Recent Progress in the Structural Understanding of Peroxisome Proliferator-Activated Receptor (PPAR)–Ligand Interaction

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that belong to the nuclear receptor superfamily comprising 48 members in human.\(^1\) PPARs include three subtypes, PPAR\(\alpha\), PPAR\(\gamma\), and PPAR\(\delta\). Although their tissue distributions, functional properties, and target genes for transcriptional regulation differ, they are all involved in glucose and lipid metabolism and their dysfunctions could lead to serious diseases such as hyperlipidemia, diabetes, and cardiovascular diseases. In 1998, the crystal structures of the PPAR\(\gamma\) ligand-binding domain (LBD) as a complex with intrinsic fatty acids derived from an expression host \(Escherichia\) (\(E\).) \(coli\) and thereafter replaces them with other higher-affinity ligands by soaking. Here we applied this method for preparation of co-crystals of PPAR\(\alpha\) LBD with its ligands that have not been obtained with the conventional co-crystallization method. We revealed the high-resolution structures of the cocrystals of PPAR\(\alpha\) LBD and the three synthetic phenylpropaonic acid derivatives: TIPP-703, APHM19, and YN4pai, the latter two of which are the first observations. The overall structures of cocrystals obtained from the two methods are identical and illustrate the close interaction between these ligands and the surrounding amino acid residues of PPAR\(\alpha\) LBD. This ligand-exchange soaking method could be applicable to high throughput preparations of co-crystals with another subtype PPAR\(\delta\) LBD for high resolution X-ray crystallography, because it also crystallizes in complex with intrinsic fatty acid(s) while not in the apo-form.

Key words nuclear receptor; crystal structure; ligand-exchange soaking method; peroxisome proliferator-activated receptor \(\alpha\); phenylpropaonic acid derivative; X-ray crystallography

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tionship (SAR) studies were conducted to explore compounds that activate two or three PPAR subtypes simultaneously.\(^{11}\) The SAR has been described for some compounds at atomic levels based on their high-resolution crystal structures.\(^{12}\) With such structural information, additional SAR study has been performed to design new partial agonists targeting PPAR\(\delta\) and PPAR\(\gamma\) that are expected to be free of adverse effects.\(^{13,14}\)

In the case of PPAR\(\alpha\), the novel compounds, which possess a molecular backbone different from phenylpropaonic acids, have been discovered to display PPAR\(\alpha\) agonist activity, and the development of candidate drugs based on the next SAR studies has been already started.\(^{15,16}\)

Cocrystallization of protein–ligand complexes is not always easy due to various reasons, even if the ligand has a high affinity towards the target protein.\(^{17}\) Particularly, PPAR\(\alpha\) and PPAR\(\delta\) have been difficult to be crystallized in their apo forms, which limited the successful protein–ligand complex crystallization. The numbers of structures of PPARs (mainly LBDs) deposited in the Protein Data Bank (PDBj: www.pdbj.org etc.) is limited to 55 and 43 for PPAR\(\alpha\) and PPAR\(\delta\), respectively, while that for PPAR\(\gamma\), the only which can be crystallized in its apo form, is 232 (as of February 17, 2021). Not all of our synthesized phenylpropaonic acid derivatives had been successfully crystallized in complexes with PPARs LBD, and there remain many compounds whose interactions with PPARs have not been resolved.

Under these circumstances, we have recently demonstrated that the recombinant PPAR\(\alpha\) LBD prepared from the *Escherichia coli* expression system maintains (through its purification process) certain amounts of host-derived fatty acids, which could act as intrinsic PPAR\(\alpha\) ligands, and that the fatty acid-bound PPAR\(\alpha\) LBD can be crystallized by cross-microseeding using the crystals complexed with WY-14643 as seeds.\(^{18,19}\) We also found that the bound fatty acids in the crystals can be replaced with other ligands by soaking the crystals in solutions containing the ligands with affinities higher than the fatty acids. This tempted us to further prepare other PPAR\(\alpha\) LBD crystals by this “ligand-exchange soaking method” (Fig. 2). Here we report the three PPAR\(\alpha\) LBD-phenylpropaonic derivative complex crystal structures, including two novel ones.

