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Prostaglandin H synthase kinetics in the two-phase aqueous-micellar system

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ABSTRACT

Reaction mixture for PGHS (prostaglandin-H-synthase) is a two-phase system including micellar hydrophobic phase and hydrophilic aqueous phase. Reagents added to the mixture are distributed between phases, thus concentrations of reagents dissolved in phases can differ significantly from their overall contents.

Using dynamic light scattering we found that the hydrophobic phase produced by tween-20 consists of micelles, which radius (4–5 nm) does not depend on either tween-20 overall content (0.1%–1% v/v) or arachidonic acid (AA) addition (10–1000 μM) or PGHS addition (1 μM).

Tween-20 overall content changing from 0.1% to 2% v/v dramatically affected COX kinetic, but accounting AA distribution between phases allowed us to estimate "true" parameters, independent of the tween-20 overall content and the concentration of another substrate: K_M^{Ox} equals 9.8 μM O_2 in the aqueous phase or 0.0074 bar in the gaseous phase, K_M^{AA} equals 5400 μM AA in the phase of tween-20 micelles and 5400/P μM AA in the aqueous phase (P is the distribution ratio for the AA between the aqueous phase and the hydrophobic phase ($P \gg 1000$)). This approach allowed to evaluate P_S, the distribution ratio for the AA between the hydrophobic phase and the PGHS active center ($P_S \sim 310$). This coefficient indicates the AA selectivity toward the cyclooxygenase active center.

1. Introduction

Water soluble enzymes are usually studied in single phase aqueous systems so components of an enzymatic reaction (enzymes, substrates, products, cofactors and other reagents) are distributed homogenously in a reaction mixture. In this case, reagents concentrations are unambiguously determined and are equal to their overall contents. Overall content is supposed to be the amount of the substance to the volume of the reaction mixture ratio.

In a case of membrane enzymes the system is frequently complicated by the presence of both hydrophobic and hydrophilic phases [1]. Membrane enzymes in vitro generally keep their activity only in the presence of a hydrophobic phase [2,3]. In vivo this phase is represented by biological membranes. During enzyme isolation such a hydrophobic phase could be saved as microsomes or replaced by artificial substitutes, such as micelles or liposomes consisting of detergents, phospholipids or other amphiphilic substances [4,5]. In general, the volume of the hydrophobic phase is a minor part of the reaction mixture.

Prostaglandin H synthase (PGHS, prostaglandin-endoperoxide synthase, cyclooxygenase, EC 1.14.99.1) is a typical representative of membrane-bound enzymes and its isolation from the membranes can be achieved by nonionic detergents [3,6,7]. PGHS catalyzes AA transformation to prostaglandin H_2, which is a precursor in biosynthesis of all prostaglandins, thromboxane and prostacyclin in most mammals including humans [8]. PGHS is localized in the lumen of the endoplasmic reticulum and the inside of the nuclear envelope [6]. For experiments with PGHS nonionic detergents like tween-20 are used as a hydrophobic component of the reaction mixture [7].

Components of the heterogeneous reaction mixture are distributed between the phases according to their chemical nature. Hydrophobic

Abbreviations: PGHS, prostaglandin H synthase; AA, arachidonic acid; COX reaction, cyclooxygenase reaction; POX reaction, peroxidase reaction; K_M, Michaelis constant in the Michaelis-Menten equation; V_m, the maximum enzymatic reaction rate in the Michaelis-Menten equation; IC_{50}, the half maximal inhibitory concentration; TMPD, *N,N,N′,N′*-Tetramethyl-p-phenylenediamine; disodium EDTA, disodium dihydrogen ethylenediamine tetraacetate; DEDTC, diethyldithiocarbamate; DEAE Sepharose, Diethylaminoethyl Sepharose.

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substances are concentrated in the hydrophobic phase, so their concentrations in this phase can significantly exceed their overall contents.

It was noticed that for both PGHS isoforms cyclooxygenase reaction rate decreased and K_M^{AA} increased with an increase of phospholipids and tween-20 overall content in the reaction mixture [4]. A similar effect was shown for the water soluble maize lipoxygenase in heterogeneous system (tween-20 micelles for AA dissipation). This suggested that the rate decreased due to the distribution of the reaction components between phases [9].

Such heterogeneity of reaction mixtures could be the reason for the large spectrum of values of kinetic parameters published for PGHS: experimental values of K_M^{AA} vary from 2 to 15 μM [10–12] and experimental values of IC_{50} vary from 0.062 to 24 μM for naproxen and from 0.0037 to 100 μM for celecoxib [13–16].

In this paper we showed the necessity of quantitative calculation of arachidonic acid (AA) distribution between phases to determine the "true" enzyme kinetic parameters. We investigated the influence of tween-20 overall content in reaction mixture on PGHS cyclooxygenase and peroxidase reaction kinetics. Distribution of tween-20 micelles by size was detected by dynamic light scattering. The obtained results confirmed the assumption that changes in the detergent overall content did not affect the catalytic properties of the enzyme, but affected the cyclooxygenase reaction kinetics due to the arachidonic acid distribution between the aqueous phase and the detergent micelles hydrophobic phase.

Using this approach it was possible to calculate "true" Michaelis constant values on the basis of concentrations of substrates in corresponding phases.

2. Materials and methods

2.1. Reagents and solutions

All chemicals were obtained from commercial sources with the highest quality. Tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol), potassium hexacyanoferrate(II) trihydrate (ferrocyanide), arachidonic acid, sodium hydroxide, sodium chloride were obtained from Sigma Aldrich. Hemin chloride, tween-20, TMPD (*N*,*N*,*N*′,*N*′-Tetramethyl-p-phenylenediamine), DEAE-Sepharose (Diethylaminoethyl Sepharose), EDTA (disodium dihydrogen ethylenediamine tetraacetate), DEDTC (diethyldithiocarbamate) were obtained from MP Biomedicals. Triton X-100 and hydrogen peroxide were obtained from Fluka. All the other chemicals were obtained from standard sources.

