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Enhancement of glutathione-dependent haemin degradation by ascorbic acid

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Abstract

In the current work, we investigated the effect of ascorbic acid on GSH-mediated haemin degradation. GSH-mediated haemin degradation in the presence of ascorbic acid in phosphate-buffered saline and in erythrocyte ghosts was determined by recording absorbance at 365 and 399 nm, respectively. Generation of intracellular H_2O_2 was measured indirectly in terms of the inactivation of endogenous catalase in erythrocytes in the presence of 3-amino-1,2,4-triazole. Although ascorbic acid itself did not induce haemin degradation, it enhanced GSH-mediated haemin degradation. Experiments with catalase showed that H_2O_2 was essential in this process. The oxidation of ascorbic acid in the presence of haemin was stimulated by GSH, suggesting that ascorbic acid can alter the mechanism of H_2O_2 generation observed with GSH and haemin alone. These results suggest that enhancement of GSH-mediated haemin degradation by ascorbic acid may be due to an increase in the production of H_2O_2 generated by GSH and haemin in the absence of ascorbic acid.

Keywords: Haemin degradation; Ascorbic acid; Glutathione; Hydrogen peroxide; Erythrocyte

1. Introduction

The most concentrated store of haem in the body resides in erythrocyte haemoglobin [1]. In some pathological conditions or under oxidative stress, haem, a product of haemoglobin denaturation, may be released [1,2]. Increased free haem levels have been observed in β-thalassaemia and sickle cell anaemia [3] as well as in glucose-6-phosphate dehydrogenase deficiency [4]. Haemin tends to intercalate into the membrane lipid bilayer as well as bind to membrane proteins [5–7]. These interactions may result in the dissociation of cell membrane skeletal proteins [8,9] and increases in membrane permeability [10] as well as cell lysis [5,10,11].

Glutathione (GSH), an intracellular reductant, has been shown to inhibit haemin-induced haemolysis [7]. The inhibitory effect of GSH on haemolysis is due to its ability to bind with haemin. Several years ago, Atamna and Ginsburg [12] reported that GSH itself could degrade haemin by an oxygen-dependent mechanism.

Ascorbic acid, another intracellular reductant, has been shown to inhibit oxidative modification of human low-density lipoprotein (LDL) by haemin and H_2O_2 [13]. Both GSH and ascorbic acid inhibit haemin-dependent lipid peroxidation in rat liver microsomes [14] and degradation of folic acid in the presence of H_2O_2 [15]. Interestingly, it has been shown that the concentration of ascorbic acid is decreased markedly in both sickle cell disease and iron overload disease [16]. In the current work, we studied the role of ascorbic acid on haemin degradation. Our results demonstrate that, although ascorbic acid itself does not induce haemin degradation, it is able to enhance GSH-dependent degradation of haemin, probably by increasing the flux of H_2O_2 through the cell.

2. Materials and methods

2.1. Materials

Haemin (ferriprotoporphyrin IX chloride), GSH, H_2O_2, 3-AT, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained

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Abbreviations: 3-AT, 3-amino-1,2,4-triazole; CAT, catalase; DFO, desferrioxamine; DTPA, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase.

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from the Sigma Chemical Co. CAT, SOD, ascorbic acid, DTPA, and DFO were purchased from the ICN Co. All other chemicals were of analytic grade.

2.2. Preparation of erythrocytes, ghosts, and haemin

Fresh erythrocytes were obtained from healthy adult sheep by venipuncture. Heparinized blood was washed three times in PBS (135 mM NaCl and 10 mM sodium phosphate, pH 7.4). The washed erythrocytes were suspended to a final haematocrit of 1%. Erythrocyte membranes were prepared from the reaction mixtures by the method of Hanahan and Ekholm [17]. Ghosts were washed with 10 mM Tris buffer (pH 7.6) until they became colourless. Erythrocyte membrane proteins were assayed by the method of Lowry *et al*. [18].

Haemin was prepared fresh at the beginning of each experiment as a 2 mM stock solution in 5 mM NaOH and was kept in the dark on ice.

