On the Relationship of Coral Allene Oxide Synthase to Catalase

A SINGLE ACTIVE SITE MUTATION THAT INDUCES CATALASE ACTIVITY IN CORAL ALLENE OXIDE SYNTHASE*

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A heme domain of coral allene oxide synthase (cAOS) catalyzes the formation of allene oxide from fatty acid hydroperoxide. Although cAOS has a similar heme active site to that of catalase, cAOS is completely lacking in catalase activity. A close look at the hydrogen-bonding possibilities around the distal His in cAOS suggested that the imidazole ring is rotated by 180° relative to that of catalase because of the hydrogen bond between Thr-66 and the distal His-67. This could contribute to the functional differences between cAOS and catalase, and to examine this possibility, we mutated Thr-66 in cAOS to Val, the corresponding residue in catalase. In contrast to the complete absence of catalase activity in wild type (WT) cAOS, T66V had a modest catalase activity. On the other hand, the mutation suppressed the native enzymatic activity of the formation of allene oxide to 14% of that of WT cAOS. In the resonance Raman spectrum, whereas WT cAOS has only a 6-coordinate/high spin heme, T66V has a 5-coordinate/high spin heme as a minor species. Because catalase adopts a 5-coordinate/high spin structure, probably the 5-coordinate/high spin portion of T66V showed the catalase activity. Furthermore, in accord with the fact that the CN affinity of catalase is higher than that of WT cAOS, the CN affinity of T66V was 8-fold higher than that of WT cAOS, indicating that the mutation could mimic the heme active site in catalase. We, therefore, propose that the hydrogen bond between Thr-66 and distal His-67 could modulate the orientation of distal His, thereby regulating the enzymatic activity in cAOS.

Allene oxides formed by the enzymatic dehydration of fatty acid hydroperoxides, the lipoxgenase (LOX)*4 products of polyunsaturated fatty acids, are involved in biosynthetic pathways of plants and invertebrates. The main plant pathway leads to jasmonic acid, which seems to be a physiological signaling molecule for several wound- and pathogen-induced responses (1). The conversion from fatty acid hydroperoxide to allene oxide in plants is catalyzed by a heme containing enzyme called allene oxide synthase (AOS) (2, 3). Plant AOS belongs to a subfamily of the fatty acid hydroperoxide metabolizing cytochrome P450s (P450s) designated as CYP74A (4–6). Despite significant sequence homology of plant AOS to those of other P450s, the reaction of plant AOS is different from those of typical P450s (7, 8). Plant AOS does not require the reductants and oxygen, and carries out the homolytic cleavage of the O-O bond in hydroperoxide, although typical P450s utilize two electrons and molecular oxygen for the hydroxylation of its substrate.

Koljak et al. (1997) discovered and isolated a cDNA encoding a fusion protein of 8R-LOX and AOS from the Caribbean sea soft coral, Plexaura homomalla (9), the first example of an AOS found in animals. The fusion protein consists of a C-terminal 8R-LOX domain (79 kDa) and N-terminal heme-containing AOS domain (43 kDa). As shown in Scheme 1, reaction (i), the 8R-LOX domain initially catalyzes the oxygenation of arachidonic acid at the 8R position, yielding the 8R-hydroperoxide of arachidonic acid (8R-HpETE). Then, the AOS domain produces allene oxide from 8R-HpETE (Scheme 1, reaction (iii)). The truncated construct of the AOS domain (cAOS) can catalyze an identical reaction to the AOS domain in the native coral fusion protein (7). Despite the functional similarity to plant AOS, cAOS does not exhibit sequence homology to plant AOS, but shows significant homology to catalase (~11% of sequence identity) (9). However, cAOS cannot catalyze the dismutation of H2O2 to water and oxygen molecule, namely the catalase reaction (Scheme 2) (7, 9), whereas cAOS, like bovine liver catalase (BLC), reacts with peracetic acid to form a ferryl oxo species with a tyrosine radical followed by the formation of a ferryl oxo porphyrin π-cation radical (compound I) species (10, 11).

