A SOLUBLE AMINO ACID INCORPORATING SYSTEM

Akira Kaji,[1] Hideko Kaji,[2] and G. David Novelli
Biology Division
Oak Ridge National Laboratory[3]
Oak Ridge, Tennessee

Received February 5, 1963

In the course of studies on the stimulatory effect of an RNA-polymerase preparation from *Escherichia coli* on C^{14}-leucine incorporation into a protein fraction by extracts of this organism, it was found that the crude RNA-polymerase fraction by itself catalyzes this incorporation without addition of the usual ribosomal and soluble components. Incorporated radioactivity was determined in the usual fashion, after extraction of materials soluble in cold trichloroacetic acid (TCA), hot TCA, and lipid solvents (Mans and Novelli, 1960). Since the fraction was isolated from a supernatant practically free of the ribosomes, the characteristics of this incorporating system were studied. The results of this investigation are briefly reported here and will be published in detail elsewhere.

E. coli B. or *E. coli* Lac$^-$ strain (ML 308) was grown in a minimal salts medium with glycerol as a carbon source. Cells were harvested at early stationary phase, washed with 0.01 *M* tris-HCl pH 7.5, 0.01 *M* magnesium acetate, and 0.01 *M* β-mercaptoethanol (medium A) and kept frozen at -20°C. Similar results were obtained when freshly harvested cells were used. A suspension of 120 g of the wet cells in 100 ml of medium A was passed once through a French pressure cell at 12,000 psi. The suspension was then made up to 700 ml by adding medium A and cellular debris was removed by centrifuging twice at 15,000 *g* for 1 hr. The supernatant (S-15) was centrifuged at 85,000 *g* for 2.5 hr and the resulting supernatant (S-85) was fractionated essentially according to Chamberlin and Berg (Chamberlin and Berg, 1962), with the following modifications: (1) All the buffers used were medium A instead of those used by the original authors. (2) The protamine precipitate was eluted (without washing) with 60 ml of 0.3 *M* ammonium sulfate in medium A, and the eluted fraction (protamine eluate) was centrifuged to eliminate any insoluble materials. To 65 ml of the protamine eluate, 27.8 ml of saturated ammonium sulfate (pH 7.2, saturated at 25°C) was added and the precipitate was collected and dissolved in 5 ml of medium A (Fraction I). To the supernatant liquid was added an additional 28.4 ml of the saturated ammonium sulfate, the precipitate was collected, and dissolved in 20 ml of medium A (Fraction II). Fractions I and II were dialized against medium A overnight

[1]Helen Hay Whitney Foundation Fellow.
[2]U.S. Public Health Service Postdoctoral Fellow.
[3]Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

at 4°C. All the experiments reported here were carried out using Fraction II. S-RNA was prepared according to Ofengand *et al.* (Ofengand *et al.*, 1961). Density gradient centrifugation experiments were carried out essentially as described by Britten and Roberts (1960).

When Fraction II was incubated in the presence of ATP, an ATP generator, amino acids, C^{14}-leucine, GTP, and S-RNA, the radioactive leucine was incorporated into the protein fraction (Table 1).

TABLE 1

Incorporation of C^{14}-leucine by Fraction II

Additions and Deletions	cpm/0.1 ml	
	20 min	40 min
Complete	1869	2538
– ATP generator and ATP	18	25
– GTP	1835	2839
– S-RNA	66	124
– Amino Acids	1465	2547
+ RNase	19	18

The complete reaction mixture contained 1.5 mg of Fraction II, 2.0 μmoles of ATP (potassium salt), 4.0 μmoles of phosphoenol pyruvate (potassium salt), 0.05 mg of pyruvate kinase from Böhringer, 50 μmoles of tris buffer pH 7.5, 416,000 cpm of C^{14}-leucine (44 $\mu c/\mu$mole), 4.8 μmoles of magnesium acetate, 1 μmole of $MnCl_2$, 2 μmoles of β-mercaptoethanol, 0.2 μmole of GTP, 80 μg of L-amino acids (except leucine) according to Wallenfels and Arens (1960), and 0.3 mg of S-RNA in a total volume of 0.5 ml. Incubation was carried out at 37°C in air. Of the reaction mixture 0.1 ml was taken at the time intervals, and incorporated radioactivity was counted as described previously (Mans and Novelli, 1960) by Packard Scintillation Counter (counting efficiency, 1 $\mu c = 10^6$ cpm). Where indicated, 75 μg of pancreatic ribonuclease was added in the reaction mixture.

It is clear that incorporation was dependent on S-RNA and the energy generator. Incorporation was almost completely inhibited by pancreatic RNase but was not influenced by deletion of either GTP or the unlabeled amino acid mixture.

Preliminary examination of the distribution of the incorporating activity in the fractionation described above revealed that this activity is found mainly in Fractions I, II, and the streptomycin precipitate (see Chamberlin and Berg, 1962). Treatment of the incorporated radioactivity with trypsin, chymotrypsin, and papain made the radioactivity hot TCA soluble completely.

