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Biochemistry

Regulation of protein synthesis in rabbit reticulocyte lysates: Purification and characterization of heme-reversible translational inhibitor

(cyclic 3′:5′-AMP-independent protein kinase/phosphorylation of Met-tRNA$_f$ binding factor/protein synthesis control)

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ABSTRACT To define the mechanism of regulation of the protein kinase that is activated in heme deficiency and that inhibits initiation of protein synthesis, we have isolated and purified the heme-reversible form of the protein kinase from rabbit reticulocytes. The inhibitory activity is found in a single band after polyacrylamide gel electrophoresis under nondenaturing conditions. It migrates as a 95,000-dalton polypeptide in 15% sodium dodecyl sulfate/polyacrylamide gels. This purified inhibitor becomes self-phosphorylated in the presence of ATP; the phosphorylated protein and the inhibitory activity copurify. The inhibitor produces characteristic biphasic kinetics of inhibition in reticulocyte lysates and phosphorylates the 38,000-dalton subunit of eukaryotic initiation factor 2 (eIF-2); the inhibition is reversed by added eIF-2. In contrast to the heme-irreversible inhibitor, this heme-reversible inhibitor is no longer inhibitory after incubation with 20 μM hemin. Incubation with hemin also inhibits self-phosphorylation. Preincubation of the heme-reversible inhibitor in the presence of ATP potentiates the inhibition of protein synthesis in the subsequent incubation, as does treatment with *N*-ethylmaleimide. Phosphorylation of the heme-reversible inhibitor and inhibition of protein synthesis in the lysate due to phosphorylation of eIF-2 appear to be related. These findings suggest that hemin acts directly on the heme-reversible inhibitor.

Protein synthesis in rabbit reticulocytes and reticulocyte lysates is dependent on the presence of hemin (1–5). In the lysate in the absence of added hemin, protein synthesis proceeds for several minutes at the control rate and then declines abruptly (biphasic kinetics). The inhibition is characterized by the rapid disaggregation of polyribosomes (6, 7) and the depletion of [40S–Met-tRNA$_f$] complexes (8–11). The inhibition of protein synthesis occurs at the level of initiation and is due to the activation of a cyclic AMP-independent protein kinase which specifically phosphorylates the smallest subunit of eukaryotic initiation factor 2 (eIF-2) (12–16). In a reconstituted system with highly purified components, phosphorylated eIF-2 is as efficient as unphosphorylated eIF-2 in the formation of 40S and 80S initiation complexes (17). However, recent evidence indicates that the formation of the ternary complex [eIF-2–GTP–Met-tRNA$_f$] and its subsequent binding to the 40S subunit involve other factors in addition to eIF-2 (18–21) and the phosphorylation of eIF-2 may inhibit its interaction with these factors (20–23). The inhibitory kinase, designated heme-regulated inhibitor (HRI, ref. 16) or heme-controlled repressor (HCR, ref. 4), is activated in the absence of added hemin from an inactive form (proinhibitor) in the postribosomal supernatant. The activation process is reversible since addition of hemin to inhibited heme-deficient lysates rapidly restores protein synthesis (24, 25). There is indirect evidence that the conversion of proinhibitor to inhibitor involves a conformational change (25) and that hemin blocks the conversion by binding to the proinhibitor (26). Incubation of the proinhibitor in the absence of hemin for prolonged periods (24) or brief treatment with sulfhydryl reagents (25) yields a form of the inhibitor that is not reversed by hemin. It is this form of the inhibitory kinase that has been purified in several laboratories (12, 16, 27). However, the heme-reversible form of the inhibitory kinase has not been purified to a high degree of purity. We describe now the isolation and purification of the the heme-reversible form of the inhibitory kinase from rabbit reticulocyte lysates. We call this inhibitory kinase reversible HRI to distinguish it from the stable form (irreversible HRI), which was earlier called HRI (16). Further, we present evidence that self-phosphorylation of the purified reversible HRI accompanies its activation in the absence of hemin.

