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# REGULATION OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATES: SEPARATION OF HEME REGULATED PROTEIN KINASE INTO A HIGH AND A LOW MOLECULAR WEIGHT SPECIES

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Summary: Protein synthesis in rabbit reticulocyte lysates is regulated by heme. In heme deficiency, a heme regulated protein kinase (HRI) is activated that phosphorylates initiation factor eIF-2. Consequently, eIF-2 is inactivated. Results described in this report show that HRI exists in crude and highly purified preparations in two forms; a high molecular weight component which sediments at a sedimentation co-efficient of 14-15S and a previously described 5.8S component (Ranu, R. S. and London, I. M. (1976) Proc. Natl. Acad. Sci. USA 73, 4349-4353). The 14-15S HRI selfphosphorylates poorly and undergoes dissociation into the 5.8S component via an intermediate of 8.5-9S. The 5.8S HRI, on weight basis, is about 5-10 times more active than the 14-15S HRI. In addition, a phosphoprotein phosphatase has been detected in lysates that dephosphorylates selfphosphorylated HRI. This observation suggests that phosphate on HRI turns over. These findings may be relevant to the mechanism of activation and inactivation of HRI in the absence and presence of heme in situ.

Protein synthesis in rabbit reticulocyte lysates is regulated by the availability of heme (1,2). In heme deficiency, a heme regulated translational inhibitor (HRI) is activated that blocks protein chain initiation (2). The HRI has been identified as an adenosine 3':5' cyclic monophosphate independent protein kinase that specifically phosphorylates the 38,000-dalton subunit of initiation factor eIF-2 (2-6). As a consequence, eIF-2 is inactivated (7-9). eIF-2 promotes the formation of a ternary complex (eIF-2.GTP.Met-tRNA$_f$) with GTP and Met-tRNA$_f$ (2), the first rate limiting reaction in the protein chain initiation cycle (10).

In a previous study we described the purification of HRI (3). The purified HRI exhibits a sedimentation co-efficient of 5.8S and a native molecular weight of 140,000-daltons (3). On electrophoresis in sodium dodecyl sulfate-polyacrylamide gel, purified HRI migrates as a single polypeptide of 80,000-daltons (6,11-13). In the presence of $Mg^{++}$-ATP, HRI undergoes a rapid selfphosphorylation. This selfphosphorylation is associated with enhancement of HRI activity (14,15). The mechanism of

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activation of HRI in heme deficiency is not known, however. In this communication, we show that both the highly purified and the crude HRI exist as a high molecular weight complex of about 15S that undergoes dissociation into the 5.8S HRI via a 8.5S-9S intermediate. We also show that lysates contain a phosphoprotein phosphatase that dephosphorylates HRI. These findings may have a bearing on the mechanism of activation and inactivation of HRI.

## MATERIALS AND METHODS

The following procedures has been described previously: preparation of rabbit reticulocyte lysates; protein synthesis mixtures; assay of protein synthesis; purification of HRI; preparation of purified eIF-2; eIF-2 protein kinase assay; SDS-polyacrylamide gel electrophoresis; and autoradiography of polyacrylamide gel (13). Sources of other materials has been described (13).

Preparation of [$^{32}$P]HRI: The purified HRI (4-6 µg) in 10 µl was incubated in buffer (Tris-HCl (pH 7.6), 10 mM; KCl, 50 mM; $Mg(Ac)_2$, 1 mM; dithiothreitol (DTT), 1 mM) containing 0.1 mM [$\gamma^{32}$P]ATP (specific activity 12,000 CPM/pmol) at 30° for 5 min. The free [$\gamma^{32}$P]ATP was removed by gel filtration on a sephadex-G25 column.

