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Molecular Oxygen (a Substrate of the Cyclooxygenase Reaction) in the Kinetic Mechanism of the Bifunctional Enzyme Prostaglandin-H-synthase

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Abstract—Prostaglandin-H-synthase is a bifunctional enzyme catalyzing conversion of arachidonic acid into prostaglandin H_2 as a result of cyclooxygenase and peroxidase reactions. The dependence of the rate of the cyclooxygenase reaction on oxygen concentration in the absence and in the presence of electron donor was determined. A two-dimensional kinetic scheme accounting for independent proceeding and mutual influence of the cyclooxygenase and peroxidase reactions and also for hierarchy of the rates of these reactions was used as a model. In the context of this model, it was shown that there are irreversible stages in the mechanism of the cyclooxygenase reaction between points of substrate donation (between donation of arachidonic acid and the first oxygen molecule and also between donation of two oxygen molecules).

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Key words: prostaglandin-H-synthase, bifunctional enzyme, cyclooxygenase activity, kinetic mechanism, oxygen, arachidonic acid, adrenaline

Prostaglandin-H-synthase (PGHS, EC 1.14.99.1) participates in the first step of biosynthesis of all prostaglandins and also thromboxane and prostacyclin, catalyzing conversion of arachidonic acid to prostaglandin H_2. Prostaglandins are modulators of cardiovascular, digestive, excretory, and reproductive systems; they are mediators of pain, inflammation, temperature increase, and allergy. That is why the enzyme catalyzing their biosynthesis is a very important pharmacological target [1, 2]. The therapeutic effect of all nonsteroidal anti-inflammatory preparations is due to their inhibition of PGHS [3, 4].

PGHS is a bifunctional enzyme catalyzing two reactions: cyclooxygenase oxidation of arachidonic acid by two oxygen molecules with formation of prostaglandin G_2 (PGG_2) and peroxidase reduction of the peroxide group of PGG_2 in the presence of an electron donor with formation of prostaglandin H_2 (PGH_2) [5]:

$$AA + 2O_2 \rightarrow PGG_2, \quad (1)$$

$$PGG_2 + 2DH \rightarrow PGH_2 + 2D + H_2O. \quad (2)$$

The two reactions proceed on one protein molecule and on two closely positioned active sites. Both reactions are heme-dependent and are accompanied by irreversible enzyme inactivation in the course of reaction [6].

Along with arachidonic acid, two oxygen molecules take part in cyclooxygenase reaction (1). However, studies on the effect of concentration of this substrate on the enzymatic reaction are practically absent. The Michaelis constant of the cyclooxygenase reaction for oxygen (5 μM) has been evaluated [7]. Judging from the results [7], this value does not depend on the concentration of arachidonic acid. Sensitivity of conversion of labeled

Abbreviations: AA) arachidonic acid; D) oxidized form of electron donor; DH) reduced form of electron donor; PGG_2) prostaglandin G_2; PGH_2) prostaglandin H_2; PGHS) prostaglandin-H-synthase; PP) protoporphyrin IX; ROOH) peroxide; Tyr) tyrosine residue.

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arachidonic acid into PGH_2 per minute to concentration of solubilized oxygen was studied in [8]; based on these studies, the authors evaluated the Michaelis constant as 10 μM.

These data are obviously insufficient for determination of the kinetic mechanism of enzymatic reaction in the case of such a complex object as PGHS. For a multisubstrate reaction catalyzed by a bifunctional enzyme, the concept of the Michaelis constant is conditional in itself, because this constant always depends in a complex manner on concentration of other participants of the reaction. It is necessary to study dependence of the rate of the cyclooxygenase reaction on concentration of its substrate, oxygen, and to determine the effect of varied concentrations of other substrates of PGHS-catalyzed reactions on these dependencies.

The goal of this work was to study the effect of concentration of solubilized molecular oxygen on the kinetics of the cyclooxygenase oxidation of arachidonic acid at varied concentrations of arachidonic acid and various electron donors and also development of a kinetic model of PGHS functioning.

All self-consistent PGHS kinetic models known to date include common material balance of all enzyme intermediates participating in the cyclooxygenase and peroxidase reactions [9, 10]; that is, suppression of the cyclooxygenase reaction when the peroxidase reaction proceeds and vice versa, suppression of the peroxidase reaction when the cyclooxygenase reaction proceeds. However, there are clear evidences for the contrary—the rate of the cyclooxygenase reaction significantly increases in the presence of electron donor [11, 12]. It is evident that models with a common material balance are in principle not applicable here.

PGHS is a bifunctional enzyme, and we have suggested [13] a two-dimensional kinetic scheme for bifunctional enzymes, which provides independent proceeding of two reactions, and their mutual influence is not excluded. Application of this model to PGHS can explain the experimental dependencies, but the dependencies of the reaction rates on concentrations are very complex expressions involving fractionally-rational functions.

Relative simplicity of the dependencies of the cyclooxygenase reaction rate on concentration of substrates and qualitative retention of these dependencies in the presence of electron donor (substrate of peroxidase reaction) as well as in the absence of electron donor are typical of results presented in this work. As shown [14], such results can be obtained based on a two-dimensional scheme [13] accounting for the hierarchy of rates of separate elementary reactions comprising the reaction mechanism.

Application of reported results [14] for PGHS allowed reduction of a general two-dimensional scheme of action of the bifunctional enzyme and simplification of expressions for the rates of enzymatic reaction. These results allowed description of such phenomena as deviation of the cyclooxygenase reaction from Michaelis–Menten kinetics, increase in the rate of the cyclooxygenase reaction in the presence of electron donor, linear dependencies in double reciprocal coordinates, dependence of the Michaelis constant for oxygen (the cyclooxygenase reaction) on concentration of electron donor (substrate of the peroxidase reaction), and some other experimental facts.

