Vol. 97, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 17, 1980 Pages 252-262

REGULATION OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATES: PURIFICATION AND INITIAL CHARACTERIZATION OF THE DOUBLE STRANDED RNA ACTIVATED PROTEIN KINASE

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Received September 30,1980

Summary: Double stranded RNA (dsRNA) activated translational inhibitor (dRI) has been purified from rabbit reticulocyte lysates. Purified dRI inhibits protein synthesis in rabbit reticulocyte lysates with inhibition kinetics that parallel those observed by the addition of dsRNA (0.1-100 ng/ml). The inhibition is overcome by initiation factor eIF-2. Purified dRI migrates as a single polypeptide of 70,000-daltons when subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gel. A molecular weight of 70,000-daltons and a sedimentation co-efficient of 3.2S is obtained by gel filtration on Sephacryl S-300 (superfine) and by glycerol density gradient centrifugation, respectively. The inhibitor activation is accompanied by the dsRNA-dependent phosphorylation of the 70,000-dalton polypeptide of dRI. Purified dRI is a cyclic-AMP independent protein kinase that phosphorylates the 38,000-dalton subunit of eIF-2.

Double stranded RNA (0.1 to 100 ng/ml) inhibits protein chain initiation in rabbit reticulocyte lysates and in extracts of other eukaryotes (1,2). Inhibition of protein synthesis is preceded by the activation of a translational inhibitor (dRI) (3,4). Farrell et al (4) have previously shown that the activation of dRI is accompanied by the double stranded RNA-dependent phosphorylation of a ribosome associated polypeptide of about 67,000-daltons and dRI preparations phosphorylate the 38,000-dalton subunit of initiation factor eIF-2 that forms a ternary complex (eIF-2·GTP·Met-tRNA$_f$) with Met-tRNA$_f$ and GTP (4-7).

There are many features of inhibition of protein synthesis produced by the addition of double stranded RNA (dsRNA) and by heme-deficiency in rabbit reticulocyte lysates which suggest that a common mechanism is involved in the inhibition of protein synthesis (reviewed in 8). These include: a, biphasic kinetics of inhibition of protein synthesis in which protein synthesis proceeds at the control rate for several minutes followed by an abrupt decline in the rate of synthesis (8); b, inhibition is preceded by a marked decline in formation of the complexes of 40S ribosomal subunits and Met-tRNA$_f$ (8); c, eIF-2 overcomes these inhibitions (8), and dRI and the heme-regulated protein kinase (HRI) that is activated in heme-deficiency phosphorylate the same site(s) of eIF-2 (4-6). These characteristics of inhibition of protein synthesis are also shared by the

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similar eIF-2 specific protein kinases that have been isolated from diverse eukaryotes (9). These findings support the contention that phosphorylation-dephosphorylation of eIF-2 is one of the major mechanisms that regulates protein biosynthesis in eukaryotes (9).

In an earlier report we (5) and subsequently others (6,10) described the partial purification of dRI. I describe here the purification and characterization of dRI. dRI has been purified to apparent homogeneity. dRI is a single polypeptide of 70,000-daltons that is phosphorylated in the presence of dsRNA. Purified dRI inhibits protein synthesis in rabbit reticulocyte lysates and phosphorylates the 38,000-dalton subunit of eIF-2.

MATERIALS AND METHODS

The following procedures have been described: preparation of rabbit reticulocyte lysates; protein synthesis mixtures; assay of protein synthesis; preparation of purified eIF-2; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the autoradiography of polyacrylamide gel; phosphorylation assay (11); preparation of eIF-2 stabilization factor (SF) and CM-eIF-2; and the ternary complex inhibition assay (12). tRNA-Sepharose and Heparin-Sepharose were prepared according to Lamed et al. (13) and Waldman et al. (14), respectively.

Assay of dRI activity: The activity of dRI in fractions from various stages of purification was assayed in a standard protein synthesis incubation mixture (25 µl) containing optimal levels of hemin (10 µM). One unit of dRI is defined as the minimal amount of dRI protein that diminishes protein synthesis by 50% in a reaction mixture incubated at 30° for 40 minutes.