Buffer solution 50 mM Tris HCl, pH 8.0 was used in all experiments.

Detergents (tween-20 and triton X-100) were added to the reaction mixture prior to the experiments. In this article the overall content of detergents means a volume of added detergent to the reaction mixture volume ratio (the v/v).

Detergent overall contents varied from 0.1 to 5% v/v.

Ferrocyanide stock solution was prepared in the buffer solution in concentration 0.1 M by weight. Ferrocyanide overall content in the reaction mixture was 1000 μM unless otherwise noted.

Hemin chloride stock solution was first prepared in 0.1 M NaOH and then diluted 20 times in the buffer solution with tween-20 overall content of 0.1% v/v. Thereafter, the solution was filtered through a glass filter. Hemin concentration in the stock solution was refined spectrophotometrically in complex with pyridine [17]. Hemin overall content in the reaction mixture was 1.7 μM.

Solution of AA in ethanol (20 mM–150 mM) was prepared by weight taking into account the volumes of AA and ethanol. This solution was added in the reaction mixture to an overall content of 5–1500 μM.

Hydrogen peroxide was used as aqueous solutions (the concentrations of stock solutions were 0.1 M or 1 M). Stock solution of TMPD in ethanol was prepared by weight. TMPD overall content in the reaction mixture was 200 μM.

Stock solutions of TMPD, ferrocyanide and hydrogen peroxide were prepared prior to experiments. Other stock solutions were stored at −20 °C. Buffer solution was stored at 4 °C.

All values in this work were obtained at 25 °C and pH 8.0 in aqueous phase.

2.2. PGHS purification

PGHS was isolated from ram seminal vesicles microsomes by solubilization with tween-20 as described previously [7]. This technique was used with minor modifications described in [18] and in centrifugation run time and speed (1st iteration - 30 min 13,000*g*, 2nd - 3 h 57,000*g*, 3rd - 2 h 90,000*g*, 4th - 2.5 h 180,000*g*).

Solubilized enzyme preparation was purified chromatographically. A glass column (13 × 1.3 cm) comprising 17 ml of DEAE-Sepharose Fast Flow was equilibrated with the elution buffer: 10 mM Tris, 10 mM bisTris, 0.1 mM EDTA, 0.1 mM DEDTC, 2 μM hemin chloride, 0.1% (v/v) tween-20, pH 8.0. Solubilized PGHS preparation was applied onto the column and washed by the elution buffer. The enzyme was eluted with a linear gradient of NaCl from 0 to 150 mM in the elution buffer. The active fractions were collected at 30–60 mM NaCl. Obtained purified PGHS preparation had electrophoretic purity >95%, and was used as a source of PGHS for experiments.

The purified PGHS preparation was stored as solution (0.5 mg protein/ml) in the elution buffer at −70 °C. The overall content of PGHS in experiments was 20 nM (based upon 72-kDa monomer), unless otherwise indicated.

In the case of experiments with Triton X-100, a solubilized enzyme preparation, obtained by solubilization with 4% v/v Triton X-100 instead of 1% tween-20 was used.

2.3. Cyclooxygenase reaction (COX reaction) registration

Oxygen concentration was detected using the amperometric detection system Expert MTX-001 (Econix-Expert Ltd., Russia) or Oxygraph (Hansatech Instruments Ltd., UK) with a Clark-type oxygen electrode [19]. COX reaction was carried out and the oxygen consumption was detected in the closed thermostated reaction cell equipped with magnetic stirrer. Reaction cell volume was 2 ml. Reaction temperature was maintained at 25 °C by liquid thermostating.

Dissolved oxygen (in contrast to other components of the enzymatic reaction mixture) was supplied to the reaction mixture from the gaseous phase (the atmosphere). The electrode was used to measure oxygen concentration in the phase which contacts with the electrode membrane (amperage is directly proportional to the oxygen concentration). Oxygen solubility in tween-20 is several times greater than in water [20]. Nevertheless the electrode response was the same for solutions containing 0–2% v/v tween-20 (data not shown). This fact indicates that the technique used determines adequately oxygen concentration directly in the aqueous phase.

Calibrations of the amperometric detection systems were performed using reference data about oxygen solubility in water and taking into account gas composition of the atmosphere (270 μM O_2 in water at 25 °C and 1 atm) [19,20].

In experiments the reaction cell was filled with a buffer solution containing detergent in required overall content, and then aliquots of the hemin and ferrocyanide stock solutions were added. After that the cell was closed and registration of oxygen concentration in aqueous phase was started.

Aliquots of the AA stock solutions were added through the channel in the reaction cell with Hamilton microsyringes in volume from 5 to 100 μl. After 2 min cyclooxygenase reaction was initiated by adding aliquots of the PGHS preparation to the reaction mixture. COX reaction was registered for at least 2 min.

The required oxygen concentration in the aqueous phase of the reaction mixture (10–270 μM) was achieved as follows. Buffer solutions

with needed tween-20 overall content were slowly bubbled with nitrogen in a glass separating funnel. Then, obtained buffer solution with low oxygen concentration was dosed to the reaction cell containing a fresh buffer solution with needed tween-20 overall content until the desired oxygen concentration.

All experiments were reproduced at least two times.

A typical oxygen consumption curve obtained by the amperometric method comprises the following stages: non-enzymatic oxygen consumption prior to the enzymatic reaction, a beginning of the reaction once an aliquot of the PGHS preparation has been added, and a progress of the reaction itself (Fig. 1). Further reaction rate decrease was associated with irreversible enzyme inactivation during the reaction [21,22] and substrates depletion.

The maximum rate of net enzymatic oxygen consumption was used as the cyclooxygenase reaction rate (V). Non-enzymatic oxygen consumption was taken into account.

