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Isolation of a Heme-Controlled Inhibitor of Translation That Blocks the Interaction between Messenger RNA and Eukaryotic Initiation Factor 2†

Sarah Knoller and Raymond Kaempfer*

ABSTRACT: A heme-controlled inhibitor of translation was isolated from the S-100 of rabbit reticulocytes by a novel procedure including chromatography on double-stranded ribonucleic acid (dsRNA)–cellulose. The inhibitor thus purified is extremely active and functionally resembles previously studied heme-controlled inhibitor preparations in terms of kinetics and extent of inhibition of translation, relief of inhibition by eukaryotic initiation factor 2 (eIF-2), relief of inhibition by 2-aminopurine, and preferential inhibition of α- over β-globin synthesis. The action of this inhibitor on translation is resistant to treatment with bacterial alkaline phosphatase, micrococcal nuclease, or trypsin and to incubation at 95 °C, pH 2 or pH 12. The inhibitor not only is retained on DEAE-cellulose, phosphocellulose, and dsRNA–cellulose but also exhibits a high affinity for the dye Cibacron Blue, properties that suggest that it may be a protein. Unlike previously described heme-controlled inhibitor preparations, or preparations that did not pass over dsRNA–cellulose, the inhibitor recovered upon dsRNA–cellulose chromatography does not exhibit eIF-2 kinase activity. The inhibitor does not block ternary complex formation between eIF-2, methionyl-$tRNA_f^{Met}$, and GTP but inhibits the ability of eIF-2 to form a complex with labeled globin mRNA. In the presence of inhibitor, the formation of mRNA/eIF-2 complexes can be restored effectively by an excess of eIF-2 but not by an excess of mRNA. The inhibitor thus appears to block the interaction between eIF-2 and mRNA not by competing with eIF-2 for a binding site on mRNA but, instead, by acting on eIF-2 itself. Since there is evidence for a specific interaction between eIF-2 and mRNA during initiation of protein synthesis, inhibition of this interaction by the inhibitor described here may be involved in translational control by heme.

Initiation of translation in lysates from reticulocytes (Bruns & London, 1965) or other eukaryotic cells (Weber et al., 1975) is inhibited in the absence of added heme. A similar inhibition is observed in reticulocyte lysates in the presence of dsRNA[1] or oxidized glutathione [reviewed by Revel & Groner (1976), Safer & Anderson (1978), Hunt (1979), Ochoa & de Haro (1979), and Austin & Clemens (1980)]; translation in extracts of interferon-treated cells is also sensitive to dsRNA (Kerr et al., 1974). In each case, the inhibition can be reversed by high levels of eIF-2 (Kaempfer, 1974; Clemens et al., 1975; Kaempfer et al., 1979a), the initiation factor that forms a ternary complex with Met-$tRNA_f$ and GTP and promotes the binding of Met-$tRNA_f$ to 40S ribosomal subunits, a necessary prerequisite for the subsequent binding of mRNA (Darnbrough et al., 1973; Trachsel et al., 1977). In addition, eIF-2 itself binds specifically to mRNA (Kaempfer, 1974; Barrieux & Rosenfeld, 1977, 1978; Kaempfer et al., 1978a,b, 1979b; Rosen & Kaempfer, 1979), recognizing a site essential for translation that has been shown to be virtually identical with the ribosome binding site sequence (Kaempfer et al., 1981; Perez-Bercoff & Kaempfer, 1982). The relevance of this interaction for protein synthesis is indicated by the finding that a direct correlation exists between the affinity of an mRNA species for eIF-2 and its ability to compete in translation (Di Segni et al., 1979; Rosen et al., 1982; Kaempfer, 1982).

Incubation of reticulocyte lysates in the absence of heme or in the presence of dsRNA leads to the appearance of translation inhibitory activity. Purified preparations of in-

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[1] Abbreviations: eIF-2, eukaryotic initiation factor 2; dsRNA, double-stranded RNA; Met-$tRNA_f$, methionyl-$tRNA_f^{Met}$; Tris, tris(hydroxymethyl)aminomethane; DEAE-cellulose, diethylaminoethylcellulose; Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IN, inhibitor; AP, 2-aminopurine; BAP, bacterial alkaline phosphatase; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

