Ribosome Precursors Accumulated by *Escherichia coli* during Incubation with Cobalt Chloride

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The ribonucleoprotein particles that accumulate during inhibition of *Escherichia coli* by CoCl_2 are ribosome precursors. The RNA that they contain can be transferred intact to completed ribosomes. The particles contain less protein than do ribosomes, but this protein appears identical with proteins from the appropriate ribosomal subunit. At least one of the particles is heterogeneous.

When the growth of *Escherichia coli* is partially inhibited by cobalt salts the bacteria synthesize an excess of RNA relative to protein (Blundell & Wild, 1969*a, b*). Much of this RNA is contained in three 'cobalt particles' whose sedimentation coefficients in Tris buffer containing 10 mM-magnesium acetate and 100 mM-KCl are about 44, 33 and 23S. The 44S and 33S components contain RNA that is similar to 23S rRNA; the RNA of the 23S particles sediments slightly faster than 16S rRNA.

We suggested (Blundell & Wild, 1969*b*) that cobalt particles are ribosome precursors. If this is so, the particles should be converted into ribosomes when the inhibition caused by Co^{2+} is relieved. Because ribosome assembly involves, *inter alia*, the sequential addition of proteins to ribosomal RNA, the particles should contain less protein than do completed ribosomes and this protein should be ribosomal protein. In this paper we confirm these predictions.

Experimental

Growth of bacteria

Escherichia coli strain M.R.E. 600 was grown at 37°C (generation time about 50 min) in a Tris-salts-glucose medium as described by Blundell & Wild (1969*b*). Cultures were shaken overnight in this growth medium containing a limiting concentration of glucose, giving an E_{450} of about 0.015; the next morning, cultures received 2 mg of glucose/ml and [^{14}C]uracil was added 15 min later. After a further 45 min, 15 μg of non-radioactive uracil/ml was added and the culture grown to give an E_{450} of 0.20–0.25. CoCl_2 (0.3 mM) was then added, together with [^3H]uracil to label the RNA, and inhibition was allowed to proceed for 30 min. The RNA of exponentially growing cells was labelled with [^{32}P]phosphate as described previously (Blundell & Wild, 1971).

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Centrifuging and radioactivity measurements

Two buffers were used, both of pH 7.4: (i) 10 mM-Tris-HCl-10 mM-magnesium acetate-100 mM-KCl (THMK buffer); (ii) 10 mM-Tris-HCl-2 mM-magnesium acetate-100 mM-KCl (TLMK buffer). Lysed cells (100 μl) were layered on to linear sucrose gradients (15–30%, w/w) and these were centrifuged at 234 000 g and 4°C in the SW50.1 rotor of a Spinco model L2-65B ultracentrifuge. About 40 fractions were collected from the bottom of a tube on to strips of glass-fibre paper (Whatman GF 82) and assayed for radioactivity that was insoluble at 0°C in 5% (w/v) trichloroacetic acid. When portions of a gradient were to be collected, about 230 drops were dripped from a tube in alternate 5-drop and 1-drop fractions. Acid-insoluble material was assayed in the 1-drop fractions.

Lysis of cells

This was done as described previously (Blundell & Wild, 1971) and essentially the method of Godson (1967) was used. When subsequent centrifuging was to be done in THMK buffer, the final concentration of MgSO_4 in the lytic mixture was 10 mM, the same as that in the buffer. When centrifuging was to be done in TLMK buffer, the mixture contained 5 mM- MgSO_4 and 100 mM-KCl; this latter procedure dissociates 70S ribosomes to their subunits.

