CONTROL OF PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES: THE EFFECT OF NUCLEOTIDE TRIPHOSPHATES ON FORMATION OF THE TRANSLATIONAL REPRESSOR.

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Received September 16,1975

Summary. High concentrations (1-2mM) of GTP completely prevented the decline in protein synthesis which occurs in a reticulocyte lysate incubated in the absence of haemin. The effect of GTP was diminished by raising the concentration of ATP. When formation of the haemin-controlled translational repressor was studied as a partial reaction using post-ribosomal supernatant, it was found that GTP acted by blocking repressor formation, whilst ATP was found to stimulate this process.

Maintenance of protein synthesis in rabbit reticulocyte cells or their lysates requires the addition of haemin (1-4). In the absence of haemin a translational repressor accumulates which blocks protein synthesis at the locus of chain initiation, apparently by preventing the association of met-$tRNA_f$ with the small ribosomal subunit (5-9). The repressor has been named "reversible inhibitor" since its activity is lost after preincubation with haemin (7). The inhibitor is a protein of molecular weight $4 \pm 1 \times 10^5$ daltons and forms from a precursor (proinhibitor) which has a similar molecular weight (5,6,10). Pro-inhibitor also gives rise to other molecules termed "irreversible inhibitor" (7,10,11) and "intermediate inhibitor" (12). Although these inhibitors appear to be derived from and closely related to reversible inhibitor their precise physiological function is not clear. However, for reasons of stability, most studies have hitherto used the irreversible

form. Gross and Rabinovitz (13) have shown that it acts catalytically, since one molecule can inactivate more than a thousand ribosomes. However, the nature of the enzymic reaction catalysed by the repressor is not known.

We previously reported (14) that high concentrations of certain purine derivatives, including 2 amino purine and 3'-5' cyclic AMP, could substitute for haemin in maintaining protein synthesis in lysates. It seemed likely that these compounds interfered with the normal control mechanism by acting as analogues of some natural component, e.g. a purine nucleotide. Accordingly, we decided to investigate whether either ATP or GTP played any role in the formation or action of the repressor. It was hoped that this approach might provide a clue to its enzymic nature. It was found that GTP could act as a substitute for haemin in maintaining protein synthesis in the lysate and the effect was diminished by raising the concentration of ATP. Analysis showed that GTP acted by blocking the formation of repressor. The significance of these results is discussed.

MATERIALS AND METHODS

Preparation of cell-free extracts The procedures for preparation of rabbit reticulocyte cells and their lysates have been previously described (15). Supernatant was obtained by spinning 5 ml portions of lysate at 50,000 r.p.m. for 2½ hr at 2° in the Beckmann SW50.1 rotor. It was freed of low molecular weight components by passage through a column of Sephadex G50 superfine equilibrated with Buffer A (10mM HEPES, pH 7.2; 25mM KCl; 10mM NaCl; 1mM $MgCl_2$; 0.4mM spermidine 0.1mM dithiothreitol). Lysates and gel-filtered supernatant were stored in liquid nitrogen.

Incubation conditions After thawing, lysates were made 25μgm/ml in creatine phosphokinase, and, where appropriate, 20μM in haemin. The protein-synthesizing system consisted of 30μl lysate, 10μl master mix, and 10μl water or other additions. Master mix contained the following ingredients to give the indicated final concentrations; creatine phosphate, 10mM; KCl, 100mM; $MgCl_2$, 0.5mM; [^{3}H] L-leucine, 16.7μCi/ml, 80μM; and a mixture of 19 unlabelled amino acids in the proportions found in rabbit globin (15). Whenever either ATP or GTP was present an equimolar amount of $MgCl_2$ was also added, over and above that already present in the master mix, in order to counteract the Mg^{2+} chelating effect of the nucleotides. This is indicated in

appropriate figure legends. Reversible inhibitor was assayed in the same system with the difference that the volume of lysate was reduced to 20 or 25 µl in order to accommodate relatively large volumes of supernatant in a final volume of 50µl. Details are given in appropriate figure legends. Incubations were at 30° unless otherwise stated and 5 or 10 µl samples were withdrawn to monitor incorporation of labelled leucine into protein. The sample was added to 1 ml of water and then mixed with 1 ml 0.6M NaOH containing 2mg/ml unlabelled L-leucine and 5% H_2O_2. After incubation at 37° for 10 min protein was precipitated by the addition of 1 ml of 25% TCA. The precipitates were collected on glass fibre filters (Whatman GF/C), washed with 8% TCA and dried with a warm air blower. The filters were placed in toluene scintillator and counted in a Packard Model 3330 liquid scintillation spectrometer with an efficiency of 60%.

RESULTS

We initially decided to examine whether lysate protein synthesis was affected by high concentrations of ATP and GTP themselves. Additional ATP up to a final concentration 2.5mM had no effect either in the presence or absence of haemin (data not shown). By contrast, GTP had no effect in the presence of haemin, but stimulated strongly in its absence (Fig. 1). Most lysates tested responded maximally to GTP in the concentration range 1-2mM although a few required as much as 4mM. A time-course experiment (Fig. 2) showed that GTP acted, like haemin (3,4) or 2 amino purine (14), by prolonging the period of linear synthesis. The effect was diminished by raising the level of ATP (Fig. 2).

