The Nature of the Proteins Present in the 'Relaxed Particles' from Methionine-starved *Escherichia coli* A19 (Hfr *rel met rns*)

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SUMMARY

The 'relaxed particles' formed during methionine starvation of *Escherichia coli* A19 (Hfr *rel met rns*) have been isolated by large-scale rate-zonal density gradient ultracentrifugation. The proteins and rRNA species associated with these particles have been examined.

The rRNA species present are precursor and mature forms of 16S and 23S rRNA. The bulk of the rRNA which accumulates during starvation is found within the particles. The proteins prepared directly from the particles give strong multiple immunoprecipitates with antisera specific to 30S and 50S ribosomal proteins. The soluble proteins, prepared and examined in the same manner, do not give this immunological reaction. Two-dimensional electrophoresis patterns of the proteins from the particles show that the proteins co-migrate with proteins from 30S and 50S ribosomes and are entirely dissimilar to the proteins prepared by the same methods from the soluble fraction of the cells. On the basis of these and other observations, it is concluded that the 'relaxed particles' are not artefacts but are arrested ribosome precursors containing both rRNA and certain ribosomal proteins.

The free pool of ribosomal proteins is low in exponential-phase cells and is not significantly increased by a 2 h period of starvation for glucose.

The implications of these observations concerning the proteins associated with 'relaxed' and 'chloramphenicol particles' are discussed in relation to ribosome biogenesis and the stabilization of rRNA.

INTRODUCTION

Methionine starvation of the relaxed auxotroph *Escherichia coli* A19 (Hfr *rel met rns*) [for abbreviations, see preceding paper (Sykes, Metcalf & Pickering, 1977)] dissociates RNA synthesis from protein synthesis. RNA synthesis continues until the cellular RNA content has reached two to three times the pre-starvation amount whilst protein synthesis is depressed to only 5 to 10 % of the normal exponential rate. Low molecular weight proteins are preferentially synthesized during starvation of these relaxed strains (Goodman, Manor & Rombauts, 1969; Brunschede & Bremer, 1971). Centrifugal fractionation of extracts from the cells as starvation proceeds reveals the steady accumulation of abnormal ribonucleoprotein particles, 'relaxed particles', sedimenting between 14 and 30S (Dagley, Turnock & Wild, 1963). During methionine starvation, all the normal cellular RNA species continue to be synthesized and since few new mature ribosomes are formed the primary reason for the steady increase in the cellular RNA content is the accumulation of p16S and p23S rRNA (Adesnik & Levinthal, 1969; Chang & Irr, 1973; Dean & Sykes, 1974) which may be sub-methylated (Osawa *et al.*, 1969). These precursor RNA forms are relatively

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stable during the starvation period, and when the required amino acid is restored, growth resumes and the precursor rRNAs are converted to their mature forms without degradation (Chang & Irr, 1973). The period immediately following the restoration of the required amino acid to the starved culture is notable for the temporary suppression of RNA synthesis, the preferential synthesis of certain ribosomal proteins and the loss of the 'relaxed particles' (Nakada, 1965; Goodman *et al.*, 1969). Nakada & Unowsky (1966) have shown that the 'relaxed particles' will combine with solubilized ribosomal proteins *in vitro* to form functional ribosomes.

The events associated with methionine starvation and recovery of the relaxed strain closely parallel those observed during chloramphenicol inhibition. However, the possibility that the 'relaxed particles' may be arrested precursors has never been fully confirmed nor seriously questioned although by inference from the earlier observations with 'chloramphenicol particles' they may be artefacts (Schleif, 1968; Yoshida & Osawa, 1968).

The properties of the rRNA species which accumulate during starvation and their behaviour on recovery are entirely consistent with their being sub-methylated but otherwise normal precursor rRNAs. These rRNAs are found within the 'relaxed particles' in association with proteins which have been reported to have similar mobilities in one-dimensional polyacrylamide gel electrophoresis to some ribosomal proteins (Nakada, 1967). There are clearly many points of similarity between the response to chloramphenicol treatment and methionine starvation in *E. coli*. With the *E. coli* A19 strain used in previous experiments with chloramphenicol (Sykes *et al.*, 1977), it is possible to produce 'relaxed particles' and so directly compare the nature of the proteins associated with the RNA in the particles produced by genetic and phenotypic relaxation in the same organism.

