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GLUTATHIONE REDUCTASE-DEPENDENT METABOLISM OF CYSTEINE-*S*-SULFATE BY *PENICILLIUM CHRYSOGENUM**

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SUMMARY

A study of the metabolism of cysteine *S*-sulfate by *Penicillium chrysogenum* revealed that (a) cysteine-*S*-sulfate can serve as the sole sulfur source for the organism, (b) a sulfur-regulated permease mediates the transport of cysteine-*S*-sulfate into the cell and (c) a series of chemical exchange reactions coupled to glutathione reductase (NADPH: glutathione oxidoreductase, EC 1.6.4.2) (to regenerate reduced glutathione) could account for any net cysteine-*S*-sulfate utilization observable *in vivo* and in crude extracts. The proposed reactions are:

$CySSO_3^- + GSH \rightleftharpoons CySSG + HSO_3^-$ (chemical reaction) (1)

$CySSG + GSH \rightleftharpoons GSSG + CySH$ (chemical reaction) (2)

$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+$ (glutathione reductase) (3)

$CySSO_3^- + NADPH + H^+ \rightleftharpoons CySH + NADP^+ + HSO_3^-$ (Sum)

and/or:

$CySSO_3^- + GSH \rightleftharpoons CySSG + HSO_3^-$ (chemical reaction) (1)

$CySSG + HSO_3^- \rightleftharpoons GSSO_3^- + CySH$ (chemical reaction) (2)

$GSSO_3^- + GSH \rightleftharpoons GSSG + HSO_3^-$ (chemical reaction) (3)

$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+$ (glutathione reductase) (4)

$CySSO_3^- + NADPH + H^+ \rightleftharpoons CySH + NADP^+ + HSO_3^-$ (Sum)

INTRODUCTION

The early work of SHEPARD[1] and HOCKENHULL[2] demonstrated that certain mutant strains of *Aspergillus nidulans* unable to utilize sulfate or sulfite could use

Abbreviations: $CySSO_3^-$, cysteine-*S*-sulfate; CySSG, the mixed disulfide of cysteine and glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; $GSSO_3^-$, glutathione-*S*-sulfate; CySH, cysteine; CySSCy, cystine.

* Some of the work described in this paper was taken from a thesis submitted to the Graduate School of the University of California by T.S.W. in partial fulfillment of the requirements for the Ph. D. degree in Comparative Biochemistry.

thiosulfate or cysteine-*S*-sulfate (cysteine-*S*-sulfonate, *S*-sulfocysteine) as their sole sulfur source. Both workers proposed cysteine *S*-sulfate as an obligate intermediate in the conversion of inorganic sulfate or thiosulfate to cysteine or methionine. More recently NAKAMURA AND SATO[3] described the properties of a new series of *A. nidulans* mutants which again suggested an important role for cysteine-*S*-sulfate ($CySSO_3^-$) in sulfate assimilation. These mutants can not grow on sulfate or sulfite but can grow on cysteine or methionine. One of these mutants, 793, is also unable to grow on $CySSO_3^-$. Strain 793 accumulates $CySSO_3^-$ intracellularly when incubated with sulfate[4]. NAKAMURA further reported that extracts of *A. nidulans* can catalyze the production of: (a) $CySSO_3^-$ when incubated with ATP, Mg^{2+}, pyridoxal phosphate, thiosulfate and serine[5], and (b) cysteine and sulfite when incubated with NADPH and $CySSO_3^-$ (ref. 6).

The metabolism of $CySSO_3^-$ by animal tissue extracts has also been studied. SÖRBO[7] and COLETTA[8] reported the *in vitro* cleavage of $CySSO_3^-$ to pyruvate and thiosulfate by a transaminase present in rat liver extracts. The reaction required a keto acid and pyridoxal phosphate. The specificity of the transaminase was not investigated. The non-enzymic reaction between alkyl thiosulfates and thiols has been investigated by CLARKE[9], KOLTHOFF AND STRICKS[10,11], SWAN[12] and SCHÖBERL AND BAUER[13]. WALEY[14] points out that the analogous decomposition of glutathione *S*-sulfate by calf lens extract may be attributed to a coupled chemical and enzymatic reaction:

$$GSSO_3^- + GSH \rightleftharpoons GSSG + HSO_3^- \qquad \text{(chemical)}$$

$$GSSG + NADPH + H^+ \rightleftharpoons NADP^+ + 2\,GSH \qquad \text{(enzymatic)}$$

The experiments described in this paper were designed to determine the pathway by which filamentous fungi converts $CySSO_3^-$ to cysteine.

