Stimulation of the Rhodanese Formation in *Trametes sanguinea* by Glycylglycine

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The formation of rhodanese (EC 2.8.1.1) by mycelia of *Trametes sanguinea* was stimulated by glycylglycine (Gly·Gly). The Gly·Gly added to the medium containing sucrose was slowly metabolized, whereas the glycine added instead of Gly·Gly was metabolized more rapidly with less formation of rhodanese. The rhodanese formation was inhibited by 2,4-dinitrophenol, antimycin A, potassium cyanide, and cycloheximide, and was significantly depressed by several surface active agents. The fungal mycelia incubated with sucrose and Gly·Gly were less active in isocitrate lyase and more active in NAD- and NADP-linked isocitrate dehydrogenase activities than those incubated with glucose and Gly·Gly. The results are discussed in regard to the mechanism of the rhodanese formation by the fungal mycelia.

Microorganisms belonging to *Thiobacillus*,¹⁾ *Streptomyces*,²⁾ and *Bacillus*³⁾ have been known to form rhodanese (EC 2.8.1.1) adaptively. *Trametes sanguinea* was found to secrete rhodanese after the mycelial growth had reached the maximum.^{4,5)} The enzyme was produced even by washed mycelia of the fungus, and the enzyme formation was greatly stimulated by Gly·Gly in the presence of sucrose added as the main carbon source.

The present study deals with the rhodanese formation by washed mycelia of *Trametes sanguinea* under various conditions, especially in regards to the effect of Gly·Gly. Gly·Gly also increased NAD-NADP-linked isocitrate dehydrogenase activities while it decreased isocitrate lyase activity. The results are discussed in connection with the stimulating effect of the rhodanese formation by Gly·Gly.

MATERIALS AND METHODS

Culture and preparation of washed mycelia of *Trametes sanguinea*. *Trametes sanguinea* IFO 6489 was cultured in the medium consisting of 1% sucrose, 0.5% yeast extract, 0.05% each of K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ (100 ml each in 500-ml flasks) with shaking (150 rpm) at 25°C for several days. The growth arrived at the maximum in 4 days, and mycelia were collected. They were washed several times with several volume of 0.05 M phosphate buffer, pH 6.0, and sus-

pended in the same buffer for examination of the rhodanese formation.

Assay of enzyme. Rhodanese activity was assayed by the method reported previously.⁴⁾ The reaction mixture contained 5 mM thiosulfate, 20 mM potassium cyanide, 45 mM potassium phosphate buffer, pH 8.0, and enzyme or the mycelial suspension (0.5 ml) in a total volume of 1.0 ml. The incubation was carried out at 37°C for 10 min. One unit of rhodanese activity was defined as the enzyme amount which produced 1 nmole of thiocyanate per min under the above conditions.

Isocitrate lyase activity was assayed according to the method of Dixon and Kornberg,⁶⁾ with slight modifications. The assay mixture consisted of 66.6 mM potassium phosphate buffer, pH 7.0, 50 mM $MgCl_2$, 3.3 mM phenylhydrazine-HCl, 2 mM cysteine-HCl, 8.3 mM sodium D,L-isocitrate and enzyme in a total volume of 3.0 ml. The glyoxylate-phenylhydrazine formed at 30°C was measured from the absorption at 324 nm. One unit of the enzyme activity was defined as the enzyme amount that formed one nmole of glyoxylate per min under the above conditions.

The activity of isocitrate dehydrogenase was determined by a modified method of Kornberg.^{7,8)} The assay mixture for the NAD-linked enzyme consisted of 33.3 mM Tris-HCl buffer, pH 8.5, 3.3 mM $MgCl_2$, 0.83 mM AMP, 1.67 mM NAD, 1.67 mM trisodium D,L-isocitrate and enzyme in a total volume of 3.0 ml. The mixture for assay of the NADP-linked enzyme was composed of 33.3 mM Tris-HCl buffer, pH 8.5, 3.3 mM $MgCl_2$, 0.33 mM NADP, 1.67 mM trisodium D,L-isocitrate and enzyme in a total volume of 3.0 ml. Both enzyme reactions were carried out at 30°C, and the

increase in absorbance at 340 nm was measured. One unit of the enzyme activity was defined as the enzyme amount that reduced one nmole of NAD or NADP per min under the above conditions.

