Interaction between Membrane Functions and Protein Synthesis in Reticulocytes

AN ELONGATION-STAGE INHIBITOR OF PROTEIN SYNTHESIS EXTRACTED FROM THE RETICULOCYTE MEMBRANE

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A component of the reticulocyte cell membrane was found to inhibit protein synthesis severely in a reticulocyte lysate system. An investigation into the mode of action of the membrane inhibitor revealed the following facts. (1) The binding of the tertiary initiation complex (methionyl-tRNA^{Met}-Initiation Factor 2-GTP) to the 40S ribosomal subunit was unaffected by the membrane inhibitor. (2) The membrane component did not interfere with the binding of the 40S initiation complex to the AUG initiation codon and subsequent attachment of the 60S ribosomal subunit. (3) Elongation of the peptide chain, as assayed by peptidyl-puromycin formation, was markedly affected by the membrane inhibitor. Surprisingly, the membrane component caused a considerable increase in peptidyl-puromycin formation. (4) Reticulocyte ribosomes that had been reisolated by high-speed centrifugation, after preincubation with the membrane component, were found to be highly defective when assayed in a cell-free protein-synthesizing system. These results indicated that an extract of the reticulocyte cell membrane inhibited protein synthesis by interacting with the ribosome and thus interfered with the correct functions of the elongation stage of protein synthesis. The implications of this conclusion are discussed in the light of data showing that a highly purified preparation of the membrane inhibitor also displayed an endonucleolytic activity highly specific for 28S RNA.

Although it is increasingly evident that the cell membrane plays a role in regulation of cellular metabolism, the actual nature of this membrane participation needs to be better defined.

The ideal system to investigate the role of the membrane would be a chemically defined membrane (perhaps as a liposome containing known purified proteins) encompassing part of the cell machinery such as the protein-synthesizing system. The effect of delicate changes at the membrane level could then be directly correlated with the effect on the protein-synthesizing system. This line of research must wait, however, for suitable techniques to be devised. Meanwhile, the rabbit reticulocyte represents an adequate substitute for this ideal system. It is a simple cell, consisting almost entirely of a limiting membrane and a highly developed intracellular ribosomal network. Other subcellular organelles such as nuclei, lysosomes and mitochondria are progressively lost during the transition of the polychromatic erythroblast to the reticulocyte (Tavassoli & Crosby, 1973; Gasko & Danon, 1972).

Significant changes occur in both the surface properties and membrane components of the cell during erythroid-cell differentiation. A unique set of membrane components that are completely

different from those on circulating erythrocytes have been found in bone-marrow nucleated erythroid cells. Most significantly, the nucleated erythroid cells completely lacked the spectrin-actin complex (Light & Tanner, 1977). Alterations in the cell surface also occur during the maturation of the reticulocyte to the erythrocyte, as shown by labelling with cationized ferritin (Danon *et al.*, 1972). Differences in the cell-surface components between reticulocytes and erythrocytes have been observed (Koch *et al.*, 1973), as well as changes in the structural organization of surface membrane during the cell maturation (Inoue *et al.*, 1977).

Changes in the reticulocyte membrane during the maturation process occur at the level of membrane proteins, glycoproteins and the lipid matrix. Shattil & Cooper (1976) found that reticulocyte maturation is accompanied by a 20–30% loss of membrane lipids, where both phospholipids and cholesterol are lost in parallel to the reticulocyte-maturation process.

Several groups have found an involvement of the membrane in regulating protein synthesis (Engelhardt, 1971; Fisher & Koch, 1976), and in normal rat liver microsomal fractions (Scornik *et al.*, 1967). It is thus surprising to note that the reticulocyte cell membrane has been almost totally ignored as a component

capable of playing an active part in erythroid-cell differentiation. Work performed in our laboratory (Herzberg *et al.*, 1974) suggested that the reticulocyte cell membrane may play an important role in regulating intracellular protein synthesis. Furthermore, we demonstrated that a soluble reticulocyte-membrane extract strongly inhibited protein synthesis in a purified cell-free system (Wreschner & Herzberg, 1976).

In the present work, we extend our experiments to a reticulocyte-lysate system, where both re-initiation and elongation occur. We show here that the specific endonucleolytic activity of the membrane inhibitor demonstrated previously (Wreschner *et al.*, 1978) results in an alteration of the tRNA-binding site on the ribosome and thus interferes with the elongation of the polypeptide chain.

