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Ascorbyl Free Radical and Dehydroascorbate Formation in Rat Liver Endoplasmic Reticulum

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The mechanism of ascorbate oxidation was studied in rat liver microsomes. A continuous consumption of the added ascorbate was observed, which was accompanied with a prompt appearance of ascorbyl free radical and dehydroascorbate. Microsomes sustained steady-state level of ascorbyl free radical and dehydroascorbate till ascorbate was present in the medium. Ascorbyl free radical formation was diminished when microsomes had been pretreated with heat or trypsine. It was also decreased by addition of quercetin, econazole or metal chelators, including the copper specific neocuproine. Enzymatic (superoxide dismutase, catalase) and nonenzymatic (dimethyl sulfoxide, mannitol) antioxidants did not modify the microsomal production of ascorbyl free radical. Investigation of the subcellular distribution of ascorbate oxidation showed that the microsomal fraction of liver had the highest activity. The decrease of ascorbate oxidation after protease treatment and the negligible increase upon permeabilization of microsomal vesicles showed that a membrane protein is responsible for the activity, which is exposed to the outer surface of the endoplasmic reticulum. The results indicate the presence of a primary enzymatic ascorbate oxidation in rat liver endoplasmic reticulum which is able to generate dehydroascorbate, an important source of the oxidizing environment in the endoplasmic reticulum.

KEY WORDS: Ascorbate; dehydroascorbate; ascorbyl free radical; protein thiol; protein disulfide; endoplasmic reticulum; copper; neocuproine.

INTRODUCTION

Protein disulfide bonds in secreted proteins and in the extracellular domains of membrane proteins are formed in the lumen of the endoplasmic reticulum (ER) in eukaryotic cells. It has been presumed that an electron transfer chain is responsible for the oxidation of protein thiols, which carries the electrons to the final electron acceptor. Several components of this chain have been identified in yeast (Frand *et al*., 2000; Kadokura and Beckwith, 2001). The system transfers electrons to molecular oxygen under aerobic conditions. Recent observations in yeast show that a FAD-binding sulfhydryl oxidase (Erv2p) can directly generate disulfides by using molecular oxygen (Gross *et al*., 2002). However, Erv2p is not essential for yeast growth indicating that it does not constitute the only pathway for disulfide bond formation. Moreover, protein disulfide formation is operative under anaerobic conditions therefore alternative oxidizing powers can also be used.

In mammalian cells the cellular mechanisms providing oxidizing equivalents to the ER has not been completely elucidated. Glutathione disulfide (GSSG) was a candidate for this function (Hwang *et al*., 1992). However, as it turned out, the production of GSSG is not an obligatory step in protein thiol oxidation; disulfide

Key to abbreviations: ESR, electron spin resonance; AFR, ascorbyl free radical; ER, endoplasmic reticulum.

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bond formation is normal in glutathione deficient yeast and GSH even competes with protein thiols for oxidizing equivalents (Cuozzo and Kaiser, 1999). Moreover, GSSG transport is practically absent through the ER membrane (Bánhegyi *et al.*, 1999). Recent observations in yeast may suggest that FAD import is responsible for the generation of the oxidizing environment (Gerber *et al*., 2001; Sevier *et al*., 2001; Tu *et al*., 2000), but a FAD transporter has not been described in the ER.

Dehydroascorbate is another possible candidate; it has been shown that dehydroascorbate oxidizes protein thiols with the mediation of protein disulfide isomerase (Nardai *et al*., 2001) and dehydroascorbate can be transported into the ER lumen (Bánhegyi *et al*., 1998). The cytosolic dehydroascorbate concentration is very low, due to the reducing environment of this compartment and to the presence of dehydroascorbate reductases. Therefore, a local ascorbate oxidizing activity should exist in the ER membrane or in its intimate neighborhood.