MATERIALS AND METHODS

The following reagents were used in this study: tris(hydroxymethyl)methylamine, Tween-20, L-adrenaline, and diethyldithiocarbamate from MP Biomedicals Inc. (Germany); potassium ferrocyanide from Merck (Germany); arachidonic acid, hemin, and hydrogen peroxide from Sigma-Aldrich (USA). Other reagents were of extra pure grade of Russian production.

Prostaglandin-H-synthase (PGHS) in the form of solubilized preparation was isolated form microsome of sheep vesicular glands obtained from the Rus breeding farm (Moscow Region) according to [15] but with minor changes. Enzyme preparation was in the following buffer: Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.1 mM diethyldithiocarbamic acid sodium salt trihydrate (DEDTC), 1% (v/v) Tween-20 (that is, there are 10 ml of Tween-20 in 1000 ml of buffer solution).

In all experiments on evaluation of the cyclooxygenase enzymatic activity we used the following standard buffer (usual when studying PGHS kinetics [16-18]): 50 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween-20. The enzymatic reaction was performed in a thermostatted polarographic cell at 25°C. Reagents were added in a buffer solution, and the reaction was initiated by enzyme addition. Arachidonic acid was added in the reaction mixture as solution in ethanol (10-150 mM), and 0.1 M adrenaline was prepared by its dissolution in 0.1 M HCl. Potassium ferrocyanide (0.1 M) was prepared in standard buffer. A weight of hemin was dissolved in a small volume of 0.1 M NaOH and then 20 times diluted with standard buffer. To determine the concentration of this solution, an 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 [19].

The cyclooxygenase reaction was monitored amperometrically via changes in concentration of dissolved oxygen using an Ekotest-120/ATC oxygen analyzer from Ekoniks (Russia) supplied with gas-diffusion platinum-silver Clark electrode [20]. This method provides a complete kinetic curve in real time. The volume of the reaction mixture was 3.3 ml and that of the added enzyme solution 10 μl (with protein concentration 4.5 mg/ml).

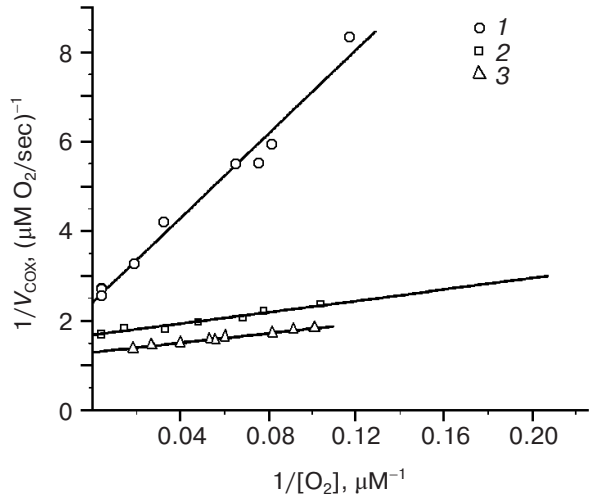


Fig. 1. Rate of the cyclooxygenase reaction versus oxygen concentration in double-reciprocal coordinates without electron donor (*1*) and in the presence of 1 mM potassium ferrocyanide (*2*) or 1 mM adrenaline (*3*). Conditions: standard buffer solution, 2 μM hemin, 33 μM arachidonic acid (AA). Linear approximation of experimental data is shown by solid lines.

In order to decrease concentration of dissolved oxygen in the reaction mixture it was partly replaced by argon. For this, 8 ml of standard buffer solution containing hemin and adrenaline (or potassium ferrocyanide) at certain concentrations was placed in a round-bottom flask 100 ml in volume supplied with magnetic stirrer; the gas phase was removed using a water pump, and an argon atmosphere was supplied. This cycle (evacuation–filling with argon) was repeated several times. The polarographic cell was filled with this mixture, and the reaction was performed as described above.

The rate of the cyclooxygenase reaction was determined as the slope of the integral curve of oxygen absorption at the initial moment.

Data were processed using the Origin 6.1 package from Microcal Software (USA).

RESULTS

Effect of electron donor type on the dependence of the cyclooxygenase reaction rate on oxygen concentration. The rates of PGHS-catalyzed cyclooxygenase reaction versus oxygen concentration under various conditions in double-reciprocal coordinates are presented in Fig. 1. In the first approximation, the dependence is linear using as electron donor both L-adrenaline (organic electron donor) and potassium ferrocyanide (inorganic electron donor) as well as in the absence of electron donor.

It should be noted that the presence of electron donor in the reaction mixture affects the slope of the curve, the Michaelis constant, and the maximal rate of the cyclooxygenase reaction (Fig. 1), although electron donor does not directly participate in the cyclooxygenase reaction but participates in PGG_2 reduction in the course of the peroxidase reaction.

Dependence of the cyclooxygenase reaction rate on oxygen concentration at various concentrations of arachidonic acid. We performed a series of experiments in which the concentrations of the two substrates of the cyclooxygenase reaction (oxygen and arachidonic acid) were varied. The conditions were as follows: the absence of electron

