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KINETICS OF GLUTATHIONE PEROXIDASE

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SUMMARY

The dependency of the reaction rate of purified glutathione peroxidase (GSH: H_2O_2 oxidoreductase, EC 1.11.1.9) on the concentration of the substrates is investigated employing methods by which the substrates involved are determined immediately. GSH is measured polarographically. H_2O_2 is estimated by enzymatic oxidation of the fluorescent dye scopoletine but in the presence of Hg^{2+} in order to prevent the GSH present in the medium from reacting with scopoletine. The results differ considerably from some data reported in the literature. An apparent K_m value for H_2O_2 of 1 μM is obtained. Within a range of 0.5–16 mM GSH, the initial rate of the enzymatic oxidation of GSH by H_2O_2 exhibits a linear proportionality to the molarity of GSH in the incubation mixture indicating that a saturation of the enzyme by the donor substrate will not be achieved. The results are discussed with regard to a hypothetical reaction mechanism. The data furthermore allow the calculation of the maximum amount of H_2O_2 eliminated *via* glutathione peroxidase at given concentrations of GSH, H_2O_2 and glutathione peroxidase. The results show that glutathione peroxidase can compete with catalase for the common substrate H_2O_2 at low concentrations under physiological conditions.

INTRODUCTION

The enzymatic breakdown of H_2O_2 by glutathione peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) in various living tissues has been extensively discussed[1–6]. However, no precise measurements have been reported of the kinetic constants of the enzyme which would enable one to estimate the biological importance of this metabolic pathway. One would expect a high affinity of this enzyme for H_2O_2 in order to quickly eliminate this highly reactive substrate and thereby prevent undesirable reactions *in vivo*.

In 1967 SCHNEIDER AND FLOHÉ[4] attempted a determination of the K_m values of purified glutathione peroxidase of bovine erythrocytes but were unsuccessful because the enzyme could not be saturated by GSH and no dependency on the H_2O_2 concn. was found in the range between 10 μM and 5 mM. Similar results were ob-

tained by several authors studying the kinetics of the enzymatic reduction of H_2O_2 (ref. 5) or lipid peroxides[7-9] by GSH in rat liver. Definite K_m values both for H_2O_2 (25 μM) and GSH (10 mM) of glutathione peroxidase from human erythrocytes have been obtained by PAGLIA AND VALENTINE[10] using a coupled enzymatic test for activity determination. These data, however, are inconsistent with the results cited above[4,5,7-9]. Investigations of the protective function of glutathione peroxidase in erythrocytes[2,3] indicate that the K_m value for H_2O_2 is appreciably lower than 25 μM. We thus reinvestigated the dependency of the reaction rate of glutathione peroxidase on substrate concentration utilizing methods which allow direct determination of the substrates involved.

MATERIALS AND METHODS

Glutathione and horseradish peroxidase were products of Boehringer and Söhne (Mannheim); scopoletine (6-methoxy 7-hydroxycumarine) was purchased from Mann Research Laboratories (New York); materials for column chromatography were obtained from Pharmacia (Uppsala) and all other reagents were products of Merck (Darmstadt).

Glutathione peroxidase of bovine erythrocytes was purified essentially by the method of SCHNEIDER AND FLOHÉ[4] and FLOHÉ AND BRAND[11] until a specific activity of 70 units/mg (1 unit = $\Delta \log c_{GSH}$/min) was obtained. The dependency of the reaction rate of glutathione peroxidase on the concentration of GSH was investigated by making use of a polarographic determination of GSH, as described in ref. 4. The experiments were performed with the polarograph PO4 of Radiometer (Copenhagen). (GSH amounts greater than 1 mM were diluted with 0.59 M $HClO_4$ prior to polarographic determination). The molarity of GSH in the incubation medium varied between 0.5 and 16 mM during these experiments. The molarity of the stock solution of H_2O_2 was controlled by manganometric titration. The initial reaction rates are obtained by graphical extrapolation of 4 (only 2 at the lowest concentrations) independent determinations of different incubation times (10, 20, 30, 40 sec and 5, 10, 15 sec, if necessary). The experiments were carried out at 37°.

