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Synthesis and Characterization of the L-Cysteine-Glutathione Mixed Disulfide

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The cysteine-glutathione mixed disulfide has been synthesized in good yield by thiolysis of the thiolsulfonate derivative of cystine ("cystine disulfoxide") with glutathione. The purified mixed disulfide, which is stable in the solid state, has been studied by NMR and IR spectroscopy, as well as by polarimetry. A method for the determination of disulfides is also described.

In recent years several low-molecular mixed disulfides of biological origin have been isolated. Some examples are the disulfides between cysteine and homocysteine,[1] cysteine and glutathione,[2–4] and coenzyme A and glutathione.[5–7] Since GSH * has been claimed to be the dominating non-protein thiol in most cells,[8] it seems reasonable to assume that it should frequently be one of the components forming an unsymmetrical disulfide. Some doubt may be cast upon the presence in living organisms of some of the mixed disulfides demonstrated, *e.g.* in the work on pantethine.[9] However, the authenticity of the mixed disulfide of cysteine and glutathione seems to be established. Its presence in tissues such as liver [2,3] and lens [4] has been claimed.

The successful synthesis of the coenzyme A-glutathione mixed disulfide, based on the thiolysis of the thiolsulfonate derivative of GSSG,[10] encouraged us to undertake the synthesis of CySSG along analogous lines. Thus, we treated glutathione with an excess of the thiolsulfonate corresponding to cystine ("cystine disulfoxide")

$$GSH + CySO_2SCy \longrightarrow CySSG + CySO_2H \qquad (1)$$

and separated CySSG from contaminating compounds by ion-exchange chromatography. Some of the properties of the purified CySSG are reported.

* Abbreviations: BSSB, 4,4'-dithiobis(benzenesulfonate); CySH, cysteine; CySSCy, cystine; CySSG, CySH-GSH mixed disulfide; $CySO_2SCy$, the thiolsulfonate analogue of cystine; $CySO_2H$, cysteinesulfinic acid; $CySO_3H$, cysteic acid; $CySSO_3H$, S-sulfocysteine; DNS, 1-dimethylaminonaphthalene-5-sulfonyl; GSH, glutathione; GSSG, glutathione disulfide; GSO_2SG, the thiolsulfonate analogue of GSSG; GSO_2H, the sulfinic acid corresponding to GSH; $GSSO_3H$, S-sulfoglutathione; TFA, trifluoroacetic acid.

EXPERIMENTAL

Materials. Fluorescein mercuric acetate was prepared according to Karush, Klinman and Marks.[11] The sodium salt of BSSB was synthesized according to Smith, Doughty and Gorin.[12] Paper electrophoresis at pH 1.9 followed by development with fluorescein mercuric acetate failed to reveal any UV-absorbing impurities. The bis(S-benzylthiuronium) salt of BSSB had a m.p. of 170°. S-Sulfoglutathione was synthesized by a preparative version of the sulfitolysis of Bailey and Cole[13] with tetrathionate as the oxidant (B. Eriksson and V. Schalén, unpublished experiments). Ion-exchange chromatography on DEAE-Sephadex A-25 with the application of a formic acid gradient, followed by precipitation of the barium salt of S-sulfoglutathione with ethanol, yielded the pure product.

GSSG was obtained from C. F. Boehringer & Soehne GmbH; GSH and DNS-chloride from Sigma Chemical Co.

Electrophoresis and thin-layer chromatography. Paper electrophoresis was carried out on Whatman No. 1 paper. Buffer systems were formic acid-acetic acid pH 1.9[14] and pyridinium acetate pH 4.0.[15] Thin-layer chromatography was performed on glass plates, 20 × 20 cm, covered with cellulose powder MN 300 (Machery, Nagel & Co., Düren, Germany), 0.2 mm thick, or Silica Gel G (Merck), 0.3 mm thick. The silica gel was activated at 110° before use. Detection was usually accomplished with ninhydrin (0.4 % in ethanol, containing 0.04 ml pyridine per 100 ml).

Synthesis of CySSG. The thiolsulfonate analogue of L-cystine was prepared according to Emiliozzi and Pichat.[16] The purified product was analyzed by the iodometric method of Toennis and Lavine,[17] and by an indirect method in which the thiolsulfonate was decomposed with alkali:

$$3\ CySO_2SCy + 4\ OH^- \longrightarrow 4\ CySO_2^- + CySSCy + 2\ H_2O \qquad (2)$$

The formed cystine was then determined by the quantitative method described below. (The calculated value is exceeded by an amount corresponding to the cystine present in the thiolsulfonate). Both methods demonstrated a purity of 78 %.