Activation of Pro-dRI: The dRI purification procedure described in the succeeding section was carried out without the preactivation of dRI in the presence of ATP and dsRNA. Hence, it was necessary to convert the inactive form of dRI to an active form for the assay of dRI activity in the protein synthesis inhibition assay. The inactive form of dRI will be referred to as Pro-dRI. The activation reaction mixture (10 µl) in buffer (Tris-HCl, 20 mM [pH 7.8]; KCl, 50 mM; Magnesium acetate, 2 mM; dithiothreitol [DTT], 1 mM containing 0.1 mM ATP and 0.34 ng dsRNA were incubated at 30° for 15 min. High concentration of dsRNA (PolyI:C, 2 µg/ml) was added to protein synthesis reaction mixture to prevent the activation of endogenous dRI by the dsRNA carried over in aliquots of activation reaction mixture used to determine the level of dRI activity (3).

Glycerol density gradient centrifugation: The sedimentation co-efficient of dRI was determined by centrifugation in a 10-30% glycerol density gradient in Buffer A (20 mM Tris-HCl, pH 7.8; KCl, 80 mM; DTT, 1 mM and EDTA 0.1 mM). Samples (100 µl) containing dRI (180 µg, step 6 preparation, Table 1) or standard protein were applied to the gradient and centrifuged at 48,000 RPM in a SW 50.1 rotor for 15.5 hr at 2°. The bottom of the tube was punctured and fractions were collected. dRI activity in an aliquot (20 µl) of each fraction was determined by the dRI catalyzed phosphorylation of the 38,000-dalton subunit of eIF-2 under limiting concentrations of dRI and by dsRNA-dependent phosphorylation of dRI. The phosphorylated reaction mixture from each fraction was subjected to SDS-polyacrylamide gel (10%) electrophoresis (11). The 38,000-dalton polypeptide of eIF-2 and the 70,000-dalton polypeptide of dRI were located in the dried gel by autoradiography. The 38,000-dalton sub-

Table I. Purification of dRI

Purification Step	Total Protein (mg)	Specific Activity Units/ml	Fold Purification
1. Ribosomal salt wash	2633	-	-
2. DEAE-cellulose	1613	-	-
3. $(NH_4)_2SO_4$, 35-50%	287	-	-
4. Phosphocellulose	87	287	1
5. Heparin-Sepharose	41	560	2
6. tRNA-Sepharose	0.5	4000	16
7. DEAE-cellulose	0.04	40,000	160

unit of eIF-2 and the 70,000-dalton protein band of dRI were cut out and the radioactivity in each fraction in the two phosphoproteins was determined by Scintillation spectrophotometry.

Determination of molecular weight by gel filtration: The molecular weight of dRI was determined by gel filtration on a Sephacryl S-300 (superfine) column (0.9 x 55 cm) preequilibrated with Buffer A (15). Samples containing dRI (380 µg, step 6 preparation, Table I) or standards were applied to the column. Proteins were filtered at a rate of 1.8 ml/hr. Fractions were assayed for the dRI catalyzed phosphorylation of the 38,000-dalton subunit of eIF-2 and the dsRNA-dependent phosphorylation of the 70,000-dalton polypeptide of dRI as described under "Glycerol density gradient centrifugation". K_{av} for the proteins was calculated from Ve-Vo/Vi-Vo where Ve is the elution volume of the protein, Vo is the void volume and Vi is the column volume (16).

Chromatography of ribosomal salt wash on DEAE-cellulose. Ribosomal salt wash from 30,000 A_{260} units of rabbit reticulocyte ribosomes was prepared (5) and was brought to 70% saturation by the addition of solid $(NH_4)_2SO_4$. The precipitate was dissolved in Buffer B (Tris-HCl, 20 mM [pH 7.8]; KCl, 50 mM; DTT, 1 mM and 5% glycerol) and dialyzed against the same buffer. The protein preparation (2633 mg) was applied to a DEAE-cellulose column (2.5 x 30 cm) preequilibrated with Buffer A. Under these conditions essentially all the dRI activity in the activated or in its inactive precursor form is present in the protein fraction that does not bind to DEAE-cellulose (5). This protein fraction was pooled and used for further purification of dRI.