Experimental

Reticulocyte-rich blood, isolation of membrane extract and cell-free protein-synthesizing system have been described extensively elsewhere (Wreschner & Herzberg, 1976; Wreschner *et al.*, 1978).

Reticulocyte lysate system

Washed reticulocytes were lysed for 1 min with 1 vol. of cold deionized water and then centrifuged at 12000*g* for 15 min at 4°C. The lysate supernatant was removed and either used immediately or stored in liquid nitrogen.

The final volume of the lysate system was 60 μ l, and this consisted of 25 μ l of lysate (as above), haemin to give a final concentration of 50 μ M, 10 μ l of water or the substance to be investigated and 20 μ l of a master mix solution. The addition of master mix solution gave the following final concentrations: 1 mM-ATP, 0.2 mM-GTP, 75 mM-KCl, 2 mM-MgCl₂, 10 mM-Tris/HCl, pH 7.8, 2 mg of phosphocreatine/ml, creatine kinase (15 units/ml) and leucine (1.5 μ Ci/ml). ATP, GTP and phosphocreatine were all previously neutralized to pH 7.0.

A stock solution of haemin (1 mM) was prepared by dissolving solid haemin in 1 ml of 50 mM-Tris/HCl, pH 7.8; 0.5 ml of 1 M-NaOH was added to return the pH to 7.8. The final pH was checked. Portions of the stock haemin solution were stored in liquid nitrogen.

The lysate system was incubated at 34°C for 40 min, after which [¹⁴C]leucine incorporation into protein was checked as previously described (Wreschner & Herzberg, 1976) and performed in duplicate.

Reticulocyte RNA extraction

RNA was extracted from isolated reticulocyte ribosomes with phenol/chloroform as previously described (Wreschner *et al.*, 1978).

Sucrose gradients

Exponential sucrose gradients were performed by the method described by Noll (1967).

Polyribosomal profiles

Isolated reticulocyte ribosomes were loaded on 12 ml exponential sucrose gradients (15–40%, w/v) in 50 mM-KCl/20 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂. Tubes were centrifuged at 32000 rev./min for 90 min at 4°C without brakes in the Beckman SW41 rotor. Fractions were either collected from the tube directly, or the contents were pumped through a spectrophotometer with simultaneous continuous absorption recording. The maximum volume of sample applied to the gradient was 0.5 ml.

When polyribosomal profiles of lysates were analysed, the reticulocyte lysate was diluted to a density equivalent to one-sixth or one-seventh of that in the intact reticulocyte. This step was found to be critically important.

Ribosomal-subunits analysis

A reticulocyte lysate (400–500 μ l), previously diluted to a suitable density (see above) with 10 mM-KCl/10 mM-Tris/HCl (pH 7.8)/1.5 mM-MgCl₂, was placed on a linear 15–30% sucrose gradient made up in the same buffer and centrifuged at 35000 rev./min for 250 min without brakes at 4°C in the Beckman SW41 rotor. The gradients were either collected into fractions or analysed through a spectrophotometer as described above.

RNA analysis on sucrose gradients

A maximum of 280 μ g and a minimum of 60 μ g of rRNA was loaded on 12 ml 15–40% exponential sucrose gradients made up in 100 mM-KCl/10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA. Maximum sample volume was 0.5 ml. Gradients were centrifuged at 37000 rev./min for 1½ h at 4°C in the Beckman SW41 rotor without brakes. The gradients were collected or analysed as above.

Preincubation of reticulocyte ribosomes with crude membrane extract

Reticulocyte ribosomes (2.4 mg) were suspended in a final volume of 4 ml of 50 mM-KCl/20 mM-Tris/HCl (pH 7.4)/4 mM-MgCl₂. One test tube contained 2 ml of TT buffer (1% Triton X-100/3 mM-Tris/HCl, pH 7.4), and the other contained membrane inhibitor at a final concentration of 500 μ g/ml. The tubes were incubated for 6 min at 37°C, chilled and the ribosomes reisolated by centrifugation through a cushion of 1 M-sucrose/5 mM-Tris/HCl (pH 7.4)/1 mM-mercaptoethanol/0.1 mM-EDTA in the SW50.1 rotor of a Beckman centrifuge at 43000 rev./min for 3 h at 4°C. The pelleted ribosomes were resuspended in a

minimal volume of 0.25M-sucrose/5mM-Tris/HCl (pH7.4)/1mM-dithiothreitol/0.1mM-EDTA.

