AN INTRACELLULAR GSH-PEROXIDASE WITH A LIPID PEROXIDE SUBSTRATE

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Studies have been made on the kinetics, products and properties of liver supernatant-catalyzed GSH oxidation by linoleic acid hydroperoxide (LAHPO). It was found that an enzymic peroxidase was principally involved and not the hemoproteins previously suggested (Little & O'Brien, 1967a). The peroxidase was specific for thiols, especially GSH, as H-donors, but probably accepted any hydroperoxide as substrate. Unlike hemoprotein peroxidases, the enzyme was not inhibited by CN^-, N_3^-, F^- or peroxide. Furthermore, it is unlikely that the enzyme was a flavoprotein since although GSH-peroxidase was heat and acid labile the activity was not restored by FMN or FAD, and also not inhibited by the flavin analog quinacrine. The enzyme was readily and irreversibly inhibited by N-ethylmaleimide and p-chloromercuribenzoate (pCMB), but not by other thiol reagents. A limited purification of the enzyme was carried out.

METHODS: LAPHO was prepared as previously described (Little & O'Brien, 1967b), and ethyl linolenate hydroperoxide prepared in a similar manner. Subcellular fractions were prepared from perfused rat liver (Sedgwick & Hübscher, 1965). Protein was determined by the method of Weichselbaum (1946). The rate of GSH oxidation was measured at 20-23° by adding an excess of GSSG reductase (obtained from Sigma London Ltd) (1 µg) and 50 µM NADPH to the oxidation system (3 ml) and measuring the rate of NADPH oxidation spectrophotometrically at 340 nm. The rates of oxidation of

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other thiols were measured by assaying the residual thiol concentrations at different times during the reaction using the method of Boyer (1954).

All work reported in this paper, with the exception of that in Table 3, was carried out on a partially purified sample of enzyme from perfused rat liver supernatant (2 vol ethanol + 1 vol supernatant) at -20°. The precipitate was centrifuged and extracted with buffer as described below. Experiments were carried out on the resultant extract.

RESULTS AND DISCUSSION: The kinetics of GSH oxidation by LAHPO in the presence of liver supernatant were studied in the range 0 - 0.2 mM GSH and 0 - 0.1 mg/ml supernatant protein and found to be typical of enzymic peroxidases (Chance, 1949):

$$\frac{-d(GSH)}{dt} = k(GSH)(\text{supernatant protein}).$$

The value of k, the second order rate constant in sec^{-1} ml, mg^{-1} supernatant protein at pH 8 (0.1 M phosphate buffer) was 150 at 23°. Attempts to obtain zero order conditions with respect to GSH were unsuccessful because the high rate of uncatalyzed GSH oxidation by LAHPO made measurements at high GSH concentrations very difficult.

The above rate equation differs from that for the hemoprotein catalyzed reaction (Little & O'Brien, 1967c):

$$\frac{-d(GSH)}{dt} = k'(ROOH)(\text{hemoprotein}).$$

Furthermore, the effect of pH on the two mechanisms was different. The rate of the enzymic reaction was optimal at approximately pH 9 and decreased at more alkaline pH, whereas the hemoprotein catalyzed reaction was maximal at pH 9.2 and did not alter at higher pH (Little, 1966).

The reaction products of LAHPO (O'Brien, unpublished work) and of GSH were quite different in the two mechanisms. In the enzymic mechanism, quantitative oxidation of GSH to GSSG occurred. The reaction was fully reversible by NADPH with GSSG reductase. One equivalent of LAHPO oxidized 2 molar equivalents of GSH. In the hemoprotein mechanism, GSH was oxidized to GSSG (60%) and sulfonic acid (40%) (Little & O'Brien, 1967c). One equivalent of

LAHPO oxidized 0.7 molar equivalents of GSH in this mechanism. In the non enzymic mechanism, large amounts of LAHPO may be consumed in attacking the hematin ring of hemoproteins (Little & O'Brien, 1968). In the enzymic mechanism, even using crude liver supernatant containing both endogenous and exogenous hemoglobin and ascrobate, 1 equivalent of LAHPO oxidized 2 equivalents of GSH. Thus, in the presence of excess GSH, the peroxidase mechanism can account for virtually all lipid peroxide decomposition in liver supernatant.

TABLE I Substrate specificities of GSH-peroxidase

Reactions were carried out at 23° in 3 ml solution containing 0.25 mM thiol, 0.15 mM hydroperoxide, 5 μg supernatant protein/ml and 0.1 M tris buffer, pH 8.5. Reaction rates have been corrected for thiol oxidation in the absence of supernatant.

