Translational control by hemin is due to binding to cyclic AMP-dependent protein kinase

(protein synthesis regulation/noncompetitive inhibition of protein kinase activation/globin–hemin interaction/ reticulocyte lysates)

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ABSTRACT   Our previous work [*Proc. Natl. Acad. Sci. USA* (1977) 74, 1463–1467, 3326–3329] is consistent with the view that (*a*) the hemin-controlled inhibitor of protein synthesis in reticulocyte lysates (active eIF-2 kinase) is formed by phosphorylation of proinhibitor (inactive eIF-2 kinase) catalyzed by cyclic AMP-dependent protein kinase (ATP-protein phosphotransferase; EC 2.7.1.37), and (*b*) hemin prevents this conversion by blocking the interaction of cyclic AMP with the kinase's regulatory subunit, thereby rendering the enzyme inactive. We now show that hemin blocks cyclic AMP binding because it itself binds specifically to the regulatory subunit. This binding is noncompetitive with respect to cyclic AMP. Whereas unlabeled hemin can displace bound [^{3}H]hemin as well as cyclic [^{3}H]AMP, unlabeled cyclic AMP can displace bound cyclic [^{3}H]AMP but not [^{3}H]hemin. This suggests that cyclic AMP and hemin bind to different sites on the protein and that hemin binding affects cyclic AMP binding in an allosteric manner.

The requirement of hemin for protein synthesis in reticulocyte lysates has been the subject of intensive study in several laboratories. Hemin prevents the conversion of an inactive precursor to an inhibitor of polypeptide chain initiation of similar molecular weight (1) and the inhibition was relieved by adding relatively large amounts of the chain initiation factor eIF-2 (2, 3). This suggested that the inhibitor interfered with the function of this factor. eIF-2 forms a ternary complex with GTP and the initiator tRNA, Met-tRNA$_i$, which, upon binding to a 40S ribosomal subunit, is converted to a 40S initiation complex (see ref. 4 for review). Recently, the inhibitor was shown to be a cyclic AMP (cAMP)-independent protein kinase (active eIF-2 kinase) that catalyzes the phosphorylation of the small (38,000 daltons) subunit of eIF-2 (5–8). The mechanism of conversion of proinhibitor to inhibitor (and therefore the mode of action of hemin) and the manner in which phosphorylation of eIF-2 led to inhibition of chain initiation remained unexplained.

We have provided answers to the above questions by showing that (*a*) proinhibitor is converted to inhibitor in the presence of ATP and cAMP-dependent protein kinase (ATP:protein phosphotransferase; EC 2.7.1.37) (9, 10); (*b*) hemin prevents this conversion by blocking the binding of cAMP to the regulatory subunit of the kinase, thus inactivating the enzyme (10); and (*c*) whereas phosphorylation of eIF-2 does not interfere with its function *per se*, it makes it insensitive to a new factor (eIF-2 stimulating protein) that, at the low concentrations of eIF-2 in reticulocyte lysates, is essential for eIF-2 activity (11). The present paper defines the mode of action of hemin by showing that it binds to the regulatory subunit of cAMP-dependent protein kinase, blocking cAMP binding in a noncompetitive fashion.