METHODS

Organism and growth conditions. The organism, materials and techniques used were as described in the preceding paper (Sykes *et al.*, 1977) except that the period of chloramphenicol treatment was replaced by a 2 h period of starvation in complete medium lacking methionine. During starvation the absorbance of the culture at 540 nm increased by 35.3 % (average of eight experiments).

All the other methods employed were as described previously, with 'relaxed particles' R1, R2 and R3 replacing references to 'chloramphenicol particles'.

RESULTS

Zonal and analytical ultracentrifugation

Figure 1(a) shows the analytical ultracentrifuge diagram for a crude cell-free extract from *E. coli* A19 cells starved for 2 h in complete medium lacking methionine. Comparison with the corresponding pattern for control, exponentially growing cells [see Fig. 1a, preceding paper (Sykes *et al.*, 1977)] shows that the 'relaxed particles' sediment in the region 10 to 30S just like the 'chloramphenicol particles' (see Fig. 2a, preceding paper). Figure 1(b) shows the E_{260} profile for the gradient recovered from the zonal ultracentrifuge run with the supernatant fraction from this crude extract. The samples from the gradient pooled to provide 'relaxed particle' preparations R1, R2 and R3 are indicated. Similarly, Fig. 1(c) shows the E_{260} profile for the pellet fraction and the samples pooled to provide further R1, R2 and R3 material and also 50S ribosomes from methionine-starved cells [50S (MET⁻)].

The analytical and zonal ultracentrifuge profiles for extracts from A19 cells starved for

Fig. 1. Analytical schlieren and zonal ultracentrifuge gradient absorbance profiles for extracts from exponential-phase cells of *E. coli* A19 after 2 h in complete medium lacking methionine. Sedimentation is from left to right.

(a) Analytical ultracentrifuge schlieren diagram for the crude cell-free extract from methionine-starved cells. Protein concentration 10.0 mg ml^{-1} ; temperature 20.9°C ; bar angle 30° ; photograph taken after 20 min at $50\,740 \text{ rev. min}^{-1}$. The 'relaxed particles' are seen, by comparison with Fig. 1 (a) (preceding paper), to be sedimenting between the leading 50S ribosome boundary and the large, slowest moving boundary.

(b) Rate-zonal ultracentrifuge density gradient absorbance profile at 260 nm (continuous line) for the supernatant fraction from (a). The samples from the gradient pooled to provide 'relaxed particles' R1, R2 and R3 are indicated. Run conditions: MSE A1-14 rotor; load 250 mg total protein; 24 h at $30\,000 \text{ rev. min}^{-1}$; 5°C ; gradient 10 to 35 % (w/v) sucrose in TMN buffer hyperbolic with radius (dashed line indicates the gradient at the end of run).

(c) Rate-zonal ultracentrifuge density gradient absorbance profile at 260 nm (continuous line) for the pellet fraction from (a). The samples from the gradient pooled to provide additional R1, R2 and R3 material are shown together with those for the 50S (MET⁻) ribosomes. Run conditions: MSE A1-14 rotor; load 264 mg ribosome equivalents; 16 h at $26\,000 \text{ rev. min}^{-1}$; 5°C ; gradient 10 to 35 % (w/v) sucrose in TMN buffer hyperbolic with radius (dashed line indicates the gradient at end of run).

glucose for 2 h were identical to those shown for control cells (see Fig. 1a, b, c preceding paper). The soluble proteins used in later experiments were prepared from the pooled zonal gradient fractions containing the large, slowly sedimenting boundary material from the centrifugation of the supernatant fractions from these cells (compare Fig. 1b, preceding paper).

rRNA species present in samples from methionine-starved E. coli A19

Comparison of the electrophoretograms for the total rRNA from fully supplemented exponential-phase cells (Fig. 3a, preceding paper) and from methionine-starved cells (Fig. 2a) indicates that precursor RNA, particularly p16S rRNA which is well resolved,

