DNA Directed Peptide Synthesis, V. The Cell-free Synthesis of a Polypeptide With β-galactosidase Activity.

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Introduction

The E. coli enzyme β-galactosidase has a molecular weight of 538,000 and is composed of four identical monomers. Previously, we have described the cell-free synthesis of a segment of the polypeptide chain of the enzyme (Zubay, Lederman, and DeVries, 1967). This synthesis requires DNA containing the β-galactosidase gene and all the components required for transcription of DNA and translation of mRNA into protein. The segment synthesized in this system is not itself enzymatically active. It can be detected only by the enzyme activity resulting from a complementation reaction with a mutant β-galactosidase which alone also lacks enzyme activity. Recently, the efficiency of the system for the synthesis of this segment has been increased 20 fold above that previously reported. The purpose of this study was to see if, by using the newly developed conditions, it might be possible to synthesize active enzyme without complementation.

Results and Discussion

Synthesis is carried out in the improved incorporation system described in Table I using sub-cellular components from either of two strains of E. coli with a deletion of the entire β-galactosidase gene and Ø80dlac DNA by incubation for one hour at 37°. The system is assayed for gross peptide synthesis by ^{14}C-leucine incorporation and for the synthesis of β-galactosidase by enzyme assay with the substrate o-nitrophenyl-β-D-galactoside (ONPG).

Table I

^{14}C-leucine Incorporation and Enzyme Activity After Synthesis in *lac* Deletion Strains

	DNA Source	Incubation System	Strain 514 cpm/ml	Strain 514 Increase in OD_{420} after 42 hours	Strain X103 cpm/ml	Strain X103 Increase in OD_{420} after 42 hours
1.	no DNA	complete	586	< 0.01	645	< 0.01
2.	Ø80dlac DNA	complete	5970	0.216	2960	0.081
3.	Ø80 DNA instead of Ø80dlac DNA	complete	5220	< 0.01	5560	< 0.01
4.	Ø80dlac DNA	complete + 10 /ml DNAse	361	< 0.01	256	< 0.01
5.	Ø80dlac DNA	complete + 2 /ml Act. C_1	814	< 0.01	1090	< 0.01
6.	Ø80dlac DNA	complete - CTP - UTP	581	< 0.01	352	< 0.01
7.	Ø80dlac DNA	complete + 10^{-2} M CAP	---	---	395	< 0.01

The strains used are 514 ($F^-\Delta$ lac, $tryp^-$, T_6r, Sm^r) and X103B (Δ pro lac, $tryp^-$, HfrH). Pre-incubated S-30 extracts are made as previously described (Lederman and Zubay, 1967), except that 1 umole/ml dithiothreitol replaces 6 umoles/ml 2-mercaptoethanol in all steps except the washing of cells and 60mM KAc replaces 60mM KCl in the dialysis buffer. The incubation mixture contains/ml: 40 umoles Tris-acetate, pH 8.2; 1.25 umoles dithiothreitol; 50 umoles KAc; 25 umoles NH_4Ac; 13.3 umoles $MgAc_2$; 6.7 umoles $CaCl_2$; 0.2 umoles 19 ^{12}C-amino acids; 2 umoles ATP; 0.5 umoles each GTP, CTP, UTP; 19 umoles PEP; 100 ug t-RNA; 25 ug pyridoxine HCl; 25 ug TPN; 25 ug FAD; 10 ug PABA; 0.1 umoles ^{14}C-leucine (10^6cpm/umole). The above ingredients are pre-incubated for three minutes at 37° with 240 ug/ml DNA with shaking before adding 6.5 mg/ml pre-incubated S-30 protein/ml and 25 ug/ml folinic acid. Incubations with shaking are for 60 minutes. Pre-incubated S-30's and folinic acid are protected from light. Assays for measuring leucine incorporation and enzyme activity are published (Lederman and Zubay, 1967; Zubay, Lederman and DeVries, 1967). All incubation mixes have an O.D. of 0.045 $\pm$ 0.01 at zero time of assay. For any individual experiment, duplicate enzyme assays are reproducible to $\pm$0.01 O.D. units.

The complete, uninhibited system gives a distinct yellow color in the enzyme assay characteristic of the breakdown of the ONPG substrate. This is best characterized by the increase of optical density at 420 mμ as shown in Table I. In order to ensure that this optical density increase is due to de novo synthesis directed by added DNA carrying the β-galactosidase gene, various control experiments have been carried out. Homologous viral DNA lacking the β-galactosidase gene has been substituted for the ϕ80dlac DNA (3. in Table). In this case, the amino acid incorporation is excellent but no enzyme activity is detectable. Other controls in which amino acid incorporation is inhibited by degradation of DNA (4. in Table), by stopping messenger RNA synthesis with Actinomycin C_1 (5. in Table), by leaving out the pyrimidine nucleoside triphosphates (6. in Table), or by stopping translation directly with chloramphenicol (7. in Table) also show no enzyme activity. The absolute amount of enzyme activity made in this system is very small indeed. From the constants provided by Craven, Steers, and Anfinsen (1965) we calculate that the enzyme activity synthesized using extracts of E. coli strain 514 (see Table) is equivalent to about 2×10^{-7} mg of β-galactosidase.

