Gulonolactone oxidase activity-dependent intravesicular glutathione oxidation in rat liver microsomes

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Abstract The orientation of gulonolactone oxidase activity was investigated in rat liver microsomes. Ascorbate formation upon gulonolactone addition resulted in higher intravesicular than extravesicular ascorbate concentrations in native microsomal vesicles. The intraluminal ascorbate accumulation could be prevented or the accumulated ascorbate could be released by permeabilising the vesicles with the pore-forming alamethicin. The formation of the other product of the enzyme, hydrogen peroxide caused the preferential oxidation of intraluminal glutathione in glutathione-loaded microsomes. In conclusion, these results suggest that the orientation of the active site of gulonolactone oxidase is intraluminal and/or the enzyme releases its products towards the lumen of the endoplasmic reticulum.

Key words: Gulonolactone oxidase; Ascorbate; Glutathione; Endoplasmic reticulum

1. Introduction

The oxidative environment of the endoplasmic reticulum lumen, supported by high oxidised/reduced glutathione ratio [1], is necessary for the disulfide bridge formation and for the proper folding of proteins transported along the secretory pathway [2]. However, the primary source of the oxidising effect(s) is unknown. It has been reported recently that hydrogen peroxide formation during ascorbate synthesis is connected to glutathione oxidation both in isolated hepatocytes and in the microsomal system [3]. γ-Gulonolactone oxidase, the enzyme catalysing the terminal step of ascorbate biosynthesis, is an integral membrane protein of the hepatic endoplasmic reticulum in mammals [4,5]. Previous observations indicate that the protein is deeply embedded into the membrane [6,7], but the orientation of the active site of the enzyme is unknown. Therefore, the question has arisen whether ascorbate and hydrogen peroxide are synthesised in the lumen or on the cytosolic surface of the endoplasmic reticulum.

2. Materials and methods

2.1. Materials

Alamethicin, α,α′-dipyridyl, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), gulonolactone, polyethylene glycol (PEG, molecular mass 8000 Da), Triton X-100 and trypsin were purchased from Sigma (St. Louis, MO, USA). All other materials were of analytical grade. μBondapak aminopropyl column (average particle size 10 μM, 300×3.9 mm I.D.) was bought from Waters Millipore (Milford, MA, USA).

2.2. Preparation and treatment of liver microsomes

Rat liver microsomal vesicles were prepared from male Sprague-Dawley rats (Charles River Ltd, Budapest, Hungary) as previously described [8] and resuspended (40–80 mg of protein/ml) in 20 mM MOPS (pH 7.2) containing 100 mM KCl, 20 mM NaCl, 3mM $MgCl_2$ (buffer A). The suspensions were frozen and stored under liquid nitrogen until used. Glutathione-loaded microsomes were obtained by the incubation of vesicles in the presence of 5 mM GSH in buffer A for 2 h at 4°C. Permeabilisation of vesicles was performed by the pore-forming alamethicin or Triton X-100 [9]. Intactness of microsomal membranes was ascertained by measuring the latency of mannose 6-phosphatase activity [10]. It was higher than 92% in all preparations used.

2.3. Light-scattering measurements

Permeability of microsomal vesicles to gulonolactone was investigated by the light-scattering technique [11] as described in detail elsewhere [12]. Briefly, microsomal vesicles (0.07–0.1 mg of protein/ml) were equilibrated in a hypotonic medium (5 mM K-PIPES, pH 7.0) and the osmotically induced changes in light-scattering were measured following the addition of a small volume (1/13–1/25 of the reaction volume) of the investigated compound (1 M) dissolved in the hypotonic buffer. Light-scattering (400-nm excitation and 400-nm emission wavelength) was measured in a fluorimeter at room temperature under continuous stirring. The light-scattering signal was fed to MacLab hardware (AD Instruments) equipped with a computer and Chart v3.2.5 software (Macintosh); traces were visualised on the monitor and stored for later printing.

2.4. Measurement of metabolites

Microsomes were incubated in buffer A at 37°C. Incubations were terminated by the addition of PEG (5% final concentration). The separation of pellet from supernatant was obtained by rapid low-speed sedimentation of PEG-aggregated microsomes as previously described [13]. Microsomal pellets were washed twice with 1.1 ml buffer A (4°C) containing 5% PEG and the anion transport inhibitor DIDS (0.5 mM) to prevent the leakage of anions from the pellet [14]. For ascorbate measurements pellets and supernatants were deproteinised by the addition of trichloroacetic acid (5% final concentration). Ascorbate content was measured by the method of Omaye et al. [15]. For the determination of intra- and extravesicular oxidised and reduced glutathione the microsomal pellet and the medium were separated as described above, then the samples were treated and their GSH and GSSG contents were measured by HPLC after specific sample preparation as described earlier [16].

