Mechanism of Action of a Microsomal Inhibitor of Protein Synthesis Potentiated by Oxidized Glutathione

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Extracts of microsomal fractions cause an inhibition of protein synthesis that is most pronounced in the presence of 0.1 mM-GSSG and 0.01 mM-GTP, and is abolished by thiol or 0.4 mM-GTP (Scornik *et al.*, 1967). Fractionation of microsomal extracts showed that this inhibition of protein synthesis was caused by an enzyme, nucleoside diphosphate phosphohydrolase. Direct inhibition of protein synthesis on detergent-treated polyribosomes by 0.1 mM-GSSG was observed under conditions of GTP limitation induced by omission of a GTP-regenerating system, or addition of a nucleoside triphosphate diphosphohydrolase. Thus GSSG potentiated the inhibition of protein synthesis caused by an enzyme that promoted removal of GTP. The inhibition was abolished by adding 4 mM-2-mercaptoethanol or 0.4 mM-GTP. Nucleoside diphosphate phosphohydrolase was thought also to act by promoting removal of GTP, thus causing an inhibition of protein synthesis that was potentiated by GSSG.

The rate of polypeptide chain elongation by ribosomes attached to mRNA can vary *in vivo* (Wool & Kurihara, 1967; Scornik, 1969; Henshaw *et al.*, 1971; Kaminskas, 1972). The mechanism by which the activity of ribosomes in polyribosomes can be varied is unknown. Possibilities include the limitation of factors required for peptide-chain elongation (Henshaw *et al.*, 1971), the presence of inoperative ribosomes attached to polyribosomes (Hunter & Korner, 1969), or of inhibitory factors. A factor that inhibits protein synthesis *in vitro* has been extracted from microsomal membranes obtained from rat liver. The effect of this inhibitory factor is enhanced by GSSG and diminished by GSH, 2-mercaptoethanol and excess of GTP (Scornik *et al.*, 1967). The inhibitor is absent from detergent-treated polyribosomes and is active *in situ* in microsomal fractions from livers of normal rats, but much less active in microsomal fractions from regenerating liver (Nolan & Hoagland, 1971).

The purpose of the work presented here was to determine the basis for the susceptibility of microsomal fractions to the effects of GSSG as an aid to establishing the possible identity and mode of action of the inhibitor.

Materials and Methods

Cell fractions

Female Wistar rats weighing about 150 g were starved for 24 h before death. Livers from the starved

animals were homogenized in 2 vol. of medium A (25 mM-KCl–5 mM-magnesium acetate–100 mM-Tris–HCl buffer, pH 7.2 at 37°C) containing 0.25 M-sucrose.

'Pure microsomes'. Microsomal fractions depleted of lysosomes ('pure microsomes') were prepared by the method of Scornik *et al.* (1967). However, to minimize lysosomal contamination of the post-mitochondrial supernatant, the homogenate was centrifuged twice at 19000g (r_{av} , 8.3 cm) for 10 min at 4°C. The microsomal pellet obtained after centrifugation of the post-mitochondrial supernatant at 107000g (r_{av} , 6.5 cm) for 90 min at 4°C was resuspended in medium A containing 1.53 M-sucrose (2.0 ml/g of liver) and centrifuged at 107000g for 2 h at 4°C. The pellicle containing residual lysosomes and mitochondria was discarded. 'Pure microsomes' remaining in suspension were sedimented by centrifugation at 107000g for 4 h at 4°C after addition of an equal volume of medium A. 'Pure microsomes' contained 5–10% of the ribosomes in the homogenate.

Crude microsomal fractions. Liver homogenate was centrifuged at 15000g for 6 min at 4°C to obtain a post-mitochondrial supernatant. A crude microsomal fraction was obtained from the post-mitochondrial supernatant by centrifugation at 107000g for 90 min at 4°C. The crude microsomal fraction contained about 30% of the ribosomes in the homogenate.

Detergent-treated polyribosomes. A post-mitochondrial supernatant was obtained by centrifugation of a liver homogenate at 19000g for 10 min at 4°C.

