Intracellular Accumulation of Trehalose during Streptomycin Formation by Streptomyces griseus

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When washed mycelium of Streptomyces griseus HUT 6037 was incubated in 0.5% NaCl solution containing 14C-glucosamine with shaking at 28°C, the activity of the mycelium to incorporate radioactivity into the cell wall decreased rapidly, while that into streptomycin increased. During this physiological change, UDP-N-acetylmuramyl-pentapeptide, UDP-N-acetylglucosamine, glutamic acid and trehalose accumulated in the mycelium. The latter two substances accumulated much more than the former two. When the time courses of the activities of incorporation of 14C-glucosamine into the four substances, mucopeptide and streptomycin were examined, a decrease in the activity into peptidoglycan led to an increase into streptomycin, glutamic acid and trehalose.

In a pH-stat batch culture with a defined medium, trehalose was accumulated in the cell before glucose was consumed. However, after glucose was consumed, the consumption of trehalose began. Streptomycin production continued until intracellular trehalose completely disappeared in spite of the lack of glucose in the culture medium.

D-Glucosamine is utilized by Streptomyces griseus for both mucopeptide synthesis and streptomycin (SM) production. In a mycelium-suspended culture of S. griseus supplemented with D-[l-14C]glucosamine, suppression of cell wall synthesis by the addition of antibiotics such as enduracidin enhanced the incorporation of radioactivity into SM.1) A similar effect of suppression of cell wall synthesis has been demonstrated in production of other aminoglycoside antibiotics such as neomycin,2) kanamycin3) and sagamicin.4)

In S. griseus, suppression of mucopeptide synthesis by enduracidin, vancomycin or bacitracin enhanced incorporation of D-[l-14C]glucosamine into SM, while suppression by cycloserine did not.5) These results suggested that although glucosamine was usually utilized for synthesis of the cell wall, its utilization was shunted toward SM production by a control mechanism which was regulated by intracellular accumulation of some specific substances owing to suppression of the phospholipid cycle in the mucopeptide-synthesizing pathway.

To understand the control mechanism, an investigation was carried out to detect the intracellular substances accumulated during the increase of SM production and we found the intracellular accumulation of trehalose which was probably correlated with SM formation.

MATERIALS AND METHODS

Strain and culture conditions. Streptomyces griseus HUT 6037 was shake-cultured at 28°C in a medium containing 25 g glucose, 2 g (NH₄)₂SO₄, 0.4 g KH₂PO₄, 1 g NaCl, 6 g K₂SO₄, 0.2 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.05 g of ZnSO₄·7H₂O, 1 g CaCO₃ and 7 g L-asparagine per liter, pH 7.0. The mycelium at the exponential growth phase (48 hr incubation) was harvested to obtain washed mycelium as previously described.1,5) For SM fermentation by a pH-stat batch culture, the medium was modified to contain 12 g glucose, 5 g (NH₄)₂SO₄ and 0.4 g...
CaCl$_2$·2H$_2$O instead of CaCO$_3$ per liter, pH 6.5. The fermentation was carried out as reported by Inoue et al.,$^9$ using a 2.6 liter jar fermentor (Marubishi MD-260; working volume: 1.5 liters, aeration: 1 vvm, agitation: 500 rpm, temp.: 28°C).

Determination of radioactivity incorporated from D-[1-$^{14}$C]glucosamine into mucopeptide and SM. Labeled mucopeptide and SM were determined by the method described previously.$^1$)

Detection of the intracellular substances labeled with D-[1-$^{14}$C]glucosamine in cells. Intracellularly accumulated substances were detected by the method of Strominger$^6$ with some modifications for extraction and separation. A 3.5 ml portion of the above mycelium-suspended culture after 30 min incubation was centrifuged for 3 min at 4,500 × g. The precipitated mycelium was washed twice with cold deionized water and heated with 3 ml of water in a boiling water bath for 10 min. After cooling quickly, the mycelial suspension was mixed with 3 ml of 10% trichloroacetic acid (TCA) and the mixture kept for more than one hour at 0°C. After filtration through a glass fiber filter (Whatman GF/C), the filtrate was shaken vigorously with the same volume of ethyl ether to remove TCA. The ether layer was discarded. After repeating this ethyl ether treatment three times, the water layer was heated in a hot water bath to remove ethyl ether completely. The solution was adjusted to pH 7.0 and freeze-dried. After dissolving the freeze-dried sample in 5 μl of deionized water, a 1 μl portion was spotted on a filter paper strip (Whatman 20 Chr.) and chromatographed using a solvent system consisting of isobutyric acid:0.5 N NH$_4$OH (5:3). The paper was radioautographed on a Fuji X ray film (KX Medical) for 7 to 20 days. The radioactive parts of the paper were cut out and the radioactivity was determined with a liquid scintillation counter.