Isopycnic centrifuging

The three cobalt particles, labelled with [^{14}C]uracil, were isolated from sucrose gradients made in a buffer equivalent to THMK buffer but containing 5 mM-triethanolamine-HCl in place of Tris. Particles (44S and 33S) were each mixed with ^{32}P -labelled 50S subunits, prepared from exponentially growing cells, and 23S particles with 30S subunits. To the samples was added 0.02 vol. of 5% (w/v) Brij-58 followed by 0.125 vol. of 36% (w/v) formaldehyde. The mixture

(1.0 ml) was added at room temperature to 2.0 ml of 65% (w/w) CsCl, was overlaid with liquid paraffin and then centrifuged for 24 h at 20°C and 189 000g in the Spinco SW 50.1 rotor. Fractions (2 drops) were collected on to glass-fibre strips for determination of radioactivity; after five 2-drop fractions, 1 drop was taken for density measurements; these were made by weighing.

Chromatography of ribosomal proteins

The methods used were derived from those of Otaka *et al.* (1968) and Osawa *et al.* (1969). Carrier ribosomes were made from *E. coli* M.R.E. 600 grown with aeration at 37°C in medium containing, per l: KH₂PO₄, 9 g; (NH₄)₂SO₄, 1 g; MgSO₄·7H₂O, 0.4 g; glucose, 10 g; peptone, 10 g. The pH was adjusted to 7.4 with NaOH. Cells were collected, resuspended in THMK buffer containing 6 mM-mercaptoethanol (2 ml of buffer/g wet wt. of cells) and broken in a French pressure cell. The crude extract was clarified by centrifuging at 35 000g for 20 min and then ribosomes were pelleted by centrifuging for 3 h at 78 000g. The ribosomes were resuspended and centrifuged twice; the pellet was finally resuspended in buffer at a concentration of 50 mg/ml. Samples (1–10 ml) for chromatography contained a mixture of ribosomal subunits and cobalt particles, both containing radioactively labelled proteins. They were mixed with 2 mg of carrier ribosomes and 0.1 vol. of 1 M-MgCl₂ (Hardy *et al.*, 1969) and then 2 vol. of acetic acid was added. After stirring for 45 min at 0°C, a sample was centrifuged at 5000g for 30 min and the supernatant was removed and dialysed at 0°C against water. Proteins were recovered by freeze-drying and dissolved in 3 ml of buffer (5 mM-sodium acetate, pH 5.6, containing 6 M-urea), then loaded on to a column (18 cm × 0.3 cm) of Whatman CM52 microgranular pre-swollen CM-cellulose. Elution (3.6 ml/h) was done with a linear gradient (70 ml) of 5–500 mM-sodium acetate, pH 5.6, containing 6 M-urea. Fractions (0.3 ml) were collected into vials to each of which was then added 3.0 ml of scintillant made from 2 vol. of 5.2% (w/v) 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole in toluene plus 1 vol. of Triton X-100.

Chemicals

Radioactive chemicals, [5-³H]uracil, [2-¹⁴C]uracil, L-[4,5-³H]lysine and L-[U-¹⁴C]lysine, were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Pancreatic ribonuclease and Triton X-100 (scintillation grade) were from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; rifampicin was from Calbiochem, London W.1, U.K.; Brij-58 was from Honeywell and Stein Ltd., Carshalton, Surrey, U.K.; Whatman products were from H. Reeve Angel and Co., London E.C.4, U.K.

Results

Recovery from inhibition by CoCl₂

The procedure was that used previously (Blundell & Wild, 1969b); CoCl₂ (final concn. 0.3 mM) was added to an exponentially growing culture that had an E₄₅₀ of 0.20–0.25. After inhibition for 30 min the three cobalt particles could then be detected in centrifuged extracts. Cells that had undergone this inhibition were collected by filtration, resuspended in fresh cobalt-free medium and portions were incubated either without further additions being made or after the addition of 2 mg of casamino acids (Difco)/ml. In minimal medium there was a lag of about 1 h before growth resumed, whereas the culture supplemented with casamino acids started to grow shortly after resuspension. This effect of casamino acids was probably not due to chelation of residual Co²⁺, since inclusion of 50 µg of L-histidine/ml in the minimal medium was without effect, although this amino acid can complex Co²⁺ (Schade, 1949). The medium that contained casamino acids was used to study recovery.

