

# The Kinetic Factors That Determine the Affinity and Selectivity for Slow Binding Inhibition of Human Prostaglandin H Synthase 1 and 2 by Indomethacin and Flurbiprofen\*

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We present here for the first time a method for determining the rate constants associated with slow binding inhibition of prostaglandin H synthase (PGHS). The rate constants were determined by a method using initial steady-state conditions, which minimize the impact of catalytic autoinactivation of the enzyme. The currently available methods for determining the kinetic constants associated with slow binding enzyme inhibition do not distinguish between rate decreases due to enzyme inhibition or due to autoinactivation of the enzyme. A mathematical model was derived assuming a rapid reversible formation of an initial enzyme-inhibitor complex ( $EI$ ) followed by a slow reversible formation of a second enzyme-inhibitor complex ( $EI^*$ ). The two enzyme inhibitor complexes are assumed to be in slow equilibrium. This method was used to evaluate the kinetic parameters associated with the binding and selectivity of the non-steroidal antiinflammatory drugs (NSAIDs), flurbiprofen and indomethacin.

The  $K_I$  values associated with the formation of the first reversible complex ( $EI$ ) for flurbiprofen with PGHS1 and PGHS2 were  $0.53 \pm 0.06$  and  $0.61 \pm 0.08$   $\mu\text{M}$ , respectively; the rate constants for the forward isomerization,  $k_2$ , into the second reversible complex ( $EI^*$ ) were  $0.97 \pm 0.99$  and  $0.11 \pm 0.01$   $\text{s}^{-1}$ , respectively, and rates of the reverse isomerization from  $EI^*$ ,  $k_{-2}$ , were  $0.031 \pm 0.004$  and  $0.0082 \pm 0.0008$   $\text{s}^{-1}$ , respectively. Indomethacin was estimated to form the  $EI$  complex with the same affinity for both PGHS1 and PGHS2,  $10.0 \pm 2.8$   $\mu\text{M}$  and  $11.2 \pm 2.0$   $\mu\text{M}$ , respectively, and dissociate from  $EI^*$  at approximately the same rate  $0.0011 \pm 0.0002$   $\text{s}^{-1}$  and  $0.0031 \pm 0.0003$   $\text{s}^{-1}$ , respectively. However, the rate of isomerization into  $EI^*$  from  $EI$  was much greater for PGHS1 than PGHS2,  $0.33 \pm 0.08$   $\text{s}^{-1}$  as compared with  $0.034 \pm 0.004$   $\text{s}^{-1}$ . These results show that the overall affinity for the inhibition of PGHS1 versus PGHS2 was 30-fold greater for indomethacin ( $K_I^* = 0.032 \pm 0.005$  and  $1.02 \pm 0.08$   $\mu\text{M}$ , respectively) and 3-fold greater for flurbiprofen ( $K_I^* = 0.017 \pm 0.002$  and  $0.045 \pm 0.004$   $\mu\text{M}$ , respectively). The results also show that for both PGHS1 and PGHS2, flurbiprofen was bound tighter to the initial  $EI$  complex than indomethacin; however, the rate of dissociation from  $EI^*$  was slower for indomethacin than flurbiprofen. The rate of the forward isomerization to  $EI^*$  is primarily responsible for the selectivity of both NSAIDs for PGHS1. This analysis shows the quantitative importance of the different kinetic parameters upon the

overall binding affinity of these NSAIDs and should greatly assist in our understanding of the structural interactions that promote enzyme-inhibitor binding.

The treatment of pain and inflammation by the inhibition of prostaglandin formation has been successfully accomplished with many currently marketed NSAIDs<sup>1</sup> including indomethacin, flurbiprofen, ibuprofen, and aspirin. These therapeutic agents are thought to elicit their action by the inhibition of prostaglandin H synthase. However, severe gastrointestinal irritation is also observed with the administration of these compounds, limiting their usage. This irritation has been associated with the inhibition of prostaglandin formation in the gastrointestinal tract (1). The recent discovery of an inducible form of prostaglandin H synthase (PGHS2) has renewed interest in discovering new NSAIDs that will be better tolerated (2). This isoform is induced at the sites of inflammation (3). The working hypothesis is that selective inhibition of the inducible enzyme will block the inflammation and the pain associated with the inflammation without serious gastrointestinal side effects (4, 5). To this end, a great deal of effort is being spent to discover PGHS2 selective compounds.

Previous work by Rome and Lands (6) with ram PGHS1 revealed that many PGHS inhibitors, including indomethacin, inhibited the enzyme in a time-dependent manner. The mechanism of the time-dependent inhibition was subsequently postulated to be associated with a slow binding mechanism involving a slow reversible isomerization of the initial enzyme-inhibitor complex ( $EI$ ) to a second enzyme-inhibitor complex ( $EI^*$ ) (see Scheme 1) (7, 8). Although the inhibition was reported to be reversible, the rate of the reverse isomerization from  $EI^*$  (dissociation rate) was not addressed in those reports. The only reported attempt to determine the reverse isomerization rates was by Walenga and co-workers (9), who observed slow reversible inhibition of human platelet cyclooxygenase by indomethacin and determined the  $t_{1/2}$  for the recovery of enzymatic activity to be between 100 and 200 min (9). This was considerably shorter than the  $t_{1/2}$  of 4–5 days for human platelet. In this manuscript we present a method to determine the rate constants associated with slow binding inhibition of PGHS1 and PGHS2, which minimizes the impact of the substrate-dependent autoinactivation upon the slow binding inhibition kinetics. This method allows for the first time the determination of the intrinsic equilibrium affinity of slow binding inhibitors for PGHSs, as defined by  $K_I^*$ , and the impact of the individual components of the reaction dynamics upon the overall inhibition.