One explanation for these results is that in the absence of haemin there is a failure to efficiently regenerate GTP. This possibility was investigated by incubating the protein-synthesizing system with [^{3}H] GTP, and then separating the radioactive nucleotides by thin layer chromatography according to the methods described by Randerath and Randerath (16). It was found that, either in the presence or absence of haemin, over 98% of the radioactivity on the chromatogram was associated with material of the same mobility as authentic GTP (data not shown). There were also no discernible

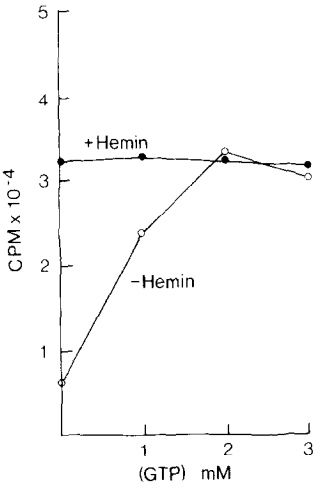

Figure 1.

Effect of GTP on protein synthesis. The protein-synthesizing system was incubated with or without haemin and different amounts of GTP plus $MgCl_2$. The latter two components were added separately in equimolar amounts since mixing the two together resulted in the formation of a precipitate. The mixtures were incubated for 60 min at 30° and a 5µl sample taken for measurement of leucine incorporation into protein; (●) plus haemin; (o) minus haemin.

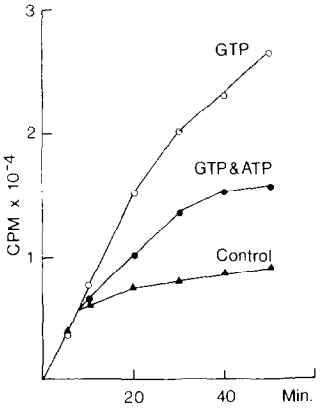

Figure 2.

Effect of ATP on the stimulation of protein synthesis by GTP The protein-synthesizing system was incubated at 30° in the absence of haemin. One sample contained 2mM GTP plus 2mM $MgCl_2$ (O); a second contained 2mM GTP, 2mM ATP and 4mM $MgCl_2$ (●); and a third was incubated without additional components (▲). Samples of 5µl were taken at the indicated times to monitor incorporation of leucine into protein.

differences in the distribution of minor radioactive components. Thus cessation of protein synthesis in the absence of haemin is not simply due to depletion of the pool of GTP.

The foregoing results suggested that ATP and GTP probably affected the formation or subsequent action of reversible inhibitor, and that most likely, their effects were opposite. We have examined the effect of the nucleotides on formation of reversible inhibitor. This process has previously been studied in lysates incubated in the presence or absence of protein synthesis (7,8), as well as in ribosome-free supernatants (7). The present experiments used supernatant which had been passed through a column of Sephadex G50 to remove free low molecular weight material. When such gel-filtered supernatant is incubated without haemin for up to 30 min at 30° reversible inhibitor is generated, and can be assayed by mixing with a complete lysate protein-synthesizing system (Fig.3). Protein synthesis becomes inhibited within 2 to 3 minutes of incubation, but the rate of amino acid incorporation later returns to normal as the inhibitor is inactivated, giving the time-course its characteristic "kinked" appearance. When the supernatant is incubated with GTP no inhibitor is detected (Fig.3). Concentrations of GTP above 0.2mM were sufficient to completely suppress inhibitory activity in gel-filtered supernatant, although addition of increasing amounts of ATP caused the GTP optimum to shift to progressively higher values (Fig.4). It is not clear from this type of experiment whether GTP is acting during the preincubation (by blocking inhibitor formation) or simply overcoming its action during the second incubation. In order to distinguish between these alternatives supernatant was first incubated with or without GTP and then gel-filtered again before assaying for inhibitory activity. The results were identical to those shown in Fig. 3, confirming that GTP acts by blocking inhibitor formation. In

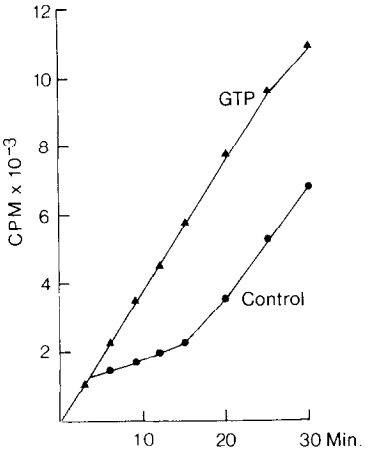

Figure 3.

Effect of GTP on the activity of reversible inhibitor. Gel-filtered supernatant (15µl) and master mix (10µl) were incubated for 30 min at 30° in the presence of 2mM GTP plus 2mM $MgCl_2$. Prewarmed, haeminated lysate (25µl) was added and the incubation continued. Samples of 5µl were withdrawn at the indicated times to monitor leucine incorporation; (▲) plus GTP; (●) minus GTP.

