Kor. J. Appl. Microbiol. Bioeng.
Vol. 17, No. 1, 46—50 (1989)

Mechanism of Phosphate Regulation of Cephalosporin C Biosynthesis in *Cephalosporium acremonium*

Choi, Sang-Ho, Kyoung Lee, Byung-Dae Yoon and Tae-Ick Mheen*

Microbial Technology Laboratory, Genetic Engineering Center,
Korea Advanced Institute of Science and Technology
P.O.BOX 131, Cheongryang, Seoul 136-791, Korea

*Cephalosporium acremonium*의 Cephalosporin C 생합성에 있어 무기인의 조절기작

최상호·이 경·윤병대·민태익*

한국과학기술원 유전공학센터 미생물공학연구실

A high concentration of inorganic phosphate (above 25 mM), which was suboptimal for vegetative growth in the minimal production medium, suppressed cephalosporin C (CPC) production in *Cephalosporium acremonium*. Results from the determination of intracellular concentrations of ATP, ADP and AMP with phosphate-starved resting cells indicated that phosphate exerted its effect indirectly by regulating the ratio of adenylated nucleotides, the so-called adenylated energy charge. It was also found that the type of phosphate regulation of CPC biosynthesis was not a repression effect but an inhibition effect.

Inorganic phosphate exerts a depressive effect on the production of many antibiotics by microorganisms (1). Phosphate generally supports extensive cell growth up to 300 mM, but high concentrations of phosphate (above 10 mM) suppress the biosynthesis of many antibiotics. Although the mechanism by which different organic or inorganic phosphate sources control secondary metabolism is not yet known, the maximum product yields in most antibiotics fermentations are obtained at phosphate concentrations that are suboptimal for vegetative growth (2).

In the last few years, the mechanism of phosphate regulation on the production of antibiotics has been studied at molecular level. It was reported that addition of phosphate rapidly increase the intracellular concentration of ATP followed by inhibition of incorporation of labelled precursors into candicidin (4). This suggested that ATP might be an intracellular effector involved in phosphate control of antibiotic biosynthesis. On the other hand, Atkinson and coworkers maintained that the ratio of adenine nucleotide concentrations is a regulatory parameter coordinating energy-utilizing and energy-regenerating metabolic pathways, and is more important than the absolute concentration of ATP (5,6).

The biosynthesis of cephalosporin C (CPC) in *C. acremonium* is also extremely sensitive to the higher concentration of inorganic phosphate that showed suboptimal growth (3). The molecular mechanism of phosphate control on CPC biosynthesis, however, is only partially understood. Even though Kuenzi (3) has reported that a combined effect was exhibited by glucose and phosphate, but this view is not generally accepted (1).

Key words: *Cephalosporium acremonium*, CPC biosynthesis, Phosphate regulation, Energy charge
*Corresponding author

An important question is whether intracellular orthophosphate is the ultimate effector or it merely regulates the level of some other intracellular effector that controls the biosynthesis of CPC. Therefore, we felt that it was important to study the intracellular level of ATP and the adenylate energy charge of CPC-producing cells to elucidate the intracellular effector mediating the phosphate effect.

Materials and Methods

Microorganisms, media and culure conditions

A superior CPC-producing strain, N-5 which was derived from *Cephalosporium acremonium* ATCC 20339 by serveral mutations was used through all experiments.

One loopful of slant culture of seed medium containing 3.0% sucrose, 1.5% beef extract, 0.5% corn steep liquor and 0.15% $CaCO_3$ was inoculated into 15 m*l* of the same medium in 100-m*l* Erlenmeyer flask as described by Kanzaki (7). After growing for 3 days at 28 °C in a rotary shaker at 250 rpm, 1 m*l* of the seed culture was transferred to 15 m*l* of minimal production medium in 100-m*l* Erlenmeyer flask.

Minimal production medium was composed as follows; 3.6% sucrose, 0.2% methyloleate, 0.75% $(NH_4)_2SO_4$, 0.4% DL-methionine and 10% (v/v) salt mixture (containing in g/*l*; Na_2SO_4 7.5, $MgSO_4 \cdot 7H_2O$ 1.8, $CaCl_2$ 0.6, $Fe(NH_4)_2SO_4 \cdot 6H_2O$ 1.5, $MnSO_4$ 0.3, $ZnSO_4 \cdot 2H_2O$ 0.3, $CaSO_4 \cdot 5H_2O$ 0.075). Phosphate was added to each media with the same ratio of KH_2PO_4 and K_2HPO_4 up to the concentrations given in the figure legend. Additionally, each media were initially buffered with 0.1M 3-(N-morpholino) prophanesulfonic acid (MOPS) at pH 7.2.