Ammonium sulfate fractionation: The protein fraction (1613 mg) was brought to 70% saturation by the addition of solid $(NH_4)_2SO_4$. The precipitate was collected and was then extracted with 50% saturated $(NH_4)_2SO_4$ solution in Buffer B. The fraction of precipitate which did not go in solution was collected and extracted with 35% saturated solution of $(NH_4)_2SO_4$ in Buffer B. The material which failed to go in solution with 35% saturated solution of $(NH_4)_2SO_4$ was discarded. The protein fraction which precipitates with 35 to 50% saturated $(NH_4)_2SO_4$ contains most of dRI activity.

Chromatography on phosphocellulose: The protein fraction from step 3 was dialyzed against Buffer C (potassium phosphate, 50 mM [pH 6.7]; DTT, 0.5 mM and 5% glycerol). The protein fraction (287 mg) was applied to a phosphocellulose column (1.5 x 27 cm) preequilibrated with Buffer C and the chromatography was performed as described (5). dRI is present in the protein fraction that elutes with 100 to 300 mM KCl in Buffer C (5).

Chromatography on Heparin-Sepharose: The dRI preparation from phosphocellulose column was brought to 50% saturation with the addition of a saturated solution of $(NH_4)_2SO_4$. The precipitate was collected and dissolved in Buffer B. The protein sample was dialyzed against the same buffer. The sample was applied to a Heparin-Sepharose column (1.5 x 12 cm) preequilibrated with

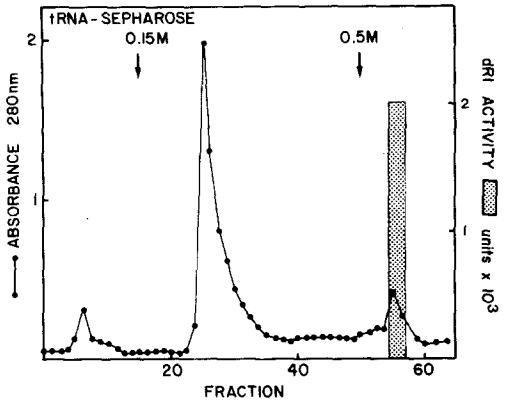

Fig. 1. Chromatography of dRI on tRNA-Sepharose: The details are provided in the "Methods Section."

Buffer B. The column was washed extensively with Buffer B. dRI bound to the column was eluted with Buffer B containing 0.23 M KCl. The leading edge of the fractions of the protein peak from the column contained no detectable dRI activity. The protein in these fractions was discarded. The fractions containing dRI activity were pooled. The remaining bound protein fraction was eluted with Buffer A containing 1 M KCl. This fraction contained no dRI activity.

Chromatography on tRNA-Sepharose: The protein preparation (41 mg) containing dRI activity from Heparin-Sepharose column was brought to 50% saturation with saturated solution of $(NH_4)_2SO_4$. The precipitate was dissolved in 5 ml of Buffer D (Potassium phosphate, 25 mM [pH 6.7]; magnesium acetate, 1 mM; β-mercaptoethanol (β-ME), 7 mM; EDTA, 0.1 mM and glycerol 10%). The sample was dialyzed against the same buffer; during dialysis there was extensive precipitation of protein. The precipitate was removed by centrifugation and discarded. The protein fraction containing dRI activity was applied to a tRNA-Sepharose column (1 x 9 cm) preequilibrated with Buffer D. Except for a very small fraction of protein most of the protein applied to tRNA-Sepharose was bound. The bound protein was eluted with Buffer D containing 100 mM potassium phosphate, followed by an elution with Buffer E [Tris-HCl, 100 mM (pH 8.0); KCl, 150 mM; magnesium acetate, 1 mM; β-ME; 7 mM and 10% glycerol] and finally with Buffer E containing 0.5 mM KCl. The elution profile of protein from tRNA-Sepharose is shown in Fig 1. It should be noted that under these conditions essentially all the dRI activity elutes with Buffer D containing 0.5 M KCl.

Chromatography on DEAE-Cellulose: The dRI from tRNA-Sepharose was phosphorylated as described under "Activation of Pro-dRI Section". A small aliquot of dRI was phosphorylated in the presence of [γ ^{32}P]ATP (specific activity 4000 CPM/pmol). The phosphorylation reaction was terminated by the addition of potassium phosphate buffer (pH 6.7) to a final concentration of 20 mM. The two fractions were combined and the sample was subjected to chromatography on a DEAE-cellulose column (prepared from a 1 ml plastic syringe with DEAE-cellulose packed up to 0.8 ml). DEAE-cellulose column was preequilibrated with Buffer F (Hepes, 5 mM (pH 7.2); KCl, 50 mM; DTT, 1 mM and 10% glycerol). After the application of the sample, the column was extensively washed with Buffer F. The bound dRI was eluted with Buffer F containing 0.2 M KCl.