MATERIALS AND METHODS

Growth of the mold and preparation of the extract

The growth and culture storage conditions have been described earlier[15].

Cell-free extracts were prepared from mycelium grown for 2 days on synthetic citrate No. 3 medium[16] containing the indicated sulfur sources. The mycelium was removed from the growth medium by suction filtration on a coarse sintered glass funnel and washed several times with deionized water by resuspension and filtration. This pad was put in an "X-Press" and frozen overnight at −20°. The frozen mycelium was then broken under pressure in a Carver press.

Purified glutathione reductase was prepared as described by WOODIN AND SEGEL[17].

Assay procedure

Protein was determined by the biuret method[18].

For routine assays, the rate of NADPH utilization was determined by observing the decrease in absorbance at 340 mμ ($\Delta A_{340\ m\mu}$) on a Cary recording spectrophotometer.

Paper electrophoresis was performed on an E-C Electrophoresis Apparatus with

dialysis tubing-filter paper contacts between the paper strips and the buffer compartment[19]. Most electrophoretic separations were run on Whatman 3 MM paper strips (18 inch × 0.5 inch) at a voltage gradient of 20 V/cm and 10–30 mA. Unless otherwise indicated all electrophoresis runs used 0.2 M Tris–chloride buffer (pH 8.2). Chromate was used as a marker. The distribution of radioactivity along the strips was determined with a Vanguard Autoscanner Model 880.

Chemicals

All chemicals unless otherwise indicated were obtained from commercial sources and used without further purification. The sodium salt of $CySSO_3^-$ was prepared by

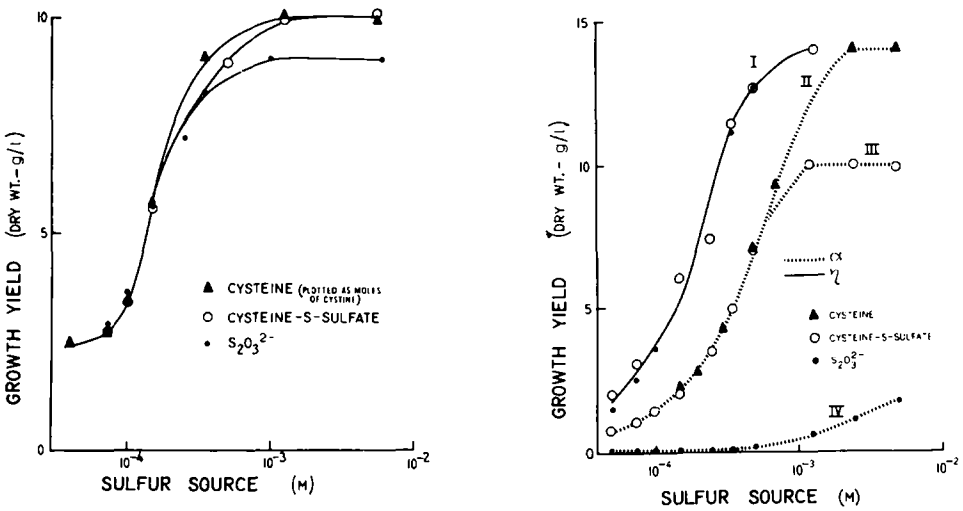

Fig. 1. Growth of *P. chrysogenum* on various sulfur sources. *P. chrysogenum* was grown for 2 days in 100 ml of citrate No. 3 containing 10 mg L-Djenkolic acid as the sole sulfur source. 3-ml aliquots of this culture were then aseptically transferred to 500 ml flasks containing 100 ml of citrate No. 3 medium and the indicated sulfur source. These flasks were incubated at 25° on a rotary shaker operating at a speed of approx. 250 rev./min and describing a 2 inch circle. After 50 h the mycelia were collected by filtration, washed with deionized water, dried for 24 h in a 100° oven and weighed. For ease of comparison the concentration of cysteine is plotted as moles of cystine (*i.e.* a solution 2 mM in cysteine is plotted as 1 mM in molarity of sulfur source).