To clarify the structure-function relationship, especially the reason for the lack of the catalase activity, several spectroscopic techniques including UV/vis, EPR, MCD, and x-ray crystallography were applied to the structural characterization of cAOS (11, 12). As expected from the sequence homology, the spectroscopic data indicated that the heme active site structure in cAOS is quite similar to that of catalase (11, 12). For example, Tyr is a heme ligand of cAOS as observed in catalase (12, 12). On the other hand, as compared with catalase, the recent crystal structure of cAOS showed the lack of hydrogen-bonding network involving the proximal tyrosinate ligand and also the remarkable plannarity of the heme (12). Oldham et al. (12) proposed that such structural properties would inhibit the reaction with H2O2, thereby showing no catalase activity in cAOS.

In addition, the crystal structures also showed remarkable differences in the heme distal sites between cAOS and catalase as displayed in Fig. 1. Although residues responsible for the catalase reaction such as the distal His and Asn are conserved in cAOS, the hydrogen-bonding pattern of the distal His in cAOS is different from that of catalase (12). In catalase,

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* The abbreviations used are: LOX, lipoxigenase; AOS, allene oxide synthase; cAOS, AOS domain of coral AOS; 8R-HpETE, 8R-hydroperoxy-5Z, 9E, 11Z, 14Z-eicosatetraenoic acid; RR, resonance Raman; BLC, bovine liver catalase; HPII, E. coli hydroperoxidase II; WT, wild type.
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Thymol-free BLC was purchased from Sigma. The purification of BLC was followed by the previous report (11). The fractions bearing the $A_{403}/A_{280}$ ratio greater than 0.8 were employed in this study. The concentration of BLC was determined by using an extinction coefficient for heme at 405 nm of 100 mM$^{-1}$ cm$^{-1}$ (22).

The substrate for cAOS, 8R-HpETE, was enzymatically synthesized. Arachidonic acid (3 mg in 300 μl of ethanol) was added to 90 ml of 50 mM Tris-HCl buffer, pH 7.5 containing 500 mM NaCl and 5 mM CaCl$_2$ with vigorous stirring. The purified 8R-LOX domain was immediately added to the solution containing arachidonic acid. The formation of 8R-HpETE was monitored in dilutions of small aliquots of the reaction by the increase in the absorbance at 235 nm. The reaction mixture was placed on ice, and acidified to pH 4.5 by the addition of 1 N HCl. The product was extracted with methylene chloride. The organic phase was washed with water, and then the methylene chloride solution containing 8R-HpETE was dried under a strong stream of nitrogen gas at room temperature. Immediately upon drying, the 8R-HpETE was dissolved in ethanol, and stored under argon at −20°C.

$H_2O_2$ Consumption Rate—Catalase activities for WT cAOS and the T66V mutant were evaluated by the $H_2O_2$ consumption rate. The assay solution (500 μl) contained 1–50 mM $H_2O_2$ in 50 mM Tris-HCl buffer, pH 8.0 containing 100 mM NaCl. The reaction was started by the addition of 1 μM (final concentration) of the cAOS samples, and the $H_2O_2$ consumption rate was determined by monitoring the decrease in the absorbance at 240 nm at 20°C with the spectrometer (Hitachi, U-3310). The concentration of $H_2O_2$ was determined by the absorbance at 240 nm (ε = 43.6 M$^{-1}$ cm$^{-1}$) (23). For comparison, the $H_2O_2$ consumption rates for BLC and hemin were also estimated.

$O_2$ Production Rate—Oxygen production upon mixing of cAOS with $H_2O_2$ was polarographically monitored with a Clark-type electrode (Yellow Springs Instruments, 5331). The assay solution (2 ml) containing 1–350 mM $H_2O_2$ and 100 mM NaCl in 50 mM Tris buffer, pH 8.0 was kept at 20°C. The reaction was started by the addition of 100 nm (final concentration) of the cAOS samples or 0.1 nm (final concentration) of BLC.

Stopped-flow Measurements—The spectral changes in the reaction of ferric cAOS with $H_2O_2$ were monitored by a rapid-scanning system equipped with a stopped-flow apparatus (Unisoku RSP-1000) at 4°C in 50 mM Tris-HCl buffer, pH 8.0 containing 100 mM NaCl. The sample solution containing 5 μM cAOS was transferred to one of the stopped-flow reservoirs. The other reservoir was filled with the solution containing 500 μM $H_2O_2$.

