Crystal Structure of the Tumor-promoter Okadaic Acid Bound to Protein Phosphatase-1*

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Protein phosphatase-1 (PP1) plays a key role in dephosphorylation in numerous biological processes such as glycogen metabolism, cell cycle regulation, smooth muscle contraction, and protein synthesis. Microorganisms produce a variety of inhibitors of PP1, which include the microcystin class of inhibitors and okadaic acid, the latter being the major cause of diarrhetic shellfish poisoning and a powerful tumor promoter. We have determined the crystal structure of the molecular complex of okadaic acid bound to PP1 to a resolution of 1.9 Å. This structure reveals that the acid binds in a hydrophobic groove adjacent to the active site of the protein and interacts with basic residues within the active site. Okadaic acid exhibits a cyclic structure, which is maintained via an intramolecular hydrogen bond. This is reminiscent of other macrocyclic protein phosphatase inhibitors. The inhibitor-bound enzyme shows very little conformational change when compared with two other PP1 structures, except in the inhibitor-sensitive β12-β13 loop region. The selectivity of okadaic acid for protein phosphatases-1 and -2A but not PP-2B (calcinemin) may be reassessed in light of this study.

The phosphorylation and dephosphorylation of proteins is vital to the regulation of many cellular pathways and processes. Two classes of enzymes in the cell that catalyze cellular dephosphorylation activity are tyrosine phosphatases and serine/threonine phosphatases (1). Classification of serine/threonine phosphatases. There are several natural toxin inhibitors of the PPP family of phosphatases since they contain extensive sequence similarity in their catalytic domains and little or no sequence homology to PP2C or to tyrosine phosphatases. There are several natural toxin inhibitors of the PPP family of enzymes. These include microcystins, calyculins, tautomycin and okadaic acid (OA) (Fig. 1) (1).

OA is a tumor-promoting Cnp polyether fatty acid produced by marine dinoflagellates (1, 3–6). OA contains acidic and hydrophobic moieties and is cyclic (via an intramolecular hydrogen bond) (6). This toxin can accumulate in filter-feeding organisms and is the principle cause of diarrhetic shellfish poisoning worldwide (4).

There have been many biochemical and modeling studies on the inhibition of the PPP family of phosphatases by the natural toxins, but the lone crystal structure is of microcystin-LR (MCLR) bound to PP1 (α isoform) (8). Here we describe the crystal structure of OA bound to the recombinant catalytic subunit of PP1 (γ isoform).

EXPERIMENTAL PROCEDURES

Crystallization—The catalytic subunit of protein phosphatase-1 γ isoform was purified as described previously (9, 10). OA was purified from Procercentrum lima (9, 10). Crystals were obtained by the hanging drop vapor diffusion method at room temperature. The enzyme and inhibitor were mixed in a 1:2 molar ratio with the concentration of protein being ~0.4 mM. The PP1-OA complex was then mixed with an equivalent volume of mother liquor, which consisted of 2 M lithium sulfate, 100 mM Tris (pH 8.0), 2% polyethylene glycol 4000, and 10 mM p-mercaptoethanol. The complex crystallized in the space group P4212 with cell dimensions a = b = 99.18 Å, c = 62.17 Å, with one complex per asymmetric unit.

Data Collection, Structure Determination, and Refinement—A first data set to 2.9 Å was collected at 100 K on a Rigaku RU-H3R rotating anode generator equipped with a Rigaku R-AXIS IV++ image plate detector. The data were processed with the HKL suite of programs (11). This initial structure was solved by molecular replacement with the program AMoRe, using the PP1-MCLR structure (with MCLR removed) as a search model (Protein Data Bank accession code 1FJM) (12). Electron density for both the protein and the inhibitor were clear from the initial map generated from the molecular replacement solution. Another data set to 1.9 Å was then collected on the same instrumenta as above. The first structure was used as a search model for another round of molecular replacement as above. OA was fit to the difference density using the crystal structure of the free inhibitor as the starting model (6). The protein-inhibitor model was subjected to rigid-body refinement in CNS prior to manually fitting the model using the program XtalView (13, 14). The model was then subjected to iterative rounds of macromolecular refinement using CNS with a maximum likelihood target. The crystallographic data are listed in Table I. The final model consisted of density for residues 6–299 and was checked for acid; MCLR, microcystin-LR; PP, superfamily of protein phosphohydrolases; r.m.s.d., root mean square deviations.

