Interaction between Substrate and Oxygen Ligand Responsible for Effective O–O Bond Cleavage in Bovine Cytochrome P450 Steroid 21-Hydroxylase Proved by Raman Spectroscopy

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We investigated structural and functional properties of bovine cytochrome P450 steroid 21-hydroxylase (P450c21), which catalyzes hydroxylation at C-21 of progesterone and 17α-hydroxyprogesterone. The uncoupled H2O2 formation was higher in the hydroxylation of progesterone (26% of NADPH consumed) than that of 17α-hydroxyprogesterone (15% of NADPH consumed), indicating that 17α-hydroxyprogesterone can better facilitate the O–O bond scission. In relation to this, it is noted that the O–O stretching mode (νO–O) of the oxygen complex of P450c21 was sensitive to the substrate; the progesterone- or 17α-hydroxyprogesterone-bound enzyme gave single (at 1137 cm⁻¹) or split νO–O bands (at 1124 and 1138 cm⁻¹), respectively, demonstrating the presence of two forms for the latter. In contrast to νO–O, no corresponding difference was observed for the Fe-O2 stretching mode between two different substrate-bound forms. The Fe-S(Cys) stretching mode in the ferric state was also identical (349 cm⁻¹) for each substrate-bound form, suggesting that modulation through the axial thiolate by the substrate is unlikely. Therefore, it is deduced that the hydroxyl group at C-17 of 17α-hydroxyprogesterone forms a hydrogen bond with the terminal oxygen atom of the FeOO complex in one form, yielding a lower νO–O frequency with higher reactivity for O–O cleavage, whereas the other form in which the substrate does not provide a hydrogen bond to the oxygen ligand is essentially the same between the two kinds of substrates. In the hydrogen-bonded species, the substrate changes the geometry of the FeOO moiety, thereby performing the hydroxylation reaction more effectively in 17α-hydroxyprogesterone than in progesterone.

Cytochrome P450 steroid 21-hydroxylase (P450c21)4 is predominantly expressed in adrenal cortex under the control of adrenocorticotropic hormone via the cAMP-dependent signaling pathway (1, 2). P450c21 catalyzes hydroxylation at C-21 of progesterone (Prog) and 17α-hydroxyprogesterone (17-OH-prog) to produce 11-deoxycorticosterone and 11-deoxycortisol, respectively, as shown in Fig. 1. These C-21 hydroxylation steps are essential for the biosynthesis of aldosterone and cortisol. Deficiency in P450c21 found in ∼1:10,000 newborn babies results in impaired steroid synthesis in which adrenal androgens are overproduced in addition to the lack of aldosterone and cortisol, as shown in Fig. 1 (3). The P450c21 deficiency accounts for more than 90% of a common genetic disease, congenital adrenal hyperplasia, with symptoms of salt wasting, simple virilizing, and nonclassical phenotypes (3). The enzyme deficiency can be attributed to mutations in the CYP21B1 gene encoding P450c21 (4, 5). For better understanding the mechanism of the deficiency of P450c21 by the mutation, it is highly desirable to elucidate the structure-function relationship in P450c21.

The structural and functional studies have been carried out with use of bovine P450c21 purified from adrenal cortex (6). P450c21 shows higher hydroxylation activity with 17-OH-prog than that of Prog in the steady state condition (7). Subsequent mechanistic study by Kominami et al. (8) found that the rate-limiting step was different in the hydroxylation reactions of two substrates and proposed that this difference could cause higher reactivity with 17-OH-prog in P450c21. However, from the structural viewpoint, the difference between the two physiological substrate-bound forms has been poorly understood.

Although the sample purified from bovine adrenal cortex was stable in the presence of detergents or lipids, its hydrophobic nature and difficulty in obtaining qualities of the purified samples have hampered further mechanistic and structural studies. Actually, despite the fact that P450c21 is known as the first functional P450 (9), the structure-based study on P450c21 has hardly progressed. Recently, Kagawa et al. (10–12) devel-

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4 The abbreviations used are: P450c21, cytochrome P450 steroid 21-hydroxylase; Prog, progesterone; 17-OH-prog, 17α-hydroxyprogesterone; RR, resonance Raman; oxy-P450c21, oxygen complex of P450c21; NOS, nitric oxide synthase; P450cam, cytochrome P450 camphor monooxygenase; MOPS, 4-morpholinepropanesulfonic acid.

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opened the Escherichia coli expression systems and purification methods for mammalian P450s in the steroid hormone biosynthetic pathway, including bovine P450c21, which enables us to carry out the structural and functional studies (10, 13).