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<sup>1</sup> The abbreviations used are: NSAID, nonsteroidal antiinflammatory drug; PGHS2, human prostaglandin H synthase 2; PGHS1, human prostaglandin H synthase 1.

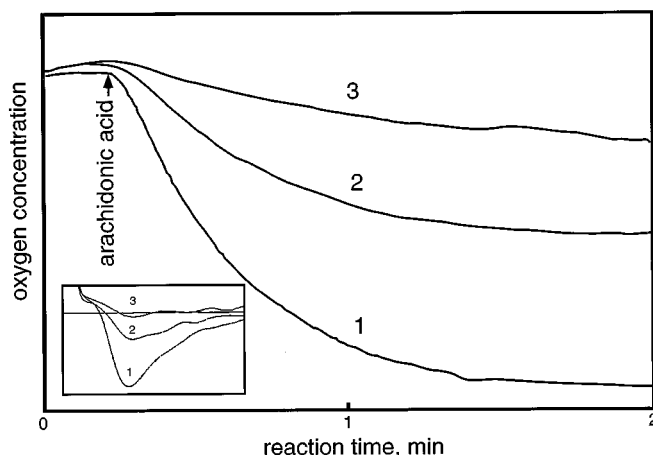


FIG. 1. Reaction profile showing the consumption of oxygen with time after the addition of 200  $\mu\text{M}$  arachidonic acid with PGHS2. Curve 1, control; curve 2, preincubation with 25  $\mu\text{M}$  indomethacin for 20 s; curve 3, preincubation with 25  $\mu\text{M}$  indomethacin for 2 min. Inset, the first derivative spectra of the first minute of the oxygen consumption curves. The average of the 5 minimum points was used to determine the maximum initial velocity.

#### EXPERIMENTAL METHODS

**Materials**—Human PGHS1 and PGHS2 were expressed and purified from a baculovirus/insect cell culture system as described previously (10). Arachidonic acid was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Hemin, phenol, and other reagents were of the highest grade available from Sigma.

**Cyclooxygenase Activity**—Oxygen consumption was measured with a YSI model 5300 biological oxygen monitor equipped with a Clark-type micro oxygen electrode. The analog output was collected and transformed to a digital signal using an external connection to a SLM-Aminco DW2000 data system. The analog output was filtered through a brick wall low pass filter at 0.2 Hz and amplified 10-fold. Enzyme (20–50 nM), was mixed with hemin (0.8  $\mu\text{M}$ ), and phenol (2 mM) to establish a base line. The reaction was initiated with arachidonic acid (200  $\mu\text{M}$ ) after preincubation with either inhibitor or vehicle. Arachidonic acid and indomethacin were added in tyloxapol to a final concentration of not greater than 0.08%. The maximum initial velocity was determined from a first derivative transformation of the reaction profile (see Fig. 1). The system was calibrated using the catalase-dependent oxidation of hydrogen peroxide.

**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. The 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 several inhibitor concentrations to the final equation. Each experiment was repeated at least twice. The apparent  $K_m$  value used for data analysis was 5  $\mu\text{M}$ . This was the approximate median of multiple determinations, and the same value was used for both enzymes (data not shown). The Nelder-Mead simplex algorithm in an adaptation of the software package PCNONLIN was used to estimate the parameters.

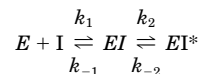
#### RESULTS AND DISCUSSION

In order to determine the rate constants associated with inhibition of PGHS by NSAIDs, an approach was needed to minimize the impact of the substrate catalyzed rate of enzyme autoinactivation. The oxidation of arachidonic acid to prostaglandin  $\text{H}_2$  by PGHS is associated with the consumption of 2 mol of oxygen consumed for every mol of product formed. The continuous progress of the reaction can be measured by monitoring the consumption of oxygen. The oxygen consumption profile (Fig. 1) is characterized by a rapid, protein-dependent decrease in oxygen concentration after addition of the substrate, arachidonic acid. The rate of decrease in oxygen concentration decreases until all the enzyme is inactivated. The max-

imum velocity,  $v_{\text{max}}$ ,<sup>2</sup> obtained in each reaction was used for the kinetic evaluations in order to minimize the effect of enzyme inactivation. We assume that at the maximum velocity no enzyme has been inactivated. The maximum velocity was determined from the first derivative of the oxygen consumption versus time profile (Fig. 1, inset).

The next issue in developing a method to estimate the rate constants was to determine the appropriate kinetic model of inhibition. The  $v_{\text{max}}$  associated with oxygen consumption was observed to decrease in a time-dependent manner following preincubation of indomethacin and flurbiprofen with both human PGHS enzymes, as reported previously (7). The reactions were initiated with 200  $\mu\text{M}$  arachidonic acid, a concentration greater than 40-fold excess of the apparent  $K_m$ . Under these conditions, all enzyme-inhibitor complexes that are in rapid equilibrium with free enzyme should rapidly bind the excess substrate and catalyze product formation. The inhibition will be the result of enzyme-inhibitor complex that is not in rapid equilibrium with free enzyme. Plots of the percent activity remaining versus preincubation time show a sharp initial decrease in activity, which eventually reaches a plateau (Fig. 2). This characteristic was also reported for the ram enzyme with indomethacin and flurbiprofen, where 4 and 6% residual activity, respectively, was observed to always remain (7). More recently Quellet and Percival (11) reported 0.6 and 13% residual activity remaining following inhibition of human PGHS2 by indomethacin and flurbiprofen, respectively. The residual activity (plateau activity) appears to saturate as inhibitor concentrations are increased. This observation is consistent with a mechanism where even at saturation a certain percentage of the inhibitor bound enzyme is in rapid equilibrium with substrate. The enzyme-inhibitor complex in rapid equilibrium with substrate (termed  $EI$ ) must also be in slow equilibrium with another enzyme-inhibitor complex (termed  $EI^*$ ) in order to observe reversible time-dependent inhibition. These observations are consistent with the proposed mechanism of slow binding inhibition, first termed by Morrison (12), where the inhibitor initially binds to the enzyme to form an equilibrium complex ( $EI$ ), which in turn slowly isomerizes to another reversible enzyme-inhibitor complex ( $EI^*$ ) (Scheme 1). The amount of residual activity will then depend on the equilibrium between  $EI$  and  $EI^*$ .

