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Kinetic Mechanism of the Bifunctional Enzyme Prostaglandin-H-synthase. Effect of Electron Donors on the Cyclooxygenase Reaction

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Abstract—Prostaglandin-H-synthase (PGHS, EC 1.14.99.1) catalyzes the first committed step in biosynthesis of all prostaglandins, thromboxanes, and prostacyclins by converting arachidonic acid to prostaglandin H_2 (PGH_2). PGHS exhibits two enzymatic activities: cyclooxygenase activity converting arachidonic acid to prostaglandin G_2 (PGG_2) and peroxidase activity reducing the hydroperoxide PGG_2 to the corresponding alcohol, PGH_2. Despite the many investigations of the kinetics of PGHS, many features such as the absence of competition and mutual activation between the cyclooxygenase and peroxidase activities cannot be explained in terms of existing schemes. In this work we have studied the influence of different electron donors (N,N,N′,N′-tetramethyl-*p*-phenylenediamine, L-epinephrine, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), potassium ferrocyanide) on the PGHS activities. The proposed scheme describes independent but interconnected cyclooxygenase and peroxidase activities of PGHS. It also explains the experimental data obtained in the present work and known from the literature.

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Key words: prostaglandin-H-synthase, bifunctional enzyme, cyclooxygenase activity, kinetic mechanism, inhibition, naproxen, TMPD

The enzyme prostaglandin-H-synthase (PGHS) catalyzes the conversion of arachidonic acid (AA) to prostaglandin H_2 (PGH_2), a universal precursor of prostaglandins and thromboxanes. Synthesis of PGH_2 mediated by this enzyme occurs in two steps [1]: 1) conversion of arachidonic acid into organic peroxide (prostaglandin G_2) via the cyclooxygenase reaction; 2) reduction of prostaglandin G_2 to prostaglandin H_2 via the peroxidase reaction. Further, following the action of specific enzymes (convertases), prostaglandin H_2 can be converted into such physiologically active compounds as prostaglandins E_2, D_2, $F_{2\alpha}$, thromboxane A_2, and prostacyclin [2]. These substances play a significant role in tumor cell growth, blood coagulation, progression of bronchial asthma, and inflammatory processes; they are also neuromodulators [3].

Therefore, prostaglandin-H-synthase is a crucial link in the biosynthesis chain for these intracellular regulators that supplies all subsequent steps with a universal "building block", prostaglandin H_2. Two isoforms of prostaglandin-H-synthase are found in mammalian cells, including PGHS-1 (it is always present in cells and provides their normal functioning) [4] and PGHS-2 (produced in response to certain stimuli, such as cytokines and growth factors) [5]. In the present work, we have studied the properties of PGHS-1 from sheep vesicular gland.

Abbreviations: AA) arachidonic acid; ABTS) 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); COX) cyclooxygenase; DEDTC) diethyldithiocarbamate; PGHS) prostaglandin-H-synthase; PGG_2) prostaglandin G_2; PGH_2) prostaglandin H_2; PPIX) protoporphyrin IX; ROH) general formula for alcohols; ROOH) general formula for peroxides; TMPD) N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

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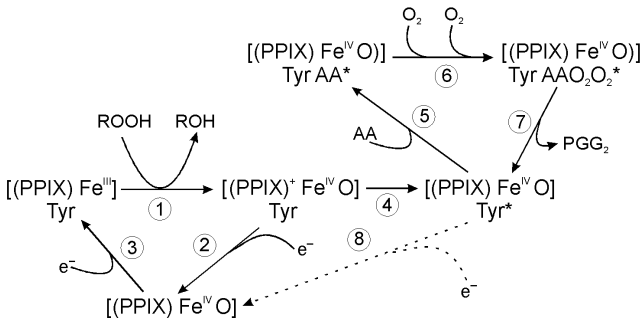
The PGHS enzyme is present in solution as a dimer, consisting of two identical subunits with molecular weight of 72 kD [6]. It is assumed that each PGHS subunit binds a molecule of hemin [7] and performs catalysis autonomously by using adjacently located active sites, one of which is responsible for the cyclooxygenase and the other for the peroxidase reaction [6]. Since the catalysis of both reactions occurs within one and the same enzyme molecule, the question rises of how these activities are connected. It has been demonstrated that the cyclooxygenase activity of the enzyme requires trace amounts of peroxides in the reaction mixture. The removal of peroxides from the reaction mixture by adding glutathione peroxidase and reduced glutathione leads to the appearance of a lag period on the kinetic curve of oxygen consumption during the cyclooxygenase reaction. The lag period can be prevented by exogenous addition of peroxides to the reaction medium [8]. This means that during the peroxidase reaction some active enzyme form (able to perform cyclooxygenase catalysis) is produced.

It has been established [9] that a radical of a tyrosine residue is formed in the peroxidase reaction. The hypothesis that a radical of Tyr385, located near the enzyme active site, initiates the cyclooxygenase reaction is now widely accepted [10].