First, stable RNA was labelled with [¹⁴C]uracil, and RNA made during inhibition by CoCl₂ in minimal medium was labelled with [³H]uracil. Cells were filtered off and then resuspended in non-radioactive medium containing uracil and casamino acids. Samples were taken immediately after resuspension and also 20 min later. Gradient centrifuging of the sample taken immediately after resuspension gave a profile in which the three cobalt particles were evident. After recovery for 20 min the corresponding profile did not show these particles; transfer of radioactivity into ribosomes had taken place because additional sampling showed no loss of acid-precipitable radioactivity from the cells. This transfer could be by direct conversion of cobalt particles into ribosomes; alternatively, there might be breakdown of the particles followed by re-synthesis of new RNA. Therefore, in another experiment, synthesis of RNA during recovery was prevented by the inclusion of 200 µg of rifampicin/ml in the resuspension medium. Samples were taken for harvesting and lysis immediately after resuspension and also 20 min later. The acid-precipitable radioactivities of the culture were also measured; there was a small loss (10%) of stable RNA, but ³H radioactivity incorporated during inhibition decreased by 28% during this 20 min. The lysate made immediately after resuspension was centrifuged and gave a normal profile (Fig. 1a) in which the three cobalt particles are displayed; the lysate made after 20 min has no such particles. (The large increase in the quantity of 70S ribosomes is due to decay of polyribosomes caused by rifampicin.) However, the loss of 28% of the ³H radioactivity might partially account for loss of the cobalt particles. Therefore additional samples of the two lysates were

briefly treated with ribonuclease before centrifuging; the conditions were such that cobalt particles were degraded, whereas ribosomes remained apparently intact (Blundell & Wild, 1969b). The legend to Fig. 1 summarizes the acid-insoluble radioactivities after ribonuclease treatment. During 'recovery' there is a 3-fold increase in the ^3H radioactivity of the ribosomes which, since no RNA synthesis takes place, is derived directly from the RNA of the cobalt particles.

Protein content of cobalt particles

Cobalt particles were labelled with [^{14}C]uracil and isolated from sucrose gradients; 44S and 33S particles were each mixed with ^{32}P -labelled 50S ribosomes, and 23S particles were mixed with 30S ribosomes. After treatment with formaldehyde and centrifuging in CsCl , 50S subunits banded at a buoyant density of 1.65; 44S and 33S particles had densities of about 1.70 and 1.78 respectively. A calculation as described by Hamilton & Ruth (1969) gave

protein contents of 36, 28 and 15 %. The 23S particles ($\rho=1.78$) were more dense than 30S ribosomes ($\rho=1.61$); these values correspond to particles that contain 15 and 43 % protein.

Proteins of cobalt particles

Proteins were labelled with [^3H]lysine during the inhibition by CoCl_2 . Isolated 44S or 33S particles were mixed with 50S ribosomal subunits from cells that had incorporated [^{14}C]lysine during exponential growth, and 23S particles were mixed with 30S ribosomes. Non-radioactive ribosomes were added as carrier, the proteins were separated from RNA and then chromatographed on CM-cellulose.

Fig. 2(a) shows co-chromatography of protein from the 23S particles and 30S subunits. The profile of ^{14}C radioactivity (from 30S ribosomes) resembles that reported by Otaka *et al.* (1968) and peaks 1 to 11 in Fig. 2(a) are numbered according to their system,