The mathematical interpretation of the theoretical kinetic model in terms of the experimental protocol is subject to two phases, a preincubation phase in which inhibitor is incubated with enzyme in the absence of substrate, and a reaction phase, which begins when the substrate is added to the inhibitor enzyme mix. The following dynamics have been assumed to be associated with the preincubation phase.



SCHEME 1.

Let  $E = E(t)$ ,  $I = I(t)$ ,  $EI = EI(t)$ , and  $EI^* = EI^*(t)$  describe the concentration of free enzyme, free inhibitor, enzyme-inhibitor complex, and isomerized enzyme-inhibitor complex at time  $t$ , respectively. Applying the laws of mass action, we describe the dynamics in Scheme 1 with the following system of differential equations:

<sup>2</sup> The  $v_{\text{max}}$  described here should not be confused with the Michaelis-Menten  $V_{\text{max}}$ . The  $v_{\text{max}}$  defined in this study is the measured initial maximum velocity in an individual reaction.

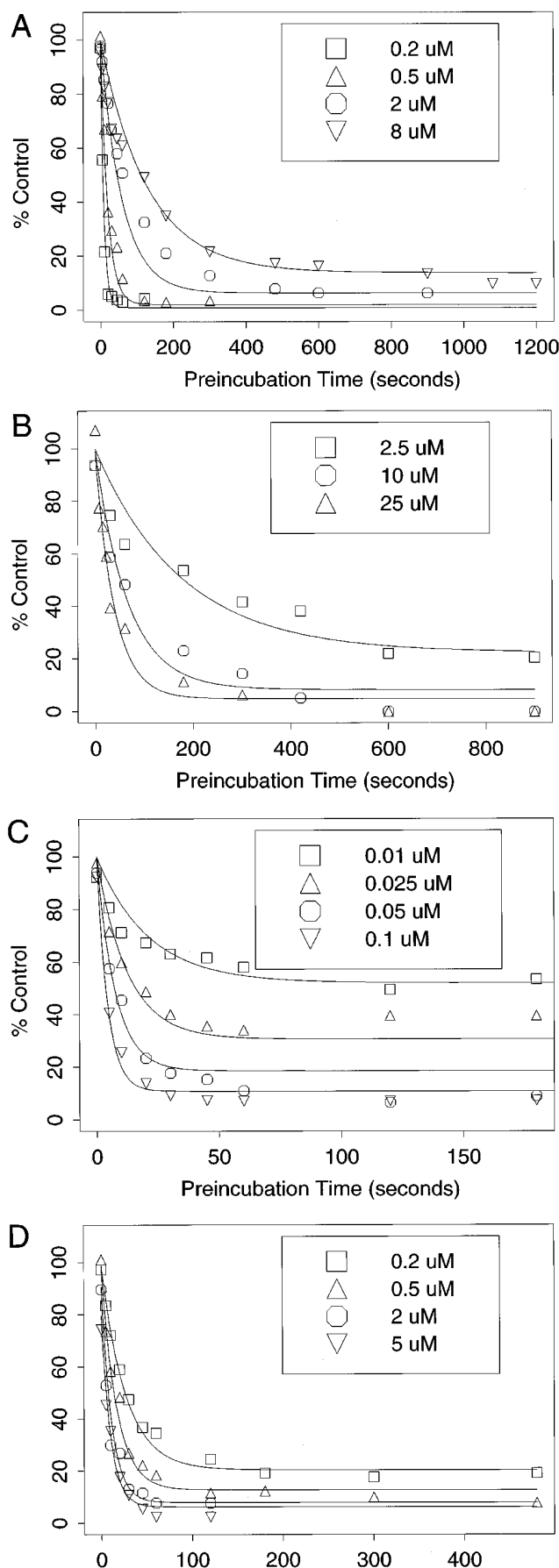


FIG. 2. Time-dependent inhibition of human PGHS1 and PGHS2 by indomethacin and flurbiprofen. Individual data points were determined from the first derivative of the oxygen consumption

$$\begin{aligned} \frac{dE}{dt} &= k_{-1}EI - k_1(I)(E) \\ \frac{dEI}{dt} &= k_{-2}EI^* + k_1(I)(E) - (k_{-1} + k_2)EI \\ \frac{dEI^*}{dt} &= k_2EI - k_{-2}EI^* \end{aligned} \quad (\text{Eq. 1})$$

We assume that (i) inhibitor is in excess of enzyme and (ii) the reaction  $E + I \rightleftharpoons EI$  is in quasiequilibrium ( $k_{\pm 2} \ll k_{\pm 1}$ ). As a result of assumption (ii), the  $E + I \rightleftharpoons EI$  equilibrium is essentially achieved by  $t \approx 0^+$ . During all of the preincubation then, we have the quasi-steady-state identity  $E = (K_I/I)EI$ , where  $K_I = k_{-1}/k_1$ . Substituting this identity into the conservation equation  $E_0 = E + EI + EI^*$ , where  $E_0$  is the initial enzyme concentration, then substituting the resulting expression for  $EI$  into the differential equation for  $EI^*$  in Equation 1, we have

$$\frac{dEI^*}{dt} = k_{app}E_0 - (k_{app} + k_{-2})EI^* \quad \text{where } k_{app} = \frac{k_2}{1 + \frac{K_I}{I}} \quad (\text{Eq. 2})$$

