STUDIES ON BIOSYNTHESIS OF KASUGAMYCIN. III

BIOSYNTHESIS OF THE D-INOSITOL MOIETY

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The incorporation of various ^{14}C-labeled compounds into the D-inositol moiety was studied. Among U-^{14}C-maltose, U-^{14}C-glucose, U-^{14}C-mannose, 1-^{14}C-glycerol, U-^{14}C-pyruvate, 1-^{14}C-acetate, U-^{14}C-myoinositol, 1-^{14}C-glycine, 2-^{14}C-glycine and ^{14}C-D-inositol, only U-^{14}C-myoinositol was incorporated at a high rate. From the dilution test of D-inositol, D-inositol was proved not to be a precursor of the D-inositol moiety of kasugamycin. The radiogaschromatography of ^{14}C-kasugamycin showed the conversion of U-^{14}C-myoinositol to the ^{14}C-D-inositol moiety of kasugamycin.

The incorporation of ^{14}C-labeled compounds into kasugamycin, the kasugamine moiety and the side chain has been reported in previous papers[1,2]. Glucose is incorporated into kasuganobiosamine and more highly into kasugamine than into the residual parts. Glycine is almost exclusively incorporated into the side chain. Myoinositol is incorporated at a high rate into kasugamycin and almost no radioactivity is detected in the kasugamine moiety and the side chain.

In this paper, the incorporation of labeled compounds into the D-inositol moiety is reported.

Methods and Materials

The procedure of the shaking culture, the processes for isolation of kasugamycin, the method of degradation of kasugamycin to D-inositol, and the methods for determination of radioactivity have been described in a previous paper[1].

(1) Gas chromatography of myoinositol and D-inositol: Gas chromatography of the hydrolysate of ^{14}C-kasugamycin was carried out by the method of YAMAKAWA and UETA[3]. Ten mg of ^{14}C-kasugamycin hydrochloride monohydrate was dissolved in 2.0 ml of methanol containing 5.0 % HCl and the solution was heated at 100°C for 4 hours in a sealed tube. After methanolysis, the solution was neutralized with IR-4B (OH^- form) which had been washed with methanol and dried. The ^{14}C-methanolysate thus obtained was dissolved in 1.0 ml of anhydrous pyridine and was shaken with 0.2 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane for about 20 seconds. It was then heated at 60~70°C for several minutes. Three ml of chloroform and 3 ml of distilled water were added and shaken. After centrifugation the upper layer was discarded. This process was repeated 3 times to remove pyridine. The chloroform layer was evaporated *in vacuo*. The trimethylsilyl-^{14}C-methanolysate thus obtained was dissolved in a small amount of chloroform and a suitable amount of the solution was chromatographed with a column of 10 % Ucon LB 550X on Gaschrom CLH at 190°C. The gas chromatograph used was of the Hitachi-Perkin Elmer F-6D. The effluent was subjected to a radioisotope flow counter

KR-2. As reference, trimethylsilyl-myoinositol and trimethylsilyl-D-inositol were subjected to the same procedure.

(2) Preparation and incorporation of ^{14}C-D-inositol: ^{14}C-D-Inositol was prepared by hydrolysis of ^{14}C-kasugamycin obtained by the shaking culture with ^{14}C-myoinositol. ^{14}C-D-Inositol thus prepared has the specific activity of 5.31 μc/m mole. The incorporation rate of ^{14}C-D-inositol into kasugamycin, especially into the D-inositol moiety was studied by addition of 20.0 mg of ^{14}C-D-inositol to 125 ml of the cultured broth at 70 hours of the shaking culture and by isolation of ^{14}C-kasugamycin 19 hours thereafter.

(3) Dilution test: The dilution effect of D-inositol (20 mg) on the incorporation of U-^{14}C-myoinositol into kasugamycin was studied. Five mcg of U-^{14}C-moyoinositol (1.0 μc) was added to 125 ml of the cultured broth at 70 hours of the shaking culture together with or without 20 mg of D-inositol, and 19 hours thereafter kasugamycin was isolated.