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hibitor generated in the absence of heme, in the presence of dsRNA, or in extracts of interferon-treated cells have been shown to contain protein kinase activities that phosphorylate the M_r 38 000 subunit of eIF-2 (Farrell et al., 1977; Gross & Mendelewski, 1977; Trachsel et al., 1978; Levin et al., 1981; Roberts et al., 1976; Lebleu et al., 1976). Phosphorylation of eIF-2 inhibits the exchange of GTP for GDP bound to eIF-2 (Clemens et al., 1982), apparently by preventing the action of a second protein that catalyzes this exchange (Siekierka et al., 1982). Consequently, recycling of eIF-2 between rounds of initiation would be impaired. However, full inhibition of protein synthesis can be observed when as little as 30% of the total eIF-2 is phosphorylated (Leroux & London, 1982), raising the question whether phosphorylation is in fact the only mechanism responsible for the inhibition of eIF-2 action. If it were the primary cause of inhibition, then the phosphorylation of eIF-2 should prevent binding of Met-tRNA$_f$ to 40S ribosomal subunits and hence the subsequent binding of mRNA, irrespective of the type of mRNA being translated. Yet, for the inhibitory effect of dsRNA on translation, we have demonstrated that this is not the case (Rosen et al., 1981). Instead, dsRNA exhibits mRNA specificity in its inhibitory effect, leading to inhibition of translation, inactivation, and phosphorylation of eIF-2 when the template is globin or total cellular mRNA, but not when it is picornavirus RNA. In that study, it was shown (i) that Mengovirus RNA protects eIF-2 against inactivation when dsRNA is present during translation, (ii) that Mengovirus RNA competitively inhibits the dsRNA-dependent phosphorylation of eIF-2, and (iii) that dsRNA competes with mRNA for eIF-2, binding it more tightly than globin mRNA, but more weakly than Mengovirus RNA (Rosen et al., 1981). This study revealed a correlation between the affinity of an mRNA species for eIF-2 and the sensitivity of its translation to inhibition by dsRNA. This led to the concept that the rate-determining event in the establishment of inhibition of translation by dsRNA involves competition with mRNA, with phosphorylation and inactivation of eIF-2 depending on the outcome of this competition.

Here, we report the isolation of a heme-controlled inhibitor of translation that does not exhibit eIF-2 kinase activity and show that this inhibitor affects the ability of eIF-2 to bind to messenger RNA. We offer an explanation for the mode of action of this inhibitor in translation and its relationship to the phosphorylation of eIF-2.

Experimental Procedures

Preparation of Heme-Controlled Inhibitor. Rabbit reticulocyte S-100 (25 mL), lacking added heme, was diluted with an equal volume of 10 mM Tris-HCl, pH 7.4, 50 mM KCl, and 10 mM 2-mercaptoethanol and incubated for 3 h at 37 °C. In some instances, incubation was for 16 h at 37 C, with identical results in terms of inhibitory activity. Proteins precipitated by $(NH_4)_2SO_4$ at 40% saturation were dialyzed against the same buffer, clarified by centrifugation, and applied, in the cold, to a column of DEAE-cellulose equilibrated with 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 10% glycerol, and 10 mM 2-mercaptoethanol. The protein eluted with buffer containing 240 mM KCl (see Figure 1A) was assayed for inhibitor activity by a translation assay (see Figure 1C). The peak of activity was diluted to 50 mM KCl in 50 mM Tris-HCl, pH 6.7, and 10 mM 2-mercaptoethanol and applied to a phosphocellulose column equilibrated with the latter buffer. Protein eluted with buffer containing 200 mM KCl (see Figure 1B) was assayed for inhibitor activity. The peak of activity was diluted to 20 mM KCl in 20 mM Tris-HCl, pH 7.8, and 10 mM 2-mercaptoethanol and applied to a 1-mL column of *Penicillium chrysogenum* dsRNA–cellulose (Kaempfer et al., 1978a; Kaempfer, 1979b) equilibrated with the latter buffer. After being washed with 10 mL of 20 mM KCl buffer, the column was developed with a 12-mL gradient from 20 to 600 mM KCl in the same buffer (Figure 1C). Fractions could be stored for several months at −20 °C without loss of activity.

Reticulocyte Lysate. Rabbit reticulocyte lysate was prepared as described by Kaempfer & Kaufman (1972). Preparation of micrococcal nuclease treated reticulocyte lysate was as described by Di Segni et al. (1979).

mRNA. Globin mRNA was prepared as described (Kaempfer, 1979a).

Cell-Free Translation. Cell-free translation mixtures were prepared, incubated, and analyzed as described by Rosen et al. (1981, 1982).

Preparation of [^{35}S]Met-tRNA$_f$ and Assay of Ternary Complex Formation. The procedures of Kaempfer et al. (1978a) and Rosen et al. (1981) were followed.

Purification of eIF-2. The procedure is described by Kaempfer (1979b), Di Segni et al. (1979), and Rosen & Kaempfer (1979). eIF-2 purified by this procedure is at least 98% pure as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis; its mRNA-binding activity is completely sensitive to competitive inhibition by Met-tRNA$_f$ and GTP but not by uncharged tRNA, showing that the only mRNA-binding component in the preparation is eIF-2 itself (Rosen & Kaempfer, 1979). The purified eIF-2 bound 0.3–1.0 pmol of Met-tRNA$_f$/μg of protein.

Radioiodination of mRNA. The method of Commerford (1971) was followed, with the modifications described by Di Segni et al. (1979) and Rosen et al. (1982).

Radioiodination of Protein. The Bolton–Hunter reagent was used to label protein with ^{125}I (Bolton & Hunter, 1973).

Binding of mRNA to eIF-2. This was assayed as described by Kaempfer (1979a) and Rosen et al. (1982).

Results

Purification of the Inhibitor. The inhibitor was purified from rabbit reticulocyte S-100 that had been incubated at 37 °C in the absence of heme. After ammonium sulfate precipitation and dialysis, inhibitor activity was recovered from a DEAE-cellulose column by stepwise elution at 240 mM KCl (Figure 1A) and subsequently from a phosphocellulose column by stepwise elution at 200 mM KCl (Figure 1B), as previously described for other heme-controlled inhibitor preparations (Ranu & London, 1976; Trachsel et al., 1978). At this point, the inhibitory activity was free of eIF-2 and of casein kinase, which elute at 700 mM KCl, and of polypeptides that do not bind to the column, including several kinases that phosphorylate ribosomal proteins (Traugh & Traut, 1974).

