Hydrogen Peroxide Detoxication by Glutathione Peroxidase and Catalase in Rat Liver Homogenates

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SUMMARY

The activity of glutathione peroxidase (H_2O_2:glutathione oxidoreductase, EC 1.11.1.9) has been studied in the soluble supernatant fractions prepared from rat liver homogenates. Enzyme activity may be demonstrated in the presence of active as well as inhibited catalase. The findings are compatible with an essential function for hepatic glutathione peroxidase in the detoxication of intracellular hydrogen peroxide, and with the view that hydrogen peroxide is the common toxic agent in the drug-induced anemia associated with a genetic deficiency of erythrocyte glucose 6-phosphate dehydrogenase. The experiments also suggest a potential role for glutathione peroxidase in regulating cellular metabolism via pentose shunt control.

INTRODUCTION

Glutathione peroxidase (H_2O_2:glutathione oxidoreductase, EC 1.11.1.9) catalyzes Reaction I. Mills (1–4) was the first to report the presence of this enzyme in a variety of tissues, including erythrocytes. He suggested that it might function to protect hemoglobin against oxidation to methemoglobin by hydrogen peroxide (Reaction II). Subsequently, Cohen and Hochstein (5, 6) provided experimental evidence favoring this concept. However, other workers (7, 8) have claimed that catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6, Reaction III) is the enzyme which affords protection of hemoglobin against peroxide. This confusion regarding the functional role of glutathione peroxidase has stemmed in part from a failure to recognize the first-order kinetics of the enzyme with respect to reduced vlutathione. The efficient continuous activity of the enzyme in intact erythrocytes depends on the presence of a sufficient supply of glucose to maintain adequate concentrations of NADPH necessary for the regeneration of GSH (Reaction IV).

Similarly, a satisfactory assay system *in vitro* for glutathione peroxidase in lysates of erythrocytes or tissue fractions requires a generating system for GSH. It is only when this condition is met that one may compare the relative activity of glutathione peroxidase with other peroxide-utilizing systems.

In this paper, we report experiments on the activity of soluble rat liver glutathione peroxidase in which the above condition has been satisfied in the sense that the steady-state concentration of GSH was maintained. We have coupled glutathione peroxidase activity to glutathione reductase, thereby permitting the spectrophotometric assay of activity by following changes in the absorption of NADPH. At the same time, GSH levels are maintained by providing for the continuous reduction of GSSG. This assay is essentially identical with the one recently described by Paglia and Valentine (9) and used by them to examine the activity of glutathione peroxi-

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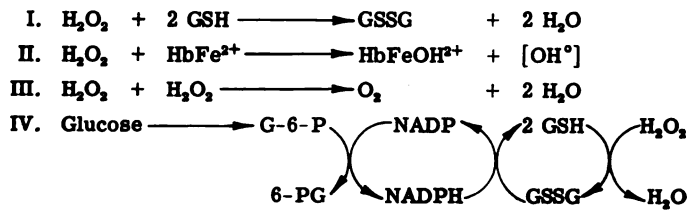

dase in erythrocyte lysates. It permits the measurement of activity in the presence and absence of reagents which inhibit catalase. Such experiments allow an estimation of the activity of glutathione peroxidase when competing with catalase for hydrogen peroxide. The results reported below show that in centrifuged rat liver homogenates, there is only a small decrease in the rate of glutathione peroxidase activity when catalase is functionally active. Particularly under conditions of slow, steady-state generation of H_2O_2, there is very little "sparing" action of catalase on glutathione peroxidase action.

MATERIALS AND METHODS

Male rats weighing approximately 150 g were killed by decapitation, and the livers were rapidly removed, chilled, and perfused with isotonic saline to remove contaminating erythrocytes. This and all subsequent steps were carried out in the cold. The livers were minced and homogenized in 9 volumes of 2.0 mM potassium phosphate buffer, pH 7.0. The homogenates were then centrifuged for 60 min at 100,000 × g. The small upper fatty layer was removed, and the clear supernatant fraction was used as a source of glutathione peroxidase and catalase. This fraction, prepared in hypotonic solution, contains more than 90% of the total catalase of the whole liver homogenate as measured by the technique of Feinstein (10).