Results

Action of the membrane inhibitor on the reticulocyte lysate system

The crude membrane extract, isolated from the reticulocyte membranes, was added to the lysate system and its effect on protein synthesis was observed.

When the crude membrane extract was added to the reticulocyte lysate under optimum conditions (i.e. with the addition of haemin to a final concentration of 30 μ M), inhibition of protein synthesis was observed (Fig. 1). This inhibition was dependent on the amount of inhibitor added and 50% inhibition was obtained with 9 μ g of added inhibitor protein. At higher concentrations, the inhibition curved off and approached a plateau.

The reticulocyte lysate system is responsive to added haemin. The above experiment was performed under optimal conditions with the addition of 30 μ M-haemin. Haemin (in the range 20–80 μ M)

may boost endogenous protein synthesis severalfold, by restricting the formation of a translational repressor, which in turn inhibits the synthesis of the ternary initiation complex composed of methionyl-tRNA, eIF-2 (initiation factor 2) and 40S subunits. It was thus important to compare the action of the crude membrane inhibitor on the haemin-stimulated system with the membrane inhibitor's action on a haemin-deprived system.

The effect of the inhibitor in the haemin-supplemented system is described above (see Fig. 1). A maximum inhibition of 73% was obtained with added inhibitor. The haemin-deprived system (Fig. 1) gave protein synthesis approx. 50% of that in the haemin-supplemented system. Membrane inhibitor added to the haemin-deprived system also caused inhibition, but the extent was much less than when it was added to the haemin-supplemented lysate. Both the haemin-deprived and haemin-supplemented lysates showed similar protein synthesis when maximum amounts of inhibitor were added.

Kinetics of the inhibition of protein synthesis caused by the membrane extract was checked by removing samples from a control lysate system and a membrane-inhibitor-supplemented lysate at different time intervals and then checking for trichloroacetic acid-precipitable radioactivity. Fig. 2 shows the results of such an experiment. The control system

Fig. 1. Concentration-dependence of membrane-inhibitor action on reticulocyte lysate

Incorporation of [¹⁴C]leucine into polypeptides was measured in the reticulocyte lysate system (see the Experimental section) in either the presence (■—■) or the absence (■---■) of 30 μ M-haemin. Different quantities of membrane inhibitor were added and incubation was at 31°C for 40 min.

Fig. 2. Time-dependence of membrane-inhibitor action on reticulocyte-lysate protein synthesis

The reticulocyte-lysate system was set up as described in the Experimental section and supplemented with 30 μ M-haemin. Membrane inhibitor (60 μ g) was added to the lysate system (final volume 160 μ l) (●). A similar amount of buffer (1% Triton X-100 in 3mM-Tris/HCl, pH7.4) was added to the control test tube (■). The tubes were incubated at 34°C and duplicate 10 μ l samples were removed at the indicated times and checked for hot-trichloroacetic acid-precipitable radioactivity, which represented [¹⁴C]leucine incorporation into polypeptides.

showed linear kinetics for the first 20 min of incubation; protein synthesis still increased for the next 20 min (until 40 min of incubation) and then curved off after 60 min of incubation. The inhibited system gave linear kinetics for the first 10 min of incubation, and then protein synthesis curved off to a plateau. It is significant that initial inhibition was only slight for the first 10 min of incubation (only 33 % inhibition after 10 min), but then drastically increased to 60 % inhibition by 20 min of incubation.

Effect of the membrane inhibitor on the initiation stage of protein synthesis

(1) *Binding of the methionyl-tRNA^{fMet}-eIF₂-GTP complex to the 40S ribosomal subunit.* In order to understand its mechanism of action, the effect of the membrane inhibitor on the primary stage of protein synthesis was investigated.

Binding of the methionyl-tRNA^{fMet}-eIF₂-GTP complex to the 40S ribosomal subunits was checked by separating the ribosomal subunits through a sucrose gradient, after incubation of the lysate (supplemented with [³⁵S]methionine) with or without the membrane inhibitor. Fractions were collected and the radioactivity bound to the ribosomes was measured.