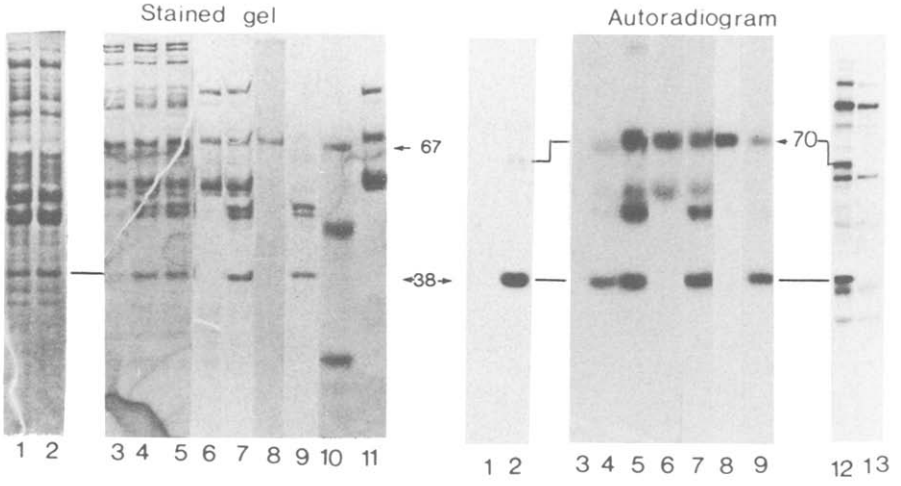

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the various dRI preparations phosphorylated in the presence of [γ^{32}P]ATP. Reaction mixture (10 µl) in 20 mM Tris-HCl, pH 7.8; 50 mM KCl; 2 mM Mg $(Ac)_2$ and 1 mM DTT containing 0.1 mM [γ^{32}P]ATP (specific activity 4000 CPM/pmol) were incubated at 30° for 25 min with: lane 1, phosphocellulose dRI preparation (11 µg) + eIF-2 (0.7 µg); lane 2, phosphocellulose dRI preparation (11 µg) + eIF-2 (0.7 µg) + dsRNA (0.34 ng); lane 3, Heparin-Sepharose dRI preparation (3 µg); lane 4, Heparin-Sepharose dRI preparation (3 µg) + eIF-2 (0.7 µg); lane 5, Heparin-Sepharose dRI preparation (3 µg) + eIF-2 (0.7 µg) + (0.34 ng); lane 6, tRNA-Sepharose dRI preparation (1 µg); lane 7, tRNA-Sepharose dRI preparation (1 µg) + eIF-2 (0.7 µg) + dsRNA (0.34 ng); lane 8, purified (step 7) dRI (0.3 µg); lane 9, purified dRI (20 ng) + eIF-2 (0.7 µg); lane 10, standard proteins (bovine serum albumin (BSA), ovalbumin and chymotrypsinogen) and lane 11, tRNA-Sepharose dRI preparation (1 µg). The samples were electrophoresed in SDS-polyacrylamide gel (10%); the proteins were stained (left) and autoradiogram (right) was prepared (11). The arrows indicate the position of 70,000-dalton polypeptide of dRI, 67,000-dalton polypeptide of BSA and the 38,000-dalton subunit of eIF-2. Lanes 12 and 13 are an autoradiogram of the Sepharose 6B ribosomes (0.2 A_{260}) phosphorylated in presence (lane 12) and absence of dsRNA (lane 13).