The reactions were measured at room temperature (23–25°) in a Beckman DK-2 recording spectrophotometer by observing the optical density changes at 340 mμ. The other conditions of the assay are described in the legends to the figures. All chemicals used were of reagent grade. Glutathione reductase and glucose oxidase were obtained from Sigma Chemical Company. Hydrogen peroxide solutions were prepared by suitable dilution of 30% Baker "analyzed reagent" peroxide.

RESULTS

As illustrated in Fig. 1, the addition of H_2O_2 to the reaction system results in a prompt and linear oxidation of GSH as measured by the disappearance of NADPH. The rate of GSH oxidation by H_2O_2 is proportional to the amount of liver supernatant fraction added, as may be noted in the figure. In the absence of supernatant enzyme there is no appreciable conversion of GSH to GSSG, or direct oxidation of NADPH by H_2O_2. This may be seen in the topmost curve in Fig. 1. It should be mentioned that at higher levels of either GSH or H_2O_2 appreciable nonenzymatic oxidation of GSH may occur. This nonenzymatic oxidation is pH-dependent, and may account for as much as 40% of the observed activity at pH 7.8 or above.

The dependence of the rate of reaction on the concentration of GSH is shown in Fig. 2. As a consequence of the high nonenzymatic oxidation of GSH when present in amounts greater than that indicated in Fig. 2, a reliable estimate of K_m with respect to the enzyme is difficult to obtain. It appears to be greater than 10^{-3} M. One may note the nonlinearity of the response to GSH between additions of 2.0 and 5.0 μmoles. It has also not been possible to obtain a value for the K_m for H_2O_2 in these experiments. At all levels of H_2O_2 at which there is detectable oxidation of GSH, the rates of reaction calculated from the straight-line portions of the curves were essentially the same, although, of course, the extent of the reactions varied. With limiting amounts of H_2O_2, there was a 1:1 stoichiometry between the quantity of H_2O_2 added and the amount of NADPH oxidized.

The experiments described in Figs. 1 and 2 were carried out in the presence of 5.0 μmoles of KCN in order to inhibit the catalase present in the enzyme preparation. When cyanide is omitted from the reaction mixture, the rate of glutathione peroxidase activity is depressed only about 20 to 30%, while the final extent of the reaction is more markedly decreased. This is shown in the upper portion of Fig. 3. Although the rates of glutathione peroxidase activity in the presence and absence of cyanide are not markedly different, the early termination of activity when catalase is not inhibited suggests a rapid turnover and depletion of H_2O_2 by catalase. The addition of a second aliquot of H_2O_2 to the same reaction cuvette reinstates glutathione peroxidase activity, which is again rapidly terminated. This "staircase" may be extended until the system is depleted of NADPH. Such experiments suggest that catalase is not inactivated during the course of these experiments. If cyanide is finally added to the cuvettes (the upper curve of Fig. 3) before the addition of H_2O_2, however, the reaction proceeds to completion at a rate identical with that observed when cyanide was present initially (the lower curve of Fig. 3). Similar results may be obtained if other catalase inhibitors (azide) are substituted for cyanide. Hydrogen donors, such as ethanol and methanol, at concentrations of 10 mM, had no effect on glutathione peroxidase activity with or without added cyanide. Thus, it seems unlikely that the