The solution of this differential equation for  $EI^*(t)$  with initial condition  $EI^*(0) = 0$  is

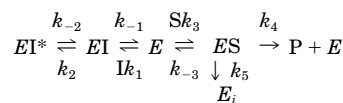
$$EI^*(t) = E_0 \frac{k_{app}}{k_{app} + k_{-2}} (1 - e^{-(k_{app} + k_{-2})t}) \quad (\text{Eq. 3})$$

Now let  $\epsilon = \epsilon(t) = E(t) + EI(t)$ . Then since  $\epsilon = E_0 - EI^*$ , from Equation 3 we have the final concentration of free enzyme and enzyme-inhibitor complex ( $E + EI$ ) at the end of  $T$  seconds of preincubation is approximately

$$\epsilon_T = \epsilon(T) = E_0 \left( 1 - \frac{k_{app}}{k_{app} + k_{-2}} (1 - e^{-(k_{app} + k_{-2})T}) \right) \quad (\text{Eq. 4})$$

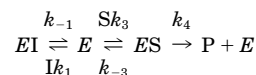
This equation will be used for the initial enzyme concentration for the subsequent reaction.

The reaction phase of product formation is initiated by adding substrate after  $T$  seconds of preincubation, and the following dynamics are assumed.



SCHEME 2.

In this diagram  $S$ ,  $P$ , and  $E_i$  denote substrate, product, and inactivated enzyme, respectively. Together with the two previous assumptions made, we assume (iii) that substrate is in excess of enzyme, so that  $Sk_3$  and  $Ik_1$  can be considered constants, (iv) that the rates  $k_{\pm 2}$  are slow relative to the rates  $k_{\pm 1}$ ,  $k_{\pm 3}$ ,  $k_4$ , and (v) that the rate  $k_5$  is slow relative to the rates  $k_{\pm 1}$ ,  $k_{\pm 3}$ ,  $k_4$ . As a result of assumptions (iv) and (v), the rates  $k_{\pm 2}$  and  $k_5$  are negligible in the initial stages of the reaction. With these assumptions, the initial dynamics of the reaction can be described with the following scheme.



SCHEME 3.

assay after preincubation of enzyme with inhibitor for the specified time. Data are represented as percent control, i.e. the maximum velocity of the reaction with inhibitor divided by the maximum velocity of the reaction without inhibitor. The curves represent the fit of the data at each inhibitor concentration over several experiments to EQN 10. A, indomethacin/PGHS1,  $n = 2$ ; B, indomethacin/PGHS2,  $n = 5$ ; C, flurbiprofen/PGHS1,  $n = 5$ ; D, flurbiprofen/PGHS2,  $n = 5$ ; where  $n$  is the number of experiments.

Letting  $S = S(t)$ ,  $ES = ES(t)$  and  $P = P(t)$  describe the concentration of substrate, enzyme-substrate complex, and product at time  $t$ , respectively, and applying the law of mass action, we describe the dynamics in Scheme 3 with the following system of differential equations:

$$\begin{aligned} \frac{dEI}{dt} &= k_1(I)(E) - k_{-1}EI \\ \frac{dES}{dt} &= k_3(S)(E) - (k_{-3} + k_4)ES \\ \frac{dE}{dt} &= k_{-1}EI + (k_{-3} + k_4)ES - k_1(I)(E) - k_3(S)(E) \end{aligned} \quad (\text{Eq. 5})$$

$$\frac{dP}{dt} = k_4ES$$

The steady-state solution for Equation 5 can be computed and depends on the length of the preincubation time  $T$ , giving the steady-state concentration for  $ES$  after  $T$  seconds of preincubation ( $ES_{ss}(T)$ ) as

$$ES_{ss}(T) = \frac{\epsilon_T}{1 + \frac{K_m}{S} \left(1 + \frac{I}{K_I}\right)} \quad (\text{Eq. 6})$$

where  $K_m = (k_{-3} + k_4)/k_3$  and  $\epsilon_T = EI + E + ES$ , the conservation equation for the reaction phase. From Equation 6 and  $dP/dt$  in Equation 5, we have the maximum velocity of product formation after  $T$  seconds of preincubation as

$$v_{\max}(T) = \frac{k_4\epsilon_T}{1 + \frac{K_m}{S} \left(1 + \frac{I}{K_I}\right)} \quad (\text{Eq. 7})$$

If there is no inhibitor (control),  $v_{\max}$  is computed by setting  $\epsilon_T = E_0$  and  $I = 0$  (in this case  $v_{\max}$  is independent of  $T$ ). Dividing Equation 7 by the control  $v_{\max}$  and using the expression for  $\epsilon_T$  in Equation 4, we obtain the equation for the maximum velocity after  $T$  seconds of preincubation as a percent of control.

$$f(T) = 100 Q \left(1 - \frac{k_{\text{app}}}{k_{\text{app}} + k_{-2}} (1 - e^{(k_{\text{app}} + k_{-2})T})\right) \quad (\text{Eq. 8})$$

$$\text{where } Q = \frac{1 + \frac{K_m}{S}}{1 + \frac{K_m}{S} \left(1 + \frac{I}{K_I}\right)}, k_{\text{app}} = \frac{k_2}{1 + \frac{K_I}{I}}$$