Starvation of intracellular phophate

The medium for phosphate starvation was derived from the minimal production medium without supplementation of phosphate. The phosphate starvation was carried out as follows; mycelia grown for 72 hr in the minimal production medium were harvested and washed twice with sterile saline. Then cells were resuspended to the starvation medium and incubated for 12 hr at 28 °C in a rotary shaker at 250 rpm. This is an attempt to eliminate the influence of the stored phosphate of the cells in the production of CPC with cell suspension.

Resting-cell Experiments

After the phosphate starvation, mycelia were harvested and washed twice with sterile phosphate-free water. These cells were suspended in 100-m*l* Erlenmeyer flasks containing 20 m*l* of 20 mM and 200 mM potassium phosphate reaction buffer, respectively. And its cell density was adjusted to 10 mg per m*l* as dry cell weight (DCW). To stabilize pH,

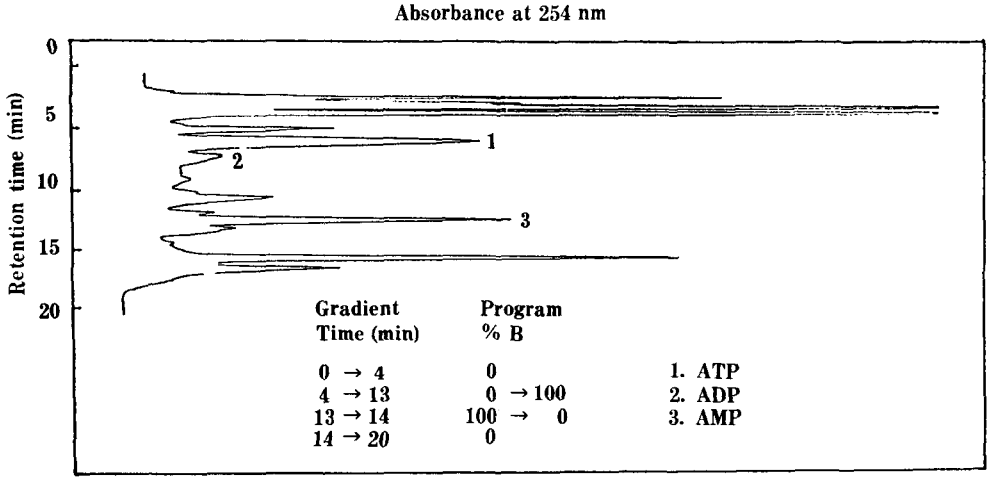

Fig. 1. Separation of nucleotides in a perchloric acid extracts of *C. acremonium*.
Solvent system, A: Potassium phosphate buffer (0.1M, pH6.0)
B: Solvent A + 10% (v/v) Methanol

0.1 M MOPS was added into each flask. These resting cells were incubated and shaked with 200 rpm at 28 °C. At time intervals, samples were removed and centrifuged at 10,000 rpm for 10 min. Supernatants were used for the analysis of the CPC titer and mycelia were extracted for the investigation of intracellular concentrations of adenylated nucleotides.

Analysis of intracellular nucleotides

The formation of cell-free extracts was carried out as previously described by Lee *et al.* (8). Adenylated nucleotides were separated and analyzed on a reverse phase (RP) C-18 HPLC column 0.39× 30cm, Lichrosorb, Merck) and monitored at 254 nm. Buffer system and other conditions were the same to the method of Crescentini *et al.* (9). Figure 1 represented the peak profile with retention times identical to those of the authentic adenylated nucleotides.

Assay of CPC

Culture filtrates were used for CPC assays through out the experiments. Amounts of CPC in culture broth was determined by the agar diffusion method using paper disc. *Alcaligenes faecalis* ATCC 8750 was used as a test organism.

