REGULATION OF PROTEIN SYNTHESIS*

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INTRODUCTION

In prokaryotic cells with short lived messenger RNAs, gene expression is probably controlled mainly at the level of transcription. In eukaryotic cells on the other hand, messenger RNAs have in general a much longer life span, and gene expression may be controlled not only at the transcriptional, but also at the translational level. The fact that there is translational control at the stage of polypeptide chain initiation may account for the greater number and the greater structural complexity of the eukaryotic initiation factors as compared with their prokaryotic counterparts. The increased complexity might be connected with a need for greater versatility and a higher degree of sophistication of the eukaryotic initiation machinery.

REGULATION OF PROTEIN SYNTHESIS BY HEMIN

In reticulocytes, globin synthesis is controlled by the level of heme, the prosthetic group of hemoglobin. It has been known for several years that protein synthesis in reticulocyte lysates is but briefly maintained in the absence of added hemin.[1] Gross and Rabinovitz[2] showed that hemin prevents the formation of an inhibitor of chain initiation from a proinhibitor of similar molecular weight. As seen in Figure 1, the synthesis of protein in a lysate of rabbit reticulocytes comes to a standstill in about 10 min if hemin is not added to the incubation mixture. Addition of the inhibitor in the presence of hemin has the same effect as omission of hemin (Figure 1).

The proinhibitor is present in the postribosomal supernatant of reticulocyte lysates. It can be converted to inhibitor by incubation for several hours at 34°C or by incubation with *N*-ethylmaleimide (NEM) for a few minutes at 30°C.[2] The molecular weight of both proinhibitor and inhibitor was found to be about 300,000 by Sephadex® G-200 filtration.[2]

TRANSLATIONAL INHIBITOR OF RETICULOCYTE LYSATES

Recently, it has been shown[3-6] that the inhibitor is a cyclic AMP-independent protein kinase that catalyzes the phosphorylation of the small (38,000 daltons) subunitof the initiation factor eIF-2. This factor forms a ternary complex with the initiator methionyl transfer RNA (Met-tRNA$_i$) and GTP which, on interaction with a 40S ribosome, gives rise to a 40S initiation complex.[7] Phosphorylation of eIF-2 renders the factor inactive in chain initiation. Thus, the translational inhibitor is an eIF-2 kinase.

ACTIVATION OF eIF-2 KINASE

The mechanism of conversion of proinhibitor (inactive eIF-2 kinase) to inhibitor (active eIF-2 kinase) was unknown. We found[8] that the conversion of proinhibitor to

* The symposium talk was entitled "From CO_2 Fixation to Protein Synthesis". It started with early work in the writer's laboratory that was closely related to Harland Wood's pioneering work and ended with more recent work on protein biosynthesis. However, for the published version of the symposium, it appeared preferable to concentrate on the most recent work.

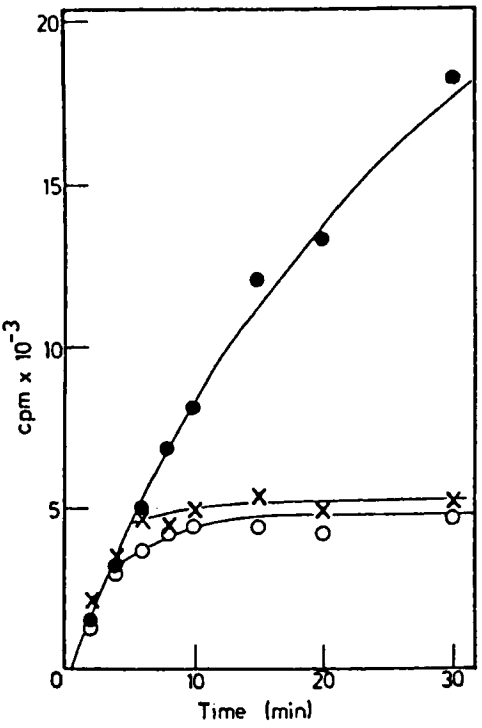

FIGURE 1. Protein synthesis in the reticulocyte lysate in the presence of added hemin (●),in the absence of added hemin (O), or in the presence of both added hemin and translational inhibitor (X). The concentation of hemin was 30 μM and of partially purified inhibitor, 25 μg. Aliquots were removed at the times indicated for assay of [^{14}C]-leucine incorporation into protein. (From Clemens, M. J., Henshaw, E. C., Rahamimoff, H., and London, I. M., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 2946, 1974. With permission.)

inhibitor can be promoted by cyclic AMP (cAMP)-dependent protein kinase or its catalytic subunit. This observation is consistent with the view that as in the case of phosphorylase kinase,[9,10] inactive eIF-2 kinase is activated by phosphorylation catalyzed by cAMP-dependent protein kinase. This is illustrated diagramatically in Figure 2. As indicated in this figure, we found[11] that hemin blocks the activation of cAMP-dependent protein kinase by cAMP and in this way, inhibits the proinhibitor-inhibitor conversion. The experimental evidence reviewed in this paper is consistent with the above model.

As seen in Table 1, protein kinases catalyze the phosphorylation of proteins by ATP (or GTP) and can be classified into two main groups, cAMP-independent and cAMP-dependent.* The action of protein kinases results in the phosphorylation of serine and threonine residues in proteins. eIF-2 Kinase belongs to the same group of cAMP-independent protein kinases as phosphorylase kinase. cAMP-dependent protein kinases have two kinds of subunits, in fact two of each kind, regulatory (R) and catalytic (C).

* Some protein kinases are activated by cGMP besides cAMP. Certain protein kinases appear to be activated by double-stranded RNA.