In this study, we attempted to elucidate the structure-function relationship in P450c21 by using the high quality sample purified from the E. coli expression system. To get insight into the functional property of P450c21, we estimated the uncoupled H2O2 formation during the hydroxylation of two different physiological substrates, which should reflect efficiency of the step of O–O bond scission (14–17). To examine the structural properties related to the function, we characterized the environment of the heme of P450c21 with and without the substrate by using resonance Raman (RR) spectroscopy, a powerful tool to explore the environment of the heme in the solution state (18–20). We found that there was less H2O2 production in the hydroxylation of 17-OH-prog than that of Prog and that the \( v_{\text{O–O}} \) mode of the oxygen complex of P450c21 (oxy-P450c21) was sensitive to the structure of the substrate. On the basis of the current data, we discuss the factor determining the structural and functional difference between two physiological substrate-bound P450c21s.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The E. coli expression and purification of the N-terminal membrane anchor truncated bovine P450c21 with His tag were carried out according to a previous report (10). The purified ferric substrate-free P450c21 in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 1.0% sodium cholate was stored at \(-80^\circ\text{C}\). Each substrate (Prog or 17-OH-prog) was dissolved in 45% (w/v) 2-hydroxypropyl-\( \beta \)-cyclodextrin in water as a \( \sim 20 \) mM stock solution.

**Spectroscopic Measurements**—UV-visible absorption spectra were recorded on a Lambda 18 UV-visible spectrophotometer (PerkinElmer Life Sciences) or U-3310 spectrophotometer (Hitachi). Substrates (50 \( \mu \)l of the stock solution) were added to 450 \( \mu \)l of the substrate-free P450c21 (10 \( \mu \)M) solution to generate the substrate-bound ferric forms. Ferrous P450c21 was prepared by the addition of 10 \( \mu \)l of \( \sim 20 \) mM sodium dithionite solution to ferric P450c21 under anaerobic condition. Oxy-P450c21 was prepared according to the method for the preparation of the oxygen adduct of P450cam (21). In brief, oxy-P450c21 was produced by mixing ferrous P450c21 (20 \( \mu \)M), which was prepared by a minimal addition of \( \sim 20 \) mM sodium dithionite solution to ferric P450c21 with equal volume of air-saturated buffer on ice. The measurements of optical absorption spectra were performed at room temperature for the ferric and ferrous samples and at 4 °C for oxy-P450c21.

RR spectra were obtained with a single polychromator (Jobin Yvon SPEX750M) equipped with a liquid nitrogen-cooled CCD detector (Spec10:400B/LN; Roper Scientific). The 406.7- and 413.1-nm lines from a Kr+ laser (BeamLok 2060; Spectra Physics) and the 363.8-nm line from an Ar+ laser (BeamLok 2080; Spectra Physics) were used for the Raman excitation. Raman shifts were calibrated with indene, acetone, and carbon tetrachloride. All of the measurements were performed at 4 °C with a quartz spinning cell (2000 rpm). The Raman samples were prepared by a method similar to those for the UV-visible absorption measurements except for oxy-P450c21. Oxy-P450c21 for the Raman measurement was prepared by incorporation of oxygen gas (\( ^{16}\text{O}_2 \) or \( ^{18}\text{O}_2 \)) into the Raman cell containing ferrous P450c21 after its degassing on ice. The sample concentration was adjusted to 20 \( \mu \)M.

**Enzymatic Activity**—The formation of H2O2 from the uncoupling reaction of P450 was determined as described (22) with a slight modification. The reaction mixture (100 \( \mu \)l) containing P450c21 (2.2 \( \mu \)M), NADPH-dependent P450 reductase (1.5 \( \mu \)M), 2.5 mM Prog or 17-OH-prog, 50 mM potassium phosphate (pH 7.4), 20% glycerol, and 0.025% Cymal 5 was preincubated at 30 °C for 2 min. The hydroxylation reaction was ini-
ated with the addition of NADPH (final 50–200 μM). After a 5-min reaction at 30 °C, 20 μl of reaction mixture was transferred, mixed with 0.5 μl of 20% SDS and 0.5 μl of 10 mg/ml proteinase K, and incubated at room temperature for 5 min. The mixture was added with a 140 μl H2O2 assay solution that contains 200 mM MOPS, pH 7.5, 200 mM sodium chloride, 5 mM 4-aminoantipyrine, 10 mM vanillic acid, and 40 units/ml horseradish peroxidase. The formation of a red quinone imine dye (ε490 = 6.99 mM⁻¹ cm⁻¹) was measured by optical spectroscopy. The consumption of NADPH was confirmed by monitoring the absorption at 340 nm (ε340 = 6.21 mM⁻¹ cm⁻¹).

RESULTS

Uncoupled H₂O₂ Formation by P450c21—A previous study by Arase et al. (10) demonstrated that bovine P450c21 shows different reactivity with its alternative substrates, Prog and 17-OH-prog, under the steady state condition but provides no information on the elementary steps. Here, to further characterize the enzymatic property in terms of the reactivity with different substrates, the efficiency of the hydroxylation for Prog and 17-OH-prog was estimated by the amount of H₂O₂ from the uncoupling reaction of P450c21. Fig. 2 displays the formation of H₂O₂ against NADPH consumed during the hydroxylation of Prog or 17-OH-prog by P450c21. As seen in Fig. 2, NADPH was more efficiently consumed for the 21-hydroxylation of 17-OH-prog compared with that of Prog. Under the experimental conditions, the percentage of uncoupled H₂O₂ formation per NADPH consumption was 26 ± 1.1 and 15 ± 0.7% for Prog and 17-OH-prog, respectively. 17-OH-prog is a better substrate for P450c21 than Prog from the catalytic coupling with consumption of NADPH.

