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Biochemistry

In vitro synthesis of a constitutive enzyme of *Escherichia coli*, 6-phosphogluconate dehydrogenase

(central intermediary metabolism/cell-free protein synthesis/*gnd*/3′:5′-cyclic AMP)

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ABSTRACT **Enzymes of central intermediary metabolism are among the constitutive proteins of *Escherichia coli*. 6-Phosphogluconate dehydrogenase [6-phospho-D-gluconate: nicotinamide adenine dinucleotide phosphate 2-oxidoreductase (decarboxylating), EC 1.1.1.44], an enzyme of the hexose monophosphate shunt, was synthesized *in vitro* in a coupled transcription–translation system directed by DNA from a specialized transducing phage carrying *gnd*, the structural gene. Enzyme synthesized *in vitro* was detected by radiochemical assay of its activity. Kinetic experiments using inhibitors of transcription and translation suggest that synthesis of the mRNA for the enzyme was initiated at the *gnd* promoter. Neither cyclic AMP nor cyclic GMP had any effect on the amount of enzyme synthesized *in vitro*.**

Pathways of central intermediary metabolism such as glycolysis and the hexose monophosphate shunt are found in most cells, prokaryotic and eukaryotic. Even in prokaryotes little is known of their molecular biology. Deficiency mutants are available for some of the enzymes, and genetic mapping in *Escherichia coli* has shown that genes governing sequential metabolic steps are not themselves adjacent. It is also known that the levels of certain such enzymes do not vary greatly with obvious metabolic need: hence they are "constitutive"—a term which reflects our ignorance about specific or general mechanisms regulating their synthesis (reviewed in ref. 1).

The present report shows cell-free synthesis of such an enzyme. We used DNA from a specialized transducing phage carrying the gene for 6-phosphogluconate dehydrogenase [6PGD; 6-phospho-D-gluconate:NADP 2-oxidoreductase (decarboxylating), EC 1.1.1.44] of *Escherichia coli* (*gnd*, Fig. 1; ref. 2). Synthesis of the mRNA for 6PGD probably was initiated at the *gnd* promoter. The amount of enzyme synthesized was not dependent on cyclic AMP or cyclic GMP, and, since the overall efficiency of the system was as high as has been reported for other genes, it is likely that *gnd* expression does not depend on any such controlling small molecules which are dialyzable. (But it is still possible that some component routinely included in the incubation mixture has such a controlling role.)

The simplest model for expression of constitutive genes has been that only promoters govern their rate of expression and that they are not subject to repressor or positive regulatory proteins. This model is still compatible with the present results, but the cell-free system is crude; if such regulatory elements are functioning, it should now be possible to reveal them by fractionation.

MATERIALS AND METHODS

Preparation of Phage DNA and Bacterial Extract for Cell-Free Synthesis of 6PGD. Strain RW181 (F^-*trpR*$^-$, *lacY*$^-$,*trpA*$^-$,*dgR*^{c}, (*edd-zwf*)Δ22,(*sbcB-his-gnd-rfb*)Δ_{P2}) lysogenized with the specialized transducing phage λ*cI857St68*h80d*gndhis* (2) and the helper phage λ*cI857St68*h80 was the source of the DNA for cell-free synthesis of 6PGD. First a lysate was prepared by heat-induction and the phage were concentrated according to Zubay (3). Then phage were purified by centrifugation twice in CsCl block gradients and λ*cI857St68*h80d*gndhis* was separated from the helper phage of lower buoyant density by centrifugation to equilibrium in CsCl as described by Greenblatt (4). DNA was extracted according to Zubay (3).

The cell-free extract for *in vitro* synthesis was prepared according to Zubay (3) as modified by Wilcox *et al.* (5). A derivative of strain RW181 was used, lac_{X74} being introduced so that the efficiency of the extract could be monitored by *in vitro* synthesis and assay of β-galactosidase.

***In Vitro* Protein Synthesis.** The components of the reaction mixture and conditions for protein synthesis were generally as described by Zubay (3) with exceptions noted in the legends to figures and tables.

