Negative Cooperativity in the Interaction of Prostaglandin H Synthase-1 with the Competitive Inhibitor Naproxen Can Be Described as the Interaction of a Non-competitive Inhibitor with Heterogeneous Enzyme Preparation

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Abstract—The kinetic mechanism of the interaction of nonsteroidal anti-inflammatory drugs (NSAIDs) with their main pharmacological target, prostaglandin H synthase (PGHS), has not yet been established. We showed that inhibition of PGHS-1 from sheep vesicular glands by naproxen (a representative of NSAIDs) demonstrates a non-competitive character with respect to arachidonic acid and cannot be described within a framework of the commonly used kinetic schemes. However, it can be described by taking into account the negative cooperativity of naproxen binding to the cyclooxygenase active sites of the PGHS-1 homodimer (the first naproxen molecule forms a more stable complex ($K_1 = 0.1$ μM) with the enzyme than the second naproxen molecule ($K_2 = 9.2$ μM)). An apparent non-competitive interaction of PGHS-1 with naproxen is due to slow dissociation of the enzyme−inhibitor complexes. The same experimental data could also be described using commonly accepted kinetic schemes, assuming that naproxen interacts was a mixture of two enzyme species with the inhibition constants $K_\alpha = 0.05$ μM and $K_\beta = 18.3$ μM. Theoretical analysis and numerical calculations show that the phenomenon of kinetic convergence of these two models has a general nature: when $K_2 \gg K_1$, the kinetic patterns (for transient kinetics and equilibrium state) generated by the cooperative model could be described by a scheme assuming the presence of two enzyme forms with the inhibition constants $K_\alpha = K_1/2$, $K_\beta = 2 \cdot K_2$. When $K_2 \ll K_1$, the cooperative model can be presented as a scheme with two inhibitor molecules simultaneously binding to the enzyme with the observed inhibition constant K ($K = K_1 \cdot K_2$). The assumption on the heterogeneity of the enzyme preparation in relation to its affinity to the inhibitor can be used instead of the assumption on the negative cooperativity of the enzyme−inhibitor interactions for convenient and easy practical description of such phenomena in enzymology, biotechnology, pharmacology, and other fields of science.

Prostaglandin H synthase (PGHS; EC 1.14.99.1), also known as cyclooxygenase (COX), plays a key role in inflammation development in mammals by catalyzing conversion of arachidonic acid into prostaglandin H_2, a starting compound in the biosynthesis of prostaglandins, thromboxane, and prostacyclin [1]. The PGHS-1 isoform is constitutively synthesized in cells, providing their normal functioning [2]; the PGHS-2 isoform is produced in response to certain stimuli, such as cytokines or growth factors [3]. PGHS is inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) widely used in pharmacology [4, 5].

It is commonly accepted that all PGHS inhibitors bind in the cyclooxygenase active site of the enzyme and compete with arachidonic acid (substrate), as undoubtedly evidenced by the X-ray structural studies [6, 7] and kinetic data for some inhibitors [8, 9].

Accumulation of prostaglandin E_2, a product of the bienzymatic reaction, within 5-15 min of the reaction has been studied in many works [10-13]. When interpreting the results of these experiments, it should be taken into account that PGHS is rapidly inactivated in the course of

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Abbreviations: COX, cyclooxygenase; NSAIDs, nonsteroidal anti-inflammatory drugs; PGHS, prostaglandin H synthase.
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the reactions [14-16], and the estimated value does not reflect the reaction rate, but rather the maximally achieved yield of the reaction product.

PGHS is a dimer composed of two identical polypeptide chains with molecular mass of 72 kDa [17]. Each subunit has two active sites: one for the cyclooxygenase reaction (oxidation of arachidonic acid to prostaglandin G_2 [18]), and the other − for peroxidase reaction (reduction of prostaglandin G_2 peroxy group to the alcohol group [19]). The two reactions result in the formation of prostaglandin H_2 [20], that is then converted to other physiologically active compounds, such as prostaglandins E_2, D_2, and $F_{2\alpha}$, thromboxane A_2, and prostacyclin [1, 21, 22], by specific convertases.

It had been believed earlier that the dimeric nature of PGHS does not manifest itself in the kinetics PGHS catalysis, although there was some evidence that the active sites in the two subunits or within the same subunit interact with each other [23-25]. In recent years, the number of such publications has increased, which led to the conclusions on the gained [26, 27] or innate [28] functional asymmetry of the PGHS dimer subunits.

We studied PGHS cyclooxygenase activity from the inhibitor effect on the initial rate of consumption of dissolved molecular oxygen, which allowed us to investigate the kinetics of interactions between PGHS-1 and its inhibitor naproxen under equilibrium conditions. We found that the competitive mechanism of inhibition by naproxen did not manifest itself in the experiments, and none of the known schemes of PGHS inhibition, including those applied for the description of interactions between PGHS and naproxen [29], could appropriately describe the experimental data. An adequate interpretation of the experimental results on the naproxen binding was possible only when taking into account the dimeric nature of PGHS. However, the same data could be interpreted without accounting for cooperativity, but assuming that the reaction system contained two enzyme forms differing in their sensitivity to the inhibitor. Theoretical analysis and numerical calculations showed that the negative cooperativity observed in our study (both in the equilibrium state and for the kinetics of inhibition) can be described with high accuracy as manifestation of the enzyme preparation heterogeneity with respect to the affinity for the inhibitor.

