Spatial Requirements for 15-(R)-Hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic Acid Synthesis within the Cyclooxygenase Active Site of Murine COX-2

WHY ACETYLATED COX-1 DOES NOT SYNTHESIZE 15-(R)-HETE*

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The two isoforms of cyclooxygenase, COX-1 and COX-2, are acetylated by aspirin at Ser-530 and Ser-516, respectively, in the cyclooxygenase active site. Acetylated COX-2 is essentially a lipooxygenase, making 15-(R)-hydroxyeicosatetraenoic acid (15-HETE) and 11-(R)-hydroxyeicosatetraenoic acid (11-HETE), whereas acetylated COX-1 is unable to oxidize arachidonic acid to any products. Because the COX isoforms are structurally similar and share approximately 60% amino acid identity, we postulated that differences within the cyclooxygenase active sites must account for the inability of acetylated COX-1 to make 11- and 15-HETE. Residues Val-434, Arg-513, and Val-523 were predicted by comparison of the COX-1 and -2 crystal structures to account for spatial and flexibility differences observed between the COX isoforms. Site-directed mutagenesis of Val-434, Arg-513, and Val-523 in mouse COX-2 to their COX-1 equivalents resulted in abrogation of 11- and 15-HETE production after aspirin treatment, confirming the hypothesis that these residues are the major isoform selectivity determinants regulating HETE production. The ability of aspirin-treated R513H mCOX-2 to make 15-HETE, although in reduced amounts, indicates that this residue is not an alternate binding site for the carboxylate of arachidonate and that it is not the only specificity determinant regulating HETE production. Further experiments were undertaken to ascertain whether the steric bulk imparted by the acetyl moiety on Ser-530 prevented the ω-end of arachidonic acid from binding within the top channel cavity in mCOX-2. Site-directed mutagenesis was performed to change Val-228, which resides at the junction of the main cyclooxygenase channel and the top channel, and Gly-533, which is in the top channel. Both V228F and G533A produced wild type-like product profiles, but, upon acetylation, neither was able to make HETE products. This suggests that arachidonic acid orientates in a L-shaped binding configuration in the production of both prostaglandin and HETE products.

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† The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; HPLC, high performance liquid chromatography; mCOX-2, mouse-cyclooxygenase-2; NSAID, non-steroidal anti-inflammatory drug; 5-HETE, 5-hydroxy-6Z,8Z,11Z,14Z-eicosatetraenoic acid; 11-HETE, 11-hydroxy-5Z,8Z,12Z,14Z-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 11-(R)-HETE, 11-(R)-hydroxy-5Z,8Z,12Z,14Z-eicosatetraenoic acid; 15-(R)-HETE, 15-(R)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid.

‡ The numbering system used is in accord with that of COX-1.
of COX-2 abrogates PGG$_2$ synthesis but forces the infrequently occurring lipoxygenase reaction. 15-($R$)-HETE and to a lesser extent 11-($R$)-HETE are the predominant products. Until recently, it was uncertain whether these aspirin-induced products were physiologically relevant, but the discovery that 15-($R$)-HETE can be converted to the lipoxins 15 epi-LXA$_4$ and 15 epi-LXB$_4$ suggests that the beneficial actions of aspirin may be partly mediated by the generation of these products (13).

The structural basis for the difference between the COX isoforms to make lipoxygenase products is unknown, but the elucidation of the COX-1 and COX-2 crystal structures complexed with inhibitors is helpful in formulating hypotheses (14–18). The major difference between the COX-1 and COX-2 active sites is that the COX-1 active site is smaller than that of COX-2 (17, 18). This is primarily due to valine to isoleucine substitutions at residues 434 and 523. The smaller Val-434 side chain in COX-2 enables the opening of a solvent accessible space, referred to as the side pocket, that increases the total volume of the cyclooxygenase active site. The other residue that differs between COX-1 and -2 in this side pocket region is at position 513, a histidine in COX-1 and an arginine in COX-2. Because this side chain points from the side pocket toward the center of the COX active site, it seems plausible that it could be an alternate binding site for the carboxylate of arachidonate. Thus, in the present study we addressed the hypothesis that the inability of acetylated COX-1 to make large amounts of 15-HETE is due to differences in the side pocket region compared with that of COX-2. We performed site-directed mutagenesis on mouse COX-2, mutating Val-434, Arg-513, and Val-523 to their COX-1 consensus residues. Our results suggest that Arg-513 is not an alternate binding determinant for the carboxylate of arachidonate and that the smaller solvent-accessible space within the COX-1 active site is the major determinant preventing the synthesis of HETE products following acetylation by aspirin.