The lysate was incubated for 5 min at 34°C, which gave a 22 % inhibition in an inhibitor-treated system. There was no significant change in the 40S-subunit-bound radioactivity between the control and the inhibitor-treated system. The bound radioactivity on both the 80S particle and 40S subunit was calculated by summing up the peak radioactivity value plus two readings on either side of the peak, and no significant difference was found between the control and inhibitor-treated system.

It was concluded that the inhibitor was not acting by inhibiting the formation of methionyl-tRNA^{fMet}-eIF₂-GTP, nor by restricting the transfer of this complex to the 40S ribosomal subunit.

(2) *AUG-induced initiation-complex formation.* The next step in the initiation stage of protein synthesis is the binding of methionyl-tRNA^{fMet}-40S subunit to the AUG initiation codon on the mRNA, followed by the attachment of the 60S subunit. The effect of the inhibitor on the formation of the 80S initiation complex was investigated by setting up the lysate system (without [³⁵S]methionine) and incubating it at 20°C for 10 min. This preliminary incubation included staphylococcal nuclease as well as CaCl₂, which is required for the nuclease activity. The nuclease degraded the polyribosomes to monoribosomes, as well as degrading free mRNA, so that the lysate was now composed of monoribosomes, small segments of mRNA and free ribosomal subunits (40S and 60S subunits that were originally in the subunit pool). The test tube was placed in ice

and EGTA added to chelate the Ca²⁺ ions and thus stop the nuclease activity. Synthetic AUG codon for initiation-complex formation and [³⁵S]methionine were then added, as well as the membrane inhibitor. Controls were performed without added AUG and without added inhibitor.

The membrane inhibitor used in this series of experiments was a highly purified preparation obtained after chromatography on Sephadex G-75 (Wreschner *et al.*, 1978). The tubes were then placed in a 32°C bath and incubated for a further 10 min. Incubation with the inhibitor in a normal lysate system under these conditions gave 40 % inhibition. After the incubation period, the lysates were centrifuged through sucrose gradients and analysed for radioactivity bound to 80S particles and subunits.

Fig. 3 shows the results of such an experiment. The control tubes that did not contain synthetic initiation codon AUG showed radioactivity on the 40S subunit; less, but significant, amounts of radioactivity were also present on the 80S ribosome. This 40S-subunit-bound radioactivity represents the methionyl-tRNA^{fMet}-40S-subunit complex, whereas the 80S-particle-bound radioactivity represents the 80S initiation complex. The 80S initiation complexes were formed owing to the presence of endogenous initiation codons. No major differences were observed between the control tube without AUG and the tube containing inhibitor without AUG. The inhibitor-treated system did have slightly lower amounts of 80S-particle-bound radioactivity, but this was considered insignificant when viewed in the light of the 40 % inhibition of total protein synthesis.

Figs. 3(b) and 3(d) show the effect of adding exogenous synthetic initiation codon AUG to the system. It is immediately apparent that there is a shift of radioactivity from the 40S subunit to the 80S initiation complex when Fig. 3(a) (-AUG) and Fig. 3(b) (+AUG) are compared. This shift is promoted by the addition of the initiation codon to give the 80S initiation complex. Addition of inhibitor did not block the shift of radioactivity from 40S subunits to form the 80S initiation complex (compare Figs. 3b and 3d). On the contrary, considerable amounts of radioactivity were bound to the 80S monoribosome, even in the presence of inhibitor.

Effect of the membrane inhibitor on the elongation stage

The experiments described above eliminated the possibility that the membrane inhibitor was interfering with the initiation stages of protein synthesis. Experiments reported earlier (Wreschner & Herzberg, 1976) have shown that the membrane inhibitor may possibly be affecting the elongation stage of protein synthesis. If, in fact, correct ribosomal functioning

Fig. 3. Effect of highly purified membrane inhibitor on AUG-induced complex-formation (methionyl-tRNA^{fMet}-40S subunit-AUG-60S subunit or 80S initiation complex)