MATERIALS AND METHODS

Assays. Binding of labeled hemin and cAMP was assayed according to the procedure of Baxter and Tomkins (12) for measuring the binding of steroids to specific receptors. As in the case of steroids, activated charcoal can adsorb free but not protein-bound hemin or 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. For determination of total binding (specific plus non-specific), samples (0.2 ml) contained potassium phosphate buffer, pH 6.0, 20 mM; [^{3}H]hemin (3000 cpm/ nmol), 9 μM unless otherwise stated, or [^{3}H]cAMP (71,000 cpm/nmol), 3 μM if not otherwise specified; and cAMP-dependent bovine heart protein kinase, 100 μg of the commercial (Sigma) preparation if not otherwise stated, homogeneous bovine heart protein kinase, or catalytic (C) or regulatory (R) subunit, as specified in the legends. Samples with the same amount of [^{3}H]hemin or [^{3}H]cAMP, plus an excess of nonradioactive hemin (usually 0.2 mM) or cAMP (usually 0.1 mM), were run simultaneously for determination of nonspecific binding. Protein kinase or its regulatory or catalytic subunit was added last. After incubation for 60 min at 4°, 0.05 ml of a suspension of activated charcoal (0.05 mg/ml) was added to each tube. The tubes were vigorously agitated on a Vortex mixer for 10 sec and centrifuged at 600 × g for 2 min. The radioactivity of an aliquot (0.15 ml) of the supernatant fluid was measured on Biofluor (New England Nuclear). The amount of hemin or cAMP specifically bound by protein kinase was obtained by subtracting the nonspecific from the total binding. No nonspecific binding was observed with cAMP. Free hemin concentrations were calculated by subtracting the radioactivity of the specifically bound hemin from that of the hemin added. [^{3}H]cAMP binding was measured occasionally by the hydroxyapatite procedure (10). Protein synthesis was assayed as described (9). Protein was determined by the method of Lowry *et al.* (13), with bovine serum albumin as the standard.

Preparations. Labeled hemin was prepared by synthesis from δ-[^{3}H]aminolevulinic acid (New England Nuclear, 5.05 Ci/mmol) by a lysate of duck blood according to the procedure of Shemin *et al.* (14). Fresh heparinized blood from one duck
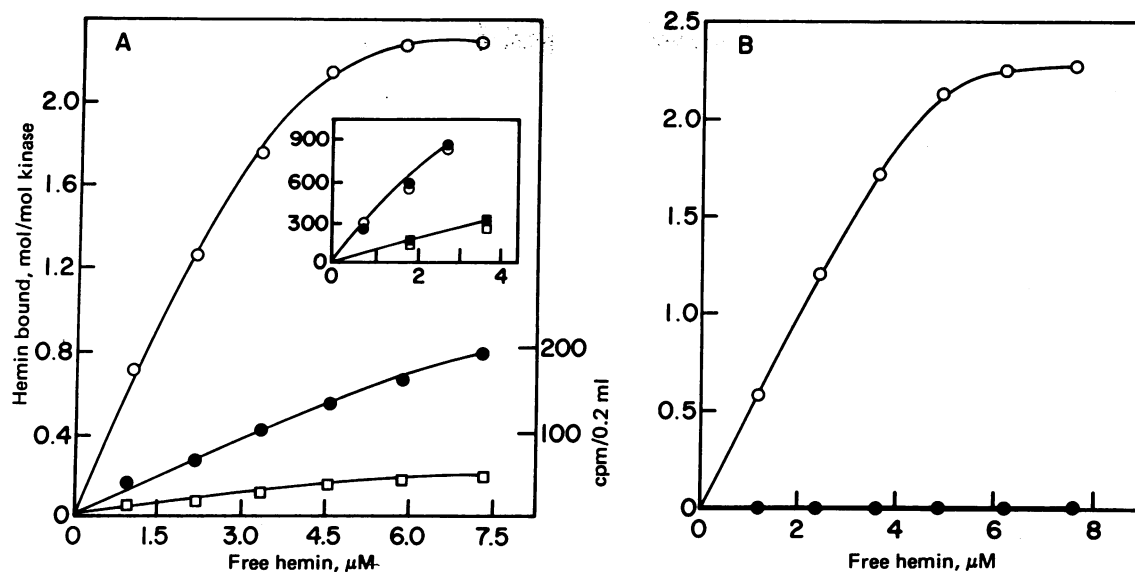

FIG. 1. Binding of hemin to protein kinase. Specific and nonspecific binding of [³H]hemin was measured. (A) Sigma bovine heart protein kinase (corresponding to 16 μg of homogeneous protein kinase/0.2 ml) was used. The specific binding of hemin (mol/mol kinase) is plotted against the concentration of free [³H]hemin (O). Nonspecific hemin binding (●) and a sample background in the absence of protein (□) are given in cpm/sample. (*Inset*) Binding of [³H]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, is plotted (in cpm/sample) against the concentration of free [³H]hemin. (B) Binding of [³H]hemin to homogeneous protein kinase (12 μg/0.2 ml) in the absence (O) and presence (●) of an excess of nonlabeled hemin.