* This work was supported by Grant MGP-37770 from the Canadian Institutes of Health Research (to M. N. G. J.) and by equipment funds from the Alberta Heritage Foundation for Medical Research. A. D. thanks the Alberta Heritage Foundation for Medical Research for the award of travel funds. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1JK) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).‡ Supported by a Doctoral Training Award from the Canadian Institutes of Health Research and supplemental funding from the Alberta Heritage Foundation for Medical Research.

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** Supported by Grant MGP-13687 from the Canadian Institutes of Health Research.

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1 The abbreviations used are: PP, protein phosphatase; OA, okadaic acid; MCLR, microcystin-LR; PP, superfamily of protein phosphohydrolases; r.m.s.d., root mean square deviations.
**TABLE I**

| Data collection and refinement statistics |
|------------------------------------------|
| Data collection                          |
| Wavelength (Å)                           | 1.5418    |
| Resolution ($\AA^\text{r.m.s.}$)         | 30–1.9    |
| Total number of reflections              | 434,544   |
| Number of unique reflections             | 24,459    |
| Completeness ($\%$)                      | 97.6 (95.4) |
| Redundancy $^a$                          | 5.46 (5.33) |
| $<\Sigma I - I^\text{c}>/\Sigma I$      | 30.0 (4.9) |
| $R_{\text{sym}}$ ($\%$)                  | 4.3 (35.8) |

| Refinement                                |
| Resolution (Å)                           | 30–1.9    |
| Atoms                                    | 2630      |
| Waters                                   | 181       |
| Waters                                   |           |
| Bond length (Å)                          | 0.007     |
| Bond angles (°)                          | 1.523     |
| Average B-factors                        |           |
| Protein atoms (Å$^2$)                    | 32        |
| Solvent (Å$^2$)                          | 44        |
| $R_{\text{sym}}$ ($\%$)                  | 19.9      |
| $R_{\text{free}}$ ($\%$)                | 22.4      |

| Data in parentheses correspond to highest resolution shell. |
| $^a$ $R_{\text{sym}}$ = $\Sigma I - I^\text{c}>/\Sigma I$, where $I$ is the observed intensity, and $(\bar{I})$ is the average intensity obtained from multiple observations of symmetry related reflections. |
| $^b$ $R_{\text{sym}}$ = $\Sigma |F_o^\prime| - |F_{\text{c}}|/\Sigma |F_o^\prime|$, where $|F_o^\prime|$ and $|F_{\text{c}}|$ are the observed and calculated structure factor amplitudes respectively. |
| $^c$ $R_{\text{sym}}$ was calculated as for $R_{\text{sym}}$ with 5% of the data omitted from structural refinement. |

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**RESULTS**

**Okadaic Acid-PP1 Structure**—The catalytic subunit of PP1 is composed of an $\alpha$-helical domain (9 $\alpha$-helices) and a $\beta$-sheet domain (14 $\beta$-strands comprising 3 $\beta$-sheets) (Fig. 1). The active site lies at one of the interfaces between the two domains. Residues within the active site are almost exclusively found on segments of random coil joining regular secondary structural elements, rather than within the regular secondary structural elements themselves. There have been two previously published structures of each of phosphatases PP1 and PP2B (8, 17–19). The overall structure obtained here with OA-bound is very similar to both the tungstate-inhibited structure of PP1 (C$\alpha$ r.m.s.d. 0.555 Å (residues 6–288) averaged over the two tungstate-PP1 complex molecules in the asymmetric unit) and the structure of PP1 with the inhibitor MCLR-bound (C$\alpha$ r.m.s.d. 0.518 Å (residues 6–285) averaged over the two PP1-MCLR complexes in the asymmetric unit) (Fig. 3).