Results and Discussion

The radioactivity of ^{14}C-kasugamycin was divided by 14 carbons and that of ^{14}C-D-inositol by 6 carbons, and radioactivity/each carbon is shown in Table 1. If a ^{14}C-compound is exclusively taken into the D-inositol moiety, then the ratio of the radioactivity/μM of carbon of D-inositol to that of kasugamycin will be 2.33. The incorporation rate of maltose, glucose, mannose and pyruvate into D-inositol was less than that into the residual parts. 1-^{14}C-Acetate, 1-^{14}C-glycine, and 2-^{14}C-glycine showed lower incorporation into the D-inositol moiety than into the other parts of kasugamycin. 1-^{14}C-Glycerol was incorporated equally into D-inositol and the residual parts. The data for the incorporation rate of glucose, glycerol, pyruvate, acetate, glycine and myoinositol are summarized and shown in Table 2. U-^{14}C-Glucose and U-^{14}C-pyruvate were more highly incorporated into kasugamine than into D-inositol. 1-^{14}C-Acetate was 6~7 times more highly incorporated into kasugamine than into D-inositol. 1-^{14}C-Glycerol was equally incorporated into kasugamine and into D-inositol. 1-^{14}C-Glycine and 2-^{14}C-glycine were incorporated almost exclusively into the side chain.

The incorporation rate of U-^{14}C-myoinositol into kasugamycin was higher than 50 % and as shown in Tables 1 and 2 most of it was taken into the D-inositol moiety. Addition of 20 mg of D-inositol to U-^{14}C-myoinositol showed no dilution effect as shown in Table 3. The incorporation rate of ^{14}C-D-inositol into kasugamycin was

Table 1. Distribution of ^{14}C in the D-inositol moiety of kasugamycin

^{14}C-compound	^{14}C added (μc)	Incorp. rate into kasugamycin (%)	Specific activity of kasugamycin ($\times 10^3$ cpm/μM)	Specific activity/μM of C		Relative incorp.* of D-inositol
				Kasugamycin	D-Inositol	
U-^{14}C-maltose	25.6	1.91	5.51	394	190	0.48
U-^{14}C-glucose	25.0	10.69	36.15	2,582	1,389	0.54
U-^{14}C-mannose	10.0	10.03	6.14	439	207	0.47
1-^{14}C-glycerol	7.5	1.41	0.96	57.9	64.2	1.11
U-^{14}C-pyruvate	10.0	1.28	0.97	69.3	37.7	0.54
1-^{14}C-acetate	6.3	0.13	0.06	4.3	1.0	0.23
U-^{14}C-myoinositol	6.0	60.29	36.74	2,624	5,815	2.22
1-^{14}C-glycine	25.0	18.52	48.27	3,448	15	0.004
2-^{14}C-glycine	10.0	22.39	22.74	1,624	8	0.005

* $\frac{\text{sp. act. of D-inositol part/}\mu\text{M of C}}{\text{sp. act. of kasugamycin/}\mu\text{M of C}}$

Table 2. Incorporation rate of U-^{14}C-glucose, 1-^{14}C-glycerol, U-^{14}C-pyruvate, 1-^{14}C-acetate, 1-^{14}C-glycine, 2-^{14}C-glycine and U-^{14}C-myoinositol into three moieties of kasugamycin

^{14}C-compound	Incorp. rate into kasugamycin % of ^{14}C added	Specific activity/μM			
		Kasugamycin	Kasugamine	Side chain	D-Inositol
U-^{14}C-glucose	10.69	2,582×14 cpm	4,522×6 cpm	342×2 cpm	1,389×6 cpm
1-^{14}C-glycerol	1.41	57.9×14	59.8×6	32.5×2	64.2×6
U-^{14}C-pyruvate	1.28	69.3×14	104.5×6	58.5×2	37.7×6
1-^{14}C-acetate	0.13	4.3×14	6.7×6	7.0×2	1.0×6
1-^{14}C-glycine	18.52	3,448×14	23×6	24,007×2	15×6
2-^{14}C-glycine	22.39	1,624×14	8×6	11,322×2	8×6
U-^{14}C-myoinositol	60.29	2,624×14	274×6	88.0×2	5,815×6