was centrifuged for 10 min at 10,000 × *g*; the cells were washed five times with isotonic saline and lysed by addition of 1.5 vol of water. After standing for 1 hr at 4°, the lysate was made 0.14 M with KCl and 2 mM with MgCl₂ by addition of the appropriate amounts of the solid salts. After δ-[³H]aminolevulinic acid (0.5 mCi) was added, the mixture was incubated with shaking for 16 hr at room temperature. For isolation of the labeled hemin (15), the incubated suspension was poured dropwise, over a period of 15–20 min, into a flask containing a mixture of 5.0 ml of glacial acetic acid and 0.04 ml of saturated sodium chloride that was kept at 95–100°. During this time the mixture was stirred vigorously. After one drop of concentrated HCl was added, the mixture was heated on a steam bath for 1 hr. The hemin crystals that formed during this time were collected by centrifugation and washed successively (twice with each reagent) with 50% acetic acid, water, alcohol, and ether, and then dried. The yield of labeled hemin was about 5 mg. As judged by thin-layer chromatography, carried out by the procedure of Falk *et al.* (16) as modified by S. Sassa (personal communication), the purity of the preparation was approximately 98%. The concentration of the labeled hemin was determined spectrophotometrically (17); its specific radioactivity was 3000 cpm/nmol. cAMP was from P. L. Biochemicals and [8-³H]-cAMP from the Radiochemical Centre, Amersham, England. The amount of [³H]cAMP in the solution used for the experiments was determined spectrophotometrically; its specific radioactivity was 71,000 cpm/nmol. Rabbit reticulocyte lysates for assay of protein synthesis were prepared as described (9). A commercial preparation (Sigma) of cAMP-dependent bovine heart protein kinase was used in most experiments; its purity, as standardized (9) with homogeneous protein kinase, was 16%. The amounts of this enzyme used are given throughout as μg of the pure protein. Homogeneous protein kinase (18) and its regulatory subunit (virtually nucleotide-free) were the kind gift of R. Rangel-Aldao and O. M. Rosen, Albert Einstein College of Medicine. Nucleotide-free regulatory subunit from cAMP-dependent protein kinase of rabbit skeletal muscle (19) was

kindly provided by J. A. Beavo and E. G. Krebs, University of Washington, Seattle, Washington. Sperm whale apomyoglobin (20) was a gift of S. Pestka of this Institute. Other preparations and materials were as in previous work (9–11).

RESULTS

Binding of hemin to bovine heart protein kinase

Both specific and nonspecific binding of hemin was observed with partially purified (Sigma) protein kinase. In Fig. 1A the specific hemin binding (upper curve) is plotted against the free hemin concentration. At saturation, approximately 2 molecules of hemin are bound specifically per molecule of protein kinase (R₂C₂) or 1 molecule per R subunit. The nonspecific binding of the preparation, i.e., the binding of [³H]hemin in the presence of an excess of nonlabeled hemin, and the [³H]hemin background, i.e., the amount of hemin not adsorbed to charcoal in the absence of protein, are also shown. Other proteins bind hemin nonspecifically, as shown by the failure of excess nonradioactive hemin to quench [³H]hemin binding. As shown in Fig. 1A *inset*, nonspecific binding is rather high with bovine serum albumin. Nonspecific hemin binding by the partially purified kinase is due to proteins other than protein kinase for, as seen in Fig. 1B, homogeneous protein kinase showed only specific hemin binding. Fig. 2A compares the specific binding of [³H]cAMP and [³H]hemin to Sigma bovine heart protein kinase as a function of their concentration. The points for both ligands fall on the same curve, the affinity of hemin for protein kinase being about one-third as high as that of cAMP. The association constant for the reaction hemin + protein kinase ⇌ hemin–protein kinase complex, calculated from a Scatchard plot (21) of the data of Fig. 2A (Fig. 2B), was 0.8 × 10⁶ M.

