COMPARISON OF THE STRUCTURE AND FUNCTION OF POLYSOMAL AND HELICAL RIBOSOMES FROM ENTAMOEBA INVADENS

THANIT KUSAMRARN, KAMOLWAT VINIJCHAIKUL,† and GORDON B. BAILEY

From the Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand. Dr. Bailey's present address is the Anemia and Malnutrition Research Center, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

ABSTRACT

Some structural and functional properties of ribosomes from polysomes and from helix aggregates of Entamoeba invadens have been compared by sucrose gradient analysis and assays of in vitro protein synthesis.

Actively growing trophozoites, lacking helices, presented normal polysome profiles in sucrose gradients. The single large ribosomal helix aggregate (chromatoid body) of cysts disappeared as the cells were disrupted. Gradient profiles of cyst extracts contained predominantly large and small ribosome subunit peaks and no evidence of remaining helix fragments or mRNA-bound polysomes. Sequential profiles of trophozoites incubated with NaF or cycloheximide (which both stimulate ribosome aggregation, but at different rates) showed that polysome breakdown occurred before aggregates appeared and, again, that helices broke down to subunits in vitro. Radioactive ribosomes synthesized during vegetative growth were collected into helices during encystation. Subunits of these ribosomes cosedimented with comparable particles isolated from trophozoites. Ribosomes from both trophozoites and cysts were active in cell-free protein synthesis, although activity in cyst extracts required the addition of trophozoite-soluble fraction. It was concluded that ribosomes from polysomes and helices in E. invadens were probably identical and that the ability to form helices was an intrinsic property of mature mRNA-free ribosomes of this organism.

During cellular differentiation of Entamoeba from vegetative trophozoites to cysts, a large symmetrical array of ribosome-containing helices is formed. This so-called chromatoid body appears to contain virtually all of the cyst ribonucleoprotein (1). Helices and smaller helix aggregates have also been observed at times in Entamoeba trophozoites, and these are ultrastructurally similar to the cyst arrays (2). In the preceding paper (4) we suggested that there was a correlation between the natural appearance of ribosome aggregates in amebas and periods of reduced metabolic activity. We demonstrated that massive aggregation could be induced by treatment of actively growing trophozoites with inhibitors of protein synthesis. These aggregates
appeared cytochemically and ultrastructurally the same as those formed naturally. The assembly of helices in inhibitor-treated trophozoites required neither protein nor nucleic acid synthesis. It appeared to depend simply on the accumulation in the cytoplasm of mRNA-free ribosomes. We proposed that the ability to form helices and arrays of helices was an intrinsic property of functional Entamoeba ribosomes and that aggregation would occur whenever these particles were unable to participate in the translational cycle. Others have suggested that the ribosomal particles comprising helices in Entamoeba represent an intermediate stage in the maturation of functional ribosomes and that these may be synthesized in large numbers during differentiation (3).

To help resolve this question and to begin to understand the physiological significance of ribosome helices in Entamoeba, it is important to determine what, if any, structural or functional differences exist between polysomal- and helix-bound ribosomes in this organism. In this report we describe the sedimentation properties of ribosomes isolated from growing and inhibitor-treated trophozoites and from the helix arrays in cysts. In addition, we have established conditions for the measurement of peptide synthesis in cell-free extracts from E. invadens trophozoites and have tested the ability of ribosomes isolated from cysts to function in this system.

MATERIALS AND METHODS

Cultures

Growth and encystation of E. invadens, strain IP-1, stimulation of ribosome aggregation by NaF and cycloheximide (CH), and visualization of aggregates by light microscopy were all accomplished as described in the preceding paper (4).

Preparation of Cell-Free Extracts

Growth and encystation cultures were chilled in ice for 5 min and then processed in the cold as follows: The amebas were collected by centrifugation at 500 g for 5 min and washed once in TKM buffer (10 mM Tris, 10 mM KCl, 5 mM MgCl₂, pH 7.5) containing 0.2 M sucrose. To rid encystation cultures of remaining trophozoites, the washed cells were suspended and agitated briefly in TKM containing 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). They were then washed three times with TKM alone to remove the detergent, and finally returned to TKM plus sucrose. Cysts prepared in this manner contained less than 2% trophozoites. Their ribosome aggregate content was unaffected by detergent treatment.