2.3. Degradation of haemin

Haemin was incubated at 37° in PBS (pH 7.4) for 30 min in the presence of GSH and/or in the presence of ascorbic acid. Haemin concentration was monitored as a decrease in absorbance at 365 nm and calculated using a millimolar extinction coefficient of 64.1 [12]. As Atamna and Ginsburg [12] pointed out, the degradation of haemin is a first order reaction with respect to haemin. The initial rate of haemin degradation (μM/min) is the rate constant × the initial concentration of haemin. The effect of the hypoxic conditions was measured by bubbling nitrogen (N_2) through PBS and the stock solutions, and the haemin degradation was followed as described above under continuous exposure to N_2.

When degradation of membrane-associated haemin was assayed, white erythrocyte ghosts were suspended in PBS (pH 7.4) containing 30 μM haemin to a concentration of 3 mg of membrane protein/mL and incubated for 60 min at 37°. Ghosts were then washed twice in 10 mM Tris buffer (pH 7.6) and finally resuspended in PBS to a concentration of 3 mg/mL (pH 7.4). Two milliliters of the ghost suspension was mixed with 1 mL PBS (pH 7.4) containing the agents for testing for 180 min at 37°. Ghosts were then washed twice with 10 mM Tris buffer (pH 7.6) and finally resuspended in PBS to a concentration of 3 mg/mL (pH 7.4). To determine membrane-associated haemin, the ghosts were dissolved by adding SDS (1% (w/v) final concentration). Haemin concentration was determined as absorbance at 399 nm and calculated using a millimolar extinction coefficient of 83.5 [12].

2.4. Catalase inactivation

Under normal *in vivo* conditions, 3-AT almost completely inhibits liver CAT, whereas it has a minimal effect on erythrocyte CAT [19]. CAT bound to H_2O_2 is inhibited irreversibly by 3-AT [20], and the extent of inhibition in erythrocytes exposed to 3-AT *in vitro* is a function of H_2O_2 flux within the cell [21]. In our experiments, 5% erythrocytes were incubated with 1 mL PBS containing 20 μM haemin, 40 mM 3-AT, 1 mM DTPA, and 2 mM GSH in the presence or absence of 0.2 mM ascorbic acid at 37° for 60 min. Erythrocytes were then washed three times with PBS, and lysed in 1 mL of lysis buffer (sodium phosphate buffer, 5 mM, pH 7.4). After centrifugation (16,000 *g*, 10 min, 4°), CAT activity was measured. Briefly, 10 μL of lysate was added to 990 μL of 6 mM H_2O_2, and decomposition of the substrate was followed by the decrease in absorbance at 236 nm [12]. Uninhibited CAT activity was estimated from the mixture containing erythrocytes and 3-AT only.

2.5. Oxidation of ascorbic acid and GSH

Ascorbic acid (0.5 mM) was incubated at 37° in PBS buffer (pH 7.4) containing 30 μM haemin and 1 mM DTPA for 30 min in the presence of 0.1 or 2 mM GSH or 100 U/mL of SOD. At various time intervals, 50 μL of the mixture was transferred into microcentrifuge tubes and added to 950 μL PBS (pH 7.4). The content of ascorbic acid was calculated by monitoring the absorbance at 265 nm [22].

GSH (2 mM) was incubated at 37° in PBS buffer (pH 7.4) containing 30 μM haemin and 1 mM DTPA for 30 min in the presence of 0.1, 0.5, and 1 mM ascorbic acid, respectively. At various time intervals, 50 μL of the mixture was added to 950 μL PBS buffer (pH 7.4) in a microcentrifuge tube. After centrifugation (16,000 *g*, 2 min, 4°), the content of GSH in the supernatant was determined according to the colorimetric method by evaluating the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) at 412 nm [23].

2.6. Statistics

Data represent means ± SD of five separate experiments. Student's paired *t*-test was used to compare mean values with and without ascorbic acid treatment. Multiple comparisons between groups at different pH values were done using the Student–Newman–Keuls test after analysis of variance was performed. A P value less than 0.05 was considered significant.

