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Preferential Synthesis of a Membrane-Associated Protein by Free Polyribosomes

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Isolated free polyribosomes from rat liver appear to synthesize NADPH–cytochome *c* reductase *in vitro* four times as efficiently as do membrane-bound polyribosomes. The observed synthesis by the latter could result from contamination with free polyribosomes, but an alternative explanation is also suggested.

There is a large body of evidence that secreted proteins are synthesized by membrane-bound polyribosomes, whereas cytosol proteins are made by free polyribosomes (Hallinan *et al.*, 1968; Ganoza & Williams, 1969; Hicks *et al.*, 1969; Redman, 1969; Takagi *et al.*, 1970; Campbell, 1970).

However, the synthesis of membrane-associated proteins has not yet been assigned exclusively to one class of particles. Ragnotti *et al.* (1969) reported that one such protein, NADPH–cytochrome *c* reductase, was made equally by both classes. Since there was reason to doubt the completeness of their polyribosome separation, and since they did not demonstrate the purity of their enzyme, we decided to re-investigate the site of synthesis of this enzyme, using modified methods.

Our results indicate that NADPH–cytochrome *c* reductase is made preferentially on free polyribosomes.

Materials and methods

Animals. Male Wistar rats (200–400g), starved overnight, were used for Expts. 1–3. In Expts. 4 and 5, Sprague–Dawley rats of the same size were injected intraperitoneally, 12h before being killed, with 100mg of phenobarbital/kg to increase the rate of synthesis of NADPH–cytochrome *c* reductase (Kuriyama *et al.*, 1969). Injected rats were starved 24h before death (Kuriyama *et al.*, 1969).

Materials. Pure ^{14}C-labelled amino acids (code no. CFB 103) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., as separate 10μCi/ml vials, and mixed before use. The specific radioactivity was approx. 25mCi/mg-atom of C. Pre-mixed amino acids (The Radiochemical Centre; code no. CFB 104), in spite of having a higher specific radioactivity (54mCi/mg-atom of C), were found to give low incorporation into total protein. Steapsin was from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. ('Lipase type II').

Preparation of free and bound polyribosomes. Livers were homogenized in 3vol. of ice-cold medium A (50mM-Tris–HCl, pH7.8, 25mM-KCl, 5mM-$MgSO_4$ and 6mM-2-mercaptoethanol), containing 0.25M-sucrose, and centrifuged for 10min at 10000$g_{av.}$ in the MSE 18 High-Speed Centrifuge. The supernatant was layered in portions of 8–13ml over 10ml each of 1.5M-sucrose and 2M-sucrose, both in medium A, and centrifuged at 130000$g_{av.}$ for 20h in the 8× 50ml angle rotor of the MSE 65 centrifuge.

The interfacial membrane layer was diluted with 1vol. of medium B (0.15M-sucrose, 35mM-Tris–HCl, pH7.8, 25mM-KCl, 10mM-$MgSO_4$ and 6mM-mercaptoethanol), and centrifuged for 1½h at 130000$g_{av.}$. The resulting pellet of bound polyribosomes was gently resuspended in the same medium. In Expts. 4 and 5 the diluted interface material was concentrated by layering on to 7ml of 2M-sucrose containing medium-B additives before centrifugation, so that no pellet was formed. This increased the incorporation of radioactive amino acids into total protein, although the difference obtained (40% increase) was much less than the threefold increase reported by Ragnotti (1971).

The pellet from the 20h centrifugation, consisting of free polyribosomes, was rinsed, and gently resuspended in medium B.

Preparation of cell sap and microsomal fraction. Livers were homogenized in 1vol. of medium B, and centrifuged for 10min at 10000$g_{av.}$. The supernatant was centrifuged for 1½h at 130000$g_{av.}$. The microsomal pellet was resuspended in medium B without mercaptoethanol, containing 3mg of non-radioactive casamino acids (Difco)/ml; this suspension was used to stop the incubation, as described below. The 130000*g* supernatant (cell sap) was passed through a 50ml column of Sephadex G-25 to remove amino acids, the volume collected being the same as that applied.

Amino acid incorporation. Incubation mixtures contained ATP (2mM), GTP (0.25mM), phosphoenolpyruvate (10mM) and GSH (4mM). These were all adjusted to pH6–8 and added in a total volume of 2ml of medium B. Polyribosome concentration was 0.15–0.58mg of RNA/ml (but see Expt. 2), with

pyruvate kinase (50μg/ml), ^{14}C-labelled amino acids as stated in Table 1, and buffered cell sap (8–32mg of protein/ml). All concentrations are given as in the final incubation mixture (10 or 20ml). In control experiments, cycloheximide (1mg/ml) was added, and ATP, GTP and phosphoenolpyruvate were omitted. Incubation was for 30min at 37°C. The reaction was stopped by putting the tubes in ice and immediately adding 5–13ml of microsomal suspension containing non-radioactive casamino acids. The microsomal membranes act as a source of carrier enzyme.

