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Inhibition of Cyclooxygenase Activity of Prostaglandin-H-Synthase by Excess Substrate (Molecular Oxygen)

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Abstract—For the cyclooxygenase reaction of prostaglandin-H-synthase isolated from ram vesicular glands, dependences of the initial reaction rate, the maximal yield of the product, and the rate constant of enzyme inactivation in the course of reaction on oxygen concentration were studied in the absence and in the presence of electron donor in the reaction medium. It is shown that in the absence of electron donor the cyclooxygenase reaction is strictly governed by Michaelis–Menten kinetics over a wide range of oxygen concentrations (5-800 μM). In the presence of electron donor in the reaction medium it was found that cyclooxygenase reaction is inhibited by an excess of dissolved oxygen: the maximal values of the initial reaction rate and yield of the product are attained at oxygen concentration 50 μM, and its increase to 500 μM causes twofold decrease in the initial rate and maximal yield. The rate constant of enzyme inactivation in the course of reaction increases on increase in oxygen concentration both in the presence and in the absence of electron donor.

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Key words: prostaglandin-H-synthase, cyclooxygenase activity, kinetic mechanism, oxygen, substrate inhibition, hexacyanoferrate(II)

Aerobic organisms cannot exist in the absence of molecular oxygen, which participates in various biochemical reactions in all organs and tissues. Oxygen-deficiency (hypoxia) or oxygen-excess (hyperoxia) results in development of pathologic processes. Nonetheless, the mechanism of participation of molecular oxygen in many enzymatic reactions is not well studied. Prostaglandin-H-synthase (PGHS, EC 1.14.99.1) catalyzing the cyclooxygenase reaction, which involves two oxygen molecules, is an example. PGHS provides the first stage of biosynthesis of all prostaglandins and also thromboxane A_2 and prostacyclin I_2, catalyzing conversion of the arachidonic acid molecule into prostaglandin H_2 (PGH_2).

PGHS is a bifunctional enzyme catalyzing cyclooxygenase oxidation of arachidonic acid (AA) 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 electron donor DH (reduced form) with formation of PGH_2 [1]:

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

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

Both reactions proceed on one and the same protein molecule in two neighboring active sites. These reactions are heme-dependent and are accompanied by irreversible enzyme inactivation [2]. The kinetic mechanism of enzyme functioning with its irreversible inactivation in the course of the catalyzed reaction was in general developed and analyzed [3]. As for oxygen, the Michaelis constant K_m for the cyclooxygenase reaction was only evaluated: 5 [4] and 10 μM [5].

In the first systematic study of the rate of oxygen in PGHS cyclooxygenase reaction O_2 concentration was varied from 5 to 270 μM (concentration of dissolved oxygen at atmospheric pressure and 25°C) [6]. It was shown

Abbreviations: AA, arachidonic acid; D and DH, oxidized and reduced form of electron donor, respectively; DEDTC, diethyldithiocarbamate; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGHS, prostaglandin-H-synthase; PP, protoporphyrin IX; ROOH, peroxide.

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that K_m for oxygen is not a constant value in a case of PGHS but significantly depends on concentration of arachidonic acid and is sensitive to the presence of an electron donor. Dependences of the cyclooxygenase reaction rate on oxygen concentration in double reciprocal coordinates were linear to the first approximation, but in the presence of electron donor (L-adrenalin) the rate of cyclooxygenase reaction slightly (10%) but reproducibly decreased in the range of dissolved oxygen concentration 50-270 μM. Nonlinearity in the double reciprocal coordinates was also found in [7].

For more detailed study of this phenomenon, in this work we investigated kinetics of the PGHS cyclooxygenase reaction in a wider range of oxygen concentrations (5-800 μM).

MATERIALS AND METHODS

The following reagents were used in this work: Tween 20 and L-adrenalin from MP Biomedicals Inc. (Germany); potassium hexacyanoferrate(II) from Merck (Germany); Tris, arachidonic acid (AA), hemin chloride from Sigma Aldrich (USA). Other reagents of extra pure grade were produced in Russia. PGHS as a solubilized preparation was isolated from microsomes of freshly obtained ram vesicular glands as described in [8] but with some modifications. The enzyme preparation was stored in 50 mM Tris-HCl, pH 8.0, containing 0.5 mM EDTA, 0.1 mM diethyldithiocarbamate (DEDTC), and 1% (v/v) Tween 20. A standard buffer (50 mM Tris-HCl, pH 8.0, containing 0.1% (v/v) Tween 20) was used in all kinetic experiments.

