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Raman Evidence for Specific Substrate-Induced Structural Changes in the Heme Pocket of Human Cytochrome P450 Aromatase during the Three Consecutive Oxygen Activation Steps[†]

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ABSTRACT: Specific substrate-induced structural changes in the heme pocket are proposed for human cytochrome P450 aromatase (P450arom) which undergoes three consecutive oxygen activation steps. We have experimentally investigated this heme environment by resonance Raman spectra of both substrate-free and substrate-bound forms of the purified enzyme. The Fe–CO stretching mode ($\nu_{\text{Fe-CO}}$) of the CO complex and Fe³⁺–S stretching mode ($\nu_{\text{Fe-S}}$) of the oxidized form were monitored as a structural marker of the distal and proximal sides of the heme, respectively. The $\nu_{\text{Fe-CO}}$ mode was upshifted from 477 to 485 and to 490 cm^{−1} by the binding of androstenedione and 19-aldehyde-androstenedione, substrates for the first and third steps, respectively, whereas $\nu_{\text{Fe-CO}}$ was not observed for P450arom with 19-hydroxyandrostenedione, a substrate for the second step, indicating that the heme distal site is very flexible and changes its structure depending on the substrate. The 19-aldehyde-androstenedione binding could reduce the electron donation from the axial thiolate, which was evident from the low-frequency shift of $\nu_{\text{Fe-S}}$ by 5 cm^{−1} compared to that of androstenedione-bound P450arom. Changes in the environment in the heme distal site and the reduced electron donation from the axial thiolate upon 19-aldehyde-androstenedione binding might stabilize the ferric peroxo species, an active intermediate for the third step, with the suppression of the formation of compound I (Fe⁴⁺=O porphyrin⁺) that is the active species for the first and second steps. We, therefore, propose that the substrates can regulate the formation of alternative reaction intermediates by modulating the structure on both the heme distal and proximal sites in P450arom.

Cytochrome P450 aromatase (P450arom),¹ CYP19, catalyzes the final step for the biosynthesis of estrogens (1, 2). The enzyme is widely distributed in gonads and extragonadal tissues, including ovary, testis, brain, skin, and adipose tissue for the regulation of hormone balance (3, 4), and is related to several hormone-dependent diseases such as breast cancer (5–7). The P450arom-catalyzed reaction exhibits a complex nature consisting of three consecutive oxygen activation steps, converting C19 androgens such as androstenedione

(AD) and testosterone (Test) to aromatic C18 estrogenic steroids (1, 2, 8–10) as shown in Figure 1. The first and second oxidative steps are hydroxylations at C19 of androgens by a ferryl porphyrin π -cation radical species, which corresponds to compound I of heme proteins including peroxidases and P450s (11, 12). The third oxidative step cleaves the C10–C19 bond in the 19-aldehyde androgen, which is nonenzymatically produced by dehydration of 19-diol androgen, with the specific removal of the 1 β and 2 β hydrogens to aromatize the steroid A-ring and the release of formate (2, 9, 10). Several lines of studies on the mechanism for the third oxidative step propose that a ferric peroxo intermediate (Fe³⁺–OO[−]) but not compound I acts as an active species, and the nucleophilic attack of the ferric peroxo species on the carbonyl carbon of the aldehyde group triggers the third oxidative reaction (2, 9, 10, 13, 14).

Similar to the P450arom-catalyzed reactions, multiple reaction steps catalyzed by multiple reactive intermediates are reported for lanosterol 14 α -demethylase (CYP51) (15), progesterone 17 α -hydroxylase/17,20-lyase (CYP17) (16–18), and nitric oxide synthase (NOS) (19, 20). In addition, compound I or ferric peroxo species possibly works as an alternative intermediate in several P450s (21, 22). While the ferric peroxo species is now widely accepted as an additional

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¹ Abbreviations: P450arom, cytochrome P450 aromatase; AD, androstenedione; Test, testosterone; 19-aldo-AD, 19-aldehyde-AD; 19-OH-AD, 19-hydroxy-AD; RR, resonance Raman; 6CLS, 6-coordinate/low spin; 5cHS, 5-coordinate/high spin; P450cam, cytochrome P450 camphor; NOS, nitric oxide synthase; CPO, chloroperoxidase; P450 BM3, cytochrome P450 from *Bacillus megaterium*.