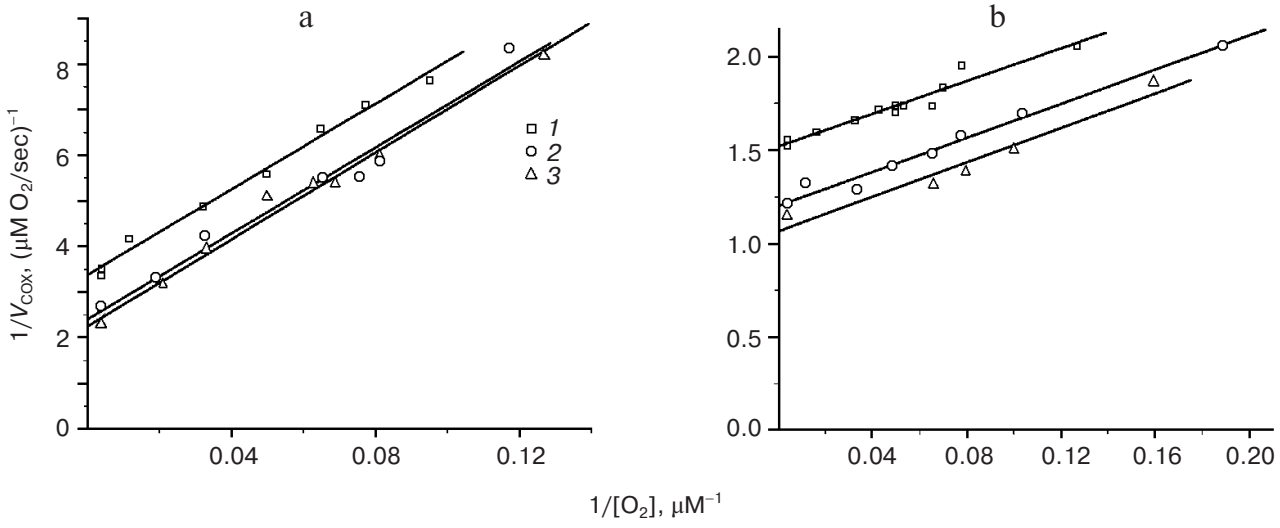


Fig. 2. Rate of the cyclooxygenase reaction versus oxygen concentration in double-reciprocal coordinates at various AA concentrations in the absence of electron donor (a) and in the presence of electron donor (potassium ferrocyanide) (b). Conditions: standard buffer solution, 2 μM hemin, 1 μM ferrocyanide (only (b)). Concentrations of AA (μM): *1*) 10; *2*) 33; *3*) 100. Linear approximation of the experimental data is shown by solid lines.

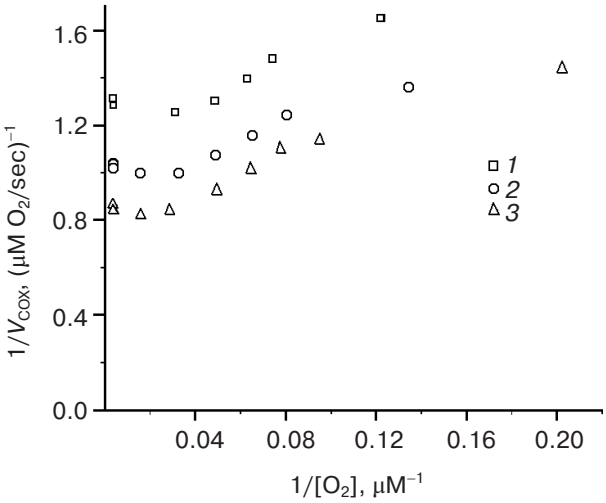


Fig. 3. Rate of the cyclooxygenase reaction versus oxygen concentration in double-reciprocal coordinates at various AA concentrations in the presence of electron donor (adrenaline). Conditions: standard buffer solution, 2 μM hemin, 1 mM adrenaline. Concentrations of AA (μM): *1*) 10; *2*) 33; *3*) 100.

donor in the reaction mixture, and the presence of organic (adrenaline) or inorganic (potassium ferrocyanide) electron donor. In the absence of electron donor in the reaction mixture, the dependence of the cyclooxygenase reaction rate on oxygen concentration at various arachidonic acid concentrations in double reciprocal coordinates is in first approximation a series of parallel lines (Fig. 2a). The presence of inorganic electron donor (potassium ferrocyanide) in the reaction mixture influenced the slopes of the lines but their parallelism is retained (Fig. 2b).

Analogous experiments with adrenaline as electron donor showed that the dependence of the rate on oxygen concentration in double-reciprocal coordinates can be considered as linear only in a first approximation. In the range of oxygen concentrations from 30 to 270 μM a slight (10% maximal rate) but reproducible deviation from linearity is observed (Fig. 3). However, the distance between curves remains constant. The maximal rate is observed at 50 μM oxygen. However, in the presence of potassium ferrocyanide and in the absence of electron donor such deviations were not observed. These deviations probably caused by the properties of adrenaline are not typical of the dependence of the cyclooxygenase reaction rate on oxygen concentration.

Dependence of the cyclooxygenase reaction rate on oxygen concentration at various adrenaline concentrations. Fixing concentration of arachidonic acid, we studied how the dependence of the cyclooxygenase reaction rate on oxygen concentration changes in double-reciprocal coordinates when adrenaline concentration is varied (Fig. 4). As a result of linear approximation of the experimental data, we obtained a bundle of lines intersecting in a single point of the second quadrant.

The nonlinear portion of the kinetic curve in double-reciprocal coordinates, which is noticeable at adrenaline concentration 1 mM (Fig. 3), disappears at its decreased concentration (Fig. 4).

Dependence of the cyclooxygenase reaction rate on concentration of arachidonic acid in the presence and in the absence of electron donor. The rate of the PGHS cyclooxygenase reaction versus concentration of arachidonic acid in double-reciprocal coordinates is presented in Fig. 5. We observed nonlinear dependence both at low

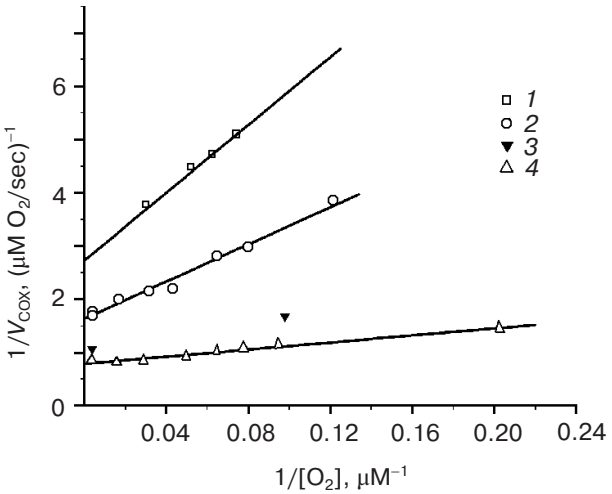


Fig. 4. Rate of the cyclooxygenase reaction versus oxygen concentration in double-reciprocal coordinates at various concentrations of electron donor (adrenaline). Conditions: standard buffer solution, 2 μM hemin, 100 μM AA. Concentrations of adrenaline (μM): *1*) 0; *2*) 30; *3*) 200; *4*) 1000. Linear approximation of the experimental data is shown by solid lines.