MATERIALS AND METHODS

Reagents. The following reagents we used: Tween-20, Tris, naproxen, EDTA, and sodium diethyldithiocarbamate (DEDTC) from MP Biochemicals (Germany); arachidonic acid, potassium ferrocyanide, hemin chloride, and sodium hydroxide from Sigma-Aldrich (USA); sodium dithionite from Panreac (Spain); DEAE-Sepharose Fast Flow from Amersham Biosciences (Austria); hydrochloric acid from Khimmed (Russia).

Other reagents of high purity grade were produced in Russia.

Enzyme preparation. PGHS-1 preparation from sheep vesicular glands was obtained by solubilization with nonionic detergent Tween-20 and purified by ion-exchange chromatography as described in [17] with minor modifications [30]. Enzyme preparation (>95% purity by PAGE) was stored in a buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1 mM DEDTC, 0.1% (v/v) Tween-20, 2 μM hemin) at −70°C.

Kinetic experiments. Buffer solution A (50 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween-20) with potassium ferrocyanide (1 mM) and hemin (2 μM) was used in all experiments on measuring the PGHS-1 cyclooxygenase activity. Enzymatic reaction was performed in the closed thermostated cell at 25°C with constant stirring. The reaction mixture volume was 1.1-1.5 ml, to which 10-40 μl enzyme solution (0.5 mg/ml) was added. Arachidonic acid (10-150 mM) was dissolved in ethanol; potassium ferrocyanide (0.1 M) was dissolved in 50 mM Tris-HCl buffer, pH 8.0; naproxen was used as 100 mM aqueous solution of its sodium salt, that was diluted to the required concentration with 50 mM Tris-HCl (pH 8.0) before the experiment. Hemin was dissolved in a small volume of 0.1 M NaOH and then diluted 20× with buffer A. To determine hemin concentration, an aliquot of the solution was mixed with 20% pyridine in 0.1 M NaOH; then sodium dithionite was added, and hemin concentration was estimated spectrophotometrically using the extinction coefficients for the reduced hemin−pyridine complex [31].

Experiments on the PGHS-1 interaction with the inhibitor were performed according to the following scheme: the inhibitor and the enzyme were sequentially added to the reaction cell containing buffer A, potassium ferrocyanide, and hemin in the concentrations indicated above. After preincubation for a time period t (in this study, the time sufficient for the equilibrium establishment; no less than 2 min), the reaction was initiated by adding the substrate (arachidonic acid). In experiments with zero preincubation time, the order of addition of arachidonic acid and PGHS-1 was switched.

When plotting the kinetic curves, we accounted for the "dead time" period (1 s) of the measuring system.

Measurement of the enzymatic reaction rate. The rate of cyclooxygenase reaction was estimated amperometrically from the oxygen consumption in the course of reaction (arachidonic acid $+ 2O_2 \rightarrow$ prostaglandin G_2) using an Expert MTX-001 (Econic Expert, Russia) and an OxyGraph (Hansatech Instruments, United Kingdom) amperometric system for oxygen detection equipped with the closed gas-diffusion platinum Clark electrodes [32] (Fig. 1).

Relative enzymatic activity. The catalytic activity of a mixed enzyme preparation preincubated with the inhibitor for a time period t was evaluated from the rate of cyclooxygenase-catalyzed oxidation of arachidonic acid (Fig. 1). To

use the data obtained in different experiments and to ensure the convenience of further calculations and modeling, we applied the concept of relative enzymatic activity. Relative activity of the enzyme is a value of the normalized rate (V_{norm}) calculated as a ratio of the enzymatic reaction rate to the rate of the same reaction in the absence of the inhibitor and consequently, at the zero time point of enzyme preincubation with the inhibitor in the reaction cell, i.e.,

$$V_{norm}(I,t) = \frac{V(S,I,t)}{V(S,0,0)}, \qquad (1)$$

where S and I are concentrations of the substrate (arachidonic acid) and the inhibitor, respectively. It should be noted that the normalized rate appears to be independent of the substrate concentration (Fig. 2) and is a function of the inhibitor concentration I and preincubation time t; that is why hereafter S is not given as an argument in all formulae containing V_{norm}. Interpretation of the V_{norm} value will be presented in more details in the "Results" section.

Limiting value of the relative enzymatic activity $V_{norm}(I)$ was determined as a limit of $V_{norm}(I, t)$ at $t \rightarrow \infty$ (PGHS-1 was preincubated with the inhibitor for no less than 2 min for the equilibrium to be established) (Fig. 2):

$$V_{norm}(I) = \frac{V(S,I)}{V(S,0)}, \qquad (2)$$

where $V(S, I)$ corresponds to the limiting value of $V(S, I, t)$ at $t \rightarrow \infty$, and $V(S, 0)$ corresponds to $V(S, 0, 0)$.
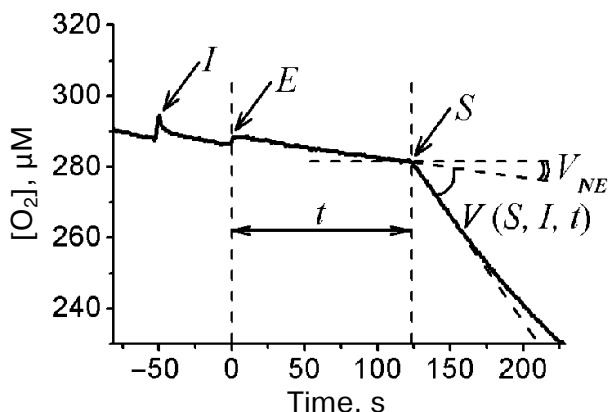