Fig. 2. Absorbance profiles at 260 nm for the electrophoretic separation of rRNA preparations in polyacrylamide gels. The rRNA preparations were extracted from the cell-free extracts and gradient fractions, electrophoresed and scanned as described in the preceding paper (Sykes *et al.*, 1977). (a) Total rRNA (50 μ g) from crude cell-free extracts of exponential-phase cells of *E. coli* A19 2 h after transfer to fresh growth medium lacking methionine. (b) rRNA (23.5 μ g) from 50S (MET⁻) ribosomes recovered from the zonal ultracentrifuge (see Fig. 1c). (c) rRNA (40 μ g) from 'relaxed particle' region R1 (see Fig. 1b, c). (d) rRNA (30 μ g) from 'relaxed particle' region R2 (see Fig. 1b, c). (e) rRNA (28 μ g) from 'relaxed particle' region R3 (see Fig. 1b, c).

accumulates within the cells as the starvation proceeds, as was found for chloramphenicol inhibition. Figure 2(b) shows that intact 23S rRNA remains within the 50S (MET⁻) ribosomes that are still intact after 2 h starvation. The electrophoretograms for the RNA from 'relaxed particles' R1, R2 and R3 (Fig. 2c, d, e) reveal that the bulk of the precursor 16S rRNA which accumulates during starvation is associated with the particles. R1 (Fig. 2c) contains a high proportion of 23S and p16S rRNA indicating the presence of precursor RNA components of 50S and 30S ribosomes in this region. R2 (Fig. 2d) is similar although in this the proportions of p16S and m16S rRNA are not the same as in R1. R3 (Fig. 2e) has a predominance of m16S rRNA and 23S rRNA.

From these electrophoretograms, we conclude that precursor rRNA species accumulate in the cells during methionine starvation, and that the RNA which accumulates is associated with the 'relaxed particles'. The relaxed particle fractions taken in these experiments are clearly complex and embrace different proportions of RNA precursors of both 30S and 50S ribosomes. As the sedimentation coefficient increases through the particle region the proportion of precursor 16S rRNA diminishes. A further notable feature is the relative stability of the RNA from region R1 of the 'relaxed particles' compared with that from the corresponding region P1 of 'chloramphenicol particles'.

Fig. 3. Immunoprecipitates formed during double diffusion in one dimension between proteins from the soluble fraction of glucose-starved *E. coli* A19 and from the 'relaxed particles' R1, R2 and R3 and antisera specific to 30S or 50S ribosomal proteins. Undiluted antiserum to 30S (right) or 50S proteins (left) was in the topmost well (12 o'clock) and serial dilutions (1:1) were arranged clockwise in the surrounding wells. Proteins were in the centre well: (a) proteins from R1; (b) proteins from R2; (c) proteins from R3; and (d) proteins prepared by ribonuclease treatment of the soluble fraction from glucose-starved A19.

Immunological properties of the proteins associated with the 'relaxed particles' and the soluble fraction from glucose-starved cells

The raising of specific antisera to 50S and 30S ribosomal proteins of *E. coli* A19 and their immunological properties are described in the preceding paper. When these antisera were tested against the proteins isolated from the 'relaxed particles' R1, R2 and R3, they gave strong multiple immunoprecipitates with each particle at all dilutions of the respective antisera (Fig. 3*a, b, c*). This result unequivocally establishes the presence of both 50S and 30S ribosomal proteins throughout the 'relaxed particle' region. This is consistent with the finding of both rRNA precursors throughout the particle region and with the electrophoresis results (see below).

The immunological and protein electrophoresis experiments with the proteins of the soluble fraction from exponentially growing cells, described in the preceding paper, suggested a low level of free ribosomal proteins. It was therefore of interest to see if the level could be raised during a period of minimal growth such as occurs in chloramphenicol-inhibited or methionine-starved cultures. This should provide a more meaningful assessment of the free ribosomal protein pool potentially available for precursor particle formation. Exponentially growing cultures of *E. coli* A19 were therefore starved for 2 h in a complete medium lacking glucose. The soluble proteins were then prepared from the appropriate pooled samples after zonal ultracentrifugation of the supernatant fraction from extracts of these cells. Glucose starvation was chosen since it does not lead to obvious ribosome breakdown and there is no detectable growth, RNA or protein synthesis using the usual turbidimetric and colorimetric assays. In these circumstances any preferential synthesis or turnover of pre-existing ribosomal proteins may be expected to swell the free pool since there is no additional rRNA to bind and no accumulation of unusual ribonucleoproteins in the cells. The immunological response to the 50S and 30S protein antisera of the soluble proteins prepared from these cells by the acetic-acid and ribonuclease procedures was extremely weak (Fig. 3*d*) and comparable with that obtained with similar preparations from exponentially growing cells. Results for the gel electrophoresis of these proteins (see below) confirm this result. The pool of free ribosomal proteins is therefore not increased significantly by a 2 h period of growth inhibition due to glucose starvation.