3. Results

3.1. Effect of ascorbic acid on GSH-mediated degradation of haemin

In our experiments, ascorbic acid alone did not mediate degradation of haemin (data not shown). By contrast, ascorbic acid remarkably enhanced GSH-mediated degradation

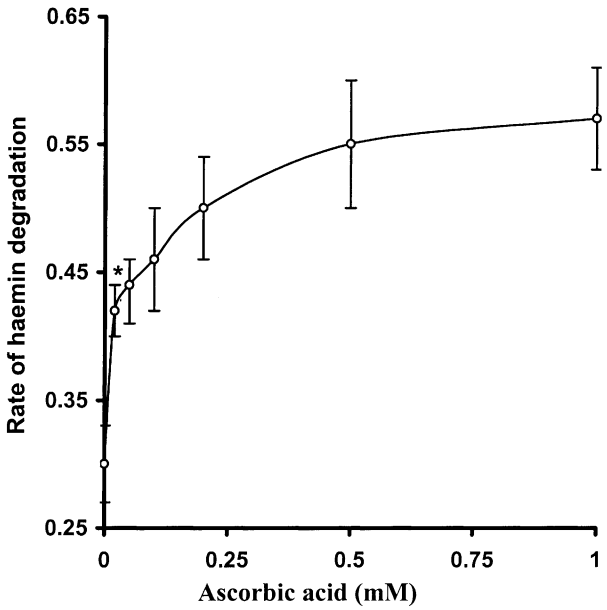

Fig. 1. Effect of ascorbic acid on GSH-mediated degradation of haemin in PBS. Haemin (20 μM) was incubated with 2 mM GSH in PBS (pH 7.4) for 30 min at 37° in the presence of ascorbic acid. Haemin concentration was monitored as a decrease in absorbance at 365 nm. The degradation of haemin is a first order reaction with respect to haemin. The initial rate of haemin degradation (μM/min) = the rate constant × the initial concentration of haemin. Key: (*) $P < 0.05$, compared with GSH + haemin (Student's paired *t*-test). Data represent means ± SD of five separate experiments.

of haemin (Fig. 1). When the concentration of haemin was 20 μM, 0.1 mM and 0.5 mM ascorbic acid increased the rate of GSH-mediated degradation of haemin by 50 and 80%, respectively (Fig. 1).

As Atamna and Ginsburg [12] pointed out, the degradation of haemin is a first order reaction. Here, we found GSH-dependent haemin degradation in the presence of ascorbic acid also to be of first order kinetics (data not shown).

The rate of haemin degradation was linear up to 1 mM GSH in the presence and absence of ascorbic acid, although it was lower in the absence of the vitamin (Fig. 2a). The extent of haemin degradation in the presence of ascorbic acid was dependent upon the haemin concentration (Fig. 2b).

Haemin, released from denatured or oxidised haemoglobin in the erythrocyte, can bind to the cell membrane [5–7]. It has been shown that GSH alone degrades membrane-associated haemin and induces release of iron from the membrane [12]. It was of interest to investigate whether ascorbic acid is able to increase GSH-dependent degradation of haemin in the cell membrane. White erythrocyte ghosts loaded with haemin were incubated with GSH (10 mM) in PBS in the presence and the absence of 1 mM ascorbic acid for 180 min. As shown in Fig. 3, ascorbic acid also remarkably increased the degradation of membrane-associated haemin in the presence of GSH.