**Inhibitor Binding**—The presence of three grooves (hydrophobic, C-terminal, and acidic) on the surface of PP1 has been reported previously (Fig. 1) (8). The double ring spiroketal moiety of OA is hydrophobic and binds into the hydrophobic groove on the surface of the protein. In the PP1-OA complex, Trp-206 and Ile-130 in the hydrophobic groove appear to be the two most important residues in this interaction due to their proximity to the hydrophobic segment of OA (Fig. 2). Other hydrophobic interactions occur between the C-4 to C-16 region of OA (which includes two six-membered rings) and the PP1 residues Phe-276 and Val-250.

The remaining sites of interaction between PP1 and OA involve hydrogen bonding. A conserved acidic motif is present in many PP1 inhibitors, either as a carboxylic acid (C-1 in OA) or as a phosphate (calyculin A) (Fig. 2). In our structure, the acid motif in OA accepts a hydrogen bond from the hydroxyl group of Tyr-272 (20). Other hydrogen bonding interactions occur between Arg-96 and the C-2 hydroxyl of the inhibitor and between Arg-221 and the C-24 hydroxyl group of OA.

A comparison of OA-bound to PP1 and the crystal structure of OA alone reveals that the two structures have a very similar overall conformation (0.397 Å r.m.s.d. over all 56 atoms). In the structure of unbound OA, a hydrogen bond between the C-24 hydroxyl and the C-1 acid keeps the inhibitor in a cyclic structure. This hydrogen bond is present in the PP1-OA complex as well. The recent proposal that, based on modeling consider-
Crucial PP1-OA Interactions—The presence of a hydrophobic moiety in many PPP family inhibitors suggests that the hydrophobic groove may play a key role in inhibitor binding. This was seen in the crystal structure of MCLR bound to PP1 where the hydrophobic β(12S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) group of MCLR contacts residues in the hydrophobic groove of PP1 and is repeated in our structure where the hydrophobic tail of OA contacts similar residues in the hydrophobic groove. The other hydrophobic interactions that occur are between the C-4 to C-16 region of OA and PP1 residues Phe-276 and Val-250. These interactions may actually be an impediment to OA inhibition since mutation of Phe-276 to a smaller and less hydrophobic cysteine reduces the K_i value (22). The loop in the MCLR complex is a secondary event accompanying the covalent bonding reaction. An important interaction between PP1 and MCLR that is not present in the active site of the PP1-OA complex. All residues of PP1 within 4 Å of the OA are shown, the closest residue-OA interactions are shown by dashed lines, and the distances of all possible hydrogen bonding interactions are labeled.

Despite the intimate interaction of the C-2 hydroxyl group of OA with the Arg-96 side chain, removal of the hydroxyl group results in only a 7-fold increase in the K_i value (24). In contrast, mutation of Arg-221 to Ser confers resistance to inhibition by OA, underlining the importance of the interaction between the Arg and the C-24 hydroxyl of OA (25).

Comparison to Other PPP Structures—As mentioned, the structure of PP1 obtained here is remarkably similar to the two structures of each of PP1 and PP2B determined previously. Even with only the phosphate-mimic tungstate present, the architecture of the active site of the tungstate-bound PP1 structure is virtually identical to our structure with OA-bound (Fig. 3). This suggests that either tungstate does not accurately mimic the phosphate product or that the binding of OA is enthalpically favorable and does not cause significant changes to the structure of the protein. In contrast, the MCLR-bound structure reveals large changes in the conformation of the active site relative to the tungstate-bound PP1 structure (Fig. 3). These changes are mainly restricted to the β12-β13 loop, which has been previously implicated in inhibitor specificity (26, 27). The loop in the MCLR structure folds back on itself, causing significant shifting of residues 273–278 relative to the tungstate-bound structure of PP1. One critical difference between MCLR and OA is the presence of a dehydroalanine residue in MCLR that covalently alkylates the S_e of Cys-273 in a time-dependent reaction (1). This covalent linkage is not the primary cause of inhibition of PP1 by MCLR (8). It is unclear whether inhibition by MCLR requires the movement of the β12-β13 loop or whether this movement occurs along with covalent bond formation. Given the strong similarity of the PP1-interacting domains of OA and MCLR, it is likely that the primary mode of inhibition of PP1 by MCLR is similar to that of OA and that the movement of the β12-β13 loop in the MCLR complex is a secondary event accompanying the covalent bonding reaction. An important interaction between PP1 and MCLR that is not present in the
PP1-OA complex is the hydrogen bond that occurs between Arg-96 (PP1) and the acid of the methyl-aspartate residue (MCLR). This interaction may account for the 100-fold greater inhibition of PP1 by MCLR over OA (28).

