Occurrence and enzymic degradation of glutathionylspermidine in *Citrobacter freundii*

TOMOHIRO HISANO,¹ SHIRO ABE,¹ NOBUE OSAMURA,² XI-BAI SONG,³ KOUSAKU MURATA^{3*} and AKIRA KIMURA³

¹ Department of Biotechnology, Kyoto Institute, Gunze Co., Ltd, Ayabe, Kyoto 623, Japan

² Department of Domestic Science, Kyoto Women's University, Kyoto, Kyoto 605, Japan

³ Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

(Received 30 October 1990; revised 11 December 1990; accepted 20 December 1990)

Cells of *Citrobacter freundii* IFO 12681 contained glutathionylspermidine at a level of 2–3 nmol (g wet cells)⁻¹. Three kinds of enzymes (E1, E2 and E3) catalysing the degradation of glutathionylspermidine were found in extracts of the bacterium. E1 was a spermidine dehydrogenase and was induced by spermidine. The enzyme hydrolysed glutathionylspermidine only in the presence of an electron acceptor. E2 and E3 were formed constitutively and required no cofactor for the degradation of glutathionylspermidine. E3 required at least two protein components for activity. The reaction products formed by E1, E2 and E3 differed from each other, but have not yet been identified. No activity generating glutathione and spermidine from glutathionylspermidine was detected in extracts of *C. freundii*.

Introduction

Glutathione (GSH) is a tripeptide consisting of L-glutamate, L-cysteine and glycine and is involved in processes for detoxification of unfavourable compounds and maintenance of cellular redox potentials (Meister & Tate, 1976; Meister & Anderson, 1983). The tripeptide is required as a cofactor for some enzyme reactions. Polyamines, especially putrescine (PUT) and spermidine (SPD), are polycationic molecules frequently found in all biological materials and regulate many important processes such as proliferation and differentiation of cells and synthesis of protein and nucleic acids. Tabor & Tabor (1975) showed the occurrence of glutathionylspermidine (GSH-SPD: N¹-mono-glutathionylspermidine) in *Escherichia coli*. The bacterium has an enzyme that catalyses the formation of GSH-SPD from GSH and SPD in the presence of adenosine 5'-triphosphate (Tabor & Tabor, 1966). GSH-SPD is accumulated when the growth of *E. coli* is arrested and disappears in association with the resumption of growth (Tabor & Tabor, 1970). Very recently, another type of the conjugate, N¹,N⁸-

bis(glutathionyl)spermidine, was isolated from an insect trypanosomatid, *Crithidia fasciculata*. The conjugate, named trypanothione (T[SH]₂), has a role in regulation of SPD level and is a substrate for the enzymes T[SH]₂-reductase and T[SH]₂-peroxidase in *C. fasciculata* (Fairlamb *et al.*, 1985; Henderson *et al.*, 1987; Fairlamb & Henderson, 1987; Shim & Fairlamb, 1988). These observations on the occurrence of a conjugate between GSH and SPD suggest an additional function of GSH and SPD. In order to learn more about the physiological function of SPD, we initiated the study on the metabolic fate of SPD in a bacterium, *Citrobacter freundii* IFO 12681, and reported the detailed properties of SPD dehydrogenase in the bacterium (Hisano *et al.*, 1990). This report describes the occurrence of GSH-SPD in *C. freundii* cells and demonstrates the presence of enzymes responsible for its degradation.

Methods

Preparation of GSH-SPD. For large-scale preparation of GSH-SPD, *Escherichia coli* B was grown aerobically at 37 °C in 40 l Vogel–Bonner minimal medium (Vogel & Bonner, 1956). When the culture reached late stationary phase, the aeration was stopped and 80 g glucose was added. After anaerobic incubation at 37 °C for 3 h, the cells (150 g wet

Abbreviations: CAD, cadaverine; GSH, glutathione; GSH-SPD, glutathionylspermidine; PUT, putrescine; SPD, spermidine; SPR, spermine.