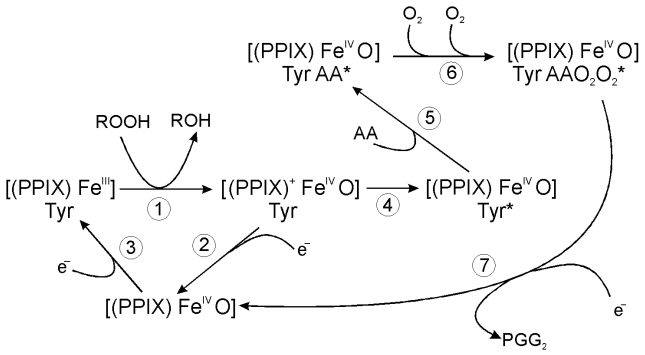
For kinetic interpretation of these facts, the so-called branched-chain mechanism was suggested for PGHS in 1988 [11]; this is shown in Scheme 1. According to this mechanism (Scheme 1), after initial oxidation by a peroxide molecule (step 1) the heme group receives an electron either from the exogenous donor (step 2) or from the tyrosine residue, resulting in the formation of tyrosyl radical Tyr* (step 4), which is presumably involved in the cyclooxygenase catalysis.

Despite the fact that the scheme explains the activation of cyclooxygenase catalysis during the peroxidase reaction, it has a significant drawback. It follows from the scheme that the enzyme molecule either carries out the cyclooxygenase reaction (steps 5-7), and thus cannot catalyze the peroxidase reaction (steps 1-3) at the same time, or performs the peroxidase reaction, and cannot simultaneously catalyze the cyclooxygenase reaction. This means that these two activities compete with each other, and the increase in the concentration of substrate for one reaction should result in a decrease in the rate of the other reaction. However, this has not been observed in practice; as shown many times, and in work [12] in particular, with increase in the concentration of electron donors the rate of the cyclooxygenase reaction also increases. To explain this fact, which cannot be described in terms of the branched-chain mechanism, an alternative tightly coupled mechanism has been suggested [12, 13] (Scheme 2).



Branched-chain mechanism of PGHS catalysis (from [11])

Scheme 1



Tightly coupled mechanism of PGHS catalysis (from [14])

Scheme 2

In terms of this mechanism the increase in the rate of cyclooxygenase reaction upon the increase in the concentration of electron donors can be explained [14]. However, it follows from Scheme 2 that cyclooxygenase oxidation of arachidonic acid can occur only after the oxidation of the heme group by the peroxide molecule (step 1). If the electron donor is absent in the reaction mixture, the mechanism predicts the absence of cyclooxygenase reaction, since the steps 3 and 7 cannot occur. This is contradictory to both the literature [15, 16] and the experimental data obtained in our work. Tightly coupled mechanism cannot explain the accumulation of prostaglandin G_2 (PGG_2) during the reaction (in the presence of electron donor) observed in [17], because the PGG_2 molecule in the absence of other peroxides is by all means consumed in the peroxidase reaction (step 1). The absence of the expected stoichiometry between reacting electron donor and arachidonic acid (the measured ratio between the concentration of consumed electron donor and the concentration of the oxidized form of arachidonic acid was 1.3, whereas the tightly coupled mechanism predicts this value to be 2.0) can neither be explained [17].

Therefore, it can be concluded that each of the suggested schemes has significant drawbacks, and neither one of them can strictly describe the kinetics of the prostaglandin-H-synthase catalyzed reaction.

The mathematical analysis of kinetic models for the mechanism of this enzyme is ongoing [18]. Due to the complexity of the system, the authors of the above publi-

cations resort to drastic simplifications, in particular, they use the material balance equation for the intermediate enzyme forms involved in the cyclooxygenase and peroxidase reactions. This automatically leads to the prediction that the increase in the rate of one of the reactions results in the decrease in the rate of the other, which, as mentioned above, is in contradiction with the experimental data. The authors mathematically investigate the behavior of the system upon the change in concentration of arachidonic acid by 20 orders of magnitude; however, the inadequacy of the initial model reduces the practical meaning of such an analysis down to zero.

Individually, the mechanisms of cyclooxygenase and peroxidase reactions of PGHS are studied in detail. The question concerning the interaction of the two activities remains open. Besides, that cyclooxygenase produces the substrate for peroxidase; both reactions occur within the same enzyme molecule, which can determine some features of the kinetics of the prostaglandin-H-synthase reaction.

