INHIBITION OF RABBIT RETICULOCYTE PROTEIN KINASES BY HEMIN *

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SUMMARY

Hemin, at concentrations optimal for globin synthesis, produces inhibition of a specific cytoplasmic protein kinase and a ribosome-associated protein kinase isolated from rabbit reticulocytes. Both enzymes are cyclic AMP-dependent. This inhibition is noncompetitive and greater when ribosomal proteins are used as the phosphate acceptors. The inhibition of these protein kinases by hemin and its partial reversal by globin suggests that hemin regulates protein kinase activity in reticulocytes.

Hemoglobin production in reticulocytes results from the synchronous synthesis of heme and globin (1,2). It is now well established that heme plays a role in the regulation of globin synthesis, but the exact mechanism is unclear (3,4). Indeed, in the absence of heme, globin synthesis is greatly inhibited (5). The recent finding that cyclic AMP reverses this inhibition suggests that a cyclic AMP-dependent protein kinase(s) plays a role in globin synthesis (6). Several protein kinases have been isolated from reticulocyte cytosol as well as from reticulocyte ribosomes, and these enzymes appear to mediate the phosphorylation of ribosomal proteins among other substrates (7,8). However, the exact role of such phosphorylation in protein synthesis remains to be clarified. In recent studies in our laboratory aimed at elucidating the possible role of heme in control of reticulocyte protein kinase activity, we found that hemin inhibits several reticulocyte cyclic AMP-dependent protein kinases in vitro. The results of these experiments and their possible significance form the basis of this report.

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MATERIALS AND METHODS

Hemin (bovine type I, Sigma) was resuspended as a 1 mM solution in 0.25% sodium carbonate at pH 7.0 and stored at -20°. A new aliquot was used for each experiment and all dilutions were done in glass-distilled water. Human globin was a generous gift of Dr. A.G. So. {γ-^{32}P}ATP (> 10 Ci/mmole) was purchased from New England Nuclear.

Reticulocyte cytosol protein kinases were purified from a ribosome-free cytoplasmic fraction obtained from female New Zealand White rabbits made polycythemic by injection of phenylhydrazine (4). The enzymes were partially purified by the method of Tao and Hackett (7). The purification steps used were DEAE-Sephadex which separated kinase I from kinaseII (7), followed by phosphocellulose column chromatography which eliminated most of the autophosphorylation seen (unpublished observations). Kinase II was then subjected to column chromatography on QAE-Sephadex which separated it into kinases IIa and IIb (7). The enzymes were then dialyzed versus buffer A (20 mM Tris-HCl, pH 7.5, 1 mM dithiothrietol) and frozen at -70°.

The ribosomal protein fraction used as a substrate in these experiments was obtained from reticulocyte ribosomes by the method of Kaempfer and Kaufman (9) which involved washing the ribosomes with 0.5 M KCl and collecting the released protein by precipitation with ammonium sulfate. This fraction was then dialyzed versus buffer A and subjected to column chromatography on DEAE-Sephadex (9). The protein fraction used was eluted from this column with buffer A containing 0.25 M KCl, recovered by precipitation with ammonium sulfate as before, and dialyzed versus buffer A. This fraction is believed to contain initiation factor IF-M_3 among other proteins (9).

Protein kinase activity was assayed in 20 µl reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM unlabeled ATP, 50 mM KCl, 1.4 µM{γ-^{32}P}ATP, and 5 µM cyclic AMP when added. Incubations were for 20 minutes at 30°. Trichloroacetic acid-precipitable ^{32}P radioactivity was determined as previously described (10).

TABLE 1

Effect of Hemin on Phosphorylation of Ribosomal Proteins

	cpm ^{32}P incorp./µg ribosomal protein				
			+cAMP		
Enzyme	-cAMP	+cAMP	+hemin: 10µM	35µM	50µM
kinase I	165	631	627(1)	618(6)	492(22)
kinase II	450	530	221(58)	180(64)	88(83)

Protein kinase activity was assayed as described in MATERIALS AND METHODS. Each assay contained 1.8 µg of ribosomal protein, 5 µg of kinase I or II, and cyclic AMP(cAMP) at 5 µM when added. Endogenous phosphorylation in the kinases was negligible in these assays and the contribution of autophosphorylation in the ribosomal protein has been subtracted from the results. Blank values were determined in the absence of ribosomal proteins and subtracted from the results before conversion of the data to the form shown above. The % inhibition of phosphorylation by hemin is in parentheses.

