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Inhibition of Protein Synthesis in Rabbit Reticulocyte Lysates by Double-Stranded RNA and Oxidized Glutathione: Indirect Mode of Action on Polypeptide Chain Initiation*

(translational control/Met-tRNA$_f^{Met}$ binding factor/initiation factor MP/reticulocyte inhibitor protein)

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ABSTRACT **In the presence of added double-stranded RNA or oxidized glutathione, protein synthesis in hemin-supplemented reticulocyte lysates declines abruptly after 8–12 min of incubation at 30°. The kinetics of amino-acid incorporation are very similar to those seen when lysates are incubated in the absence of added hemin. The inhibitory effects of double-stranded RNA (dsRNA) and oxidized glutathione (GSSG) are partially overcome by a homogeneous initiation factor, IF-MP, which also stimulates protein synthesis in hemin-deficient lysates. This factor is involved in the binding of Met-tRNA$_f^{Met}$ to 40S ribosomal subunits during protein chain initiation. However, neither dsRNA alone nor GSSG alone significantly inhibits formation of [40S subunit-Met-tRNA$_f$] complexes in a fractionated *in vitro* system. These results suggest that the loss of native [40S subunit-Met-tRNA$_f$] complexes induced in reticulocyte lysates by dsRNA or GSSG involves one or more components present in the lysates but absent from the fractionated *in vitro* system. Such components may be related to the translational inhibitor that is active in hemin-deficient lysates.**

Addition to rabbit reticulocyte lysates of either of two seemingly unrelated compounds, double-stranded RNA (dsRNA) and oxidized glutathione (GSSG), results in a sharp decline in amino-acid incorporation into protein after several minutes of incubation under conditions otherwise optimal for protein synthesis (2–5). This sudden decrease in the rate of protein synthesis is accompanied by disaggregation of polysomes, suggesting a block at the level of polypeptide chain initiation. The kinetics of amino-acid incorporation in the presence of either of these inhibitors are very similar to those seen in reticulocyte lysates which are not supplemented with added hemin (6, 7). A further common feature of the inhibition of protein synthesis by dsRNA, GSSG, or hemin deficiency is the marked diminution in the number of native 40S ribosomal subunits carrying Met-tRNA$_f$ (8–10). These findings suggest a possible common pathway for regulation of initiation of translation by dsRNA, GSSG, and hemin. We have shown that a partially purified preparation of an initiation factor, IF-MP, which forms a ternary complex with Met-tRNA$_f$ and GTP (11;¶) and is probably involved in the binding of Met-tRNA$_f$ to 40S subunits, stimulates protein synthesis in hemin-deficient incubation (12). It was, therefore, of interest to examine the effects of this factor on lysates to which dsRNA or GSSG had been added. Homogeneous IF-MP is now available and was used for these studies.

Recently an assay has been established for measuring binding of Met-tRNA$_f$ to 40S subunits in a fractionated system (13). Using this system, we have reported that preparations of an inhibitory protein, thought to mediate the effects of hemin on protein synthesis in lysates (14–16), reduces binding of Met-tRNA$_f$ to 40S subunits (12). In seeking to define the mechanism of action of dsRNA and GSSG, we have investigated whether these compounds have an inhibitory effect in the fractionated system similar to that observed with the inhibitor protein preparations.

MATERIALS AND METHODS

Methods for the preparation of reticulocyte lysates and the conditions of incubation for assay of protein synthesis *in vitro* have been described previously (7, 12). The labeled amino acid was [^{14}C]leucine (273 Ci/mol), 4 μCi/ml. Incorporation of radioactivity into protein following incubation at 30° was estimated by the filter disc method of Mans and Novelli (17).

Partial purification of the reticulocyte translational inhibitor from the ribosome-free supernatant was performed as previously described (12).

Assays for initiation-factor-dependent binding of [^{35}S]Met-tRNA$_f$ to salt-washed 40S subunits were also carried out as previously reported (12). Subunits were analyzed for radioactivity after formaldehyde fixation and CsCl equilibrium buoyant density gradient centrifugation (18). Individual variations from the published procedure are described in Tables 1 and 2.

Double-stranded RNA from *Penicillium chrysogenum* was a gift of Dr. H. D. Robertson. Oxidized glutathione was purchased from Sigma Chemical Co. The Met-tRNA$_f$ binding factor (IF-MP) was purified to homogeneity as reported

Abbreviations: dsRNA, double-stranded RNA; GSSG, oxidized glutathione; IF, initiation factor.