Then 0.1 vol. of 10% (v/v) Triton X-100 was added to the post-mitochondrial supernatant and 3.0 ml of the detergent-treated solution was layered over a discontinuous sucrose gradient consisting of 1.5 ml of medium B (0.5 M-NH₄Cl–10 mM-magnesium acetate–100 mM-Tris–HCl buffer, pH 7.2 at 37°C) containing 0.7 M-sucrose and 3.0 ml of medium B containing 2.0 M-sucrose. The detergent-treated polyribosomes were sedimented by centrifugation at 157 000g (r_{av} , 6.1 cm) for 7 h at 4°C and comprised about 20% of the ribosomes in the homogenate.

Free polyribosomes. Free polyribosomes (those not membrane-bound *in vivo*) were prepared by the method of Blobel & Potter (1967) and comprised 10% of the ribosomes in the homogenate.

High-speed supernatant. This was obtained by centrifugation of a post-mitochondrial supernatant for 90 min at 107 000g at 4°C. The resulting supernatant was centrifuged again at 107 000g at 4°C for 2 h to remove any residual ribosomes. The ribosome-free supernatant (30 ml) was passed through a Sephadex G-25 column (bed vol. 210 ml) equilibrated with medium A containing 0.25 M-sucrose, and concentrated 5-fold by freeze-drying and redissolving in water.

Microsomal inhibitor. The microsomal inhibitor of protein synthesis was extracted from 'pure microsomes'. Pellets of 'pure microsomes' obtained from 25 g of liver were suspended in 18 ml of medium A at 0°C and 0.04 vol. of butan-1-ol was added. After stirring for 4 min the suspension was added slowly to 48 vol. of acetone at –10°C. After stirring for 6 min the precipitate was collected by filtration and washed with 2 vol. of acetone at –10°C. The washed precipitate was placed in a vacuum desiccator for 1 h and then suspended in 5 ml of 0.2 M-NH₄HCO₃ adjusted to pH 8.4 with aq. NH₃. The supernatant, which contained inhibitor, was separated from undissolved material by centrifugation at 107 000g for 1 h at 4°C.

Nucleoside diphosphate phosphohydrolase. This microsomal enzyme was purified by a slightly modified method of Kuriyama (1972). A crude microsomal fraction was prepared from 60 g of liver. The microsomal fraction was suspended by homogenization in 90 ml of medium I (0.25 M-sucrose–10 mM-Tris–HCl, pH 7.5 at 20°C) and adjusted to pH 10.7 in the presence of 0.05% sodium deoxycholate by adding 3 M-NH₃ with vigorous stirring. After 5 min at 0°C the suspension was adjusted to pH 7.5 with 3 M-acetic acid. The suspension was centrifuged at 100 000g for 30 min at 4°C. The supernatant was fractionated with (NH₄)₂SO₄ after addition of 0.2 mM-dithiothreitol. The precipitate formed at 35% saturation was discarded and the supernatant was adjusted to 75% saturation by addition of solid (NH₄)₂SO₄. The resultant precipi-

tate was collected by centrifugation at 100 000g for 30 min at 4°C and dissolved in 12 ml of medium II (20 mM-Tris–HCl, pH 8.0 at 20°C, 1.0 mM-dithiothreitol). This solution was passed through a Sephadex G-25 column (bed vol. 210 ml) equilibrated with medium II containing 0.1 M-KCl.

The filtrate containing protein was applied to a column (bed vol. 12 ml) of DEAE-cellulose (DE 52 from Whatman Biochemicals, Maidstone, Kent, U.K.) equilibrated with medium II containing 0.1 M-KCl. Unretarded material was removed by washing with 50 ml of medium II containing 0.1 M-KCl. Absorbed protein was eluted with a linear gradient of 0.1–0.35 M-KCl in medium II. Total elution volume was 100 ml and the flow rate 0.5 ml/min.

Fractions (1.8 ml) were dialysed against 100 vol. of 20 mM-Tris–HCl buffer (pH 8.0 at 20°C) for 3 h at 4°C. The phosphohydrolase activity of 10 μ l samples was determined by using 1.0 mM-GDP as substrate, and 10–30 μ l portions were assayed for their effect on protein synthesis.