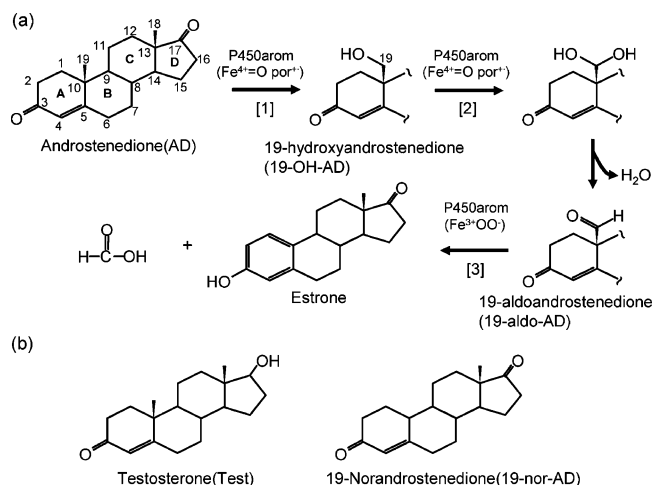


FIGURE 1: Proposed reaction mechanism for the aromatization of androstenedione by P450arom (a) and the chemical structures of a substrate and analogue used in this study (b). While a ferryl oxo porphyrin π cation radical ($\text{Fe}^{4+}=\text{O}$ porphyrin⁺), compound I, is supposed to be an active species for the first and second steps ([1] and [2]) in the aromatization reaction, the ferric peroxo species ($\text{Fe}^{3+}\text{OO}^-$) is proposed to act as the active species in the final step ([3]).

reaction intermediate in the P450 chemistry (23), the regulation mechanism for the formation of alternative reaction intermediates has been poorly understood.

To interpret the mechanism for the three consecutive oxygen activation steps in P450arom, Graham-Lorence et al. constructed the structural model for P450arom (24). They proposed that the 19-aldehyde androgen could induce the rearrangement of the conserved distal Thr, which is crucial for the formation of compound I, through a hydrogen-bonding interaction. Such displacement of the distal Thr could suppress the formation of compound I and stabilize the ferric peroxo species only in the third oxidative step (24). However, there is no structural evidence that the substrate modulates the distal heme environment for formation of the alternative reaction intermediates in P450arom. It is still unclear how P450arom regulates the formation of the alternative intermediates for the successive multiple oxidative steps mainly due to the lack of structural information on the heme active site in P450arom.

Although it is desirable to characterize the structure of the heme active site in P450arom for understanding the mechanism of the formation of alternative intermediates, so far, the difficulty in obtaining high-quality samples has hampered spectroscopic characterization of P450arom. Recently, Kagawa et al. developed an *Escherichia coli* expression system of human P450arom and purified it as a stable form (25, 26), allowing us to examine the heme active site structure by utilizing spectroscopic techniques.

In this study, we applied resonance Raman (RR) spectroscopy to structural characterization of human P450arom. RR spectroscopy is a powerful tool to explore the heme environment in the solution state and requires a moderate amount of sample (e.g., 100 μL of 50 μM solutions). Besides the porphyrin skeletal vibrations, which reflect the coordination structure and spin state of the heme (27, 28), the CO-associated vibrations in the CO adduct are useful to characterize the environment of the heme distal site and the property of an axial ligand (29–31). Furthermore, the iron–

axial ligand stretching mode ($\nu_{\text{Fe-S}}$) should serve as a structural marker at the heme proximal site. We found that the environments of both heme distal and proximal sides depend on the substrates by comparing the Raman lines for substrate-free with those of the substrate-bound forms of P450arom, where substrates include AD, Test, 19-hydroxy-AD (19-OH-AD), and 19-aldehyde-AD (19-aldo-AD) and an analogue (19-nor-AD), in which C19 is absent (Figure 1b). Because the distal environment and the strength of the Fe–S bond could be essential for the formation of a specific reaction intermediate (32), the finding in this study should produce new insights into the multiple oxidative reactions by P450s such as P450arom.

MATERIALS AND METHODS

Materials. Substrates 19-OH-AD and 19-aldo-AD were purchased from Steraloids. The solution of 45% (w/v) 2-hydroxypropyl- β -cyclodextrin in water was obtained from Sigma. Isotopically labeled CO, $^{13}\text{C}^{18}\text{O}$, was acquired from Cambridge Isotope Laboratories, Inc. (Andover, MA). All other reagents with highest purity were purchased from Wako (Osaka, Japan) and Nacalai tesque (Kyoto, Japan).

Expression and Purification of P450arom. Human P450arom is found to be polymorphic at residue 264 (Arg/Cys) (33). Both forms were expressed as functional P450arom but the Arg form is more stable (25, 26) and is used in subsequent studies. The *E. coli* expression and purification of P450arom were carried out as described (25, 26). The purified substrate-free P450arom in 100 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 1% Tween 20, 1% sodium cholate, 0.1 mM EDTA, and 0.1 mM DTT, was stored at -80°C .

Optical Spectroscopy. UV/vis absorption spectra were recorded on a U-3310 spectrometer (Hitachi). Each substrate was dissolved in 45% (w/v) 2-hydroxypropyl- β -cyclodextrin in water and stored as a 50 mM stock solution at 4°C . Substrates (10 μL of the stock solution) were added to 490 μL of substrate-free P450arom (10 μM) to generate substrate-bound forms. The ferrous–CO form of P450arom was produced by the addition of 1 μL of the dithionite solution (~ 100 mM) under CO atmosphere.

