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Prostaglandin H synthase

Inactivation of the enzyme in the course of catalysis is accompanied by fast and dramatic changes in protein structure

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Prostaglandin H synthase (PGHS) as apo-PGHS, holo-PGHS, and holo-PGHS, inactivated in the course of catalysis was studied using chemical modification with diethyl pyrocarbonate (DEPC). The exhausted reaction with DEPC corresponded to the modification of 7 histidine residues in apo-PGHS and 4 in holo-PGHS. All 18 histidine residues became accessible for modification with DEPC in the enzyme, inactivated in the course of catalysis. The velocities of tryptic cleavage of all the three forms into two fragments were fairly different but independent of modification. Based on the results we hypothesize fast and dramatic changes in the protein structure in the course of the substrate conversion.

Prostaglandin H synthase; Diethyl pyrocarbonate; Inactivation in the course of the reaction; Chemical modification

1. INTRODUCTION

Prostaglandin H synthase (PGHS) (EC 1.14.99.1) is the first and rate-limiting enzyme in the pathway in which polyunsaturated fatty acids (PUFA) are converted into prostanoids [1–3]. The enzyme displays cyclooxygenase and peroxidase activities and requires heme [1–3].

The requirements of the enzyme active centre for the PUFA nature are very strict [3–5]. In addition, great demands are known for the structural correlation between enzyme and cofactor [6–12].

Several lines of evidence suggest an important role of histidine residues in the catalysis by PGHS [6–8]. We pointed out in preliminary studies [6,7] that their chemical modification with DEPC is accompanied by inactivation of the enzyme. The loss of enzyme activity was also shown to result from site-directed replacement of histidine residues with alanine or glutamine [7]. The authors [7] assumed His-309 as the axial ligand for heme binding (numeration of amino acid residues here and further is given according to ref. [13]). An alternative version based on the data of the chemical modification and the properties of mutant PGHS implies a prominent role in heme binding of Tyr-385 [9,10].

In a previous report [11] we demonstrated that the substitution of the vinyl groups of protohemin IX with ethyl groups or hydrogen affects neither binding of the prosthetic group to the apo-enzyme nor catalytic properties of holo-PGHS; however, the bulkier substitutes (hydroxyethyl or acetyl groups) markedly decreased the holo-enzyme stability and catalytic activity. In addition protohemin IX with both carboxyl groups substituted lost the cofactor ability while the modification of one carboxyl did not influence the enzyme activity [12].

PGHS displays a phenomenon of fast and irreversible self-inactivation in the course of the reaction [14]. It has been suggested that two of at least seven intermediates in the process of the catalysis were responsible for the inactivation [14–17]. The enzyme inactivation is accompanied by hemoprotein radicals formation [18] and relatively slow destruction of the protein–heme complex [19].

In this report we demonstrate the results of the chemical modification of histidine residues with DEPC in apo- and holo-PGHS and in holo-PGHS inactivated in the process of arachidonic acid conversion into prostaglandin H_2 and analysis of the content of the modified residues in 33 and 38 kDa fragments.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Arachidonic acid (AA), L-adrenaline and acetonitrile were products of Merck (Germany); hemine, L-histidine, diethyl pyrocarbonate (DEPC) were from Serva (USA), trypsin was from Worthington Biochemicals (USA), hydroxylamine from Fluka (Switzerland), *p*-APMSF from Calbiochem (USA).

2.2. *Preparation of PGHS patterns*

Homogenous apo-PGHS was obtained from ram seminal glands as

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described previously [32,33]. Holo-PGHS was prepared by adding 100 μl 200 μM hemine and 20 μl 100 mM adrenalin to 1 ml $6 \cdot 10^{-6}$ M PGHS in Tris buffer pH 7.8 with 0.1% Tween 20 at 25°C. The enzyme was inactivated in the course of the reaction was holo-PGHS incubated with $1.5 \cdot 10^{-5}$ M AA for 5 min at 25°C.

2.3. *Assay of enzyme activities*

Spectrophotometric assay of PGHS combined cyclo-oxygenase plus peroxidase and peroxidase activities was done using saturating concentrations of either AA or H_2O_2 with adrenalin as an electron donor. Both activities were detected at 480 nm (JASCO or Shimadzu) [8,12]. The enzyme concentration was $5–7 \cdot 10^{-8}$ M. The activities were estimated as initial velocities taken from the recorded kinetics at the first seconds after mixing.

2.4. *Modification with DEPC*

The enzyme solutions were adjusted to pH 6.0 and fresh prepared DEPC solution was added to each pattern (molar ratio 40:1). UV differential spectra were recorded with a JASCO spectrophotometer at wavelengths 200–300 nm. The quantities of modified histidine residues were calculated using an absorption coefficient 3,200 $M^{-1} \cdot cm^{-1}$ at 240 nm [20]. Aliquots were taken periodically and assayed for PGHS activities.

