Published in final edited form as:

J Struct Biol. 2015 January ; 189(1): 62–66. doi:10.1016/j.jsb.2014.11.005.

THE STRUCTURE OF IBUPROFEN BOUND TO CYCLOOXYGENASE-2

Benjamin J. Orlando, Michael J. Lucido, and Michael G. Malkowski*

Department of Structural Biology, The State University of New York at Buffalo and the Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY, 14203, USA

Abstract

The cyclooxygenases (COX-1 and COX-2) catalyze the rate-limiting step in the biosynthesis of prostaglandins, and are the pharmacological targets of non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors (coxibs). Ibuprofen (IBP) is one of the most commonly available over-the-counter pharmaceuticals in the world. The anti-inflammatory and analgesic properties of IBP are thought to arise from inhibition of COX-2 rather than COX-1. While an x-ray crystal structure of IBP bound to COX-1 has been solved, no such structure exists for the cognate isoform COX-2. We have determined the crystal structure of muCOX-2 with a racemic mixture of (*R/S*)-IBP. Our structure reveals that only the *S*-isomer of IBP was bound, indicating that the *S*-isomer possesses higher affinity for COX-2 than the *R*-isomer. Mutational analysis of Arg-120 and Tyr-355 at the entrance of the cyclooxygenase channel confirmed their role in binding and inhibition of COX-2 by IBP. Our results provide the first atomic level detail of the interaction between IBP and COX-2.

Keywords

Cyclooxygenase; Ibuprofen; Nonsteroidal anti-inflammatory drugs; prostaglandin H₂ synthase; crystal structure

1. Introduction

Cyclooxygenases (COX-1 and COX-2) catalyze the rate-limiting step in the biosynthesis of prostaglandins, prostacyclins, and thromboxanes (Smith et al., 2011). These potent lipid-signaling molecules regulate “housekeeping” functions required for normal physiological activities. Changes in COX-mediated prostaglandin production are associated with various disease pathologies, including inflammation, cardiovascular disease, and cancer (Smyth et al., 2009). COX-1 and COX-2 are the pharmacological targets of nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors (coxibs). These compounds

© 2014 Elsevier Inc. All rights reserved.

*Corresponding Author at: Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, New York, USA 14203. Tel: (716) 898–8624; Fax: (716) 898–8660; malkowski@hwi.buffalo.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

are some of the most heavily utilized pharmaceuticals in the world, used to decrease acute and chronic inflammation and protect against adverse cardiovascular events (Blobaum and Marnett, 2007).

COX inhibitors fall into four different categories based on their mechanism of inhibition (Gierse et al., 1999; Smith et al., 2011). Time-independent inhibitors bind to COX in a rapidly reversible manner resulting in competitive inhibition. Tight binding, time-dependent inhibitors initially bind to the COX active site rapidly and reversibly but then undergo a time-dependent transition to an EI* complex in which the inhibitor dissociates slowly. Mixed inhibitors display an initial time-dependent decrease in enzyme activity that ultimately levels off without completely inhibiting the enzyme, and covalent inhibitors chemically modify the cyclooxygenase active site. Ibuprofen (IBP) has classically fallen into the time-independent class of COX inhibitors as it binds rapidly and reversibly to COX and acts as a competitive inhibitor of arachidonic acid (AA) oxygenation (Gierse et al., 1999; Prusakiewicz et al., 2009).

COX-1 preferentially oxygenates AA (Laneuville et al., 1995), whereas COX-2 efficiently oxygenates numerous fatty acids including AA, eicosapentaenoic acid, and linoleic acid (Vecchio et al., 2010; Vecchio et al., 2012). In addition, COX-2 oxygenates endocannabinoid substrates including 2-arachidonoyl glycerol and arachidonoyl ethanolamide (Kozak et al., 2004; Vecchio and Malkowski, 2011b). Recent studies have demonstrated that certain NSAIDs can act as substrate selective inhibitors such that they prevent the oxygenation of endocannabinoid substrates but not AA in COX-2 (Duggan et al., 2011; Prusakiewicz et al., 2009). A mechanism for substrate selective inhibition by IBP has been proposed wherein the binding of IBP to one monomer of COX-2 results in the inhibition of endocannabinoid but not AA oxygenation in the partner monomer. Thus, while IBP acts as a simple competitive inhibitor when AA is the substrate, it acts in a non-competitive, allosteric manner to prevent endocannabinoid oxygenation. The nature of the allosteric communication pathway between monomers is unknown.

