Glutathione Peroxidase of Calf Trabecular Meshwork

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Glutathione peroxidase was extracted from calf trabecular meshwork. The kinetics of this enzyme were examined, varying the substrates hydrogen peroxide (H$_2$O$_2$), tert-butylhydroperoxide (tBHP), and glutathione. The activity of the enzyme in nonpurified homogenates was 596 nmole H$_2$O$_2$ reduced/min/gm wet weight and 680 nmole tBHP reduced/min/gm wet weight (27-28 nmole peroxide reduced/min/mg protein). The apparent Michaelis-Menten constants for H$_2$O$_2$, tBHP, and GSH were 0.012, 0.102, and 2.89 mM, respectively. These data, together with published levels of glutathione in trabecular meshwork, suggest that the trabecular endothelial cell actively detoxifies H$_2$O$_2$. Invest Ophthalmol Vis Sci 25:599–602, 1984

Hydrogen peroxide (H$_2$O$_2$) is a normal constituent of the aqueous humor in many species. Spector and Garner found mean levels of 26 μM and 25 μM in normal human and bovine aqueous, respectively. Both samples showed normal distribution. However, in a population of 17 cataract patients, they found a highly right-skewed distribution with a median value of 40 μM compared with the normal human median of 25 μM. They suggest that excessive levels of H$_2$O$_2$ in aqueous humor may impair lens membrane permeability and contribute to cataract formation.

Since the bulk of aqueous humor leaves the eye via the trabecular meshwork (TM), this tissue also must be exposed to these high levels of H$_2$O$_2$. Therefore, it was of interest to see if TM possessed a mechanism for H$_2$O$_2$ detoxification, both to protect itself and more distal portions of the aqueous outflow system.

Biochemically, H$_2$O$_2$ is detoxified by two major enzymatic mechanisms: one uses catalase and the second uses a dienzymatic redox cycling of the sulfhydryl compound glutathione (GSH) by glutathione peroxidase (GSH-Px), which catalyses the reaction:

\[ H_2O_2 + 2GSH \rightarrow 2H_2O + GSSH \]

and glutathione reductase (GR), which catalyses:

\[ \text{NADPH} + H^+ + GSSH \rightarrow \text{NADP}^+ + 2GSH. \]

Giblin and McCready have shown that this system is present in rabbit lens and appears to be involved intimately in the detoxification of H$_2$O$_2$. In order to characterize this possible detoxification mechanism, we examined the enzyme glutathione peroxidase obtained from calf trabecular meshwork. We determined its activity and the apparent K_m values for the substrates hydrogen peroxide, tert-butylhydroperoxide, and glutathione.

Materials and Methods

Hydrogen peroxide (30%) was obtained from Mallinckrodt (St. Louis, MO). Glutathione reductase, tBHP, NADPH, and GSH were obtained from Sigma Chemical Corporation (St. Louis, MO). All other chemicals were reagent grade. Enzyme assays were carried out on a Cary 210 Spectrophotometer, Varian Instrument (Palo Alto, CA). Calf eyes were obtained from Joseph Trelegan and Company (Cambridge, MA). The animals were killed by exsanguination. Shortly after death, the eyes were removed and placed in iced saline for transportation. The trabecular meshwork was excised within 6 hr of enucleation. Immediately after excision the tissue was blotted on Whatman No. 1 filter paper, frozen on dry ice, and placed in tared vials for storage at ~80°C. Trabecular meshwork contaminated with blood was discarded.

Tissue homogenates were prepared by combining tissue at a ratio of 1 g/5 ml with ice-cold buffer containing 140 mM KCl, 5 mM potassium phosphate (pH 7.0), 2.5 mM MgSO$_4$, 22 mM NH$_4$Cl, and 1 mM EDTA, then homogenizing in a Tissumizer (Model SDT 100N; Tekmar, Cincinnati, OH). The homogenate was centrifuged for 10 min at 1800 × g. The supernatant was then collected and kept at 0°C for same day use or stored at ~80°C for use within 1 week. No change in enzyme activity was observed following such storage of the supernatant. The protein content of this preparation was determined by a dye-binding

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method using a globulin standard. A kit from Biorad Laboratories (Richmond, CA) was used. Preparations made in this way do not contain detectable amounts of hemoglobin, even when concentrated 100-fold. Because of the limited amount of material available, it was not possible to purify the preparation further and still have enough activity to do the kinetic study.