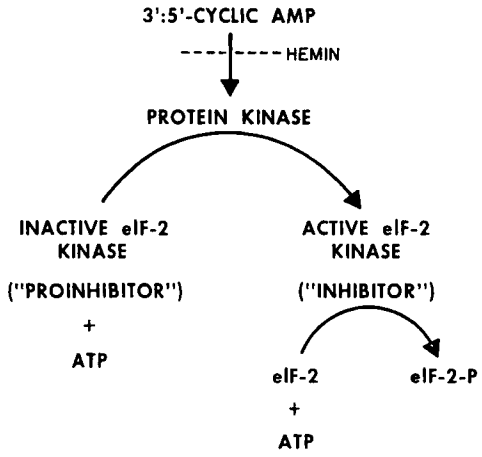

FIGURE 2. Regulation of polypeptide chain initiation by cAMP. (From Datta, A., de Haro, C., Sierra, J. M., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1463, 1977. With permission.)

Note that the activation by cAMP is due to its binding to R with release of the active C. Phosphorylase kinase was known to exist in an inactive, nonphosphorylated form and to be converted to an active form by phosphorylation catalyzed by cAMP-dependent protein kinase.[9,10]

We found[8] that protein synthesis in hemin-containing reticulocyte lysates is inhibited not only by the cAMP-independent eIF-2 kinase (inhibitor), but also by cAMP-dependent protein kinase and, more strongly, by its catalytic subunit. Typical results are shown in Figure 3. Panel A compares the effect of eIF-2 kinase (solid circles) and cAMP-dependent protein kinase (squares) from bovine heart muscle (BHK). Panel B compares the effect of the absence of hemin (solid circles) with the effect of two different concentrations of BHK catalytic subunit (squares) in the presence of hemin. The effect of kinase and catalytic subunit as a function of their concentration is shown on Figure 3C and 3D. Both are strong inhibitors, but the catalytic subunit is much more so. Thus, 50% inhibition is obtained with about 1 μg of BHK, whereas the same inhibition requires only 0.15 μg of catalytic subunit.

The above results raised the question whether eIF-2 can be phosphorylated by both cAMP-independent eIF-2 kinase and cAMP-dependent protein kinase or whether the effect of the latter enzyme is indirect. To answer this question, we used a ternary complex formation assay. We found that formation of the ternary complex eIF-2·GTP·Met-tRNA$_i$ is inhibited when eIF-2 is briefly preincubated with eIF-2 kinase and ATP. However, this is true only when partially purified, but not highly purified eIF-2 is used. We interpreted this to mean that eIF-2 is not inactivated by simple phosphorylation of its 38,000 daltons subunit, but by interaction of the phosphorylated factor with another protein (factor X) present as a contaminant of eIF-2 preparations. We found later that this interpretation was wrong, but nevertheless we could use the ternary complex assay for our purpose provided we utilized relatively crude preparations of eIF-2. As seen in Figure 4, incubation with eIF-2 kinase and ATP rapidly reduces the capacity of eIF-2 to form a ternary complex (Figure 4A). With short incubation times, the inactivation of eIF-2 is proportional to the eIF-2 kinase concentration (Figure 4b). The inactivation of eIF-2 is clearly a consequence of the phosphorylation of the factor for, as seen in Figure 4A, there is no inhibition if ATP is omitted. As

TABLE 1

Protein Kinases (ATP [GTP] + Protein → ADP [GDP] + Phosphoprotein)

cAMP-Independent	cAMP-Dependent (R_2C_2 + 2 cAMP ⇌ $R_2(cAMP)_2$ + 2 C)
Type A	Phosphate donorP
Phosphate donor: ATP or GTP	Substrates:
Substrates:	basic proteins (protamines, histones, ribosomal proteins), enzymes (glycogen synthetase, hormone-sensitive lipase, phosphorylase kinase, eIF-2 kinase)
Acidic proteins (casein, phosvitin), certain subunits of some initiation factors	
Type B	
Phosphate donorP	
Substrates:	
phosphorylase (phosphorylase kinase), 38K subunit of initiation factor eIF-2 (eIF-2 kinase)	

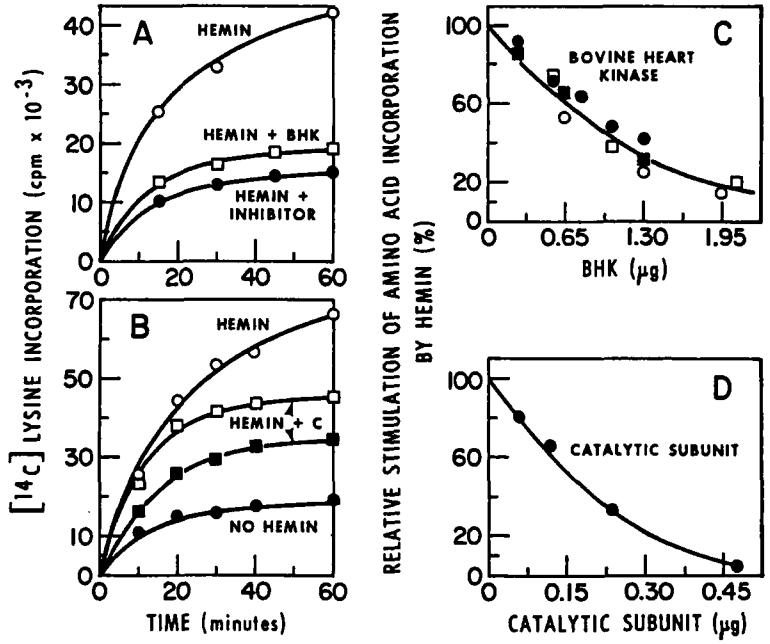

FIGURE 3. Inhibition of protein synthesis in hemin-containing reticulocyte lysates by cAMP-dependent bovine heart protein kinase (BHK) and its catalytic subunit (C). Protein synthesis was assayed through the incorporation of [^{14}C]lysine into acid-insoluble material. The concentration of hemin was 34 μM, that of cAMP, 10 μM. The concentration of BHK in the middle curve of panel A was 13 $\mu g/50\ \mu l$ and that of partially purified inhibitor (active eIF-2 kinase), 10 $\mu g/50\ \mu l$. The concentration of C in the two middle curves of panel B was 0.15 (□) and 0.3 (■) $\mu g/50\ \mu l$. (From Datta, A., de Haro, C., Sierra, J. M., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1463, 1977. With permission.)

further shown in Figure 4A, cAMP-dependent protein kinase, whether from bovine heart or rabbit reticulocytes or the catalytic subunit, are inactive in this assay. It is clear from these results that the inhibition of translation in reticulocyte lysates by cAMP-dependent protein kinase is indirect.