Fig. 1. Estimation of the enzymatic reaction rate from the kinetic curve of dissolved molecular oxygen consumption. The moment of enzyme addition to the reaction mixture was taken as a zero time point. The reaction rate corresponds to the slope of the tangent to the kinetic curve at the moment of reaction initiation (for calculations, we used approximation of the initial portion of the curve (5-10 s) minus the rate of non-enzymatic reaction). t, time of the enzyme and the inhibitor preincubation in the reaction cell; I, inhibitor; E, enzyme; S, substrate; V_{NE}, non-enzymatic reaction rate; $V(S, I, t)$, enzymatic reaction rate. The moments of addition of the corresponding reagents are shown with arrows.
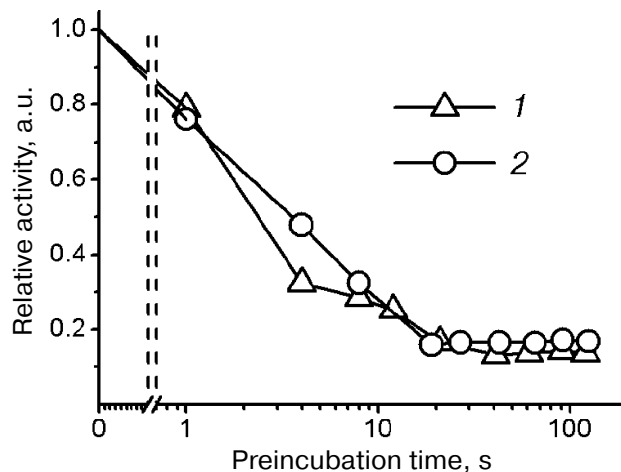
Fig. 2. Dependence of the relative activity (V_{norm}) on the time (t) of PGHS-1 preincubation with naproxen for two arachidonic acid concentrations (µM): *1*) 5; *2*) 100. Naproxen concentration, 2.5 µM.

Mathematical treatment of experimental data. Analysis and graphical presentation of kinetic data were done using the Origin 7.5 software package. Approximation of the whole data set was made using the MatLab software package (Fig. 3a).

Multiple approximations of experimental data (Fig. 4) were performed by finding the global minimum of the corresponding sums of the squared deviations in the space of the estimated parameters. For this, we made an exhaustive search of randomly generated parameter sets with sequential decrease in the search space. At the final stage, the extremum was determined within the narrow range using the *leastsq* function of the SciPy Optimize software package [33] (Python programing language). Confidence intervals were determined independently for all the estimated parameters by simulations (more than 50,000) using 6% standard deviation for V_{norm}. For all the parameters, dispersion did not exceed 15%.

When approximating the experimental dependence of the cyclooxygenase reaction rate on the arachidonic acid concentration at various naproxen concentrations (Fig. 3a), the desired values were determined by minimizing the sum of the rate squared deviations (*Sum*):

$$Sum = \sum_j (V_j^{theor} - V_j^{exp})^2, \qquad (3)$$

where V_j^{exp} is experimentally found value of the cyclooxygenase reaction rate, and V_j^{theor} is the corresponding theoretical value calculated by the formula for a general case of mixed inhibition:

$$V = \frac{V_M \cdot S}{K_M \cdot (1 + \dfrac{I}{K_i}) + S(1 + \dfrac{I}{K_i^*})}. \qquad (4)$$
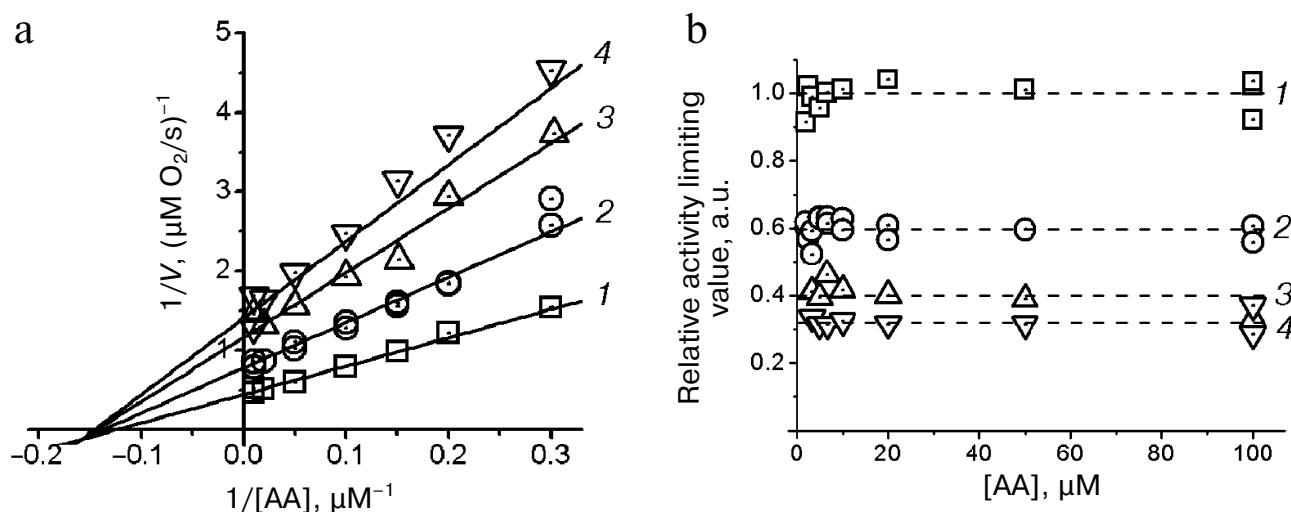
Fig. 3. Dependence of the limiting values of (a) cyclooxygenase reaction rate (V) (in the double reciprocal coordinates) and (b) relative activity (V_{norm}) on the arachidonic acid (AA) concentration for different naproxen concentrations (μM): *1*) 0; *2*) 0.07; *3*) 0.15; *4*) 0.2. In all experiments, the time of enzyme preincubation with the inhibitor was 3 min. Graphs in panel (a) are plotted by data approximation by nonlinear regression according to Eq. (4) for the general case of mixed inhibition. Graphs in panel (b) correspond to the average values of relative activities obtained for a given naproxen concentration. Relative activity (see "Materials and Methods") was determined as a ratio of the enzymatic reaction rate to the theoretical reaction rate under the same conditions obtained by approximation of experimental dependence using nonlinear regression according to the Michaelis–Menten equation in the absence of the inhibitor.