Fig. 1. Fate of cobalt particles

A culture (75 ml) was labelled with 20 nCi of [^{14}C]uracil/ml and was grown with carrier uracil until an E_{450} of 0.23 was reached. It was then incubated for 30 min with 0.3 mM- CoCl_2 and 4 μCi of [^3H]uracil/ml. The cells were collected by filtration and resuspended in medium containing, per ml, 2 mg of casamino acids, 15 μg of uracil and 200 μg of rifampicin. Immediately after resuspension (a) and 20 min later (b) the cells from 25 ml of culture were harvested, lysed and a portion was centrifuged for 2.5 h at 234 000 g in sucrose gradients made in THMK buffer. Fractions were collected for measurement of the ^3H (●) and ^{14}C (○) radioactivities that were insoluble in 5 % trichloroacetic acid. In addition, 0.3 ml of each lysate was incubated for 20 min at room temperature with 1 μg of ribonuclease, then chilled to 0°C and portions (100 μl) were immediately centrifuged as described above. No cobalt particles were apparent. Radioactivities (c.p.m.) in fractions of these latter gradients were summed (fractions 1–28 contained ribosomes, fractions 29–38 contained low-molecular-weight material). For the sample taken immediately the values were: ribosomes: $^3\text{H}=6000$, $^{14}\text{C}=15930$; low-molecular-weight material: $^3\text{H}=6820$, $^{14}\text{C}=2770$. For the sample taken after 20 min the values were: ribosomes: $^3\text{H}=18450$, $^{14}\text{C}=15960$; low-molecular-weight material: $^3\text{H}=5270$, $^{14}\text{C}=2710$.

Fig. 2. Chromatography of the proteins of 23S particles

(a) A culture (50 ml) was grown with carrier uracil until an E_{450} of 0.1 was reached. [^{14}C]Lysine (260 nCi/4 μg per ml) was then added and the cells were grown until an E_{450} of 0.5 was reached and were then harvested and lysed. The extract was centrifuged for 20 h at 53 500g (SW 25.1 rotor) in a sucrose gradient made in TLMK buffer and fractions containing 30S ribosomes were collected. Another culture (200 ml) was grown with carrier uracil until an E_{450} of 0.23 was reached, then it was incubated for 30 min with 0.3 mM- CoCl_2 , [^3H]lysine (540 nCi/0.5 μg per ml) and [^{14}C]uracil (100 nCi/ml). The cells were harvested and lysed, and the extract was centrifuged for 28 h at 53 500g (SW 25.1 rotor) in a sucrose gradient made in THMK buffer. One-quarter of each fraction containing 23S particles was combined and mixed with the [^{14}C]lysine-labelled 30S ribosomes. The proteins of this mixture were isolated (see the Experimental section) and then they were chromatographed on CM-cellulose. —, ^3H ; ----, ^{14}C . (b) The remainder of the fraction comprising the more-slowly sedimenting portion of the 23S particle peak was mixed with [^{14}C]lysine-labelled 30S ribosomes, the proteins were isolated and then chromatographed.

but 12 and 13 are added. The major peaks of ^3H -labelled particle proteins coincide with the subunit proteins but the ratio of $^3\text{H}/^{14}\text{C}$ radioactivities in the different components differs by about 8-fold; no group of proteins that shares a common value is apparent. The proportionalities between ratios in the various peaks were reproducible in different experiments.

One explanation of the variation in the radioactivity ratios might be that 23S particles are heterogeneous. In the experiment that gave the pro-

file of Fig. 2(a), a fraction was also isolated from the slower-sedimenting region of the 23S particle peak and proteins from this fraction were chromatographed (Fig. 2b). This slower-sedimenting fraction is more deficient in some components (particularly 7, 8, 11 and 12) than the total preparation. Peak 4 has a low $^3\text{H}/^{14}\text{C}$ ratio in both preparations; thus there is no appreciable contamination caused by 30S ribosomes that are formed during the inhibition.

When both RNA and protein that were made during inhibition were labelled, the ratio of radioactivities

Fig. 3. *Chromatography of the proteins of 33S particles*

The particles (labelled with [^{14}C]uracil and [^3H]lysine) were isolated from a sucrose gradient. They were mixed with [^{14}C]lysine-labelled 50S ribosomes. The proteins were isolated (see the Experimental section) and were then chromatographed on CM-cellulose. —, ^3H ; ----, ^{14}C .

in fractions containing 23S particles differed by about 2-fold across the peak, slower-sedimenting material having less protein. Moreover, when samples of 23S particles from different parts of the peak were re-centrifuged in sucrose gradients, their sedimentation rates remained distinct. These results also imply that 23S particles are heterogeneous.