The most likely explanation for the inhibition of translation in lysates by cAMP-dependent protein kinase is that it acts as a kinase to catalyze the conversion of proinhibitor to inhibitor by transfer of phosphate from ATP. This would be in strict anology

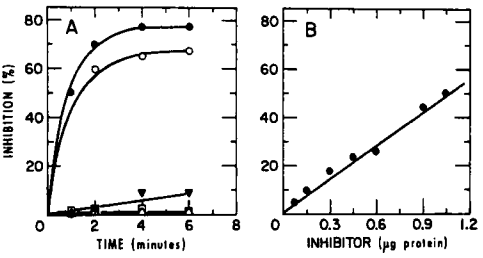

FIGURE 4. Assay of inhibitor (active eIF-2 kinase) through its effect on ternary complex formation. (A) Kinetics of inhibition. Partially purified eIF-2 was incubated for various times at 30° with the indicated supplements before GTP and [^{35}S]Met-tRNA, were added. The samples were then incubated for a further 5 min and assayed for ternary complex formation by the Millipore® filtration procedure. (●), eIF-2 kinase, 1.5 μg of protein, ATP, 0.4 m*M*; (○), eIF-2 kinase, 0.75 μg of protein, ATP, 0.4 m*M*; (▼), BHK, 0.35 μg, cAMP, 5 μ*M*, ATP, 0.4 m*M*; (□), eIF-2 kinase, 1.5 μg of protein (no ATP); (Δ), either BHK catalytic subunit, 0.3 μg, ATP, 0.4 m*M*, or cAMP-dependent rabbit reticulocyte protein kinase (partially purified), 2 μg of protein, cAMP, 5 m*M*, ATP, 0.4 m*M*; (B) Inhibition of ternary complex formation as a function of the concentration of eIF-2 kinase. eIF-2 was incubated with ATP, 0.4 m*M*, and without or with the indicated amounts of eIF-2 kinase (inhibitor) for 1 min at 25° before assay. (From Datta, A., de Haro, C., Sierra, J. M., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1463, 1977. With permission.)

to the activation of phosphorylase kinase by cAMP-dependent protein kinase.[9,10] To test this hypothesis, crude proinhibitor (inactive eIF-2 kinase) prepared from fresh lysate by chromatography on CM-Sephadex® C-50 according to Gross and Rabinovitz[2] was incubated without or with catalytic subunit and ATP, and the reaction mixtures were assayed for inhibitor (active eIF-2 kinase) formation with the ternary complex assay. As seen in Figure 5, there was significant inhibition when the proinhibitor was incubated with both catalytic subunit and ATP, but not with either alone, thus providing convincing evidence for the involvement of phosphorylation in this reaction. Since NEM produces quantitative conversion of proinhibitor to inhibitor, the data obtained with 0.45 μg of proinhibitor (Figure 5) show that the enzymatic conversion was quantitative.

MODE OF ACTION OF HEMIN

Hemin has been reported to inhibit the activity of cAMP-dependent protein kinase of rabbit reticulocytes.[12] We confirmed this observation, but found that hemin had no effect when catalytic subunit rather than the whole protein kinase was used.[11] These results, together with our finding that eIF-2 kinase is activated by phosphorylation catalyzed by cAMP-dependent protein kinase, suggested that hemin prevents this activation by blocking the dissociation of cAMP-dependent protein kinase by cAMP as shown below.

$$C_2 R_2 + 2\,cAMP \xrightarrow[\text{hemin}]{} R_2\,cAMP_2 + 2C$$

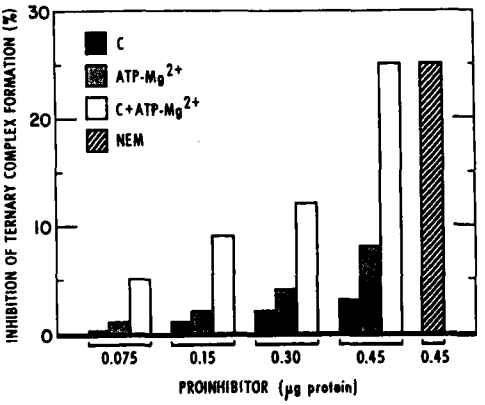

FIGURE 5. Activation of eIF-2 kinase by BHK catalytic subunit: requirement for catalytic subunit and ATP. The experiment was carried out in two stages, the first to form active eIF-2 kinase by phosphorylation of the inactive enzyme, the second to assay for inhibition of ternary complex formation. Samples contained crude inactive eIF-2 kinase by phosphorylation of the inactive enzyme, the second to assay for inhibition of ternary complex formation. Samples contained crude inactive eIF-2 kinase (proinhibitor) freshly prepared from the postribosomal supernatant of reticulocyte lysate by chromatography on CM-Sephadex C-50® in the amounts indicated. Three samples, all containing inactive eIF-2 kinase, were prepared containing: (1) catalytic subunit (C), 3.6 μg; (2) Mg^{2+}, 2 m*M*, ATP, 0.4 m*M*. (3) catalytic subunit, 3.6 μg, Mg^{2+}, 2 m*M*, ATP, 0.4 m*M*. After incubation for 8 min at 30°, the samples were chilled and each was separately chromatographed on a small CM-Sephadex® column to remove the catalytic subunit. This also removes more than 99% of the ATP. In the second stage, the formation of active eIF-2 kinase was assayed through inhibition of ternary complex formation. In the last bar, the total eIF-2 kinase content of the preparation was assayed with NEM which causes the quantitative conversion of inactive to active eIF-2 kinase (2). Comparison of the last two bars shows that the enzymatic conversion was complete. (From Datta, A., de Haro, C., Sierra, J. M., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 1463, 1977. With permission.)