Gel electrophoresis of proteins prepared from normal 30S and 50S ribosomes, 'relaxed particles' R1, R2 and R3 and the soluble cell proteins from control and glucose-starved cells

The two-dimensional gel electrophoresis patterns for normal 30S and 50S ribosomal proteins and the soluble proteins from exponentially growing cells are shown in Fig. 5(*a, b, c*) in the preceding paper. The corresponding patterns for the proteins isolated from 'relaxed particles' R1, R2 and R3 are shown in Fig. 4(*a, b, c*). A comparison of these patterns shows that the proteins from the 'relaxed particles' are *in toto* quite distinct from those in the soluble protein fraction of the cell and closely resemble the 30S and 50S ribosomal protein patterns. Most of the proteins in the particle regions are basic, and have relatively low molecular weights. These results, in conjunction with the immunological observations, establish that the proteins associated with the 'relaxed particles' are entirely ribosomal in nature.

The number of proteins associated with the rRNA species in the particles increases with the increasing sedimentation coefficient of the particles. Particle R1 (Fig. 4*a*) has five clearly discernible basic proteins and one acidic one in association with the mainly p16S

Fig. 4. Two-dimensional electrophoresis patterns for the proteins prepared from the 'relaxed particles' from methionine-starved *E. coli* A19 and the soluble fraction from glucose-starved cells. All the proteins were prepared as described in the preceding paper (Sykes *et al.*, 1977) and electrophoresis followed the procedure of Kaltschmidt & Wittmann (1970). (a) Proteins from 'relaxed particle' R1, 180 μ g total protein loaded. (b) Proteins from 'relaxed particle' R2, 200 μ g total protein loaded. (c) Proteins from 'relaxed particle' R3, 240 μ g total protein loaded. (d) Soluble proteins prepared via ribonuclease treatment from glucose-starved *E. coli* A19, 219 μ g total protein loaded.

rRNA and 23S rRNA present in this region. Particle region P2 (Fig. 4b) has 12 basic proteins and two to three acidic ones, and P3 (Fig. 4c) has 14 basic proteins and three acidic ones clearly resolved.

The majority of the proteins in region R3 co-migrate with the 30S proteins. This observation is consistent with the high proportion of m16S rRNA in this region and probably indicates the presence of mature 30S and late precursors of 30S ribosomes in this fastest sedimenting 'relaxed particle' region. The proteins in region R1 also co-migrate mainly with the 30S proteins. Since p16S rRNA predominates in this region (Fig. 2c) R1 is biased towards a 30S ribosome precursor.

50S proteins are not as evident in the electrophoretograms of the proteins from the relaxed particles' as they are in those from the 'chloramphenicol particles'. Chloramphenicol particle region P3 embraces far more ribosomal protein species than 'relaxed particle' region R3 (compare Fig. 4c with Fig. 5f in the preceding paper) although P3 has relatively more p16S rRNA and less 23S rRNA than R3 (compare Fig. 2e with Fig. 3f in

the preceding paper). Nevertheless 23S rRNA is present throughout the 'relaxed particle' region and the immunological evidence shows the presence of 50S proteins throughout the region.

Figure 4(*d*) shows the two-dimensional electrophoretic pattern of proteins obtained from the soluble fraction of *E. coli* A19 following a period of 2 h starvation for glucose in an otherwise complete growth medium. A comparison of this Figure with Fig. 5(*a*) in the preceding paper indicates that a period of zero growth does not raise the pool of free ribosomal proteins to a level detectable by electrophoretic or immunological analysis.