AOS Activity—The consumption of 8R-HpETE by cAOS was estimated by the decrease in absorbance at 235 nm of 8R-HpETE (ε = 23 mM$^{-1}$ cm$^{-1}$) using a Hitachi, U-3310 spectrometer at room temperature (7). The reaction was initiated by the addition of 1 nm (final concentration) cAOS to the assay solution (500 μl) containing 10 μM 8R-HpETE and 100 mM NaCl in 50 mM Tris-HCl buffer, pH 8.0.
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**FIGURE 1.** Heme active site structures in cAOS (1U5U) (12) (A) and Cat F, catalase from Pseudomonas syringae (1MT7) (14) (B). Dotted lines indicate the hydrogen bonds between distal His and neighboring residues.

Resonance Raman Spectroscopy—RR spectra for ferric cAOS and BLC were obtained with a charge-coupled device (CCD) (Roper Scientific, Spec10:400B/LN) attached to a single polychromator (Jobin Yvon, SPEX750M). The 413.1-nm line from a krypton laser (Spectra Physics, BeamLok 2060) and the 488.0-nm line from an argon laser (NEC, GLG 3200) were used for the excitation source. Typically, 100 μl of the solution containing ~15–50 μM cAOS or BLC was transferred to the quartz Raman cell. All measurements were carried out at room temperature with the spinning cell (2,000 rpm) with a diameter of 8 mm. After the RR measurements, no denaturation upon the laser irradiation was confirmed with the absorption spectrum. The calibration of Raman lines was performed with indene, acetone, and carbon tetrachloride.

**CN Binding**—UV/vis absorption spectra of the cAOS enzymes with various concentrations of potassium cyanide were recorded in 50 mM Tris-HCl buffer, pH 8.0 containing 100 mM NaCl at room temperature. The dissociation constants (K_d) were obtained for the reaction using the following Equation 1 that assumes the reversible binding of CN to a single binding site (24).

\[
A_{\text{obs}} = A_0 + (A_c - A_0) \frac{K_d + [\text{cAOS}] + [\text{CN}]}{2[\text{cAOS}]} - \frac{(K_d + [\text{cAOS}] + [\text{CN}])^2 - 4 \cdot [\text{cAOS}][\text{CN}]}{2[\text{cAOS}]} 
\]

(Eq. 1)

where \(A_{\text{obs}}\) denotes the observed absorbances at 406 nm during the titration, \(A_c\) and \(A_0\) represent the absorbance at 406 nm for ligand-free and CN-ligated cAOS, respectively. [cAOS] and [CN] correspond to the total concentrations of cAOS and CN in the sample solution, respectively.

**RESULTS**

**Catalase Activity of the T66V cAOS Mutant**—To examine the catalase activity of the T66V mutant, we evaluated the H_2O_2 consumption rate by monitoring the absorbance change of H_2O_2 after mixing 1 μM T66V mutant and 10 mM H_2O_2. Fig. 2A shows a time course for the decrease of the H_2O_2 absorption of 240 nm at 20 °C. The absorbance change of H_2O_2 upon the addition of 1 μM WT cAOS or free heme to the solution containing 10 mM H_2O_2 was negligible, which is consistent with the report by Boutaud and Brash (7). In sharp contrast to this, the decrease of the absorbance of H_2O_2 was observed with the initial velocity of 86 μM sec^{-1} in the T66V mutant (Fig. 2A, squares). Furthermore, we observed bubbles probably derived from the oxygen evolution in the reaction mixture only for the T66V mutant (data not shown). These results indicate that the T66V mutant has catalase activity.

To further characterize the catalase reaction of the T66V mutant, we estimated the kinetic parameters of the reaction. The initial rates of the H_2O_2 consumption obtained from linear fits to data points for the decrease in absorption at 240 nm by the T66V mutant at the various concentrations (from 1 mM to 50 mM) of H_2O_2 are plotted as a function of the H_2O_2 concentration (Fig. 2B). The hyperbolic dependence of the initial rate of the H_2O_2 consumption on the H_2O_2 concentration in the T66V mutant allows us to determine the kinetic parameters, \(k_m\) and \(k_{cat}\), by fitting the data using the Michaelis-Menten equation. The estimated \(k_m\) and \(k_{cat}\) values for the catalase reaction in the T66V mutant cAOS are summarized in Table 1 together with those of catalases (25) and the other heme enzymes possessing catalase activity (26–28). As seen in Table 1, the \(k_m\) value for the T66V mutant is within the range of those of catalases. On the other hand, \(k_{cat}\) in the T66V mutant is 2–3 orders of magnitude lower than those of catalases.