The enzymatic reaction was performed in thermally stabilized polarographic cell at 25°C. The reaction was initiated by addition of the enzyme. Arachidonic acid was added to the reaction mixture as 150 mM solution in ethanol; its concentration in a cell was 100 μM in all experiments. L-Adrenalin solution (0.1 M) was prepared by weight dissolving in 0.1 M HCl, potassium hexacyanoferrate(II) (0.1 M) and phenol (0.1 M) solutions were prepared by weight dissolving in the standard buffer solution. Hemin chloride solution was prepared by weight dissolving in a small volume of 0.1 M NaOH; the resulting solution was 20 times diluted with the standard buffer. To determine concentration, hemin solution aliquot was mixed with 20% pyridine solution in 0.1 M NaOH with addition of sodium dithionite. Hemin concentration was measured spectrophotometrically using the known molar extinction coefficients for the reduced hemin–pyridine complex [9]. In all experiments concentration of exogenous hemin in the reaction mixture was 2 μM.

Absorption of dissolved oxygen in the course of the cyclooxygenase reaction was measured amperometrically using an Ekotest-120/ATC oxygen analyzer from Ekoniks (Russia) supplied by a Clark gas-diffusion Pt/Ag electrode [10]. The reaction mixture volume was 3.3 ml, concentration of the added enzyme solution 4.5 mg/ml, the final enzyme concentration in cell 10-20 nM.

To obtain various concentrations of dissolved oxygen (5-800 μM) in the reaction mixture, gases dissolved in buffer solution were replaced by argon–oxygen mixture of the given ratio. For this, 40 ml of buffer solution were placed in a round-bottom flask (100 ml) with a magnetic stirrer, vacuum-treated using a water jet pump, then argon or pure oxygen atmosphere was produced. The vacuum-treatment gas-filling cycle was repeated several times. Thus, depending on whether pure oxygen or argon was used, two solutions – with high (~1000 μM) and low (~5 μM) oxygen concentration, respectively were obtained. All other oxygen concentrations were obtained by mixing these initial solutions in the desired ratio.

The polarographic cell was filled by the mixture, and the reaction was performed as described above. Oxygen concentration in the cell was detected polarographically using a Clark electrode.

In the course of the experiment integral curves of oxygen absorption were obtained. The data were treated using the Origin 7.0 package from MicroCal Software (USA). The beginning of reaction was taken as the initial point (t = 0). The simultaneously proceeding nonenzymatic reaction caused by oxygen absorption by the electrode typical of the polarographic method was taken into account.

The initial experimental curves of oxygen absorption were obtained as $G(t) = S_0 - S(t)$, where S_0 is the initial substrate (oxygen) concentration and $S(t)$ is its running concentration. If substrates of the enzymatic reaction are poorly consumed, $G(t)$ was approximated according to equation:

$$G(t) = P_\infty(1 - e^{-\Lambda t}) + \mu t. \quad (3)$$

Function (3) accounts for contributions of enzymatic reaction accompanied by irreversible inactivation of the enzyme (the first term of the sum) [11] and the nonenzymatic reaction that proceeds even after complete inactivation of the enzyme (the second term of the sum, μt) [12]. For each thus treated curve, the following parameters were determined: $V_0 = P_\infty\Lambda$, where V_0 is the initial rate of enzymatic reaction, P_∞ is the limiting yield of reaction product, and Λ is the rate constant of enzyme inactivation during the reaction. Integral curves presented in figures are the initial experimental curves with the subtracted term of nonenzymatic reaction: $P(t) = G(t) - \mu t$.

If oxygen concentration in the reaction medium is <50 μM, the above described method of determination of reaction characteristics is incorrect because of significant consumption of substrate. For this range of oxygen concentrations the limiting yield of reaction product and inactivation constant were not determined and the initial rate was evaluated as the tangent of the $G(t)$ curve at the initial point.

RESULTS

Effect of electron donor on kinetics of the cyclooxygenase reaction. Typical integral curves of oxygen absorption by PGHS in the presence and in the absence of electron donor are presented in Fig. 1. If substrates are poorly consumed, curves are well approximated by Eq. (3). In all experiments the presence of electron donor in the reaction medium increased the initial reaction rate V_0 and the limiting yield of reaction product P_∞ but had negligible effect on the rate constant of enzyme inactivation during the reaction Λ. In the presence and in the absence of electron donor $V_0 = 0.41$ and 0.25 μM O_2/sec, $P_\infty = 18.6$ and 7.8 μM O_2, $\Lambda = 0.032$ and 0.022 sec^{-1}, respectively. Oxygen concentration at the beginning of the reaction was 270 μM.

