Modulation of Subcellular Particles of the Rat Small Intestine and Liver by Ebselen

Tadashi MIZOGUCHI,^{*,a} Tohru NISHINAKA,^a Hirofumi NANJO,^a Kiyohito YAGI,^a and Hideo HAKUSUI^b

Laboratory of Bio-Functional Engineering, Faculty of Pharmaceutical Sciences, Osaka University,^a 1-6 Yamada-oka, Suita 565, Japan and Developmental Research Laboratories, Daiichi Pharmaceutical Co., Ltd.,^b 16-13, Kita-Kasai 1-chome, Edogawa-ku, Tokyo 134, Japan. Received November 18, 1994; accepted December 2, 1994

Reactions between ebselen and subcellular particles of rat liver were investigated by monitoring the activity of mitochondrial glutamate dehydrogenase and microsomal glucose 6-phosphate dehydrogenase. Rat small intestine lactate dehydrogenase was purified and was also used in the reaction between cytosolic protein and ebselen. Glutamate dehydrogenase in intact rat liver mitochondria was completely resistant to ebselen, but the enzyme was significantly inactivated in broken mitochondria mediated by Triton X-100, reflecting the fact that ebselen was not transported through the mitochondrial membrane into the matrix. Glucose 6-phosphate dehydrogenase in rat liver microsomes was inactivated by ebselen, accompanied by a slight decrease in the thiol groups of microsomal membrane protein. Purified cytosolic lactate dehydrogenase was inactivated concentration- and time-dependently by ebselen. The activity of rat small intestine lactate dehydrogenase abolished by ebselen was significantly restored by incubation with purified rat small intestine thioltransferase in the presence of reduced glutathione (GSH). The level of thiol groups in rat liver microsomal membrane protein decreased by ebselen was partially restored by an incubation with purified rat liver thioltransferase in the presence of GSH. The results suggested that thioltransferase can cleave the Se-S conjugates between ebselen and cytosolic proteins or microsomal membrane proteins in the presence of GSH.

Key words ebselen; glutamate dehydrogenase; lactate dehydrogenase; microsome; seleno-sulfide; thioltransferase

Thioltransferase plays a role in the SH/SS exchange reaction *in vitro*, especially that of low molecular disulfides,¹⁾ but the actual physiological role of the enzyme remains to be elucidated. We demonstrated the inhibition of bovine leukocyte thioltransferase by anti-inflammatory and anti-histaminic drugs and suggested that thioltransferase plays physiological roles during inflammatory stages.²⁾ Using rat small intestine, we showed that enzymes which were not inactivated under Fe²⁺-EDTA-mediated oxidative stress, *i.e.* thioltransferase *etc.*, may play an important role in cellular antioxidant defenses in the small intestine.³⁾ We also pointed out the involvement of thioltransferase in the modulation of enzyme activities, judging from the release by purified human placenta thioltransferase of the incorporated half-[¹⁴C]cystine from glutathione S-transferase- π ⁴⁾ and the reduced activity of thioltransferase against the fructose 1,6-bisphosphatase activated.⁵⁾

Ebselen is a selenium-containing heterocyclic compound which exhibits glutathione peroxidase-like activity.⁶⁾ It forms a seleno-sulfide linkage with the sulfhydryl group of bovine serum albumin, as shown in reaction (Chart 1), and the restoration of the sulfhydryl group of bovine serum albumin can be induced with more than 0.5 mM of reduced glutathione (GSH).⁷⁾ In this study, we demonstrated the cleavage of the seleno-sulfide linkage between ebselen and proteins by thioltransferase, as well as the modulation of rat liver and small intestinal subcellular particles by ebselen.

