On the Synthesis and Enzymatic Reduction of the Coenzyme A-Glutathione Mixed Disulfide

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Evidence for the natural occurrence of CoASSG * is accumulating.[1,2] So far, CoASSG has been prepared by the thiol-disulfide interchange of CoA and GSSG. The yield of this reaction is determined by the equilibrium constants of the reactions between the different thiol and disulfide species in equilibrium. The expensiveness of CoA makes its stoichiometrical conversion into the mixed disulfide highly desirable. This is theoretically possible by the reaction between CoA and an excess of the thiolsulfonate analogue of GSSG:

$$CoASH + GSSO_2G \rightarrow CoASSG + GSO_2H$$

The present communication describes the synthesis of CoASSG by this reaction. In principle, it should be possible to isolate CoASSG from the reaction mixture by adsorption on a charcoal column. However, contamination of the eluted mixed disulfide necessitated further purification by ion-exchange chromatography. The final yield of the purified CoASSG was 43 % (corrected for 10 % impurities in the CoA preparation).

Synthesis. GSSG (Boehringer), 125 mg, was dissolved in 1 ml of 98–100 % formic acid and oxidized with 60 µl of 30 % H_2O_2 in the presence of 20 µl of conc. HCl. The yield of $GSSO_2G$ by this reaction is about 50 %.[3] 90 min after the start of the oxidation the reaction mixture was concentrated in a rotary evaporator. The product was dissolved in 1 ml of water and carefully adjusted to pH 3 with 1 M NaOH. CoA, 30.0 mg, (Sigma, containing 90 % CoA when assayed with 3-hydroxyacyl-CoA dehydrogenase) dissolved in 0.4 ml of water was added. The nitroprusside test was negative within a few minutes. 15 min after the addition of CoA the solution was poured on a charcoal column, 0.8 × 14 cm (Nuchar C 190, deactivated with stearic acid, 6 % w/w,[4] degassed in 1 M HCl *in vacuo* and washed with water). The column was rinsed with 100 ml of water, and the mixed disulfide was then eluted with 60 ml of 5 % pyridine. The effluent was extracted with 30 ml of chloroform and the aqueous phase was evaporated *in vacuo*. The residue was dissolved in 1.5 ml of water, and after adjustment to pH 2 with 1 M HCl CoASSG was precipitated by the addition of a fivefold excess of acetone. The precipitate was collected by centrifugation and dried to constant weight over silica gel *in vacuo*. The product, 32.6 mg, was contaminated with GSSG, CoASSCoA and a few minor impurities as demonstrated by paper electrophoresis.

Purification of CoASSG. The impure mixed disulfide was dissolved in 2 ml of water and adsorbed on a DEAE-Sephadex A-25 column, Cl^--form (Pharmacia), 2 × 10 cm, packed in water. A linear gradient, with 1000 ml of water in the mixing vessel and 1000 ml of 1 M NaCl in the reservoir, separated CoASSG from 5 other UV-absorbing substances. The pooled fractions containing the mixed disulfide were concentrated by evaporation, dissolved in 10 ml of water and freed from NaCl by gel filtration on a Sephadex G-10 column (Pharmacia), 1.5 × 85 cm, eluted with water. The CoASSG containing fractions were combined, 19.5 ml, and analyzed for adenine, CoA and disulfide content (Table 1).

Analyses. The CoA part of the product was determined in a modified 3-hydroxyacyl-CoA dehydrogenase test (*cf.* Ref. 5). The CoASSG sample, 500 µl, was diluted with 500 µl of water and treated with 10 µl of thioglycolic acid. The mixture, kept in an ice bath, was adjusted to pH 9 with 100 µl of 2 M KOH. After 20 min 10 µl of diketene was vigorously stirred into the sample. 5 min later the formed acetoacetyl-CoA was determined spectrophotometrically in a system consisting of: 2800 µl of pyrophosphate buffer (0.1 M, pH 7.3), 200 µl of the pretreated sample, 20 µl of reduced NAD (10 mg/ml, dissolved in 1 % $NaHCO_3$), and 5 µl of 3-hydroxyacyl-CoA dehydrogenase (Sigma, 2 mg/ml). Corrections for absorption changes by the addition of the enzyme were not found necessary.

* Abbreviations: CoA (CoASH), coenzyme A; CoASSCoA, CoA disulfide; CoASSG, CoA-glutathione mixed disulfide; GSSG, glutathione disulfide; $GSSO_2G$, the thiolsulfonate analogue of GSSG; GSO_2H and GSO_3H, the sulfinic and sulfonic acid analogues of glutathione; NAD(P), nicotinamide-adenine dinucleotide (phosphate); EDTA, ethylenediaminetetraacetate.

Table 1. Enzymatic analyses of CoASSG.

Component	Concentration	
	μmole/ml	μmole/μmole adenine [a]
CoA	0.776 0.768	1.00
Disulfide group	0.771 0.776	1.00

[a] Assuming an extinction coefficient of 16 $cm^2/\mu mole$ at 260 nm, the adenine content of the sample (in water, pH 4.5) was 0.770 μmole/ml.

The disulfide content of the mixed disulfide was determined by reduction in a system consisting of: 2600 μl of phosphate buffer (0.1 M, pH 7.6, 1 mM with respect to EDTA), 300 μl of reduced NADP (1 mg/ml, dissolved in phosphate buffer), 100 μl of the CoASSG sample, and 5 μl of glutathione reductase (Boehringer, 1 mg/ml). The oxidation of reduced NADP was followed spectrophotometrically at 340 nm. The initial rate was 7.3 nmole/min, and the reaction required about 100 min for completion.

According to the analyses the yield of the purified CoASSG was 15.0 μmole.

The UV-absorption had a maximum at 258 nm and a minimum at 229 nm.

Paper electrophoresis at pH 1.9 (formic acid-acetic acid buffer) and 4.0 (pyridinium acetate buffer) resolved CoASSG from possible contaminants: GSSG, GSO_2H, GSO_3H, CoA, and CoASSCoA. No impurities could be detected after the ion-exchange chromatography, and the mixed disulfide showed the expected reactions when developed on the electropherograms. Positive tests were obtained with ninhydrin, nitroprusside-KCN, fluorescein mercuric acetate,[6] iodoplatinate, and $FeCl_3$-sulfosalicylic acid.

Interaction with glutathione reductase. The observed rate of the CoASSG reduction, 1.46 μmole/min/mg glutathione reductase, was considerably higher than that reported by Ondarza and Martínez.[7] The finding that an amount of glutathione equimolar to CoASSG did not accelerate the reduction, seems to exclude the possibility that glutathione or other thiols in the commercial glutathione reductase preparation catalyzed the reaction by thiol-disulfide interchange.

A 1400-fold purified glutathione reductase from porcine erythrocytes [8] showed a very low activity with CoASSG. However, the mixed disulfide caused a time-dependent inhibition of the enzymatic reduction of GSSG. No effect was observed when CoASSG and GSSG were added simultaneously, but incubation of glutathione reductase with CoASSG and reduced NADP before the addition of GSSG resulted in an inhibition, which increased with time. This finding demonstrates that interaction between CoASSG and the enzyme actually occurs. It is assumed that the inhibition is due to the formation of a mixed disulfide between CoA and a sulfhydryl group at the active site of glutathione reductase.

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Received April 1, 1966.