Ascorbic acid can be oxidized to dehydroascorbic acid in enzymatic or nonenzymatic reactions. The process involves two one-electron transfers; the oxidation of ascorbate to ascorbyl free radical (AFR) followed by its further oxidation to dehydroascorbate or by its very rapid disproportionation to dehydroascorbate and ascorbate (Bánhegyi *et al*., 1997). While enzymatic oxidation of ascorbate by ascorbate oxidases and peroxidases is well characterized in plants (Horemans *et al*., 2000; Smirnoff and Wheeler, 2000), ascorbate oxidation is regarded as an eventual nonenzymatic reaction induced by free radicals, ions of transitory metals and other oxidants in animal tissues. Only sporadic data are available on enzymatic ascorbate oxidation in some tissues; such activities were reported in thyroid (Nakamura and Ohtaki, 1993) and liver (Sun *et al*., 1984) microsomal fractions.

The aim of the present study was the investigation of ascorbate oxidation and its mechanism in rat liver microsomes. Experiments were undertaken to clarify the enzymatic or nonenzymatic nature of the process. For this purpose the effect of antioxidant enzymes and compounds and the inhibitors of enzymes possibly involved in ascorbate oxidation was determined. The subcellular and membrane localization of ascorbate oxidase activity was also studied. The results suggest that ascorbate oxidation is an obligatory step in ascorbate-dependent protein thiol oxidation. The enzymatic process catalyzed by a microsomal metalloenzyme, presumably by a copper oxidase, is able to maintain high local AFR and dehydroascorbate levels. The interrelationship between ascorbate oxidation and protein thiol consumption was also verified.

MATERIALS AND METHODS

Chemicals

Econazole, quercetin, 1,10-phenanthroline, neocuproine (2,9-dimethyl-1,10-phenanthroline), diphenyleneiodonium chloride, 4-morpholinepropanesulfonic acid (MOPS), catalase, alamethicin, and mannitol were obtained from Sigma. Superoxide dismutase was from Oxis Health Products Inc. All the other chemicals were of analytical grade.

Preparation and Treatment of Microsomes and Other Subcellular Fractions

Microsomes were prepared from male Wistar rats (180–230 g) as described in (Bánhegyi *et al*., 1998). Animals were obtained from Charles River Ltd., Budapest, Hungary. Microsomal fractions were resuspended in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM MOPS, pH 7.2. The suspensions were rapidly frozen and maintained under liquid N_2 until required. The intactness of liver microsomal membrane was checked by the measurement of the latencies of mannose-6-phosphatase (Burchell *et al*., 1988) and *p*-nitrophenol UDP-glucuronosyltransferase (Fulceri *et al*., 1994) activities, which were greater than 90 and 95%, respectively. Intactness of microsomal vesicles was also detected on the basis of their impermeability towards sucrose, checked by light scattering technique (Bánhegyi *et al*., 1998). Heat-treated microsomes were boiled for 15 min; trypsine-treated microsomes were preincubated in the presence of 3.5% trypsine for 60 min in the incubation buffer. Microsomes were permeabilized by the addition of alamethicin (0.1 mg/mg protein).

Subcellular fractions were obtained by standard differential centrifugation from rat liver homogenate and maintained under liquid N_2 until used. Subcellular fractions were characterized by measuring marker enzyme activities (Benedetti *et al*., 1988; Fulceri *et al*., 1994). The following activities were observed in mitochondrial and microsomal fractions, respectively: cytochrome *c* oxidase 397 ± 20 vs. 51 ± 33, glucose-6-phosphatase 59 ± 3 vs. 116 ± 2, and UDP-glucuronosyltransferase 14 ± 2 vs. 26 ± 1 (nmol/min/mg protein, means $\pm$ SD, $n = 4$).