This hypothesis could be demonstrated directly using crude proinhibitor prepared by CM-Sephadex® chromatography of fresh postribosomal supernatant from reticulocyte lysate.[11] This preparation, as assayed with histone as substrate, contained a very active protein kinase highly dependent on cAMP. In the experiments of Figure 6, activation of eIF-2 kinase was assayed as before through inhibition of ternary complex formation. As seen in Figure 6A, eIF-2 kinase activation required besides ATP, either cAMP (Bar 2) or catalytic subunit (Bar 4). Moreover, the conversion promoted by cAMP (through dissociation of endogenous protein kinase) was completely abolished by 45 μ*M* hemin (Bar 3), but that elicited by the catalytic subunit was not significantly affected by the porphyrin (Bar 5). Figure 6B shows the effect of the hemin concentra-

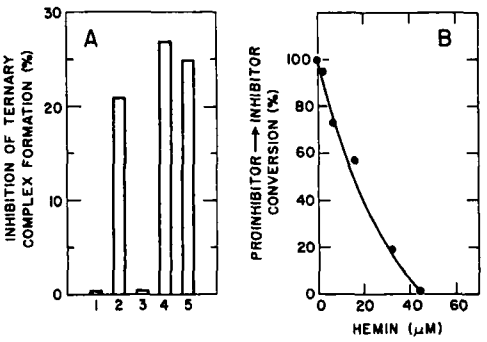

FIGURE 6. (A) Effect of hemin on the activation of eIF-2 kinase. Activation was promoted either by endogenous cAMP-dependent protein kinase upon the addition of cAMP or by BHK catalytic subunit and was measured by the ternary complex formation assay. All samples contained inactive eIF-2 kinase (0.5 μg of protein) and ATP (0.67 m*M*). (1) No further additions; (2) cAMP (15 μ*M*); (3) cAMP (15 μ*M*) and hemin (44.6 μ*M*); (4) catalytic subunit (0.04 μg); (5) catalytic subunit (0.04 μg) and hemin (44.6 μ*M*). (B) Inhibition of the activation of eIF-2 kinase as a function of the hemin concentration. Conditions of (A) with inactive eIF-2 kinase with 0.2 μg of protein, 0.67 m*M* ATP, 15 μ*M* cAMP, and the indicated concentrations of hemin. The inhibition of ternary complex formation in the absence of hemin was taken as 100% activation of eIF-2 kinase (proinhibitor ⟶ inhibitor conversion). (From Datta, A., de Haro, C., Sierra, J. M., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3326, 1977. With permission.)

tion on the activation of eIF-2 kinase. Under the conditions of the experiment, the conversion was 100% blocked by 45 μ*M* hemin. The 50% value was about 15 μ*M*. Whereas relatively high concentrations (15 μ*M*) of the cyclic nucleotide were used in the experiments of Figure 6, it should be emphasized that cAMP promotes the activation of eIF-2 kinase, i.e., the proinhibitor-inhibitor conversion, at physiological concentrations. We found that the concentration of cAMP in rabbit reticulocyte lysates is about 0.1 μ*M*. Under the conditions of the protein synthesis assay,[8] the concentration of cAMP is around 0.05 μ*M*. Figure 7 shows that in the presence of a phosphodiesterase inhibitor, 0.05 μ*M* cAMP caused 65% conversion of proinhibitor to inhibitor. A conversion of 50% was produced by about 0.02 μ*M* cAMP. Hemin prevents the dissociation of the protein kinase holoenzyme by cAMP by blocking the binding of the cyclic nucleotide to the regulatory subunit.[11] As shown in Figure 8, the binding of [^{3}H]cAMP to bovine heart protein kinase, bovine heart kinase regulatory subunit, and cAMP-dependent protein kinase from rabbit reticulocytes was severely curtailed by hemin.

The above experiments suggested that hemin binds to the regulatory subunit of cAMP-dependent protein kinase and blocks the binding of cAMP. This was shown by studying the binding of [^{3}H]hemin to bovine heart kinase and its regulatory subunit.[13] Binding of labeled hemin and labeled cAMP was assayed by the procedure of Baxter and Tomkins[14] for measuring the binding of steroids to specific receptors. As in the case of steroids, activated charcoal adsorbs free, but not protein-bound hemin or

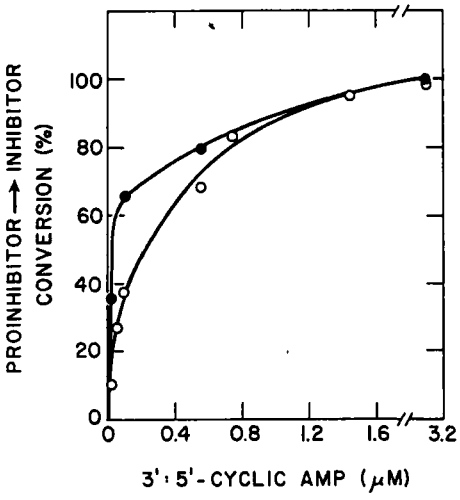

FIGURE 7. Enzymatic activation of eIF-2 kinase as a function of the concentration of cAMP. The conditions were those of Figure 6B without hemin, with 0.24 μg of inactive eIF-2 kinase protein, and various concentrations of cAMP, either without (○) or with (●) the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (41.7 μ*M*). The highest inhibition of ternary complex formation was taken as 100% activation of eIF-2 kinase; it was reached at about 3 μ*M* cAMP, both in the absence and presence of phosphodiesterase inhibitor. (From Datta, A., de Haro, C., Sierra, J. M., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3326, 1977. With permission.)

