THE EFFECT OF CYCLIC AMP AND RELATED COMPOUNDS ON THE CONTROL OF PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES.

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SUMMARY: Cyclic AMP and a variety of purines are able to overcome the inhibition of the initiation of protein synthesis caused by incubation of the lysate in the absence of added hemin or with double-stranded RNA or oxidised glutathione. These three inhibitions show similar kinetics and are each accompanied by the disappearance of a complex between the 40S ribosomal subunits and met-tRNA$_f$. A translational repressor has been implicated in the inhibition seen in the absence of hemin and we suggest that the link between these three inhibitions is the accumulation of this repressor.

The initiation of protein synthesis in rabbit reticulocyte lysates becomes inhibited in three conditions of possible physiological significance : when dsRNA* (1) or GSSG (2) are added to incubations or when hemin is omitted from them (3). In each case, the rate of initiation is unaffected for the first few minutes but then declines abruptly. Three further findings link the inhibitions caused by dsRNA and lack of hemin : inhibition is accompanied by disappearance of a complex between met-tRNA$_f$ and the 40S ribosomal subunits (4,5), it affects the synthesis of all the proteins made by the lysate (6-9), and it can be prevented by the addition of large amounts

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ABBREVIATIONS : CTAB, cetyl trimethyl ammonium bromide; 2AP 2-amino purine; GSH, reduced glutathione; GSSG, oxidised glutathione.

* dsRNA, double-stranded RNA - this term is used to denote RNA:RNA duplexes held together by 'Watson-Crick' base pairing, the strands having a length of at least 50 bases and exactly complementary sequences.

of an initiation factor preparation (10,11). We show here that the inhibition caused by GSSG is similarly accompanied by loss of the 40S/met-tRNA$_f$ complex. We have also followed up the observation by Mager and Giloh (12) that 3'5'cAMP can overcome the inhibition caused by GSSG or lack of hemin - we find that besides 3'5'cAMP, a variety of purines can overcome both of these inhibitions and also the inhibition caused by dsRNA. We would like to suggest that the accumulation of a translational repressor is common to these three inhibitions and that 3'5'cAMP and the active purines work by preventing either the accumulation or the action of this repressor.

MATERIALS AND METHODS

The preparation and incubation of rabbit reticulocyte lysates was as previously described (13) except that the creatine phosphate concentration has now been increased to 10 mM, the added $MgCl_2$ has been reduced to 0.5 mM, and ATP and GTP are no longer added to incubations since the lysate contains adequate supplies of both. Methods for monitoring the synthesis of protein were also as previously described (13) - in all cases incubations were at 30°, the sample size was 5 µl and the radioactive amino acid was L 1-[^{14}C] leucine (62 µCi/µM) at a final concentration of 5 µCi/ml. The dsRNA used was a gift from Dr. D. Planterose, Beecham Research Laboratories and was prepared from Penicillium chrysogenum. Other compounds added to the lysate were obtained from commercial sources as follows - from Sigma: 3'5'cAMP, 3'5'cIMP, 3'5'cXMP, FAD, 3'5'ADP, purine, 2-amino purine, theobromine, caffeine and theophylline; from Boehringer: 3'AMP, 3'5'cGMP, NAD, NADP, S-adenosyl methionine, and Coenzyme A; from B.D.H.: 2'3'cAMP, dithiothreitol, cytosine, and thiamine; from Calbiochem: 3'5'cUMP; from Waldhof: mixed 2' & 3' AMP; from Koch-Light Labs: adenine.

RESULTS

The addition of 1-10 mM 3'5'cAMP to the lysate results in a considerable reduction in its sensitivity to dsRNA (Fig 1a).

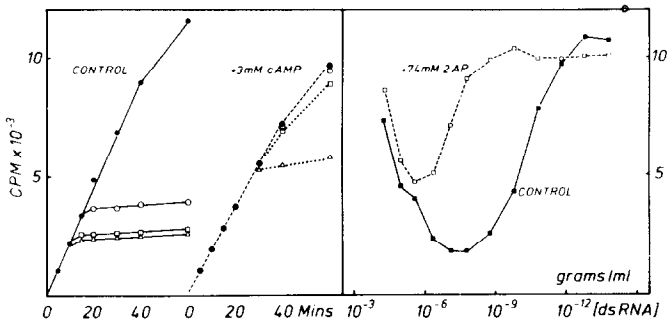

Fig. 1 Susceptibility of the lysate to dsRNA in the presence of (a) 3'5'cAMP and (b) 2-amino purine.

a) Incorporation of [^{14}C] leucine into protein was monitored when lysate was incubated with the following amounts of dsRNA :- no dsRNA (●——●), 200 pg/ml (o——o), 2 ng/ml (□——□), and 20 ng/ml (△——△). Solid lines represent incubations with no further additions; broken lines, incubations made 3 mM in 3'5'cAMP. Note - coincident points are shown singly.
b) Lysate was incubated for 45 min with the indicated amounts of dsRNA before sampling. The solid line represents incubations with no further additions, the broken line, incubations containing 7.4 mM 2-amino purine. With no added dsRNA, counts incorporated into protein were: 10,600 (control) and 9,800 with added 2AP.

