Prostaglandin H synthases (PGHSs) catalyze the conversion of arachidonic acid to prostaglandins. In this report, we describe the effect of a PGHS2 Y355F mutation on the dynamics of PGHS2 catalysis and inhibition. Tyr355 is part of a hydrogen-bonding network located at the entrance to the cyclooxygenase active site. The Y355F mutant exhibited allosteric activation kinetics in the presence of arachidonic acid that was defined by a curved Eadie-Scatchard plot and a Hill coefficient of 1.36 ± 0.05. Arachidonic acid-induced allosteric activation has not been directly observed with wild type PGHS2. The mutation also decreased the observed time-dependent inhibition by indomethacin, flurbiprofen, RS-57067, and SC-57666. Detailed kinetic analysis showed that the Y355F mutation decreased the transition state energy associated with slow-binding inhibition ($E_{IS}^\ddagger$) relative to the energy associated with catalysis ($E_{IS}^\neq$) by 1.33, 0.67, and 1.06 kcal/mol, respectively, for indomethacin, flurbiprofen, and RS-57067. These observations show Tyr355 to be involved in the molecular mechanism of time-dependent inhibition. We interpret these results to indicate that slow binding inhibitors and the Y355F mutant slow the rate and unmask intrinsic, dynamic events associated with product formation. We hypothesize that the dynamic events are the equilibrium between relaxed and tightened organizations of the hydrogen-bonding network at the entrance to the cyclooxygenase active site. It is these rearrangements that control the rate of substrate binding and ultimately the rate of prostaglandin formation.

Prostaglandins are formed from arachidonic acid by constitutive prostaglandin H synthase 1 (PGHS1) and inducible prostaglandin H synthase 2 (PGHS2) (1). They are important cellular mediators of many biological functions, including inflammation, pyresis, and algesia (2, 3). Recent evidence suggests that prostaglandins formed by PGHS2 mediate inflammation (3, 4). PGHS2 is also implicated in the pathology of Alzheimer’s disease and colon cancer (5–7).

The latest methodologies in structure-based drug discovery are being used to identify PGHS2-selective medicines. Inhibitor-bound structures of PGHS1 and PGHS2 have been solved (8–11). The structures and additional mutagenesis data have identified a number of important features (12–17) (Fig. 1). The inhibitors bind in a long channel whose entrance is flanked by three residues capable of creating a hydrogen-bonding network, Arg330, Glu324, and Tyr355. Arg330 is required for binding the carboxylic acid moiety of fatty acid substrates and nonsteroidal anti-inflammatory drugs (12, 13). Tyr355 is proposed to be a determinant of specificity in the 2-phenylpropionic class of inhibitors; inhibition of the PGHS1 phenylalanine mutant by ibuprofen produced a change in the stereochemical specificity but not potency (13). The channel ends at residue Tyr385, a residue required for cyclooxygenase catalytic activity (14). The channel is bordered by Ser326, the site of aspirin acetylation and a side pocket that nonsteroidal anti-inflammatory drugs can occupy in PGHS2 but not PGHS1. This pocket is the result of a change in PGHS1H513 to PGHS2V523 and PGHS1H513 to PGHS2R513 and is important for the specificity of some PGHS2-selective inhibitors (15–17).

Dynamics have been shown to play critical roles in the mechanism and inhibition of PGHSs. Many inhibitors of both PGHS isoforms are time-dependent (18–21), and the enzymes are allosterically activated by arachidonic acid (22). Selective PGHS2 inhibition in many cases is correlated with the time-dependent inhibition of PGHS2 and a lack of time-dependent PGHS1 inhibition (20, 21). A loss of PGHS1 binding affinity coinciding with conformational changes induced by the allosteric activation of PGHS1 also results in an increase in PGHS2 selectivity (22). Additionally, the allosteric regulation is proposed to contribute to regulation and selectivity of prostaglandin formation (22). The factors that relate these dynamic changes to protein structure are unknown.