wt) were washed once in chilled 0.85% saline solution, resuspended in 600 ml of 5% (w/v) trichloroacetic acid (TCA), and then incubated for 1 h at 25 °C to extract GSH-SPD. The extract (650 ml) was applied to an Amberlite CG-50 column (2.5 × 25 cm) and the conjugate was eluted with 1 M-acetic acid (150 ml) according to the method of Tabor & Tabor (1975). The acetic acid solution containing GSH-SPD was concentrated to 20 ml by evaporation at 30 °C and 2 µl of the concentrate was used for the analysis of polyamines as shown below. To detect GSH-SPD in *C. freundii* IFO 12681, the cells were cultured aerobically at 30 °C in 1:5:1 Vogel–Bonner minimal medium. When the culture reached late stationary phase, it received 3 g glucose and was then incubated anaerobically at 30 °C for 3 h. Approximately 9 g wet wt cells was obtained from 1:5:1 culture. The subsequent conditions for TCA extraction and Amberlite CG-50 column (1.0 × 10 cm) chromatography were the same as those described above, except that the volumes of liquids to be processed were reduced to one-twentieth. The eluate with 1 M-acetic acid was concentrated to 1 ml as above and 2 µl of the concentrate was subjected to analysis of polyamines.

Analysis of polyamine derivatives. Polyamine and GSH-SPD in the eluate after Amberlite CG-50 column chromatography of TCA extracts were analysed by ion-paired, reversed-phase high-performance liquid chromatography (HPLC) according to the method of Suzuki *et al.* (1990), except that a µBondasphere 5 µ C-18 100Å column (Waters, Tokyo, Japan) was used in place of a Shim-Pak CLC-ODS column (Shimadzu, Kyoto, Japan).

Enzyme assay. GSH-SPD-degrading activity was assayed in a mixture (50 µl) consisting of 0.2 mM-GSH-SPD, 100 mM-potassium phosphate buffer (pH 7.2) and enzyme samples. GSH-SPD was isolated from *E. coli* B cells as described in Results and Discussion. When SPD dehydrogenase was used, 1 mM-K₃Fe(CN)₆ was added to the mixture. After incubation at 37 °C for 2 h, 4 µl of the reaction mixture was chromatographed on Kieselgel 60 F₅₄ (Merck) (TLC) with solvent system I [n-butanol/acetic acid/H₂O/pyridine (4:2:2:1, by vol.)] or II [methanol/acetic acid/28% (w/v) ammonium hydroxide (2:2:1, by vol.)]. Products were visualized with ninhydrin. Protein was determined by the Lowry procedure.

Column chromatography of enzymes. Cells of *C. freundii* IFO 12681 (43 g wet wt), previously grown aerobically in 4 l Vogel–Bonner minimal medium at 30 °C for 41 h, were washed in chilled 0.85% saline solution, resuspended in 5 mM-potassium phosphate buffer (pH 7.2), and sonicated at 9 kHz, 0 °C for 5 min. The homogenates were centrifuged at 25000 g for 30 min and the clear supernatants were used as enzyme sources. The cell extract (2.8 g protein, 65 ml) was applied to a DEAE-cellulose column (4.4 × 44 cm) equilibrated with 5 mM-potassium phosphate buffer (pH 7.2) and eluted with a linear gradient of NaCl (0–1 M, 2 l). Fractions (15 ml) were collected every 10 min. Active fractions, which were eluted at about 0.35 M-NaCl, were pooled, concentrated to 28.2 ml (477 mg protein) by using an Amicon PM10 membrane, and then applied to a hydroxylapatite column (2.7 × 15 cm) equilibrated with 5 mM-potassium phosphate buffer (pH 7.2) (5–500 mM, 300 ml) and 4 ml fractions were collected every 12 min. The active fractions, which were eluted at 0.25 M-buffer, were combined and concentrated to 7 ml as above. The concentrate (169 mg protein) was loaded onto a Sephadex G-150 column (2 × 80 cm) equilibrated with 5 mM-potassium phosphate buffer (pH 7.2). The enzymes were eluted with the same buffer and 4 ml fractions were collected every 10 min. The active fractions, which were eluted as V_e (elution volume)/ V_0 (void volume) = 1.9, were combined (99 mg protein) and applied to a DEAE-Sepharose CL-6B column (1 × 28 cm) equilibrated with 5 mM-potassium phosphate buffer (pH 7.2) and the enzymes were eluted with a linear gradient of NaCl (0–0.6 M, 100 ml). Fractions (2 ml) were collected every 6 min.

Chemicals. Polyamines were purchased from Sigma.

