Vol. 83, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1978 Pages 822-828

# ATP AND ADENYLATE ENERGY CHARGE DURING PHOSPHATE-MEDIATED CONTROL OF ANTIBIOTIC SYNTHESIS

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Received February 22,1978

SUMMARY: The addition of inorganic phosphate or guanosine-5'-monophosphate to a phosphate-limited mycelial system of Streptomyces griseus inhibited candicidin production. Accompanying the inhibition was a rapid increase in intracellular ATP concentration. Adenylate energy charge increased only slightly indicating that ATP is a more likely intracellular effector than energy charge in mediating phosphate control of antibiotic biosynthesis.

Inorganic phosphate exerts a depressive effect on the synthesis of many antibiotics (1). Antibiotics belonging to different biosynthetic groups are susceptible to such phosphate regulation, including peptide antibiotics, polyene macrolides, tetracyclines and biosynthetically complex antibiotics. Industrial production of these antibiotics is often carried out at concentrations of inorganic phosphate which are limiting for growth. Phosphate in the range of 0.3-300 mM generally supports extensive cell growth, but concentrations of 10 mM and above suppress the biosynthesis of many antibiotics.

Because the various antibiotics subject to phosphate regulation are synthesized via different pathways, many different mechanisms must exist, or else a common regulatory effector must act upon the different biosynthetic pathways (2). Phosphate addition not only inhibits antibiotic synthesis but also results after several hours in a reversal of non-growing, antibiotic-producing cells back to a growing, non-producing state (3).

0006-291X/78/0833-0822$01.00/0

In the last few years, studies have been carried out on the regulatory effect of phosphate on the biosynthesis of the polyene macrolide candicidin, by Streptomyces griseus (3, 4, 5). A phosphate-limited mycelial system that synthesizes candicidin in absence of growth and is highly sensitive to phosphate inhibition has been developed (6). Addition of phosphate to this system, at a concentration above 1 mM, results in a rapid concentration-dependent inhibition of the synthesis of antibiotic. An important question is whether intracellular orthophosphate is the ultimate effector or whether it merely regulates the level of some other intracellular effector that controls expression of antibiotic biosynthesis.

We felt that it was important to study the intracellular level of ATP and the adenylate energy charge of candicidin-producing cells and phosphate-inhibited non-producing cells to establish the relationship between the energetics of the cell and antibiotic production.

## MATERIALS AND METHODS

Resting cell system. Streptomyces griseus was grown and suspended in phosphate-limited synthetic medium as reported previously (6). Incorporation of labeled precursors into candicidin and purification of labeled candicidin was carried out as described elsewhere (5).

ATP determination. One-milliliter culture samples were rapidly pipetted (within 15 sec) into 0.2 ml of ice-cold 35% perchloric acid. After 2 min at 0ºC, the suspension was rapidly frozen in liquid nitrogen and kept frozen until assayed. After thawing, the suspension was thoroughly mixed and centrifuged at 10,000 g for 5 min. The supernatant fluid was neutralized with 0.4 ml of 2.6 M KOH. The final pH was adjusted to 7.4 with 100 µl of 1 M phosphate buffer, pH 7.4. After at least 30 min at 0ºC, the $KClO_4$ precipitate was removed by centrifugation. Recovery of externally added ATP was 85%. Aliquots of the supernatant nucleotide extract were assayed within hours of neutralization, and the rest was frozen in liquid nitrogen. No loss of ATP was observed when samples or standards were kept frozen for up to 2 weeks. ATP was determined by the luciferase reaction by use of the scintillation counter (Packard, Tricarb 3230) as photometer. One vial of crystalline luciferin-luciferase (Dupont de Nemours and Co.) was dissolved in 3 ml of morpholinopropane sulfonic acid (MOPS) buffer, pH 7.4. Prior to the enzymatic reaction, the enzyme solution was diluted 1/10 with distilled water. A reaction cuvette containing 100 µl of enzyme solution, 500 µl of assay buffer (0.2 M glycine-NaOH buffer, pH 7.4), and 500 µl of 15 mM magnesium chloride, was placed in the belt of the scintillation counter and 10-100 µl of ATP extract were rapidly injected into the vial. Standard ATP solutions in the range $10^{-7}$ to $10^{-5}$ M ATP (10 pmoles to 1 nmole per 100-µl sample) gave a reproducible linear response. The concentration of ATP in the extracellular fluid of the resting culture (Millipore filtration) was always

two orders of magnitude lower than the intracellular levels, and in most cases was undetectable.