**MATERIALS AND METHODS**

**PPAR\(\alpha\) Ligands** The three phenylpropaonic acid derivatives, APHM19, TIPP-703, and YN4pai, were synthesized. Figure 1 illustrates their chemical structures and the EC\(_{50}\) values for each PPAR subtype that have been estimated from PPAR-GAL4 chimeric reporter assays. We followed the structure-unit definitions that have been used for developing several compounds based on the SAR study.\(^{10}\) The hydrophilic unit containing the carboxy group that interacts with the Tyr sidechain (residue 464 for PPAR\(\alpha\), 473 for PPAR\(\gamma\) and PPAR\(\delta\)) on H12 is designated as the “head”; the hydrophobic part located on the opposite side as the “tail”; the part protruding from the central phenyl group as the “branch” (n-alkoxy groups in this study); and a connecting unit between the central phenyl and the tail part as the “linker.” PPARs possess the Y-shaped ligand-binding pockets, with the head and branches attached to the left and right arms of “Y,” and the tail located in the leg part of Y.\(^{22}\)

APHM19 is one of the few derivatives that retain the same linker backbone as KCL, and was synthesized to possess a bulkier hydrophobic unit in the tail with two fluorophenyl groups in tandem connected by an oxygen atom. While KCL and APHM19 are PPAR\(\alpha\)-specific, the replacement of the linker from –CO–NH–CH\(_3\) (head to tail) to –CH\(_3\)–NH–CO– enabled them to acquire the PPAR\(\alpha\)/PPAR\(\delta\) dual agonist ac-
Fig. 2. Ligand-Exchange Soaking
(a) PPARα LBD is co-crystallized with endogenous fatty-acids; (b) the obtained crystals are soaked into solutions containing the ligands with higher affinities; (c) the exchanged ligands are visible in the electron density maps. (Color figure can be accessed in the online version.)