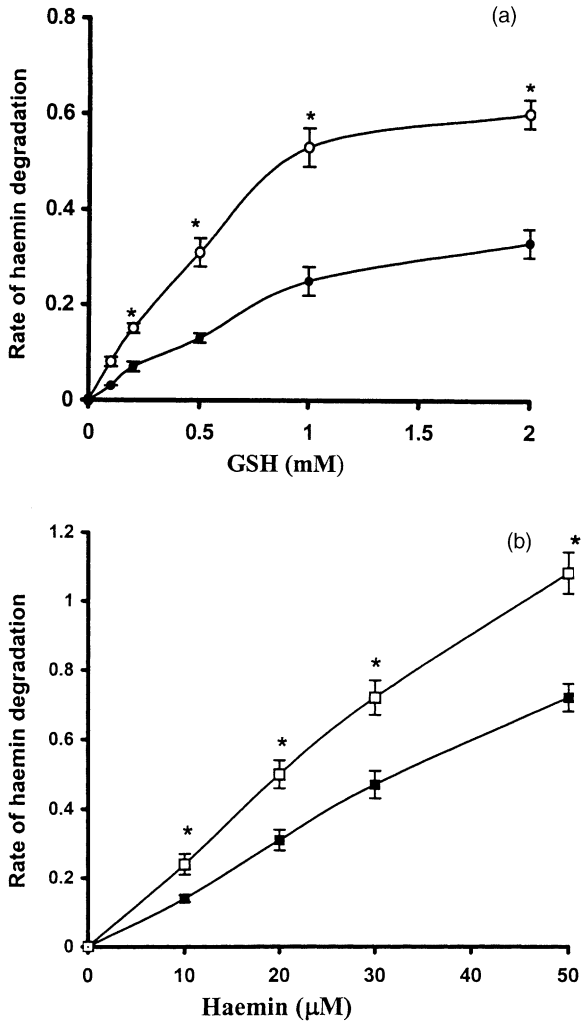

Fig. 2. Dependence of haemin degradation on GSH and haemin concentration in PBS. Haemin was incubated with GSH in PBS for 30 min at 37° in the presence of 0.5 mM ascorbic acid (open circles or squares) and in the absence of ascorbic acid (solid circles or squares). Haemin concentration was monitored as a decrease in absorbance at 365 nm. (a) Increasing GSH concentration in the presence of 20 μM haemin. (b) Increasing haemin concentration in the presence of 2 mM GSH. Key: (*) $P < 0.05$, compared with GSH + haemin (Student's paired *t*-test). Data represent means ± SD of five separate experiments.

3.2. Roles of oxygen species, iron, and pH on degradation of haemin by GSH and ascorbic acid

In our experiments, several factors that may potentially exert an influence on the rate of haemin degradation were investigated (Table 1). In the presence of N_2, degradation of haemin by GSH and ascorbic acid in PBS (pH 7.4) was minimal, indicating that O_2 is involved in this process. CAT (100 U/mL) completely inhibited the degradation of haemin by GSH and ascorbic acid in PBS (Table 1). SOD (100 U/mL) partially inhibited the degradation of haemin by GSH in the presence of ascorbic acid (about 33%). By contrast, SOD (100 U/mL) inhibited by about 58% the degradation of haemin by GSH alone. At higher SOD concentrations, we did not observe any additional

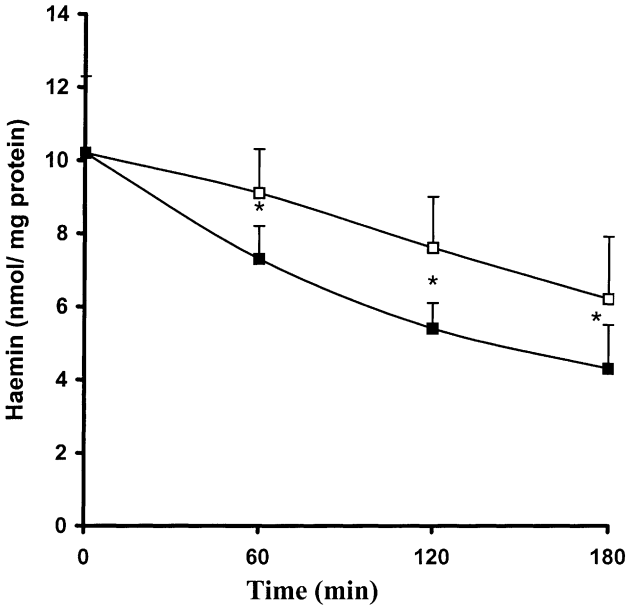

Fig. 3. Effect of ascorbic acid on GSH-mediated degradation of membrane-associated haemin. White erythrocyte ghosts (containing 3 mg of membrane protein/mL) were loaded at 37° for 60 min with 30 μM haemin. After the removal of non-associated haemin, the membranes were incubated with 10 mM GSH in PBS buffer (pH 7.4) at 37° for 180 min in the presence (solid squares) or absence (open squares) of 1 mM ascorbic acid. Haemin content was assayed by dissolving the white ghosts in 1% SDS and measuring the absorbance at 399 nm. Key: (*) $P < 0.05$, compared with GSH + haemin (Student's paired *t*-test). Data represent means ± SD of five separate experiments.