The aim of the present work was to study the kinetics of interaction between the peroxidase and cyclooxygenase activities of PGHS using polarographic and spectrophotometric techniques for registration of the reaction rate. Interaction between the two activities is understood as the effect of the concentration of substrate for one reaction on the rate of the other. In the present work, the effect of electron donors (substrates for the peroxidase reaction) on the initial rate of cyclooxygenase reaction of PGHS, as well as the effect of arachidonic acid and an inhibitor of cyclooxygenase (naproxen) on the peroxidase reaction of PGHS has been investigated. The natural electron donor for PGHS is not known [19]. Several compounds widely used studies of PGHS kinetics [20-22] were employed in our work as electron donors. N,N,N′,N′-Tetramethyl-*p*-phenylenediamine (TMPD) is a well known one-electron donor [20], 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) is a one-electron donor; both TMPD and ABTS are hydrophobic molecules, but are well dissolved in the reaction medium used in our work since it contains nonionic detergent Tween-20. Ferrocyanide is a one-electron inorganic donor, and L-adrenalin is a two-electron organic donor. Ferrocyanide and L-adrenalin are water-soluble molecules. Therefore, the selected electron donors represent a large group of various substances. The peroxidase activity of PGHS is characterized by the absence of strict specificity towards peroxide substrates. PGHS catalyzes the reduction of the peroxide group in a large variety of compounds with different structures, including hydrogen peroxide [22] used in this work.

MATERIALS AND METHODS

Tris, Tween-20, TMPD, ABTS, naproxen, and L-adrenalin were acquired from MP Biomedicals Inc. (Germany), potassium ferrocyanide from Merck (Germany), and arachidonic acid and hydrogen peroxide were purchased from Sigma-Aldrich (USA). Other chemicals of highly pure grade were of domestic production.

The preparation of prostaglandin-H-synthase (PGHS) solubilized from microsomes of sheep vesicular gland (produced by livestock breeding farm Rus', Moscow Region) was isolated according a technique described in [23] with minor changes. The enzyme preparation was made up using the following buffer solution: Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1 mM diethyldithiocarbamate (DEDTC), 1% (v/v) Tween-20 (i.e. 1000 ml of buffer contained 10 ml of Tween-20).

The standard buffer containing 50 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween-20 (that is usually used for the studies of PGHS kinetics [9, 11]) was used in the kinetic experiments. The temperature of the reaction mixture was 25°C. The reagents were added to the buffer and the reaction initiated by addition of the enzyme. Solutions of arachidonic acid (10-150 mM), TMPD (5-100 mM), and naproxen (0.1 M) were prepared and added to the reaction as ethanolic solutions; 0.1 M L-adrenalin solution was prepared by dissolving L-adrenalin in 0.1 M HCl. The solution of potassium ferrocyanide (0.1 M), ABTS (75 mM), and hydrogen peroxide (0.5 M) were prepared in the standard buffer. Hemin was prepared by dissolving dry weight in a small amount of 0.1 M NaOH, followed by 20-fold dilution with the standard buffer. To determine the concentration, an aliquot of this solution was mixed with 20% pyridine in 0.1 M NaOH and sodium dithionite was added. Hemin concentration was determined spectrophotometrically using the known extinction coefficients of reduced hemin–pyridine complex [24].

Peroxidase reaction. To perform the peroxidase reaction, hydrogen peroxide, TMPD or ABTS, and the enzyme were added to the reaction mixture. Accumulation of the oxidized form of electron donors was monitored. In the case of TMPD the stoichiometric equation is as follows:

$$H_2O_2 + 2TMPD + 2H^+ \rightarrow 2H_2O + 2TMPD^{*+}.$$

The extent of the peroxidase reaction of PGHS was monitored spectrophotometrically by accumulation of oxidized TMPD form using a Cary 100 spectrophotometer (Varian Inc., USA). The wavelength for detection was 611 nm, extinction coefficient for oxidized TMPD molecule (the stoichiometric ratio of which to hydrogen peroxide is 2 : 1 [21]) is 13,500 $M^{-1} \cdot cm^{-1}$. The complete kinetic curve for the oxidation of donor was registered in real time. The volume of the reaction mixture was 2.5 ml; the volume of added enzyme solution (protein concentration 4.5 mg/ml) was usually 10 μl.

Cyclooxygenase reaction. Electron donor, arachidonic acid, and enzyme were added to the reaction mix-

ture, and the change in the concentration of dissolved oxygen was registered:

$$AA + 2O_2 > PGG_2.$$

The extent of the cyclooxygenase reaction was monitored amperometrically using an Ecotest-120/ATC oxygen analyzer (NPP Econics Ltd., Russia), equipped with a platinum/silver gas diffusion Clark-type electrode [25]. The total kinetic curve for utilization of dissolved oxygen was registered in real time. The volume of the reaction mixture was 1.7 ml, and the volume of added enzyme solution (protein concentration 4.5 mg/ml) was usually 10 μl.

Inhibition of cyclooxygenase reaction by naproxen. Naproxen was used as an inhibitor of cyclooxygenase at the concentration of 1 mM in the reaction mixture. Naproxen was added to the reaction mixture either *prior to* the addition of enzyme or 5 or 10 sec after the start of the reaction for the following PGHS-catalyzed reactions:

– for cyclooxygenase reaction: ABTS, hemin, arachidonic acid, and 5 μl of enzyme solution (protein concentration 9 mg/ml) were added to the buffer. Measurements were performed using an AKPM-02-05 polarograph (Alfa BASSENS Ltd., Russia) and a platinum/silver gas diffusion Clark-type electrode [25]. The cell volume was 3 ml.