Fig. 2. Growth of *A. nidulans* mutants on various sulfur sources. *A. nidulans* was grown for 2 days in 100 ml of citrate No. 3 containing 10 mg L-Djenkolic acid as the sole sulfur source. 3-ml aliquots of this culture were then aseptically transferred to 500 ml flasks containing 100 ml of citrate No. 3 and the indicated sulfur source. These flasks were then incubated for 50 h at 34° on a rotary shaker operating as described in Fig. 1. The mycelia were collected, dried and weighed as described in Fig. 1. The concentration of cysteine is plotted as moles of cysteine (*i.e.* a solution 1 mM in cysteine is plotted as 1 mM in molarity of sulfur source).

the method of SEGEL AND JOHNSON[20]. Radioactive $CyS^{35}SO_3^-$ was prepared in the same manner using 1 g quantities of starting materials and high specific activity sulfite-^{35}S (Nuclear Chicago). The preparation of the mixed disulfide of cysteine and glutathione is described in detail in the text.

RESULTS AND DISCUSSION

Use of $CySSO_3^-$ as the sole sulfur source of P. chrysogenum and A. nidulans

Fig. 1 shows that *P. chrysogenum* can use $CySSO_3^-$, cysteine or thiosulfate with essentially equal efficiency as sole sulfur sources. As shown in Fig. 2, Curve I, strain η, an *A. nidulans* mutant lacking PAPS reductase (and thus unable to grow on sulfate), grew equally well on either $CySSO_3^-$ or thiosulfate. (The growth curve for a wild type of *A. nidulans* on either $CySSO_3^-$ or thiosulfate is similar to that of η.) It is thus evident that neither sulfur of $CySSO_3^-$ is metabolized to sulfate.

α, a strain unable to grow well on sulfite or sulfide as indicated by its inability to utilize cysteic or cysteine sulfinic acids or either sulfur of thiosulfate (Curve IV, Fig. 2), can utilize $CySSO_3^-$ as its sole sulfur source. The growth yield of α per mole of cysteine or $CySSO_3^-$ is the same at low concentrations of sulfur source; however, a mole of $CySSO_3^-$ contains twice as much sulfur as a mole of cysteine. Therefore, the results in Fig. 2 suggest that only half of the sulfur in $CySSO_3^-$ can be used by a mutant blocked between sulfide and cysteine. The fact that increasing the concentrations of $CySSO_3^-$ in the growth medium above 10^{-3} M does not increase the total growth of α suggests that the mutant may accumulate a nonmetabolizable product of $CySSO_3^-$ metabolism that is toxic (*e.g.* sulfite).

Sulfur-regulated transport of $CySSO_3^-$

The presence of a sulfur-regulated permease able to transport $CySSO_3^-$ into *P. chrysogenum* is suggested by the data in Table I. The specificity of the $CySSO_3^-$

TABLE I

TRANSPORT RATES OF $CySSO_3^-$ IN *P. chrysogenum* GROWN ON VARIOUS SULFUR SOURCES

Transport rates were determined according to Method II of BENKO, WOOD AND SEGEL[16] at an external substrate concentration of 10^{-4} M. The sulfur-starved mycelium was grown for 2 days on citrate No. 3 medium containing 1 g/l Na_2SO_4 as sole sulfur-source and then sulfur-starved for 12 h in the same medium *minus* a sulfur source. The rate of [^{35}S]methionine uptake was also determined as a control to establish that the cells were truly sulfur starved.

Mycelium	*Rate of $CyS^{35}SO_3^-$ transport (μmoles/g · min)*	*Rate of [^{35}S]methionine transport (control) (μmoles/g · min)*
Sulfur starved	0.76	1.3
$CySSO_3^-$-grown (1 g/l)	<0.1	0.1
SO_4^{2-}-grown (1 g/l)	<0.1	0.1

permease was not investigated. However, it is probably not identical to the sulfate[15] or methionine[16] permeases as $CySSO_3^-$ does not inhibit uptake of either sulfate or methionine.

The $CySSO_3^-$ permease appears to saturate at 10^{-4} M as the transport rate does not increase when the concentration of $CySSO_3^-$ is increased to $5 \cdot 10^{-4}$ M. This lends further support to the assumption that the uptake of ^{35}S results from $CyS^{35}SO_3^-$

transport rather than possible trace impurities of $^{35}SO_3^{2-}$ or $^{35}SO_4^{2-}$ present in our preparation and undetectable by chromatography or electrophoresis.