DISCUSSION

The results in this and the preceding paper show that the parallelism between phenotypic and genetic relaxation and restoration of the control of RNA synthesis in *E. coli* extends beyond the points described in the Introductions to the papers. In both types of relaxation the particles which accumulate contain ribosomal rRNA species, particularly the precursor forms, in association with ribosomal proteins. Proteins with the two-dimensional electrophoretic and immunological characteristics of those found in the soluble fraction of the cell are not found in the particles which accumulate during relaxation. The electrophoretic and immunological analyses show that all the proteins in the particles are ribosomal, either 30S or 50S proteins. The particles are therefore not artefacts formed between the rRNA synthesized during relaxation and the soluble proteins of the cell, as previously concluded (Schleif, 1968; Yoshida & Osawa, 1968). The proteins in the unusual ribonucleoproteins produced during relaxation of RNA control by Co^{2+} have also been shown to be ribosomal (Blundell & Wild, 1973). All the evidence concerning the formation and behaviour of the particles formed during the phenomena of phenotypic or genetic relaxation and restoration of control of RNA synthesis is therefore consistent with those particles being arrested ribosome precursors as suggested by Dagley *et al.* (1962). During relaxation, RNA synthesis continues unabated and protein synthesis drops to 5 to 10 % of the exponential rate. The proportion of this synthesis diverted to ribosomal proteins is twice as great during methionine starvation as the corresponding proportion during exponential growth. The proportions of the ribosomal proteins produced are also distorted compared with the co-ordinated synthesis of exponential growth since a few proteins are produced at very high relative rates (Goodman *et al.*, 1969; Goodman, 1970). The rRNA accumulating during relaxation is formed in the usual precursor forms within the particles although it may be sub-methylated in methionine-starved cultures (Adesnik & Levinthal, 1969; Lowry & Dahlberg, 1971; this paper). Recent studies on the specificity of interaction and the chronology of association of ribosomal proteins with mature and nascent rRNA *in vitro* (see Zimmermann, 1974, and Nomura & Held, 1974, for reviews; Nikolaev & Schlessinger, 1974) and *in vivo* (Nierhaus, Bordsch & Homann, 1973) show that an association between the nascent precursor rRNA and ribosomal proteins would be more likely than a non-specific association between the rRNA and the soluble proteins of the cell. In this case the particles steadily accumulating during relaxation would have all the characteristics of ribosome precursors, i.e. precursor rRNA in association with an increasing but selected number of ribosomal proteins. The usual orderly ribosome biogenesis sequence would, therefore, be arrested at a series of different points during relaxation depending on the availability of certain ribosomal proteins (Sykes, 1966; Chang & Irr, 1973; Marvaldi *et al.*, 1974). The points of arrest would not necessarily be the same in all relaxed states since they would depend on the size and composition of the free ribosomal protein pool (see below), the

pattern and extent of residual protein synthesis and turnover. The RNA:protein ratios of the components of the particle region would therefore vary. The evidence in these experiments shows that the pattern of both rRNA and protein distribution differs across the particle region for methionine-starved and chloramphenicol-induced relaxation. This is also consistent with earlier observations concerning the different stabilities of these particles (Dagley *et al.*, 1963). The stabilization of the nascent rRNA synthesized during relaxation may depend on its association with a critical number of specific ribosomal proteins as suggested by Chang & Irr (1973). This, coupled with a limited supply of ribosomal proteins, would account for the abrupt halt in RNA accumulation in relaxed states. This usually occurs when the RNA content of the cell has reached two to three times the pre-relaxation level (Dalgarno & Gros, 1968). It is therefore likely that these arrested states do not correspond to the kinetic 'hold-up' points detected for normal ribosome biogenesis since the latter are not primarily determined by a limited availability of certain ribosomal proteins. Ribosomal protein synthesis is fully co-ordinated during normal growth. The particles formed during relaxed states are therefore a heterogeneous collection of nascent 50S and 30S ribosome intermediates. They may be titrated with ribosomal proteins *in vitro* to produce functional ribosomes (Nakada & Unowsky, 1966) or *in vivo* converted to mature ribosomes by a preferential, skewed and complementary pattern of ribosomal protein synthesis (Nakada, 1965; Davis & Sells, 1969).

