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.

\[
\text{R}_C + 2 \text{cAMP} \rightarrow \text{R}_C \text{AMP} + 2 \text{C}
\]

**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–tRNA1 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 [35S]Met–tRNA; 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 II A) 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 

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Abbreviations: cAMP, adenosine 3':5'-cyclic monophosphate; Heps, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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\[\text{Histone phosphorylation}^*\]

<table>
<thead>
<tr>
<th>Proinhibitor, (\mu g)</th>
<th>(-cAMP)</th>
<th>+cAMP</th>
<th>+cAMP/-cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>0</td>
<td>7.88</td>
<td>—</td>
</tr>
<tr>
<td>2.0</td>
<td>0.33</td>
<td>19.35</td>
<td>58.6</td>
</tr>
</tbody>
</table>

The standard assay for protein kinase was used.

* pmol of \(^{32}\text{P}\) incorporated per 8 min.

\(\mu M\), or bovine heart protein kinase catalytic subunit, 0.04 \(\mu M\), were incubated for 6 min at 30\(^\circ\)C. After incubation the samples were brought to 90 \(\mu l\) with 3.7 mM Heps buffer, pH 7.2, and 4-\(\mu l\) aliquots were used for stage (b). The stage (b) samples, 40 \(\mu l\), containing Heps 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 \(\mu g\) of protein; and 4 \(\mu l\) of incubated, diluted stage (a) sample with 0.5 \(\mu g\) of protein, were incubated for 2 min at 30\(^\circ\)C. For stage (c) the samples were further supplemented with KCl, 100 mM; A. salina \[^{32}\text{S}\text{Met-TRNA}\] (25,000 cpm/pmole), 1.8 pmol; and GTP, 0.14 mM (final volume 50 \(\mu l\)) and incubated for 5 min at 30\(^\circ\)C. The formation of ternary complex was assayed as previously described (14). At highest concentration higher than 10 \(\mu M\), hemin has some inhibitory effect on the formation of the ternary complex. However, its highest concentration at stage (c) was 1.2 \(\mu M\), 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 (R. D. Webb, unpublished). The samples (0.2 ml) contained potassium phosphate buffer, pH 6.0, 10 \(\mu M\); Mg(OAc)\(_2\), 1 \(\mu M\); \[^{3}H\text{cAMP}\] (Schwarz Bioresearch, 16.3 Ci/mmole), 3 pmol; protein kinase inhibitor (Sigma), 40 \(\mu g\); and cAMP binding protein (cAMP-dependent protein kinase or regulatory subunit). After incubation for 90 min at 4\(^\circ\), 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 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 \(\mu M\).

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 \(\mu g\) of histone per 50 \(\mu l\) (conditions of the standard protein kinase assay) 50 \(\mu M\) hemin inhibited phosphorylation about 40\% whereas, with 5 \(\mu g\) of histone per 50 \(\mu l\), 30 \(\mu M\) 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.

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. 2A, 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 \(\mu M\) 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. 2B. Under the conditions of the experiment the conversion was 100\% blocked by 45 \(\mu M\) hemin. The 50\% value was about 15 \(\mu M\).

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 \[^{3}H\text{cAMP}\] to bovine heart...
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. 2A, 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.

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. 5B. This figure, a replot of Figs. 2B and 5A, shows that the hemin concentration dependence of [3H]cAMP binding to reticulocyte protein kinase, inhibition of the proinhibitor-inhibitor conversion, is promi-
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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