heme in OxdB is thought to be the active site for the dehydration reaction. OxdB is the first example of a heme protein catalyzing the dehydration reaction physiologically, although many functions of hemeproteins have been elucidated, including oxygen storage/transport, electron transfer, gas molecule sensor, and redox catalysis of various substrates.

For hemeproteins, a heme can exhibit its physiological function via three functional patterns. (i) An exogenous ligand binds to and dissociates from a heme reversibly. Oxygen storage/transport proteins (myoglobins and hemoglobin (8)) and heme-based sensor proteins (FixL (9–12), HemAT (9, 11–13), CooA (9–11, 14, 15), etc.) belong to this category. (ii) A heme acts as an electron transfer agent. Typical examples are various b-type and c-type cytochromes (16, 17). (iii) A substrate must bind directly to heme iron for the enzymatic reaction to proceed. Heme iron has an oxidation state of 2+ or 3+ before the reaction starts. One of these two states is the resting form to which a substrate cannot bind, and the other is active and can bind a substrate. For example, P450-type monooxygenase (18–20) and cytochrome c oxidase (21, 22) bind their substrate, molecular oxygen, to Fe(II) heme. On the other hand, hydrogen peroxide, the substrate of peroxidase (23, 24) and catalase (25–27), is bound only to Fe(III) heme. Thus, the oxidation state of iron heme is an important factor for whether a substrate can bind to the heme or not.

In this work, we report that both Fe(II) and Fe(III) hemes in OxdB bind the substrate, PAOx, using a different coordination mode. As described in this paper, ferrous OxdB is active, but ferric OxdB is not. This demonstrates a novel mechanism by which the activity of heme enzymes is regulated, that is, the heme oxidation state controls in the coordination structure of the substrate heme complex. Rapid scanning spectroscopy using stopped-flow apparatus reveals a reaction intermediate in which the nitrogen atom of PAOx is bound to the ferrous heme. His-306 in OxdB is identified as the catalytic residue responsible for assisting the elimination of the PAOx hydrogen atom. Based on the experimental results obtained in this study, we

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¶ To whom correspondence may be addressed. Tel.: 81-564-59-5575; Fax: 81-564-59-5576; E-mail: aono@ims.ac.jp.

¶¶ To whom correspondence may be addressed. Tel.: 81-766-56-7500 (ext. 530); Fax: 81-766-56-2498; E-mail: asano@pu-toyama.ac.jp.

* The abbreviations used are: OxdB, phenylacetaldoxime dehydratase from Bacillus sp. Oxd-1; PAOx, Z-phenylacetaldoxime; EPR, electron paramagnetic resonance; DMF, dimethylformamide; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
propose the reaction mechanism of OxdB catalysis.

**EXPERIMENTAL PROCEDURES**

**Expression of OxdB in Escherichia coli—**OxdB was expressed in *E. coli* JM109 under the control of the lac promoter in pUC18. The cultivation of *E. coli* cells was carried out as reported previously (2, 4). The harvested cells were stored at −80 °C until use. The recombinant OxdB with a His6-tag at the N terminus was prepared and used in this work. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene).

**Purification of OxdB—**All purification procedures were performed at 4 °C. Purification of the recombinant OxdB was carried out as follows. The *E. coli* cells were resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl. The cells were disrupted by sonication and centrifuged to remove the undisrupted cells and cell debris. Cell-free extract was loaded on a Co2+-loaded metal-ion chelating column (Talon®). After washing the column with 50 mM potassium phosphate buffer (pH 7.0) containing 300 mM NaCl, OxdB was eluted from the column with 50 mM potassium phosphate buffer containing 50 mM imidazole (pH 7.0). The fractions containing OxdB were combined and subsequently purified using a Superdex 200 column. The gel filtration column was operated with 50 mM potassium phosphate buffer (pH 7.0).

**Spectral Measurements—**The electronic absorption spectra were measured on an Agilent 8453 UV-visible spectrometer. EPR spectra at low temperatures were measured with a Bruker X-band spectrometer (Bruker ESP300E) attached to a cryostat (Oxford ESR900) and a temperature controller (Oxford Model ITC4). The g values were calculated from the magnetic field and the microwave frequency, which were measured using a NMR tesla meter (Bruker ER035M) and a microwave frequency counter (Hewlett Packard 5352B), respectively. EPR measurements were carried out under non-saturating microwave power conditions.