Crystal structures of COX-1 and COX-2 in complex with a myriad of inhibitors and substrates have been determined (Kurumbail et al., 1996; Vecchio and Malkowski, 2011a; Vecchio and Malkowski, 2011b; Vecchio et al., 2012). While a crystal structure of IBP bound to COX-1 has been determined (Selinsky et al., 2001), no such structure exists for the cognate isoform COX-2. Importantly, the analgesic and anti-inflammatory effects of IBP are thought to arise from the inhibition of COX-2 rather than COX-1 (Laneuville et al., 1994). In order to compare the binding mode of IBP to COX-2 versus COX-1, and to reveal a possible mechanism of IBP-mediated substrate selective inhibition, we determined the crystal structure of murine (μ) COX-2 in complex with IBP.

2. Materials and methods

2.1 Crystallization and data collection

Wild type, R120A, and Y355F μ COX-2 constructs were engineered and expressed in baculovirus-infected insect cells and the apo enzyme purified as previously described (Vecchio et al., 2010; Vecchio et al., 2012). Prior to crystallization, wild-type μ COX-2

was concentrated to 5.6 mg/mL, reconstituted with a 2-fold molar excess of Fe³⁺-protoporphyrin IX and dialyzed overnight at 4°C against 20mM TRIS, pH 8.0, 100mM NaCl, and 0.6% (w/v) n-octyl β-D-glucopyranoside (βOG). A 5-fold molar excess of racemic (*R/S*)-IBP was added to the reconstituted enzyme and allowed to incubate on ice for 30 minutes before dispensing into crystallization trays. Crystallization trials were set up at 23°C using the sitting drop vapor diffusion method. 3 μL of protein solution was combined with 3 μL of a drop solution consisting of 23–34% polyacrylic acid 5100, 100mM HEPES, pH 7.5, and 20mM MgCl₂ and 0.6% (w/v) βOG and equilibrated over 0.5mL reservoir solution of 23–34% polyacrylic acid 5100, 100mM HEPES, pH 7.5, and 20mM MgCl₂. Prior to data collection, crystals were harvested from the drop and soaked in 30% polyacrylic acid 5100, 100mM HEPES, pH 7.5, 20mM MgCl₂, and 0.6% βOG supplemented with 10% ethylene glycol for cryopreservation. The crystals were then looped and frozen directly in a gaseous nitrogen stream cooled to 100K. Diffraction data were collected on beamline A1 at the Cornell High Energy Synchrotron Source (Ithaca, NY) using an Area Detector Systems CCD Quantum-210. Data collection statistics are summarized in Table 1.

2.2 Structure solution and refinement

The diffraction data were processed in the orthorhombic space group I222 using HKL2000 (Otwinowski and Minor, 1997). The structure was solved by molecular replacement (MR) using the program PHASER (McCoy et al., 2007) and a truncated search model of muCOX-2 derived from PDB entry 3HS5 (Vecchio et al., 2010), with residues 33–144, 320–325, 344–391, 500–553, and all ligands, cofactors, and waters removed. Two monomers were located in the crystallographic asymmetric unit. Phases from MR were input into ARP/wARP (Langer et al., 2008), which successfully built 99% of the model. Iterative model building in COOT (Emsley and Cowtan, 2004) followed by refinement in PHENIX (Adams et al., 2010) was carried out to place remaining residues, ligands, and waters. Translation libration screw (TLS) refinement (Winn et al., 2001), utilizing the TLSMD web server (Painter and Merritt; Painter and Merritt, 2006), was carried out during the final rounds of refinement. The final model consists of residues 33–582, Fe³⁺-protoporphyrin IX, carbohydrate moieties linked to Asn-68, Asn-144, and Asn-410, and *S*-IBP bound in the cyclooxygenase channel of each monomer. Three βOG molecules, 14 ethylene glycol molecules, 4 acrylic acid molecules, and 1170 waters were also modeled into the electron density. Refinement statistics are summarized in Table 1.

