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STEROID HYDROPEROXIDES AS SUBSTRATES FOR GLUTATHIONE PEROXIDASE

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SUMMARY

1. Several steroid hydroperoxides were found to serve as substrates for glutathione peroxidase (glutathione:H_2O_2 oxidoreductase, EC 1.11.1.9), with progesterone 17α-hydroperoxide being particularly effective. An apparent K_m of $4 \cdot 10^{-5}$ M was obtained with this substrate at pH 8 using 0.25 mM GSH.

2. Cholesterol 25-hydroperoxide would not function as a substrate and is the first hydroperoxide so far reported that cannot be metabolized by glutathione peroxidase.

3. The enzyme was found to occur in several steroid metabolizing tissues.

INTRODUCTION

GSH peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) is a very active enzyme able to catalyze the reduction of a wide range of hydroperoxides. With lipid hydroperoxides, the enzyme produces hydroxides without forming the highly reactive free radical intermediates which occur when transition metal compounds react with hydroperoxides[1–4]. The physiological hydroperoxides, hydrogen peroxide[5] and lipid hydroperoxides[6] are rapidly reduced by the enzyme. Thus, GSH peroxidase has been suggested to be an intracellular detoxifier of hydroperoxides[7], partially responsible for controlling hydrogen peroxide levels[8] and protecting cell membranes from oxidative damage[1]. In addition, steroid hydroperoxides have been suggested to occur physiologically as intermediates in steroid hydroxylations[9–13]. The main aim of the present work was to determine whether steroid hydroperoxides might serve as sub-

Trivial names used for steroids: progesterone 17α-hydroperoxide, 4-pregnene-3,20-dione-17αhydroperoxide; allopregnanolone 17α-hydroperoxide, 5α-pregnan-3β-ol, 20-one-17α-hydroperoxide; pregnenolone 17α-hydroperoxide, 5-pregnen-3β-ol, 20-one-17α-hydroperoxide; cholesterol 7β-hydroperoxide, 3β-hydroxycholest-5-ene-7β-hydroperoxide; 6-ketocholestanol 5α-hydroperoxide, 5α-cholestan-3β-ol-6-one-5α-hydroperoxide; cholesterol 25-hydroperoxide, 3β-hydroxycholest-5-ene-25-hydroperoxide; 3β-acetoxycholesterol, 3β-acetoxycholest-5-ene.

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strates for GSH peroxidase and if so, then to examine the characteristics of the reaction. Because of its relative ease of preparation, progesterone 17α-hydroperoxide was used in most experiments.

MATERIALS AND METHODS

Materials

GSH peroxidase was prepared from pig erythrocytes as described by Little *et al.*[14]. Progesterone 17α-hydroperoxide, allopregnanolone 17α-hydroperoxide, pregnenolone 17α-hydroperoxide and 6-ketocholestanol 5α-hydroperoxide were prepared by the method of Bailey *et al.*[15]. Cholesterol 25-hydroperoxide was prepared by the autoxidation of cholesterol as described by van Lier and Smith[16]. Cholesterol 7β-hydroperoxide was prepared by the autoxidation of 3β-acetoxycholesterol (E. G. Hrycay, P. J. O'Brien, G. Kan and J. E. van Lier, unpublished). Linoleic acid hydroperoxide was prepared as described by Little and O'Brien[17]. Cumene hydroperoxide was obtained from Matheson, Coleman and Bell and hydrogen peroxide from J. T. Baker Chemical Company. All biochemicals were obtained from Sigma. Chromatographic solvents and other chemicals used were of analytical reagent grade of purity.

Enzyme assay

GSH peroxidase was assayed at 23 °C by the method of Paglia and Valentine[18], as modified by Little *et al.*[14]. The enzyme is coupled to NADPH *via* GSSG reductase (EC 1.6.4.2) and the rate of NADPH oxidation is measured spectrophotometrically at 340 nm. Unless otherwise stated, the reaction mixture (1 ml) contained 0.12 mM NADPH, 0.1 M Tris–HCl buffer (pH 8), 0.25 mM GSH, 3 mM EDTA, 0.08 mM hydroperoxide and a large excess of GSSG reductase. The reaction was started by the addition of GSH peroxidase (approx. 0.2 μg). Throughout the experiments repeated checks were made to ensure that the reductase level was not rate-limiting. In the absence of peroxidase, a small uncatalyzed reaction rate was observed. Except where hydrogen peroxide was used, the uncatalyzed rate was $<5\%$ of the total rate. Enzymic reaction rates were calculated from: Enzymic rate = total rate − rate without peroxidase. To assist steroid solubility, the assay medium contained 15% (w/v) ethanol. In view of the high stability of GSH peroxidase in aqueous ethanol[14], the presence of 15% (w/v) ethanol inhibited the enzyme by $<10\%$.

