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Cyclooxygenase and Peroxidase Inactivation of Prostaglandin-H-Synthase during Catalysis

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Abstract—Prostaglandin-H-synthase (PGHS) is a bifunctional enzyme catalyzing cyclooxygenase and peroxidase reactions and undergoing irreversible inactivation during catalysis. A new method for kinetic studies of both PGHS activities in the course of cyclooxygenase as well as peroxidase reactions and also preincubation with hydroperoxides is suggested. It is shown that peroxidase activity is retained after complete cyclooxygenase inactivation and cyclooxygenase activity is retained after complete peroxidase inactivation. Two-stage cyclooxygenase inactivation occurs on preincubation of PGHS with hydrogen peroxide. Studies on inactivation under various conditions indicate that chemical mechanisms of cyclooxygenase and peroxidase inactivation are different. The data allow development of kinetic models.

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Prostaglandin-H-synthase (PGHS, EC 1.14.99.1) catalyzes conversion of arachidonic acid into prostaglandin H_2, which is a parent compound in biosynthesis of all prostaglandins, thromboxane, and prostacyclin in most mammals including humans [1, 2]. PGHS has two active sites for catalyzing cyclooxygenase (oxidation of arachidonic acid to prostaglandin G_2 [3]) and peroxidase (reduction of the peroxide group of prostaglandin G_2 to hydroxyl group [4]) reactions, respectively. Prostaglandin H_2 is formed as a result of two reactions [5]; it can be further converted into other physiologically active compounds such as prostaglandins E_2, D_2, $F_{2\alpha}$, thromboxane A_2, and prostacyclin [1, 2, 6, 7] by the action of specific enzymes – convertases.

In solution, PGHS exists as a dimer composed of two equal subunits with molecular mass 72 kD [8]. Each PGHS subunit binds a hemin molecule and catalyses cyclooxygenase and peroxidase reactions in a complex with hemin [9]. As shown by kinetic [10] and crystallographic [11] studies, cyclooxygenase and peroxidase active sites in PGHS subunit are spatially separated and located on each side of the heme group plane. In mammals, prostaglandin-H-synthase is persistently in cells, providing their normal functioning; this isoform was named PGHS-1 [12]. Another isoform (PGHS-2) is produced in response on certain stimulants such as cytokines and growth factors [13]. In this work, we studied the properties of PGHS-1 from sheep vesicular gland.

The interrelations between the two PGHS activities are rather complex: the cyclooxygenase reaction does not proceed in the absence of peroxides [14], whereas the peroxidase reaction proceeds in the presence as well as in the absence of arachidonic acid and also in the presence of cyclooxygenase inhibitors [4, 15].

Free radicals were detected by EPR in the reaction mixture in the course of cyclooxygenase and peroxidase reactions [16]. The tyrosine radical formed in the peroxidase reaction initiates the cyclooxygenase reaction [17]: replacement of Tyr385 by Phe results in complete inactivation of the cyclooxygenase [18]. The branched-chain mechanism of PGHS reactivity is based on these data [19].

Both PGHS reactions are accompanied by fast irreversible inactivation of the enzyme [16, 20, 21]. So far the mechanism of PGHS inactivation has not been elucidated.

Abbreviations: AA) arachidonic acid; ABTS) 2,2′-azino-di-3-ethyl-benzotiazoline-6-sulfonic acid; DEDTC) diethyldithiocarbamate; PGG_2) prostaglandin G_2; PGHS) prostaglandin-H-synthase; PPIX) protoporphyrin IX; TMPD) N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

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PGHS cyclooxygenase inactivation is described by the first-order kinetic equation in relation to the active enzyme concentration [21]. As reported in [22], only the holoenzyme is inactivated during the cyclooxygenase reaction. It is suggested that the rate constant of PGHS cyclooxygenase inactivation depends on the nature of the fatty acid (arachidonic, eicosapentaenoic) – substrate for the cyclooxygenase reaction [20, 23].

Peroxidase inactivation is supposed to be a result of reactions involving one of the states of the heme group [24, 25]. The studies of spectral properties of hemin in the enzyme composition during PGHS incubation with ethylhydroperoxide in the absence of electron donor showed [26] that the rate constant of peroxidase inactivation is comparable with the rate constant of transfer of the suggested enzyme state [(PPIX)Fe^{4+}O]Tyr* (the state of heme group and tyrosine residue is shown, the unpaired electron is designated by the asterisk) into some state [(PPIX)Fe^{4+}O]X*$^{+}$, where X is an unknown amino acid residue. The hemin molecules isolated from the inactivated enzyme were chemically unchanged [26]. The rate of holoenzyme destruction monitored via the changes of PGHS optical absorption spectrum was five times lower and the rate of hemin concentration decrease under the cyclooxygenase reaction conditions determined by the hemochromogenic method was seven times lower than the rate of cyclooxygenase inactivation under the same conditions [14]. Consequently, decreased hemin concentration is most probably not the origin of inactivation but its consequence.

EPR studies demonstrated that peroxidase inactivation is accompanied by changes in the environmental structure of tyrosyl radical, although the properties and significance of these changes are unknown [27].

Some data [28] indicate that byproducts of oxidation of arachidonic acid may be the origin of PGHS inactivation; these byproducts are present in the reaction mixture during the cyclooxygenase reaction and can covalently bind to the enzyme molecule.