2.3 Structure validation and functional analysis of mutant constructs

Model validation was carried out in MOLPROBITY (Davis et al., 2007) and PROCHECK (Laskowski et al., 1993). Simulated annealing omit maps were created using PHENIX. Figures were generated using PYMOL (Version 1.5.0.4; Schrodinger, LLC). Cyclooxygenase activity was determined using an oxygen electrode and 100μM arachidonic acid as the substrate as described in (Vecchio et al., 2012). Inhibition studies using IBP were carried out as described in (Orlando et al., 2014). Thermal shift assays were carried out with apo muCOX-2 using a Stratagene Mx3005P real-time PCR instrument as described in (Koszelak-Rosenblum et al., 2008). Coordinates and structure factors have been deposited in the protein data bank (PDB id 4PH9).

3. Results and Discussion

3.1 Overview of the muCOX-2:IBP Complex

We determined the structure of muCOX-2 in complex with IBP (muCOX-2:IBP) to 1.8Å resolution using synchrotron radiation. There are two monomers in the crystallographic asymmetric unit that form the canonical dimer, which is consistent with other crystal structures of muCOX-2 elucidated with bound substrate or inhibitor (Vecchio and Malkowski, 2011a; Vecchio et al., 2010; Vecchio et al., 2012). The domain makeup of each monomer, including the N-terminal epidermal growth factor-like domain, the membrane binding domain, and the C-terminal catalytic domain, is identical to that observed in other muCOX-2 crystal structures and there is well resolved electron density for the Fe³⁺-protoporphyrin IX and N-linked carbohydrate moieties. Interpretable electron density is present for IBP bound within the cyclooxygenase channel in each monomer (Figure 1; Suppl. Figure 1). To confirm the stereo-chemical identity of IBP in the structure, either the *R* or *S* isomer of IBP was modeled into the electron density before refinement in PHENIX. Analysis of the resultant peaks in the F_o-F_c difference electron density maps, as well as simulated annealing omit electron density maps confirm that only the *S* isomer of IBP is bound within the cyclooxygenase active site (Suppl. Figure 2). The root mean square deviation (rmsd) between Ca atoms of each monomer is 0.15Å. The conformation of IBP in monomer A will be used to describe the enzyme-inhibitor interactions and in comparison to the crystal structure of Ibuprofen bound to ovine (ov) COX-1 (Selinsky et al., 2001). As a matter of convention, cyclooxygenase residues are labeled according to the ovCOX-1 numbering scheme (Smith et al., 2011).

The structure of IBP bound to ovCOX-1 has previously been determined to 2.6Å (Selinsky et al., 2001). Comparison of the structures of IBP bound to COX-1 vs. COX-2 reveals that the inhibitor binds identically to both isoforms, suggesting that the *S*-isomer of IBP is preferred by COX-1 as well (Supp. Figure 3). IBP occupies an area of the enzyme between the substrate channel opening and the apex of the active site (Figure 1). The carboxylate moiety of IBP forms a salt bridge with the guanidinium group of Arg-120, and a hydrogen bond with the hydroxyl group of Tyr-355. All other interactions formed between IBP and the active site residues of COX-2 consist of hydrophobic interactions (Supp. Table 1 and 2). The majority of these hydrophobic interactions are between the benzyl and isobutyl groups of IBP and residues near the apex of the cyclooxygenase channel. The benzyl group of IBP makes five contacts with Ala-527 and three contacts with Val-349. A total of thirteen contacts are made between the isobutyl group of IBP and Trp-387, Met-522, Val-523, Gly-526, Ala-527, and Ser-530. Additionally, the α-methyl group of IBP makes one contact with both Val-349 and Leu-359. The *R*-isomer of IBP would be expected to bind to muCOX-2 in a very similar fashion and with similar contacts as *S*-IBP in our structure, as has previously been demonstrated for other propionic acid class NSAIDs (Duggan et al., 2011).

3.2 Role of Arg-120 and Tyr-355 in the binding and inhibition of COX-2 by IBP

Our crystal structure suggests that the major determinants of IBP binding to muCOX-2 are the hydrophilic interactions between Arg-120 and Tyr-355 at the entrance of the

cyclooxygenase channel. Previous kinetic characterizations of R120A and Y355F mutants of muCOX-2 have shown that AA is oxygenated by these mutants (Vecchio and Malkowski, 2011b; Vecchio et al., 2012). The R120A mutation has no effect on k_{cat} and results in a 3.4 fold increase in k_m (Vecchio et al., 2012). The Y355F mutation results in a 45% decrease in k_{cat} and a 2.4 fold increase in k_m (Vecchio and Malkowski, 2011b). Cyclooxygenase activity assays in the presence of IBP were conducted to analyze the role of Arg-120 and Tyr-355 in the inhibition of muCOX-2. Wild-type (WT), R120A, and Y355F constructs were incubated with 100 μ M (*R/S*)-IBP on ice for 30 minutes before injection into a reaction cuvette containing 100 μ M AA and 100 μ M (*R/S*)-IBP. IBP resulted in a 40% decrease in cyclooxygenase activity with WT muCOX-2, consistent with previous studies (Dong et al., 2011; Orlando et al., 2014). Conversely, neither the R120A nor the Y355F constructs were inhibited by (*R/S*)-IBP (Figure 2).