The reticulocyte-lysate system was set up as described in the Experimental section, supplemented with 40 μ M-haemin but without added [³⁵S]methionine. Staphylococcal nuclease and CaCl₂ were added to give final concentrations of 80 units/ml and 1 mM respectively. This lysate was incubated at 20°C for 10 min and then placed in ice. EGTA and AUG were added to the lysate to give final concentrations of 2 mM and 0.6 A_{260} unit/ml, respectively. Highly purified membrane inhibitor was added to give an equivalent of 40% inhibition in hot-trichloroacetic acid-precipitable radioactivity. Finally 5 μ Ci of [³⁵S]methionine was added to each test tube and incubation continued for 10 min at 32°C. Appropriate controls were performed without AUG and without inhibitor. After incubation, 200 μ l of cold 10 mM-KCl/10 mM-Tris/HCl (pH 7.8)/1.5 mM-MgCl₂ was added to each tube and a 300 μ l sample loaded on to a 5 ml 15–40% isokinetic exponential 15–40% sucrose gradient prepared in the above buffer. The tubes were centrifuged at 4°C for 130 min at 45000 rev./min. The gradients were then pumped through a spectrophotometer set at 260 nm with simultaneous continuous recording, and fractions were subsequently collected and counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter after addition of 5 ml of dioxan-based scintillator. (a) Without AUG, without inhibitor (horizontal arrow denotes sedimentation); (b) with AUG, without inhibitor; (c) without AUG, with inhibitor; (d) with AUG, with inhibitor. ●, Radioactivity (c.p.m.); —, A_{260} .

in the elongation stage was impaired, then three conceivable possibilities could arise:

(i) peptidyltransferase inhibition by the membrane inhibitor: then the incoming tRNA would be blocked

in the A site as the transfer of the peptidyl chain to the new amino acid was inhibited;

(ii) translocase inhibition by the membrane inhibitor: thus there would be a transfer of the

peptidyl chain to the aminoacyl-tRNA, but translocation of the tRNA with the nascent peptide chain into the P site would not occur;

or (iii) both the peptidyltransferase and the translocation reactions are not inhibited, but the membrane inhibitor has caused a structural defect in the ribosome, thus effecting a conformational change of the A site, which then, after one translocation, does not accept incoming aminoacyl-tRNA.

These three possibilities are represented in Fig. 4.

To differentiate between these three possibilities, use was made of [^3H]puromycin, and formation of peptidyl-puromycin was observed. If the membrane inhibitor was affecting either of stages (i) or (ii), then the formation of peptidyl-puromycin would be expected to be less than that found in the control. If the membrane inhibitor was affecting stage (iii), there would be considerably higher peptidyl-puromycin values than in the control, as more peptidyl-tRNA could react with the radioactive puromycin.

Peptidyl-puromycin formation

[^3H]Puromycin was added to cell-free systems (derived from rabbit reticulocyte) and the tube incubated at 37°C , in the presence of 20 non-radioactive amino acids. Formation of peptidyl-[^3H]puromycin was tested by precipitation with cold 10% (w/v) trichloroacetic acid and then scintillation counting.

In addition to the effect of the membrane inhibitor

on peptidyl-puromycin formation, the effects of pancreatic ribonuclease B, which would presumably degrade polyribosomes to monoribosomes, and of cycloheximide, a classical inhibitor of elongation, were also checked. The influence of the above compounds was also tested, in separate test tubes, on total protein synthesis, as measured by hot-trichloroacetic acid precipitation of [^{14}C]leucine incorporated into protein.

Fig. 5 shows the results of such an experiment. Cross-hatched bars show peptidyl-puromycin formation expressed as a percentage of the control, whereas lightly stippled bars show [^{14}C]leucine incorporation into protein. The membrane inhibitor used in this experiment was a DEAE-cellulose-purified fraction (Wreschner *et al.*, 1978). This fraction ($2.5\mu\text{l}$) caused 50% inhibition of protein synthesis (Fig. 5b). Surprisingly, this amount of inhibitor stimulated peptidyl-puromycin formation to 166% of the control value. The same experiment was repeated with the use of a larger quantity of membrane inhibitor: $10\mu\text{l}$ of the inhibitor caused 89% inhibition of total protein synthesis, but stimulated the formation of peptidyl-puromycin to 205% of the control (Fig. 5c).

Pancreatic ribonuclease B caused a 52% inhibition of protein synthesis, when used at a final concentration of $0.1\mu\text{g/ml}$. This amount of ribonuclease B did not cause any appreciable change in peptidyl-puromycin formation (93% of the control) (Fig. 5d).