RESULTS

The purification data are summarized in Table 1. Previously I described the purification of dRI up to the phosphocellulose step which results in a purification of 100 fold (5). Further purification of dRI to homogeneity results in an additional 160 fold purification, thus a net purification of 16,000 fold is obtained. The SDS-polyacrylamide gel electrophoretic patterns of protein preparations from the phosphocellulose step of dRI to the final purification step are presented in Fig 2. The dsRNA-dependent phosphorylation of the 70,000-dalton polypeptide of dRI and the phosphorylation of the 38,000-dalton subunit of eIF-2 is clearly evident in the phosphocellulose fraction (Fig 2, lanes 1 and 2) and in the Heparin-Sepharose dRI preparation (Fig 2, lanes 3-5). After the chromatography of dRI on tRNA-Sepha-

rose this dependence on dsRNA is lost (Fig 2, lanes 6 and 7). In other preparations this dsRNA-dependence is preserved provided the assay is carried out shortly after the chromatography on tRNA-Sepharose (data not shown). In spite of the loss of dsRNA-dependence, the close association of the phosphorylation of the 70,000-dalton polypeptide of dRI and the phosphorylation of the 38,000-dalton subunit of eIF-2, however, remains (Fig 2, lanes 6 and 7). Purified dRI migrates as a single polypeptide with a molecular weight of 70,000 (Fig 2, lane 8). Since a fraction of this protein was phosphorylated in the presence of [γ^{32}P]ATP, the co-migration of the [^{32}P]phosphoprotein with the stained protein band is clearly evident from the autoradiogram (Fig 2, lane 8). This close association of the [^{32}P]phosphoprotein with the stained protein band of 70,000-daltons is also demonstrable in the less purified dRI fractions (Fig 2, lanes 3-8). Purified dRI phosphorylates the 38,000-dalton subunit of eIF-2 (Fig 2, lane 9; for an eIF-2 control see lane 1) and does not require cyclic-AMP (results not shown).

A molecular weight of 70,000-daltons is obtained by gel filtration of dRI on Sephacryl S-300 (Fig 3, B). The actual elution profile of dRI is presented in (Fig 3, A). Data show that the elution profile the 70,000-dalton polypeptide of dRI (labelled with [^{32}P]) and the eIF-2 38,000-dalton subunit specific protein kinase activity of dRI co-chromatograph. A sedimentation coefficient of 3.2S is calculated from glycerol density gradient centrifugation of dRI (Fig 4). The results show that the 70,000-dalton polypeptide of dRI and the eIF-2 38,000-dalton subunit specific protein kinase activity of dRI co-sediment (Fig 4).

The purified dRI is highly effective in inhibiting protein synthesis in lysates (Fig 5, A) with inhibition kinetics that parallel those observed in the presence of dsRNA (1). The inhibition of protein synthesis by purified dRI is overcome (Fig 5, B) and reversed (Fig 5, C) by eIF-2. Treatment of dRI with 1 mM and 5 mM N-ethylmaleimide leads to a loss of 50% and 100% of the inhibitor activity, respectively (results not shown). In the presence of eIF-2 stabilization factor (SF), dRI inhibits the ternary complex formation capacity of eIF-2 (Table II). The inhibitory effect of dRI requires fully activated dRI and ATP (Table II).

DISCUSSION

The double stranded RNA activated protein kinase has been purified. The increase in specific activity of dRI during purification is accompanied by the increase in stainable protein band of 70,000-daltons at the expense of

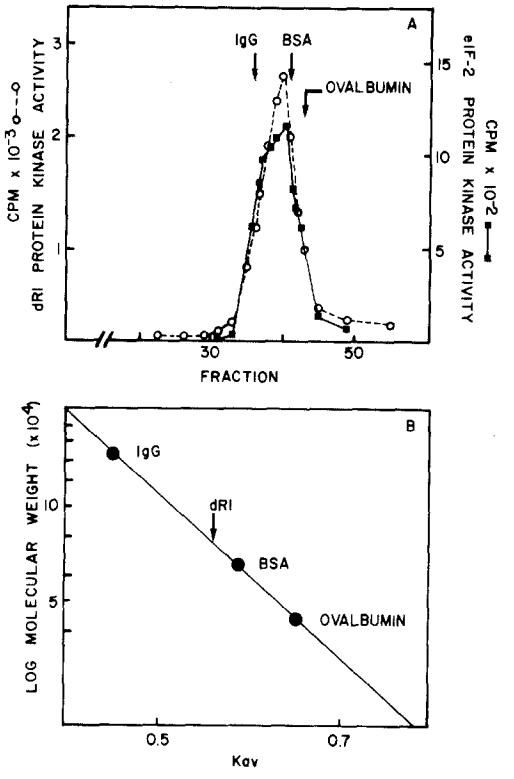

Fig. 3. Determination of molecular weight of dRI by gel filtration on Sephacryl S-300 (Superfine). The elution profile of dRI and the dRI catalyzed phosphorylation of eIF-2 are shown in A, and a plot of the K_{av} (data obtained from A) and Log Molecular weight is presented in B.

