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Translational control by protein kinases in *Artemia salina* and wheat germ

(regulation of protein synthesis/protein phosphorylation/translational inhibitor/Met-tRNA$_i$ binding factor/ternary complex formation)

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ABSTRACT **The catalytic subunit of cyclic 3′:5′-AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) inhibits translation in *Artemia salina* and wheat germ extracts. It acts, as in reticulocyte lysates [Datta, A., de Haro, C., Sierra, J. M. & Ochoa, S. (1977) *Proc. Natl. Acad. Sci. USA* 74, 1463–1467] by catalyzing the conversion of a proinhibitor to an inhibitor of polypeptide chain initiation. Addition of ATP and either cyclic AMP or catalytic subunit promotes the proinhibitor–inhibitor conversion in crude proinhibitor preparations from *A. salina* embryos. The effect of cyclic AMP is due to stimulation of cyclic AMP-dependent protein kinase, present in such preparations, and is inhibited by hemin. In similar preparations from wheat germ, addition of ATP and catalytic subunit promoted proinhibitor–inhibitor conversion, but addition of ATP and cyclic AMP has little or no effect. As assayed with histone as substrate, wheat germ preparations exhibit a protein kinase activity that is not stimulated by the addition of cyclic AMP or cyclic GMP. Our results suggest that a translational control system, similar to that existing in rabbit reticulocytes and other mammalian cells, is present in organisms evolutionarily far removed from mammals.**

In rabbit reticulocyte lysates, initiation of protein synthesis is controlled by phosphorylation of the small (38,000-dalton) subunit of the initiation factor eIF-2, an event that leads to inactivation of the factor and inhibition of translation. The translational inhibitor (active eIF-2 kinase), a cyclic AMP (cAMP) independent protein kinase (1–4), is formed from a proinhibitor (inactive eIF-2 kinase) of similar molecular weight, and this conversion is inhibited by hemin (5). This proinhibitor–inhibitor conversion is promoted (6, 7), in the presence of ATP, by cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) and the available evidence is consistent with the view that eIF-2 kinase, like phosphorylase kinase (ATP:phosphorylase-*b* phosphotransferase, EC 2.7.1.38) (8, 9), is activated by phosphorylation catalyzed by cAMP-dependent protein kinase. A similar translational inhibitor is present in other mammalian cells, e.g., ascites tumor (10), liver (11), and Friend leukemia cells (12). In this paper we report the presence of analogous translational control systems in organisms, such as *Artemia salina* and wheat germ, that are far removed from mammals in the evolutionary scale.

MATERIALS AND METHODS

Preparations. Brine shrimp eggs (Long Life Aquarium Products, Harrison, NJ, or Metaframe Co., San Francisco, CA) and wheat germ (Niblack, Rochester, NY) were purchased locally. The preparation of ribosomes and postribosomal supernatants from undeveloped or developing *A. salina* embryos (13) and of wheat germ S30 extracts (14) has been described. Crude proinhibitor was prepared by the procedure of Gross and Rabinovitz (5). The *A. salina* postribosomal supernatant used for this purpose was prepared as described (13) but omitting the ammonium sulfate precipitation. The wheat germ postribosomal supernatant used for the same purpose was prepared from S30 extract by centrifugation for 90 min at 4° and 230,000 × *g* in the Spinco 75 Ti rotor. The supernatants (2.5 ml) were dialyzed in the cold room against 3.7 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer at pH 7.2, applied to a column (1.5 × 28 cm) of carboxymethyl-Sephadex G-50 (Pharmacia), and eluted with the same buffer. The leading A_{280}-absorbing fractions were pooled and, if not used right away, stored in liquid nitrogen in 0.1-ml aliquots. The proinhibitor content of the fractions was determined by treatment of an aliquot with *N*-ethylmaleimide, which converts proinhibitor to inhibitor (5), and the latter was assayed with the eIF-2 ternary complex formation assay (6, 7). The *A. salina* protein kinase preparation used for [^{3}H]cAMP binding assays was the DEAE-cellulose peak II obtained from postribosomal supernatant of developing embryos by the procedure of Traugh *et al.* (15). Other preparations and materials were as in previous work (6, 7, 13).