METHODS

Purification of Reversible HRI. *Step 1. pH 5 precipitation.* The postpolyribosomal supernate was prepared as described (28). This supernate, 1250 ml from about 40 rabbits in 20 mM Tris·HCl, pH 7.6/0.25 M sucrose/3 mM $Mg(OAc)_2$/3 mM 2-mercaptoethanol (MSH), was diluted to 2500 ml with 3 mM $Mg(OAc)_2$/1 mM MSH. The pH was brought to 5.1 by addition of 1 M HOAc with stirring at 0°. The precipitate was collected by centrifugation at 6000 rpm for 10 min in the Sorvall GSA-type rotor at 0° and was dissolved in 20 mM Tris base/10 mM Tris·HCl, pH 7.6/0.25 M sucrose/50 mM KCl/6 mM $Mg(OAc)_2$/14 mM MSH (30–40 A_{280}/ml, ~2500 A_{280} total).

Step 2. Ammonium sulfate fractionation. The fractionation was done as described (29). The 25–40% saturation cut (350–400 A_{280}) was dissolved in 20 mM Tris·HCl, pH 7.6/100 mM KCl/0.1 mM EDTA/14 mM MSH/10% glycerol and was dialyzed against 2 liters of this buffer overnight. The protein concentration was about 20 A_{280}/ml.

Step 3. DEAE-cellulose chromatography. The procedure of Ranu and London (16) developed for the purification of HRI was followed, except that the buffers contained 20 mM Tris·HCl (pH 7.6) and 14 mM MSH instead of 3 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.2) and 0.1 mM dithiothreitol. The activity of reversible HRI was assayed as described below. Reversible HRI was eluted between 0.19 and 0.23 M KCl. The fractions containing reversible HRI were pooled and precipitated by addition of $(NH_4)_2SO_4$ to 70% saturation. The precipitate was collected, resuspended in 5 ml of 30 mM phosphate buffer, pH 6.8/40 mM KCl/0.1 mM EDTA/14 mM MSH/10% glycerol (55 A_{280} total protein), and dialyzed overnight against this buffer.

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Abbreviations: HRI, heme-regulated inhibitor; MalNEt, *N*-ethylmaleimide; MSH, 2-mercaptoethanol; $NaDodSO_4$, sodium dodecyl sulfate.

Step 4. Phosphocellulose chromatography. The chromatography was done as described by Ranu and London (16) except that phosphate buffer (pH 6.8) was used instead of Tris buffer. About 80% of the reversible HRI activity was eluted in the 0.2 M KCl step. Fractions containing reversible HRI were precipitated as described above, and the precipitated protein (13 A_{280}) was dissolved in 4 ml of 20 mM phosphate buffer, pH 7.5/50 mM KCl/0.1 mM EDTA/14 mM MSH/10% glycerol and dialyzed against this buffer.

Step 5. Hydroxylapatite chromatography. The reversible HRI from step 4 was applied to a hydroxylapatite column (0.9 × 10 cm) equilibrated with 20 mM phosphate buffer, pH 7.5/50 mM KCl/0.1 mM EDTA/14 mM MSH/10% glycerol. The column was washed with this buffer; the bound protein was then eluted stepwise with the above buffer containing 80 mM phosphate buffer and 200 mM phosphate buffer. Most of the reversible HRI was eluted with 200 mM phosphate buffer.

Step 6. DEAE-Sephadex A50 chromatography. The HRI from step 5 (2 A_{280}) was directly applied to a DEAE-Sephadex A50 column (0.9 × 5 cm) equilibrated with 20 mM Tris·HCl, pH 7.6/100 mM KCl/0.1 mM EDTA/14 mM MSH/10% glycerol. The column was washed with the above buffer and was then step-eluted with the above buffer containing 270 mM KCl and 400 mM KCl. The reversible HRI was eluted at the 400 mM KCl step. The fractions containing reversible HRI were pooled and dialyzed overnight against 80% saturated, degassed ammonium sulfate containing 14 mM MSH. The precipitated protein was collected by centrifugation and dissolved in 30 mM Tris·HCl, pH 7.6/100 mM KCl/0.1 mM EDTA/14 mM MSH/5% glycerol and dialyzed against this buffer.