**RESULTS**

**Electronic Absorption Spectra—**The electronic absorption spectra of OxdB are shown in Fig. 1. Ferric OxdB had a Soret peak at 407 nm and small absorption bands at 503, 539, and 630 nm in 50 mM potassium phosphate buffer (pH 7.0) (Fig. 1). This spectrum is similar to that of H2O-bound Fe(III) myoglobin (8). The reduction of OxdB with sodium dithionite resulted in a shift of the Soret peak from 407 to 432 nm. Ferrous OxdB showed Q-bands at 508 nm with a small shoulder band at 588 nm (Fig. 1). Although the Soret peak at 432 nm was typical for five-coordinate Fe(II) hemeproteins, a shoulder band at 588 nm was not reduced by sodium dithionite. A cell-free extract showed the Soret peak at 407 nm, suggesting that an accidental, but reproducible, conformational change occurred in OxdB during purification using an ion-exchange column in the presence of 2-mercaptoethanol. These results indicate that a high spin form showing the Soret peak at 407 nm in the ferric state is the native form of OxdB.

**Rapid Scanning Spectra—**A solution of 4.7 or 0.9 mM ferrous OxdB, containing ~2 mM sodium dithionite, was mixed with 10 mM PAOx dissolved in 50 mM potassium phosphate buffer containing 10% DMF at 10.0 K. The reduction of OxdB with sodium dithionite resulted in a shift of the Soret peak from 407 to 432 nm. The fractions containing OxdB were combined and subsequently purified using a Superdex 200 column. The gel filtration column was operated with 50 mM potassium phosphate buffer (pH 7.0).

**EPR Spectra of Ferric OxdB—**EPR spectra of ferric OxdB in 50 mM potassium phosphate buffer (pH 7.0) at 10.0 K. A, EPR spectrum of ferric OxdB in the presence of 10% DMF at 10.0 K. B, EPR spectrum of ferric OxdB in 50 mM potassium phosphate buffer containing 10% DMF at 10.0 K. C, EPR spectrum of ferric OxdB in the presence of 500 eq of 2-phenylethylamine in 50 mM potassium phosphate buffer containing 10% DMF at 10.0 K.

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suggests that a six-coordinate Fe(II) form existed in a small fraction in ferrous OxdB. When CO gas was added to ferrous OxdB, the Soret peak shifted to 418 nm, indicating the formation of CO-bound OxdB (Fig. 1). The resulting spectrum was typical of CO-bound hemeproteins with a histidine as the proximal ligand.

The Dependence of OxdB Activity on the Oxidation State of the Heme and on pH—The specific activity of OxdB was measured in both the ferric and ferrous state to clarify the active form. The activities of ferric and ferrous OxdB were 2.0 and $2.3 \times 10^7$ turnovers $^{-1}$ heme at pH 7.0, respectively. Ferrous OxdB was 1150-fold more active than ferric OxdB, indicating that ferrous OxdB is the active form. Some cytochrome P450 (28) and very recently reported aldoxime dehydratase from Pseudomonas chlororaphis B23 (OxdA) (29, 30) catalyze the dehydration reaction of aldoxime, where the ferrous form is also reported to be the active form.

The activity of OxdB was dependent on the pH of the assay solution, and this is shown in Fig. 2. The optimum pH range was between 7 and 8, and the activity decreased gradually below pH 7 and above pH 8. Denaturation of OxdB was observed below pH 6.0 and over pH 10.0.

EPR Spectra—EPR spectroscopy provides useful information about the coordination structure of ferric heme. The EPR spectrum of ferric OxdB is shown in Fig. 3A. Ferric OxdB exhibited high spin signals with three-line splitting around $g = 6$ ($g_1 = 6.52, g_2 = 6.15, g_3 = 5.66$) and a small anisotropic signal at $g_\text{iso} = 1.99$. The former were perpendicular components containing a histidine residue as the proximal ligand, and the latter was the parallel component of the high spin heme. The signals around $g = 6$ were presumably because of two different conformers of the high spin heme. Although we tried to specify the $g$ values of the two conformers by EPR experiments under various temperature and microwave power conditions, it was not successful. The signals at $g_4 = 2.30$ and $g_5 = 2.05$ were probably because of a low spin form of OxdB that existed in a small fraction.

Substrate Binding to Ferric OxdB—Although ferric OxdB was inactive, the substrate (PAOx) was bound to the heme in ferric OxdB. The addition of PAOx to ferric OxdB resulted in a red shift of the Soret peak, as shown in Fig. 4. The Soret peak finally shifted to 418 nm on the addition of 500 eq of PAOx. A high spin marker band at 630 nm disappeared concomitantly with increasing $\alpha$ and $\beta$ bands at 574 and 542 nm, respectively, when PAOx was added. These results indicated the formation of a six-coordinate low spin ferric heme by the coordination of PAOx with heme iron. The dissociation constant of PAOx for ferric OxdB, $K_\text{d}$, was estimated to be ~65 $\mu$M.

