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TRANSFER OF PERSULFIDE SULFUR FROM THIOCYSTINE TO RHODANESE

RASUL ABDOLRASULNIA * and JOHN L. WOOD **

Department of Biochemistry, College of Basic Medical Sciences, University of Tennessee Center for the Health Sciences, Memphis, TN 38163 (U.S.A.)

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Summary

Thiocystine (bis-[2-amino-2-carboxyethyl]trisulfide) is a natural substrate for rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1). Analogs of thiocystine were prepared by eliminating the carboxyl or amino group or by lengthening the carbon chain. Of these only homothiocystine (bis-[2-amino-2-carboxypropyl]trisulfide) had appreciable activity as a substrate. At pH 8.6, the optimum for rhodanese, transfer of sulfane sulfur to cyanide in the presence of rhodanese was nonspecific.

Only the sulfane sulfur of ^{35}S-labeled thiocystine was transferred to rhodanese. Thus, thiocystine and thiosulfate both produce a rhodanese persulfide as a stable intermediate in sulfur transfer.

Introduction

Thiocystine (bis-[2-amino-2-carboxyethyl]trisulfide) is a stable trisulfide analog of cystine which is produced in the hydrolysis of proteins [1]. It also occurs in the natural state of *Rhodopseudomonas spheroides* [2]. Szczepkowski and Wood found thiocystine to be formed by the action of cystathionase (EC 4.4.1.1) on cystine [3]. They also showed that thiocystine can function as a substrate for rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) which transfers sulfane sulfur to thiophilic acceptors. The present study demonstrates thiocystine to transfer its sulfane sulfur to rhodanese to form an intermediate enzyme-persulfide compound. A structural specificity for thiocystine as a substrate for rhodanese has been established.

* Present address: Department of Biochemistry, Medical School, Pahlavi University, Shiraz, Iran.
** To whom inquiries should be addressed.

Materials and Methods

Thiocystine, trithiodipropionic acid and homothiocystine (bis-[2-amino-2-carboxypropyl]trisulfide) were prepared as described Savige et al. [4]. Trithiodiacetic acid was prepared according to the method of Schöberl and Wagner [5]. Diacetylthiocystamine, (bis-[2-acetamidoethyl]trisulfide), was prepared by the method of Buckman and Field [6]. Ethyl trisulfide was obtained from Eastman Organic Chemicals. The trisulfides were characterized by the cold cyanolysis method of Fletcher and Robson [1]. Thiocystine was shown to be free of cystine by paper chromatography. L-[^{35}S]Cystine was obtained from Amersham and carrier-free [^{35}S]sulfate from New England Nuclear. The latter was converted to sulfide by the method of Johnson and Nishita [7]. Mercaptosuccinic acid was recrystallized from a preparation obtained from National Aniline Company. DL-isocitric acid was obtained from Sigma Chemical Company. Crystalline beef liver rhodanese was prepared essentially according to the method of Horowitz and De Toma with modifications made by Westley [8]. Other chemicals were reagent grade from commercial sources.

Enzyme assays. Rhodanese activity was measured by the rate of thiocyanate formation from thiocystine and cyanide based on a modified procedure of Szczepkowski and Wood [3]. The incubation mixture contained 20 μmol thiocystine (or substrate analog) in 0.2 N HCl, 900 μmol glycine, 350 μmol potassium phosphate buffer, 1.75 μmol EDTA and 0.2 N NaOH equivalent to the HCl used. The total volume was 4.5 ml at pH 7.4 and 25°C. Rhodanese in 1.8 M $(NH_4)_2SO_4$ was diluted with 1 M glycine buffer (pH 7.9). In some experiments, a mixture of 0.012 M sodium thiosulfate and 0.025% bovine serum albumin was used as diluent. 80 μmol cyanide was added just before the reaction was initiated by addition of a 1—10 μl quantity of enzyme solution. The reaction was stopped after 1 min at 25°C by addition of 0.5 ml 18% formaldehyde. 7 ml Golstein's ferric nitrate reagent [9] was added and the absorbance measured in a Zeiss PMQ II or a Bausch and Lomb Spectronic 20 spectrophotometer. Results were expressed in μmol thiocyanate formed per min, by comparison to standards prepared from potassium thiocyanate. The results were corrected for cyanolysis of the substrate by omitting the enzyme from an assay. When the assays were done at pH 8.6, 200 μmol bis-[1-hydroxymethyl]-aminomethane buffer were substituted for phosphate, and glycine was omitted from the medium. This procedure provided the same amount of thiocyanate as obtained in Sörbo's assay [10].