Several explanations could be advanced to account for the very high levels of 3'5'cAMP required: 1)it could be a simple ionic effect, 2) the 3'5'cAMP might be broken down rapidly in the lysate, or 3) the 3'5'cAMP might be an analogue of (or be contaminated by) some substance active at much lower concentrations. Accordingly, other compounds were tested and the following were found to be active in the range 0.5 - 10 mM ; caffeine, adenine, theophylline, purine and 2-amino purine. The following had no effect at similar concentrations ; NH_4Cl, KCl, 2'AMP, 3'AMP, 5'AMP, 2'3'cAMP, 3'5'cGMP, 3'5'cIMP, 3'5'cUMP, 3'5'cXMP, 3'5'ADP, FAD, NAD, NADP, adenosine, cytosine, thiamine, theobromine, Coenzyme A and S-adenosyl methionine.

The effective purines do not seem to act by preventing the breakdown of 3'5'cAMP since there is no potentiation between the two : the effects are no more than additive. Most active of the purine derivatives was 2-amino purine making the lysate about

1000 times less sensitive to dsRNA (Fig 1b). Further study showed that 2-amino purine was also capable of delaying the onset of the inhibitions caused by GSSG or lack of hemin and, in all three cases, was capable of reactivating an inhibited lysate (Fig 2). Note that there are differences between the kinetics of reactivation according to which agent originally caused the inhibition.

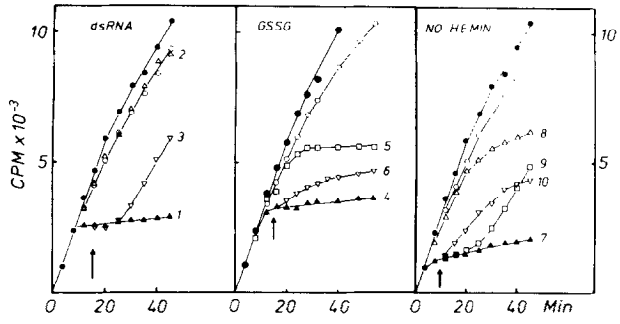

Fig. 2 Effect of 2-amino purine on the inhibitions caused by (a) dsRNA, (b) GSSG, and (c) lack of hemin.

The incorporation of [^{14}C] leucine into protein was followed in incubations of the lysate containing 20 μM hemin (unless otherwise stated) and with the following additions where indicated: 2AP (7.4 mM), dsRNA (1 ng/ml), GSSG (0.75 mM). In each series there is a control incubation (●——●) and a further control with 2AP added (o—o). Other incubations were as follows - left panel: (1) with dsRNA, (2) with dsRNA and 2AP, (3) with dsRNA from zero time, 2AP added after 15 min; centre panel: (4) with GSSG, (5) with GSSG and 2AP, (6) with GSSG from zero time, 2AP added after 15 min; right panel: (7) no hemin, (8) no hemin, with 2AP, (9) hemin added after 10 min, (10) no hemin, 2AP added after 10 min.

There is evidence that when the lysate is incubated without hemin, a translational repressor accumulates which inhibits initiation by preventing the association of met-tRNA$_f$ with the native 40S ribosomal subunits (5). Lysates inhibited by dsRNA also lack the 40S/met-tRNA$_f$ complex (4) and Fig. 3 shows an experiment which suggests that dsRNA also acts by causing the accumulation of such a translational repressor.

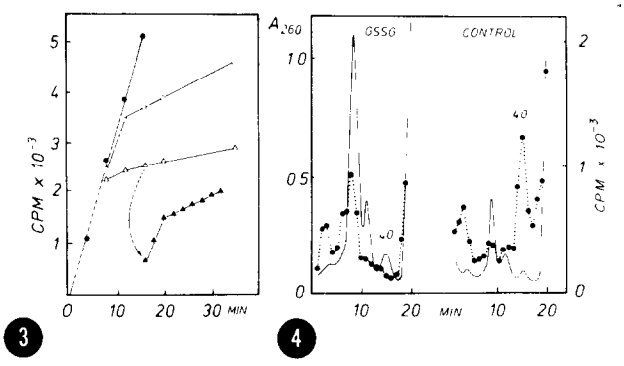

Fig. 3 Evidence for the accumulation of a repressor after incubation with dsRNA.