Preparation of samples for identification of the radioactive substances accumulated in cells. In order to identify the four radioactive substances, the cell extract with hot water was subjected to PPC (Whatman 3MM, 10 cm × 18 cm: a solvent system of isobutyric acid:0.5 N NH$_4$OH (5:3)). After drying the paper, substance III was visualized as a fluorescent linear area by UV ray irradiation. None of fluorescence and radioactivity into four pieces. The fluorescent radioactive areas were detected with a radiochromatogram scanner. The paper was cut along the bands of fluorescence and radioactivity into four pieces. The radioactive substance on each piece was extracted with water and concentrated in vacuo. Each sample was further purified by PPC using solvent system B (ethanol:1 M ammonium acetate=7.5:3) and also by high voltage paper electrophoresis (PIP) using 0.2 M acetate buffer (pH 5.0) at 50 V/cm for 25 min.

Determination of dry cell weight, glucose, SM and trehalose on SM fermentation using a growing culture. In SM fermentation using a growing culture, 10 ml of culture broth was taken at the indicated time. Dry cell weight was measured as follows; 5 ml of culture broth was centrifuged at 5,000 × g for 20 min and the wet cells were washed twice with deionized water and dried for 24 hr at 105°C. SM produced was assayed by the agar diffusion method using Bacillus subtilis IFO 3134 as a test organism. Glucose in culture broth was determined by the glucostat method$^9$ (reagents; Fujisawa Medical Supply). Trehalose accumulated in mycelium was determined as follows; a 2.5 ml portion of culture broth was treated in the same way as described above to obtain the deproteinized cell free extract. The extract at pH 7.0 was passed through connected columns of Amberlite IRC 120 (H form, 0.4 ml) and IRA 400 (OH form, 0.8 ml). After washing the columns with 1.5 ml of water, the effluent and washing water were mixed and dried in vacuo. The dried sample was dissolved in 50 μl and subjected to high pressure liquid chromatography (column: Finepak GEL SC-220, Japan Spectroscopic Co., Ltd., eluant: distilled water, detector: Shodex RI, Showa Denko Co.) for determination of trehalose.

Reagents. D-[1-$^{14}$C]Glucosamine hydrochloride was obtained from Amersham and UDP-N-acetylglucosamine and D-glucosamine-6-phosphate barium salt were from Sigma Chem. Co. N-Acetyl-D-glucosamine and D-glucosamine hydrochloride were of special grade available commercially. The 5′-uridine monophosphate disodium salt was kindly supplied by Yamasa Shoyu Co.

RESULTS

Accumulation of the labeled substances derived from D-$^{14}$C-glucosamine

As reported in a previous paper,$^1$ D-glucosamine is incorporated into both the cell wall and streptomycin. Suppression of the former incorporation by antibiotics such as enduracidin and bacitracin led to an increase in the latter incorporation. These facts suggested that intracellular accumulation of certain substances relating to cell wall synthesis gave rise to an increase in production of SM. Accordingly it was first investigated what kind of substance accumulated in the cell when the cell wall synthesis was suppressed by enduracidin or bacitracin. Washed mycelium suspensions with or without an antibiotic were incubated with D-[1-$^{14}$C]glucosamine and the 14C-labeled substance accumulated in cells was analyzed (Fig. 1). In the presence and absence of an antibiotic, all samples gave four distinct
radioactive substances (designated as I, II, III and IV) on a paper radioautogram.

By using a mycelium-suspended culture without addition of an antibiotic, changes of activities of incorporation of D-[1-14C]glucosamine into the four substances, SM and cell walls were examined during 12 hr incubation as shown in Fig. 2. During the first two hr, the cell wall-synthesizing activity rapidly decreased while streptomycin-synthesizing activity rapidly increased. The activities accumulating I and II were low and constant through out 12 hr incubation. In contrast, the activities accumulating III and IV increased rapidly in the first two hours. At this stage, the mycelium accumulated a larger amount of III than IV. Equally the activity incorporating 14C-glucosamine into streptomycin was also highest in the mycelium after 4 hr incubation. The strong activities accumulating III and IV remained even after 12 hr incubation.

