IN VITRO STUDIES ON THE METABOLIC PATHWAY OF SQ 14225 (CAPTOPRIL) AND MECHANISM OF MIXED DISULFIDE FORMATION

TORU KOMAI, TOSHIHIKO IKEDA, KENJI KAWAI, EMI KAMEYAMA AND HIDEYO SHINDO

Central Research Laboratories, Sankyo Co., Ltd., Hiromachi 1-2-58, Shinagawa-ku, Tokyo 140, Japan

(Received March 6, 1981)

The metabolic pathway of SQ 14225 (Captopril) and the mechanism of the mixed disulfide formation with endogenous sulfhydryl compounds were studied in *in vitro* cell free systems. In the rat liver $9000\times g$ supernatants, SQ 14225- ^{14}C was metabolized to one major metabolite, glutathione-SQ 14225 mixed disulfide (GSSQ), and two minor metabolites including SQ 14551, a symmetrical disulfide of SQ 14225. The formation of GSSQ was markedly accelerated by the addition of oxidized glutathione (GSSG), but not affected by the addition of reduced glutathione (GSH), indicating that GSSQ was formed by the thiol-disulfide interchange between SQ 14225 and GSSG. Although the thiol-disulfide interchange was also observed between SQ 14225 and L-cystine, L-homocystine and SQ 14551 as well as GSSG, only the formation of GSSQ was a rapid reaction and markedly decreased by heat treatment of the liver $9000\times g$ supernatants. These findings demonstrate that the formation of GSSQ is catalyzed by a GSSG specific enzyme which is supposed to be thioltransferase (Glutathione: Disulfide Oxidoreductase).

Although GSSQ was stable in the rat liver $9000\times g$ supernatants, it was rapidly hydrolyzed to cysteine-SQ 14225 mixed disulfide (CySSQ) in the rat kidney $9000\times g$ supernatants. A specific inhibitor of γ -glutamyltranspeptidase, anthglutin, inhibited the hydrolysis. GSSQ- ^{14}C administered to a beagle dog was excreted into the urine in the form of CySSQ.

Thus, it was speculated that CySSQ excreted into the urine as a major metabolite of SQ 14225 was derived from GSSQ formed in the liver followed by hydrolysis in the kidney.

Keywords—SQ 14225, Captopril, glutathione-SQ 14225 mixed disulfide, cysteine-SQ 14225 mixed disulfide, metabolic pathway, *in vitro*, antihypertensive, sulfhydryl compound

SQ 14225 (1-(3-Mercapto-2-D-methyl-1-oxo-propyl)-L-proline, Captopril) is a strong inhibitor of angiotensin converting enzyme developed in the Squibb Institute for Medical Research, USA, as an antihypertensive agent (Fig. 1).¹⁾ In our previous paper,²⁾ it was shown that SQ 14225- ^{14}C was metabolized to SQ 14225 (symmetrical disulfide of SQ 14225), glutathione-SQ 14225 mixed disulfide (GSSQ), cysteine-SQ 14225 mixed disulfide (CySSQ), N-acetylcysteine-SQ 14225 mixed disulfide, S-methyl-SQ 14225, S-methyl-SQ 14225 sulfoxide and a few other minor metabolites after the oral administration into rats and dogs.

In the present investigation, the metabolic

pathway of SQ 14225 and the mechanism of the mixed disulfide formation with endogenous sulfhydryl compounds were studied in *in vitro* cell free systems.