An excess of thiolsulfonate (3.00 g) was added to a solution of GSH (1.00 g, 3.26 mmole) in 100 ml of 0.01 M formic acid. The mixture was thoroughly stirred, and after 120 min solid material was removed by centrifugation. Washing of the residue with 0.01 M formic acid was performed four times. The supernatant from the reaction mixture and the combined washings (60 ml) were then applied to a Dowex 1-formate column (4 × 15 cm, X2, 50—100 mesh). Cystine and $CySO_2SCy$ were not absorbed and were washed out with water. When about 1400 ml of effluent had been collected, the eluent was changed to 0.15 M formic acid. The mixed disulfide appeared immediately after the void volume (140 ml). GSSG was eluted by raising the formic acid concentration to 0.27 M (Fig. 1), whereas the more acidic cysteinesulfinic acid still was retained by the column. Electrophoresis verified the identity of the eluted substances.

The fractions containing CySSG were pooled (490 ml) and concentrated in a rotary evaporator. The syrupy residue, *ca.* 5 ml, was diluted with 5 ml of water, and CySSG was then precipitated by the addition of 100 ml of acetone. The precipitate was collected by centrifugation and dried to constant weight over silica gel in vacuum. The dry product (1.27 g, 2.98 mmole) was obtained as a white powder. Yield 91 %; m.p. 234° (decomp.).

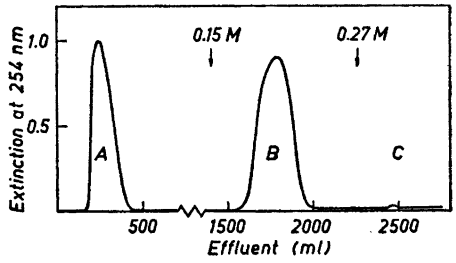

Fig. 1. Ion-exchange chromatographic separation of cystine (A), CySSG (B), and GSSG (C) on a Dowex 1-formate column (4 × 15 cm). Elution was carried out stepwise with water, 0.15 M formic acid, and 0.27 M formic acid.

(Found: C 36.8; H 5.6; N 12.8. Calc. for $C_{13}H_{22}N_4O_8S_2$: C 36.6; H 5.2; N 13.1). The disulfide content of the substance was determined by the procedure described below and was found to be 0.99 mole per mole (M.W. 426.5). CySSG was stable when stored in the refrigerator. The solubility in deionized water was 0.8 g per 100 ml.

Paper electrophoresis and thin-layer chromatography, which resolve CySSG from possible impurities (Tables 1 and 2), demonstrated the homogeneity of the purified product. Positive reactions were obtained with ninhydrin, nitroprusside-KCN,[18] and fluorescein mercuric acetate [11] development.

RESULTS

Determination of amino acid composition. The product was labelled with DNS-groups in N-terminal positions and was then hydrolyzed with the formic acid reagent of Hanes *et al.*[19] The DNS-amino acids in the hydrolyzate were identified by thin-layer chromatography on silica gel [20] and by paper electrophoresis (Table 3). The mixed disulfide was also hydrolyzed before treatment with the DNS-reagent to give DNS-derivatives of all constituent amino acids. The results were consistent with the anticipated structure. Further proof was provided by sulfitolysis [13] followed by paper electrophoresis, which demonstrated the formation of S-sulfocysteine and S-sulfoglutathione:

$$CySSG + 2\ SO_3^{2-} + S_4O_6^{2-} \longrightarrow CySSO_3^- + GSSO_3^- + 2\ S_2O_3^{2-} \quad (3)$$

Table 1. Thin-layer chromatography on cellulose powder of compounds related to CySSG. Systems: A, acetic acid-butanol-water, 3:12:5 (by vol.); B, acetic acid-butanol-water-phenol, 8:15:15:5 (v/v/v/w); C, acetic acid-butanol-water-pyridine, 3:15:12:10 (by vol.); D, acetic acid-butanol-water-phenol-acetone, 9.2:16.8:26:4:2.5 (v/v/v/w/v).

Compound	R_F-Values			
	System			
	A	B	C	D
CySSCy	0.23	0.37	0.23	0.55
CySSG	0.15	0.41	0.22	0.63
GSSG	0.13	0.50	0.21	0.71
GSH	0.35	0.61	0.43	0.72

Table 2. Paper electrophoresis of disulfides related to CySSG. Migration expressed as distance moved towards the cathode, corrected for electroosmosis; 10 V/cm, 180 min.

Compound	Migration (cm)	
	pH 1.9	pH 4.0
CySSCy	5.6	0.2
CySSG	4.8	−2.0
GSSG	4.2	−3.2

Table 3. Paper electrophoresis of DNS-derivatives. Migration expressed as distance moved towards the cathode at pH 1.9, not corrected for electroosmosis; 10 V/cm, 345 min.