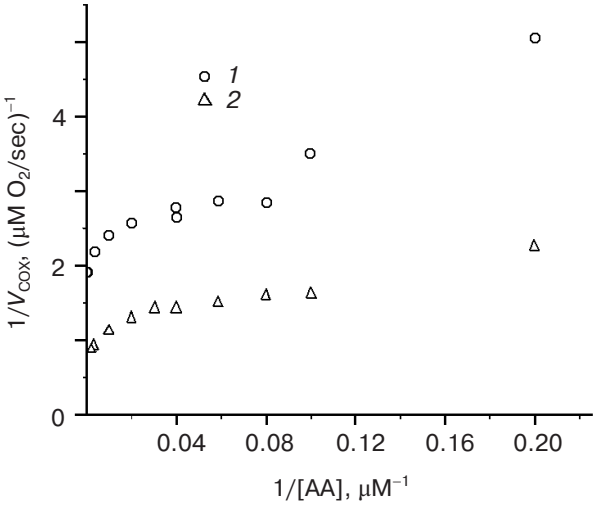


Fig. 5. Rate of the cyclooxygenase reaction versus concentration of arachidonic acid in double-reciprocal coordinates in the absence and in the presence of electron donor. Conditions: standard buffer solution, 2 μM hemin, 260 μM O_2. Concentrations of adrenaline (μM): *1*) 0; *2*) 1000.

(data not presented here) and high concentrations; this indicates that the reaction kinetics are rather complex. Such results are obtained in the presence as well as in the absence of electron donor, the distance between curves being constant in the range of AA concentration from 10 to 300 μM (Fig. 5).

DEVELOPMENT OF THE KINETIC MODEL

In the simplest case, enzyme kinetics are governed by the Michaelis–Menten equation, and the dependence of the reaction rate on substrate concentration is linear in double-reciprocal coordinates. For bifunctional multisubstrate enzymes, this linearity may be violated: quadratic, S-shaped, or some other dependence may be observed in double reciprocal coordinates. As shown in Fig. 1, the dependence of the cyclooxygenase reaction rate on oxygen concentration in double reciprocal coordinates is in a first approximation linear. Linearity is retained when various electron donors (organic (adrenaline) and inorganic (potassium ferrocyanide)) are used and also in the absence of electron donor; however, the slopes, the apparent Michaelis constant for oxygen, and the maximal rate of the cyclooxygenase reaction are changed. Thus, it is shown that the proceeding of the peroxidase reaction caused by the presence of electron donor does not influence the general view of the kinetic curves of the cyclooxygenase reaction, but changes their quantitative parameters. This is true for the complete set of our data. If the dependence is linear in the presence of electron donor, it remains linear also in the absence of the latter (Fig. 1). If deviation from linearity is observed in the presence of electron donor, it remains so also in the absence of the latter (Fig. 5).

So, the kinetic model of functioning of prostaglandin-H-synthase should fulfill the following requirements:

– account for independent proceeding and mutual influence of the cyclooxygenase and peroxidase reactions. Qualitatively kinetic properties of a model of the cyclooxygenase oxidation of arachidonic acid should be the same in the presence as well as in the absence of electron donor;

– suggest relatively simple theoretical dependencies of the rate of the cyclooxygenase reaction on concentration of substrates retaining their general view both in the absence and in the presence of the peroxidase reaction;

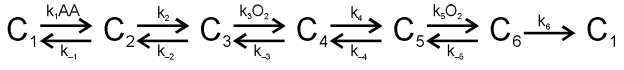
– agree with the following set of experimental data: (i) dependence of the cyclooxygenase reaction rate on oxygen concentration in the absence of electron donor is linear in double-reciprocal coordinates (Fig. 1), the slope remaining constant on varying concentration of arachidonic acid (Fig. 2a); (ii) dependence of the cyclooxygenase reaction rate on oxygen concentration in the presence of electron donor is linear in double-reciprocal coordinates (Fig. 1), the slope remaining constant on varying concentration of arachidonic acid (Fig. 2b); (iii) on varying electron donor concentration, linear dependencies of the rate of the cyclooxygenase reaction on oxygen concentration in double-reciprocal coordinates form a bundle of lines intersecting in a single point of the second quadrant (Fig. 4); (iv) in double-reciprocal coordinates, the distance between curves presenting the dependence of the cyclooxygenase reaction rate on arachidonic acid concentration in the presence and in the absence of electron donor remains constant (Fig. 5).

The two-dimensional kinetic scheme suggested in [13] for bifunctional enzymes such as PGHS completely satisfies all the above-mentioned requirements but results in rather complicated expressions. However, the use of such a two-dimensional scheme is the only possible way of adequate description of the kinetics of any bifunctional enzyme. The solution of the problem is in simplification of the general scheme, accounting for the real properties of a certain enzyme.

First, let us consider specific cases.

Case 1. Only the cyclooxygenase reaction proceeds, the peroxidase reaction does not proceed (electron donor is absent from the reaction mixture). Kinetic mechanism of the cyclooxygenase reaction should include the following stages [21] (see Scheme 1).