The inhibitory activity was next subjected to chromatography on a dsRNA–cellulose column (Kaempfer et al., 1978a; Kaempfer, 1979b). The activity was bound quantitatively. On application of a gradient from 20 to 600 mM KCl, a peak of inhibitory activity was eluted around 140 mM KCl that reduced translation in a reticulocyte lysate by 75% (Figure 1C). It will be seen below that this extent of reduction represents maximal inhibition.

Role of Heme. If the active inhibitor is involved in the regulation of protein synthesis in reticulocytes by heme, one should expect to recover only low amounts from lysates, prepared in the presence of heme, that actively synthesize protein. Indeed, as seen in Figure 2, when inhibitor is prepared from the S-100 fraction of such a lysate, only traces of inhibitory activity are eluted from the dsRNA–cellulose column, but the dramatic decrease in translation observed in Figure

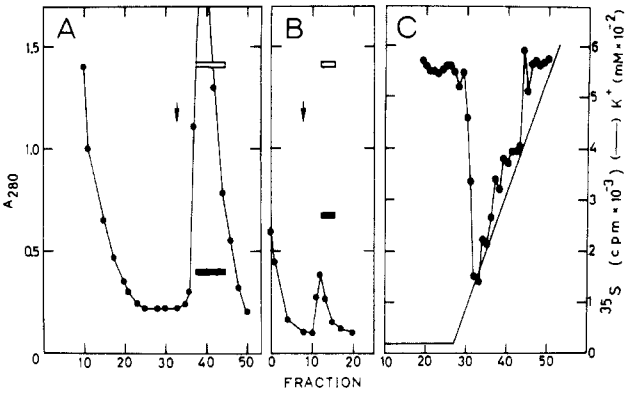

FIGURE 1: Purification of heme-controlled inhibitor. Inhibitory activity was purified from S-100 of a reticulocyte lysate lacking added heme that had been incubated for 3 h at 37 °C. (A) DEAE-cellulose column (volume 2.5 mL); (B) phosphocellulose column (1 mL); (C) dsRNA–cellulose column (1 mL). Arrows denote buffer change. In (A) and (B), the A_{280} profile is shown. Inhibitor activity is indicated by filled horizontal bars for pooled fractions 38–44 (A) and 12–16 (B) and for individual fractions in (C). Open bars represent translation in the absence of inhibitor. To measure inhibitor activity, 20-μL aliquots [representing, for (A), (B), and (C), respectively, 0.6%, 1%, and 6% of total activity in the fraction] were added to reaction mixtures (final volume 50 μL) for translation, containing 25 μL of reticulocyte lysate, 30 μM heme, and 0.5 μCi of [^{35}S]methionine, that were adjusted to 90 mM of added KCl. After 50 min at 30 °C, Cl_3CCOOH-precipitable radioactivity was determined in 40-μL samples. Background (170 cpm) was subtracted.

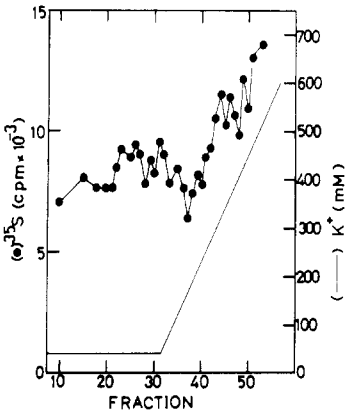

FIGURE 2: dsRNA–cellulose elution profile of inhibitory activity from heme-containing S-100. The procedure used in Figure 1 to purify inhibitor was followed, but the starting material for the S-100 was lysate containing 30 μM heme. The dsRNA–cellulose step is presented (cf. Figure 1C).

1C does not occur. Apparently, the inhibitor is formed specifically upon incubation of S-100 in the absence of heme.

Kinetics of Inhibition. Figure 3 depicts the kinetics of translation in a reticulocyte lysate supplemented with heme, in the presence of increasing amounts of the purified inhibitor. It is seen that inhibition is manifested after a 10-min period of unabated synthesis, yielding biphasic curves comparable to those described for the heme-controlled inhibitory activities purified by other methods (Safer & Anderson, 1978; Hunt, 1979; Austin & Clemens, 1980). The escape synthesis accounts for the observation that inhibition never reaches 100% (Figure 3*B*; cf. Figure 1C).