Identification of the radioactive substances derived from D-14C-glucosamine

Fig. 1. Paper Radioautogram of the Intracellular Substances Derived from 14C-Glucosamine during Incubation with and without Antibiotics.

Twelve ml of 0.5% NaCl and 3 ml of washed mycelium were mixed and shaken at 28°C in a test tube (24 mm x 200 mm) containing 0.3 μCi/ml D-[1-14C]glucosamine with or without enduracidin (100 μg/ml) or bacitracin (250 μg/ml).

Fig. 2. Changes of the Activities of Incorporation of Radioactivity from D-14C Glucosamine into Cell Walls (CW), Streptomycin (SM) and Intracellular Substances in a Mycelium-suspended Culture.

Two cultures were prepared in 500 ml Sakaguchi shaking flasks with 80 ml 0.5% NaCl solution and 20 ml washed mycelium by incubation at 28°C for 12 hr. Every two hr, 7 ml portions of the two cultures were taken and combined in a test tube. 10 ml of the combined mycelial culture was mixed with 1 μCi of D-[1-14C]glucosamine (0.5 μCi/μmol) and the mixture incubated for 30 min at 28°C with shaking.

The four radioactive substances accumulated in cells were partially purified by the procedure described in MATERIALS AND METHODS and used for their identification. Substance I exhibited an ultraviolet absorption maximum at 262 nm. It was hydrolyzed in 1 N HCl at 100°C for 60 min, dried in vacuo and subjected to thin layer chromatography on silica gel G (Merck, Type 60) which was developed with methanol : conc. HCl : water = 7:2:1. When detected at 254 nm UV adsorption, the hydrolyzate gave a spot with the same Rf value as authentic UMP. The hydrolyzate in 6 N HCl at 100°C for 24 hr was found to contain muramic acid by PPC (solvent system B) and PIP. The latter hydrolyzate
was further analyzed with an amino acid analyzer after removal of HCl by drying *in vacuo* in a desiccator containing NaOH. As shown in Fig. 3, glutamic acid, alanine and diaminopimeric acid were detected in a ratio of 1:3:1. Therefore, substance I was identified as UDP-\(\text{N}-\text{acetylglucosamine}\). Component II-2 showed the same *Rf* and *Rm* values as those of authentic UDP-\(\text{N}-\text{acetylglucosamine}\) on PPC and PIP, respectively. Component II-2 gave a radioactive spot of glucosamine on hydrolysis with 6\(\text{N}\) HCl at 100°C for 6 hr and also gave both \(\text{N}-\text{acetylglucosamine}\) and glucosamine on hydrolysis with 1\(\text{N}\) HCl at 100°C for 1 hr. On the basis of these results, component II-2 was identified as UDP-\(\text{N}-\text{acetylglucosamine}\).

Substance II was separated into two spots, II-1 and II-2, by PPC using solvent system B. The major component (II-2) showed an *Rf* value of 0.28 and the other (II-1) one of 0.04. Both components gave an UV adsorption pattern which agreed with that of authentic UDP-\(\text{N}-\text{acetylglucosamine}\). Component II-2 showed the same *Rf* and *Rm* values as those of authentic UDP-\(\text{N}-\text{acetylglucosamine}\) on PPC and PIP, respectively. Component II-2 gave a radioactive spot of glucosamine on hydrolysis with 6\(\text{N}\) HCl at 100°C for 6 hr and also gave both \(\text{N}-\text{acetylglucosamine}\) and glucosamine on hydrolysis with 1\(\text{N}\) HCl at 100°C for 1 hr. On the basis of these results, component II-2 was identified as UDP-\(\text{N}-\text{acetylglucosamine}\).

Minor substance II-1 gave muramic acid and equal amounts of glutamic acid and alanine on hydrolysis with 6\(\text{N}\) HCl at 100°C for 6 hr. Thus, minor component II-1 was considered to be UDP-\(\text{N}-\text{acetylmuramyl-glutamate}\).

Substance III was also separated into two substances, III-1 and III-2, by PPC using solvent system B. The former (*Rf* 0.24) was the minor component and the latter (*Rf* 0.5) was the major one judging from the extent of radioactivity. The minor component, III-1, showed a positive phosphomolybdate reaction and negative Elson-Morgan, Ninhydrin and alkaline silver nitrate reactions. Component III-1 gave radioactive \(\text{N}-\text{acetylglucosamine}\) on hydrolysis with alkaline phosphatase of *Escherichia coli*. Thus, III-1 was identified as \(\text{N}-\text{acetylglucosamine}-1\)-phosphate. Major component III-2 corresponded to glucosamine-6-phosphate on PPC and PIP using solvent system A, but it showed a different

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**Fig. 3.** Amino Acid Composition of the Substance I-Hydrolysate Analyzed with an Amino Acid Analyzer. Amm, ammonia; Glu, glutamic acid; Ala, alanine; DAP, diaminopimeric acid.