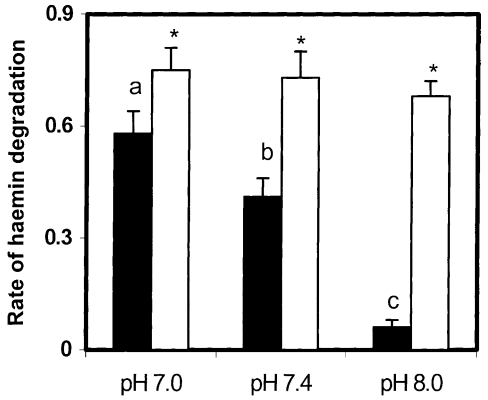

Fig. 4. Effect of ascorbic acid on GSH-mediated degradation of haemin in PBS at three pH values. Haemin (20 μM) was incubated with 2 mM GSH in PBS at 37° for 30 min in the presence (open bars) and absence (solid bars) of 0.5 mM ascorbic acid at pH 7.0, 7.4, and 8.0, respectively. Haemin concentration was monitored as a decrease in absorbance at 365 nm. Key: (*) $P < 0.05$, compared with GSH + haemin at corresponding pH (Student's paired *t*-test); and (a, b, and c) mean values with different letters are significantly different ($P < 0.05$, Student–Newman–Keuls test). Data represent means ± SD of five separate experiments.

inhibition of haemin degradation by GSH in the presence and absence of ascorbic acid (data not shown) indicating that SOD was saturating at 100 U/mL. DFO completely inhibited degradation of haemin by GSH and ascorbic acid while DTPA had little effect, indicating that haemin-iron rather than free iron is essential in the process.

It has been shown that haemin degradation is pH-dependent in PBS or 0.2 M HEPES buffer, peaking at pH 7.0, and almost undetectable in PBS at pH 8.0 [12]. Our data indicated that the rates of haemin degradation by GSH alone at pH 7.0, 7.4, and 8.0 were significantly different (0.59 ± 0.02, 0.41 ± 0.03, and 0.06 ± 0.02 μM/min, respectively) (Fig. 4). In this regard, our data are consistent with the results of Atamna and Ginsburg [12]. By contrast, there were no significant differences in the rates of haemin degradation by GSH in the presence of ascorbic acid at pH 7.0, 7.4, and 8.0 (Fig. 4). Similar results were also observed in 0.2 M HEPES buffer (data not shown).

3.3. Enhancement of intracellular H_2O_2 by GSH and haemin in the presence of ascorbic acid

It has been shown that haemin and GSH yield H_2O_2 intracellularly and that H_2O_2 is essential for haemin

Table 1
The effect of antioxidants on the rate of haemin degradation

Sample	Rate of haemin degradation (μM/min)
20 μM haemin + 2 mM GSH	0.31 ± 0.03
20 μM haemin + 2 mM GSH + 0.5 mM ascorbic acid	0.55 ± 0.04*
20 μM haemin + 2 mM GSH + 0.5 mM ascorbic acid (under N_2)	0.05 ± 004**
20 μM haemin + 2 mM GSH + 100 U/mL SOD	0.18 ± 0.02*
20 μM haemin + 2 mM GSH + 0.5 mM ascorbic acid + 100 U/mL SOD	0.38 ± 0.05*
20 μM haemin + 2 mM GSH + 0.5 mM ascorbic acid + 100 U/mL CAT	0.03 ± 001**
20 μM haemin + 2 mM GSH + 0.5 mM ascorbic acid + 1 mM DFO	0.02 ± 0.02**
20 μM haemin + 2 mM GSH + 0.5 mM ascorbic acid + 1 mM DTPA	0.53 ± 0.06

Haemin (20 μM) was incubated at 37° in PBS (pH 7.4) for 30 min in the presence of 2 mM GSH and/or in the presence of 0.5 mM ascorbic acid. Haemin degradation was monitored as the decrease in absorbance at 365 nm. Haemin degradation is a first order reaction. The initial rate of haemin degradation = the rate constant × the initial concentration of haemin.

* Significantly different from 20 μM haemin + 2 mM GSH ($P < 0.05$, Student's paired *t*-test).

** Significantly different from 20 μM haemin + 2 mM GSH + 0.5 mM ascorbic acid ($P < 0.05$, Student's paired *t*-test). Data represent means ± SD of five separate experiments.