Resonance Raman Spectroscopy. RR spectra were obtained with a single polychromator (Jobin Yvon, SPEX750M) equipped with a liquid nitrogen-cooled CCD detector (Roper Scientific, Spec 10:400B/LN). The 406.7 nm line from a Kr⁺ laser (Spectra Physics, BeamLok 2060) and the 441.6 nm line from a He–Cd laser (Kinmon Electric, model CD4805R) were used for the excitation of ferric and ferrous–CO forms of P450arom, respectively. To observe the Fe³⁺–S stretching mode ($\nu_{\text{Fe-S}}$), the 363.8 nm line from an Ar⁺ laser (Spectra Physics, BeamLok 2080) was used as the excitation source. The laser power was adjusted to 1–2 mW at the sample point. Raman shifts were calibrated with indene, acetone, and carbon tetrachloride. All measurements were performed at $\sim 4^\circ\text{C}$ with a spinning cell (2000 rpm) for sample concentrations of 30–60 μM .

RESULTS

UV/vis Absorption Spectra of Ferric P450arom. To characterize the heme environment of P450arom with and without a substrate, we measured the UV/vis absorption

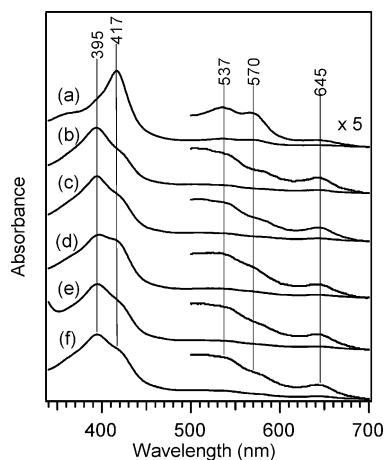


FIGURE 2: Optical absorption spectra of ferric P450arom in the absence (a) and presence of AD (b), Test (c), 19-OH-AD (d), 19-aldo-AD (e), and 19-nor-AD (f).

spectra. UV/vis absorption spectra for ferric forms of substrate-free P450arom and AD-, Test-, 19-OH-AD-, and 19-aldo-AD-bound P450arom are shown in Figure 2. As previously reported (25), substrate-free P450arom exhibits a Soret maximum at 417 nm, an α -band at 570 nm, and a β -band at 537 nm, indicating that the heme iron in substrate-free P450arom is in a 6-coordinate/low-spin (6cLS) state. The binding of substrates, AD, Test, and 19-aldo-AD, showed the transition of the coordination structure and the spin state of the heme iron to a 5-coordinate/high-spin (5cHS) state, which is evident from the blue shift of the Soret peak to \sim 395 nm and the appearance of a charge-transfer band at 645 nm (34). This structural transition of the heme is due to the dissociation of the sixth ligand such as a water molecule upon the binding of the substrates.

Contrary to the other substrate-bound forms of P450arom, the 19-OH-AD-bound enzyme exhibits a mixture of the 6cLS and 5cHS states (Figure 2d). Further addition of 19-OH-AD to P450arom showed no further spectral change (data not shown), indicating that the remaining 6cLS state did not arise from the substrate-free enzyme. It is, therefore, likely that in the 6cLS form the 19-hydroxyl group might stabilize a water ligand at the sixth coordination position through a hydrogen bond or that the 19-hydroxyl group might directly interact with the heme iron as the hydroxyl group of the product for P450cam weakly interacts with the heme iron (35).

We measured the UV/vis absorption spectrum of P450arom with 19-nor-AD, in which the C19 group is absent, to examine the role of the C19 group in the structural transition upon the binding of substrates. As shown in Figure 2f, the addition of 19-nor-AD also induced the formation of the 5cHS state. We can conclude that the C19 group is not crucial for the structural transition of the spin state and coordination structure in the heme upon substrate binding and suggest that the steroid ring could lie in close proximity to the heme iron.

Resonance Raman Spectra of Ferric P450arom. To obtain further insight into the structural environment of the heme of P450arom, we measured their RR spectra. Figure 3 displays RR spectra of ferric P450arom in the absence and presence of substrates and analogue with 406.7 nm excitation. In the high-frequency region of RR spectra (Figure 3A),