Summation was performed simultaneously for all results (*j*) of all experimental series with varying values of inhibitor concentration.

When approximating the experimental dependence of the relative activity limiting value on the naproxen concentration (Fig. 4), the desired values were determined by minimizing the sum of the squared deviations of the relative values (Q):

$$Q = \sum_j \frac{1}{(V_{norm_j}^{exp})^2} \cdot (V_{norm_j}^{theor} - V_{norm_j}^{exp})^2, \qquad (5)$$

where $V_{norm_j}^{exp}$ is experimentally determined limiting value of the relative enzyme activity; $V_{norm_j}^{theor}$ is the corresponding theoretically calculated limiting value of the relative enzyme activity determined by Eq. (8) when searching for approximation parameters for the simple model (7) of the enzyme–inhibitor interaction; by Eq. (13) – for the cooperative model (9); and by Eq. (18) – for the bienzymatic model (14).

Since the region of high inhibitor concentrations and, correspondently, small experimentally determined values (Fig. 4) is crucial for discrimination between the considered models, we varied PGHS-1 concentration to provide the optimal relative error (~6%) of the relative enzyme activity, which was reflected in the approximation algorithm (Eq. (5)).

We also checked for the correspondence between the theoretical models and experimental data using the Fisher criterion. The following formula was used for model comparison [34]:

$$F = \frac{(Q_1 - Q_2)(n - p_2)}{Q_2(p_2 - p_1)}, \qquad (6)$$

where Q_1 and Q_2 are sums of the squared deviations for models 1 and 2, respectively, n is a number of experimen-

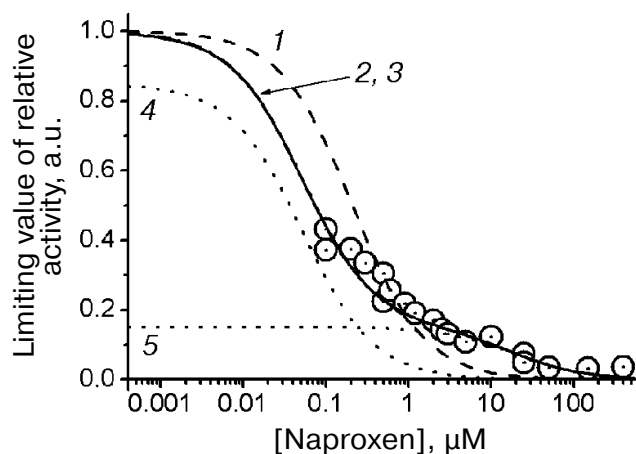**Fig. 4.** Dependence of the relative activity limiting value ($V_{norm}(I)$) on the naproxen concentration. In all the experiments, the time of enzyme preincubation with the inhibitor was 2 min; arachidonic acid concentration was 100 μM. Graphs correspond to the approximation of data performed using various equations: *1*) Eq. (8); *2*) Eq. (13); *3*) Eq. (18). Parameters K, K_1, K_2, K_α, K_β, γ and χ obtained by approximation are presented in the table. For convenience, the figure shows dotted lines plotted using Eq. (18) for constants $K_\alpha = 0.05$ μM (*4*) and $K_\beta = 18.3$ μM (*5*) and $\chi = 0.178$ separately for each addend of this equations.

tal points, p_1 and p_2 are the numbers of parameters for models 1 and 2, respectively.

The obtained F value was compared to the critical F values for (f_1, f_2) degrees of freedom (where $f_1 = p_2 - p_1$; $f_2 = n - p_2$) at significance levels of 0.05 and 0.01. If the F value did not exceed the critical one, zero hypothesis was accepted (i.e., model 2 does not describe experimental data better than model 1). In the opposite case, zero hypothesis was rejected.