MATERIALS AND METHODS

Materials D-Glucose 6-phosphate (G6P) and pyruvate were purchased from Sigma Chemical Co. Chlorodinitrobenzene, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and guanidine hydrochloride were products of Wako Pure

Chemical Co., Ltd. Glutathione reductase, GSH, NADP⁺, NADPH and NADH were obtained from Oriental Yeast Co., Ltd. GSH-Sepharose 4B was prepared from cyanogen bromide-activated Sepharose 4B according to method of Cuatrecasas.⁸⁾ S-Sulfocysteine was prepared according to the method of Segel and Johnson.⁹⁾ All other chemicals and solvents were reagent grade from standard sources. Ebselen was dissolved in dimethylformamide before use and added to the reaction mixture. All reaction mixtures contained less than 2.0% dimethylformamide and this amount did not affect the activities of lactate dehydrogenase, thioltransferase, glutamate dehydrogenase or G6P dehydrogenase.

Enzyme Assay The activities of thioltransferase were measured following a decrease of the absorbance at 340 nm using S-sulfocysteine³⁾ as a substrate in the presence of GSH, glutathione reductase and NADPH by the method previously described.²⁾ Other enzyme activities were measured as described in the figure legends.

Subcellular Fractionation of Rat Liver Wistar rats weighing 180–200 g were fasted overnight and anesthetized with ether. The ascites were opened, blood was taken from the aorta, and the tissues, small intestine and liver, were then isolated. After being washed with cold saline, the liver was homogenized in four volumes of cold 0.25 M

Chart 1

* To whom correspondence should be addressed.

sucrose containing 0.25 mM EDTA and 0.9 mM potassium phosphate buffer, pH 7.0 (sucrose solution). The pellet obtained by centrifugation at $2500 \times g$ for 10 min from the homogenates was used as rat liver mitochondria after several resuspensions in sucrose following centrifugation at 1000 – $10000 \times g$. The supernatant was further centrifuged at $100000 \times g$ for 60 min and the resulting pellet was used as rat liver microsomes after several resuspensions in sucrose following ultracentrifugation to remove the cytosol. Thioltransferase was purified from the cytosol of rat liver obtained after the centrifugation at $100000 \times g$ for 60 min.

Isolation of Rat Liver Microsomal and Mitochondrial Membrane Protein Fresh microsomal and mitochondrial suspensions in sucrose were sonicated 5 times for 5 s in a Heat System Sonifier (Missouri) and centrifuged at $10000 \times g$ for 30 min. The pellets were separately washed with 10 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM EDTA and dissolved in 17 mM potassium phosphate buffer, pH 8.0, containing 6.4 M guanidine HCl and 0.2 mM EDTA. After centrifugation at $10000 \times g$ for 30 min to remove insoluble materials, the supernatant solutions were used as rat liver microsomal and mitochondrial membrane proteins, respectively.

Thiol and Protein Estimation in Rat Liver Microsomes and Mitochondria Membranes The thiol content was estimated according to the procedure of Ellman,¹⁰⁾ using $E_{cm, 412}$; 13.6 in 15.3 mM potassium phosphate buffer, pH 8.0, containing 5.7 M guanidine HCl and 0.18 mM EDTA. The protein concentration was measured by the method of Bradford¹¹⁾ in 15.3 mM potassium phosphate buffer, pH 8.0, containing 5.7 M guanidine HCl and 0.18 mM EDTA with bovine serum albumin as the standard.

Purification of Rat Small Intestine Lactate Dehydrogenase and Thioltransferase All purification procedures were performed at 0 – 4°C . Rat small intestine was homogenized with 4 vol. of 20 mM Tris-HCl, pH 7.3, containing 0.2 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at $11000 \times g$ for 20 min and the supernatant was applied to Sephadex G-75 (2.2×96 cm) equilibrated with 10 mM potassium phosphate, pH 6.5, containing 10 mM 2-mercaptoethanol, 200 mM NaCl and 0.2 mM EDTA. The lactate dehydrogenase fraction was concentrated by 0.65% saturated ammonium sulfate and the thioltransferase fraction was evaporated to a small volume under reduced pressure at 42°C in the presence of few drops of octylalcohol. After dialysis against 10 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM EDTA overnight, lactate dehydrogenase and thioltransferase were eluted by means of Q-Sepharose column chromatography (1.3×24 cm) a 0 – 500 mM NaCl gradient. Lactate dehydrogenase was finally purified by hydroxyapatite column chromatography (1.2×3.3 cm) using a gradient of 10 – 100 mM potassium phosphate, pH 7.4. The thioltransferase fraction from Q-Sepharose column chromatography was finally eluted from GSH-Sepharose 4B affinity chromatography by 500 mM NaCl in 10 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA. Rat liver thioltransferase was also purified from the cytosol by a similar procedure. Purified lactate dehydrogenase and thioltransferase were stored in a