DNS-derivative	Migration (cm)
$CySO_3H$	0.5
Glu	8.2
N-Terminal amino acids of CySSG	0.5, 8.0
Gly	9.2
CySSCy	9.5

Determination of disulfide content. The assay for disulfide bonds was based on reduction with sodium borohydride, followed by the reaction between the formed thiol and BSSB. The liberated thiobenzenesulfonate was then determined spectrophotometrically. The thiol-disulfide exchange proceeds to completion above pH 7 (*cf.* Ref. 21). The system for the determination of thiols had the following composition: 2.00 ml of 0.33 mM BSSB (sodium salt) in phosphate buffer (0.02 M, pH 7.5, 1 mM with respect to EDTA) + 1.00 ml of the sample. The blank was identical except for the substitution of water or buffer for the sample. After stabilization, which requires a few minutes, the extinction at 285 nm was read in a Beckman DB spectrophotometer with the blank in the reference compartment.

Calibration with cysteine showed that the extinction was directly proportional to the thiol concentration in the range of 0 to 7×10^{-5} M, a cysteine concentration of 5×10^{-5} M in a 1-cm cuvette giving an extinction of 0.700. This sensitivity is slightly higher than that obtained with the reagent described by Ellman.[22]

Reduction of disulfides was carried out by the addition of solid $NaBH_4$ to an alkaline solution of the disulfides. To 1 ml of 0.6 mM disulfide, dissolved in 10 mM NaOH, 40 mg of $NaBH_4$ was added with stirring. This corresponds to a 1700-fold molar excess of the reductant, and gives a quantitative reduction within 10 min. The mixture was diluted with a few ml of water 10 min after the start of the reduction and was brought to pH 7 by the slow addition of 0.075 M formic acid (about 5 ml). The final volume was determined, and an 1 ml aliquot was immediately transferred to a cuvette containing the BSSB solution.

Cystine samples were analyzed by this procedure, and the extinction values obtained coincided with the values calculated from the cysteine standardization.

Infra-red spectrum. IR spectra of CySSG (Fig. 2), GSH, GSSG (*cf.* Ref. 23), and cystine (*cf.* Ref. 24) were recorded with the substances in KBr discs. Great similarities between the spectra of CySSG and GSH are apparent, especially in the region 5—8 μm containing the C—O stretching and N—H deformation absorptions. The distinct peak at 9.27 μm is also found in GSH, whereas the absorption at 11.73 μm is not. The latter, however, can be correlated with the dominating band (11.79 μm) in the corresponding region of the cystine spectrum. The two peaks at 8.17 and 8.41 μm in Fig. 2 seem to

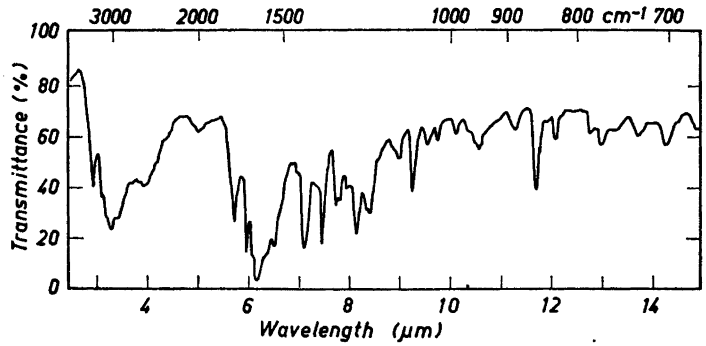

Fig. 2. Infra-red spectrum of CySSG in a KBr disc.

be rather characteristic for CySSG. The former peak has a counterpart at 8.12 μm in the spectrum of GSSG (which has a poor resolution, however, probably owing to the ethanol present) but is not found in the spectra of cystine or GSH.

NMR measurements. Spectra of CySSG, GSH, GSSG, cystine, and cysteine were obtained with a Varian Associates A-60 nuclear magnetic resonance spectrometer. The samples were dissolved to a concentration of 50 to 100 mg/ml in conc. TFA, 2.5 M TFA in D_2O, or 1 M TFA in D_2O. Chemical shifts were determined with 3-trimethylsilylpropionic acid as an internal standard.