Incubation of Microsomes and Measurement of Metabolites

For the determination of ascorbate oxidation intact microsomal vesicles were incubated in the presence of

ascorbate in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 20 mM MOPS, pH 7.2. Econazole and quercetin were dissolved in dimethyl sulfoxide; the final dimethyl sulfoxide concentration in incubations containing econazole or quercetin was 1%. Incubations were terminated by the addition of 0.75 volume of 5 mM metaphosphoric acid. Ascorbate contents and dehydroascorbate formation were measured by reverse phase HPLC after specific sample preparation as described in (Harapanhalli *et al*., 1993). The isocratic analyses were carried out with a Waters Series 2690 Separations Module and Waters Model 2487 dual absorbance UV detector at 254 nm. The separations were carried out on a Jones Chromatography APEX Octadecyl column (average particle size 5 μm, 15 cm $\times$ 4.6 mm). Mobile phase was 0.1 M NaH_2PO_4 and 0.2 mM Na_2EDTA, adjusted to pH 3.1 with orthophosphoric acid. Protein thiols were measured by the Ellman method as described in details earlier (Csala *et al*., 1999). Protein concentration of microsomes was determined using BioRad protein assay solution with bovine serum albumin as a standard according to manufacturer instructions. All data were expressed as means $\pm$ SD.

Electron Spin Resonance (ESR) Measurements

ESR spectra were obtained using an X-Band computer controlled spectrometer constructed by Magnettech GmbH (Berlin, Germany). Spectra of all samples were recorded at room temperature using a quartz flat cell. Instrument settings were: 100 kHz modulation frequency, 0.1762 mT modulation amplitude, 5 mW microwave power, 5 min scan time, 1.02 s time constant, and 2 mT field sweep. For evaluation, the method of double integration of the ESR signals was used.

RESULTS

Ascorbate Oxidation in Rat Liver Microsomes

The oxidation of ascorbate was investigated by high pressure liquid chromatography and electron spin resonance spectroscopy in rat liver microsomal vesicles. In the first set of experiments, microsomes (1 mg protein/mL) were incubated in the presence of a cytosol-like concentration of ascorbate (0.1 mM) at 37°C. Ascorbate and dehydroascorbate content of the incubation medium was measured by HPLC. A continuous decrease in ascorbate concentration was observed for 3 h (Fig. 1). After 3 h the rate of ascorbate consumption declined due to the very low ascorbate concentration in the medium

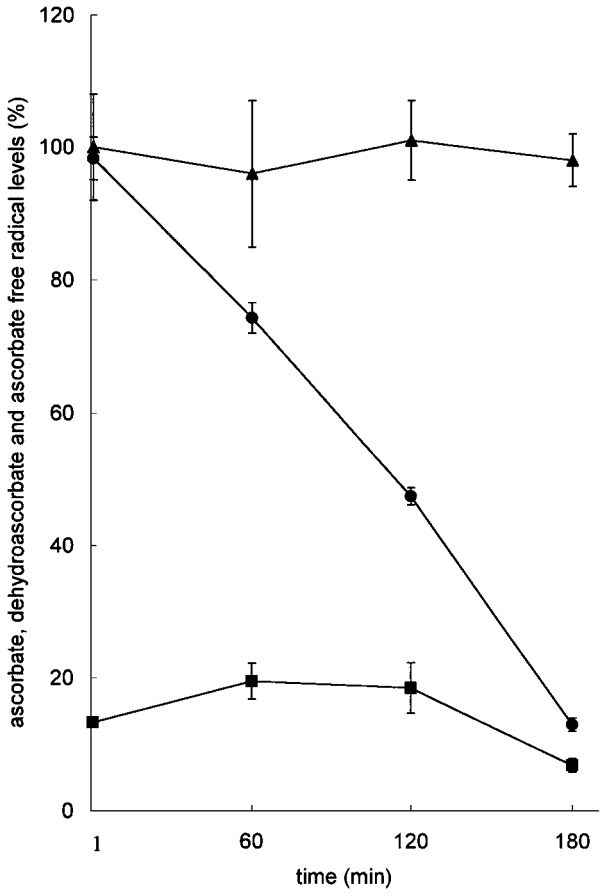

Fig. 1. Time course of ascorbate consumption, ascorbyl free radical generation, and dehydroascorbate formation in rat liver microsomal vesicles. Microsomes (1 mg protein/mL) were incubated in the presence of 100 μM ascorbate at 37°C. Ascorbic acid (●) and dehydroascorbic acid (■) levels were measured by reverse phase HPLC, AFR (▲) level was determined by ESR spectroscopy. Ascorbate and dehydroascorbate levels are expressed as percents of initial ascorbate concentration (100 μM), AFR levels are expressed as percents of the 1 min value. Data are means $\pm$ SD, $n = 6$.