Step 7. Glycerol gradient centrifugation. Reversible HRI from step 6 (1.5 A_{280}) was applied to a 12-ml, 15–50% glycerol gradient in 30 mM Tris·HCl, pH 7.6/250 mM KCl/0.1 mM EDTA/5 mM MSH. The gradient was centrifuged for 67 hr at 39,000 rpm in a Spinco SW 41 Ti rotor at 4°. Fractions of 0.5 ml were then collected and reversible HRI activity was assayed. The fractions containing reversible HRI (1.5 ml, 0.5 mg of protein total) were frozen in aliquots in liquid nitrogen.

Step 8. Polyacrylamide gel electrophoresis under nondenaturing conditions. The electrophoresis was performed by omitting sodium dodecyl sulfate ($NaDodSO_4$) from system (*ii*) of Schreier *et al.* (29). The separating gel contained 10% (wt/vol) acrylamide and 0.13% (wt/vol) bisacrylamide. Samples were dialyzed overnight against 50 mM Tris·HCl, pH 6.8/14 mM MSH/10% glycerol at 4°. Electrophoresis was performed at 25 mA at 4°. The region of the gel where HRI was located was cut into 1.5-mm slices and each slice was eluted at 0° in 50 μl of 50 mM Tris·HCl, pH 6.8/14 mM MSH/10% glycerol. Reversible HRI activity was determined in the eluate.

Assay of Protein Synthesis and Reversible HRI Activity. The procedures for the preparation of reticulocytes, reticulocyte lysates, and incubation mixtures for protein synthesis have been described (3). Protein synthesis in reaction mixtures was measured by the incorporation of [^{14}C]leucine (specific activity 150 mCi/mol) in 5-μl aliquots (3). Reversible HRI activity was measured in incubation mixtures containing 20 μM hemin. One unit of reversible HRI is defined as the amount of reversible HRI that reduces protein synthesis to 50% in a 20-μl hemin-supplemented incubation mixture incubated for 20 min at 30°. The concentration of reversible HRI was usually so high that the free hemin present did not inactivate a significant portion of the reversible HRI. When the amount of reversible HRI assayed was low, considerable inactivation took place during the incubation in the lysate. This effect can be avoided by pretreatment of aliquots of the fractions to be tested with *N*-ethylmaleimide (MalNEt): 1 μl of 50 mM MalNEt (dissolved in H_2O) was added to a 10-μl aliquot of the fraction to be tested. The mixture was incubated for 3 min at 30°; then 1 μl of 0.1 M MSH was added. An aliquot of the MalNEt-treated reversible HRI was assayed.

Protein Kinase Assays and $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Protein kinase assay mixtures (20 μl) contained 20 mM Tris·HCl (pH 7.6), 60 mM KCl, 2 mM $Mg(OAc)_2$, and 0.1 mM [γ-^{32}P]ATP (1–2 Ci/mmol). For other additions see figure legends. After incubation the samples were diluted by addition of 30 μl of $NaDodSO_4$ sample buffer (29) and heated at 100° for 1 min. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed in system (*ii*) (29), the protein in the gel was stained with Coomassie brilliant blue, and the gel was autoradiographed (13). Stained gels were scanned at 550 nm.

Isolation and Purification of Protein Synthesis Initiation Factors. The initiation factors were prepared from rabbit reticulocytes as described (29).