A preliminary attempt has been made to determine the size of the polypeptide made in the synthetic system. This has been done by determining the sedimentation constant of the species with enzyme activity in a sucrose gradient using an internal alkaline phosphatase marker. The results presented in Figure 1 are on an $(NH_4)_2SO_4$ concentrated fraction of the incubation mixture. Several experiments done directly on incubation mixtures give qualitatively the same results. Figure 1 shows a major peak of activity with a sedimentation constant of 7.4 Svedbergs (S) and a minor peak with a sedimentation constant of 11.4 S. Although the minor peak is small it has appeared in several gradients. When losses of enzyme activity due to standing in sucrose are taken into account, the recovery of activity from the gradient is close to 100 percent.

Martin and Ames (1961) have suggested a formula for the estimation of molecular weight from sedimentation constants which states that the sedi-

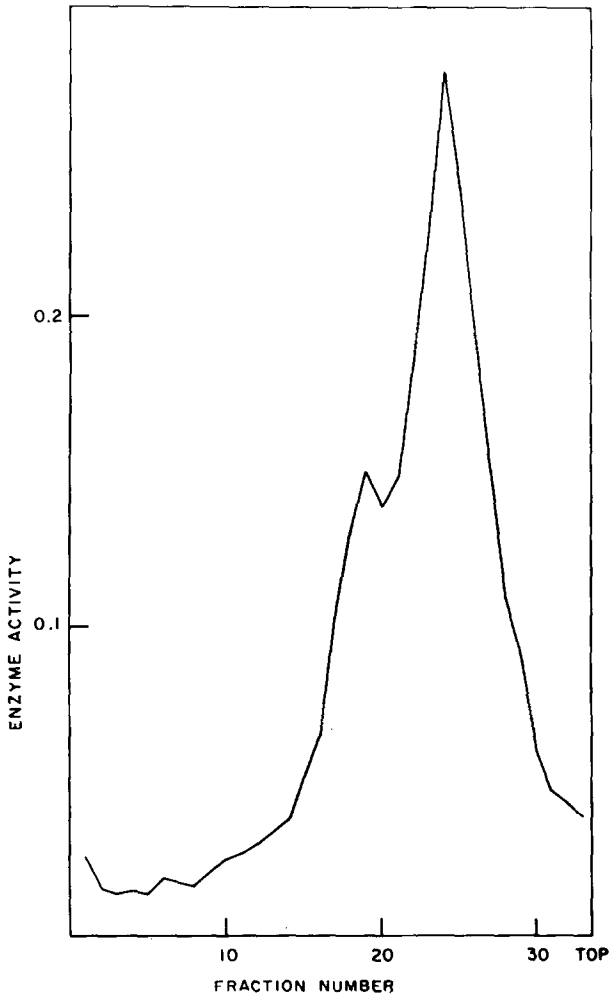

Figure 1: An incubation in strain 514 primed by ø80dlac DNA was centrifuged at 78,000 x g for 90 min. Saturated $(NH_4)_2SO_4$ was added to 40% saturation, the precipitate collected and resuspended in one-tenth its original volume in 0.1M Na_2HPO_4, pH 7.3, 1mM $MgAc_2$, 0.14 M 2-mercaptoethanol. After dialysis against this buffer, an aliquot was layered on a 5-20% linear sucrose gradient in 0.01 M Na_2HPO_4, 1 mM $MgAc_2$, 0.14 M 2-mercaptoethanol. Centrifugation was for 20 hrs at 24,000 rpm in the Spinco SW 25.1 rotor. 1 ml aliquots were collected and assayed for enzyme activity with 1 ml 0.70 mg/ml ONPG in 0.1 M Na_2HPO_4, pH 7.3, 0.14 M 2-mercaptoethanol for 44 hours at 28°. Similar gradients on incubations done without added DNA show uniform low background ($OD_{420} \simeq 0.02$ after 44 hours) over the entire gradient.

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mentation constant is proportional to the two-thirds power of the molecular weight. Using the value of 16.6 S for the tetramer of β-galactosidase, we

may estimate the sedimentation constants of the monomer, dimer, and trimer of the naturally occuring enzyme as 6.6, 10.4 and 13.7 S respectively. Comparison of these calculated values with the observed value for the main peak in the sucrose gradient suggests that an enzymatically active polypeptide close in size to β-galactosidase monomer is being synthesized. The subsidiary peak is close in size to dimer. It is important to note that whereas the polypeptides made in the cell-free system have enzyme activity, species of naturally occurring β-galactosidase smaller than tetramer have never been shown to have activity. It is entirely possible that experiments necessary to demonstrate activity of monomer and dimer of β-galactosidase may never have been done since tetramer is formed at very low concentrations of the subunits. Further work comparing the properties of the cell-free synthesized polypeptide with the in vivo synthesized enzyme is in progress.

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References

1. Craven, G. R., Steers, E. and Anfinsen, C. B. (1965), J. Biol. Chem. 240, 2468.
2. Lederman, M. and Zubay, G. (1967), Biochem. Biophys. Acta 149, 253.
3. Martin, R. G. and Ames, B. N. (1961), J. Biol. Chem. 236, 1372.
4. Zubay, G., Lederman, M. and DeVries, J. K. (1967), Proc. Natl. Acad. Sci. U.S. 58, 1669.