2.4. Peroxidase reaction (POX reaction) registration

POX reaction was monitored spectrophotometrically at 611 nm by the accumulation of the oxidized form of TMPD. TMPD is a one electron donor giving a stable colored cation radical after oxidation. Extinction coefficient for this radical is $\varepsilon_{611} = 13{,}500\ M^{-1}\ cm^{-1}$. The reduction of one peroxide molecule requires two molecules of TMPD [23]. Absorbance was measured with a spectrophotometer "Cary 100" (Varian Inc., USA) in a quartz cell with 1 cm optical path. Assay reaction mixture volume was 2.5 ml. The cell was equipped with a magnetic stirrer. Buffer solution with additions of required tween-20 overall content and aliquots of TMPD and hydrogen peroxide stock solutions were added to the cell. Reaction was initiated by enzyme addition to the reaction mixture. TMPD, hydrogen peroxide and PGHS aliquots were added with Hamilton microsyringes. Reaction temperature was maintained at 25 °C using the spectrophotometer temperature control system.

All experiments were reproduced at least two times. Peroxidase reaction rate (V) was determined from the curve of the reaction product accumulation analogously to the COX reaction rate.

2.5. Data processing

Parameters of multidimensional functions (7.1) and (7.2) were obtained by search method of finding a global minimum of minimization function using MATLAB software (USA). The following expression was used as the minimization function:

$$f = \sum_j \left({V_j}^{theor} - {V_j}^{exp} \right)^2, \quad (1)$$

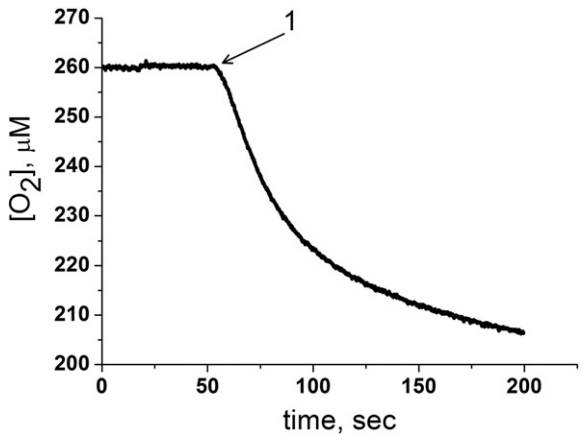

Fig. 1. The time course for the oxygen concentration during cyclooxygenase reaction. Conditions: 0.1% v/v tween-20, 100 μM AA, 1000 μM ferrocyanide. 1 – PGHS preparation addition. The other conditions are in Materials and methods section.

where ${V_j}^{exp}$ is the COX reaction rate defined in the j-th experiment, ${V_j}^{theor}$ is the corresponding COX reaction rate calculated theoretically. All experiments of the used dataset were summed.

Mathematical processing of other experimental data was carried out using the Origin 7.5 package from MicroCAL Software (USA).

Hyperbolic dependences of the enzymatic reaction rate V on one of the substrates concentration S were approximated in terms of the Michaelis-Menten Eq. (2). The concentrations of other reaction components were constant.

$$V = {V_m}^{obs} \cdot S / \left({K_M}^{obs} + S \right), \quad (2)$$

where K_M^{obs} and V_m^{obs} are observed parameters depending on concentrations of other enzymatic reaction components.

2.6. Particle size distribution

Particle size distribution was determined by dynamic light scattering method using Zetasizer Nano ZS MALV-1012-01 (Malvern instruments Ltd.) with scattering angle 173°. The dynamic light scattering method is based on determination of diffusion coefficients of particles dispersed in the medium by analysis of the characteristic time of scattered light intensity fluctuations [24]. Zetasizer Nano ZS has He-Ne laser of wavelength 633 nm. Disposable low volume polystyrene cuvettes (ZEN0112) were used. Temperature was maintained at 25 °C. Refractive index and the absorption capacity were taken as 1.6 and 0.01, respectively. All liquids were filtered through filters with pore size 0.2 μm. Sample volume was 600 μl. Measurement duration was 10 s. Each sample was subjected to at least 8 measurements. Temperature equilibration continued for 2 min. Data were processed automatically using software attached to Zetasizer Nano ZS and resulted in a size distribution graph.

3. Results

3.1. Tween-20 micelle phase characterization

The particle size distribution was measured by dynamic light scattering in buffer solutions with tween-20 and additions of AA and PGHS. In these experiments tween-20 overall content varied from 0.1% to 1% v/v. Unimodal distributions with the same characteristics within experimental error were obtained in the range of 1–10 nm (Fig. 2). The resulting distributions in semilogarithmic plot were symmetrical peaks with a maximum at 4–5 nm and about 4 nm width at half peak height. This fact gave us reason to consider that tween-20 forms stable micelles with equal radiuses of 4–5 nm.

The micelle size did not depend on the tween-20 overall content. Therefore, the number of micelles must be a linear function of the micelles volume fraction in mixture.

AA did not form its own stable micelles in the buffer solution with particle radius in the range 1–10 nm. This conclusion was based on repeated measurements of particle size distribution in mixtures containing AA (100–1000 μM) in the buffer with absence of tween-20 (data not shown).

In systems containing tween-20 (0.1–1% v/v) and AA (0–1000 μM) particle size distribution by the scattered light intensity in the range of 0.1–10 nm was characterized by a sustained peak. It was almost identical to the peak obtained in the case of tween-20 mixture in the absence of other additives (Fig. 2).

In the case of PGHS addition up to 1 μM to a mixture containing 0.1%–1% v/v tween-20, the particle size distribution by the scattered light intensity also remained without visible changes (Fig. 2, D illustrates the result when tween-20 overall content was 1%).

Therefore, changes of tween-20 overall content (0.1–1% v/v) and AA or PGHS additions did not affect particle size in the analyzed mixtures.