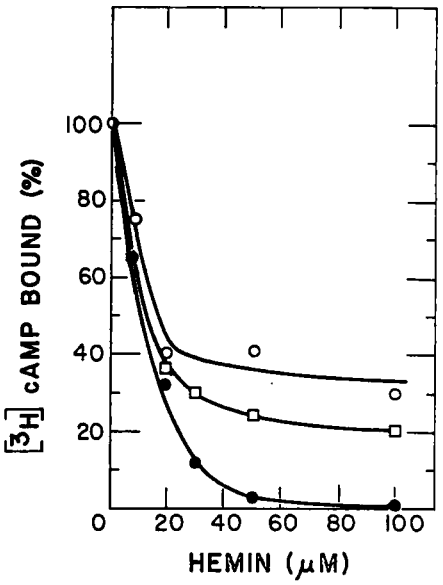

FIGURE 8. Blocking by hemin of cAMP binding to the regulatory subunit of cAMP-dependent protein kinase. The binding of [³H]cAMP to cAMP-dependent protein kinase or its regulatory subunit is given as a function of the hemin concentration. (○), Homogeneous bovine heart protein kinase, 1.0 μg; (□), bovine heart protein kinase regulatory subunit, 0.4 μg; (●), cAMP-dependent rabbit reticulocyte protein kinase (partially purified), 4.8 μg. (From Datta, A., de Haro, C., Sierra, J. M., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 3326, 1977. With permission.)

cAMP. Thus, when a solution of radioactive hemin or cAMP is shaken with charcoal and then centrifuged, only a small fraction of the radioactivity remains in the supernatant. However, if the radioactive compound is first incubated with cAMP-dependent protein kinase or its regulatory subunit, considerably more radioactivity is recovered in the supernatant. As shown by Baxter and Tomkins for steroids,[14] if the binding of the radioactive ligand is specific, it is completely eliminated by the presence of a large excess of nonradioactive ligand; nonspecific binding is not affected. Thus, incubation of two parallel samples, one of which contains the radioactive ligand and the other the same amount of radioactive plus a large excess of nonradioactive ligand, permits the determination of specific binding. We found[13] that there is some nonspecific binding of labeled hemin to various proteins, including partially purified commercial BHK, but there is only specific binding of labeled hemin as well as labeled cAMP to homogenous BHK. In Figure 9A, the specific hemin binding (upper curve) is plotted against the free hemin concentration. At saturation, approximately 2 mol of hemin are bound specifically per mole of BHK (R_2C_2) or 1 mol per R (regulatory) subunit. The middle curve shows the nonspecific binding of the commercial BHK preparaion, i.e., the binding of [³H]hemin in the presence of an excess of nonlabeled hemin. The lower curve gives the [³H]hemin background, i.e., the amount of hemin not adsorbed to charcoal in the absence of protein. The inset in the same figure shows that other proteins bind hemin nonspecifically as shown by the failure of excess nonradioactive hemin to block

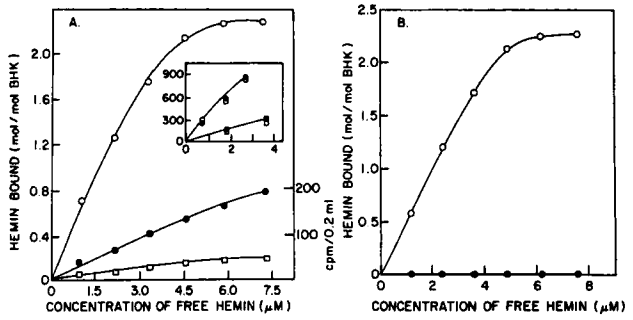

FIGURE 9. Binding of hemin to BHK. Specific and nonspecific binding of [^{3}H]hemin was measured as outlined in the text. (A), main figure. Partially purified BHK (corresponding to 16 μg of homogeneous BHK in 0.2 mℓ reaction mixture). The specific binding of hemin (in mol/mol BHK) is plotted against the concentration of free [^{3}H]hemin (O). Nonspecific hemin binding (●) and the background in the absence of protein (□) are given in cpm/sample. (A), inset. Binding of [^{3}H]hemin to bovine serum albumin (circles) or *Escherichia coli* protein (squares) in the absence (solid symbols) and presence (open symbols) of an excess of nonlabeled hemin plotted (in cpm/sample) against the concentration of free hemin. (B) Binding of [^{3}H]hemin to homogeneous BHK (12 μg/0.2 mℓ) in the absence (O) and presence (●) of an excess of nonlabeled hemin. (From Datta, A., de Haro, C., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1148, 1978. With permission.)

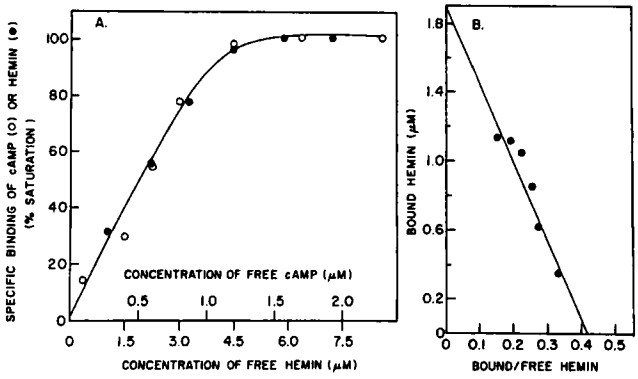

FIGURE 10. (A), Specific binding of ^{3}[H]cAMP (O) or hemin (●) to BHK as a function of their concentration (conditions of Figure 9). (B), Scatchard plot of hemin-binding. (From Datta, A., de Haro, C., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1148, 1978. With Permission.)

[^{3}H]hemin binding. Nonspecific binding is rather high with bovine serum albumin. The nonspecific hemin binding by the partially purified kinase is due to proteins other than BHK for, as seen in Figure 9B, homogeneous BHK shows only specific hemin binding. Figure 10A compares the specific binding of [^{3}H]cAMP and [^{3}H]hemin to BHK as a function of their concentration. The points for both ligands fall on the same curve, the affinity of hemin for BHK being about one third as high as that of cAMP. The dissociation constant for the reaction hemin + BHK ⇌ hemin − BHK complex, calculated from a Scatchard plot of the data of Figure 10A (Figure 10B) was 0.8×10^{-6}