[1-¹⁴C]Leucyl-tRNA. Rat liver tRNA was deacylated by incubation in 0.5 M-Tris–HCl (pH 8.0 at 37°C) for 45 min. Aminoacyl-tRNA labelled with L-[1-¹⁴C]leucine was prepared from the deacylated tRNA by the method of Hoagland & Comly (1960) and separated from contaminating rRNA by chromatography on DEAE-cellulose by the method of Yang & Novelli (1971). L-[1-¹⁴C]leucine (sp. radioactivity 62 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivity of the ¹⁴C-labelled aminoacyl-tRNA obtained was 1.45×10^5 – 1.54×10^5 c.p.m./mg of RNA. Radioactivity measurements were performed under the same conditions as those used for measuring the incorporation of [¹⁴C]leucine into protein. A 10 μ l portion of ¹⁴C-labelled aminoacyl-tRNA was placed on a Whatman GF/C 25 mm filter disc and dried before being measured for radioactivity in a scintillation counter as described below.

All cell fractions were stored at –20°C.

Analytical methods

Incorporation of [¹⁴C]leucine into protein. The complete reaction mixture of 0.1 ml contained microsomal fractions (about 72 μ g of RNA) or polyribosomes (about 40 μ g of RNA), 25–35 μ g of aminoacyl-tRNA labelled with [¹⁴C]leucine, 440 μ g of freeze-dried supernatant protein, 10 μ g of pyruvate kinase, 1.0 μ mol of phosphoenolpyruvate, 10 μ mol of Tris–HCl buffer (pH 7.2 at 37°C), 2.5 μ mol of K⁺, 0.5 μ mol of Mg²⁺ and various amounts of GTP (0–100 nmol). The amount of freeze-dried supernatant that was used contained an amount of transfer enzymes that was 80% of the amount for optimum

activity, as determined under conditions of GTP limitation and excess. The reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by addition of 5 ml of 5% (w/v) trichloroacetic acid containing 0.1% DL-leucine. After heating at 90°C for 20 min to hydrolyse residual aminoacyl-tRNA, the precipitated protein was collected on Whatman GF/C 25 mm filter discs. The precipitate was washed with 25 ml of 5% (w/v) trichloroacetic acid containing 0.1% DL-leucine. After drying, the filter discs were immersed in 10 ml of scintillation fluid [4 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene] and radioactivity was measured in a Nuclear-Chicago liquid-scintillation counter (series 720).

Phosphohydrolase activity. Enzyme activity was measured in a reaction mixture of 1.0 ml containing 100 μ mol of Tris-HCl buffer (pH 7.2 at 37°C), 5 μ mol of Mg^{2+} , 25 μ mol of K^+ and 1.0 μ mol of nucleoside 5'-triphosphate or nucleoside 5'-diphosphate as substrate. The reaction mixture was incubated for 5 min at 37°C. The assay conditions used were therefore comparable with those used in incorporation measurements. The reaction was stopped with 1 ml of cold 10% (w/v) trichloroacetic acid and the tubes were cooled in ice for 5 min. Precipitated protein was removed by centrifugation and 1.0 ml of the supernatant was used for determination of P_i . Phosphohydrolase activity was expressed in terms of μ mol of P_i liberated/min per mg of protein.

The identity of products was determined by t.l.c. on plastic-backed polyethyleneimine cellulose sheets (Macherey-Nagel Polygram cel 300 PEI from Camlab, Cambridge, U.K.). A reaction mixture of 0.2 ml containing 20 μ mol of Tris-HCl (pH 7.2 at 37°C), 1.0 μ mol of Mg^{2+} , 5.0 μ mol of K^+ , 1 μ mol of GDP or UDP and 22 μ g of protein containing inhibitor was incubated for 5 min at 37°C. The reaction was terminated by adding an equal volume of 3 M-formic acid, and 8 μ l of this mixture (containing 20 nmol of nucleotide) was applied to a sheet that had previously been soaked for 5 min in water and methanol and then dried. Chromatograms were developed with 1.0 M-LiCl. Products were detected under a u.v. lamp and identified by comparison with standard compounds.

Apyrase (ATP diphosphohydrolase, EC 3.6.1.5), grade I, was obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Other analytical methods. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Koch-Light, Colnbrook, Bucks., U.K.) as a standard. The concentration of the bovine serum albumin was determined by measuring its absorbance at 280 nm ($E_{1\%}^{1\text{cm}}$ 6.0). RNA was measured by the Schmidt-Thannhauser method as modified by Munro & Fleck (1966). P_i was measured by the method of Fiske & SubbaRow (1925).