Characteristics of the Inhibitor. The inhibitory activity is resistant to treatment with micrococcal nuclease (Figure 4A). In this experiment, inhibitor was added to a native reticulocyte lysate, and the mixture was subjected to micrococcal nuclease digestion under conditions that caused complete elimination of globin mRNA template activity, as judged by the fact that after termination of digestion with EGTA, translation had

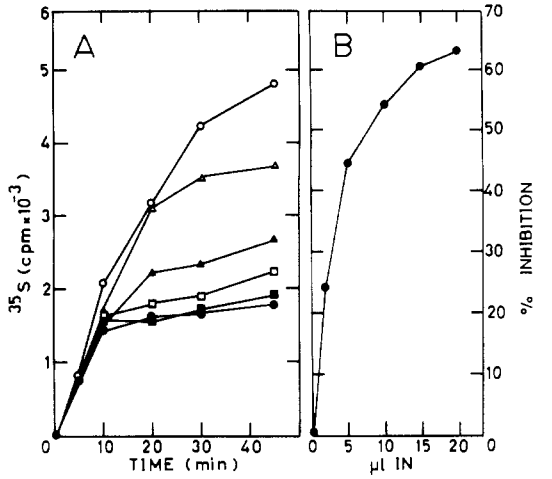

FIGURE 3: Kinetics of translation in the presence of purified inhibitor. (A) Reaction mixtures for translation (see Figure 1C) contained the following microliter amounts of purified inhibitor (IN): none (○), 2 (△), 5 (▲), 10 (□), 15 (■), and 20 (●). At intervals, Cl_3CCOOH-precipitable radioactivity was determined in 5-μL samples. In (B), the extent of inhibition is expressed as the decrease in incorporation at 50 min, relative to the control (○).

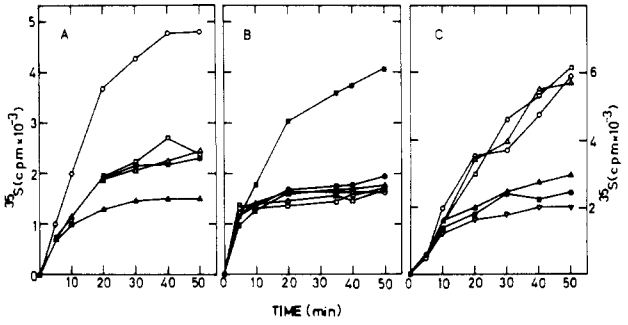

FIGURE 4: Effect of micrococcal nuclease, high temperature, and pH on inhibitor activity. (A) Kinetics of translation in reaction mixtures containing micrococcal nuclease-treated rabbit reticulocyte lysate and 1.5 μg of rabbit globin mRNA in the presence of no (○), 10 μL (□), or 20 μL (△) of purified inhibitor that was added at the start of translation. In parallel, aliquots of reticulocyte lysate were treated with micrococcal nuclease (20 μg/mL) in the same manner, but in the presence of 10 (■) or 20 μL (▲) of inhibitor; they were made 4 mM in EGTA and received 1.5 μg of globin mRNA before the start of translation. No incorporation of ^{35}S was observed when globin mRNA was omitted. (B) Kinetics of translation in reaction mixtures containing reticulocyte lysate and no inhibitor (■), 20 μL of control inhibitor (●), or 20 μL of inhibitor that had been incubated for 15 min at 30 (○), 45 (△), 60 (□), or 95 °C (▲) and cooled rapidly. (C) Kinetics of translation in reaction mixtures containing reticulocyte lysate and no inhibitor (○), 18 μL of inhibitor (▽), or 18 μL of inhibitor (▲, ■) or buffer (△, □) that was made pH 2 (▲, △) or pH 12 (■, □), incubated for 1 h at 30 °C, and then neutralized to pH 7.5 before addition.

become totally dependent on exogenous mRNA.

To eliminate the possibility that the inhibitory activity might be caused by dsRNA liberated from the dsRNA-cellulose column, we examined its sensitivity to micrococcal nuclease. Digestion of dsRNA with the enzyme, either in a native reticulocyte lysate or separately, abolished the inhibition of translation by 5 ng/mL dsRNA, an amount sufficient for maximal inhibition, thus confirming the results of Ehrenfeld & Hunt (1971).

The inhibitory activity is resistant to heat treatment up to 95 °C (Figure 4B) and only slightly diminished by treatment at pH 2 or pH 12 (Figure 4C).

As seen from Figure 5A, the inhibitory activity is resistant to treatment with trypsin. In a similar experiment, treatment with papain also failed to diminish inhibitor activity (not

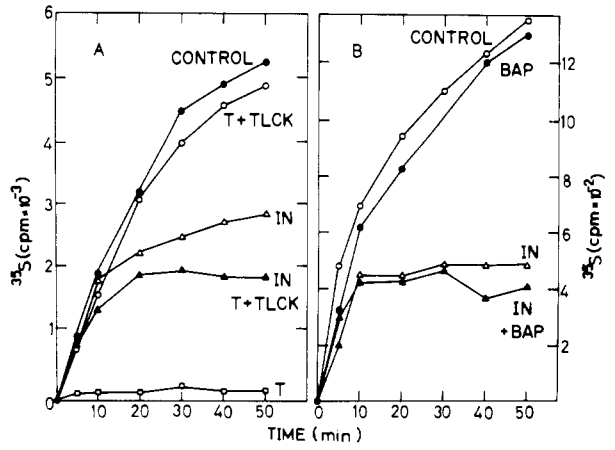

FIGURE 5: Effect of trypsin (T) and bacterial alkaline phosphatase (BAP) on inhibitor activity. (A) Kinetics of translation in reaction mixtures containing reticulocyte lysate and no addition (●), 1 μg of trypsin (9700 units/mg; Sigma) (□), 20 μL of inhibitor (△), or 20 μL of inhibitor (▲) or buffer (○) that were incubated with 0.05 μg of trypsin for 20 min at 30 °C and then with 1 μg of TLCK for 10 min at 30 °C before addition. (B) Kinetics of translation in reaction mixtures containing reticulocyte lysate and no addition (○), 25 μL of inhibitor (△), or 25 μL of inhibitor (▲) or buffer (●) that were incubated with 0.4 unit of BAP for 1 h at 37 °C before addition. BAP activity was confirmed by 5′-dephosphorylation of DNA and dsRNA; 1.6 units of BAP inhibited translation (not shown).