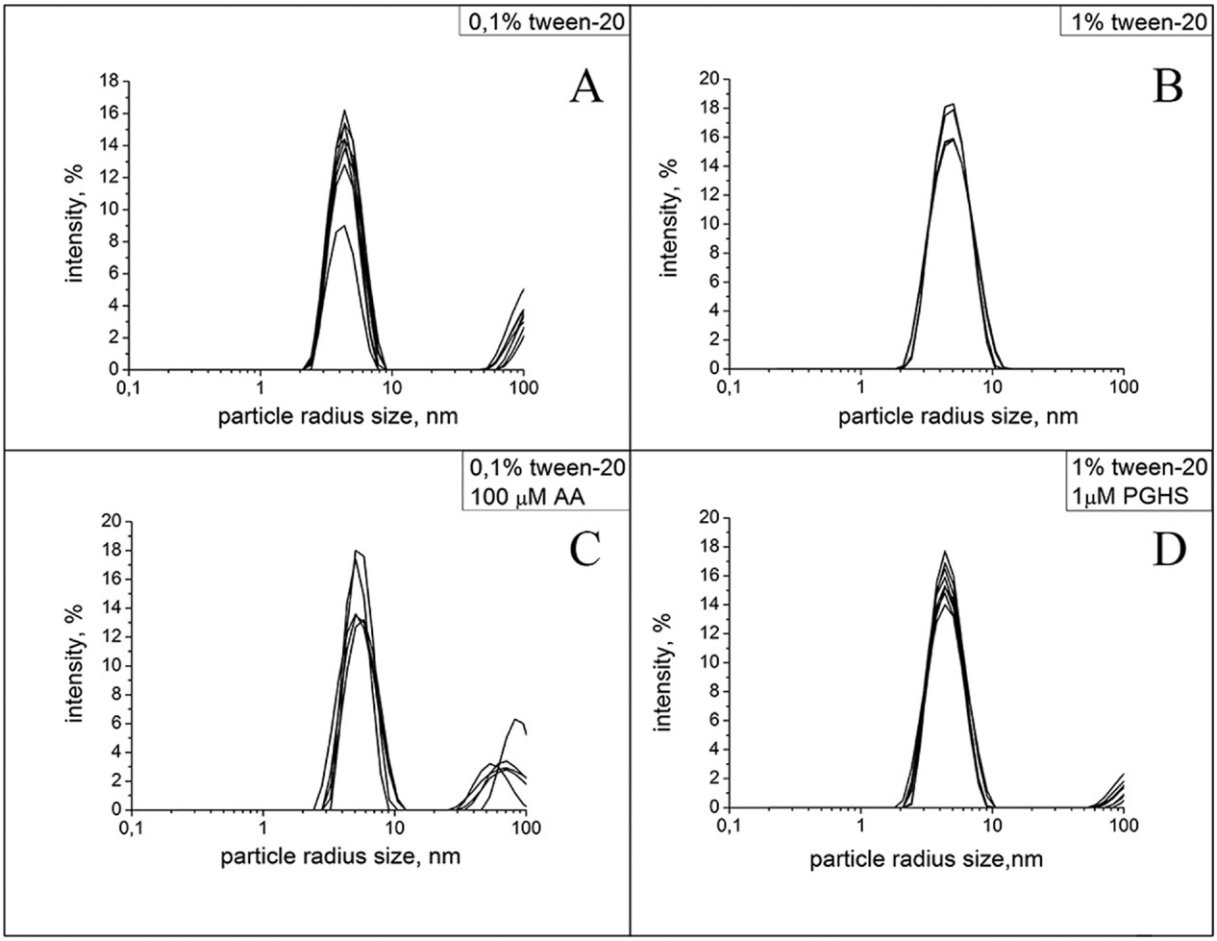

Fig. 2. Particle size distribution by the intensity of scattered light in buffer solution for the following systems: A) 0.1% v/v tween-20, B) 1% v/v tween-20, C) 0.1% v/v tween-20, AA overall content 100 μM, D) 1% v/v tween-20, PGHS overall content 1 μM.

Distributions in the radius range over 10 nm were irreproducible.

According the results of experiments described above, we concluded that tween-20 forms micelles with 4–5 nm radius in water solutions. This conclusion allowed us to give some quantitative estimates for experimental systems used in the present investigation. According to this probable particle radius, tween-20 molar weight (≈1228 g/mol) and density (1.1 g/ml) (according to the manufacturer) we estimated aggregation number (N) of tween-20 micelles as N = 140–280. Thus in 0.1% tween-20 mixture micelle overall content is 3–6 μM in molar units. When AA overall content is 100 μM in 0.1% tween-20 mixture there would be 15–30 AA molecules per one tween-20 micelle (thus, AA volume concentration in micelle would be up to 0.1 M and AA surface concentration in micelle up to 1.25–1.59 nmole/dm^2). Thereby when PGHS concentration in the reaction mixture is 20 nM there would be 150–300 tween-20 micelles per one monomer of enzyme.

3.2. The dependence of the COX reaction rate on AA overall content at various detergent contents

The dependences of the cyclooxygenase reaction rate on AA overall contents (5–1500 μM) at various overall contents of tween-20 (0.1–2% v/v) in the reaction mixture were examined. The dependences were linearized in double reciprocal plots and could be formally governed by the Michaelis–Menten equation (Fig. 3).

Value of the experimentally observed K_M^{obs} for AA increased if tween-20 overall content increased (Table 1). At the same time the V_m^{obs} value did not change. A similar effect was observed in the case of cyclooxygenase reaction in the absence of any electron donor. Supplementary studies were performed using Triton X-100 as detergent. In this case the K_M^{obs} value also increased and the maximum rate value was constant when detergent overall content increased (Table 1).