– for peroxidase reaction: ABTS, hemin, arachidonic acid, and 1.5 μl of enzyme solution (protein concentration 9 mg/ml) were added to the buffer. The solution of arachidonic acid was added to the reaction mixture for the peroxidase reaction in some experiments. Measurements were carried out using a Cary 100 spectrophotometer. The detection wavelength was 405 nm, and the extinction coefficient for ABTS molecule in stoichiometric ratio to hydrogen peroxide of 2 : 1 is 36,800 $M^{-1} \cdot cm^{-1}$ [26]. The complete kinetic curve for the oxidation of donor was registered in real time.

The control experiments were performed under the same conditions without an inhibitor. Reagent concentrations are given in figure legends.

Mathematical analysis of the data was performed using the Origin 6.1 software package (Microcal Software, USA).

RESULTS AND DISCUSSION

Since PGHS catalyzes the cyclooxygenase and peroxidase reactions only as the holoenzyme (complexed with hemin) [7], it is necessary to settle a question of addition of exogenous hemin to the reaction mixture. In the experiments concerning the dependence of the rate of the cyclooxygenase reaction of PGHS on the concentration of hemin, it has been determined that 83% of solubilized PGHS preparation is in the form of holoenzyme, and 17% as the apoenzyme. In the present work, we normally used solubilized PGHS preparation without addition of exogenous hemin. Cases when exogenous hemin was added to the reaction mixture are noted in the figure legends.

The increase in TMPD concentration up to a certain value increases the rate of the cyclooxygenase reaction, but further increase decreases it (Fig. 1). Stimulation of the cyclooxygenase activity at low concentrations and inhibition at high concentrations is described for many donors: MK-447 [15], phenol [16], guaiacol [27], acetaminophen [28], dimethylphenylenediamine [27], and methylphenylsulfide [29]. However, inhibition of the cyclooxygenase reaction by electron donor at high concentrations does not occur for all donors: L-adrenalin, ferrocyanide, and ABTS do not display this property over a large concentration range (Fig. 2). Therefore, the ability to inhibit the cyclooxygenase reaction is not an essential feature of all electron donors, but it depends on their nature. Hypotheses about possible reasons for an inhibitory effect of the electron donors on the cyclooxygenase reaction are presented in the literature. First, it can be associated with the fact that the presence of the donors in solution results in non-enzymatic depletion of peroxide [27], which is an activator for cyclooxygenase. Second, the donors can interact with radicals, including tyrosyl radical, the presence of which in the active site of cyclooxygenase is necessary for catalysis of the cyclooxygenase reaction [8]. Third, the donors can competitively interact with the substrate-binding site of cyclooxygenase [30]. It is also possible that the effect results from a combination of these reasons.

It was found (data not shown) that the addition of hydrogen peroxide at the concentration of 140 μM does

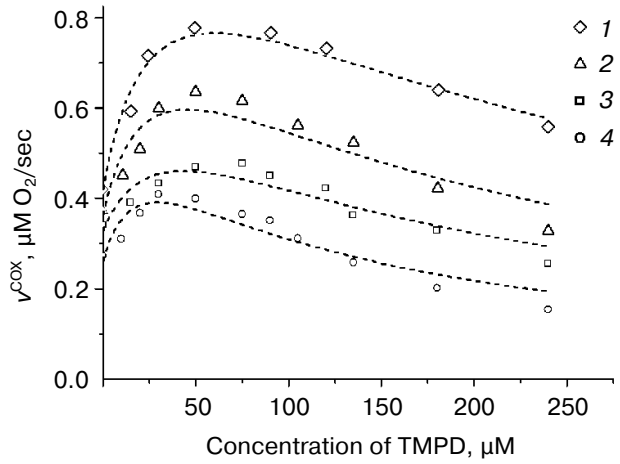


Fig. 1. Dependence of initial rate of cyclooxygenase reaction on the concentration of TMPD at 180, 100, 75, and 40 μM of arachidonic acid (*1-4*, respectively). Conditions: see "Materials and Methods". Dashed lines were plotted using approximation of the experimental data according to Eq. (9), and the respective parameter values are given in the table.