(data not shown). Measurable amounts of dehydroascorbate appeared in the medium immediately and its concentration remained roughly the same during the whole incubation period (Fig. 1). Both the rate of ascorbate consumption and the steady-state level of dehydroascorbate were temperature-dependent: at room temperature the corresponding values were lower than at 37°C (188 ± 31 pmol/min/mg protein and $12.1 \pm 3.6\mu$M, respectively). In the absence of microsomes the rate of ascorbate consumption was negligible (less than 20 pmol/min).

Ascorbate oxidation was also studied by electron spin resonance spectroscopy. Ascorbate autooxidation and the corresponding AFR signal could be observed in ascorbate solution (100 μM ascorbate in MOPS buffer) containing

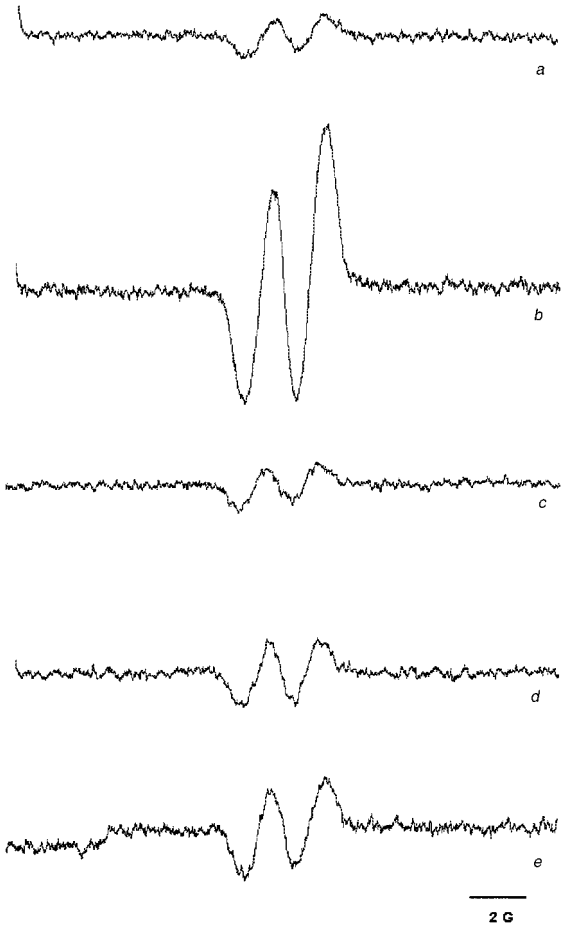

Fig. 2. Enzymatic formation of ascorbyl free radicals in rat liver microsomes. Ascorbate (100 μM) was incubated in MOPS buffer, pH 7.2 in the absence of microsomes (spectrum *a*); in the presence of microsomes (1 mg protein/mL, spectrum *b*); in the presence of microsomes heat-treated microsomes (spectrum *c*) or 100 μM neocuproine (spectrum *d*); or in the presence of trypsine-treated microsomes (spectrum *e*). ESR spectra were registered after 10 min incubation at room temperature. Spectra are representative ones of 4–16 registrations.

no microsomes showing an ESR spectrum consisting of a doublet with hyperfine splitting $a^{H4} = 1.8$ G (Fig. 2, spectrum *a*).

