Measurements of Rates of Adenosine 3'5'-Cyclic Monophosphate Synthesis in Intact Escherichia coli B  
(adenylate cyclase/glucose utilization)  
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ABSTRACT A method for labeling adenosine 3'5'-cyclic monophosphate in vivo from precursor adenosine followed by quantitative analysis of the labeled nucleotide is described. When the labeling period is short and the specific activity of the ATP pool is determined, the rate of incorporation of radioactivity corresponds to a determination of adenylate cyclase activity in vivo. In E. coli B the specific activity of adenylate cyclase in vivo varies under different growth conditions. Under all conditions tested, the adenylate cyclase activity in vivo is great enough to account for the pattern of accumulation of cyclic AMP. This is in contrast to previous adenylate cyclase assays in vitro, where the measured enzyme activities were insufficient to account for the amounts of cyclic AMP accumulated.

We have previously reported that Escherichia coli B accumulates substantial amounts of cyclic AMP (cAMP) extracellularly (1). During typical growth conditions in glucose-minimal medium, the extracellular cAMP concentration increases dramatically at the end of exponential growth. The coincidence of cAMP accumulation and glucose depletion of the medium (1, 2) indicates that cAMP metabolism is influenced by glucose. In our previous study, we explored the possibility that glucose exhaustion derepresses the synthesis of adenylate cyclase. However, measurements of adenylate cyclase activity in extracts of logarithmic or stationary phase cells gave no indication of higher adenylate cyclase activity in stationary or glucose-starved cells than was found in extracts of logarithmic phase cells. These data were, however, complicated by the fact that the measured activities of adenylate cyclase in extracts were insufficient to account for the amounts of cAMP which accumulated.

With the intention of further studying the mode of regulation of adenylate cyclase under conditions where the enzyme activity is maximally expressed, we have devoted our recent attention to studies in intact cells. The present report details a method whereby rates of cAMP formation can be measured in intact cells. We have termed this the "in vivo" assay for adenylate cyclase. Under these conditions, we show that adenylate cyclase activity is sufficiently high to account for the rate of accumulation of the nucleotide under various growth conditions. Further, we demonstrate that when glucose is depleted from growth medium, there is an increase in the adenylate cyclase activity in vivo sufficient to fully account for the increased accumulation of cAMP.

A portion of this work has been published in abstract form (3).

METHODS

All growth experiments reported here were done with E. coli B cultured in a minimal salts medium (4) supplemented with the indicated concentrations of glucose. Growth was determined turbidimetrically at 650 nm in a Gilford model 300 spectrophotometer. Concentrations of unlabeled cAMP were determined by the Gilman procedure (5) as previously described (1). ATP concentrations were measured by the luciferase procedure, with firefly lantern extract (Sigma) by the procedure of Klofat et al. (6). Radioactivity in ATP was determined after thin-layer chromatography of an aliquot of the HCOOH extract of cells by the method of Cashel et al. (7). [3H]Adenosine (17.7 Ci/mmol) and [14C]cAMP (52.5 Ci/mol) were obtained from New England Nuclear Corp. Dowex-50, analytical grade, was obtained from Bio-Rad. Beef-heart 3'5'-cyclic nucleotide phosphodiesterase was a Sigma product. Polyethyleneimine thin-layer chromatography plates (Polygram Cel-300 PEI) were from Brinkmann.

Assay for cAMP formation in intact cells

A 10-ml aliquot of E. coli B in salts medium (4) supplemented with glucose was placed in a 50-ml beaker and subjected to shaking at 37°C in a reciprocating water-bath shaker. Under continuous shaking, an aliquot (0.5 ml) of [3H]adenosine (17.7 Ci/mmol, 1 mCi/ml) was added to the sample. For determination of ATP concentration and specific activity, samples (0.1-ml) were withdrawn into tubes containing 0.05 ml of 2 N HCOOH at 20-sec intervals during the reaction. The balance of the incubation was terminated usually at 1 min by addition of 5 ml of 2 N HCOOH. The acidified samples were frozen and thawed, then centrifuged to remove the cell debris.