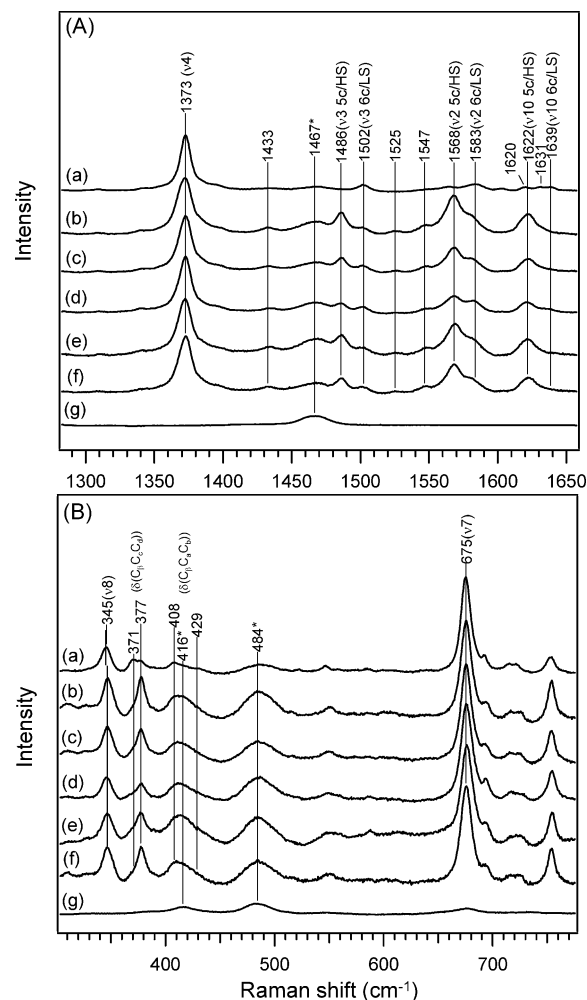


FIGURE 3: Resonance Raman spectra of ferric P450arom excited at 406.7 nm. Panels A and B represent the high- and low-frequency regions of the resonance Raman spectra, respectively. Traces shown were obtained from ferric P450arom in the absence of substrate (a) and in the presence of AD (b), Test (c), 19-OH-AD (d), 19-aldo-AD (e), and 19-nor-AD (f) and the spectrum of buffer used in this measurement (g). The Raman lines from glycerol in the buffer are denoted by an asterisk. The laser power was 1.5 mW at the sample point.

several porphyrin marker lines, ν_4 , ν_3 , ν_2 , and ν_{10} , are useful to characterize the heme active site structure (27, 28). For the substrate-free P450arom [Figure 3A(a)], the oxidation state marker, ν_4 , appears at 1373 cm^{-1} . The core size markers, ν_3 and ν_2 , which are sensitive to the spin and coordination states of the heme iron, are observed at 1502 and 1583 cm^{-1} , respectively. We assigned the Raman line at 1639 cm^{-1} to the ν_{10} vibration from its depolarized character in the polarization experiment (data not shown). The ν_4 , ν_3 , ν_2 , and ν_{10} vibrations at 1373, 1502, 1583, and 1639 cm^{-1} , respectively, are characteristic of the 6cLS heme. Consistent with the optical spectrum, these frequencies are indicative of the 6cLS state in the heme iron for substrate-free P450arom.

Although the Raman lines arising from the 6cLS heme were still detected as a minor component, the Raman lines at 1486, 1568, and 1622 cm^{-1} assignable to the ν_3 , ν_2 , and ν_{10} vibrations of the 5cHS heme, respectively, were predominant for the substrate- and analogue-bound forms of P450arom [Figure 3A(b–f)]. In 19-OH-AD-bound P450arom, the fraction of the 6cLS heme was relatively large, as

Table 1: Frequencies (cm^{-1}) of the Heme Marker Lines for P450arom and Other Thiolate-Ligated Enzymes

sample	ν_4	ν_3	ν_2	ν_{10}	state	ref
P450arom	1373	1502	1583	1639	6cLS	this study
+AD	1369 ^a	1486	1568	1622	5cHS	this study
+Test	1369 ^a	1486	1568	1622	5cHS	this study
+19-OH-AD	1369 ^a	1486	1568	1622	5cHS	this study
+19-aldo-AD	1373	1502	1583	1639	6cLS	this study
+19-nor-AD	1369 ^a	1486	1568	1622	5cHS	this study
P450cam	1373	1503	1584	1635	6cLS	34
+D-camphor	1368	1488	1570	1623	5cHS	34
P450 BM3	1374	1502	1583	1638	6cLS	46
+arachidonic acid	1372	1487	1570	nd ^b	5cHS	46
MtCYP51 ^c	1373	1504	1584	1638	6cLS	62
+2PhIm ^d	1372	1503	1583	1639	6cLS	62
	nd ^b	1488	nd ^b	nd ^b	5cHS	62
TXAS ^e	1373	1503	1584	1635	6cLS	54
+CTA ₂ ^f	1373	1503	1586	1638	6cLS	54
	1371	1487	1568	nd ^b	5cHS	54
eNOS ^g	1374	1503	1579	1635	6cLS	48
+L-Arg	1370	1489	1563	nd ^b	5cHS	48
CPO ^h	1374	1505	nd ^b	1640	6cLS	63
	1369	1490	1564	1627	5cHS	64

^a The Raman line at 1373 cm^{-1} observed with 406.7 nm excitation (Figure 3) is probably due to the contribution from the 6cLS species. The ν_4 vibration for the 5cHS state was detected at 1369 cm^{-1} upon excitation at 363.8 nm (data not shown). Such observation was also reported in other P450s (65). ^b nd: not determined. ^c Sterol 14 α -demethylase cytochrome P450 from *Mycobacterium tuberculosis*. ^d 2-Phenylimidazole. ^e Thromboxane synthase. ^f Carbocyclic thromboxane A₂. ^g Endothelial nitric oxide synthase. ^h Chloroperoxidase.