The second aspect of HETE formation we addressed is the position of the $\omega$-end of arachidonic acid in the cyclooxygenase active site. Our recent modeling and site-directed mutagenesis studies (19) suggest that arachidonate binds within the COX active site with its $\omega$-end extending above Ser-530, into a solvent-filled top channel (Fig. 1B). It is clear that acetylation of Ser-530 (Fig. 1A) will force arachidonate into an alternate binding conformation, but, it is uncertain whether arachidonic acid will retain its L-shaped conformation or bind in a more hairpin or U-shaped orientation. Through site-directed mutagenesis of Val-228 at the junction of the main cyclooxygenase channel and top channel and of Gly-533, which is in the top channel, we provide evidence that in acetylated mouse COX-2 arachidonic acid binds in an L-shaped orientation in a manner similar to that proposed for the synthesis of PGG$_2$.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid) and [14C]arachidonic acid were purchased from Nu-Chek-Prep (Elysian, MN) and NEN Life Science Products, respectively. Standards of 5-HETE, 11-HETE, 12-HETE, and 15-HETE were purchased from Cayman Chemical Company (Ann Arbor, MI). Electrophoresis and chroma-
Arachidonic Acid Binding in Acetylated COX-2

RESULTS

Wild type and mutant COX-2 proteins were expressed in insect cells from baculovirus vectors and then purified by ion exchange and gel filtration. All of the purified proteins were shown by densitometric scanning of a 7.5% SDS-polyacrylamide gel to be at least 80% pure (Table I). The purified V228F, G533A, G533V, and G533L mutants and the triple V434I/R513H/V523I mutant exhibited at least 87% of the wild type peroxidase activity, suggesting that no major conformational change had occurred as a result of the site-directed mutation (Table I). Both the total oxygen consumption and initial rate of oxygen uptake by the V434I/R513H/V523I mutant were similar to those of wild type enzyme, whereas the V228F mutant consumed only 68% of the oxygen consumed by wild type enzyme and displayed a 5-fold reduced initial rate. Of the Gly-533 mutants, only the G533A enzyme was able to metabolize arachidonic acid, but the initial rate was only 8% of that observed with wild type COX-2, and the total products were 26% of wild type (Table I).

The effect of aspirin acetylation on the conversion of arachidonic acid to products was assessed by reacting 500 μM aspirin with COX-2 protein for 1 h followed by incubation with [3H]arachidonic acid and HPLC analysis of products. Reactions were carried out at 25 °C to correspond to previous reports (20, 21). As expected, the products formed by acetylated COX-2 were significantly different than those formed by control enzyme (Fig. 2, B and C). Most of the products generated by wild type enzyme coeluted with prostaglandin standards, although a small percentage of products were detected that coeluted with 11- and 15-HETE standards. In contrast, acetylated mCOX-2 produced mainly HETEs, the major product being 15-HETE with a small amount of 11-HETE. Small amounts of prostaglandins were detected that reflect the presence of residual uninhibited enzyme. The product profile observed with the V434I/R513H/V523I mutant was similar to that observed with wild type mCOX-2. However, following acetylation, very little metabolism took place and only a small amount of 15-HETE was observed. Because the V434I/R513H/V523I triple mutation was so detrimental to HETE synthesis, we performed assays on the single mutant proteins to establish what effect each had on HETE production. Each of the individual mutant proteins made predominantly 15-HETE following acetylation, but the amounts were approximately one-third those seen with the wild type enzyme (Fig. 3).