Results and Discussion

Isolation of GSH-SPD from *E. coli* B

When compounds in the eluate after Amberlite CG-50 column chromatography of TCA extracts prepared from *E. coli* B were analysed by HPLC, three unknown peaks (I, II and X) were observed in addition to PUT, cadaverine (CAD), SPD and spermine (SPR) (Fig. 1*b*). TLC of the eluate showed the presence of six ninhydrin-positive compounds [SPR (a), SPD (b) and unknown spots c, d, e and f] (Fig. 1*c*). These unknown spots were extracted from chromatograms and analysed by HPLC (data not shown). Spot c showed the same retention time as that of peak I. Spot d was separated into three peaks with the same retention times as those of PUT, CAD and peak II. Contamination of PUT in spot d was suggested from the result of TLC (Fig. 1*c*). CAD also migrates to just above PUT on TLC (data not shown). Thus, the material in peak I was recovered in spot c as a single compound and that in peak II was in spot d together with PUT and CAD. These materials in spots c and d were designated compounds c (for peak I) and d (for peak II) for further analysis. The nature of the materials in peak X and spots e and f is not yet clear.

The structures of the material in peaks I and II were determined using compounds c and d obtained from TLC. Amino acid analysis of acid-hydrolysed compound c yielded equimolar amounts of glutamate, cysteine and glycine residues (Table 1). HPLC of the acid-hydrolysed compound c confirmed the presence of a polyamine having the same retention time as that of SPD (Fig. 2*d*) and the amount of the polyamine was stoichiometric with that of the amino acid residues liberated (Table 1). Acid hydrolysis of compound c after dansylation revealed the presence of dansylglutamate (data not shown), thus indicating that the amino-terminal amino acid of compound c is glutamate. These results indicate that compound c (material in peak I) is the reduced form of GSH-SPD:

Likewise, the structure of compound d was analysed. The analysis of acid-hydrolysed compound d yielded equimolar amounts of SPD, glutamate, cysteine and glycine (Table 1). When compound d was treated with dithiothreitol, it gave a molecule having the same retention time as that of compound c (Fig. 2*b*), and showed the same properties as those of compound c. From these results, compound d (peak II) was identified as an oxidized form of compound c (peak I).

To prepare GSH-SPD (i.e. compound c) from *E. coli* B, the eluate after Amberlite CG-50 column chromato-

Fig. 1. Separation of compounds in TCA extracts by HPLC and TLC. TCA extracts prepared from *E. coli* B were analysed after Amberlite CG-50 column chromatography. (a) Separation of standard polyamines by HPLC; (b) separation of compounds in TCA extracts by HPLC; (c) separation of compounds in TCA extracts by TLC with solvent system I. Abbreviations of polyamines are indicated in the text, except for HIS (histamine).

Table 1. Amino acid and polyamine analyses of compounds c, d and c*

Compounds analysed (µg)†	Source	Amount liberated (nmol)			
		L-Glutamate	L-Cysteine	Glycine	Spermidine (SPD)
c (26.1)	<i>E. coli</i> B	55.1	49.8	57.9	56.5
d (32.2)‡	<i>E. coli</i> B	17.6	13.9	20.5	19.7
c* (12.3)	<i>C. freundii</i>	23.3	21.6	22.0	21.8

c*: material from *C. freundii* having the same mobility on TLC as that of compound c.

† Determined using SPD dehydrogenase.

‡ Sample contains putrescine and cadaverine.

graphy of TCA extracts was concentrated and compounds in the concentrate were separated by TLC as shown in Fig. 1(c). Compound c was extracted with water from the chromatograms, concentrated and used as a substrate for the study of enzymes responsible for the degradation of GSH-SPD in *C. freundii* IFO 12681 cells. By this method, approximately 3 µmol of GSH-SPD was isolated from 150 g wet wt *E. coli* B.

Detection of GSH-SPD in *C. freundii*

The level of GSH-SPD in *E. coli* has been shown to increase when the cultures of the bacterium become anaerobic and slightly acidic (Tabor & Tabor, 1966, 1970, 1975). In order to increase the GSH-SPD content of *C. freundii*, aeration of the culture was stopped at late stationary phase and it was incubated with added glucose

Fig. 2. Analysis of polyamine in compounds c and d. Compounds c (left) and d (right) obtained by extraction from chromatograms shown in Fig. 1(c) were analysed by HPLC. (a) Compound c or d; (b) compound c or d after treatment with dithiothreitol; (c) compound c or d mixed with SPD; (d) compound c or d after acid hydrolysis; (e) acid-hydrolysed compound c mixed with SPD.