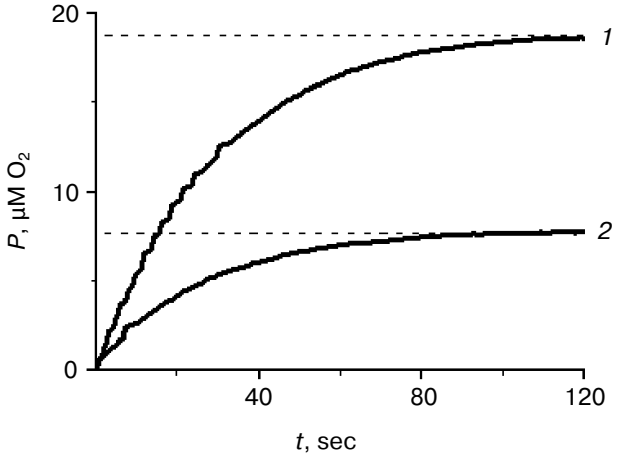


Fig. 1. Integral curves of oxygen absorption by PGHS in the presence (*1*) and in the absence (*2*) of electron donor. Concentrations: PGHS, 10 nM; O_2, 270 μM; potassium hexacyanoferrate(II) (μM): *1*) 1000; *2*) 0.

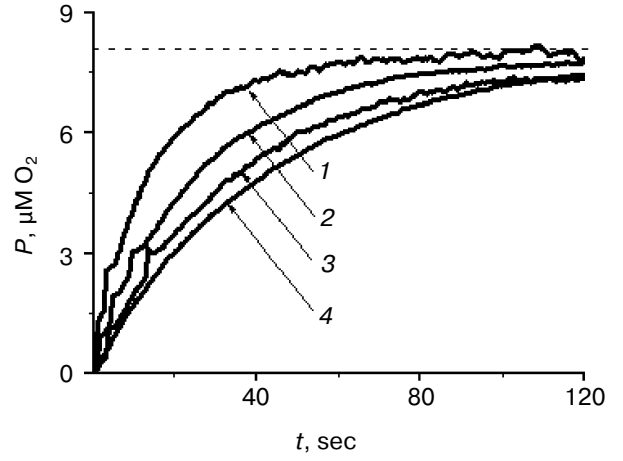


Fig. 2. Integral curves of oxygen absorption by PGHS in the absence of electron donor at various oxygen concentrations in the reaction medium. Concentrations: PGHS, 10 nM; potassium hexacyanoferrate(II), 0 μM; O_2 (μM): *1*) 610; *2*) 270; *3*) 150; *4*) 50.

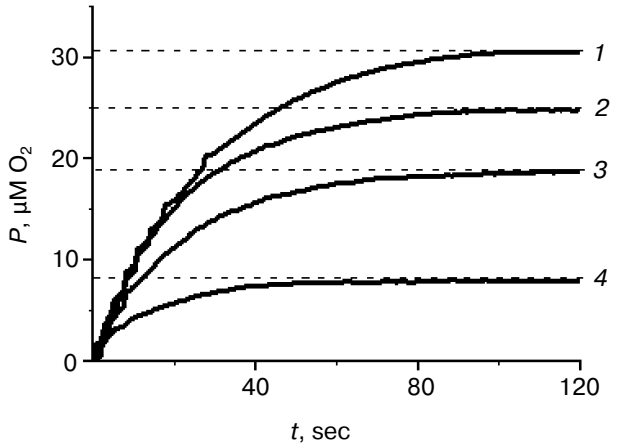


Fig. 3. Examples of integral curves of oxygen absorption by PGHS in the presence of electron donor at various oxygen concentrations in the reaction medium. Concentrations: PGHS, 10 nM; potassium hexacyanoferrate(II), 1000 μM; O_2 (μM): *1*) 70; *2*) 200; *3*) 270; *4*) 850.

Effect of oxygen concentration in reaction medium on kinetics of cyclooxygenase reaction. In the absence of electron donor the limiting yield of the reaction product was almost constant while oxygen concentration in the reaction medium was varied from 50 to 800 μM, whereas the initial reaction rate and the rate constant of enzyme inactivation significantly changed during the reaction (typical curves are presented in Fig. 2).