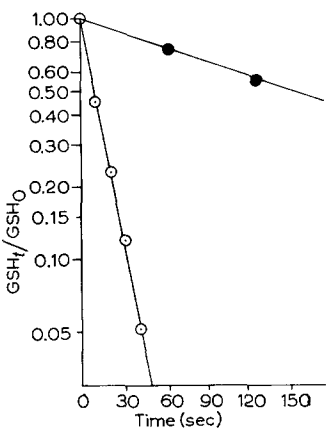
The influence of the concentration of H_2O_2 on the rate of reaction of glutathione peroxidase was studied by determining H_2O_2 by a modification of the method applied by AEBI[12] to estimate catalase activity. This method is based on the enzymatic oxidation of the fluorescent dye scopoletine by H_2O_2, as described by ANDREAE[13]. The modification became necessary since the GSH present in the incubation medium strongly interfered with the determination of H_2O_2.

The experiments are carried out as follows: 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing EDTA (1 mM) and 0.05 ml of enzyme solution are equilibrated for 15 min at 37°. Then 0.05 ml of 4 mM GSH is added, and the reaction is begun by addition of a H_2O_2 solution to a final concentration of 13–0.52 μM in the incubation medium (final volume, 2 ml). The reactions are terminated by rapid addition of 1 ml of 1.18 M $HClO_4$ after 10, 20, 30 and 40 sec or 5, 10, 15 and 20 sec at initial H_2O_2 concentrations lower than 2 μM. The solution is partially neutralized by addition of 1 ml of 1 M NaOH in order to adapt the samples to the subsequent reaction. Subsequently the following mixture is prepared: 1.0 ml of 0.2 M sodium acetate buffer (pH 4.7) containing EDTA (1 mM), 0.5 ml of horse-radish peroxidase

solution (50 μg/ml), 0.5 ml of 4 μM scopoletine, 4 mM solution of mercuriacetate in equimolar amounts to the GSH present. At this point an aliquot of the incubation medium containing H_2O_2, such that the final H_2O_2 concentration can be easily determined (about 0.2 μM), is added to the above mixture and the total volume is brought to 5 ml with H_2O. (A slight excess of mercuriacetate does not influence the test system. We therefore calculated the amount of mercuriacetate to be added on the basis of the GSH initially present in the above incubation medium. Thus, the added amount of the mercuriacetate solution depends on the volume of the aliquot of the incubation medium.) After a 20-min incubation at room temperature, 5.0 ml of 0.15 M borate buffer (pH 10) are added, and the fluorescence intensity is read in an Eppendorf photometer using the fluorescence equipment 1030 (primary filter 313–366 mμ; secondary filter 380–3000 mμ). The concentrations of H_2O_2 are obtained by using a calibration curve. All vessels and pipets are finally washed with quartz-distilled water containing EDTA. Control experiments are performed in the same manner but without GSH or glutathione peroxidase, respectively.

RESULTS AND DISCUSSION

Glutathione peroxidase has been shown to follow first-order kinetics with regard to GSH by COHEN AND HOCHSTEIN[2], SCHNEIDER AND FLOHÉ[4], LITTLE AND O'BRIEN[8] and CHRISTOPHERSEN[9], whereas PAGLIA AND VALENTINE[10] described a saturation of glutathione peroxidase by GSH at high concentration. HOCHSTEIN AND UTLEY[5] recently reported deviations from first-order kinetics at high GSH concentrations, suggesting a K_m(GSH) value of 10 mM or higher. We were able to show, however, that the applicability of the test systems of PAGLIA AND VALENTINE[10] and HOCHSTEIN AND UTLEY[5] are restricted to a narrow range of substrate concentrations[11]. A reinvestigation of the dependency on GSH of the glutathione peroxidase reaction rate