RESULTS

Experimental data. *Kinetics of naproxen binding to PGHS-1.* As seen from the data on the naproxen binding to PGHS-1 (Fig. 2), the experimentally determined relative enzyme activity slowly decreases in the course of incubation with the inhibitor, thereby indicating that the mechanism of naproxen interaction with PGHS-1 involves some slow processes. We found (Figs. 2 and 3b) that the relative activity of PGHS-1 in the presence of naproxen does not depend on the arachidonic acid concentration, indicating that no fast, reversible, and competitive (with respect to arachidonic acid) binding of naproxen to PGHS-1 takes place.

In other words, the procedure for the estimation of the PGHS-1 relative activity used in the present work does not introduce changes in the enzyme–inhibitor system: the substrate does not have a chance to displace inhibitor in the cyclooxygenase binding site. Therefore, for the "slow" inhibitor naproxen, the relative activity value in our system is a function of the concentration of the enzyme active sites unoccupied by the inhibitor at the moment of arachidonic acid introduction. This fact was used for comparing the experimental data and theoretical models.

The relative activity of PGHS-1 after preincubation with naproxen was also independent on the enzyme concentration (data not shown).

Dependence of the limiting values of cyclooxygenase reaction rate and enzyme relative activity on the arachidonic acid concentration at varying naproxen concentrations. As can be seen in Fig. 3a, the graphs corresponding to the approximation of experimental data according to the equation for a general case of mixed incubation (Eq. (4)) cross each other almost at the x axis. We conclude that no competition between naproxen and the substrate takes place under given conditions. In order to determine the type of cyclooxygenase reaction rate dependence on the naproxen concentration, we performed experiments on the PGHS-1 inhibition at a constant substrate concentration (Fig. 4).

The dependence of the relative activity limiting value (V_{norm}) on the concentration of arachidonic acid at varying naproxen concentrations (Fig. 3b) was obtained from the data plotted in Fig. 3a. It appeared that V_{norm} is almost

independent on the arachidonic acid concentrations (10-100 µM), which corroborates the data presented in Fig. 2. Dashed lines parallel to the x axis (Fig. 3b) correspond to the average values of relative activity for the tested naproxen concentrations.

Dependence of the limiting value of PGHS-1 relative activity on the naproxen concentration. The limiting value of the enzyme relative activity characterizes the equilibrium state and corresponds to the relative activity of existing active enzyme forms after establishment of equilibrium in the enzyme–inhibitor system.

Figure 4 shows the dependence of the limiting value of PGHS-1 relative activity on a wide range of inhibitor concentrations at a constant substrate concentration. For convenience, the data are presented in semi-logarithmic coordinates. The dashed line is plotted based on the experimental data in accordance with the simple model (7) of enzyme–inhibitor interactions (Fig. 4, line *1*); the solid line corresponds to the cooperative model (9) that accounts for the interaction between cyclooxygenase active sites of the enzyme dimer subunits (Fig. 4, line *2*). The dot-dashed line (that almost coincides with line *2*) corresponds to the bienzymatic model (14). According to this model, PGHS-1 interaction with naproxen is determined by the existence of two enzyme forms with different inhibition constants (0.05 and 18.3 µM) simultaneously present in the reaction mixture (Fig. 4, line *3*). For illustration purposes, Fig. 4 shows dotted lines (lines *4* and *5*) that are hypothetical inhibition curves corresponding to the naproxen interaction with each of the two "imaginary" enzyme forms.

As shown in Fig. 4, the simple model (7) does not describe all the experimental data (Fig. 4, line *1*). However, these data are equally well interpreted using either cooperative (9) or bienzymatic models (14) (Fig. 4, lines *2* and *3*, respectively). This is also confirmed by the higher value of the sum of squared deviations between the theoretical and experimental values for the simple model as compared to those for the cooperative and bienzymatic models (for the last two models, the sums of squared deviations coincide) (table). Approximations based on the cooperative and bienzymatic models do not virtually differ (lines *2* and *3* in Fig. 4 almost coincide within all the inhibitor concentration range); and numerical values of the dissociation constants are governed by the ratios $K_\alpha = K_1/2$ and $K_\beta = 2K_2$ (see "Theoretical results" below).

Theoretical results. Below we present three kinetic schemes for the enzyme–inhibitor interactions. For all the schemes, the solution is given for the equilibrium state, presuming that the inhibitor concentration significantly exceeds the concentration of PGHS-1 (i.e., changes in the inhibitor concentration resulting from its interaction with the enzyme could be neglected; $I =$ const). All expressions are given only for the enzyme forms with the unoccupied binding sites (potentially active enzyme forms); expressions for completely inactive

enzyme–inhibitor complexes can be easily derived from the material balance equation for the enzyme. The $V_{\text{norm}}(I)$ value was determined from Eq. (2) and used as the relative activity limiting value in all the cases discussed below.

The rate of the reaction catalyzed by each of the enzyme forms can be defined as a product of multiplication of the concentration of this form and the corresponding observed catalytic rate constant that depends on the substrate concentration ($k^{\text{cat}}(S)$). Since $V_{\text{norm}}(I)$ is not a function of the substrate concentration or the reaction time (Figs. 2 and 3b), by using solutions for the concentration of intermediates, one can obtain general expressions for the enzymatic reaction rate in the cases of simple (7), cooperative (9), and bienzymatic (14) models as a linear combination of concentrations of intermediates with unoccupied active sites.