Protein was assayed by the method of Lowry *et al.*⁽⁹⁾

Preparation of mycelial extract. The washed mycelia obtained as above were subjected to disruption with a French press at 18,000 psi for one min at 4°C. The disrupted mixture was centrifuged at $10^4 \times g$ for 20 min. The supernatant fluid was used for assays of isocitrate lyase, NAD-, NADP-linked isocitrate dehydrogenase.

Analysis of nonvolatile acids in the incubation mixture for rhodanese formation. The incubated mixture for the rhodanese formation by mycelia was centrifuged, the supernatant was extracted three times with equal volumes of ether, and the aqueous phase was brought to dryness under the reduced pressure. The composition of nonvolatile acids of the residue was investigated by thin layer chromatography using bromophenol blue as a locator. Oxalic and glyoxylic acids were directly determined by the methods of Lewis *et al.*⁽¹⁰⁾ and MacFadden *et al.*⁽¹¹⁾ respectively. Since citric acid was the main component of other acids than the above two, its amount was tentatively calculated from the alkali-titration value subtracting the acidity due to oxalic and glyoxylic acids.

Assay of Gly·Gly and glycine in the medium. After centrifugation, the supernatant of the mycelial incubation mixture was subjected to amino acid analysis using a Hitachi amino acid analyzer KLA 5. The amount of Gly·Gly was determined from the glycine content after hydrolysis of the supernatant with 6 N HCl at 110°C for 18 hr, subtracting the glycine content before the acid hydrolysis.

The chemical reagents used were of pure grade commercially available.

RESULTS

1. Rhodanese formation by the washed mycelia and the effect of glycylglycine

The washed mycelial suspension secreted rhodanese on aerobic incubation in the presence of sugar as a carbon source. The enzyme formation was observed with various sugars. However, in the medium supplemented with Gly·Gly, the enzyme formation was prominently stimulated by sucrose, as shown in Table I. Further, the enzyme formation increased in proportional to the concentration of Gly·Gly added (Table II). Gly·Gly·Gly were as effective as Gly·Gly, while free amino acids

TABLE I. EFFECTS OF SUGARS ON THE RHODANESE FORMATION WITH OR WITHOUT GLYCYLGLYCINE

A mycelial suspension (30 mg as dry weight) of 30 ml containing 30 mM sugar and 0.05 M phosphate buffer, pH 6.0, was incubated at 25°C for 48 hr with or without 22.7 mM glycyl-glycine.

Gly·Gly added Sugars added	Rhodanese activity, units/ml	
	—	+
Glucose	3.23	7.09
Fructose	3.23	5.80
Galactose	1.60	3.00
Mannose	1.70	3.40
Sorbitol	0.80	3.40
Xylose	0.70	2.60
Inositol	1.00	1.20
L-Arabinose	1.00	3.40
Sucrose	3.00	16.2
Glucose+Fructose	3.50	7.09
Maltose	3.60	4.00
Lactose	4.00	4.50
Dextrin	1.60	1.60
Cellulose	0.80	3.00

TABLE II. EFFECTS OF GLYCYLGLYCINE AND GLYCINE ON THE RHODANESE FORMATION

The conditions were the same as in Table I, but 30 mM sucrose was employed, and amounts of Gly·Gly and glycine were varied.

Additions, mM	Rhodanese activity, units/ml	
	Gly	Gly·Gly
None	3.23	3.23
0.73	3.23	9.68
7.30	4.00	10.2
22.7	5.80	16.2
37.8	9.68	25.8

except valine and other glycine-containing peptides tested were ineffective (Table III).

The time courses of the rhodanese formation and of the consumptions of Gly·Gly or glycine, and sucrose, are shown in Figs. 1A and 1B. The Gly·Gly was metabolized slowly, and most of it remained even after 72 hr of incubation. The glycine, on the other hand, was rapidly metabolized and completely consumed in 48 hr. The sucrose in the medium with glycine was readily hydrolyzed and consumed up in 48 hr. However, only a half of the sucrose added to the medium with Gly·Gly was consumed in

TABLE III. EFFECTS OF SEVERAL AMINO ACIDS AND GLYCYPEPTIDES ON THE RHODANESE FORMATION

The conditions were the same as in Table I, but with amino acids or glycylopeptides (22.7 mM), and with 30 mM sucrose.

