Ascorbate-mediated electron transfer in protein thiol oxidation in the endoplasmic reticulum

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Abstract Addition of, or gulonolactone oxidase-dependent in situ generation of, ascorbate provoked the oxidation of protein thiols, which was accompanied by ascorbate consumption in liver microsomal vesicles. The maximal rate of protein thiol oxidation was similar upon gulonolactone, ascorbate or dehydroascorbate addition. Cytochrome P450 inhibitors (econazole, proadifen, quercetin) decreased ascorbate consumption and the gulonolactone or ascorbate-stimulated thiol oxidation. The results demonstrate that the ascorbate/dehydroascorbate redox couple plays an important role in electron transfer from protein thiols to oxygen in the hepatic endoplasmic reticulum, even in gulonolactone oxidase deficient species.

Key words: Ascorbate; Dehydroascorbate; Protein thiol; Hydrogen peroxide; Gulonolactone oxidase; Endoplasmic reticulum

1. Introduction

The endoplasmic reticulum (ER) of the cell is the site of synthesis, post-translational modification and folding of proteins transported along the secretory pathway. The oxidizing environment in the lumen of the ER is necessary for the formation of disulfide bonds and for the proper folding of these proteins (for recent comprehensive reviews on the topic, see [1,2]). The formation of native disulfide bonds is catalyzed by protein disulfide isomerase [3]. The effect of unknown oxidant(s), necessary for its activity, is reflected in and supported by the glutathione redox buffer. The ratio of glutathione (GSH) and glutathione disulfide (GSSG) is around 2:1 within the lumen of ER and along the secretory pathway, whilst the cytosolic ratio ranges from 30:1 to 100:1 [4]. However, the primary source(s) and the mechanism of the oxidative environment remain to be elucidated. Recent observations suggest two possible mechanisms. First, the preferential uptake of the oxidized member of a redox couple through the ER membrane and/or the efflux (or exocytosis) of its reduced form could ensure the oxidative environment. Alternatively, enzymes resident in the membrane or lumen of the ER could produce oxidizing compounds (e.g. reactive oxygen species) towards the lumen. Experimental evidences support both mechanisms. On the one hand, the preferential transport of dehydroascorbate (the oxidized form of ascorbate) has been described in rat liver microsomal vesicles [5]. In contrast, GSSG transport seems to be negligible [6]. On the other hand, several microsomal enzymes (cytochrome P450s, NADPH cytochrome P450 reductase, gulonolactone oxidase, microsomal iron protein, NADPH-dependent oxidase, sulfhydryl oxidase, flavin-containing monooxygenase etc.) can produce reactive oxygen species [7–14]. The recent exploration of yeast ER oxidase protein (Ero1p) and its role in the protein folding supports also the latter mechanism [15,16]. However, little direct information is available on the enzymatic mechanism and redox partners of Ero1p. There are no data about either the source of the oxidizing equivalents or the target to which it transfers them. It can oxidize an intermediate carrier (e.g. GSH) or directly the reduced proteins.

The role of ascorbate in the electron transfer from proteins has been suggested for a long time [17]. Topological coincidences support this view in case of disulfide bond formation: the last steps of ascorbate synthesis are located in the ER [18]. Gulonolactone oxidase, an ER resident flavoenzyme catalyzing the final reaction of ascorbate biosynthesis, is known to produce the oxidant hydrogen peroxide as a byproduct [18]. Ascorbate itself can also behave as a pro-oxidant under some circumstances [19]. It has been observed that gulonolactone oxidase activity stimulated by gulonolactone addition results in the oxidation of GSH both in isolated hepatocytes and in the microsomal system [20]. In GSH-loaded microsomal vesicles, the reaction led to intraluminal GSSG formation [7]. Since recent observations indicate that GSH can compete with protein thiols for the oxidizing agent [21], it can be supposed that the oxidizing agent generated by gulonolactone oxidase activity contributes to the disulfide bridge formation in proteins. In the present work, therefore, the effect of ascorbate and its metabolism on the oxidation of protein thiols of the ER has been investigated.

