Tyrosine-385 Is Critical for Acetylation of Cyclooxygenase-2 by Aspirin

G. Phillip Hochgesang, Jr., Scott W. Rowlinson, and Lawrence J. Marnett*

Departments of Biochemistry and Chemistry Center in Molecular Toxicology, and the Vanderbilt-Ingram Cancer Center Vanderbilt University School of Medicine Nashville Tennessee 37232-0146

Received February 2, 2000

Aspirin has been in use as a pharmaceutical agent for over 100 years.[1] The molecular target for its antiinflammatory and analgesic action is the cyclooxygenase isoform, COX-2, that converts arachidonic acid to prostaglandin endoperoxides.[2] Aspirin is unique among COX inhibitors because it covalently modifies the proteins and irreversibly inactivates them.[3] Aspirin transfers its acetyl group to Ser-530, which prevents proper binding of arachidonic acid in the cyclooxygenase active site (eq 1).[4−7] No

other residues on either COX-1 or COX-2 are acetylated. We and others have been interested in the factors that lead to the selectivity of acetylation of Ser-530 by aspirin and have previously identified the heme prosthetic group and the fatty acid carboxylate-binding residue, Arg-120, as important determinants of acetylation.[8−11] The heme prosthetic group restricts protein conformational mobility and may facilitate enzyme−aspirin interactions whereas Arg-120 is positioned adjacent to Ser-530 in the COX-2 active site.[12]

We have begun a systematic investigation of the importance of other amino acid residues in aspirin acetylation of COX-2 utilizing site-directed mutagenesis. Residues were chosen for mutation that are located in the vicinity of Ser-530 or have been implicated in the specific inhibition of COX-2 by other classes of non-covalent inhibitors. Mutant COX-2 proteins were expressed in insect cells and purified to near homogeneity.[13] Table 1 lists the purity of each protein preparation estimated by electrophoretic analysis in addition to the cyclooxygenase and peroxidase activities of the mutant proteins relative to wild-type enzyme. The high relative peroxidase activity exhibited by all of the mutants indicates that no gross structural changes occurred in the expressed proteins. Most of the mutant COX-2's retained significant cyclooxygenase activity with the exception of the Tyr385Phe mutant. The lack of cyclooxygenase activity in this protein is consistent with previous mutagenesis experiments with COX-1 and with the critical role of the tyrosyl radical of Tyr-385 in the oxidation of arachidonic acid.[14−16]

Table 1. Properties of Mutant COX-2 Enzymes and Their Acetylation by [^{14}C-Acetyl]-aspirin

mutant	purity %	cyclooxygenase activity[a] (wt %)	peroxidase activity (wt %)	aspirin labeling[b] (wt %)
Ser530Ala	85	49 ± 1	92 ± 6	0 ± 0 (0)
Tyr385Phe	95		134 ± 10	7 ± 1 (10)
Arg120Gln	97	86 ± 4	79 ± 10	27 ± 1 (25)
Arg120Ala	90	23 ± 0.8	92 ± 5	19 ± 2
VRV[c]	92	104 ± 4	109 ± 6	94 ± 5 (111)
Tyr348Phe	95	75 ± 1	94 ± 9	42 ± 2

[a] Catalytic activities are reported relative to the activity of wild-type enzyme. Assays were conducted at saturating concentrations of arachidonic acid (cyclooxygenase activity) or H_2O_2 (peroxidase activity). [b] Acetylation was determined as described and compared to the extent of acetylation of wild-type enzyme under identical conditions. Each value represents the average of 8−18 separate experiments ± standard error of the mean. The values in parentheses represent the relative extent of acetylation determined in the absence of hydroxylamine treatment. Measurements were not made in the absence of hydroxylamine for the Arg120Ala or Tyr348Phe mutants. [c] VRV = Val523Ile/Arg513His/Val434Ile

Recombinant proteins were reacted with [^{14}C-acetyl]-aspirin and the extent of acetylation quantitated by gel electrophoresis.[17] The incorporation of [^{14}C] into each mutant was compared to the extent of acetylation of wild-type enzyme. To minimize potential nonspecific labeling, each acetylated protein was treated with hydroxylamine to remove chemically labile acetyl groups. The relative labeling of each mutant enzyme is presented in Table 1. As expected, no acetylation of the Ser530Ala mutant was detected. The importance of acetylation on Ser-530 for inhibition of cyclooxygenase activity was verified by the observation that the Ser530Ala mutant was completely resistant to the inhibitory action of aspirin.