The state of tyrosine 385 (Tyr), which plays a key role in the enzyme functioning, changes in the course of the cyclooxygenase reaction [21]. Formation of tyrosine radical (Tyr*) during the peroxidase reaction is necessary for initiation of the cyclooxygenase reaction. That is why in Scheme 1 we designated the states of cyclooxygenase active site as follows: C_1 = [Tyr*] (enzyme form with radical at tyrosine 385), C_2 = [Tyr*]AA, C_3 = [Tyr]AA* (radical of arachidonic acid at the 11th position), C_4 = [Tyr]AAO_2* (11-peroxy-radical of arachidonic acid), C_5 = [Tyr]AAO_2 (radical of endoperoxide of arachidonic acid at the 15th position), C_6 = [Tyr]AAO_2O_2* (15-peroxy-radical of PGG_2 at the 15th position), k_i are rate constants. For this scheme, calculation of the cyclooxygenase reaction rate in a steady state yielded quadratic dependence of the rate on oxygen concentration in double-reciprocal coordinates (A1). However, parameters of the parabola depended on arachidonic acid concentration. These facts are in contradiction with real experimental dependencies, which are a set of parallel lines in a first approximation (Fig. 2a). Agreement between experiment and theory is attained if we suggest that there are irreversible stages between the donation points of substrates



Cyclooxygenase reaction

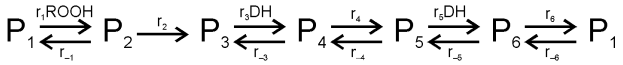
Scheme 1

[22]. In this case, constants k_{-2} and k_{-4} in Scheme 1 are equal to zero, and theoretical and observed dependencies of the cyclooxygenase reaction rate on oxygen concentration agree well with each other (A2). Equality of constant k_{-4} to zero provides linearity of rate dependence on oxygen concentration in double-reciprocal coordinates, and equality of constant k_{-2} to zero provides parallelism of these lines.

Case 2. Only the peroxidase reaction proceeds, the cyclooxygenase reaction does not proceed (peroxide and electron donor—substrates of the peroxidase reaction—are present in the reaction mixture, while arachidonic acid is absent). The kinetic mechanism of the peroxidase reaction should include the following main stages [21] (see Scheme 2).

In the course of the peroxidase reaction, the oxidation state of iron, a constituent of the heme, changes ($Fe^{3+} \rightarrow Fe^{4+}$); heme is a necessary element (cofactor) for catalytic activity of PGHS [21]. At one of the stages of the peroxidase reaction, a radical is formed on the protoporphyrin ring of the heme ($PP \rightarrow PP^*$) [21]. That is why in Scheme 2 we designated the states of the peroxidase active site as follows: $P_1 = [(PP)Fe^{3+}]$, $P_2 = [(PP)Fe^{3+}]ROOH$, $P_3 = [(PP^*)^+Fe^{4+}O]$, $P_4 = [(PP^*)^+Fe^{4+}O]DH$, $P_5 = [(PP)Fe^{4+}O]$, $P_6 = [(PP)Fe^{4+}O]DH$, r_j are rate constants. For this scheme, calculation of the peroxidase reaction rate in a steady state yielded quadratic dependence of the rate on concentration of electron donor in double-reciprocal coordinates (A3). However, parameters of the parabola depended on peroxide concentration [ROOH]. These facts are in contradiction with observed dependencies, which are a set of lines parallel in a first approximation [23]. Agreement between experiment and theory is attained, if we suggest that there are irreversible stages between the donation points of substrates [22]. In this case, constants r_{-4} and r_{-6} in Scheme 2 are equal to zero, and theoretical and observed dependencies of the peroxidase reaction rate on electron donor concentration agree well with each other (A4). Equality of constant r_{-4} to zero provides linearity of rate dependence on oxygen concentration in double-reciprocal coordinates, and equality of constant r_{-6} to zero provides parallelism of these lines.

We considered two cases when PGHS exhibits only one of its activities and concluded that some stages of cyclooxygenase and peroxidase reactions are irreversible. One can suggest that the mechanism of each reaction (reversibility–irreversibility) will be retained also in the case of their simultaneous proceeding.