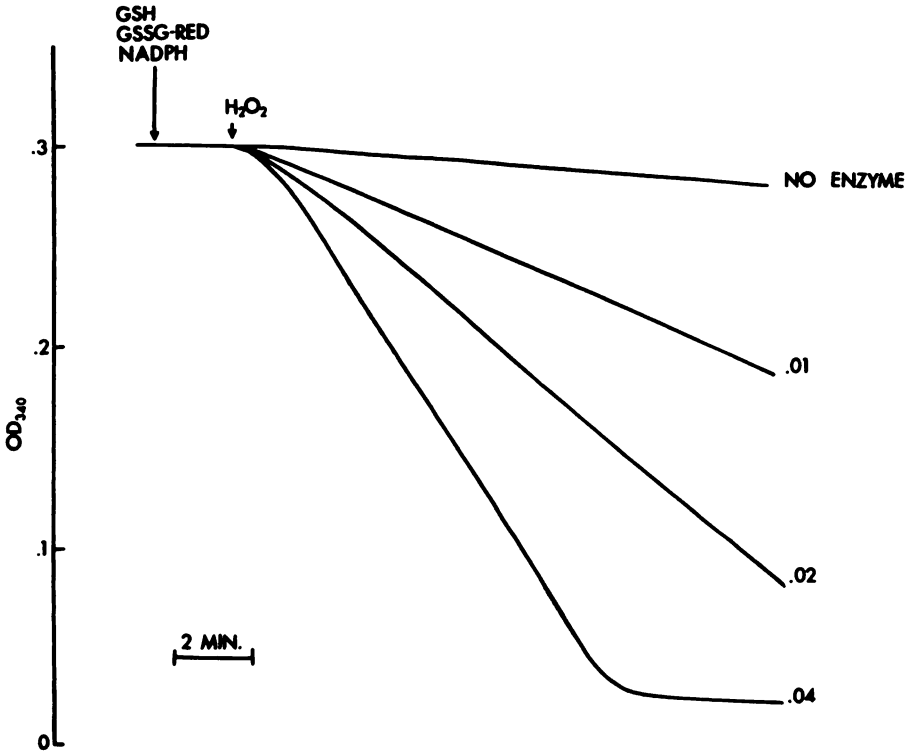

FIG. 1. *Glutathione peroxidase activity with varying quantities of rat liver supernatant fraction*

The complete system consisted of the following materials: potassium phosphate buffer, pH 6.8, 50 μmoles; GSH, 5.0 μmoles; sufficient glutathione reductase (GSSG-RED) to reduce 25 μmoles of GSSG per minute at 25°; KCN, 5.0 μmoles; NADPH, 0.5 μmole. Liver supernatant fraction was added in 10-, 20-, or 40-μl amounts as indicated. Reactions were initiated by the addition of 20 μl of a solution containing 1.0 μmole of H_2O_2. The final fluid volume of the reaction system was 2.85 ml. In this and all other experiments, the blank cuvette contained the complete system minus NADPH and H_2O_2.

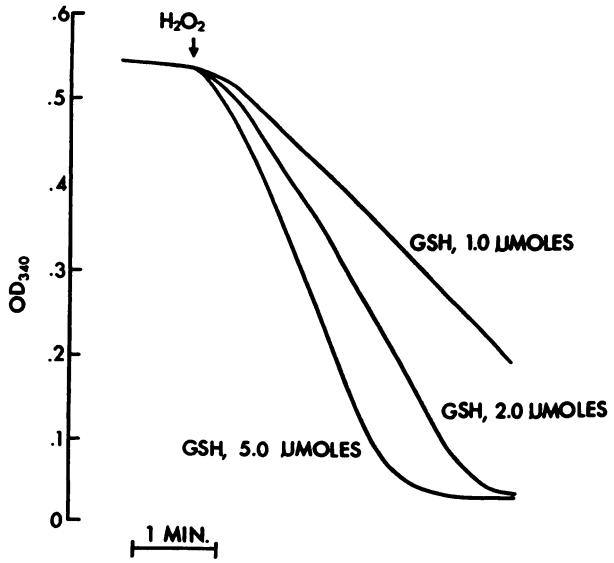

FIG. 2. *The effect of GSH concentration on glutathione peroxidase activity*

The conditions were the same as those described in Fig. 1, except that 1.0 μmole of NADPH and 1.5 μmoles of H_2O_2 were utilized. The GSH concentration was varied as indicated.

peroxidatic activity of catalase is more effective than glutathione peroxidase in removing H_2O_2 from this system.