The incubation of ascorbate in the presence of rat liver microsomal vesicles (1 mg protein/mL) resulted in a significantly enhanced AFR level (Fig. 2, spectrum *b*). A sustained AFR level was observed during the 3-h incubation period; there was no significant difference between the ESR signals in the 1st or 180th min of incubation (Fig. 1).

It was reported that gulonolactone, the substrate of gulonolactone oxidase, is able to promote protein thiol oxidation in rat liver microsomes (Csala *et al*., 1999). Therefore, AFR generation upon gulonolactone addition was also detected. AFR generation could be detected in the presence of 0.1 mM gulonolactone and the intensity of the signal was at least 60% of that observed in the presence of ascorbate (Table I). The AFR level reached a plateau value quickly.

Table I. Ascorbyl Free Radical Generation Upon Gulonolactone Addition in Rat Liver Microsomal Vesicles

Incubation time (min)	AFR signal (A.U.)
5	152 ± 31
30	285 ± 8
60	274 ± 22

Note. Microsomes (1 mg protein/mL) were incubated in the presence of 0.1 mM gulonolactone at room temperature. AFR level was measured by ESR spectroscopy. Results are expressed as means $\pm$ SD of five experiments. A.U., arbitrary units.

Localization of Ascorbate Oxidizing Activity

The localization of the ascorbate oxidizing activity was examined by measuring AFR generation in various subcellular fractions gained by differential centrifugation. AFR formation was characteristic of the microsomal fraction of the liver. Only a minor increase in AFR generation was seen in the mitochondrial and cytosolic fractions of rat liver (Table II). The microsomal topology of ascorbate oxidase activity was also investigated. Trypsine treatment of liver microsomes diminished the ascorbate oxidizing capacity indicating that the enzyme is exposed to the outer surface of the vesicles. Permeabilization of microsomal vesicles by the pore-forming agent alamethicin elevated AFR formation only slightly, showing that the

Table II. Localization of Ascorbate Oxidase Activity

	AFR signal (A.U.)
(a) *Subcellular localization of ascorbate oxidase activity*	
Rat liver total homogenate	460 ± 75 (4)
Mitochondria	253 ± 10 (4)
Cytoplasm	176 ± 96 (4)
Microsomes	548 ± 69 (13)
(b) *Microsomal localization of ascorbate oxidase activity*	
Native rat liver microsomes	548 ± 69 (13)
Plus 3, 5% trypsine	207 ± 44 (4)
Plus alamethicin (0.1 mg/mg protein)	670 ± 41 (4)

Note. The 0.1-mM ascorbate were incubated in the presence of microsomes or other subcellular fractions (1 mg protein/mL) at room temperature for 10 min. Alamethicin was added at the beginning of the incubation, trypsine-treated microsomes were preincubated in the presence of trypsine for 60 min. AFR levels were determined by ESR spectroscopy. Data are means $\pm$ SD (*n*). A.U., arbitrary units.

place of oxidation is easily accessible for ascorbate (Table II).

Inhibiton of Ascorbate Oxidation in Rat Liver Microsomes

Ascorbate oxidation proved to be heat- and protease-sensitive in rat liver microsomes supporting the presence of a protein-mediated process. AFR generation was decreased to the basal autooxidation level after boiling of microsomes (Fig. 2, spectrum *c*, Table III). Protease treatment (3.5% trypsine, see above) also broke down AFR generation to the autooxidation level (Table II, Fig. 2, spectra *e*). The enzymatic nature of the oxidation was further investigated by the addition of inhibitors of candidate enzymes. Hemoproteins, flavoproteins, and metalloenzymes were the most suspicious means of ascorbate oxidation. Econazole and quercetin (known cytochrome P-450 inhibitors), which have been reported to decrease ascorbate oxidation and its consecutive uptake into the ER lumen (Csala *et al*., 2000), reduced effectively the intensity of the AFR signal both in the presence or absence of microsomes (Table III). Addition of the flavoprotein inhibitor diphenylene iodonium and the hemoprotein inhibitor sodium azide was ineffective. On the other hand, addition of various metal chelators, such as dipyridyl, 1,10-phenanthroline or its copper-specific analogue neocuproine (Fig. 2, spectrum *d*) reduced effectively the AFR signal. These compounds did not diminish the autooxidation of ascorbate either in the absence of microsomes or in the presence of heat-treated microsomes (Table III). Decreased AFR generation was reflected in ascorbate consumption: 1,10-phenanthroline, dipyridyl, and neocuproine inhibited ascorbate consumption measured by HPLC (Table IV).

