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*

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Examination of the crystal structure of the ovine prostaglandin endoperoxide synthase-1 (PGHS-1)†-flurbiprofen complex (Picot, D.; Loll, P. J.; and Garavito, R. M. (1994) Nature 367, 243-249) suggests (a) that the carboxyl group of arachidonic acid interacts with the arginino group of Arg120, (b) that Arg120 forms an important salt bridge with Glu524, and (c) that Tyr355, which is in close proximity to Arg120, could determine the stereospecificity of PGHS-1 toward 2-phenylpropionic acid inhibitors. To test these concepts, we used site-directed mutagenesis to prepare ovine PGHS-1 mutants having modifications of Arg120 (R120K, R120Q, R120E), Glu524 (E524D, E524Q, E524K), and Tyr355 (Y355F) and examined the properties of the mutant enzymes expressed in COS-1 cells. All of the mutants retained at least part of their cyclooxygenase and peroxidase activities except the R120E mutant, which had no detectable activity. The $K_m$ values of the R120K and R120Q mutants with arachidonic acid were 67 and 3300 $\mu M$, respectively, versus 4 $\mu M$ for native PGHS-1. The R120Q mutant failed to undergo suicide inactivation during catalysis or time-dependent inhibition by flurbiprofen. These results are consistent with Arg120 binding the carboxylic group of arachidonate and suggest that interaction of the carboxylic group of substrates and inhibitors with Arg120 is necessary for suicide inactivation and time-dependent inhibition, respectively. The $K_m$ values for the E524D, E524Q, and E524K mutants were not significantly different from values obtained for the native PGHS-1, suggesting that this residue is not importantly involved in catalysis or substrate binding. The effect of modifications of Arg120 and Tyr355 on the stereospecificity of inhibitor binding were determined. Ratios of $IC_{50}$ values for cyclooxygenase inhibition by o-and l-ibuprofen, a competitive cyclooxygenase inhibitor, were 32, 67, and 71 for native PGHS-1, R120Q PGHS-1, and Y355F PGHS-1, respectively. The decreased stereospecificity observed with the Y355F PGHS-1 mutant suggests that Tyr355 is a determinant of the stereospecificity of PGHS-1 toward inhibitors of the 2-phenylpropionic acid class.

Prostaglandin endoperoxide H synthase-1 (PGHS-1)† catalyzes the committed step in the conversion of arachidonic acid to prostaglandins and thromboxanes (1). The processed form of ovine PGHS-1 is an N-glycosylated hemoprotein having 576 amino acids and an apparent subunit molecular mass of 72 kDa (2–6). PGHS-1 exhibits both a cyclooxygenase activity involved in converting arachidonate to PGG2 and a hydroperoxidase activity that reduces the hydroperoxy group of PGG2 to PGH2 (7–9). Although a single heme molecule is essential for both activities, the cyclooxygenase and peroxidase substrate binding sites are functionally and spatially distinct (10–15).

Examination of the crystal structure of the ovine PGHS-1/s-flurbiprofen inhibitor complex indicates that the cyclooxygenase active site is a long hydrophobic channel extending from the putative membrane binding domain into the center of the globular catalytic domain (Fig. 1) (10). In the crystal structure, the arginino group of Arg120 located near the channel opening is complexed with the carboxylic group of flurbiprofen. The carboxylic group of Glu524 appears to form a salt bridge with the arginino group of Arg120. Arachidonate is postulated to bind with its ω-methyl group extending into the core of the channel and its carboxylic group complexed to Arg120. The phenolic side chain of Tyr355 is present near the mouth of the channel opposite Arg120. This bulky group places a constriction on one side of the channel which may determine the stereoselectivity of PGHS-1 for the S-isomers of 2-phenylpropionic acid inhibitors (16), such as ibuprofen and flurbiprofen. Finally, some PGHS-1 and PGHS-2 inhibitors, including flurbiprofen, cause a time-dependent, slowly reversible inhibition of cyclooxygenase activity (17–21); however, the methyl esters of these types of compounds are freely reversible (although less potent) inhibitors (17). This latter observation suggests that modification of the amino acid involved in binding the carboxylic group of time-dependent inhibitors such as flurbiprofen would affect the mechanism of inhibition. To begin testing the functions of Arg120, Glu524, and Tyr355 in the binding of fatty acid substrates and 2-phenylpropionic acid inhibitors, we prepared appropriate mutants of ovine PGHS-1 and examined their kinetic properties.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium was from Life Technologies, Inc. Fetal calf serum and calf serum were obtained from Hyclone. Bovine hemoglobin, hematin, guaiacol, DEAE-dextran, and penicillin G were from Sigma. Arachidonic acid, PGG2, PGE2, and PGF2α were purchased from Cayman Chemical Co.