Protein determinations were done spectrophotometrically by ultraviolet absorption with the use of an extinction coefficient of $E^{1\%}_{cm} = 1.75$ at 280 nm [11]. For measurements of radioactivity of ^{35}S, a 10 μl aliquot of the sample was dissolved in 0.5 ml water and 5 ml Bray's scintillation fluid [12] and was counted in a Nuclear Chicago Mark II liquid scintillation spectrometer.

Results

Kinetic studies were limited by the insolubility and instability of thiocystine. The concentration of a saturated solution in 0.2 N HCl was approx. 60 mM. At

pH 7.4 thiocystine, in the presence of cyanide or rhodanese, proved to be unstable for incubations longer than one min. Clouding of the solution indicated decomposition of thiocystine to free sulfur and cystine. By discarding cloudy solutions it was possible to utilize thoiocystine in the assay medium up to 11.1 mM. However, the practical concentration for routine enzyme assays was 4.4 mM.

Data for K_m and V determinations were subjected to statistical analyses using a BASIC program for a PDP 11 computer, which was provided by Dr. R.J. Hill. An apparent K_m (Fig. 1) obtained under the above limitations was $8.4 \pm 1.7 \cdot 10^{-3}$ M and the apparent V was 1.9 ± 0.3 μmol/min thiocyanate formed when the cyanide concentration was 17.7 mM. At lower concentrations of cyanide the results were erratic. The apparent K_m for cyanide was $2.5 \pm 0.7 \cdot 10^{-2}$ M and the V was 1.6 ± 0.4 μmol/min thiocyanate formed when thiocystine was 4.4 mM.

A comparative study was performed under the assay conditions of Szczepkowski and Wood [3], who found thiocystine to provide approximately the same amount of thiocyanate as a 7-fold concentration of thiosulfate. Incubations were done with the substrates in the presence of 18 mm phosphate buffer, 0.8 mm EDTA and 5.5 mM cyanide at pH 7.4 and 25°C for 5 min. In agreement with the results of Szczepkowski and Wood, 16 units enzymes and 0.17 mM thiocystine produced the same amount of thiocyanate as 1.1 μM thiosulfate. With 24 or 30 units rhodanese, however, 1.1 mM thiosulfate produced twice as much thiocyanate as 0.17 mM thiocystine.

The inhibition of rhodanese by DL-isocitrate was compared with respect to

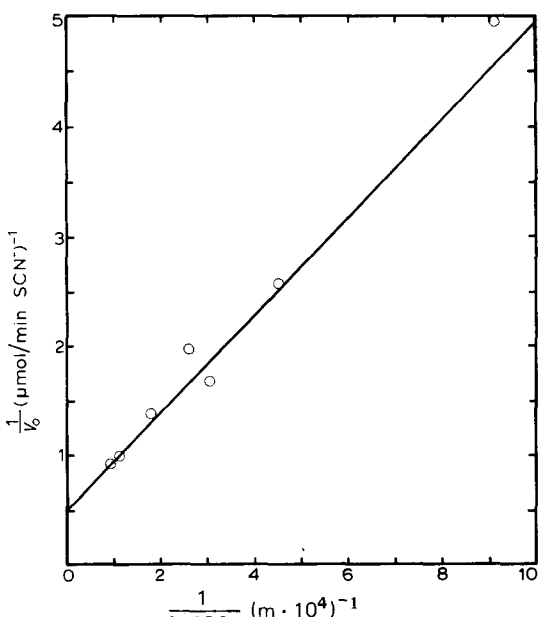

Fig. 1. Double reciprocal plot of the rhodanese-catalyzed reaction of thiocystine with cyanide. Initial velocities of thiocyanate formation were obtained at 25°C with $1.77 \cdot 10^{-2}$ M sodium cyanide in 0.2 M phosphate buffer, pH 7.4.