A 73% reduction in acetylation was observed in the Arg120Gln mutant consistent with the role of Arg-120 in ion-pairing to the salicylate moiety of aspirin. An even greater reduction in acetylation and loss of cyclooxygenase activity was observed in the Arg120Ala mutant. Since this mutation eliminates both ion-pairing and hydrogen bonding, the higher extent of labeling and

* Address correspondence to this author at: Department of Biochemistry, Vanderbilt University School of Medicine, Nashville TN 37232-0146. Telephone: (615) 343-7329. Fax: (615) 343-7534. E-mail: marnett@toxicology.mc.vanderbilt.edu.

(1) Vane, J. R.; Flower, R. J.; Botting, R. M. *Stroke* **1990**, *21(suppl IV)*, IV-12−IV-23.

(2) Vane, J. R.; Bakhle, Y. S.; Botting, R. M. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 97−120. There are two COX isoforms that exhibit differential cellular regulation and tissue distribution. In the present contribution, we use the abbreviation COX to refer to the proteins and the unabbreviated cyclooxygenase to refer to the catalytic activity or the active site where the reaction occurs.

(3) Rome, L. H.; Lands, W. E. M.; Roth, G. J.; Majerus, P. W. *Prostaglandins* **1976**, *11*, 23−30.

(4) Van Der Ouderaa, F. J.; Buytenhek, M.; Nugteren, D. H.; Van Dorp, D. A. *Eur. J. Biochem.* **1980**, *109*, 1−8.

(5) DeWitt, D. L.; El-Harith, E. A.; Kraemer, S. A.; Andrews, M. J.; Yao, E. F.; Armstrong, R. L.; Smith, W. L. *J. Biol. Chem.* **1990**, *265*, 5192−5198.

(6) Lecomte, M.; Laneuville, O.; Ji, C.; DeWitt, D. L.; Smith, W. L. *J. Biol. Chem.* **1994**, *269*, 13207−13215.

(7) Rowlinson, S. W.; Crews, B. C.; Goodwin, D. C.; Schneider, C.; Gierse, J. K.; Marnett, L. J. *J. Biol. Chem.* **2000**, *274*, 6586−6591.

(8) Chen, Y.-N. P.; Marnett, L. J. *FASEB J.* **1989**, *3*, 2294−2297.

(9) Wells, I.; Marnett, L. J. *Biochemistry* **1992**, *31*, 9520−9525.

(10) Loll, P. J.; Picot, D.; Garavito, R. M. *Nat. Struct. Biol.* **1995**, *2*, 637−642.

(11) Bhattacharyya, D. K.; Lecomte, M.; Rieke, C. J.; Garavito, R. M.; Smith, W. L. *J. Biol. Chem.* **1996**, *271*, 2179−2184.

(12) By convention, the COX-1 numbering system is used for all residues.

(13) Rowlinson, S. W.; Crews, B. C.; Lanzo, C. A.; Marnett, L. J. *J. Biol. Chem.* **1999**, *274*, 23305−23310.

(14) Shimokawa, T.; Kulmacz, R. J.; DeWitt, D. L.; Smith, W. L. *J. Biol. Chem.* **1990**, *265*, 20073−20076.

(15) Tsai, A.-L.; Palmer, G.; Xiao, G.; Swinney, D. C.; Kulmacz, R. J. *J. Biol. Chem.* **1998**, *273*, 3888−3894.