Nonlinear regression as described under "Experimental Methods" was used to fit the time-velocity curves to  $f(T)$  in Equation 8. This estimates three parameters  $k_2$ ,  $k_{-2}$ , and  $K_I$ , using the known  $K_m/S$  and several inhibitor concentrations  $[I]$ . The overall inhibition is defined by the following equation.

$$K_I^* = K_I \frac{k_{-2}}{k_2} \quad (\text{Eq. 9})$$

The estimated rate constants are shown in Table I. Comparison of the two inhibitors with two enzymes shows that all three kinetic parameters must be examined in order to understand which factors contribute to the overall affinities. For example, the  $K_I^*$  values for flurbiprofen and indomethacin with PGHS1 are similar, 0.017 and 0.032  $\mu\text{M}$ , respectively. However, the individual rate constants are vastly different;  $K_I$  is 20-fold lower for flurbiprofen and  $k_{-2}$  is 30-fold slower for indomethacin. Whereas  $K_I$  and  $k_{-2}$  were the critical kinetic parameters in explaining the similarities in affinities for flurbiprofen and indomethacin for PGHS1, the 30-fold selectivity of indomethacin for PGHS1 versus PGHS2 is accounted for by the difference

TABLE I  
Rate constants associated with indomethacin and flurbiprofen inhibition of PGHS1 and PGHS2

Rate constants	Enzyme	Inhibitor	
		Indomethacin	Flurbiprofen
$k_2$ ( $\text{s}^{-1}$ )	PGHS1	$0.334 \pm 0.08^a$	$0.97 \pm 0.99$
	PGHS2	$0.034 \pm 0.004$	$0.105 \pm 0.008$
$k_{-2}$ ( $\text{s}^{-1}$ )	PGHS1	$0.0011 \pm 0.0002$	$0.031 \pm 0.004$
	PGHS2	$0.0031 \pm 0.00033$	$0.0082 \pm 0.0008$
$K_I$ ( $\mu\text{M}$ )	PGHS1	$10.0 \pm 2.8$	$0.53 \pm 0.06$
	PGHS2	$11.2 \pm 2.0$	$0.61 \pm 0.075$
$K_I^*$ ( $\mu\text{M}$ )	PGHS1	$0.032 \pm 0.005$	$0.017 \pm 0.002$
	PGHS2	$1.02 \pm 0.08$	$0.047 \pm 0.004$

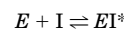
<sup>a</sup>  $\pm$  standard errors.

in  $k_2$ . The  $K_I$  and  $k_{-2}$  are similar, while there is a 10-fold difference in  $k_2$  for the two enzymes,  $0.334 \text{ s}^{-1}$  for PGHS1 and  $0.034 \text{ s}^{-1}$  for PGHS2.

The plateau phase of the curves upon casual inspection appears to approach saturation (Fig. 2). Indeed, this is consistent with the predictions of the model. The plateau represents the amount of inhibitor in the  $EI^*$  complex after equilibrium has been achieved in the preincubation phase. Saturation of the enzyme with inhibitor results in the saturation of the  $EI^*$  complex and accordingly, the plateau. Computing the limit of  $\epsilon_T$  in Equation 4 as  $I$  and  $T \rightarrow \infty$ , the percentage of enzyme in  $EI^*$  at saturation, termed  $\% EI_{\text{sat}}^*$ , can be expressed as:

$$\% EI_{\text{sat}}^* = \frac{k_2}{k_2 + k_{-2}} \times 100 \quad (\text{Eq. 10})$$

The  $\% EI_{\text{sat}}^*$  for flurbiprofen was determined to be 97 and 92.7% for PGHS1 and PGHS2, respectively. Therefore, the amount of activity remaining at saturation is 3 and 7.3%, respectively. The  $\% EI_{\text{sat}}^*$  for indomethacin was 99.7 and 91.6% for PGHS1 and PGHS2, respectively. These data indicate that even when the enzyme is fully saturated with inhibitor, not all of the enzyme is in the  $EI^*$  complex. This is consistent with an equilibrium between  $EI$  and  $EI^*$  that is not affected directly by inhibitor concentration. This is inconsistent with a single-step reaction in which a rapid equilibrium is not established prior to the formation of  $EI^*$  (Scheme 4).



SCHEME 4.

The  $K_I$  and forward isomerization rates were determined by Kulmacz and Lands (7) for the inhibition of ram PGHS1 by flurbiprofen and indomethacin assuming irreversible inhibition. The results they reported were similar to those reported here for human PGHS1. For flurbiprofen and indomethacin, Kulmacz and Lands (7) reported  $K_I$  values of 0.2 and 1.7  $\mu\text{M}$ , respectively, and forward isomerization rates of 0.27 and 0.25  $\text{s}^{-1}$ , respectively. The similarity in the data is surprising given the difference in methods and enzymes. These data suggest that the interactions that promote the slow binding kinetics may be similar for the human and ram PGHS1, although no definitive conclusions can be made without determining the off rates for the ram enzyme. More recently, Quellet and Percival (11) used the same type analysis to determine the rate constants associated with inhibition of human PGHS2 by indomethacin and flurbiprofen. The  $K_I$  values were 114 and 0.17  $\mu\text{M}$ , respectively, and the rate of  $k_{\text{on}}$  ( $k_2$ ) was 0.035 and 0.018  $\text{s}^{-1}$ , respectively.