Glutathione peroxidase activity and K_m’s for H_2O_2 and tert-butylhydroperoxide (tBHP) were determined using a modification of the coupled method of Paglia and Valentine. An 80-μl sample of the supernatant enzyme solution was incubated at 25°C with 2.65 ml of 50 mM phosphate buffer (pH 7.0), containing 5 mM EDTA, 4 mM NaN_3, 0.44 M GSH, 0.29 mM NADPH, and 0.56 μl/mgl glutathione reductase.

The reaction mixture was equilibrated at 25°C in the quartz cuvette of the spectrophotometer. The reaction was started by addition of the peroxide substrate (H_2O_2 or tBHP) to the sample cuvette. The progress of the reaction was followed by monitoring the disappearance of NADPH at 340 nm. Substrate solutions were prepared by precise dilution of stock and the range of final concentrations used in each experiment was 0–0.1 mM for H_2O_2 and 0–1 mM for tBHP. The final volume was adjusted to 3.0 ml with deionized water. Since some nonenzymatic oxidation of GSH occurs following the addition of substrate, separate blanks were prepared for each concentration of substrate by replacing the enzyme solution with water. No reaction occurred in the absence of peroxide. Because H_2O_2 is unstable in dilute solutions, the concentration of GSH was varied over the range 2.5–5.0 mM. The reaction was started by addition of peroxide to final concentration of 0.1 mM tBHP. The blanks were again run by replacing homogenate with water. No reaction occurred in the absence of peroxide. Because H_2O_2 is unstable in dilute solutions, the concentration of the working solution was determined by titration with acid KMnO_4.

In order to determine the K_m of GSH, it was necessary to remove endogenous GSH. The enzyme preparation was passed over a Bio-gel P-6DG desalting column. The concentration of GSH was varied over the range 2.5–5.0 mM. The reaction was started by addition of peroxide to final concentration of 0.1 mM tBHP. The blanks were again run by replacing homogenate with water. No reaction occurred in the absence of GSH.

Following the recommendation of Wilkinson, we have plotted our kinetic data as s/v versus s. (See Figs. 1A–C). Unlike the double-reciprocal plot this form yields a valid, unweighted, least squares fit. Data obtained on different days were sufficiently close to allow simple averaging of s/v. The plotting routine provided SDs of slope and vertical intercept. The SDs of the K_m’s were estimated using the approximation:

\[ CV^2(A/B \text{ or } A \times B) \approx CV^2(A) + CV^2(B) \]

where CV = SD/mean.

Results

Apparent K_m’s for each substrate of GSH-Px examined (H_2O_2, tBHP, GSH) were calculated from plots of s/v versus s (Figs. 1A–C, respectively). We obtained values of 0.012, 0.102, and 2.89 mM for H_2O_2, tBHP, and GSH, respectively (Table 1). In all cases, the K_m value for H_2O_2 is an order of magnitude lower than that for tBHP, indicating a higher affinity of the enzyme for the natural substrate. The curve for GSH was nonlinear at lower concentrations of substrate. A Hill plot (Fig. 1D) gave a slope of 1.43, which is consistent with a positive cooperativity between two binding sites.

The V_max from Figures 1A–C was used to calculate the activity of the enzyme in vivo. These results are given in Table 2. The activity per gram wet wt found for GSH was 240 nmole/min versus 680 nmole/min for tBHP. This low activity is clearly due to the desalting procedure performed prior to the GSH experiments (see Methods and Materials); therefore, the value was omitted from the table; the activities reported per mg protein are nearly identical for all three experiments.

Discussion

Hydrogen peroxide found in the aqueous humor is reported to originate from a light catalyzed oxidation of ascorbic acid. Elevated levels of H_2O_2 could exert toxic effects on intraocular tissues through its strong oxidizing properties. For instance, Spector and Garner found that the aqueous humor from human eyes with cataractous lenses frequently contained elevated levels of H_2O_2, suggesting that these lenses may be less efficient in destroying it.

As aqueous humor leaves the eye principally via the TM, this tissue also is continuously exposed to high levels of H_2O_2. It would appear that TM may require an efficient system for destroying H_2O_2, not only to protect itself, but also the endothelial lining of Schlemm’s canal (aqueous plexus), the collector channels, and the aqueous veins. Conceivably, a breakdown in this protective mechanism could lead to tissue injury, resulting in a changed resistance to aqueous outflow.

