Reaction of Prostaglandin Endoperoxide Synthase with cis,cis-Eicosa-11,14-dienoic Acid*

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The pre-steady-state phase of the oxygenase reaction of prostaglandin endoperoxide synthase with cis,cis-eicosa-11,14-dienoic acid has been studied using stopped flow techniques. Because some intermediate forms of prostaglandin endoperoxide synthase are spectrally indistinguishable, the enzyme and substrate transformations were monitored in parallel to simplify the interpretation of the kinetics. Over a wide range of conditions, the formation of the enzyme intermediate II, the form of compound I containing the tyrosyl radical, precedes substrate oxidation. This result supports the occurrence of a unimolecular conversion of compound I into intermediate II. Furthermore, the rate of intermediate II formation was stimulated by increased concentration of dienoic acid, perhaps because of increased occupation of the fatty acid binding site. The importance of the unimolecular formation of intermediate II was confirmed by simulated kinetics of the oxygenase reaction. These results provide evidence that intermediate II is the primary oxidant in the reaction of prostaglandin synthase with the dienoic acid, as it is with arachidonic acid.

Prostaglandin H endoperoxide synthase (PGH synthase) catalyzes the first step in the biosynthesis of prostaglandins. This step consists of two reactions: the conversion of arachidonic acid into prostaglandin G_2 (cyclooxygenase reaction) and the reduction of prostaglandin G_2 to prostaglandin H_2 (peroxidase reaction) (1–3). Extensive investigation of these reactions has revealed enzymatic intermediates analogous to the classic peroxidase compounds I and II as well as free radical forms of the enzyme (4–6). Two reaction mechanisms have been proposed for this system, differing first of all in relation to the initial events of the cyclooxygenase reaction. The branched chain mechanism considers the tyrosyl radical of PGH synthase with the dienoic acid, as it is with arachidonic acid, which in principle can detect the free radical intermediate, is unable to follow the reaction on a millisecond time scale, typical for enzyme conversion. In such a situation, parallel kinetic monitoring of both enzyme and substrate behavior could be useful.

cis,cis-Eicosa-11,14-dienoic acid (20:2) has been used in previous studies because it is the only PGH synthase substrate producing easily measurable conjugated diene products that are readily detected spectrophotometrically (10–15). Another advantage of this substrate is that the reaction is relatively slow and can be measured using micromolar concentrations of enzyme. This provides a rare opportunity to observe the enzyme and substrate transformations in parallel, which could give additional information about the PGH synthase reaction mechanism, compared with results obtained with its natural substrate arachidonic acid.

EXPERIMENTAL PROCEDURES

Materials were purchased from the following sources. cis,cis-Eicosa-11,14-dienoic acid, hematin, diethyldithiocarbamate were from Sigma; phenol was from British Drug House; DE-53 ion exchange chromatography gel was from Whatman; and Tween 20 was from J. T. Baker Chemical Co.

PGH synthase was isolated from ram seminal vesicles microsomes using the method of Marnett et al. (16), modified by MacDonald and Dunford (17). Cyclooxygenase activity was measured with a Clark-type polarographic electrode by monitoring oxygen consumption in the reaction of arachidonic acid with PGH synthase in 0.1 M phosphate buffer, pH 8.0, containing 200 μM arachidonic acid, 1 μM hematin, and 1 mM phenol. Oxygen calibration was performed according to Robinson and Cooper (18), and protein content was measured by the Bio-Rad protein assay using bovine serum albumin as a standard. Preparations of PGH synthase contained 30–50% holoenzyme and consumed 20–40 μmol of oxygen/min/mg of protein; for kinetic measurements, they were reconstituted with the appropriate amount of hematin (19). The kinetic experiments were conducted on an Applied Photophysics SX.17MV (Micro-Volume) stopped flow reaction analyzer. Parallel kinetic runs at 234 nm (absorption peak of conjugated diene products), 414 nm (isosbestic point between compound I and resting enzyme) were performed, when necessary, by passing the enzyme solution through Sephadex G-25 (medium) preequilibrated with 0.1 M phosphate buffer, pH 8.0, containing 30% glycerol (24). Data were analyzed using a stopped flow reaction analyser kinetic software and a pro Fit data analysis program (Cherwell Scientific, UK).

RESULTS

The spontaneous oxygenase reaction was studied using 0.75 μM PGH synthase and 10–170 μM 20:2. Typical kinetic traces of the initial phase at different concentrations of 20:2 and 20:2

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1 The abbreviations used are: PGH synthase, prostaglandin endoperoxide synthase; Porph, protoporphyrin IX; TyrOH, tyrosyl residue; 20:2, cis,cis-eicosa-11,14-dienoic acid; DDC, diethyldithiocarbamate.
hydroperoxide are shown in Fig. 1. The common feature of all these experiments is the almost simultaneous formation of compound I and intermediate II and considerable delay in appearance of conjugated diene products. Because addition of oxygen to the fatty acid radical is very fast, formation of the conjugated diene products reflects the rate of hydrogen atom abstraction from 20:2 by PGH synthase.