Degradation of $CySSO_3^-$ by crude extracts of P. chrysogenum

In an effort to detect a $CySSO_3^-$ reductase in *P. chrysogenum* similar to that reported by NAKAMURA for *A. nidulans*[6] we incubated $CySSO_3^-$, NADPH, and crude extracts of *P. chrysogenum* and observed the decrease in $A_{340\ m\mu}$ over relatively long periods of time, 15-30 min. No $CySSO_3^-$-stimulated oxidation of NADPH was observed. Identical experiments without EDTA or with extracts from cells grown on 1 g/l SO_4^{2-} or $CySSO_3^-$ or from cells which had been "sulfur starved"[16] gave essentially the same results, *i.e.* no evidence for a NADPH-dependent $CySSO_3^-$ reductase. Identical results were also obtained at pH 8.0 in 0.1 M Tris–chloride buffer.

In another effort to determine the metabolic fate of $CySSO_3^-$, crude extracts of *P. chrysogenum* (grown on citrate No. 3 medium, containing 1 g/l SO_4^{2-} or $CySSO_3^-$) were prepared as described above. The pellet obtained from the "X-Press" was diluted 1:1 with 0.1 M phosphate buffer (pH 7.2), centrifuged for 30 min at 10 000 × *g* in a Sorvall refrigerated centrifuge (4°) and the supernatant solution collected. The supernatants contained about 20 mg/ml protein. Reaction mixtures containing $CyS^{35}SO_3^-$ (0.5 μmoles), extract (1 mg protein), GSH (0.5 μmoles) and an NADPH generating system (glucose-6-phosphate dehydrogenase, $NADP^+$ and glucose 6-phosphate) in a total of 0.165 ml of 0.1 M phosphate buffer (pH 7.2), were incubated at 25° for 30 min. The reaction was stopped by placing the tubes in a boiling-water bath for 3 min. The reaction mixtures were centrifuged and 0.02 ml of the supernatant solution spotted on Whatman 3 MM paper strips (1 cm wide) and electrophoresed for 2 h. Under the same conditions a chromate standard moved 9 cm from the origin. Two radioactive spots were detected: one corresponding to $CyS^{35}SO_3^-$ and one having a mobility identical to that of $^{35}SO_3^{2-}$ (0.7 with respect to $^{35}SO_4^{2-}$). In similar experiments with reaction mixtures lacking either an NADPH generating system or GSH, or with these cofactors replaced by pyridoxal phosphate and α-ketoglutarate, no radioactive or ninhydrin positive degradation products were observable. This was also true of a boiled control. These results confirm those of the growth studies which indicate that cysteine-*S*-sulfate is metabolized to cysteine and sulfite. However, they give no indication of the nature or specificity of the enzyme system involved. The results could be explained equally well by (a) GSH-stimulated, NADPH-dependent $CySSO_3^-$ reductase, or (b) a chemical reaction between GSH and $CySSO_3^-$ followed by further exchange reactions (as shown below) and then an NADPH-dependent enzymatic reaction with any one or more of the reaction products:

$$CySSO_3^- + GSH \rightleftharpoons CySSG + HSO_3^- \quad (1)$$

$$CySSG + HSO_3^- \rightleftharpoons CySH + GSSO_3^- \quad (2)$$

$$CySSG + GSH \rightleftharpoons GSSG + CySH \quad (3)$$

$$CySSCy + GSH \rightleftharpoons GSSCy + CySH \quad (4)$$

$$GSSO_3^- + GSH \rightleftharpoons GSSG + HSO_3^- \quad (5)$$

(Because the K_{eq} of $RSSO_3^- + R'SH \rightleftharpoons RSSR' + HSO_3^-$ is $\sim 10^{-3}$ at pH 6.7 (ref. 20), the NADPH-generating system is necessary in order to observe a net production of SO_3^{2-}.)