Both cysts and trophozoites, suspended in 1.5 vol of TKM plus sucrose, were disrupted by 10 strokes of a motor-driven Teflon pestle in a close-fitting glass homogenizer. After centrifugation at 20,000 g, the supernatant solution (S-20) was removed for use in sucrose gradient analyses or protein synthesis. When extracts were prepared for the latter experiments, 10 mM mercaptoethanol was added to TKM buffer.

Sucrose Density Gradient Analyses

Ribosome patterns in S-20 extracts were analyzed in 15-40% linear sucrose gradients prepared in TKM buffer. Before being layered on the gradients, samples were made 0.1% in Triton X-100. Centrifugation was carried out for 90 min at 40,000 rpm in a Beckman Spincwo SW 41 rotor (Beckman Instruments, Spincwo Div., Palo Alto, Calif.) at 5°. The sedimentation profiles were scanned at 254 nm using ISCO (Instrument Specialities Co., Lincoln, Neb.) equipment and a 5-mm light path.

The sedimentation coefficients of E. invadens ribosome particles were estimated by sucrose gradient centrifugation using Escherichia coli and rat liver ribosomes as standards and using techniques described by Noll (8). The standard ribosomes were centrifuged through separate, identical sucrose gradients simultaneously with the ameba particles.

Preparation and Cosedimentation of Radioactive Ribosomes from Trophozoites and Cysts

Growth media were prepared containing either 3 μCi/ml [14C]inosinic phosphate (Atomic Energy Commission of Thailand) or 10 μCi/ml [3H]uridine (New England Nuclear, Boston, Mass.). The [3P] medium was inoculated with trophozoites 48 h before the [H] medium. After approximately 2.5 generations (before the end of growth phase), the [3P]-labeled trophozoites were harvested, washed twice, and then suspended in encystation medium which contained 5 mM nonradioactive inorganic phosphate. The vegetative cells contained no ribosome aggregates at the time of transfer to encystation medium. After 48 h, encystation was completed. At this time both the [3P]-labeled cysts and the [3H]-labeled trophozoites (now at late growth stage) were harvested. The cysts were purified as described above and S-20 fractions were prepared from both the trophozoites and cysts. During fractionation the MgCl₂ concentration was reduced to 0.1 mM to allow dissociation of polysomes and mono-
Analysis of RNA and Protein peaks from sucrose gradients was assayed by the GF/A disks. The filters were washed with 5% TCA containing approximately equal amounts of trichloroacetic acid (TCA)-precipitable radioactivity were then mixed and treated with Triton X-100 as above. Samples were centrifuged in 10–30% sucrose gradients for 120 min at 40,000 rpm in the SW 41 rotor.

Autoradiography

Amebas were grown in the medium containing [3H]uridine and encysted as described above. The encystation medium contained approximately 50 μg/ml of nonradioactive uridine.

After harvest and purification, the cysts were fixed and embedded in Araldite as described in the preceding paper (4). Approximately 2-μm sections were cut and dried onto microscope slides at 30°C. The slides were dipped in Kodak NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed for 2 wk. After developing, the sections were stained for 1 min with 1% toluidine blue in 1% sodium borate at 60°C to reveal the chromatoid bodies, and stained briefly with D’Antoni’s iodine to show the nuclei.

In Vitro Protein Synthesis

Ribosomal and soluble fractions for cell-free protein synthesis assays were prepared by centrifugation of 6 ml of S-20 extract through a discontinuous gradient (2 ml, 2 M sucrose; 3 ml, 0.5 M sucrose in TKM plus 10 mM mercaptoethanol) for 3.5 h at 40,000 rpm in the SW 41 rotor. The upper layer (S-150) was immediately dialyzed for 5 h against TKM plus mercaptoethanol. The middle layer was discarded, and the remainder was retained as the ribosomal fraction.