nitrogen-saturated 100 mM phosphate buffer, pH 6.6, containing 0.3 mM EDTA at 4°C .

RESULTS

Rat small intestine lactate dehydrogenase and thioltransferase were purified according to the simple procedures summarized in Tables 1 and 2, respectively. The molecular weights of lactate dehydrogenase and thioltransferase, estimated by Sephadex G-75 gel filtration, were 140 and 11 kDa, respectively. Electrofocusing proceeded on Pharmalyte at a pH range between 3.0 and 10.0. The pI values for lactate dehydrogenase and thioltransferase were 6.6 and 9.1, respectively. These enzymes migrated as single bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue G-250 staining, reflecting the tetramer and monomer of lactate dehydrogenase and thioltransferase, respectively.

Rat small intestine lactate dehydrogenase was inactivated dose- and time- dependently by ebselen (Fig. 1). This reflected the seleno-sulfide bond formation, like that of Chart 1, between ebselen and the representative SH enzyme, lactate dehydrogenase. After incubation with $20 \mu\text{M}$ ebselen for 30 min, the ebselen-bound lactate dehydrogenase, which had activity level of about 70% of the control, was isolated by gel-filtration on Sephadex G-25 and used for a reaction with purified rat small intestine thioltransferase. The activity of lactate dehydrogenase inhibited by ebselen was completely restored by an incubation for 30 min with purified rat small intestine thioltransferase in the presence of GSH, but $500 \mu\text{M}$ GSH alone could not recover the lost activity (Fig. 2). The concentration of GSH in the living cell has been reported in a range between 0.5 – 3.0 mM.¹²⁾ But, in consideration of the easy decrease of GSH under oxidative conditions, GSH cannot serve the restoration of the enzyme *in vivo*.

Table 1. Purification of Rat Small Intestine Lactate Dehydrogenase

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Extracts	561	1570	2.8	100
Sephadex G-75	127	1040	8.2	66.1
(Am) ₂ SO ₄	80.9	932	11.4	59.2
Q-Sepharose	3.5	483	134.4	30.7
Hydroxyapatite	0.6	218	355.0	13.9

The results represent the values for purification from 21.9 g of rat small intestine.

Table 2. Purification of Rat Small Intestine Thioltransferase

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Extracts	1670	39.0	0.02	100
Sephadex G-75	101	28.6	0.28	73.2
Evaporation	65.3	24.6	0.37	63.1
Q-Sepharose	40.6	21.6	0.53	55.5
GSH-Sepharose 4B	0.14	10.6	75.71	27.2

The results represent the values for purification from 65.7 g of rat small intestine.

Fig. 1. Decrease in Lactate Dehydrogenase Activity during Incubation with Ebselen

Rat small intestine lactate dehydrogenase (40 μ g protein) was incubated with 10 to 100 μ M ebselen in a total volume of 0.5 ml of potassium phosphate buffer, pH 6.5, containing 0.12 mM EDTA, 5.0% dimethylformamide at 25 °C. At various times, aliquots of the reaction mixture (0.3–0.6 μ g protein) were assayed photometrically for lactate dehydrogenase activity in 50 mM sodium phosphate buffer, pH 7.0, containing 0.6 mM pyruvate, 50 mM NADH and 0.5 mM EDTA, without the removal of ebselen. The amount of ebselen did not affect the enzyme reaction at time 0. The activities are expressed as a percentage of intact enzyme activity. Each value is the average of two separate experiments. Ebselen concentrations: $\circ$, 0; $\triangle$, 10; $\square$, 20; $\blacksquare$, 50; $\bullet$, 100 μ M.