RESULTS

The postribosomal supernate was the starting material for the preparation of reversible HRI. In order not to produce the stable form of the inhibitor (irreversible HRI), this material was not incubated or treated with MalNEt. The postribosomal supernate was precipitated at pH 5 and the precipitate was fractionated with ammonium sulfate. The conditions of DEAE-cellulose, phosphocellulose, hydroxylapatite, and DEAE-Sephadex chromatography were essentially those described (16) for irreversible HRI. In step 7, reversible HRI was purified further by glycerol gradient centrifugation. Reversible HRI sediments at about 6 S. A similar sedimentation coefficient was determined previously for irreversible HRI (16). The last step in the purification procedure was electrophoresis under nondenaturing conditions (Fig. 1). The main stained protein band (Fig. 1*a*) contained the inhibitory activity (Fig. 1*c*). Although other step 8 preparations sometimes displayed more than one stained protein band, inhibitory activity was always associated with the protein band with the same electrophoretic mobility. When reversible HRI was incubated in the presence of [γ-^{32}P]ATP prior to electrophoresis, the protein band with the inhibitory activity became phosphorylated (Fig. 1*b*). The inhibitory activity eluted from the gel slices displayed some of the characteristics of irreversible HRI (Fig. 2). The extracted material inhibits protein synthesis when added to heme-supplemented reticulocyte lysates with biphasic kinetics; on addition of eIF-2, protein synthesis is resumed in the inhibited lysate and the inhibitory activity phosphorylates the smallest subunit of eIF-2 (Fig. 2, *inset c*). On the autoradiograph of a $NaDodSO_4$ gel (*inset*, Fig. 2), a band with a molecular weight of approximately 95,000 is phosphorylated. This represents phosphorylation of the inhibitory kinase itself (self-phosphorylation). The phosphorylation of the 50,000-dalton subunit of eIF-2 is due to a contaminating protein kinase in the eIF-2 preparation (Fig. 2, *inset b*). The amount of reversible HRI producing about 50% inhibition (2 μl of reversible HRI, Fig. 2) in a 40-μl reaction mixture was in the range of 2–5 ng of the 95,000-dalton polypeptide. The purification procedure is summarized in Table 1. Purified reversible HRI was further analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3). It migrates as a single band with a molecular weight of 95,000 in a 15% gel (acrylamide/bisacrylamide 174:1). Analysis of the same preparation of reversible HRI on 10% $NaDodSO_4$/polyacrylamide gels (acrylamide/bisacrylamide 37:1), however, yields a lower apparent molecular weight of this band, in the range of 80,000 (results not shown) (22). Irreversible inhibitor has been shown (12–16) to be a cyclic AMP-independent protein kinase specific for the smallest subunit of

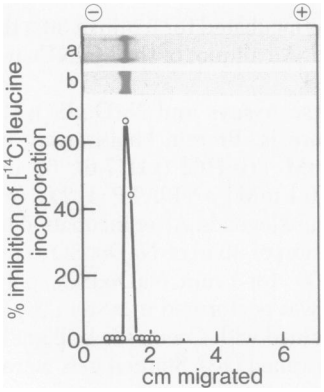

FIG. 1. Polyacrylamide gel electrophoresis under nondenaturing conditions. Reversible HRI from step 7 (25 μg) was subjected to electrophoresis with or without preincubation in the presence of 0.1 mM [γ-^{32}P]ATP (1 Ci/mmol) for 5 min at 30°. (*a*) Stained gel; (*b*) autoradiograph of sample preincubated with [γ-^{32}P]ATP; (*c*) the unstained gel was sliced and aliquots (1 μl) of eluted protein were assayed in 15 μl of protein synthesis reaction mixture. Incubation was at 30° for 20 min. Control sample incorporated 14,052 cpm in 5 μl.

eIF-2. The reversible HRI also phosphorylates the 38,000-dalton subunit of eIF-2 and does not phosphorylate ribosomes or the initiation factors eIF-3, 4A, 4C, and eIF-5 in the absence (Fig. 4) or presence of 10 μM cyclic AMP (results not shown). The preparations of partially purified factors contain endogenous kinase activities (Fig. 4).

In contrast to the preparations of irreversible inhibitor (generated by MalNEt treatment or prolonged incubation in the absence of hemin), reversible inhibitor is no longer inhibitory in the reticulocyte lysate when it is preincubated in the presence of 20 μM hemin (Fig. 5*a*). Preincubation of reversible HRI in the presence of ATP tends to potentiate the inhibition in the subsequent incubation, as does treatment with MalNEt. In this respect reversible HRI behaves in a manner similar to