[3H]Phenylalanine incorporation was measured in a total volume of 0.5 ml which included 0.1 ml each of the S-150 ribosomal fractions, (or 0.2 ml of S-20 extract) plus 0.3 ml of a mixture which yielded final concentrations as follows: 10 mM Tris, pH 7.5; 10 mM KCl, 10 mM MgCl₂, 10 mM potassium phosphoenolpyruvate, 1 mM ATP, 50 μM GTP, 2 U/ml pyruvate kinase (Sigma Chemical Co., St. Louis, Mo.), 10 μM each L-amino acid (excluding phenylalanine), 1 μCi L-[2,3-3H]phenylalanine, (4.5 Ci/mol, New England Nuclear) and 50 μg/ml polyuridylic acid (poly U, Sigma Chemical Co.).

Reactions were incubated at 30°C for 30 min and stopped with 2.5 ml cold 5% TCA containing 3 μg/ml phenylalanine. Samples were heated at 90°C for 10 min, placed in ice for 30 min, and then filtered on Whatman GF/A disks. The filters were washed with 5% TCA containing phenylalanine, dried, and counted in a toluene-based scintillation fluid.

Analysis of RNA and Protein

The total RNA in cell-free extracts and in ribosome peaks from sucrose gradients was assayed by the Schmidt-Thannhauser procedure as described by Munro and Fleck (7) using E. coli RNA as a standard. The RNA-containing peaks from sucrose gradients were collected after spectrophotometric analysis, and the RNA was hydrolyzed directly without prior precipitation. Protein was analyzed by the method of Lowry et al. (5) using crystalline bovine serum albumin as a standard.

RESULTS

The Instability of Ribosome Helices

When inhibitor-treated trophozoites or cysts, both of which contained large ribosome aggregates (4), were disrupted by any of a variety of methods, over 90% of the total cell RNA remained in the S-20 fraction after centrifugation. If whole or even partially fragmented ribosome arrays survived in the cell homogenates, they should have been sedimneted by this force. Addition of aldehyde fixatives immediately after cell lysis did not preserve aggregates large enough to be detected by light microscopy and did not alter the distribution of RNA during centrifugation at 20,000 g.

When inhibitor-treated trophozoites were lysed with detergent, or cysts were broken by pressure while being observed under the microscope, the large aggregates (visible by phase-contrast optics within fresh cells) were seen to disintegrate instantaneously as the amebas were disrupted and their cellular contents escaped. Thus, the ribosome helices in both cysts and inhibited trophozoites appeared extremely fragile, at least under the conditions employed to prepare cell-free extracts. Unable to isolate the aggregates present within the cells, we proceeded to analyze the degree of ribosome aggregation in S-20 fractions by gradient centrifugation.

Ribosome Profiles

The ribosome profile of growing trophozoites which lacked helix aggregates is shown in Fig. 1a. A typical pattern was seen with minor subunit peaks, a significant monosome peak and a broad distribution of polysomes. Brief treatment of the S-20 fraction with pancreatic RNase (Worthington Biochemical Corp., Freehold N.J.) before centrifugation destroyed most of the polymer peaks and caused an increase in the proportion of monomers (Fig. 1b). Preincubation with an energy-generating system (Fig. 1c) caused a shift to a mixture of monosomes and subunits, and puromycin treatment (Fig. 1d) produced almost exclusively subunit peaks. Thus, the only aggregated ribosomes appearing in growing trophozoites behaved as active mRNA-bound poly
are shown in Fig. 2. When extracts were prepared and centrifuged in the presence of 5 mM MgCl₂ (as for trophozoites), only monosome and subunit peaks were observed, with a heavy predominance of the latter. There was no evidence of mRNA-bound ribosomes, nor was there any indication of remaining ribosome helices, which should have appeared as heavier peaks or at the bottom of the tube. Raising the MgCl₂ concentration from 5 to 10 mM during isolation and centrifugation of cyst extracts preserved monosomes (Fig. 2 b). However, this or higher concentrations did not yield larger aggregates.