For measurement of incorporation of radioactivity into total protein, 1–2ml incubation mixtures were used, and the reaction was stopped with 2vol. of ice-cold 0.3M-$HClO_4$ containing 1mg of non-radioactive casamino acids/ml.

Extraction and purification of NADPH–cytochrome c reductase. After incubation for amino acid incorporation, the mixture was centrifuged for 1½h at 130000$g_{av.}$. The microsomal pellet was resuspended in 50mM-potassium phosphate buffer (pH7.8) containing 1mM-EDTA and 1mg of casamino acids/ml, and re-centrifuged. Then the pellet was resuspended in 10mM-potassium phosphate buffer (pH7.8) containing 1mg of non-radioactive casamino acids/ml, to a volume of 1ml/g of liver equivalent. Steapsin was added to an experimentally determined optimal concentration of 0.7mg/ml, and the mixture left at 4°C for 12–16h, after which it was centrifuged for 1½h at 130000$g_{av.}$. The supernatant was freeze-dried, and re-dissolved in 1–2ml of water. This solution was passed through a 1cm×50cm column of Sephadex G-100, equilibrated with 10mM-Tris–HCl, pH7.8 (Expts. 1 and 2), or 10mM-potassium phosphate buffer, pH7.8 (all other experiments). The most enzymically active fractions were used for the next stage of purification.

For the second stage, two methods were used.

(a) Method used for Expts. 1 and 2. The preparative electrophoresis method of Shuster & Schrier (1967) was used without modification, except that the electrophoresis-chamber diameter was 1cm.

(b) Method used for Expts. 3–5. Although the first method gave results indicating that free polyribosomes might be more active than bound in synthesizing NADPH–cytochrome *c* reductase, it gave unreliable recovery in our hands and thus was eventually abandoned in favour of ion-exchange chromatography.

Appropriate Sephadex fractions were combined and loaded on a 1cm×10cm column of DEAE-cellulose, equilibrated with 10mM-potassium phosphate buffer, pH7.8. During stepwise elution with 50mM-potassium phosphate buffer containing increasing concentrations of KCl, the enzyme emerged with 0.18M-KCl.

Each fraction was tested for purity by gel electrophoresis (Davis, 1964). The fractions constituting the central area of the enzyme peak showed only one band after staining with Coomassie Brilliant Blue (Bennett & Scott, 1971); parallel staining with neotetrazolium chloride (Ichihara *et al.*, 1972) demonstrated that this band was NADPH–cytochrome *c* reductase.

Table 1. *Radioactive labelling of NADPH–cytochrome c reductase in vitro by free and bound polyribosomes*

For experimental details see the text. The results of six experiments are shown, Expts. 5a and 5b having been performed in parallel, by using polyribosomes from separate rats but cell sap and microsomal fraction from a pooled homogenate. In Expts. 4 and 5, the animals were treated with phenobarbital, and the bound polyribosomes were prepared by centrifuging on to a cushion of 2M-sucrose. For amino acid incorporation, [^{14}C]leucine (0.82μCi/ml final concentration) was used in Expt. 1, and a ^{14}C-labelled amino acid mixture (1–2μCi/ml final concentration) was used for Expts. 2–5. The final reductase purification was by electrophoresis in Expts. 1 and 2, and by DEAE-cellulose chomatography in Expts. 3–5; homogeneity was demonstrated by polyacrylamide-gel electrophoresis in Expts. 4 and 5. The differences between columns A and B for each experiment were tested by Student's *t* test, and the increase in the free/bound incorporation ratio was found to be significant ($0.02 < P < 0.05$). The results of Expt. 2 were not included in the test, for the reason given in the text. Incorporation is expressed as c.p.m./mg of rRNA.

	Incorporation into total protein			Incorporation into reductase		
Class of polyribosomes ...	Free	Bound	Free/bound (A)	Free	Bound	Free/bound (B)
Expt. no.						
1	35600	19700	1.8	275	41	6.7
2	96400	93000	1.0	162	218	0.7
3	192500	95600	2.0	20.1	4.4	4.6
4	186700	130500	1.4	36	50	0.7
5a	304500	267500	1.1	308	104	3.0
5b	255500	206500	1.2	376	51	7.4

Measurement of radioactivity. Samples for determination of incorporation into total proteins were washed twice in 0.2M-$HClO_4$, incubated for 1h in 0.3M-NaOH to hydrolyse RNA, precipitated and re-washed with acid and dissolved in 0.1M-NaOH. A 1ml portion was added to 10ml of scintillant and counted for radioactivity after fluorescence had subsided.