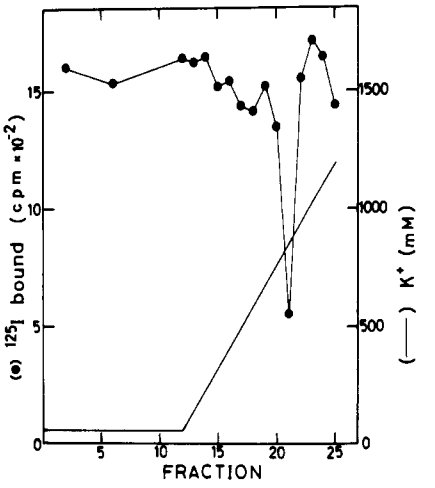

FIGURE 6: Chromatography of inhibitor on Cibacron Blue–Sephadex. Material from the peak of inhibitor activity (Figure 1C) was applied to a 0.2-mL column of Cibacron Blue FG3-A coupled to Sephadex G-50 (fine) (Böhme et al., 1972) that was washed with 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM KCl, and 10 mM 2-mercaptoethanol and developed with a 3.5-mL gradient from 50 to 1200 mM KCl in the same buffer. The ability to block complex formation between eIF-2 and ^{125}I-labeled globin mRNA (input 2400 cpm; 4 × 10^5 cpm/μg of RNA) was assayed (see Figure 12). Translation inhibitory activity eluted in the same fraction.

shown). The inhibitor is also resistant to treatment with bacterial alkaline phosphatase (Figure 5B). Moreover, treatment with neuraminidase did not affect inhibitor activity (not shown).

Highly active inhibitor preparations, purified as in Figure 1, possessed no detectable A_{280} or A_{260}, nor was protein detected by staining (Bradford, 1976) or by radioiodination with the Bolton–Hunter reagent, in spite of the fact that all three subunits of purified eIF-2 were readily visualized on Na-DodSO$_4$–polyacrylamide gels by either method.

The chromatographic properties of the inhibitor, that is, its retention on DEAE-cellulose, phosphocellulose, and dsRNA–cellulose columns and its elution from each of these, are, however, most readily compatible with a protein nature. Additional support for this interpretation is provided by the reproducible and effective retention of the inhibitory activity on a column of Cibacron Blue coupled to Sephadex (Figure 6). A relatively high salt concentration (0.7 M) is needed to elute the inhibitor, attesting to its high affinity for this dye.

eIF-2 and 2-Aminopurine Relieve the Action of the Inhibitor on Translation. An eIF-2 preparation purified to about 98% (Rosen & Kaempfer, 1979; Di Segni et al., 1979), able to relieve completely the inhibition of translation observed in a reticulocyte lysate in the absence of added heme (Figure 7A) or in the presence of inhibiting concentrations of dsRNA (panel B), effectively reversed the effect of inhibitor purified through the phosphocellulose step (panel C). The inhibition by dsRNA–cellulose-purified inhibitor could also be relieved by the same amount of eIF-2, but in this case, reversal was observed only in the presence of an amount of inhibitor that gave partial inhibition of translation, reflecting the high activity of the inhibitor preparation (panel D).

Inhibitor Acts at Initiation. The inhibition of translation in reticulocyte lysates observed in the absence of heme or in the presence of low concentrations of dsRNA occurs with kinetics similar to those seen in the presence of the inhibitor and can be overcome by purine derivatives such as 2-aminopurine (Figure 8A; Legon et al., 1974). The action of the inhibitor likewise is reversed completely by 2-aminopurine (Figure 8B).