2. Materials and methods

2.1. Materials

L-Gulono-γ-lactone, 5,5′-dithio-bis(2-nitrobenzoic acid), econazole, proadifen, quercetin, alamethicin, α,α′-dipyridyl and ascorbate were obtained from Sigma. Dehydroascorbate was prepared as described earlier [5]. All other chemicals were of analytical grade.

2.2. Preparation of liver microsomes

Microsomes were prepared from male Wistar rats (180–230 g) or male guinea pigs (700–750 g) as described in [22]. Animals were obtained from Charles River (Budapest, Hungary). Human liver microsomes were prepared from the marginal normal tissues of liver tumors removed by surgical operation. The Ethical Committee of the Uni-

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Abbreviations: ER, endoplasmic reticulum; GSH, glutathione; GSSG, glutathione disulfide; Mops, morpholinepropanesulfonic acid; HPLC, high-pressure liquid chromatography

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versity approved the use of human samples. Microsomal fractions were resuspended in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM morpholinepropanesulfonic acid (Mops), pH 7.2. The suspensions were rapidly frozen and maintained under liquid N_2 until required. The intactness of microsomal membrane was checked by the measurement of the latencies of mannose-6-phosphatase [23] and *p*-nitrophenol UDP-glucuronosyltransferase [24] activities, which were greater than 90 and 95%, respectively.

2.3. Incubation of microsomes and the measurement of metabolites

For the determination of protein thiol oxidation and ascorbate synthesis/consumption, intact or permeabilized microsomal vesicles (usually 1 mg protein/ml) were incubated in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM Mops, pH 7.2, at 37°C. For permeabilization, microsomes were treated with alamethicin (0.05 mg/mg protein) [24]. Incubations were terminated by the addition of 0.05 volume of 100% trichloroacetic acid. Ascorbate contents were measured in trichloroacetic acid soluble supernatants by the method of Omaye et al. [25], based on the reduction of Fe^{3+} with the oxidation of ascorbate and the subsequent determination of the Fe^{2+}-α,α′-dipyridyl complex. Protein thiols were measured in the washed and resuspended pellets by the Ellman method [26]. Microsomal ascorbate and dehydroascorbate formation upon gulonolactone addition was also measured by reverse phase high-pressure liquid chromatography (HPLC) after a specific sample preparation as described earlier [27]. The protein concentration of microsomes was determined by using Bio-Rad protein assay solution with bovine serum albumin as standard according to the manufacturer's instructions. All data were expressed as means ± S.D.

3. Results

3.1. Ascorbate-dependent oxidation of protein thiols in liver microsomes

Rat, guinea pig or human liver microsomal vesicles were incubated in the presence of a cytosol-like concentration of ascorbate (0.1 mM). The disappearance of protein thiols and the consumption of ascorbate were measured. In the absence of ascorbate, the amount of protein thiols was unchanged up to 1 h incubation. Ascorbate addition resulted in a decrease of the protein thiol concentration (Table 1), which was due to their oxidation, since they could be reduced back with mercaptoethanol (data not shown). In rat liver microsomes, 1 h incubation resulted in the oxidation of about 50% of protein thiols (19.9 ± 2.4 nmol/mg protein, mean ± S.D., $n = 7$), which increased to 70% (28.8 ± 5.1 nmol/mg protein, mean ± S.D., $n = 6$) after 2 h. No significant further oxidation could be observed at later times, although ascorbate was still present in the medium. The thiol oxidation was accompanied by ascorbate consumption, which suggested that ascorbate was oxidized in the process, so the effect of dehydroascorbate was also investigated. It caused thiol oxidation while it was reduced to ascorbate but it was less effective than ascorbate even at a concentration of 1 mM (Table 1). Acceleration of ascorbate oxidation by hydrogen peroxide increased ascorbate consumption but the oxidation of protein thiols did not increase proportionally (Table 1). In control experiments, hydrogen peroxide in a 1 mM concentration was almost ineffective (Table 1). Even at 10 mM, it caused only a negligible decrease in protein thiols in the absence of added ascorbate (data not shown).

