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Mechanism of translational control by hemin in reticulocyte lysates

(regulation of protein synthesis/3′:5′-cyclic AMP-dependent protein kinases/translational control inhibitor formation)

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ABSTRACT **The formation of translational inhibitor (active eIF-2 kinase) from proinhibitor (inactive eIF-2 kinase) in reticulocyte lysates, known to be controlled by hemin, can, as we recently reported, be induced by 3′:5′-cyclic AMP(cAMP)-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) or its catalytic subunit. We find that in crude preparations from rabbit reticulocyte lysates, hemin inhibits the conversion of proinhibitor to inhibitor catalyzed by endogenous cAMP-dependent protein kinase upon addition of cAMP, but not that caused by the addition of free protein kinase catalytic subunit. Hemin prevents the binding of cAMP to the regulatory subunit of cAMP-dependent protein kinase and blocks the cAMP-induced dissociation of regulatory and catalytic subunits of the enzyme whereby the enzyme is inactivated. The mechanism by which hemin prevents the formation of the inhibitor and maintains protein synthesis in reticulocyte lysates is thus explained.**

Protein synthesis in reticulocyte lysates is but briefly maintained in the absence of added hemin (1). Under these conditions an inhibitor of polypeptide chain initiation is produced from a proinhibitor of similar molecular weight (2). The inhibitor is a 3′:5′-cyclic AMP (cAMP)-independent protein kinase that catalyzes the phosphorylation of the small (38,000 daltons) subunit of the initiation factor eIF-2 (3–6), a modification which eventually renders this factor inactive in chain initiation. The inhibitor (eIF-2 kinase) has been extensively purified (7). The conversion of proinhibitor (inactive eIF-2 kinase) to inhibitor appears to involve a phosphorylation catalyzed by cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) (8) similar to the conversion of inactive phosphorylase kinase to active phosphorylase kinase (9, 10).

Hemin was known to prevent the conversion of proinhibitor to inhibitor (ref. 2, see also ref. 6), but as long as the mechanism of this conversion remained obscure, there was little chance of clarifying its mode of action. The finding that the conversion involves cAMP-dependent protein kinase suggested that hemin probably interferes with the activity of this enzyme and, in previous work (8), we obtained indirect evidence for this view. This evidence was consistent with an earlier report (11) that hemin inhibits the activity of cAMP-dependent protein kinases from rabbit reticulocytes. We confirmed that hemin inhibits histone phosphorylation by cAMP-dependent protein kinases but found that it has no effect on the activity of the free catalytic subunit, suggesting that it blocks the dissociation of cAMP-dependent protein kinase by cAMP.

In this paper we show that hemin inhibits the conversion of proinhibitor to inhibitor catalyzed by endogenous protein kinase upon addition of cAMP, but not that elicited by free catalytic subunit. We further show that this is due to the fact that hemin apparently competes with cAMP for binding to the regulatory subunit of the enzyme. Thus, hemin blocks the cAMP-induced dissociation of the regulatory and catalytic subunits of cAMP-dependent protein kinase as shown in Eq. 1 below.

$$R_2C_2 + 2\,cAMP \xrightarrow[hemin]{} R_2cAMP_2 + 2C \qquad [1]$$

MATERIALS AND METHODS

Preparations. Crude proinhibitor was prepared by carboxymethyl-Sephadex chromatography of postribosomal supernatant of rabbit reticulocyte lysate (2) as previously described (8). The proinhibitor content of the preparations was determined by treatment of an aliquot with *N*-ethylmaleimide, which quantitatively converts proinhibitor to inhibitor (2); the inhibitor was assayed with the eIF-2–GTP–Met-tRNA$_i$ ternary complex formation assay as described (8). The preparations contain active protein kinase(s) highly dependent on cAMP (Table 1). The following were as previously used or prepared (8): *Artemia salina* DE-180 eIF-2 and [^{35}S]Met-tRNA$_i$; cAMP-dependent bovine heart protein kinase, either homogeneous (gift of R. Rangel-Aldao and O. M. Rosen, Albert Einstein College of Medicine) or partially purified (Sigma), and its catalytic subunit; and cAMP-dependent protein kinase (pkIIb) from rabbit reticulocytes. Protein kinase activity was assayed with histone (Sigma, type IIA) as substrate as in previous work (8). Protein was determined by the method of Lowry *et al.* (12). The specific activities of the kinase preparations and catalytic subunit were those previously stated (8). The amounts of bovine heart protein kinase and catalytic subunit used (whether homogeneous or not) are given throughout as μg of the pure protein. A sample of regulatory subunit (cAMP binding protein) from bovine heart protein kinase was obtained from David R. Webb of this Institute. The cAMP binding activity of this preparation was about 1% of the calculated value. We are indebted to A. Blume of this Institute for a sample of 3-isobutyl-1-methylxanthine (Aldrich Chemical Co.), a potent inhibitor (13) of cAMP phosphodiesterase (3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17).