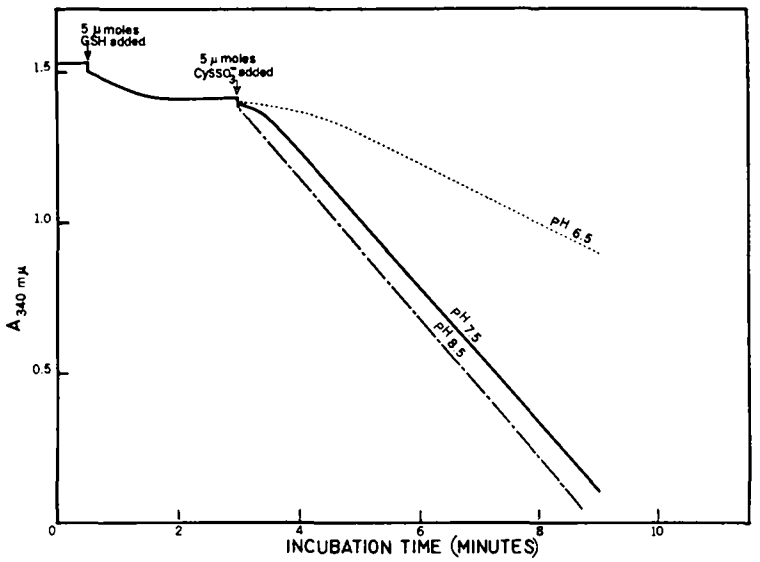

Fig. 3. The rate of oxidation of NADPH by $CySSO_3^-$ *plus* GSH. Absorption at 340 mμ ($A_{340\ m\mu}$) was plotted by the Cary recording spectrophotometer. Tests were carried out in 1 ml of the indicated buffers. At pH 6.5 and 7.5, 0.1 M phosphate was used. At pH 8.5, 0.1 M Tris–chloride was used. The cuvettes also contained 5 μmoles GSH, 5 μmoles $CySSO_3^-$ and 0.24 μmoles NADPH.

TABLE II

RATE OF NADPH OXIDATION BY $CySSO_3^-$ *plus* GSH AS A FUNCTION OF ENZYME CONCENTRATION

The rate of oxidation of NADPH at 25° was followed by noting the $\Delta A_{340\ m\mu}$ in a Cary recording spectrophotometer, Model 14. Reaction mixtures contained 5 μmoles $CySSO_3^-$, 5 μmoles GSH, 0.25 μmole NADPH and 0.5 μmole EDTA in a volume of 1 ml buffer. The buffer used at pH 6.5 and 7.5 was 0.1 M phosphate; at pH 8.5, 0.1 M Tris–chloride was employed. Crude extracts of *P. chrysogenum* grown on 1 g $S_2O_3^{2-}$/l were centrifuged at 1000 000 × *g* at 4° in the Beckman Model L Ultracentrifuge. The supernatant solution was diluted 1:1 with the appropriate buffer to give a protein concentration of 10 mg/ml. Purified glutathione reductase was diluted with the appropriate buffer so that its activity with GSSG was the same as the glutathione reductase activity of an equal volume of crude extract (50 mμmoles NADPH oxidized/min · 50 μl at pH 7.5, 37 mμmoles/min · 50 μl at pH 6.5, and 37 mμmoles/min · 50 μl at pH 8.5). In all cases the pH of the reaction mixture was measured before and after the reaction and was within ± 0.05 units of the original value.

Enzyme used (μl)	*NADPH oxidized (mμmoles/min)*					
	pH 6.5		*pH 7.5*		*pH 8.5*	
	Purified enzyme	*Crude extract*	*Purified enzyme*	*Crude extract*	*Purified enzyme*	*Crude extract*
10	2.6	2.6	5.0	4.0	5.9	5.9
20	—	—	10.0	11.5	—	—
25	6.3	6.3	12.5	12.5	15.0	18.5
50	13.0	13.0	25.0	25.0	30.0	30.0
70	—	—	—	30.0	—	—
80	—	—	35.0	35.0	—	47.5
100	26.0	26.0	—	—	50.5	53.7

In an effort to detect the substrate or substrates reduced by NADPH in the crude extract, extracts from mycelium grown on a variety of sulfur sources were assayed for their ability to use NADPH to reduce any of the products listed above. *P. chrysogenum* was grown on a variety of sulfur sources ($S_2O_3^{2-}$, $CySSO_3^-$, SO_4^{2-} and L-Djenkolic acid) at 10 mg/l, 100 mg/l, and 1 g/l. Extracts were also prepared from "sulfur-starved" mycelium.

Crude extracts were prepared as described earlier, but to minimize possible buffer and dilution effects, the pellet from the "X-press was" centrifuged for 1 h at 100 000 × *g* at 4° in the Beckman Model L Ultracentrifuge. The clear supernatant solution (virtually pure cell sap) which contained about 20 mg/ml of protein was used for all subsequent assays. The pH of this cell sap extract varied from 6.2 6.7.