HRI phosphoprotein phosphatase assay: The phosphoprotein phosphatase activity that dephosphorylates HRI was assayed in lysates. Reaction mixture (30 µl) containing 15 µl of lysates; hemin (20 µM); [$^{32}$P]HRI (12,000 CPM) and appropriate concentration of various components of protein synthesis mixtures (13) was incubated at 30°. At intervals aliquots (3 µl) were removed and transferred to 15 µl of denaturing solution (Tris-HCl, 50 mM (pH 7.0); 2% SDS and 5% β-mercaptoethanol). The samples were heated at 100° and were then subjected to electrophoresis in 10% SDS-polyacrylamide gel. After the gel has been autoradiographed, the 80,000-dalton HRI polypeptide region of the gel was excised and the radioactivity associated with the polypeptide was determined.

Preparation of crude HRI for chromatography on Sephacryl-S300 superfine. Ribosome free lysate supernate (12 ml containing 40 mg/ml of protein) was brought to 40% saturation by the addition of saturated solution of $(NH_4)_2SO_4$. After 10 min in an ice bath, the precipitate was collected. It was dissolved in 0.8 ml of Buffer A (Hepes, 10 mM (pH 7.2), KCl, 50 mM; DTT, 1 mM; EDTA, 0.1 mM and 10% glycerol). The protein was dialyzed against the same buffer. An aliquot of this HRI protein preparation (0.45 ml (34 mg/ml)) was activated by incubation with 7 mM N-ethylmalemide (NEM) at 30° for 15 min. The excess NEM was inactivated by the addition of 11 mM DTT.

## RESULTS AND DISCUSSION

In an earlier work we described the purification of HRI (3). The purified HRI exhibited a sedimentation co-efficient of 5.8S and a native molecular weight of 140,000-daltons. It is an open question, however, whether molecular species other than the 5.8S component are also present in HRI preparations. In the course of purification of a preparation of HRI by our standard purification procedure, which involves: $(NH_4)_2SO_4$ precipitation (0-40% cut), DEAE-cellulose and phosphocellulose chromatographies, followed by a glycerol density gradient centrifugation (3,12), a high molecular weight species of HRI was detected. This HRI component sedimented faster than catalase (sedimentation co-efficient, 11.3S). In