(16) Goodwin, D. C.; Gunther, M. H.; Hsi, L. H.; Crews, B. C.; Eling, T. E.; Mason, R. P.; Marnett, L. J. *J. Biol. Chem.* **1998**, *273*, 8903−8909.

(17) Wild-type or mutant murine COX-2 (40 μg) was reconstituted with 2 equiv of hematin and incubated for 3 h at 25 °C with 100 equiv of [1-^{14}C-acetyl]-salicylic acid in 100 mM tris-hydroxymethylaminomethane (pH 7.0) buffer containing 500 μM phenol in a final volume of 70 μL. After the initial 3 h incubation with aspirin, 10 μL of 1.05 M hydroxylamine was added, and the solution was incubated for an additional 1 h at 25 °C. Finally, 20 μL aliquots of the solution were added to each of three separate tubes containing 10 μL of sample buffer (3% sodium dodecyl sulfate, 10% glycerol, 62.5 μM tris-hydroxymethylaminomethane, 50 μM dithiothreitol) and then heated to 95 °C for three min prior to loading onto a 7.5% sodium dodecyl sulfate−polyacrylamide gel. Following electrophoresis, the gels were dried and autoradiographed on an Instant Imager (Packard Instruments, Meriden, CT).

10.1021/ja0003932 CCC: $19.00
Published on Web 06/20/2000

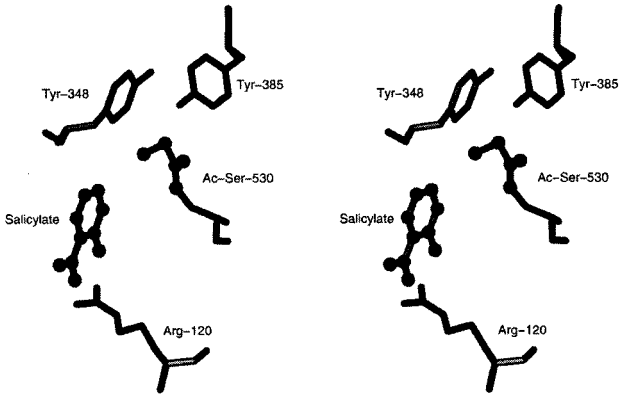

Figure 1. Stereo drawing of the active site of COX-1 bromoacetylated on Ser-530. Taken from ref 10.

cyclooxygenase activity of the Arg120Gln mutant indicates that hydrogen-bonding is important in COX-2 interactions with carboxylates. No reduction in acetylation was observed in the Val523Ile/Arg513His/Val434Ile triple mutant. This triple mutation introduces COX-1 residues at positions shown to be important for binding diarylheterocycles to COX-2.[18,19] Obviously, these residues make no contribution to the selectivity of aspirin for Ser-530 of COX-2.

The most dramatic finding of the present series of experiments was the significant decrease in acetylation observed in the Tyr385Phe mutant. As mentioned above, Tyr-385 plays an essential role in the oxidation of arachidonic acid, but there has been no indication that it is important for inhibitor binding. In fact, the decrease in acetylation of the Tyr385Phe mutant is greater than that detected in the Arg120Gln and Arg120Ala mutants and nearly as great as that observed in the Ser530Ala mutant. Acetylation of the Tyr348Phe mutant also was significantly decreased relative to wild-type but not to the same extent as in the Tyr385Phe mutant.