3.2. Gulonolactone oxidase activity-dependent oxidation of microsomal protein thiols

Gulonolactone, the substrate of gulonolactone oxidase, was added to rat microsomal vesicles to study the putative role of in situ ascorbate synthesis in microsomal protein thiol oxidation. Ascorbate synthesis from gulonolactone resulted in the oxidation of protein thiols (Table 2). The apparent Michaelis constant for the reaction was 8.1 μM and the apparent maximal rate was 315 pmol/min/mg protein (Fig. 1a). The time course of the reaction measured in the presence of 1 mM gulonolactone showed that after an initial lag phase (≈15 min), the rate of protein thiol disappearance was linear up to 1 h (Fig. 1b). The oxidizing effect was due to the metabolism of gulonolactone. The effect was totally absent in human or guinea pig liver microsomes, which do not have gulonolactone oxidase activity (Fig. 1b). Ascorbate generated by gulonolactone oxidase was further oxidized and dehydroascorbate formation could be observed (Fig. 2).

3.3. Inhibition of ascorbate-mediated microsomal protein thiol oxidation by cytochrome P450 inhibitors

The enzymatic nature of electron transfer was investigated by using inhibitors of the possible enzymes involved. It has

Table 1
Effect of ascorbate (AA) on the oxidation of protein thiols in rat, guinea pig and human liver microsomal vesicles

Addition	Rat liver microsomes		Guinea pig liver microsomes		Human liver microsomes	
	Oxidation of protein thiols	Ascorbate consumption	Oxidation of protein thiols	Ascorbate consumption	Oxidation of protein thiols	Ascorbate consumption
	pmol/min/mg protein					
None	12 ± 34 (8)	30 ± 14 (8)	18 ± 23 (3)	ND	2 ± 2 (4)	ND
1 mM H_2O_2	26 ± 43 (6)	NM	52 ± 5 (3)	ND	7 ± 16 (3)	ND
0.1 mM AA	332 ± 40 (7)	362 ± 68 (8)	135 ± 81 (4)	149 ± 59 (4)	149 ± 35 (7)	407 ± 128 (7)
0.1 mM AA+1 mM H_2O_2	448 ± 46 (4)	808 ± 161 (4)	NM	NM	174 ± 28 (3)	610 ± 84 (3)
0.1 mM AA+10 mM H_2O_2	325 ± 15 (5)	762 ± 63 (3)	164 ± 34 (3)	1 653 ± 137 (3)	217 ± 49 (4)	1 541 ± 271 (4)
1 mM dehydroascorbate	259 ± 10 (5)	−410 ± 163 (5)	82 ± 4 (3)	−295 ± 34 (3)	101 ± 26 (3)	−294 ± 55 (3)
0.1 mM AA+0.1 mM econazole	181 ± 19 (6)	131 ± 8 (6)	31 ± 31 (3)	9 ± 13 (3)	86 ± 5 (3)	93 ± 70 (3)
0.1 mM AA+0.1 mM proadifen	62 ± 5 (3)	6 ± 11 (3)	NM	NM	NM	NM
0.1 mM AA+0.1 mM quercetin	119 ± 28 (3)	137 ± 17 (3)	NM	NM	NM	NM
0.1 mM AA+10 mM H_2O_2+0.1 mM econazole	69 ± 33 (3)	301 ± 16 (3)	92 ± 3 (3)	733 ± 102 (3)	172 ± 4 (4)	933 ± 331 (4)

Microsomes (1 mg protein/ml) were incubated in the presence of the indicated compounds for 60 min at 37°C. The change in the amount of protein thiols and ascorbate was measured. The initial protein thiol contents were 40.2 ± 1.8 nmol/mg protein (mean ± S.D., $n = 16$), 52.2 ± 4.4 (6) and 42.3 ± 2.8 (8) nmol/mg protein, respectively. Data are means ± S.D. (*n*). AA, ascorbate; ND, not detectable; NM, not measured.