EPR spectroscopy also confirmed that a six-coordinate, low spin heme in OxdB was formed when PAOx was added to ferric OxdB. The EPR spectrum of ferric OxdB in the presence of 500 eq of PAOx is shown in Fig. 3C. The addition of PAOx gave rise to the appearance of signals around the $g = 2$ region because of a low spin heme (31, 32), accompanied by decreasing intensity of the signal around $g = 6$ corresponding to a high spin heme. The signal with three $g$ values at $g_1 = 2.53, g_\text{iso} = 2.18$, and $g_3 = 1.87$, shown in Fig. 3C, was assigned to a low spin heme that was formed by the coordination of PAOx to high spin ferric heme. The remaining high spin signals in Fig. 3C were because of multiple high spin Fe(III) species, which were also present in the absence of PAOx in potassium phosphate buffer containing 10% DMF, as shown in Fig. 3B. DMF causes a conformational change around the heme, which will cause a further signal splitting in the $g = 6$ region. The formation of the low spin species was consistent with the visible spectral change on adding PAOx.

The crystal field parameters, $\Delta/\alpha$ (tetragonal field) and $V/\alpha$ (rhombic field), calculated from the $g$-values by EPR allowed
the identification of the heme iron axial ligands (33–35). In a
graphic representation of the rhombicity, $V/\Delta$, versus
tetragonality, $\Delta\alpha$, the data points associated with each type of axial
coordination cluster in relatively well defined regions of the
diagram (33, 34). The crystal field parameters of PAOx-bound
OxdB gave 0.55 and 6.71 for rhombicity and tetragonality,
respectively. These values are close to type O coordination
where a histidine and an oxygen atom are the axial ligands of
a heme rather than the type H and B coordination where a
histidine and a nitrogen atom are the axial ligands (35–37).

2-Phenylethylamine, a PAOx analogue bound to the heme
via N-coordination, was bound to ferrous OxdB. 2-Phenylethyl-
amine-bound OxdB showed an EPR spectrum with $g = 3.18$
and 2.11, as shown in Fig. 3D. The EPR spectrum of 2-phenyl-
ethylamine-bound OxdB with a bis-N-coordinated heme was
completely different from that of PAOx-bound OxdB. These
results indicate that PAOx binds to the ferric heme in OxdB via
the coordination of the PAOx oxygen atom. It is reported that
the oxygen atom of N-hydroxyl moiety (–C–N–Oʼ) is bound
to the ferric heme in the model systems (38, 39), which indi-
cates that the binding of PAOx via its oxygen atom is not
exceptional.

Substrate Binding to Ferrous OxdB—When substrate-bind-
ing experiments were carried out for ferrous OxdB using a
UV-visible spectrometer, no spectral change was observed be-
cause of the very fast enzymatic reaction rate. The stopped-flow
technique was therefore used to observe the reaction interme-
diate(s). Typical transient absorption spectra are shown in Fig.
5A, which were observed at regular intervals with stopped-flow
apparatus after mixing ferrous OxdB with PAOx. A reaction
intermediate that showed the Soret peak at 415 nm was ob-
served immediately after mixing ferrous OxdB with PAOx.
This reaction intermediate was the ES complex in which PAOx
is bound to the ferrous heme. This ES complex showed $\alpha$ and $\beta$
bands at 555 and 524 nm, respectively, with the Soret peak at
415 nm, which was a typical spectrum of a bis-N coordinate-
ed Fe(II) heme (40, 41). As shown in Fig. 5B, absorbance at 415
nm because of the ES complex immediately increased to a
constant value within the mixing dead time after mixing OxdB
with PAOx. This shows that the approach to steady state [ES]
was too rapid to be observed.

His-306 as a Catalytic Residue—The substrate, PAOx, was
directly bound to the ferrous heme in OxdB during catalysis, as
described above. Generally, a catalytic residue(s) at a distal
heme pocket where a substrate is bound plays a key role in the
enzymatic reaction of a hemeprotein. To identify a possible
candidate(s) for a catalytic residue(s) in OxdB, we carried out
alanine-scanning mutagenesis on amino acid residues that can
work as an acid or base catalyst. These experiments revealed
that His-306 was a possible candidate for a catalytic residue in
OxdB, because the H306A mutant showed very low activity.

Although H306A OxdB showed almost the same electronic
absorption spectra as wild-type OxdB, the specific activity of
the H306A mutant decreased dramatically. The substrate-
binding experiment showed that PAOx was bound to the fer-
rrouss heme in the H306A mutant, as shown in Fig. 6. The Soret
peak shifted from 432 to 416 nm when PAOx was added to
ferrous H306A OxdB. The resulting spectrum was identical to
that observed for wild-type OxdB by stopped-flow spectroscopy
(Fig. 5A). However, this spectral change for the H306A mutant
could be observed on a standard UV-visible spectrometer.