2.5. *Tryptic cleavage of PGHS*

All the three patterns of the enzyme and the respective modified forms were treated in Tris buffer pH 8.0 at 10°C with trypsin using 10:1 molar ratio of 70 kDa subunit to trypsin. 20 μl aliquots were taken for electrophoretic assay and 150 μl aliquots for HPLC assay of the cleavage velocity. The reaction was stopped by adding *p*-APMSF (1:1).

2.6. *HPLC assay of the tryptic digests*

The assay was done as described previously [9] at Machery Nagel C4 Nucleosil 250 × 4 mm column using Beckman System Gold or Altex 420/100 chromatographs. The detection was performed at 280 and 206 nm (Altex) or 230 nm (Beckman). For spectrophotometric assay peptide samples from several experiments were pooled.

2.7. *Spectrophotometric assay of modified peptides*

UV spectra of 33 and 38 kDa fragments were recorded with a JASCO spectrophotometer at 200–300 nm. 1 ml Tris buffer with 0.24 mg of 33 kDa peptide or 0.34 mg of 38 kDa peptide from the native enzyme were used as a reference. The concentrations of modified peptides were adjusted to reference solution by dilution or concentration.

3. RESULTS AND DISCUSSION

The purpose of this study was to investigate the regularities of DEPC interaction with holo-PGHS inactivated in the course of catalysis as compared with apo- and holo-PGHS. All three forms of the enzyme reacted with DEPC under conditions most specific for modification of histidine residues [20,21]. The most impressive changes in UV spectra of the reaction media at 240 nm occurred during the first 3–5 min and finished after 20 min of incubation. As seen in Fig. 1 the maximal absorptions corresponded to the modification of 4–5 histidine residues in holo-PGHS and 7–8 in apo-PGHS. In the case of holo-PGHS inactivated in the course of the reaction all the 18 histidine residues present in the enzyme molecule became accessible for modification with DEPC. The concert and practically complete loss of the combined cyclooxygenase plus peroxidase and peroxidase activities was observed within 2 min for the enzyme modified to the apo-form (Fig. 2). The respective activity changes in the holo-enzyme were not significant. The degree of modification during 2 min corresponded to the involvement of 5–6 histidine residues in apo-PGHS and 1–2 in holo-PGHS. Treatment of the enzyme patterns with hydroxylamine at pH 7 was accompanied by the removal of some groups and the partial reduction of the enzyme activities (data not shown).

Trypsin [22,23], proteinase K and chymotrypsin [23]

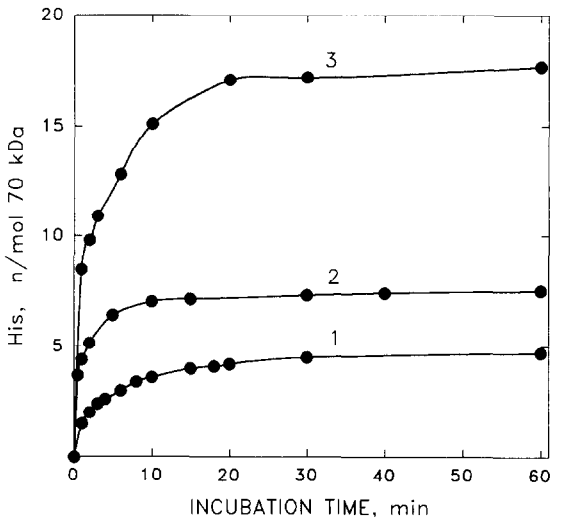

Fig. 1. Modification of PGHS by DEPC. PGHS ($5.2–8.0 \cdot 10^{-6}$ M) as holo- (curve 1), apo- (curve 2) and holo-enzyme inactivated in the course of the reaction (curve 3) was incubated with DEPC (molar ratio 1:40) at pH 6 and 25°C. The values on the ordinate represent the number of modified His residues per 1 mol of 70 kDa protein.