The cysts used for this preparation were free of trophozoites, and nearly all contained a single large chromatoid body in which most of the cell RNA is localized (see reference 1 and Results below). After homogenization and initial centrifugation, over 95% of the cyst RNA remained in the S-20 fraction. After sucrose gradient centrifugation, almost 90% of this material was recovered from the monosome and subunit peaks. It must be concluded, therefore, that most of the ribosome particles in these peaks derived from the helices of the chromatoid bodies, and that these aggregates collapsed completely to unit particles when the cysts were disrupted.

The profiles of extracts prepared from trophozoites treated for different times with NaF and CH were next analyzed (Figs. 3 and 4). Both of

some, indicating that these amebas contained ribosomes distributed as expected in metabolically active eucaryotic cells.

Ribosome patterns in S-20 fractions from cysts
these inhibitors stimulate massive aggregation of *E. invadens* vegetative ribosomes (4). However, the rate of aggregate formation is considerably faster with NaF than with CH. We proposed that this rate differential indicated that ribosomes had to be released from mRNA before assembly into helices, since release would be expected to occur more rapidly with NaF than with CH. This proposal was supported by the results shown in Figs. 3 and 4. After 15 min of treatment with NaF, when 65% of the trophozoites contained numerous small ribosome arrays, few polysome peaks remained and the ribosomes appeared as a mixture of monosomes and subunits (Fig. 3 b). In contrast, trophozoites treated with CH still presented a normal polysome pattern 15 min after addition of inhibitor (Fig. 4 b). At this time ribosome aggregates were not yet visible in the cells. However, by 3 h, when aggregation had reached 60% in the CH-treated cells, polysomes had disappeared.

As aggregation proceeded in the presence of both of these inhibitors, there was a progressive decrease in the monosome peak with a concomitant increase in the subunit peaks. When aggrega-

FIGURE 3 Gradient profiles of ribosomes in S-20 extracts from trophozoites treated with 50 mM NaF. Inhibitor was added to growing cultures, and samples were taken to prepare S-20 extracts at the times indicated. The percentage of the cells containing numerous ribosome aggregates at each time was: 0 min, 0%; 15 min, 65%; 45 min and 2 h, 100%. Each gradient was layered with 8 A260 U of S-20. Other details were as described in Fig. 1.

FIGURE 4 Gradient profiles of ribosomes in S-20 extracts isolated from trophozoites treated with 100 μg/ml CH. The percentage of cells containing numerous ribosome aggregates at each time was: 0 min and 15 min, 0%; 3 h, 60%; 8 h, 100%. Each gradient was layered with 8 A260 U of S-20. Other details were as described in Fig. 3.
tion appeared complete (3 h for NaF, 8 h for CH), the ribosome profiles were similar to the pattern from cysts, primarily subunit peaks (Figs. 3 d, 4 d). Thus the natural and induced helices behaved similarly when cells were disrupted again, suggesting that they had similar structures in vivo.

**Comparison of Cyst and Trophozoite Ribosome Sedimentation Coefficients**

The sedimentation coefficients of monosome and ribosomal subunits from helix-free trophozoites and from cysts were determined as described in Materials and Methods. The average values from two runs of trophozoite particles were 80S, 59S, and 38S for monosomes, large subunits, and small subunits, respectively. The average values for comparable particles from cysts were 79.5S, 62S, and 38S. The breadth of experimental error in the separate determinations made it impossible to conclude whether or not any actual differences in the sedimentation values of the related particles from these two sources existed. A more accurate comparison was obtained by cosedimentation of differentially radioactive ribosome subunits from trophozoites and cysts. The labeled particles were prepared as described in Materials and Methods. The cosedimentation profile is shown in Fig. 5. There was no detectable difference between the sedimentation rates of either the large or small labeled subunits of trophozoite polysomes and cyst helix aggregates.