Assay of 6PGD. 6PGD activity was determined by a radiochemical assay similar to one described by Brown and Wittenberger (6). 6-Phospho-[1-^{14}C]gluconate was synthesized enzymatically from [1-^{14}C]gluconate (Nuclear Chicago) and ATP with yeast gluconokinase (Boehringer). Assays were done in 50 ml Erlenmeyer flasks fitted with a center well. The main compartment contained 50 μmol of Tris-chloride, pH 7.5, 10 μmol of $MgCl_2$, 0.4 μmol of 6-phospho-[1-^{14}C]gluconate (88 cpm/nmol), and 0.01–0.05 ml of *in vitro* synthesis mixture in a volume of 0.9 ml. The center well contained 0.5 ml of Protosol (New England Nuclear). Serum bottle stoppers were inserted into the flasks which were then incubated at 30° for 15 min to permit temperature equilibration. The reaction was initiated by injecting NADP (0.1 ml of a 2 mM solution) through the stopper with a syringe. Flasks were incubated at 30° for 15 min to 3 hr, after which time the reaction was terminated by injection of 0.2 ml of 5 N H_2SO_4 into the main compartment. Flasks were transferred to 37° and incubation was continued for 1 hr at 160 rpm to ensure complete trapping in the center well of evolved $^{14}CO_2$. The contents of the center well were transferred quantitatively to scintillation vials that contained 10 ml of 0.4% Omnifluor (New England Nuclear) in toluene

Abbreviations: 6PGD, 6-phosphogluconate dehydrogenase; cAMP, cyclic AMP; cGMP, 3′:5′-cyclic GMP; CAP, cyclic AMP receptor protein.

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Table 1. *In vitro* synthesis of 6-phosphogluconate dehydrogenase

Conditions	Enzyme activity (U/ml of synthesis mixture)
Complete	19.5
Complete — DNA	0.0
Complete, zero time	0.0

The components of the reaction mixture and conditions for protein synthesis were as described by Zubay (3) except that the DNA concentration was 6 μg/ml, and the concentration of cell-free extract was 6.0 mg/ml. cAMP, NADP, FAD, pyridoxine·HCl, and *p*-aminobenzoic acid had no measurable effect on synthesis of 6PGD and were omitted. A Mg^{++} concentration of 12.7 mM gave optimal results. The final volume was 0.1 ml and synthesis was at 36° for 30 min. Synthesis was initiated by the addition of S-30 and terminated by chilling and adding chloramphenicol to 100 μg/ml.

and counted in a Packard Tri-Carb liquid scintillation spectrophotometer. Twenty-two cpm were released nonenzymatically from assays lacking NADP or the cell-free extract and were subtracted from all enzyme assays. Initial rates were linear for more than 1 hr and were proportional to enzyme concentration in the ranges used. Enzyme activity is expressed as units of activity per ml of protein-synthesizing mixture, where one unit of enzyme is defined as the amount that catalyzes the release of 1 nmol of $^{14}CO_2$ per min at 30°.

RESULTS

In vitro synthesis of 6PGD

In vitro studies on the mechanism of expression of a particular gene are done best with a source of DNA enriched for the gene of interest, a cell-free extract (S-30) prepared from a strain carrying a deletion of the gene, and a sensitive assay for the gene product. To detect the small amount of 6PGD synthesized *in vitro* we first prepared 6-phospho[*1*-^{14}C]gluconate to serve as substrate and developed a radiochemical method for quantitating the amount of $^{14}CO_2$ released as a product of the enzymatic reaction. The strain used for the preparation of the cell-free extract carried deletions of both *gnd* and *edd* and thus lacks both of the enzymes known to use 6-phosphogluconate as a substrate (1). DNA enriched for *gnd*, the structural gene for 6PGD (ref. 7; unpublished results), comes from λ*cI857St68*h80d*gndhis*. (See Fig. 1).

The results presented in Table 1 demonstrate the DNA-directed synthesis of 6PGD *in vitro*. In accord with the source of the cell-free extract the synthesis mixture lacking exogenous DNA did not contain an activity that could catalyze the release of $^{14}CO_2$ from 6-phospho[*1*-^{14}C]gluconate, nor was such an activity present at the time of addition of

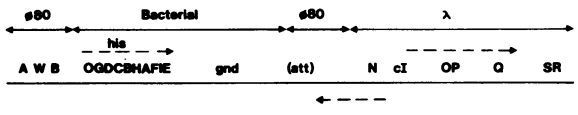

FIG. 1. Genetic map of λ*cI857St68*h80d*gndhis*. The map was determined by a marker rescue experiment of the progenitor phage, φ80d*gndhis* (2) and deletion analysis in lysogens (to be reported). φ80 genes are named like their λ counterparts. The precise junction of bacterial and phage DNA is not known, nor is it known whether the phage actually carries *att*. Relevant directions of transcription, where known, are noted by: — — — →.