Results and Discussion

Effect of Phosphate on the CPC Production

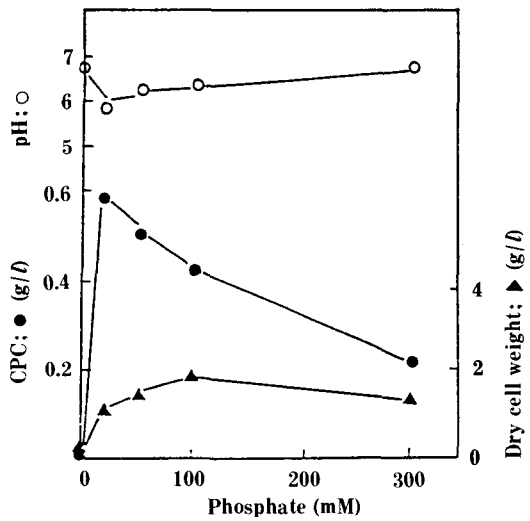

Fig. 2. Cephalosporin C biosynthesis in the minimal production media by different phosphate levels.

Figure 2 showed that phosphate in the concentration of 100 mM was required to support maximum growth, but the maximum production of CPC occured at 25 mM phosphate in the minimal production media. And CPC production was drastically decreased by increasing of the phosphate concentration.

One of the problems involved in the determination of the effect of phosphate concentrations was varying pH. Even though pH dropped to 5.0-6.0 in spited of the presense of MOPS buffer as shown in Fig. 2, it had been found by previous work in our research group that this range of pH variation could not cause considerable depresson of CPC biosynthesis (10). As a conclusion, in the presence of MOPS, we were able to detect an inhibitory effect of phosphate on CPC production, and this effect clearly was not due to pH changes.

Phosphate inhibition in the biosynthesis of CPC by resting-cell system

It was interesting to establish whether the phosphate effect was due to a repression or an inhibition of CPC-synthesizing enzymes related to CPC biosynthetic pathway. Cells grown in low (20 mM) and high (200 mM) concentration of potassium phosphate were suspended in phosphate-free medium for 12 hr and were used to the experiments of phosphate starvation. Phosphate-starved cells were resuspended to reaction buffer containing low (20 mM) and high (200 mM) concentration of phosphate. In these experiments, we investigated the inhibitory and the repressive levels of the CPC-synthesizing enzymes in the presence of the different content of phosphate as described above.

Table 1. Phosphate inhibition in biosynthesis of cephalosporin C by *C. acremonium* in buffer with low and high phosphate contests

Phosphate in media for growth	Cephalosphorin C (μg/g-DCW)*	
	Phosphate added	
	20 mM	200 mM
20 mM	2950	N.D**
200 mM	2840	N.D

*; Cephalosporin C titer was determined after 12 hr in the reaction buffer.
**; No-detected in bioassay

Table 2. Intracellular levels of adenylated compounds and energy charges in *C. acremonium* in buffer with low and high phosphate contents

Phosphate in media for growth	Adenylated compound	Concentration of adenylated compound (μmole/g-DCW)				
		0 hr (Control)	3 hr		6 hr	
			Phosphate added			
			20 mM	200 mM	20 mM	200 mM
20 mM	ATP	2.42	2.27	1.68	2.04	1.27
	ADP	0.32	0.40	0.19	0.58	0.00
	AMP	0.32	0.68	0.34	0.72	0.23
	EC*	0.84	0.73	0.80	0.70	0.85
200 mM	ATP	1.75	2.06	1.56	3.25	1.06
	ADP	0.00	0.31	0.21	0.61	0.00
	AMP	0.00	0.78	0.23	1.11	0.18
	EC	1.00	0.70	0.83	0.71	0.85

*; Energy chargy = $\frac{[ATP] + 1/2[ADP]}{[ATP] + [ADP] + [AMP]}$

Table 1 represented that the biosynthesis of CPC was severely reduced by the high (200 mM) concentration of exogeneous phosphate regardless of the cells which were grown in low or high concentration of phosphate. These results suggested that phosphate inhibits the activities of preformed CPC-synthesizing enzymes, or decreases the overall flux of precursors through the cephalosporin biosynthetic pathway by the same way as the suggestion of Martin *et al.* (1).

In conclusion, the reduction of CPC production by the phosphate was exerted by an inhibition effect, not by a repression effect. As a matter of fact, the determination of phosphate concentration during the fermentation illustrated that production of CPC was started only after depletion of the phosphate in culture broth (11).