Peroxidase reaction

Scheme 2

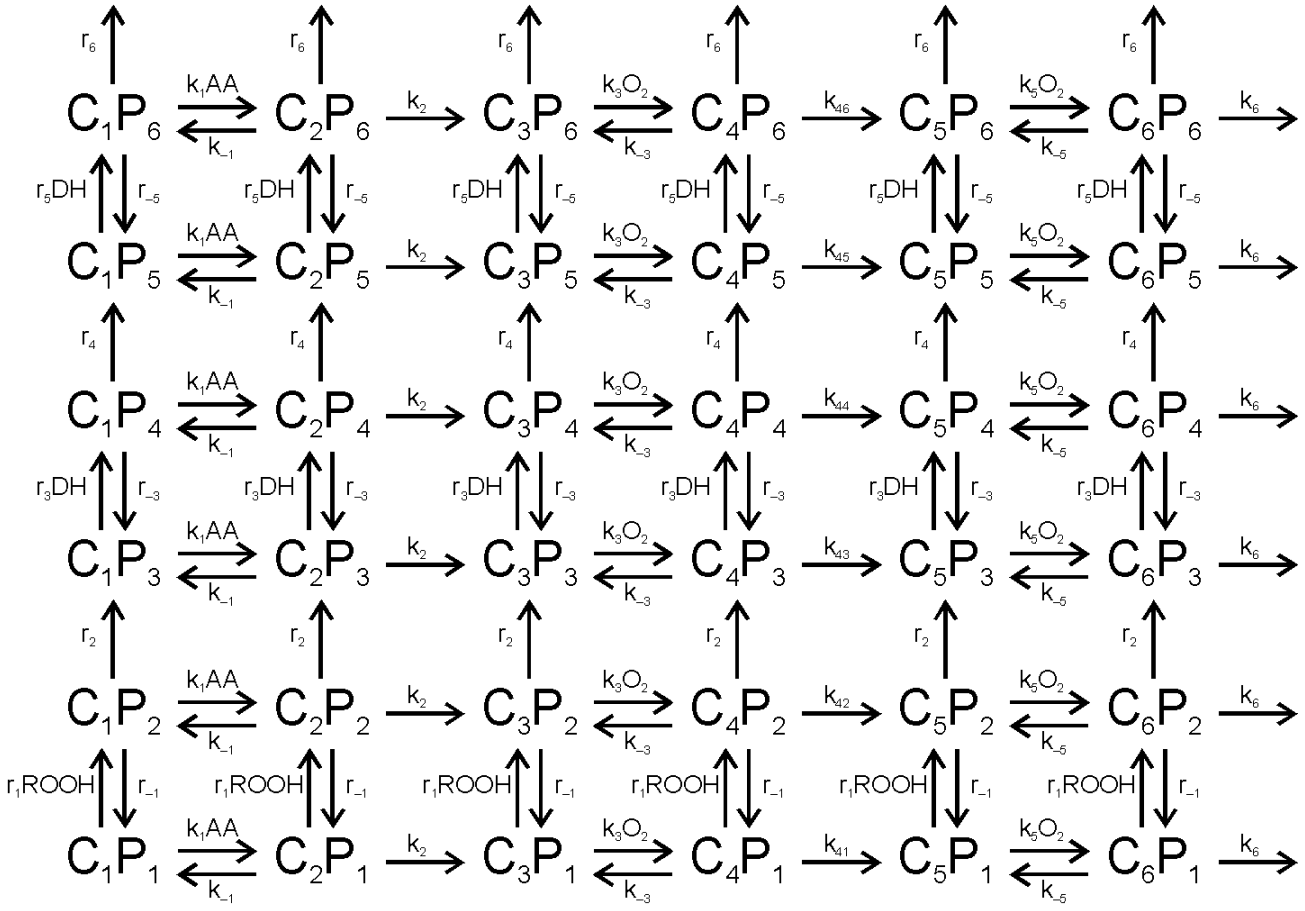
Let us consider this case: the enzyme catalyzes proceeding of both reactions; then accounting for the above formulated conclusions about irreversibility of some stages, a two-dimensional scheme for the bifunctional enzyme [13] will look as follows as applied to PGHS (see Scheme 3). Designations suggested earlier (Schemes 1 and 2) are retained, but the stage of two active sites (cyclooxygenase and peroxidase) on one enzyme molecule is presented simultaneously.

The effect of the cyclooxygenase reaction on the peroxidase reaction can be neglected, because the peroxidase reaction proceeds in the absence of arachidonic acid or in the presence of cyclooxygenase inhibitors. Addition of nonsteroidal anti-inflammatory preparations, which inhibit PGHS cyclooxygenase activity into the reaction mixture of the peroxidase reaction, has practically no influence on the initial rate of the peroxidase reaction [24, 25] and only negligibly decreases the rate of peroxidase inactivation in the course of the reaction [25]. Then the rate constants of elementary stages of the peroxidase reaction r_i do not depend on the state of the active site of the cyclooxygenase reaction as accounted in Scheme 3.

Since the presence of electron donor influences parameters of the cyclooxygenase reaction, the rate constants of elementary stages of the cyclooxygenase reaction should depend on the state of the peroxidase active site. For sake of simplicity, let us consider that the peroxidase reaction influences only one of six stages of the cyclooxygenase reaction presented in Scheme 1, for example, stage 4; that is, k_4 depends on the state of the active site of the peroxidase reaction, whereas k_1, k_2, k_3, k_5, and k_6 do not depend. That is why the second index accounting for the state of the peroxidase active site is ascribed for the rate constant of the fourth stage of the cyclooxygenase reaction (Scheme 3).

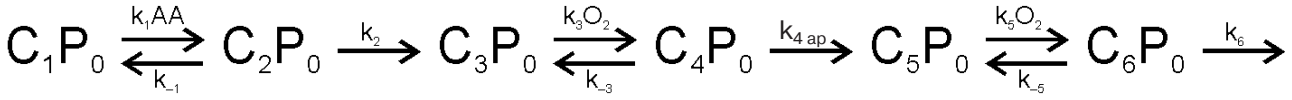
Literature data on PGHS kinetics [12, 26-28] and our data indicate that PGHS peroxidase activity significantly increases its cyclooxygenase activity, so one can suggest that a steady state establishing for intermediates of the peroxidase reaction is achieved rapidly as compared with the time of steady-state establishment for intermediates of the cyclooxygenase reaction and does not change in the course of the cyclooxygenase reaction. Then, according to [14], Scheme 3 can be simplified if all k_{4j} ($j = 1, ..., 6$) are changed for one constant $k_{4\ ap}$, which is expressed via the rate constants of the elementary stages and substrate concentrations of the peroxidase reaction (r_j and [ROOH], [DH]) and the rate constants of the fourth stage of the cyclooxygenase reaction k_{4j} ($j = 1, ..., 6$) (A7). Thus, Scheme 3 is transformed into Scheme 4.

Accounting for all the above-mentioned suggestions, calculation of Scheme 4 in a steady state yields the following equation for the rate of the cyclooxygenase reaction:



Cyclooxygenase and peroxidase reactions

Scheme 3



Cyclooxygenase and peroxidase reactions after simplification

Scheme 4

$$\frac{C_0}{V_{cox}} = \frac{\eta}{[AA]} + \left(\theta + \frac{\rho}{[DH]+\mu}\right)\frac{1}{[O_2]} + \frac{\nu\rho}{[DH]+\mu} + \omega \ , \quad (3)$$

where η, θ, ρ, μ, ν, and ω are expressed via the rate constants of the elementary stages of the peroxidase and cyclooxygenase reactions and the peroxide concentration ROOH ((A11)-(A16)).