We utilized a fluorescent dye based thermal shift assay to quantify the contribution that Arg-120 and Tyr-355 had on the binding of IBP in the cyclooxygenase channel. WT, R120A, or Y355F muCOX-2 was incubated with 100 μ M (*R/S*)-IBP for 30 minutes on ice before addition of the thiol specific fluorescent dye N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) and subsequent thermal denaturation as described in Materials and Methods. Binding of IBP to WT muCOX-2 results in a 12°C shift in enzyme melting temperature (Supp. Figure 4). No shift in melting temperature was observed upon incubation of R120A muCOX-2 with IBP. This result indicates that the salt bridge formed between the carboxylate moiety of IBP and the guanidinium moiety of Arg-120 is required for binding of the inhibitor. IBP also induced a thermal melting shift in the Y355F mutant (10°C) indicating that the inhibitor can still bind to the mutant enzyme despite the lack of a stabilizing hydrogen bond, but does not afford the same level of stabilization against thermal denaturation as in the WT construct. It is important to note that although the thermal denaturation data shows that IBP can bind to and stabilizes the Y355F mutant (Supp. Figure 4), this binding results in no inhibition of AA oxygenation (Figure 2).

4. Summary

IBP is one of the most readily available and commonly utilized pharmaceuticals in the world. The anti-inflammatory and analgesic properties of IBP are thought to arise from inhibition of COX-2 rather than COX-1 (Laneuville et al., 1994). IBP possesses a chiral center and thus exists as both the *R* and *S* stereoisomer. Commonly available over-the-counter preparations of IBP (ex: Advil®) are sold as the racemic mixture. Until recently the *S*-isomer of IBP has been considered the pharmacologically active isomer since only this isomer inhibits the oxygenation of AA by COX-2. However, recent results have demonstrated that the *R*-isomers of propionic acid class NSAIDs act as potent inhibitors of endocannabinoid oxygenation in COX-2 (Duggan et al., 2011; Prusakiewicz et al., 2009). Effectively, these results have reclassified the *R*-isomer of IBP from a biologically inactive compound to a potent substrate selective inhibitor of COX-2. A working model has been proposed in which binding of IBP to one monomer of COX-2 results in allosteric inhibition of endocannabinoid oxygenation in the partner monomer (Duggan et al., 2011).

The conformation of IBP in muCOX-2 was found to be identical to the conformation found within the ovCOX-1 active site (Selinsky et al., 2001) (Supp. Figure 2). In both COX isoforms IBP binds with a salt bridge between the carboxylate moiety of the inhibitor and the guanidinium group of Arg-120. Our inhibition (Figure 2) and thermal denaturation (Supp. Figure 4) assays confirm that this salt bridge interaction is required for inhibition of muCOX-2 by IBP, as the drug was unable to inhibit AA oxygenation or induce a shift in melting temperature with the R120A mutant. Previous studies have shown that this salt bridge interaction with Arg-120 is also required for inhibition of COX-1 by carboxylic acid containing NSAIDs (Bhattacharyya et al., 1996; Mancini et al., 1995).

In both COX-1 and COX-2 the carboxylate of IBP forms a hydrogen bond with the hydroxyl group of Tyr-355. Mutations of Tyr-355 in COX-1 alter the ability of the enzyme to discriminate between *R* and *S* isomers of IBP, with the ratio of IC₅₀'s between stereoisomers approaching one as the size of the Tyr-355 side chain is reduced (Thuresson, 2000). Our crystallographic studies confirm that the *S* isomer of IBP possesses greater affinity for the muCOX-2 active site than the *R* isomer, as only the *S*-isomer bound within the active site in our structure. Interestingly, while IBP can still bind to and stabilize Y355F muCOX-2 against thermal denaturation (Supp. Figure 4), we found that the drug was unable to inhibit AA turnover in this mutant (Figure 2). We interpret these results to suggest that while IBP can still bind to the Y355F mutant, the affinity of the inhibitor must be significantly reduced such that it can no longer act as a competitive inhibitor against AA. Importantly, the Y355F mutant in COX-1 results in a reduced IC₅₀ for both the *S* and *R* isomers of IBP when AA is used as the substrate (Thuresson, 2000). Thus, the Y355F mutation has divergent effects in regards to IBP mediated inhibition in COX-1 versus COX-2.