Binding to regulatory subunit

Hemin binds specifically to the protein kinase regulatory but not the catalytic subunit (Table 1, Exps. 3 and 4). C was assayed for histone phosphorylation in the standard assay (9), in the
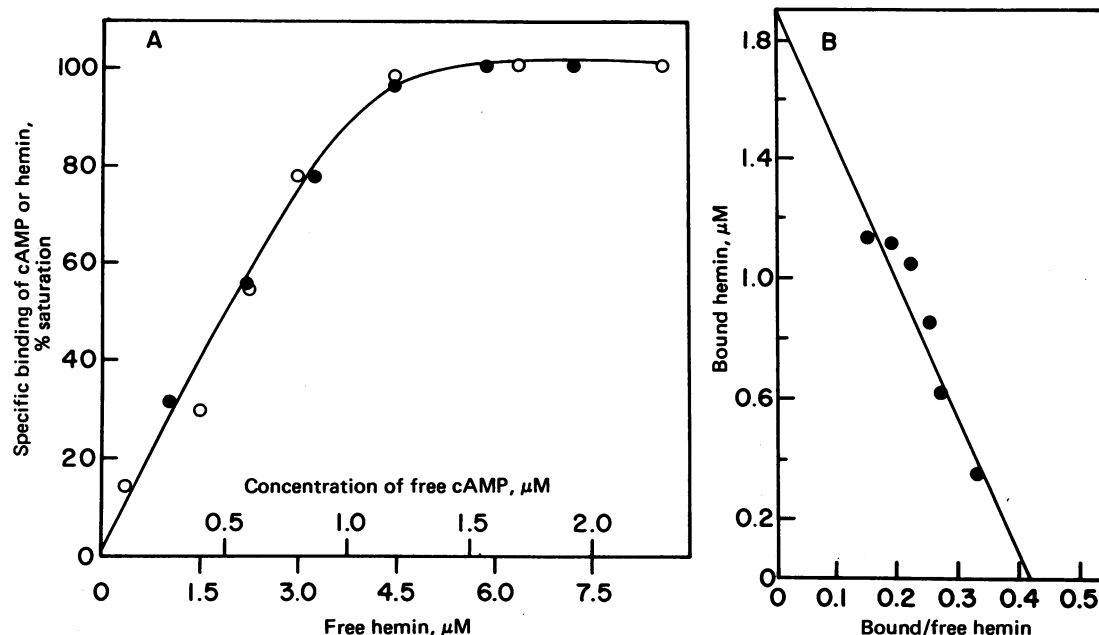
FIG. 2.   (A) Specific binding of [³H]cAMP (O) or hemin (●) to protein kinase as a function of their concentration. (B) Scatchard plot of hemin binding. Binding of cAMP and hemin was measured by the charcoal procedure. Sigma bovine heart protein kinase was used.

absence of cAMP, just before it was assayed for hemin binding, and it was highly active. Since protein kinase binds 2 molecules of hemin per molecule of enzyme, the molar binding ratio for R should be 1. The fact that it was found to be 2 or higher is probably due to the large margin of error involved in measuring the specific binding of small amounts of R (2.2 μg/0.2-ml sample) in the presence of large amounts of bovine serum albumin which, as already noted, has high nonspecific binding of hemin. As observed earlier with a less active R preparation (10), hemin blocks the binding of cAMP to this subunit (Table 1, Exp. 5).

Kinetics of association and dissociation

The time course of association and dissociation at 4° of the hemin–kinase complex is shown in Fig. 3. The reaction is readily reversible, since addition of an excess of nonradioactive hemin to the complex rapidly displaced kinase-bound radioactivity. The binding of cAMP to the regulatory subunit of cAMP-dependent protein kinase is also readily reversible (Fig. 4). As shown in Fig. 4, [³H]cAMP bound to protein kinase was displaced by adding not only an excess of nonlabeled cAMP, but also an excess of nonlabeled hemin. On the other hand (Fig. 4 *inset*), addition of an excess of nonradioactive cAMP failed

Table 1.   Specific binding of hemin and cAMP to cAMP-dependent protein kinase and its regulatory subunit