Interaction between Substrate and O₂ Ligand in P450c21

Raman Characterization of Ferric P450c21—To elucidate the reason for the different reactivity with two substrates, Prog and 17-OH-prog, in bovine P450c21, we explored the heme active site structure of P450c21 with the substrates along the catalytic cycle. Because the first step of the P450-catalyzed reaction is the binding of the substrate to the ferric enzyme (23), we have focused on the characterization of the environment of the heme of ferric P450c21 with Prog and 17-OH-prog as well as the substrate-free form by RR spectroscopy.

Fig. 3A displays the high frequency region of RR spectra for ferric P450c21 in the absence and presence of the substrates with 406.7-nm excitation. The assignments (Table 1) of the porphyrin marker lines, ν₁, ν₃, ν₂, and ν₁₀, sensitive to the oxidation state, spin state, and coordination structure (24, 25), were carried out by analogy to the Raman spectra of other P450s (13, 26, 27) and polarization experiment (data not shown). Consistent with previous spectroscopic data (10), substrate-free ferric heme adopts a six-coordinate/low spin structure, which is indicated by the ν₁, ν₃, ν₂, and ν₁₀ modes at 1371, 1501, 1581, and 1637 cm⁻¹, respectively. A water molecule
probably ligates to the heme as a sixth ligand in the substrate-free enzyme.

As expected from previous data on the optical spectra (10), the binding of Prog or 17-OH-prog induces the structural transition of the heme from six-coordinate/low spin to five-coordinate/high spin, which was evident from the appearance of the Raman lines at 1487, 1567, and 1624 cm\(^{-1}\) assignable to \(v_{\alpha}, v_{\beta},\) and \(v_{\alpha 10}\) modes, respectively, for a five-coordinate/high spin heme (Fig. 3, a and c). It should be noted here that the six-coordinate/low spin heme is still observed in Prog-bound P450c21 (Fig. 3, a, b, and c), contrary to the almost complete spin conversion observed in 17-OH-prog-bound P450c21 (Fig. 3, a, c). Further addition of Prog to the enzyme did not increase the population of the high spin species (data not shown), suggesting that the remaining low spin species did not arise from the substrate-free enzyme. It is plausible that the additional 17-hydroxyl group in 17-OH-prog could be in proximity to the water ligand and facilitate the dissociation of the water ligand from the heme iron.

Raman lines observed in the low frequency region involve the bending vibrations of heme peripheral groups as well as the porphyrin skeletal vibrations (28, 29) (Fig. 3B) and would provide information on the environment of the heme peripheral groups (30–32). On the basis of the complete assignment for myoglobin (29), the Raman lines at 344, 379, 423, and 676 cm\(^{-1}\) were assigned to the \(v_{\alpha}, \delta(C_{p}C_{p}C_{p}C_{p})\) (bending mode of the heme propionate groups), \(\delta(C_{p}C_{p}C_{p}C_{p})\) (bending mode of the heme vinyl groups), and \(v_{s}\) modes, respectively, for substrate-free P450c21 (Fig. 3B, a). As seen in Fig. 3B (b), the binding of substrate, Prog, did not alter the peak positions of the bending vibrations of heme peripheral groups. In addition, the spectrum for 17-OH-prog-bound P450c21 is almost indistinguishable from that of Prog-bound P450c21 (Fig. 3B, c). The substrate binding would not perturb the structure of the heme peripheral group in ferric P450c21.

The iron-thiolate stretching vibration \(v_{Fe-S}\) was reported to be also detectable in the low frequency region around \(\sim 350\) cm\(^{-1}\) for ferric five-coordinate/high spin species upon excitation at the iron-thiolate charge-transfer band (33, 34). The \(v_{Fe-S}\) mode should serve as a structural marker at the heme proximal site. Fig. 4 displays the low frequency region of the RR spectra for ferric P450c21 in the absence and presence of the substrates with 363.8-nm excitation corresponding to the iron-thiolate charge transfer band. The intensities of almost all Raman lines were drastically decreased upon 363.8-nm excitation in the substrate-free form (Fig. 4a) as compared with that with 406.7 nm excitation (Fig. 3a, a). In contrast to this, a new Raman line at 349 cm\(^{-1}\) was detected for substrate-bound P450c21 (Fig. 4, b and c). This Raman line at 349 cm\(^{-1}\) was not observed by 406.7-nm excitation as shown in Fig. 4 (inset). The fact that the Raman line at 349 cm\(^{-1}\) was detectable only with 363.8-nm excitation for the five-coordinate/high spin heme enables us to assign this Raman line to the \(v_{Fe-S}\) mode. The \(v_{Fe-S}\) mode at 349 cm\(^{-1}\) is within the range of those of P450s (\(v_{Fe-S} = 344 \sim 356\) cm\(^{-1}\)) (13, 27, 33, 35) and higher than those of nitric oxide synthases (NOSs) (\(v_{Fe-S} = 337 \sim 342\) cm\(^{-1}\)) (36, 37). The environment around the axial thiolate ligand of P450c21 could be of the typical P450 type. It should be stressed that the \(v_{Fe-S}\) mode was insensitive to the structures of the substrates. We therefore suggest that the environment around the proximal thiolate ligand is essentially the same between Prog- and 17-OH-prog-bound ferric P450c21.