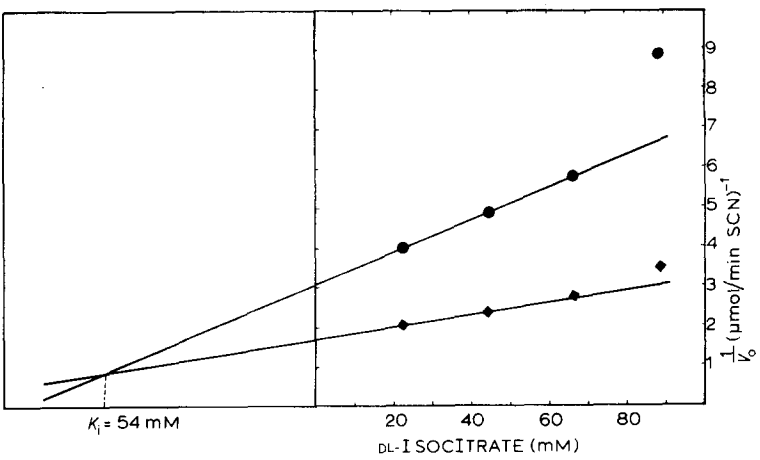

Fig. 2. Competitive inhibition of the rhodanese-thiocystine system by DL-isocitrate. Assays were done at pH 7.4 as described in Methods. Thiocystine concentrations of 2.2 mM (●) and 4.4 mM (♦) were used. The enzyme was $6.8 \cdot 10^{-8}$ M (30 units). DL-isocitrate concentration was varied as indicated.

thiocystine and thiosulfate [13]. 50% inhibition of 30 units enzymic activity on thiocystine was obtained with $7.4 \cdot 10^{-2}$ M DL-isocitrate, which is equivalent to an inhibitor-substrate molar ratio of 16. To obtain the same inhibition of enzyme action on thiosulfate required $9 \cdot 10^{-2}$ M DL-isocitrate, an inhibitor-substrate ratio of 23. Use of a Dixon plot showed DL-isocitrate inhibited the enzyme competitively with thiocystine (K_i = 0.054 M) (Fig. 2).

Table I shows the relative activity of analogs of thiocystine when employed as substrates for the rhodanese-cyanide system. Systematic alterations in the structure developed no analog with more than a fraction of the activity of thiocystine. Removal of the carboxyl group greatly diminished substrate activity and removal of the amino group was even more effective. Lengthening the carbon chain (homothiocystine) likewise reduced the rate of persulfide sulfur

TABLE I

TRISULFIDE COMPOUNDS AS SUBSTRATES FOR RHODANESE

All assays were corrected for non-enzymatic cyanolysis (a). At pH 8.6, the concentration of the substrates was 1.1 mM and the enzyme, $2.3 \cdot 10^{-8}$ M (10 units) (b). Assay conditions (c) were the same as (b) except that the reaction mixture was made 25 percent in ethanol. Non-enzymatic thiocyanate formation by cold cyanolysis (d) under the same assay conditions as (b).

Substrate	(μmol SCN^- per min)			
	pH 7.4		pH 8.6	
	(a)	(b)	(c)	(d)
Thiocystine	0.77	1.38	2.03	0.14
Homothiocystine	0.16	0.896	—	0.08
Diacetylthiocystamine	0.096	2.680	—	0.02
Trithiodiacetic acid	0.024	0.096	—	0.01
Trithiodipropionic acid	0.0	—	0.096	0.0
Ethyl trisulfide	0.0	—	0.64	0.01

transfer. Wood and Szczepkowski (unpublished data) found glutathione trisulfide also transferred its sulfane sulfur to cyanide via rhodanese. Thus insertion of thiocystine into a small peptide does not interfere with its reaction with rhodanese.