Fig. 4. Inhibition of protein synthesis at the elongation level: possible sites of action

The A and P sites on the 60S ribosomal subunit represent the aminoacyl and peptidyl sites respectively. Further details about this Figure are found in the Results section. A' represents the aminoacyl site that has been modified by inhibitor action, and aa aa denotes the nascent peptide chain.

Fig. 5. Effect of purified membrane inhibitor on peptidyl-puromycin in formation

Cell-free protein-synthesizing systems containing all 20 non-radioactive amino acids were set up as detailed in the Experimental section. [³H]Puromycin (0.5 µCi) was added to each test tube and the tubes were incubated for 40 min at 37°C. At the end of this incubation period, peptidyl-[³H]puromycin was measured by counting cold-trichloroacetic acid-precipitable radioactivity. Similar incubations were performed with [¹⁴C]leucine (without [³H]puromycin) and amino acid incorporation into protein was checked by hot-trichloroacetic acid precipitation. Results were expressed as percentages of control values. Control peptidyl-puromycin gave a value of 599 c.p.m. and control protein synthesis was 2354 c.p.m. Cross-hatched bars represent peptidyl-puromycin formation, and lightly stippled bars show [¹⁴C]leucine incorporation. (a) Control; (b) 2.5 µl of purified membrane inhibitor added; (c) 10 µl of purified membrane inhibitor added; (d) 2.5 µl of a 2 µg/ml solution of pancreatic ribonuclease B added; (e) cycloheximide (final concn. 14 µg/ml) added.

Cycloheximide caused a 31% inhibition of protein synthesis (when used at a final concentration of 14 µg/ml), and a similar inhibition of peptidyl-puromycin formation (33% inhibition) (Fig. 5e).

This experimental evidence (Fig. 5) confirmed that the action of the membrane inhibition was indeed inducing state (iii), as inhibitor-treated systems gave 2–3-fold increased values for peptidyl-puromycin formation.

Inhibitor action on ribosomes

The stimulation of peptidyl-puromycin formation, effected by the reticulocyte membrane extract, could be reconciled by assuming that the membrane inhibitor was interacting with the ribosome and inactivating (or changing) some structural requirement essential for efficient and correct working of the elongation stage. This membrane-inhibitor-treated ribosome would then display abnormal functions when tested at the elongation stage.

This hypothesis was tested by preincubating reticulocyte ribosomes with the membrane inhibitor or with control buffer, reisolating these treated ribosomes by centrifugation through a 1M-sucrose cushion and then testing them for their activity in protein synthesis.

Table 1 shows the results of such an experiment. Preincubated and unincubated control ribosomes gave protein synthesis equivalent to 3157 and 3389 c.p.m. respectively. Protein synthesis by control ribosomes in the presence of membrane inhibitor was inhibited by 51%, whereas that of ribosomes pretreated with inhibitor was inhibited by 62%.

As shown above, it was proposed that the membrane inhibitor was causing a conformational change in the ribosome, thus distorting the aminoacyl site (A site) and in such a way inhibiting the normal functions of protein synthesis (stage iii, Fig. 4). This hypothesis requires a change in ribosome structure. The membrane inhibitor was, however, found to alter neither the protein content of the ribosome nor the ribosomal density as tested on CsCl buoyant-density gradients (results not shown). However, as shown previously (Wreschner *et al.*, 1978), when the RNA of membrane-inhibitor-treated ribosomes was extracted and analysed on a sucrose gradient, it was both qualitatively and quantitatively different from RNA of control ribosomes. New RNA species sedimented between 10S and 14S, being degradation products of a specific cleavage of the 28S RNA found on the 60S subunit.

The crude membrane extract was found to contain, besides the potent inhibitory activity towards protein synthesis, a specific endonucleolytic activity. When challenged with 28S RNA the highly purified inhibitor displayed the specific endonucleolytic cleavage. It was thus concluded that the inhibitor was indeed interfering with protein synthesis by

Table 1. Effect of preincubating ribosomes with membrane inhibitor

Reticulocyte ribosomes were pretreated with either TT buffer (preincubated control ribosomes) or with crude membrane extract (crude-membrane-extract-preincubated ribosomes) and then reisolated, exactly as described in the Experimental section. These ribosomes, as well as control ribosomes that had not been preincubated, were assayed in the cell-free protein-synthesizing system as described in the Experimental section.