RESULTS

The experiments shown in TABLE 1 reveal that hemin, at physiological concentrations (11), specifically inhibited cytosol cyclic AMP-dependent protein kinase II from rabbit reticulocytes in its phosphorylation of a protein fraction obtained from 0.5 M KCl-washed ribosomes. Both kinase I and kinase II were cyclic AMP-dependent in their phosphorylation of the ribosomal protein, although kinase I was activated to a greater extent by cyclic AMP. Kinase I was mildly inhibited by 10 or 35 μM hemin while kinase II was inhibited markedly at these concentrations. Fifty μM hemin resulted in 83% inhibition of phosphorylation by kinase II.

Figure 1 shows the effect of hemin on the time course of ribosomal protein phosphorylation by kinase II. Substantial inhibition by hemin of kinase II as well as the endogenous protein kinase activity found in the ribosomal wash was seen. Both protein kinases were cyclic AMP-dependent.

It was next of interest to determine if hemin could inhibit the phos-

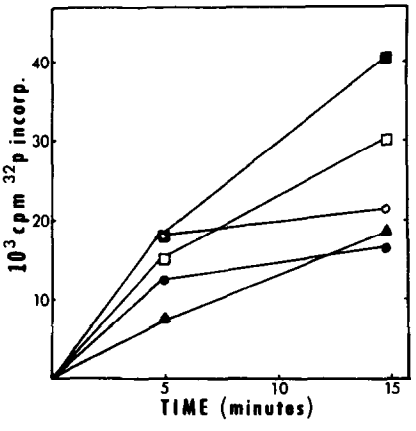

Figure 1: Effect of hemin on the time course of phosphorylation by kinase II. Protein kinase activity was assayed as described in MATERIALS AND METHODS. Each assay contained 27 μg of kinase II, 2.7 μg of ribosomal protein, 50 μM hemin when added, and 5 μM cyclic AMP when added. (●—●) ribosomal proteins alone; (○—○) ribosomal proteins+cyclic AMP; (□—□) kinase II+ribosomal proteins; (■—■) kinase II+ribosomal proteins+cyclic AMP; (▲—▲) kinase II+ribosomal proteins+cyclic AMP+50 μM hemin.

phorylation of another substrate for the reticulocyte kinases, histone. Both kinases were cyclic AMP-dependent in their phosphorylation of this substrate. Kinase II was much more sensitive to inhibition by hemin than kinase I. At 100 μM, hemin inhibited kinase II by 49% and kinase I by 1% (not shown).

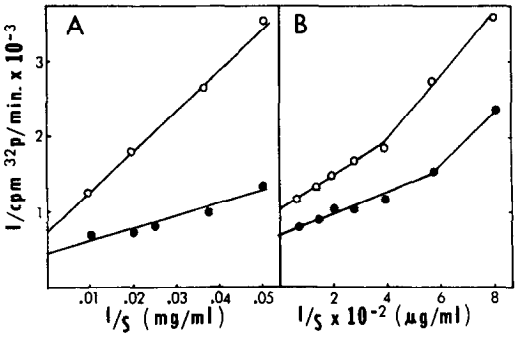

Figure 2: Lineweaver-Burk analysis of hemin inhibition of histone and ribosomal protein phosphorylation by kinase II. Each assay contained 5 μg of kinase II and increasing concentrations of histone (panel A) or ribosomal protein (panel B) as indicated; protein kinase activity was assayed as described in MATERIALS AND METHODS. Panel A: hemin at 100 μM. Panel B: hemin at 50 μM. In both panels: (●—●) -hemin; (○—○) +hemin.

Figure 2 depicts Lineweaver-Burk plots of the effects of hemin on the phosphorylation of histone (panel A) and ribosomal proteins (panel B) by kinase II. Inhibition by hemin in both cases was noncompetitive with respect to the substrate. Hemin resulted in a decrease in the apparent K_m for histone but no change in the apparent K_m for ribosomal proteins. In both cases, the V_{max} of the reaction was decreased.