If nonsteroid antiinflammatory preparations inhibiting PGHS cyclooxygenase activity are added into the peroxidase reaction mixture, this has practically no effect on the initial rate of the peroxidase reaction [4, 15] and only negligibly decreases the rate of peroxidase inactivation in the course of the reaction [15].

The rate constant of peroxidase inactivation during the PGHS peroxidase reaction is reported to increase on increased peroxidase concentration [24]. On pre-incubation with peroxides, their nature does not effect the rate constant of peroxidase inactivation, which is on the average 24 min^{-1} at 24°C [26].

Inactivation of PGHS in whose molecule the iron atom of protoporphyrin is replaced with a manganese atom (Mn-PGHS) has been studied [4]. Mn-PGHS is also inactivated in the course of the peroxidase and cyclooxygenase reactions. Similar to PGHS, Mn-PGHS inactivation is a first-order reaction; the rate constant of Mn-PGHS peroxidase inactivation is one order of magnitude lower than that of PGHS and is independent of the nature of the peroxide [4].

On the whole, PGHS inactivation was studied as inactivation in the course of reaction of the same name; for example, inactivation of cyclooxygenase was monitored during the cyclooxygenase reaction. However, it would be worthwhile to study the effect of the cyclooxygenase reaction on the peroxidase activity and vice versa. Only once using the purified PGHS preparation it was shown that more than 60% of PGHS peroxidase activity was retained after the complete loss of cyclooxygenase activity [25]; the data suggest that cyclooxygenase and peroxidase inactivation are different processes.

Studies of PGHS inactivation in the course of catalysis are not systematic and do not allow unambiguous conclusions. Correlation between peroxidase and cyclooxygenase inactivation is still not elucidated.

MATERIALS AND METHODS

Reagents. In this study we used Tris, bis-Tris, Tween-20, N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), 2,2′-azino-di-3-ethyl-benzotiazoline-6-sulfonic acid (ABTS), EDTA, and diethyldithiocarbamate (DEDTC) from MP Biomedicals Inc. (Germany); potassium ferrocyanide from Merck (Germany); arachidonic acid (AA) and hydrogen peroxide from Sigma-Aldrich (USA); DEAE-Sepharose fast flow from Amersham Biosciences (Austria). Other reagents were of extra pure grade and of Russian production.

Isolation and purification of PGHS. Sheep vesicular glands were obtained from the Rus breeding farm (Moscow Region). Solubilized preparation of prostaglandin-H-synthase from washed microsome of sheep vesicular gland was obtained as described in [29] but with minor changes: in order to avoid significant enzyme loss, Tween-20 was not added to buffer solution for washing of microsomes. Solubilized enzyme preparation was in the following buffer: 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1 mM DEDTC, and 2% (v/v) Tween-20 (that is, there are 20 ml of Tween-20 in 1000 ml of buffer). The solubilized preparation was purified chromatographically. A column (13 × 1.5 cm) with DEAE-Sepharose fast flow was equilibrated with the following buffer: 10 mM Tris, 10 mM bis-Tris, 0.1 mM EDTA, 0.1 mM DEDTC, 0.1% (v/v) Tween-20, pH 8.0 (buffer 1). Solubilized PGHS preparation (25-50 mg) was applied onto the column and washed with buffer 1. Then the column was washed with buffer 2 (salt content as in buffer 1 but pH 6.5). The enzyme was eluted with a linear gradient of buffer 2 and buffer 3 (buffer 2 containing 150 mM NaCl). The active fractions eluted at 30-60 mM NaCl were pooled, and the purified PGHS preparation was thus obtained.

In kinetic experiment, we used a standard buffer (50 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween-20), which is usually used for PGHS kinetic studies [17, 30]. All kinetic experiments were performed at 25°C. Solutions of 150 mM arachidonic acid (AA), 60 mM TMPD, and 2.5 M *tert*-butylperoxide in ethanol were added into the reaction mixture. Solutions of 0.1 M potassium ferrocyanide, 75 mM ABTS, and 0.5-1.2 M hydrogen peroxide were prepared in standard buffer. A weight of hemin was dissolved in small volume of 0.1 M NaOH and then 20 times diluted with standard buffer. To determine concentration of this solution, its aliquot was mixed with 20% pyridine solution in 0.1 M NaOH with addition of sodium dithionite. Hemin concentration was determined spectrophotometrically using the known molar extinction coefficients for reduced hemin–pyridine complex (ε_{418} = 191,500, ε_{526} = 17,500, and ε_{557} = 34,400 $M^{-1}\cdot cm^{-1}$) [31].

PGHS peroxidase reaction. Enzyme was added to a solution of hydrogen peroxide (1.5 mM) and donor of electrons (60 μM TMPD or 1.5 mM potassium ferrocyanide) in standard buffer, and the complete kinetic curve of electron donor oxidation was recorded. The final concentrations of compounds in the reaction mixture are given.

For TMPD, the stoichiometric equation is as follows:

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

The PGHS peroxidase reaction was monitored spectrophotometrically via accumulation of the oxidized forms of TMPD or potassium ferrocyanide using a Cary 100 spectrophotometer from Varian Inc. (USA). For TMPD, λ = 611 nm, ε = 13,500 $M^{-1}\cdot cm^{-1}$ for the oxidized TMPD molecule [32]; for potassium ferrocyanide, λ = 420 nm, ε = 1040 $M^{-1}\cdot cm^{-1}$ for the oxidized potassium ferrocyanide molecule [33]. Extinction coefficients are given for a 2 : 1 ratio of electron donor molecule to hydrogen peroxide molecules. Volume of reaction mixture was 2.5 ml.