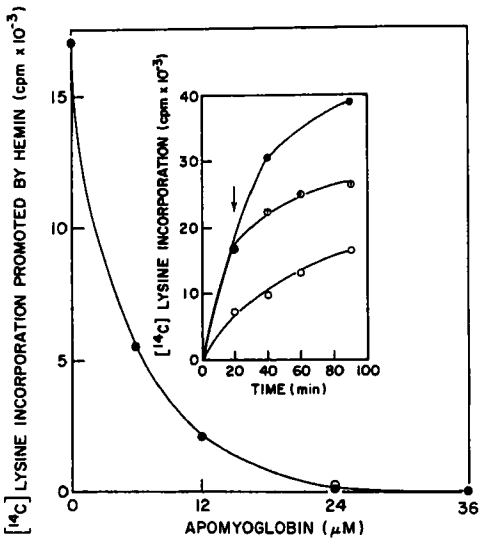

FIGURE 11. Reversal by globin of the effect of hemin on protein synthesis in reticulocyte lysates. Samples were incubated for 60 min at 34°, (1) in the absence of hemin and (2) in the presence of 27 μM hemin with various concentrations of apomyoglobin. The difference in incorporation of [^{14}C]lysine into protein between hemin-containing and non-hemin-containing samples is plotted against the apomyoglobin concentration (●). In one experiment (○), the samples were preincubated for 15 min at 0°C prior to adding 10 μg of apomyoglobin (final concentration, 24 μM to one of the hemin-containing samples. The inset gives the incorporation of [^{14}C]lysine into protein as a function of time for one incubation without added hemin (○), one with 27 μM hemin (●), and one with 2 μM hemin to which 10 μg of apomoglobin (final concentration, 24 μM) was added, as shown by arrow, 20 min after the start of incubation (⊙). (From Datta, A., de Haro, C., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 1148, 1978. With permission.)

M. In other experiments not documented here, we showed that hemin binds to the regulatory but not to the catalytic subunit of cAMP-dependent protein kinase. Moreover, whereas an excess of nonlabeled hemin can displace both labeled hemin and cyclic AMP bound to protein kinase, an excess of nonlabeled cAMP can displace bound labeled cAMP, but not labeled hemin.[13] This may be interpreted to mean that cAMP and hemin bind to different sites of the regulatory subunit. We had suggested[11] that if hemin binds to the regulatory subunit, it must bind at a site other than the cAMP binding site for hemin, unlike cAMP, does not activate protein kinase. These results are consistent with the view that hemin prevents cAMP binding to the catalytic subunit by eliciting a change of conformation of the molecule whereby the cAMP binding site is allosterically blocked. Whereas cAMP cannot displace protein kinase bound-hemin and therefore cannot reverse the translational effect of hemin, globin can completely reverse this effect.[13] We found (Figure 11) that the level of protein synthesis in the presence of 27 μM hemin is brought back to the nonhemin level by an equimolar concentration of apymyoglobin. A kinetic experiment (Figure 11, inset) in

which the apomyoglobin was added to a hemin-containing sample 20 min after the start of translation showed that the reversal is virtually instantaneous. Globin has a very high affinity for hemin and effectively competes with the regulatory subunit for hemin-binding. Thus, the reticulocyte synthesizes globin as long as hemin is available, and synthesis stops when enough globin has been produced to bind all of the hemin present.

MECHANISM OF TRANSLATIONAL INHIBITION

As already noted, the presence of active eIF-2 kinase and ATP in hemin-containing reticulocyte lysates leads in a few minutes to a cessation of protein synthesis. Since under such conditions the small subunit of the initiation factor eIF-2 is phosphorylated, it would appear that phosphorylated eIF-2 fails to function in initiation. However, treatment of purified eIF-2 with eIF-2 kinase and ATP does not seem to interfere with its ability to form ternary or 40S initiation complexes.[3,8,15] On the other hand, formation of the complex is impaired, as already mentioned, when partially purified eIF-2 is used.[8,15] This suggested that inhibition of translation involves another factor(s) present in lysates and in partially purified eIF-2 preparations. Using inhibition of ternary complex formation as the assay, we separated eIF-2 from another factor, present together with eIF-2 in ribosomal salt washes. This factor is required for inhibition when eIF-2 is incubated with eIF-2 kinase and ATP.[16] To our surprise, the new factor proved to be a potent stimulator of the activity of eIF-2.[16] It stimulates both ternary and 40S complex formation. We refer to this factor as ESP for eIF-2-stimulating protein.[16,17] Although ESP enhances the ability of unphosphorylated eIF-2 to form ternary or 40S initiation complexes, it has no effect on phosphorylated eIF-2. At the low concentrations present in reticulocyte lysates, eIF-2 is virtually inactive without ESP. Thus, the hemin-controlled translational inhibitor, i.e., eIF-2 kinase, acts by abolishing the stimulating effect of ESP.[16,17] As seen in Figure 12A, ESP has no effect in the absence of eIF-2, but markedly stimulates ternary complex formation by eIF-2. In some cases, at the lower concentrations of eIF-2, ESP increased ternary complex formation nearly 40-fold (Table 2). Figure 12B shows that incubation with eIF-2 kinase and ATP is without effect on ternary complex formation with eIF-2 alone lower curve of Figure 12B), but markedly inhibits the stimulation normally elicited by ESP (Figure 12B, compare upper and middle curves). The time course of ternary complex formation in the absence or presence of ESP and/or, eIF-2 kinase and ATP is shown in Figure 13. ESP increases both the extent and the rate of ternary complex formation. Again, incubation with eIF-2 kinase and ATP has no effect on complex formation by eIF-2 alone, but severely depresses the ability of ESP to enhance this reaction. Figure 14 (A and B) shows that what is true of ternary complex is also true of 40S initiation complex formation. Thus, the small amount of complex formed in the absence of ESP is not affected by preincubation with eIF-2 kinase and ATP (Bars and Panels 1 and 2), whereas such preincubation virtually abolishes the considerable stimulation produced by ESP (Bars and Panels 3 and 4). It is of interest to note that like eIF-2, ESP is sensitive to SH-binding reagents, such as *N*-ethylmaleimide and *p*-chloromercuribenzene sulfonate (data not shown).