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TABLE 1. *Binding of Met-tRNA$_f$ to 40S subunits in the presence of dsRNA*

Exp. no.	Source of Met-tRNA$_f$ binding activity	dsRNA (μg/ml)	[^{35}S]Met-tRNA$_f$ bound to subunits (cpm $\times$ 10^{-3})
1	1 M KCl ribosomal salt wash	0	4.85
		0.1	3.91
		0.2	4.04
2	DEAE-cellulose-treated salt wash	0	4.42
		0.1	5.83
		0.5	4.27
3	Homogeneous IF-MP	0	36.2
		0.1	42.3

The conditions of incubation were as follows: Exp. 1: One A_{260} unit of salt-washed 40S subunits was incubated in the presence or absence of dsRNA with 5.7 $\times$ 10^4 cpm of [^{35}S]Met-tRNA$_f$ (specific activity 90 $\times$ 10^3 Ci/mol of methionine) and 0.2 mM GTP for 2 min at 30° in a reaction volume of 1 ml. A 1 M KCl reticulocyte ribosomal salt wash (235 μg) was then added and incubation was continued for a further 15 min. Exp. 2: 0.5 A_{260} unit of 40S subunits was incubated in a volume of 1 ml in the presence or absence of dsRNA with 0.2 mM GTP for 5 min at 30°. 1.3 $\times$ 10^6 cpm of [^{35}S]Met-tRNA$_f$ was then added together with 50 μg of a ribosomal salt wash which had been chromatographed on DEAE-cellulose and eluted with an 0.1 M–0.4 M KCl step. Incubation was continued for a further 15 min. Exp. 3: One A_{260} unit of 40S subunits was incubated with 3.35 $\times$ 10^5 cpm of [^{35}S]Met-tRNA$_f$ and 0.2 mM GTP for 5 min at 30° in a reaction volume of 0.1 ml. Homogeneous IF-MP (4.2 μg) was then added and incubation was continued for a further 15 min. All samples were fixed with formaldehyde and analyzed by CsCl buoyant density centrifugation as described previously (12, 13, 18).

elsewhere (11,¶). Edeine was purchased from Calbiochem Ltd.

RESULTS

Similarities in the mode of inhibition of protein synthesis by dsRNA, GSSG, and the inhibitor protein

When a reticulocyte lysate is incubated with 0.1 μg/ml of dsRNA in the presence of hemin, protein synthesis virtually ceases after 12 min at 30° (Fig. 1). If hemin is omitted from the incubation mixture or if the reticulocyte inhibitor protein (12, 14–16) is added, a similar abrupt change in the rate of amino-acid incorporation also occurs at about 10 min. However, dsRNA in the presence of the inhibitor protein has no effect beyond that seen with inhibitor alone (Fig. 1).

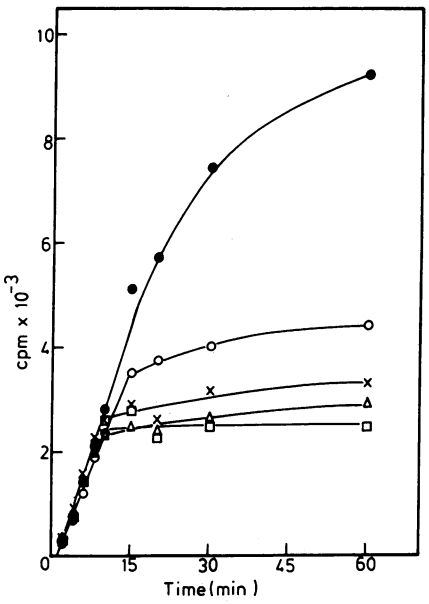

FIG. 1. Effects of hemin deficiency, dsRNA, and the translational inhibitor on protein synthesis in a reticulocyte lysate. Reaction mixtures (100 μl) were incubated at 30° as described previously (7, 12) in the presence or absence of hemin (40 μM), dsRNA (0.1 μg/ml), or partially purified translational inhibitor (100 μg of protein per ml). Aliquots (10 μl) were removed at the times indicated for estimation of [^{14}C]leucine incorporation into acid-insoluble material. (●——●), + hemin control; (×——×), minus hemin; (○——○), + hemin and dsRNA; (△——△), + hemin and translational inhibitor; (□——□), + hemin, dsRNA, and translational inhibitor.

A similar observation regarding the effects of GSSG and hemin deficiency is shown in Fig. 2. GSSG (0.5 mM) inhibits protein synthesis to almost the same extent and with similar kinetics as does omission of hemin from the incubation. Hemin deficiency and GSSG do not immediately inhibit initiation in the reticulocyte lysate, however, since amino-acid incorporation continues for several minutes longer than in the presence of 0.5 μM edeine. The latter is a specific inhibitor of chain initiation (19) and shuts off protein synthesis completely within 1.5 min at 30° (Fig. 2).