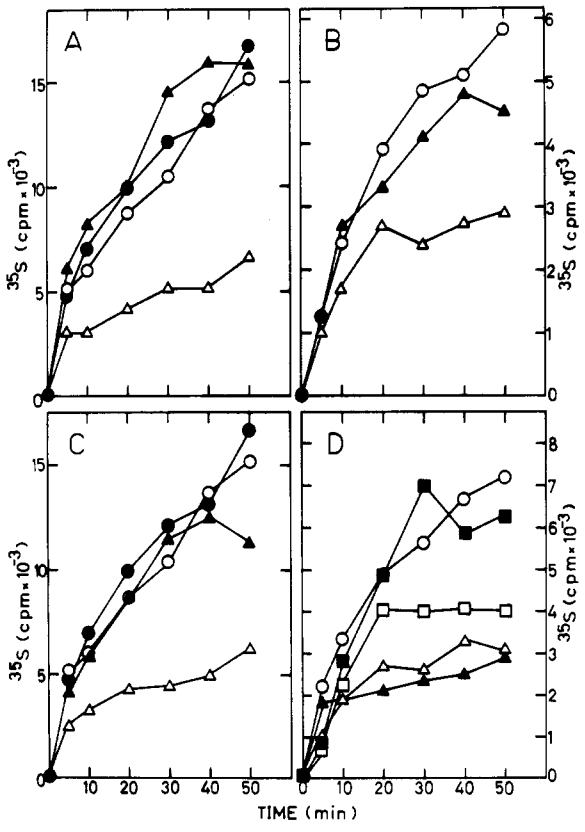

FIGURE 7: Relief by eIF-2 of translational inhibition in the absence of heme (A) and in the presence of dsRNA (B), phosphocellulose-purified inhibitor (C), or dsRNA–cellulose-purified inhibitor (D). Reaction mixtures for translation were as for Figure 1C. (A) With (○, ●) or without (△, ▲) 30 μM heme and with (●, ▲) or without (○, △) 1.4 μg of eIF-2. (B) With (△, ▲) or without (○) 5 ng/mL dsRNA and with (▲) or without (△, ○) 2.2 μg of eIF-2. (C) With (△, ▲) or without (○, ●) 8 μL of phosphocellulose-purified inhibitor (see Figure 1B) and with (●, ▲) or without (○, △) 1.4 μg of eIF-2. (D) With no (○), 3 μL (□, ■) or 11 μL (△, ▲) of dsRNA–cellulose-purified inhibitor (see Figure 1C) and with (■, ▲) or without (○, □, △) 1.1 μg of eIF-2.

The kinetics of translation during inhibition (Figure 3) and the relief of inhibition by eIF-2 (Figure 7D) suggest that the

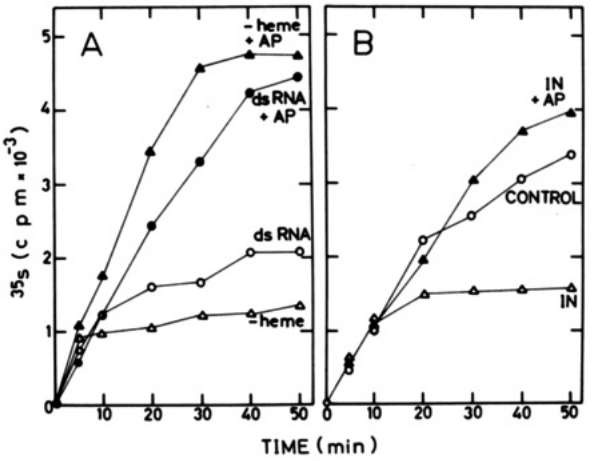

FIGURE 8: Relief of inhibition by 2-aminopurine. Kinetics of translation as for Figure 3, in the presence of 30 μM heme, and 1 ng/mL *P. chrysogenum* dsRNA, 1 mg/mL 2-aminopurine (AP), and 15 μL of purified inhibitor (IN) where indicated.

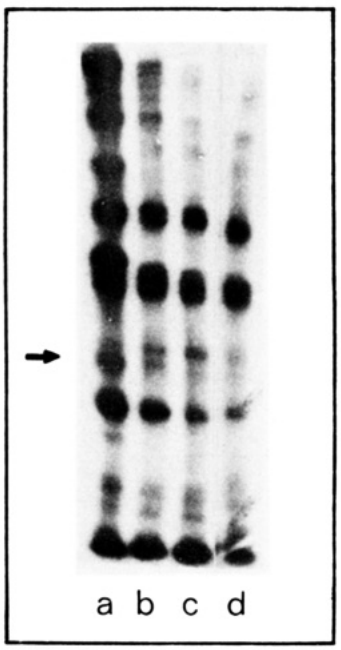

FIGURE 9: Assay of eIF-2 kinase activity. Crude reticulocyte initiation factors [0.1–0.22 M DEAE-cellulose fraction (Kaempfer, 1979a)] were incubated with 1 μCi of [γ-^{32}P]ATP and, in lanes a–c, with 5 μL of peak inhibitor activity from the columns of Figure 1A–1C, respectively. Control (lane d) contained no inhibitor. The autoradiogram of a NaDodSO$_4$–polyacrylamide gel (Laemmli, 1970) of the reaction mixtures is shown. Arrow denotes position of the M_r 38 000 polypeptide as determined by the migration of cross-linked hemoglobin and cross-linked bovine albumin markers (Sigma).

inhibitor acts at the initiation step. Indeed, synthesis of α-globin is much more sensitive to the inhibitor than is synthesis of β-globin, as manifested by a decrease in the α/β synthetic ratio from 0.80 in a control to 0.52 in the presence of inhibitor, when total translation was reduced by 57%.