**The Presence of Trophozoite Ribosomes in the Chromatoid Body of Cysts**

The design and interpretation of the above cosedimentation experiment assumed that the 32p-labeled ribosomes isolated from cysts had been synthesized during vegetative growth and were contained within the helices of the cyst chromatoid body. This was confirmed by autoradiography of cysts prepared exactly as described for the cosedimentation experiment, but labeled with [3H]uridine rather than [32P]phosphate. As can be seen in Fig. 6, virtually all the radioactivity was localized within the chromatoid bodies. Thus, most if not all of the cyst ribosomes must be collected into this aggregate, and it can be concluded that the 32p ribosomes from cysts labeled in a similar manner also came primarily from chromatoid helices.

Furthermore, most if not all of these ribosomes must have been synthesized during vegetative growth when the exogenous radioactive precursor was present. For if the radioactive ribosomes isolated from cysts had been synthesized only during encystation with labeled degradation products of trophozoite RNA, it would have been necessary (a) that nearly all vegetative RNA had been destroyed during encystation, and (b) that the radioactive catabolites from RNA breakdown had been utilized for resynthesis in overwhelming preference to the exogenous nonradioactive precursor present in the encystation medium. Seng has shown that exogenous uridine and inorganic phosphate are both readily incorporated during the early stages of encystation. Therefore, it is unlikely that both of these limitations were fulfilled and reasonably certain that the radioactive ribosomes present in cyst chromatoid bodies were synthesized during vegetative growth.

**Protein Synthesis in Cell-Free Extracts from Trophozoites and Cysts**

In order to determine the biological activity of ribosomes isolated from helix aggregates in cysts, conditions were first sought to assay poly U-directed polyphenylalanine synthesis in trophozoite cell-free extracts. Optimal conditions

---

2 Seng, L. Manuscript in preparation.
were established using an S-20 fraction from actively growing trophozoites. Distinct optima were found for the concentrations of MgCl₂ (10 mM), KCl (10 mM), and temperature (30°C). At these optima, [³H]phenylalanine incorporation was linear for 30 min.

Successful separation of active soluble (S-150) and ribosomal fractions was accomplished by centrifugation of the trophozoite S-20 fraction through sucrose followed by dialysis. The dependence of incorporation on the presence of both these fractions and poly U is shown in Table I. Over 96% of the complete system activity was lost when either the S-150 or the ribosomal fraction was omitted from the assay mixture. Low but significant activity was detected in the absence of poly U, indicating the presence of some translatable endogenous mRNA, probably in the polysome-containing ribosomal fraction.

The S-20 fraction prepared from cysts was essentially inactive when assayed in the same way as the trophozoite extract (Table I, expt. 2). However, marked incorporation occurred when the dialyzed soluble (S-150) fraction from trophozoites was added to the cyst S-20. Since the trophozoite soluble fraction was inactive alone, this incorporation must have been dependent upon cyst ribosomes. Thus, at least some of the ribosomes from helices in cysts were biologically active.

| Expt.   | Fraction                  | [³H]phenylalanine synthesis | cpmp* |
|---------|---------------------------|-----------------------------|-------|
| I       | Troph S-150 + troph rib   | 16,150                      |       |
|         | Troph S-150 + troph rib   | 2,700                       |       |
|         | (minus poly U)            |                             |       |
|         | Troph S-150               | 600                         |       |
|         | Troph rib                 | 600                         |       |
| II      | Cyst S-20                 | 130                         |       |
|         | Troph S-150               | 200                         |       |
|         | Cyst S-20 + troph S-150   | 4,000                       |       |
|         | Cyst S-20 + troph rib     | 150                         |       |

Cell-free extracts were prepared and assayed as described in Materials and Methods. Assays contained 0.5 mg trophozoite S-150 protein, 1 A₂₆₀₅ unit of trophozoite ribosomes, and 0.5 mg cyst S-20 protein as appropriate.

The restoration of activity in the cyst S-20 by the trophozoite soluble fraction implied that during encystation there was a loss of activity of one or more soluble factors required for protein synthesis.
This may be an important event in the mechanism of encystation and could explain why potentially functional ribosomes aggregate in cysts.