Calculation of the binding energies associated with the kinetic constants using the Eyring equation gives further insight into the factors that affect the selectivity. The calculated free energies,  $\Delta G$ , are plotted against the reaction coordinate in Fig. 3. The transition state energy in going from  $EI$  to  $EI^*$  is

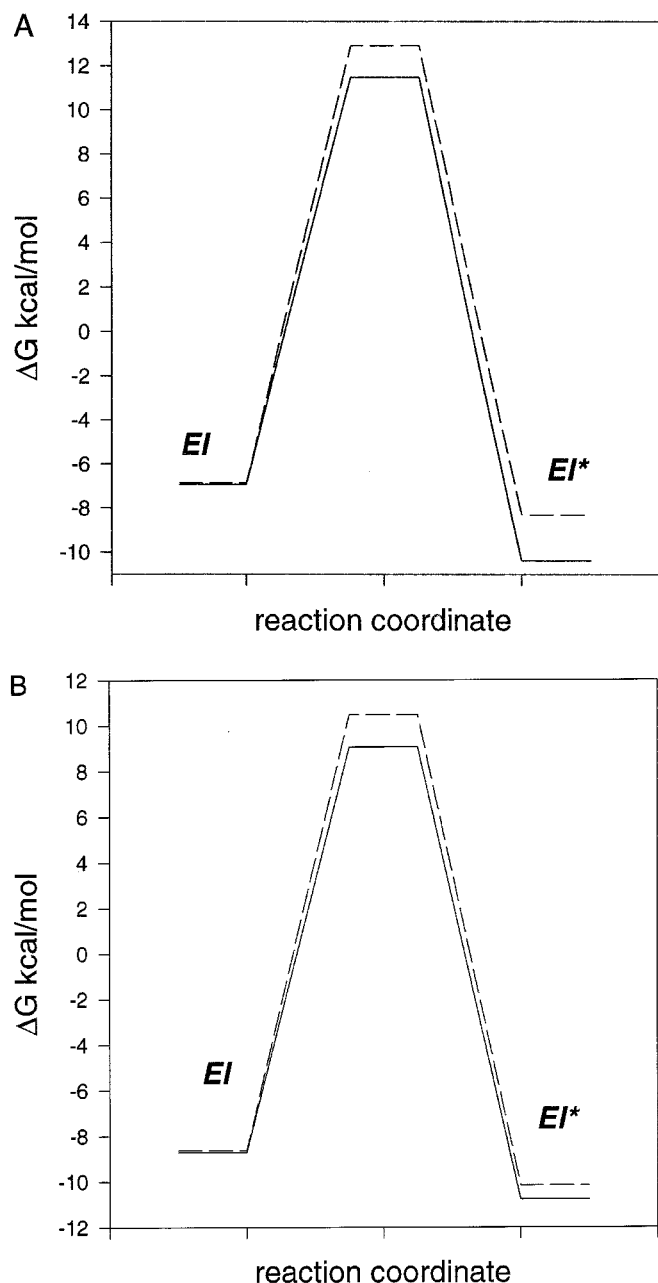


FIG. 3. Reaction coordinate associated with the inhibition of PGHS1 and PGHS2 by indomethacin (A) and flurbiprofen (B).

lower for PGHS1 by approximately 1.4 kcal/mol for both inhibitors. The ground state energy for  $EI^*$  is 2.07 kcal/mol lower for PGHS1 with indomethacin and 0.61 kcal/mol lower with flurbiprofen. Accordingly, indomethacin selectivity results from a combination of interactions from the transition state between  $EI$  and  $EI^*$  and the  $EI^*$  ground state. The selectivity observed with flurbiprofen appears to be exclusively the result of transition state interactions.

One obvious question from this analysis is what structurally is the difference between  $EI^*$  and  $EI$  and what interactions influence the transition state structure? The recently described crystal structure by the Garavito and co-workers (13) of flurbiprofen bound to ram PGHS1 is most likely the  $EI^*$  complex. The identity of  $EI$  is unknown. The difference between  $EI$  and  $EI^*$  could be the displacement of a water molecule, as has been proposed for slow binding inhibition of stromelysin, thermolysin, and pepsin (14–16) or a larger protein-inhibitor conformation change. Further work will be needed to define the struc-

tural factors that contribute to these kinetic observations.

Characterizing and quantitating the factors important to the inhibition of PGHSs for use in drug design has been a challenging undertaking. It has not been resolved which *in vitro* parameters are most predictive of *in vivo* efficacy, and in addition, it has been difficult to correlate *in vitro* enzyme data with cell-based data. An example of this is seen when comparing the  $IC_{50}$  values determined for the inhibition of human PGHS1 and PGHS2 by indomethacin under a variety of different conditions (Table II). The variability in the results is not surprising when one considers that there are at least five dynamic processes involved in the slow binding inhibition of PGHS and the results are but a single snapshot of the dynamics. The five dynamic processes are (i) the association and (ii) dissociation rates of inhibitor with  $EI^*$ , (iii) the rate of enzyme autoinactivation, (iv) the rate of substrate turnover, and (v) the rate of catalytic activation of the enzyme. Instead of a single snapshot, our analysis of the reactions has led to the quantitation of the effect of the rates of association and dissociation of the inhibitor upon the overall inhibition of substrate turnover by minimizing the impact of the autoinactivation of the enzyme.