Amino acids or glycylopeptides added	Rhodanese activity, units/ml
Glycine	5.80
Leucine	7.00
Tyrosine	5.00
Phenylalanine	5.00
Valine	10.0
Gly·Gly	16.2
Gly·Gly·Gly	16.0
Gly·Leu	5.0
Gly·Tyr	5.0
Ala·Gly·Gly	6.0
Leu·Gly·Phe	4.0
Gly·Phe	3.0

FIG. 1. Time Course of the Rhodanese Formation and Change in the Composition of Medium during Incubation of Washed Mycelia of *Trametes*.

The mycelial suspension of 30 ml containing 30 mM sucrose and 0.05 M phosphate buffer, pH 6.0, was incubated at 25°C with shaking.

A, with Gly·Gly; B, with glycine.

△—△, Gly·Gly or glycine; ○—○, rhodanese; ●—●, total sugar; ●---●, reducing sugar; ○—○, protein.

48 hr, though the sucrose was hydrolyzed as rapidly as in the case with glycine. Secretion of proteinous materials in the Gly·Gly medium was almost double that in the medium with glycine.

2. Effects of surface active agents on the rhodanese formation

To test the possibility that Gly·Gly acted

TABLE IV. EFFECTS OF SEVERAL SURFACE ACTIVE AGENTS ON THE RHODANESE FORMATION

The conditions were the same as in Table I.

Surface active agents added,	mg/ml	Rhodanese activity, units/ml
None	—	3.00
Glycylglycine	3.0	16.2
Spermidine phosphate	1.0	5.20
Spermine phosphate	1.0	3.55
Desoxycholate	0.5	3.55
Sodium lauryl sulfate	0.5	8.70

as a surface active agent, the effects of several other surface active agents on the rhodanese formation were examined. As shown in Table IV, sodium laurylsulfate and spermidine phosphate were effective in that order, though their effects were much less than Gly·Gly. The supernatant of the media supplemented with a surface active agent except Gly·Gly became viscous and colored in yellow.

3. Effects of some antibiotics and antimetabolites on rhodanese formation

The results described above suggest that the effect of Gly·Gly was not due to its surface active property. Thus, the effects of several antibiotics and antimetabolites were examined. Cycloheximide, antimycin A, 2,4-dinitrophenol, and potassium cyanide severely depressed the enzyme formation, as shown in Table V.

TABLE V. EFFECTS OF SEVERAL ANTIBIOTICS AND ANTIMETABOLITES ON THE RHODANESE FORMATION IN THE PRESENCE OF GLYCYLGLYCINE

The conditions were the same as in Table I, but with 22.7 mM Gly·Gly and with or without inhibitors.

Inhibitors added,	μg/ml	Rhodanese activity, units/ml
None	—	16.2
Antimycin A	40	2.25
Chloramphenicol	10	11.3
Cycloheximide	40	4.68
2,4-Dinitrophenol	150	4.35
KCN	323	3.39

The inhibitory effect of chloramphenicol was not so pronounced under the conditions used. These results may indicate that the formation

and secretion of rhodanese by the fungal mycelia were dependent upon the *de novo* protein synthesis on the ribosomal level with the energy supply through oxidative phosphorylation *via* cytochromes.

4. Formation of citric, oxalic and glyoxylic acid from sugar in the presence of Gly·Gly

The media supplied with Gly·Gly were observed to be gradually acidified during incubation, and thus the acids formed were analyzed. The thin-layer chromatography of the supernatant of the media after the extraction with ether revealed that citric, oxalic and glyoxylic acids were main acids produced.

TABLE VI. EFFECT OF GLYCYLGLYCINE ON THE FORMATION OF CITRIC, OXALIC AND GLYOXYLIC ACIDS

The conditions were the same as in Table I, but with or without 22.7 mM Gly·Gly and 30 mM of sucrose, glucose or fructose.

Additions	Acid accumulated, $\mu\text{g/ml}$		
	Citric acid	Oxalic acid	Glyoxylic acid
Sucrose	90	200	12
Sucrose + Gly·Gly	295	113	12
Glucose + Gly·Gly	275	216	23
Fructose + Gly·Gly	285	303	15
Glucose } + Gly·Gly	290	290	20
Fructose }			

Table VI shows that the citric acid formation from sucrose was greatly stimulated, while the oxalic acid formation was significantly depressed, by the addition of Gly·Gly. The citric acid formation from glucose or fructose or their mixture in the presence of Gly·Gly was comparable with that from sucrose in the presence of Gly·Gly. However, the oxalic acid formation under the latter conditions was almost double or more than under the former conditions. The glyoxylic acid formation was remarkable from glucose. As shown in Table I, the stimulative effect of Gly·Gly in the formation of rhodanese was clear in the presence of sucrose, but was not so clear in the presence of glucose or fructose instead of sucrose. It was thus suggested that there is a parallelism

between the accumulation of citric acid and the rhodanese formation. Thus, the enzymes involving in the metabolism of isocitric acid which is an intermediate to citric, oxalic, and glyoxylic acids were investigated.