Fig. 2. Restoration by Purified Rat Small Intestine Thioltransferase of the Lactate Dehydrogenase Activity Decreased by Ebselen

Lactate dehydrogenase (0.2 μ g protein), partially inactivated by ebselen (remaining activity, 69.5%), was incubated in a total volume of 0.2 ml of 200 mM potassium phosphate buffer, pH 7.6, containing 2.5 mM EDTA at 25 °C for 30 min without any additions ($\square$); with 10.1 munits of purified rat small intestine thioltransferase in the absence ($\blacksquare$) and presence of 500 μ M GSH ($\blacksquare$). Lactate dehydrogenase activity was determined as described in Fig. 1, without the removal of thioltransferase and GSH. Quadruplicate determinations were performed and averages calculated. The values are the means of these averages $\pm$ the standard error of the means.

Preliminary studies of the reaction between ebselen and purified rat small intestine thioltransferase revealed no inactivation of thioltransferase. However, further detailed studies are necessary to clarify this. Rat liver mitochondria in 0.25 M sucrose solution were incubated with 200 μ M ebselen, and the activity of glutamate dehydrogenase was measured, which is a typical mitochondria matrix enzyme. The total amount of membrane protein thiols slightly exceeded the amount of ebselen used here to avoid the membrane disorder induced by a large excess of ebselen. Glutamate dehydrogenase was completely resistant, even after a 60 min incubation with 200 μ M ebselen. However, in the presence of 2.0% Triton X-100, the inactivation of glutamate dehydrogenase was time-dependent (Fig. 3). In contrast to the mitochondria enzyme, incubation of rat

Fig. 3. Decrease of Glutamate Dehydrogenase Activity in Rat Liver Mitochondria during Incubation with Ebselen in the Absence or Presence of Triton X-100

A suspension of rat liver mitochondria (12–14 mg protein) was incubated at 25 °C with 0.2 mM ebselen in a total volume of 1.0 ml of 0.25 M sucrose containing 0.5 mM EDTA and 1.9% dimethylformamide in the absence ($\circ$) and presence ($\bullet$) of 2.0% Triton X-100. Glutamate dehydrogenase activity was determined photometrically in 100 mM potassium phosphate buffer, pH 7.6, containing a rat liver mitochondria suspension (1.0–1.5 mg protein), 1.2% Triton X-100, 0.15 mM NAD, 50 mM L-glutamate, 1.2 mM EDTA and 50 mM sucrose, without the removal of ebselen. The amount of ebselen did not affect the enzyme reaction at time 0. The activities are expressed per ml of incubation mixture. Each value is the average of two experiments.

Fig. 4. Decrease of Glucose 6-Phosphate Dehydrogenase Activity in Rat Liver Microsome during Incubation with Ebselen in the Absence or Presence of Triton X-100

A suspension of rat liver microsome (4.38 mg protein) was incubated at 25 °C in a total volume of 1.0 ml of 0.25 M sucrose containing 0.5 mM EDTA, 1.9% dimethylformamide, 0.9 mM potassium phosphate buffer, pH 7.0, with 0.2 mM ebselen in the absence ($\bullet$) and presence of 2.0% Triton X-100 ($\triangle$). A control without ebselen was also run in the presence of Triton X-100 ($\circ$). Glucose 6-phosphate dehydrogenase activity was photometrically determined in 25 mM Tris-HCl buffer, pH 7.6, containing rat liver microsome (0.5–0.6 mg protein), 1.4% Triton X-100, 2.5 mM NADP⁺, 1 mM D-glucose 6-phosphate, 20 mM MgCl₂, 0.25 mM EDTA and 10 mM sucrose without removing ebselen. The activities are expressed per ml of incubation mixture. Each value is the average of two experiments.

liver microsomes with ebselen revealed the time-dependent inactivation of microsomal G6P dehydrogenase (Fig. 4). The inactivation of G6P dehydrogenase by ebselen was more effective in the presence of Triton X-100.