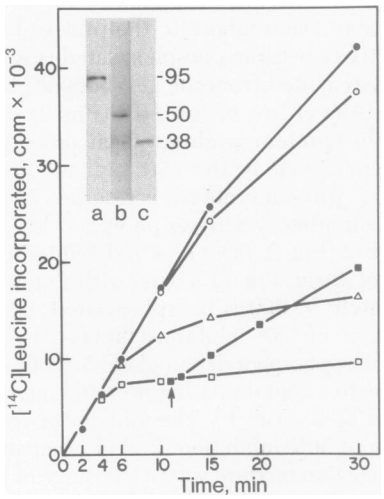

FIG. 2. Characterization of inhibitory activity eluted from nondenaturing polyacrylamide gel slice 5 from Fig. 1. Inhibitory activity was assayed in 40 μl of protein synthesis reaction mixture. ●, Control; ○, 1 μl (about 2 ng); △, 2 μl; □, 3 μl; ■, 3 μl of eluate from slice 5. After 11 min of incubation, 14 pmol of eIF-2 was added to one of the incubations (■). (*Inset*) Autoradiograph of samples electrophoresed in NaDodSO$_4$/polyacrylamide gel: (*a*) step 8 preparation of reversible HRI (about 25 ng) was incubated in 20 mM Tris-HCl (pH 7.6), 60 mM KCl, 1 mM Mg(OAc)$_2$, and 0.1 mM [γ-^{32}P]ATP (1 Ci/mmol) for 10 min at 30°; (*b*) eIF-2 (2.6 μg) was added to the medium described in *a*; (*c*) step 8 preparation of reversible HRI (about 5 ng) and eIF-2 (1.3 μg) were incubated in the medium described in *a*.

Table 1. Purification of reversible HRI

Step	Total protein, mg	Specific activity, units/mg	Purification, -fold
1. pH 5 fraction	3500	89	1
2. $(NH_4)_2SO_4$, 25–40%	400	340	3.8
3. DEAE-cellulose	55	1,090	12.2
4. Phosphocellulose	13	2,307	26
5. Hydroxylapatite	2	13,500	152
6. DEAE-Sephadex A50	1.5	15,333	172
7. Glycerol gradient	0.5	41,500	466
8. Nondenaturing gel	0.1	205,300	2306

that of crude preparations of reticulocyte postribosomal supernatant containing proinhibitor (Fig. 5*b*). Addition of crude proinhibitor preparations preincubated in the absence of hemin leads only to a transient inhibition of protein synthesis in the lysate. With purified reversible HRI at low concentrations we also observe inhibition of its activity by the free hemin in the lysate. The degree of reversibility depends on the relative concentrations of hemin and reversible HRI (Table 2).

Reversible HRI becomes phosphorylated in the presence of [γ-^{32}P]ATP (Figs. 1 and 2 and Fig. 6, slot 1). When reversible HRI is preincubated with hemin, its inhibitory activity for protein synthesis in the reticulocyte lysate disappears (Fig. 5*a*) and the self-phosphorylation of this polypeptide is diminished (Fig. 6, slot 4). Concentrations of hemin at 2–20 μM gradually inhibit the self-phosphorylation (Fig. 6, slots 2–4). Pretreatment of reversible HRI with MalNEt slightly enhances the self-phosphorylation (Fig. 6, slot 5); this phosphorylation is inhibited only slightly (but reproducibly) by hemin (Fig. 6, slot 6). Preincubation of reversible HRI with hemin or high concentrations of cyclic AMP (10 mM) not only inhibits its self-phosphorylation (Fig. 6, slots 4 and 7), but also diminishes in a similar fashion the labeling of added eIF-2 (Fig. 6, slots 8, 9, and 11). MalNEt-treated reversible HRI, however, is not inhibited in its kinase activity towards eIF-2 (Fig. 6, slot 10). These data suggest that phosphorylation of reversible HRI and inhibition of protein synthesis in the lysate by the phosphorylation of eIF-2 may be related.

The molecular weight of reversible HRI was estimated by glycerol gradient centrifugation (Fig. 7). Reversible HRI sediments faster than bovine serum albumin and at a rate very similar to that of eIF-2 (molecular weight 140,000). A similar value was obtained with irreversible HRI by gel filtration (16). Preincubation of reversible HRI in the presence of hemin and sedimentation in a gradient containing hemin do not detectably change the sedimentation coefficient of this protein (Fig. 7).