5. Isocitrate lyase, isocitrate dehydrogenase activities of mycelia incubated with sucrose and Gly·Gly

NAD-linked and NADP-linked isocitrate dehydrogenase activities of the mycelia incubated with sucrose and Gly·Gly were higher than those of the mycelia incubated with glucose or fructose in the presence of Gly·Gly. However, isocitrate lyase of the mycelia incubated with glucose or fructose was more active than that of the mycelia incubated with sucrose, as shown in Table VII.

TABLE VII. ACTIVITIES OF ISOCITRATE LYASE, NAD- AND NADP-LINKED ISOCITRATE DEHYDROGENASES OF THE MYCELIA

The conditions were the same as in Table I, but with or without 22.7 mM Gly·Gly. After centrifugation, mycelia of the incubated mixture was washed with distilled water twice, and then disrupted with a French press. The supernatant of the disrupted mixture was used as enzyme. Enzyme assay was carried out by the method described in the text.

Additions	Enzyme activity, units/ml		
	Isocitrate lyase	NAD-linked isocitrate dehydrogenase	NADP-linked isocitrate dehydrogenase
Sucrose	21.5	6.6	7.2
Sucrose + Gly·Gly	17.5	13.5	22.8
Glucose + Gly·Gly	43.3	7.1	8.9
Fructose + Gly·Gly	30.0	5.0	7.0
Glucose } + Gly·Gly	20.0	6.0	12.0
Fructose }			

DISCUSSION

The biochemical system for the active transport of Gly·Gly in cells of *Escherichia coli* has been clarified to some extent.¹²⁾ The uptake of Gly·Gly by the cells is inhibited by oxidative phosphorylation uncouplers, and is also regulated by the concentration of ATP in cells. In the present experiments, the consumption rate of Gly·Gly by the fungal mycelia was

significantly smaller than that of glycine (Fig. 1). This fact indicates that the mycelial cells were much less active in transporting Gly·Gly. On the other hand, the addition of Gly·Gly resulted in depression of the energy metabolism of the mycelial cells, as shown in Fig. 1A.

Newcomb *et al.*¹³⁾ have reported that *Polyporus* fungus shows tricarboxylic acid cycle reactions. The fungus exhibited a respiratory quotient (RQ) of 1.0 when glucose is supplied to the medium. The sugar might be assimilated to cellular reserves. Oxalic acid that often appears as an end product in the aerobic oxidation of glucose was not so rapidly utilized by the fungus. Cytochromes were detected in the mycelia of *Trametes* by the direct vision spectroscopic test.¹⁴⁾ Kobayashi *et al.*¹⁵⁾ have also reported that a species of *Trametes* accumulated oxalic, citric, malic and gluconic acids in the media containing sucrose and polypeptone. Also, the present results of the effects of several inhibitors on the rhodanese formation showed that, to obtain energy, *Trametes sanguinea* depends on the tricarboxylic acid cycle system to a considerable extent. The increase in NAD- and NADP-linked isocitrate dehydrogenase activities and the depression in isocitrate lyase activity of the mycelia on incubation with Gly·Gly suggest that the rhodanese formation is correlated to certain step(s) of the system of TCA cycle. The above results also suggest that Gly·Gly regulates the metabolism described above to favor the rhodanese formation by its certain inhibitory effect. Of importance is the fact that under the conditions where less accumulation of oxalic acid with more activities of NAD- and NADP-linked isocitrate dehydrogenase and less activity of isocitrate lyase of the mycelial cells, occurred a good formation of rhodanese.

It is uncertain that the rhodanese formation proceeded by the *de novo* synthesis of protein. Therefore, the formation and the secretion of rhodanese by mycelial cells of *Trametes sanguinea* may correlate to certain steps of the protein synthesis through the energy supply process(es) or metabolite(s) produced intermedially whose effectiveness for the enzyme formation is greatly influenced by the kind of sugar and Gly·Gly.

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