The spectrum of the reaction intermediate for H306A OxdB,
showing the Soret peak at 415 nm, was finally converted into
that of the ferrous form of this mutant after 30–40 min (data
not shown). These results indicated that the mutation of His-
306 did not result in a complete loss of OxdB activity, although
H306A activity was very low compared with wild-type OxdB.

DISCUSSION

OxdB is the first example of a hemeprotein that can catalyze
a dehydration reaction physiologically. In this paper, we have
characterized OxdB by means of spectroscopic, kinetic, and
mutagenesis studies. The electronic absorption spectra of OxdB
were similar to those of myoglobin in the ferric, ferrous, and
CO-bound forms, suggesting that the heme environmental
structure of OxdB was similar to that of myoglobin. The EPR
spectrum of ferric OxdB shows characteristic three-line signals
around $g = 6 (g_1 = 6.52, g_2 = 6.15, g_3 = 5.66)$, because of two
different conformers of a high spin heme possessing a histidine
as the proximal ligand. Two of three lines were attributable to
one rhombic species and the third arose from a second species.
A similar rhombic EPR signal in the low field region was
observed for myoglobin mutants such as V68E myoglobin ($g_1$
= 6.76, $g_2 = 6.0, g_3 = 5.2$) (42) and H64Y myoglobin ($g_1$
= 6.63,
g_2 = 5.83, g_3 = 5.43 (43). In these myoglobin mutants, the distortion of the heme-bound water and/or of the distal heme pocket around the heme causes a rhombic EPR signal in the g = 6 region. Similar distortion around the distal heme pocket would be included in OxdB.

The heme in OxdB is the active site for the dehydration of its substrate and binds directly the substrate. The enzymatic activity of OxdB is controlled by the oxidation state of heme iron; that is, ferrous OxdB is active but ferric OxdB is not. It is well known in heme enzymes, where a substrate is bound directly to a heme, that one of the two oxidation states, Fe(III) or Fe(II), is active and the other is the resting form (44). In these cases, only the active form of a heme can bind a substrate, indicating that whether or not a substrate is bound to a heme regulates enzymatic activity.

EPR, UV-visible, and stopped-flow spectroscopy revealed that the substrate, PAOx, is bound to the ferric (resting form) and ferrous (active form) hemes via O- and N-coordination, respectively. Although the PAOx-ferrous OxdB complex with O-coordination is inactive in the dehydration reaction, dehydration proceeds when PAOx is bound to the ferrous heme with N-coordination. These results show that the heme oxidation state controls the atom through which the substrate binds to the heme. To our knowledge, this is a novel mechanism by which the enzymatic activity of a hemeprotein is regulated.

Although ferric OxdB binds the substrate, PAOx, to ferric heme iron, it shows very little activity, and this can be explained as follows. To carry out the dehydration reaction of PAOx bound to the heme via O-coordination, the cleavage of the N–O bond of PAOx or the elimination of the \( \beta \) hydrogen atom is required. However, both reactions are problematic, because there is no driving force (Scheme 1A).

On the other hand, PAOx is bound to the ferrous heme in OxdB via the nitrogen atom of PAOx. Rapid scanning spectroscopy using a stopped-flow system reveals a reaction intermediate that shows the Soret peak at 415 nm. This reaction intermediate is an ES complex where the nitrogen atom of PAOx is bound to the heme (40, 41). An important role of the ferrous heme is to tether PAOx in the appropriate orientation toward a catalytic residue(s) in the distal heme pocket. Mutagenesis and substrate binding studies show that His-306 is a catalytic residue for PAOx dehydration.

During the preparation of this manuscript, Konishi et al. (30) reported that His-320 is a crucial histidine for enzymatic activity in another aldoxime dehydratase, OxdA. They propose that His-320 acts as a general acid catalyst in OxdA catalysis. The position of His-320 in OxdA corresponds to that of His-306 in OxdB in amino acid sequence alignment. Although OxdB shows homology with the amino acid sequence (32% identity) to OxdA, there are some differences in enzymatic properties between OxdB and OxdA. Although the enzymatic activity of OxdA decreases gradually above \( pH = 5.5 \) (30), the \( pH \) dependence of OxdB activity is bell-shaped, as shown in Fig. 2. This \( pH \) profile for OxdB activity suggests that two catalytic residues with acidic and basic \( pK_a \)s, respectively, are involved in OxdB catalysis. His-306 in OxdB is assigned to a residue with an acidic \( pK_a \), which acts to assist the elimination of the PAOx hydrogen atom (Scheme 1B). In addition to His-306, a proton source is required to assist the elimination of the OH group in PAOx. In OxdB catalysis, His-306 and a side chain of an amino acid residue, which act as a proton donor, work concertedly as catalytic residues for the dehydration of PAOx to proceed. We are now in the process of identifying a catalytic residue that assists the elimination of the OH group of PAOx.