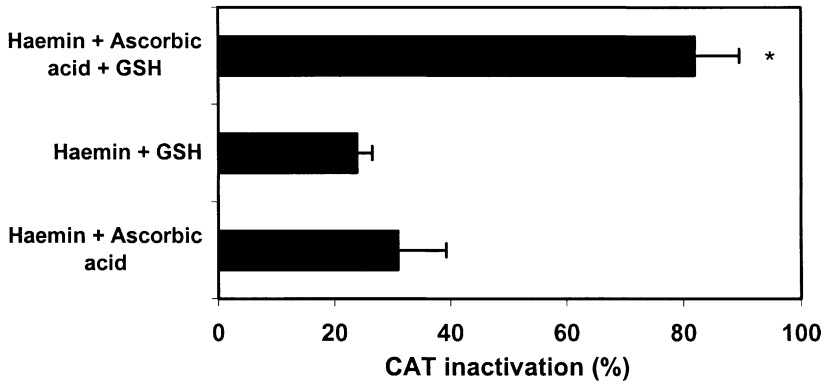

Fig. 5. Effect of ascorbic acid and GSH on CAT inactivation in the presence of 3-AT. Erythrocyte suspensions (1%) were incubated with 20 μM haemin and 40 mM 3-AT in PBS containing 2 mM GSH and 1 mM DTPA at 37° for 60 min in the presence or absence of 0.2 mM ascorbic acid. Key: (*) $P < 0.05$, compared with GSH + haemin (Student's paired *t*-test). Data represent means ± SD of five separate experiments.

degradation [12]. We therefore investigated the effect of ascorbic acid on the production of H_2O_2 induced by haemin and GSH. CAT inactivation in the presence of 3-AT has been used to demonstrate H_2O_2 flux in erythrocytes [21]. Fig. 5 shows that either GSH alone or ascorbic acid alone in the presence of haemin can induce CAT inactivation in erythrocytes, indicating H_2O_2 generation. In the presence of GSH and ascorbic acid plus haemin, CAT in erythrocytes was inhibited to a much greater extent. CAT inactivation was 82% in the presence of ascorbic acid and GSH, compared with that of 31% in the presence of ascorbic acid alone or 24% in the presence of GSH alone (Fig. 5). Clearly, ascorbic acid synergistically increased H_2O_2 production induced by GSH and haemin.

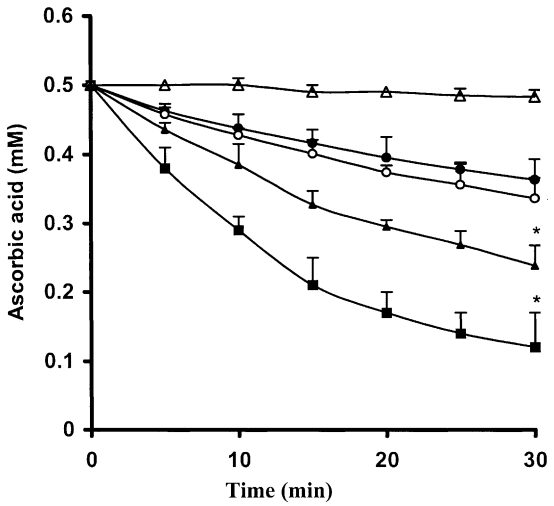

Fig. 6. Effect of GSH on the oxidation of ascorbic acid by haemin. Ascorbic acid (0.5 mM) was incubated at 37° in PBS containing 30 μM haemin and 1 mM DTPA for 30 min in the presence of 0.1 or 2 mM GSH or 100 U/mL of SOD. Ascorbic acid alone (open triangles); ascorbic acid + haemin (solid circles); ascorbic acid + haemin + 2 mM GSH + SOD (open circles); ascorbic acid + haemin + 0.1 mM GSH (solid triangles); ascorbic acid + haemin + 2 mM GSH (solid squares). Key: (*) $P < 0.05$, compared with ascorbic acid + haemin (Student's paired *t*-test), and (**) $P < 0.05$, compared with ascorbic acid + haemin + 2 mM GSH (Student's paired *t*-test). Data represent means ± SD of five separate experiments.

3.4. Effect of GSH on the oxidation of ascorbic acid

To further the understanding of the interaction between GSH and ascorbic acid in the degradation of haemin, the oxidation of ascorbic acid and GSH in the presence of haemin was investigated. In our experiments, we found that autoxidation of ascorbic acid was very slow in PBS in the absence of haemin (Fig. 6, open triangles). The oxidation of ascorbic acid that occurred in the presence of haemin (Fig. 6, solid circles) was greatly accelerated by GSH in the presence of haemin (compare Fig. 6, solid circles with solid triangles and solid squares). GSH at 2 mM was more effective than at 0.1 mM. SOD remarkably inhibited the oxidation of ascorbic acid in the presence of haemin and GSH (Fig. 6, open circles) but had no effect on the oxidation of ascorbic acid in the presence of haemin alone (data not shown).