The kinetic mechanism of many time-dependent PGHS inhibitors is reversible slow binding inhibition. The inhibitors bind to form an initial enzyme-inhibitor complex ($E_1$) that is in slow equilibrium with another enzyme-inhibitor complex ($E_1^\ddagger$) (Scheme 1) (23, 24). Intrinsic to this kinetic mechanism are two competing equilibria: an intermolecular equilibrium between the inhibitor and substrate for enzyme and an intramolecular equilibrium between the two enzyme-inhibitor binding complexes. It is the rates of these equilibria relative to the rate of product formation that will determine if these equilibria can be kinetically observed.

\[
EP \leftrightarrow ES \leftrightarrow E \leftrightarrow E_1 \leftrightarrow E_1^\ddagger
\]

Scheme 1

We have previously described a method to determine the kinetic constants associated with the reversible slow binding inhibition of PGHS (24). The method enabled us to determine
the transition state energy between the two enzyme inhibitor complexes, $E_1$ and $E^{1*}$. The transition state energies for indomethacin and flurbiprofen were both approximately 1.4 kcal/mol lower for PGHS1 than PGHS2.

We have used this methodology to evaluate the influence of structure on the functional dynamics of PGHS2 inhibition. The dynamics associated with inhibition by flurbiprofen, indomethacin, and RS-57067 and the kinetics associated with allosteric activation by arachidonic acid were evaluated with the PGHS2 Y355F mutant. The time-dependent inhibition and allosteric activation are shown to involve the phenolic group of Tyr355. These dynamics are postulated to be kinetically detectable due to the unmasking of an equilibrium between a relaxed and tightened organization of the hydrogen-bonding network located at the entrance to the cyclooxygenase binding pocket.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Arachidonic acid, [3H]Anadamide, and [3H]Prostaglandin E2 were purchased from NEN Life Science Products; unlabeled arachidonic acid was from Nu-Chek-Prep, Inc. (Elysian, MN); and unlabeled anadamide, indomethacin, and flurbiprofen were from Cayman Chemical Co. (Ann Arbor, MI). RS-57067 and SC-57666 were synthesized at Roche Bioscience. Hemin and tyloxapol were obtained from Sigma. All other chemicals were of the highest grade available.

**Rate Measurements**—Prostaglandin formation was determined in reactions that contained [3H]arachidonic acid (50 nM to 20 µM; 0.5 µCi) or [3H]Anadamide, tyloxapol (0.05%), phenol (1.2 mM), hemin (0.6 µM), and potassium phosphate buffer (100 mM, pH 8.0) in a total volume of 0.1 ml unless otherwise stated. All reactions were incubated at 30 °C. The reactions containing arachidonic acid were incubated for 30 s (3 min for anadamide) and stopped with 0.4 ml of ice-cold ethanol and placed on ice. After approximately 30 min on ice, the reactions were evaporated to dryness and reconstituted in 0.2 ml of 50:50:1 water/methanol/acetic acid, and 0.1 ml was injected into the HPLC for analysis. The substrate solution was prepared by combining unlabeled arachidonic acid in ethanol, [3H]arachidonic acid in ethanol, and 10 µl of a 0.5% solution of tyloxapol in acetone and evaporating to dryness. This was reconstituted in 0.05 ml of water. The reactions were initiated by adding the detergent-solubilized substrate solution to the enzyme mix containing buffer, phenol, and hemin, which had been preincubated at room temperature for 1–5 min.

Oxygen consumption was measured with a YSI model 5300 Biological Oxygen Monitor equipped with a Clark-type micro oxygen electrode. Data was collected and transformed using an external connection to a SLM-Aminco DW2000 data system. Enzyme (20–50 µM) was mixed with hemin (0.8 µM) and phenol (2 mM) to establish a baseline. The reaction was initiated with arachidonic acid (200 µM) after preincubation with either inhibitor or vehicle. Arachidonic acid was added in tyloxapol; the final concentration of tyloxapol was not greater than 0.08%. The maximum velocity was determined from a first derivative transformation of the reaction profile. The system was calibrated using the catalase-dependent oxidation of hydrogen peroxide.