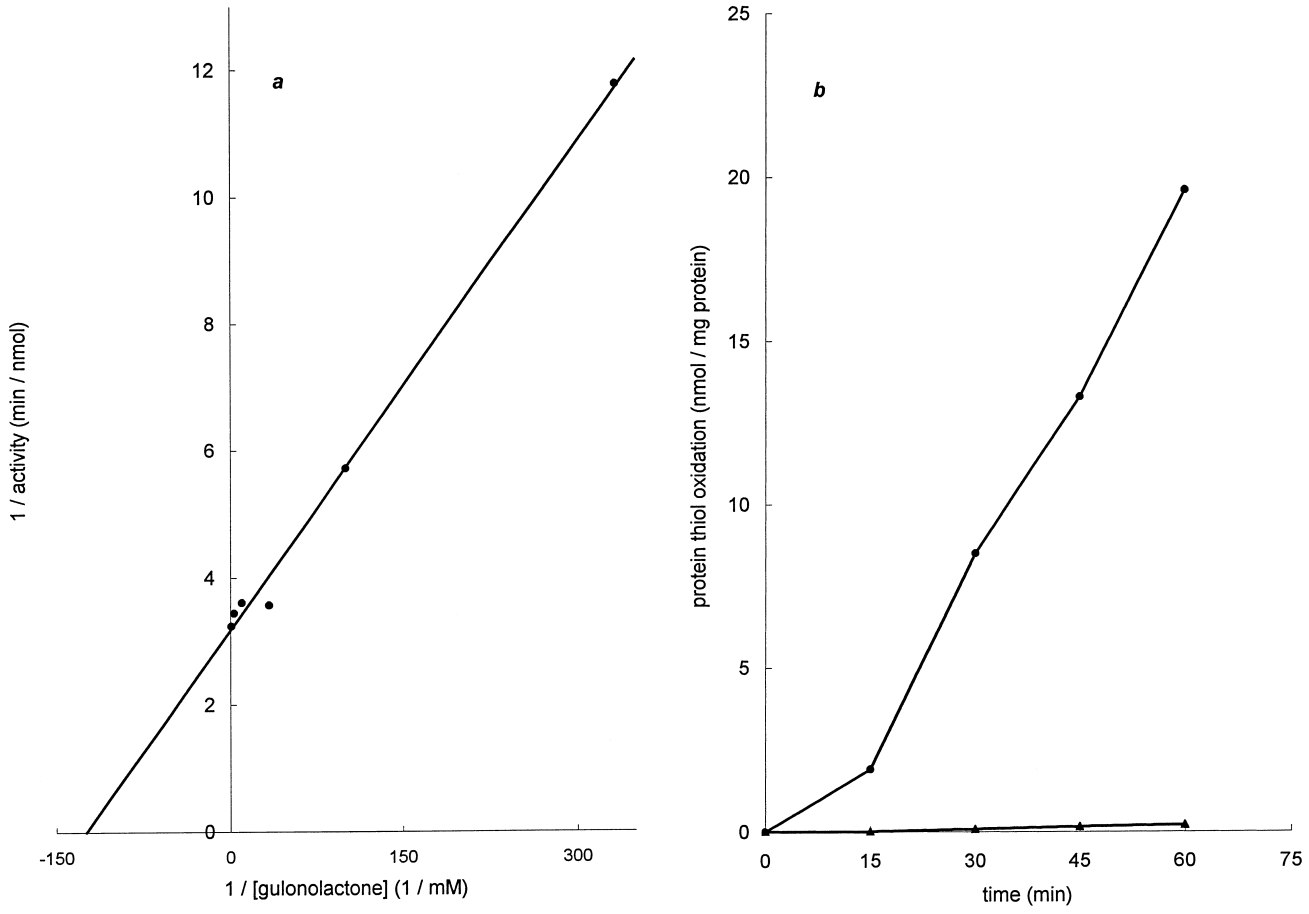

Fig. 1. Gulonolactone concentration and time-dependence of protein thiol oxidation in rat liver microsomal vesicles. Microsomes (1 mg protein/ml) were incubated in the presence of various concentrations of gulonolactone for 60 min (a) or in the presence of 1 mM gulonolactone for the indicated times (b, circles) at 37°C. The disappearance of protein thiols was measured. The initial protein thiol content of microsomes was 40.2 ± 1.8 nmol/mg protein (mean ± S.D., $n = 16$). For comparison, the absence of gulonolactone effect in guinea pig liver microsomes is also shown (b, triangles). Data are means of 8–16 experiments, S.D. values were less than 10% of means.

been found that various cytochrome P450 inhibitors (econazole, proadifen, quercetin) decreased both ascorbate consumption and protein thiol oxidation in rat liver microsomes (Table 1). Econazole effectively diminished the oxidation of protein thiols and ascorbate consumption also in (ascorbate or ascorbate plus hydrogen peroxide-treated) guinea pig and human microsomes (Table 1).