(see Methods). TCA extracts were prepared from such cells, fractionated on an Amberlite CG-50 column and the eluate used for the analysis of polyamine derivatives in *C. freundii*. TLC of the eluate indicated the presence of a ninhydrin-positive spot at the same migration position as that of compound c isolated from *E. coli* B (data not shown). The amino acid analysis of the acid-hydrolysed compound in the spot indicated the presence of equimolar amounts of glutamate, cysteine and glycine residues (Table 1). The HPLC analysis of the acid-hydrolysate of this compound confirmed the presence of SPD, and the amount of SPD was closely similar to those of amino acid residues (Table 1). These results indicate the presence of GSH-SPD in *C. freundii*.

In the case of *E. coli* B, the GSH-SPD level substantially increased, from 8 nmol (g wet wt cells)⁻¹ to 60 nmol (g wet wt cells)⁻¹, when late stationary phase cells were made anaerobic (data not shown). However, the GSH-SPD level in *C. freundii* cells was only 2–3 nmol (g wet wt cells)⁻¹ even after anaerobic incubation. Therefore, GSH-SPD was prepared from *E. coli* B cells as mentioned before. This is the first report showing the occurrence of GSH-SPD in a prokaryote other than *E. coli*.

Degradation of GSH-SPD by extracts of *C. freundii*

When GSH-SPD isolated from *E. coli* B was incubated with crude cell extracts of *C. freundii* grown in Vogel-Bonner minimal medium, three kinds of ninhydrin-positive products (P1, P2 and P3) were found (Fig. 3a). In order to elucidate the enzymes responsible for the formation of these products, the cell extracts were fractionated by column chromatography (Fig. 4). On the DEAE-cellulose (Fig. 4a) and hydroxylapatite (data not shown) columns, enzyme activities catalysing the formation of P1, P2 and P3 were eluted unseparated with 0.35 M-NaCl (Fig. 4c) and 0.25 M-potassium phosphate buffer (pH 7.2), respectively. These activities were also eluted at the same position upon further chromatography on Sephadex G-150 (data not shown). The enzyme sample was then fractionated on a DEAE-Sepharose CL-6B column (Fig. 4b). However, no fractions catalysing the formation of P1, P2 and P3 were found (Fig. 4d). The fractions were combined into three pools (f1, f2 and f3) (Fig. 4b), and activity in all possible combinations was determined (Fig. 3c). As a result, activity giving rise to two ninhydrin-positive products (P1* and P3*) was found only with the combination of pools f2 and f3. No

Fig. 3. Separation of GSH-SPD degradation products. Reaction products were separated by TLC with solvent system I (for *a*, *b* and *c*) or II (for *d*). Standard compounds are shown in the Figure by abbreviations, all of which are indicated in text, except for DAP (1,3-diaminopropane). (*a*) Products formed by the reaction with cell extracts in the presence (lane 1) or absence (lane 2) of GSH-SPD. (*b*) Products formed by the reaction with purified SPD dehydrogenase (lane 3). (*c*) Products formed by the reaction with combined pools (f1, f2 and f3; see Fig. 4*b*). Combinations of these pools as enzyme sources are indicated in the figure. (*d*) Products formed by the reaction with f2 + f3 (lane 4).

Fig. 4. Elution of GSH-SPD-degrading enzymes in *C. freundii*. Conditions for DEAE-cellulose (*a*) or DEAE-Sepharose CL-6B (*b*) column chromatography are described in Methods. Activity in each fraction after DEAE-cellulose (*c*) and DEAE-Sepharose CL-6B (*d*) column chromatography was checked by separating reaction products by TLC with solvent system I.

activity forming a P2-like product was found in any possible combination. The activity requiring components in f2 and f3 to produce P1* and P3* was designated E3 and that responsible for the formation of P2 in cell extracts was designated E2.

The products of E2 and E3 reactions were compared with those of reactions catalysed by crude cell extracts and SPD dehydrogenase, which we call E1, purified from *C. freundii* cells grown in Vogel-Bonner minimal medium supplemented with SPD (Hisano *et al.*, 1990)