We also observed that, although the oxidation of GSH occurred in the presence of haemin, it was unaffected by ascorbic acid (data not shown).

4. Discussion

It has been shown that GSH can form a complex with haemin through its thiol group [7] and induce haemin degradation [12]. Unlike GSH alone, our results show that ascorbic acid alone does not mediate the degradation of haemin (data not shown). In our experiments, ascorbic acid did, however, significantly stimulate the degradation of haemin mediated by GSH alone (Figs. 1–3). It has been shown previously that intracellular GSH is able to induce haemin degradation in intact human erythrocytes [12].

The homeostatic level of ascorbic acid in human erythrocytes is about 0.1 mM [24]. Our data presented here show that as little as 0.02 mM of added ascorbic acid can increase by about 40% haemin degradation by GSH ($P < 0.05$). Thus, it is reasonable to conclude that the combination of GSH and ascorbic acid may have a synergistic effect on haemin degradation under prevailing physiological conditions.

The mechanism behind the GSH-mediated degradation of haemin is not clear [12]. The data from Atamna and Ginsburg [12] have shown that: (i) the reaction is oxygen-dependent since haemin degradation does not occur in the absence of O_2; (ii) since DFO completely inhibits haemin degradation by GSH, haemin-iron in the Fe^{3+} state can be reduced to Fe^{2+} by GSH in this process; (iii) the degradation of haemin is maximal at pH 7 and almost undetectable at a higher pH (pH 8) in PBS; and (iv) CAT inhibits haemin degradation completely and SOD inhibits haemin degradation by 91%, indicating that both $O_2^{\bullet -}$ and H_2O_2 are involved in the process.

O_2 is also involved in GSH-mediated haemin degradation in the presence of ascorbic acid since the reaction does not occur under N_2 (Table 1). In our experiments, SOD inhibited haemin degradation by GSH in the presence of ascorbic acid by about 33%. Although inhibition by SOD of GSH-mediated haemin degradation in PBS in our study (about 58%) was lower than that reported by Atamna and Ginsburg in HEPES buffer [12], we nonetheless report a significant difference in the inhibition of haemin degradation by SOD in PBS in the presence and absence of ascorbic acid ($P < 0.05$). Furthermore, in stark contradistinction to haemin degradation by GSH alone, no difference was seen in the rate of haemin degradation in the presence of ascorbic acid at pH 7.0, 7.4, and 8.0 (Fig. 4). Taken together, these data imply that the mechanism of GSH-mediated haemin degradation in the presence of ascorbic acid is fundamentally different from that in the absence of ascorbic acid.

It has been suggested that GSH can prevent the oxidation of ascorbic acid by the copper ion [25]. By contrast, our results show that GSH remarkably increased the oxidation of ascorbic acid (Fig. 6), whereas ascorbic acid did not affect the oxidation of GSH in the presence of haemin (data not shown). The effect of GSH on the oxidation of ascorbic acid in the presence of haemin was inhibited profoundly by SOD (Fig. 6), indicating that $O_2^{\bullet -}$ is an essential intermediate for the oxidation of ascorbic acid in the presence of haemin and GSH. However, SOD failed to inhibit the oxidation of ascorbic acid in the presence of haemin alone (data not shown). These results imply that GSH, at least partially, changes the mechanism of the oxidation of ascorbic acid in the presence of haemin.

Chiu *et al*. [26] have reported that haemin oxidizes GSH, yielding thiyl radicals ($GS^{\bullet}$). These authors have suggested that haemin iron is converted from Fe^{3+} to Fe^{2+} by GSH, thus promoting the $O_2^{\bullet -}$-generating cycle. These data may help us to further understand both the mechanism of the oxidation of ascorbic acid and the enhancement of haemin degradation by ascorbic acid.