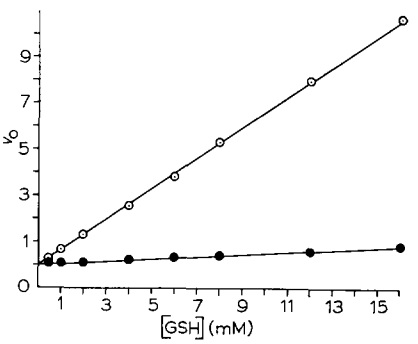


Fig. 1. Oxidation of GSH by H_2O_2 in the presence (○—○) and absence (●—●) of glutathione peroxidase at 37° and pH 7.0. The graph shows the relative amounts of GSH *versus* incubation time *t*. The initial concentrations were 8 mM GSH and 5 mM H_2O_2.

Fig. 2. Initial velocity of the oxidation of GSH by H_2O_2 in the presence (○—○) and absence (●—●) of glutathione peroxidase at varying concentrations of GSH (details see under MATERIALS AND METHODS).

by our polarographic method revealed exact first-order kinetics at all concentrations of GSH tested. As illustrated in Fig. 1, the time–reaction curve of the glutathione peroxidase reaction at 8 mM GSH is a straight line in a logarithmic scale. Correspondingly, it was found that the initial reaction rate exhibits a linear proportionality to the molarity of GSH even at the highest concentrations investigated (Fig. 2).

With regard to the evaluation of the K_m value for H_2O_2, it is first necessary to discuss our experimental procedure. No reliable results have been obtained by means of the polarographic test[4] at H_2O_2 concentrations below 10 μM. However, measurements in this range of concentration are necessary for the evaluation of K_m. Furthermore, HOCHSTEIN AND UTLEY[5] were unable to estimate the $K_m(H_2O_2)$ of rat-liver glutathione peroxidase by the procedure of PAGLIA AND VALENTINE[10] which was designed to operate even in the low molarity range of H_2O_2. In our experiments we attempted to determine the exact concentration of H_2O_2 during the enzyme reaction which is certainly a more direct approach for the evaluation of the K_m for H_2O_2. By using our method we also were able to exclude the presence of catalase in the reaction mixture by replacing GSH by H_2O. Under these conditions no decrease in H_2O_2 concentration was found.

The determination of trace amounts of H_2O_2 by the unmodified method of AEBI[12] was not feasible because GSH apparently competes with scopoletine for horseradish peroxidase. (A nonenzymatic reaction of oxidized scopoletine with GSH was not detectable even at high pH.) The masking of GSH by Hg^{2+}, however, completely restored the recovery of H_2O_2. No significant decomposition of H_2O_2 by Hg^{2+} took place during the time needed for the experiments, probably due to the low pH of the medium and the presence of EDTA. Neither is the activity of horseradish peroxidase influenced by Hg^{2+}. Fig. 3 shows a calibration curve obtained under the conditions given in MATERIALS AND METHODS replacing the incubation medium of the glutathione peroxidase assay by an equivalent GSH solution and samples containing definite amounts of H_2O_2.

The results of our kinetic studies using the scopoletine test are summarized in Fig. 4. A decrease in reaction rate is only observed at H_2O_2 concentrations below 10 μM which is in agreement with earlier results[4]. The large deviations of the values