The mechanism of inhibition of protein synthesis by the hemin-controlled translational inhibitor (eIF-2 kinase) is now clear. Phosphorylation of the 38,000 daltons subunit of eIF-2, catalyzed by eIF-2 kinase in the presence of ATP, abolishes the action of ESP, a protein essential for eIF-2 activity at physiological concentrations of the factor. ESP may act by complexing with intact, but not with phosphorylated eIF-2, thus displacing the equilibrium of ternary complex formation in favor of this reaction.

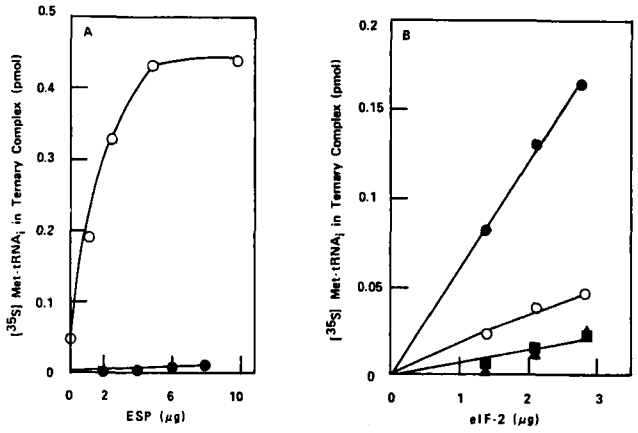

FIGURE 12. (A), Ternary complex formation as a function of the concentration of ESP. The assay was conducted with 2.5 pmol of [^{35}S]Met-tRNA$_i$ (20.075 cpm/pmol), 0.14 m*M* GTP, and increasing amounts of ESP, without (●) or with (○) 1.9 μg of eIF-2. (B), Effect of ESP and eIF-2 kinase on ternary complex formation as a function of the concentration of eIF-2. Assay with [^{35}S]Met-tRNA$_i$, GTP, ATP (0.4 m*M*), increasing amounts of eIF-2, and further additions as follows: (■), none, (▲), eIF-2 kinase (0.7 μg), (●) ESP (3.6 μg), (○) eIF-2 kinase and ESP. (From de Haro, C. and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 2713, 1978. With permission.)

TABLE 2

Effect of ESP on Ternary Complex Formation at Different eIF-2 Concentrations

ESP (μg/50 μl)	eIF-2 (μg/50 μl)	[^{35}S]Met-tRNA$_i$ in ternary complex (pmol)		Stimulation by ESP fold
		Without ESP	With ESP	
3.6	1.4	0.01	0.36	36
3.6		0.03	0.49	16
3.6	2.8	0.07	0.53	7
10	2.0	0.04	0.72	18
10	7.0	0.30	0.89	3
10	12.0	0.54	0.96	2

From de Haro, C. and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 243, 1978. With permission.

eIF-2 consists of three subunits and has a total molecular weight of about 160,000. The molecular weight of ESP is about 200,000. Thus, a complex of eIF-2 and ESP would have a mass of over 350,000 daltons.

PROINHIBITOR-INHIBITOR PAIR IN OTHER CELLS

A similar proinhibitor-inhibitor system is present in cells other than reticulocytes,[18-20] including cells that are far removed from mammalian cells in the evolutionary scale, e.g., *Artemia salina* and wheat germ.[21] As shown in Figure 15, the BHK catalytic subunit is a potent inhibitor of translation in the *A. salina* cell-free system. In view of our data with the reticulocyte system, these results can only mean

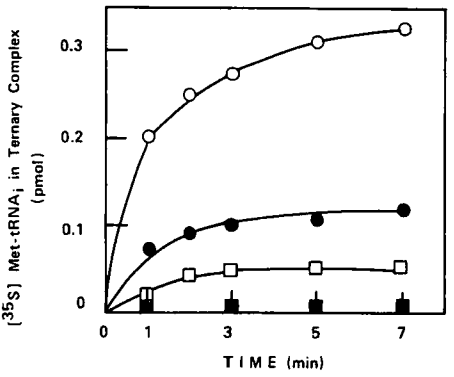

FIGURE 13. Kinetics of ternary complex formation in the absence of presence of ESP and/or eIF-2 kinase. Assays as in Figure 12B with [^{35}S]Met-tRNA, GTP, ATP, and other additions as follows: (■) ESP, (□) eIF-2 either without or with eIF-2 kinase, (●) eIF-2, eIF-2 kinase, and ESP, (○) eIF-2 and ESP. (From de Haro, C. and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 2713, 1978. With permission.)

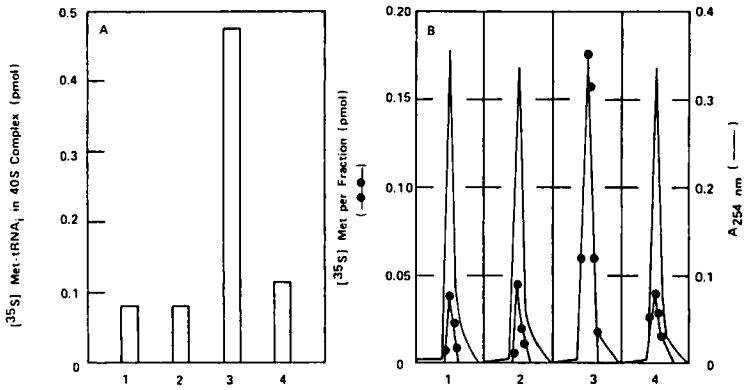

FIGURE 14. Effect of ESP and eIF-2 kinase on 40S complex formation. (A), [^{35}S]Met-tRNA, bound in the 40S region of the sucrose gradient. (B), Sucrose density gradient centrifugation profiles. All samples had ATP and eIF-2. Other additions were as follows: (1) none; (2) eIF-2 kinase; (3) ESP; (4) eIF-2 kinase and ESP. (From de Haro, C. and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 75, 2713, 1978. With permission.)