TABLE 2. *Binding of Met-tRNA$_f$ to 40S subunits in the presence of GSSG*

GSSG (mM)	[^{35}S]Met-tRNA$_f$ bound to subunits (cpm $\times$ 10^{-3})
0	4.15
1	3.81
2	3.66

A 0.5 A_{260} unit of salt-washed 40S subunits was incubated in the presence or absence of GSSG with 3.9 $\times$ 10^5 cpm of [^{35}S]Met-tRNA$_f$, 0.2 mM GTP, and 115 μg of 1 M KCl reticulocyte ribosomal salt wash for 15 min at 30°. Samples were fixed with formaldehyde and analyzed by CsCl buoyant density centrifugation as described previously (12, 13, 18).

Relief of the dsRNA and GSSG inhibitions by an initiation factor preparation

It has been shown that crude IF-M$_3$ (20) and the Met-tRNA$_f$ binding factor (IF-MP) which has been purified from crude IF-M$_3$ preparations (11;¶) are able to stimulate protein synthesis in hemin-deficient lysates (12, 21). Crude IF-M$_3$ also partially reverses the inhibitory effect of dsRNA (21). It was, therefore, of interest to test the ability of homogeneous IF-MP to stimulate protein synthesis in lysates which are inhibited by dsRNA or GSSG. As shown in Fig. 3, IF-MP partially overcomes the inhibition observed in lysates in the presence of dsRNA or GSSG as well as in hemin-deficient lysates; thus, IF-MP prevents the abrupt shut-off of leucine incorporation which occurs in each of the inhibited lysates

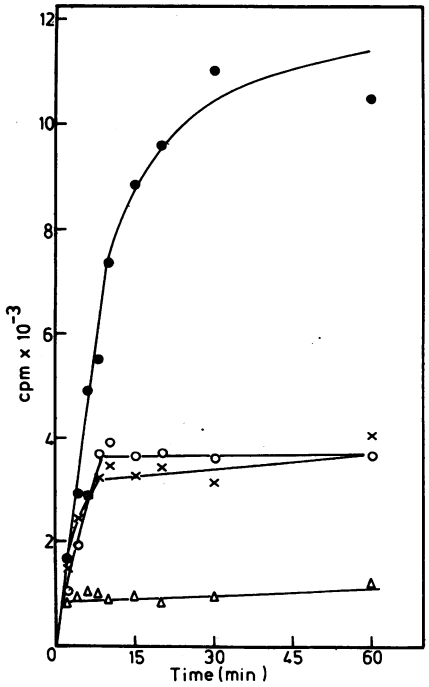

FIG. 2. Effects of hemin deficiency and addition of GSSG on protein synthesis in a reticulocyte lysate; comparison with the effect of edeine. Reaction mixtures were incubated as described in Fig. 1 in the presence or absence of hemin (30 μM), GSSG (0.5 mM), or edeine (0.5 μM). (●——●), + hemin control; (×——×), minus hemin; (○——○), + hemin and GSSG; (△——△), minus hemin, plus edeine.

after a few minutes. However, it should be stressed that complete reversal of inhibition was rarely observed; optimal concentrations of the initiation factor preparation usually restored protein synthesis to about 50% of the uninhibited rate in all three cases, although in some experiments up to 90% reversal was obtained.

Fig. 4 shows the effect of one concentration of IF-MP on protein synthesis over a range of dsRNA and GSSG concentrations. It can be seen that IF-MP has no effect in hemin-supplemented lysates in the absence of the inhibitors. It is also apparent that the stimulatory effect of IF-MP is constant over a wide range of dsRNA and GSSG concentrations. This suggests that IF-MP is not simply titrating out the inhibitors in the incubation. As noted by others (9) the inhibitory effect of dsRNA is considerably reduced at high dsRNA concentrations; at the same time the stimulatory effect of IF-MP is diminished under these conditions. The concentration curve for the GSSG inhibition is biphasic, probably reflecting inhibition of chain elongation as well as of initiation by concentrations of GSSG above 0.5 mM. In the presence of 1 mM GSSG IF-MP is unable to overcome the inhibition of protein synthesis.