At pH 8.6, the optimum for rhodanese activity, all of the compounds transfered sulfane sulfur to cyanide. Non-enzymic cyanolysis under the conditions of the assay was small but measureable. Notably, diacetylthiocystamine produced twice the amount of thiocyanate as thiocystine.

Fluorescent studies on the reaction of thiocystine with rhodanese were conducted according to the conditions of Finazzi Agro et al. [14] which minimized the contribution of the tryptophan residues in the molecule (Fig. 3). Addition of cyanide, which removed persulfide sulfur from the enzyme molecule, resulted in an increase in fluorescence. Restoration of the enzyme-persulfide sulfur complex by addition of an excess of thiocystine quenched the fluorescence. The cycle could be repeated but the enhancement of fluorescence by cyanide decreased with each cycle. Similar observations were made by Cannella et al. [15] when rhodanese was titrated with thiosulfate and cyanide.

Since the stable form of rhodanese in the presence of thiosulfate is an enzyme-persulfide complex [16,17], labeling experiments were done to determined the nature of the product when thiocystine was the source of the sulfur. Sulfane-free rhodanese was prepared by treatment with a 10-fold molar excess of cyanide followed by precipitation with $(NH_4)_2SO_4$ according to the method of Bonomi et al. [18]. To 0.27 μmol rhodanese dissolved in 1 ml cold 1 M glycine buffer (pH 7.9) was added a 30-fold excess of isotopically-labeled thiocystine (Cy-S-^{35}S-S-Cy) with a specific activity of $4.7 \cdot 10^5$ cpm/μmol. After equilibration at 0°C for 10 min, the enzyme was precipitated by addition of 2.4

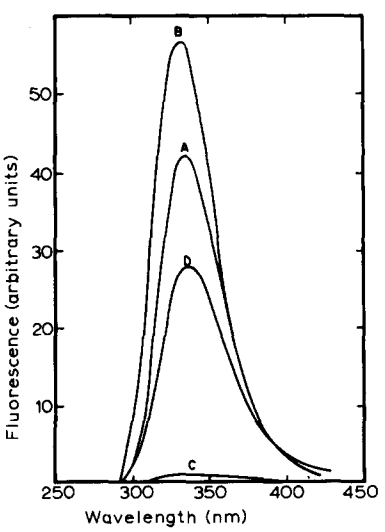

Fig. 3. Effect of cyanide and thiocystine on rhodanese fluorescence. A; 5 μM rhodanese (sulfur-containing form) in 1 M glycine/NaOH buffer, pH 7.9; B; 50 μM KCN added to enzyme; C; 3.7 μmol solid thiocystine added to cyanide-treated enzyme solution; D; 8 μmol KCN added to thiocystine-treated enzyme. Excitation wavelengths, 278 nm; the whole spectrum was obtained just after addition of reagents, at 25°C.

TABLE II

LABELING OF RHODANESE WITH SUBSTRATE ^{35}S

Rhodanese was stripped of its persulfide sulfur with cyanide and recharged with labeled thiocystine.

Substrate	Treatment	^{35}S-incorporated (μmol/μmol enzyme)
Cy-S-^{35}S-S-Cy	1st column chromatography	10
	1st recrystallization	0.88
	2nd recrystallization	0.72
	2nd column chromatogram fraction	1.4
	cyanide followed by gel filtration *	0
Cy-^{35}S-S-^{35}S-Cy	2nd column chromatography	0.074

* Thiocyanate was isolated from the column and identified by paper chromatography. It was highly radioactive. The protein fraction had no radioactivity.