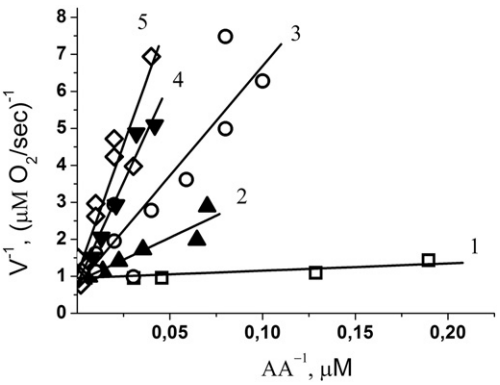

Fig. 3. COX reaction rate (V) versus AA overall content in double-reciprocal plots: 1–0.1% v/v tween-20, 2–0.5% v/v tween-20, 3–1% v/v tween-20, 4–1.5% v/v tween-20, 5–2% v/v tween-20. Dissolved oxygen concentration in aqueous phase was 270 μM. Ferrocyanide (electron donor) overall content was 1000 μM. Linear approximations of the experimental data are shown by solid lines. The other conditions are in Materials and methods section.

Table 1
The experimentally observed values of the Michaelis-Menten equation parameters for COX reaction at various detergent overall contents. Results are expressed as the mean ± SE.

		Tween-20					Triton X-100	
	Detergent overall content (% v/v)	0.1	0.5	1	1.5	2	1	5
In the presence of electron donor (1000 μM ferrocyanide)	K_M^{obs}, μM AA	2.1 ± 1.2	27.7 ± 6.7	78.2 ± 50.9	134.8 ± 31.4	133.9 ± 45.5	22.8 ± 1.8	109.1 ± 10.3
	V_m^{obs}, μM O_2/s	1.1 ± 0.1	1.17 ± 0.1	1.3 ± 0.6	1.25 ± 0.2	0.91 ± 0.2	1 ± 0.06	1.1 ± 0.2
In the absence of electron donor	K_M^{obs}, μM AA	5.9 ± 0.7	22.4 ± 3.3	50.7 ± 4.1	62.7 ± 5.5	n/d	n/d	n/d
	V_m^{obs}, μM O_2/s	0.5 ± 0.02	0.4 ± 0.03	0.41 ± 0.1	0.4 ± 0.02	n/d	n/d	n/d

n/d – no data.

3.3. The COX reaction rate dependence on oxygen concentration in aqueous phase at various tween-20 overall contents

Molecular oxygen is the other substrate of the COX reaction. The dependences of the cyclooxygenase reaction rate on oxygen concentration in double reciprocal plots were linearized and could be formally governed by the Michaelis–Menten equation. Linear approximation lines were parallel. Observed values of the Michaelis-Menten equation parameters were calculated and appeared to depend on tween-20 overall content (Fig. 4).

A similar result was obtained in case of the COX reaction in the absence of any electron donor (data not shown).

3.4. The POX reaction rate dependence on peroxide overall content at various detergent overall contents

One possible hypothesis about detergent influences on PGHS kinetics was that changes in tween-20 overall content might directly affect the properties of this protein and, consequently, affect catalytic properties of PGHS. In order to test this hypothesis we experimentally checked whether tween-20 overall content had any effects on kinetics of peroxidase reaction catalyzed by PGHS.

The dependences of the peroxidase reaction rate on peroxide overall content at various overall contents of tween-20 in the reaction mixture were examined (Fig. 5). The peroxidase reaction rate varied in <20%, while overall content of tween-20 varied from 0.01 to 2% v/v. To the first approximation, the kinetic characteristics of the peroxidase reaction did not depend on detergent overall content in the reaction mixture, in contrast with the cyclooxygenase reaction rate which changed by 5 times (Fig. 3).

This indicates that changes in tween-20 overall content are not likely to have any direct influence on the catalytic properties of PGHS, although different reactions might be influenced selectively.

3.5. The influence of reaction mixture heterogeneity on the kinetics of cyclooxygenase reaction

As was shown above (Fig. 2), the changes in detergent overall content in the reaction mixture did not affect the size of the micelles. The true concentration of free detergent, dissolved in the aqueous phase (within the studied range of tween-20 contents) should remain constant and equal to the critical micelle concentration (CMC) [25]. Consequently, when the detergent overall content exceeds CMC (0.007% v/v [26]), hydrophobic environment of the enzyme remains unchanged. Therefore, the PGHS catalytic properties should not directly depend on tween-20 overall content.

This conclusion was also confirmed by the results of experiments on the effect of tween-20 on the POX reaction kinetics (Fig. 5).

We assumed that influence of detergent overall content on the PGHS kinetics was the result of AA distribution between the aqueous phase and the hydrophobic micellar phase formed by detergent micelles. The molecular oxygen true concentration in the aqueous phase was directly measured with amperometric techniques (see 2.3 section). So possible oxygen distribution between the phases should not be taken into account under the calculations. Hemin and TMPD (in the case of peroxidase reaction) were used in saturating contents, so a hypothetical changing in their concentrations due to distribution between the phases should not have a significant effect on the enzymatic reactions rate.

The distributions of the hydrogen peroxide and potassium ferrocyanide between phases were not considered because the concentrations of these substances in the aqueous phase are almost independent of the tween-20 overall content in the range used (calculated value of distribution coefficient for hydrogen peroxide between water and octanol is 0.37 [27], ferrocyanide is soluble in the aqueous phase only).

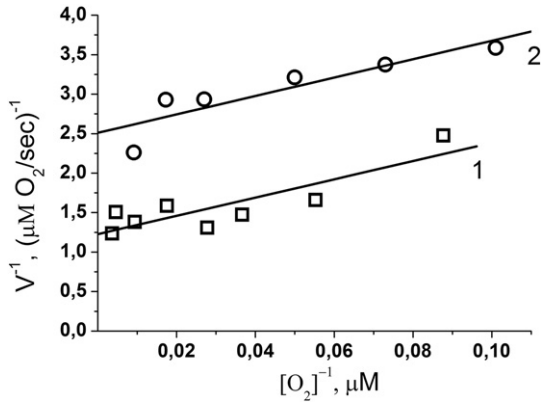

Fig. 4. Cyclooxygenase reaction rate (V) versus molecular oxygen concentration in double-reciprocal plots: 1–0.5% tween-20 v/v, 2–1.5% tween-20 v/v. Overall contents were: 1000 μM potassium ferrocyanide; 50 μM AA. The other conditions are in Materials and methods section. Linear approximations of the experimental data are shown by solid lines.