The formation of intermediate II and oxidation of 20:2 at different concentrations of 20:2 and constant concentration of 20:2 hydroperoxide is shown in Fig. 2. An increase in 20:2 concentration stimulates both formation of intermediate II and oxidation of 20:2, but again, transformation of substrate noticeably lags the enzyme conversion.

The reaction of PGH synthase with 20:2 was performed under single turnover conditions using an approximately equimolar amount of 20:2 hydroperoxide to prevent the reaction of excess hydroperoxide with the compound I to produce compound II (19, 25). Kinetic traces for the system containing 0.77 \( \mu \)M PGH synthase, 0.83 \( \mu \)M 20:2 hydroperoxide, 20:2, 9 \( \mu \)M; Formation of reaction products was monitored at 234 nm (absorption peak of conjugated dienes), formation of compound I at 414 nm (isosbestic point between compound I and intermediate II), and formation of intermediate II at 426 nm (isosbestic point between resting enzyme and compound I). Dotted lines represent original kinetic traces, and solid lines represent traces averaged over five successive points (total number of recorded points = 400).

**FIG. 2.** Influence of [20:2] on the formation of intermediate II (solid lines) and oxidation of 20:2 (dotted lines). The reaction mixture contained 0.75 \( \mu \)M PGH synthase, 1.9 \( \mu \)M 20:2 hydroperoxide, and the following concentrations of 20:2: 44.7 \( \mu \)M (traces marked 1), 88.4 \( \mu \)M (traces marked 2), and 131.5 \( \mu \)M (traces marked 3). Kinetic traces consist of averages of each successive five points (total number of recorded points = 400).

**FIG. 3.** Kinetics of interaction of PGH synthase and 20:2 at stochiometric level of 20:2 hydroperoxide. PGH synthase, 0.77 \( \mu \)M; hydroperoxide of 20:2, 0.83 \( \mu \)M; 20:2, 9 \( \mu \)M. Formation of reaction products was monitored at 234 nm (absorption peak of conjugated dienes), formation of compound I at 414 nm (isosbestic point between compound I and intermediate II), and formation of intermediate II at 426 nm (isosbestic point between resting enzyme and compound I). Dotted lines represent original kinetic traces, and solid lines represent traces averaged over five successive points.

**FIG. 4.** Effect of DDC on intermediate II formation. \( k_{obs} \) values were found from exponential fits of kinetic traces of intermediate II formation at 426 nm. Reaction conditions: PGH synthase, 0.73 \( \mu \)M; 20:2 hydroperoxide, 0.86 \( \mu \)M; 20:2, 4.2 \( \mu \)M; DDC 3.1, 6.8, and 11.0 \( \mu \)M.
absorbance traces can be compared on an approximately equimolar basis.

DDC is commonly used as a stabilizer during purification and storage of PGH synthase. Under our conditions, it was present in the final mixture in micromolar concentrations, and its reducing action could result in formation of compound II (24). This possibility was tested by performing the reaction at different levels of DDC. The kinetics of appearance of compound II/intermediate II were fitted to a single exponential function, and $k_{obs}$ values obtained in this way were plotted against [DDC] (Fig. 4). This plot indicates that both DDC-dependent and -independent reactions contribute to the observed reactions. The DDC-dependent part corresponds to reduction of compound I to compound II, whereas the DDC-independent part corresponds to the spontaneous conversion of compound I to intermediate II.

To achieve greater clarity on the role of DDC, it was replaced by glycerol as stabilizing agent. This resulted in a considerable decrease of the rate of change in absorbance. However, the lag between the kinetics of appearance of intermediate II and oxidation of 20:2 remained. As a typical example Fig. 5 shows kinetic traces for the reaction of PGH synthase with 1.3 equivalents of 20:2 hydroperoxide and 5 equivalents of 20:2 in a DDC-free medium.