DISCUSSION

Although it was not possible to isolate wholly or partially intact ribosome aggregates from cysts or inhibitor-treated trophozoites of *E. invadens*, it is quite certain that the ribosome subunit peaks observed in sucrose gradient profiles of extracts from these cells represented particles derived principally from helices. Barker and Sviha (1) first provided convincing evidence that the ribosomes in cysts were localized primarily in the chromatoid body when they showed by UV microscopy that most of the cellular material absorbing light at 265 nm (nucleic acid) was concentrated in this structure. Our autoradiographs showing that [3H]uridine in cysts was present almost exclusively in the chromatoid body when they showed by UV microscopy that most of the cellular material absorbing light at 265 nm (nucleic acid) was concentrated in this structure. Our autoradiographs showing that [3H]uridine in cysts was present almost exclusively in the chromatoid body when they showed by UV microscopy that most of the cellular material absorbing light at 265 nm (nucleic acid) was concentrated in this structure. Some profiles indicating that helices collapsed completely to free large and small subunits when the cells were disrupted. These subunits were biologically active and could be preserved as monosomes by raising the magnesium concentration. Nevertheless, it appeared that at some stage during aggregation, ribosomes lost some property which allowed the interaction of subunits before helix formation. This may have involved the addition or removal of extraribosomal factors beyond mRNA.

It was clear from the sequential sucrose gradient analyses of extracts from trophozoites treated with NaF and CH that polysome breakdown occurred before, and was therefore probably required for ribosome aggregation. It follows, then, that mRNA was absent from the helix aggregate structures. Cysts and inhibited trophozoites in which aggregation was maximal presented ribosome profiles indicating that helices collapsed completely to free large and small subunits when the cells were disrupted. These subunits were biologically active and could be preserved as monosomes by raising the magnesium concentration. Nevertheless, it appeared that at some stage during aggregation, ribosomes lost some property which allowed the interaction of subunits before helix formation. This may have involved the addition or removal of extraribosomal factors beyond mRNA.

Aggregates disintegrated rapidly when the amebas were disrupted. It has been suggested that this is the result of enzymatic destruction (6). We feel that an equally probable explanation is that the chemical environment after cell lysis was inappropriate for continued strong interaction between the ribosome particles. If so, further study may reveal conditions which will allow isolation of intact arrays or possibly permit stimulation of symmetrical aggregation of *Entamoeba* ribosomes in vitro.

We thank Dr. Douglas C. Barker for helpful comments during the course of these studies. Two of us, Thanit Kusamrarn and Gordon B. Bailey, express deep sorrow at the untimely passing of our enthusiastic colleague and coauthor, Dr. Kamolwat Vinijchaikul.
REFERENCES

1. Barker, D. C., and G. T. Svihla. 1964. Localization of cytoplasmic nucleic acid during growth and encystment of Entamoeba invadens. J. Cell Biol. 20:389-398.

2. Barker, D. C., and L. S. Swales. 1972 a. Comparison of trophozoite helical polysomes with cyst ribosomogen microcrystals in axenic Entamoeba sp. Cell Diff. 1:307-315.

3. Barker, D. C., and L. S. Swales. 1972 b. Characteristics of ribosomes during differentiation of trophozoites to cysts in axenic Entamoeba sp. Cell Diff. 1:297-306.

4. Kusamrarn, T., and G. B. Bailey. 1975. The mechanism of formation of inhibitor-induced ribosome helices in Entamoeba invadens. J. Cell Biol. 65:529-539.

5. Lowry, O. H., N. J. Rosebrough, R. J. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

6. Morgan, R. S., H. S. Slayter, and D. L. Weller. 1968. Isolation of ribosomes from cysts of Entamoeba invadens. J. Cell Biol. 36:45-51.

7. Munro, H. N., and A. Fleck. 1966. The determination of nucleic acids. In Methods of Biochemical Analysis. D. Glick, editor. Interscience Publishers, Inc., New York. 14:113-176.

8. Noll, H. 1969. Polysomes: analysis of structure and function. In Techniques in Protein Biosynthesis. P. N. Campbell and J. R. Sargent, editors. Academic Press, (Inc.), London. 2:101-179.