The structure of PP1-OA is strikingly similar to the structure of PP2B (Fig. 3) despite the fact that OA does not strongly inhibit PP2B. In the catalytic domain of PP2B, the protein backbone around the active site is virtually identical to that in PP1, including the β12–β13 loop. However, the overall Cα r.m.s.d. is 1.04 Å (residues 6–272), indicating some significant differences in other parts of the protein, namely in the less structured N and C termini regions.

Several attempts to model the PP1-OA structure based on the other PPP family structures have been made (29–32). These models have largely been based on the MCLR-bound structure in which the conformation of the β12-β13 loop differs from PP1 with tungstate-bound and our structure with OA-bound. The OA-PP1 structure presented here will greatly facilitate models based on PP1 inhibitors structurally related to OA (e.g., tautomycin).

OA Binding to Other Protein Phosphatases—OA exhibits different inhibitory potential on the structurally similar PPP family members. Okadaic acid inhibits PP2A most strongly (IC_{50} ~0.1 nM), PP1 less well (IC_{50} ~10 nM), and PP2B weakly (IC_{50} ~1–2 μM) (28, 33). The primary difference in the active site region between PP1 and PP2A is in the β12-β13 loop where PP1 contains the residues GEFD (residues 274–277) but PP2A has YRCG (residues 267–270) in the equivalent positions (Fig. 2). While mutation of Phe-276 to Cys (in PP2A) clearly facilitates inhibition by OA, there are likely additional reasons for the enhanced inhibition of PP2A by OA. The side chain of Tyr-267 in PP2A should point into solvent, away from the active site, if it is in the same position as the equivalent Gly-274 in PP1, so this change should not be expected to be very significant. However, the allowed torsion angles of a tyrosine would constrain the β12–β13 loop to a larger degree (Gly-274 is in the third position of a type II β-turn and is currently in a region of the Ramachandran plot that is disallowed for Tyr residues), and perhaps the loop is brought into a position to allow for a hydrogen bond to occur between the tyrosine and a hydroxyl group on OA (C-7–OH). The tyrosine could also produce a favorable hydrophobic interaction with the C-10 methyl group of OA. Further interactions in PP2A may result from substitution of Glu-275 in PP1 with Arg-268 in PP2A. Hydrogen bonding interactions may occur between this arginine and the C-7–OH group or hydrophobic interactions with the C-10 methyl group. The enhanced inhibitory potency of OA with PP2A is likely a combination of these effects.
The decreased inhibition of PP2B with OA is more difficult to interpret. Major active site differences between the two proteins are the replacement of Ile-133 in PP1 with Tyr-159 in PP2B, Tyr-134 in PP1 with Phe-160 in PP2B, Cys-273 in PP1 with Leu-312 in PP2B, and Phe-276 in PP1 with Tyr-315 in PP2B. Of these, changing Phe-276 in PP1 to a tyrosine in PP2B may make the greatest impact on OA inhibition. The portion of the inhibitor that resides in this part of the active site is quite hydrophobic, interacting with Phe-276, the methylene carbon of Cys-273, the side chain of Val-250, and the aromatic ring of Tyr-272. The ring of Phe-276 points almost directly at one of the double ring systems of OA (carbons 4–12 (Fig. 2), and the introduction of a hydroxyl group in PP2B may be enough to disrupt the interaction between OA and the protein, thus reducing the inhibitory potency of OA for PP2B. The resistance of PP2B to OA may therefore arise from the combination of this residue change and subtle structural changes in key OA contact residues in the potential OA binding site. Determination of the PP1-OA structure presented here will now facilitate rational mutagenesis of the PP2B catalytic subunit to test this hypothesis.

Acknowledgment—We would like to thank Dr. David Barford for providing the coordinates of the tungstate-bound structure of PP1.

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