Assays. For mRNA translation in the *A. salina* system, the reaction mixtures (50 μl) contained 20 mM Hepes buffer (adjusted to pH 7.6 with KOH), 80 mM KCl, 2 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 0.15 mM spermine, 1 mM ATP, 0.3 mM GTP, 10 mM creatine phosphate, 5 μg (0.7 units) of creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2), 12 μg of *A. salina* tRNA, 0.3 μCi of L-[^{14}C]leucine (specific radioactivity, 320 mCi/mmol), 0.02 mM each of the remaining, unlabeled L amino acids, 1.0 A_{260} unit of 0.5 M KCl-washed ribosomes from undeveloped embryos, S105 supernatant from developing embryos with 0.75 mg of protein, and, when present, 3 μg of *A. salina* mRNA, isolated from polysomes of developing embryos (one *A* unit is the amount of material giving an absorbance of 1 when dissolved in 1 ml and the light path is 1 cm). Incubations were for 60 min at 25° and the radioactivity incorporated into hot trichloroacetic acid-insoluble material was determined as described (13). Chain elongation was similarly assayed except that polysomes (3.7 A_{260} units) from developing embryos were substituted for both KCl-washed ribosomes and mRNA, and S105 supernatant (0.54 mg of protein) from undeveloped embryos was substituted for the same supernatant from developed embryos (13). Incubation

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Abbreviations: cAMP, cyclic AMP; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; BHK, bovine heart protein kinase.

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was for 45 min at 25°. This assay was insensitive to edeine, a specific inhibitor of chain initiation, and sensitive to sparsomycin, an inhibitor of elongation.

Poly(U) translation samples (50 μl) contained 50 mM Hepes buffer at pH 7.6, 120 mM KCl, 7 mM $Mg(OAc)_2$, and 2 mM dithiothreitol. Their composition was otherwise similar to that of mRNA translation samples except for the omission of spermine, amino acids, and tRNA, the substitution of poly(U) (0.8 A_{260} unit) for mRNA, and that of [^{14}C]Phe-tRNA (25 pmol, specific radioactivity, 900 cpm/pmol) for [^{14}C]leucine. Incubation was for 30 min at 25°. The translation assay with wheat germ S30 extract was as previously described (14) with mRNA (3 μg/50 μl) from *A. salina* as above. Incubation was for 60 min at 25°.

Protein kinase activity was assayed by measuring the phosphorylation of histone (Sigma, type IIA) by γ-^{32}P-labeled ATP in the standard assay previously described (6) except that no bovine serum albumin was added. The amounts of bovine heart protein kinase (BHK) catalytic subunit used in the proinhibitor–inhibitor conversion experiments are given throughout as μg of the pure protein. The estimated specific activity of BHK catalytic subunit was 195 pmol of ^{32}P transferred per min/μg of protein (6). Protein was determined by the procedure of Lowry *et al.* (16) with bovine serum albumin as standard.

The conversion of proinhibitor to inhibitor requires, besides ATP, either cAMP or catalytic subunit (cf. ref. 7). The effect of cAMP is due to activation of cAMP-dependent protein kinase present in the proinhibitor preparations. The conversion was analyzed by the ternary complex formation assay. This was conducted in stepwise fashion, as described in a previous paper (7), except that the concentration of Hepes buffer in step *a* was 33.3 mM, that of $Mg(OAc)_2$ was 5 mM, and the samples contained 3.33 mM dithiothreitol. The amounts of proinhibitor, cAMP, and catalytic subunit are given in the figure legends.

RESULTS

Inhibition of translation by protein kinase catalytic subunit

The catalytic subunit of cAMP-dependent protein kinase is a potent inhibitor of translation not only in reticulocyte lysates (6) but also in *A. salina* and wheat germ extracts. Figs. 1*A* and 2*A* show the time course of the reaction in *A. salina* and wheat germ extracts, respectively. Figs. 1*B* and 2*B* illustrate the inhibition by catalytic subunit as a function of its concentration. Comparison with reticulocyte lysates (6) shows similar kinetics and sensitivity. The inhibition of translation occurs at the level of chain initiation because, as seen in Fig. 1*B* for the *A. salina* system, the catalytic subunit does not inhibit chain elongation or poly(U) translation that, at high Mg^{2+} concentration (7 mM), is not initiation factor dependent.

It appears that, as in reticulocyte lysates (6), the inhibition of chain initiation by the catalytic subunit of cAMP-dependent protein kinase could be due to the presence in *A. salina* and wheat germ extracts of a translational proinhibitor (inactive eIF-2 kinase) that is converted to inhibitor (active eIF-2 kinase) by phosphorylation. In order to analyze these systems further, we looked for the presence of protein kinases in proinhibitor-containing preparations from *A. salina* and wheat germ and studied the requirements for conversion of proinhibitor to inhibitor.