To investigate the possibility that 15-HETE synthesis required the carboxylate of arachidonate to bind preferentially to Arg-513 and not Arg-120, we analyzed the chirality of the 15-HETE produced by the wild type and R513H COX-2 enzymes. We deemed this experiment necessary because it has recently been shown that the Kₐ for arachidonate oxygenation is more dependent on Arg-120 in COX-1 than in COX-2 (22). Although we found that the amount of 15-HETE made by the R513H mutant was less than that produced by wild type mCOX-2, no difference in the chirality of the products was observed because both enzymes made only 15-(R)-HETE (Fig. 4).
studies that arachidonic acid binds in an L-shaped orientation, with the \( \omega \)-end extending above Ser-530 into a region we term the top channel (Fig. 1B) (19). It is clear that acetylation of Ser-530 will impart steric constraints upon arachidonic acid binding, because the substrate would no longer be able to lie directly above the Ser-530 side chain (Fig. 1). However, it is unclear whether arachidonic acid could still enter the top channel cavity of the acetylated enzyme. To test this possibility, we designed the V228F mutant to fill the solvent accessible space at the junction of the main cyclooxygenase channel and top channel, the region where we predicted arachidonic acid would bind after displacement by the acetyl moiety on aspirin-acetylated Ser-530. This mutant had 90% of the wild type peroxidase activity (Table I) and also had a wild type mCOX-2-like product profile (Fig. 5A), indicating that the V228F mutation had little effect on the structure of the enzyme and the binding of the substrate in the unacetylated active site. Aspirin treatment, however, abolished the lipoxygenase activity of this mutant (Fig. 5B), suggesting that the space filled by the larger phenyl side chain is important in production of 11- and 15-HETE. To further test the hypothesis that the \( \omega \)-end of arachidonic acid binds in the top channel in the acetylated enzyme, three Gly-533 mCOX-2 mutants (G533A, G533V, and G533L) were tested for their ability to make 15-HETE. Only G533A was able to make normal prostaglandin products (Fig. 5C), but the specific activity of this protein was much lower than that of wild type mCOX-2. Upon acetylation, G533A was unable to make any products (Fig. 5D). Similarly, both acetylated and nonacetylated G533V and G533L were unable to metabolize arachidonic acid (results not shown).

**DISCUSSION**

The discovery of a second isoform of COX, the expression of which is induced by a number of cytokines and growth factors (23–26), has been the catalyst for significant efforts by numerous academic and pharmaceutical groups to isolate isoform selective inhibitors. One of the best known and oldest NSAIDs, aspirin, along with the newer COX-2-selective version of aspirin, APHS (7), differ from all other NSAIDs in that they covalently modify the same serine residue (Ser-530) in both COX-1 (6) and COX-2 (7, 21, 27). Biochemical and structural studies suggest that residues at the mouth of the substrate access channel, specifically Arg-120 and Tyr-355, facilitate binding of aspirin in this region of the protein by ionic and hydrogen-bonding interactions (14, 15, 17, 18, 20, 22, 28). This increases the probability of reaction of the acetyl group with the hydroxyl group of Ser-530.

The current model for arachidonate/COX binding predicts an L-shaped configuration for the substrate (19, 22). The carboxylate moiety is anchored to Arg-120 and Tyr-355, and the fatty acid backbone extends upwards toward the top of the COX active site, with the 13-pro-S hydrogen of the substrate positioned close to Tyr-385 (32). The \( \omega \)-end of arachidonate extends into the region we have termed the top channel, with carbons 17–20 lying above Ser-530. Acetylation of Ser-530 should restrict access of arachidonate to the top channel and prevent its oxygenation by cyclooxygenase. In fact, aspirin completely
blocks arachidonate oxygenation by sheep COX-1, and the crystal structure of sheep COX-1 inactivated by bromoacetylsalicylic acid reveals steric congestion in this region (15). Although aspirin blocks arachidonate oxygenation to PGG2 by mouse and human COX-2, the acetylated enzyme is able to convert arachidonate to 15-(R)-HETE (11, 12). We attempted to determine the protein residues responsible for this catalytic difference and whether the ω-end of arachidonate binds in a different region of acetylated COX-2 to enable oxygenation at carbon-15.