These results reveal that Tyr-385 and, to a lesser extent Tyr-348, are important for aspirin acetylation. It is likely that Tyr-385 is acting as a hydrogen bond donor to position the acetyl group adjacent to the hydroxyl group of Ser-530 and to increase the reactivity of aspirin. However, it is also possible that Tyr-385 is acting as a nucleophile and forming a transient *O*-acetyl-Tyr-385 that transfers acetyl to Ser-530. To test this, we carried out the acetylation of the Ser530Ala mutant and several other mutants without hydroxylamine treatment. Gel electrophoretic analysis revealed that no incorporation of [^{14}C] occurred in the Ser530Ala mutant protein. This suggests that Tyr-385 was not acetylated by aspirin in the Ser530Ala mutant. To test the stability

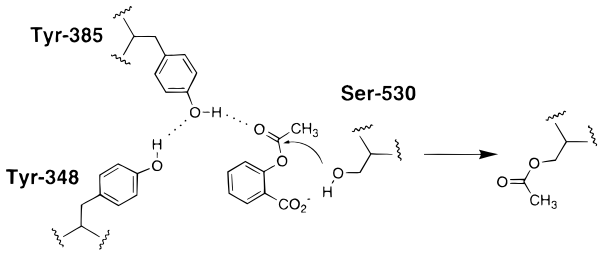

Figure 2. Proposed H-bonding between Tyr-385 and the carboxyl oxygen of aspirin during acetylation of Ser-530.

of acetylated tyrosine, *N*,*O*-diacetyltyrosine was exposed to the standard reaction and workup conditions. HPLC analysis of the recovered *N*,*O*-diacetyltyrosine demonstrated that it only underwent 15% hydrolysis. Thus, if COX-2 had been acetylated on Tyr-385, it should have survived workup and analysis. Quantitation of the acetylation of several other mutants without hydroxylamine treatment revealed no significant differences from the values measured with hydroxylamine treatment (Table 1). Thus, the extent of nonspecific acetylation of the various COX-2 proteins was extremely low.

The present experiments reveal a new and very important determinant of the selectivity of aspirin for COX-2. Inspection of the crystal structure of COX-1, covalently modified by bromoacetylsalicylic acid, reveals that the phenolic oxygen of Tyr-385 is 3.2 Å from the carbonyl oxygen of the bromoacetyl group, placing the two oxygens within hydrogen-bonding distance (Figure 1).[10] We propose that Tyr-385 hydrogen bonds to the acetyl group of aspirin, helping to localize it near Ser-530 and increasing its reactivity by stabilizing the incipient negative charge of the tetrahedral intermediate of acetylation (Figure 2). In the latter fashion, Tyr-385 acts in a manner analogous to the peptide N−H's of the oxyanion hole of the serine proteases.[20] In COX-1 and COX-2 crystal structures, the phenolic hydroxyl of Tyr-348 is hydrogen-bonded to the phenolic hydroxyl of Tyr-385, which would increase the hydrogen bonding capacity of the latter.[18,19,21] This may account for the significant reduction in acetylation of the Tyr348Phe mutant. Together, Tyr-385 and Tyr-348 constitute a hydrogen-bonding network that is critical for the precise positioning and increased reactivity of the acetyl group of aspirin in the vicinity of Ser-530.

Acknowledgment. This work was supported by a research grant and center grants from the National Institutes of Health (CA47479, ES00267, and CA68485). S.W.R. was supported by a C. J. Martin Fellowship from the National Health and Medical Research Council of Australia.

JA0003932

(18) Luong, C.; Miller, A.; Barnett, J.; Chow, J.; Ramesha, C.; Browner, M. F. *Nat. Struct. Biol.* **1996**, *3*, 927−933.
(19) Kurumbail, R. G.; Stevens, A. M.; Gierse, J. K.; McDonald, J. J.; Stegeman, R. A.; Pak, J. Y.; Gildehaus, D.; Miyashiro, J. M.; Penning, T. D.; Seibert, K.; Isakson, P. C.; Stallings, W. C. *Nature* **1996**, *384*, 644−648.
(20) Menard, R.; Storer, A. C. *Biol. Chem. Hoppe Seyler* **1992**, *373*, 393−400.
(21) Picot, D.; Loll, P. J.; Garavito, R. M. *Nature* **1994**, *367*, 243−249.
(22) Odenwaller, R.; Maddipati, K. R.; Marnett, L. J. *J. Biol. Chem.* **1992**, *267*, 13863−13869.