In addition to the H_2O_2 consumption, the oxygen evolution was monitored by a Clark-type oxygen electrode. The initial rates of the oxygen evolution by wild type and the T66V mutant of cAOS are plotted against the H_2O_2 concentration in Fig. 3. Similar to the H_2O_2 consumption, the data in the T66V mutant can be fitted by the Michaelis-Menten equation. The \(k_m\) and \(k_{cat}\) values calculated from the oxygen evolution rate are summarized in Table 1. As seen in Table 1, \(k_{cat}\) from the oxygen evolution in the T66V mutant is 5-fold that of the H_2O_2 consumption and a similar trend was observed in BLC. Although we have no satisfactory explanation for this, we note that on account of the UV saturation at 240 nm, measurements were made over different ranges of H_2O_2 concentration in the two methods, 0–50 mM for the UV assay, 0–400 mM for oxygen evolution. The \(k_{cat}\) values are in reasonable agreement between the two assays in BLC, indicating that, for the T66V mutant, the 4-fold slower rate of the oxygen evolution (as H_2O_2 degraded) relative to the rate of the H_2O_2 consumption could truly reflect uncoupling between oxygen evolution and H_2O_2 consumption. Participation of the compound II pathway might explain the uncoupling between H_2O_2 consumption and oxygen evolution, because compound II can revert back to the resting ferric state without producing molecular oxygen (29).

Actually, the increase in the absorbance around 440 nm, which is characteristic of the formation of compound II (10), was observed after mixing the T66V mutant with H_2O_2 (Fig. 4B), supporting the participation of the compound II pathway. Thus, although the T66V mutant can show the catalase reaction, its rate is considerably slower than that of catalase and more than one pathway may participate in H_2O_2 degradation.

We also measured the optical absorption spectra for wild type and the T66V mutant of cAOS during reaction with H_2O_2 at 4 °C by using a stopped-flow system to examine the reaction mechanism for the T66V mutant. In the case of catalase, the formation of compound I upon mixing of H_2O_2 with the ferric enzyme is the first step in the decomposition of H_2O_2 as shown in Scheme 2. In WT cAOS, the addition of H_2O_2 induced no spectral changes, even though the amount of H_2O_2 corresponds to 100 eq. over the enzyme concentration (Fig. 4A). As expected from the result on the H_2O_2 consumption and the oxygen evolution of the T66V mutant; however, a remarkable decrease in the intensity of the Soret band was observed by the addition of 100 eq. of H_2O_2 to the T66V mutant (Fig. 4B), which is characteristic of the formation of compound I (30).

To observe the compound I species, we examined the reaction between cAOS and peracetic acid. The use of peracetic acid instead of H_2O_2 should simplify the catalase reaction in the T66V mutant by inhibition of the reduction of compound I. In the T66V mutant, the addition
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Figure 2. H₂O₂ consumptions by WT and the T66V mutant cAOS. A represents the time courses of absorbance at 240 nm during the catalase reaction in WT (circles) and the T66V mutant of cAOS (squares). Triangles indicate the time course for the reaction between hemin and H₂O₂ for comparison. The time courses were obtained by mixing 1 μM enzymes and 10 mM H₂O₂ at 20 °C in 50 mM Tris buffer, pH 8.0 containing 100 mM NaCl. The inset represents the time courses of absorbance at 240 nm after the addition of 1 μM T66V mutant to the solution containing 1, 5, 10, 20, 30, and 40 mM H₂O₂. B represents the initial rates of the H₂O₂ consumptions of WT (circles) and the T66V mutant (squares) against the concentration of H₂O₂. The rates were determined from the time courses of absorbance at 240 nm. The solid curve is the result from the calculation with the Michaelis-Menten equation and the fitted values of Kₘ = 28 mM and k₅ = 312 s⁻¹.