Table 2: Frequencies (cm^{-1}) of the Heme Skeletal and Peripheral Vibrations Observed in the Low-Frequency Region

sample	ν_8	$\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\alpha)$	$\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\beta)$	ν_7
P450arom	345	371, 377	408, 429	675
+AD	346	377	$\sim 416^a$	676
+Test	346	377	$\sim 416^a$	676
+19-OH-AD	346	377	$\sim 416^a$	676
+19-aldo-AD	346	377	$\sim 416^a$	676
+19-nor-AD	346	377	$\sim 416^a$	676

^a We cannot determine the specific peak position due to the overlapping with the Raman line from glycerol.

indicated from the optical spectrum. These assignments of Raman lines are summarized in Table 1 together with those of other P450s and NOSs.

In the low-frequency region (Figure 3B), we can detect the Raman lines arising from the vibrations including the heme peripheral groups as well as the porphyrin skeletal vibrations (36, 37). Thus, the Raman lines observed in the low-frequency region can provide information on the interaction between the heme prosthetic group and protein environment. The intense Raman lines at 675 and 345 cm^{-1} in substrate-free P450arom can be assigned to ν_7 and ν_8 , respectively [Figure 3B(a)]. By analogy to the Raman spectra of other heme proteins (36, 37), the Raman lines at 371 and 377 cm^{-1} are assigned to the bending modes of the heme propionate groups, $\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\alpha)$, and the Raman lines at 406 and 429 cm^{-1} can be assigned to the bending modes of the heme vinyl groups, $\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\beta)$. These assignments are summarized in Table 2.

As shown in Figure 3B, the binding of the substrates and analogue to P450arom results in the changes in the bending modes of the heme vinyl and propionate groups without noticeable changes in other Raman lines observed in this

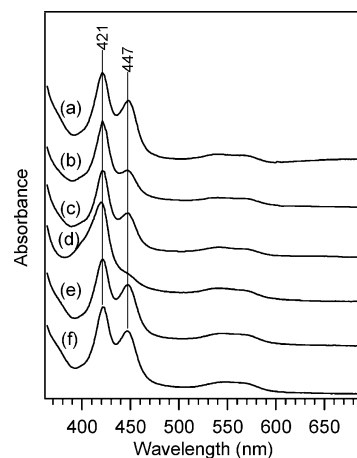


FIGURE 4: Optical absorption spectra of ferrous-CO P450arom in the absence (a) and presence of AD (b), Test (c), 19-OH-AD (d), 19-aldo-AD (e), and 19-nor-AD (f).

region. The peak position of the bending mode of the heme vinyl group cannot be determined due to the overlapping with the glycerol peak. The bending mode of the heme propionate at 371 cm^{-1} in substrate-free P450arom was missing while the peak at 377 cm^{-1} remains upon the addition of the substrates and analogue. It has been demonstrated that the frequency of the bending mode for the heme propionate is sensitive to the hydrogen-bonding interaction with the protein environment (38, 39) and that the disruption of the hydrogen-bonding network lowers the bending frequency of the heme propionate by $\sim 10 \text{ cm}^{-1}$ (38). On the basis of these studies, the Raman lines at 377 and 371 cm^{-1} can be assigned to the bending modes of heme propionate with and without hydrogen-bonding interaction with the protein environment, respectively. We, therefore, propose that substrate binding might generate a hydrogen-bonding network involving the heme propionate group.

It should be stressed that all substrates and analogue gave essentially the same spectral changes in the low-frequency region of RR spectra. While the UV/vis absorption and high-frequency region of RR spectrum for 19-OH-AD-bound P450arom showed that the coordination structure and the spin state were different from other substrate- and analogue-bound P450arom, its binding effect on the low-frequency Raman spectrum was similar to those in other substrate- and analogue-bound P450arom. This observation suggests that the binding mode for the steroid ring would be conserved among the substrates and analogue.

Resonance Raman Spectra of Ferrous-CO P450arom. Our current results on ferric P450arom indicate that the effects of substrate binding on the structural environment of the heme in P450arom are similar among the substrates and analogue. Each substrate-bound P450arom, however, exhibits different reactivity to the molecular oxygen and different sensitivity to the inhibition by CO (40), allowing us to speculate that the heme environment of P450arom would depend on the substrates. To confirm this, we measured the absorption spectra for the ferrous-CO form of P450arom and tried to detect the CO-associated vibrations that are sensitive to the distal environment and the electronic property of the proximal ligand (29–31).