The simplest kinetic scheme of the enzyme–inhibitor interactions (simple model). The simplest kinetic scheme of the reversible interaction of the inhibitor (I) with the enzyme monomer active site (E):
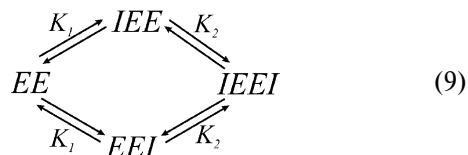
$$E \xrightleftharpoons{K} EI \qquad (7)$$

gives:

$$V_{norm}(I) = \frac{K}{K + I}, \qquad (8)$$

where K is the equilibrium (dissociation, inhibition) constant.

Kinetic scheme of the inhibitor interaction with the enzyme homodimer that accounts for cooperation between the active sites (cooperative model). Here, we present an equilibrium solution for the kinetic scheme of the inhibitor (I) reversible interaction with the active sites of the homodimeric enzyme (EE) that accounts for the cooperative interaction between the enzyme active sites (9). This interaction manifests itself in the fact that kinetic constants that describe the interactions of the inhibitor with the free form of the enzyme (EE) generally differ from the kinetic constants describing inhibitor interactions with the enzyme dimer, in which one of the active sites is occupied with the inhibitor (EEI or IEE).

$$\begin{array}{ccc} & \xrightarrow{K_1} IEE \xrightarrow{K_2} & \\ EE & & IEEI \\ & \xrightarrow{K_1} EEI \xrightarrow{K_2} & \end{array} \qquad (9)$$

Taking into account that *IEE* and *EEI* are the same compound (besides, their concentrations are equal due to the symmetry of the system), we can designate the sum of the *IEE* and *EEI* concentrations as *EEI** to obtain:

$$EE(I) = \frac{K_1 \cdot K_2 \cdot EE_0}{I^2 + 2 \cdot K_2 \cdot I + K_1 \cdot K_2}, \qquad (10\_1)$$

$$EEI^*(I) = \frac{2 \cdot K_2 \cdot I \cdot EE_0}{I^2 + 2 \cdot K_2 \cdot I + K_1 \cdot K_2}, \qquad (10\_2)$$

where K_1, K_2 are the equilibrium (dissociation, inhibition) constants, and EE_0 is a total concentration of enzyme dimers.

For the enzymatic reaction rate:

$$V(S,I) = 2 \cdot k_1^{cat}(S) \cdot EE(I) + k_2^{cat}(S) \cdot EEI^*(I), (11)$$
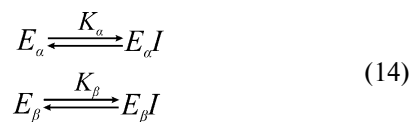
$$V(S,0) = 2 \cdot k_1^{cat}(S) \cdot EE_0, \qquad (12)$$

where $k_1^{\text{cat}}(S)$ and $k_2^{\text{cat}}(S)$ are observed catalytic rate constants of the enzymatic reaction for the free monomer in the *EE* and *IEE* (or *EEI*) complexes, respectively. Coefficient "2" in Eqs. (11) and (12) reflects the fact that both monomers exhibit catalytic activity in the completely free enzyme dimer. Let us introduce $\gamma = k_2^{\text{cat}}(S)/k_1^{\text{cat}}(S)$, the ratio of the activity of the free monomer in *IEE* (or *EEI*) complex to the activity of the monomer in *EE* complex. Taking into account (2) and (10)-(12), we obtain:

$$V_{norm}(I) = \frac{K_2 \cdot (K_1 + \gamma \cdot I)}{I^2 + 2 \cdot K_2 \cdot I + K_1 \cdot K_2}. \qquad (13)$$

It should be noted that the experimental results (Figs. 2 and 3b) indicate that the γ value calculated in the framework of the cooperative model is independent of S.

Kinetic scheme for the inhibitor simultaneous interaction with the two enzyme forms (bienzymatic model). Here, we present the equilibrium solution for a case when two forms of the enzyme (E_α and E_β) are present in the reaction mixture, and each of them interacts with the inhibitor (I) independently according to the simplest kinetic scheme of the reversible inhibitor interaction with the active site (14). However, in a general case, the equilibrium inhibition constants (K_α and K_β) for these enzymes are different. From

$$\begin{aligned} E_\alpha &\xrightleftharpoons{K_\alpha} E_\alpha I \\ E_\beta &\xrightleftharpoons{K_\beta} E_\beta I \end{aligned} \qquad (14)$$

we obtain:

$$V(S,I) = k_\alpha^{cat}(S) \cdot E_\alpha(I) + k_\beta^{cat}(S) \cdot E_\beta(I), \qquad (15)$$

$$V(S,0) = k_\alpha^{cat}(S) \cdot E_{\alpha 0} + k_\beta^{cat}(S) \cdot E_{\beta 0}, \qquad (16)$$

where $k_\alpha^{\text{cat}}(S)$ and $k_\beta^{\text{cat}}(S)$ are the observed catalytic rate constants of the enzymatic reaction for E_α and E_β, respectively. Let us introduce designation:

$$\chi = \frac{k_\beta^{cat}(S) \cdot E_{\beta 0}}{k_\alpha^{cat}(S) \cdot E_{\alpha 0}}, \qquad (17)$$

where χ is a ratio between the activities of two enzymes E_α and E_β. It is worth noting that the experimental results

(Figs. 2 and 3b) indicate that the χ value calculated in the framework of the bienzymatic model is independent on S. Then accounting for (2) and (15)-(17), we obtain:

$$V_{norm}(I) = \frac{1}{(1+\chi)} \cdot \frac{K_\alpha}{K_\alpha + I} + \frac{\chi}{(1+\chi)} \cdot \frac{K_\beta}{K_\beta + I}. \quad (18)$$

Approximation of experimental data and comparison of the models. Equations (8), (13), and (18) corresponding to the simple, cooperative, and bienzymatic models, respectively, were used for approximation of the experimentally obtained dependences of the relative enzyme activity on the naproxen concentration (Fig. 4).