Since it is known the reactions of alkyl thiosulfates with thiols are highly pH-dependent[9–13], we tested all extracts for the ability to oxidize NADPH or NADH at 3 different pH's: pH 6.5, 0.1 M phosphate; pH 7.5, 0.1 M phosphate and pH 8.5, 0.1 M Tris–chloride. The following substrates were employed: $CySSO_3^-$, $CySSO_3^-$ *plus* GSH, CySSG, CySSG *plus* CySSCy, CySSCy *plus* GSH, $S_2O_3^{2-}$, $S_2O_3^{2-}$ *plus* GSH, $S_2O_3^{2-}$ *plus* FAD, SO_3^{2-}, SO_3^{2-} *plus* GSH. All tests were performed in a total of 1 ml buffer containing 0.24 μmoles of pyridine nucleotide, 5 μmoles substrat e and 0.1–1.0 mg of protein.

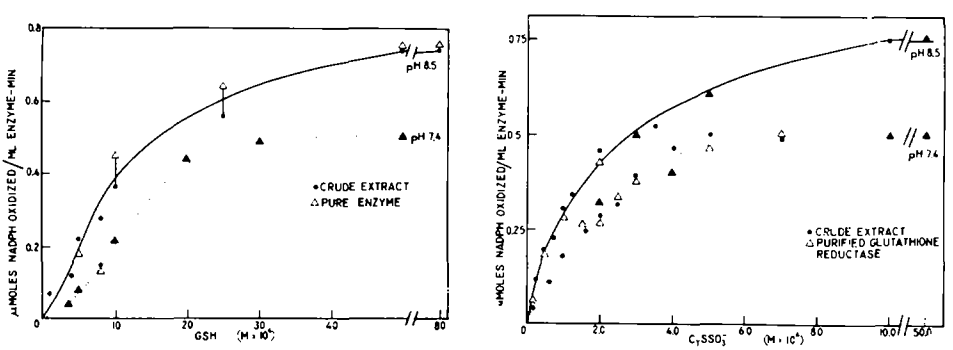

Fig. 4. The rate of NADPH oxidation by $CySSO_3^-$ *plus* GSH as a function of GSH concentration with crude extracts or purified glutathione reductase. Reaction mixtures contained 5 μmoles $CySSO_3^-$, 0.15 μmoles NADPH, and 0.5 μmoles EDTA in a volume of 1 ml 0.1 M phosphate (pH 7.4) or 0.1 M Tris-chloride (pH 8.5). Crude extracts of *P. chrysogenum* were grown on 1 g sulfate/l and were prepared in the "X-Press" as described; 0.5 mg (50 μl) of protein was used for each assay. Pure glutathione reductase was diluted so that 50 μl of it gave the same rate of NADPH oxidation (with GSSG as substrate) as 50 μl of crude extract (0.03 μmoles NADPH oxidized/min at pH 7.4). All rates are normalized so that the saturation rate represents the rate which would be observed if 1 ml of the enzyme (pure or crude) had a v_{max} (with GSSG as substrate) of 1 mmole NADPH oxidized/min.

Fig. 5. The rate of NADPH oxidation by $CySSO_3^-$ *plus* GSH as a function of $CySSO_3^-$ concentration with purified glutathione reductase or crude extracts. Reaction mixtures contained 5 μmoles GSH, 0.15 μmoles NADPH and 0.5 μmoles EDTA in a volume of 1 ml 0.1 M phosphate (pH 7.4) or 0.1 M Tris–chloride (pH 8.5). Crude extracts of *P. chrysogenum* grown on 1 g sulfate/l were prepared in the "X-Press" as described and 0.5 mg (50 μl) of extract used for each assay. Purified glutathione reductase was diluted so that 50 μl of solution gave the same rate of NADPH oxidation (with GSSG as substrate) as 50 μl of the crude extract (0.03 μmoles NADPH oxidized/min at pH 7.4). All rates are normalized so that the saturation rate represents the rate which would be observed if 1 ml of the enzyme (pure or crude) had a v_{max} (with GSSG as substrate) of 1 mmole NADPH oxidized/min.

In all tests containing GSH and NADPH, reactions were started by adding the NADPH and GSH to the enzyme and buffer and observing the $A_{340\ m\mu}$. Because all solutions of GSH contained small amounts of GSSG, the reaction mixture was incubated until the $A_{340\ m\mu}$ was stable and then more NADPH was added to make the final solution 0.24 mM in NADPH. Only incubation mixtures containing GSH *plus* CySSG, GSH *plus* cystine, GSH *plus* $CySSO_3^-$ or GSSG alone showed any NADPH oxidation. In no case was NADH oxidation observed. Since Cleland's reagent, cysteine or reduced lipoate could not substitute for GSH it was apparent that the system we were observing was either a GSH-dependent reductase or involved a non-enzymatic reaction followed by enzymatic reduction of GSSG.