Determination of [3H]cAMP. The supernatant solutions (usually about 15 ml), after dilution to 20 ml, were adjusted to pH 1.54-1.6 with HCl. An aliquot (0.01 ml) of [14C]cAMP (52.5 Ci/mol, 10 μCi/ml) was added, and the sample was applied to a column of Dowex-50 (AG50W-X2, 200-400 mesh), 1.5 × 23 cm, that had been equilibrated with HCl solution (pH 1.54-1.6). All column equilibrations, washings, and elutions were with this same HCl solution. 10-ml Fractions were collected. Those fractions containing the [14C]cAMP were pooled and lyophilized (see Fig. 1A). The dried sample was dissolvled in 6 ml of 0.05 M Tris-HCl buffer (pH 7.5). Aliquots (2.5-ml) of this solution were incubated with 0.05 ml of 0.1 M MgCl₂ in the presence or absence of 0.4 mg of 3':5'-cyclic nucleotide phosphodiesterase from beef heart (0.45 units/mg
of protein) for 30 min at 30°. After this incubation period, about 60 \underline{A}_{260} units of unlabeled 5'-AMP was added to each sample. 15 ml of HCl was added, and the solutions were adjusted to pH 1.57 with HCl. These samples were applied to columns (0.9 × 21 cm) with an attached reservoir (3 × 20 cm) containing a 9-ml volume of Dowex-50 (AG50W-X8, 100–200 mesh) equilibrated with HCl. The columns were washed with 175 ml of HCl; then a further fraction of 150 ml was collected. On some occasions, as in Fig. 1 B and C, 3.5-ml fractions were collected.

Measurement of the \underline{A}_{260} of the second fraction usually indicated that 90–100% of the added 5'-AMP was recovered. Aliquots (3-ml) of the fractions containing 5'-AMP were counted in a scintillation counter, under conditions that detected both \underline{14}C and \underline{3}H. After correction for recovery of the added 5'-AMP, the difference in the \underline{4}H counts between the enzyme-treated and untreated samples was calculated. This difference was assumed to correspond to the amount of labeled cAMP originally in that aliquot. The \underline{14}C radioactivity recovered was used to calculate the amount of radioactive cAMP in the total original sample.

**RESULTS**

**Measurement of in vivo cAMP formation**

The assay for detection of radioactive cAMP synthesized in short time intervals in intact cells was based on the extraction of acid-soluble compounds from cells followed by chromatographic isolation of a fraction enriched in cAMP. Isolation of the 5'-AMP produced by treatment of this fraction with 3':
5'-cyclic nucleotide phosphodiesterase was used as a final purification step. A determination of the specific activity of the ATP pool that served as the precursor of the measured cAMP provided the basis for calculating the amount of newly synthesized cAMP.

Fig. 1 shows the results of a representative fractionation. The major portion of the tritium-labeled radioactivity was resolved from cAMP in the first column fractionation (panel A). The cAMP fraction was treated as described in Methods and rechromatographed. Fig. 1B shows the behavior of the standard [14C]cAMP. In the absence of phosphodiesterase treatment, the radioactivity was eluted in the first fraction, characteristic of cAMP. After phosphodiesterase treatment, the radioactivity eluted later in the second fraction, characteristic of 5'-AMP. The radioactivity in the 5'-AMP region that specifically resulted from phosphodiesterase treatment was taken as a measure of the amount of cAMP in the original 3H-labeled extract.

**Kinetics of cAMP formation in intact cells**

Using the radioactive labeling method described in the preceding section, we determined the time course of labeled cAMP synthesis after a single addition of radioactive adenosine (Fig. 2). The kinetics are curvilinear for 2–3 min. The departure from linearity after that time probably reflects an equilibrium of the radioactivity in the ATP pool with that in the cAMP pool. The specific activity of the ATP pool remains essentially constant (about 45 cpm/pmol) from the period 1 through 5 min. To avoid complications due to nonlinear incorporation, the studies to be described were done with 1-min labeling periods.