Conversion of Proinhibitor to Inhibitor. This was analyzed by the ternary complex formation assay (8). Reaction and assay were conducted in three stages: (*a*) enzymatic conversion of proinhibitor (inactive eIF-2 kinase) to inhibitor (active eIF-2 kinase); (*b*) phosphorylation of eIF-2 by ATP catalyzed by active eIF-2 kinase; and (*c*) ternary complex formation assay. Suitable dilutions of proinhibitor, freshly prepared by carboxymethyl-Sephadex chromatography, were used for stage (*a*). Samples (30 μl) containing (in order of addition) *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, pH 7.6, 20 mM; Mg(OAc)$_2$, 4 mM; ATP, 0.67 mM; proinhibitor, 12 μg of protein; and, when present, cAMP, 15.0

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Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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Table 1. cAMP-dependent protein kinase in proinhibitor preparations from rabbit reticulocyte lysates

Proinhibitor, μg	Histone phosphorylation* −cAMP	+cAMP	+cAMP/−cAMP
0.7	0	7.88	—
2.0	0.33	19.35	58.6

The standard assay for protein kinase was used.
* pmol of ^{32}P incorporated per 8 min.

μM, or bovine heart protein kinase catalytic subunit, 0.04 μg, were incubated for 6 min at 30°. After incubation the samples were brought to 90 μl with 3.7 mM Hepes buffer, pH 7.2, and 4-μl aliquots were used for stage (*b*). The stage (*b*) samples, 40 μl, containing Hepes buffer, pH 7.6, 25 mM; $Mg(OAc)_2$, 3.75 mM; dithiothreitol, 2.5 mM; ATP, 0.5 mM; DE-180 eIF-2 with 22.5 μg of protein; and 4 μl of incubated, diluted stage (*a*) sample with 0.5 μg of protein, were incubated for 2 min at 30°. For stage (*c*) the samples were further supplemented with KCl, 100 mM; *A. salina* [^{35}S]Met-tRNA$_i$ (25,000 cpm/pmol), 1.8 pmol; and GTP, 0.14 mM (final volume 50 μl) and incubated for 5 min at 30°. The formation of ternary complex was assayed as previously described (14). At concentrations higher than 10 μM, hemin has some inhibitory effect on the formation of the ternary complex. However, its highest concentration at stage (*c*) was 1.2 μM, well below the inhibitory level.

cAMP Binding to Protein Kinase. The binding assay was performed according to the procedure of Gilman (15) with some modifications (D. R. Webb, unpublished). The samples (0.2 ml) contained potassium phosphate buffer, pH 6.0, 10 μmol; $Mg(OAc)_2$, 1 μmol; [^3H]cAMP (Schwarz Bioresearch, 16.3 Ci/mmol), 3 pmol; protein kinase inhibitor (Sigma), 40 μg; and cAMP binding protein (cAMP-dependent protein kinase or regulatory subunit). After incubation for 90 min at 4°, 12.5 mg of freshly prepared hydroxyapatite solution (in 0.1 ml of 20 mM potassium phosphate buffer, pH 6.0) was added. The precipitate was collected by centrifugation at 2500 rpm for 5 min, washed with 4 ml of 20 mM potassium phosphate buffer, pH 6.0, and dissolved in 0.1 ml of 3.0 M HCl. Radioactivity was determined in Aquasol (New England Nuclear, Boston, MA). A sample without cAMP binding protein served as the blank. cAMP was assayed as described by Gilman (15) using a commercial preparation (Sigma) of cAMP-binding protein. The concentration of cAMP in rabbit reticulocyte lysates averaged 0.1 μM.