(Fig. 3). When SPD dehydrogenase was incubated with GSH-SPD in the presence of $K_3Fe(CN)_6$, only one ninhydrin-positive product, P2*, was formed (Fig. 3*b*, lane 3); this was identified as glutathionyl-1,3-diaminopropane according to the method of Tabor & Tabor (1975). On the other hand, E3 reaction yielded two kinds of products, P1* and P3* (Fig. 3*c*). Comparison of R_F values of the reaction products indicated that P1 and P3 formed by the reaction with crude cell extracts (Fig. 3*a*) correspond to P1* and P3* formed by E3 reaction (Fig. 3*c*). The structures of these products are not yet clear, although we have confirmed by TLC that P1* and P3* were neither SPD nor GSH (Fig. 3*c*, *d*). On the other hand, P2 formed by the reaction with crude cell extracts (Fig. 3*a*) showed almost the same R_F value as that of P2* formed by SPD dehydrogenase (E1) reaction (Fig. 3*b*, lane 3). However, E2 is different from SPD dehydrogenase (E1), because synthesis of E2 is independent of SPD, although that of SPD dehydrogenase (E1) requires the presence of SPD in the growth medium (Hisano *et al.*, 1990). E2 requires no cofactors to produce P2. On the other hand, SPD dehydrogenase requires electron acceptors to produce P2*.

Thus, *C. freundii* IFO 12681 was found to contain at least three kinds of enzymes catalysing the degradation of GSH-SPD. One is a cofactor-dependent, inducible enzyme E1 (SPD dehydrogenase). The other two (E2 and E3) are cofactor-independent, constitutive enzymes. E3 is different from E2 in stability and catalytic properties. None of these enzyme activities catalysed the liberation of GSH and/or SPD from GSH-SPD, and no such activities were detected in extracts of *C. freundii* grown in Vogel-Bonner minimal medium. These results indicate that GSH-SPD is not merely a storage form of GSH and SPD in *C. freundii* IFO 12681, and the reactions catalysed by enzymes E1, E2 and E3 give alternative routes for the regulation of cellular levels of GSH and SPD. To determine the role of GSH-SPD and precise routes for GSH-SPD degradation in the bacterium, we are isolating and characterizing enzymes E2 and E3 and their reaction products P1* and P3*.

We thank Ms Tamiko Fukuda for her assistance in experiments and in preparation of the manuscript.

References

- FAIRLAMB, A. H. & HENDERSON, G. B. (1987). Metabolism of trypanothione and glutathionylspermidine in trypanosomatids. In *Host-Parasite Molecular Recognition and Interaction in Protozoal Infections*, pp. 29-40. Edited by K.-P. Chang & D. Snary. Heidelberg: NATO ASI Series.
- FAIRLAMB, A. H., BLACKBURN, F., ULRICH, P., CHAIT, E. T. & CERAMI, A. (1985). Trypanothione: a novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. *Science* **227**, 1485-1487.
- HENDERSON, G. B., FAIRLAMB, A. H. & CERAMI, A. (1987). Trypanothione dependent peroxide metabolism in *Crithidia fasciculata* and *Trypanosoma brucei*. *Molecular and Biochemical Parasitology* **24**, 39-45.
- HISANO, T., ABE, S., WAKASHIRO, M., KIMURA, A. & MURATA, K. (1990). Microbial spermidine dehydrogenase: purification and characterization of the enzyme from *Pseudomonas aeruginosa* and *Citrobacter freundii*. *Journal of Fermentation and Bioengineering* **69**, 335-340.
- MEISTER, A. & ANDERSON, M. E. (1983). Glutathione. *Annual Review of Biochemistry* **52**, 711-760.
- MEISTER, A. & TATE, S. S. (1976). Glutathione and related γ -glutamyl compounds: biosynthesis and utilization. *Annual Review of Biochemistry* **45**, 559-604.
- SHIM, H. & FAIRLAMB, A. H. (1988). Levels of polyamines, glutathione and glutathione-spermidine conjugates during growth of the insect trypanosomatid *Crithidia fasciculata*. *Journal of General Microbiology* **134**, 807-817.
- SUZUKI, S., KOBAYASHI, K., NODA, J., SUZUKI, T. & TAKAMA, K. (1990). Simultaneous determination of biogenic amines by reversed-phase high performance liquid chromatography. *Journal of Chromatography* **508**, 225-228.
- TABOR, H. & TABOR, C. H. (1966). Preliminary studies on the enzymatic metabolism of spermidine by *Escherichia coli* extracts. *Federation Proceedings* **25**, 879-880.
- TABOR, C. H. & TABOR, H. (1970). The complete conversion of spermidine to a peptide derivative in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **41**, 232-238.
- TABOR, H. & TABOR, C. H. (1975). Isolation, characterization, and turnover of glutathionylspermidine from *Escherichia coli*. *Journal of Biological Chemistry* **250**, 2648-2654.
- VOGEL, H. J. & BONNER, D. M. (1956). Acetylornithinase of *Escherichia coli*: partial purification and some properties. *Journal of Biological Chemistry* **218**, 97-106.