Results

Effects of GSSG, 2-mercaptoethanol and GTP on protein synthesis occurring on microsomal fractions and detergent-treated polyribosomes

Interpretation of the effects of GSSG and 2-mercaptoethanol on protein synthesis occurring in the presence of the microsomal inhibitor was hampered initially by the use of a transfer-enzyme preparation whose activity was dependent on added thiol. However, when freeze-dried high-speed supernatant was used as the source of transfer enzymes protein synthesis occurring on detergent-treated polyribosomes was essentially unaffected by addition of 4 mM-2-mercaptoethanol (Fig. 1*b*). This source of transfer enzymes was therefore used for all subsequent experiments.

The effect of GSSG on protein synthesis occurring on 'pure microsomes' and detergent-treated polyribosomes was determined at various initial concentrations of GTP, and in the presence of a GTP-regenerating system (phosphoenolpyruvate and pyruvate kinase) (Fig. 1). Addition of 0.1 mM-GSSG caused an inhibition of protein synthesis occurring on 'pure microsomes' that was most marked at low initial concentrations of GTP. Addition of 0.4 mM-GTP or 4 mM-2-mercaptoethanol abolished the effect of GSSG. In contrast protein synthesis on detergent-treated polyribosomes was unaffected by 0.1 mM-GSSG or 4 mM-2-mercaptoethanol, except in the absence of added GTP. In the absence of a GTP-regenerating system (Fig. 2) protein synthesis on 'pure microsomes' and detergent-treated polyribosomes was diminished (compare Figs. 1 and 2), presumably owing to failure to maintain an adequate GTP concentration. The greater effect of omission of the GTP-regenerating system on protein synthesis occurring on 'pure microsomes' correlated well with the observed differences in the ability of 'pure microsomes' and detergent-treated polyribosomes to hydrolyse GTP (Table 1). Under these conditions of GTP limitation, 0.1 mM-GSSG inhibited protein synthesis on both 'pure microsomes' and detergent-treated polyribosomes, although to different extents (Fig. 2). The effects persisted at an initial concentration of 0.4 mM-GTP but were abolished by 4 mM-2-mercaptoethanol.

Identical series of experiments were performed on a crude microsomal fraction and on free polyribosomes. The crude microsomal fraction behaved like 'pure microsomes', and free polyribosomes behaved like detergent-treated polyribosomes. Thus all the polyribosome-containing fractions that were obtained were sensitive to GSSG under conditions of GTP limitation. However, as the recovery of polyribosomes from the homogenate was never greater than 30% of the total these experiments do not

Fig. 1. Effects of 0.1 mM-GSSG on protein synthesis occurring on (a) 'pure microsomes' and (b) detergent-treated polyribosomes in the presence of various amounts of added GTP and a GTP-regenerating system

The reaction mixture of 0.1 ml contained microsomal fractions (about 72 μ g of RNA) or polyribosomes (about 40 μ g of RNA), about 25 μ g of aminoacyl-tRNA labelled with [¹⁴C]leucine, 440 μ g of freeze-dried high-speed supernatant protein, 10 μ g of pyruvate kinase, 1.0 μ mol of phosphoenolpyruvate, 10 μ mol of Tris-HCl buffer, pH 7.2 at 37°C, 2.5 μ mol of K⁺, 0.5 μ mol of Mg²⁺ and various amounts of GTP. Incubation was for 30 min at 37°C. Protein synthesis was measured in the absence of further additions (○), in the presence of 0.1 mM-GSSG (Δ), 4 mM-2-mercaptoethanol (□) and 0.1 mM-GSSG + 4 mM-2-mercaptoethanol (●).

Fig. 2. Effects of 0.1 mM-GSSG on protein synthesis occurring on (a) 'pure microsomes' and (b) detergent-treated polyribosomes in the presence of various amounts of added GTP and in the absence of a GTP-regenerating system

The reaction mixture and conditions of incubation were as described in Fig. 1 except that pyruvate kinase and phosphoenolpyruvate were omitted. Protein synthesis was measured in the absence of further additions (○), in the presence of 0.1 mM-GSSG (Δ), 4 mM-2-mercaptoethanol (□) and 0.1 mM-GSSG + 4 mM-2-mercaptoethanol (●).

exclude the presence of a population of polyribosomes resistant to GSSG.