Econazole inhibited the gulonolactone oxidase-dependent thiol oxidation without decreasing the rate of ascorbate synthesis in rat liver microsomes. Moreover, it seems to make ascorbate synthesis more active, probably due to the lower ascorbate consumption (Table 2).

4. Discussion

In intact hepatocytes, the protein thiols of newly synthesized secretory proteins continuously undergo an oxidation in the lumen of the ER. The fact that protein disulfide bridge formation does not proceed in ER-derived liver microsomal vesicles suggests that the process needs a cytosolic factor or a membrane-permeable compound that is lost during the preparation of microsomes. GSSG has long been thought to act as an electron acceptor in the process. However, recent observations seem to exclude this possibility: protein disulfide formation is normal in GSH deficient yeast [15], GSH can even

Table 2
Effect of gulonolactone oxidase activity on the oxidation of protein thiols in rat liver microsomal vesicles

Addition	Oxidation of protein thiols	Ascorbate production
	pmol/min/mg protein (n)	
None	12 ± 34 (8)	−30 ± 14 (8)
1 mM gulonolactone	249 ± 18 (8)	1 854 ± 153 (8)
0.1 mM econazole	−15 ± 18 (5)	127 ± 85 (4)
1 mM gulonolactone+0.1 mM econazole	50 ± 22 (5)	2 014 ± 27 (5)

Microsomes (1 mg protein/ml) were incubated in the presence of the indicated compounds for 60 min at 37°C. The change in the amount of protein thiols and ascorbate was measured. The initial protein thiol content was 40.2 ± 1.8 nmol/mg protein (mean ± S.D., $n = 16$). Data are means ± S.D. (n).

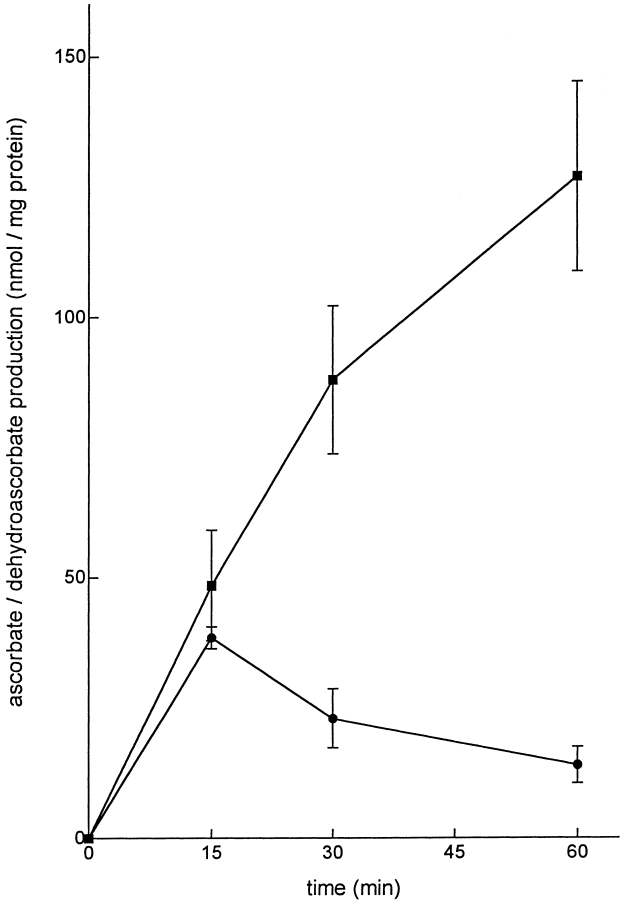

Fig. 2. Ascorbate and dehydroascorbate formation in rat liver microsomes upon gulonolactone addition. Microsomes (1 mg protein/ml) were incubated in the presence of 1 mM gulonolactone at 37°C. The formation of ascorbate (squares) and dehydroascorbate (circles) was measured by HPLC as described under Section 2. Data are means ± S.D., $n = 3$.

compete with protein thiols for oxidizing agents [21] and GSSG is hardly transported through the ER membrane [6]. The present paper demonstrates that ascorbate may play a crucial role in this electron transfer chain.