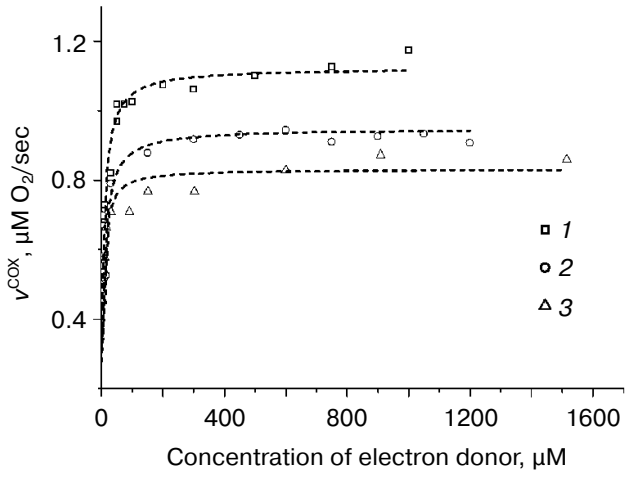


Fig. 2. Dependence of initial rate of cyclooxygenase reaction on the concentration of electron donors. Conditions: see "Materials and Methods". The concentration of arachidonic acid is 100 μM and hemin 2 μM. Dashed lines were plotted using approximation of experimental data according to Eq. (9). *1*) ABTS, $K_3 = 0$, $V_1/V_2 = 2.2$, $K_1/K_2 = 28$, $V_2 = 0.58$; *2*) L-adrenalin, $K_3 = 0$, $V_1/V_2 = 1.6$, $K_1/K_2 = 3$, $V_2 = 0.52$; *3*) ferrocyanide, $K_3 = 0$, $V_1/V_2 = 1.8$, $K_1/K_2 = 15$, $V_2 = 0.5$.

not eliminate the inhibitory effect of TMPD. This means that the hypothesis about the deficit of peroxide (an activator of cyclooxygenase) at the high concentrations of electron donor does not explain the experimental data in this case. Another fact that also indicates that there is no shortage of peroxide in the described experiments is that there was no lag period observed on the kinetic curves (see further Fig. 4) (upon removal of peroxide from the reaction mixture by addition of glutathione peroxidase and reduced glutathione a lag period was observed on the kinetic curves for oxygen consumption [8]).

From the hypothesis about completely non-competitive interaction of TMPD with the PGHS active site in regard to arachidonic acid, it follows that (upon decrease in the concentration of tyrosyl radical) the change in the concentration of arachidonic acid should not influence the pattern of TMPD inhibitory effect. However, in practice increase in the concentration of arachidonic acid leads to maximal reaction rate being reached at higher TMPD concentrations (Fig. 1). Therefore, the hypothesis about pure non-competitive inhibition is inconsistent.

Hence, for explanation of the inhibitory effect of high concentrations of electron donor TMPD on the cyclooxygenase reaction of PGHS, it is necessary to take into account the possibility of competitive (with regard to AA) interaction of TMPD with the binding site for the substrate of the cyclooxygenase reaction.

The dependence of the initial rate of the cyclooxygenase reaction on the concentration of arachidonic acid (upon fixed concentration of TMPD) in double reciprocal coordinates is shown in Fig. 3. At given values of TMPD concentration inhibition of cyclooxygenase reaction is observed (see Fig. 1). If pure non-competitive inhibition took place, intersection of the X-axis would have been observed, which does not correspond to the experimental data. Therefore, the fact of intersection in the top right quadrant (Fig. 3) confirms the assumption concerning the character of the interaction of the TMPD molecule with the binding site for the substrate of the cyclooxygenase reaction. It is seen from the figure that the dependence is linear in the double reciprocal coordinates. Based on this fact it can be concluded that the mechanism of enzyme interaction with arachidonic acid can be described in terms of the Michaelis model.

The effect of an inhibitor of the cyclooxygenase reaction (naproxen) on the course of the cyclooxygenase and peroxidase reactions is illustrated in Fig. 4. Naproxen is a non-steroidal anti-inflammatory drug and is a rapid, reversible inhibitor of the cyclooxygenase reaction of PGHS [3]. As seen from Fig. 4a, the addition of naproxen causes virtually complete inhibition of the cyclooxygenase activity of PGHS. The presence of naproxen during the peroxidase reaction has no effect on the initial rate (Fig. 4b), but decreases the constant of PGHS inactivation in the course of the peroxidase reaction compared to the control value.

The presence of arachidonic acid (and arachidonic acid together with naproxen) during the peroxidase reaction has no effect on the initial rate and decreases the constant of inactivation of PGHS in the course of the peroxidase reaction compared to the control (Fig. 4b).

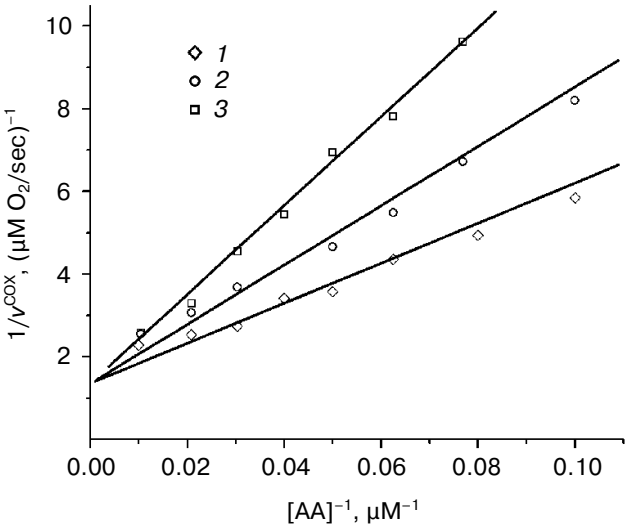


Fig. 3. Dependence of the initial rate of the cyclooxygenase reaction on the concentration of arachidonic acid with fixed concentrations of TMPD (60, 90, and 135 μM) (*1-3*, respectively) in double reciprocal coordinates. Conditions: see "Materials and Methods". Solid lines were plotted using approximation of experimental data according to Eq. (9): $K_1/K_2 = 2$, $V_1 = 0.75$, $V_2 = 0.22$, $K_3 = 1.3$, $K_4 = 0.44$.