Since Fraction II was derived from S-85 which is practically free of ribosomes, the sedimentation behavior of this fraction was studied in comparison with S-15 which contains ribosomes. It was found that the sedimentation pattern of Fraction II was radically different from that of S-15 and that the most rapidly sedimenting material present in Fraction II was a trace of a 30 S (Svedberg unit) component. This 30 S material constitutes less than 5% of the total.

That the physical behavior of Fraction II is markedly different from S-15 was further confirmed by the distribution of activity after centrifugation of Fraction II. In this experiment, Fraction II was centrifuged for 2 hr at 105,000 *g*, and both supernatant and pellet were tested for the incorporating activity. It was found that most of the activity remained in the supernatant fraction and that reconstitution of the pellet with the supernatant did not cause stimulation.

Unequivocal proof that the incorporating activity of Fraction II does not depend on the trace of 30 S ribosomes was obtained from the sucrose gradient centrifugation experiment shown in Fig. 1. It can be seen from this figure that the peak of incorporating activity was found at the 24th tube which corresponds to approximately 10-S. No activity was found in the small amount of pellet obtained after the centrifugation. Combination of this pellet and the active fractions did not cause any stimulation of the incorporating activity.

Although these observations clearly show that incorporation of C^{14}-leucine into the protein fraction takes place in the absence of ribosomal particles, an important question remains as to the nature of the product. Preliminary experiments showed that treatment with carboxypeptidase did not make the incorporated radioactivity hot TCA soluble. The incorporated radioactivity behaved as a macromolecule on a Sephadex-G25 column. Furthermore, radioactivity from S-RNA–C^{14}-leucine prepared according to Zubay (Zubay, 1962) was incorporated by Fraction II into the protein fraction. Puromycin, at the level of 2×10^{-4} *M*, caused 60% inhibition. Together with the observation that the incorporated radioactivity is susceptible to proteolytic enzymes, these observations suggest that radioactivity is incorporated into the internal linkage of peptide chains. On the other hand, the observation that omission of non-radioactive amino acids had no influence on the extent of incorporation of C^{14}-leucine suggests that the product may be different from conventional protein.

In connection with this point, out of twelve labeled amino acids tested, only leucine and phenylalanine were incorporated to an extent comparable to that incorporated by the ordinary ribosome – supernatant system. Studies on the nature of the product and the biological significance of this incorporating system are in progress.

Acknowledgement

The authors would like to express appreciation to Dr. Peter Pfuderer for his help in performing the ultracentrifugal analysis.

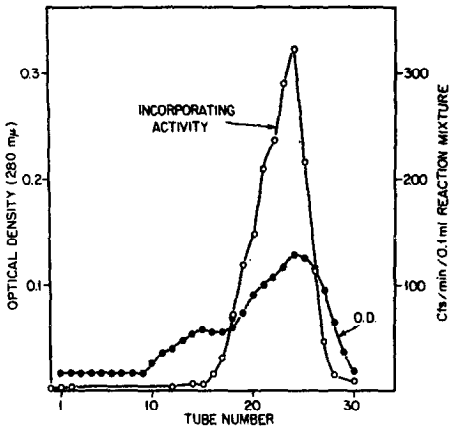

Figure 1. Density gradient centrifugation of Fraction II.

0.3 ml of Fraction II (14 mg/ml) in medium A containing 2% sucrose was layered on top of 4.7 ml of a linear sucrose gradient (3% to 20%) in medium A. The tube was centrifuged for 3 hr and 45 min at 39,000 rpm in the rotor SW 39L of the Spinco Model L centrifuge at about 4°C. After centrifugation, 2-drop fractions were collected in test tubes by puncturing the bottom of the centrifuge tube. The test tubes are numbered from the bottom fraction to the top fraction. The pellet was suspended in 0.3 ml of medium A. Of each fraction 0.1 ml was tested for its incorporating activity. The reaction mixture for incorporation was identical to that of Table 1 except for the amount of protein and the C^{14}-leucine added (130 μc/μmole leucine, 219,400 cpm). Radioactivity incorporated in the protein fraction after 60 min incubation is plotted against the tube number. For UV absorption measurement, each fraction was diluted with 2.5 ml of distilled water and the OD at 280 mμ was measured against water as a blank.

References

Britten, R. J. and R. B. Roberts, Science **131**, 32 (1960).
Chamberlin, M. and P. Berg, Proc. Natl. Acad. Sci., U.S. **48**, 81 (1962).
Mans, R. J. and G. D. Novelli, Biochem. Biophys. Res. Communs. **3**, 540 (1960).
Ofengand, E. J., M. Dieckmann, and P. Berg, J. Biol. Chem. **236**, 1741 (1961).
Wallenfels, K. and A. Arens, Biochem. Z. **332**, 247 (1960).
Zubay, G., J. Mol. Biol. **4**, 347 (1962).