Ascorbate can derive from two sources in the lumen of the hepatic ER. Exogenous ascorbate (or its oxidized form, dehydroascorbate) enters from the cytosol in ascorbate non-synthesizing species (e.g. guinea pig and human). Both compounds are transported through the ER membrane [5]. In animals that express a functioning gulonolactone oxidase (e.g. rat), ascorbate is de novo synthesized directly in the lumen of the ER [7]. In both cases, ascorbate must be oxidized to become an electron acceptor. It might be the function of a microsomal ascorbate oxidase activity, which has already been reported [28]. Accordingly, oxidation of the added or in situ produced ascorbate was found in our experimental systems. Gulonolactone addition also gave rise to dehydroascorbate, the ultimate product of ascorbate oxidation.

Ascorbate addition provoked protein thiol oxidation in the liver microsomes of all the studied species. Dehydroascorbate, in agreement with previous findings [29,30], also oxidizes protein thiols with a simultaneous ascorbate formation. The maximal rate of protein thiol oxidation was similar in the case of gulonolactone, ascorbate, ascorbate plus hydrogen peroxide or dehydroascorbate addition, suggesting that the rate limiting step in the electron transfer is between protein thiols and ascorbate, e.g. the activity of protein disulfide isomerase.

It is also demonstrated that gulonolactone oxidase activity results in the oxidation of protein thiols in the hepatic ER of ascorbate-synthesizing species. Although the concentration of gulonolactone in hepatocytes is not known, the very low virtual K_M of the gulonolactone-provoked thiol oxidation strongly suggests the physiological relevance of the process. The oxidizing effect could logically be attributed to hydrogen peroxide, an oxidant produced by gulonolactone oxidase. However, the addition of hydrogen peroxide even in a high concentration (10 mM, which is about two orders of magnitude higher than the one produced from gulonolactone oxidation in the microsomal vesicles during 1 h incubation) cannot mimic the effect. This observation indicates that the other product of the enzyme, ascorbate, must play a role.

We have found that three typical cytochrome P450 inhibitors of various structures (econazole, proadifen and quercetin [31–33]) inhibit the electron flow from protein thiols to oxygen. In their presence, protein thiols and ascorbate remained in the reduced form. In other words, they decreased ascorbate oxidation and consequently gulonolactone, ascorbate or ascorbate plus hydrogen peroxide-dependent thiol oxidation, but did not affect gulonolactone oxidase activity. It suggests that the electron transfer from ascorbate to oxygen (and/or hydrogen peroxide) is an enzymatic process possibly mediated by a cytochrome P450 isozyme. Ascorbate as an electron donor in cytochrome P450-catalyzed drug oxidation has already been reported [34].

The overall process with some modifications supports the model for the proposed role of dehydroascorbate in the protein disulfide isomerase-catalyzed formation of protein disulfides [35]. Ascorbate oxidase and peroxidase activities present in the microsomal vesicles oxidize ascorbate to dehydroascorbate. Dehydroascorbate (formed in or transported into the lumen of the ER) can be reduced by protein disulfide isomerase [36], oxidizing the active center dithiols of the enzyme. Oxidized protein disulfide isomerase reacts with reduced nascent proteins, yielding protein disulfides and catalytically regenerating protein disulfide isomerase.

The participation of small molecular weight compounds (quinones) has been reported in the electron transfer chain of prokaryotic protein disulfide formation [37]. In the present paper, a similar role of ascorbate is shown in the mammalian ER. The oxidation of protein thiols in the presence of very low concentrations of gulonolactone or cytosolic-like concentrations of ascorbate strongly suggests the physiological relevance of these effects.

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