MATERIALS AND METHODS

Compounds—SQ 14225 labeled with ^{14}C in the side chain was obtained from Daiichi Pure Chemical Co. Ltd. (Fig. 1).³⁾ The specific radioactivity was $14.7 \mu Ci/mg$ ($3.2 mCi/mmol$) and the radiochemical purity was determined by a reverse isotope dilution method to be 98.4%. Non-radioactive SQ 14225 was obtained from the Squibb Institute for Medical Research, USA. Glutathione-L-(glycine-2- 3H), (GSH- 3H), was

purchased from New England Nuclear Corp., Boston, USA. GSSQ- ^{14}C , SQ 14551, GSSQ, CySSQ, N-acetylcysteine-SQ 14225, S-methyl-SQ 14225, and SQ 14225-N-ethylmaleimide adduct (SQ 14225-NEM) were prepared in our laboratories as described previously.²⁾ N-Ethylmaleimide, reduced glutathione (GSH), oxidized glutathione (GSSG), dithiothreitol (DTT), L-cystine and L-homocystine were purchased from Sigma Chemical Co., Mo., USA. Anthglutin, a specific inhibitor of γ -glutamyltranspeptidase⁴⁾ was supplied by Dr. S. Minato in the Fermentation Research Laboratories of this company. Other reagents were all commercially available and of reagent grade.

Preparation of the 9000 $\times$ g Supernatant of Liver and Kidney and Incubation Condition—Male Sprague-Dawley rats weighing about 200 g were sacrificed by exsanguination after 18 h fasting. The liver and the kidney were excised immediately, and the blood contained in the tissues was eliminated by a cold saline perfusion. The tissue was homogenized in three volumes of 1.15% KCl. The homogenates were centrifuged at 9000 $\times$ g for 20 min at 2°C and the supernatant was used. To obtain the heat denatured supernatant, the 9000 $\times$ g supernatant was placed in boiling water for 5 min and then centrifuged at 9000 $\times$ g for 10 min. Dialysis of the 9000 $\times$ g supernatant was carried out using Visking tube against a hundred volumes of 10mM Na-K-phosphate buffer containing 1.15% KCl, pH 7.4, for 24 h at 4°C.

FIG. 1. The Structure of SQ 14225 (1-(3-Mercapto-2-D-methyl-1-oxopropyl)-L-proline, Captopril)
*) the position labeled with ^{14}C .

FIG. 2. Thin Layer Autoradiograms of SQ 14225- ^{14}C incubated in the 9000 $\times$ g Supernatant of Rat Liver

Arrows show the position of authentic standards.

*) 10 mM DTT was added to the medium 30 min after the incubation.

FIG. 3. Formation of GSSQ- ^{14}C from SQ 14225- ^{14}C in Rat Liver 9000 $\times$ g Supernatant

The concentration of SQ 14225- ^{14}C and GSH were 0.46 and 0.65 mM, respectively.

—●—: in the liver supernatant.

—○—: in the liver supernatant added with GSH.

—△—: in the Na-K-phosphate buffer added with GSH.

The incubation mixture consisted of 50 μ l of the solutions of the radioactive substrates, 200 μ l of 50mM Na-K-phosphate buffer, pH 7.4, and 250 μ l of the 9000 $\times$ *g* supernatant and incubated with shaking at 37°C. The final concentration of the substrate was 0.46 mM, 0.19 mM and 0.19 mM for SQ 14225- 14 C, GSSQ- 14 C and GSH- 3 H, respectively. The final concentrations of GSH, GSSG, DTT and anthglutin were 0.64 mM, 0.33 mM, 10 mM and 1 mM, respectively, unless otherwise specified. They were dissolved in 50 mM Na-K-phosphate buffer, pH 7.4, and 200 μ l of the solution was added in place of the buffer.

After the incubation, 50 μ l of NEM solution in acetone (250 mg/ml) was added to the incubation medium for both termination of the reaction and stabilization of sulfhydryl substrates or products such as SQ 14225 or GSH. Then, 2 ml of ethanol was added to the medium and the precipitates were centrifuged at 9000 $\times$ *g* for 10 min at 4°C. The ethanol extracts were supplied for determination of the metabolites. The incubation was carried out at least in duplicate.

Intravenous Injection of GSSQ- 14 C to a Beagle Dog and Urine Collection—Three ml of a GSSQ- 14 C solution was intravenously administered into a male beagle dog weighing approximately 11 kg after 20 h fasting at the dose of 2 mg/kg. The urine was collected for the first 24 h period into a plastic beaker containing 500 mg of NEM in 10 ml of 2M Na-K-phosphate buffer, pH 6.0.