Table 1. Kinetic parameters for the catalase reaction in the T66V mutant of cAOS, catalases and other heme enzymes

| Protein                  | K_m (mM) | k₅ (sec⁻¹) | Ref.     |
|--------------------------|----------|------------|----------|
| T66V of cAOS (H₂O₂ consumption) | 28 ± 8   | 312 ± 34   | This study|
| T66V of cAOS (O₂ evolution)          | 140 ± 12 | 78 ± 2.8   | This study|
| BLC (H₂O₂ consumption)              | 40 ± 1   | (7.23 ± 0.10) × 10⁴ | This study|
| BLC (O₂ evolution)                  | 160 ± 24 | (10.6 ± 0.70) × 10⁴ | This study|
| BLC                               | 93       | 2.12 × 10⁴  | (25)     |
| HPDII                            | 64       | 7.0 × 10⁴   | (25)     |
| Kat G                            | 5.18     | 1.01 × 10⁴  | (28)     |
| HRP                             | 4.0      | 1.78       | (26)     |
| HS2Q of CeP                      | 0.45     | 30         | (27)     |

* H₂O₂ degraded per second.
* E. coli hydroperoxidas II.
* Catalase-peroxidase.
* Horseradish peroxidase.
* Cytochrome c peroxidase.

AOS Activity of the T66V Mutant—We examined the effects of the T66V mutation on the inherent AOS enzymatic activity. Fig. 5 displays the time course of A₅₃₀ of the substrate at 235 nm, following the addition of cAOS to the solution containing 8R-HpETE at room temperature. In the case of WT cAOS, an immediate decrease in the absorbance at 235 nm was observed with the initial rate of 0.083 ± 0.008 μM sec⁻¹ (Fig. 5, circles). Whereas the T66V mutant also exhibits the consumption of 8R-HpETE (Fig. 5, squares), its initial rate (0.012 ± 0.002 μM sec⁻¹) corresponds to ~one-seventh that of WT cAOS. Therefore, the mutation of Thr-66 to Val suppresses the enzymatic activity of the allene oxide production.

Resonance Raman Spectra of cAOS—Even though WT cAOS shows no catalase activity, the mutation of Thr-66 to Val in cAOS enables the enzyme to decompose H₂O₂ similar to catalase. On the other hand, the mutation decreases the AOS activity. To elucidate the origin of such drastic changes in the functional properties of cAOS by the mutation of Thr-66 to Val, the mutational effects on the heme active site structure were examined by RR spectroscopy.

Fig. 6 displays the high frequency region of the RR spectra for ferric WT cAOS upon excitation at 413.1 (Soret region) (a) and 488.0 nm (Q-band) (b). Several Raman lines observed in this region contain the heme skeletal vibrations involving ν₁, ν₂, ν₃, and ν₄ which are sensitive to the oxidation, coordination and spin states of the heme iron (31, 32). The oxidation state marker, ν₂, at 1373 cm⁻¹ for ferric WT cAOS is consistent with the ferric heme iron. The ν₃ marker band is useful for determination of the coordination and spin states of the heme iron (31). The ν₄ band at 1482 cm⁻¹ in ferric cAOS suggests that ferric cAOS has a 6c/HS structure. As expected from ν₅, 1561 cm⁻¹ of ν₅ supports the 6c/HS structure in ferric cAOS. Furthermore, the 1613 cm⁻¹ line in ferric
Cao is likely to have arisen from the $v_{10}$ mode of a 6c/HS heme (31), because the Raman line at 1613 cm$^{-1}$ was depolarized and maximally enhanced with visible, not Soret, excitation. Although the heme in WT cAOS was shown to have a 5-coordinate heme from the crystal structure (12), the set of $v_{1}$, $v_{2}$, and $v_{10}$ frequencies suggests that ferric cAOS has a 6c/HS heme, implying that a water molecule or a hydroxide anion would coordinate to the heme iron as the 6th ligand in solution. These Raman lines observed in the high frequency region are summarized in Table 2 with those of catalases.