Theoretically, under equilibrium conditions, the  $IC_{50}$  values determined with preincubation should approximate  $K_I^*$ . However, when the rate of autoinactivation is faster than the other dynamic processes, an equilibrium can never be established, and the  $IC_{50}$  will not approximate  $K_I^*$ . The methodology required to accurately determine a meaningful  $IC_{50}$  associated with the slow binding inhibition of PGHS by indomethacin requires the preincubation and/or incubation time to be long enough to allow for the association of indomethacin into  $EI^*$  ( $k_2$ ), and the incubations must be long enough to allow for equilibrium dissociation from  $EI^*$  ( $k_{-2}$ ). Therefore, equilibrium will only be established after five half-lives of the slowest process,<sup>3</sup> which for indomethacin is the dissociation from  $EI^*$ . The rates of dissociation from  $EI^*$  were  $0.0011\text{ s}^{-1}$  and  $0.0031\text{ s}^{-1}$  for PGHS1 and PGHS2, respectively. The corresponding half-lives are 630 and 224 s, respectively. Accordingly, it will take approximately 50 and 20 min, respectively, for slow binding equilibrium to be established with indomethacin. The reactions must proceed at least this long for the  $IC_{50}$  to be a good estimate of  $K_I^*$ . This is impossible *in vitro* because of the rapid autoinactivation. Consequently, the  $IC_{50}$  values can only represent pre-equilibrium inhibition because the dissociation rates are much slower than the rates of autoinactivation. This being the case, the apparent  $IC_{50}$  values will vary depending on preincubation time, incubation time, and factors that affect the rate of enzyme autoinactivation. In general,  $IC_{50}$  determinations in which preincubation times are less than 5 times the  $EI^*$  association half-life will underestimate the affinity. Reactions in which the  $EI^*$  dissociation rate is greater than the inactivation rate or incubation time will overestimate the affinity because the enzyme will be bound in  $EI^*$  and unable to compete with substrate. Considering the complexity of the system, it is not unexpected that the  $IC_{50}$  values determined with different assay protocols will vary considerably (Table II). The reported values for inhibition of human PGHS1 by indomethacin following preincubations from 5 to 30 min range from 13 nM to  $1.7\text{ }\mu\text{M}$ , and those for inhibition of human PGHS2 range from 74 nM to  $25\text{ }\mu\text{M}$ . The selectivity ranged from 2.3 to 17. We determined an intrinsic selectivity of 30.

The apparent  $K_I$  represents the affinity of inhibitor for the first equilibrium complex,  $EI$ .  $IC_{50}$  values determined by inhibition of the initial velocities should be independent of the

<sup>3</sup> We assume it requires five half-lives to saturate a biochemical process.

TABLE II  
Summary of published  $IC_{50}$  values associated with the inhibition of human PGHS1 and PGHS2 by indomethacin and flurbiprofen

Preincubation time	$IC_{50}$ values ( $\mu M$ )		PGHS2 PGHS1	Ref.
	PGHS1	PGHS2		
	$\mu M$			
Indomethacin/ <i>in vitro</i>				
5 min	0.6	1.4	2.3	O'Neil <i>et al.</i> (17) <sup>a</sup>
10 min	1.7	25	15	Barnett <i>et al.</i> (10) <sup>b</sup>
30 min	0.013	0.074	5.7	Glaser <i>et al.</i> (18) <sup>c</sup>
5 s	8	15	2	This study
None	13.5	>1000	>74	Laneuville <i>et al.</i> (19) <sup>d</sup>
None	30	>300	>10	This study
Indomethacin/whole cell				
None	0.7	0.36	0.5	Patrignani <i>et al.</i> (20) <sup>e</sup>
15 min	0.005	0.010	2	Chan <i>et al.</i> (21) <sup>f</sup>
Flurbiprofen/ <i>in vitro</i>				
10 min	0.04	0.51	13	Barnett <i>et al.</i> (10) <sup>b</sup>
5 s	0.1	0.3	3	This study
None	0.5	3.2	6.4	Laneuville <i>et al.</i> (19) <sup>d</sup>
None	3	10	3	This study

<sup>a</sup> Microsomal suspensions from transfected COS7 cells were incubated with 2  $\mu M$  arachidonic acid at room temperature for 40 min, and products were determined for radioimmunoassay quantitation of total prostaglandin  $E_2$ .

<sup>b</sup> Enzyme purified from baculovirus/insect cell expression system was incubated with 20  $\mu M$  arachidonic acid at room temperature for 45 s.

<sup>c</sup> PGHS2 from baculovirus/insect cell system and solubilized microsomal human platelet PGHS1 were incubated for 35 s at 37 °C following addition of 30  $\mu M$  arachidonic acid. Reaction products were converted to Prostaglandin  $E_{2\alpha}$  and quantitated by radioimmunoassay.

<sup>d</sup> Microsomal suspensions from transfected COS1 cells were incubated with 10  $\mu M$  arachidonic acid at 37 °C, and  $IC_{50}$  values were determined from the initial rate of oxygen consumption.

<sup>e</sup> Activities of platelet PGHS1 and LPS-induced monocyte PGHS2 in human whole blood were followed after incubating with indomethacin for 60 min at 37 °C.

<sup>f</sup> PGHS1 in human U-937 cells and PGHS2 in human osteosarcoma cells were incubated with 10  $\mu M$  arachidonic acid for 10 min at 37 °C following a 15-min preincubation in the presence of indomethacin. Prostaglandin  $E_2$  levels were measured by radioimmunoassay.