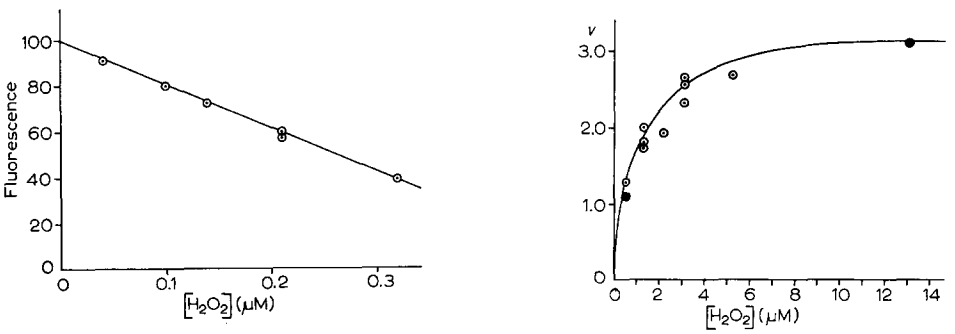


Fig. 3. Calibration curve of the determination of H_2O_2, as described under MATERIALS AND METHODS. 100 is equivalent to a scopoletine concentration of 0.4 μM.

Fig. 4. Dependency of the glutathione peroxidase reaction rate on the concentration of H_2O_2. The velocity, v, is given in nmoles H_2O_2 reduced per min per ml of the sample. The initial concentration of GSH was 0.1 mM. ●, duplicate determinations.

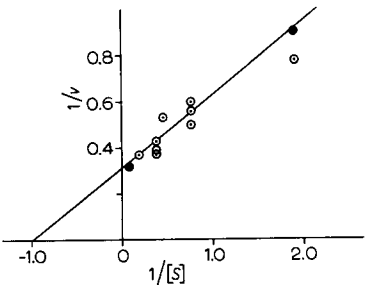


Fig. 5. Lineweaver–Burk plot using the values of Fig. 4. The velocity, v, is defined as in Fig. 4. The H_2O_2 concentration, $[S]$, is given in μM.

are primarily due to the short incubation times which were necessary due to the high turnover of the enzymatic reaction and the low concentrations of the substrate. The Lineweaver–Burk plot (Fig. 5) nevertheless allows one to calculate an apparent K_m value of about 1 μM. This value differs considerably from the $K_m(H_2O_2)$ found by PAGLIA AND VALENTINE[10]. Although our results may not be directly compared to those of PAGLIA AND VALENTINE[10] because enzymes of different species have been investigated, we think our value is more reliable since it is not feasible to apply the coupled enzymatic test of PAGLIA AND VALENTINE[10] at extreme concentrations of substrates[11]. In addition we recently were able to show that $K_m(H_2O_2)$ of glutathione peroxidase of another species (rat liver, rat erythrocytes) is nearly identical with that of bovine erythrocytes (L. FLOHÉ AND W. SCHLEGEL, unpublished results (1969)).

Summarizing our results, we can say that glutathione peroxidase behaves similarly to other peroxidases insofar as it possesses a high affinity for the peroxide, whereas a saturation of the enzyme by the hydrogen donor does not occur. We propose the following tentative reaction mechanism which is partially analogous to the one given by BRILL[14] for horseradish peroxidase:

$$E_{red} + H_2O_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_{ox} + H_2O \qquad (1)$$

$$E_{ox} + GSH \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E_{ox}GSH \qquad (2)$$

$$E_{ox}GSH + GSH \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E_{ox}(GSH)_2 \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E_{red} + GSSG \qquad (3)$$

This hypothesis is difficult to confirm with respect to the absence of any spectral characteristics of the enzyme which possibly would allow the evaluation of the individual kinetic constants. However, some support for our working hypothesis is as follows. As can be estimated from the low apparent K_m value, the affinity of the enzyme for the peroxide is nearly as high as the one of other peroxidases. Since the formation of E_{ox} or perhaps an enzyme–H_2O_2 complex is favored, it is unlikely that H_2O_2 and the donor molecules are randomly attached to the enzyme. Pursuing further the analogy to horseradish peroxidase, we may conclude that the apparent K_m for