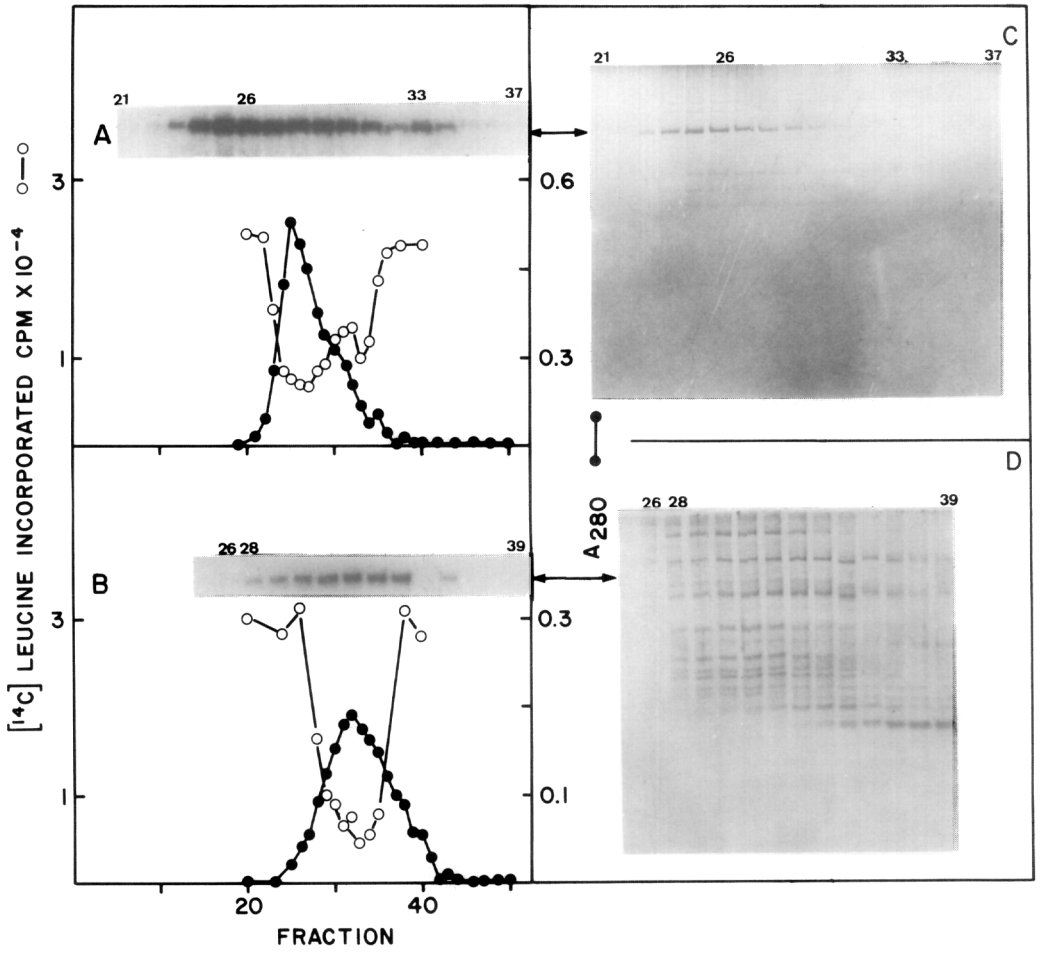


Fig 1.Gel filteration of low and high molecular weight form of HRI on Sephacryl-S300 superfine. High (3 mg in 0.43 ml) or low (1 mg in 0.5 ml) molecular weight form of HRI were chromatographed separately by gel filteration on a Sephacryl-S300 superfine column (0.9 X 55 cm) equilibrated with Buffer B (Tris-HCl, 10 mM (pH 7.6); KCl, 100 mM: DTT, 0.5 mM, EDTA, 0.1 and 5% glycerol). The column was developed at a rate of 2 ml/hr. An aliquot (2 µl) of each fraction was assayed for HRI activity in a protein synthesis assay (10 µl). Incubation was at 30° for 40 min and 5 µl aliquots were assayed (13). Fractions were also assayed (5 µl each of high and 10 µl each of low molecular weight form of HRI) for selfphosphorylation as described before (13) and were then subjected to electrophoresis in 10% SDS-polyacrylamide gel. The proteins in gel were stained with Coomassie brilliant Blue (C, high molecular weight and D, low molecular weight HRI) and were then autoradiographed (results shown in the inset in A and B). NB: The lack of phosphorylation in the autoradiogram in fraction 35 (panel B) is due to an inadvertent lack of addition of [$\gamma^{32}$P]ATP mixture to the fraction.

order to determine the relationship between the high molecular weight component and the standard 5.8S HRI, the two fractions were pooled, separately. Judging from the stained gel polypeptides at this stage of purification, the high molecular weight HRI was nearly 60% pure; whereas

the 80,000-dalton polypeptide in the 5.8S HRI was hardly detectable. The two fractions were brought to 50% saturation with saturated solution of $(NH_4)_2SO_4$. The protein precipitate was dissolved in 0.4 ml of buffer B (See Fig 1). The protein fractions were dialyzed against the buffer.

The high and low molecular weight HRI components were subjected to gel filteration on Sephacryl-S300 superfine, separately. Data in Fig 1 show that the high molecular weight HRI chromatographed well ahead of the low molecular weight HRI (Fig 1, A and B). The high molecular weight HRI fraction also exhibited an inhibitory fraction that cochromatographed with the low molecular weight component (see fraction 33 in Fig 1A). The major protein fraction in these two molecular species also cochroma-