In the presence of electron donor the dependence of the form of integral curves on oxygen concentration is completely different (Fig. 3). On increase in oxygen concentration in the reaction medium from 50 to 800 μM, the initial rate of cyclooxygenase reaction decreases more than twofold, and the limiting yield of the product also decreases.

Dependence of initial rate of PGHS cyclooxygenase reaction on concentration of dissolved oxygen (see Fig. 4). In the absence of electron donor in the reaction medium the dependence of the initial rate on oxygen concentration is strictly hyperbolic and can be linearized in double reciprocal coordinates (Fig. 5), that is, this dependence is governed by the Michaelis–Menten equation. Theoretical dependence is depicted by the solid line in Fig. 4 (approximation curve for (*2*)). The value of the Michaelis constant for oxygen (K_m = 35 μM) was determined from this theoretical dependence.

The presence of electron donor in the reaction medium completely changes the studied dependence (Fig. 4). The dependence of the initial rate on oxygen concentration cannot be linearized in the double reciprocal coordinates in this case (Fig. 6). The maximal initial rate is observed at oxygen concentrations 50-100 μM. At con-

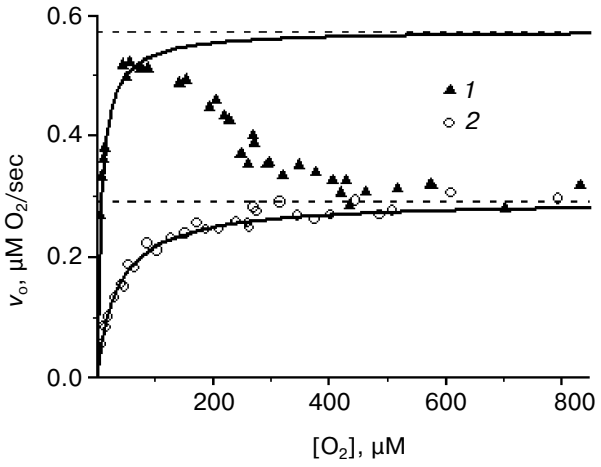


Fig. 4. Initial rate of PGHS cyclooxygenase reaction versus concentration of oxygen dissolved in the reaction medium in the presence (*1*) and in the absence (*2*) of electron donor in solution. Theoretical dependences according to the Michaelis–Menten equation are depicted by solid lines. The maximal rates for theoretical dependences are depicted by broken lines. Concentrations: PGHS, 10 nM; potassium hexacyanoferrate(II) (μM): *1*) 1000; *2*) 0.

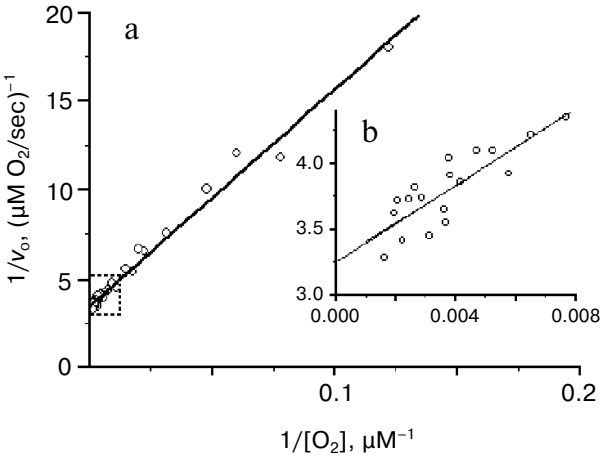


Fig. 5. Initial rate of PGHS cyclooxygenase reaction in the absence of electron donor in solution versus dissolved oxygen concentration in the reaction medium in the double reciprocal coordinates. The area framed on a plot (a) is presented in the inset (b) with enlarged scale. Linear approximation of the dependences is drawn with solid lines. Concentrations: PGHS, 10 nM; potassium hexacyanoferrate(II), 0 μM.

centrations 1-50 μM the dependence of the initial rate on oxygen concentration can be described by the Michaelis–Menten equation, and the theoretical dependence obtained from the experimental data in this concentration range is presented in Fig. 4 (approximation curve for (*1*)). For this dependence, the Michaelis constant for oxygen is 9 μM.