**HPLC Conditions**—The products of arachidonic acid and anadamide oxidation were separated by reverse-phase HPLC using a 25-cm, 5 µm, Jones Chromatography apex octadecyl column and detected using a Packard Flo-one A-500 radioflow detector with a scintillant mixture: HPLC eluant ratio of 3:1. The strong component of the mobile phase was 0.1% acetic acid/ammonium hydroxide buffer (pH 6.1), and the eluting solvent was methanol. The flow rate was 1 ml/min. The following elution profile was used: 0–5 min 20% methanol; 6–15 min 7% convex gradient to 60% methanol; 16–20 min 80% methanol; 21–30 min 90% methanol; 31–35 min 80% methanol; 36–45 min 100% methanol. The prostaglandins eluted around 25 min, arachidonic acid at 44 min, anadamide-derived prostaglandins at 22 min, and anadamide at 38 min (25).

**Mathematical Methods**—A mathematical model was developed, resulting in a final three-parameter equation describing the initial maximum velocity of the enzyme-substrate reaction as a function of the enzyme-inhibitor preincubation time (24). Time-velocity curves were obtained for various inhibitor concentrations, and the three parameters and their asymptotic standard errors were estimated by simultaneously fitting the time-velocity curves for all inhibitor concentrations to the final equation. The Nelder-Mead simplex algorithm in an adaptation of the software package PCNONLIN used to estimate the parameters,

$$f(T) = 100 \left(1 + \frac{k_{app}}{K_i} \left(e^{-k_{app}T} - 1 \right) \right)$$

where

$$Q = \frac{(K_i/S) + 1}{(K_i/S)(1 + (K_i/S)) + 1}$$

and

$$k_{app} = \frac{k_2}{1 + (K_i)}$$

Fig. 1. Structure of the active sites of PGHS1 (A) and PGHS2 (B) with key amino acids highlighted. The PGHS1 structure is bound with flurbiprofen (8), and the PGHS2 structure is bound with RS-57067 (10). These two views show two possible arrangements of the hydrogen bonding network: one involving Arg120, Glu524, Tyr355 (PGHS1) and the other Glu524, Tyr355, and Arg513 (PGHS2).
The enzymes were incubated with arachidonic acid for 30 s or anadamide for 3 min, and the amount of prostaglandins formed was determined by HPLC. The statistics are the S.E. determined from best fit of a single representative experiment. \[ \Delta G^\circ_T = -RT \ln \left( \frac{k_{cat}/K_m}{\text{mut}/K_m} \right) \] (Eq. 7) The free energy of ES was calculated by subtracting the free energy associated with \( k_{cat} \), determined by the Eyring equation, from the free energy associated with ES. The activation energies between free enzyme and EI and ES were assumed to be small relative to those associated with ES and EI.

The apparent \( K_m \) and \( k_{cat} \) values associated with prostaglandin formation from arachidonic acid and its ethanamide analog, anadamide (25), catalyzed by purified recombinant human PGHS2 and the site-directed mutant PGHS2 Y355F are shown in Table I. The fit of the data to the Michaelis-Menten equation showed the Y355F mutation to decrease the \( k_{cat} \) associated with arachidonic acid oxidation by 2-fold, without a large effect on the apparent \( K_m \). Anadamide oxidation by the wild type enzyme showed a 6-fold increase in apparent \( K_m \) and a 3-fold decrease in \( k_{cat} \) as compared with arachidonic acid. The Y355F mutation decreased the apparent \( K_m \) associated with anadamide 2-fold relative to wild type.