Samples from various stages of enzyme purification, with bovine serum albumin added as a carrier, were acid-precipitated and washed, and dissolved in 0.1M-NaOH. Portions (1.0–1.4ml) were added to 10ml of scintillant.

All samples were counted by the emulsion method of Patterson & Greene (1965), by using a Triton X-100/toluene ratio of 1:2. This was found to give almost constant quench, samples containing 5mg of protein causing a decrease in efficiency of less than 3%.

It was not possible to add the DEAE-cellulose fractions directly to scintillant, because KCl and phosphate were found to interfere with clearing of the emulsion.

The radioactivity found in the pure enzyme was corrected for the recovery of enzyme activity, to give the counts in the reductase in the steapsin supernatant.

Other assays. NADPH–cytochrome *c* reductase was assayed as by Ragnotti *et al.* (1969), except that 100mM-potassium phosphate buffer was used.

RNA was measured by the method of Fleck & Begg (1965). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as standard.

Results

Ragnotti *et al.* (1969) separated free from bound polyribosomes by centrifuging the post-mitochondrial supernatant through steps of 0.5M- and 1.6M-sucrose. Since Dallner *et al.* (1963) showed that rough endoplasmic reticulum sediments through 1.5M-sucrose, we considered that 1.6M-sucrose might not be sufficiently dense to prevent the sedimentation of some polyribosomes into the 'free polyribosome' pellet. Two experiments, using [^{14}C]choline as a membrane marker (Hallinan *et al.*, 1969) in one case and direct phospholipid assay in the other, both showed that although slightly more RNA was found in the pellet from a 4h centrifugation through 0.5M-/1.6M-sucrose steps than in that from a 20h centrifugation through 1.5M-/2M-sucrose steps, six times as much membrane was found in the former pellet as in the latter. This membrane was 3–7% of the total membrane from the post-mitochondrial supernatant and may have consisted of denser fragments of the rough endoplasmic reticulum, that is, with most ribosomes attached. If this were the case, up to 15% of the bound polyribosomes could sediment into the free-polyribosome pellet if 1.6M-sucrose were used.

Table 1 shows the incorporation of ^{14}C-labelled amino acids into NADPH–cytochrome *c* reductase by free and bound polyribosomes. The enzyme appears to be synthesized predominantly, although not exclusively, on free particles. The incorporation by the two classes of particle is approximately equal in Expts. 2 and 4. For Expt. 2, this can be explained: the concentration of free polyribosomal RNA (1.12mg/ml) was nearly three times that of bound RNA (0.45mg/ml) in their respective incubation mixtures. Since we found, in agreement with Ragnotti (1971), that the efficiency of amino acid incorporation decreases with increasing RNA/cell-sap ratio, it follows that RNA concentrations must be not only as low as is consistent with measurable incorporation, but also equal for comparative purposes. Thus the free polyribosomes were working at a lower efficiency than the bound in Expt. 2, and so the result is biased in favour of the bound particles, and is therefore not included in the statistics. In Expt. 4, however, the RNA/cell-sap ratio was low, and equal for both classes of polyribosomes; the result was therefore considered genuine.

Parallel incubations in the presence of cycloheximide and the absence of ATP, GTP and phosphoenolpyruvate showed no radioactivity in the pure enzyme, whichever class of particle was used.

Discussion

In spite of the preferential synthesis of the enzyme by free polyribosomes, there is significant activity by the bound-polyribosome fraction. This may be accounted for in two ways.

Some free particles may remain trapped at the 1.5M-/2M-sucrose interface, among the rough endoplasmic reticulum, in spite of the rats having been starved (Lowe *et al.*, 1970). The other possibility is that membrane-associated proteins are made by free polyribosomes, but are attached to the membrane before completion, so that synthesis is continued and terminated on what are apparently membrane-bound polyribosomes.

Synthesis of enzyme was followed by monitoring the enzymic activity of the reductase and measuring the radioactivity that accompanied it through the purification procedure. This method introduces a bias, in that only molecules complete enough to resemble the active carrier molecules in size and charge are studied. This difficulty might be overcome by an alternative method of purification, by using an antibody directed against the *N*-terminal segment cleaved with trypsin from the detergent-isolated enzyme (Ichihara *et al.*, 1972). This would detect not only whole molecules of holoenzyme and apoprotein, but also recently initiated chains of the latter.

We venture to predict that this method would indicate a greater difference between synthesis by free and bound polyribosomes than we could show.

Another question which remains unanswered is the site and timing of addition of the flavin prosthetic group to the apoprotein. Peptide synthesis and prosthetic-group addition could be completely separate processes, as with cytochrome *c* (Kadenbach, 1970); or the flavin might be attached to the apoprotein during protein synthesis.

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