Figure 4 illustrates the activity of glutathione peroxidase when, instead of being added directly, peroxide was slowly generated by the action of glucose oxidase on glucose. The amount of H_2O_2 generated in a 10-min period (0.5 μmole) is approximately equal to that added initially in the

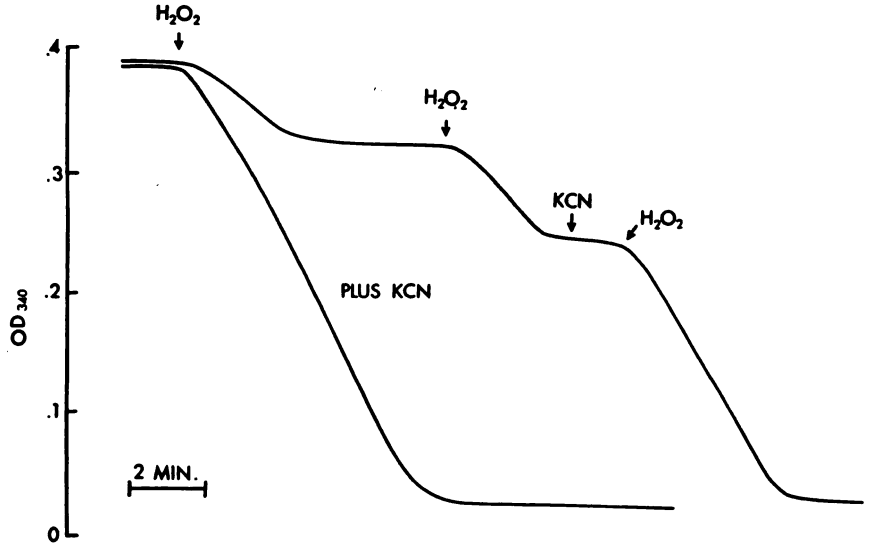

FIG. 3. *The effect of cyanide on glutathione peroxidase activity*

The conditions were the same as those described in the legend to Fig. 1, except that 50 μl of liver supernatant fraction were present and KCN was not added to the cuvette depicted in the upper curve until the time indicated by the arrow. An initial addition of 1.0 μmole of H_2O_2 was made to each cuvette. Each sequential addition, indicated on the upper curve, contained the same amount of H_2O_2.

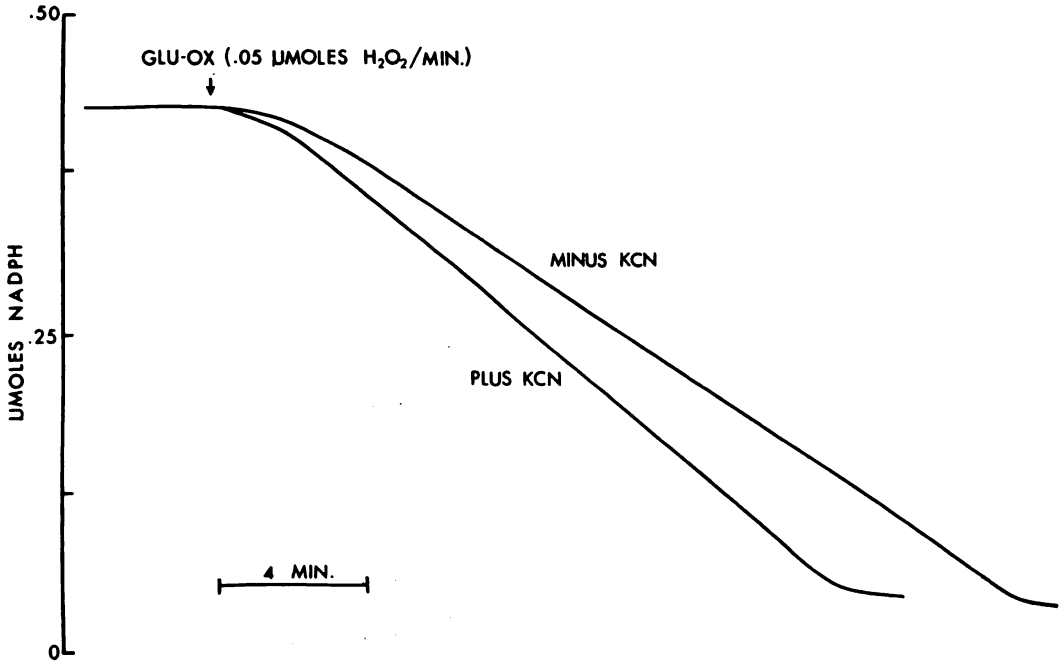

FIG. 4. *The activity of glutathione peroxidase in the presence of an H_2O_2-generating system*