Relationship between the energetics of the cell and CPC production

As observed in Table 2, regardless of the phosphate concentration in which cells were grown, the intracellular ATP levels were roughly 2.0-3.0 μmole/g-DCW by the cells in 20 mM phosphate buffer and 1.0-1.5 μmole/g-DCW in 200 mM phosphate buffer, respectively. The result from the decreasing levels of ATP followed by the addition of phosphate was just the opposite to the data reported for other microorganisms. Silaeva *et al.* (12) and Janglova *et al.* (13) reported that the ATP levels were lower in high antibiotics producer of *Bacillus brevis* and *Streptomyces aureofaciens* than in low producer of these microorganisms. Previous work by Martin *et al.* (4) also pointed out that phosphate inhibition of candicidin production by *Streptomyces griseus* was accompanying a rapid increase in intracellular ATP concentration.

Table 2, however, illustrated that the intracellular leves of ADP and AMP increased in the buffer of 20mM phosphate buffer and decreased in the 200 mM buffer. Therefore, the energy change (EC) defined by Atkinson and Walton (5) [i.e., EC = ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]) was a more likely than ATP concentration in mediating phosphate control of CPC biosynthesis. As shown in Table 2, the energy charges of *C. acremonium* were about 0.70 in 20 mM phosphate buffer and 0.85 in 200 mM phosphate buffer, respectively. This possible role of energy charge in phosphate regulation is in agreement with the data from Atkinson and his coworkers (5,6). So, they suggested that energy charge may for some purposes be considered analogous to an electrochemical storage cell as an energy-handling device. And their results also indicated that growth are more closely related to the energy charge value than to the ATP concentration.

As a conclusion, these results suggested that the adenylated energy charge rather than the intracellular level of ATP should be the intracellular ef-

fector madiating phosphate effect on the biosynthesis of CPC. In point of energetics, the low level of energy charge is required for CPC production in *Cephalosporium acremonium*.

요 약

최소 생산배지에 포함된 25 mM 이상의 무기인(inorganic phosphate)은 *Cephalsphorium acremonium*의 생육은 증대시켰으나, cephalosporin C(CPC) 생산은 감소시켰다. Phosphate-starved 휴지세포계를 이용하여 균체내의 ATP, ADP와 AMP를 측정하였다. 그 결과로부터 무기인은 adenylated nucleotide의 농도의 비율로 표시되는 energy chargy를 조절하여 CPC 생산성에 영향을 주는 것으로 밝혀졌다. 또한, 무기인에 의한 CPC 생합성은 repression 효과에 의한 것이 아니라 inhibition 효과에 의해 조절받는 것을 알 수 있었다.

References

1. Martin, J.F.: in "Advances in Biochemical Engineering" (Ghose, T.K. *et al.*, eds). vol. 6, p. 105 Springer-verlag, Berlin (1977).
2. Weinberg, E.D.: *Dev. Ind. Microbiol.*, **15**, 70 (1974).
3. Kuenzi, M.T.: *Arch. Microbiol.*, **128**, 78 (1980).
4. Martin, J.F., P. Lias and A.L. Demain: *Biochem. Biophy. Res. Commun.*, **83**, 822 (1978).
5. Atkinson, D.E. and G.M. Walton: *J. Biol. Chem.*, **242**, 3239 (1967).
6. Swedes, J.S., R.J. Sedo and D.E. Atkinson: *J. Biol. Chem.*, **250**, 6930 (1975).
7. Kanzaki, T., T. Fukita, K. Katamoto, K. Nara and Y. Nakao: *J. Ferment. Technol.*, **54**(10), 720 (1976).
8. Lee, K., S.H. Choi, J.J. Lee and T.I. Mheen: *Kor. J. Appl. Microbiol. Bioeng.*, **15**(5), 36 (1987).
9. Crescentini, G. and V. Stocchi: *J. Chromatography*, **290**, 393 (1984).
10. KAIST Report, BSE 707-2446-5 (1986).
11. KAIST Report, BSN 7039-92-3 (1988).
12. Silaeva, S.A., V.M. Glazer, S.V. Shestakov and M.A. Protofiev: *Biokhimiya*, **30**, 947 (1965).
13. Janglova, Z., J. Suchy and Z. Vanek: *Folia. Microbiol.*, **14**, 208 (1969).

(Received November 7, 1988)