**TABLE 1**

| Frequency (cm\(^{-1}\)) of the heme marker lines for P450c21 |
|---------------------------------|
| **Sample**     | \(v_{\alpha}\) | \(v_{\beta}\) | \(v_{\alpha 10}\) | **State**           |
|-----------------|----------------|---------------|-----------------|---------------------|
| **Ferric P450c21** |                |               |                 |                     |
| Substrate-free  | 1371           | 1501          | 1581            | Six-coordinate/low spin |
| With Prog       | 1368            | 1487          | 1567            | Five-coordinate/high spin |
| With 17-OH-prog | 1368            | 1487          | 1567            | Five-coordinate/high spin |
| **Ferrous P450c21** |                |               |                 |                     |
| Substrate-free  | 1344           | 1467          | 1561            | Five-coordinate/high spin |
| With Prog       | 1344           | 1467          | 1561            | Five-coordinate/high spin |
| With 17-OH-prog | 1344           | 1467          | 1561            | Five-coordinate/high spin |
| **Oxy-P450c21** |                |               |                 |                     |
| With Prog       | 1371           | 1501          | 1582            | Six-coordinate/low spin |
| With 17-OH-prog | 1371           | 1501          | 1582            | Six-coordinate/low spin |

\(\alpha\) The \(v_{\alpha}\) line for the five-coordinate/high spin state was detected at 1368 cm\(^{-1}\) upon excitation at 363.8 nm (data not shown). The intense Raman line observed at 1371 cm\(^{-1}\) with 406.7-nm excitation (Fig. 3, a, b, and c) is supposed to be the contribution from the six-coordinate/low spin species. Such an observation was reported in other P450s (13, 64).

\(\beta\) Due to the significant contribution from the 6-coordinate/low spin species, the peak of the \(v_{\alpha}\) line for the five-coordinate/high spin state is not clear in Fig. 3a, b. Its peak top was determined from polarization experiment (data not shown).

\(\gamma\) ND, not determined.

**FIGURE 4. Low frequency region of RR spectra for ferric P450c21 with 363.8-nm excitation.** Traces shown were obtained from ferric P450c21 without substrate (a) and with Prog (b) and 17-OH-prog (c) and the buffer used in this measurement (d). The Raman lines arising from glycerol in the buffer are denoted by asterisks. The inset shows the Fe-S stretching frequency region of RR spectra for ferric Prog-bound P450c21 with 406.7 nm (e) and 363.8-nm excitation (f). The spectra were taken at \(-4\) °C. The laser power was 2 milliwatts at the sample point. The sample condition is the same as described in the legend to Fig. 3.

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substrate-free ferrous enzyme is off the pathway for catalysis, we examine its environment of the heme for comparison with that of the substrate-bound enzyme.

Despite the addition of an ~100-fold molar excess amount of dithionite to ferric substrate-free P450c21, we could not obtain a completely reduced form of P450c21 but produced a partially reduced form, as judged from the absorption spectrum (data not shown). Therefore, the RR spectrum of ferrous substrate-free P450c21 (Fig. 5a) was obtained by subtracting the contribution of the ferric enzyme. The $\nu_4$ vibration is observed in the lower frequency at 1344 cm$^{-1}$ than those of other heme proteins whose axial ligand is histidine ($\nu_3$; 1354–1359 cm$^{-1}$), indicating a strong Fe$^{2+}$-d$\pi$ back bonding to the porphyrin ring and a presence of the strong electron donation from axial thiolate to the heme iron (38). The $\nu_3$, $\nu_2$, and $\nu_{10}$ modes at 1467, 1561, and 1602 cm$^{-1}$, respectively, indicate that the heme adopts a five-coordinate/high spin structure and is typical of P450 in the ferrous state (26, 27).

The substrate-bound forms were easily reduced by the addition of dithionite solution. As reported in the P450cam study (39), the redox potential of the Fe$^{3+}$/Fe$^{2+}$ couple of the substrate-bound form with a high spin heme is supposed to be higher than that of the substrate-free form with a low spin heme. The RR spectra of both ferrous Prog- and 17-OH-prog-bound P450c21 are superimposable to that of substrate-free P450c21 (Fig. 5A) and are characteristic of ferrous five-coordinate/high spin heme. There is no clear difference between the RR spectra of Prog- and 17-OH-prog-bound ferrous P450c21s.