Let us analyze Eq. (3) for the rate of the cyclooxygenase reaction. Coefficients θ, ρ, μ, and ω depend on ROOH concentration, so they are not constant. However, data [29] indicate that peroxide influences parameters of the cyclooxygenase reaction to a significantly lesser extent than electron donor. This suggests that θ, ρ, μ, and ω coefficients can be considered constant in a first approximation. If so, Eq. (3) agrees well with data presented in Figs. 1-5. In fact, Eq. (3) will describe linear dependence of the rate on oxygen concentration in double-reciprocal coordinates. When changing AA concentration, we obtain a series of parallel lines in the same coordinates (Fig. 2, a and b). This is true at any electron donor concentration and also in the absence of electron donor.

The suggestion that proceeding of the peroxidase reaction influences only the rate constant of the fourth stage of cyclooxygenase reaction was one of our simplifications when deriving Eq. (3). However, it is easy to show that only in a case when the peroxidase reaction influences the 4th or 6th stages (Scheme 3), theoretical description of lines intersecting in a single point (Fig. 4) becomes possible (A17).

DISCUSSION

In the case when one of the substrates twice participates in the reaction, quadratic dependence of the reaction rate on concentration of this substrate in double-reciprocal coordinates would be expected. However, if linear dependence is observed in double-reciprocal coordinates, this indicates the presence of an irreversible stage between donating points of this substrate [22].

The data presented here (Fig. 2a) indicate the presence of an irreversible stage in the cyclooxygenase reaction between donation points of oxygen twice participating in the reaction. It is possible that the irreversible stage between donation points of oxygen (4th stage in Scheme 1) corresponds to a significant change in the substrate conformation [21] after formation of the endoperoxide bridge between the C-8 and C-12 atoms during conversion of 11-peroxyarachidonic intermediate in the C-15 radical.

The dependence of the cyclooxygenase reaction rate on oxygen concentration in double-reciprocal coordinates is linear, whereas its dependence on arachidonic acid concentration is nonlinear (Fig. 5). This cannot be explained in the framework of our kinetic model of PGHS functioning but is not in contradiction with it. The fact that the distance between curves describing dependence of the rates of the cyclooxygenase reaction on concentration of arachidonic acid in the presence and in the absence of substrates of the peroxidase reaction remains constant in double-reciprocal coordinates (Fig. 5) agrees well with Eq. (3). Deviation from linearity by itself can be described by Eq. (3) if we suggest that η is not a constant but is a function of arachidonic acid concentration.

We observed deviations from linear dependence at low concentrations of arachidonic acid (less than 3 μM) described earlier [11]. It was shown [23] that this effect decreases or completely disappears on addition of organic peroxide into the reaction mixture. Activation by excess substrate (Fig. 5) is first described; we failed to find similar effects for PGHS in the literature. It is possible that activation of the cyclooxygenase reaction by excess arachidonic acid is caused by the effect of arachidonic acid at high concentrations on micelle formation in the reaction mixture.

It was already mentioned that formal agreement of theory and experiment is achieved when the peroxidase reaction influences the 4th or 6th stages of the cyclooxygenase reaction (Scheme 3). It should be mentioned here that the effect of the state of peroxidase active site on the 6th stage seems to be more probable, because at this stage the tyrosine residue is oxidized by the PGG_2 radical, and since Tyr385 is in the immediate vicinity of the heme group, the state of the latter may affect this process [21]. The processes described by the 4th stage proceed without participation of tyrosine residue and away from the peroxidase active site.

We observed that the concentration of electron donor markedly influences the quantitative parameters of the oxygen dependencies of the cyclooxygenase reaction. For example, at oxygen concentration 5-10 μM, comparable with intracellular concentrations of dissolved oxygen [8], the rate of the cyclooxygenase reaction was 6-8 times less in the absence of electron donor than in its presence. This can play a significant role in cell functioning, especially under hypoxia, and this fact should evidently be accounted for in modeling intracellular processes and drug design.

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APPENDIX

Deduction of Scheme 1:

$$\frac{[C_{0i}]}{V_{COX}} = \frac{k_{-1}k_{-2}k_{-3}k_{-4}(k_6+k_{-5})}{k_1k_2k_3k_4k_5k_6}\frac{1}{[O_2]^2[AA]} + \frac{k_{-1}k_{-2}(k_4+k_{-3})}{k_1k_2k_3k_4}\frac{1}{[O_2][AA]} + \frac{k_{-3}k_{-4}(k_{-2}+k_2)(k_6+k_{-5})}{k_2k_3k_4k_5k_6}\frac{1}{[O_2]^2} + \frac{k_2+k_{-1}}{k_1k_2}\frac{1}{[AA]} + \frac{k_5k_6(k_{-2}+k_2)(k_4+k_{-3})+k_2k_3(k_{-4}+k_4)(k_6+k_{-5})}{k_2k_3k_4k_5k_6}\frac{1}{[O_2]} + \frac{k_4k_6+k_2k_6+k_2k_4}{k_2k_4k_6}. \quad (A1)$$

If $k_{-2} = 0$ and $k_{-4} = 0$, then:

$$\frac{[C_0]}{V_{COX}} = \frac{k_2+k_{-1}}{k_1k_2}\frac{1}{[AA]} + \left(\frac{k_4+k_{-3}}{k_3k_4}+\frac{k_6+k_{-5}}{k_5k_6}\right)\frac{1}{[O_2]} + \frac{1}{k_2}+\frac{1}{k_4}+\frac{1}{k_6}. \quad (A2)$$