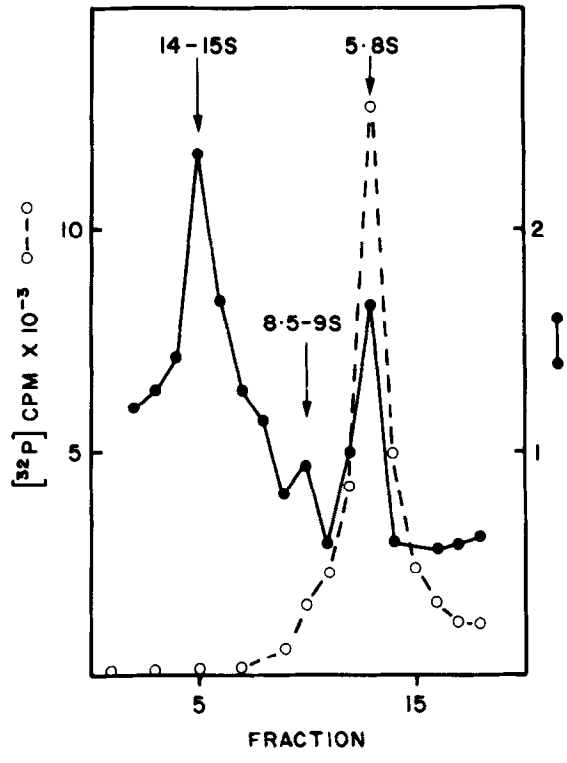


Fig 2. Glycerol density gradient centrifugation of low and high molecular weight forms of HRI: Aliquots (55 µl) of peak HRI fraction from low and high molecular weight forms of HRI (from a Sephacryl-S300 column) were selfphosphorylated in the presence of 0.1 mM [$\gamma^{32}$P]ATP (specific activity 10,000 CPM/pmol) as described in methods section. The samples were applied on two separate, 10-40% glycerol density gradients prepared in Buffer B. The samples (along with standard proteins; myoglobin, bovine serum albumin and catalase) were centrifuged at 48,000 rpm for 15 hr at 2° in a Spinco SW 50.1 rotor. The gradients were fractionated and 100 µl aliquots of each fraction were used to determine trichloroacetic acid precipitable CPM and 45 µl aliquots of fraction were denatured in 10% SDS and 5% β-mercaptoethanol and were subjected to electrophoresis in 10% SDS-polyacrylamide gel. The gels were autoradiographed and counted. O--O, low and ●—●, high molecular weight forms of HRI.

tographed with protein synthesis inhibitory and selfphosphorylating activity of HRI (Fig 1A and B and the inset). The analysis of each fraction by electrophoresis in SDS-polyacrylamide gel showed that nearly 60-70% of the total protein in the high molecular weight fraction consisted of the 80,000-dalton polypeptide of HRI (Fig 1C). Whether these other polypeptides are contaminants or are a part of the HRI complex is not clear at this time. There was barely detectable HRI stained polypeptide in the low molecular weight component (Fig 1 D). Based on the intensity of staining of HRI polypeptide in SDS-polyacrylamide gel (Fig C & D), the degree of selfphosphorylation (inset Fig 1 A and B), and the protein synthesis inhibitory activity associated with these fractions (Fig 1 A and B), the results suggest that the low molecular weight HRI is about 5-10 times more active than the high molecular weight HRI.

When the high and the low molecular weight HRI fractions were at first selfphosphorylated in the presence of [$\gamma^{32}$P]ATP and were then