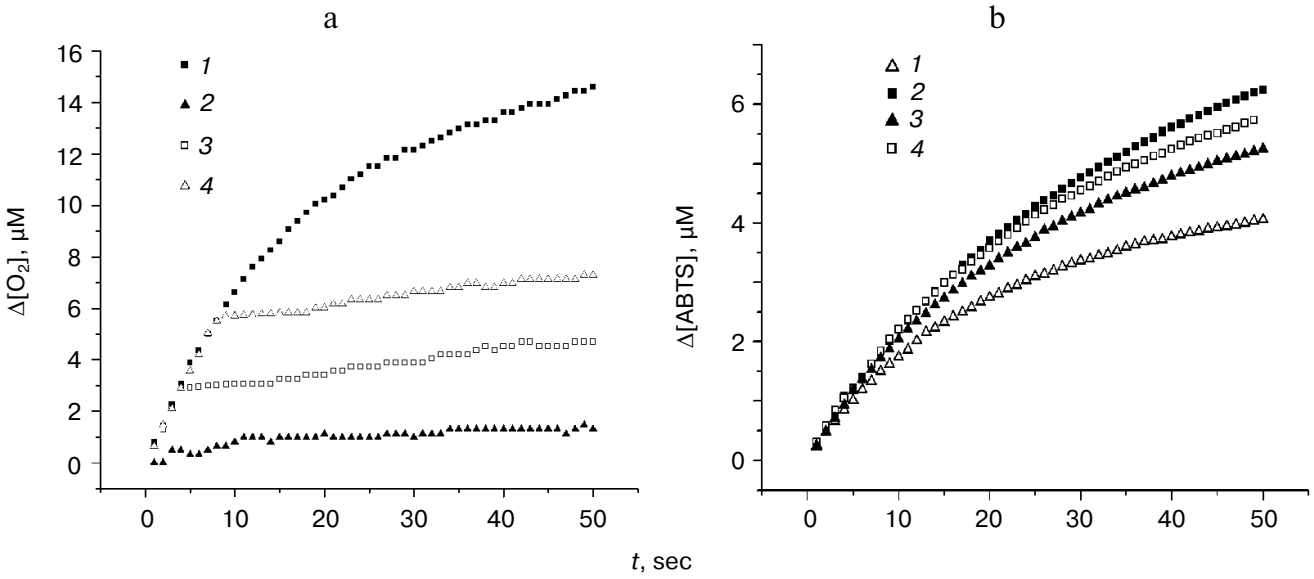
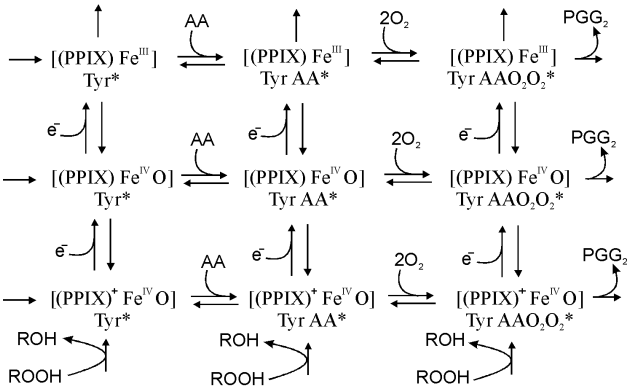


Fig. 4. Effect of naproxen on kinetics of the cyclooxygenase reaction (a) and peroxidase reaction in the presence of arachidonic acid (b). a: *1*) 750 μM ABTS, 150 μM AA, 2 μM hemin, 15 μg/ml PGHS (control); *2*) control + naproxen (1 mM, added *prior to* addition of enzyme); *3*) control + naproxen (1 mM, added 5 sec after the start of the reaction); *4*) control + naproxen (1 mM, added 10 sec after the start of the reaction). b: *1*) 750 μM ABTS, 1.5 mM H_2O_2, 5.4 μg/ml PGHS (control); *2*) control + arachidonic acid (150 μM, added before the addition of enzyme); *3*) control + naproxen (1 mM, added before the addition of enzyme); *4*) control + 150 μM arachidonic acid and 1 mM naproxen added before addition of the enzyme.