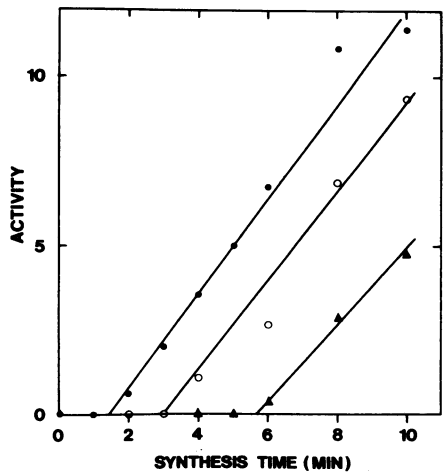

FIG. 2. Time course of the initiation, synthesis, and translation of the mRNA for 6PGD. The conditions for protein synthesis and assay of 6PGD activity (units/ml) were as described in Table 1. Separate flasks were used for each time point. Synthesis was initiated by addition of cell-free extract, and rifampicin (4 μg/ml), actinomycin D (4 μg/ml), or chloramphenicol (100 μg/ml) were added at each time point shown, and incubation was continued for an additional 30 min before chilling. ●, rifampicin; ○, actinomycin D; ▲, chloramphenicol.

λ*cI857St68*h80d*gndhis* DNA. However, 6PGD activity was detectable after incubation of the complete mixture. That activity, like authentic 6PGD, was dependent on 6-phosphogluconate and NADP and released $^{14}CO_2$ at a linear rate for more than 1 hr. The amount of activity synthesized increased in proportion to the DNA concentration in the range of 0–10 μg/ml (data not shown).

Time course of enzyme synthesis and of the capacity to synthesize the mRNA

We next wanted to determine the extent to which the *in vitro* synthesis of 6PGD represented the *in vivo* expression of *gnd*. With other systems the usual approach has been to attempt to reproduce *in vitro* a regulatory event thought to occur *in vivo*, e.g., the repression of β-galactosidase synthesis by *lac* repressor, or its stimulation by cAMP (8). However, such an approach is not possible for a gene whose regulatory properties are unknown. Thus we have studied instead the time course for the initiation, synthesis, and translation of the mRNA for 6PGD (Fig. 2), the rationale being that the time for transcription and for enzyme synthesis would be a measure of the distance between the promoter at which transcription was initiated and the end of the structural gene. Inhibitors of either RNA or protein synthesis were added, in parallel experiments, at various times after the initiation of *in vitro* synthesis, and incubation was continued for 30 min in order to permit the completion of synthesis in progress and/or assembly of active enzyme. Appearance of 6PGD activity became resistant to chloramphenicol within 6 min. Thus this is the time required to complete the synthesis of a 6PGD molecule. Indeed it is about the time required for the synthesis of β-galactosidase (9) and arabinose isomerase (5) in similar systems where transcription was shown by other criteria to have been initiated correctly, and considerably less than the time for synthesis of anthranilate synthetase Component I under conditions where transcription was known to be initiated at a phage promoter (10).

We next estimate the number of nucleotides in the mRNA for 6PGD synthesized *in vitro* and correlate it with the

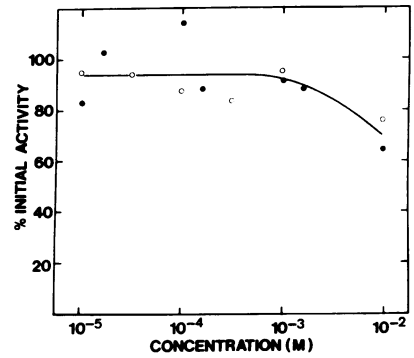

FIG. 3. Effect of cAMP and cGMP on *in vitro* synthesis of 6PGD. The conditions for protein synthesis were as described in Table 1 except that the incubation mixture contained the amount of cAMP or cGMP indicated on the abscissa. The 100% value is the activity obtained in the absence of cyclic nucleotide. ●, cAMP; ○, cGMP.

number necessary to encode the 6PGD monomer. By extrapolation from the linear initial rates, the capacity to synthesize the mRNA for 6PGD becomes resistant to rifampicin by 1.5 min and to actinomycin D by 3 min (Fig. 2). Thus, since rifampicin and actinomycin D inhibit the initiation and propagation of RNA synthesis, respectively, it follows that the time to synthesize the mRNA for 6PGD was about 1.5 min (3.0–1.5 = 1.5). At an *in vitro* transcription rate of about 19 nucleotides/sec (10) a mRNA approximately 1710 nucleotides long could be made in 1.5 min. The monomer of 6PGD is about 60,000 daltons (11, 12), which would require a mRNA of 1500 nucleotides. Therefore, since the estimated length of the mRNA made *in vitro* is about the same as that required to encode the 6PGD monomer, it seems likely that in our cell-free system transcription was initiated at the *gnd* promoter.