reaction dynamics and approximate the apparent  $K_I$ . Laneuville and co-workers (19) reported  $IC_{50}$  values associated with instantaneous inhibition determined from the inhibition of the initial rate of oxygen consumption (19). A good correlation was observed between the  $IC_{50}$  reported by Laneuville *et al.* (19) and  $K_I$  values reported here for PGHS1. However, there was a large discrepancy between the two PGHS2 sets of data. The  $IC_{50}$  values for instantaneous inhibition by indomethacin and flurbiprofen were nearly 100 and 5 times greater, respectively, than the  $K_I$  values. We used the method of Laneuville *et al.* (19) and calculated  $IC_{50}$  values nearly identical to those they had reported (Table II). Surprisingly, when we determined the  $IC_{50}$  values by adding the inhibitors immediately prior to addition of the substrate instead of simultaneously (preincubation less than 5 s), the  $IC_{50}$  values corresponded to the  $K_I$  values (Table II). The only dynamic process we are aware of that could potentially influence these determinations is the rate of catalytic activation of the enzyme. It is well documented that the activation of PGHSs by hydroperoxides must proceed the cyclooxygenase activity and oxygen consumption. This results in a lag in enzyme activity after the addition of arachidonic acid as a result of the time required to synthesize sufficient quantity of the hydroperoxide product, prostaglandin  $G_2$ , needed to activate all the PGHS in the reaction mixture. When inhibitor is added to an incubation prior to arachidonic acid, it will interact only with unactivated enzyme, whereas when inhibitor and arachidonic acid are added simultaneously the inhibitor will be competing with arachidonic acid for binding to a dynamic mixture of activated and unactivated enzyme. We can envision two possible scenarios to account for the differences in  $IC_{50}$  values between the two methods: (i) preincubation of inhibitor with enzyme, even for 5 s, delays the activation and increases the apparent affinity and/or (ii) the inhibitors have a different affinity for activated and unactivated enzyme. While we cannot rule out the first scenario, our laboratory does have preliminary evidence from other studies that supports the hypothesis of an inhibitor sensitive allosteric activation of the PGHS cy-

clooxygenase activity.<sup>4</sup> Our data suggests that there is a separate SAR for the activated and unactivated states of both PGHS1 and PGHS2. Therefore, the selectivity will be determined by the state of enzyme activation and the sensitivity of the inhibitors to the activation state of the enzyme. Indomethacin and flurbiprofen appear to be sensitive to the activation state of PGHS2, not PGHS1.

The real value of any simplified *in vitro* methodology is the ability to predict what will occur in a more complex whole cell *in vivo* system. In the preceding paragraphs, we presented an argument for why the kinetic constants we have determined are a more accurate measure of the overall inhibition, as defined by  $K_I^*$ , than  $IC_{50}$  values determined after preincubation. We have also discussed factors that may effect  $K_I$  and the  $IC_{50}$  values associated with instantaneous inhibition. The next question to answer is whether these kinetic parameters have any relevance to inhibition in whole cells and *in vivo*. Interestingly, the two reported studies of the inhibition human PGHSs by indomethacin in whole cells had very different protocols and results. Patrignani *et al.* (20) measured the inhibition of platelet PGHS1 by indomethacin for 1 h and compared it with the inhibition of lipopolysaccharide-induced monocyte PGHS2 for 4 h in human whole blood with no addition of exogenous arachidonic acid and reported  $IC_{50}$  values of 0.7 and 0.36  $\mu M$ , respectively. Chan *et al.* (21) measured the inhibition of PGHS1 in U-937 cell and PGHS2 in osteosarcoma cells following a 15-min preincubation and a 10-min incubation with arachidonic acid and determined  $IC_{50}$  values of 5 and 10 nM, respectively. One major difference between these two protocols is the amount of time in which indomethacin is incubated with the enzymes. In the experiments by Chan *et al.* (21) the reaction times are much shorter than 5 times the half-life for dissociation, therefore the enzyme will stay bound in the  $EI^*$  complex during the reaction and will not be able to equilibrate with the substrate. This protocol should and does enhance the apparent affinity. In the

<sup>4</sup> A. Y. Mak and D. C. Swinney, unpublished result.

experiments by Patrignani *et al.* (20) the reaction times are much longer than the time needed to dissociate from the enzyme, therefore we would expect the  $IC_{50}$  values to approach the  $K_I^*$  values. This is the case for PGHS2 ( $IC_{50} = 0.36 \mu M$ ;  $K_I^* = 1.02 \mu M$ ) but not for PGHS1 ( $IC_{50} = 0.7 \mu M$ ;  $K_I^* = 0.03 \mu M$ ).

Since we do not know the rates of autoinactivation and catalytic activation in these cells, we cannot determine if these dynamic processes play a role in the discrepancies between the observed  $IC_{50}$  value and the  $K_I^*$ . Unfortunately it is these factors that may play the most important role in determining the efficacy of inhibitors *in vivo*. First, if the rate of enzyme inactivation is less than the dissociation rate, then the dissociation rates ( $k_{-2}$ ), not the overall affinity ( $K_I^*$ ) may be a better predictor of *in vivo* activity. And second, if the affinity of the inhibitor is different for activated and unactivated enzyme, the *in vivo* efficacy will depend upon whether an inhibitor is introduced to a cell whose PGHS system is activated or unactivated.

Not all time-dependent PGHS inhibitors are reversible slow binding inhibitors. Another class of time-dependent PGHS inhibitors, exemplified by NS-398 and DuP 697, was recently described by Copeland and co-workers (22) to be selective, irreversible inhibitors of PGHS2. Similar to the slow binding inhibitors, indomethacin, and flurbiprofen, these compounds could be recovered unaltered from the reaction mixture; however, the enzyme was not active after these compounds dissociated from the enzyme. These compounds appear to form an enzyme-inhibitor complex, which promotes inactivation of the enzyme in the absence of substrate. The advantage to be gained by inhibiting PGHS2 *in vivo* with a time-dependent irreversible inhibitor as compared to a time-dependent reversible inhibitor remains to be determined.

We have presented a methodology for evaluation of intrinsic kinetic parameters for slow binding inhibitors. This allows for the first time the determination of the dissociation rates from the  $EI^*$  complex ( $k_{-2}$ ), the evaluation of the critical enzyme-inhibitor structural interactions for  $K_I$ ,  $k_2$ , and  $k_{-2}$ , the determination of the overall affinity of a slow binding inhibitor ( $K_I^*$ ), and the impact of these kinetic parameters upon the cell-based and *in vivo* efficacy of PGHS inhibitors. This should allow us to

determine what factors affect the potency and selectivity of slow binding inhibitors.

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