†The abbreviations used are: PGHS, prostaglandin endoperoxide H synthase; PG, prostaglandin; 12-HOTrE, 12-hydroxy- (9Z,13E/9Z,15Z)-octadecatrienoic acids.
[1-14C]Arachidonic acid (40–60 mCi/mmol), [1-14C]a-linolenic acid (53.0 mCi/mmol), and [35S]methionine (1000–1500 Ci/mmol) were from Du Pont NEN. ECL Western blotting reagents were from Amersham Corp. T4 DNA ligase and T4 DNA polymerase were purchased from Boehringer Mannheim. Other reagents were purchased from common commercial sources.

Preparation of Ovine PGHS-1 Mutants by Site-directed Mutagenesis—Mutants of ovine PGHS-1 (R120K, R120Q, R120E, E524K, E524Q, E524D) were prepared starting with M13mp19-PGHSov-1, which contains the coding region of ovine PGHS-1 (22), using a Bio-Rad kit essentially as described previously (23). Listed in Table I are the oligonucleotides used for the preparation of each mutant. Phage samples were sequenced using the dideoxy method (24) to identify mutants. The 2.3-kilobase insert from the replicative form of M13mp19-PGHSov-1 containing the desired mutation was isolated after digestion with Sall and subcloned into the Sall site of pSVT7 (23, 25). The correct orientation of the insert was determined by restriction digestion with PstI. The Y355F mutant of ovine PGHS-1 was as described previously (12). Plasmids used for transfection were purified by CsCl density gradient ultracentrifugation.

Measurement of Prostaglandin Formation by Transfected COS-1 Cells—COS-1 cells (ATCC CRL-1650) were grown to near confluence in Dulbecco’s modified Eagle’s medium containing 8% calf serum and 2% fetal calf serum, streptomycin (0.1 g/liter), and penicillin G (0.1 g/liter) in a water-saturated 5% CO2 atmosphere. About 16 h before transfection, the cells were subcultured 1:2. The COS-1 cells were transfected as described previously (22, 23), using the DEAE-dextran/chloroquine method.

Assays of prostaglandin formation by transfected cells were performed essentially as described previously (26). Approximately 40 h post-transfections, the cells were removed from culture dishes using a rubber policeman, collected by centrifugation at 1000 x g for 10 min, and resuspended in 0.5 ml of Dulbecco’s modified Eagle’s medium/three 100-mm culture plates. The resuspended cells were incubated with 60 µM [1-14C]arachidonic acid or 10 µM [1-14C]a-linolenic acid for 15 min at 37°C. To terminate the reactions, the cells were centrifuged for 5 min at 1000 x g, the medium was removed, and 4 volumes of ice-cold acetone were added to the medium. After removal of denatured protein by centrifugation, the supernatant containing the radioactive prostaglandin products and unreacted arachidonic acid was added with 0.5 volume of 0.2 M HCl and extracted with 6 volumes of chloroform. The organic phase was evaporated to dryness under a stream of nitrogen, resuspended in chloroform, and applied to a silica gel 60 thin-layer chromatography plate. The lipid products were separated by chromatography twice in benzene:dioxane:acetic acid:formic acid (82:14:1:1, v/v/v/v), and the thin-layer chromatography plates were then exposed to Kodak XAR-5 film, typically for 40 h. Autoradiographic bands were quantified by densitometry twice in benzene:dioxane:acetic acid:formic acid (82:14:1:1, v/v/v/v), and the thin-layer chromatography plates were then exposed to Kodak XAR-5 film, typically for 40 h. Autoradiographic bands were quantified by densitometry using a Visage 110 Image Analyzer. The percentage of radioactive arachidonate converted to prostaglandins was calculated by dividing the density attributable to PGF2α plus PGE2 plus PGD2 by the total density of all bands and multiplying by 100.