The effect of cAMP and cGMP on the synthesis of 6PGD *in vitro*

cAMP in conjunction with the cAMP receptor protein (CAP) is required for maximal expression of a number of inducible genes, but its effect on genes for enzymes of glycolysis and the hexose monophosphate shunt has not been reported (13). Data presented in Fig. 3 show that cAMP is without effect on the amount of 6PGD synthesized *in vitro*. Under the same conditions the synthesis of β-galactosidase as directed by φ80d*lac*$_{III}$ DNA (kindly supplied by J. Hopkins) was stimulated about 10-fold by 10^{-3} M cAMP (data not shown), indicating that CAP was functional in the cell-free extract.

Concentrations of cGMP in *E. coli* are highest under conditions of growth when cAMP concentrations are low (14), but the physiological function of this molecule is unknown. Fig. 3 shows that cGMP is not a regulator of 6PGD synthesis *in vitro*.

DISCUSSION

The present communication marks the synthesis of an enzyme of central intermediary metabolism in a DNA-directed, cell-free system. Moreover, among the constitutive proteins of *E. coli*, only 6PGD, the β subunit of RNA polymerase (15, 16) and some of the ribosomal proteins (17, 18) have been synthesized *in vitro*. Based on previous work with genes for amino-acid biosynthesis and carbohydrate degradation, it seems likely that studies using systems like these will help to elucidate the mechanisms that regulate the synthesis of this important class of proteins.

Our *in vivo* studies (to be reported) with λ*cI857St68*h80d*gndhis* lysogens indicated that the phage carries the structural gene for 6PGD, and that synthesis of the enzyme from the prophage is constitutive and not under control of a phage promoter. The experiments reported here have shown that in our cell-free system the synthesis of the mRNA for 6PGD was probably initiated at the *gnd* promoter, a necessary condition if the system is to be useful in subsequent study of the mechanisms that regulate expression of the gene. It should be pointed out, however, that the kinetic experiments (Fig. 2) do not rule out the possibility that readthrough transcription contributes to the total amount of enzyme made. Nonetheless, since at least some transcription was initiated at the *gnd* promoter, and since the synthesis of β-galactosidase with the same cell-free extract showed normal cAMP dependence, the fact that cAMP was without effect on the *in vitro* synthesis of 6PGD strongly suggests that it is not a regulator of *gnd in vivo*. Moreover, because similar results were obtained for the *in vitro* synthesis of ribosomal proteins (17) and the β subunit of RNA polymerase (15), it would appear that in general the synthesis of constitutive proteins is not subject to regulation by the cAMP-CAP system.

We next calculate the efficiency of our *in vitro* system and compare it to other systems. The initial rate of appearance of 6PGD activity, as determined from the slope of the curve in the rifampicin experiment (Fig. 2), was about 90 units/hr per ml of synthesis mixture. The DNA concentration in the experiment was 6 μg/ml. Thus the rate per genome was about 7.5×10^{-10} units/hr, given Avogadro's number and assuming the molecular weight of λ*cI857St68*h80d*gndhis* DNA to be 30×10^6 and that all genomes were functional. The *in vivo* rate per genome would be about 7.5×10^{-9} units/hr for cells growing at a rate of one mass doubling per hr where the specific activity is about 150 units/mg (19), assuming 2 genomes and 10^{-10} mg of soluble protein per cell. (We note that the value would vary according to the effect of growth rate on the *in vivo* rate of enzyme synthesis.) Thus the approximate efficiency of our system (10%), expressed as the percent of the *in vivo* rate per genome, is as high as any previously reported [arabinose isomerase, 5% (5); tryptophan synthetase, 2–10% (20); galactokinase, about 0.1% (21); *N*-α-acetyl ornithinase, 0.09% (22)]. However, since the rate-limiting step in the *in vitro* synthesis of 6PGD is unknown, the possibility cannot be ruled out that the overall rate is limited by the action of a repressor, or the lack of action of a positive regulator. Subsequent studies using such a system may help determine what mechanisms actually regulate the synthesis of 6PGD.

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