Table 1. X-Ray Diffraction Data Collection and Refinement

| Crystal | PPARα LBD−TIPP-703 (soaking) | PPARα LBD−APHM19 (cocystal) | PPARα LBD−APHM19 (soaking) | PPARα LBD−YN4pai (soaking) |
|---------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Data collection | | | | |
| Beams line | SPring-8 BL26B1 | SPring-8 BL26B1 | SPring-8 BL26B1 | SPring-8 BL26B1 |
| Detector | EIGER X 4M | EIGER X 4M | EIGER X 4M | EIGER X 4M |
| Wavelength (Å) | 1.0000 | 1.0000 | 1.0000 | 1.0000 |
| Space group | P2₁ | P2₁ | P2₁ | P2₁ |
| Unit cell constants | | | | |
| a (Å) | 44.89 | 44.60 | 44.72 | 44.84 |
| b (Å) | 62.16 | 62.92 | 62.00 | 61.80 |
| c (Å) | 53.37 | 53.15 | 53.12 | 53.13 |
| β (deg) | 105.78 | 106.54 | 106.50 | 106.16 |
| Resolution (Å) | 50.0–1.79 (1.83–1.79) | 50.0–1.66 (1.69–1.66) | 50.0–1.58 (1.61–1.58) | 50.0–1.66 (1.69–1.66) |
| Average mosaicity (deg) | 0.16 | 0.50 | 0.09 | 0.07 |
| No. of total reflections | 87784 (5480) | 107560 (5074) | 130074 (6682) | 113535 (5551) |
| No. of unique reflections | 26328 (1548) | 32593 (1569) | 37911 (1873) | 32994 (1590) |
| Rmerge | 0.029 (0.285) | 0.027 (0.306) | 0.025 (0.316) | 0.046 (0.321) |
| Rmeas | 0.041 (0.393) | 0.037 (0.425) | 0.035 (0.445) | 0.063 (0.440) |
| Rfree | 0.022 (0.214) | 0.025 (0.294) | 0.025 (0.314) | 0.044 (0.300) |
| Completeness (%) | 98.6 (99.4) | 99.2 (97.8) | 99.2 (99.1) | 99.9 (100.0) |
| Completeness (%) | 98.6 (99.4) | 99.2 (97.8) | 99.2 (99.1) | 99.9 (100.0) |
| Redundancy | 3.3 (3.5) | 3.3 (3.2) | 3.4 (3.6) | 3.4 (3.5) |
| CC1/2 | 0.999 (0.924) | 0.999 (0.894) | 0.999 (0.912) | 0.997 (0.906) |
| tσ(f) | 12.3 (2.7) | 19.6 (3.4) | 12.8 (2.3) | 14.1 (3.8) |
| Refinement | | | | |
| Resolution range (Å) | 50.0–1.79 | 50.0–1.66 | 50.0–1.58 | 50.0–1.66 |
| Rwork/Rfree | 0.1960/0.2289 | 0.2028/0.2389 | 0.2068/0.2210 | 0.2038/0.2290 |
| No. of atoms | 2249 | 2299 | 2312 | 2269 |
| Protein | 2105 | 2162 | 2169 | 2118 |
| Ligand | 37 | 34 | 34 | 38 |
| Water | 107 | 103 | 109 | 113 |
| r.m.s.d. | | | | |
| Bond lengths (Å) | 0.005 | 0.013 | 0.007 | 0.005 |
| Angles (deg) | 0.78 | 1.30 | 0.85 | 0.76 |
| Average B-factor (Å) | 29.38 | 28.86 | 28.66 | 20.79 |
| Protein/ligand/water | 29.36/24.35/31.58 | 28.91/20.71/30.39 | 28.60/24.24/31.22 | 20.79/20.64/20.53 |
| Ramachandran plot (%) | | | | |
| Favored/allowed/outliers | 98.85/1.15/0.00 | 98.08/1.92/0.00 | 98.47/1.53/0.00 | 98.85/1.15/0.00 |
| PDB code | 7E5F | 7E5H | 7E5I | 7E5G |

a) Values in parentheses indicate those for the highest resolution shell.
tivity. In addition, TIPP-703, in which an adamantyl group is introduced into the tail and showing pan-agonist activity, became significantly potent against all PPARs at sub µM EC₅₀ concentrations.\(^{21}\)

A series of compounds, in which the adamantyl group at the tail of TIPP-703 was replaced with a fluorescent pyrene ring, showed different agonist activities against PPARα and PPARδ, depending on the length of the branched n-alkoxy group. They include APHM13S\(^{22}\) and YN4pai, and are expected to be used for intracellular imaging of PPARs. APHM13S with a methoxy group on the branch is a dual PPARα/δ agonist. The crystal structure of the APHM13S bound PPARα LBD has been determined\(^{23}\) and its unique fluorescence properties have been analyzed.\(^{24}\) YN4pai with an n-butoxy group on the branch is a PPARδ-specific agonist, but has not successfully been co-crystallized with PPARδ LBD yet. Although less potent than on PPARδ, YN4pai acts on PPARα at µM order (unpublished data). YN4pai could possibly form a complex with PPARα, which may give only less potent interactions, and thus was included as a target for the present structure analysis.