In the absence of GSH, the oxidation of ascorbic acid occurred in the presence of haemin (Fig. 6). In this process, haemin is more likely to serve as a catalyst than an oxidant since the absorption band of haem-Fe^{2+} at 580 nm [7] was not observed in the presence of haemin and ascorbic acid in our experiments (data not shown). It has been shown that $O_2^{\bullet -}$ is not involved in the oxidation of ascorbic acid in the presence of transition metals [27,28]. Since $O_2^{\bullet -}$ is not an intermediate for the oxidation of ascorbic acid, SOD does not inhibit the reaction. If GSH exists in the system, haemin can react with GSH, generating $GS^{\bullet}$ [26]. The dimerization of $GS^{\bullet}$ to oxidised glutathione (GSSG) is expected to contribute very little to the removal of the thiyl radicals, for a high steady-state concentration of these radicals is required for a bimolecular collision [29]. Under normal circumstances, $GS^{\bullet}$ undergoes conjugation reaction with thiolate (GS^-) [29] (reaction 1; a small percent of GSH ($pK_a = 9$) will be present as GS^- at physiological pH).

$$GS^{\bullet} + GS^- \rightarrow [GSSG]^{\bullet -} \quad (1)$$

$[GSSG]^{\bullet -}$ is a very strong reducing agent and can react with O_2 to generate $O_2^{\bullet -}$ (reaction 2) [29].

$$[GSSG]^{\bullet -} + O_2 \rightarrow GSSG + O_2^{\bullet -} \quad (2)$$

Indeed, many studies have shown that the $O_2^{\bullet -}$ is produced in the presence of GSH and transition metals [30–33] or haemin [26,34]. $O_2^{\bullet -}$ can function as an oxidant to react with ascorbic acid [28], and the following reaction (reaction 3) can take place:

$$O_2^{\bullet -} + H^+ + \text{ascorbic acid} \rightarrow H_2O_2 + \text{dehydroascorbic acid} \quad (3)$$

Here, $O_2^{\bullet -}$ becomes an essential intermediate for the oxidation of ascorbic acid. This explains the observation that SOD can remarkably inhibit the oxidation of ascorbic acid in the presence of haemin and GSH (Fig. 6). On the other hand, the oxidation of ascorbic acid catalysed by haemin alone still occurs even in the presence of SOD. This again provides a ready explanation of our observation that SOD did not completely inhibit the oxidation of ascorbic acid in the presence of GSH and haemin (Fig. 6).

H_2O_2 is essential in GSH-dependent haemin degradation in the presence or absence of ascorbic acid since CAT inhibits both reactions (Table 1). This may provide an explanation for the ascorbic acid enhancement of GSH-dependent haemin degradation. Compared with the production of H_2O_2 by haemin and GSH alone, the reaction is stimulated in the presence of ascorbic acid (Fig. 5). In the absence of ascorbic acid, the probable pathway that produces H_2O_2 by GSH and haemin proceeds as follows (reaction 4):

$$O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 \quad (4)$$

The fact that haemin degradation by GSH alone decreases with an increase in pH and almost ceases at pH 8 in PBS ([12] and Fig. 4) supports this view since the non-enzymatic dismutation of $O_2^{\bullet -}$ decreases with an increase in pH [29]. In the presence of ascorbic acid, the production of H_2O_2 is mainly via reaction 3 above. Our observation that there is no difference in haemin

degradation in the presence of ascorbic acid at pH 7.0, 7.4, and pH 8.0 (Fig. 4) supports this point.

It has been shown that H_2O_2 can degrade haem [35–37] and that the initial reaction involves haem peroxidation [38–40]. If ascorbic acid is present in this system, the complex can react with it to release free haem [40]. This may explain why no apparent degradation of haemin was observed in the presence of ascorbic acid alone in our experiments. GSH can degrade haemin in the presence or absence of ascorbic acid. This means that GSH-mediated haemin degradation is essentially different from H_2O_2-mediated haemin degradation, although H_2O_2 is proven to be involved in both reactions. Other data support this view. The product of haemin degradation by H_2O_2 is biliverdin [35], while the product of haemin degradation by GSH is not biliverdin [12].

The $^{\bullet}OH$ radical that can be generated from $O_2^{\bullet -}$ and H_2O_2 via the Haber–Weiss reaction is unlikely to be involved in GSH-mediated haemin destruction, as the rate of degradation in the presence of the $^{\bullet}OH$ radical scavenger HEPES is faster than in PBS [12]. Clearly, the mechanism of GSH-mediated degradation and particularly the role of H_2O_2 need to be investigated further in light of our current results.

Acknowledgments

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