The effects of GSSG on protein synthesis occurring on microsomal fractions could be explained in terms of the action of a membrane component (Nolan & Hoagland, 1971). The effect of GSSG on protein synthesis occurring on detergent-treated

and free polyribosomes could not be explained on a similar basis, and must have been due to an effect of GSSG on a normal component of the protein-synthesizing apparatus. The effect of GSSG was manifest only under conditions of GTP limitation. Thus the observed differences between the sensitivity of protein synthesis occurring on detergent-treated polyribosomes and microsomal fractions to GSSG could have been due to different effective GTP concentrations in the respective systems caused by the presence in microsomal fractions of an enzyme (or enzymes) that promoted removal of GTP. Such an enzyme could be formally identical with the component in microsomal extracts that causes inhibition of protein synthesis occurring on detergent-treated polyribosomes *in vitro* in the presence of 0.1 mM-GSSG (Scornik *et al.*, 1967).

Phosphohydrolase activity

The phosphohydrolase activity of 'pure microsomes' and extracts of 'pure microsomes' was investigated (Table 1) to determine whether enzymes were present that could promote removal of GTP and cause an inhibition of protein synthesis *in vitro* that was potentiated by 0.1 mM-GSSG. Extracts of 'pure microsomes' contained a nucleoside diphosphate phosphohydrolase that hydrolysed GDP and UDP but not ADP or CDP. Chromatography on polyethyleneimine-impregnated cellulose showed that the product of hydrolysis of GDP and UDP was the nucleoside 5'-monophosphate in each case. The nucleoside diphosphate phosphohydrolase was

Table 1. *Phosphohydrolase activity of 'pure microsomes' and microsomal extracts*

The reaction mixture of 1.0 ml contained 'pure microsomes' (about 200 μ g of protein) or microsomal extract (about 22 μ g of protein), 100 μ mol of Tris-HCl buffer (pH 7.2 at 37°C), 5 μ mol of Mg^{2+} , 25 μ mol of K^+ , 1.0 μ mol of nucleoside 5'-triphosphate or nucleoside 5'-diphosphate. Incubation was for 5 min at 37°C.

Substrate	Phosphohydrolase activity (μ mol of P_i liberated/min per mg of protein)		
	Extracts of 'pure microsomes'	'Pure microsomes'	Detergent-treated polyribosomes
ATP	—	0.059	—
GTP	—	0.111	—
GDP	3.9	0.462	—
UDP	3.86	0.424	—
ADP	—	—	—
CDP	—	—	—

also detected in 'pure microsomes', as were enzymes that hydrolysed ATP and GTP. Hydrolysis of nucleoside di- and tri-phosphates by detergent-treated polyribosomes was not detected. This result probably reflected the relative insensitivity of the method, as hydrolysis of GTP by polyribosomes has been demonstrated by release of radioactivity from [γ - ^{32}P]GTP (Raeburn *et al.*, 1971; Felicetti & Lipmann, 1968).

The properties of the nucleoside diphosphate phosphohydrolase (EC 3.6.1.6) in microsomal extracts were investigated with 1.0 mM-GDP as substrate (Table 2). The enzyme was partially inhibited by 4.0 mM-ATP, -ADP and -GTP and completely inhibited by 0.1 M-KF. Slight activation of the enzyme by 0.2 mM-ATP was observed. Similar results were obtained by Plaut (1955) and by Yamazaki & Hayaishi (1968). In addition, 0.1 mM-GSSG and 4.0 mM-2-mercaptoethanol were shown to have no effect on the activity of the nucleoside diphosphate phosphohydrolase.

Inhibitory activity of nucleoside diphosphate phosphohydrolase

Nucleoside diphosphate phosphohydrolase was the only phosphohydrolase observed in extracts of 'pure microsomes' and was the most active phosphohydrolase in 'pure microsomes'. This enzyme, by destroying GDP and so preventing regeneration of GTP, might readily promote removal of GTP during protein synthesis *in vitro* and so be responsible for the inhibition of protein synthesis occurring on detergent-treated polyribosomes in the presence of 0.1 mM-GSSG, 0.01 mM-GTP and a GTP-regenerating system that is caused by the addition of a microsomal extract (Scornik *et al.*, 1967).

To test this hypothesis, nucleoside diphosphate phosphohydrolase was partially purified by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose (Fig. 3). Co-chromatography of the

Table 2. *Properties of the nucleoside diphosphate phosphohydrolase in extracts of 'pure microsomes'*

Phosphohydrolase activity was measured as described in Table 1 with 1.0 mM-GDP as substrate.