The spectra run in conc. TFA did not show much fine structure (*cf.* Ref. 25), but the bulky peaks could usually be correlated with the different types of protons (Table 4). It was found that the CySSG spectrum contained a weak but very sharp peak at 2.42 ppm (not shown in Table 4), which on integration was shown to be equivalent to about 0.5 of a proton of CySSG. We interpret this as being due to remaining acetone in the preparation. Since acetone contains 6 protons, this corresponds to about 0.08 mole per mole CySSG, or 1 % of the weight of CySSG. This may explain why the elementary analyses yielded percentages, which were higher for carbon and hydrogen and lower for nitrogen than the expected values.

The GSSG spectrum was complicated by impurities in the commercial GSSG preparation. Quantitative assays for disulfide content as described above or with glutathione reductase and NADPH (B. Eriksson, unpublished experiments) have demonstrated that GSSG represents only 80 % of the weight. The peaks not belonging to the GSSG spectrum were easily recognized, however, being very narrow in comparison with the authentic ones. Thus, a strong quartet and a strong triplet centered around 4.58 and 1.47 ppm, respectively, were ascribed to ethanol, whereas a weak quartet at 4.03 and a weak triplet at 1.38 ppm could be due to diethyl ether. These multiplets, which had splittings of 7 to 8 cps, were omitted from Table 4. It was evident that the quartet at 4.58 ppm, which was skew, was hiding the α-CH absorption of the glutamyl residues of GSSG.

Table 4. NMR chemical shifts of compounds related to CySSG dissolved in conc. TFA.

Compound	Chemical shift (ppm)							
	$-NH_3^+$ $-NHCO-$	α-CH Cys	α-CH Glu	α-CH Gly	β-CH_2 Cys	β-CH_2 Glu	γ-CH_2 Glu	SH
CySSG	7.93	5.13 4.85	4.52	4.37	3.40[a]	2.98	2.63	
GSSG	7.95	5.15	see text	4.37	3.31	2.98	2.63	
GSH	7.92	4.92[b]	4.57[b]	4.37[c]		3.01[d]	2.62	1.85[e]
CySSCy	7.82	4.88			3.65 3.51[f]			
CySH	7.75	4.75[b]			3.40			1.88[e]

[a] The broad peak is asymmetric (the four β-hydrogens of the different half-cystines are not equivalent), and a component corresponding to one of the hydrogens of the cysteine residue * appears at 3.57 ppm. [b] Doublet split 6 to 7 cps. [c] Doublet split 5 to 6 cps. [d] This peak corresponds to four protons and includes the β-CH_2 absorption of the cysteinyl residue. In GSSG the peak due to the latter is shifted downfield, since the neighbouring —SS— is more electronegative than —SH (*cf.* cystine and cysteine). [e] Triplet split 8 to 9 cps. [f] The intensity of the peak at 3.65 ppm being about three times that at 3.51 ppm.

Fig. 3 demonstrates the spectrum of CySSG in 2.5 M TFA. At this acid concentration the absorption due to the α-CH of the cysteinyl residue * of CySSG was resolved from the solvent peak. This was not the case when 1 M TFA was used, which in other respects offered a better resolution. It can be seen that the main features of the spectrum in conc. TFA are retained (*cf.* Table 4),

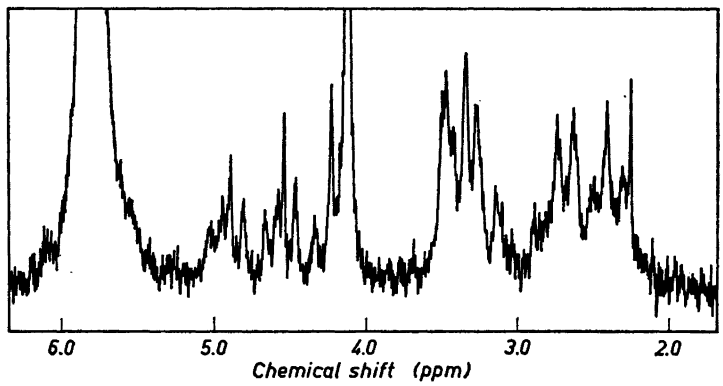

Fig. 3. Nuclear magnetic resonance spectrum of CySSG dissolved in 2.5 M TFA. Trimethylsilylpropionic acid was used as an internal standard.

* In this paper we denote the cysteine linked to glutamic acid and glycine by the "cysteinyl" residue, whereas the other cysteine of CySSG is referred to as the "cysteine" residue.

but that the absorptions are shifted upfield due to the lower acidity of the solvent. Protons bonded to nitrogen, however, cannot be observed at this acidity.