We also measured the low frequency region of the RR spectra for ferrous P450c21 (Fig. 5B). As described above, the RR spectrum of ferrous substrate-free P450c21 was obtained by subtracting the contribution of the ferric enzyme. The $\nu_\alpha$, $\delta(C_pC_\alpha)$ (bending mode of the heme propionate groups), $\delta(C_pC_\beta)$ (bending mode of the heme vinyl groups) and $\nu_7$ modes were observed at 347, 381, ~410, and 677 cm$^{-1}$, respectively, for ferrous substrate-free P450c21. Contrary to the ferric state, the Raman lines observed in the low frequency region were shifted by substrate binding, as shown in Fig. 5B. Because the $\delta(C_pC_\alpha)$ mode is sensitive to the hydrogen bonding interaction between heme propionate groups and surroundings (31, 32), the substrate-induced downshift of the $\delta(C_pC_\alpha)$ mode by 5 cm$^{-1}$ indicates that the substrate could alter the environment around the heme peripheral groups in the ferrous state. The substrate-induced frequency shifts were also detected in the $\delta(C_pC_\beta)$ mode. In the substrate-bound form, the $\delta(C_pC_\beta)$ modes were observed at 408 and 419 cm$^{-1}$, whereas the peak position was ~410 cm$^{-1}$ in substrate-free P450c21. These observations indicate that the substrate affects the environment of the heme peripheral groups in the ferrous state but not in the ferric state. Although this oxidation-state-dependent response to the substrate binding is an interesting issue, we have no clear explanation for it at this moment. On the basis of the fact that the substrate approaches closer to the heme iron upon reduction from the ferric to ferrous state in CYP102 (40), we speculate that the location of the substrate in the heme pocket of the ferrous state might be different from that of the ferric state in P450c21. However, it should be emphasized that the effects of substrate binding on the heme peripheral groups are conserved between Prog and 17-OH-prog in ferrous P450c21, which is evident from the same spectra of ferrous Prog- and 17-OH-prog-bound P450c21s.

**Raman Characterization of Oxy-P450c21**—After the formation of the ferrous state, the binding of dioxygen to the heme iron produces metastable oxygen adduct (23). The successive intermediates in the catalytic cycle, such as hydroperoxo and ferryl-oxo species, were not detected. Although the oxygen adduct is not a direct reaction intermediate responsible for the hydroxylation of substrates, the structural analysis of the oxygen adduct is useful to deduce the structural elements controlling the reactivity of P450 (41, 42). Therefore, we attempted to characterize oxy-P450c21 by spectroscopic techniques.

We tried to observe oxy-P450c21 with the substrate, Prog, by reacting the ferrous enzyme with O$_2$ on ice (21). As shown in Fig. 6A, we could obtain the species whose Soret peak and Q-band were observed at 418 and ~555 nm, respectively, at 4°C. This spectral feature is distinct from those of ferric ($\lambda_{\max}$ = 3712 JOURNAL OF BIOLOGICAL CHEMISTRY
~390 nm) and ferrous ($\lambda_{\text{max}} \approx 410$ nm) P450c21 with Prog (10) but quite similar to those of the oxygen complexes of P450cam (21) and NOS (43, 44). We therefore conclude that the optical absorption spectrum with the Soret maxima at 418 nm is derived from oxy-P450c21. Oxy-P450c21 was also detected for the alternative substrate (17-OH-prog)-bound form, and its Soret peak and Q-band were identical to those of Prog-bound oxy-P450c21 (data not shown). Unfortunately, oxy-P450c21, however, was not produced in the substrate-free enzyme due to the difficulty in the reduction of the ferric substrate-free form, as described above.

We further characterized the environment of the active site in oxy-P450c21 by using RR spectroscopy. Fig. 6B displays the high frequency region of the RR spectrum for Prog-bound oxy-P450c21 excited at 413.1 nm together with those of ferric and ferrous states. Again, the porphyrin marker lines, such as $v_4$, $v_3$, and $v_2$, indicated that the heme is five-coordinate/high spin in both the ferric and ferrous states. In oxy-P450c21 with Prog, $v_4$ at 1371 cm$^{-1}$ and $v_3$ at 1501 cm$^{-1}$ show that the heme adopts a ferric six-coordinate/low spin structure. Oxy-P450c21 with 17-OH-prog also has a ferric six-coordinate/low spin heme, which was confirmed by the $v_4$ and $v_3$ modes (data not shown). Oxy-P450c21, therefore, has a ferric superoxo character similar to the oxygen complexes of P450cam (45) and NOSs (46, 47).

To identify the O$_2$-related vibrational modes, we measured the RR spectra for oxy-P450c21 with $^{16}$O$_2$ and $^{18}$O$_2$ (Fig. 7, A and B). As evident from the difference spectrum obtained from the isotope substitution for O$_2$, three isotope-sensitive bands were obtained in the spectrum for oxy-P450c21 with Prog (Fig. 7A, a). We assigned the intense isotope-sensitive band at 1137 cm$^{-1}$ to the $v_{\text{O-O}}$ mode, because the intense $v_{\text{O-O}}$ mode around $\sim 1135$ cm$^{-1}$ was reported in P450cam (45, 48, 49) and NOSs (41, 42, 46, 47). The 66-cm$^{-1}$ downshift upon the isotope substitution is in good agreement with the theoretical value ($\Delta v_{\text{O-O}} = 65$ cm$^{-1}$) expected for a diatomic harmonic oscillator, further supporting our assignment. The other isotope-sensitive bands around $\sim 533$ and $\sim 393$ cm$^{-1}$ (Fig. 7B, a) are assignable to the $v_{\text{FeOO}}$ and $\delta_{\text{FeOO}}$ modes, respectively, from the fact that the $v_{\text{FeOO}}$ and $\delta_{\text{FeOO}}$ modes are observed at 540 and 410 cm$^{-1}$, respectively, for the oxygen complex of P450cam (45, 48, 50). These assignments for the O$_2$-related modes are
TABLE 2
Frequencies (cm$^{-1}$) of the O$_2$-related vibrations for oxy-P450c21 and the oxygen adducts of heme-thiolate complexes