Separation and Determination of Metabolites—An aliquot of ethanol extracts or the urinary samples was spotted on a silica gel thin-layer plate (Precoated, F 254, 0.25 mm thick, Merck Co.) with authentic standards, and developed in a solvent system consisting of benzene: glacial acetic acid (3:1, v/v). The position of authentic standards was detected by UV absorption and the radioactive metabolites were located by autoradiography after exposure on X-ray film for 10 days. The radioactive spots were scraped off into counting vials and after extraction with 0.5 ml of 70% ethanol, 15 ml of scintillation cocktail (toluene 500 ml: ethanol 500 ml: POP 6 g:

dimethyl-POPOP 200 mg) was added. The radioactivity was measured by a Packard Tri-Carb liquid scintillation spectrophotometer (Model 3380). Counting efficiency was determined with automatic external standardization using a previously prepared quench curve.

When GSH- 3 H was incubated in the liver 9000 $\times$ *g* supernatant, the ethanol extracts were developed on silica gel thin-layer plates in a solvent system of *n*-butanol:glacial acetic acid:water (4:1:1, v/v). The amount of GSH- 3 H bound to protein was estimated as a difference between the amount of added GSH- 3 H and the amount ex-

FIG. 4. Conversion of GSH- 3 H to GSSG and Protein-bound Glutathione in Rat Liver Supernatant. Initial concentration of GSH was 0.19 mM and trace amount of GSH- 3 H (5 μ Ci) was added to the medium.

- : unchanged GSH.
- : oxidized form of glutathione (GSSG).
- △—: protein-bound glutathione.

tracted with ethanol. The concentrations of endogenous GSH and total glutathione (GSH + GSSG) in the liver were determined by Ellman's method,⁵⁾ as mentioned by Sasame *et al.*⁶⁾

FIG. 5. Effect of GSSG Addition on GSSQ-¹⁴C Formation from SQ 14225-¹⁴C

—●—: in the liver supernatant added with GSSG (0.33 mM).

—○—: in the liver supernatant added with GSH (0.64 mM).

—▲—: in the heat denatured supernatant added with GSSG.

—□—: in the Na-K-phosphate buffer added with GSSG.

RESULTS

Metabolic Change of SQ 14225-¹⁴C in the Rat Liver 9000× g Supernatant

When SQ 14225-¹⁴C (0.46 mM) was incubated in the rat liver 9000× g supernatant at 37°C, as shown in Fig. 2, the amount of SQ 14225-¹⁴C was decreased, and was approximately 21 and 4% of the total radioactivity in the ethanol extracts after 1 and 3 h of the incubation, respectively. Accompanying the decrease of SQ 14225-¹⁴C, the formation of three metabolites was detected. The major metabolite had the lowest *R_f* value among them and accounted for more than 60% of the total radioactivity 1 h after the start of the incubation.

In the previous paper,²⁾ it was shown that some of urinary metabolites of SQ 14225 in rats and dogs were mixed disulfides between SQ 14225 and endogenous sulfhydryl compounds and that the disulfide linkage of these metabolites were readily reduced by the addition of DTT. Thus, 10 mM of DTT was added to an incubation medium after 30 min of the incubation. As shown in Fig. 2 (1 h^{a)}), all of the *in vitro* metabolites disappeared on further 30 min incubation, suggesting that these metabolites would be disulfide-type metabolites.