that in the *A. salina* system, the catalytic subunit also mediates the transfer of phosphate from ATP to a proinhibitor to form an inhibitor of polypeptide chain initiation. This conclusion is strengthened by the fact that, as shown in Figure 15 (right panel), the catalytic subunit does not inhibit chain elongation or poly(U) translation which at relatively high Mg^{2+} concentrations does not involve initiation factors. Inhibition of translation by the catalytic subunit was also observed in the wheat germ systems (Figure 16). However, even though hemin can prevent the proinhibitor-inhibitor conversion generally (e.g., in cell-free preparations from *A. saline*), cell-free extracts other than reticulocyte lysates do not seem to require addition of hemin for maintenance of protein synthesis. Perhaps through development of a protein kinase regulatory subunit

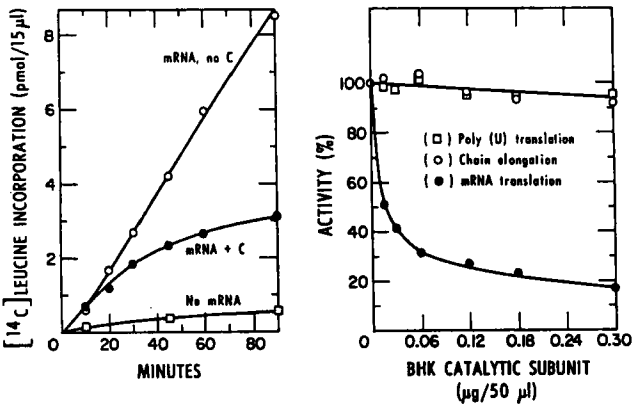

FIGURE 15. Inhibition of translation in the *Artemia salina* system by BHK catalytic subunit (C). The translation system consisted of ribosomes, tRNA, and postribosomal supernatant, with or without *A. salina* mRNA or poly (U), for assaying total translation or polysomes from developed *A. salina* embryos for assaying chain elongation. Incubations at 25°C. The amount of C, when present, in left panel experiments was 0.3 µg/50 µl reaction mixture. The incubation time in the right panel experiments was 45 min (□, ○) or 60 min (●). (From Sierra, J. M., de Haro, C., Datta, A., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4356, 1977. With permission.)

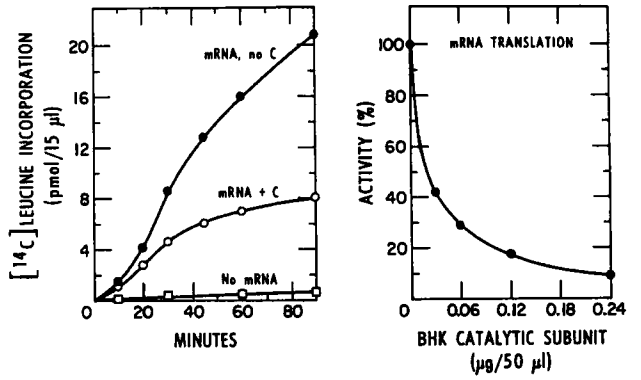

FIGURE 16. Inhibition of translation in wheat germ extracts by BHK catalytic subunit (C). Conditions were the same as in Figure 15 except for the substitution of wheat germ S30 extract for the *Artemia salina* ribosomes, tRNA, and postribosomal supernatant. (From Sierra, J. M., de Haro, C., Datta, A., and Ochoa, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4356, 1977. With permission.)

exquisitely senstive to both cAMP and hemin, the reticulocyte has evolved as a cell adjusted to synthesize globin only as long as hemoglobin can be assembled, i.e., as long as heme is present. The presence of iron is not necessary for the effect first observed with hemin. Protoporphyrin IX and mesoporphyrin IX are as active as hemin in maintaining protein synthesis in reticulocyte lysates.[27]

Protein Synthesis and Cell Growth

An important fact arising from our studies is that protein synthesis can be, and

probably is, regulated by cAMP. It has been observed as far back as 1960 that glucagon and cAMP inhibit protein synthesis in liver slices,[22] but the significance of this finding could not be assessed at that time. In 1972 Bloxham and collaborators[23] demonstrated inhibition by cAMP of protein synthesis in cell-free preparations of rat liver. Their more recent studies[24] strongly support the involvement of cAMP-dependent protein kinase in translational control.

The inverse relationship between cell growth and cAMP levels disclosed by the work of Pastan and others[25] can now be explained in view of the identical relationship between protein synthesis and cAMP levels suggested by the work reported here. Tomkins and collaborators[26] showed that cAMP and prostaglandin E_1 (which raises the intracellular level of cAMP) inhibited the growth of cultured mouse lymphoma S49 cells, but were much less effective on a variant that had low levels of cAMP-dependent protein kinase. Thus, the inhibition by cAMP of both protein synthesis and cell growth is mediated by cAMP-dependent protein kinase. Probably inhibition of cell growth is a consequence of inhibition of protein synthesis.

SUMMARY

A system of translational control in eukaryotes consists of (1) a proinhibitor and (2) an inhibitor of polypeptide chain initiation. The inhibitor (active eIF-2 kinase), a cAMP-independent protein kinase, catalyzes the phosphorylation by ATP of the small subunit of the polypeptide chain initiation factor eIF-2. This blocks the interaction of eIF-2 with eIF-2, stimulating protein (ESP) without which eIF-2 is unable to form an initiation complex, a prerequisite for translation. Our observations are consistent with the view that the proinhibitor (inactive eIF-2 kinase) is converted to the inhibitor by phosphorylation catalyzed by a cAMP-dependent protein kinase. This is analogous to the conversion of inactive phosphorylase kinase to active phosphorylase kinase. As in the case of phosphorylase kinase and phosphorylase, the modification of activity produced by phosphorylation of eIF-2 kinase and eIF-2 itself is probably reversed by dephosphorylation catalyzed by specific protein phosphatases, but no evidence bearing on this aspect of the problem is yet available. Hemin inhibits the cAMP-induced dissociation of the regulatory and catalytic subunits of cAMP-dependent protein kinase by binding to the regulatory subunit of the enzyme and blocking, through an allosteric effect, the binding of cAMP. Thus, hemin prevents the activation of eIF-2 kinase by inhibiting the cAMP-dependent protein kinase.

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