Chromatographic procedure

Thin-layer chromatography was conducted on 20 cm × 20 cm chromatoplates of silica gel N-HR (Machery-Nagel Company) of 0.2 mm thickness. Visualization was effected by spraying the air dried chromatoplate with 50% aqueous sulfuric acid followed by heating at 110 °C for 15 min and noting the color display during this period. Alternatively, for detection of hydroperoxides, the chromatoplates were sprayed with ammonium thiocyanate–ferrous sulfate solution (Waldo No. 5 spray)[19].

Tissue preparation

Tissues were obtained from Sprague–Dawley rats, 8 to 10 weeks old and were homogenized in 0.3 M sucrose solution and sonicated for 2 min prior to enzyme assay. The pooled tissue of 6 rats, either all male or all female, were used.

Protein was assayed by the Biuret method.

TABLE I

GSH PEROXIDASE ACTIVITY IN THE PRESENCE OF VARIOUS STEROIDAL AND OTHER HYDROPEROXIDES

GSH peroxidase was assayed at pH 8 as described in Materials and Methods. The assay system contained 15% ethanol, $8 \cdot 10^{-5}$ M hydroperoxide and 0.26 mM GSH. Steroid hydroperoxides were introduced into the assay system as solutions in 95% ethanol.

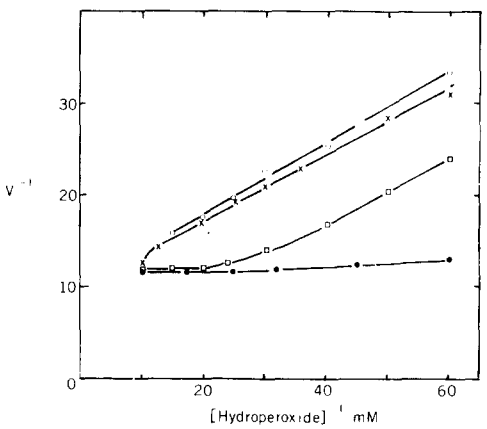
Hydroperoxide	*GSH peroxidase activity (GSH oxidation, Moles/min × 10^5)*
Hydrogen peroxide	8.7
Linoleic acid hydroperoxide	8.5
Cumene hydroperoxide	6.7
Progesterone 17α-hydroperoxide	6.3
Allopregnanolone 17α-hydroperoxide	1.6
Pregnenolone 17α-hydroperoxide	1.6
Cholesterol 7β-hydroperoxide	0.4
6-Ketocholestanol 5α-hydroperoxide	0
Cholesterol 25-hydroperoxide	0

RESULTS

In the presence of GSH peroxidase, progesterone 17α-hydroperoxide caused a rapid oxidation of GSH to GSSG, clearly demonstrating that this hydroperoxide can serve as a substrate for the enzyme. In order to assess how good a substrate this is for the enzyme, the reaction rate with progresterone 17α-hydroperoxide was compared with that for several other hydroperoxides. Table I shows that under the assay conditions used, progesterone 17α-hydroperoxide gave a reaction rate very similar to that obtained with cumene hydroperoxide and about 75% of the rate with hydrogen peroxide or linoleic acid hydroperoxide. Compounds such as allopregnanolone 17α-hydroperoxide and pregnenolone 17α-hydroperoxide which are closely related to progesterone 17α-hydroperoxide both gave rates about 25% of that obtained with the latter. Three hydroperoxides related to cholesterol were also used. Of these, only cholesterol 7β-hydroperoxide gave a reliably measurable rate. 6-Ketocholestanol 5α-hydroperoxide caused a rapid denaturation and precipitation of the enzyme. Even the addition of hydrogen peroxide to assay systems containing 6-ketocholestanol 5α-hydroperoxide gave no activity. In view of this effect, it was not possible to decide whether or not this compound could be metabolized by the enzyme. Cholesterol 25-hydroperoxide gave no reaction with the peroxidase and also caused no denaturation, since when hydrogen peroxide was added to an assay system already containing cholesterol 25-hydroperoxide, the usual rate for hydrogen peroxide was obtained. Additionally, samples from the same batch of cholesterol 25-hydroperoxide were active with cytochrome P450 (E.G. Hrycay, P. J. O'Brien, G. Kan and J. E. van Lier, unpublished), indicating that the inactivity with GSH peroxidase cannot be ascribed to previous decomposition of the hydroperoxide. Clearly, therefore, cholesterol 25-hydroperoxide cannot serve as a substrate for GSH peroxidase.