The values of the constants for the three kinetic models derived by approximation of the experimentally obtained dependences are summarized in the table.

The table shows the constants K, K_1, K_2, K_α, K_β, γ and χ, the sum of squared deviations (Q) calculated by Eq. (5) (see "Materials and Methods"), the number of experimental points (n) and model parameters (p) for the corresponding approximations, and F values calculated by Eq. (6) (see "Materials and Methods") for comparison of the simple and cooperative models and also of the simple and heterogeneous models.

Based on the comparison of the calculated F values with the critical F values for the significance levels 0.05 ($F = 3.55$) and 0.01 ($F = 6.01$), we rejected the zero hypothesis in all the cases, thereby proving that the cooperative/heterogeneous models describe PGHS-1 interactions with naproxen better than the simple model.

Relation between cooperative and bienzymatic models. It is easy to see (see Supplement to this paper on the site of the journal http://protein.bio.msu.ru/biokhimiya and Springer site http://link.springer.com) that if $K_1 \ll K_2$, the equation for the relative enzymatic activity limiting value for the cooperative model (13) can be written with an accuracy of the dimensionless value K_1/K_2 as:

$$V_{norm}(I) = \left(1 - \frac{\gamma}{2}\right) \cdot \frac{K_1/2}{K_1/2 + I} + \frac{\gamma}{2} \cdot \frac{2 \cdot K_2}{2 \cdot K_2 + I}, \quad (19)$$

i.e., if $K_1 \ll K_2$, the right parts of Eqs. (13) and (19) are equal.

In turn, Eq. (19) coincides with analogous Eq. (18) for the bienzymatic model if:

$$K_\alpha = K_1/2, \quad (20)$$

$$K_\beta = 2K_2, \quad (21)$$

$$\chi = \frac{\gamma}{2 - \gamma}. \quad (22)$$

The values obtained by approximation of the experimental data (table) correlate well with the expressions (20)-(22).

The last equation (22) clearly demonstrates a limited character of the bienzymatic model as compared to the cooperative one; the former can be applied only if $0 \le \gamma < 2$; therefore, it cannot be used, if I is a strong activator of the cooperative enzyme (occupation of one of the two active sites in the enzyme homodimer results in the total gain in the catalytic activity of the dimer at $\gamma > 2$).

Moreover, in the case of negative cooperativity of the interaction between the enzyme active sites ($K_1 \ll K_2$; Eq. (9)), the bienzymatic model (14) appropriately describes the kinetics of interaction with the inhibitor for the cooperative model as well (see Supplement; the ratios between the constants for both models).

Note the case, when $K_1 \gg K_2$. It is evident that concentrations of *IEE* and *EEI* intermediates in this case could be neglected within all the range of I concentrations, and expression for $V_{norm}(I)$ will be written as:

$$V_{norm}(I) = \frac{K_1 K_2}{K_1 K_2 + I^2}, \quad (23)$$

Values of constants determined by approximation of experimental data presented in Fig. 4 using Eqs. (8), (13), and (18)

Simple model (7)						
K, µM	–	–	Q	n	p	–
0.21	–	–	7.77	21	1	–
Cooperative model (9)						
K_1, µM	K_2, µM	γ	Q	n	p	F
0.10	9.2	0.307	1.38	21	3	41.67
Bienzymatic model (14)						
K_α, µM	K_β, µM	χ	Q	n	p	F
0.05	18.3	0.178	1.38	21	3	41.67

thereby demonstrating positive cooperativity with the Hill coefficient of 2.

DISCUSSION

PGHS inhibitors are very important in pharmacology. A discovery that cyclooxygenase is a target for nonsteroidal anti-inflammatory drugs was rewarded with the Nobel prize in 1982 [4]. Since then, the number of NSAIDs has significantly increased, and selective inhibitors of PGHS-1 and PGHS-2 have been developed [5]. Nonetheless, the mechanism of PGHS inhibition by these compounds is still not completely understood.

Various models are used for description of NSAID action on PGHS. PGHS inhibitors can be fast and slow, reversible and irreversible [35]. Along with the one-step models [35], the two-step models with rapid equilibrium competitive first step and slow irreversible [36-38] or slow reversible [39] second step are used for the description of the NSAID interaction with PGHS. The three-step inhibition scheme was also proposed [40]. The use of different methods for the enzymatic activity estimation under different experimental conditions resulted not only in significant differences in the quantitative characteristics of the inhibitors properties [41], but also in contradictions in the inhibitor classification. For example, naproxen is described as a slow [29, 41] as well as a rapid inhibitor [35].