H_2O_2 will approach the quotients k_{+2}/k_{+1} or k_{+3}/k_{+1} rather than K_s of Eqn. 1. (The assumption that k_{-1} can be neglected implies that K_m depends on the concentration of the donor. This question remains to be investigated.) We may further exclude that the first and second donor molecule combine with the product of Eqn. 1 in a random mechanism because in this case the reaction would obey the following equation:

$$\frac{d[GSH]}{dt} = k[E_{ox}]\,[GSH]^2 \qquad (4)$$

In contrast to Eqn. 4, our results actually fit Eqn. 5:

$$\frac{d[GSH]}{dt} = k[E_{ox}]\,[GSH] \qquad (5)$$

Finally, a ternary complex $E_{ox}(GSH)_2$ will not accumulate, *i.e.* k_{+4} can not be limiting under normal conditions. If we want to avoid the hypothesis that free radicals of GSH are released during the reaction of E_{ox} with the donors, we must assume that Eqn. 2 is the limiting step of the overall sequence (at high peroxide concentration at least). Otherwise again a Michaelis–Menten-like intermediate (E_{ox}GSH) would accumulate which would be inconsistent with the observation that the first-order velocity constant (at constant $[E]$) remains unaltered over the whole range of GSH concentrations investigated. However, minor deviations from the type of reaction described by Eqn. 5 at very low concentration of GSH may be obscured in our experimental conditions so that a definite conclusion cannot be given.

We may now consider our results in regard to the calculation of COHEN AND HOCHSTEIN[2], AEBI *et al.*[3] and HOCHSTEIN AND UTLEY[5] which revealed that the glutathione peroxidase reaction is the primary mechanism for removal of H_2O_2 at low concentrations. The role of catalase which competes with glutathione peroxidase for the common substrate H_2O_2 physiologically is, as yet, somewhat uncertain. It has been discussed by the authors cited above[2,3,5] that catalase will have no significant catalatic function *in vivo* at concentrations below 3 μM H_2O_2. For a long time, however, it has been known that catalase may act as a peroxidase with a large variety of substrates[14–17]. However, the quantitative physiological importance of the peroxidatic function of catalase has not been elucidated[5,6].

In 1965 NICHOLLS[6] took an opposite position. He attempted to solve the problem by comparing the molecular reaction rates of catalase and glutathione peroxidase with H_2O_2. By these investigations the subsequent conclusions were drawn: (1) In erythrocytes at least, catalase has a catalatic function or none at all. (2) Glutathione peroxidase is of no importance in protecting hemoglobin from oxidative damage by H_2O_2 in erythrocytes. The arguments which suggest the latter conclusion, however, suffer from the poor information available on glutathione peroxidase at that time. The calculation of the molecular reactivity is based on data obtained in a crude enzyme preparation by MILLS[18]. Although our recent preparations (ref. 4 and L. FLOHÉ AND W. SCHLEGEL, unpublished results, 1969) had a considerably higher specific activity, the degree of purity of any glutathione peroxidase sample has not yet been determined. Thus a calculation of molecular reaction rates is impossible. Furthermore, we cannot agree with the assumption of NICHOLLS[6] that the enzyme is saturated by GSH at physiological concentrations of GSH (see above and refs. 5, 8, 9). Our kinetic data may contribute to the solution of the question insofar as they

allow a calculation of the maximum of turnover by glutathione peroxidase at given concentrations of GSH, H_2O_2 and enzyme. Considering the data of ref. 4 (activity of glutathione peroxidase: 2150 units/l blood) and those of NICHOLLS[6] on catalase (first-order velocity constant $3.6 \cdot 10^8\ M^{-1} \cdot min^{-1}$; catalase = 3.3 μM), we may estimate the initial rate of the elimination of H_2O_2 by either enzyme according to the following equations*:

(a) Catalase:

$$\frac{d[H_2O_2]}{dt} = k'_c\,[H_2O_2]\,[\text{catalase}] \tag{6}$$

or

$$\frac{\Delta[H_2O_2]}{\Delta t} = k'_c\,[H_2O_2]_{t_0}\,[\text{catalase}] \tag{7}$$

assuming that H_2O_2 is kept at the initial concentration during the reaction.