By comparing their *R_f* values with those of authentic standards, the major metabolite was identified as GSSQ and one of two minor metabolites as SQ 14551. The formation of CySSQ was not significant even after 5 h incubation in the rat liver 9000× g supernatant,

TABLE I. Formation of Mixed Disulfides between SQ 14225-¹⁴C and Various Disulfides in Rat Liver 9000× g Supernatant

Disulfide ^{a)}	Formation of mixed Disulfide (%)	
	In untreated supernatant	In heat denatured supernatant
GSSG	30.2 ± 2.1 ^{b)}	11.9 ± 1.5
SQ 14551	10.5 (10.6, 10.5) ^{c)}	10.9 (10.9, 10.9)
L-Homocystine	1.57 (1.35, 1.79)	1.60 (1.09, 2.10)
L-Cystine	12.81 (11.7, 14.0)	14.4 (14.0, 14.8)

a) Concentration of the disulfide used was 0.33 mM. Incubation was carried out for 10 min.

b) The mean ± S.E. from four experiments.

c) Results are quoted as the average for two experiments, with single values in parentheses.

although CySSQ was a major metabolite in the urine after oral administration of SQ 14225 to rats and dogs.²⁾

Effect of Reduced and Oxidized Glutathione on GSSQ Formation in Rat Liver 9000× g Supernatant

Since the major metabolite was a mixed disulfide between SQ 14225 and glutathione, the effect of reduced glutathione (GSH) on the GSSQ formation was examined in the rat liver 9000× g supernatant. The addition of 0.65 mM GSH did not affect the rate of the GSSQ formation as shown in Fig. 3, suggesting that reduced GSH is not a direct reactant for the GSSQ formation.

It was also noted that the formation of GSSQ took place in two different steps. A small amount of GSSQ was produced in the earliest period and after a lag time of several minutes, a larger amount of GSSQ was produced. The presence of

the plateau between the two steps suggested that an active reactant might be produced during the plateau. Since the amount of SQ 14225 remained relatively constant during the plateau time, the change of reduced glutathione in the rat liver 9000× g supernatant on incubation was examined by adding a trace amount of GSH-³H. As revealed in Fig. 4, it was found that the concentration of GSH was rapidly decreased in the earliest period of the incubation and the concomitant increase of those of oxidized glutathione (GSSG) and protein-bound glutathione in the supernatant was observed. The concentration of GSH was almost constant when incubated with the heat denatured liver supernatant. Thus, the effect of oxidized glutathione on the formation of GSSQ was examined.

As depicted in Fig. 5, the formation of GSSQ was significantly accelerated by the addition of GSSG to the incubation medium, and the plateau phase of the GSSQ formation was no longer observed. These results indicated that GSSG was the reactant which directly react with SQ 14225. Although the thiol-disulfide interchange between SQ 14225 and GSSG took place both in the buffer and in the heat denatured supernatant, the extent of the GSSQ formation was much lower than that in the untreated supernatant, suggesting that most of GSSQ is formed by an enzymatic reaction.

Thiol-Disulfide Interchange Between SQ 14225-¹⁴C and Various Disulfides

The thiol-disulfide interchange between SQ 14225 and various disulfides was examined in the rat liver 9000× g supernatant and in the heat treated supernatant. As listed in Table I, the thiol-disulfide interchange was observed not only with GSSG, but also with L-cystine, L-homocystine and SQ 14551. However, the rate of the interchange with the latter three disulfides was slow and not affected by the heat treated liver supernatant. In contrast to these disulfides, the rate of thiol-disulfide interchange between SQ 14225 and GSSG was high in the untreated supernatant and significantly decreased on heat denaturation. These results demonstrated that the enzyme

FIG. 6. GSSQ-¹⁴C Formation from SQ 14225-¹⁴C in Dialyzed and Nondialyzed Liver Supernatants
 —●—: GSSQ-¹⁴C in nondialyzed liver supernatant.
 —○—: GSSQ-¹⁴C in dialyzed liver supernatant.

FIG. 7. Hydrolysis of GSSQ- ^{14}C to CySSQ- ^{14}C in the $9000 \times g$ Supernatant from Liver and Kidney. The concentration of GSSQ- ^{14}C was 0.19 mM.
 —○—: in kidney supernatant.
 —●—: in kidney supernatant with anthglutin.
 —△—: in liver supernatant.