The data presented in this work (Figs. 2 and 3) indicate that inhibition by naproxen detected from the initial rate of PGHS-1 cyclooxygenase reaction is not competitive with respect to arachidonic acid, which contradicts the fact that naproxen forms a complex with the enzyme active site [6, 7]. Our work demonstrates that the PGHS-1−naproxen complexes form slowly, and this is the reason of the apparent absence of competition between naproxen and arachidonic acid (naproxen does not have enough time to leave PGHS-1 active site during estimation of the cyclooxygenase reaction initial rate).

As follows from the data depicted in Fig. 4, PGHS-1 inhibition by naproxen cannot be described by the simple binding model (7). To bring the experimental data into compliance with the kinetic model, we accounted for the dimeric nature of PGHS-1 and found that the cooperative model (9) that implies the dependence of the dissociation constant of the naproxen complex with the enzyme active site on the presence of naproxen in the active site of the neighboring monomer (line *2*) described well the experimental data (Fig. 4).

It should be emphasized that it is principally impossible to kinetically distinguish the scheme, in which the first and the second inhibitor molecules bind to the PGHS-1 dimer with different affinities (9) from the scheme that assumes the presence of two enzyme forms in the enzyme preparation that differ in their affinity to naproxen (14) (lines *2* and *3* in Fig. 4 coincide).

It has been already noticed in the studies on the negative cooperativity of enzymes and receptors, that negative cooperativity is poorly distinguishable from the initial heterogeneity of the binding sites [42-46]. As shown in the present work, if $K_2 \gg K_1$, the cooperative model used by us (9) suggests the presence of two enzyme forms in the enzyme preparation that differ in their affinity to naproxen (14) within the whole range of inhibitor concentrations for both the equilibrium state and the kinetics of inhibition. The determined ratios of the kinetic and equilibrium constants result in a complete convergence of these two models.

So, in our case the negative cooperativity of the enzyme homodimer interaction with the inhibitor ($K_1 = 0.1\ \mu M$, $K_2 = 9.2\ \mu M$) suggests that the enzyme preparation is heterogeneous in its affinity to the inhibitor and consists of 85% molecules with $K_\alpha = 0.05\ \mu M$ and 15% molecules with $K_\beta = 18.3\ \mu M$ (percentage content reflects catalytic activity of the components), which mathematically describes the idea that PGHS-1 exists as a functional heterodimer [28]. However, it is unlikely, because PGHS is encoded by a single unique DNA sequence [47], and no heterogeneity in other properties of this enzyme has been observed before. In our opinion, the character of PGHS-1 inhibition by naproxen reflects the cooperative interaction of the active sites in enzyme dimers; however, for a practicing doctor or a pharmacologist, an idea of preparation heterogeneity is more familiar and less complicated than the idea of cooperative interactions. Since both models describe the observed properties of the inhibitor to an equal extent, the use of the bienzymatic model can be considered a "mnemonic rule".

The problem of complete coincidence of kinetic behavior of the two models ((9) and (14)) is beyond the scope of the studies of PGHS-1 inhibition by naproxen and has a more general character; the problem of discrimination between the two mechanisms in practice should be solved in principle. As was mentioned above, at the given ratio of dissociation constants, the obtained expressions completely coincide, that is why increasing the accuracy of experiments and calculations will fail to succeed. For successful discrimination, it is important to remember that the cooperative model suggests initially homogeneous enzyme preparation, in which heterogeneity arises when the enzyme interacts with the inhibitor. In contrast to it, the bienzymatic model assumes initially heterogeneous (at least, in its affinity to the inhibitor) enzyme preparation.

It should be expected that the initial heterogeneity of the enzyme will necessarily manifest itself in other properties (e.g., kinetics of substrate or inhibitor binding, pH dependence, effects of temperature and ionic strength, etc.) and could be possibly revealed in the corresponding experiments. Thus, it was shown that PGHS-1 interaction with other inhibitors (indomethacin and diclofenac) under analogous conditions is described by the simple

mechanism [48]. It can be suggested that if the enzyme were really heterogeneous, its heterogeneity would be observed in the interactions with other inhibitors, not only naproxen. However, there are reasons to believe that manifestations of cooperative interactions depend on the nature of the bound ligand [23-25]. Moreover, any heterogeneous preparation can be in principle separated into its homogeneous parts, and the initial cause of heterogeneity − significant difference in affinity to the inhibitor − can be a basis for the development of a method for fractionation of such preparation by affinity chromatography using the same inhibitor (or its analog) chemically linked to a resin as an active component of the steady-state phase.

The $[I]_{50}$ value that is commonly used in pharmacological studies is insufficient to describe inhibitors exhibiting similar behavior in their interactions with enzymes. The degree of cooperativity (a $[I]_{90}/[I]_{10}$ ratio) was suggested for this purpose in [49]. Although it has not received a wide acceptance, we believe that the degree of cooperativity can be very useful for characterizing inhibitors. Thus, in our case to, to decrease the PGHS-1 activity from 90 to 10%, naproxen concentration should be increased ~1500 times (Fig. 4, line *3*), whereas in the case of a regular inhibitor interacting by the simple mechanism, similar inhibition can be achieved by increasing inhibitor concentration 81 times (Fig. 4, line *1*).

Such a degree of cooperativity indicates that naproxen exhibits its pharmacological effects within in a wider concentration range than regular inhibitors. This allows us to classify naproxen as a "soft" inhibitor, that acts in a wide concentration range and does not leave any traces of its activity after removal from the organism due to the completely reversible nature of its action. These characteristics of naproxen should be considered, when it is prescribed as a medication.

Acknowledgments

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