**Fig. 4.** Infrared Absorption Spectra of Substance III and Trehalose. Dotted line, trehalose; solid line, substance III.
Glucosamine Metabolism during Streptomycin Formation

![Infrared Absorption Spectra of Substance IV and L-Glutamate](image)

**Fig. 5.** Infrared Absorption Spectra of Substance IV and L-Glutamate.
Dotted line, L-glutamic acid monosodium salt; solid line, substance IV.

*Rf* value from glucosamine-6-phosphate on PPC using solvent system B. After deionization with ion-exchange resins (Amberlite CG-50 and CG-400) and concentration in *vacuo*, III-2 was applied on a cellulose column (Avicell, Asahikasei Kogyo Co.) and eluted with a solvent system of methanol–formate–water (85:15:5). The radioactive substance showed the same *Rf* and *Rm* values as those of trehalose on PPC (solvent systems A and B) and PIP, respectively. Moreover, III-2 gave only glucose after hydrolysis with hydrochloric acid and an identical infrared absorption spectrum to authentic trehalose (Fig. 4).

Substance IV was applied to an Amberlite CG-120 (H form) column, then washed with 0.1 M pyridine formate solution (pH 3.1) and eluted with 0.2 M of the same solution. The fraction of substance IV was further purified by column chromatography using Amberlite CG-400. After washing the column with water, the substances was eluted by gradient elution with 0 to 0.13 M formate–pyridine solution (pH 3.1). Substance IV was identified as L-glutamic acid by examination with an amino acid analyzer and from the infrared absorption spectrum shown in Fig. 5.

**Intracellular accumulation of trehalose in a growing culture**

The increase of SM-synthesizing activity was coordinated with that of radioactive substance III containing trehalose and substance IV containing glutamic acid. Glutamic acid formation in *S. griseus* has already been reported. On the other hand, since the correlation between intracellular trehalose accumulation and SM production was a new finding, it was further investigated in SM fermentation using a growing culture (Fig. 6). Cell
mass and trehalose in cells increased to the maximum levels until glucose was completely consumed and then decreased. The production of SM was coordinated with trehalose accumulation until complete consumption of glucose and further continued even after trehalose began to be consumed. Thus, SM production after consumption of glucose seemed to be due to the utilization of trehalose.

DISCUSSION

In the previous papers,\(^1,5\) a close biosynthetic correlation between cell wall and streptomycin was suggested on the basis of the facts that exogenous D-glucosamine was incorporated into streptomycin and cell walls and that interruption of the phospholipid cycle in mucoprotein synthesis led to an increase in utilization of glucosamine for streptomycin production. In the present investigation, exogenous \(^{14}\)C-glucosamine was found to be incorporated to a significant extent into four substances accumulated in the cell when cell wall synthesis was suppressed. These substances were identified as \(N\)-acetylmuramylpentapeptide, UDP-\(N\)-acetylglucosamine, trehalose and L-glutamic acid.

Trehalose has been reported to be a reserve substance in spores of fungi\(^{11-15}\) and yeast.\(^{16}\) As shown in Fig. 2, the ability of incorporation of \(^{14}\)C-glucosamine into SM, glutamic acid and trehalose rapidly increased while cell wall synthesis was suppressed. These substances were identified as \(N\)-acetylmuramylpentapeptide, UDP-\(N\)-acetylglucosamine, trehalose and L-glutamic acid.

Figure 6 shows the correlation between SM production and intracellular accumulation and consumption of trehalose in a growing culture in a medium containing glucose as a carbon source. Hunter and Hockenfull\(^{17}\) demonstrated that the SM molecule was derived from glucose. It is of interest that SM production still continued even after consumption of glucose in the culture broth. On the other hand, intracellular trehalose increased as long as glucose remained and immediately decreased after the complete consumption of glucose. There is an apparent coincidence in time lapse between SM production and trehalose consumption. Therefore, it seems likely that trehalose accumulated in cells is utilisable as a precursor or/and energy source for SM production after consumption of glucose.

REFERENCES