| Sample                      | $\nu_{O-O}$ | $\nu_{Fe-O}$ | $\delta_{FeOO}$ | Reference |
|-----------------------------|-------------|--------------|-----------------|-----------|
| P450c21/Prog                 | 1137        | 533          | 393             | This study|
| P450c21/17-OH-prog           | 1124        | 529          | 394             | This study|
| P450cam/o-camphor            | 1138        | 540          | 401             | 45, 48-50 |
| P450cam/adamanantone         | 1139        | 537          | NR              | 45        |
| D251N P450cam/o-camphor      | 1136        | 536          | 399             | 48        |
| D251N P450cam/o-camphor + Pdx$^6$ | 1129 | 521          | 399             | 48        |
| iNOSox$^x$                  | 1137        | 536          | 399             | 48        |
| iNOSox/$\lambda$-Arg        | 1126        | NR           | 42              |          |
| iNOSox$^{N-hydroxy-Arg}$    | 1132        | NR           | 42              |          |
| nNOSox$^c$                  | 1135        | NR           | 19, 47          |          |
| nNOSox/$\lambda$-Arg        | 1129        | NR           | 19, 47          |          |
| nNOSox$^{N-hydroxy-Arg}$    | 1132        | NR           | 19, 47          |          |
| saNOS$^f$                   | 1135        | 517          | NR              | 46        |
| saNOS/$\lambda$-Arg         | 1135        | 517          | NR              | 46        |
| saNOS$^{N-hydroxy-Arg}$      | 1135        | 530          | NR              | 41        |
| bsNOS/$\lambda$-Arg$^g$     | 1135        | 517          | NR              | 41        |
| bsNOS$^{N-hydroxy-Arg}$      | 1137        | 530          | NR              | 41        |
| TpVp(C$_5$H$_5$S)$_2$         | 1140        | 527          | NR              | 59        |
| TpVp(C$_8$F$_5$S)$_2$        | 1147        | 536          | NR              | 59        |

$^x$ NR, not reported.
$^y$ Pdx, putidaredoxin, an electron donor for P450cam.
$^f$ iNOSox, oxygenase domain of inducible NOS.
$^g$ nNOSox, oxygenase domain of neuronal NOS.
$^h$ This peak was observed as a shoulder peak.
$^i$ saNOS, NOS from S. aureus.
$^j$ bsNOS, NOS from Bacillus subtilis.
$^k$ TPvP, picket fence porphyrin.

summarized in Table 2 together with those of the heme-thiolate complexes.

We also measured RR spectrum for oxy-P450c21 with 17-OH-prog to examine the effects of the structural difference in the substrates on the FeOO moiety. In oxy-P450c21 with 17-OH-prog, the $\nu_{O-O}$ mode was observed at 1138 cm$^{-1}$ (Fig. 7A, c) like that observed for the enzyme with Prog (Fig. 7A, a). Interestingly, in addition to this, a new Raman line appeared at 1124 cm$^{-1}$, which was downshifted to 1059 cm$^{-1}$ in the 18O$_2$ complex, as shown in Fig. 7A, d. The size of the isotope shift ($\Delta \nu_{O-O} = 65$ cm$^{-1}$) allows us to assign this Raman line to the second $\nu_{O-O}$ mode. Unlike the $\nu_{O-O}$ mode, we could not detect the clear split signals of the $\nu_{Fe-O}$ ($\sim 529$ cm$^{-1}$) and $\delta_{FeOO}$ ($\sim 394$ cm$^{-1}$) modes, but the $\nu_{Fe-O}$ mode might be downshifted in 17-OH-prog-bound oxy-P450c21 (Fig. 7B, d). No significant line broadening in the $\nu_{Fe-O}$ mode of 17-OH-prog-bound P450c21 compared with that of Prog-bound P450c21 suggests single $\nu_{Fe-O}$ mode in 17-OH-prog-bound P450c21, whereas it is difficult to judge from the current data due to the low resolution around the $\nu_{Fe-O}$ mode. Thus, the O$_2$-related vibrations, especially the $\nu_{O-O}$ mode, are sensitive to the structure of substrates, which might be associated with the different reactivities of P450c21 with each substrate.

DISCUSSION

In this study, we found that bovine P450c21 showed different catalytic coupling with consumption of NADPH in the hydroxylation of Prog and 17-OH-prog. In relation to these different reactivities of P450c21 to Prog and 17-OH-prog, RR spectra revealed structural differences between Prog- and 17-OH-prog-bound P450c21. In particular, a marked difference was detected regarding the $\nu_{O-O}$ mode of oxy-P450c21 in two different substrate-bound forms. Below, we discuss the structure of the heme active site in oxy-P450c21 with two different substrates and its relationship to the enzymatic property of bovine P450c21.