**Protein Preparation and Crystallization**

Protein expression, purification, and crystallization were carried out according to the recent structural analysis of PPARα LBD in complex with fibrates, endogenous fatty acids, and other synthetic ligands,\(^{18,19}\) rather than the previous crystallographic analysis for PPAR LBDs using phenylpropanoic acid agonists.\(^{12}\) The protein was overexpressed as an N-terminal (histidine (His)₆ tag fusion using the *E. coli* pET expression system. The first affinity column chromatography was performed using a cobalt chelating column (HisTALON Superflow Cartridge 5 mL, Clontech). Following the cleavage of the His-tag by thrombin protease, the protein was purified to homogeneity suitable for crystallization by an anion exchange column chromatography (HiTrap Q HP 5 mL, Cytiva) and a gel filtration chromatography (HiLoad 16/60 Superdex 75 HR, Cytiva). The protein was crystalized by the hanging drop vapor diffusion at 273 K. Buffer containing 0.1 M Bis-Tris (pH 6.5) and 25% (w/v) PEG3350 was used as a reservoir solution. After obtaining the crystals of the PPARα LBD–WY-14643 complex, they were crushed into micro crystals for seeding and added to fresh crystallization drops to obtain single crystals of PPARα LBD as the fatty acid-bound form.

**Ligand-Exchange Soaking**

This experiment was performed as previously described\(^{19}\) (Fig. 2). A series of reservoir solutions, each containing 1 mM of ligand, were prepared. The above-mentioned fatty acid-bound crystals were soaked in the respective ligand-containing reservoir solutions and incubated at 277 K for up to two weeks.

**X-Ray Diffraction Data Collection and Refinement**

All X-ray diffraction data were collected at 100 K on the SPring-8 BL26B1 beamline (Harima, Hyogo, Japan). The crystals were cryo-protected by soaking into a reservoir solution supplemented with 20% (v/v) glycerol for seconds and then flash-cooled in liquid nitrogen. Diffraction spots were recorded on the EIGER X 4M pixel array detector (DECTRIS). A fine slicing strategy with an oscillation angle of 0.1° per frame was used. Data were processed by the XDS\(^{25}\) and AIMLESS\(^{26}\) programs. The initial structures were determined using the PHASER program in the PHENIX package,\(^{27}\) using the protein structure in the PPARα LBD–TIPP-703 (PDB ID: 2ZNN)\(^{12}\) complex as a probe. Manual building of the atomic model of the complexes were performed on the Coot pro-

![Fig. 3. Structure of the PPARα LBD-Phenylpropanoic Acid Derivative Complex](image-url)
gram. Ligand stereochemical restriction parameters were generated using the JLigand program. Crystallographic refinement with Phenix.refine and manual model rebuilding were iterated to convergence. Data collection and refinement statistics are shown in Table 1.

RESULTS AND DISCUSSION

Summary of the Ligand-Exchange Soaking It has been reported that the recombinant PPARδ LBD prepared in the E. coli expression system was fixed in an active form by binding with the host-derived fatty acid. Although we considered the possibility of fatty acid binding to PPARα LBD in the previous study, neither did we analyze the fatty acid binding to the purified proteins nor try to crystallize them as a fatty acid-bound form, because we successfully obtained the two crystal structures by co-crystallization. Whereas, in the recent study targeting PPARα LBD, it was possible to prepare 6 out of 17 PPARα LBD–ligand complexes by ligand-exchange soaking. On the other hand, the exchange soaking using ligands with binding affinities lower than the bound fatty acids or less soluble ligands was unsuccessful. In this study, we tried the

Fig. 4. Comparison of the Ligand-Binding Mode in the Binding Pocket of PPARα LBD

TIPP-703, APHM19, YN4pai, APHM13S, and pemafibrate are colored cyan, green, violet, yellow, and grey, respectively. Superimposed structures of (a) TIPP-703 and pemafibrate (b) TIPP-703 and APHM19 (c) APHM13S and YN4pai are shown in stereoview. (Color figure can be accessed in the online version.)
been determined. We previously tried to co-crystallize PPARα and LBD of PPARα having the same linker as KCL, including KCL itself, has been obtained not shown). In this study, however, we were able to obtain the PPARα LBD–APHM19 complex crystals not only by the conventional cocrystallization but also by the ligand-exchange soaking, leading to the high-resolution structure determination. As in the case of PPARα LBD–TIPP-703, the PPARα LBD–APHM19 complexes obtained by cocrystallization and the ligand-exchange soaking showed no significant structural difference. The r.m.s.d. on the superposition of the structures was 0.14Å for the corresponding 264Cα atoms.

