Regulation of N-Acetylkanamycin Amidohydrolase in the Idiophase in Kanamycin Fermentation

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Formation of N-acetylkanamycin amidohydrolase (NAKA) in a strain of *Streptomyces kanamyceticus* proceeded in parallel with the increase of both kanamycin (KM) and N-acetylkanamycin (NAK) in the initial period. NAKA formation then suddenly began to decrease in NAK but KM continued to increase before reaching a maximum level. The study on conversion of intracellular NAK into KM using washed cells has shown that the endogenous activity of NAKA is most efficiently induced by acetate together with the activity of isocitrate lyase and N-acetyltyrosine amidohydrolase. Sclerin (SCL) stimulated their induction in the same manner, while N-acetyltyrosine repressed NAKA. Glucose effect was relieved by SCL and some amino acids. From these results, change in the chemical composition of cells in idiophase was well elucidated.

It has been shown previously that the activity of KM acetyltransferase (KAT) essential in KM biosynthesis in the strain of *S. kanamyceticus* is associated with citrate lyase.^{1,5)} Citrate lyase, an anaplerotic enzyme, produces acetate and oxalacetate from citrate, and the acetate must be used by KAT for acetylation of KM-composing aminoglycoside. Oxalacetate inhibits the KAT reaction, as reported previously.¹⁾ Accumulation of acetyl~CoA then stimulates phosphoenolpyruvate (PEP) carboxylase,^{2~4)} elevating the concentration of oxalacetate. A high concentration of oxalacetate favors the carbohydrate synthesis in the amphibolic pathway. Thus, the formation of NAK, a precursor of KM, may be preceded by synthesis of a carbohydrate component in cells of trophophase. Conversely, the accumulation of NAK in the cells at idiophase stimulates aerobic decomposition of the carbohydrate removing the inhibition by acetyl~CoA on pyruvate dehydrogenase.^{2~4)} On the other hand, the function of NAKA in the idiophase might restore the gluconeogenesis from the acetate by liberating the acetate group from NAK. It is supposed that NAKA participates in the anaplerotic pathway in carbohydrate metabolism. In the present

paper, the effect of carbon-sources and SCL on the regulation of NAKA in idiophase was investigated using a washed-cell system. Changes in the chemical composition of cells during KM fermentation were also examined.

MATERIALS AND METHODS

Strain and culture condition. These were the same as in previous papers,^{1,5)} using *S. kanamyceticus* K. 133-28.

Preparation and incubation of washed-cell suspensions. About 1 g (moist weight) of cells grown for 48~72 hr was centrifuged, washing twice with 0.1 M Tris-HCl buffer (pH 8.5), suspended in 10 ml of 0.1 M Tris-HCl buffer (pH 8.5) in 100 ml Erlenmeyer flask, and incubated at 28°C for 24 hr with shaking.

Determination of KM and NAK (endogenous activity of NAKA). Production of KM in growing cells was measured as previously reported.⁵⁾ Conversion of intracellular NAK into KM in washed-cell suspensions was determined as follows: Cell suspensions were disrupted by ultrasonication (10 kc/sec for 5 min) at 3°C, adjusted at pH 2.0, centrifuged and neutralized with NaOH (free-KM). Then, the supernatant was hydrolyzed by 2 N NaOH at 100°C for 60 min and neutralized (total KM after alkali hydrolysis). KM potency in each neutralized solution was determined as previously reported.^{1,5)} NAK was calculated as total KM minus free-KM. Endogenous activity of NAKA

expressed as the conversion rate (%) of intracellular NAK into KM.

Activity of NAKA in cell-free extracts. The cells were collected by centrifugation at $3000 \times g$ for 10 min, washed twice with 0.1 M Tris-HCl buffer (pH 8.0), disrupted by a motor grinder with powdered glass, suspended in the same buffer and centrifuged at $30,000 \times g$ for 30 min. The supernatant was dialyzed against 0.1 M Tris-HCl buffer (pH 8.0) at 5°C for 24 hr and used as the enzyme solution. A reaction mixture containing enzyme solution (4~5 mg protein/ml) and 0.1 mM NAK in 10 ml of 0.1 M Tris-HCl buffer (pH 8.0) was incubated at 28°C for 180 min. After incubation, the amount of KM formed was determined as previously reported.^{1,5)} One unit (u) of the enzyme activity was expressed as, μmoles of KM liberated/100 mg dry cells/60 min.