**In Vivo cAMP synthesis as a function of the growth stage**

By use of the assay just described, the initial rates of cAMP synthesis at different stages of growth and under different growth conditions were determined (Table 1). As we have previously shown (1), cells in the logarithmic phase accumulate only small amounts of cAMP (72 pmol/hr per mg of protein). During the transition to stationary phase, in concert with depletion of glucose, there was a marked increase in cAMP accumulation (1425 pmol/hr per mg of protein). This increased accumulation rate was transient, since after overnight incubation, the accumulation rate was decreased to about the logarithmic level (46 pmol/hr per mg of protein). Measurements of the adenylate cyclase activity in vivo showed a parallel to the pattern of cAMP accumulation. At the logarithmic stage of growth, the in vivo activity was 1740 pmol/hr per mg of protein, a value substantially higher than necessary to account for the accumulated nucleotide. We interpret this to mean that the 1-min measurement in vivo describes the initial rate of adenylate cyclase uncomplicated by degradation, while the measurement of the accumulated nucleotide reflects both synthetic and degradative processes. Therefore, the reason that logarithmic cells show no accumulation of cAMP is that their synthetic capacity (1700 pmol/hr per mg of protein) is balanced by an equivalent degradative activity.

<table>
<thead>
<tr>
<th>Stage of growth</th>
<th>Logarithmic</th>
<th>Stationary</th>
<th>Overnight</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3% Glucose medium</td>
<td>0.51 2.2 2.2</td>
<td>ATP concentration (M X 10^-4) 3.8 1.1 0.4</td>
<td>ATP specific activity* 716 135 46</td>
</tr>
<tr>
<td>3% Glucose medium</td>
<td>0.46 2.4 2.4</td>
<td>ATP concentration (M X 10^-4) 3.3 0.9 0.4</td>
<td>ATP specific activity 868 606 25</td>
</tr>
</tbody>
</table>

* Specific activity is expressed as cpm/pmol.
† Determined as described in Methods. The unit of specific activity is pmol/hr per mg of protein.
‡ Determined by the procedure of Gilman (5). For comparison with the in vivo adenylate cyclase activity, the data are also expressed as pmol/hr per mg of protein. While an aliquot of the total culture is sampled, most of the cAMP is found in the medium.

At the stationary phase of growth, the adenylate cyclase activity in vivo was markedly increased (3972 pmol/hr per mg of protein). This activity was clearly great enough to account for the measured increased rate of accumulation of cAMP characteristic of stationary phase. The data suggest that if the degradative activity were essentially unchanged in the transition from logarithmic to stationary phase, the increase in synthetic activity (3972–1740 = 2232) could reasonably explain the increase in accumulation rate (1425–72 = 1353).

Our previous study (1) showed that the concentration of cAMP rose substantially for a brief period at stationary phase and then remained stable. This marked decrease in the rate of cAMP accumulation in overnight cultures (46 pmol/hr per mg of protein) was paralleled by a similar marked decrease in the synthetic capacity (18 pmol/hr per mg of protein).

We have shown before that E. coli grown in salts-medium supplemented with 3% glucose have a growth curve similar to that of cells grown in 0.3% glucose medium. However, in the high glucose medium the characteristic burst of cAMP accumulation in stationary phase was absent (1). Measurements of the adenylate cyclase activity in vivo at different stages of growth in the high glucose medium provided an explanation for this inhibition. At logarithmic phase, the adenylate cyclase activities in vivo of 0.3 and 3% glucose cultures were comparable and both cultures had correspondingly low accumulation patterns. This finding suggests a similarity in the balance between synthesis and degradation in logarithmic phase independent of the glucose concentration in the medium. However, in stationary phase, the in vivo adenylate cyclase activity decreased in the high glucose medium compared to an increase in the low glucose medium. It should be noted that glucose was exhausted from the medium at stationary phase only in the 0.3% glucose culture. Overnight
cultures of cells grown in either medium had low activities of adenylate cyclase in vitro. Overnight cultures of E. coli grown in 3% glucose were relatively inactive in their capacity to convert \([H]TTP\) to \([H]ATP\) (note the low specific activity of ATP, 25 cpm/pmol). Thus, the value for adenylate cyclase activity in overnight cells grown in 3% glucose is relatively unreliable.