| Substrate | Enzyme  | \( K_m \) \( \mu M \) | \( k_{cat} \) \( \mu M^{-1} s^{-1} \) | \( k_{cat}/K_m \) | \( \Delta G^\circ_T \) \( kT \) | \( \Delta G_T^\circ \) \( kT \) |
|----------------|---------|---------------------|-------------------|----------------|-----------------|----------------|
| AA            | PGHS2   | 10.0 ± 1.1          | 13.0 ± 0.68       | 1.3 ± 0.6     | -0.30           | 0.04           |
| Y355F         |         | 7.76 ± 0.58         | 6.06 ± 0.16       | 0.79 ± 0.6    | -1.80           | -0.14          |
| Anadamide     | PGHS2   | 61.6 ± 11.3         | 4.0 ± 0.38        | 6.5 ± 4.6     | 0.04            | 0.04           |
| Y355F         |         | 36.1 ± 3.5          | 2.49 ± 0.15       | 6.9 ± 4.6     | -1.47           | -0.14          |

Results

Substrate Oxidation Kinetics—The apparent \( K_m \) and \( k_{cat} \) values associated with prostaglandin formation from arachidonic acid and its ethanamide analog, anadamide (25), catalyzed by purified recombinant human PGHS2 and the site-directed mutant PGHS2 Y355F were shown in Table I. The fit of the data to the Michaelis-Menten equation showed the Y355F mutation to decrease the \( k_{cat} \) associated with arachidonic acid oxidation by 2-fold, without a large effect on the apparent \( K_m \). Anadamide oxidation by the wild type enzyme showed a 6-fold increase in apparent \( K_m \) and a 3-fold decrease in \( k_{cat} \) as compared with arachidonic acid. The Y355F mutation decreased the apparent \( K_m \) associated with anadamide 2-fold relative to wild type.

A more complete evaluation of the data showed a curved Eadie-Scatchard plot for oxidation of arachidonic acid by the Y355F mutant (Fig. 2). The data was replotted to the Hill equation (Fig. 2). The Hill plot appeared to be biphasic, with cooperativity observed below 0.5 \( \mu M \) arachidonic acid (\( n = 1.36 < 0.5, r^2 = 0.994 \)). No cooperativity was observed above 0.5 \( \mu M \) arachidonic acid (\( n = 1.01 ± 0.03, r^2 = 0.994 \)). Fitting the Hill equation to the entire data set (\( n = 1.10 ± 0.03, r^2 = 0.996 \)) resulted in a nonrandom distribution of the residuals as compared with the biphasic fit (Fig. 2, upper left panel). These results are consistent with positive cooperative activation of PGHS2 Y355F by arachidonic acid. Arachidonic acid has been directly observed to induce allorsetic activation of PGHS1, not PGHS2. However, fluorescent quenching experiments indirectly indicated that PGHS2 was activated at very low arachidonic acid concentrations (22). These results suggest that the Y355F mutant is less responsive to the arachidonic acid-induced activation than the wild type enzyme. No direct evidence for allorsetic activation was observed with the Y355F mutant using anadamide as substrate or with any of the other PGHS2 site-directed mutants that we have investigated (R120Q, R153H, E524Q, V523I, and the double mutant R513H/V523I) (data not shown).

Changes in transition state energy (\( \Delta G^\circ_T \)) due to changes in an R group or a mutation can be calculated from Equation 7 (30). \( \Delta G^\circ_T \) represents the change in free energy required to reach the transition state complex (ES) from free enzyme and substrate (E + S).

\[ \Delta G^\circ_T = -RT \ln \left( \frac{k_{cat}/K_m}{K_m/\text{mut}} \right) \] (Eq. 7)

The Y355F mutation decreased the transition state energy for prostaglandin formation from arachidonic acid by 0.3 kcal/mol; no change was observed with anadamide. This suggests that the phenolic group of the Tyr355 does not contribute to the rate-limiting step in catalysis. The change of the carboxylic
hibitors were incubated for various times prior to the addition of PGHS2 Y355F mutation on time-dependent inhibition with the PGHS2 (●) and PGHS2 Y355F (○) are shown in the lower right. In the upper left panels are the residuals associated with the fit of the data to the Hill equation. The left is the fit to the entire data set, the middle is below 0.5 μM arachidonic acid, and the right is above 0.5 μM. The scale of the y axis residuals is ±0.015.