(b) Glutathione peroxidase:

$$\frac{d[GSH]}{dt} = k'_p\,[GSH] = 2.302 \text{ units } [GSH] \tag{8}$$

$$\frac{d[GSH]}{dt} = 0.5\,\frac{d[H_2O_2]}{dt} \tag{9}$$

or

$$\frac{\Delta[GSH]}{\Delta t} = 2.302 \text{ units } [GSH]_{t_0} \tag{10}$$

if GSH is regenerated to the initial concentration, as happens physiologically *via* glutathione reductase. Eqn. 10 holds for varying concentrations of H_2O_2 if the latter exceed 10 μM. Only at lower concentrations does the dependency on H_2O_2 concentration have to be considered. Under these conditions, the H_2O_2 must be kept constant during the reaction time.

Assuming a H_2O_2 concentration of 1 mM and 1 μM and a GSH concentration of 2 mM and using Eqns. 7 and 10, respectively, we obtain the following initial reaction rates (pseudo zero-order):

(a) $\frac{\Delta H_2O_2}{\Delta t} = 1.2\ M \cdot min^{-1}$ at $c_{H_2O_2} = 1$ mM

(b) $\frac{\Delta H_2O_2}{\Delta t} = 0.0012\ M \cdot min^{-1}$ at $c_{H_2O_2} = 1\ \mu M$

} for the catalatic reaction

(c) $\frac{\Delta H_2O_2}{\Delta t} = 4.45\ M \cdot min^{-1}$ at $c_{H_2O_2} = 1$ mM

(d) $\frac{\Delta H_2O_2}{\Delta t} = 2.23\ M \cdot min^{-1}$ at $c_{H_2O_2} = 1\ \mu M$

} for the glutathione peroxidase reaction

* Abbreviations: k'_c: pseudo first-order velocity constant for catalase in $M^{-1} \cdot min^{-1}$; t: time in min; t_0: zero time; k'_p: pseudo first-order velocity constant in min^{-1} for glutathione peroxidase; units: activity units ($\Delta \log c_{GSH}$/min); all concentrations in M.

We do not conclude from these calculations that the glutathione peroxidase reaction may have an importance even at high concentrations of H_2O_2 for under these conditions GSH would be consumed physiologically in a short time. Besides, k'_c used in Eqns. 6 and 7 represents the lowest limit of values estimated[6], and the K_m value for glutathione peroxidase may depend on the donor concentration (see above). In any case, at low concentrations of H_2O_2 glutathione peroxidase clearly has an advantage over catalase as the overall velocity of the reaction is only lowered to $v_{max}/2$ at 1 μM H_2O_2 (= apparent K_m of glutathione peroxidase). A further decrease in H_2O_2 concentration does not influence the ratio of catalatic to peroxidatic reaction rate. Thus, if NICHOLLS[6] is correct in stating that catalase in erythrocytes has only a catalatic function, our results on the kinetics of glutathione peroxidase may confirm the hypothesis of COHEN AND HOCHSTEIN[2], AEBI *et al.*[3] and HOCHSTEIN AND UTLEY[5] that the main metabolic pathway of H_2O_2 at low concentrations is the enzymatic reduction by GSH. Furthermore, it is interesting to note that hereditary deficiencies involving a variety of enzymes, *e.g.* glucose 6-phosphate:$NADP^+$ oxidoreductase[19–22], 6-phosphogluconate:$NADP^+$ oxidoreductase[23], NADPH:glutathione oxidoreductase[24,25] and γ-L-glutamyl-L-cysteine–glycine ligase[26,27], which finally result in a decreased level of GSH, produce very similar pictures of hematologic diseases. These may all stem from the inability of red cells to detoxify, at a sufficient rate, H_2O_2 or other peroxides *via* glutathione peroxidase. Nevertheless, we should consider the possibility that large species differences in catalase[28] or glutathione peroxidase levels or deviations in the K_m values of glutathione peroxidase may significantly alter the ratio of catalatic to peroxidatic elimination of peroxide.

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