Figure 4b shows a comparison of the binding modes between TIPP-703 and APHM19 in the ligand pocket. In APHM6, the carbonyl group of the linker is directly connected to the 3-position of the central phenyl group, and the N atom adjacent to the carbonyl group is placed in the plane as the phenyl group probably to avoid steric hindrance with the methoxy group attached to the 4-position of the phenyl group. Although it is hypothetical, the ligand binding with this partially restricted conformation may be suitable for PPARα, but not for PPARγ or PPARδ, to generate the subtype-selective ligands.

Binding Mode of YN4pai The only structural difference between APHM13S (PPARα/δ dual agonist) and YN4pai (PPARδ-specific agonist) is the length of the alkoxy group in the branch portion; APHM13S and YN4pai have a methoxy and n-butoxy group, respectively (Fig. 1). In order to understand how this difference affects their affinity to PPARα, we determined the crystal structure of the PPARα LBD–YN4pai complex and compared it with that of the previously solved PPARα LBD–APHM13S (PDB ID: 3Vl8). As in the case of PPARα LBD–TIPP-703, the crystal structure of PPARα LBD–YN4pai obtained by co-crystallization could not be determined due to its weak diffraction quality. However, the ligand-exchange soaking enabled the binding of YN4pai to the ligand binding pocket and the crystal structure determination of the complex at high resolution.

As shown in Fig. 4c, both APHM13S and YN4pai are bound to the ligand-binding pocket in a similar manner, but small differences are also observed. Although the structure and arrangement of the propaonic acid portion of the head are almost the same, the central phenyl group of YN4pai is rotated about 9° so that the methoxy group oxygen is shifted away from the branch-binding subpocket by 0.9Å compared with that of APHM13S. As a result, the butoxy group of YN4pai can be fit into this subpocket. However, the nearest-neighbor interatomic distances between Met355 and lysine (Lys)358 and the YN4pai butoxy group were 3.3 and 3.4Å, respectively. These may be close contacts for ideal hydrophobic interactions and account for a less potent activity of YN4pai on PPARα than on PPARδ. This structure-function relationship in PPAR agonism is consistent with that for another series of phenylpropionic acid derivatives. TIPP-401 has a methoxy group on the branch and is a PPARα/δ dual agonist (EC50 of the compound synthesized as a racemic form is 8.2nM for PPARα and 21nM for PPARδ), while TIPP-204, with an n-butoxy group instead of the methoxy group of TIPP-401, exhibits a PPARδ selective agonistic activity with the corresponding EC50 values of 280nM for PPARα and 1.9nM for PPARδ.

CONCLUSION

We have determined the three crystal structures of three PPARα LBD-phenylpropanoic acid derivative complexes, two of which are novel structures, by the ligand-exchange soaking. The PPARα LBD–TIPP-703 complex structure prepared by

Overall Structure of the PPARα LBD–Ligand Complex

All crystals belong to the space group P21, and contain one molecule in the asymmetric unit (Fig. 3a). The C-terminal α-helix H12 adopts the active conformation and the phenyl oxygen of the Tyr464 sidechain forms a hydrogen bond with the carboxy group of the bound ligand. In the three complex crystal structures, the clear electron density maps correspond to the exchanged ligands in the ligand-binding pockets were observed (Figs. 3b–d).

