THE STIMULATORY EFFECT OF CYCLIC ADENOSINE 3′5′-MONOPHOSPHATE ON DNA-DIRECTED SYNTHESIS OF β-GALACTOSIDASE IN A CELL-FREE SYSTEM

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Abstract.—A cell-free system allowing for synthesis of β-galactosidase enzymatic activity has been developed. This system requires DNA containing the β-galactosidase gene, a cell-free extract of *Escherichia coli* bacteria, and the low-molecular-weight components necessary for transcription of the DNA and translation of the resulting messenger RNA. Such a system is useful for studying enzyme synthesis, as well as its regulation. The gene for β-galactosidase is part of the lac operon whose expression is under the control of the lac repressor. In whole cells the lac repressor inhibits almost all of the gene expression for β-galactosidase. In the cell-free system, we had previously been able to repress about half the gene expression. Adding cyclic adenosine 3′5′-monophosphate to the cell-free system improved the yield of β-galactosidase enzymatic activity by 8 to 30 times and the efficiency of repression from 50 to 95 per cent.

Introduction.—A DNA-directed cell-free system capable of producing β-galactosidase activity has been reported.[1-3] This system requires the presence of DNA containing the gene for β-galactosidase, a cell-free extract of *E. coli* bacteria, and the low-molecular-weight components necessary for transcription of the DNA and translation of the resulting mRNA. Conditions can be varied such that it is necessary to synthesize only the first quarter of the β-galactosidase peptide chain, termed α, to obtain enzyme activity.[1, 2] This synthesis is accomplished through use of a cell-free extract from a mutant *E. coli* strain containing M15 protein, a defective β-galactosidase that complements with the newly formed α to give the active enzyme. Complementation between α and M15 protein occurs concomitantly with the synthesis of α.[4] Synthesis of the active enzyme can also be made to occur in the absence of M15 protein or other complement of α.[3] In this case, the entire enzymatically active polypeptide must be synthesized *de novo* by the cell-free system. In addition to examination of the unrepressed synthesis of α and whole β-galactosidase, studies of the inhibition of α synthesis by lac repressor have been made.[2, 5] The lac repressor, which must be present at the start of synthesis, was supplied in one of two ways: either it was a normal component of the bacterial strain from which the cell-free extract was made, or, where strains contain no endogenous repressor, it was added in a partially purified concentrated form. Partial inhibition of α synthesis was observed in the presence of the lac repressor, and this inhibition could be overcome by addition of isopropyl-1-thio-β-D-galactopyranoside, a strong inducer of the lac operon.[2, 5] These observations of repression and derepression are in qualitative agreement with the chemical behavior of the lac system in whole cells. It seems likely that the inhibiting effect of the repressor

on the synthesis of α is due to the binding of the repressor to the lac operator, as postulated by Jacob and Monod.[6]

Heretofore, only about half the synthesis of α was repressible in the cell-free system.[2, 5] Attempts have been made to optimize conditions, both for the gross amount of enzyme synthesis and for the effectiveness of repression. In the present study, we report the effect of the addition of cyclic adenosine 3′5′-monophosphate (3′5′-AMP) to the cell-free system. This compound produces a dramatic increase in the total synthesis of the enzyme, as well as in the effectiveness of repression. We were led to test the effect of 3′5′-AMP in the cell-free system by the report of Pearlman and Pastan that the compound stimulates β-galactosidase synthesis in whole cells.[7]

Results and Discussion.—Description of the cell-free system and assays: The details involved in synthesis of partial and total enzyme activity have been described elsewhere.[1–3, 8] Usually all ingredients except the cell-free bacterial extract (S-30) are mixed in the cold and then preincubated at 37°C for three minutes. The S-30 is added to the mix and incubated at 37°C for one hour with mild shaking. After this time, an aliquot is removed and assayed for β-galactosidase activity. In experiments where gross RNA or peptide synthesis was measured, ^{14}C-ATP or ^{14}C-leucine, respectively, was added at the start of synthesis. RNA synthesis was assayed by measuring radioactive incorporation into the cold trichloroacetic acid precipitate; to observe peptide synthesis, the hot trichloroacetic acid insoluble precipitate was measured in a similar fashion. The S-30 extracts used in these studies were prepared from three different *E. coli* strains: Z19i^q, Z19i$^-$, and 514 (described in detail elsewhere[3, 5]). Z19i^q contains the repressor gene i^q and the M15 β-galactosidase gene on both chromosome and episome. Strain Z19i$^-$ is identical to Z19i^q, except that it does not contain a lac repressor gene. Strain 514 contains a deletion of the entire region occupied by the lac operon and the gene for lac repressor. DNA was obtained from the ϕ80dlac virus.