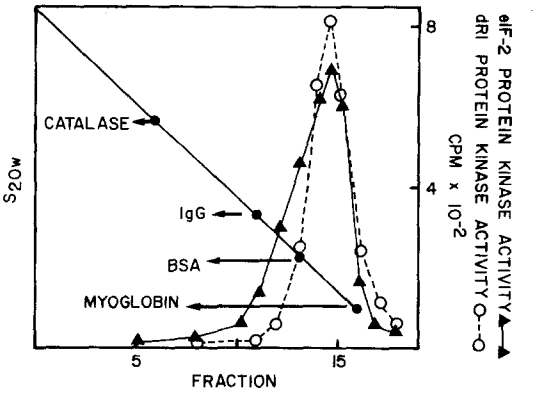

Fig. 4. Determination of sedimentation co-efficient of dRI by glycerol density gradient centrifugation. The sedimentation profile of dRI and the dRI catalyzed phosphorylation of eIF-2 are shown.

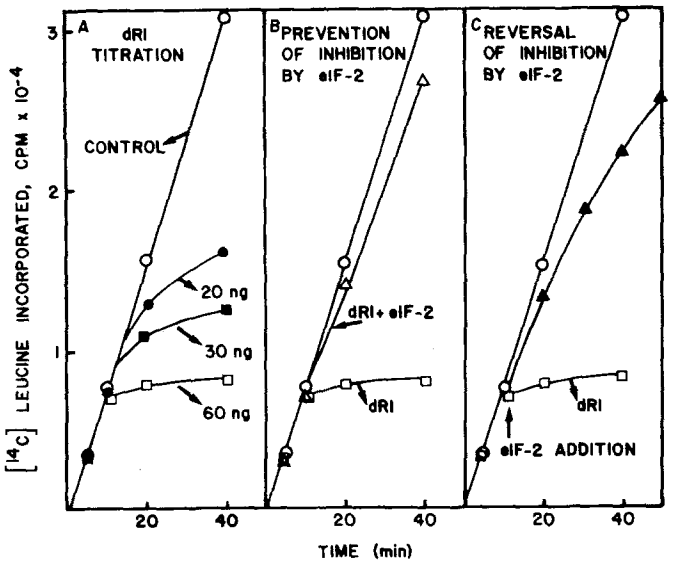

Fig. 5. Kinetics of inhibition of protein synthesis by dRI and its rescue by eIF-2. Protein synthesis reaction mixtures (25 µl) containing 20 µM hemin were incubated with: A, indicated concentrations of purified dRI; B, dRI (60 ng) or dRI (60 ng) and eIF-2 (1 µg) added at the start of incubation, and C, dRI (60 ng) or dRI (60 ng) added at zero time and eIF-2 (1 µg) added after 10 min of incubation. At intervals 5 µl aliquots were removed and assayed for protein synthesis (11).

other proteins. The purified dRI preparation contained only this polypeptide of 70,000-daltons (Fig 2, lane 8). This same polypeptide also gets phosphorylated in crude and purified preparations of dRI in the presence of dRNA (Fig 2). The dsRNA-dependent phosphorylation of the 70,000-dalton polypeptide of dRI is associated with the activation of the protein kinase activity of dRI that phosphorylates eIF-2. Farrell et al (4) and subsequently others (5,6,10) have suggested that the phosphorylated 70,000-dalton poly-

Table II. dRI catalyzed inhibition of ternary complex formation (eIF-2·GTP·Met-tRNA$_f$) in presence of SF

Additions	[^{3}H]Met-tRNA$_f$ Bound (CPM)
eIF-2	177
eIF-2 + SF	4001
eIF-2 + SF + ATP	3777
eIF-2 + SF + dRI	3623
eIF-2 + SF + ATP + dRI	2959
eIF-2 + SF + ATP + dRI + dsRNA	1685