Activity of N-acetyltyrosine amidohydrolase and isocitrate lyase. N-Acetyltyrosine amidohydrolase was measured by intact cells. A reaction mixture containing 1 g cells and 1 mM N-acetyltyrosine in 10 ml of 0.01M phosphate buffer (pH 8.5) was incubated at 28°C for 60 min with shaking. After incubation, the amount of tyrosine liberated was determined by the Ninhydrin method,⁶⁾ and 1 u of the enzyme activity was expressed as mg tyrosine released/100 mg dry cells/60 min. Isocitrate lyase was measured according to the method described by Daron and Ganalus,⁷⁾ by preparing a cell-free extracts similar to that described above but using a 0.1 M Tris-HCl buffer (pH 7.9) containing 0.005 M dithiothreitol. One unit (u) of the enzyme activity was expressed as μmoles of glyoxylate formed/100 mg dry cells/10 min.

Analysis of cell components. The contents of lipid, protein and carbohydrate in the cells were analyzed by the method of Schmidt-Thanhauser-Schneider.^{8~10)} Moreover, the carbohydrate content was checked by other methods,^{11~14)} and shown to contain approximately 60~95% sugars determined as glucose or glucosamine after hydrolysis. Total-N was determined by the method of Kjeldahl.¹⁵⁾

RESULTS

Formation of NAKA during KM fermentation and conversion of intracellular NAK into KM

As presented in Fig. 1, the activity of NAKA in *S. kanamyceticus* cell-free extracts during KM fermentation appeared just at 48 hr and increased for a while in parallel with the production of KM and NAK. The activity suddenly began to decrease NAK at 72 hr and gradually declined after attaining a peak at

96 hr. The production of KM increased linearly until the end. If the production of NAK did not occur after 72 hr, the linear increase of KM in the latter half of idiophase must depend on stimulation of the endogenous activity of NAKA.

FIG. 1. Time Course of the Formation of NAKA and Increase of NAK and KM in *S. kanamyceticus* during KM Fermentation.

○—○, KM, $\mu\text{g}/\text{mg}$ dry cells; Δ — Δ , NAK, $\mu\text{g}/\text{mg}$ dry cells; $\times$ — $\times$, activity of cell-free extracts, $10 \times \text{u}/100$ mg dry cells.

SCL has previously been found to stimulate the formation of KM and NAKA.⁵⁾ The effect of SCL on conversion of intracellular NAK into KM in washed-cell suspensions was examined. As shown in Table I, when growing cells were harvested at 48 hr for preparing the

TABLE I. EFFECT OF SCL ON CONVERSION OF INTRACELLULAR NAK INTO KM IN WASHED-CELL SUSPENSIONS DURING INCUBATION FOR 24 HR

Age of cells hr	SCL ^{a)} ppm	NAK $\mu\text{g}/\text{ml}$		KM formed $\mu\text{g}/\text{ml}$	Conversion ^{b)} rate %
		Initial	Remaining		
48	0	82	90	13	16
48	5 ^{c)}	82	100	17	21
72	0	117	50	62	53
72	5	117	30	90	78

^{a)} SCL was added in 0.1 M Tris-HCl buffer (pH 8.5) containing 0.5% glucose and 0.01 M MgSO_4 .

^{b)} KM formed/NAK initial $\times 100$.

^{c)} 5 ppm = 2.1×10^{-5} M.

Washed-cell suspensions were prepared and incubated as described in MATERIALS AND METHODS.

washed cells, intracellular NAK increased during incubation for 24 hr, and its conversion rate into KM was very low. At this period SCL had only a slight effect. With cells grown for 72 hr, SCL markedly stimulated the conversion of NAK into KM. The effect of SCL may have a close relationship to the metabolic pattern or the structure of cells in the transition period from trophophase to idiophase. As shown in Table II, when cells were pre-

TABLE II. EFFECT OF PRETREATMENT OF CELLS ON ENDOGENOUS ACTIVITY OF NAKA

Pretreatment	Endogenous activity ^{a)} of NAKA
Control	16
Freeze-drying ^{b)}	32
Acetone ^{b)}	61
Toluol ^{c)}	100

^{a)} Conversion rate (%) of intracellular NAK into KM was computed by the same formula as in Table I.

^{b)} Dried cells at 70 mg corresponding to 1 g moist weight were used.

^{c)} Washed cells were stirred for 1 hr in 0.01 M phosphate buffer containing 2% toluol and filtrated. After treatment, 1 g of moist cells was collected and used.