Table III. Inhibition of Ascorbyl Free Radical Formation in Rat Liver Microsomal Vesicles

	AFR signal (A.U.)	
	−Microsomes	+Microsomes
Control	152 ± 11 (6)	548 ± 69 (13)
Boiled microsomes	—	235 ± 65 (4)*
1% dimethyl sulfoxide	—	608 ± 74 (3)
0.1 mM econazole	48 ± 32 (3)*	240 ± 52 (10)*
0.1 mM quercetin	68 ± 26 (3)*	125 ± 44 (4)*
5 mM mannitol	—	443 ± 57 (7)
600 U/mL superoxide dismutase	—	498 ± 129 (5)
900 U/mL catalase	—	509 ± 69 (4)
0.1 mM diphenylene iodonium	—	441 ± 74 (3)
10 mM sodium azide	—	394 ± 32 (3)
0.1 mM 1,10-phenanthroline	264 ± 21 (4)	202 ± 11 (4)*
Boiled microsomes +0.1 mM 1, 10-phenanthroline	—	164 (2)
0.1 mM neocuproine	192 ± 18 (4)	200 ± 16 (4)*
Boiled microsomes +0.1 mM neocuproine	—	214 (2)
0.1 mM dipyridyl	144 ± 36 (3)	198 ± 55 (3)*
Boiled microsomes +0.1 mM dipyridyl	—	143 (2)

Note. Microsomes (1 mg protein/mL) were incubated in the presence of 0.1 mM ascorbate and various compounds at room temperature for 10 min. AFR levels were determined by ESR spectroscopy. Data are means ± SD (*n*). A.U., arbitrary units.
*Significantly different with respect to control values ($p < 0.05$).

Table IV. Effect of the Inhibitors of Ascorbate Oxidation on Ascorbate and Protein Thiol Consumption in Rat Liver Microsomal Vesicles

	(pmol/min/mg protein)	
Treatment	Ascorbate consumption	Protein thiol consumption
None	463 ± 32	325 ± 58
0.1 mM 1,10-phenanthroline	243 ± 39*	<10*
0.1 mM dipyridyl	NM	<10*
0.1 mM neocuproine	270 ± 8*	25 ± 16*
Boiled microsomes	191 ± 36*	NM

Note. Microsomes (1 mg protein/mL) were incubated in the presence of 0.1 mM ascorbate and inhibitors of ascorbate oxidation at 37°C for 60 min. Ascorbate and protein thiol concentrations were measured as described in Materials and Methods section. Results are expressed as means ± SD of 4–6 experiments NM; not measured.
*Significantly different from control value, $p < 0.05$.

To estimate the involvement of reactive oxygen species in ascorbate oxidation, the effect of enzymatic and nonenzymatic antioxidants was also checked. The superoxide scavenger superoxide dismutase, the hydrogen peroxide metabolizing catalase, and the general free radical (mostly hydroperoxyl radical) scavengers mannitol and dimethyl sulfoxide did not decrease either ascorbate consumption or AFR level suggesting that reactive oxygen species are not involved in the oxidation procedure (Table III).