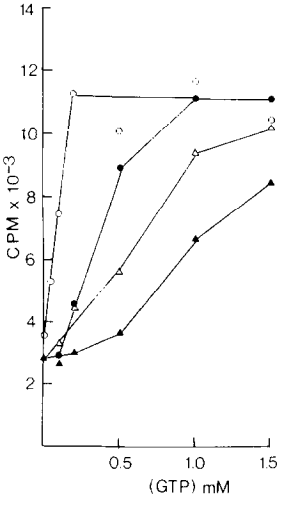

Figure 4.

Effect of different levels of ATP and GTP on formation of reversible inhibitor. Gel-filtered supernatant (15µl) was incubated for 30 min at 30° with master mix (10µl) and different amounts of GTP, ATP and $MgCl_2$ (5µl). The concentration of added $MgCl_2$ was equal to the sum of the concentrations of ATP and GTP. Prewarmed, haeminated lysate (20µl) was added and the incubation continued for a further 15 min. Samples of 10µl were then removed to measure leucine incorporation This was used as an index of the activity of reversible inhibitor. The GTP concentration was varied as indicated; (○) no ATP; (●) 0.2mM ATP; (△) 0.5mM ATP; (▲) 1.0mM ATP.

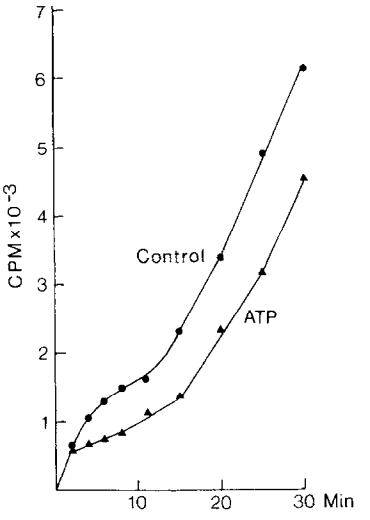

Figure 5

Effect of ATP on formation of reversible inhibitor. Gel-filtered supernatant (15µl) was incubated with master mix (10µl) in the presence or absence of 2.0mM ATP plus 2.0mM $MgCl_2$ in a final volume of 30µl. After 10 min at 30° prewarmed, haeminated lysate (20µl) was added and the incubation continued. Samples of 5µl were withdrawn at the indicated times to monitor leucine incorporation; (▲) plus ATP; (●) minus ATP.

a similar assay we found that 2 amino purine (4mM) did not suppress inhibitor formation. Therefore, the effect of 2 amino purine in supporting protein synthesis in the absence of haemin (14) is not mediated by any effect on formation of reversible inhibitor. However, the effect of GTP in blocking this partial reaction clearly accounts for its effect on lysate protein synthesis. The relatively high level of GTP required is not surprising in view of the presence of a pool of ATP in the crude lysate.

When supernatant was incubated with ATP increased amounts of reversible inhibitor were formed, as evidenced by earlier cessation of protein synthesis and an increase in the length of the inhibited phase (Fig. 5). However, there is clearly no absolute requirement for ATP in this reaction, in agreement with the data of Gross (11).

DISCUSSION

During recent years considerable evidence has been obtained to support the model whereby haemin prolongs protein synthesis in reticulocytes by preventing the formation of a catalytically-acting inhibitor of polypeptide chain initiation (5-10,11,13). The new data presented here show that inhibitor formation is influenced not only by haemin, but also by the relative concentrations of ATP and GTP. The fact that the effects of the nucleotides on inhibitor formation correlate with effects on protein synthesis provides further support for this model.

The present experiments were undertaken in an attempt to determine how high levels of various purines stimulated protein synthesis in the absence of haemin (14). The finding that GTP acted similarly was a by-product of this investigation. However, it appears that the simple purines do not act by blocking inhibitor formation as analogues of GTP, attractive though this possibility may seem. Recently we have developed an assay for the action of the inhibitor independent of protein synthesis (17). Under these conditions binding of the initiator tRNA to the 40S ribosomal subunit is blocked by the inhibitor only when ATP is also present. It appears that the inhibitor functions as a protein kinase, bringing about the phosphorylation of certain ribosome-bound polypeptides at the expense of the γ-phosphate group of ATP, and 2 amino purine was found to antagonise ATP in this function (17).

The fact that activation of the inhibitory activity is affected, as we have shown, by ATP and GTP is not surprising in view of its protein kinase nature. Since the inhibitor is a high molecular weight protein (13) it probably contains both catalytic and regulatory subunits. ATP and GTP might therefore simply be allosteric effectors which bind to a regulatory subunit and influence the conformation of the catalytic site. A similar mechanism has been envisioned for the

action of haemin (11). Proof of these hypotheses will require further purification of the repressor and knowledge of its subunit nature.

This work was supported by a grant from the Medical Research Council. K.B. was a fellow of the Science Research Council and T.H. was a Beit Memorial Fellow. We thank Anne Brayley and Jack Brittain for technical assistance and Tony Hunter for helpful discussion.

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