These results indicate that loading of the binding site of the cyclooxygenase reaction with arachidonic acid and inhibitor has practically no effect on the initial rate of the peroxidase reaction. The effect of naproxen and arachidonic acid on the enzyme inactivation constant in the course of the peroxidase reaction is not discussed within the scope of this work. Therefore, it can be concluded that the initial rate of the peroxidase reaction of PGHS does not depend on the extent of the cyclooxygenase reaction. In individual experiments, it has been demonstrated (data not shown) that along with the peroxidase reaction, upon the addition of arachidonic acid to the corresponding reaction mixture (see Fig. 4b) the cyclooxygenase reaction also takes place. The latter can be inhibited by 10-20% by hydrogen peroxide (compared to the cyclooxygenase reaction without hydrogen peroxide). These facts will be used for construction of a kinetic model.

Our experimental results as well as the data of other researchers require the following experimental results to be explained: the cyclooxygenase reaction occurs in the absence of electron donor; the rate of the cyclooxygenase reaction increases in the presence of electron donor; for some donors the increase transforms into inhibition. Inhibition of the cyclooxygenase reaction does not lead to a change in the rate of the peroxidase reaction of PGHS. The cyclooxygenase and peroxidase reactions occur simultaneously, and there is no clear evidence of competitive interactions between them.

Construction of kinetic models. As shown above, the existing schemes of branched-chain and tightly coupled mechanisms cannot explain all the experimental data known for this enzyme. Moreover, any kinetic model for PGHS that includes total material balance for all enzyme intermediates in the cyclooxygenase and peroxidase reaction will be characterized by competition between the two activities, i.e. inhibition of the cyclooxygenase reaction in the course of the peroxidase reaction and vice versa. However, as shown above, there is clear evidence of quite the opposite: the rate of the cyclooxygenase reaction is significantly increased in the presence of electron donor. It becomes obvious that the models with total material balance are not principally suitable here; kinetic models for PGHS should take into account that the two reactions occur independently, which however does not exclude their influence on each other. Based on theoretical elaboration of kinetic models for bifunctional enzyme [31, 32] the kinetic model for PGHS providing for the two independent reactions within the same enzyme molecule, but not postulating the absence of mutual influence of the reactions, is suggested in the present work (Scheme 3). In the process of enzyme activation by peroxide, the formation of tyrosyl radical in a quantity sufficient for the cyclooxygenase reaction occurs rapidly (1-2 sec after the beginning of the reaction) [33]. The formation of tyrosyl radical is not illustrated in Scheme 3 because we consider the enzyme mechanism in a stationary approach. The correctness of this approach is



Putative scheme of prostaglandin-H-synthase catalyzed reaction

Scheme 3

confirmed by the absence of the lag period in our experiments.

As a result of cycle completion in the vertical direction (Scheme 3) there is one turn of the peroxidase reaction; as a result of the cycle completion in the horizontal direction there is one turn of the cyclooxygenase reaction. The principal difference of this scheme from all other schemes suggested previously is that the material balance is considered separately for all possible enzyme intermediates, and the reactions can occur via different pathways.

The dependence of the initial rate of the cyclooxygenase reaction on the concentration of electron donor in a stationary mode is expressed for this scheme as follows [32]:

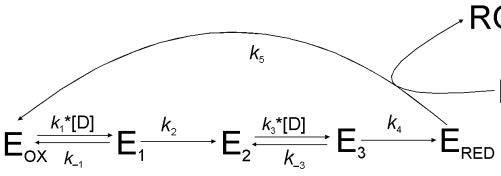
$$v^{COX} = \frac{e_0 + e_1[D] + e_2[D]^2 + e_3[D]^3 + e_4[D]^4}{d_0 + d_1[D] + d_2[D]^2 + d_3[D]^3 + d_4[D]^4}, \quad (1)$$

where [D] is the concentration of electron donor; constants e_i and d_i are expressed through the constants of elementary steps and concentrations of other components of the reaction, where $e_i \neq 0$ $(0 \leq i \leq 4)$, $d_i \neq 0$ $(0 \leq i \leq 4)$.

Under certain ratios between the constants e_i and d_i the dependence presented by the above formula describes the stimulation of the cyclooxygenase reaction with increase in the concentration of electron donors. Scheme 3 does not contradict the fact of accumulation of prostaglandin G_2 in the reaction mixture. Thus, this approach describes the known pattern of PGHS kinetics better than the branched-chain or tightly coupled mechanisms. In principle, formula (1) can describe not only the increase in the rate of the cyclooxygenase reaction under low concentrations of electron donor, but also its decrease under higher concentrations. However, as already mentioned, the ability to inhibit the cyclooxygenase reaction depends on the nature of electron donor and is not inherent for all donors. Therefore, this phenomenon should be described separately.

Scheme 3 explains all known experimental data. However, it is fairly complex for analysis. We will perform the quantitative study of the scheme using experimentally valid simplifications. Let us suppose that the course of the cyclooxygenase reaction has no effect on the rate of the peroxidase reaction. This is confirmed by the literature data [34, 35] and experimental results obtained by us (see Fig. 4).

