Direct Evidence for Catalase as the Predominant H₂O₂-Removing Enzyme in Human Erythrocytes

By Sebastian Mueller, Hans-Dieter Riedel, and Wolfgang Stremmel

Decomposition of hydrogen peroxide (H₂O₂) at physiological levels was studied in human erythrocytes by means of a recently developed sensitive H₂O₂ assay. The exponential decay of H₂O₂ in the presence of purified erythrocyte catalase was followed down to 10⁻³ mol/L H₂O₂ at pH 7.4. H₂O₂ decomposition by purified erythrocyte glutathione peroxidase (GPO) could be directly observed down to 10⁻⁷ mol/L H₂O₂. No enzyme inhibition was observed at these low H₂O₂ concentrations. Catalase and GPO activities can be determined separately in a titrated mixture of purified enzymes, which simulates the conditions of H₂O₂ removal by the erythrocyte. Experiments with fresh human hemolysate allowed us to determine H₂O₂ decomposition by catalase and GPO using these enzymes in their original quantitative ratio. The different kinetics of these enzymes are shown: H₂O₂ decomposition by catalase depends linearly on H₂O₂ concentration, whereas that by GPO becomes saturated at concentrations above 10⁻⁶ mol/L H₂O₂. Even at very low H₂O₂ concentrations GPO reaches only approximately 8% of the rate at which catalase simultaneously degrades H₂O₂. These data indicate an almost exclusive role for catalase in the removal of H₂O₂ in normal human erythrocytes.

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where $k'$ is the specific catalase activity. Catalase is completely inhibited by 1 mmol/L NaN₃ as can be shown after a second addition of H₂O₂. At these low substrate concentrations no enzyme inactivation or gaseous oxygen release is observed.

The effect of purified GPO on H₂O₂ was studied at a GSH concentration of 2 mmol/L, which corresponds to the level found in erythrocytes and which permits determination of submicromolar H₂O₂ concentrations without significant interference with the assay method. Figure 2 shows that the addition of GPO elicits a rapid degradation of H₂O₂. Under saturating conditions, the decomposition follows a zero order kinetics and the degradation rate depends linearly on GPO concentrations (not shown). After a second addition of H₂O₂, GPO activity is not altered at these low H₂O₂ concentrations (Fig 2). Heat inactivation of the enzyme (boiling for 10 minutes) prevented H₂O₂ degradation. Because GSH concentration requires maintenance at a constant level, we studied the effect of a GSH-regenerating system (NADPH and glutathione reductase) on GPO activity. GSH regeneration did not change GPO-mediated H₂O₂ degradation. It may be surmised that GSH concentration is not significantly changed under these conditions, most likely because of the high [GSH]/[H₂O₂] ratio (>200). The small decrease in GSH concentration during the reaction does not affect H₂O₂ decomposition rate. Conversely, experiments performed with [GSH]/[H₂O₂] ratio (<10) are associated with a significant GSH depletion and a consequent decrease in GPO activity (data not shown).

The rate of H₂O₂ degradation by purified erythrocyte GPO as a function of GSH concentration is shown in Fig 3. The reaction rate depends linearly on GSH concentration within the concentrations examined. These findings are consistent

![Diagram of exponential decomposition of H₂O₂ by purified catalase](image-url)
CATALASE AS THE PREDOMINANT H₂O₂-REMOVING ENZYME

Fig 3. H₂O₂ decomposition rate by GPO (0.02 U/mL) as a function of GSH concentration. All values are the means (standard deviation < 8%) of four experiments.

with ping-pong mechanisms, inherent in the GPO activity.\(^{31-33}\), 

2 GSH + ROOH → GSSG + ROH + H₂O.

In summary, the luminol/hypochlorite assay allows a measurement of H₂O₂ removal by GPO at submicromolar concentrations of H₂O₂ and at concentrations of GSH normally found in erythrocytes. Under these conditions, regeneration of glutathione via glutathione reductase is not a requisite condition.

**Determination of individual GPO and catalase activities in a mixture of purified enzymes at physiological H₂O₂ concentrations.** Catalase and GPO are present in the erythrocyte cytoplasm. An experimental model was designed to determine the individual catalase and GPO activity in a mixture containing both enzymes and mimicking the ability of a hemolysate or erythrocyte cytoplasm to decompose H₂O₂.