ml cold 3.6 M $(NH_4)_2SO_4$ (pH 7.9). The product was recovered by centrifugation and washed with 10 ml 3.25 M $(NH_4)_2SO_4$ (pH 7.9) dissolved in 1 ml glycine buffer and placed on Sephadex G-25 column to remove excess substrate. It was eluted with 0.05 M glycine buffer (pH 7.9 at 4°C). The protein-containing fractions were detected by ultraviolet absorption. Two fraction containing most of the enzyme were concentrated and the enzyme was crystallized from 2.8 M $(NH_4)_2SO_3$. After recrystallization, the enzyme was again chromatographed on Sephadex G-25. The enzyme contained radioactive sulfur by transfer from thiocystine (Table II). The fraction containing 0.05 μmol enzyme was treated with a 20-fold molar excess of cyanide at pH 7.9. After 15 min, 0.5 mg carrier potassium thiocyanate was added and protein and thiocyanate were separated on a Sephadex G-25 column. Radioactivity measurements were made on the protein fracions at each step (Table II). The final protein fraction after cyanide treatment contained no radioactivity. The thiocyanate fraction was purified by paper chromatography and identified by the ferric nitrate reaction and by comparison with the migration of an authentic sample. It was found to be highly radioactive.

A similar experiment was performed in which the thiocystine was labeled in the non-sulfane sulfurs (Cy-^{35}S-S-^{35}S-Cy). After removal of ^{35}S-labeled thiocystine by two successive gel filtrations, the protein concentration was determined by ultraviolet absorption and the radioactivity of the enzyme was measured. As seen in Table II, the radioactivity remaining with the enzyme was at a contamination level. This value is insignificant with respect to the stoichiometric relationship of the rhodanese to the substrate.

No evidence was obtained to suggest the formation of a stable thiocystine-rhodanese complex. As with thiosulfate transfer, only the sulfane sulfur was linked to the isolated rhodanese.

Discussion

The present studies support the conclusion of Szczepkowski and Wood that thiocystine is a more efficient sulfur donor than thiosulfate. With cyanide as the acceptor, the differential was 7-fold at the lowest enzyme concentration

studied but even at higher concentrations of the enzyme, 0.17 μM thiocystine produced half as much thiocyanate as 1.1 μM thiosulfate. Wider de Xifra et al. [19] found similar relationships when rhodanese from *R. spheroides* was used with the two substrates.

The substrates for rhodanese which presently have been recognized are disulfide ion [20], thiosulfate [21], aryl and and alkyl thiosulfonates [22,23] and thiocystine [3]. Of these, only thiosulfate, thiotaurine (an alkyl thiolsulfonate) and thiocystine have been produced by enzymatically-catalyzed systems. It is apparent that, if any of the above contact rhodanese in a mitochondrion, transfer of persulfide sulfur to an available thiophilic compound will take place. It seems possible that formation of thiocystine yields an active source of persulfide sulfur in biological systems. Furthermore, it is more sensitive to DL-isocitrate inhibition than thiosulfate.

The mechanism of the decomposition of trisulfides by cyanide is discussed elsewhere (unpublished data). The cyanide-catalyzed decomposition does not constitute a cyanolysis because the yield of thiocyanate is not significant at pH 7.4 and is only a small proportion of the enzyme-catalyzed yield at pH 8.6. It was found that the most active catalysis was produced by sulfhydryl ions generated by the action of cyanide, hydroxyl or sulfite ion or by enzyme action at the higher pH.

The data in Table I suggest that the initial step in utilization of thiocystine is conversion to a persulfide, thiocysteine, $CySS^-$, by catalytic action of rhodanese. This could be nucleophilic attack on the trisulfide group by the enzyme sulfhydryl.

$$EnzS^- + CySSSCy \rightleftharpoons EnzSSCy + CySS^-$$

$$EnzSSCy + CySS^- \rightleftharpoons EnzSS^- + CySSCy$$

$$EnzSS^- + CN^- \rightarrow EnzS^- + SCN^-$$

The data on analogs indicate that thiocystine attaches to the enzyme at three pointes via the carboxyl, amino and trisulfide groups. When the distance between the trisulfide and the amino or carboxyl groups was increased (homothiocystine) the activity was greatly decreased. Loss of the carboxyl or amino group practically obliterated substrate activity and there was none with trisulfides which were structurally remote from thiocystine. Thus at pH 7.4, thiocystine was unique among the trisulfides as a substrate for rhodanese. At pH 8.6, the pH optimum of rhodanese, there was no clear relationship between substrate activity and structure. There was the complicating factor of cold cyanolysis. Under conditions of the assay, cold cyanolysis did not account for the rates of reaction seen in Table I. Other factors such as stability of the trisulfide group as affected by substituent groups on the molecule or by perturbations of the solvent predominate [24]. In alkaline solution the enzyme sulfhydryls alone were sufficient to initiate persulfide exchange. Diacetylthiocystamine proved more active than thiocystine as a substrate. It was not possible to compared thiocystamine directly since it is unstable in water.