The maximum inhibition will saturate as the inhibitor concentration is increased (24). At saturation, the relative equilibrium between $E_1$ and $E_1^*$ can be determined graphically by comparing the fraction of enzyme that is inhibited ($E_1^*$) and to the fraction that is uninhibited ($E_1$). The graphical results are essentially identical to the values obtained mathematically. The observation of saturable inhibition using this experimental protocol is the strongest evidence for the proposed kinetic mechanism and emphasizes the importance of an equilibrium between $E_1$ and $E_1^*$ that is independent of inhibitor concentration.

The impact of the Y355F mutation on the kinetic parameters associated with slow binding inhibition by flurbiprofen, indomethacin, and RS-57067 is shown in Table II. The carboxylic acids, flurbiprofen and indomethacin, form the $E_1$ complex with a similar affinity for both enzymes (similar $K_i$) but rearranged more rapidly to the $E_1^*$ complex with PGHS2 Y355F (increase in $k_2$) (Table II). The mutation increases the rate of equilibration between $E_1$ and $E_1^*$ without increasing the apparent binding affinity. RS-57067, a non-carboxylic acid inhibitor, bound tighter to $E_1$ with PGHS2 Y355F. The reverse isomerization rate from $E_1^*$ increased 3-fold with PGHS2 Y355F, while the forward rate did not change.

In order for the Y355F mutation to influence the isomerization between $E_1$ and $E_1^*$, it must lower the transition state between the two forms of the inhibitor-bound enzyme ($E_1^*$). The extent of the mutation’s effect on the transition state is not directly evident from the magnitude of the individual rate constants; therefore, we constructed free energy profiles to evaluate the effect of the mutation on the transition state energies. (The reaction coordinate for indomethacin is shown in Fig. 5.) Calculation of the influence of the Y355F mutation on binding free energies ($\Delta G$) is shown in Tables III and IV. Indomethacin and flurbiprofen binding to PGHS2 Y355F had minimal effect on the free energy associated with the initial enzyme-inhibitor complex ($E_1$); however, the change lowered the energy associated with the $E_1$ to $E_1^*$ transition state by 1.63 and 0.97 kcal/mol, respectively (Table III, Fig. 5). The ground state energy associated with $E_1^*$ was stabilized by 0.25 kcal/mol for flurbiprofen and 1.46 kcal/mol for indomethacin. RS-57067 binding to PGHS2 Tyr355 was stabilized by 1.29 and 0.83 kcal/mol in $E_1$ and $E_1^*$, respectively, and the transition state energy was lowered by 1.36 kcal/mol. The mutation decreased the entire RS-57067 reaction coordinate by the approximately 1 kcal/mol.

The mutation increased the rate at which the inhibitors approached the $E_1/E_1^*$ equilibrium by increasing the forward rate for indomethacin, both the forward and reverse rates for flurbiprofen, and the reverse rates for RS-57067. $\Delta G_{TS} - $ Phe-induced Decrease in Slow Binding Inhibition—Slow binding kinetic behavior is proposed to occur when $k_s$ is slower than $k_{cat}$ (31). The $k_s$ values determined for inhibition of PGHS2 by flurbiprofen (0.105 s\(^{-1}\)), indomethacin (0.034 s\(^{-1}\)), and RS-57067 (0.21 s\(^{-1}\)) compared with the $k_{cat}$ reported for PGHS2 (13 s\(^{-1}\)) are consistent with this proposal. The data in
Table IV show quantitatively the reason for the observed decrease in the time-dependent inhibition associated with the Y355F mutant and also provide a thermodynamic explanation for the kinetics of slow binding inhibition. The Y355F mutation decreases the relative difference between the $E_I^\dagger$/ES$^\dagger$ transition state energies by 1.33, 0.67, and 1.06 kcal/mol for indomethacin, flurbiprofen, and RS-57067, respectively (Table IV).