After incubation with 0.2 mM ebselen for 60 min, the membrane protein thiol groups in rat mitochondria (223 nmol SH/mg protein) and microsomes (53.6 nmol SH/mg protein) decreased to about 65% and 20% of the control, respectively, despite the absence of Triton X-100 (Fig. 5). The thiol groups in rat liver microsome membrane protein that were decreased by ebselen were partially restored by incubation with purified rat liver thioltransfer-

Fig. 5. Decrease in SH Groups in Rat Liver Mitochondria (A) and Microsome (B) Membrane Proteins after Incubation with Ebselen

Rat liver mitochondria (4 mg protein) and microsome (12 mg protein) in a total volume of 2.0 ml of 0.25 M sucrose containing 0.5 mM EDTA, 1.9% dimethylformamide, 0.9 mM potassium phosphate buffer, pH 7.0, were separately incubated for 60 min without (□) and with 0.2 mM ebselen (■) as described in the Fig. legend to 3. Mitochondria and microsome membrane proteins were prepared, and protein and thiol contents were estimated as described in the text. Quadruplicate determinations were performed and averages were calculated. The values shown are the means of these averages $\pm$ the standard error of the means. The numbers indicated on the figure are expressed as a percentage of intact mitochondria and microsome thiol contents.

Fig. 6. Restoration of SH Groups in Ebselen-Bound Rat Liver Microsome Membrane Protein by Thioltransferase in the Presence of GSH

A suspension of rat liver microsome (12 mg protein) was incubated with 0.2 mM ebselen in a total volume of 1.1 ml of 0.25 M sucrose containing 0.5 mM EDTA, 0.9 mM potassium phosphate buffer, pH 7.0, and 1.9% dimethylformamide at 25 °C for 60 min. After being washed with sucrose by ultracentrifugation twice, the pellet, including ebselen-bound microsomes, was resuspended in sucrose to about 0.8 mg protein per ml and was immediately incubated at 25 °C for 30 min without additions (■); and with 280 μ M GSH in the absence (□) and presence (▨) of 17.7 m units of purified rat liver thioltransferase. The preparation of microsome membrane protein, membrane protein-determination and thiol content-estimation were performed as described in the text. The microsome membrane protein thiol content was concomitantly measured after the incubation without ebselen (□). Quadruplicate determinations were performed and averages calculated. The values shown are the means of these averages $\pm$ the standard error of the means. The numbers indicated on the figure are expressed as a percentage of intact microsome thiol contents.

ase in the presence of GSH for 30 min, but 280 μ M GSH alone could not recover the decreased thiol groups of the membrane protein (Fig. 6). There was no recovery of the thiol groups in mitochondria membrane proteins decreased by ebselen, despite the incubation with thioltransferase in the presence of GSH (data not shown).

DISCUSSION

The aim of this study was to elucidate the reactivity of ebselen with subcellular particles, *i.e.* mitochondria and microsomes, and soluble proteins, and also the involvement of thioltransferase in cleaving Se-S bonds of ebselen-conjugated proteins. Our data provided evidence that the representative SH enzyme, lactate dehydrogenase, reacted easily with ebselen accompanied by a reduction in the enzyme activity and the reversible restoration of