PGHS cyclooxygenase reaction. The cyclooxygenase reaction was monitored via change in concentration of dissolved oxygen according to equation:

$$AA + 2O_2 \rightarrow PGG_2.$$

Changes in concentration of dissolved oxygen (solution contains 270 μM oxygen in equilibrium with atmospheric oxygen at 25°C) were monitored amperometrically using a AKPM-02-05 polarograph from Alpha BASSENS (Russia) supplied with gas-diffusion platinum-silver Clark electrode [34]. Enzyme was added to a solution of arachidonic acid (150 μM) in standard buffer in the polarograph cell. The complete kinetic curve of consumption of dissolved oxygen was recorded. Volume of the reaction mixture was 3 ml.

Cyclooxygenation reaction in the presence of electron donor. Enzyme was added to solution of arachidonic acid (150 μM) and ABTS (750 μM) in standard buffer in the polarograph cell (3 ml). Changes in dissolved oxygen concentration were monitored polarographically as described above.

Processing of kinetic curves. The rates of cyclooxygenase and peroxidase reactions were determined via the slopes of kinetic curves. Changes in substrate concentrations during these reactions were not more than 10%, that is, significant decrease in the rate was only due mostly to inactivation. The relative activity A_{rel} equal to the present rate divided by the initial rate was used as a quantitative characteristic of the cyclooxygenase and peroxidase reactions.

Catalase activity. To determine catalase activity, enzyme was added to a solution of hydrogen peroxide (1.5 mM) in standard buffer. The complete kinetic curve of oxygen release was recorded using the polarograph as described above.

Peroxidase activity of PGHS incubated under the cyclooxygenase reaction conditions. *Incubation in the absence of electron donor.* Enzyme and 150 μM arachidonic acid were incubated in a spectrophotometer cuvette (2.5 ml) for a given time period, then 1.5 mM hydrogen peroxide and electron donor (60 μM TMPD or 750 μM ABTS) were added simultaneously and the peroxidase reaction was monitored spectrophotometrically as described above. For ABTS, λ = 405 nm, ε = 36,800 $M^{-1}\cdot cm^{-1}$ for the oxidized ABTS molecule and ABTS/H_2O_2 ratio = 2 : 1 [33]. The initial rate of peroxidase reaction was determined as change in slope of the kinetic curve after addition of substrates (hydrogen peroxide and electron donor).

Incubation in the presence of electron donor. Enzyme, 150 μM arachidonic acid and electron donor (750 μM ABTS) were incubated in a spectrophotometer cuvette (2.5 ml) for a given time period, then 1.5 mM hydrogen peroxide was added and the peroxidase reaction was monitored spectrophotometrically as described above. The initial rate of the peroxidase reaction was determined after addition of hydrogen peroxide as described above.

To calculate the relative peroxidase activity after proceeding of the cyclooxygenase reaction, the initial rate of the peroxidase reaction was divided by the initial rate of the same reaction under the same conditions but without incubation, that is, when enzyme was the last to be added (relative activity B_{rel}).

Cyclooxygenase activity of PGHS incubated under the peroxidase reaction conditions. Purified enzyme preparation, electron donor (60 μM TMPD or 1.5 mM potassium ferrocyanide), 1.5 mM hydrogen peroxide, and 2 μM hemin were incubated in the polarograph cell (3 ml) for a given time period, then 150 μM arachidonic acid was added, and change in concentration of dissolved oxygen was monitored polarographically as described above. The

initial rate of the cyclooxygenase reaction was determined as the change in slope of the kinetic reaction curve after addition of arachidonic acid.

To calculate the relative cyclooxygenase activity after proceeding of the peroxidase reaction, the initial rate of the cyclooxygenase reaction was divided by the initial rate of the same reaction under the same conditions but without incubation, that is, when enzyme was the last to be added (relative activity B_{rel}).

PGHS preincubation with hydrogen peroxide. *Peroxidase reaction*. Enzyme and 1.5 mM hydrogen peroxide were incubated in the spectrophotometer cuvette (2.5 ml) for a given time period, then electron donor (60 μM TMPD or 750 μM ABTS) was added and the peroxidase reaction was monitored spectrophotometrically as described above.

Cyclooxygenase reaction. Purified PGHS, 1.5 mM hydrogen peroxide, and 2 μM hemin were incubated in the polarograph cell (3 ml) for a given time period, then 150 μM arachidonic acid was added and change in concentration of dissolved oxygen was monitored as described above. In indicated cases, electron donor (1 mM potassium ferrocyanide) was added together with arachidonic acid. Analogously, enzyme was preincubated with 5 mM *tert*-butylperoxide.

To calculate cyclooxygenase and peroxidase activity after preincubation with peroxides, the initial reaction rate was divided by the initial rate obtained without incubation (relative activity C_{rel}). Concentrations at incubation and reaction monitoring differed by not more than 2% as a result of dilution with added reagents.

Protein concentration was determined according to Lowry [35]; enzyme concentration in the reaction mixture is given in the figure captions.