Protein kinases

Postribosomal supernatants from *A. salina* embryos, assayed with histone as substrate, have rather high protein kinase activity. Fractions enriched in either cAMP-independent or cAMP-dependent activity have been prepared by DEAE-cellulose and phosphocellulose chromatography (unpublished). Fig. 3*A* shows histone kinase activity in proinhibitor preparations from undeveloped and developing *A. salina* embryos. It may be seen from the specific activities given in the legend that histone phosphorylation was stimulated 3- to 4-fold by cAMP addition. The histone kinase activity of proinhibitor preparations from wheat germ is shown in Fig. 3*B*. The activity was the same in the absence or presence of added cAMP or cGMP. Thus, the protein kinase(s) in wheat germ preparations is cyclic nucleotide-independent. The specific protein kinase activity of the wheat germ preparations (0.1) was the same as that of proinhibitor preparations from developing *A. salina* embryos assayed in the absence of added cAMP.

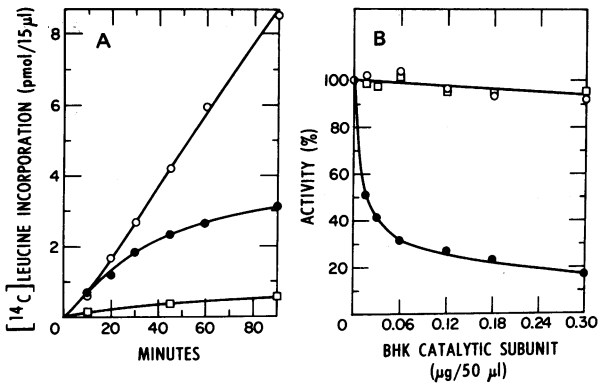

FIG. 1. Inhibition of protein synthesis in *A. salina* extracts by bovine heart protein kinase catalytic subunit. (*A*) Time course: ○, mRNA; ●, mRNA and catalytic subunit (0.3 μg/50 μl); □, no mRNA. (*B*) Translation as a function of the concentration of catalytic subunit: ●, mRNA translation; □, poly(U) translation; ○, chain elongation. Translation in the absence of catalytic subunit was taken as 100%. The actual values (in pmol/50 μl) were as follows. mRNA translation ([^{14}C]leucine incorporation): with added mRNA, 19.9; without mRNA addition, 1.5; poly(U) translation ([^{14}C]phenylalanine incorporation): with poly(U), 21.4; without poly(U), 0.15; chain elongation: with no additions, 19.9; with 0.9 μM edeine, 19.4; with 0.24 mM sparsomycin, 3.5. Net messenger translation values (after subtracting blanks without added mRNA, without added poly(U), or in the presence of sparsomycin) were plotted.

Conversion of proinhibitor to inhibitor

The conversion of proinhibitor to inhibitor in crude preparations from postribosomal supernatants of *A. salina* embryos and

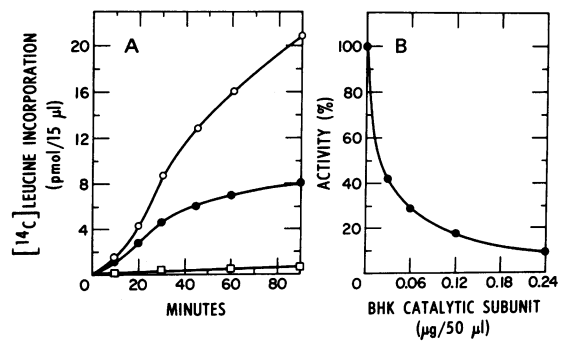

FIG. 2. Inhibition of protein synthesis in wheat germ extracts by bovine heart protein kinase catalytic subunit. (*A*) Time course: ○, mRNA; ●, mRNA and catalytic subunit (0.18 μg/50 μl); □, no mRNA. (*B*) Translation as a function of the concentration of catalytic subunit. The [^{14}C]leucine incorporation values (pmol/50 μl), in the absence of added catalytic subunit were: with mRNA, 65.1; without mRNA, 1.1. The net value due to mRNA, 64 pmol, was taken as 100% translation.