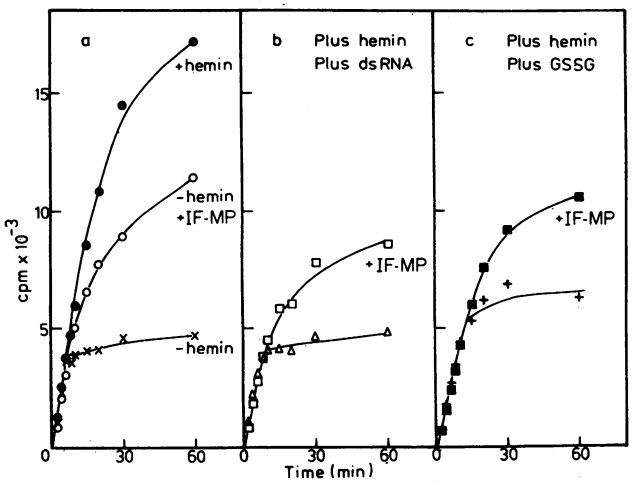

FIG. 3. Effect of IF-MP on the rate of protein synthesis in lysates inhibited by (a) hemin deficiency; (b) addition of dsRNA, or (c) GSSG. Reaction mixtures were incubated as described in Fig. 1 in the presence or absence of hemin (40 μM), dsRNA (0.1 μg/ml), or GSSG (0.5 mM). Homogeneous IF-MP was added where indicated to give a final concentration of 128 μg/ml.

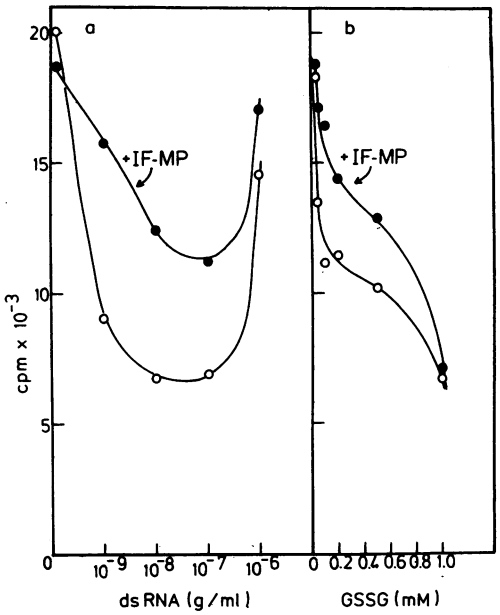

FIG. 4. Concentration curves for inhibition of protein synthesis by (a) dsRNA or (b) GSSG in a reticulocyte lysate in the presence or absence of IF-MP. Reaction mixtures (25 μl) were incubated for 30 min at 30° in the presence or absence of homogeneous IF-MP (84 μg/ml). [^{14}C]Leucine incorporation into protein was estimated on duplicate 10 μl aliquots.

dsRNA and GSSG do not inhibit binding of Met-tRNA$_f$ to 40S subunits directly

When salt-washed 40S ribosomal subunits are incubated with Met-tRNA$_f$, GTP, and a reticulocyte ribosomal salt wash containing initiation factors, proteins in the salt wash become associated with the subunits and give rise to particles with buoyant densities of 1.40 and 1.49 g/cm^3 as determined in CsCl density gradients (12, 13, 18). The Met-tRNA$_f$ is bound only to the lighter fraction of the subunits, which has more protein associated with it (13). We have used this assay system to examine the effects of dsRNA and GSSG on Met-tRNA$_f$ binding in the absence of the other components of the reticulocyte lysate. As shown in Table 1, we have been unable to observe any significant inhibition of binding of the initiator tRNA to the subunits by dsRNA in concentrations at which it inhibits protein synthesis in crude lysates. This was the case in experiments which employed either crude salt washes or a homogeneous preparation of IF-MP. A preincubation period of up to 5 min with dsRNA before addition of the initiation factors did not reveal any inhibitory effect. In contrast, under

the same conditions, preparations of the reticulocyte translational inhibitor did reduce the formation of 40S subunit–Met-tRNA$_f$ complexes, as reported previously (12).

GSSG was also not markedly inhibitory for Met-tRNA$_f$ binding when present in concentrations as high as 2 mM (Table 2). This is in spite of the fact that reduced thiol groups have been reported to be essential to the activity of the Met-tRNA$_f$ binding factor (22).