It is important to distinguish between the equilibrium rate of $E_I$ and $E_I^*$ and the observation of slow binding inhibition. The observation of slow binding inhibition occurs when the equilibrium rate is similar or slower than the rate of catalysis. The equilibrium can occur in the absence of observable slow binding inhibition if it is more rapid than the rate of catalysis.

**TABLE II**

Kinetic constants associated with inhibition of PGHS2 and PGHS2 Y355F by indomethacin, flurbiprofen, and RS-57067

The rate constants associated with inhibition of wild type PGHS2 by indomethacin and flurbiprofen were previously reported by Callan et al. (24), and those associated with RS-57067 were reported by Swinney et al. (22). %$E_I^*$ represents the percentage of inhibitor complexed in $E_I^*$ at saturation. $k_2$ and $k_3$ are expressed in s$^{-1}$, $K_i$ in $\mu$M.

|            | PGHS2   | PGHS2Y355F |
|------------|---------|------------|
| Indomethacin |         |            |
| $k_2$      | 0.034 ± 0.004 | 0.23 ± 0.039 |
| $k_3$      | 0.0031 ± 0.00033 | 0.0041 ± 0.001 |
| $K_i$      | 11.2 ± 2.0 | 5.1 ± 1.2 |
| %$E_I^*$   | 91.6%    | 98.2%      |
| Flurbiprofen |         |            |
| $k_2$      | 0.105 ± 0.008 | 0.603 ± 0.186 |
| $k_3$      | 0.0082 ± 0.0008 | 0.027 ± 0.009 |
| $K_i$      | 0.61 ± 0.075 | 0.70 ± 0.25 |
| %$E_I^*$   | 92.7%    | 86.4%      |
| RS-57067   |         |            |
| $k_2$      | 0.21 ± 0.09 | 0.24 ± 0.06 |
| $k_3$      | 0.083 ± 0.033 | 0.20 ± 0.054 |
| $K_i$      | 16.9 ± 14.7 | 2.0 ± 0.35 |
| %$E_I^*$   | 70%      | 55%        |

Table IV show quantitatively the reason for the observed decrease in the time-dependent inhibition associated with the Y355F mutant and also provide a thermodynamic explanation for the kinetics of slow binding inhibition. The Y355F mutation decreases the relative difference between the $E_I^\dagger$/ES$^\dagger$ transition state energies by 1.33, 0.67, and 1.06 kcal/mol for indomethacin, flurbiprofen, and RS-57067, respectively (Table IV). It is important to distinguish between the equilibrium rate of $E_I$ and $E_I^*$ and the observation of slow binding inhibition. The observation of slow binding inhibition occurs when the equilibrium rate is similar or slower than the rate of catalysis. The equilibrium can occur in the absence of observable slow binding inhibition if it is more rapid than the rate of catalysis.

**Loss of Apparent Irreversibility with SC-57666**

SC-57666 is a selective, time-dependent reversible PGHS2 inhibitor (32). Compounds of this class have been proposed to induce inactivation of PGHS2 (21). Preincubation of wild type PGHS2 with SC-57666 resulted in incomplete consumption of oxygen as compared with control (Fig. 6, top). We have interpreted incomplete oxygen consumption to correlate with the inactivation of PGHS2 or extremely slow dissociation from PGHS2. Incubation of SC-57666 with PGHS2 Y355F only slightly delays the total oxygen consumption (Fig. 6, bottom). These results suggest that SC-57666 binding to PGHS2 Y355F is rapidly reversible. This is a dramatic contrast to the extremely slow dissociation observed with wild type PGHS2.