COS-1 cells expressing human PGHS-1 (18), human PGHS-2, ovine PGHS-1 (18), or ovine R120Q PGHS-1 were incubated with 10 µM [1-14C]a-linolenic acid for 15 min at 37°C and the products extracted and separated by radio-thin-layer chromatography essentially as described above. Products chromatographing as 12-hydroxy-9,13,15-octadecatrienonic acid (12-HOTIE) were quantified by densitometry (27).

Oligonucleotides used for site-directed mutagenesis of ovine PGHS-1

The superscripts indicate the nucleotide number of the cDNA for ovine PGHS-1 (5); the original codons are CGT for Arg120 and GAA for Glu524. The base substitutions are underlined.

| Mutation | Mutagenic oligonucleotides |
|----------|-----------------------------|
| Arg120→Lys | 445 | 5′-GACTCAGTGGTGAGGCGCTCTTA-3′ |
| Arg120→Gln | 445 | 5′-GACTCAGTGGTGAGGCGCTCTTA-3′ |
| Arg120→Glu | 445 | 5′-GACTCAGTGGTGAGGCGCTCTTA-3′ |
| Glu248→Lys | 1657 | 1683 |
| Glu248→Gln | 1657 | 1683 |
| Glu248→Asp | 1657 | 1683 |

FIG. 1. Stereo view of the amino acid side chains which create the cyclooxygenase active site channel in ovine PGHS-1. The active site lies between the heme group (in red) at the top and the channel mouth. Bound within the cyclooxygenase active site is the NSAID flurbiprofen (yellow) which lies near Ser530 (orange), the site of aspirin acetylation. The putative radical donor, Tyr385 (blue), lies between the heme and flurbiprofen. The carboxylate of flurbiprofen ligands Arg120 (green) and Tyr355 (blue). Glu524 (red) may also interact with arginine 120.
harvested as described above. The isolated cells were resuspended in 0.1 M Tris-HCl, pH 7.4, and used to prepare microsomal membrane fractions as described previously (22, 23). Cyclooxygenase activities were assayed using microsomal preparations made within 3–4 h of their isolation by monitoring the initial rate of O$_2$ uptake at 37 °C (200 μM O$_2$) using an oxygen electrode (22, 23). Kam values for arachidonate were determined using arachidonate in the presence of varying concentrations of inhibitor added directly to the assay mixture prior to the addition of enzyme (18). To measure time-dependent inhibition of cyclooxygenase activity by flurbiprofen, microsomal preparations of PGHS-1 or R120Q PGHS-1 were incubated at 37 °C for 0–20 min in the presence and absence of various concentrations of flurbiprofen. Aliquots of these preincubation mixtures were then assayed for cyclooxygenase activity.

Western Transfer Blotting—Solubilized microsomal membranes were resolved by one-dimensional SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to BA85 nitrocellulose filters (0.45 μm), essentially as detailed previously (29, 30). The filters were washed and incubated for 1–2 h with a 1:2000 dilution of an affinity-purified rabbit antibody directed against the sequence LMHYPRGIPPQ-C, corresponding to residues Leu$^{272}$–Gln$^{283}$ of ovine PGHS-1 (30). The filters were then washed and incubated with a 1:2000 dilution of a goat anti-rabbit IgG-horseradish peroxidase conjugate. The filters were then washed and incubated with 1:5 min with Amersham ECL Western blotting detection reagents and then blot-dried and exposed to XAR-5 film.