	Membrane extract (30 µg)	[¹⁴ C]Leucine incorporated (c.p.m.)
Control ribosomes	—	3389
	+	1677
Preincubated control ribosomes	—	3157
Crude-membrane-extract-preincubated ribosomes	—	1184

cleaving the 28S rRNA on the 60S subunit of the ribosome.

Discussion

The reticulocyte activity synthesizes haemoglobin on a highly developed ribosomal network. After 48 h in the circulatory system, it differentiates to become a cell that does not demonstrate any protein-synthetic activity, the erythrocyte. The reticulocyte must thus logically have inbuilt mechanisms controlling the extent and rate of intracellular protein synthesis. Several possibilities have been presented in an attempt to explain the progressive slow-down of protein synthesis in the reticulocyte. These include a haemin-controlled repressor of protein synthesis (Gross & Rabinovitz, 1973), decreased stability of poly(A)-less globin mRNA (Huez *et al.*, 1974), decreased activity of initiation factors correlated with reticulocyte maturation (Herzberg *et al.*, 1969), and finally translational repressor bound to messenger ribonucleoprotein particles (Civelli *et al.*, 1976). The actual mechanism regulating protein synthesis in the reticulocyte may indeed be a conglomerate of all the above proposals. We do believe, however, that a central element of the reticulocyte has been overlooked, namely the cell membrane itself.

The present work has shown that an extract of the reticulocyte cell membrane can severely inhibit the extent of protein synthesis in a lysate system. This inhibitor did not affect any part of the initiation stage of protein synthesis (Fig. 3). Both the formation of the ternary complex (methionyl-tRNA^{Met}-eIF₂-40S subunit) and the binding of this complex to the initiation codon with the subsequent attachment of the 60S subunit were found to be unaffected. However, an investigation with labelled puromycin demonstrated that the elongation stage was inhibited by the membrane extract. The results obtained with labelled puromycin indicated that, after the addition of the membrane inhibitor, peptidyl-tRNA was confined to the ribosomal P sites, from which it was readily removable by puromycin, via the peptidyl-transferase reaction (Fig. 5). It has been shown (Cundliffe & McQuillen, 1967) that the only way a substance can stop polypeptide-chain elongation without affecting the puromycin reaction is by blocking the A site and in such a way prevents aminoacyl-tRNA binding to that site. This is obviously the case with the membrane inhibitor. Indeed, treatment with the membrane extract significantly stimulated peptidyl-puromycin formation.

As shown in previous work, the membrane inhibitor contained a specific endonucleolytic activity in addition to its potent inhibitory action (Wreschner *et al.*, 1978). This cleavage of rRNA could conceivably account for the observed inhibition of protein synthesis. It is to be expected that the mRNA

with its attached ribosomes would be most vulnerable to any endonucleolytic attack: this was indeed the case with pancreatic ribonuclease (results not shown). In marked contrast, however, the membrane inhibitor only caused cleavage of the rRNA; no degradation of mRNA (as tested by polyribosomal breakdown) was evidenced.

We have purified the inhibitor from the crude membrane extract and shown it to be 95% homogeneous. The specific endonucleolytic cleavage activity co-purified with the inhibitory activity through all stages of purification (Wreschner *et al.*, 1978). The scheme presented in Fig. 6 may explain the mode of action of the inhibitor. The specific cleavage of 28S rRNA effected by the membrane inhibitor may in turn cause a structural change in the aminoacyl site. A new incoming aminoacyl-tRNA will thus be unable to attach to the ribosome. This mechanism is compatible with both the results presented and with our previous data (Wreschner & Herzberg, 1976).

Alternatively, the cleavage on the 28S rRNA may restrict the binding of EF1 (elongation factor 1) to the ribosome. This would prevent subsequent aminoacyl-tRNA binding on the A site.

In either case, these data show that the structural integrity of the 28S rRNA is a prerequisite for correct ribosomal function in the elongation stage of protein synthesis. It may indeed be that only a certain segment of the 28S rRNA must be intact for the efficient working of the ribosome. Multiple cleavage at sites on the 28S rRNA other than at the specific segment may not be detrimental to ribosomal function.