3. Results

Latency is a common feature of several intraluminal enzymatic activities in the endoplasmic reticulum, therefore, the latency of gulonolactone oxidase was examined. Addition of membrane detergent Triton X-100 to intact microsomes re-

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Abbreviations: GSH, glutathione (reduced form); GSSG, glutathione (oxidised form); PEG, polyethylene glycol; MOPS, 4-morpholinepropanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid

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sulted in a concentration dependent increase in gulonolactone oxidase activity measured at 10 mM substrate concentration. Maximal activation (38%) was reached at an unusually high concentration (5 mg Triton X-100/mg protein). Permeabilisation of native microsomes with alamethicin (0.1 mg/mg protein) caused only 7.5% increase in ascorbate production and the activity could not be further increased by the elevation of alamethicin concentration. Further (unsuccessful) attempts were made to determine the orientation of the active site by the inhibition of the enzyme with trypsin digestion. Gulonolactone oxidase activity proved to be highly resistant to proteolytic treatment both in native and in disrupted microsomes (data not shown).

The slight latency can be explained by the extraluminal orientation of the active site of gulonolactone oxidase, or alternatively, by the intraluminal orientation combined with an easy permeation of the substrate through the membrane. Therefore, the permeation of the substrate and the product of the enzyme was investigated using the light-scattering technique. The measurements indicated the rapid penetration of gulonolactone into the microsomal vesicles (Fig. 1), while ascorbate (in accordance with previous observations) was less permeable. These experiments did not solve the problem of the orientation of the enzyme, but the relatively slow transport of ascorbate through the microsomal membrane gave an opportunity to investigate separately the appearance of ascorbate in the intra- and extravesicular compartments during the enzymatic reaction.

The time course of the intra- and extravesicular appearance of ascorbate upon gulonolactone addition (10 mM) was investigated in rat liver microsomes. Ascorbate levels were detected in the supernatant and in the PEG precipitated pellet of microsomes after their rapid separation. Intraluminal ascorbate concentrations – calculated on the basis of the intravesicular water space (3.5 μl/mg protein, [14]) – rapidly increased and approached a steady state level within 1 min after the addition of gulonolactone. The appearance of ascorbate in the supernatant was continuous and steady (Fig. 2). However, the intravesicular ascorbate concentrations were

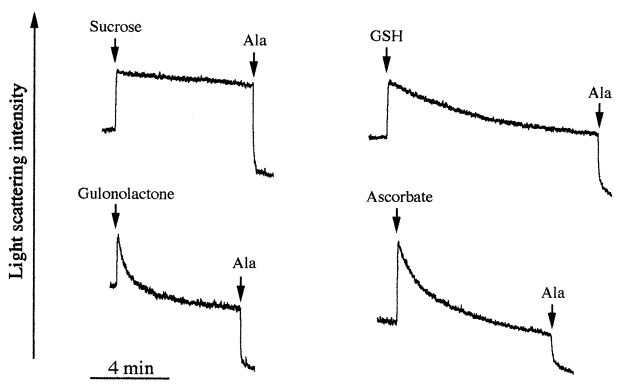

Fig. 1. Influx of gulonolactone, ascorbate and GSH into rat liver microsomal vesicles. Osmotically induced changes in light-scattering intensity of liver microsomal vesicles were detected as described in Section 2. Concentrated solutions (1 M) were added giving 75 mM (sucrose, gulonolactone), 50 mM (ascorbate) or 40 mM (GSH) final concentrations. The addition of the poorly permeant sucrose is shown as a control to demonstrate vesicular integrity. Alamethicin (Ala; 20 μg) was added to fully permeabilise the microsomal vesicles. Traces are representative of three individual measurements on different microsomal preparations.