Figure 4 illustrates the optical absorption spectra for the ferrous-CO forms of P450arom in the absence and presence

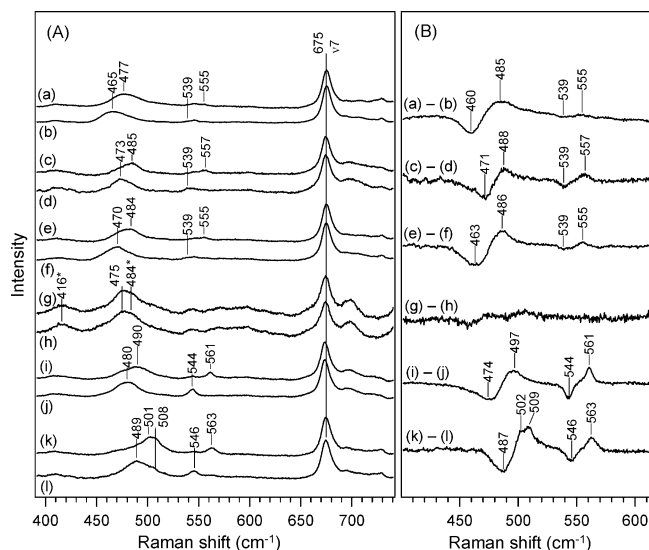


FIGURE 5: Low-frequency region of resonance Raman spectra of ferrous-CO P450arom with 441.6 nm excitation. Panel A represents the spectra obtained from $^{12}\text{C}^{16}\text{O}$ -bound P450arom in the absence of substrate (a) and in the presence of AD (c), Test (e), 19-OH-AD (g), 19-aldo-AD (i), and 19-nor-AD (k) and from $^{13}\text{C}^{18}\text{O}$ -bound P450arom in the absence of substrate (b) and in the presence of AD (d), Test (f), 19-OH-AD (h), 19-aldo-AD (j), and 19-nor-AD (l). The Raman lines from glycerol in the buffer are denoted by an asterisk in traces g and h. The laser power was 1 mW at the sample point. Panel B represents the difference spectra ($^{12}\text{C}^{16}\text{O} - ^{13}\text{C}^{18}\text{O}$) in the Fe-CO stretching and Fe-C-O bending frequency region.

of substrate. As shown in Figure 4a, the Soret peaks were observed at ~ 420 and ~ 450 nm in the substrate-free P450arom. The higher absorbance at ~ 420 nm than that at ~ 450 nm indicates that the inactive P420 form is a major species in the ferrous-CO state. Because the P420 form is not a major species in the ferric state, which was confirmed by the structural transition from 6cLS to 5cHS by the binding of substrate in the current work, it is plausible that the reduction of the heme iron could destabilize the thiolate coordination and increase in the population of the P420 form.

In AD- and Test-bound P450arom (Figure 4b,c), the population of the active P450 species was relatively low as compared with that of the substrate-free form. Interestingly, as shown in Figure 4d, the absorbance at ~ 450 nm was observed as a shoulder peak in 19-OH-AD-bound P450arom. Furthermore, the contribution of a 5-coordinated ferrous heme was also detected in 19-OH-AD-bound P450arom, which is evident from the shoulder peak at ~ 410 nm. In contrast to AD-, Test-, and 19-OH-AD-bound P450arom, the population of the active P450 species of ferrous-CO P450arom was comparable to that of substrate-free P450arom in the presence of 19-aldo-AD or 19-nor-AD (Figure 4e,f).

Although some fraction of P450arom is easily converted to the inactive P420 species upon reduction and substrates such as AD-, Test-, and 19-OH-AD-bound P450arom still contain the nonligated 5-coordinate ferrous state as shown in Figure 4, it should be noted here that the 441.6 nm line from a He-Cd laser can selectively excite the active form of ferrous-CO P450arom. Figure 5A illustrates the RR spectra for the ferrous-CO form of P450arom excited at 441.6 nm. In substrate-free P450arom [Figure 5A(a,b)], a broad line centered at 477 cm^{-1} and a weak line at 555 cm^{-1} were assigned to $\nu_{\text{Fe-CO}}$ and δ_{FeCO} , respectively, on the basis

Table 3: Frequencies (cm^{-1}) of the Iron-Ligand Vibrations for P450arom and Other Thiolate-Ligated Heme Proteins

sample	$\nu_{\text{Fe-CO}}$	δ_{FeCO}	$I(\delta_{\text{FeCO}})/I(\nu_{\text{Fe-CO}})$	$\nu_{\text{Fe-S}}$	ref
P450arom	477	555	0.17	nd ^a	this study
+AD	485	557	0.29	349	this study
+Test	484	555	0.29	349	this study
+19-OH-AD	nd ^a	nd ^a	nd ^a	346	this study
+19-aldo-AD	490	561	0.45	344	this study
+19-nor-AD	501	563	0.38	349	this study
P450cam	464	556	nr ^b	nd ^a	34
+D-camphor	481	558	nr ^b	351	34, 45
P450 BM3	471	558	nr ^b	nd ^a	66
+arachidonic acid	nr ^b	nr ^b	nr ^b	356	46
P450scc ^c	476	nr ^b	nr ^b	nr ^b	42
+cholesterol	482	nr ^b	nr ^b	nr ^b	42
+22(R)-OH-cholesterol	479	nr ^b	nr ^b	nr ^b	42
TXAS + CTA ₂	nr ^b	nr ^b	nr ^b	350	54
CPO	485	560	nr ^b	347	43, 47
eNOS + L-Arg	512	567	nr ^b	338	44, 48
iNOS ^d	487	560	nr ^b	nr ^b	44
+L-Arg	512	567	nr ^b	nr ^b	44
nNOS ^e	491	562	nr ^b	nr ^b	43
+L-Arg	503	566	nr ^b	nr ^b	43

^a nd: not determined. ^b nr: not reported. ^c Cytochrome P450 side-chain cleavage. ^d Inducible NOS. ^e Neuronal NOS.

of the difference spectrum obtained from the isotope substitution for CO [Figure 5, (a) - (b)], which is illustrated in Figure 5B.