Effect of 3′5′-AMP on enzyme synthesis: It was first noted that 3′5′-AMP has a beneficial effect on α synthesis with S-30 extracts prepared from strain Z19i$^-$. The concentration dependence of the effect (see Figure 1) showed a broad optimum above 0.5 mM 3′5′-AMP. In subsequent studies, 3′5′-AMP was used at a concentration of 1 mM. It must be added before the start of synthesis. No effect was obtained if the compound was added after synthesis, nor could adenosine 2′-monophosphate, adenosine 3′-monophosphate, or cyclic adenosine 2′3′-monophosphate substitute for 3′5′-AMP.

The presence of 3′5′-AMP improved the observed amount of enzyme synthesis by from 8 to 13 times when S-30's from strains Z19i$^-$ or Z19i^q were used (see Figures 1 and 2). With Z19i^q, 5×10^{-5} *M* of isopropyl-1-thio-β-D-galactopyranoside inducer must be present to derepress the system. It should be noted that both these strains contain the α complement. 3′5′-AMP improved the observed enzymatic activity 20- to 30-fold when strain 514 was used to prepare the S-30 (see Figure 2). Since strain 514 contains a deletion of the entire lac region, to obtain activity in this case the enzymatically active polypeptide must have been synthesized in its entirety. In the absence of 3′5′-AMP, more enzyme activity

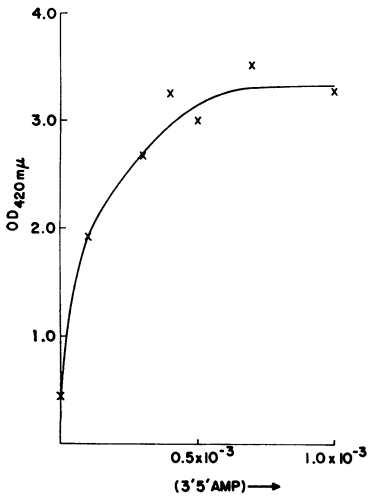

FIG. 1.—Enzyme activity as a function of 3'5'-AMP: The *ordinate* shows the optical density at 420 mμ normalized to an assay time of 20 hr. The abscissa represents the concentration of 3'5'-AMP during synthesis. Except for slight modifications described herein, all procedures used for synthesis, enzyme assay, and preparation of bacterial extracts and DNA have been described in detail elsewhere.[1-3,8] The procedures for synthesis and assay will be reviewed here. The incubation mixture contains per ml: 44 μmoles Tris-acetate, pH 8.2; 1.37 μmoles dithiothreitol; 55 μmoles KAc; 27 μmoles NH_4Ac; 14.7 μmoles $MgAc_2$; 7.4 μmoles $CaCl_2$; 0.22 μmole amino acids; 2.2 μmoles ATP; 0.55 μmole each GTP, CTP, UTP; 21 μmoles phosphoenolpyruvic acid; 100 μg tRNA; 27 μg pyridoxine·HCl; 27 μg triphosphopyridine nucleotide; 27 μg flavine adenine dinucleotide; 11 μg *p*-aminobenzoic acid. The above ingredients are preincubated for 3 min at 37°C with 65 γ/ml DNA with shaking before 6.5 mg S-30 protein extract is added. Incubations with shaking are allowed to continue for 60 min at 37°C. Strain Z19i⁻ was used to prepare the S-30 extract used for the experiments recorded in Fig. 1. Varying quantities of 3'5'-AMP, obtained from California Biochemical Corp., were added to the incubation mixtures. After the synthesis had been completed, a 0.2-ml aliquot was removed and mixed with 1.5 ml of assay buffer containing 0.53 mg O-nitrophenyl β-D-galactoside, 0.1 *M* sodium phosphate, pH 7.3, and 0.14 *M* β-mercaptoethanol. After a suitable length of time, the mixture was treated with one drop of glacial acetic acid, chilled, and centrifuged to remove precipitate. The supernatant was mixed with an equal volume of 1 *M* sodium carbonate and read against water in a 1-cm quartz cell at a wavelength of 420 mμ. A zero time value of .035 was subtracted from all readings. All optical density readings have been normalized to an assay time of 20 hr. The assay is known to be linear over this period of time.

has always been obtained with S-30's prepared from α-complementing strains than from lac deletion strains such as 514 (for example, see Fig. 2 and ref. 3). This attests to the efficacy of the complementation process. The data of Figure 2 suggest that in the presence of 3'5'-AMP this situation is reversed. Our tentative explanation for the reversal is that in the noncomplementing system in the presence of 3'5'-AMP, many more complete chains of β-galactosi-

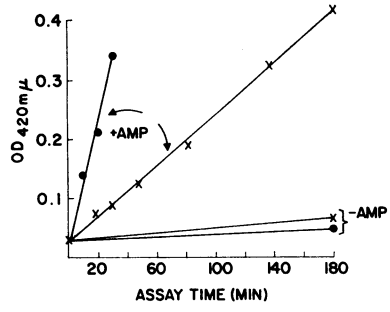

FIG. 2.—Optical density as a function of assay time: The ordinate shows the optical density at 420 mμ, using the standard assay procedure as described in the caption for Figure 1. The time of assay is varied, and this is recorded on the abscissa. Over the period of times studied, the optical density bears a linear relationship to assay time. The optical density is read against a tube containing water, and no subtractions have been made. The conditions for synthesis of enzymatic activity have been described in the caption for Figure 1. When 3'5'-AMP is used, the final concentration during synthesis is 1 mM. In all experiments involving S-30 extracts of strain Z19i^q, isopropyl-1-thio-β-D-galactopyranoside is present at a concentration of 5×10^{-5} *M* during synthesis.