The incorporation of [^{14}C] leucine into protein was monitored when the lysate was incubated with no dsRNA (●——●), with 250 pg/ml dsRNA (o——o) or with 1 ng/ml dsRNA (△——△). After 16 min, a portion of the latter incubation was mixed with three volumes of fresh incubation mixture containing no dsRNA and the incubation was then continued (▲——▲).

Fig. 4 The effect of inhibition of initiation by GSSG on the 40S/met-tRNA$_f$ complex.

Two incubations of 100 μl were made at 30° each containing 25 μCi/ml of [^{35}S] methionine (16 Ci/mMole), one incubation (a) containing 1.5 mM GSSG. After 16 min, incubations were stopped by tenfold dilution with ice-cold gradient buffer for analysis on sucrose density gradients (3 hours at 40,000 rpm in a Beckman SW41 rotor) as previously described (13). Polysomes are pelleted by this procedure and the continuous absorbance trace at 260 nm (———) shows only the single ribosomes and subunits. Counts in CTAB precipitable material (i.e. RNA) were determined in each fraction of the gradients as previously described (13)(o----o). Note, more GSSG has to be added when labelled methionine is used since the label contains mercapto--ethanol.

A lysate was incubated with 1 ng/ml dsRNA until initiation had become inhibited at which time it was diluted by mixture with three volumes of lysate lacking dsRNA. Although the concentration of dsRNA is now only 0.25 ng/ml, the burst of uninhibited protein synthesis is less than in the first incubation and is far less than in a control containing 0.25 ng/ml dsRNA from the start of incubation. Either the dsRNA

has been converted into a more inhibitory form during the first incubation or its presence in the first incubation has led to the accumulation of an inhibitor of protein synthesis. We have tended to favour the latter explanation since we have been unable to detect any change in the nature or amount of dsRNA during such incubations. Further, we find that the amount of this 'potentiated inhibitory activity' is not a function of the amount of dsRNA in the first incubation, the effect being lost if the dilution is as much as ten-fold, no matter how much dsRNA was present in the original incubation.

The relation of the GSSG inhibition to the other two is not entirely clear. We find that the inhibition is accompanied by loss of the 40S/met-$tRNA_f$ complex in a manner indistinguishable from that produced by dsRNA or lack of hemin (Fig 4). In some experiments we have found evidence for the presence of a translational repressor after incubations with GSSG in the same way as after incubations with dsRNA but results have been variable. The problem has been that lysates differ surprisingly widely in their capacity to reduce the added GSSG to the non-inhibitory GSH making the selection of GSSG concentration and lysate critical to the result.

DISCUSSION

There appear to be extensive similarities between the three kinds of inhibition we have studied and we take this as an indication of a possible common mechanism. In the case of the inhibition caused by lack of hemin, it is now recognised that the failure of initiation results from the accumulation of a translational repressor (5,14). This is termed the 'reversible repressor' since the inhibition is reversed by the addition of hemin. After prolonged incubation, an 'irreversible repressor' forms (15), seemingly from the 'reversible repressor' (14). Like the 'reversible repressor', this inhibits initiation with concomitant loss of the 40S/ /met-$tRNA_f$ complex (S.Legon, unpublished data). As yet,

there is no clear indication of how these repressors form, nor of precisely how they inhibit initiation.

We would like to suggest that dsRNA acts by causing the 'reversible repressor' to form. The dsRNA might act by stabilising the repressor in its inhibitory form, or it might act more directly either by promoting the formation or by inhibiting the inactivation of the repressor. Although the binding of the dsRNA to an initiation factor might be a part of the repressor forming process, it is important to stress that the data shown in Fig.3 are inconsistent with proposals that inhibition is merely a consequence of the sequestration of an initiation factor which falsely recognises and binds to the dsRNA (10).

The inhibition produced by GSSG may be explained in a similar fashion. However, GSSG clearly has some irreversible effects since the inhibition it produces cannot be reversed by the delayed addition of dithiothreitol or glucose + NADP (2), both of which can convert the added GSSG to the non-inhibitory GSH. On the other hand, the inhibition is readily reversed by the addition of ribosome-free cytoplasm (2) and so cannot simply be due to the 'irreversible repressor' which can accum-ulate in the absence of hemin (15).

We can see no reason why, at concentrations far in excess of likely cellular levels, 3'5'cAMP and the various purine derivatives should be capable of preventing all three inhibitions. The high concentrations required would seem to rule out any direct involvement of these compounds in the normal regulatory process but one might speculate that they are acting as analogues of some small molecule which is directly involved. At present however, the only inference that may reasonably be drawn from the effects described is that a common mechanism is involved when the initiation of protein synthesis is inhibited by either dsRNA or GSSG or by lack of hemin.

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