Table 3. Incorporation of U-^{14}C-myoinositol and ^{14}C-D-inositol

^{14}C-compound	U-^{14}C-myoinositol	U-^{14}C-myoinositol	^{14}C-D-inositol
Dilution	none	D-inositol	none
Specific activity	36.0 mc/mM	36.0 mc/mM	5.31 μc/mM
Total amount of ^{14}C added	1.0 μc	1.0 μc	0.591 μc
^{14}C of kasugamycin recovered	1.19×10^6 dpm	1.35×10^6 dpm	0.006×10^6 dpm
Incorporation rate	54.09 %	61.36 %	0.46 %

very low, 0.46 %, as shown in Table 3. Gas chromatography of the hydrolysate of kasugamycin labeled by addition of U-^{14}C-myoinositol during the fermentation also shows the utilization of myoinositol for the D-inositor moiety (Fig. 1).

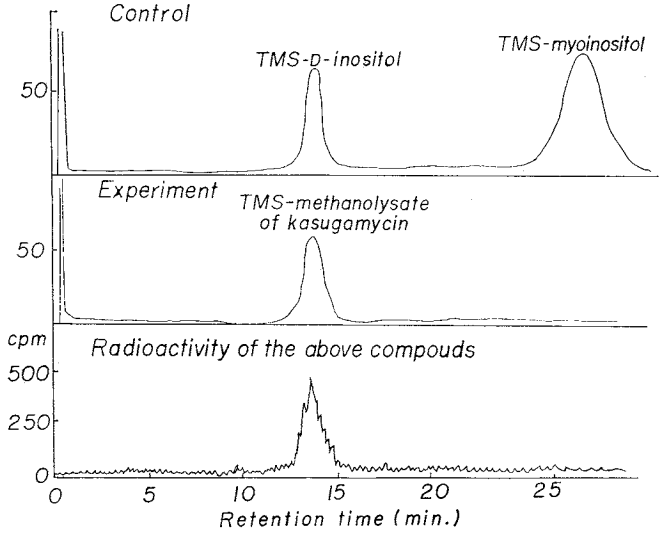

Fig. 1. Gas-liquid chromotography of HCl-methanolysate of kasugamycin
10% Ucon LB 550X on Gaschrom CLH, 190°C

In plants[4], animals[5] and yeasts[6,7], myoinositol is derived from the reductive ring closure of C-1 and C-6 of glucose through glucose-6-phosphate. The higher incorporation rate of glucose into D-inositol than 1-^{14}C-glycerol or U-^{14}C-pyruvate and the high incorporation of myoinositol into D-inositol suggest the biogenesis of myoinositol from glucose. When U-^{14}C-myoinositol was added, and the lipid fraction and the extracts of mycelium were analyzed by gas chromatography, D-inositol was not detected. ^{14}C-D-inositol was not incorporated into kasugamycin. Two possibilities are considered for the pathway of myoinositol to D-inositol. Myoinositol and D-inositol are different in stereochemistry of one carbon, an equatorial hydroxyl group in the former and axial in the latter. In one of the possible pathways, this equatorial hydroxyl methine of myoinositol is oxidized to a carbonyl group or the hydroxyl group is removed by

dehydration and the axial hydroxyl group of the methine of D-inositol is formed by reduction of the carbonyl group or hydration of the double bond. In this case, the axial hydrogen atom on this carbon is removed and the equatorial hydrogen atom is attached. Another possible pathway is that the hydroxyl group is phosphorylated. If, instead of the P-O fission, the O-C fission takes place in the hydrolysis of phosphate, then it is possible that after removal of phosphate the hydroxyl group from water binds with that carbon atom in the axial relation. In this case, the hydrogen atom on the hydroxyl methine is not removed. It is known that the C-O fission occurs in simple organic phosphate depending on the conditions of hydrolysis. As discussed in a previous paper[2], a type of biosynthesis resembling biosynthesis of cell wall can be considered for the biosynthesis of kasugamycin. Nucleotide kasugamine is first formed from glucose and then the side chain is formed from glycine. This nucleotide-deinositol-kasugamycin binds with myoinositol in lipid with concurrent separation of nucleotide, and forms kasugamycin. Though the experimental data are not sufficient, the comparative studies on the biogenesis of kasugamycin and other aminoglycosidic antibiotics may be helpful to find the general principle of biogenesis of aminoglycosidic antibiotics.

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