Addition	Change in activity (%)
ADP (4.0 mM)	-18.3
ATP (4.0 mM)	- 6.6
GTP (4.0 mM)	- 8.5
KF (0.1 M)	-97.2
ATP (0.2 mM)	+ 7.9
GSSG (0.1 mM)	0.0
2-Mercaptoethanol (4.0 mM)	0.0

Fig. 3. Purification of the microsomal nucleoside diphosphate phosphohydrolase and inhibitor of protein synthesis

Protein was extracted from a crude microsomal fraction and fractionated with $(\text{NH}_4)_2\text{SO}_4$ as described in the Materials and Methods section. The protein fraction precipitated between 35% and 75% saturation with $(\text{NH}_4)_2\text{SO}_4$ was applied to a column of DEAE-cellulose (bed vol. 12ml) and eluted with a linear KCl gradient (---). Fractions (1.8ml) were collected and dialysed against 20mM-Tris-HCl buffer (pH 8.0 at 20°C). The nucleoside diphosphate phosphohydrolase activity of various dialysed fractions (Δ) was measured as described in Table 1 and was expressed as μmol of P_i liberated from GDP/min per mg of protein at pH 7.2 at 37°C. The effect of various dialysed fractions on protein synthesis was determined in the presence of 0.01 mM-GTP + 0.1 mM-GSSG ($\square$), and 0.01 mM-GTP + 0.1 mM-GSSG + 4 mM-2-mercaptoethanol ($\circ$). Protein synthesis on detergent-treated polyribosomes was measured as described in Fig. 1. A unit of inhibitory activity was defined as the reciprocal of the amount of protein (mg) required to inhibit protein synthesis by 50%. Transmittance at 280 nm ($\cdot - \cdot -$) was measured with an LKB Uvicord II instrument.

enzyme and of an inhibitory activity sensitive to 4mM-2-mercaptoethanol was observed. Inhibitory activity was determined by measuring the effect of samples of the various fractions on protein synthesis occurring on detergent-treated polyribosomes in the presence of 0.1mM-GSSG, 0.01mM-GTP and a GTP-regenerating system. Additional experiments on fraction 54, which exhibited high nucleoside diphosphate phosphohydrolase and inhibitory activity, showed that the inhibition of protein synthesis was increased 4.8-fold by 0.1mM-GSSG and was abolished by 0.4mM-GTP, as well as by 4mM-2-mercaptoethanol. Since such properties are characteristic of the inhibitor present in microsomal extracts (Fig. 4a) it was concluded that nucleoside diphosphate phosphohydrolase was the microsomal-associated inhibitor of protein synthesis described by Scornik *et al.* (1967).

The effect of the nucleoside diphosphate phosphohydrolase on the concentration of GTP during protein synthesis *in vitro* was investigated by using ^{14}C -labelled GTP in the reaction mixture in place of unlabelled GTP. Chromatography of portions of the reaction mixture on polyethyleneimine-impregnated cellulose showed that the concentration of GTP was greatly decreased by the addition of the nucleoside diphosphate phosphohydrolase. Since the activity of the nucleoside diphosphate phospho-

hydrolase was unaffected by 0.1mM-GSSG or 4mM-2-mercaptoethanol (Table 2) the effects of GSSG and 2-mercaptoethanol on the inhibition of protein synthesis caused by this enzyme must have been secondary to the effect of this enzyme on GTP concentration. To obtain more information on the role of GSSG under conditions of GTP limitation the effect of 0.1mM-GSSG on protein synthesis occurring in the presence of apyrase, a non-specific nucleoside triphosphate diphosphohydrolase, was studied.