(●) refers to the synthetic system in which S-30 extract is prepared from strain 514.

(×) refers to the synthetic system in which S-30 extract is prepared from strain Z19i^q.

dase polypeptide are made with a higher specific activity than that of the enzyme produced by α complementation.

The amount of enzyme activity obtained with S-30 extract prepared from strain 514 (Fig. 2) was equivalent to 25×10^{-9} gm/ml of β-galactosidase. In a parallel experiment, it was found that 2.5 mμmoles of leucine were incorporated per mg of S-30 protein. If we assume that seven per cent of the peptide synthesized contains leucine, it can be calculated that 23×10^{-6} gm of peptide per ml were synthesized in the cell-free system. This means that about 1 part per 1000 of the peptide synthesized in the system was found in functional β-galactosidase. The remainder may be incorporated into incomplete β-galactosidase chains, as well as other products encoded by the ϕ80dlac DNA used to direct the synthesis.

Effect of 3′5′-AMP on repression of enzyme synthesis: The beneficial effect of 3′5′-AMP on enzyme synthesis was not reflected by a rise in gross RNA or peptide synthesis (compare lines 2 and 4, Table 1). A clue to the action of

TABLE 1. *Enzyme activity and CTP and leucine incorporation in the cell-free system when using S-30 extract prepared from strain Z19i^q.*

	Incubation system	3′5′ = AMP	IPTG*	Enzyme activity†	Leucine incorporation mμmoles/mg S-30 protein	ATP incorporation mμmoles/mg S-30 protein
1.	−DNA	+	+	$<2.5 \times 10^{-6}$	0.52	1.7
2.	Complete	+	+	4.6×10^{-3}	2.2	5.1
3.	Complete	+	−	0.23×10^{-3}		
4.	Complete	−	+	0.34×10^{-3}	2.3	4.5
5.	Complete	−	−	0.18×10^{-3}		

* IPTG, isopropyl-1-thio-β-D-galactopyranoside.

† Expressed in International Units. One unit of β-galactosidase is defined as that amount of enzyme producing 1 μmole of O-nitrophenol per minute at 28°C and pH 7.3. The specific activity of ^{14}C-leucine used was 3.3×10^5 cpm/μmole and of the ^{14}C-ATP used was 0.62×10^5 cpm/μmole. Enzyme assays are reproducible to 5%. Radioactivity incorporation assays are reproducible to 15%. The enzyme assays and radioactivity incorporation assays represent separate experiments executed in parallel. The synthetic system has been described in the caption for Figure 1. When IPTG is used, the final concentration is 5×10^{-5} *M*. When 3′5′-AMP is used, the final concentration is 10^{-3} *M*.

3′5′-AMP is suggested by the effect it has on repression in the cell-free system. The S-30 extract containing repressor prepared from strain Z19i^q was used for these studies. In the absence of 3′5′-AMP the effect of adding inducer was to increase the observed enzyme activity about twofold (compare lines 4 and 5, Table 1). In the absence of inducer, the addition of 3′5′-AMP increased the enzyme synthesis by about one third (compare lines 3 and 5, Table 1). The main effect of 3′5′-AMP was on the derepressed system in the presence of inducer. In this case, addition of 3′5′-AMP resulted in a 13-fold increase in enzyme activity (compare lines 2 and 4, Table 1). Since 3′,5′-AMP exerts its main effect on that portion of the synthesis that is repressible, the addition of 3′5′-AMP has greatly improved the system for studying repression. Whereas previously only 50 to 65 per cent of the enzyme synthesis was repressible,[2, 5] now 95 per cent of the synthesis is repressible. The existence of irrepressible synthesis suggests that transcription is beginning at points other than the

true initiation points. 3′5′-AMP appears to have greatly improved the quality of transcription, so that a much higher percentage is repressor-sensitive.

The compound 3′5′-AMP is known to have a wide variety of physiological effects.[7, 9] This study represents the first time it has been shown to be directly involved in the cell-free process of RNA and protein synthesis. We feel that an understanding of the effect of 3′5′-AMP in this system will be most valuable for the elucidation of mechanisms in cell synthesizing systems.

Note added in proof: Makman and Sutherland (*J. Biol. Chem.*, **240,** 1309 (1965)) have shown that a rise in cyclic AMP occurs as the glucose level decreases in a growing culture of *E. coli.* This observation, coupled with the results presented here, leads us to the working hypothesis that cylic AMP triggers the synthesis of catabolite enzymes such as β-galactosidase. We are currently investigating the possibility that the primary action of cyclic AMP is on the RNA polymerase.

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