TABLE II. Urinary Metabolites of GSSQ- ^{14}C after Intravenous Administration to a Beagle Dog

Metabolite	% of the total urinary radioactivity
Origin	0.94
GSSQ	3.15
CySSQ	46.10
SQ 14551	8.69
SQ 14225	5.34
S-methyl SQ 14225	3.52
NAcCySSQ ^{a)}	24.61

a) Contains unknown metabolites.

which involved in the thiol-disulfide interchange was specific to GSSG.

Formation of GSSQ in the Dialyzed $9000 \times g$ Supernatant of Rat Liver

The effect of dialysis of the $9000 \times g$ supernatant of the rat liver on the GSSQ formation was examined. The concentration of total glutathione (reduced + oxidized) in the supernatant before and after the dialysis was $340 \mu\text{g/ml}$ and $53 \mu\text{g/ml}$, respectively. As shown in Fig. 6, the rate of GSSQ formation was significantly reduced by dialysis. This result suggested that most of the liver glutathione involved in the GSSQ formation was dialyzable, although it has been reported⁷⁾ that a portion of glutathione in the liver is bound to proteins.

Hydrolysis of GSSQ to CySSQ in the Rat Kidney $9000 \times g$ Supernatant

As reported in our previous paper,²⁾ CySSQ was one of major metabolites in the urine from rats and dogs after oral administration of SQ 14225, and GSSQ was rather a minor metabolite. Thus, it was speculated that GSSQ formed in the liver might be hydrolyzed to CySSQ in other tissues than the liver. In order to examine the possibility, GSSQ- ^{14}C was incubated in the $9000 \times g$ supernatant from the kidney as well as from the liver. As depicted in Fig. 7, no hydrolysis occurred in the liver supernatant, whereas a rapid hydrolysis was observed in the kidney supernatant to form CySSQ.

When 1 mM anthglutin, a specific inhibitor of γ -glutamyltranspeptidase, was added into the kidney $9000 \times g$ supernatant, the formation of CySSQ was markedly decreased, indicating that the hydrolysis of GSSQ is catalyzed by γ -glutamyltranspeptidase.

Urinary Metabolites of GSSQ- ^{14}C after Intravenous Administration into a Beagle Dog

In order to ascertain whether or not GSSQ could be an intermediate of CySSQ formation from SQ 14225 *in vivo*, GSSQ- ^{14}C was intravenously injected into a beagle dog and the urinary metabolites were analyzed by thin-layer chromatography.

More than 99% of the dosed radioactivity was

excreted into the urine within the first 24 h period. As listed in Table II, the main metabolite was CySSQ, which accounted for approximately 46% of the total urinary metabolites, whereas unchanged GSSQ was as low as 3.1% of the total urinary radioactivity.

DISCUSSION

In our previous paper,²⁾ it was shown that SQ 14225-¹⁴C administered orally into dogs and rats was metabolized into SQ 14551, GSSQ, CySSQ, N-acetylcysteine-SQ 14225, S-methyl-SQ 14225 and other metabolites. Among them, CySSQ was one of the main metabolites in dogs and rats urine. Thus, the metabolic pathway of SQ 14225 and the mechanism of the formation of mixed di-

sulfides were studied mainly in cell free systems in rats. When SQ 14225-¹⁴C was incubated in the $9000 \times g$ supernatant of the rat liver, the main metabolite was found to be GSSQ and the formation of CySSQ was negligible. The formation of GSSQ was markedly accelerated by the addition of oxidized glutathione (GSSG), while reduced glutathione (GSH) added had no effect. This result demonstrated that GSSQ was formed by a thiol-disulfide interchange between SQ 14225 and GSSG. Although the interchange occurred between SQ 14225 and other disulfides such as L-cystine, L-homocystine and SQ 14551 in the liver supernatant, all the reactions were insensitive to the heat treatment of the liver supernatant. In contrast, GSSG reacted rapidly with SQ

FIG. 8. Postulated metabolic pathway of SQ 14225

E1: Thioltransferase. E2: GSH peroxidase. γ-GTP: γ-glutamyltranspeptidase.