One of the goals of our crystallization experiments was to reveal a possible mechanism of IBP mediated substrate selective inhibition in COX-2. Previous studies have indicated that many different NSAIDs (including IBP) bind tightly to only one monomer of the COX-2 dimer and allosterically inhibit substrate oxygenation in the partner monomer (Dong et al., 2011; Duggan et al., 2011; Prusakiewicz et al., 2009; Yuan et al., 2006; Yuan et al., 2009). The current working model for substrate selective inhibition with IBP postulates that IBP binding in one monomer of the COX-2 dimer results in allosteric inhibition of endocannabinoid but not AA oxygenation in the partner monomer. Thus, we reasoned that the x-ray structure of COX-2 bound to IBP may provide insight into the mechanism of cross-monomer allosteric inhibition. However, our structure clearly reveals that *S*-Ibuprofen is bound with full occupancy in both monomers of COX-2. Furthermore, the two monomers in the muCOX-2:IBP structure are virtually superimposable. This conundrum between solution based biochemical data suggesting binding of inhibitors to only one monomer of COX-2, and x-ray crystal structure analysis clearly showing inhibitor molecules bound to both monomers has proven consistent with all NSAIDs that have been analyzed via x-ray structure determination to date. The nature of this discrepancy between structural and solution states remains elusive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health grant R01 GM077176 from the National Institute of General Medical Sciences. This work is also based upon research conducted at the Cornell High Energy Synchrotron Source (CHESS), which is supported by the National Science Foundation under NSF award DMR-0225180, using the Macromolecular Diffraction at CHESS (MacCHESS) facility, which is supported by award RR-01646 from the National Institutes of Health, through its National Center for Research Resources.

The abbreviations used are

COX	cyclooxygenase
mu	murine
ov	ovine
AA	arachidonic acid
IBP	ibuprofen
MR	molecular replacement
NSAIDs	non-steroidal anti-inflammatory drugs
βOG	n-octyl β-D-glucopyranoside
TLS	translation libration screw

References

- Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung LW, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr*. 2010; 66:213–221. [PubMed: 20124702]
- Baas BJ, Denisov IG, Sligar SG. Homotropic cooperativity of monomeric cytochrome P450 3A4 in a nanoscale native bilayer environment. *Arch Biochem Biophys*. 2004; 430:218–228. [PubMed: 15369821]
- Bhattacharyya DK, Lecomte M, Rieke CJ, Garavito M, Smith WL. Involvement of arginine 120, glutamate 524, and tyrosine 355 in the binding of arachidonate and 2-phenylpropionic acid inhibitors to the cyclooxygenase active site of ovine prostaglandin endoperoxide H synthase-1. *J Biol Chem*. 1996; 271:2179–2184. [PubMed: 8567676]
- Blobaum AL, Marnett LJ. Structural and functional basis of cyclooxygenase inhibition. *J Med Chem*. 2007; 50:1425–1441. [PubMed: 17341061]
- Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB 3rd, Snoeyink J, Richardson JS, Richardson DC. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res*. 2007; 35:W375–383. [PubMed: 17452350]
- Dong L, Vecchio AJ, Sharma NP, Jurban BJ, Malkowski MG, Smith WL. Human cy-clooxygenase-2 is a sequence homodimer that functions as a conformational heterodimer. *J Biol Chem*. 2011; 286:19035–19046. [PubMed: 21467029]
- Duggan KC, Hermanson DJ, Musee J, Prusakiewicz JJ, Scheib JL, Carter BD, Banerjee S, Oates JA, Marnett LJ. (R)-Profens are substrate-selective inhibitors of endocannabinoid oxygenation by COX-2. *Nat Chem Bio*. 2011; 7:803–809. [PubMed: 22053353]

- Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr*. 2004; 60:2126–2132. [PubMed: 15572765]
- Gierse JK, Koboldt CM, Walker MC, Seibert K, Isakson PC. Kinetic basis for selective inhibition of cyclo-oxygenases. *Biochem J*. 1999; 339(Pt 3):607–614. [PubMed: 10215599]
- Koszalak-Rosenblum M, Krol AC, Simmons DM, Goulah CC, Wroblewski L, Malkowski MG. His-311 and Arg-559 are key residues involved in fatty acid oxygenation in pathogen-inducible oxygenase. *J Biol Chem*. 2008; 283:24962–24971. [PubMed: 18596034]
- Kozak KR, Prusakiewicz JJ, Marnett LJ. Oxidative metabolism of endocannabinoids by COX-2. *Curr Pharm Des*. 2004; 10:659–667. [PubMed: 14965328]
- Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, Gildehaus D, Miyashiro JM, Penning TD, Seibert K, Isakson PC, Stallings WC. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature*. 1996; 384:644–648. [PubMed: 8967954]
- Laneuville O, Breuer DK, Dewitt DL, Hla T, Funk CD, Smith WL. Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J Pharmacol Exp Ther*. 1994; 271:927–934. [PubMed: 7965814]
- Laneuville O, Breuer DK, Xu N, Huang ZH, Gage DA, Watson JT, Lagarde M, DeWitt DL, Smith WL. Fatty acid substrate specificities of human prostaglandin-endoperoxide H synthase-1 and -2. Formation of 12-hydroxy-(9Z, 13E/Z, 15Z)- octadecatrienoic acids from alpha-linolenic acid. *J Biol Chem*. 1995; 270:19330–19336. [PubMed: 7642610]
- Langer G, Cohen SX, Lamzin VS, Perrakis A. Automated macromolecular model building for X-ray crystallography using ARP/wARP version 7. *Nat Protoc*. 2008; 3:1171–1179. [PubMed: 18600222]
- Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK - a program to check the stereochemical quality of protein structures. *J Appl Crystallogr*. 1993; 26:283–291.
- Mancini JA, Riendeau D, Falgout JP, Vickers PJ, O'Neill GP. Arginine 120 of prostaglandin G/H synthase-1 is required for the inhibition by nonsteroidal anti-inflammatory drugs containing a carboxylic acid moiety. *J Biol Chem*. 1995; 270:29372–29377. [PubMed: 7493972]
- McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J Appl Crystallogr*. 2007; 40:658–674. [PubMed: 19461840]
- Orlando BJ, McDougall DR, Lucido MJ, Eng ET, Graham LA, Schneider C, Stokes DL, Das A, Malkowski MG. Cyclooxygenase-2 catalysis and inhibition in lipid bilayer nanodiscs. *Arch Biochem Biophys*. 2014; 546:33–40. [PubMed: 24503478]
- Otwinowski Z, Minor W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. *Meth Enz*. 1997; 276:307–326.
- Painter J, Merritt EA. TLSMD web server for the generation of multi-group TLS models. *J Appl Crystallogr*. 39:109–111.
- Painter J, Merritt EA. Optimal description of a protein structure in terms of multiple groups undergoing TLS motion. *Acta Crystallogr D Biol Crystallogr*. 2006; 62:439–450. [PubMed: 16552146]
- Prusakiewicz JJ, Duggan KC, Rouzer CA, Marnett LJ. Differential sensitivity and mechanism of inhibition of COX-2 oxygenation of arachidonic acid and 2-arachidonoylglycerol by ibuprofen and mefenamic acid. *Biochemistry*. 2009; 48:7353–7355. [PubMed: 19603831]
- Selinsky BS, Gupta K, Sharkey CT, Loll PJ. Structural analysis of NSAID binding by prostaglandin H2 synthase: time-dependent and time-independent inhibitors elicit identical enzyme conformations. *Biochemistry*. 2001; 40:5172–5180. [PubMed: 11318639]
- Smith WL, Urade Y, Jakobsson PJ. Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis. *Chem Rev*. 2011; 111:5821–5865. [PubMed: 21942677]
- Smyth EM, Grosser T, Wang M, Yu Y, FitzGerald GA. Prostanoids in health and disease. *J Lipid Res*. 2009; 50(Suppl):S423–428. [PubMed: 19095631]
- Thurensson ED. Prostaglandin Endoperoxide H Synthase-1. The functions of cyclooxygenase active site residues in the binding, positioning, and oxygenation of arachidonic acid. *J Biol Chem*. 2000; 276:10347–10357. [PubMed: 11121412]
- Vecchio AJ, Malkowski MG. The structure of NS-398 bound to cyclooxygenase-2. *J Struct Biol*. 2011a; 176:254–258. [PubMed: 21843643]