Contrary to WT cAOS, the frequencies of the porphyrin marker lines indicate that the heme in BLC adopts a 5c/HS structure (Fig. 6, trace d), which is consistent with the reported result (33). The previous spectroscopic and x-ray crystallographic studies on cAOS suggested that the coordination structure of ferric cAOS is almost the same as that of catalase (11, 12). Our current finding, however, shows the different heme coordination structures between cAOS (6c/HS) and catalase (5c/HS).

Fig. 6, trace c displays the high frequency region of the RR spectra for the ferric T66V mutant excited at 413.1 nm. It is apparent from the $v_{1}$, $v_{2}$, and $v_{10}$ lines in Fig. 6 that a 6c/HS heme is present as a major species of the T66V mutant similar to WT cAOS. In addition to the Raman lines arising from a 6c/HS heme, we could detect the Raman lines assignable to a 5c/HS heme as a minor species. For example, the $v_{10}$ line arising from a 5c/HS heme was observed at 1492 cm$^{-1}$ as a shoulder. Furthermore, the intensity of the Raman line around $\sim$1630 cm$^{-1}$ in the T66V mutant was higher than that of WT cAOS probably because of overlapping with the $v_{10}$ line from a 5c/HS heme ($\sim$1627 cm$^{-1}$). The mutation has, therefore, affected the heme environment, and induced a change in the heme coordination structure of cAOS. The Raman lines in the low frequency region (Fig. 7) are comprised of several heme in-plane and out-of-plane vibrational modes including heme propionate, vinyl, and iron-ligand vibrational modes. Thus, the Raman lines observed in the low frequency region can provide information on the interaction between the heme prosthetic group and the protein environment. By comparing with the assignments of other hemoproteins (34, 35), we assigned the several Raman lines as indicated in Fig. 7. The low frequency region of the Raman spectrum of the T66V mutant, which is shown in Fig. 7b, is indistinguishable from that of wild type (Fig. 7, trace a) except for the bending modes of the heme propionates ($\delta(C_{5}C_{5}C_{3})$). This mode appeared at 384 cm$^{-1}$ in the T66V mutant (391 and 381 cm$^{-1}$ for WT cAOS). It has been demonstrated in previous studies on heme proteins such as myoglobin that the frequency of the bending mode for the heme propionate is sensitive to the hydro-

FIGURE 4. Immediate time-dependent absorption spectral changes in WT (A) and the T66V mutant of cAOS (B) after addition of 100 eq of H$_2$O$_2$ at 4 °C in 50 mM Tris buffer, pH 8.0, containing 100 mM NaCl. Gray traces represent the absorption spectra for the compound I-like species produced by mixing the enzymes and peracetic acid. Arrows in B indicate spectral changes with time. The inset represents the time courses of absorbance at 406 nm after mixing the enzymes with H$_2$O$_2$.

FIGURE 5. Consumption of BR-HpETE by WT and the T66V mutant of cAOS. Traces represent the time courses of absorbance of BR-HpETE (235 nm) before (solid curve) and after the addition of 1 nM of WT (circles) and the T66V mutant of cAOS (squares) to the solution containing 10 μM of BR-HpETE at room temperature.

FIGURE 6. Resonance Raman spectra in the high frequency region for ferric cAOS and catalase. Traces shown are the resonance Raman spectra for WT cAOS with the 413.1 nm excitation (a) and with the 488.0 nm excitation (b) (parallel (l) and perpendicular (⊥) polarizations), and for the T66V mutant of cAOS (c) and BLC (d) with the 413.1 nm excitations. The protein concentrations were ~50 μM in the 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. Laser power was 1 and 5 milliwatt for the 413.1 and 488.0 nm excitations, respectively, at the sample point.
obtained as (the isotope substitution of $^{16}$OH to $^{18}$OH in the hydroxide bound ferric for WT cAOS in the H$_2$O (c) and H$_2$O (d) based buffer solutions. The spectrum (e) represents the difference spectrum obtained as (d) minus (c). The protein concentrations were $\sim$50 $\mu$M in the 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. Laser power was 1 mW at the sample point.