RESULTS

Effect of Hemin on cAMP-Dependent Protein Kinases. The report (11) that hemin inhibits cAMP-dependent protein kinases from rabbit reticulocytes was of interest to us because of our finding that cAMP-dependent protein kinase is involved in the conversion of proinhibitor to inhibitor in reticulocyte lysates (8) and the knowledge that hemin prevents this conversion (2, 6). As shown in Fig. 1, hemin does inhibit histone phosphorylation by cAMP-dependent bovine heart and rabbit reticulocyte protein kinases but not by the free catalytic subunit. The reticulocyte kinase, which is more dependent on cAMP than the heart enzyme, is more sensitive to hemin (Fig. 1). The inhibition is higher at low histone concentrations. Thus, with 50 μg of histone per 50 μl (conditions of the standard protein kinase assay) 50 μM hemin inhibited phosphorylation about 40% whereas, with 5 μg of histone per 50 μl, 30 μM hemin caused almost 80% inhibition. Bilirubin and biliverdin (data not shown) were about half as active as hemin in inhibiting histone phosphorylation by cAMP-dependent protein kinase and in maintaining protein synthesis in reticulocyte lysates.

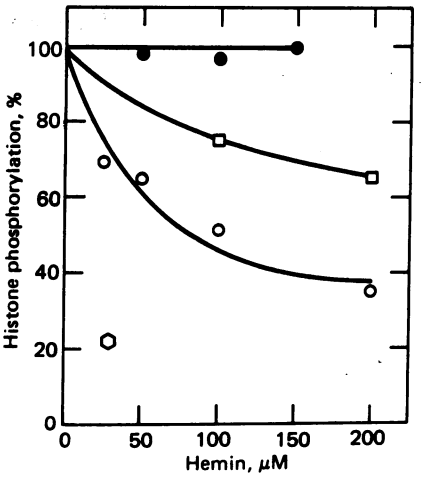

FIG. 1. Effect of hemin on histone phosphorylation by cAMP-dependent protein kinase. Standard 50-μl assay with reticulocyte protein kinase pkIIb (2 μg of protein) (○), bovine heart protein kinase (0.4 μg) (□), or bovine heart protein kinase catalytic subunit (0.154 μg) (●). The phosphorylation rate in the absence of hemin was taken as 100%. It amounted to 56.4, 31.8, and 30.3 pmol of ^{32}P/min for pkIIb, bovine heart protein kinase, and catalytic subunit, respectively. When the histone concentration was reduced from 50 μg to 5 μg/50 μl the 100% phosphorylation rate with 3 μg of pkIIb protein (12.4 pmol of ^{32}P/min) was reduced to 3 pmol/min, or 22%, by 30 μM hemin (○).

Effect of Hemin on Conversion of Proinhibitor to Inhibitor. The results of the preceding section (inhibition of the holoenzyme, no inhibition of the catalytic subunit) suggested that hemin inhibits cAMP-dependent protein kinase by blocking the cAMP-mediated dissociation of the holoenzyme into regulatory and catalytic subunits (see Eq. 1). We had tentatively reached the same conclusion previously from our observation that in hemin-containing reticulocyte lysates the catalytic subunit was a much more effective inhibitor of translation than protein kinase + cAMP, but that protein kinase + cAMP were equally effective in promoting the conversion of proinhibitor to inhibitor in the absence of hemin (8). It can in fact be shown that hemin prevents the conversion of proinhibitor to inhibitor catalyzed by endogenous protein kinase upon addition of cAMP, but not that caused by addition of free catalytic subunit. The proinhibitor used for these experiments was freshly prepared from postribosomal supernatant of rabbit reticulocyte lysate by carboxymethyl-Sephadex chromatography. It contained active protein kinase(s) highly dependent on cAMP (Table 1). As seen in Fig. 2*A*, the conversion 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 μM hemin (bar 3), but that elicited by the catalytic subunit was not significantly affected by the porphyrin (bar 5). The effect of the hemin concentration on the proinhibitor–inhibitor conversion is shown in Fig. 2*B*. Under the conditions of the experiment the conversion was 100% blocked by 45 μM hemin. The 50% value was about 15 μM.

Competition of Hemin with cAMP for Binding to Regulatory Subunit. Hemin blocks the dissociation of the protein kinase holoenzyme by cAMP through competition with the cyclic nucleotide for binding to the regulatory subunit. As shown in Fig. 3, the binding of [^3H]cAMP to bovine heart