Data processing. Time dependence of A_{rel}, B_{rel}, and C_{rel} was approximated using the empirical equation, which governs enzyme inactivation in the course of the reaction [36, 37]:

$$V = V_\infty + V_1 e^{-\Lambda t}, \quad (1)$$

where V_∞ is a residual activity, V_1 is an increment of activity, and Λ is an experimentally determined constant of inactivation.

Choice of kinetic models was based on approximation by Eq. (1).

Approximation of data obtained for models of inactivation in the course of reaction (Eqs. (2) and (3)) and of inactivation during preincubation (Eqs. (4) and (5)) is presented in the figures:

$$A_{rel} = e^{-\alpha\lambda_1 t}, \quad (2)$$

$$B_{rel} = \frac{\alpha\lambda_1}{\alpha(\lambda_1+\lambda_2)-\lambda_3} e^{-\lambda_3 t} + \frac{\alpha\lambda_2-\lambda_3}{\alpha(\lambda_1+\lambda_2)-\lambda_3} e^{-\alpha(\lambda_1+\lambda_2)t}, \quad (3)$$

$$C_{rel}^{PO} = e^{-k_1 t}, \quad (4)$$

$$C_{rel}^{CO} = \frac{k_1 e^{-k_2 t} - k_2 e^{-k_1 t}}{k_1 - k_2}, \quad (5)$$

where k_i and λ_i are experimentally determined constants, α is a fraction of inactivated form of the enzyme, and indices PO and CO designate peroxidase and cyclooxygenase activities, respectively.

To avoid local minima of the sum of quadratic deviations, the initial parameter values were varied over a wide range; as a result, the solutions correspond with absolute minimum. Deviations of experimental data from theoretical approximation were tested by a plot of difference between experimental and theoretical value (Δ).

RESULTS AND DISCUSSION

Solubilized PGHS preparation had the following properties: the initial rate of the cyclooxygenase reaction in the presence of electron donor was 0.5 μM O_2/sec, that of catalase reaction – 1.1 μM O_2/sec at concentration of solubilized PGHS preparation 5 μg/ml and saturating concentration of substrates. Holoenzyme content in the preparation was 70%.

Purified PGHS preparation had the following properties: electrophoretic purity 95%, the initial rate of cyclooxygenase reaction in the presence of electron donor was 2 μM O_2/sec, that of catalase reaction – 0.04 μM O_2/sec at concentration of purified PGHS preparation 5 μg/ml and saturating concentrations of substrates. Holoenzyme content in the preparation was 35%.

To study mechanisms of PGHS cyclooxygenase and peroxidase inactivation, we compared the rates of PGHS cyclooxygenase inactivation caused by proceeding of the cyclooxygenase reaction (that is, inactivation in the course of the reaction) with the rate of PGHS peroxidase inactivation caused by proceeding of the cyclooxygenase reaction. And vise versa, we compared the rates of PGHS peroxidase inactivation caused by proceeding of the peroxidase reaction (that is, inactivation in the course of the reaction) with the rate on PGHS cyclooxygenase inactivation caused by proceeding of the peroxidase reaction. We also studied the effect of PGHS preincubation with hydrogen peroxide and *tert*-butylperoxide on PGHS cyclooxygenase and peroxidase activities.

Effect of PGHS incubation time on peroxidase and cyclooxygenase activities under cyclooxygenase reaction conditions. Enzyme was incubated in the presence of substrates for the cyclooxygenase reaction (arachidonic acid, dissolved oxygen) in standard buffer. To determine PGHS peroxidase activity, after the specified cyclooxygenase reaction time hydrogen peroxide and electron donor were

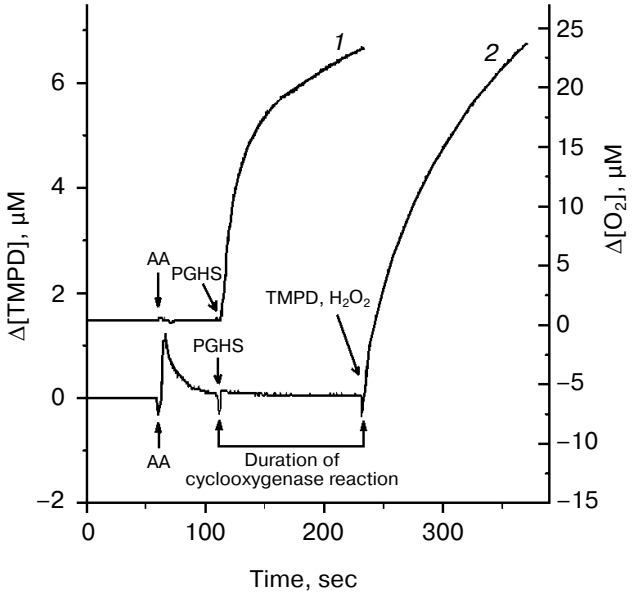

Fig. 1. Kinetics of change in oxygen concentration (curve *1*) and electron donor concentration (TMPD as an example) (curve *2*) during incubation of solubilized PGHS preparation with substrates for peroxidase and cyclooxygenase reactions. Curves: *1*) detection of oxygen concentration, 150 μM AA, 75 μg/ml PGHS; *2*) detection of oxidized TMPD concentration, 150 μM AA, 1.5 mM H_2O_2, 60 μM TMPD, 18 μg/ml PGHS. Moments of addition of reagents are shown by arrows.

simultaneously added into the same reaction mixture. Processes were monitored spectrophotometrically and polarographically. Change in oxygen concentration (Fig. 1, curve *1*), which corresponds to cyclooxygenase reaction after addition of PGHS, was monitored polarographically. Accumulation of the oxidized from of electron donor (Fig. 1, curve *2*) was monitored spectrophotometrically. As shown in Fig. 1, change in concentration of the oxidized form of electron donor due to the peroxidase reaction is observed after addition of electron donor and hydrogen peroxide. The initial rate of the peroxidase reaction was determined and B_{rel} (the relative activity) was used as a quantitative characteristic of the peroxide activity (see "Materials and Methods").