**Intracellular ATP concentration as a function of the growth stage**

The mechanism by which the in vitro adenylate cyclase activity varies in the three stages of growth is not clear. We did examine the possibility that the variations in enzyme activity resulted from changes in ATP levels. This hypothesis predicts that cells with low adenylate cyclase activity in vitro (logarithmic cells in either medium or stationary cells in 3% glucose medium) should have lower ATP levels than cells with high adenylate cyclase activity in vitro (stationary cells in 0.3% glucose medium). This prediction was not borne out by the data shown in Table 1. Intracellular ATP concentration progressively decreased through the growth stages, whether the cells were grown in 0.3 or 3% glucose. Thus, a limitation in ATP did not explain the lower adenylate cyclase activity in logarithmic cells than in stationary cells.

**DISCUSSION**

The assay method described here for measuring cAMP synthesis has several advantages. The use of the radioactive label allows us to measure small amounts of synthesis in the presence of a substantial pool of cAMP, a feature not offered by methods based on competition of binding (e.g., ref. 5). Since growing cells can be pulsed with a trace amount of radioactive adenosine with no apparent metabolic perturbations, it is possible to make measurements of adenylate cyclase activity under physiological conditions. Adenylate cyclase activity can thus be measured under various experimental conditions.

A procedure for determining levels of cAMP after prior labeling with \([3-14C]TTP\) was previously described by Kuo and De Renzo (8). The procedure described here, while more time consuming, should be more universally applicable on two grounds. The authors measured only radioactivity in cAMP without correction for changes in specific activity of the ATP pool. We incorporate a determination of the ATP specific activity in our calculations. More importantly, the isolation of radioactivity in cAMP reported here is designed to separate very small amounts of radioactive cAMP from a large pool of radioactive contaminants. When we tried a BaSO\(_4\) fractionation procedure (9) similar to that used by Kuo and De Renzo for purification of radioactive cAMP formed in short-term studies in E. coli, the cAMP fraction was still considerably contaminated. Therefore, the double-column procedure used here, which takes advantage of the specificity of 3',5'-cyclic nucleotide phosphorylase, has a greater resolution potential than the BaSO\(_4\) procedure.

Our previous attempts to measure adenylate cyclase in vitro (1) were not satisfactory on several grounds. Assays in cell extracts tended to be variable and, at best, were too low to account for the level of cAMP that the cells actually accumulated. The in vitro assay does not suffer from these limitations. Not only are the determinations reproducible, but the specific enzyme activities are always high enough to account for the accumulation pattern. These studies have shown that the enzyme activity varies as a function of growth stage as well as the conditions for growth. This is compatible with the idea that the observed regulation of cAMP levels is due to changes in adenylate cyclase activity.

The observed difference in the in vitro adenylate cyclase activity and the amount of cAMP accumulated points up the presence of an active turnover or degradation mechanism. This may be due, in part, to cAMP phosphodiesterase activity. Our demonstration that a substantial part of the cAMP that accumulates is extracellular (1) and resistant to degradation (unpublished results) provides another parameter by which cAMP levels can fluctuate.

These studies do not provide an explanation for the changes in activity of adenylate cyclase under various growth conditions. Variations in ATP concentration clearly do not explain the changes in enzyme activity. The intracellular ATP concentrations are similar throughout growth in both high and low glucose medium, even though the enzyme activities vary markedly in the two growth media. The ATP concentration in stationary phase does, however, appear to be limiting for maximum enzyme activity (unpublished experiments) and provides some possibility for metabolic control.

The increase in adenylate cyclase activity in the transition from logarithmic to stationary phase might be due to (a) an increase in the amount of enzyme or (b) an inhibition of the enzyme activity by glucose or one of its metabolites. The depletion of glucose in stationary phase might then allow the enzyme to express its full activity.

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