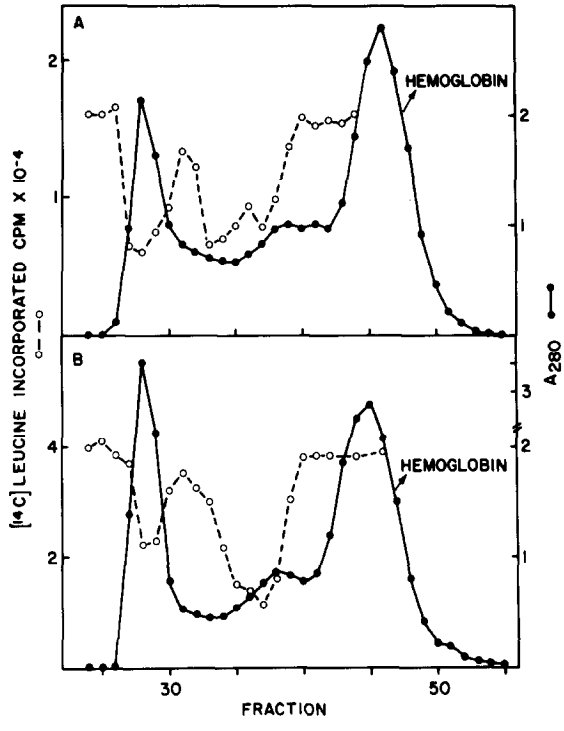


Fig 3. Chromatography of crude HRI on Sephacryl-S300 superfine. Crude HRI with or without activation with NEM (15 mg of protein in 0.45 ml) was applied to a Sephacryl-S300 superfine column (0.9 x 58 cm) preequilibrated with Buffer A. The column was developed at a rate of 2 ml/hr. Aliquots (0.5 µl of NEM activated and 3 µl of nonactivated) of each fraction were assayed for HRI activity in a 10 µl lysate protein synthesis mixture containing 20 µM hemin (13). The incubation was at 30° for 40 min and 5 µl aliquots were assayed for protein synthesis (13). A, nonactivated HRI; B, NEM activated HRI.

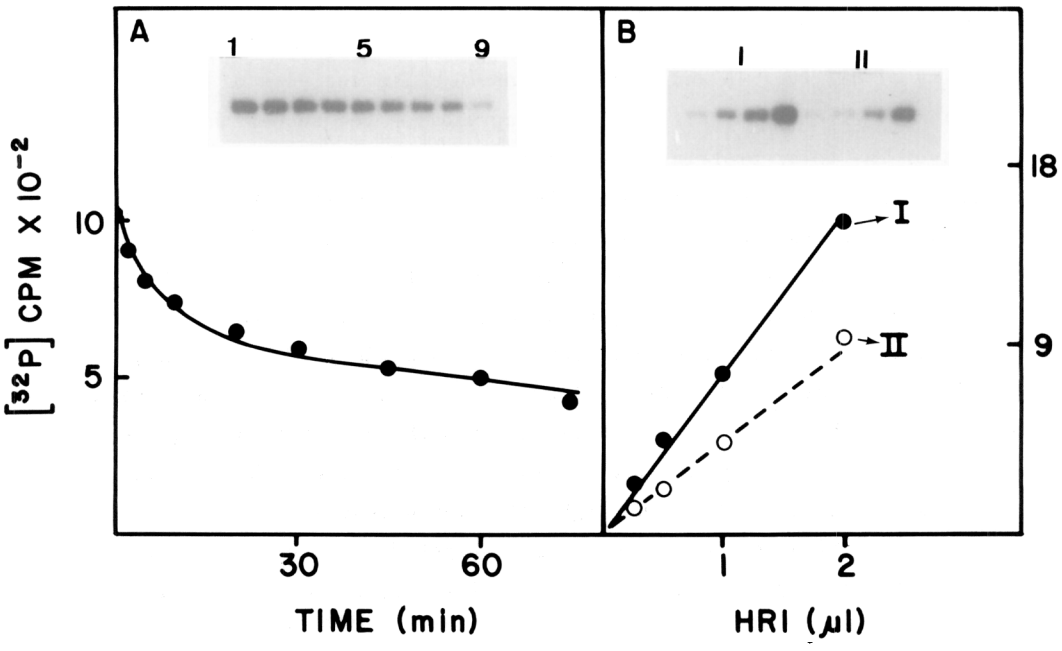


Fig 4. Dephosphorylation of HRI in lysates: Lysate protein synthesis reaction mixtures (30 µl in A and 10 µl in B) were incubated at 30° with [$^{32}$P]HRI (10,000 CPM in 4 µl in A and as indicated in B). At intervals, aliquots (3 µl) were removed and transferred to 15 µl of denaturing solution. The samples were subjected to electrophoresis in 10% SDS-polyacrylamide gel. The proteins in gel were stained with Coomassie brilliant blue and an autoradiogram was prepared. The 80,000-dalton HRI polypeptide from the gel was excised and radioactivity associated with the polypeptide was determined. The inset is an autoradiogram of the data in the figures. A; time course of dephosphorylation of HRI: samples 1 to 9 were incubated for 0, 2, 5, 10, 20, 30, 45, 60 and 75 min, respectively. B; effect of HRI concentrations on dephosphorylation; I, unincubated controls and II, samples that were incubated for 45 min at 30°.