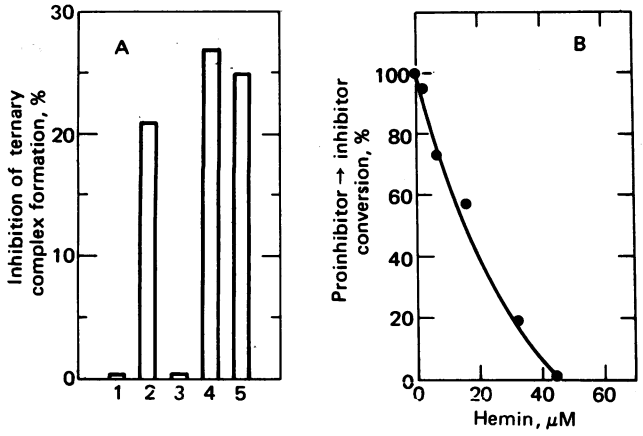

FIG. 2. (*A*) Effect of hemin on the conversion of proinhibitor to inhibitor. Conversion was promoted either by endogenous cAMP-dependent protein kinase upon the addition of cAMP or by bovine heart protein kinase catalytic subunit, and was measured by the ternary complex formation assay. All samples contained proinhibitor (0.5 μg of protein) and ATP (0.67 mM). 1, No further additions; 2, cAMP (15 μM); 3, cAMP (15 μM) and hemin (44.6 μM); 4, catalytic subunit (0.04 μg); 5, catalytic subunit (0.04 μg) and hemin (44.6 μM). The retention of ^{35}S radioactivity by ternary complex formation in a control sample without proinhibitor was 10,898 cpm. The preparation of crude proinhibitor (containing cAMP-dependent protein kinase), the experimental plan and conditions, and the ternary complex formation assay are described in *Materials and Methods*. Other experiments with amounts of proinhibitor between 0.08 and 0.8 μg of protein/sample were carried out with similar results. However, the proportionality between proinhibitor concentration and inhibition of ternary complex formation is generally poor. This may be due to the presence of protein phosphatase in the crude preparations of proinhibitor (8). (*B*) Inhibition of the proinhibitor–inhibitor conversion as a function of the hemin concentration. Conditions of (*A*) with proinhibitor containing 0.5 μg of protein, 0.67 mM ATP, 15 μM cAMP, and different concentrations of hemin. The inhibition of ternary complex formation in the absence of hemin was taken as 100% conversion of proinhibitor to inhibitor. The retention of ^{35}S radioactivity by ternary complex formation at 0% proinhibitor → inhibitor conversion was 10,663 cpm; at 100% conversion, 9231 cpm.

protein kinase, bovine heart kinase regulatory subunit, and cAMP-dependent protein kinase from rabbit reticulocytes was severely curtailed by hemin. However, although hemin appears to compete with cAMP for binding to the regulatory subunit of cAMP-dependent protein kinase, unlike cAMP it does not dissociate the holoenzyme. Thus, the phosphorylation of histone by reticulocyte protein kinase pkIIb in the standard assay was stimulated 11-fold by 10 μM cAMP but was not affected by 40 μM hemin in the absence of cAMP.

DISCUSSION

We have shown that cAMP-dependent protein kinase, present in crude proinhibitor preparations from rabbit reticulocyte lysates, catalyzes, in the presence of ATP, the conversion of proinhibitor to inhibitor upon addition of cAMP. Whereas high concentrations (15 μM) of the cyclic nucleotide were used in the experiments of Fig. 2*A*, we wish to emphasize that cAMP promotes the proinhibitor–inhibitor conversion at physiological concentrations. We find that the concentration of cAMP in rabbit reticulocyte lysates, prepared as described (8), is about 0.1 μM. Under the conditions of the protein synthesis assay (8), with 25 μl of lysate in a final volume of 50 μl, the cAMP concentration is therefore around 0.05 μM. Fig. 4 shows that, in the presence of phosphodiesterase inhibitor, 0.05 μM cAMP caused 65% conversion of proinhibitor to inhibitor. Fifty percent conversion was produced by about 0.02 μM cAMP.