The relative cyclooxygenase activity (A_{rel}) as a function of duration of the cyclooxygenase reaction is presented in Fig. 2a. PGHS peroxidase activity estimated as described above versus time of enzyme incubation under the cyclooxygenase reaction conditions is also presented in Fig. 2a.

Each value of the relative peroxidase activity presented in Fig. 2a was obtained in a separate experiment exemplified in Fig. 1 (curve *2*). One set of experiments was performed using TMPD as electron donor, another – using ABTS.

As shown in Figs. 1 and 2a, when PGHS is incubated under the cyclooxygenase reaction conditions which it

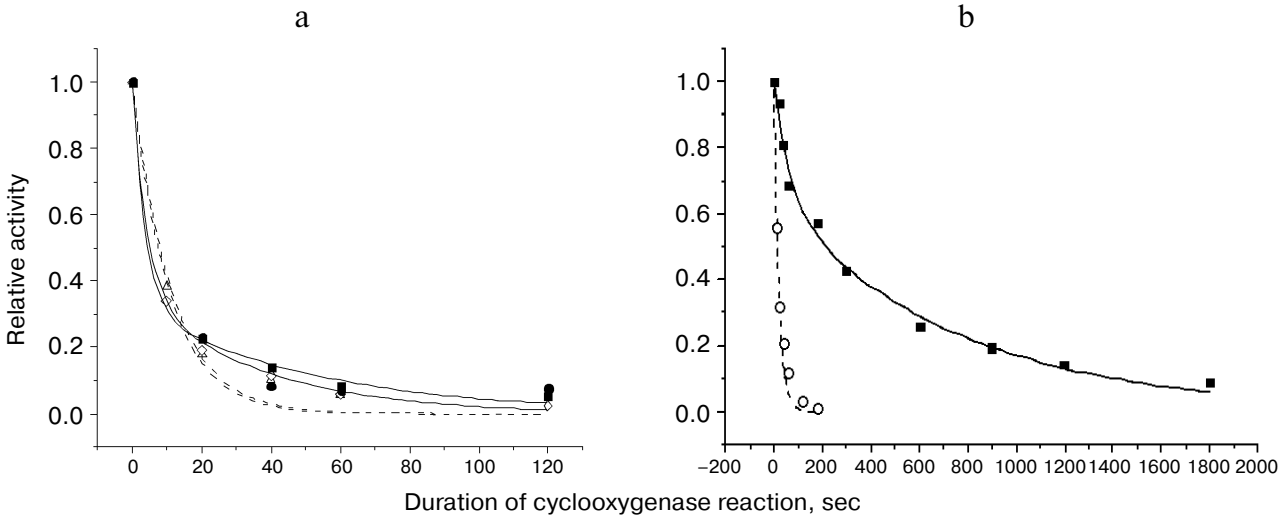

Fig. 2. a) Relative peroxidase activity (B_{rel}) (squares, circles) and relative cyclooxygenase activity (A_{rel}) (triangles, rhombuses) of solubilized PGHS preparation versus duration of cyclooxygenase reaction. Squares: 18 μg/ml PGHS, 150 μM AA, O_2. Peroxidase activity was detected after addition of 150 μM H_2O_2 and 60 μM TMPD into the reaction mixture. Circles: 9 μg/ml PGHS, 150 μM AA, O_2. Peroxidase activity was detected after addition of 150 μM H_2O_2 and 750 μM ABTS. Triangles: 45 μg/ml PGHS, 150 μM AA, O_2. Rhombuses: 75 μg/ml PGHS, 150 μM AA, O_2. Solid lines are plotted by approximation of data (squares, circles) using Eq. (3), dashed lines (triangles, rhombuses) – using Eq. (2). b) Relative peroxidase activity (B_{rel}) (squares) and relative cyclooxygenase activity (A_{rel}) (circles) of solubilized enzyme preparation versus duration of cyclooxygenase reaction in the presence of electron donor (ABTS). Squares: 9 μg/ml PGHS, 150 μM AA, O_2, 750 μM ABTS. Peroxidase activity was detected after addition of 150 μM H_2O_2 into the reaction mixture. Circles: 15 μg/ml PGHS, 150 μM AA, 750 μM ABTS, O_2. The solid line is plotted by approximation of data using Eq. (3), dashed line – using Eq. (2).

catalyzes, both cyclooxygenase and peroxidase inactivations are observed. The inactivation plots (Fig. 2a) look similar. Cyclooxygenase and peroxidase activities progressively decrease almost to zero with increased PGHS incubation time under the cyclooxygenase reaction conditions (Fig. 2a).