Inhibitor Does Not Exhibit eIF-2 Kinase Activity. As reviewed in the introduction, a number of studies have revealed a heme-controlled inhibitor activity that copurifies with an eIF-2 kinase specific for the 38 000-dalton subunit of eIF-2, and it has been proposed that this activity is itself the kinase. Figure 9 depicts an autoradiogram of an NaDodSO$_4$–polyacrylamide gel of reaction mixtures, containing crude initiation factors and [γ-^{32}P]ATP, that had been incubated with inhibitor preparations taken at various stages of purification. It is seen that phosphorylation of eIF-2 (arrow) is extensive in the

Table I: Filter Assay of eIF-2 Kinase Activity[a]

inhibitor	–eIF-2	+eIF-2
none	3641	3577
phosphocellulose purified	4078	11834
dsRNA-cellulose purified	2928	3415

[a] Values shown are cpm of ^{32}P retained on nitrocellulose membrane filters. The kinase assay system (60 μL) contained 50 ng of highly purified eIF-2 as indicated, [γ-^{32}P]ATP (156,000 cpm), 0.1 mM ATP, 10 mM Tris-HCl, pH 7.4, 70 mM KCl, 1 mM dithiothreitol, and, when present, 20 μL of inhibitor. After 20 min at 37 °C, 1 mL of 10 mM Tris-HCl, pH 7.4, and 50 mM KCl was added, and the sample was filtered and washed 3 times with the same amount of buffer. Filters were soaked in buffer containing 50 μM ATP.

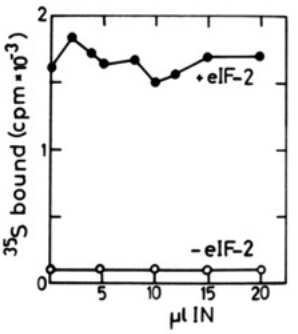

FIGURE 10: Effect of inhibitor on ternary complex formation. Ternary complex formation was assayed with GTP and [^{35}S]Met-tRNA$_f$ (input 7000 cpm; 2.8 × 10^6 cpm/mg of tRNA), with (●) or without (○) purified eIF-2 (1 ng), in the presence of the indicated amounts of purified inhibitor (IN). See Figure 3A for the translation inhibitory activity of the inhibitor preparation. Background (200 cpm) was subtracted.

presence of an inhibitor preparation purified through DEAE-cellulose chromatography (Figure 9, lane a), still present in a preparation purified through phosphocellulose chromatography (Figure 9, lane b), but not detectable after chromatography on dsRNA-cellulose (Figure 9, lane c), in spite of the fact that the latter preparation inhibits translation more effectively (cf. Figure 7C,D).

It may be noted in Figure 9 that phosphorylation of a band moving just above the arrow is increased upon purification of the inhibitor, but absent in lane d. The possibility that this band corresponds to the 38 000-dalton subunit of eIF-2 can be rejected on the basis of the following experiment. When eIF-2 purified to 98% (Rosen & Kaempfer, 1979) is incubated with [γ-^{32}P]ATP and inhibitor in conditions for phosphorylation and then passed through a nitrocellulose membrane filter, eIF-2–kinase activity is observed in the presence of an inhibitor preparation taken before the dsRNA–cellulose step but is not detectable after this step (Table I). We conclude that the kinase activity is not retained on dsRNA–cellulose.

Inhibitor Does Not Block Ternary Complex Formation. As seen in Figure 10, the formation of ternary complexes between eIF-2, [^{35}S]Met-tRNA$_f$, and GTP is not affected by amounts of inhibitor sufficient to block translation completely.

Inhibitor Does Not Bind mRNA. Complex formation between mRNA and eIF-2 can be studied through the ability of eIF-2 to cause labeled mRNA to be retained on nitrocellulose membrane filters (Kaempfer, 1974, 1979a; Kaempfer et al., 1978a). Figure 11A depicts the binding curve for ^{125}I-labeled globin mRNA. Binding is apparently first order in eIF-2 (Kaempfer et al., 1978a) and is consistent with formation of an equimolar complex (Kaempfer et al., 1979b). The labeled mRNA used in these experiments binds to eIF-2 with an affinity equal to that of the native, unlabeled species

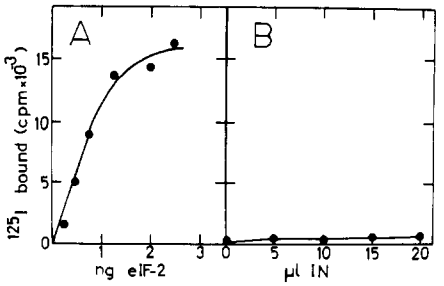

FIGURE 11: Complex formation between globin mRNA and eIF-2 (A) or inhibitor (B). Binding of ^{125}I-labeled rabbit globin mRNA (input 2.6 × 10^4 cpm; 1.7 × 10^6 cpm/μg of RNA) was measured in the presence of the indicated amounts of eIF-2 or inhibitor (IN). See Figure 3A for the translation inhibitory activity of the inhibitor preparation. Background (40 cpm) was subtracted.

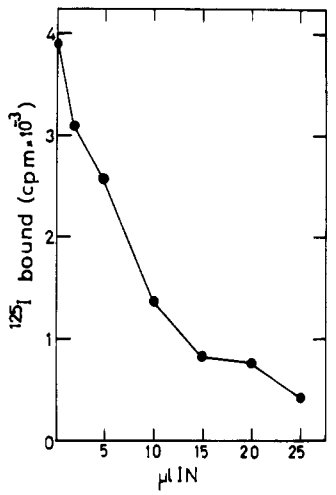

FIGURE 12: Effect of inhibitor on complex formation between eIF-2 and globin mRNA. Complex formation between ^{125}I-labeled globin mRNA (input 6700 cpm; 1.7 × 10^6 cpm/μg of RNA) and a limiting amount of eIF-2 was assayed as for Figure 11, in the presence of the indicated amounts of inhibitor. See Figure 3A for the translation inhibitory activity of the inhibitor preparation. Background (90 cpm) was subtracted.