- Vecchio AJ, Malkowski MG. The Structural Basis of Endocannabinoid Oxygenation by Cyclooxygenase-2. *J Biol Chem*. 2011b; 286:20736–20745. [PubMed: 21489986]
- Vecchio AJ, Simmons DM, Malkowski MG. Structural Basis of Fatty Acid Substrate Binding to Cyclooxygenase-2. *J Biol Chem*. 2010; 285:22152–22163. [PubMed: 20463020]
- Vecchio AJ, Orlando BJ, Nandagiri R, Malkowski MG. Investigating substrate promiscuity in cyclooxygenase-2: the role of Arg-120 and residues lining the hydrophobic groove. *J Biol Chem*. 2012; 287:24619–24630. [PubMed: 22637474]
- Winn MD, Isupov MN, Murshudov GN. Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr D Biol Crystallogr*. 2001; 57:122–133. [PubMed: 11134934]
- Yuan C, Rieke CJ, Rimón G, Wingerd BA, Smith WL. Partnering between monomers of cyclooxygenase-2 homodimers. *Proc Natl Acad Sci, USA*. 2006; 103:6142–6147. [PubMed: 16606823]
- Yuan C, Sidhu RS, Kuklev DV, Kado Y, Wada M, Song I, Smith WL. Cyclooxygenase Allosterism, Fatty Acid-mediated Cross-talk between Monomers of Cyclooxygenase Homodimers. *J Biol Chem*. 2009; 284:10046–10055. [PubMed: 19218248]

Figure 1. IBP Bound in the Cyclooxygenase Channel of COX-2

Stereo view of IBP bound within the cyclooxygenase channel of monomer A of the muCOX-2:IBP crystal structure. $F_o - F_c$ simulated annealing omit map electron density (light blue), contoured at 3.5σ , is shown with the final refined model of IBP (pink). Residues lining the cyclooxygenase channel, along with the spatial locations of the channel opening (O), channel apex (A), and COX-2 specific side pocket (S) are labeled accordingly. Carbon atoms of residues lining the channel are colored green, while nitrogen, and oxygen atoms are colored blue and red, respectively.

Figure 2. Inhibition of COX activity by IBP

COX-2 was incubated with 100 μ M (R/S)-IBP on ice for 30 minutes before assaying residual COX activity at 37°C with a Clark-type oxygen electrode. The reaction cuvette contained 3mL of 100mM Tris (pH 8), 1mM phenol, 5 μ M Fe³⁺-PPIX, 100 μ M AA, and 100 μ M (R/S)-IBP. Reactions were initiated via injection of 5 μ g of COX-2, and were repeated in triplicate.

Table 1

Summary of data collection and refinement statistics.

Crystallographic Parameter	muCOX-2:IBP
Space group	I222
No. in Asymmetric Unit	2
Unit cell length (Å)	
a	120.94
b	132.23
c	180.46
$\alpha=\beta=\gamma(^{\circ})$	90°
Wavelength (Å)	0.9759
Resolution (Å)	40.00–1.81
Highest resolution shell (Å) ^a	1.84–1.81
Rmerge ^b	5.6 (43.5)
Total observations	604868 (29897)
Total unique ^c	128926 (11767)
I/ σ (I)	13.6 (2.5)
Completeness (%)	98.5 (90.1)
Multiplicity	4.7 (2.4)
Wilson B factor (Å ²)	24.6
Number of atoms in refinement	10517
Rwork	0.155 (0.233)
Rfree ^d	0.197 (0.272)
Average B factor, protein (Å ²)	29.3
Average B factor, solvent (Å ²)	39.3
Average B factor, inhibitor (Å ²):	
Monomer A (Å ²)	29.2
Monomer B (Å ²)	26.0
Mean positional error (Å) ^e	0.188
RMSD in bond length (Å)	0.019
RMSD in bond angle (°)	1.620
Ramachandran Plot (%)	
Favored	98.0
Allowed	2.0
Disallowed	0

^a values in parentheses represent the values in the outermost resolution shell.^b R_{MERGE} as defined in HKL2000.^c Represents reflections with $F > 0 \sigma F$ used in the refinement.^d 5.0% of the total reflections were used to generate the test set.

^e Coordinate error as calculated by Luzatti plot.