Experiment no.	Protein in assay (μg/ml)	Total (pmol/ml	Specific (pmol/ml)	(mol/mol protein
	[³H]Hemin binding			
1	Sigma BHK (80)		1122	2.4
	Homogeneous BHK (300)		3190	1.85
2	BSA (500)	2647		
	BSA (500) + homogeneous BHK (60)	3413	766	2.2
3	BSA (500)	2593		
	BSA (500) + R (11)	3027	434	2.2
	BSA (500) + R (11)	3133	540	2.7
4	BSA (500)	2267		
	BSA (500) + C (10)	2273	6	0.02
	[³H]cAMP binding			
5	R (27.4)		559	
	R (27.4) + hemin (30 μM)		31	
	Inhibition of cAMP binding by hemin		94%	

Hemin binding was assayed by the standard charcoal procedure, cAMP binding by the hydroxyapatite method (10). The concentration of R was determined by cAMP binding (charcoal procedure) on the basis that one molecule of cAMP is bound per subunit. Specific hemin binding in Exp. 1 was determined as described in *Materials and Methods*. For determination of hemin binding to R (Exp. 3) or C (Exp. 4), bovine serum albumin (BSA) was added to the samples as indicated. The specific hemin binding in this case was taken as the difference between samples containing BSA + R or BSA + C and BSA alone. Comparison of Exp. 2, in which BSA was added to a sample containing homogeneous bovine heart protein kinase (BHK), with Exp. 1 proves the validity of the procedure used to measure specific binding in Exps. 3 and 4. In Exp. 3, R was from homogeneous bovine heart protein kinase; in Exp. 5, from rabbit skeletal muscle protein kinase.
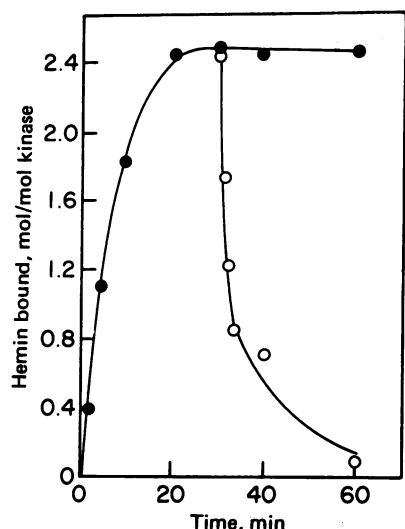
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FIG. 3. Kinetics of hemin association to and dissociation from protein kinase. Samples were incubated with kinase (16 μg) and 9 μM hemin for the indicated times, either without (●) or with (○) addition of 0.2 mM nonradioactive hemin after a 30-min incubation. Sigma bovine heart protein kinase was used.

to displace bound [³H]hemin from the hemin–kinase complex.