These data are well interpreted using empirical Eq. (1); the values of experimentally observed inactivation rate constants (Λ) were determined by this equation (approximations are not presented here). The inactivation rate constant of the peroxidase activity was 5 min^{-1} and that of the cyclooxygenase reaction in various experiments was 4.5-5.5 min^{-1} (Fig. 2a). The coincidence of these constants suggests that peroxidase inactivation of PGHS incubated under the cyclooxygenase reaction conditions and cyclooxygenase inactivation itself under the same conditions may have a common mechanism. Approximations presented in Fig. 2a are made according to Eqs. (2) and (3).

There was shown in separate experiments (Fig. 2a, triangles, rhombuses) that experimentally observed inactivation rate constants in the course of reaction and also experimentally observed inactivation rate constants under the alternative reaction conditions are independent of PGHS concentration. That is why the experimentally observed rate constants of peroxidase inactivation under the cyclooxygenase reaction conditions and that of cyclooxygenase inactivation can be compared.

The presence of electron donor in the reaction medium under cyclooxygenase catalysis conditions decreases the rate of cyclooxygenase inactivation [16]. To study peroxidase inactivation under the cyclooxygenase reaction conditions in the presence of electron donors, enzyme was incubated in the presence of substrates for the cyclooxygenase reaction (arachidonic acid, dissolved oxygen) and electron donors (ABTS) (Fig. 2b). The difference between this experiment and that presented in Fig. 2a is in PGHS incubation with arachidonic acid in the presence of electron donor. Enzyme was incubated under these conditions for a certain time, then H_2O_2 was added and the initial rate of peroxidase-catalyzed reduction of H_2O_2 was evaluated via change in the slope of the kinetic curve. The initial rate was considered as PGHS peroxidase activity in the given system. Data are presented in Fig. 2b.

Data (Fig. 2b) are well described by empirical Eq. (1) (approximation not presented here). The experimentally observed inactivation rate constant of PGHS peroxidase activity obtained by Eq. (1) is 0.2 min^{-1}, and experimentally observed inactivation rate constant of cyclooxygenase activity is 2.9 min^{-1} (Fig. 2b).

In contrast to the peroxidase activity of solubilized PGHS preparation incubated under the cyclooxygenase activity conditions without electron donor (Λ = 5 min^{-1}) (Fig. 2a), addition of electron donor during the cyclooxygenase reaction results in significant decrease in experimentally observed inactivation rate constant of PGHS peroxidase activity (Λ = 0.2 min^{-1}) (Fig. 2b). As also shown in Fig. 2b, peroxidase inactivation in the course of the cyclooxygenase reaction (Λ = 0.2 min^{-1}) is slower in the presence of electron donor than inactivation of the cyclooxygenase reaction (Λ = 2.9 min^{-1}) under the same conditions.

Electron donor also prevents cyclooxygenase inactivation in the course of the cyclooxygenase reaction. The experimentally observed inactivation rate constant is 4.5-5.5 min^{-1} in the absence of electron donor and 2.7-3.7 min^{-1} in the presence of electron donor (ABTS, potassium ferrocyanide, TMPD).

Figure 2b indicates that mechanisms of peroxidase and cyclooxygenase inactivation under the cyclooxygenase reaction conditions are different and that electron donor protects peroxidase activity from inactivation to a larger extent than cyclooxygenase activity.

Effect of PGHS incubation time on cyclooxygenase and peroxidase activity under the peroxidase reaction conditions. Enzyme was incubated in the presence of substrates for the peroxidase reaction (H_2O_2, electron donor). To evaluate the cyclooxygenase activity, arachidonic acid was added to the same reaction mixture after a prescribed peroxidase reaction time. The processes were monitored spectrophotometrically and polarographically. Change in concentration of the oxidized form of electron donor, which corresponds to the peroxidase reaction after addition of PGHS, was monitored spectrophotometrically. Change in oxygen concentration was monitored polarographically, and the initial rate of the cyclooxygenase reaction was determined. The relative activity (B_{rel}) was used as a quantitative characteristic of the cyclooxygenase activity (see "Materials and Methods"). The relative peroxidase activity (A_{rel}) as a function of peroxidase reaction time is presented in Fig. 3. PGHS cyclooxygenase activity evaluated as described above versus enzyme incubation time under the peroxidase reaction conditions is also presented in Fig. 3.

As follows from Fig. 3, both PGHS peroxidase and cyclooxygenase activity are inactivated during enzyme incubation under the peroxidase reaction conditions. However, cyclooxygenase inactivation is slower than peroxidase inactivation, and this is numerically proved by the inactivation rate constants.

The data (Fig. 3) are well described by empirical Eq. (1) (approximation not presented here). Experimentally observed inactivation rate constants were obtained for two electron donors. When TMPD was used as electron donor, the experimentally observed inactivation rate constant of PGHS cyclooxygenase activity obtained by Eq. (1) was 1.1-1.4 min^{-1} and that of peroxidase activity was 3.0-3.9 min^{-1} (Fig. 3). When potassium ferrocyanide was used as electron donor, the experimentally observed inactivation rate constant of PGHS cyclooxygenase activity obtained by Eq. (1) was 0.5-1.0 min^{-1} and that of peroxidase activity was 2.0-2.7 min^{-1} (Fig. 3).