lactate dehydrogenase activity by thioltransferase in the presence of GSH. Nikawa *et al.*¹³⁾ have demonstrated the restoration of glutathione S-transferase activity inactivated by ebselen, when incubated with 5 mM dithiothreitol (DTT) or 5 mM 2-mercaptoethanol. However, 0.5 mM GSH alone could not recover the lactate dehydrogenase inactivated by ebselen. This suggested that thioltransferase plays a role in the reversible gain of thiols from ebselen-bound soluble proteins. Rat liver mitochondrial glutamate dehydrogenase was not inactivated by ebselen, although its amount was a little bit smaller than the amount of membrane protein thiols. This indicated that ebselen did not pass through the mitochondrial membrane, but that a large amount of ebselen may disorder the membrane system by its extreme hydrophobicity. Protein thiols in rat liver mitochondria and microsome membranes were decreased significantly when the particles were incubated with ebselen in 0.25 M sucrose. It is clear that ebselen can react not only with microsome membrane protein, but also with membrane proteins of the mitochondrial surface. The microsome membrane thiols removed by ebselen were partially restored when ebselen-bound microsomes in sucrose were incubated with rat liver thioltransferase in the presence of GSH, showing the reversible restoration of membrane protein thiols. We considered the formation of GSH-ebselen adducts to be inferred by the SH/SS exchange of the thioltransferase, but ebselen was not transferred from microsome membrane protein to any other proteins, judging from the experiment. Nikawa *et al.*¹⁴⁾ have suggested a non-enzymatic transfer of ebselen from albumin to other cytosolic or microsomal proteins. Judging from characteristic SH/SS exchange reaction of low molecular disulfides,¹⁾ and no cleavage of protein disulfides (data not shown), thioltransferase does not function in the ebselen-transfer among soluble and microsomal proteins, including mitochondrial membrane proteins. Regardless, cytosolic proteins and the proteins from microsome particles may be significantly susceptible to reactive ebselen, whereas mitochondrial particles may be resistant to ebselen by means of its trap in membrane surface protein thiols and characteristic rigid membrane system, reflecting the lack of an ebselen effect upon mitochondrial respiratory chains localized on the inner side.

REFERENCES

- 1) Hatakeyama M., Tanimoto Y., Mizoguchi T., *J. Biochem.* (Tokyo), **95**, 1811 (1984); Hatakeyama M., Lee C., Chon C., Hayashi M., Mizoguchi T., *ibid.*, **97**, 893 (1985); Gan Z.-R., Wells W. W., *J. Biol. Chem.*, **261**, 996 (1986); *idem*, *Anal. Biochem.*, **162**, 265 (1987).
- 2) Mizoguchi T., Nishinaka T., Uchida G., Mizuta J., Uchida H., Terada T., Toya H., *Biol. Pharm. Bull.*, **16**, 840 (1993).
- 3) Mizoguchi T., Morita Y., Nanjo H., Terada T., Nishihara T., *Biol. Pharm. Bull.*, **17**, 607 (1994).
- 4) Terada T., Maeda H., Okamoto H., Nishinaka T., Mizoguchi T., Nishihara T., *Arch. Biochem. Biophys.*, **300**, 495 (1993).
- 5) Terada T., Hara T., Yazawa H., Mizoguchi T., *Biochem. Mol. Biol. Int.*, **32**, 239 (1994).
- 6) Wendel A., Fausel M., Safayhi H., Tiegs G., Otter R., *Biochem. Pharmacol.*, **33**, 3241 (1984).
- 7) Nomura H., Hokusui H., Takegoshi T., "Selenium in Biology and Medicine," ed. by Wendel A., Springer-Verlag, Berlin, Heidelberg, 1989, p. 189.

- 8) Cuatrecasas P., *J. Biol. Chem.*, **245**, 3059 (1970).
- 9) Segel I. H., Johnson M. J., *Anal. Biochem.*, **5**, 330 (1963).
- 10) Ellman G. L., *Arch. Biochem. Biophys.*, **82**, 70 (1959).
- 11) Bradford M. M., *Anal. Biochem.*, **72**, 248 (1976).
- 12) Kosower N. S., Kosower E., *Int. Rev. Cytol.*, **54**, 109 (1978).
- 13) Nikawa T., Schuch G., Wagner G., Sies H., *Biochem. Pharmacol.*, **47**, 1007 (1994).
- 14) Nikawa T., Schuch G., Wagner G., Sies H., *Biochem. Mol. Biol. Int.*, **32**, 291 (1994).