The saturation characteristics of the enzyme with respect to the steroid hydroperoxide substrates were examined using progesterone 17α-hydroperoxide. The dependence of rate on substrate concentration was examined for four hydroperoxides and the data plotted after the fashion of Lineweaver and Burk (Fig. 1). It is apparent

that cumene hydroperoxide and progesterone 17α-hydroperoxides are equally good substrates for the enzyme. The apparent K_m for each is approximately $4 \cdot 10^{-5}$ M, which is substantially higher than that for hydrogen peroxide. At substrate levels above 10^{-4} M, a rate enhancement occurs with cumene hydroperoxide causing deviations from a linear Lineweaver–Burk plot[14]. The possibility of such behaviour with the steroid hydroperoxide could not be examined because of enzyme denaturation at high steroid concentrations.



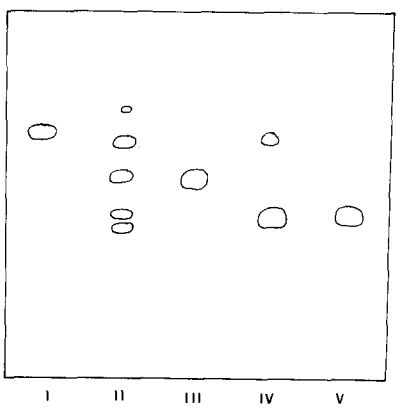


Fig. 1. Lineweaver–Burk plot for GSH peroxidase. The enzyme was assayed at pH 8 and 0.25 mM GSH as described in Materials and Methods. The assay system contained 15% (w/v) ethanol. Hydroperoxide substrates used were progesterone-17α-hydroperoxide (○), cumene hydroperoxide (×), linoleic acid hydroperoxide (□) and hydrogen peroxide (●). The reaction was started by the addition of 0.42 μg peroxidase. The reaction rate, v, was expressed as 10^{-1} × change in absorbance at 340 nm/min.

Fig. 2. Decomposition patterns of progesterone 17α-hydroperoxide. I. Progesterone 17α-hydroperoxide marker. II. 0.1 mM hydroperoxide incubated at 30 °C for 3 min with 5 μM cytochrome *c* in 0.1 M Tris–HCl buffer (pH 8). The solution was then extracted three times with one vol. of ethyl acetate. The extract was evaporated to near dryness by a jet of nitrogen. III. The hydroperoxide was gently heated until it had all melted and then cooled and dissolved in a little ethyl acetate. IV. 0.1 mM hydroperoxide, 0.1 M Tris–HCl buffer (pH 8), 0.3 mM GSH, and 0.2 mg GSH peroxidase were incubated at 30 °C for 30 min and extracted as with II. V. Progesterone 17α-hydroxide marker. The chromatogram was developed in benzene–ethyl acetate (3:2, v/v) and visualized with 50% H_2SO_4 spray. The figure represents a sketch of the developed visualized chromatogram.

To characterize further the reaction between the steroid hydroperoxide and the peroxidase, the decomposition products of progesterone 17α-hydroperoxide were examined by thin-layer chromatography. Fig. 2 represents a sketch of a thin-layer chromatogram of progesterone 17α-hydroperoxide after treatment with GSH peroxidase *plus* GSH, cytochrome *c* and heat, with certain markers being included. Peroxidase treatment yielded two spots. The faster moving component had an R_F identical to that of the hydroperoxide marker and, like the marker, gave a yellow colour with 50% H_2SO_4 spray and a positive reaction with the ammonium thiocyanate–ferrous sulfate spray. The slower moving component had an R_F identical to that of 17α-hydroxyprogesterone and, like this marker, produced a brown colour with 50% H_2SO_4 spray. Similar results were obtained when four different solvent systems were