Adenylate energy charge determination. For adenylate energy charge determination, the nucleotides were extracted and ATP determined as before. ADP was converted to ATP in a reaction mixture containing 100 µl of sample extract, 50 µl of 75 mM phosphate buffer (pH 7.4), 50 µl of 50 mM magnesium chloride, 25 µl of 0.5 mM phosphoenolpyruvate, and 20 µg of pyruvate kinase in a total reaction volume of 270 µl. Pyruvate kinase and phosphoenolpyruvate were purchased from Sigma Chemical Co. ADP and AMP were converted to ATP to determine total adenylate in a reaction mixture containing 100 µl of sample extract, 50 µl of 75 mM phosphate buffer, 50 µl of 50 mM magnesium chloride, 25 µl of 0.5 mM phosphoenolpyruvate, 20 µg of pyruvate kinase, and 250 µg of adenylate kinase (myokinase) in a total reaction volume of 270 µl. Adenylate kinase (Sigma, Grade V) was dialyzed against 50 mM potassium phosphate buffer, pH 7.4, for 24 h at 4ºC before use. The two reaction mixtures and a control reaction mixture lacking phosphoenolpyruvate, pyruvate kinase, and adenylate kinase were incubated at 30ºC for 15 min and then kept in ice until assayed. The ATP content of 100-µl aliquots of the three reaction mixtures was determined as before. ADP and AMP were determined by difference. Energy charge was calculated as (ATP) + ½ (ADP)/(ATP) + (ADP) + (AMP).

## RESULTS AND DISCUSSION

Phosphate addition inhibits the incorporation of the candicidin precursors ($^{14}$C)propionate and p-($^{14}$C)aminobenzoic acid, into candicidin (Table 1). The inhibition is observed after 15 min following addition of 10 mM phosphate.

As fig 1 illustrates, the intracellular steady-state ATP level of the phosphate-limited resting cells remained roughly constant at 2.0-3.0 µmole/g of dry weight over a period of several hours (Fig. 1, controls). This value is very similar to the ATP levels reported in Escherichia coli (7). The intracellular ATP level increased rapidly following addition of 10 mM phosphate to the antibiotic-producing cells; it doubled within 5 min of phosphate addition. This increase occurred prior to the inhibition of antibiotic synthesis, which is usually first seen 15 min after phosphate addition. The rate of protein or RNA synthesis does not change during this time (4).

The possible involvement of ATP in controlling antibiotic biosynthesis was suggested by the data of Silaeva et al (8) and Janglova et al (9). These investigators reported that the ATP levels were lower in high-producing strains of Bacillus brevis and Streptomyces aureofaciens than in low-producing strains of these microorganisms. A recent report by Fynn and

Table 1 Phosphate Inhibition of the Incorporation of Labeled Precursors into Candicidin[1]

| Time | Addition of ($^{14}$C)propionate | | Addition of p-($^{14}$C)aminobenzoic acid | |
|---|---|---|---|---|
| | Control | + 10 mM phosphate | Control | + 10 mM phosphate |
| (min) | ($10^3$ cpm) | ($10^3$ cpm) | ($10^3$ cpm) | ($10^3$ cpm) |
| 0 | 0 | 0 | 0 | 0 |
| 15 | 36 | 28 | 8 | 7 |
| 45 | 68 | 35 | 17 | 11 |
| 80 | 89 | 40 | 28 | 17 |
| 120 | 122 | 44 | 47 | 21 |

[1] ($^{14}$C)propionate, a precursor of the macrolide ring, and p-($^{14}$C)aminobenzoic acid, a precursor of the aromatic moiety of candicidin, were added as final concentrations of 1 μCi/ml and 0.5 μCi/ml, respectively, to the resting cell system, as described previously (4, 6). Radioactive candicidin was extracted, purified by thin-layer chromatography, and counted (4).

Davison (10) also points out the possible involvement of ATP in the regulation of antibiotic biosynthesis. We have previously described the inhibition of antibiotic biosynthesis by ribonucleotides (including cyclic nucleotides) of adenine, guanine, cytosine, and uracil, but not by ribonucleosides or the respective bases (11). Uptake studies indicate that the nucleotides are cleaved during uptake and their effect is due to the released phosphate (12). It was thus of interest to determine whether addition of a nucleotide results in an elevation of intracellular ATP concentration. As shown in Fig. 1, this effect did occur with guanosine-5'-monophosphate (GMP).