Addition of a mixture of purified GPO and catalase to a 10⁻⁵ mol/L H₂O₂ solution (Fig 4A) resulted in an exponential decay of H₂O₂, which was ascribed to catalase activity based on the sensitivity of the signal to NaN₃ (Fig 4B) and the subsequent lack of removal of a second addition of H₂O₂ (Fig 4C). Catalase activity calculated from these data tallies with the activity calculated from data in Fig 1. The addition of 2 mmol/L GSH (Fig 4D) and 10⁻⁴ mol/L H₂O₂ (Fig 4E) to the above mixture permits to evaluated GPO-dependent H₂O₂ degradation: The rates obtained matched those calculated from data in Fig 2. These experiments were performed with GPO activities higher than those in erythrocytes (with respect to catalase) to improve the kinetic characterization of both enzymes.

**Determination of catalase and GPO activity in erythrocyte at physiological H₂O₂ concentrations.** We applied the procedure described previously to fresh human hemolysate to study H₂O₂ removal in human erythrocytes. Because, in contrast with other cells (eg, hepatocytes), catalase and GPO are freely distributed in the cytoplasm of erythrocytes, a

**Fig 4.** Determination of GPO and catalase activity in a mixture of purified enzymes. (A) After the addition of enzyme mixture (GPO, 0.01 U/mL; catalase, \(k_{cat} = 0.034 \text{s}^{-1}\)) only the catalase-mediated H₂O₂ decay is observed. (B) Inhibition of catalase by 1 mmol/L NaN₃. (C) Second addition of 10⁻² mol/L H₂O₂ shows complete inhibition of catalase. (D) Addition of 2 mmol/L GSH and recalibration of the measuring system (see Mueller et al\(^{25}\)). (E) The GPO-mediated decomposition of H₂O₂ is observed after the addition of 10⁻⁴ mol/L H₂O₂. These experiments were performed with GPO activities higher than those in erythrocytes with respect to catalase to improve the kinetic characterization of both enzymes.

**Fig 5.** Determination of GPO and catalase activity in human hemolysate. The same procedure is applied as shown in Fig 4. (A) Addition of highly diluted hemolysate (9.4 × 10⁻² g Hb/L). (B) Inhibition of catalase by 1 mmol/L NaN₃. (C) Second addition of 10⁻³ mol/L H₂O₂. (D) Addition of 2 mmol/L GSH and calibration of the measuring system. (E) Addition of 10⁻⁴ mol/L H₂O₂. A 10-fold higher concentrated hemolysate was used (for D to E) to better characterize the GPO-mediated decomposition of H₂O₂.
Hemolysate reflects intracellular conditions in respect to H2O2 decomposition.

H2O2 removal by fresh human hemolysate is shown in Fig 5: After the addition of H2O2 (Fig 5A), an exponential decay of H2O2 is observed (r² = .995) ascribed to catalase activity as inferred by the effect of NaN3 (Fig 5B) and the lack of H2O2 consumption when added subsequently (Fig 5C). Of note, negligible GSH levels were present in this highly diluted hemolysate (1/1,000) preventing any detectable GPO activity. Further addition of 2 mmol/L GSH and H2O2 (as described for Fig 4) permitted evaluation of GPO activity, which exhibited a kinetic pattern different from catalase. This degradation pattern was similar to that observed with purified GPO. The H2O2 degradation rate depended linearly on GSH concentration (as shown in Fig 3 for the purified enzyme).

The H2O2 degradation rate of catalase and GPO was plotted against H2O2 concentration (Fig 6). These data showed a prevalence of catalase at low H2O2 concentrations. GPO reaches only approximately 8% of the rate at which catalase simultaneously decomposes H2O2. H2O2 degradation by catalase depends linearly on H2O2 concentration, whereas GPO becomes saturated at concentrations of H2O2 greater than 10⁻⁶ mol/L. At H2O2 concentrations above 10⁻⁴ mol/L, catalase contributes almost exclusively to the overall turnover of H2O2. The relative activities of catalase and GPO at different H2O2 concentrations are listed in Table 1. In H2O2 concentrations less than 10⁻⁸ mol/L, GPO becomes unsaturated: Under these conditions, the degradation rate of H2O2 depends linearly on H2O2 concentration. At 2 mmol/L GSH, the Kₘ of GPO for H2O2 is calculated as 2 × 10⁻⁵ mol/L H2O2 in agreement with previous reports.

DISCUSSION

H2O2 degradation in erythrocytes has been studied for several decades in connection with the high oxygen turnover of these cells and the toxic properties of ROS derived from H2O2 metabolism. Interest in this area was renewed by articles describing a role for H2O2 in signal transduction.