The conditions were the same as those described in the legend to Fig. 1, except that 50 μmoles of glucose were present and the reactions were initiated by the addition of sufficient glucose oxidase (GLU-OX) to generate about 0.05 μmole of H_2O_2 per minute. Glucose oxidase activity was measured by following the oxidation of dianisidine in the presence of horseradish peroxidase.

previous experiments. It may be seen from Fig. 4 that under these conditions glutathione peroxidase activity is only slightly decreased (15–20%) in the absence of cyanide, or, more specifically, in the presence of active catalase. It follows that when steady-state levels of H_2O_2 are maintained, glutathione peroxidase is able to compete quite effectively with catalase for their common substrate.

DISCUSSION

These experiments indicate the potential for hepatic glutathione peroxidase to metabolize hydrogen peroxide in the presence of active catalase. Intracellular peroxide is produced by almost all mammalian cells as a consequence of the activity of a variety of flavin enzymes (e.g., xanthine oxidase) and through the autoxidation of many natural metabolites and drugs (e.g., polyphenolic substances and hydrazo derivatives). It is reasonable to assume that when peroxide is produced in discrete morphological association with catalase, as in the peroxysome (11), it may be metabolized through the action of that enzyme. However, the kinetic data presented in this paper suggest that glutathione peroxidase may also have a central role in peroxide degradation in the cytosol. Thus, as illustrated in the present studies, the rate of hydrogen peroxide destruction by glutathione peroxidase is not substantially decreased in the presence of active catalase. This appears to be the case even when peroxidizable substrates for catalase are present.

An interesting prospect of these experiments is that the activity of glutathione peroxidase may lead to an increase in the turnover of NADPH (or, in those tissues with an active NADH-glutathione reductase, to an increased turnover to NADH). Since the hexose monophosphate shunt is usually rate-limited by the availability of NADP, one consequence of glutathione peroxidase activity may be to increase the

oxidation of glucose through the shunt and thereby increase the intracellular pool of pentose intermediates. This has been demonstrated to occur in erythrocytes.[2] Thus, the detoxication of intracellular peroxide may not merely represent a metabolic dead end; rather, it may be an important control point in cellular metabolism. The potential role for hydrogen peroxide in anabolic control is further emphasized by the recent suggestion that peroxysomes may play a role in gluconeogenesis (12).

Finally, it should be mentioned that these experiments confirm those previously published on the function of glutathione peroxidase in erythrocytes (5, 6). In this tissue it may be readily demonstrated that the detoxication of peroxide is dependent on the metabolism of glucose to maintain adequate levels of reduced glutathione for glutathione peroxidase activity. Individuals who have a genetic deficiency of the enzyme glucose 6-phosphate dehydrogenase, and hence cannot generate NADPH, also have impaired ability to detoxify peroxide despite the presence of normal amounts of catalase. Such individuals are sensitive to a large number of drugs that may be shown to generate hydrogen peroxide either directly or through their metabolites (13). We view the hematological consequences of this disorder as a manifestation of peroxide toxicity *in vivo*. It may also be noted that individuals have been identified who lack erythrocyte glutathione peroxidase (14), and others have been reported who cannot synthesize erythrocyte glutathione (15). The erythrocytes of such individuals are sensitive to the toxic effects of peroxide and peroxide-forming drugs. These genetically abnormal cells offer additional biological evidence on the central role of glutathione and glutathione peroxidase in the metabolism of hydrogen peroxide.

[2] P. Hochstein, manuscript in preparation.

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