At oxygen concentrations 50-800 μM in the presence of electron donor we observed an initial rate decrease (~2 times) in the range 50-500 μM. Then up to 800 μM a plateau is observed. It should be mentioned that in the range of oxygen concentrations 500-800 μM the initial rates of the cyclooxygenase reaction in the presence as well as in the absence of electron donor are very similar. That is, increase in the initial rate of cyclooxygenase reaction caused by the presence of electron donor in the reaction medium is completely eliminated on increase in oxygen concentration from 50 to 800 μM.

Analogous results were obtained when L-adrenalin or phenol were used as electron donor (data not presented here).

Dependence of limiting yield of product of PGHS cyclooxygenase reaction on concentration of dissolved oxygen (see Fig. 7). In the absence of electron donor in the reaction medium the limiting yield has almost no dependence on oxygen concentration. A slight linear growth of P_∞ is observed at oxygen concentrations 50-800 μM. In the presence of electron donor the dependence is nonlinear and the limiting yield of reaction product more than twofold decreases at 50-800 μM. At oxygen concentrations 0-50 μM the dependence is not determined (see "Materials and Methods").

Dependence of rate constant of PGHS inactivation during the cyclooxygenase reaction on dissolved oxygen concentration. This constant (from here on the inactivation constant) increases on increase in oxygen concentration from 50 to 800 μM in the presence as well as in absence of electron donor (Fig. 8). The inactivation con-

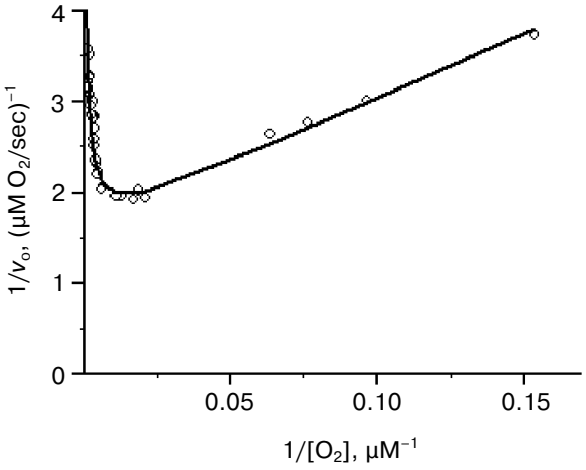


Fig. 6. Initial rate of PGHS cyclooxygenase reaction in the presence of electron donor (potassium hexacyanoferrate(II)) versus concentration of dissolved oxygen in the reaction medium in double reciprocal coordinates. The dependence is approximated by a curve. Concentrations: PGHS, 10 nM; potassium hexacyanoferrate(II), 1000 μM.

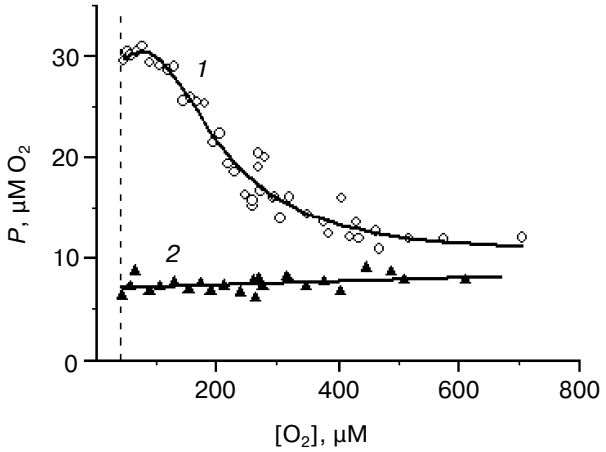


Fig. 7. Limiting yield of product of PGHS cyclooxygenase reaction versus concentration of oxygen dissolved in the reaction medium in the presence (*1*) and in the absence (*2*) of electron donor in solution. Solid lines, approximation of experimental data; broken line, right border of area of incorrect determination of parameter. Concentrations: PGHS, 10 nM; potassium hexacyanoferrate(II) (μM): *1*) 1000; *2*) 0.

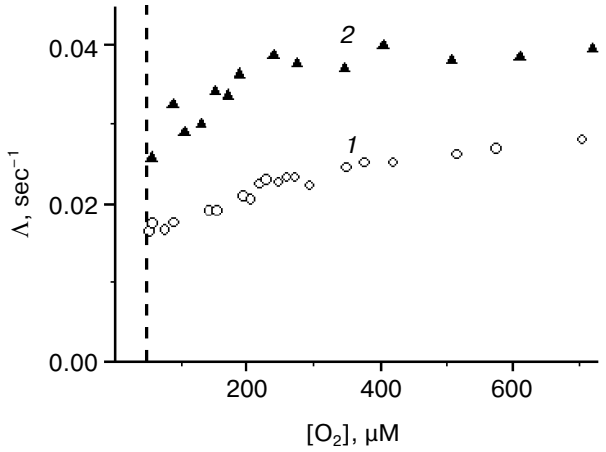


Fig. 8. Rate constant of PGHS inactivation during the cyclooxygenase reaction versus concentration of oxygen dissolved in the reaction medium in the presence (*1*) and in the absence (*2*) of electron donor. Broken line, right border of the area of incorrect determination of parameter. Concentrations: PGHS, 10 nM; potassium hexacyanoferrate(II) (μM): *1*) 1000; *2*) 0.