When $CySSO_3^-$ was added to the test mixture under the above conditions a lag was observed before a steady state rate of NADPH oxidation was observed. As can be seen in Fig. 3 this lag was longest at pH 6.5. The steady-state rate was taken as the rate of oxidation of NADPH.

The lag in rate points to both a non-enzymatic and an enzymatic reaction with GSSG as the substrate of the enzyme, although it does not exclude other possibilities. At lower pH values the chemical reaction builds up GSSG more slowly. At any pH the chemical reaction does not proceed fast enough to saturate the enzyme system with substrate but the whole system eventually reaches a point where GSSG can be supplied at a steady rate. When the $CySSO_3^-$ is used up to an appreciable degree the rate of NADPH oxidation again decreases.

Since the lag in NADPH oxidation was approximately the same whether crude extracts or purified glutathione reductase was used it appears that no transhydrogenase is active in our systems.

The rate at which NADPH was oxidized by GSH *plus* $CySSO_3^-$ in the presence of glutathione reductase was compared to the rates obtained with crude extract (Table II). All rates were essentially linear with respect to extract concentration or purified glutathione reductase concentration at the three pH values tested. The results were similar for extracts from *P. chrysogenum* grown on several different sulfur sources. The ratio of the rate of NADPH oxidation in the presence of GSH *plus* $CySSO_3^-$ to the rate of oxidation by GSSG was 0.30–0.35 at pH 6.5, 0.5 at pH 7.5 and 0.75–0.85 at pH 8.5. All the results indicate that glutathione reductase could account for any observed enzymatic activity with $CySSO_3^-$ *plus* GSH as substrates.

We then determined the effect of varying substrate concentration (Figs. 4 and 5) on the rate of NADPH oxidation in the presence of $CySSO_3^-$ *plus* GSH with either crude extract or purified glutathione reductase. The kinetics of the reaction are similar for the pure and crude systems, again indicating that no other enzyme but glutathione reductase is responsible for the observed reactions.

The mixed disulfide of cysteine and glutathione

As part of our attempts to find enzymatic reactions with any of the products of the reaction between GSH and $CySSO_3^-$, we prepared the mixed disulfide of cysteine and glutathione by a method similar to one subsequently reported by CHANG AND WILKEN[21]*. Cysteine, 1.2 g (10 mmoles) and GSH, 1.0 g (3.3 mmoles) were dissolved in

* A different method for the preparation of CySSG was subsequently reported by ERIKSSON AND ERIKSSON[22].

10 ml of water and 4 ml of H_2O_2 (30%) were added. The solution was allowed to sit at 25° for 3 h. A heavy precipitate was produced. Tests with dithionitrobenzoate showed no thiol present after 3 h. The solution was centrifuged and the precipitate (probably cystine) discarded. The supernatant solution (pH 2.9) was applied to a 21 cm × 2.5 cm Dowex-1 acetate column equilibrated with water. The column was developed with 150 ml 3.0 M acetic acid followed by 200 ml 1.0 M acetic acid, and then 100 ml 5.0 M acetic acid. 5-ml fractions were collected. Each fraction was tested qualitatively for amino acid content by spotting an aliquot on filter paper and spraying with a solution of 0.3% ninhydrin in acetone. The ninhydrin positive tubes from each eluate were pooled and an aliquot from each spotted on $^1/_2$ inch Whatman 3 MM paper strips and electrophoresed in 0.2 M acetate buffer (pH 4.5), for 4 h. The strips were sprayed with ninhydrin and all amino acid spots noted. Material eluted by 0.3 M acetic acid moved identically to a cystine standard (2 cm from the origin). Material eluted by 5.0 M acetic acid moved identically to a GSSG standard (14.5 cm from the origin). The material eluted by 1.0 M acetic acid moved intermediate to the other two (9.5 cm from the origin).

The 1.0 M fraction was lyophilized and tested for purity by paper chromatography in pyridine–butanol–acetic acid–water (10:15:3:12, by vol.), by electrophoresis at pH 4.5 in 0.2 M acetate and at pH 8.2 in 0.2 M Tris–chloride, and by chromatography on a Phoenix amino acid analyzer. Amino acid analysis of the unhydrolyzed sample revealed only one peptide present. The sample of CySSG was also hydrolyzed by incubation with 6 M HCl for 24 h at 100° (ref. 23) and then analyzed on the amino acid analyzer. Cysteine, glutamic acid and glycine in the ratio 2:1:1 were observed. These were the only amino acids present. A similar hydrolysate of a sample oxidized by performic acid[24] and analyzed on the amino acid analyzer contained only cysteic acid, glutamic acid and glycine in the ratio 2:1:1. CHANG AND WILKEN[21] tested for the purity of their samples by subjecting them to performate oxidation and paper electrophoresis. They stated that only the expected sulfonic acids were present in the ratio 1:1. If a sample of CySSCy were contaminated with equal amounts of the component symmetrical disulfides, CySSCy or GSSG, or if the asymmetric disulfide rearranges readily in solution, this method will not detect such impurities.