The formation of a rhodanese-persulfide compound by transfer from thiocystine was confirmed by fluorescence measurements in which persulfide groups on the enzyme were removed by cyanide followed by restoration of the

persulfide structure by addition of thiocystine. The quenching of fluorescence by formation of persulfide groups on rhodanese has been attributed by Davidson and Westley [25] to interaction of the sulfur with an indole group of the tryptophan residue in the active site. Finazzi Agro et al. [14] have argued the quenching of intrinsic fluorescence was due to long range energy transfer. In any case, thiocystine reacted with rhodanese similarly to thiosulfate to form an enzyme persulfide. It was observed (Fig. 3) that, after charging the enzyme with thiocystine, enhancment of fluorescence by addition of an excess of cyanide did not restore the original levels. The same result was noted by Finazzi Agro et al. when thiosulfate was used as the source of persulfide sulfur. Similar results were noted in the ultraviolet absorbance of rhodanese persulfide when the enzyme was titrated with cyanide. Restoration of fluorescence [14] or ultraviolet absorption [15] was achieved by recrystallization of the enzyme. This could be due to an induced enzyme fit produced by the substrate as proposed by Jarabak and Westley [26] or by reversible denaturation of the enzyme.

Thiocysine transferred only its persulfide sulfur to rhodanese as evidenced by studies with labeled substrate. When crystalline rhodanese was incubated with CyS-^{35}S-SCy, the enzyme was labeled with radioactivity. On the other hand, when the substrate was Cy-^{35}S-S-^{35}S-Cy there was no transfer of radioactivity to the enzyme and hence no stable complex was formed between rhodanese and thiocystine. With rhodanese labeled with ^{35}S, the ratio of atoms of bound substrate sulfur to protein was about 1. Previous work has shown that the enzyme may crystallize with less than the theoretical two atoms of sulfur. Westley and Nakamoto [27] in similar loading experiments with high concentrations of thiosulfate obtained a ratio of 1.7 whereas Cannella et al. [15] obtained 1.35 and Sörbo [17] 1. Volini and Wang [28] have noted that rhodanese persulfide slowly loses it sulfur when stored in solution. This is a property characteristic of persulfides. In purification of labeled rhodanese persulfide on a Sephadex column there was some fractionation of the persulfide form from the persulfide-free enzyme. Thus the ratio of sulfur to protein in the several fractions ranged from 0.35 to 1.4. All the radioactivity was removed by treatment with cyanide and the thiocyanate resulting was radioactive. The residual enzyme was not radioactive.

The results firmly establish the reaction product of rhodanese with thiocystine to be a persulfide of similar characteristics to the enzyme persulfide compound formed with thiosulfate as the substrate. Thus rhodanese should be capable of transferring the sulfane sulfur of thiocystine in any reaction in which thiosulfate can be utilized.

Sandy et al. [2] have shown thiocystine as well as the corresponding glutathione derivatives are activators of aminolevulinate synthetase from *Rhodopseudomonas spheroides*. Other systems in which sulfane sulfur (from thiosulfate) has been demonstrated to serve as an activator are ferridoxin [29,30], aldehyde oxidase [31], xanthine oxidase [32], malate dehydrogenase [33], succinic dehydrogenase [34] and nitrate reductase [35].

Thiocystine and the glutathione-derived trisulfide have been detected as constituents of *R. spheroides* [2]. Massey et al. [38] have detected small amounts of trisulfide in isolated samples of glutathione. Although the amount in tissues

must be rather small, it is possible that the thiocystine provides an intermediate storage compound for sulfane sulfur.

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