**DISCUSSION**

A published mechanism of the oxygenase reaction of PGH synthase (E) with 20:2 (AH) includes the following initial steps up to the rate-limiting formation of eicosadienoyl radical (12–14),

$$E + \text{AOOH} \rightarrow E-I + \text{AOH} \quad \text{(Reaction 1)}$$

$$E-I + AH \rightarrow E-II + A' \quad \text{(Reaction 2)}$$

**Scheme 1**

where E-I is compound I and E-II is compound II. The initiation role of compound I in fatty acid oxidation, proposed by this scheme, disagrees with the observed asynchronous oxidation AH and formation of E-II. Possible alternatives, based on the unimolecular transformation of E-I into intermediate II, E-I-
Tyr, which contains the same number of oxidizing equivalents as compound I (6), or reaction of E-I with a second molecule of hydroperoxide (19), are shown below.

\[
E + AOOH \rightarrow E-I + AOH \quad (\text{Reaction 1})
\]

\[
E-I \rightarrow E-I-Tyr' \quad (\text{Reaction 3})
\]

\[
E-I-Tyr' + AH \rightarrow E-II + A' \quad (\text{Reaction 4})
\]

**Scheme 2**

\[
E + AOOH \rightarrow E-I + AOH \quad (\text{Reaction 1})
\]

\[
E-I + AOOH \rightarrow E-I-Tyr' + AOOH \quad (\text{Reaction 5})
\]

\[
E-I-Tyr' + AH \rightarrow E-II + A' \quad (\text{Reaction 4})
\]

**Scheme 3**

Reaction 5 in Scheme 3 is the sum of the following two consecutive reactions.

\[
E-I + AOOH \rightarrow E-II + AOO' \quad (\text{Reaction 6})
\]

\[
E-II + AOO' \rightarrow E-I-Tyr' + AOOH \quad (\text{Reaction 7})
\]

**Scheme 4**

To determine the rate constant for compound I formation (Reaction 1) kinetic traces at 414 nm were obtained over a wide range of concentrations of 20:2 hydroperoxide. Initial parts of these curves were fitted to a single exponential function, and the following rate constants for reactions (Reactions 3–5) were estimated from the literature (6, 12, 19, 26), and the following values were used in the simulations: for Scheme 2, \( k_3 = 65 \text{ s}^{-1} \), \( k_4 = 3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \); for Scheme 3, \( k_5 = 3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \), \( k_6 = 3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \).

The behavior of these reaction schemes in relation to 20:2 consumption and production of intermediate II and compound II was tested with a Monte-Carlo method using a CKS simulation program (IBM). Fig. 7 demonstrates experimental and simulated kinetics, using the above rate constants for initiation of the oxygenase reaction with 1.2 or 6.7 eq of 20:2 hydroperoxide. It shows that the asynchronous oxidation of substrate and appearance of intermediate II at excess of hydroperoxide can be qualitatively predicted by both Schemes 2 and 3, but for a stoichiometric level of hydroperoxide, the rate of oxidation of 20:2 is far too small according to Scheme 3.

Reactions of compound I of PGH synthase are of crucial importance for understanding the catalytic mechanism of this enzyme. Since compound I can convert into the spectrally indistinguishable compound I-tyrosyl radical and compound II, the interpretation of the kinetics of compound I decay becomes difficult. Originally, the enzyme product of compound I decay in the oxygenase reaction of PGH synthase with 20:2 was interpreted as production of compound II, based on the fact that conversion of compound I was a function of fatty acid concentration, indicating a bimolecular reaction. This led to the proposal of Reaction 2 (12) with a delayed formation of the tyrosyl radical in Reactions 6 and 7 (19). However, when both participants of the reaction are monitored, it is apparent that appearance of intermediate II precedes oxidation of fatty acid, though both reactions are functions of the concentration of 20:2. This suggests that dependence of intermediate II production on the concentration of 20:2 is an indirect effect. The stimulation of intermediate II formation by 20:2 could be the result of increased occupation of the fatty acid binding site. This could be the basis of a switching mechanism which would favor initiation of cyclooxygenase cycle and prevent conversion of compound I to compound II when peroxidase reducing substrates are present.

In this light, conversion of compound I into compound II/intermediate II in the reaction of PGH synthase with 20:2 could be caused by one the following: (a) reduction of compound I by DDC used as stabilizing agent (24); (b) reaction of compound I with a second molecule of hydroperoxide (19); or (c) intramolecular electron transfer from Tyr-385 to the porphyrin \( \pi \)-cation radical (6).

Participation of DDC in intermediate II formation was excluded by the results obtained in a DDC-free medium. The relative significance of bimolecular and unimolecular paths for formation of intermediate II (reasons b and c) apparently depends on the level of hydroperoxide. However, the presence of a large excess of hydroperoxide in vivo appears unlikely, which leaves the intramolecular electron transfer as the most likely route to generate the tyrosyl radical.

From the crystal structure, the arachidonic acid binding site was shown to be located on the proximal side of the heme (27). The role of the Tyr-385 radical in the oxidation of arachidonic acid is now firmly established (28). From the present results, it would appear that the initiation of the oxidation of the eicosadienoic acid proceeds by a similar mechanism.

**REFERENCES**


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doi: 10.1074/jbc.273.11.6046

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