The biphasic nature of the curves shown in Figure 2B can be explained by the presence of two kinase activities in kinase II with different sensitivities to hemin and/or different affinities for the substrate. When kinase II was separated into kinases IIa and IIb by chromatography on QAE-Sephadex, it was found that kinase IIa was much more sensitive to hemin than

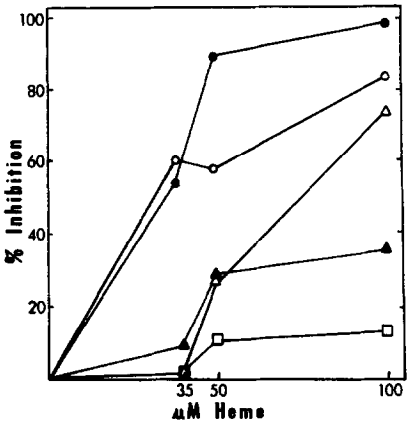

Figure 3: Inhibition of protein phosphorylation as a function of the concentration of hemin. Protein kinase activity was assayed as described in MATERIALS AND METHODS. Each assay contained 5.6 μg of kinase IIa or IIb, 2.4 μg of ribosomal protein when added, 50 μg of histone when added, and 5 μM cyclic AMP. (▲—▲) phosphorylation of histone by kinase IIa; (□—□) phosphorylation of histone by kinase IIb; (○—○) phosphorylation of ribosomal protein by kinase IIa; (△—△) phosphorylation of ribosomal protein by kinase IIb; (●—●) phosphorylation of ribosomal protein by the endogenous protein kinase.

TABLE 2

Reversal of Hemin Inhibition by Globin

	cpm ^{32}P incorp./ 20 minutes			
Additions	-hemin	+hemin (100 μM)	+hemin(100 μM) +globin: 0.3μM	1.5μM
ribosomal protein	4050	872(78)	547(86)	625(85)
ribosomal protein+kinase IIa	6025	1182(80)	1625(73)	1732(71)
ribosomal protein+kinase IIb	5400	1860(66)	1935(64)	3007(44)

Protein kinase activity was assayed as described in MATERIALS AND METHODS. Each assay contained 2.4 μg of ribosomal wash protein, 5.6 μg of kinase IIa and IIb, and cyclic AMP at 5 μM. Hemin and globin were added as indicated. The blanks in these experiments ranged from 200-400 cpm ^{32}P bound/20 minutes and this has been taken into consideration in the table. The incorpoation data reported for kinases IIa and IIb represent incorporation over and above the endogenous phosphorylation in the ribosomal protein fraction. The % inhibition of phosphorylation by hemin is in parentheses.

was kinase IIb when phosphorylating ribosomal proteins and histone. This is shown in Figure 3. Hemin inhibited the phosphorylation of ribosomal proteins to a much greater extent than histone and kinase IIa was more sensitive to hemin than kinase IIb when phosphorylating both substrates. In addition, the endogenous protein kinase in the ribosomal wash was markedly inhibited by hemin.

Inhibition of ribosomal protein phosphorylation by kinase IIb was partially reversed by globin at 1.5 μM (TABLE 2). Phosphorylation catalyzed by the endogenous ribosomal protein kinase and kinase IIa was not reversed by globin at this concentration. These results suggest that kinase IIb has either lower affinity for hemin or higher affinity for globin than the other enzymes.

DISCUSSION

Reticulocyte protein kinases appear to phosphorylate ribosomal proteins, but the role of this phosphorylation in the control of protein synthesis is unknown. The ribosomal protein fraction used in these experiments is believed to contain protein synthesis initiation factor IF-M_3 among other proteins (9). However, at this time we can only speculate that phosphorylation of certain initiation factors or other ribosomal proteins may play a role in the control of protein synthesis. A reticulocyte ribosome-associated protein kinase similar to that reported here has been described previously which also phosphorylates ribosomal proteins (12).

It has long been known that hemin regulates globin synthesis and that the inhibition of globin synthesis that occurs in the absence of heme is due to the appearence of a translational inhibitor (13, 14). The recent finding that cyclic AMP reverses this inhibition of globin synthesis suggests that a protein kinase(s) may be involved in the control of protein synthesis. Levin, et al. (14) have recently reported that cyclic AMP-dependent protein kinase activity is associated with the translational inhibitor mentioned above. The experiments described here show that hemin

inhibits kinase II which may correspond to the translational inhibitor (14). Further studies are in progress to determinewhether the heme effect on globin synthesis seen in reticulocytes is due to the inhibition of the protein kinase activity associated with the translational inhibitor. Whether hemin and globin interact to regulate reticulocyte protein kinase activity is being investigated as well.

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