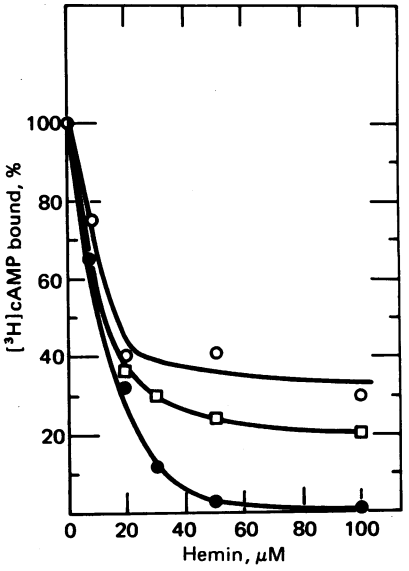

FIG. 3. Competition of hemin with cAMP for binding to the regulatory subunit of cAMP-dependent protein kinase. Binding of [^3H]cAMP to cAMP-binding protein (kinase holoenzyme or regulatory subunit) as a function of the hemin concentration. Binding assay was carried out as described in *Materials and Methods*. ○, Homogeneous bovine heart protein kinase, 1.0 μg; □, bovine heart protein kinase regulatory subunit, 0.4 μg; ●, cAMP-dependent rabbit reticulocyte protein kinase (pkIIb), 4.8 μg. The [^3H]cAMP binding in the absence of hemin (taken as 100%) was bovine heart protein kinase, 10,942 cpm; regulatory subunit, 659 cpm; pkIIb, 8883 cpm.

There seems to be little doubt that blocking by hemin of cAMP binding to the regulatory subunit of cAMP-dependent protein kinase is responsible for inhibition of the proinhibitor–inhibitor conversion and for the maintenance of protein synthesis in reticulocyte lysates. This statement is borne out by Fig. 5*B*. This figure, a replot of Figs. 2*B* and 5*A*, shows that the hemin concentration dependence of [^3H]cAMP binding to reticulocyte protein kinase, inhibition of the proinhibitor–in-

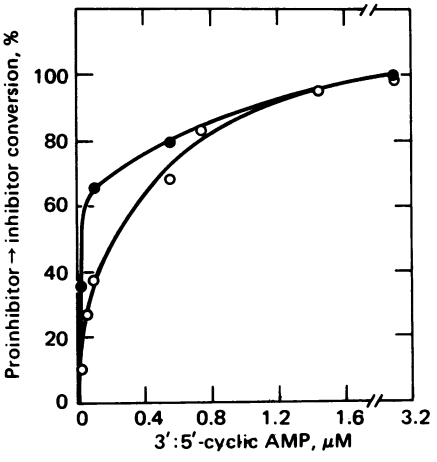

FIG. 4. Conversion of proinhibitor to inhibitor as a function of the concentration of cAMP. The conditions were those of Fig. 2*B* without hemin, with 0.42 μg of proinhibitor protein, and varying concentrations of cAMP, either without (○) or with (●) the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (41.7 μM). The highest inhibition of ternary complex formation was taken as 100% conversion of proinhibitor to inhibitor; it was reached at about 3 μM cAMP, both in the absence and presence of phosphodiesterase inhibitor. The retention of ^{35}S radioactivity by ternary complex formation at 0% proinhibitor → inhibitor conversion was 10,862 cpm; at 100% conversion, 9387 cpm.

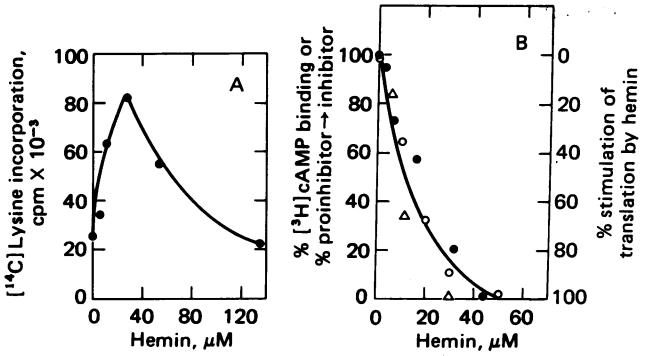

FIG. 5. (*A*) Translation in reticulocyte lysate as a function of the hemin concentration. Standard assay (8) in the absence and presence of increasing concentrations of hemin. (*B*) Comparison of the effects of hemin on the conversion of proinhibitor to inhibitor (●), [^{3}H]cAMP binding to cAMP-dependent rabbit reticulocyte protein kinase (○), and translation in reticulocyte lysate (△). Data for (●) and (○) are replotted from Fig. 2*B* (●) and 3 (●), respectively. The triangles are from the data of (*A*) plotted as % stimulation of translation ([^{14}C]-lysine incorporation) by hemin between the limits of 0 and 30 μM concentration (taken as 0 and 100%, respectively).

hibitor conversion, and stimulation of protein synthesis in lysates fall roughly on the same curve.

Note Added in Proof. We have recently become aware that Bloxham and collaborators have shown inhibition by cAMP of protein synthesis in cell-free preparations from rat liver as far back as 1972. Their more recent studies (16) strongly support the involvement of cAMP-dependent protein kinase in translational control.

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