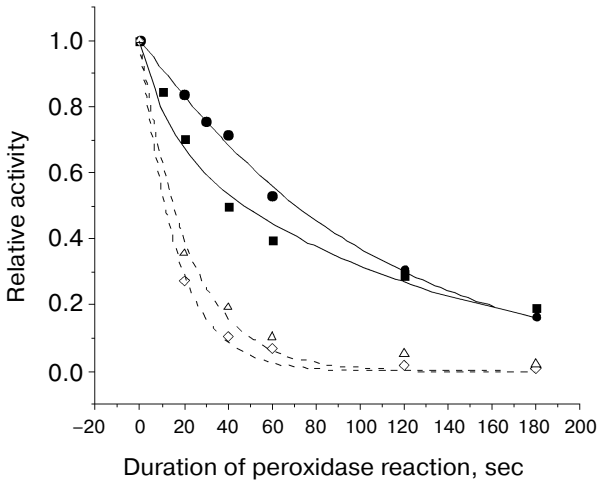

Fig. 3. Relative cyclooxygenase activity (B_{rel}) of purified (squares, circles) and the relative peroxidase activity (A_{rel}) of solubilized PGHS preparation (triangles, rhombuses) versus peroxidase reaction time. Squares: 5.3 μg/ml PGHS, 2 μM hemin, 60 μM TMPD, 150 μM H_2O_2. Cyclooxygenase activity was detected after addition of 150 μM AA into the reaction mixture. Circles: 5 μM PGHS, 2 μM hemin, 1.5 mM potassium ferrocyanide, 150 μM H_2O_2. Cyclooxygenase activity was detected after addition of 150 μM AA. Rhombuses: 18 μg/ml PGHS, 60 μM TMPD, 150 μM H_2O_2. Triangles: 216 μg/ml PGHS, 1.5 mM potassium ferrocyanide, 150 μM H_2O_2. Solid lines are plotted by approximation of data using Eq. (3), dashed lines – using Eq. (2).

As shown in Fig. 3, for the two electron donors used, cyclooxygenase inactivation during the peroxidase reaction is slower than peroxidase inactivation under the same conditions.

When potassium ferrocyanide is used, the inactivation rate constants of the peroxidase and cyclooxygenase reactions are lower than when TMPD is used. This indicates that the inactivation rate constants of peroxidase and cyclooxygenase reactions vary when various electron donors are used.

For this and some other experiments, purified PGHS preparation was used (see "Materials and Methods"). Solubilized PGHS preparation contains protein impurities possessing catalase activity. Catalase reaction results in oxygen release which hinders monitoring of cyclooxygenase activity in the presence of H_2O_2.

Earlier we showed [15] that the presence of arachidonic acid during the peroxidase reaction results in decrease in experimentally observed inactivation rate constant of peroxidase reaction (by 20-25%) and has practically no effect on the rate of the peroxidase reaction; consequently, peroxidase inactivation is not related with the presence of arachidonic acid.

The effect of duration of the cyclooxygenase reaction on the peroxidase activity presented in Fig. 2a was studied in the absence of exogenously added hemin. The experimentally observed inactivation rate constant was 5 min^{-1} when ABTS and TMPD were used as electron donors. We performed experiments with addition of exogenous hemin in the reaction mixture analogous to those presented in Fig. 2a. Experimentally observed inactivation rate constants of peroxidase activity of PGHS incubated under the cyclooxygenase reaction conditions in the presence of exogenously added hemin are as follows: $\Lambda = 5$ and 4.4 min^{-1} for ABTS and TMPD, respectively. These values are close to those obtained in the absence of exogenously added hemin; the dependences look the same. Consequently, the presence of exogenously added hemin in the reaction mixture does not effect the dependence characteristics of the peroxidase inactivation.

As shown in separate experiments, the presence of H_2O_2 in the cyclooxygenase reaction mixture with the purified PGHS preparation (both in the absence and in the presence of electron donor) decreases the initial rate of cyclooxygenase reaction by 15-20% and increases the inactivation rate constant of cyclooxygenase activity by 20-25%.

Effect of PGHS preincubation time with hydrogen peroxide and *tert*-butylperoxide on its peroxidase and cyclooxygenase activities. Hydrogen peroxide is a substrate for the PGHS peroxidase reaction. In several experiments (Fig. 3) enzyme, H_2O_2, and electron donor were incubated in buffer for a given time period. It is worthwhile to study how electron donor and H_2O_2 separately influence PGHS activity.

PGHS incubation with electron donor influences neither cyclooxygenase nor peroxidase activity (data not presented here). PGHS incubation with H_2O_2 results in progressive decrease in both cyclooxygenase and peroxidase activities (1.5 mM H_2O_2 was used for incubation with enzyme as well as for the peroxidase reaction) (Fig. 4).

Cyclooxygenase activity was detected by two methods: in the absence (Fig. 4, squares) and in the presence of electron donor (Fig. 4, rhombuses). The similar character of these dependences should be noted.

Data were approximated by empirical Eq. (1) (approximation not presented here). As a result, experimentally observed inactivation rate constants of cyclooxygenase (Λ = 0.7-1.1 min^{-1}) and peroxidase (Λ = 4-4.6 min^{-1}) activities of enzyme preincubated with H_2O_2 were obtained.

The data indicate that experimentally observed inactivation rate constant of the cyclooxygenase activity on preincubation with H_2O_2 is significantly lower than that of the peroxidase reaction.

Systematic deviations are observed (Fig. 5a) when the effect of H_2O_2 on cyclooxygenase activity (Fig. 4, rhombuses, squares) is described by Eq. (1). Using Eq. (5), a more adequate approximation of this dependence is obtained. For the last approximation, deviations are randomly distributed around the average zero value (Fig. 5b).