The addition of substrates and analogue dramatically alters the peak positions and shapes for the Raman lines associated with CO as observed for other P450s (34, 41, 42) and NOSs (43, 44). The $\nu_{\text{Fe-CO}}$ line was shifted to 485 (for AD-bound P450arom) and 484 cm^{-1} (for Test-bound P450arom), and the δ_{FeCO} line was intensified by the addition of AD or Test [Figure 5A(c,e)]. On the other hand, in the 19-OH-AD-bound enzyme, neither $\nu_{\text{Fe-CO}}$ nor δ_{FeCO} lines were detected [Figure 5A(g)]. The isotope difference spectrum clearly shows this [Figure 5B, (g) - (h)]. Because the absorbance of the CO complex of 19-OH-AD-bound P450arom at ~ 450 nm was smaller than those of AD- and Test-bound P450arom (Figure 4), we could not detect the CO-associated modes. Therefore, it is ascribed to the low population of the CO complex for 19-OH-AD-bound P450arom.

The binding of 19-aldo-AD or 19-nor-AD induced an upshift of the $\nu_{\text{Fe-CO}}$ line by more than 10 cm^{-1} with remarkable intensification of the δ_{FeCO} line [Figure 5A(i,k)]. For 19-nor-AD-bound P450arom, two peaks were observed at 501 and 508 cm^{-1} in the $\nu_{\text{Fe-CO}}$ region [Figure 5A(k)]. Because the Raman line at 508 cm^{-1} was still detected in the $^{13}\text{C}^{18}\text{O}$ complex as a shoulder peak [Figure 5A(l)], it is likely that the Raman line at 508 cm^{-1} would be attributed to a porphyrin vibration but not to the Fe-CO vibration. The peak positions for $\nu_{\text{Fe-CO}}$ and δ_{FeCO} for P450arom in the absence and presence of the substrates and analogue are summarized in Table 3 together with those of thiolate-ligated enzymes. We have also tried to detect the $\nu_{\text{C-O}}$ band expected in the $1900\text{--}2000\text{ cm}^{-1}$ region. Unfortunately, we failed to observe the CO stretching modes for P450arom with and without substrates due to the strong fluorescence.

Iron-Axial Ligand Stretching Modes. Because the substrate binding site is in the heme distal site at close proximity

the geometry of the FeCO unit, the environment around CO might be more polar and positive, resulting in the upshift of $\nu_{\text{Fe-CO}}$ in AD- and Test-bound P450arom (Figure 7b). Because the hydroxyl groups of the conserved Thr and water molecules are observed around the oxygen atom of the FeCO moiety in the crystal structure of the CO complex of P450cam with its substrate (49, 50), the conserved Thr (Thr-310) and/or water molecules might be in close proximity to the CO ligand in AD- and Test-bound P450arom, which induces the upshift of $\nu_{\text{Fe-CO}}$. Considering that the hydroxyl group of Thr-310 possibly interacts with the aldehyde group in 19-aldo-AD-bound P450arom (see following discussion) (24), we can suggest that the water molecule rather than Thr-310 is present near the CO ligand in the AD- and Test-bound P450arom (Figure 7b).

Contrary to AD-, Test-, and 19-OH-AD-bound P450arom, the population of the ferrous-CO form in 19-aldo-AD-bound P450arom was comparable to those of substrate-free and 19-nor-AD-bound P450arom as seen in Figure 4. This observation raises the possibility that the orientation of the C19 group in 19-aldo-AD-bound P450arom would be different from those of other substrate-bound P450aroms and would not block the CO binding. Graham-Lorence et al. proposed that the conserved Thr (Thr-310) at the heme distal site could be hydrogen-bonded to the oxygen atom of the aldehyde group in 19-aldo-AD to enhance the positive character on the carbonyl carbon and promote the nucleophilic attack of the ferric peroxo species in the third oxidative step (24). It is, therefore, likely that the hydrogen-bonding interaction between 19-aldo-AD and Thr-310 could induce the displacement of the C19 group in the 19-aldo-AD-bound P450arom to reduce the steric hindrance for CO binding (Figure 7d). However, similar to AD- and Test-bound P450arom, the $I(\delta_{\text{FeCO}})/I(\nu_{\text{Fe-CO}})$ value in 19-aldo-AD-bound P450arom [$I(\delta_{\text{FeCO}})/I(\nu_{\text{Fe-CO}}) = 0.45$] was greater than that of substrate-free P450arom [$I(\delta_{\text{FeCO}})/I(\nu_{\text{Fe-CO}}) = 0.17$] (Table 3), indicating that the FeCO unit adopts a bent conformation (Figure 7d). It is, therefore, likely that 19-aldo-AD could still be located at close proximity to the CO ligand and that the positive character on the carbonyl carbon of 19C would induce the upshift of $\nu_{\text{Fe-CO}}$.