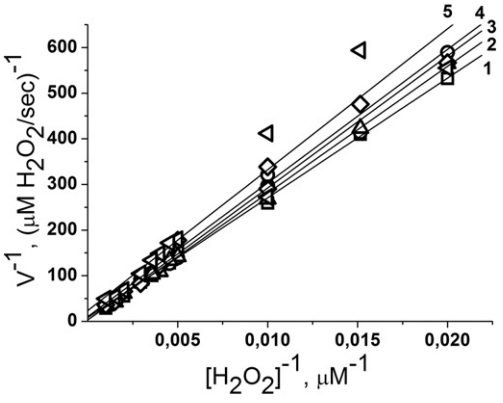

Fig. 5. Peroxidase reaction rate (V) versus peroxide overall content in double-reciprocal plots: 1 – in this case tween-20 was added only as a component of PGHS preparation and its overall content was about 0.01% v/v, 2–0.1% v/v tween-20, 3–0.3% v/v tween-20, 4–0.5% v/v tween-20, 5–2% v/v tween-20. TMPD overall content was 200 μM. The other conditions are in Materials and methods section. Linear approximations of the experimental data are shown by solid lines.

3.6. Quantitative accounting of the AA distribution between the hydrophobic and aqueous phases

In this study we took into account the distribution of AA between micelles, water and the active centers of the PGHS (Fig. 6). We accepted that AA was evenly distributed in the volume of micelles, so we could define volume concentration of AA in tween-20 micelles. We also made several approximations:

1. AA in the aqueous phase and AA in micelles were in equilibrium despite enzymatic AA consumption.
2. The amount of AA bound to PGHS was negligible in comparison with AA total amount;
3. The volume of added detergent was negligible in comparison to the volume of reaction mixture; as a consequence the volume of the aqueous phase was taken equal to the total volume of the reaction mixture;
4. The volume fraction of micelles in the reaction mixture (α) was taken equal to the tween-20 overall content.
   The last claim is truthful because tween-20 have a low solubility (0.007% v/v [26]) and even in the case of minimum tween-20 content (0.1% v/v) the calculated micelles volume fraction in the reaction mixture, expressed in a dimensionless units (α), equals 0.00093.

In view of the above approximations, the material balance of AA could be written as:

$$AA_0 = [AA]_m \cdot \alpha + [AA]_w, \tag{3}$$

where AA_0 is AA overall content in the reaction mixture, $[AA]_m$ and $[AA]_w$ are concentrations of AA in micelles (hydrophobic phase) and in water (hydrophilic aqueous phase). Consequently, α is, as mentioned above, the volume fraction of micelles in the reaction mixture, expressed in dimensionless units.

The values of AA concentrations in water and in micelles are associated by equation:

$$[AA]_m/[AA]_w = P, \tag{4}$$

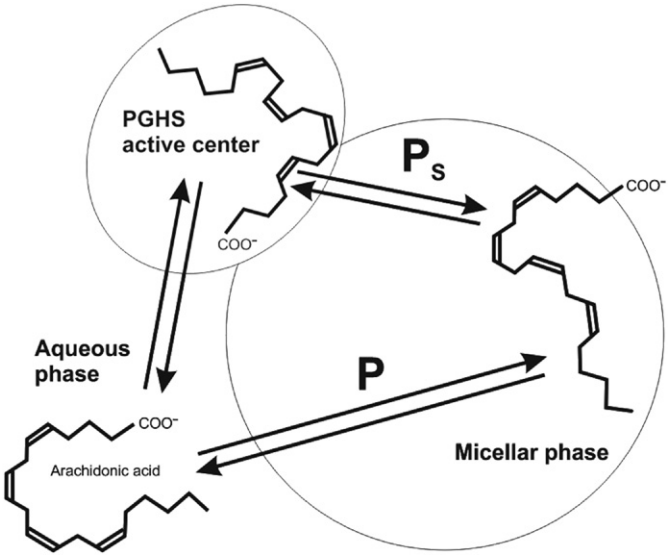

Fig. 6. AA distribution in the reaction mixture. See text for details.

where P is the distribution ratio for the AA between the aqueous phase and the hydrophobic phase.

Transformation of the Eqs. (3) and (4) results in the following equations, which associate the values of AA concentrations in micelles and the values of AA concentrations in water with AA overall content:

$$[AA]_m = AA_0 \cdot P/(1 + P \cdot \alpha), \tag{5.1}$$

$$[AA]_w = AA_0/(1 + P \cdot \alpha). \tag{5.2}$$

It should be noted that the developed equations are valid not only for the AA distribution in the two phase system but also for any substance added to such system.

3.7. Quantitative accounting of AA distribution between two phases for cyclooxygenase kinetics

Reciprocal COX reaction rate is a linear combination of reciprocal AA concentration and reciprocal oxygen concentration [28]:

$$1/V = 1/V_m \cdot \left(1 + K_M{}^{AA}/[AA] + K_M{}^{Ox}/[O_2]\right), \tag{6}$$

where coefficients of Eq. (6) (V_m, $K_M{}^{AA}$, $K_M{}^{Ox}$) are functions of elementary reaction rate constants, enzyme concentration and temperature. For convenience they were denoted in terms of the Michaelis-Menten equation, wherein [AA], and $[O_2]$ are the concentrations of AA and O_2 in the phase from which the substrates enter the active site of PGHS, respectively.