Effect of the Inhibitors of Ascorbate Oxidation on Protein Thiol Consumption in Rat Liver Microsomes

Ascorbate addition, as it had already been reported, resulted in the oxidation of thiol groups in microsomal proteins (Csala *et al*., 1999). Compounds, which diminish ascorbate consumption and AFR and dehydroascorbate formation (quercetin, econazole), decreased also protein thiol consumption (Csala *et al*., 2000). However, the inhibitory mechanism of these agents could not been clarified since they decreased AFR level even in the absence of microsomes (Table III). Therefore, we have investigated

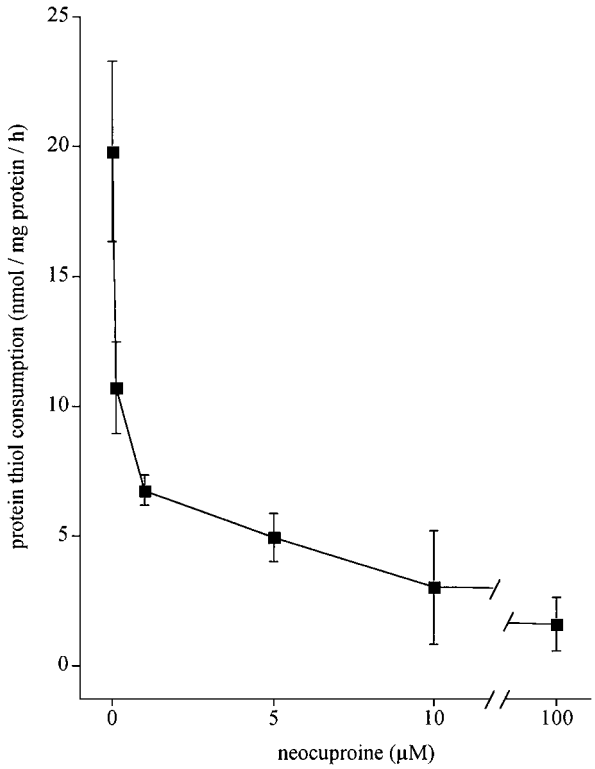

Fig. 3. Inhibition of protein thiol oxidation by neocuproine in rat liver microsomes. Microsomes (1 mg protein/mL) were incubated in the presence of 100 μM ascorbate and the indicated concentrations of neocuproine for 30 min at 37°C. Protein thiols were measured at the beginning and at the end of the incubations. Data are means ± SD, $n = 3$–6.

the effect of metal chelators, which inhibited enzymatic ascorbate oxidation more specifically. Dipyridyl, 1,10-phenanthroline, or neocuproine almost completely inhibited ascorbate-dependent protein thiol oxidation at 100 μM concentration (Table III). Neocuproine caused a concentration-dependent inhibition; more than 50% inhibition was already observed at 1 μM concentration (Fig. 3).

DISCUSSION

The results presented here indicate that ascorbate can undergo an enzymatic oxidation in rat liver microsomes. The physiological importance of the finding is that dehydroascorbate as a small molecular weight electron acceptor can participate in the machinery of the oxidative folding of proteins in the ER lumen. The role of dehydroascorbate in protein disulfide isomerase-mediated protein thiol oxidation has already been demonstrated (Nardai *et al*., 2001), just as dehydroascorbate transport through the ER membrane (Bánhegyi *et al*., 1998). The missing component, a local ascorbate oxidase activity was investigated in the present study.

Results on microsomal ascorbate oxidation detected by HPLC and ESR methods suggest that the oxidation process is enzyme-mediated: (i) in the presence of rat liver microsomes AFR level is at least three times higher than the autoxidation level; (ii) heat- or trypsine-pretreated microsomes lost their AFR generating activity, i.e., AFR formation remained at autoxidation level; (iii) addition of specific inhibitors decreased ascorbate consumption and AFR generation significantly; and (iv) ascorbate oxidation seems to be specific to the microsomal fraction.