In addition to the heme peripheral vibrations, the Fe-OH stretching frequency region of the resonance Raman spectra for ferric cAOS WT and the T66V mutant, respectively, at room temperature (data not shown). Thus, the T66V mutant has a $\sim$8-fold higher affinity for CN$^-$ than that of WT cAOS. As Abraham et al. (11) have already mentioned, the drastically lower CN affinity of WT cAOS as compared with that of catalase by a factor of $\sim$1,000 would be attributable to differences between the environments of the heme active site of cAOS and catalase. The relatively higher CN affinity of the T66V mutant than that of WT cAOS allows us to suggest that the mutation partially mimics the heme active site of catalase.

**DISCUSSION**

In the current study, we found that Thr-66 is a key player in regulation of the catalytic activity in cAOS. The mutation of Thr-66 to Val enabled the enzyme to show catalase activity with a concomitant decrease in the AOS activity. In addition, RR spectra and the CN affinity indicate that the mutation alters the heme distal environment. In the following, we discuss the reason for the lack of catalase activity in cAOS based on the structural information.

We hypothesized from the crystal structures of cAOS and catalase (Fig. 1) that WT cAOS cannot generate the compound I species upon the addition of H$_2$O$_2$ and cannot show the catalase activity owing to a flipped distal His. Consistent with this proposal, the stopped flow experiment (Fig. 3) clearly showed that the addition of H$_2$O$_2$ to WT cAOS cannot produce compound I. In contrast to WT cAOS, the T66V mutant showed catalase activity through the formation of compound I. Because the significance of the location of the distal His in compound I formation was demonstrated by using myoglobin mutants (19), it is plausible that the disruption of the hydrogen bond between Thr-66 and His-67 would render the imidazole ring of the distal-His more flexible and induce the flipping of it in the T66V mutant. We surmise that this flipped imidazole ring of the distal-His in the T66V mutant would be further stabilized by the formation of a new hydrogen bond between distal His-67 and Ser-118 corresponding to Ser-116 in catalase.

The structural changes in the heme distal site upon the mutation are clear in the RR spectra. Whereas a water-ligated 6c/HS structure is a major species in the T66V mutant similar to WT cAOS, a 5c/HS species was observed as a minor component in the T66V mutant. The fact that the water coordination to the heme iron was stabilized by a hydrogen bond and/or a negatively charged group in other heme proteins (41–43) suggests that the mutation of Thr-66 possibly affects the distal environment. Furthermore, the CN affinity was increased to 8-fold of that of WT cAOS by the mutation, which also provides evidence for the structural perturbation at the heme distal site upon the mutation. One of the key factors controlling the CN affinity is the deprotonation of HCN in the distal heme pocket (39, 40). In cytochrome c peroxidase, the distal His was suggested to be a protonation site for a dissociated proton from HCN during CN binding (44). The vital role of the distal His in the CN binding for catalase was also demonstrated by the fact that the mutation of the distal His to Asn lowered the CN affinity to less than 1:1000 of that estimated from Equation 1 to be 31 and 3.8 mM for WT cAOS and the T66V mutant, respectively, at room temperature (data not shown).

**TABLE 2**

| Raman shifts (cm$^{-1}$) of the porphyrin marker lines of ferric cAOS and catalases |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Protein                         | $v_2$           | $v_3$           | $v_2$           | $v_{10}$        | Structure       | Ref.            |
| WT cAOS                         | 1373            | 1482            | 1561            | 1612            | 6c/HS           | This study      |
| T66V                            | 1373            | 1492            | ND$^a$          | $\sim$1630      | 5c/HS           | This study      |
| BLC$^b$                         | 1373            | 1490            | 1569            | 1625            | 5c/HS           | This study and (33) |
| ANC$^c$                         | 1373            | 1489            | 1574            | 1625            | 5c/HS           | (47)            |
| MLC$^d$                         | 1374            | 1487            | 1570            | 1626            | 5c/HS           | (47)            |

$^a$ Not determined.

$^b$ Aspergillus niger catalase.

$^c$ Micrococcus luteus catalase.

$^d$ Lactobacillus casei catalase.

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FIGURE 7. Resonance Raman spectra in the low frequency region for ferric cAOS WT (a) and the T66V mutant (b) excited at 413.1 nm. The inset depicts the Fe-OH stretching frequency region of the resonance Raman spectra for WT cAOS in the H$_2$O (c) and H$_2$O (d) based buffer solutions. The spectrum (e) represents the difference spectrum obtained as (d) minus (c). The protein concentrations were $\sim$50 $\mu$M in the 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. Laser power was 1 mW at the sample point.