**DISCUSSION**

This work describes the effect of the Y355F mutation on the relative equilibria that comprise the slow binding inhibition of prostaglandin formation by PGHS2. The slow binding inhibition is the result of an intramolecular equilibrium between two kinetic states, $E_I$ and $E_I^*$, that competes with the rate of
prostaglandin formation. The results show that increasing the rate of equilibration between $E_1$ and $E_1\$ relative to the rate of product formation decreases the likelihood of observing the slow binding inhibition. The overall impact of the Y355F mutation on the competition between prostaglandin formation and slow binding inhibition is to decrease the time it takes to establish the $E_1/E_1\$ equilibrium. These results suggest a role for Tyr^{355} in the molecular mechanism of time-dependent inhibition of PGHSs.

This work also describes the effect of the Y355F mutation on the equilibrium between the allosterically activated and unactivated enzyme forms. We directly observed arachidonic acid to induce the allosteric kinetics associated with the PGHS2 Y355F mutant (Fig. 2). As we noted earlier, allosteric activation by arachidonic acid has been directly observed with PGHS1, not PGHS2. However, fluorescent quenching experiments indirectly indicate that PGHS2 is activated at very low arachidonic acid concentrations (22). One explanation for these results is that the Y355F mutation slows the rate of activation by arachidonic acid, thereby allowing it to be kinetically detectable. These results suggest a role for Tyr^{355} in the molecular mechanism of allosteric activation.

To observe a kinetic event, it must be partially rate-limiting. A practice used to delineate mechanisms of catalysis is to employ substrate analogs and isotopes to decrease the rates of reaction. In some instances, this will expose or unmask previously undetectable kinetic steps. We propose that the observations of slow binding inhibition and allosteric activation associated with the PGHS2 Y355F mutant are kinetically detectable because they unmask kinetic steps intrinsic to the activation and binding of arachidonic acid to PGHSs.

Tyr^{355} can potentially participate in the time-dependent inhibition and allosteric activation through its interactions with the substrate, the inhibitor, and/or the hydrogen bonding network. The structures of PGHS2 show at least two possible hydrogen bonding conformations near the entrance to the cyclooxygenase site: one consisting of Arg^{513}, Glu^{524}, and Tyr^{355} and another consisting of Arg^{218}, Glu^{224}, and Tyr^{355}. We propose that it is the equilibrium between these two hydrogen bonding arrangements that is responsible for the allosteric activation and that the disruption of this equilibrium contributes to the slow binding inhibition. We envision a mechanism whereby the hydrogen bonding network at the entrance to the cyclooxygenase site is relaxed in the activated enzyme and tightened in the unactivated and inhibitor bound forms (Fig. 7). The Arg^{513}/Glu^{224}/Tyr^{355} hydrogen bonding network may predominate in the relaxed form, leaving Arg^{218} to freely interact with substrate, whereas in the tightened conformation the Arg^{120}/Glu^{224}/Tyr^{355} hydrogen bonding network locks the substrate into a catalytically competent conformation (Fig. 7). For PGHS2, the rates of these transitions are normally too rapid relative to catalysis to be observed; however, inhibitors or mutations that interact with the reorganization of the hydrogen bonding network will slow the reorganization rates relative to catalysis and make the reorganization of the hydrogen bonding network partially rate-limiting. This hypothesis is consistent with the proposal by Browner and co-workers (10). They suggested from analysis of the structural data that it is the ability of the channel to move between open and closed conformations
that allows both substrates and inhibitors to reach the internal binding site. Anadamide is the ethanolamide derivative of arachidonic acid. Anadamide was recently shown to be selectively metabolized by PGHS2 to a novel class of prostaglandins (25). We did not observe allosteric activation using anadamide as substrate with either wild type PGHS2 or the Y355F mutant. The free energy required for catalysis is increased by greater than 1.4 kcal/mol for anadamide relative to arachidonic acid masks the intrinsic equilibria associated with the allosteric activation and of anadamide oxidation relative to arachidonic acid. The position of the equilibrium between relaxed and tightened forms of the hydrogen bonding network at the entrance to the cyclooxygenase binding site. The position of the equilibrium between the relaxed and tightened forms will control substrate binding and ultimately the rate of prostaglandin formation.

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