Age of cells used was 48 hr.

treated by toluol, intracellular NAK was completely converted by NAKA into KM under the same conditions as described above. Pretreatment of cells by acetone or freeze-drying also stimulated the endogenous activity of NAKA to some extent.

Effect of sugars and SCL on endogenous activity of NAKA

As shown in Table III, when washed cells were incubated with a high concentration (1%) of sugars such as glucose, fructose, mannose, maltose and lactose, the endogenous activity of NAKA was markedly repressed while sucrose and gluconate had no repressive effect. Raffinose and starch had a rather stimulating effect. As shown in Table IV, the repression of NAKA by glucose was reversed by SCL and some amino acids, such as aspartic and glutamic acids. Though cyclic AMP

TABLE III. EFFECT OF SUGARS ON ENDOGENOUS ACTIVITY OF NAKA

Sugars at 1%	Endogenous activity ^{a)} of NAKA
Glucose	18
Fructose	2
Mannose	25
Galactose	42
Lactose	0
Maltose	30
Sucrose	57
Raffinose	82
Starch	62
Gluconate	57
Control (glucose 0.5%)	53

^{a)} Method of computation is same as Table II for detail see MATERIALS AND METHODS.

TABLE IV. EFFECT OF SCL AND AMINO ACIDS ON GLUCOSE REPRESSION OF NAKA

Addition	Endogenous activity ^{a)} of NAKA at glucose concentration(%)		
	0.5	1.0	2.0
None	53	18	12
SCL (2×10^{-5} M)	78	49	45
SCL (4×10^{-5} M)	53	40	32
c-AMP (0.9×10^{-5} M)	50	50	45
Asp (0.1%)	55	70	25
Glu (0.1%)	65	65	25
Asp + SCL (4×10^{-5} M)	—	—	85
Glu + SCL (4×10^{-5} M)	—	—	60

^{a)} The same as in Table III.

abolished the glucose effect, it had no stimulatory effect on NAKA at a low glucose concentration.

Effect of acetate on endogenous activity of NAKA and the activity of isocitrate lyase and N-acetyltyrosine amidohydrolase

As shown in Table V, among carbon sources other than sugars, acetate specifically stimulated the endogenous activity of NAKA at a low concentration. Also, as shown in Table VI, acetate induced the activity of isocitrate lyase and N-acetyltyrosine amidohydrolase in the washed-cell suspensions. Moreover, as shown in Table VII, SCL stimulated the activity of both isocitrate lyase and N-acetyl-

TABLE V. EFFECT OF ACETATE, ETHANOL AND OXALACETATE ON ENDOGENOUS ACTIVITY OF NAKA

Addition at 5 ppm	Endogenous activity ^{a)} of NAKA
None	42
Acetate	81
Ethanol	64
Oxalacetate	51

^{a)} The same as in Table III.

TABLE VI. EFFECT OF ACETATE ON INDUCTION OF ISOCITRATE LYASE AND N-ACETYLTYROSINE AMIDOHYDROLASE IN WASHED-CELL SUSPENSIONS

Washed-cell suspensions were incubated for 24 hr as described in the text.

Acetate	Activity of	
	Isocitrate ^{a)} lyase	N-Acetyltyrosine ^{b)} amidohydrolase
0	0.69	0.95
2.5	0.89	—
5.0	1.02	1.90

^{a)} u/100 mg dry cells/10 min (Age of cells was 72 hr).

^{b)} u/100 mg dry cells/60 min.

TABLE VII. EFFECT OF SCL ON THE ACTIVITY OF ISOCITRATE LYASE AND N-ACETYLTYROSINE AMIDOHYDROLASE

SCL ppm	Activity ^{a)} of	
	Isocitrate lyase	N-Acetyltyrosine amidohydrolase
0	1.15	1.06
0.1	1.48	1.96
1.0	1.89	1.97
10.0	1.56	1.99

^{a)} The activity was calculated in the same manner as in Table VI, but age of cells used was 96 hr.