Reversal of translational effect of hemin

Since cAMP is unable to dissociate the hemin–protein kinase complex, the cAMP level probably has no control over the translational effect of hemin. For this reason we considered whether the effect of hemin was reversed by globin and found
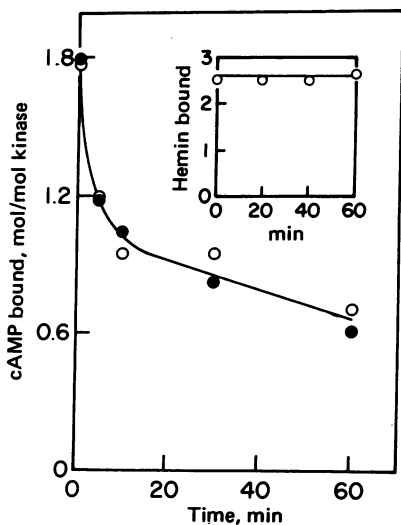
FIG. 4. Kinetics of dissociation of protein kinase-bound labeled cAMP by nonlabeled cAMP or hemin. [³H]cAMP, 3 μM, was first bound to kinase, 16 μg, by incubation for 90 min at 4°. At this time (time zero) 0.1 mM nonlabeled cAMP (●) or 0.2 mM unlabeled hemin (○) was added and the amount of bound cAMP was determined after incubation at 4° for the times indicated. (*Inset*) Nondissociation of protein kinase-bound labeled hemin by nonlabeled cAMP. [³H]Hemin, 9 μM, was allowed to bind to kinase, 16 μg, by incubation for 60 min at 4°. At this time (time zero) 0.1 mM nonlabeled cAMP was added and the amount of bound hemin was determined after incubation at 4° for the times indicated. The ordinate gives [³H]hemin bound in mol/mol of kinase. The charcoal procedure was used for determination of bound cAMP and hemin. Sigma bovine heart protein kinase was used.
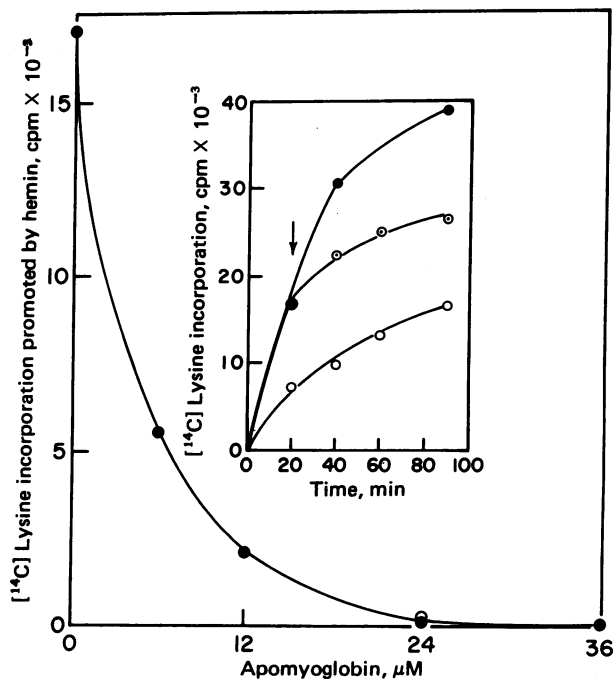
FIG. 5. Reversal by globin of the effect of hemin on protein synthesis in reticulocyte lysates. The protein synthesis assay was used. Samples (25 μl) were incubated for 60 min at 34°, in the absence of hemin and in the presence of 27 μM hemin, with various concentrations of apomyoglobin. The difference in incorporation of [¹⁴C]lysine into protein between samples containing hemin and samples not containing hemin is plotted against the myoglobin concentration (●). In one experiment (○), the samples were preincubated for 15 min at 0° before 10 μg of apomyoglobin (final concentration, 24 μM was added to one of the hemin-containing samples. (*Inset*) Incorporation of [¹⁴C]lysine into protein as a function of time for one incubation without added hemin (○), one with 27 μM hemin (●), and one with 27 μM hemin to which 10 μg of apomyoglobin (final concentration, 24 μM) was added, as shown by arrow, 20 min after the start of incubation (☉).

(Fig. 5) that the level of protein synthesis in the presence of 27 μM hemin was brought back to the nonhemin level by an equimolar concentration of apomyoglobin. A kinetic experiment (Fig. 5 *inset*) in which the apomyoglobin was added to a hemin-containing sample 20 min after the start of translation showed that the reversal was virtually instantaneous.

DISCUSSION

The results presented in this and previous papers from this laboratory provide an explanation for the molecular mechanism of translational control by hemin. cAMP-dependent protein kinase activates eIF-2 kinase (9, 10), an inhibitor of polypeptide chain initiation (5–8, 11), and hemin inhibits the activation of cAMP-dependent protein kinase by cAMP by blocking cAMP binding to the R subunit (10). In this paper we show that hemin blocks cAMP binding because it itself binds to R. The binding of cAMP and hemin is noncompetitive. The fact that hemin can displace bound cAMP but cAMP cannot displace bound hemin may be interpreted to mean that cAMP and hemin bind to different sites of the protein. It was previously suggested (10) that if hemin binds to R, it must bind at a site other than the cAMP binding site since hemin, unlike cAMP, does not activate protein kinase. The results are consistent with the view that hemin prevents cAMP binding to R 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, we find that globin (apomyoglobin) can completely reverse this effect. Globin has a high affinity for hemin and effectively competes with the regulatory subunit for hemin binding. The reticulocyte then synthesizes globin as long as hemin is available and synthesis stops when enough globin has been produced to bind all of the hemin present.

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