The proteins of [^3H]lysine-labelled 33S and 44S cobalt particles co-chromatograph with [^{14}C]lysine-labelled 50S ribosomal proteins. Results with 33S particles are shown in Fig. 3. The radioactivity ratio in the peaks differs by 5-fold, but no groups of components share common values. This difference was consistent in different experiments. Significant contamination of 33S particles by labelled 30S subunits was excluded in control experiments.

Discussion

The particles accumulated in *E. coli* during inhibition by CoCl_2 are ribosome precursors. They contain RNA that resembles rRNA as regards sedimentation (Blundell & Wild, 1969b). Their protein content is less than that of completed ribosomes but the proteins that are present appear to be identical with proteins from the appropriate subunit. The RNA in the particles is transferred intact into completed ribosomes when inhibition is stopped, since this occurs under conditions in which synthesis of RNA is prevented. Thus cobalt particles are more akin to 'natural' ribosome precursors than are, for example, the ribonucleoprotein particles produced during inhibition of *E. coli* by fluorouracil; RNA from 'fluorouracil particles' can appear in completed ribosomes

when inhibition is stopped but does not do so when RNA synthesis is prevented during recovery (Hills & Horowitz, 1966).

Chromatography shows that distinct subclasses of the 23S cobalt particle may be partially resolved by sedimentation. The particle is therefore heterogeneous. However, cultures inhibited by CoCl_2 are not in a steady state and the ribosome precursors that accumulate may differ from those detected in exponentially growing cells or cells under other conditions. The heterogeneity of the 23S cobalt particle might be peculiar to cultures inhibited by cobalt salts or could reflect more general features of ribosome assembly in *E. coli*. Further work is clearly required to establish the relationship of cobalt particles to other ribosome precursors, such as those isolated from exponentially growing bacteria (Homann & Nierhaus, 1971; Nierhaus *et al.*, 1973) or from cold-sensitive mutants incubated at a restrictive temperature (Nashimoto *et al.*, 1971). A detailed examination of the RNA of cobalt particles and a more adequate protein catalogue are particularly desirable.

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References

- Blundell, M. R. & Wild, D. G. (1969a) *Biochem. J.* **115**, 207–212
- Blundell, M. R. & Wild, D. G. (1969b) *Biochem. J.* **115**, 213–223
- Blundell, M. R. & Wild, D. G. (1971) *Biochem. J.* **121**, 391–398

- Godson, G. N. (1967) *Methods Enzymol.* **12A**, 503–516
- Hamilton, M. G. & Ruth, M. E. (1969) *Biochemistry* **8**, 851–856
- Hardy, S., Kurland, C. G., Voynow, P. & Mora, G. (1969) *Biochemistry* **8**, 2897–2905
- Hills, D. C. & Horowitz, J. (1966) *Biochemistry* **5**, 1625–1632
- Homann, H. E. & Nierhaus, K. H. (1971) *Eur. J. Biochem.* **20**, 249–257
- Nashimoto, H., Held, W., Kaltschmidt, E. & Nomura, M. (1971) *J. Mol. Biol.* **62**, 121–138
- Nierhaus, K. H., Bordsch, K. & Homann, H. E. (1973) *J. Mol. Biol.* **74**, 587–597
- Osawa, S., Otaka, E., Itoh, T. & Fukui, T. (1969) *J. Mol. Biol.* **40**, 321–351
- Otaka, E., Itoh, T. & Osawa, S. (1968) *J. Mol. Biol.* **33**, 93–107
- Schade, A. L. (1949) *J. Bacteriol.* **58**, 811–822