When a fresh sample of CySSG was prepared and 1 μmole of this was electrophoresed at pH 4.5 as soon after preparation as possible (12 h from the start of the preparation to electrophoresis of the sample), 3 ninhydrin spots appeared. One very large dark spot corresponded to the CySSG and two barely visible spots corresponded to GSSG and cystine. The intensity of the light spots were visually estimated (by comparison with standards) to be equal to 0.01–0.05 μmoles. This slight contamination was undetectable when the usual sample size (0.01–0.1 μmole) was analyzed. The possibility, as suggested by CHANG AND WILKIN[25], ONDARZA AND MARTINEZ[26] and PIHL, ELDJARN AND BREMER[27] that glutathione reductase has slight activity with CySSG can not be discounted. However, the ease with which GSSG can be formed by rearrangement of the CySSG in solution makes it almost impossible to determine the activity of glutathione reductase with CySSG.

We tested the ability of purified glutathione reductase or crude extracts to catalyze the reduction of the mixed disulfide by NADPH. The rate of NADPH oxidation observed was only 5–10% of the rate observed with GSSG as the oxidant. The system was not saturated even when the concentration of CySSG in the assay was

increased to 1 M. A Lineweaver–Burk plot of the data indicated a K_m for CySSG of about 1.6 M. The (apparent) low affinity of glutathione reductase for the mixed disulfide suggested that the true substrate was probably contaminating GSSG. Furthermore, because the rate of NADPH oxidation by CySSG was the same in both the crude extracts and with purified glutathione reductase it seemed likely that there was no mixed disulfide reductase present in our extracts.

Although CySSG is a poor substrate for glutathione reductase, we had not discounted the possibility that CySSG could inhibit the rate of oxidation of NADPH by non-saturating levels of GSSG. Accordingly we incubated glutathione reductase, NADPH, CySSG and nonsaturating levels of GSSG. If glutathione reductase has any affinity for CySSG under these conditions the observed rate of oxidation of NADPH would be less than when no CySSG was present.

TABLE III

THE INFLUENCE OF CySSG ON THE RATE OF OXIDATION OF NADPH BY GSSG

Reaction mixtures contained 0.15 μmoles NADPH, 0.5 μmoles EDTA, 4 mμg glutathione reductase and the indicated amounts of CySSG and GSSG in 1 ml of 0.1 M phosphate (pH 7.4). The figures in parentheses are the rates to be expected if the CySSG sample was contaminated with 3% GSSG.

CySSG added	*Rate of NADPH oxidation ($\Delta A_{340\ m\mu}$/30 sec)*		
	10^{-4} *M GSSG*	$5 \cdot 10^{-5}$ *M GSSG*	10^{-5} *M GSSG*
10^{-3} M	0.07 (0.075)	0.055 (0.06)	0.0325 (0.04)
$2 \cdot 10^{-3}$ M	0.0875 (0.087)	0.07 (0.07)	0.06 (0.06)
$4 \cdot 10^{-3}$ M	0.10 (0.10)	0.092 (0.085)	0.085 (0.08)
10^{-2} M	0.105 (0.10)	0.108 (0.11)	0.10 (0.10)

Our results (Table III) indicate that CySSG does not inhibit glutathione reductase but actually appears to stimulate the oxidation of NADPH by GSSG. Results of electrophoresis indicating that samples of the mixed disulfide were contaminated with 1–5% GSSG have already been cited. The data in Table III further suggests that our samples were contaminated with 3% GSSG.

CONCLUSIONS

Our results indicate that $CySSO_3^-$, in the presence of GSH, is converted *in vivo* and *in vitro* to cysteine and sulfite. However, no $CySSO_3^-$-specific enzyme could be detected. The conversion can be accounted for by a series of chemical exchange reactions to yield CySH, HSO_3^- and GSSG. The driving force of the net reaction is the NADPH-dependent reduction of GSSG by glutathione reductase.

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