RESULTS

Prostaglandin Formation by Native and Mutant Ovine PGHS-Ss-1—Transfected COS-1 cells expressing either native ovine PGHS-1 or ovine PGHS-Ss-1 muta$\text{nt}$s having substitutions of Arg$^{120}$ or Glu$^{524}$ were incubated with 60 μM [1-14C]arachidonate for 15 min. The major radioactive prostaglandin products (PGE$_2$, PGF$_{2\alpha}$, and PGE$_{3\alpha}$) were separated by thin-layer chromatography and quantified (Fig. 2). Prostaglandins were formed by cells transfected with all of the PGHS-1 mutants except R120Q mutant; however, significantly less product formation occurred with cells expressing the R120Q PGHS-1 mutant. The radioactive prostaglandin and hydroxy fatty acid products appeared to be formed in the same relative proportions in all cases, indicating that none of the mutations other than the R120E mutation affected the normal mechanism of oxygenation of arachidonate. Western transfer blotting of equal amounts of microsomal protein from COS-1 cells expressing each of the mutants and the native PGHS-1 indicated that all of the enzymes except the R120E PGHS-1 mutant were expressed at comparable levels and that R120E PGHS-1 was expressed at about 22% of the level of native PGHS-1 (data not shown).

Ovine native PGHS-1 and the various PGHS-S-1 mutants were expressed transiently in COS-1 cells, and microsomal membranes prepared from the cells were assayed for cyclooxygenase activity, using 100 μM arachidonate, and peroxidase activity, using H$_2$O$_2$ and guaiacol as the cosubstrates (Fig. 3). Again, the R120E mutant lacked detectable cyclooxygenase and peroxidase activities. The R120K mutant exhibited only 10% of cyclooxygenase activity of native PGHS-1, but increased levels of peroxidase activity. The R120Q mutant had only 5% of the cyclooxygenase activity of the native enzyme but 60% of native peroxidase activity. Mutations of Glu$^{524}$ had little effect on either the cyclooxygenase or peroxidase activities of PGHS-1.

The rate of oxygenation by native ovine PGHS-1 falls by greater than 90% within 2 min of adding enzyme to the assay chamber (Fig. 4); this is due to suicide inactivation of native ovine PGHS-1 (1). In contrast, the rate of oxygenation of arachidonic acid by the R120Q mutant remained relatively constant for several minutes after mixing enzyme and substrates. The V$_\text{max}$ values for suicide inactivation of native PGHS-1 and R120Q PGHS-1 were estimated by plotting the log of activity at different times after initiation of oxygenation versus time and found to be approximately 15 and 330 s, respectively (Fig. 4).

Clearly, R120Q PGHS-1 undergoes suicide inactivation at a much slower rate than native enzyme; this difference between rates of suicide inactivation accounts for the rather small (2-fold) difference in prostaglandin product formation observed after a 15-min incubation (Fig. 2) versus the rather large (20-fold) difference in initial velocities of oxygenation (Fig. 3) when comparing native PGHS-1 and R120Q PGHS-1.

The K$_\text{m}$ values determined for arachidonic acid with native PGHS-1 and various mutant PGHS-Ss-1 are presented in Table I. Native ovine PGHS-1 had a K$_\text{m}$ value of 4 μM with arachidonate. In the case of the R120K mutant, this value increased about 20-fold to 87 μM. An even more dramatic increase of roughly 1000-fold was observed for the R120R mutant. In contrast, the E524K, E524Q, and E524D mutants of ovine PGHS-1 exhibited K$_\text{m}$ values in the low micromolar range, similar to that of native PGHS-1.

α-Linolenic acid is a C$\text{18}$ fatty acid that can be oxidized to 12-HOTrE by either human PGHS-1 or PGHS-2 (27); however, α-linolenic acid exhibits a particularly low K$_\text{m}$/K$_\text{m}$ value with human PGHS-1 (27). Shown in Fig. 5 is a comparison of 12-HOTrE formation from [1-14C]linolenic acid by COS-1 cells expressing human PGHS-1, human PGHS-2, ovine PGHS-1, or the R120Q mutant of ovine PGHS-1. Although ovine PGHS-1 formed product from α-linolenic acid, the R120Q mutant failed to form any product. Thus, modification of Arg$^{120}$ dramatically
affects the oxygenation of this C18 fatty acid substrate as well as arachidonate. To determine whether elimination of the positively charged Arg120 group permits PGHS-1 to use fatty acid methyl esters as substrates, we also incubated microsomal preparations of COS-1 cells expressing native PGHS-1 and the R120Q PGHS-1 mutant with methyl arachidonate. However, no oxygenation of the methyl ester was observed with either enzyme preparation as determined by O2 electrode assays.