CN Affinity—To get further information on the heme environment of the T66V mutant, we estimated the affinity for CN$^-$, which is sensitive to the distal environment of the heme (39, 40), for wild type and the T66V mutant of cAOS. By monitoring the optical absorption for the titration of cAOS with CN, the dissociation constants ($K_d$) for CN were...
of wild-type *E. coli* catalase (HPII) (45). Because the distal His-67 in WT cAOS appears to be flipped relative to that of catalase, it is thus unable to provide the protonation site for CN binding. We, therefore, suggest that the orientational change enables the distal His to promote the deprotonation of HCN, thereby increasing in the CN affinity of the T66V mutant. The mutational effects on the RR spectrum and CN affinity in cAOS, however, are smaller than those expected from the difference between WT cAOS and catalase. In addition, the catalase activity of the T66V mutant is 2–3 orders magnitude lower than that of catalase. These results raise the possibility that the orientation of the distal His in the major species in the T66V mutant might be the same as in the WT cAOS. In contrast to catalase, the distal Asn-137 in cAOS is located closer to the heme iron by ~1.0 Å than that of catalase, and forms a hydrogen bond to distal His-67 (Fig. 1) (12). Therefore, the distal His could not be flipped in the major species of the T66V mutant due to the hydrogen bond between Asn-137 and His-67, despite the disruption of the hydrogen bond between Thr-66 and His-67. From this idea and two $r_1$ lines observed in the T66V mutant (Fig. 6), we propose that two conformations with different orientation of the distal His are in equilibrium in the T66V mutant as shown in Scheme 3. In Scheme 3, the minor species B whose distal His is flipped to the same orientation as that of catalase can generate compound I upon the addition of H$_2$O$_2$, whereas the major species A which has a similar heme active site structure to that of WT cAOS cannot react with H$_2$O$_2$. The low population of the conformation B could explain why the structural effects of the mutation are relatively small and the T66V mutant shows quite low catalase activity. Although it is quite difficult to determine the population of the two conformations with different orientation of the distal His, we can safely conclude that the presence of hydrogen bond between Thr-66 and distal His-67 is one of the reasons for the lack of the catalase activity in WT cAOS.

It should be noted here that, in contrast to the fact that the mutation of Thr-66 enables the enzyme to possess catalase activity, the rate for the AOS activity was decelerated. There are multiple outcomes possible upon homolysis of a fatty acid hydroperoxide moiety (46), yet in both WT cAOS and, as we confirmed here, in the T66V mutant also, the sole product is the allene oxide (data not shown). Thus the results imply that Thr-66 significantly facilitates catalysis, albeit not in a crucial role. Whereas further studies should be required for demonstrating the catalytic roles of Thr-66, it is plausible that Thr-66 is one of the key residues resulting in the functional difference between two homologous enzymes, cAOS and catalase.

**CONCLUSION**

On the basis of the structural difference in two structurally homologous heme enzymes, cAOS and catalase, we mutated the distal Thr-66 of cAOS to Val, the corresponding residue in catalase, for the purpose of understanding the origin of the functional difference between the two enzymes. In contrast to WT cAOS, we found that the mutation of Thr-66 to Val allowed the enzyme to react with H$_2$O$_2$ to form compound I, and to exhibit catalase activity. On the other hand, the mutation decelerated the formation rate of allene oxide from $R^\cdot$-HpETE to one-seventh of that of WT cAOS. In relation to the mutational effects on the functional properties, the RR spectra and the CN affinity suggested that the T66V mutant contains a conformation whose structural properties are similar to that of catalase. The mutation of Thr-66 would disrupt the hydrogen bond with distal His-67, and change the orientation of the imidazole ring of distal His-67 with formation of a new hydrogen bond between the distal His-67 and its neighboring Ser-118. Because of such structural changes in the heme distal site, the minor species of the T66V mutant showed similar structural and functional properties to those of catalase. These observations allow us to propose that the modulation of the orientation of the imidazole ring of distal His by the hydrogen bonding interactions with neighboring residues is a crucial factor controlling the enzymatic activity.

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