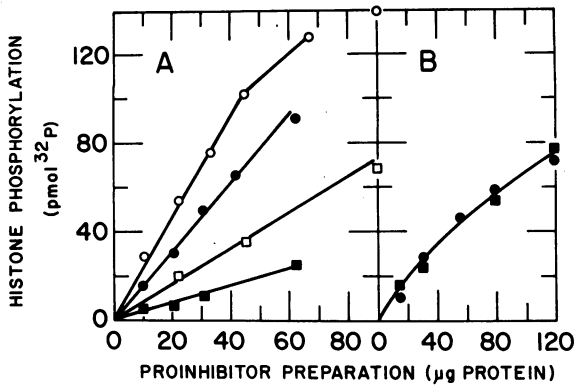

FIG. 3. Protein kinase activity in crude proinhibitor preparations from *A. salina* and wheat germ extracts. Histone phosphorylation was assayed as previously described in the presence of 10 μM cAMP (6). The preparation of proinhibitor-containing fractions is described in *Materials and Methods*. (*A*) *A. salina:* solid symbols, undeveloped embryos, without (■) and with (●) cAMP addition; open symbols, developing embryos, without (□) and with (○) cAMP addition. (*B*) Wheat germ: ■, without cyclic nucleotide addition; ●, with addition of either cAMP or cGMP. Values are expressed per 50-μl reaction mixture. The protein kinase specific activities; (pmol ^{32}P transferred/min per μg of protein) of the various preparations were as follows. Undeveloped *A. salina* embryos: without cAMP, 0.05; with cAMP, 0.2; developing *A. salina* embryos: without cAMP, 0.1; with cAMP, 0.3; wheat germ (with or without cyclic nucleotide addition), 0.1.

wheat germ was assayed as in previous work (6, 7) with use of the ternary complex formation assay.

With *A. salina* preparations the proinhibitor–inhibitor conversion occurs upon addition of ATP and either BHK catalytic subunit or cAMP. The effect of cAMP is due to activation of cAMP-dependent protein kinase that, as seen in the preceding section, is present in the proinhibitor-containing preparations. Fig. 4*A* gives the results of a typical experiment with proinhibitor from developing embryos; similar results are obtained with undeveloped embryos. Addition of ATP alone has little or no effect but ATP + catalytic subunit or ATP + cAMP promote the conversion. As with reticulocyte lysates (7), the conversion caused by addition of cAMP is inhibited by hemin. Fig. 4*B* gives the proinhibitor–inhibitor conversion and the binding of [^{3}H]cAMP by an *A. salina* protein kinase of low specific activity as a function of the hemin concentration. Comparison of Fig. 4*B* with earlier data (figure 2*B* of ref. 7) shows that hemin inhibits the proinhibitor–inhibitor conversion to similar extents in the rabbit reticulocyte and *A. salina* systems. As in the case of BHK, BHK regulatory subunit, and cAMP-dependent protein kinase from rabbit reticulocytes (7), hemin interferes with the binding of [^{3}H]cAMP to *A. salina* protein kinase (Fig. 4*B*). This explains, as previously discussed (7), why hemin inhibits the cAMP-dependent conversion of proinhibitor to inhibitor. Hemin appears to block cAMP binding to the *A. salina* kinase less effectively than it blocks binding to other enzymes previously tested (7), but this may be more apparent than real, possibly due to nonspecific binding of the porphyrin to other proteins present in the rather crude *A. salina* kinase preparation used.

Proinhibitor preparations from wheat germ postribosomal supernatant contained some inhibitor because, as seen in Fig. 5, addition of ATP alone caused significant inhibition of ternary complex formation. In contrast to the reticulocyte (7) and *A. salina* preparations, addition of ATP + cAMP had little or no effect beyond that produced by addition of ATP alone. Addition of ATP + catalytic subunit, on the other hand, caused an increase in the amount of inhibitor, i.e., further conversion of