Deduction of Scheme 2:

$$\frac{P_0}{V_{PER}} = \frac{r_{-3}r_{-4}r_{-5}r_{-6}(r_2+r_{-1})}{r_1r_2r_3r_4r_5r_6}\frac{1}{[DH]^2[ROOH]} + \frac{r_{-3}r_{-4}(r_6+r_{-5})}{r_3r_4r_5r_6}\frac{1}{[DH]^2} + \frac{r_{-5}r_{-6}(r_{-4}+r_4)(r_2+r_{-1})}{r_1r_2r_4r_5r_6}\frac{1}{[DH][ROOH]} + \frac{r_5r_6(r_4+r_{-3})+r_3(r_{-4}+r_4)(r_6+r_{-5})}{r_3r_4r_5r_6}\frac{1}{[DH]} + \frac{(r_6+r_{-6})(r_2+r_{-1})}{r_1r_2r_6}\frac{1}{[ROOH]} + \frac{r_4r_6+r_2r_6+r_2r_4}{r_2r_4r_6}. \quad (A3)$$

If $r_{-4} = 0$ and $r_{-6} = 0$, then:

$$\frac{P_0}{V_{PER}} = \frac{r_5r_6(r_4+r_{-3})+r_3r_4(r_6+r_{-5})}{r_3r_4r_5r_6}\frac{1}{[DH]} + \frac{(r_2+r_{-1})}{r_1r_2}\frac{1}{[ROOH]} + \frac{1}{r_2}+\frac{1}{r_4}+\frac{1}{r_6}. \quad (A4)$$

Deduction of $k_{4\ ap}$ (Scheme 3):

$$k_{4\ ap} = \frac{k_{41}[C_4P_1]+k_{42}[C_4P_2]+k_{43}[C_4P_3]+k_{44}[C_4P_4]+k_{45}[C_4P_5]+k_{46}[C_4P_6]}{[C_4P_0]}, \quad (A5)$$

where

$$[C_4P_0] = \sum_{i=1}^{6}[C_4P_i]. \quad (A6)$$

Then

$$k_{4\ ap} = \frac{k_{41}a[DH]+(k_{42}b+k_{44}d+k_{46}f)[ROOH][DH]+(k_{43}c+k_{45}e)[ROOH]}{a[DH]+(b+d+f)[ROOH][DH]+(c+e)[ROOH]}, \quad (A7)$$

where

$$a = r_3r_4r_5r_6(r_2+r_{-1}),\ b = r_1r_3r_4r_5r_6,$$
$$c = r_1r_2r_5r_6(r_4+r_{-3}),\ d = r_1r_2r_3r_5r_6,$$
$$e = r_1r_2r_3r_4(r_6+r_{-5}),\ f = r_1r_2r_3r_4r_5. \quad (A8)$$

Deduction of Scheme 4:

$$\frac{[C_0]}{V_{COX}} = \frac{k_2+k_{-1}}{k_1k_2}\frac{1}{[AA]} + \left(\frac{k_{-3}}{k_3}\frac{1}{k_{4\ ap}}+\frac{1}{k_3}+\frac{k_6+k_{-5}}{k_5k_6}\right)\frac{1}{[O_2]} + \frac{1}{k_2}+\frac{1}{k_{4\ ap}}+\frac{1}{k_6}. \quad (A9)$$

Let us substitute expression for $k_{4\ ap}$ (A7) in (A9) and rearrange:

$$\frac{C_0}{V_{COX}} = \frac{\eta}{[AA]} + \left(\theta+\frac{\rho}{[DH]+\mu}\right)\frac{1}{[O_2]} + \frac{\nu\rho}{[DH]+\mu}+\omega, \quad (A10)$$

where

$$\eta = (k_2+k_{-1})/k_1k_2, \quad (A11)$$

$$\theta = \frac{k_{-3}}{k_3}\frac{a+(b+d+f)[\mathrm{ROOH}]}{k_{41}a+(k_{42}b+k_{44}d+k_{46}f)[\mathrm{ROOH}]}+\frac{1}{k_3}+\frac{k_6+k_{-5}}{k_5k_6}, \quad (A12)$$

$$\rho = \frac{k_{-3}}{k_3}\frac{(k_{43}c+k_{45}e)[\mathrm{ROOH}]}{k_{41}a+(k_{42}b+k_{44}d+k_{46}f)[\mathrm{ROOH}]}\times$$

$$\times\left[\frac{c+e}{k_{43}c+k_{45}e}-\frac{a+(b+d+f)[\mathrm{ROOH}]}{k_{41}a+(k_{42}b+k_{44}d+k_{46}f)[\mathrm{ROOH}]}\right], \quad (A13)$$

$$\mu = \frac{(k_{43}c+k_{45}e)[\mathrm{ROOH}]}{k_{41}a+(k_{42}b+k_{44}d+k_{46}f)[\mathrm{ROOH}]}, \quad (A14)$$

$$\nu = k_3/k_{-3}, \quad (A15)$$

$$\omega = \frac{a+(b+d+f)[\mathrm{ROOH}]}{k_{41}a+(k_{42}b+k_{44}d+k_{46}f)[\mathrm{ROOH}]}+\frac{1}{k_2}+\frac{1}{k_6}, \quad (A16)$$

a, b, c, d, e, and f are expressed via the rate constants of elementary stages of the peroxidase reaction (A8).

Equation (10) may be rearranged as follows:

$$\frac{C_0}{V_{COX}} = \left(\theta+\frac{\rho}{[\mathrm{DH}]+\mu}\right)\left(\frac{1}{[O_2]}+\nu\right)+$$

$$+\frac{\eta}{[\mathrm{AA}]}+\omega-\nu\theta. \quad (A17)$$

When [DH] is varied, this equation yields a set of lines in coordinates $\{1/V_{COX}; 1/[O_2]\}$ intersecting in a single point. (Compare $y = p\cdot(x - x_0) + y_0$, where $p = (\theta + \rho/([\mathrm{DH}] + \mu))$, $x_0 = -\nu = -k_3/k_{-3}$, $y_0 = \eta/[\mathrm{AA}] + \omega - \nu\theta$. As obvious, coordinates of an intersection point (x_0, y_0) do not depend on [DH].)