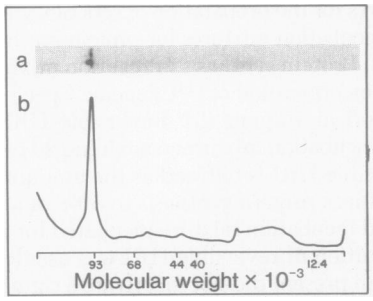

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of reversible HRI from step 8. Gel slice 5 (Fig. 1), containing reversible HRI, was incubated with 1% NaDodSO$_4$ and was then electrophoresed. The molecular weight markers were: cytochrome *c*, ovalbumin, aldolase, bovine serum albumin, and phosphorylase A. (*a*) Stained gel; (*b*) absorbance profile of stained gel.

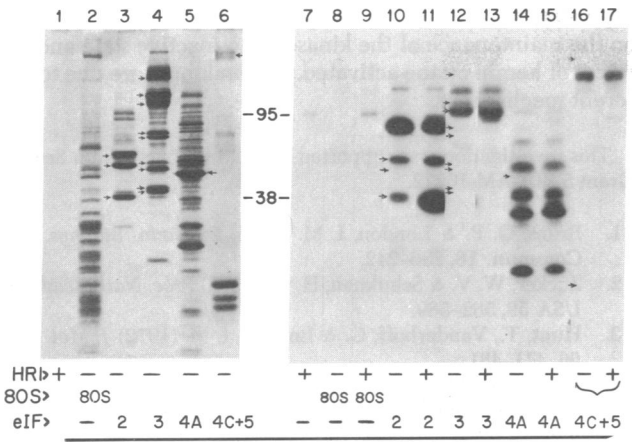

FIG. 4. Substrate specificity of purified reversible HRI from step 8. Protein kinase was assayed (20 μl) and the samples were analyzed by autoradiography of the stained NaDodSO$_4$/polyacrylamide gels. Slots 1–6, the strained gel; slots 7–17, autoradiographs. Arrows indicate the positions of polypeptides of the various initiation factors. Slots 1, 7, 9, 11, 13, 15, and 17 contained reversible HRI from step 8 (about 10 ng). Other additions were: slots 1 and 7, none; slots 2, 8, and 9, 0.3 A_{260} unit of 80S ribosomes; slots 3, 10, and 11, eIF-2 (3 μg); slots 4, 12, and 13, eIF-3 (6 μg); slots 5, 14, and 15, eIF-4A (6 μg); slots 6, 16, and 17, mixture of eIF-4C and eIF-5 (2 μg).

From these data we conclude that the activation-inactivation reaction of the inhibitor does not result in a detectable change in its molecular weight. Since reversible HRI sediments at a rate similar to that of eIF-2, the question of whether it is a single polypeptide or a dimer of the 95,000- (80,000 in a different gel system) dalton polypeptide remains to be answered.

DISCUSSION

Incubation of reticulocyte lysates in the absence of hemin for short periods of time results in the activation of an inhibitor of protein chain initiation. The inhibitor formed is readily inactivated by addition of hemin (24, 25). The purification procedure described here yields an inhibitor preparation that is inactivated by the free hemin in the lysate (Table 2) or by