Since in this investigation we determined oxygen concentration directly in the aqueous phase (see 2.3 section), there was no need to consider the molecular oxygen distribution between the aqueous and hydrophobic phases. We assumed that oxygen reacted with the enzyme through aqueous phase, so for calculations we used the experimentally determined oxygen concentration in the aqueous phase.

However, it can be assumed that the molecular oxygen enters the active site of PGHS from a hydrophobic phase. In this case, the O_2 concentration in the hydrophobic phase, and, accordingly, the value of $K_M{}^{Ox}$ could be simply recalculated using the distribution ratio for the molecular oxygen between the hydrophobic phase and water.

Using the Eqs. (5.1) and (5.2), the expression for the cyclooxygenase reaction rate (6) can be transformed into Eqs. (7.1) and (7.2). Eq. (7.1) describes the kinetics of cyclooxygenase reaction if the AA interacts with the enzyme through a hydrophobic phase (7.1), Eq. (7.2) describes the kinetics of cyclooxygenase reaction if the AA interacts with the enzyme through the aqueous phase:

$$1/V = 1/V_m \cdot \left(1 + \left(K_M{}^{AA}/AA_0 \cdot P\right) \cdot (1 + P \cdot \alpha) + K_M{}^{Ox}/[O_2]\right), \tag{7.1}$$

$$1/V = 1/V_m \cdot \left(1 + \left(K_M{}^{AA}/AA_0\right) \cdot (1 + P \cdot \alpha) + K_M{}^{Ox}/[O_2]\right). \tag{7.2}$$

In the Eqs. (7.1) and (7.2) the overall content of AA in the reaction mixture (AA_0), the oxygen concentration in the aqueous phase ($[O_2]$) and the volume fraction of micelles in the reaction mixture (α) were variables. V_m, $K_M{}^{AA}$, $K_M{}^{Ox}$ and P were sought parameters. In the case of Eq. (7.1) the parameter $K_M{}^{AA}$ was measured in units of AA concentration in hydrophobic phase, and in Eq. (7.2) the parameter $K_M{}^{AA}$ was measured in units of AA concentration in aqueous phase. Eqs. (7.1) and (7.2) successfully described all the experiments with variations of α, AA_0 and $[O_2]$. In order to determine the parameters V_m, $K_M{}^{AA}$, $K_M{}^{Ox}$ and P, the method of finding a global minimum was used as described in the 2.5 section. It was found that in the case of P there was no distinct minimum of minimization function (Eq. (1)) but there was a wide range of possible values of P, so-called "ravine". This fact is illustrated by Fig. 7, which shows the dependence of minimization function values (Eq. (1)) on preset values of parameter P in the case of using Eq. (7.2). The higher

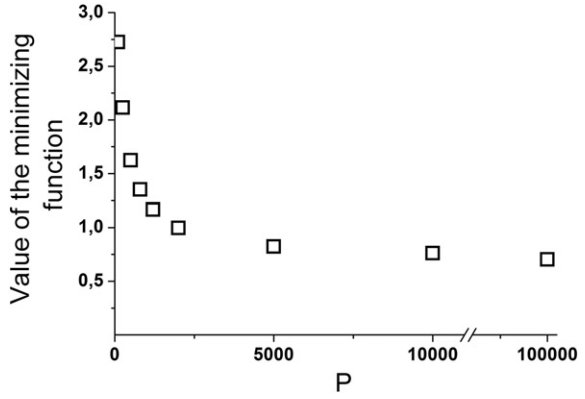

Fig. 7. Value of the minimizing function (Eq. (1)) after dataset approximation versus preset values of parameter P for Eq. (7.2). See text for details.

the values of parameter P the lower the value of minimization function. When the value of parameter P increased significantly, the quality of the approximation ceased to depend on the value of P (Fig. 7). In the case of Eq. (7.1) the result was the same (data not shown).

This observation can be explained by the structure of the Eqs. (7.1) and (7.2). For large values of P, when $P \cdot \alpha \gg 1$, Eq. (7.1) acquires the following form and ceases to depend on P:

$$1/V = 1/V_m \cdot \left(1 + \left(K_M^{AA}/AA_0\right) \cdot \alpha + K_M^{Ox}/[O_2]\right); \quad (8.1)$$

and Eq. (7.2) acquires the form:

$$1/V = 1/V_m \cdot \left(1 + \left(K_M^{AA} \cdot P/AA_0\right) \cdot \alpha + K_M^{Ox}/[O_2]\right), \quad (8.2)$$

which also does not depend explicitly on the P (in Eq. (8.2) the product of two sought parameters K_M^{AA} and P appeared instead of P itself). Since in this investigation α varied in the range 0.001–0.02, the condition $P\alpha \gg 1$ meant $P \gg 1000$.

In this range of P ($P \gg 1000$) the set of parameters V_m, K_M^{AA} and K_M^{Ox} can be determined assuming that AA interacts with the enzyme through the hydrophobic phase (Eq. (7.1)); the set of parameters V_m, $K_M^{AA} \cdot P$ and K_M^{Ox} can be determined assuming that AA interacts with the enzyme through the aqueous phase (Eq. (7.2)): $V_m = 1.1$ μM O_2/s, $K_M^{AA} =$ 5400 μM AA in hydrophobic phase, $K_M^{AA} = 5400/P$ μM AA in the aqueous phase ($P \gg 1000$), $K_M^{Ox} = 9.8$ μM O_2 in the aqueous phase (9.8 μM O_2 in the aqueous phase is equivalent to the oxygen partial pressure 0.0074 bar in the gaseous phase).

3.8. The selectivity of PGHS cyclooxygenase active center toward the AA

We can consider the formation of the enzyme-substrate complex as distribution of substrate between the reaction mixture and the active site of enzyme. In a case of substrate concentrations substantially below enzyme saturation, this process could be characterized by the distribution ratio for the substrate between the respective phase of the reaction mixture and the enzyme active center phase (P_S). It is easy to show that this distribution ratio is equal to:

$$P_S = 1/K_S \cdot \nu, \quad (9)$$

where K_S is the dissociation constant for the enzyme-substrate complex and ν is the molar volume of the enzyme active centers. Let us take as an estimate of the dissociation constant for PGHS-AA complex (K_S) obtained in this work the value of K_M^{AA}, which equals 5400 μM AA in hydrophobic phase.