stant in the presence of electron donor is less than that in the absence of electron donor in all the studied range of oxygen concentrations (50-800 μM). In the range 0-50 μM the dependence was not determined (see "Materials and Methods").

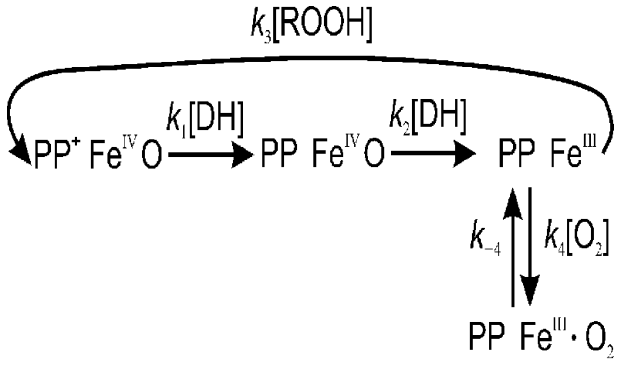
DISCUSSION

Electron donor is necessary for the PGHS peroxidase reaction. The rate of the PGHS cyclooxygenase reaction measured at the ordinary oxygen concentration (270 μM) significantly depends on the presence of electron donor in the medium [13, 14], which increases the rate of the cyclooxygenase reaction and the limiting yield of the product. It is shown that on increase in oxygen concentration from 50 to 800 μM an increase in the initial rate and the limiting yield of the product of cyclooxygenase reaction caused by the presence of electron donor in the reaction medium is eliminated.

The effect of increase in the cyclooxygenase activity of bifunctional enzyme PGHS in the presence of substrate of peroxidase reaction (electron donor) and also elimination of this effect on increase in oxygen concentration can be rationalized in the framework of the scheme [15]. This scheme illustrates the state of heme group of PGHS during the peroxidase reaction and also a complex of the reduced form of heme group with molecular oxygen (PP $Fe^{III}\cdot O_2$) postulated in the present work.

In the absence of electron donor in the medium and in the presence of prostaglandin G_2, the product of the cyclooxygenase reaction, the heme is in the oxidized form ($PP^+Fe^{IV}O$). PGHS cyclooxygenase activity in the absence of electron donor is supposed to be caused by the properties of this form. Appearance of electron donor in the medium increases the steady-state concentration of the PP Fe^{III} form. We rationalize the increase in the rate of cyclooxygenase reaction on increase in electron donor concentration by this phenomenon, that is, the PP Fe^{III} form has the higher catalytic cyclooxygenase activity than the PP^+ $Fe^{IV}O$ and PP $Fe^{IV}O$ forms.

We suggest that oxygen may reversibly bind to the heme when the latter is in the reduced form as it occurs in some oxygen-carrying proteins (hemoglobin, myoglobin, etc.). The result of such process is presented by PP $Fe^{III}\cdot O_2$ form in the Scheme. Increase in oxygen concentration in the medium decreases the steady-state concentration of PP Fe^{III} form and increases concentration of PP $Fe^{III}\cdot O_2$



Scheme of PGHS peroxidase reaction. Stage PP $Fe^{III}\cdot O_2$ accounts for supposed effect of dissolved oxygen on heme group of peroxidase active site

form. The latter should have lower catalytic activity in the cyclooxygenase reaction than the PP Fe^{III} form but probably comparable with those of the PP^+ $Fe^{IV}O$ and PP $Fe^{IV}O$ forms.

So, increase in the initial rate of cyclooxygenase reaction caused by the presence of electron donor in the reaction medium is eliminated on increase in oxygen concentration.

This scheme accounts for the absence of inhibition effect on increase in oxygen concentration in the absence of electron donor in the medium: in the absence of electron donor the heme is always in the oxidized form independent of oxygen concentration.

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