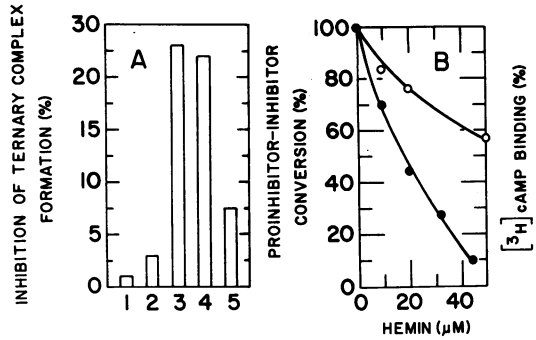

FIG. 4. (*A*) Conversion of proinhibitor to inhibitor in *A. salina* embryo preparations and effect of hemin thereon. Crude proinhibitor was prepared and the formation of inhibitor was assayed as described in *Materials and Methods*. In addition to the components listed in *Materials and Methods*, the step *1* samples contained proinhibitor (2.2 μg of protein) from developing embryos with other supplements as follows. (*1*) No further additions. (*2*) ATP (0.67 mM). (*3*) ATP (0.67 mM) and BHK catalytic subunit (0.04 μg). (*4*) ATP (0.67 mM) and cAMP (16.6 μM). (*5*) ATP (0.67 mM), cAMP (16.6 μM), and hemin (40 μM). The retention of ^{35}S radioactivity by ternary complex formation in a control sample without proinhibitor was 3655 cpm.

(*B*) Inhibition of conversion of proinhibitor to inhibitor and of [^{3}H]cAMP binding to protein in *A. salina* embryo preparations by increasing concentrations of hemin. ●, Proinhibitor → inhibitor conversion assayed as in *A*. Each sample contained 2.1 μg of crude proinhibitor protein from developing embryos and the indicated concentrations of hemin. The protein kinase specific activity of the proinhibitor preparation in the standard assay (pmol ^{32}P/min per μg of protein) was 0.3, and the total number of units/sample was 0.3 × 2.1 = 0.63. The retention of ^{35}S radioactivity by ternary complex formation in a control sample without proinhibitor was 14,596 cpm. ○, [^{3}H]cAMP binding. Each sample contained 15.8 μg of partially purified protein kinase from developing embryos and the indicated concentrations of hemin. The specific protein kinase activity of the enzyme preparations was 0.7 and the total number of units/sample was 15.8 × 0.7 = 11.1. The binding of [^{3}H]cAMP in the absence of added hemin (1788 cpm) was taken as 100%.

proinhibitor to inhibitor, as expected from the inhibition of translation by catalytic subunit (Fig. 2) described in a previous section. The lack of a cAMP effect on the proinhibitor–inhibitor conversion is consistent with the absence of cAMP-dependent protein kinase activity in the wheat germ preparations.

DISCUSSION

The results presented in this paper show that cells of organisms evolutionarily distant from mammals, such as *A. salina* and wheat, possess a similar system of control of protein synthesis. In both cases an inhibitor of chain initiation can be formed from

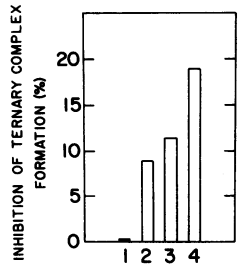

FIG. 5. Conversion of proinhibitor to inhibitor in wheat germ preparations. In addition to the components listed in *Materials and Methods*, the step *1* samples contained proinhibitor (1.3 μg of protein) with other supplements as follows. (*1*) No further additions. (*2*) ATP (0.67 mM). (*3*) ATP (0.67 mM) and cAMP (16.6 μM). (*4*) ATP (0.67 mM) and BHK catalytic subunit (0.04 μg). The retention of ^{35}S radioactivity by ternary complex formation in a control sample without proinhibitor was 3065 cpm.

an inactive proinhibitor by a process that appears to involve phosphorylation catalyzed by a protein kinase. As in reticulocyte lysates (6), the proinhibitor–inhibitor conversion in *A. salina* embryo extracts is catalyzed by a cAMP-dependent protein kinase. Wheat germ extracts, on the other hand, contain nucleotide-independent kinase(s), and cAMP has little or no effect on the proinhibitor–inhibitor conversion. Evidence for the presence of an analogous system in these extracts rests on the fact that the conversion is promoted by BHK catalytic subunit, but the mechanism governing the activation of proinhibitor in wheat germ is unknown.

As further shown in this paper, the conversion of proinhibitor to inhibitor in *A. salina* embryo preparations is inhibited by hemin just as it is in reticulocyte lysates and by the same mechanism (6, 7). It may be mentioned in this connection that hemoglobin is the oxygen-carrying pigment in *A. salina* (17). The fact that mRNA translation in fractionated *A. salina* systems proceeds normally without hemin addition (data not shown) may be due to low levels of cAMP in the postribosomal supernatants [prepared from S105 extracts by ammonium sulfate precipitation and extensive dialysis (13)] and/or low affinity of *A. salina* protein kinase(s) for cAMP. This matter requires further investigation.

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