In the present study, we have determined both catalase and GPO activities in hemolysate at physiological H2O2 concentrations by using a novel H2O2 assay. Because erythrocyte catalase and GPO are apparently not compartmentalized, the studies performed on hemolysates may reflect a situation similar to that in the erythrocyte. The luminol/hypochlorite method may be used to determine H2O2 degradation by GPO in the 10⁻⁷ mol/L H2O2 range and at GSH concentrations normally found in erythrocytes.

The assumption that the glutathione-GPO system has greater affinity for its substrate led both Keilin and Hartree to suggest that at H2O2 concentrations below 10⁻⁷ mol/L, catalase played almost no role in H2O2 detoxification. However, the studies herein indicated a clear predominance of catalase in removing H2O2 and they provided understanding of the increasing data underlying the function of catalase in erythrocytes. Considering these and the data herein, catalase needs to be regarded as the major H2O2-decomposing enzyme in normal erythrocytes. It decomposes H2O2 without generation of free radicals by minimizing one-electron-transfers. Hence, a protective role against free radicals may be the main physiological function in these cells. The existence of healthy acatalasic individuals cannot serve as an argument against the physiological importance of this enzyme. Most patients do not show a real “acatalasia” but a hypocatalasia. Additionally, Japanese acatalasic patients show evidence for increased

Table 1. Degradation Rates of H2O2 for Catalase and GPO in Human Erythrocytes at Different H2O2 Concentrations

<table>
<thead>
<tr>
<th>H2O2 (mol/L)</th>
<th>Catalase</th>
<th>GPO</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁷</td>
<td>4.5 × 10⁻⁸ ± 1.1 × 10⁻⁸</td>
<td>3.9 × 10⁻³ ± 0.8 × 10⁻³</td>
<td>7.8%</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>5.3 × 10⁻⁷ ± 0.7 × 10⁻⁷</td>
<td>4.0 × 10⁻⁴ ± 1.7 × 10⁻⁴</td>
<td>8.0%</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>4.9 × 10⁻⁷ ± 0.6 × 10⁻⁷</td>
<td>1.5 × 10⁻⁷ ± 0.5 × 10⁻⁷</td>
<td>3.0%</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>4.4 × 10⁻⁸ ± 0.9 × 10⁻⁸</td>
<td>5.0 × 10⁻⁷ ± 0.9 × 10⁻⁷</td>
<td>1.0%</td>
</tr>
</tbody>
</table>

*Degradation rates are related to gram hemoglobin (g⁻¹). Means ± standard deviations were calculated from samples of five healthy volunteers.
oxidative damage, and Swiss acatalasic variants show, at least in vitro, an enhanced sensitivity to a number of H$_2$O$_2$-generating agents. According to our data, a remaining activity of 1% catalase would still contribute largely to the overall H$_2$O$_2$ removal (approximately 50% at 10$^{-7}$ mol/L H$_2$O$_2$).

Otherwise, the hexosemonophosphate shunt in erythrocytes lacking detectable catalase activity is significantly increased because of glutathione use via GPO. Because the steady-state levels of H$_2$O$_2$ in tissues are considered to be between 10$^{-10}$ and 10$^{-7}$ mol/L, and were recently estimated to be approximately $2 \times 10^{-10}$ mol/L H$_2$O$_2$ in erythrocytes, GPO is not supposed to be saturated under these conditions. It may be surmised that GPO can undertake a substantial part of catalase activity in acatalasic erythrocytes. The contribution of hemoglobin to H$_2$O$_2$ degradation is minimal because of the low reaction rates. A major function of GPO in normal erythrocytes may be the disposal of organic peroxides and the maintenance of protein thiols in their reduced state.

It is worth mentioning that some discrepancies in the literature may be bridged by considering effects of azide and cyanide on erythrocyte metabolic pathways other than a unique inhibition of catalase. Additionally, it was long thought that GPO was the sole H$_2$O$_2$-removing enzyme in erythrocytes depending on NAPDH. The discovery of the role of catalase-bound NADPH unified the concept of two different mechanisms for disposing of H$_2$O$_2$ (catalase and the glutathione reductase/peroxidase pathway) by showing that both mechanisms are dependent on NAPDH.

In summary, these data strengthen the notion that H$_2$O$_2$ removal in normal erythrocytes is mainly the domain of catalase. On a methodological note, the luminol/hypochlorite assay may be a useful tool to study H$_2$O$_2$ degradation in other cells and tissues, which is a notion supported by previous preliminary studies with hepatocytes.

ACKNOWLEDGMENT

We are indebted to Dr E. Cadenas of the University of Southern California, Los Angeles, CA, for critical reading of the manuscript.

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