Stereochemical Selectivity of Native and Mutant PGHSs-1 with D- and L-Ibuprofen. In the crystal structure of the ovine PGHS-1/S-flurbiprofen complex, the phenolic side chain of Tyr355 is located in a position which permits the carboxylate group of S-flurbiprofen to bind to Arg120; however, molecular modeling of the binding of R-flurbiprofen suggests that the phenolic group of Tyr355 would interact with the α-methyl group of this inhibitor, so as to interfere with the binding of the carboxylate group to Arg120; this provides an explanation for why R-isomers of 2-phenylpropionic acid derivatives are always much poorer inhibitors of PGHS-1 than the corresponding S-isomers. We tested this concept using D- and L-ibuprofen and an ovine Y355F PGHS-1 mutant prepared previously (12); IC50 values were determined for native PGHS-1, Y355F PGHS-1, and R120Q PGHS-1 mutants (Fig. 6). The ratios of the IC50 values determined with D- and L-ibuprofen were 32, 7, and 67 for native PGHS-1, Y355F PGHS-1, and R120Q PGHS-1, respectively. Thus, there is a partial loss of stereospecificity with the Y355F mutant. This result is consistent with the proposal that the phenolic side chain of Tyr355 does influence the stereospecificity of binding of 2-phenylpropionic acid derivatives to ovine PGHS-1.

Time-dependent Inhibition by Flurbiprofen—Flurbiprofen causes a time-dependent inhibition of PGHS-1 (17, 18),
whereas the methyl ester of flurbiprofen does not (17). The following experiments were performed to determine if the converse were true (i.e., whether the R120Q mutant of PGHS-1 was refractory to time-dependent inhibition by flurbiprofen). First, the IC50 values were determined to be 5 μM and 1 mM for "instantaneous" inhibition by flurbiprofen of native PGHS-1 and R120Q PGHS-1, respectively (data not shown); the value for native PGHS-1 is very similar to that reported earlier (17, 22). Fig. 7 shows the effects of incubating R120Q PGHS-1 with 1 mM flurbiprofen for different times. No time-dependent inhibition of R120Q PGHS-1 was observed upon treatment of this mutant enzyme with flurbiprofen. However, as reported previously, flurbiprofen caused a time-dependent inhibition of native PGHS-1 activity (17, 27); with 10^-6 M flurbiprofen, the t1/2 for inactivation of PGHS-1 was about 2.5 min (data not shown).

DISCUSSION

The studies reported here were designed to examine postulated roles for Arg120, Glu524, and Tyr355 (10) in the binding of fatty acids and nonsteroidal anti-inflammatory drugs to the cyclooxygenase active site of ovine PGHS-1. Our results support the concept that Arg120 is involved in binding the carboxylate moieties of arachidonate and two different 2-phenylpropionate inhibitors, ibuprofen and flurbiprofen, but that Glu524 is not involved in either catalysis or substrate binding (Fig. 1). Placing a negative charge in the form of the side chain of a glutamate at residue 120 reduced cyclooxygenase activity to undetectable levels. However, this loss of cyclooxygenase activity was accompanied by a corresponding loss of peroxidase activity, suggesting that the R120E mutant does not fold properly (2, 13); also consistent with this conclusion was the observation of substantially diminished expression of the R120E mutant compared with all the catalytically active PGHS-1 mutants examined in this study. When Arg120 was replaced with a glutamine residue having a neutral side chain, the R120Q PGHS-1 mutant retained detectable levels of cyclooxygenase described in the text. Enzyme was added to the assay mixture containing substrates and inhibitor to determine instantaneous inhibition. The maximal activities in the absence of inhibitor (100%) were 140, 12, and 21 nmol of arachidonate consumed per min/mg of microsomal protein for native ovine PGHS-1, R120Q PGHS-1, or Y355F PGHS-1, respectively. The experiments depicted in this figure were performed twice with similar results.
activity (5%) and peroxidase activity (approximately 60%), but the $K_v$ value for arachidonate was about 1000-fold higher than that seen for the native enzyme. The lack of activity of the R120Q PGHS-1 toward arachidonate is also important for understanding the roles of the various functional groups based on their abilities to cause simple competitive inhibition.