14225 and the reaction was markedly reduced by the heat treated liver supernatant. These findings suggested that the thiol-disulfide interchange between SQ 14225 and GSSG is catalyzed by a GSSG specific enzyme. Askelöf *et al.*⁸⁾ have proved that there is an enzyme which catalyzes the thiol-disulfide interchange between sulfhydryl compounds and GSSG, and named it thioltransferase (GSSG: Disulfide Oxidoreductase). On the other hand, it has been reported that the content of GSSG in the liver is approximately 7% of total liver glutathione by Sasame *et al.*⁶⁾ Thus, it is most likely that GSSQ can be formed *in vivo* as a main metabolite in the liver.

GSSQ was very readily hydrolyzed to CySSQ in the 9000×*g* supernatant of the rat kidney. The fact that the hydrolysis was inhibited by anthglutin, a specific inhibitor of γ -glutamyl-transpeptidase, demonstrated that GSSQ is hydrolyzed by this enzyme which was shown to reside on the luminal surface of the renal tubule epithelium.⁹⁾ The *in vivo* conversion of GSSQ-¹⁴C to CySSQ was also ascertained.

Thus, it was speculated that CySSQ in the urine was derived from GSSQ formed in the liver followed by hydrolysis in the kidney, as schematically shown in Fig. 8.

Acknowledgement The authors wish to express their deep gratitudes to Prof. S. Tsurufuji of Tohoku University for his valuable discussions and to Dr. K. Murayama, the director of this laboratories, for his kind encouragement. The authors are also grateful to Dr. S. Minato, Fermen-

tation Research Laboratories of this company, for supplying anthglutin and Miss Wakako Kawamata of this laboratories for her technical assistance.

REFERENCES

- 1) M.A.Ondetti, D.Rubin and D.W.Cushman: Design of specific inhibitors of angiotensin converting enzyme, new class of orally active antihypertensive agents, *Science*, **196**, 441–444 (1977).
- 2) T.Ikeda, T.Komai, K.Kawai and H.Shindo: Urinary metabolites of 1-(3-mercapto-2-D-methyl-1-oxo-propyl)-L-proline (SQ 14225), a new antihypertensive agent, in rats and dogs, *Chem. Pharm. Bull.*, **29**, 1416–1422 (1981).
- 3) M.Hasegawa, S.Oyabu, T.Ueda, and M.Yamaguchi: The synthesis of SQ 14225-¹⁴C. *J. Labeled Compd.*, **18**, 643–650 (1981).
- 4) S.Minato: Isolation of anthglutin, an inhibitor of γ -glutamyl-transpeptidase from *Penicillium oxalicum*, *Arch. Biochem. Biophys.*, **192**, 235–240 (1979).
- 5) G.L.Ellman: Tissue sulfhydryl groups, *Arch. Biochem. Biophys.*, **82**, 70–77 (1959).
- 6) H.A.Sasame and M.R.Boyd: Paradoxical effects of cobaltous chloride and salts of other divalent metals on tissue levels of reduced glutathione and microsomal mixed-function oxidase components, *J. Pharmacol. Exp. Ther.*, **205**, 718–724, (1978).
- 7) J.Issacs and F.Binkley: Glutathione dependent control of protein disulfide-sulfhydryl content by subcellular fractions of hepatic tissue, *Biochem. Biophys. Acta*, **497**, 192–204 (1977).
- 8) P.Askelöf, K.Axeleson, S.Eriksson, B.Mannervik: Mechanism of action of enzymes catalyzing thiol-disulfide interchange, thioltransferase rather than transhydrogenase, *FEBS Lett.*, **38**, 263–267 (1974).
- 9) S.Horiuchi, M.Inoue and Y.Morino: γ -Glutamyl-transpeptidase: sidedness of its active site on renal brush-border membrane, *Eur. J. Biochem.*, **87**, 429–437 (1978).