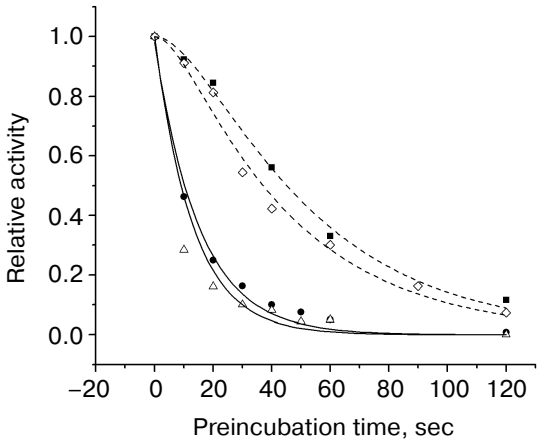

Fig. 4. Relative cyclooxygenase activity of purified (squares, rhombuses) and relative peroxidase activity of solubilized PGHS preparations (circles, triangles) (C_{rel}) versus preincubation time with H_2O_2. Squares: 6.7 μg/ml purified PGHS, 2 μM hemin, 1.5 mM H_2O_2. Cyclooxygenase activity was detected after addition of 150 μM AA into the reaction mixture. Rhombuses: 4.7 μg/ml purified PGHS, 2 μM hemin, 1.5 mM H_2O_2. Cyclooxygenase activity was detected after addition of 150 μM AA and 1 mM potassium ferrocyanide into the reaction mixture. Circles: 9 μg/ml solubilized PGHS, 1.5 mM H_2O_2. Peroxidase activity was detected after addition of 60 μM TMPD into the reaction mixture. Triangles: 9 μg/ml solubilized PGHS, 1.5 mM H_2O_2. Peroxidase activity was detected after addition of 750 μM ABTS into the reaction mixture. Solid lines are plotted by approximation of data using Eq. (4), dashed lines – using Eq. (5).

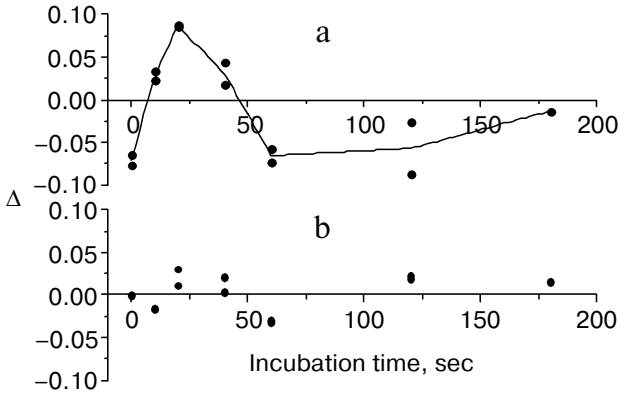

Fig. 5. Difference between experimental values of the relative rate of cyclooxygenase reaction obtained on PGHS preincubation with H_2O_2 and theoretical values obtained by approximation (Δ). Approximation made by Eq. (1) (a) and Eq. (5) (b). Conditions see in Fig. 4 caption (rhombuses).

We also studied the effect of PGHS preincubation time with 5 mM *tert*-butylperoxide (this concentration corresponds to 50% saturation for the peroxidase reaction) on the inactivation rate constants of the cyclooxygenase and peroxidase reactions. Approximating the data by Eq. (1), experimentally observed inactivation rate constants of cyclooxygenase (4.8 min^{-1}) and peroxidase (5 min^{-1}) activities were obtained. On preincubation with hydrogen peroxide, these values obtained by Eq. (1) were 0.7-1.1 and 4.0-4.6 min^{-1}, respectively. For PGHS cyclooxygenase activity, the data are well approximated by Eq. (1) on preincubation with *tert*-butylperoxide (in contrast to incubation with H_2O_2). The inactivation rate constants of peroxidase activity are similar for *tert*-butylperoxide and H_2O_2; in case of the cyclooxygenase reaction, these values differ.

Data approximation by Eq. (1) allows comparison of the rates of various processes and suggestion of kinetic mechanisms.

As shown in Figs. 2-4, in some cases experimentally observed inactivation rate constants of the cyclooxygenase and peroxidase activities significantly differ. A plot presented in Fig. 2a shows a similar inactivation mode of cyclooxygenase and peroxidase. When experimental conditions are changed (electron donor is added), inactivation modes differ from each other. After complete cyclooxygenase inactivation, 70% of the peroxidase activity is retained (Fig. 2b). After complete peroxidase inactivation, 30-40% of the cyclooxygenase activity is retained (Fig. 3). The nature of peroxide with which enzyme is preincubated effects the inactivation rate constant of the cyclooxygenase reaction but does not effect the inactivation rate constant of the peroxidase reaction. The fact that it is impossible to describe the process of cyclooxygenase inactivation at preincubation with H_2O_2 by one exponent (Fig. 4) (systematic deviations are presented in Fig. 5) indicates that the mechanism of cyclooxygenase inactivation involves at least two sequential inactivation stages. All these facts indicate that the peroxidase and cyclooxygenase inactivation mechanisms are different. Since free PGHS is not inactivated, inactivation occurs during reaction, thus it obviously proceeds via intermediates. Phenomenological description of dependences by Eq. (1) made in this study gives grounds for development of kinetic models explaining these phenomena.

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