Assuming that the rate of the cyclooxygenase reaction catalyzed by the enzyme depends on the state of an active site of the peroxidase reaction, let us consider the following scheme of peroxidase reaction:



(2)

Here and further D denotes the electron donor TMPD; E characterizes the state on a peroxidase active site: E_{RED} – $[(PPIX)Fe^{III}]$, E_{OX} – $[(PPIX^*)^+Fe^{IV}O]$, E_1 and E_3 denote enzyme–substrate complexes with electron donor: E_1 – $[(PPIX^*)^+Fe^{IV}O]D$, E_3 – $[(PPIX)Fe^{IV}O]D$, and E_2 denotes semireduced form of the enzyme $[(PPIX)Fe^{IV}O]$. It is envisaged that each of the enzyme intermediates is able to perform the cyclooxygenase catalysis.

Let us calculate the stationary concentrations for each of the enzyme intermediate forms involved in the peroxidase reaction. Let us divide the enzyme intermediates in two groups based on how their concentration depends on the concentration of electron donors. Then E_{OX} and E_2 will fall into one group, and E_{RED}, E_1, and E_3 into another. Having introduced the following designations:

$$[E^1] = [E_1] + [E_3] + [E_{RED}], \quad (3)$$

$$[E^2] = [E_{OX}] + [E_2], \quad (4)$$

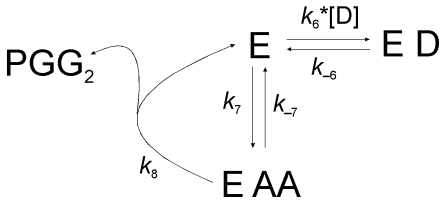
we get:

$$[E^1] = [E_0]\frac{K_2[D]}{K_1 + K_2[D]}, \quad (5)$$

$$[E^2] = [E_0]\frac{K_1}{K_1 + K_2[D]}, \quad (6)$$

where constants K_1 and K_2 are expressed through the rates of elementary steps of the peroxidase reaction.

For the cyclooxygenase reaction, considering that the electron donor can competitively interact with the binding site for arachidonic acid (7),



(7)

we introduce the following abbreviations: E is the enzyme molecule with tyrosyl radical, v^{COX} is the observed stationary rate of the cyclooxygenase reaction, v^1 is stationary rate of the cyclooxygenase reaction catalyzed by enzyme forms belonging to the first group, v^2 is the stationary rate of cyclooxygenase reaction catalyzed by enzyme forms belonging to the second group. In this case:

$$v^{cox} = v^1 + v^2 . \quad (8)$$

Let us assume that the rates of cyclooxygenase catalysis are equal for the enzyme forms belonging to one group. Also, that the quasiequilibrium approximation is fulfilled, i.e. that $k_8 << k_{-7}$ for each form of the enzyme, and all the rates of the cyclooxygenase reaction for each state of the enzyme are equal (except for k_8 that determines the rate of accumulation of prostaglandin G_2). Upper indexes 1 and 2 will be assigned to the constants k_8 for the first and second enzyme groups, respectively. Then:

$$v^{cox} = \frac{K_1V_2 + K_2V_1[D]}{K_1 + K_2[D]} \frac{[AA]}{[AA] + K_3(1 + K_4[D])} , \quad (9)$$

where

$$V_1 = k_8^1[E_0] , \quad V_2 = k_8^2[E_0] , \quad K_3 = \frac{k_{-7}}{k_7} , \quad K_4 = \frac{k_6}{k_{-6}} . \quad (10)$$

According to Eq. (9), the dependence of the initial rate of the cyclooxygenase reaction on the concentration of arachidonic acid is linear in double reciprocal coordinates, which is in agreement with the experimental data (Fig. 3).

Equation (9) contains six coordinates. For quantitative estimation of their values, a large number of experimental data (some of them are shown in Fig. 1) was approximated according to the formula using nonlinear regression. To diminish the risk of finding a local minimum, the initial values of constants were varied during approximation over a large range, and the same result was obtained. This indicates that Eq. (9) is not contradictory to the obtained experimental data and describes them very well under given values of constants (Figs. 1-3). This confirms the correctness of our assumptions concerning the mechanism of TMPD effect on the cyclooxygenase reaction. The results of approximation are given in the table.

Average values and standard deviations for the constants V_1, V_2, K_1, K_2, K_3, K_4 calculated based on the treatment of experimental data concerning the dependence of initial rate of the cyclooxygenase reaction on the concentrations of TMPD and arachidonic acid according to Eq. (9)

Constant	Units of measurement	Average value	Standard deviation
V_1	μM/sec	0.66	0.15
V_2	μM/sec	0.22	0.05
K_1	$\mu M^{-2}\cdot sec^{-4}$	12.00	3.00
K_2	$\mu M^{-3}\cdot sec^{-4}$	0.81	0.14
K_3	μM	1.30	0.30
K_4	μM^{-1}	0.44	0.08

In the present work, the kinetic scheme for PGHS catalysis envisages the occurrence of two independent enzymatic reactions within one enzyme molecule, accompanied by the mutual effect of the reactions on each other. The experimental data are described by the simplified model that takes into account the increase in cyclooxygenase activity of PGHS during the peroxidase reduction of the heme group by an electron donor.

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