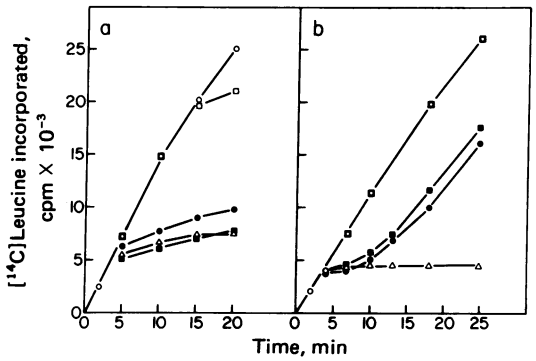

FIG. 5. Effect of preincubation of reversible HRI with hemin on its inhibitory activity in lysates. (*a*) Reversible HRI from step 7 (0.1 μg) was preincubated in 20 μl of reaction mixture (as in the protein kinase assay) for 3 min at 30° and 4 μl (20 ng) was assayed in 30 μl of protein synthesis reaction mixture. ○, Control; □, preincubated with 20 μM hemin; ●, preincubated in the absence of hemin; ■ preincubated with 0.1 mM ATP; △, preincubated with 2.5 mM MalNEt and then with 5 mM MSH. (*b*) Reticulocyte postribosomal supernate was used as a source of reversible HRI. Supernate (750 μg of protein in 15 μl) was preincubated as described above and was then assayed in 35 μl of protein synthesis reaction mixture. Preincubation was for 10 min at 30°. ○, Control; ●, preincubated in the absence of hemin; □, preincubated with 20 μM hemin; ■, preincubated with 0.5 mM ATP; △, preincubated with 2.5 mM MalNEt and then with 5 mM MSH.

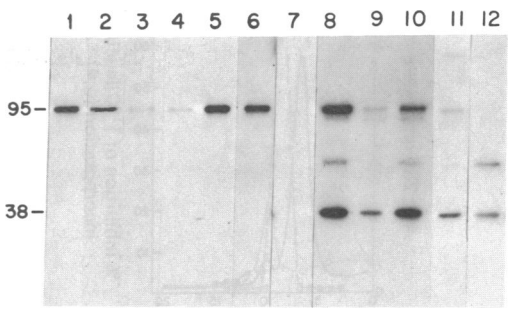

FIG. 6. Self-phosphorylation of reversible HRI and phosphorylation of eIF-2. The protein kinase assays (20 μl) were performed as described (*Methods*) except that the incubation was done in two steps: Preincubation with various reagents was followed by a second incubation in the presence of 0.1 mM [γ-^{32}P]ATP (1 Ci/mmol). Both incubations were for 5 min at 30°, and 100 ng of reversible HRI from step 7 (slots 1–7 and 11) or 10 ng of reversible HRI from step 8 (slots 8–10) was added to each reaction mixture. (Slot 1) Preincubated without hemin; (slot 2) preincubated with 2.5 μM hemin; (slot 3) preincubated with 10 μM hemin; (slot 4) preincubated with 20 μM hemin; (slot 5) preincubated with 2.5 mM MalNEt for 2 min, then 5 mM MSH was added; (slot 6) preincubated as in slot 5 and then 20 μM hemin was added; (slot 7) preincubated with 10 mM cyclic AMP; (slot 8) preincubated without hemin, and then 3 μg of eIF-2 was added; (slot 9) preincubated with 20 μM hemin, and then 3 μg of eIF-2 was added; (slot 10) preincubated with 2.5 mM MalNEt for 2 min, then 5 mM MSH was added, the sample was further incubated with 20 μM hemin, and then 3 μg of eIF-2 was added; (slot 11) preincubated with 10 mM cyclic AMP and then 3 μg of eIF-2 was added; (slot 12) preincubated without reversible HRI and then 3 μg of eIF-2 was added.

preincubation with 10–20 μM hemin (Figs. 5 and 6). At high concentrations of reversible HRI the free hemin in the lysate, however, is not sufficient to inactivate all of the added reversible HRI, and preincubation with higher hemin concentrations is required (results not shown). Preincubation with hemin also blocks the phosphorylation of reversible HRI in the presence of [γ-^{32}P]ATP (self-phosphorylation). Although the active inhibitor phosphorylates itself whereas hemin-inactivated inhibitor does not, these findings do not prove that phosphorylation of reversible HRI is necessary for its activation.

The behavior of reversible HRI during purification by ion-

Table 2. Inactivation of reversible HRI by hemin

Reversible HRI, μg	Protein synthesis, cpm		
	Untreated	MalNEt treated	Hemin treated
Exp. 1			
1.5	3,340	2,872	—
0.45	6,677	3,693	—
0.15	24,926	5,890	—
0.045	23,300	26,849	—
—	24,042	24,042	—
Exp. 2			
0.075	4,743	—	5,560
0.0225	10,787	—	22,378
0.0075	27,464	—	33,667
—	32,376	—	32,376

MalNEt treatment was carried out in 20 mM Tris·HCl, pH 7.6/60 mM KCl/2 mM Mg(OAc)$_2$ in the presence of 2.5 mM MalNEt. Incubation was at 30° for 3 min and then 5 mM MSH was added. Reversible HRI from step 4 was used in Exp. 1. Hemin treatment (20 μM) was performed with reversible HRI from step 7 at 30° for 3 min. Aliquots of inhibitor preparations were assayed in 15 μl (Exp. 1) or 35 μl (Exp. 2) of protein synthesis reaction mixture containing 20 μM hemin. Protein synthesis (in 5-μl aliquots) was assayed after incubation at 30° for 25 min (Exp. 1) or 30 min (Exp. 2).