Mutation of residues in the side pocket adjacent to Arg-120 and Tyr-355 of COX-2 to the corresponding residues in COX-1 eliminates the ability of acetylated enzyme to generate 15-HETE. The triple mutant V434I/R513H/V523I completely eliminates 15-HETE synthesis, whereas each of the single mutants causes partial loss of activity. The V434I mutant had the least effect on 15-HETE production (Fig. 3), whereas a more significant change was observed with the valine substitution at position 523. The effect this mutant has on arachidonate oxygenation is consistent with the effect of this side chain on the binding of COX-2-selective inhibitors (29–31). A similar decrease in the amount of HETE products is observed with the R513H enzyme, but we cannot be certain whether this is due to steric or electrostatic interactions. Electrostatic effects are favored because overlay of the COX-1 and COX-2 structures shows that the Arg-513 side chain extends further into the COX active site than the histidine side chain, appearing to actually decrease the solvent accessible space. Because the Arg-513 has been implicated in a network of electrostatic interaction that are important in regulating the opening and orientation of the mouth of the COX active site (17), we propose that this side chain may help stabilize the conformation of the active site so that arachidonic acid can bind in an orientation that is favorable for HETE synthesis. The hypothesis that the Arg-513 may be an alternate carboxylate binding site for arachidonate appears untenable because the R513H mutant is still able to make HETE products (Fig. 5).

The results of our study further establish that the ω-end of arachidonate binds in the top channel of acetylated COX-2. HPLC analysis of the unacetylated V228F mutant shows a wild type product profile (Fig. 5A), whereas the aspirin-treated V228F enzyme produced no HETE products (Fig. 5B). Further support for the hypothesis that 15-HETE production involves binding of arachidonic acid within the top channel is provided by results obtained with Gly-533 mutants. If arachidonic acid binds in a U-shaped or hairpin conformation with its ω-end projecting back down toward Arg-120 and Tyr-355, then adding bulk to the 533 position should not influence 11- or 15-HETE formation. This is clearly not the case because neither G533V nor G533L was able to oxygenate arachidonate in the presence or absence of aspirin. G533A is able to make prostaglandin products and small amount of HETE products (Fig. 5C) but is unable to make products when acetylated (Fig. 5D).

The present results suggest that arachidonate binds to acety-
lated COX-2 in the same regions of the protein as it binds to in unacetylated enzyme. However, to access the top channel of acetylated COX-2, arachidonate must alter its conformation, which is accomplished by the increased volume near the carboxylate-binding region of COX-2. It is possible that arachidonate also binds to acetylated COX-1 but that the conformational changes it must make to do so moves carbon-13 too far from Tyr-385 to be oxidized. Thus, acetylation of Ser-530 not only forces the ω-end of arachidonate to bind in an alternate conformation but also imparts longer range compensatory movement on the arachidonate molecule from carbon-13 to its carboxylate. This movement requires the solvent-accessible space in the side pocket region of COX-2. Because this space is not accessible in COX-1, no 15-(R)-HETE production can occur.