It is interesting to compare these results with recent work performed with alpha sarcin, a potent inhibitor of eukaryotic protein synthesis. Hobden & Cundliffe (1978) have found that alpha sarcin inhibits elongation of the peptide chain by restricting

Fig. 6. Possible dependency of a functionally active aminoacyl site on the structural integrity of 28S rRNA. This scheme is described in the Discussion section. Definition of the symbols is in the legend to Fig. 4.

the entry of a new aminoacyl-tRNA in the A site. Both peptidyltransferase and the translocation reaction were found to be unaffected. Parallel to this, alpha sarcin has been found to cleave specifically rRNA of the 60S subunit of the ribosome (Schindler & Davies, 1977). These data seem similar to the results that we have presented here, i.e. a potent inhibitor of protein synthesis that acts by specifically cleaving the 28S rRNA.

The results that we have previously described with regard to reticulocyte-membrane-bound ribosomes (Wreschner *et al.*, 1977) favour the possibility that ribosomes are inactivated by their attachment to the cell membrane in the course of differentiation. This possibility would require a differentiation-dependent movement of ribosomes from the interior of the cytoplasm to the cellular periphery. This hypothesis has gained support from Lönn & Edström (1977), who proposes the existence of a ribosomal gradient extending from the cell nucleus to the cell membrane. This proposal maintains that the closer the ribosome is situated to the cell membrane, the less active it will be in protein synthesis. The activity of the ribosome will thus be proportional to its distance from the cell nucleus.

The biological significance *in vivo* of this factor is difficult to assess from the data presented here, but it is obvious that the membrane inhibitor described, isolated and characterized above represents an efficient means of effecting cessation of protein synthesis, and could well be a membrane-located regulator of cellular metabolism.

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References

- Civelli, O., Vincent, A., Bari, J. F. & Scherrer, K. (1976) *FEBS Lett.* **72**, 71–76
- Cundliffe, E. & McQuillen, D. (1967) *J. Mol. Biol.* **30**, 137–146
- Danon, D., Goldstein, L., Marikovsky, Y. & Skutelsky, E. S. (1972) *J. Ultrastruct. Res.* **38**, 500–510
- Engelhardt, D. L. (1971) *J. Cell. Physiol.* **78**, 333–344
- Fisher, L. E. & Koch, G. (1976) *Biochem. Biophys. Res. Commun.* **72**, 1229–1236
- Gasko, O. & Danon, D. (1972) *Exp. Cell Res.* **75**, 159–169
- Gross, M. & Rabinovitz, M. (1973) *Biochem. Biophys. Res. Commun.* **50**, 823–838
- Herzberg, M., Revel, M. & Danon, D. (1969) *Eur. J. Biochem.* **11**, 148–153
- Herzberg, M., Breitbart, H. & Atlan, H. (1974) *Eur. J. Biochem.* **65**, 161–170
- Hobden, A. N. & Cundliffe, E. (1978) *Biochem. J.* **170**, 57–61
- Huez, G., Harbaix, G., Hubert, E., Leclercq, M., Nudel, V., Soreq, H., Salomon, R., Lebleu, B., Revel, M. & Littauer, U. Z. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3143–3146
- Inoue, M., Okajima, K., Ito, K., Istumi, K. & Sano, S. (1977) *Biochim. Biophys. Acta* **467**, 130–145
- Koch, P. A., Gardner, R. H. & Carter, J. R. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1296–1299
- Light, N. D. & Tanner, M. J. A. (1977) *Biochem. J.* **164**, 565–578
- Lönn, U. & Edström, J.-E. (1977) *J. Cell Biol.* **73**, 696–704
- Noll, H. (1967) *Nature (London)* **215**, 360–363
- Schindler, D. G. & Davies, J. E. (1977) *Nucleic Acids Res.* **4**, 1077–1110
- Scornik, O. A., Hoagland, M. B., Pfefferkorn, L. C. & Bishop, E. A. (1967) *J. Biol. Chem.* **242**, 131–139
- Shattil, S. J. & Cooper, R. A. (1976) *J. Lab. Clin. Med.* **79**, 215–221
- Tavassoli, M. & Crosby, W. H. (1973) *Science* **179**, 912–913
- Wreschner, D. & Herzberg, M. (1976) *Eur. J. Biochem.* **64**, 399–404
- Wreschner, D., Melloul, D. & Herzberg, M. (1977) *FEBS Lett.* **77**, 83–86
- Wreschner, D., Melloul, D. & Herzberg, M. (1978) *Eur. J. Biochem.* **85**, 233–240