Investigation of the subcellular localization of ascorbate oxidase activity showed that the highest increase in AFR signal was observed upon the addition of the microsomal fraction. Mitochondrial fraction also increased AFR formation slightly, in accordance with the finding of Li *et al*. (2001). However, this effect might also be due to the intimate association of microsomes and mitochondria, i.e., the presence of ER membrane in the mitochondrial fraction (Lightowlers and Lill, 2001). In fact, typical microsomal marker enzymes are present in the mitochondrial fraction with relatively high activities. Permeabilization of microsomal vesicles hardly modified the intensity of the signal indicating that the membrane does not form a barrier for ascorbate, i.e., the ascorbate oxidase activity is faced towards the cytosol. The decrease in ascorbate oxidation upon protease treatment further strengthened this assumption.

It can be supposed that ascorbate oxidation and AFR formation are secondary processes mediated by reactive oxygen species generated in the microsomal system. Thus we have investigated the effect of different antioxidants on ascorbyl radical level. Antioxidant enzymes (superoxide dismutase, catalase) did not alter AFR generation indicating that superoxide anion or hydrogen peroxide formation does not play a role in the mechanism. The nonenzymatic hydroxyl radical scavengers mannitol and dimethyl sulfoxide were also ineffective. These findings show that the primary process is not superoxide anion or hydroxyl free radical generation.

The enzyme(s) responsible for microsomal ascorbate oxidation cannot be identified on the basis of the present experiments, but the limits of possible candidates can be restricted. Liver microsomes are abundant in various cytochrome P450 isozymes. The effect of two typical cytochrome P450 inhibitors, econazole and quercetin, on microsomal ascorbate oxidation might suggest that one of these isozymes catalyzes the reaction. However, econazole and quercetin decreased the AFR signal even in the absence of microsomes. Therefore, their action as enzyme inhibitor cannot be clearly estimated. As another

cytochrome inhibitor, sodium azide, was poorly effective, the involvement of cytochrome P450s seems to be unlikely. Various flavoproteins also have oxidase activity. However diphenylene iodonium, a typical flavoenzyme inhibitor did not affect the generation of the AFR signal. Inhibitory effect was observed upon the addition of metal chelators dipyridyl, 1,10-phenanthroline, and neocuproine; both AFR generation and ascorbate consumption were decreased. These compounds were effective only in the presence of native microsomes, i.e., a decrease in AFR generation could not be observed in the absence of microsomes or in the presence of heat-inactivated vesicles. Therefore, their effect can be attributed to the inhibition of a metalloenzyme rather than to the chelation of transitory metal ions present in the buffer or to the reduction of AFR. The compounds used in the study can chelate Fe^{2+}, Fe^{3+}, Cu^{+}, and Cu^{2+} ions with different affinity (Lovstad, 1988). The effect of the relatively copper-specific neocuproine (Al-Sa'doni *et al*., 1997; Gocmen *et al*., 2000; Pinchuk *et al*., 2001) may suggest the participation of a copper oxidase in the process, but the involvement of other metalloenzymes cannot be unequivocally excluded. It is worth to note that plant ascorbate oxidases are also cuproenzymes.

Rat liver microsomes are able to generate a sustained AFR signal while ascorbate is present in the medium. Similarly, dehydroascorbate concentration was constant after the first minutes of incubation. AFR and dehydroascorbate are unstable compounds; therefore their constant levels must be maintained by a continuous ascorbate oxidation. In this respect, our measurements showed that ascorbate consumption is continuous and linear in time. The ascorbate precursor gulonolactone was also able to maintain a similar AFR level in the microsomes, which supports our previous observations on gulonolactone oxidase-dependent protein thiol oxidation (Csala *et al*., 1999, 2001; Puskás *et al*., 1998). Compounds which decrease ascorbate oxidase activity (metal chelators) or AFR level (quercetin, aconazole) inhibits protein thiol oxidation, demonstrating the connection between extravesicular ascorbate oxidation and intravesicular disulfide bond formation.

In summary, our results indicate the existence of an enzymatic ascorbate oxidation process in rat liver microsomes, which is able to maintain high local AFR and dehydroascorbate levels necessary for the generation of the oxidizing environment in the ER lumen.

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