THIOL SPECIFICITY WITH LAHPO		PEROXIDE SPECIFICITY WITH GSH	
Thiol	Reaction rate (μM SH oxidized/min)	Hydroperoxide	Reaction rate (E_{340}/min)
GSH	13	LAHPO	0.04
Cysteamine	5.6	H_2O_2	0.028
Cysteine	2.7	Cumene-OOH	0.052
Thioglycolic acid	1.3	t-Butyl-OOH	0.036
		Ethyl linolenate-OOH	0.024

It can be seen (Table I) that in the enzymic reaction, all hydroperoxides gave a fairly similar reaction rate. In the hemoprotein mechanism, different hydroperoxides caused very different reaction rates (Little, 1966, Little & O'Brien, 1968). Menadione, ferricyanide and o-iodosobenzoate would not participate in either mechanism, although they did cause uncatalyzed oxidation of GSH. Thus, each mechanism was specific for peroxy electron donors. The enzymic mechanism also showed a clear preference for GSH as H-donor, whilst the rate of the hemoprotein catalyzed reaction varied with the pK_{SH} of the thiol used (Little & O'Brien, 1967c). The rate of the enzymic reaction was first order with respect to thiol concentration and showed little specificity for any particular hydroperoxide. Probably therefore, the rate-determining step in this mechanism involved a reaction with the thiol rather than with the hydroperoxide.

TABLE 2 Inhibitors of GSH-peroxidase

0.56 mg supernatant protein/ml was incubated at pH 8.5 and 20° for 5 min with the following and the residual activity of an aliquot was measured.

Inhibitor	% inhibition
50 mM KCN, NaN_3 or NaF	0
3 mM LAHPO or linoleate	16
3 mM $CdCl_2$ or $NaAsO_2$	0
3 mM iodoacetate or iodoacetamide	0
3 mM pCMB	69
3 mM N-ethylmaleimide	64
10 mM quinacrine	0

The enzymic activity was severely inhibited by preincubation of supernatant with pCMB or N-ethylmaleimide, but unaffected by iodoacetate or iodoacetamide or the dithiol reagents, Cd^{+2} and arsenite (Table 2). Inhibition by pCMB was not reversed by subsequent treatment of the enzyme with large excesses of GSH or cysteine, nor, surprisingly was the enzyme protected from inactivation by excess GSH or LAHPO. The slight inhibition of the enzyme by LAHPO was probably due to the surface activity of the lipid peroxide since linoleate was an equally effective inhibitor.

The catalytic activity of supernatant probably did not involve an enzymic hemoprotein peroxidase since the supernatant enzyme, unlike normal hemoprotein peroxidases (see Saunders et al., 1964) was not inhibited by 5 min incubation at pH 8.5 with 50 mM CN^-, N_3^- or F^- and was fairly resistant to damage by hydroperoxides.

The enzyme was probably not a flavoprotein since 5 min incubation at pH 8.5 and 20° with 10 mM quinacrine, a flavin analog did not inhibit. Although a 0.5 mg/ml supernatant fraction was 70% inhibited by 5 min incubation at pH 3.5 and 23° or by 5 min incubation at 60° and 8.5, reactivation did not occur on subsequent incubation for 15 min at pH 8.5 and 23° with 0.5 mM FMN or FAD. Moreover, the presence of FMN or FAD did not stabilize

the enzyme to acid or heat inactivation. Also, unlike the flavoprotein peroxidase, NADH-peroxidase (Dolin et al., 1961) the enzyme would not accept ferricyanide or menadione as electron acceptors.

The enzyme was purified by ethanol precipiration at -20° of the whole homogenate from 3 perfused rat livers (2 vol ethanol + 1 vol homogenate). The mixture was centrifuged for 15 min at 10,000 rpm and the pellet extracted with buffer (0.1 M tris, pH 7, 5 mM EDTA and 3 μM GSH) for 1 hr at 2°. The extract was then fractionated using ammonium sulfate at 2° and the protein precipitated between 40% - 65% saturation was collected after centrifugation for 15 min at 10,000 rpm. The pellet was redissolved in the above buffer and loaded onto a Sephadex G-100 column (2.5 x 45 cm). The protein was eluted with the above buffer and the specific activity of the eluted enzyme peak found to be 1,400-fold greater than that of whole homogenate. No flavin absorption was detected in the purified enzyme preparation (0.15 mg/ml).

TABLE 3 Tissue and subcellular distribution in liver of GSH-peroxidase

Reaction mixtures contained 0.25 mM GSH, 0.15 mM LAHPO, 0.1 M tris-HCl buffer pH 8.5. Reactions were carried out in 3 ml solution at 20°C.

Tissue	E_{340}/min/mg protein	Fraction	E_{340}/min/mg protein
Blood	0.72	Whole homogenate	0.52
Liver (perfused)	0.52	Mitochondrial	0.62
Intestinal mucosa	0.02	Lysosomal	0.29
		Microsomal	0.15
		Supernatant	0.7

In the rat, high specific activities of GSH-peroxidase were found in liver and in blood with very little in intestinal mucosa (Table 3). The enzyme is therefore distributed in a manner similar to the GSH-peroxidase reported by Mills (1960). In a subcellular distribution, the present enzyme was found to have the highest specific activities in the mitochondrial and supernatant fractions (Table 3). The enzyme is therefore somewhat

similar in distribution to mitochondrial contraction factor I of rat liver (Neubert et al., 1962a), later identified as a GSH-peroxidase (Neubert et al, 1962b). The present enzyme is also similar in properties to the erythrocyte GSH-peroxidase studied by Paglia & Valentine (1967).

In view of the above results, GSH oxidation accompanying lipid peroxidation in subcellular fractions (Christophersen, 1966) probably involves GSH-peroxidase. It is also probable that the GSH-peroxidase system is the major mechanism in many cells for the detoxification of lipid peroxides.

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