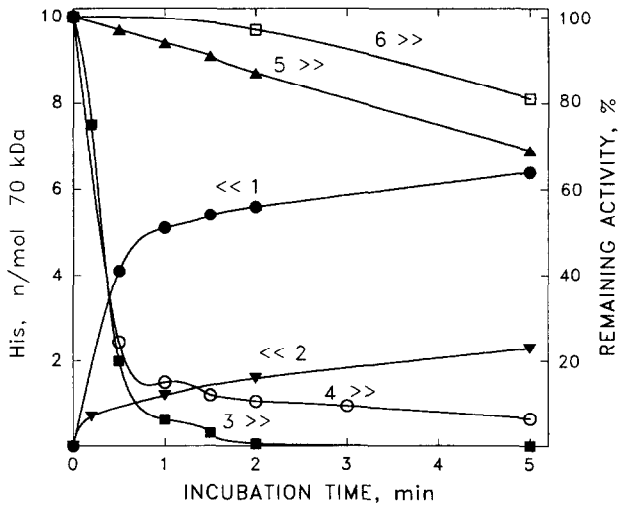

Fig. 2. Kinetics of histidine residue modification with DEPC (left ordinate) and PGHS inactivation (right ordinate). For reaction conditions see Fig. 1. Curve 1, apo-PGHS + DEPC; curve 2, holo-PGHS + DEPC; curve 3, combined cyclo-oxygenase plus peroxidase activity corresponded to curve 1; curve 4, peroxidase activity corresponded to curve 1; curve 5, combined cyclooxygenase plus peroxidase activity corresponded to curve 2; curve 6, control of combined cyclo-oxygenase plus peroxidase activity of holo-PGHS.

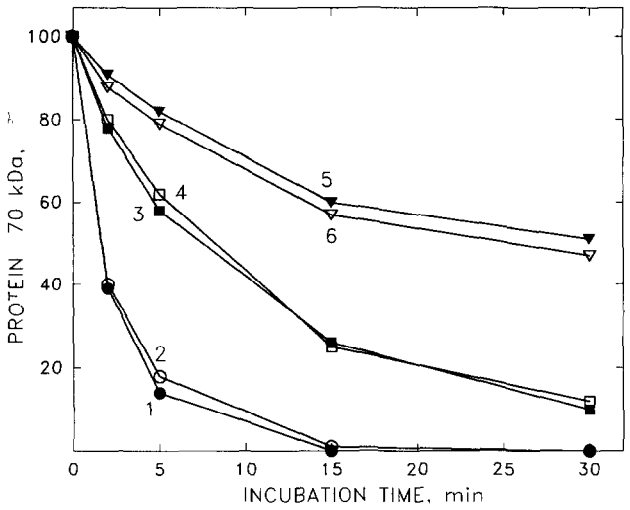

Fig. 3. Kinetics of PGHS cleavage into fragments 33 and 38 kDa by trypsin. Curves 1, 3 and 5 correspond to modified patterns of holo-PGHS inactivated in the course of reaction, apo-PGHS and holo-PGHS respectively. Curves 2, 4 and 6 correspond to non-modified patterns 1, 3 and 5.

have been reported to split apo-PGHS (70 kDa) into two fragments of 33 and 38 kDa. Under our experimental conditions (see section 2), trypsin (Worthington Biochemicals) hydrolysed all three forms of PGHS and its modified forms (Fig. 3). The cleavage velocity of the enzyme inactivated in the course of the reaction was remarkably high as compared with apo- and holo-enzyme. Our results demonstrate that the modification of the different enzyme patterns did not affect the velocity of their cleavage by trypsin.

Spectrophotometric data on the 33 and 38 kDa fragments, assayed by the HPLC method for the modified histidine residues in them, are listed in Table I. The results obtained show that two of the three modified residues are located in the C-terminal fragment of the enzyme.

Our experimental results and comparison of the most homologous regions for histidine of PGHS from various sources [13] with the known heme binding regions in oxidoreductases [24] allow us to suggest that heme is most likely to shield the His-309 and His-388 residues. This supposition is in line with the data on site-directed replacement of histidine residues by alanine or glutamine [7].

We have shown that all 18 histidine residues are accessible to DEPC modification of the enzyme, inactivated in the course of the reaction, i.e. after 2–3 min incubation in the full reaction media. This suggests that the enzyme inactivation through reaction is accompanied by fast (comparable with time of inactivation) and impressive changes in the protein structure.

From the data on abnormally high rates of trypsin hydrolysis of the enzyme inactivated in the course of reaction, and those on a faster hydrolysis of the homogeneous PGHS samples with a low specific activity [25] it follows that the enzyme inactivation in vivo prepares PGHS for its rapid utilisation by proteolytic enzymes. Obviously, quick structural changes in the course of the reaction are a necessary link in the sequence of events regulating the level of highly physiologically active compounds in mammalian tissues and organs.

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Table I

The number of modified histidine residues per molecule of PGHS and its fragments of tryptic cleavage (His, *n*)

Enzyme form	70 kDa			33 kDa			38 kDa		
	D_{240}	C (μM)	His, *n*	D_{240}	C (μM)	His, *n*	D_{240}	C (μM)	His, *n*
Holo-PGHS inactivated in the course of catalysis	0.295	5.2	18	0.233	7.3	9	0.286	8.9	9
Holo-PGHS	0.108	8.0	4	0.047	7.3	2	0.058	8.9	2
Apo-PGHS	0.166	6.7	7	0.049	7.3	2	0.143	8.9	5

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