Heme Active Site Structure of Oxy-P450c21—Our current observation of the split $\nu_{O-O}$ mode at 1124 and 1138 cm$^{-1}$ indicates that there are two conformations of the FeOO moiety in 17-OH-prog-bound oxy-P450c21 (Fig. 7A). In the Prog-bound form, on the other hand, the FeOO moiety adopts one conformation, which is expected from the single $\nu_{O-O}$ mode at 1137 cm$^{-1}$ (Fig. 7A). From the peak position of the $\nu_{O-O}$ mode, we can conclude that the component with higher $\nu_{O-O}$ mode (1137–1138 cm$^{-1}$), which is designated as conformation A, is insensitive to the structure of two different substrates, but the other component with lower $\nu_{O-O}$ mode (1124 cm$^{-1}$), conformation B, is specific to the 17-OH-prog-bound form. In sharp contrast to the $\nu_{O-O}$ mode, no corresponding shift of the $\nu_{Fe-O}$ mode was observed (Fig. 7B). Thus, 17-OH-prog modulates the $\nu_{O-O}$ mode in conformation B, but not $\nu_{Fe-O}$.

Previous Raman studies on the oxygen complex of heme-containing proteins revealed that hydrogen-bonding interactions between the oxygen ligand and surroundings are a major determinant for the $\nu_{O-O}$ and $\nu_{Fe-O}$ modes (41, 42, 51–54). The hydrogen-bonding interaction with the terminal oxygen atom affects the $\nu_{O-O}$ mode rather than the $\nu_{Fe-O}$ mode, whereas the hydrogen bonding to the proximal oxygen atom affects the $\nu_{Fe-O}$ mode (41, 51, 54). For example, in the case of NOS from Staphylococcus aureus, Chartier et al. (46) found that the substrate ($\lambda$-Arg)-bound form gave a new $\nu_{O-O}$ mode at 1123 cm$^{-1}$ in addition to that at 1135 cm$^{-1}$, which was also observed in the substrate-free form. However, the $\nu_{Fe-O}$ mode at 517 cm$^{-1}$ was not perturbed by the $\lambda$-Arg binding (46). The low frequency shift of the $\nu_{O-O}$ mode by 12 cm$^{-1}$ in the presence of the substrate, $\lambda$-Arg, in S. aureus NOS was interpreted in terms of an increase in $\pi$ back-bonding interaction by which $\lambda$-Arg can hydrogen-bond to the terminal oxygen in the oxygen complex (41, 46). Similar to the oxygen complex of S. aureus NOS in the presence of $\lambda$-Arg, the terminal oxygen can be hydrogen-bonded by the substrate or surrounding amino acid residues in the conformation B of 17-OH-prog-bound oxy-P450c21. As compared with Prog, 17-OH-prog has an additional hydroxyl group at C-17 (Fig. 1), and this hydroxyl group would be in proximity to the heme iron, which is suggested from more effective spin conversion of the ferric heme from low to high spin upon 17-OH-prog binding than that induced by Prog binding (Fig. 3A). We therefore suggest that the hydroxyl group at C-17 in 17-OH-prog could form a hydrogen bond to the terminal oxygen atom, thereby inducing the low frequency shift of the $\nu_{O-O}$ mode by 13–14 cm$^{-1}$ without shifting the $\nu_{Fe-O}$ in conformation B as compared with that in conformation A.

Another possible factor affecting the $\nu_{O-O}$ mode may be the steric effect imposed by a substrate (45). Hu et al. reported that P450cam with the substrate analogue, adamanantone, showed splitting of $\nu_{O-O}$ into those at 1139 and 1147 cm$^{-1}$, whereas that with the substrate, n-camphor, gave a single $\nu_{O-O}$ at 1140 cm$^{-1}$.
FIGURE 8. Possible models for the heme active site of bovine oxy-P450c21 based on the Raman data. A, a structural model for 17-OH-prog-bound oxy-P450c21. This model was created by using the crystal structure of the oxygen complex of P450eryF (CYP101A; Protein Data Bank code 1Z8O) (55) as a template (gray stick). The orientation of 17-OH-prog (white stick) was determined under restrictions. (i) C-21 of 17-OH-prog is in close proximity to C-6 of 6-deoxyerythronolide B (6-DEB), a hydroxylation site by P450eryF. (ii) 17-OH group of 17-OH-prog can be spatially overlapped with the 5-OH group of 6-deoxyerythronolide B, which forms a hydrogen bond to the terminal oxygen atom of the oxygen ligand of heme. B, schematic presentation of the plausible structure of the heme active site of bovine oxy-P450c21. The dotted lines indicate the presumed hydrogen bonds. The conformation A in the 17-OH-prog-bound enzyme has the same conformation of the FeOO moiety as that in the Prog-bound enzyme. In conformation B, the hydroxyl group at C-17 of the substrate forms a hydrogen bond to the terminal oxygen atom, yielding the lower νO−O species with effective O−O bond scission.