Whether ATP concentration or the adenylate energy charge (EC) of the cell is the actual regulatory effector of any enzyme function is controversial at the present time. The energy charge as defined by Atkinson and Walton (13) is one-half the number of anhydride-bound phosphates per adenosine moiety (i.e., EC = (ATP) + $\frac{1}{2}$ (ADP)/(ATP) + (ADP) + (AMP). The energy charge is a linear measure of the amount of energy stored at any time in the adeny

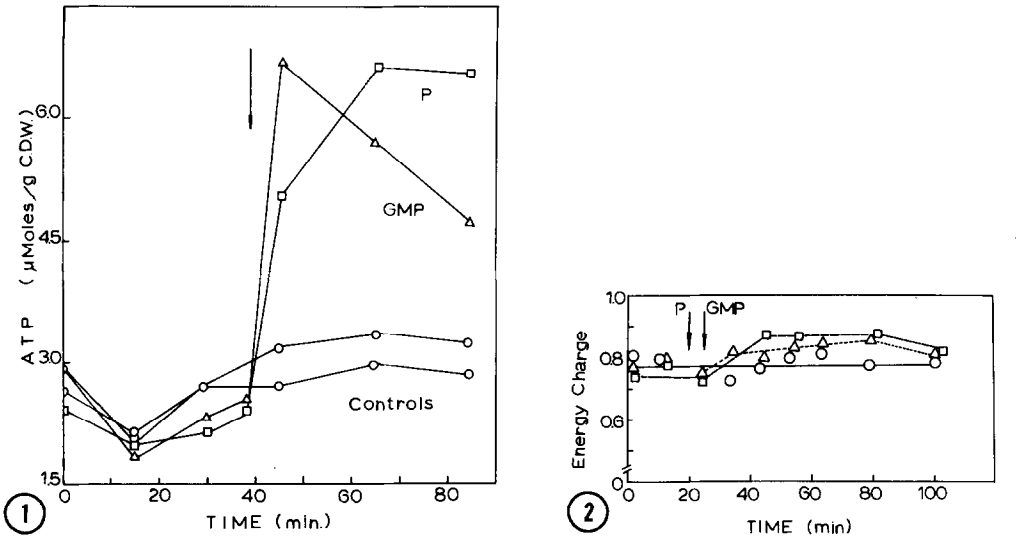


Fig. 1. ATP level (O) in resting cells of S. griseus during antibiotic production and the response to phosphate ( □ ) or GMP (△) addition. Cells were grown and suspended in phosphate-limited production medium as previously described (6). Orthophosphate (10 mM) or GMP (10 mM) was added at the time indicated by the arrow. ATP was extracted and determined as indicated in Methods.

Fig. 2. Adenylate energy charge (O) in resting cells of S. griseus during antibiotic production and the response to 10 mM phosphate ( □ ) and 10 mM GMP (△) addition. The nucleotides were extracted and determined as indicated in Methods.

late system. Its normal value during logarithmic growth of Escherichia coli (7), Bacillus subtilis (14) or Saccharomyces cerevisiae (15) is 0.8, 0.7, or 0.8-0.9, respectively. Atkinson and coworkers maintain that this 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 (13, 15; 16), but this view is not universally accepted (17). It must be kept in mind that conditions which markedly decrease the concentrations of intracellular ATP and ADP often do not result in significant changes in the adenylate energy charge (16, 18).

The energy charge of antibiotic-producing resting cells of S. griseus was found to be about 0.8 (Fig. 2), in agreement with values

reported for other microorganisms. After the addition of 10 mM phosphate or GMP to the system, the energy charge increased slightly to about 0.85. The response of the energy charge to phosphate and GMP was thus much smaller than the increase in ATP concentration. These results suggest that the intracellular level of ATP is more likely than the adenylate energy charge to be the intracellular effector mediating phosphate control of antibiotic synthesis. This postulated role of ATP agrees with data from time-course studies on ATP concentration during antibiotic fermentations; a rapid decrease in intracellular ATP takes place before the onset of antibiotic synthesis (19). We conclude that a low intracellular ATP level is required for antibiotic synthesis. The increase in ATP following phosphate addition is therefore inhibitory for candicidin synthesis. Whate ver the true regulatory effector is, it interacts with candicidin biosynthesis at the level of transcription (19) and regulates also the activity of preformed antibiotic synthases (20).

We thank Prof. J.R. Villanueva for continuous support.

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