In 19-nor-AD-bound P450arom, the largest upshift of $\nu_{\text{Fe-CO}}$ indicates that the CO ligand could be in a highly polar environment. Wang et al. reported in the study on inducible NOS that the substrate, L-Arg, binding induces the upshift of $\nu_{\text{Fe-CO}}$ by 25 cm^{-1} (44), which is closely similar to that induced by the binding of 19-nor-AD in P450arom (24 cm^{-1}). The infrared study on the CO complex of inducible NOS by Jung et al. provided the evidence for the hydrogen-bonding interaction between ligated CO and L-Arg (51). We, therefore, suggest that the CO ligand could directly interact with the surrounding amino acid residue such as Thr-310 through the hydrogen-bonding interaction in 19-nor-AD-bound P450arom (Figure 7e). Because the C19 group is absent in contrast to the substrates, we can assume that the hydroxyl group of Thr-310 directly hydrogen-bonds to the CO ligand in 19-nor-AD-bound P450arom. Such hydrogen-bonding interaction would result in the bent conformation of the FeCO unit in 19-nor-AD-bound P450arom as depicted in Figure 7e, which could enhance the intensity of δ_{FeCO} . This view is consistent to the presence of the hydrogen bond between 19-aldo-AD and Thr-310.

Besides the changes in the distal environment, the lower electron donation from the axial thiolate might also contribute to the upshift of $\nu_{\text{Fe-CO}}$ in 19-aldo-AD-bound P450arom. While $\nu_{\text{Fe-S}}$ was observed at the ferric state, the $\nu_{\text{Fe-S}}$ mode would correlate with the $\nu_{\text{Fe-CO}}$ mode in some cases (43, 52, 53). Indeed, the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{C-O}}$ correlation plot showed that the electron donation from thiolate to the heme iron in NOSs ($\nu_{\text{Fe-S}} = 338\text{ cm}^{-1}$ for L-Arg-bound eNOS) is lower than those of P450s ($\nu_{\text{Fe-S}} = \sim 350\text{ cm}^{-1}$) (45, 46, 48, 52, 54) and that the $\nu_{\text{Fe-CO}}$ modes in NOSs were observed at the frequency region higher than those of P450s. On the other hand, a recent systematic study on P450 BM3 by Chen et al. reported that the $\nu_{\text{Fe-S}}$ frequency did not change in the series of mutants whose redox potentials varied over 180 mV (46), raising the possibility that the $\nu_{\text{Fe-S}}$ frequency would not be a reliable indicator of the electron-donating capacity of the thiolate ligand. This is in conflict with our proposal. However, because the redox potential is modulated not only by the electron donation from the axial ligand but also by several factors including the solvent accessibility at the redox cofactor and the redox state-dependent dynamics, the redox potential might not have a correlation with the $\nu_{\text{Fe-S}}$ mode in P450 BM3. Although further study is required for the clarification of the correlation between the electron-donating property of thiolate and the $\nu_{\text{Fe-S}}$ mode, the lower electron donation from the axial thiolate in addition to the more positive distal environment might result in the relatively higher $\nu_{\text{Fe-CO}}$ in 19-aldo-AD-bound P450arom than those of AD- and Test-bound P450aroms.

Implication for the Molecular Mechanism of P450arom Catalysis. The current data indicated that the distal and proximal environments were dependent on the substrates, providing new aspects of the P450arom-catalyzed reaction. One is that the substrate-dependent structural changes in the heme distal site possibly modulate the formation of the alternative reaction intermediates. While, contrary to the FeCO moiety, the FeOO moiety of the oxygen adduct generally adopts a bent conformation (55–59), the environment of the O₂ ligand also might be expected to depend on the substrates. The geometry difference in the FeOO moiety among different substrates might enable the enzyme to form the alternative reaction intermediates.

The view that the substrate-dependent structural rearrangement in the heme distal site regulates the formation of the alternative reaction intermediates can be consistent with the “Thr switch” model which was originally proposed by Graham-Lorence et al. (24). In the Thr switch model, 19-aldo-AD would induce the displacement of distal Thr-310 through the hydrogen-bonding interaction for the formation of ferric peroxo species instead of compound I (24). On the basis of the Thr switch model and our current results, we propose the heme active site structure of the oxygen complex of P450arom as shown in Figure 8. Because extensive mechanistic studies on P450cam demonstrated that compound I formation requires protonation of the distal oxygen atom in the oxygen complex by the water molecule hydrogen-bonded to the conserved Thr (56, 60, 61), the O₂ ligand would be hydrogen-bonded to the water molecule in the first and second oxidative steps in P450arom (Figure 8a). On the other hand, the plausible hydrogen-bonding interaction between the aldehyde group in 19-aldo-AD and Thr-310 could induce the displacement of Thr-310 and the water

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