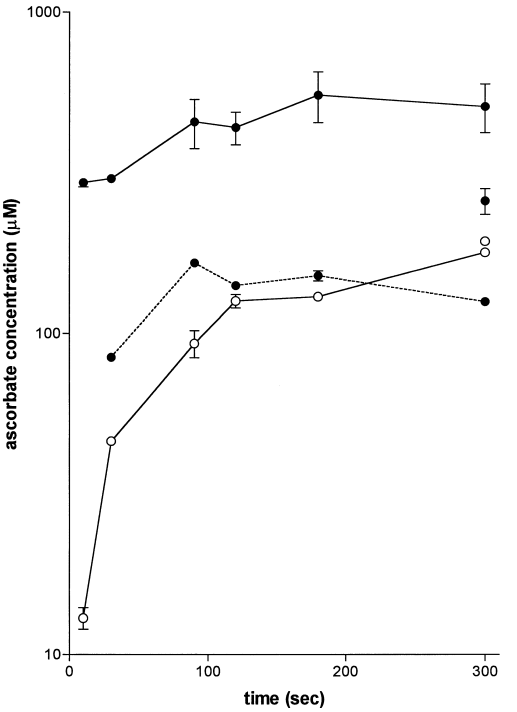

Fig. 2. Time course of intra- and extravesicular ascorbate appearance upon gulonolactone addition in rat liver microsomal vesicles. Microsomes (10 mg protein/ml) were incubated in buffer A at 37°C in the presence of 10 mM gulonolactone. At the indicated times microsomal vesicles and their medium were separated as described in Section 2. Ascorbate contents were measured in the pellets (filled circles) and in the supernatants (open circles) of control (continuous line) and alamethicin-treated (0.1 mg/mg protein; dotted line) microsomal vesicles. Single points represent the result of alamethicin addition to control microsomes at 5 min of the incubation. Data are means ± S.D., $n = 4$–8. Error bars are not visible when S.D. values were smaller than the symbol size.

higher in each time. The presence of alamethicin during the incubation caused a significant decrease in the intravesicular ascorbate concentrations, while its addition at 5 min resulted in the efflux of the accumulated ascorbate into the medium (Fig. 2).

Hydrogen peroxide, the other product of the reaction catalysed by gulonolactone oxidase, presumably easily permeates the microsomal membrane. To overcome this situation GSH-loaded microsomes were employed utilising the microsomal glutathione peroxidase activity for the generation of GSSG at the site of hydrogen peroxide formation [3]. On the basis of light-scattering experiments GSH relatively slowly enters the intravesicular space (Fig. 1). The long-lasting loading procedure resulted in complete equilibration of glutathione; however, the intraluminal glutathione pool became more oxidised, probably due to the oxidising effect of protein disulfides present in the lumen. Incubation for 5 min at 37°C caused a further oxidation. Stimulation of ascorbate synthesis by gulonolactone addition, in accordance with our previous observations [3], resulted in the oxidation of GSH. Both GSH disappearance and GSSG formation were more expressed in the lumen (Table 1).

4. Discussion

A common feature of several enzymes of the endoplasmic

Table 1
Effect of gulonolactone oxidase activity on the intra- and extravesicular oxidation of glutathione in glutathione-loaded rat liver microsomes

		GSH (mM)	GSSG (mM)
0′	intravesicular	4.19	0.65
	extravesicular	3.28	0.15
5′, without gulonolactone	intravesicular	3.59 ± 0.71	1.63 ± 0.63
	extravesicular	5.20 ± 0.10	0.16 ± 0.12
5′, 10 mM gulonolactone	intravesicular	2.03 ± 0.33	2.69 ± 0.43
	extravesicular	5.33 ± 1.06	0.75 ± 0.11

Rat liver microsomes (10 mg/ml) were incubated in buffer A containing 5 mM glutathione for 2 h at 4°C. Then samples were transferred to 37°C and they were incubated for 5 min in the presence or absence of 10 mM gulonolactone. Microsomal vesicles and their supernatant were separated as described in Section 2. GSH and GSSG were measured. Data are means ± S.D., $n = 2–6$.

reticulum is the latency: the enzyme activity is lower in native microsomal vesicles than in disrupted ones. This phenomenon is usually explained by the intraluminal position of the active site and the limited permeability of the substrates through the membrane; or alternatively, by the inhibitory interactions between the enzyme and its membranaceous microenvironment.

Gulonolactone oxidase, the key enzyme of ascorbate synthesis, is also located in the hepatic endoplasmic reticulum. Previous results [6,7] and our present observations indicate that the enzyme is remarkably resistant to proteolytic effects. It suggests that gulonolactone oxidase is deeply embedded into the microsomal membrane. In accordance with this, the enzyme activity was partially latent in native vesicles. The relatively moderate latency could be explained by the rapid permeation of gulonolactone through the membrane, revealed by light-scattering experiments.

The separate measurement of ascorbate associated to the vesicles or present in the extravesicular incubation medium showed that the concentration of microsome-associated ascorbate at the initial phase of synthesis is much higher than the extravesicular one. The majority of microsome-associated ascorbate can be regarded as intraluminal, since it could be released upon alamethicin addition. Moreover, the intravesicular values are systematically underestimated due to the possible efflux during the washing procedure and the not necessarily even distribution of gulonolactone oxidase in the vesicles. In accordance with the present results, the ascorbate concentration in rat liver rough and smooth microsomes has been reported to be at least one order of magnitude higher than its total hepatic concentration [17], supporting the possibility of the intraluminal synthesis (and/or indicating a very effective mechanism of accumulation).