Effects of apyrase and a microsomal extract on protein synthesis occurring on detergent-treated polyribosomes in the presence and absence of GSSG

Apyrase was activated by 5mM- Ca^{2+} or by 5mM- Mg^{2+} , and hydrolysed ATP and GTP with equal efficiency. The activity of apyrase was unaffected by 0.1mM-GSSG or 4mM-2-mercaptoethanol. At an initial concentration of 0.01mM-GTP and in the presence of a GTP-regenerating system, addition of 0.06mg of apyrase had little effect on protein synthesis occurring on detergent-treated polyribosomes in the absence of GSSG, but caused a marked inhibition when 0.1mM-GSSG was present (Fig. 4b). Since the activity of apyrase was unaffected by 0.1mM-GSSG it was evident that GSSG itself was causing an inhibi-

tion of protein synthesis under the conditions of GTP limitation that were induced by the action of apyrase. Addition of 4mM-2-mercaptoethanol,

Fig. 4. Effects of (a) an extract of 'pure microsomes' and (b) apyrase on protein synthesis occurring on detergent-treated polyribosomes in the presence and absence of 0.1 mM-GSSG

The reaction mixture and conditions of incubation were identical with those described in Fig. 1. Protein synthesis was measured in the presence of 0.01 mM-GTP (○), 0.01 mM-GTP + 0.1 mM-GSSG (Δ), 0.01 mM-GTP + 0.1 mM-GSSG + 4.0 mM-2-mercaptoethanol (□), 0.01 mM-GTP + 0.1 mM-GSSG + 0.4 mM-ATP (●) and 0.4 mM-GTP + 0.1 mM-GSSG (▲).

0.4 mM-GTP or 0.4 mM-ATP abolished the effect of apyrase that was observed in the presence of GSSG. The stimulation of protein synthesis that was observed in the absence of apyrase after the GTP concentration was increased from 0.01 to 0.4 mM reflected the fact that 0.01 mM-GTP was a sub-optimum concentration even in the presence of a GTP-regenerating system.

The effects of apyrase and the microsomal extract on protein synthesis differed only in their sensitivity to addition of ATP. In the presence of apyrase, ATP was thought to act to maintain the concentration of GTP. Because ATP and GTP are both substrates for apyrase, ATP and GTP will compete for substrate-binding sites on the enzyme. Thus, addition of a 40-fold molar excess of ATP will decrease the rate of hydrolysis of GTP by apyrase and so cause an increase in the rate of protein synthesis. The activity of nucleoside diphosphate phosphohydrolase, however, was little affected by ATP, which is not a substrate for this enzyme. Thus ATP would not be expected to abolish inhibition caused by nucleoside diphosphate phosphohydrolase and this enzyme could also act by promoting removal of GTP.

Discussion

The similarity of the effects of apyrase and of the nucleoside diphosphate phosphohydrolase in the microsomal extract on protein synthesis occurring on detergent-treated polyribosomes suggested a common mode of action. The most simple explanation of the effects is that both enzymes cause a decrease in the concentration of GTP that renders protein synthesis sensitive to 0.1 mM-GSSG. The susceptibility of protein synthesis *in vitro* occurring on microsomal fractions but not on detergent-treated polyribosomes to the effect of GSSG in the presence of phosphoenolpyruvate and pyruvate kinase is explained on a similar basis. Microsomal fractions contain enzymes not found on detergent-treated polyribosomes that promote removal of GTP. However, protein synthesis on detergent-treated polyribosomes can be made sensitive to 0.1 mM-GSSG by omission of pyruvate kinase and phosphoenolpyruvate, resulting in a progressive decrease in the GTP concentration. In all situations the effect of GSSG is abolished by increasing the GTP concentration.

GSSG probably causes oxidation of the component with which it interacts, since addition of 2-mercaptoethanol prevents the effect. Two effects of GTP can be envisaged. The components oxidized by GSSG might normally bind GTP during the course of its action and have a lesser affinity for GTP when oxidized. Support for this possibility was derived

from observations on protein synthesis occurring on detergent-treated polyribosomes by using other transfer-enzyme preparations that were fully active only in the presence of thiol. When such preparations were used addition of 4mM-2-mercaptoethanol greatly decreased the amount of GTP required for maximum activity. Alternatively, GTP might interact with the component susceptible to GSSG and protect it from oxidation. Sutter & Moldave (1966) showed that transferase II was inactivated by preincubation in 22mM-GSSG and was protected from this inhibition by 0.4mM-GTP. Thus, transferase II could be the component affected by 0.1mM-GSSG under conditions of GTP limitation.

Nucleoside diphosphate phosphohydrolase is apparently located on the cisternal side of the endoplasmic-reticulum membrane (Kuriyama, 1972). Since polyribosomes are attached to the other side of this membrane or are cytoplasmic, it is uncertain how this enzyme could regulate protein synthesis *in vivo*.

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