On the basis of the PGHS active center volume (about 1000 Å^3 [29]) it is easy to calculate that ν equals 0.6 l/mol. As a result we obtained that the distribution ratio (P_S) for arachidonic acid between hydrophobic phase and the PGHS active center phase equals 310.

It is also possible to calculate the distribution ratio for arachidonic acid between aqueous phase and the PGHS active center phase. In this case, the distribution ratio equals 310·P, where P is the distribution ratio for the AA between the aqueous phase and the hydrophobic phase ($P \gg 1000$).

4. Discussion

Accounting of AA distribution between the aqueous phase and hydrophobic phase formed by micelles of tween-20 helps to explain how the COX reaction kinetics depends on the overall content of detergent. This approach allows us to get "true" values of the PGHS kinetic parameters, which do not depend on the contents of the other components of enzymatic reaction and to assess the distribution ratio for AA between the aqueous phase (pH 8.0) and the micellar phase (P), as $P \gg 1000$. Experiments in this research cannot provide a more accurate value of P, but this estimate is consistent with the value for the distribution ratio for the AA between aqueous solution (pH 7.4) and 1-octanol, which equals 7900 [30].

We found that the Michaelis constant for AA equaled 5400 μM AA in tween-20 when the AA entered the active center of PGHS from the hydrophobic phase, and it equaled 5400/P μM AA in the water, when AA entered the active center of PGHS from the aqueous phase. It is very important to point out which phase the Michaelis constant definition is used for. At first sight, the values of 5400 μM AA in micellar phase and of significantly <5.4 μM AA in aqueous phase are numerically incomparable. However, we have to remember that chemical potentials of AA in these phases are equal to each other at these concentrations.

During data processing in the current study we assumed that AA was uniformly distributed in the detergent micelles volume. It allowed us to use such a notion as volume concentration of AA in micelles and to calculate K_M^{AA} in terms of volume concentration. At the same time, it is acceptable to assume that the AA can be distributed on the surface of the micelle, or both on the surface and in the bulk. The corresponding equations describing such distributions of AA and such dependences of the COX reaction rate on the overall content of AA can be easily deduced. In our study we found that the micelles radius did not depend on the overall content of detergent, so that the total micelles surface was directly proportional to the total volume of the micelles. For this reason, the equations describing the COX reaction rate dependence on the AA overall content in the case of surface AA distribution would be qualitatively the same as Eqs. (7.1) and (7.2). Thus, this approach will not answer the question whether AA enters the active center of PGHS from the aqueous phase, or from the volume of micelle, or from the surface of micelle, or simultaneously from several of them.

We can also assume that the substrate of COX reaction, O_2, may enter the PGHS active center from the hydrophobic phase. Thus, taking into account that the distribution ratio for the molecular oxygen between the water and 1-octanol equals to 4.47 [31], we can obtain an estimate of K_M^{Ox} equals to 43.8 μM O_2 in the hydrophobic phase. In the case of oxygen this equivocality in the values of K_M^{Ox} can be avoided if the value of K_M^{Ox} is defined in units of the partial pressure of molecular oxygen in the gaseous phase. According this study, the calculated value of K_M^{Ox} was 0.0074 bar of O_2 (or 5.6 mm Hg). The value of K_M^{Ox} expressed in units of partial pressure of molecular oxygen in gaseous phase is valid irrespectively of from what phase oxygen enters the active center.

The distribution ratio for AA between the hydrophobic phase and the PGHS active centers (P_S) is of special interest. This dimensionless coefficient can be used as a "true" characteristic of active center selectivity toward AA. In the case of AA passing from hydrophobic phase to hydrophobic active center there is no need to take into

account the possible influence of hydrophobic interactions typical for an aqueous phase. If the active center of the PGHS absorbed the AA only due to unselective hydrophobic interactions, then P_S would not exceed 1. Value of P_S 310 > 1 indicates the presence of some specific interactions between AA and the amino acid residues of the PGHS active center. These interactions must compensate for entropic losses due to "freezing" of AA conformation in the enzyme active center and would lead to the total decrease of the free energy upon enzyme-substrate complex formation.

Accounting for the distribution of substances added to the reaction mixture between aqueous and micellar phases helps to explain many observations. Thus it has already been indicated that an increase of detergent (phospholipids with varying acyl chain structures and physical states) overall content in the reaction mixture resulted in a decrease of the COX reaction rate [4]. This effect could be the result of the substrate dilution by increasing the hydrophobic phase volume, as in our study.

Research of PGHS inhibitors also requires accounting for AA distribution between phases as well as accounting for the inhibitor distribution. The dependence of IC_{50} values for different inhibitors of PGHS on the volume fraction of the hydrophobic phase was shown [32]. It is remarkable that for some substances observed the bigger the volume fraction of the hydrophobic phase was, the higher IC_{50} value was, while for others there was the inverse dependence. These results can be explained by taking into account both AA and inhibitor distribution between the phases.

Approach for accounting of substrate distribution between the phases proposed in our study may be used as base for a method of determining the distribution ratio P for enzymatic experimental systems. The distributed substance should be a substrate or inhibitor for any experimentally observed enzyme reaction.

It should be noted that the approach give valid results in the case when the range of α covers the area within which $P \cdot \alpha \approx 1$.

At the conclusion of the discussion we would like to say that the current work on the example of PGHS made us demonstrate that the changes in detergent overall content in biphasic systems produced a paradoxical effect of hydrophobic substrate depletion both in the hydrophobic, and in the aqueous phase. We propose at least to keep in mind this effect.

Transparency document

The Transparency document associated with this article can be found, in online version.

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