subjected to a glycerol density gradient centrifugation, the low molecular weight HRI sedimented as a standard 5.8S component (3). The high molecular weight HRI, however, exhibited three peaks of activity; one sedimenting at about 14-15S, a second peak which cosedimented with the standard 5.8S HRI, and a third peak of intermediate sedimentation co-efficient of 8.5-9S. The presence of a 5.8S and an intermediate of 8.5-9S in high molecular weight HRI in spite of the fact that the preparation used in this experiment was taken from the peak fraction obtained by gel filteration (Fig 1A), suggests that during incubation with [$\gamma^{32}$P]ATP at 30°, a fraction of 14-15S component underwent dissociation into 5.8S component via an intermediate of 8.5-9S.

The finding of a high molecular weight HRI in highly purified HRI preparations in high proportion raised the possibility that similar components may be present in crude preparations of HRI. A crude preparation of HRI (a 0-40% $(NH_4)_2SO_2$ saturation cut) was, therefore, subjected to

gel filteration with or without activation with NEM (Fig 3 A & B). Once again as with the purified HRI preparation, two main peaks of inhibitory activity of HRI were observed in NEM treated sample (Fig 3 B). The sample which was not treated with NEM showed in addition to the low and high molecular weight HRI activities, an activity that chromatographed between the low and high molecular HRI components (Fig 3A). The pretreatment of HRI with NEM completely converts this intermediate into low molecular weight HRI (Fig 3B). On glycerol density gradient centrifugation, the crude HRI preparation also gave results similar to those described for the highly purified HRI preparation in Fig 2 (results not shown).

The selfphosphorylation of HRI suggests that inactivation of HRI may involve a phosphoprotein phosphatase that dephosphorylates the phosphorylated HRI. The presence of such a phosphatase was determined by examining the rate and extent of dephosphorylation of [$^{32}$P] labelled HRI. The results are presented in Fig 4, A & B (the inset is an autoradiogram of the data in the figure). The data show a slow and progressive dephosphorylation of HRI. This finding suggests that the phosphate on active HRI is not stable and that it undergoes turnover. We have observed that hemin does not increase the rate of this dephosphorylation reaction, and, unlike the eIF-2 phosphoprotein phosphatase (16), NEM inhibits the HRI phosphatase poorly (results not shown).

In an earlier study we showed that HRI selfphosphorylation is associated with an increase in protein sysnthesis inhibition and eIF-2 phosphorylation activities of HRI (3,12,14,15). The present finding of a high molecular weight HRI that selfphosphorylates less efficiently and exhibits low protein synthesis inhibition activity suggests that in reticulocytes HRI may exist primarily in this high molecular weight form. This observation is relevant in view of the fact that in intact cells hemin addition restores protein synthesis rapidly and without a significant lag period (17). In extreme heme deficiency which is generated in lysates *in vitro*, the high molecular weight form of HRI may dissociate into 5.8S form via the 8.5-9S intermediate. Since 5.8S HRI is able to selfphosphorylate much more efficiently, the enhanced selfphosphorylation activity may be responsible for a marked increase in protein synthesis inhibition activity of this form of HRI. These observation coupled with the finding of a phosphoprotein phosphatase that dephosphorylates HRI would suggest that in the presence of hemin the dephosphorylation and reassociation of low molecular weight HRI into the high molecular weight HRI complex may be associated with the inactivation of HRI. The dephosphorylation and reassociation kinetics may represent a rate limiting step in the inactivation of HRI. It is possible that hemin may be

effective only in the reassociation of dephosphorylated low molecular weight HRI. This observation may explain the increase in lag period that is observed in the restoration of synthesis when hemin addition to hemin deficient lysates is progressively delayed (11). The presence of a low affinity heme binding fold(s) in HRI analogous to the heme binding folds in hemoglobin is now an open possibility (18). These observations also support the previous studies of others which suggest direct effect of heme in maintaining HRI in an inactive form (3,11).

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