The structure of the current PPARα LBD–TIPP-703 complex prepared by soaking was analyzed independently from the previous cocrystal structure(23) in order to analyze the differences between the cocrystal and exchange-soaked structures. The root mean square distance (r.m.s.d.) on the superposition of the structures was calculated as 0.31Å for the corresponding 256Cα atoms, and the structure of TIPP-703 was very similar between them, indicating that the complex structures are essentially the same regardless of the crystal preparation methods. The electron density map corresponding to the adamantyl group at the tail end was clearer than in the previous study, and the structure of the adamantyl group, which may bind in multiple conformations due to its approximate spherical symmetry of the group, was well-defined. (Fig. 3b). Although the PPARα LBD–TIPP-703 complex crystals were also obtained by co-crystallization, the diffraction quality of those crystals was so poor that no cocrystal structure was determined for PPARα LBD–TIPP-703 in this study.

Figure 4a shows a comparison of the binding mode of TIPP-703 with that of pemafibrate (PDB ID: 6KAZ) in the ligand pocket. A drastically different binding was observed on the branch between them, while the hydrophilic head and hydrophobic tail are comparable. The structure of TIPP-703 (and throughout the other phenylpropionate derivatives), the branched propoxy group pushes the methionine (Met)355 sidechain out of the pocket. On the other hand, the benzoxazole group of pemafibrate, which corresponds to the propoxy group of TIPP-703, is positioned at the opposite side of leucine (Leu)321 (as if it acts as a sorting flag), whereas the Met355 sidechain points toward the ligand-binding pocket. The sub pocket for binding of the pemafibrate-benzoxazole group is also used for the cyclohexylacetamide group of GW7647 (PDB ID: 6KB8). On the other hand, in the structures of the protein complexed with TIPP-703 and other phenylpropionic acid derivatives, there is no protein–ligand contact, but is instead a complexed with TIPP-703 and other phenylpropanoic acid derivatives. TIPP-401 has a methoxy group on the branch and is a PPARα/δ dual agonist (EC50 of the compound synthesized as a racemic form is 8.2nM for PPARα and 21nM for PPARδ), while TIPP-204, with an n-butoxy group instead of the methoxy group of TIPP-401, exhibits a PPARδ selective agonistic activity with the corresponding EC50 values of 280nM for PPARα and 1.9nM for PPARδ.

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CONCLUSION

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the soaking was shown to be identical to the previously determined co-crystal structure. Structure comparison of PPARα LBD–APHM19 with the related phenylpropanoic acid derivative-bound complexes indicated that a minor difference in the linker part causes a largely different conformational restriction when bound to the ligand binding pockets of PPARs leading to the altered subtype preference. The PPARα LBD–YN4pai complex showed an apparently unfavorable close contact between PPARα and YN4pai at the branch that may reduce the potency of YN4pai for PPARα.

Overall, the study demonstrated that the preparation of the PPARα LBD–ligand complex crystals by the recently developed ligand-exchange soaking method was also applied to those using the phenylpropanoic acid derivatives under certain conditions. Notably, all of TIPP-703, APHM19, and YN4pai possess bulky residues at their tail parts (Fig. 1), whereas the other three compounds, which were not successfully replaced by ligand-exchange soaking, have much smaller residues in the respective regions (our unpublished data; their structures are confidential at this moment). Such structural differences in ligands may be related to the success or failure of our attempts to replace the ligand. The structures of the complexes with the phenylpropanoic acid derivatives which could not be determined in this study may be solved by using another unique method of “delipidation” which has also been developed in the recent study.8) Since PPARδ LBD prepared in the E. coli expression system may also be purified with endogenous fatty acids bound to the protein,9) the similar ligand-exchange soaking and delipidation methods for the structure determination of PPARδ LBD–ligand complexes could be possible as PPARα LDB. If this is the case, the high-throughput crystal structure determination of complexes between PPARs and phenylpropanoic acid derivatives contribute to the development of more detailed selective-, dual-, and pan-agonism of the compounds for PPARs.

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Conflict of Interest  The authors declare no conflict of interest.

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