A final point in connection with the formation of these unusual ribonucleoproteins during the relaxed control condition concerns the origin of the ribosomal proteins which are associated with the rRNA. There is very little information available on the composition of the particles and on the basis of early estimates for 'chloramphenicol particles' (Dagley & Sykes, 1960; Nomura & Watson, 1959) it is frequently assumed that their composition is approximately 75 % RNA and 25 % protein (e.g. Schleif, 1968) although lower protein values of 15 to 18 % have been recorded (Yoshida & Osawa, 1968). Furthermore, since the particles material is equal to at least half the mass of ribosomes in the cell extract (see, for example, Fig. 1*a*), by making two further assumptions i.e. that all the protein in the particles is present in the cell before relaxation commences and that it is in the form of a free pool of ribosomal protein, one can conclude that the size of this pool must be equivalent to 25 % of the total ribosomal protein in the cell. This reasoning has led to an examination of the size of the free ribosomal protein pool in growing cells by two main approaches: direct measurement in particle-free supernatant fractions by immunological or electrophoretic analysis and by the kinetics of appearance of radioactive amino acids in ribosomes in pulse-chase experiments. The results have varied widely. The former methods tend to give higher estimates of the pool size as a percentage of the total ribosomal protein than the latter. Thus immunological analysis has given values of between 1 and 20 % (Santer *et al.*, 1968; Gupta & Singh, 1972; Stöffler, cited by Voynow & Kurland, 1971) and direct electrophoretic analysis has given values of between 3 and 10 % (Voynow & Kurland, 1971; Subramanian, 1974). The observations in this paper support the lower estimates obtained by direct examination of the particle-free supernatants from exponentially growing cells. This is also in agreement with most estimates of the pool size made by measuring the kinetics of radioactive labelling; these fall in the range 2 to 4 % (Schleif, 1968; Gierer & Gierer, 1968; Gupta & Singh, 1972; Marvaldi *et al.*, 1974; Gausing, 1974). The pool of free ribosomal proteins in exponentially growing cells therefore appears unlikely to be sufficient to satisfy the apparent protein requirement for producing the particles even though the fraction in the pool increases linearly with growth rate and its absolute concentration is proportional to the square of the growth rate (Gausing, 1974). The pool size does not appear to be significantly enlarged in

the non-growing state of glucose starvation (this paper) and the pool size is not the same for all ribosomal proteins (Marvaldi *et al.*, 1974).

Since all the proteins associated with the particles are ribosomal (this paper), the total protein complement of the particles must arise from a combination of three potential sources. (i) The free ribosomal protein pool. From the estimates of its size in both growing and non-growing cells it seems unlikely that this could account for more than 50 % of the total protein found in the particles. (ii) Breakdown of pre-existing ribosomes or selective protein detachment. No firm evidence has been found in these experiments for selective protein detachment from intact 50S ribosomes. However, during chloramphenicol inhibition extensive breakdown of pre-existing ribosomes has been reported (Lefkovits & di Girolamo, 1969; Young & Nakada, 1971). (iii) Residual preferential synthesis of ribosomal proteins in the relaxed states coupled with an increased rate of protein turnover known to take place in low-growth conditions. The residual protein synthesis in the methionine-starved relaxed state is 5 to 10 % of the full exponential rate and is skewed towards the production of a limited number of the ribosomal proteins (Goodman, 1970). Added to this, in methionine starvation the rate of protein turnover is increased to $3.6\% \text{ h}^{-1}$ (Borek, Pontecorvo & Rittenberg, 1958), and in chloramphenicol inhibition it is known that protein synthesis is not totally abolished unless exceptionally high levels (in excess of $400 \mu\text{g ml}^{-1}$) of the drug are used (Aronson & Spiegelman, 1961). A combination of these three potential sources could therefore give rise to the limited supply of selected ribosomal proteins essential for stabilization of the precursor rRNAs in the variety of arrested precursors seen in these relaxed states. The halt in net rRNA accumulation after a period of relaxation would then be attributed to an exhaustion of these supplies of ribosomal proteins, particularly the early-binding proteins.

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