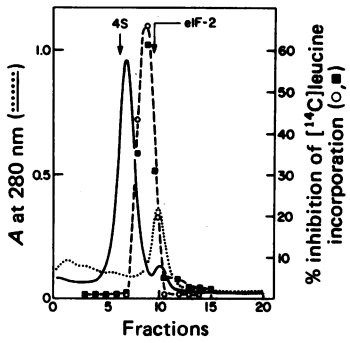

FIG. 7. Estimation of the molecular weight of reversible HRI by sedimentation in glycerol gradients. The centrifugation conditions were as described (*Methods*) except that the KCl concentration in the gradient was 100 mM. ○, Reversible HRI from step 7 (30 μg) was applied to a gradient. After centrifugation, 1 μl of every fraction was assayed for inhibitory activity in a 15-μl reaction mixture; ■, Reversible HRI from step 7 (30 μg) was incubated for 3 min at 30° with 20 μM hemin and was then applied to a glycerol gradient containing 20 μM hemin. After centrifugation, every fraction was treated with 2.5 mM MalNEt for 3 min at 30° and then 5 mM MSH was added. Aliquots (1 μl) were assayed for inhibitory activity in a 15-μl reaction mixture; the control (0% inhibition) was 22,611 cpm. (. . . .) Glyceraldehyde-3-phosphate dehydrogenase, 0.5 mg; (—) bovine serum albumin, 1.5 mg. Arrows indicate the positions of 4S reticulocyte tRNA and eIF-2.

exchange chromatography and glycerol gradient sedimentation is very similar to that described for the irreversible inhibitor (12, 16). Moreover, Farrell *et al.* (12) observed that the inhibitory activity was associated with a protein (or proteins) that gave rise to two to three closely running bands in $NaDodSO_4$ gels ranging in molecular weight from 86,000 to 96,000. The 96,000-dalton protein became phosphorylated in the presence of [γ-^{32}P]-ATP.

Reversible HRI, like the stable inhibitor, is a cyclic AMP-independent protein kinase which specifically phosphorylates the smallest subunit of eIF-2 (Fig. 4). Preincubation with hemin inactivates this kinase (Fig. 6), a finding that supports the concept that in heme deficiency, the activated kinase phosphorylates eIF-2 and causes the inhibition of protein chain initiation (12, 16, 22).

The blocking of reversible HRI by preincubation with hemin does not result in a measurable change of its sedimentation rate (Fig. 7), and the reported S value for irreversible HRI (16) is very similar to that of reversible HRI. The simplest explanation for these findings is that reversible HRI is able to bind hemin and that the [hemin–reversible HRI] complex is not inhibitory. It was suggested earlier that hemin might directly interact with the reversible form of the inhibitor and inactivate it by introducing a conformational change (24, 25). Activation of the inhibitor would then be due to the loss of hemin from the hemin-reversible HRI complex or proinhibitor, with the result that its conformation would change and self-phosphorylation would occur. Prolonged incubation in the absence of hemin or treatment with sulfhydryl reagents (in the presence or absence of hemin) of reversible HRI could change its conformation, or lead to a modification, so that it is unable to interact with hemin.

Our data with purified reversible HRI suggest that hemin acts directly on reversible HRI. We recognize, however, the possibility that in the crude reticulocyte lysate additional component(s) may be associated with this protein kinase which may be involved in its activation. It has been suggested that the inhibitory protein kinase may be activated by a cyclic AMP-dependent kinase (30). It is also possible that the effect of hemin on the maintenance of the kinase in an inactive state and the effect of hemin on the activated protein kinase are due to different mechanisms.

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