Kahn et al have found that the TM of normal healthy calf is very resistant to acute damage from even high levels of H_2O_2. Only if TM GSH was removed completely by prior perfusion with BCNU and diamide could H_2O_2 cause an appreciable increase in outflow resistance. Our results show that one of the two enzymes involved in detoxifying H_2O_2: GSH-Px, is present in calf TM at a level of 600 nmoles/min/g wet weight. If one assumes an anterior chamber volume in the calf of 1 ml, then at 25 μM, there are 25 nmoles H_2O_2 in the anterior chamber. Assuming a TM mass of 20 mg.
Fig. 1. Kinetics of glutathione peroxidase from calf trabecular meshwork. A, Substrate H₂O₂. Intercept \( I/V = 0.282 \); slope = 25.3. Estimate of \( K_m = 0.011 \pm 0.003 \). B, Substrate tert-butylhydroperoxide. Intercept \( I/V = 2.34 \); slope = 22.98. Estimate of \( K_m = 0.102 \pm 0.013 \). C, Substrate glutathione. Intercept \( I/V = 191 \); slope = 66.1. Estimate of \( K_m = 2.89 \pm 0.53 \). Points shown as solid circles were not used for fitting line. D, Substrate glutathione: Hill plot. Vertical intercept = -3.57; slope = 1.43.

at 600 nmole/min/g, TM could remove all the anterior chamber H₂O₂ in 2 min.

As the rate of aqueous outflow from the calf eye is probably of the order of 30 μl/min there is obviously a great deal of excess capacity for H₂O₂ removal. Of course the other enzymes of the system may not have such a high capacity, so a sustained rate of 600 nmole/min/g wet weight may not be possible. Kahn et al.13 give a figure of 0.4 μmole/g wet weight for the GSH content of TM. With the above assumptions, this would last 5 min without replenishment.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Calf TM</th>
<th>Bovine lens</th>
<th>Human erythrocyte</th>
<th>Rat brain</th>
<th>Human platelet</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>0.012</td>
<td>0.045</td>
<td>0.025</td>
<td>2.94</td>
<td>2.3</td>
</tr>
<tr>
<td>tBHP</td>
<td>0.102</td>
<td>0.540</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>2.89</td>
<td>2.90</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values given in mM.

* For Figs 1A–1C, data was plotted as \( s/V \) versus \( s \), where \( s \) is the substrate concentration in mM and \( V \) is the rate of reaction in μmoles/min. The \( K_m \) is given by the intercept of the line on the \( s \) axis: \( K_m = -\text{intercept}_s / \text{slope} \). Figure 1D is a Hill plot of the data of Figure 1C, ie, \( \log \left( \frac{V}{V_m - s} \right) \) versus log \( s \). Data were plotted, and straight lines were fitted, using routines from PROPHET, a national computer resource sponsored by the Division of Research Resources, NIH. Errors are given as ±SD. See Materials and Methods for further details.
Table 2. Activity of calf TM GSH-Px with various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Per g wet weight</th>
<th>Per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>H_2O_2</td>
<td>596</td>
<td>25.5</td>
</tr>
<tr>
<td>tBHP</td>
<td>280</td>
<td>28</td>
</tr>
<tr>
<td>GSH</td>
<td>27.8</td>
<td></td>
</tr>
</tbody>
</table>

Units are nmole/min per unit weight as indicated.

The K_m's of GSH-Px for H_2O_2, tBHP, and GSH are comparable with those of the enzyme from bovine lens. Martinez et al noted a sigmoidal activity versus [GSH] curve for the enzyme from human platelets. The other workers cited probably failed to notice the departure from Michaelis-Menten kinetics because of the tendency of the Lineweaver-Burke plot to conceal nonlinearities. The TM and lens GSH-Px have K_m's for H_2O_2 of one-half and double the aqueous concentration of H_2O_2, respectively. The cooperativity of GSH-Px seen with GSH is consistent with a detoxifying role for this enzyme. It allows the levels of H_2O_2 to be driven very low, yet prevents a sudden influx of H_2O_2 from drawing down GSH levels to where it becomes unavailable for other reactions.

For the future, it will be of interest to determine the activity in calf TM of glutathione reductase, which is the other component of this detoxification system, and also to examine these same enzymes in human TM to see if this tissue is as vigorously guarded from oxidative damage as is that of calf.

Key words: glutathione peroxidase, kinetics, calf, trabecular meshwork, hydrogen peroxide

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References