REFERENCES
1. Smith, W. L., and Marnett, L. J. (1991) Biochim. Biophys. Acta 1083, 1–17.
2. Marnett, L. J., and Maddipati, K. R. (1991) in Peroxidases in Chemistry and Biology (Everse, J., Everse, K. E., and Grisham, M. B., eds) Vol. 1, pp. 293–334, CRC Press, Boca Raton, FL.
3. Herschman, H. R., Xie, W. L., and Reddy, S. (1995) BioEssays 17, 1031–1037.
4. Herschman, H. R. (1996) Biochim. Biophys. Acta Lipids Lipid Metab. 1299, 125–140.
5. Smith, W. L., and DeWitt, D. L. (1996) Adv. Immunol. 65, 265–280.
6. Roth, G. J., Machuga, E. T., and Ozols, J. (1983) Biochemistry 22, 4672–4675.
7. Kalugutkar, A. S., Crews, B. C., Rowlinson, S. W., Garner, C., Seibert, K., and Marnett, L. J. (1998) Science 280, 1268–1270.
8. Shimokawa, T., and Smith, W. L. (1992) J. Biol. Chem. 267, 23305–23310.
9. Mancini, J. A., O’Neill, G. P., Bayly, C., and Vickers, P. J. (1994) Adv. Exp. Med. Biol. 332, 33–37.
10. Hamberg, M., and Samuelsson, B. (1967) J. Biol. Chem. 242, 5326–5343.
11. Lecomte, M., Laneuville, O., Ji, C., DeWitt, D. L., and Smith, W. L. (1994) J. Biol. Chem. 269, 13207–13215.
12. Xiao, G., Tsai, A.-L., Palmer, G., Boyar, W. C., Marshall, P. J., and Kulmacz, R. J. (1997) Biochemistry 36, 1830–1845.
13. Serhan, C. N., Takano, T., and Maddox, J. F. (1999) Adv. Exp. Med. Biol. 447, 133–149.
14. Picot, D., Loll, P. J., and Garavito, R. M. (1994) Nature 367, 243–249.
15. Loll, P. J., Picot, D., and Garavito, R. M. (1995) Nat. Struct. Biol. 2, 637–642.
16. Loll, P. J., Picot, D., Ekabo, O., and Garavito, R. M. (1996) Biochemistry 35, 7330–7340.
17. Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M. F. (1996) Nature Struct. Biol. 3, 927–933.
18. Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. D., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1996) Nature 384, 644–648.
19. Rowlinson, S. W., Crews, B. C., Lanaz, C. A., and Marnett, L. J. (1999) J. Biol. Chem. 274, 23305–23310.
20. O’Neill, G. P., Mancini, J. A., Kargman, S., Yergy, J., Mei Yee Kwan, Falgueyret, J.-P., Abramovitz, M., Kennedy, B. P., Ouellet, M., Curnlish, W., Culp, S., Evans, J. F., Ford-Hutchinson, A. W., and Vickers, P. J. (1998) Mol. Pharmacol. 45, 245–254.
21. Mancini, J. A., O’Neill, G. P., Bayly, C., and Vickers, P. J. (1994) FEBS Lett. 342, 33–37.
22. Rieke, C. J., Mulichak, A. M., Garavito, R. M., and Smith, W. L. (1999) J. Biol. Chem. 274, 17109–17114.
23. Raz, A., Wyche, A., and Needleman, P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1657–1661.
24. Fu, J.-Y., Masferrer, J. L., Seibert, K., Raz, A., and Needleman, P. (1990) J. Biol. Chem. 265, 16737–16740.
25. Xie, W., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2692–2696.
26. Masferrer, J. L., Zweifel, B. S., Seibert, K., and Needleman, P. (1990) J. Clin. Invest. 86, 1375–1379.
27. Capdevila, J. H., Morrow, J. D., Belosludtsev, Y. Y., Beauchamp, D. R., DuBois, R. N., and Falck, J. R. (1995) Biochemistry 34, 3325–3337.
28. Laneuville, O., Breuer, D. K., Xu, N., Huang, Z. H., Gage, D. A., Wayson, J. T., Lagarde, M., DeWitt, D. L., and Smith, W. L. (1995) J. Biol. Chem. 270, 19320–19326.
29. Gierse, J. K., McDonald, J. J., Hauser, S. D., Erikson, R. L., and Simmons, D. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2692–2696.
30. Xie, W., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2692–2696.
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