As it has been reported previously [3], ascorbate synthesis stimulated by gulonolactone addition is accompanied by GSH consumption due to hydrogen peroxide formation both in microsomal incubations and in isolated hepatocytes. The selective measurements of extra- and intravesicular GSH consumption and GSSG formation revealed that GSH oxidation occurs mainly in the lumen, suggesting that hydrogen peroxide is also formed intravesicularly. Enhanced GSSG formation could also be detected in the lumen despite the more rapid transport of GSSG through the microsomal membrane [18].

In conclusion, our data suggest that the orientation of the active site of gulonolactone oxidase is intraluminal and the activity is supported by transporters for the substrate and product, or the active site is intramembranaceous and releases preferentially its products towards the lumen of the endoplasmic reticulum. It is not yet possible to determine whether gulonolactone oxidase fits to the 'substrate-transport' [19] or the 'combined conformational flexibility-substrate transport' [20] model of the endoplasmic reticulum enzymes. The intraluminal formation of ascorbate necessitates the recently described existence of ascorbate (and dehydroascorbate) transporters in the endoplasmic reticulum membrane [21]. Besides more general mechanisms, e.g. dehydroascorbate uptake and reduction [21], the supposed preferential uptake of GSSG and local oxidation processes mediated by other enzymes (such as Ero1p [22]) the intraluminal glutathione oxidation by gulonolactone oxidase activity may be an important source of the oxidative environment in the lumen of the endoplasmic reticulum in ascorbate-synthesising species.

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References

[1] Hwang, C., Sinskey, A.J. and Lodish, H.F. (1992) Science 257, 1496–1502.
[2] Gething, M.J. and Sambrook, J. (1992) Nature 355, 33–45.
[3] Bánhegyi, G., Csala, M., Braun, L., Garzó, T. and Mandl, J. (1996) FEBS Lett. 381, 39–41.
[4] Nishikimi, M. and Yagi, K. (1996) in: Subcellular Biochemistry, Vol. 25, Ascorbic Acid: Biochemistry and Biomedical Cell Biology (Harris, J.R., Ed.) pp. 17–39, Plenum, New York, NY.
[5] Bánhegyi, G., Braun, L., Csala, M., Puskás, F. and Mandl, J. (1997) Free Radic. Biol. Med. 23, 793–803.
[6] Kiuchi, K., Nishikimi, M. and Yagi, K. (1982) Biochemistry 21, 5076–5082.
[7] Nishikimi, M., Tolbert, B.M. and Udenfriend, S. (1976) Arch. Biochem. Biophys. 175, 427–435.
[8] Henne, V. and Söling, H.D. (1986) FEBS Lett. 202, 267–273.
[9] Fulceri, R., Bánhegyi, G., Gamberucci, A., Giunti, R., Mandl, J. and Benedetti, A. (1994) Arch. Biochem. Biophys. 309, 43–46.
[10] Burchell, A., Hume, R. and Burchell, B. (1988) Clin. Chim. Acta 173, 183–192.
[11] Meissner, G. (1988) Methods Enzymol. 157, 417–437.
[12] Fulceri, R., Bellomo, G., Gamberucci, A., Scott, H.M., Burchell, A. and Benedetti, A. (1992) Biochem. J. 286, 813–817.
[13] Bánhegyi, G., Braun, L., Marcolongo, P., Csala, M., Fulceri, R., Mandl, J. and Benedetti, A. (1996) Biochem. J. 315, 171–176.
[14] Bánhegyi, G., Marcolongo, P., Fulceri, R., Hinds, C., Burchell, A. and Benedetti, A. (1997) J. Biol. Chem. 272, 13584–13590.
[15] Omaye, S.T., Turnbull, J.D. and Sauerberlich, H.E. (1979) Methods Enzymol. 62, 3–7.
[16] Matsui, M. and Nagai, F. (1980) Anal. Biochem. 105, 141–146.
[17] Devasagayam, T.P.A. (1986) FEBS Lett. 205, 246–250.
[18] Söling, H.-D., Weilandt, A. and Wasmuth, M. (1994) in: Transport in the Liver, Falk Symposium 74 (Keppler, D. and Jungermann, K., Eds.) pp. 127–136, Kluwer, Dordrecht.

[19] Arion, W.J., Wallin, B.K., Lange, A.J. and Ballas, L.M. (1975) Mol. Cell. Biochem. 6, 75–83.
[20] Berteloot, A., St-Denis, J.-F. and van de Werve, G. (1995) J. Biol. Chem. 270, 21098–21102.
[21] Bánhegyi, G., Marcolongo, P., Puskás, F., Fulceri, R., Mandl, J. and Benedetti, A. (1998) J. Biol. Chem. 273, 2758–2762.
[22] Pollard, M.G., Travers, K.J. and Weissman, J.S. (1998) Mol. Cell 1, 171–182.