TABLE IX. CHANGES IN THE CHEMICAL COMPOSITION OF CELLS DURING KM FERMENTATION WITH OR WITHOUT ADDITION OF SCL

Age ^{a)} of cells hr	None				Addition SCL (5 ppm)			
	Lipid	Carbohydrate	Protein	Total-N	Lipid	Carbohydrate	Protein	Total-N
24	16.6	26.4	24.8	11.8	13.5	40.1	23.3	9.8
48	28.8	15.9	21.2	8.8	25.8	13.4	20.6	8.3
72	28.7	16.3	20.9	8.3	25.9	16.6	20.3	8.0
96	25.5	17.3	16.2	7.8	22.9	21.6	15.2	7.8
120	25.4	24.3	14.0	7.8	21.6	35.1	11.5	7.3

^{a)} The cells were grown in a medium containing 2% starch, 2% maltose, 1% NaNO₃ and 0.01% CaCO₃ in 3% soybean meal extract (pH 7.0) at 28°C with shaking.

tyrosine amidohydrolase. With the latter enzyme activity, there was a question on identity with NAKA. As shown in Table VIII, the

TABLE VIII. EFFECT OF N-ACETYLTYROSINE ON ENDOGENOUS ACTIVITY OF NAKA

N-Acetyltyrosine ^{a)} mM	Activity ^{b)} of N-Acetyltyrosine amidohydrolase	NAKA
0	1.02	56
0.1	0.95	14
1.0	0.95	10
10.0	1.00	6

^{a)} N-Acetyltyrosine was added to 0.01 M phosphate buffer (pH 8.5) containing 0.5% glucose and 0.01 M MgSO₄.

^{b)} Calculated by same method as in Tables III and VI.

addition of N-acetyltyrosine markedly repressed the endogenous activity of NAKA in the washed-cell suspensions. This indicates the presence of N-acetyl amino acid amidohydrolase other than NAKA in the cells.

Changes in the chemical composition of cells and isocitrate lyase level during KM fermentation

Changes in the chemical composition of cells during KM fermentation were investigated to examine the role of isocitrate lyase in the cells. As shown in Table IX, among several cell components, carbohydrate exhibited a characteristic change, and the change was prominent especially with cells grown in the presence of SCL. The carbohydrate content of cells in trophophase increased rapidly within

24 hr growth, but decreased and fell to a minimum at 48 hr. The content in idiophase then increased gradually and was restored to near the initial level. On the other hand, as shown in Table X, the level of isocitrate lyase

TABLE X. CHANGE IN THE LEVEL OF ISOCITRATE LYASE DURING KM FERMENTATION WITH OR WITHOUT ADDITION OF SCL

Age ^{a)} of cells hr	Activity ^{b)}	
	No addition	SCL (5 ppm)
24	2.43	2.16
48	1.53	0.88
72	0.81	1.30
96	1.12	1.33
120	1.42	1.58

^{a)} The same as in Table IX.

^{b)} u/100 mg dry cells/10 min.

during KM fermentation changed in accordance with the carbohydrate content of cells.

DISCUSSION

As shown in the present experiments, the formation of NAKA in *S. kanamyceticus* is associated with a gluconeogenic metabolism via glyoxylate bypass, the key enzyme of which is isocitrate lyase. NAKA provides acetate to the glyoxylate cycle and NAK serves as the acetate pool in the cells. It is of significance that the endogenous activity of NAKA in the washed-cell suspensions is induced by acetate along with the activity of isocitrate lyase and N-acetyltyrosine amidohydrolase. Acetate first stimulates the enzymes of glyoxylate cycle, depressing the level of isocitrate, and the loss of isocitrate or citrate in the cells may in turn induce the activity of NAKA. N-Acetyltyrosine represses the activity of NAKA in washed cells having the activity of N-acetyltyrosine amidohydrolase. This may be due to the inhibition of the reductive process of gluconeogenesis by some degradative reaction of tyrosine which requires NADPH.

SCL stimulates the activity of isocitrate lyase *per se* and increases the carbohydrate

synthesis in cells in early trophophase and in the latter half of idiophase, as described above. The effect of SCL may involve the formation of acetyl~CoA through the activity of citrate lyase, acetate kinase and phosphotransacetylase, as reported previously.¹⁾ On the other hand, the carbon source has a relationship to the regulation of NAKA, where the adenylate energy charge may be significant, because cyclic AMP releases the repression of NAKA by glucose. Since SCL has a stimulating effect on amino acid incorporation into the protein fraction of plant and animal tissues, the reverse effect by the combination of SCL and some amino acids, such as aspartic and glutamic acids, on catabolite repression of NAKA may result from the compensation of the adenylate energy consumed for protein synthesis. The change in chemical composition of cells during KM fermentation may involve a structural change in the cells. The effect of toluol-treatment on the endogenous activity of NAKA appears to have a profound relationship to the structure of the cell wall or cell membrane.

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