DISCUSSION

Protein synthesis in reticulocyte lysates can be inhibited by: (*i*) absence of added hemin; (*ii*) addition of crude or partially purified preparations of a translational inhibitor protein which is found in the ribosome-free supernatant (12, 14–16); (*iii*) addition of dsRNA in concentrations in the range of 10^{-10} to 10^{-7} g/ml (2, 3, 21); and (*iv*) addition of GSSG in concentrations in the range of 0.05–0.5 mM (4, 5). There are several features common to all four inhibitions, which may be summarized as follows: (*a*) the initial rate of amino-acid incorporation is not diminished for the first several minutes of incubation; (*b*) the rate of protein synthesis declines abruptly after this initial period; (*c*) at the time of shut-off, polysomes disaggregate as nascent polypeptide chains are completed and released from the ribosomes; (*d*) the number of native 40S subunits carrying Met-tRNA$_f$ is severely reduced; (*e*) the rate of protein synthesis is maintained, at least partially, by the addition of low concentrations of an initiation factor (IF-MP) which can bind Met-tRNA$_f$ to 40S ribosomal subunits. These facts suggest that hemin, the translational inhibitor, dsRNA, and GSSG might all regulate synthesis by very similar mechanisms. It is likely that a major part of this regulation directly involves IF-MP. Several laboratories have reported the preparation of a fraction which contains ribosome-independent Met-tRNA$_f$ binding activity similar to that observed with IF-MP [IF-E2, Schreier and Staehelin (23); IF-1, Dettman and Stanley (24); IF-1, Gupta *et al.* (25); IF-L3, Levin *et al.* (26)].

It is possible to estimate the number of rounds of translation which take place in the absence of hemin, or in the presence of the inhibitors, from the data on amino-acid incorporation in the presence of edeine. The time courses shown in Fig. 2 indicate that when initiation is blocked by this antibiotic, synthesis of nascent globin chains is completed within 1.5 min at 30°. In the absence of hemin or in the presence of dsRNA, GSSG, or the translational inhibitor, protein synthesis shuts off between 5 and 12 min of incubation. Thus there must be several rounds of translation in this period of resistance to inhibition. Similar conclusions have been reached by others (7, 27). One possible explanation for the early resistance to inhibition is that IF-MP is initially present in the reticulocyte lysate in excess and that inhibition is only observed after this pool has been exhausted. Alternatively, the factor may exist in more than one functional state (e.g., free in solution or complexed with 40S subunits), only one of which is sensitive to the inhibitors, and conversion from one state to another may require synthesis to take place. It is not clear at present how the effects of IF-MP in maintaining protein synthesis in the reticulocyte lysate relate to those of high concentrations of 3′:5′-cyclic AMP and similar compounds described by Legon *et al.* (9). It is conceivable that addition of cyclic AMP somehow reactivates the factor either directly or via inactivation of an inhibitor (see below).

Kaempfer and Kaufman have described an initiation factor preparation, which they have called IF-3, which overcomes the inhibition of protein synthesis caused by hemin deficiency (28) or addition of dsRNA (29). This factor preparation is probably identical to the crude IF-M$_3$ of Prichard *et al.* (20). Kaempfer (30) has also recently confirmed our observations (using a highly purified preparation of IF-MP from the same purification from which the homogeneous IF-MP studied here was obtained) that it is IF-MP which is the component of crude IF-3 or crude IF-M$_3$ which reverses the effects of hemin deficiency or of dsRNA in reticulocyte lysates. Crude IF-3 was reported to be required in stoichiometric amounts to restore protein synthesis in the presence of dsRNA and was also shown to bind to dsRNA *in vitro* (29). However, our own observations show that the enhancement of protein synthesis mediated by IF-MP in limiting concentration is virtually constant over a 100-fold range of dsRNA concentrations (Fig. 4). These findings lead us to suggest that dsRNA does not inhibit initiation as a result of binding IF-3 as proposed by Kaempfer and Kaufman (29). A further piece of evidence which conflicts with their suggestion is the very marked distinction between the effect of dsRNA as well as GSSG on binding of Met-tRNA$_f$ to 40S subunits in crude reticulocyte lysates (8, 9) and in the fractionated system employed in our experiments (Tables 1 and 2). It is suggested that one or more components present in the whole lysate but absent from our Met-tRNA$_f$ binding assays are responsible for the dsRNA- or GSSG-mediated inhibitions and that dsRNA and GSSG do not act directly.

The regulation by hemin of protein synthesis in the lysate, which has several features in common with the action of dsRNA or GSSG, has been postulated to occur through the action of the translational inhibitor protein which can be isolated from the ribosome-free supernatant (12, 14–16). Partially purified preparations of this inhibitor reduce binding of Met-tRNA$_f$ to washed 40S subunits in the ribosomal salt-wash-dependent assay system. It is, therefore, a possibility that this same inhibitor mediates the effects of dsRNA and GSSG on protein synthesis. Such a proposal has been made by Legon *et al.* (9) on the basis of evidence for the accumulation of an inhibitor in dsRNA-treated lysates.

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