When IC$_{50}$ values were determined using the same concentration of arachidonate, the concentration of $i$-ibuprofen required to cause half-maximal inhibition of R120Q PGHS-1 was 10–20 times higher than that required to inhibit the native enzyme (Fig. 6). Because the difference in $K_v$ values for arachidonate and the native PGHS-1 and R120Q PGHS-1 differ by about 1000-fold (Table II), this observation suggests that Arg$_{120}$ is relatively more important for arachidonate binding than ibuprofen binding.

Previous studies had shown that Tyr$_{355}$ of ovine PGHS-1 can be nitrated in the presence but not the absence of either indomethacin or ibuprofen (12, 31). These findings indicated that Tyr$_{355}$ could reside in or near the cyclooxygenase active site of the enzyme; nonetheless, the Y355F PGHS-1 mutant retained appreciable activity, indicating that Tyr$_{355}$ is not essential for catalysis (12). When the crystal structure of the ovine PGHS-1/5-fluribuprofen complex was solved, Tyr$_{355}$ was found to be nitrated near the mouth of the cyclooxygenase channel and to neighbor the $\alpha$-methyl group of S-fluribuprofen. Accordingly, Garavito and co-workers (10) proposed that modification of this Tyr$_{355}$ might alter the stereospecificity of PGHS-1 toward inhibitors of the 2-phenylpropionic acid group such as ibuprofen and fluribuprofen. Indeed, the Y355F PGHS-1 mutant, which has a relatively small decrease in side chain size at this position, exhibited considerably less specificity toward S- and L-ibuprofen than did the native PGHS-1.

Nonsteroidal anti-inflammatory drugs fall into two major functional groups based on their abilities to cause simple competitive versus time-dependent, competitive inhibition (19, 21). Ibuprofen is a freely reversible competitive inhibitor, whereas flurbiprofen and indomethacin cause a time-dependent inhibition which is only slowly reversible (17–20, 32). However, the methyl esters of time-dependent inhibitors are freely reversible, competitive inhibitors (17). In the case of flurbiprofen, esterification would be expected to diminish the ability of the inhibitor to bind Arg$_{120}$. Our results indicate that the converse is also true since the R120Q mutant of PGHS-1 did not undergo a time-dependent inhibition even in the presence of high concentrations (10$^{-3}$ M) of flurbiprofen. Thus, binding of the carboxylate group of inhibitors is one important characteristic of time-dependent inhibition. We speculate that one requirement for time-dependent inhibition is that the inhibitor remain in the cyclooxygenase active site long enough for some secondary NSAID-induced change in protein structure to occur and that the occupancy time for binding of nonsteroidal anti-inflammatory drugs such as flurbiprofen to the active site of the R120Q mutant is too short to permit such structural changes to occur.

A final unexpected characteristic of the R120Q PGHS-1 mutant is its incapacity to undergo suicide inactivation. This feature has been observed with several other forms of ovine PGHS-1, including the Mn$^{3+}$-heme PGHS-1 (33) and several mutants having modification near the active site tyrosine, notably H386A/Q ovine PGHS-1 (13) and H372A human PGHS-2. However, unlike these mutants, the R120Q PGHS-1 mutant exhibits considerable peroxidase activity. One characteristic of peroxidase-deficient forms of PGHS-1 is that they fail to turn over a seesaw radical in abundance (33, 34). It will be of interest to determine if the same is true of R120Q PGHS-1.

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