It is known that restricted rotation about the bond between the α and β carbons of a half cystine makes the methylene protons magnetically non-equivalent.[26-28] This seems to be the case for CySSG as well, and it is probable that some interaction between the glutamyl and cysteine residues is stabilizing a particular rotamer. The possibility of hydrogen bond formation between these residues has been demonstrated by molecular model building.[29] Thus the splittings of the α-CH absorptions of the cysteinyl (4.90 ppm) and cysteine (4.57 ppm) residues could be explained as resulting from unequal coupling to the protons of their corresponding β-CH_2 groups. The α-CH of the glutamyl residue, on the other hand, gives rise to a triplet (4.23 ppm), one component of which is partially buried in the peak corresponding to the methylene protons of the glycine residue (4.12 ppm). This is seen more clearly at a lower acid concentration, since the glutamyl α-CH absorption is more sensitive to changes in pH and is moved further upfield than the methylene peak. It may be added that the α-CH absorptions of the cysteinyl and glutamyl residues of GSH both are split into triplets at the same acidity.

Absorptions corresponding to the different methylene hydrogens of the cysteine residue appear at 3.47 and 3.35 ppm, the first of which is split into a triplet. In 1 M TFA the triplet was well resolved with a splitting of 2 to 3 cps. We assume that the proton is magnetically coupled to the methylene hydrogens of the cysteinyl residue across the disulfide bridge. The peaks at 3.27 and 3.13 ppm are due to the methylene group of the cysteinyl residue. Further upfield absorptions corresponding in turn to the γ-CH_2 and β-CH_2 groups of the glutamyl residue appear. It should be noted, however, that the narrow peak at 2.26 ppm probably is due to acetone.

Polarimetric measurements. The optical activity of CySSG dissolved in 1.0 M HCl was measured in 1 dm tubes. A constant value of $[\alpha]_D^{23} = -112°$ for concentrations down to 0.25 g per 100 ml was obtained by dilution of a solution containing 2.0 g CySSG per 100 ml. At lower concentrations the specific rotation decreased. If the acid strength was decreased along with the CySSG concentration by a series of dilutions with water, the specific activity was still constant in the mentioned range, and then deviated in the same way as in the measurements with a constant HCl concentration. Similar experiments with cystine resulted in an increased specific rotation upon dilution with water.[30] If CySSG was dissolved to a concentration of 0.25 g per 100 ml in 0.01 M HCl or 0.1 M phosphate buffer pH 7.2, however, $[\alpha]_D^{25}$ was found to be −160° and −168°, respectively. Paper electrophoresis demonstrated that CySSG had not decomposed during any of the measurements. We therefore suggest that this discrepancy is due to stabilization by protonation of a particular conformation of CySSG when it is dissolved in 1 M HCl. This conformation is retained on dilution. The interpretation of the NMR spectra supports this hypothesis. When CySSG is dissolved at a lower acidity, however, the molecule may appear in another conformation with a different optical activity.

DISCUSSION

To our knowledge no synthesis of CySSG yielding an amount sufficient for a more careful characterization of the substance has previously been described. The formation of CySSG, when glutathione and cysteine were co-oxidized, was observed chromatographically.[31,32] This reaction and the thiol-disulfide interchange between cysteine and glutathione disulfide have been utilized.[2,3,33] Since cysteine is rapidly oxidized by air, all thiol species present will eventually be oxidized. The yield of CySSG will then be determined by the concentrations of the different disulfides in equilibrium:

$$\mathrm{CySSCy} + \mathrm{GSSG} \rightleftharpoons 2\ \mathrm{CySSG} \qquad (4)$$

Kolthoff *et al.*[34] have reported a value of 3.0 at 25° for the equilibrium constant of this reaction.

A reaction, which probably will give a better yield is the reaction between glutathione and S-sulfocysteine:[35]

$$\mathrm{GSH} + \mathrm{CySSO_3^-} \rightleftharpoons \mathrm{CySSG} + \mathrm{HSO_3^-} \qquad (5)$$

It is necessary, however, to have an excess of S-sulfocysteine or to remove the formed sulfite to carry the reaction to completion.

The thiolysis of $\mathrm{CySO_2SCy}$ with GSH (Eqn. 1), as well as the analogous reaction between CySH and the thiolsulfonate of GSSG,

$$\mathrm{GSO_2SG} + \mathrm{CySH} \longrightarrow \mathrm{CySSG} + \mathrm{GSO_2H} \qquad (6)$$

has previously been used for an analytical purpose.[36] As a sulfinic acid is less S-nucleophilic than a thiol,[37] the reaction is displaced to the right and the yield of mixed disulfide could be expected to be good. This anticipation was borne out by the experiments described.

We are presently investigating the enzymatic reduction of CySSG by rat liver. There is evidence for a transhydrogenase reaction dependent on GSH, which is coupled to the glutathione reductase catalyzed reduction of GSSG formed.

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