used to develop the chromatogram (benzene–ethyl acetate (3:2, v/v); benzene–ethyl acetate (1:3, v/v); chloroform–acetone (9:1, v/v); diethyl ether–benzene (1:1, v/v)). On the basis of these chromatographic studies, it was tentatively concluded that GSH peroxidase reduced the steroid hydroperoxide to the corresponding hydroxysteroid and that despite the presence of excess GSH and long incubation times, some undecomposed hydroperoxide remained. Presumably enzyme denaturation by the steroid prevented the reaction from going to completion. The very simple decomposition pattern resulting from enzymic treatment of the steroid hydroperoxide is in marked contrast to the range of products following cytochrome *c* catalyzed decomposition. These results are similar to those obtained with linoleic acid hydroperoxide which is converted to the corresponding hydroxy compound by the peroxidase and to a complex range of products by hemoproteins[1,3]. Thermal decomposition of progesterone 17α-hydroperoxide, however, yielded a single unidentified spot on the chromatoplate.

Since certain of the steroid hydroperoxides tested seemed good substrates for GSH peroxidase, the possibility that steroid hyperoxides might be normal metabolic substrates for the enzyme was considered. Thus, GSH peroxidase levels in several steroid metabolizing tissues were measured and compared with the activity in blood. Because of the effect of sex factors on the level of this enzyme[20], males and females were examined separately. It is apparent from Table II that in the rat, whilst testis

TABLE II

GSH PEROXIDASE ACTIVITY IN STEROID METABOLIZING TISSUES

GSH peroxidase was assayed as described in Materials and Methods. $4 \cdot 10^{-4}$ M cumene hydroperoxide, 0.25 mM GSH and a pH of 7.0 were used. The rate of GSH oxidation was calculated from the rate of NADPH oxidation.

Tissue	*GSH peroxidase activity (rate of GSH oxidation, Moles/min per mg per ml protein × 10^4)*	
	Males	*Females*
Liver	4.2	7.0
Kidney	2.8	2.5
Adrenals	2.4	2.2
Blood	1.8	3.2
Testis	0.6	—
Uterus	—	0.9

and uterus contain relatively low, but still significant levels of enzyme, liver, adrenals and kidney are rich in activity. Thus, all five steroid metabolizing tissues contain significant levels of GSH peroxidase. It may also be noted that consistent with the results of Pinto and Bartley[20], activity levels in liver and blood are significantly higher in females than in males. However, no corresponding differences were noted in kidney and adrenals.

DISCUSSION

It is apparent that certain steroid hydroperoxides can serve as substrates for GSH peroxidase, with progesterone 17α-hydroperoxide acting as a particularly good

one. This is further evidence for the remarkably low specificity shown by the peroxidase towards hydroperoxide substrates. Four of the six steroid hydroperoxides used were clearly able to act as substrates for the enzyme. The strong denaturant effect of 6-ketocholestanol-5α-hydroperoxide made it difficult to determine whether this compound could act as a substrate. The remaining hydroperoxide, namely cholesterol 25-hydroperoxide did not denature the enzyme, yet gave no detectable reaction rate. This is the first hydroperoxide so far reported that is definitely not metabolized by GSH peroxidase, an enzyme which accepts as substrate hydroperoxides as diverse as those of nucleic acids[21], lipids[7], hydrogen peroxide[5] and some steroid hydroperoxides. It is also of interest to note that cholesterol 25-hydroperoxide is a poor substrate relative to other steroid hydroperoxides for cytochrome P450 (E. G. Hrycay, P. J. O'Brien, G. Kan and J. E. van Lier, unpublished). Presumably some feature of the molecule renders the hydroperoxy group of cholesterol 25-hydroperoxide particularly inert. The present work indicates that amongst the steroid hydroperoxides, some are excellent substrates, others poor substrates and some unable to act as substrates at all. These findings contradict previous assumptions that GSH peroxidase metabolizes hydroperoxides of any structure at comparable rates[22].

In the rat, the steroid metabolizing tissues contain significant levels of GSH peroxidase and the possibility must be considered that steroid hydroperoxides are normal physiological substrates of the enzyme. The exact physiological relevance of such a reaction is not clear, however, hydroperoxides have been postulated as intermediates in the hydroxylation of steroids[9–13] and compounds such as fluorene[23] and tetralin[24]. Although no exhaustive analysis of the reaction products was carried out, the present results, together with those of Christophersen[1,2] are consistent with the view that the peroxidase quantitatively reduces hydroperoxides to the corresponding hydroxy derivatives. It is thus possible that one of the physiological roles of the enzyme may be the conversion of such intermediates to hydroxides.

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