Assay for the inhibition of ternary complex formation was performed as described (12). The concentrations CM-eIF-2, SF, dRI and dsRNA in the assay were 1 µg, 30 µg, 1 µg (step 5) and 0.34 ng, respectively.

peptide may be the activated form of dRI [previously identified as a 67,000-dalton polypeptide in Sepharose 6B ribosome preparations (4) (Fig 2, lanes 12 and 13); the more precise determinations by the use of purified dRI show an apparent molecular weight of 70,000 (Fig 2, lanes 8, 10 and 11) for this polypeptide]. Our results with purified dRI provide a definitive answer that this phosphoprotein is the dsRNA activated protein kinase. The molecular weight of 70,000-daltons by SDS-gel electrophoresis and a similar value obtained by gel filtration suggest that the native dRI and the pro-dRI is a single polypeptide. Hence, the activation of dRI from its inactive precursor involves a dsRNA-dependent change in conformation with a concomitant self-phosphorylation. The contention that the 70,000-dalton polypeptide of dRI which is phosphorylated in the presence of dsRNA is the protein kinase is also supported by the glycerol density gradient centrifugation and by the gel filtration data (Fig 3 and 4). These results show that the eIF-2-specific protein kinase activity of dRI and the dsRNA-dependent self-phosphorylation of 70,000-dalton polypeptide of dRI co-sediment and co-chromatograph.

The role of dsRNA-dependent self-phosphorylation of dRI not only in its activation but also in the modulation of dRI activity, however, remains to be fully explored. Recently, we have isolated a phosphoprotein phosphatase that dephosphorylates the activated dRI (R. S. Ranu, unpublished results) with a concomitant loss of dRI activity. The readdition of dsRNA and ATP results in activation and the phosphorylation of the 70,000-dalton polypeptide of dRI. These findings raise the possibliity that in the cell a reversible process exists by which activation and deactivation of dRI is modulated by the phosphorylation-dephosphorylation of dRI.

The purified dRI is highly effective in inhibiting protein synthesis in rabbit reticulocyte lysates (Fig 5) and phosphorylates the 38,000-dalton subunit of eIF-2 (Fig 2). The ability of eIF-2 to restore protein synthesis implies (17-19) that dRI catalyzed phosphorylation of eIF-2 is associated with the inactivation of eIF-2. The SF-dependent dRI catalyzed inhibition of ternary complex formation supports this view (Table II). This finding is in agreement with similar results obtained with the heme-regulated protein kinase (HRI) (12, 20-22) and is consistant with the fact that dRI and HRI phosphorylate the same site(s) of eIF-2 (4-6). The cyclic-AMP independent nature of dRI has already been established (4-6).

Previously, we and others (4,5,10,19) drew attention to the many similarities of the interferon induced and dsRNA-dependent activation of an eIF-2 specific

protein kinase (23-26) and dRI activity elicited by the addition of dsRNA to lysates. Many similarities in the chromatographic properties of the two inhibitors were also noted (5). Further work is necessary to show that these inhibitors are related.

Finally, we take a note of the recent finding of Stringer et al. (27) and our own (R. S. Ranu, unpublished results) that the purified eIF-2 consists of two polypeptides. The middle 50,000-dalton subunit (also known as β subunit) that is phosphorylated by a casein cyclic-AMP independent protein kinase (28) is not a component of eIF-2 (R. S. Ranu, unpublished results and Chaudhuri, A. and Maitra, U., Albert Einstein College of Medicine, personal communication). Previously it has been shown that the phosphorylation of this polypeptide plays no role in protein synthesis inhibition by HRI or dRI (4) or in the partial reaction of protein chain initiation (21). In order to conserve eIF-2, experiments in Fig 2 were done with eIF-2 preparation containing three polypeptides.

ACKNOWLEDGEMENTS: This work was supported by an NIH Biomedical Research Support Grant 2 S07 RR-05458-18. The author wishes to thank Dr. J. Bagby, Deans R. D. Phemister and F. Murphy for encouragement and support. Technical assistance of P. E. Ranu is gratefully acknowledged.

NOTE ADDED IN PROOF: After the completion of this manuscript I learned that Grosfeld, Haim and Ochoa, Severo (personal communication and- manuscript in press in the Proc. Natl. Acad. Sci. USA) have also purified dRI with results which are similar to those described in this communication.

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