Contrary to the case of NOSs, the alteration of the νO−O mode with different substrates in P450cam was interpreted as a steric effect (45), because both adamantanol and d-camphor do not have a hydroxyl group. 17-OH-prog, which is larger than Prog, might also impose the steric hindrance to the oxygen ligand, leading to the negative shift of the νO−O mode.

Accordingly, we then tested our model, in which the 17-OH group forms a hydrogen bond to the terminal oxygen atom, by constructing a structural model for oxy-P450c21 with 17-OH-prog (Fig. 8A). To construct the model for oxy-P450c21, we utilized the crystal structure of oxygen complex of P450eryF in which the hydroxyl group of substrate forms a hydrogen bond to the terminal oxygen atom (55). The position of 17-OH-Prog was determined under the following restrictions. (i) C-21 of 17-OH-prog is in close proximity to C-6 of 6-deoxyerythronolide B, a hydroxylation site by P450eryF. (ii) 17-OH group of 17-OH-prog can be spatially overlapped with 5-OH group of 6-deoxyerythronolide B, which forms a hydrogen bond to the terminal oxygen atom of the oxygen ligand of heme. As shown in Fig. 8A, 17-OH-prog can adopt the orientation supporting the present model, in which the 17-OH group forms a hydrogen bond to the terminal oxygen atom. Although we cannot exclude the possibility that the steric effect caused by substrate affects the νO−O mode in oxy-P450c21 in the absence of further detailed structural information, such as crystal structure, it is more plausible that the hydrogen bond from the substrate induces negative shift of the νO−O mode in this case.

In summary, we propose a possible structure of the heme active site in bovine oxy-P450c21 with Prog or 17-OH-prog, as illustrated in Fig. 8B. In the Prog-bound form, which corresponds to the conformation A, the oxygen ligand has no hydrogen-bonding interaction with the substrate. In the 17-OH-prog-bound form, conformation A takes essentially the same structure as that of the Prog-bound form. In conformation B, on the other hand, the hydroxyl group at C-17 in 17-OH-prog interacts with the terminal oxygen atom through hydrogen bonding. Consequently, the νO−O mode of conformation B is lower than that of conformation A by 13–14 cm⁻¹. In addition, the terminal oxygen atom possibly interacts with a water molecule and/or conserved distal Thr involved in the proton shuttle machinery for the O−O bond scission in both conformations. Although the Thr mutant of P450cam, in which the proton shuttle machinery is perturbed, mainly produced H₂O₂ instead of the hydroxylated product (58), P450c21 produced H₂O₂ only 26 and 15% of NADPH consumed for the hydroxylation of Prog and 17-OH-prog, respectively. It is highly plausible that the proton shuttle machinery is conserved in both conformations A and B.

It should be noted here that the correlation between the νO−O and νFe−OO modes for the oxygen complex may be positive in the thiolate-ligated heme, as can be seen in Fig. 9, which was also reported earlier by Chartier et al. (41, 46), contrary to Fe-CO and Fe-NO species (59). No negative correlation between the νO−O and νFe−OO modes was found for all heme proteins with a histidine ligand (51, 60). Chartier and Couture (41, 46) recently proposed that the positive correlation between the νO−O and νFe−OO modes could be derived from the combined effects of the π back-bonding interaction and the σ-competition by the axial thiolate ligand. In other words, the strength of the iron-thiolate bond, in addition to the heme distal environment, could be modulated by the binding of substrate. However, we could not detect any difference in the νFe−S mode between Prog- and 17-OH-prog-bound ferrie P450c21 as shown in Fig. 4. Although the νFe−S mode of the ferric state might not reflect the strength of the iron-axial thiolate bond in the oxygen complex, it is unlikely that the substrate modulates the strength of the iron-axial thiolate bond in P450c21.
Interaction between Substrate and O₂ Ligand in P450c21

Functional Implication for the Substrate-dependent Environment of the FeO₂ Moiety—The formation of the uncoupling product, such as H₂O₂, should reflect the efficiency of the O–O bond scission. Earlier studies on NOSs suggested that the P₄₅₀ hydroxylation than that of Prog. In NOSs, 17-OH-prog facilitates the O–O bond scission. This observation could be explained by the presence of conformation B with the weaker ν(O–O) mode in 17-OH-prog-bound oxy-P₄₅₀c21 than that of the Prog-bound form, because recent studies on NOSs suggested that the ν(O–O) mode is a good indicator of the reactivity of oxygen adduct (41, 42). In NOSs, the Arg binding induces the low frequency shift of the ν(O–O) mode or increases in population of the component with lower ν(O–O) mode, which is suggested to be responsible for the easier O–O bond scission responsible for the formation of H₂O₂ (62, 63). Thus, it is likely that this view is also supported by study of P₄₅₀cam. The binding of the redox reagent, putidaredoxin, to the D251N mutant of P₄₅₀cam increases the species with lower ν(O–O) mode (48). This putidaredoxin-induced structural change in P₄₅₀cam was supposed to be related to an effector function of putidaredoxin, which possibly promotes the electron transfer to oxy-P₄₅₀cam and the hydroxylation of the substrate through the terminal oxygen atom and promotes the O–O bond scission for more effective hydroxylation than that of Prog.

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