(Kaempfer, 1979a; Kaempfer et al., 1979b; Rosen et al., 1981). By contrast, a highly active inhibitor preparation exhibits no detectable mRNA-binding activity (Figure 11B). Although this result does not rule out the possibility that the inhibitor might bind to mRNA but pass with it through the filter, it will be seen below that this is not the case.

Inhibitor Blocks Binding of eIF-2 to mRNA. The fact that the inhibitor by itself does not bind detectably to mRNA allowed us to analyze if the inhibitor is able to block the interaction between eIF-2 and mRNA. Our finding is that this is indeed the case: in binding reaction mixtures containing ^{125}I-labeled globin mRNA and a limiting amount of eIF-2, the addition of increasing amounts of inhibitor leads to a drastic decrease in the amount of radioactive mRNA retained on the filter (Figure 12).

The blocking of complex formation between mRNA and eIF-2 by the inhibitor could be explained in three ways. The inhibitor could compete with eIF-2 for a binding site on mRNA and cause inhibitor-bound mRNA to pass through the filter. In this case, the addition of mRNA in excess should make mRNA available for binding to eIF-2. On the other hand, the inhibitor could compete with mRNA for a binding site on eIF-2, either in a direct way or in an allosteric manner. In that case, the addition of either mRNA or eIF-2 in excess should restore binding of mRNA. Finally, the inhibitor could prevent the binding of mRNA to eIF-2 in a noncompetitive manner. In that case, only the addition of eIF-2 in excess should restore binding of mRNA.

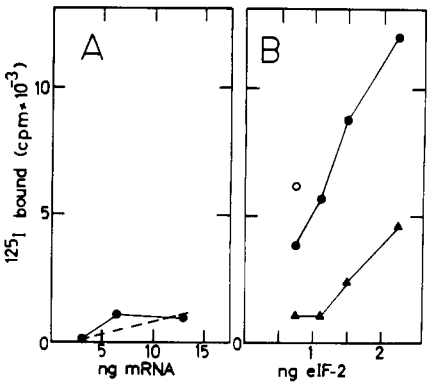

FIGURE 13: Effect of excess mRNA (A) or eIF-2 (B) on effect of inhibitor on complex formation between mRNA and eIF-2. Binding assays in (A) contained 20 μL of inhibitor, 0.75 ng of eIF-2, and the indicated amounts of ^{125}I-labeled globin mRNA (1160 cpm/ng); the broken line denotes background radioactivity. In (B), assays contained no (○), 10 μL (●), or 20 μL (▲) of inhibitor, ^{125}I-labeled globin mRNA (3570 cpm), and the indicated amounts of eIF-2; background (70 cpm) was subtracted. See Figure 3A for the translation inhibitory activity of the inhibitor preparation.

As seen in Figure 13A, the addition of excess ^{125}I-labeled globin mRNA to a binding reaction mixture containing inhibitor and a limiting amount of eIF-2 fails to restore the retention of labeled mRNA on the filter beyond the background level. By contrast, upon addition of excess eIF-2, effective relief of the inhibition is observed (Figure 13B). The extent of relief by a given amount of eIF-2 is more pronounced the lower the inhibitor concentration. Even the effect of an amount of inhibitor sufficient to give 85% inhibition of eIF-2/mRNA complex formation is relieved by an excess of eIF-2 (lower curve).

These findings show that the inhibitor acts to prevent the interaction between globin mRNA and eIF-2. It does this not by affecting the mRNA but by affecting the ability of eIF-2 to bind mRNA.

In agreement with the results of Figure 9 and Table I, the action of inhibitor purified through the dsRNA–cellulose step on complex formation between globin mRNA and eIF-2 could not be reversed by including in the reaction mixture the nonhydrolyzable ATP analogue, 5′-adenylyl imidodiphosphate (AMP-PNP), up to a concentration of 1 mM (not shown), yet AMP-PNP effectively inhibits the action of the ATP-dependent eIF-2 kinase (Ranu & London, 1979).

Discussion

These experiments report on the isolation of a heme-controlled inhibitor of translation that does not act to phosphorylate eIF-2 but instead affects the ability of this initiation factor to bind messenger RNA. Since there is evidence that a specific interaction between eIF-2 and mRNA occurs during initiation of protein synthesis, the present findings suggest that inhibition of that interaction by this inhibitor may be a plausible mechanism of its action.

In previous studies (see the introduction), a heme-controlled inhibitor activity was isolated that copurified with eIF-2 kinase activity and was thought to be identical with it. The introduction of a novel purification step, involving chromatography on dsRNA–cellulose (Figure 1), revealed the existence of a heme-controlled inhibitor that effectively blocks protein synthesis (Figure 3) yet possesses no detectable eIF-2-kinase activity (Figure 9 and Table I). The inhibitor thus purified functionally resembles previously studied heme-controlled inhibitor preparations (Safer & Anderson, 1978; Hunt, 1979; de Haro & Ochoa, 1979; Austin & Clemens, 1980) in terms

of the kinetics and extent of inhibition of translation (Figure 3), relief of this inhibition by eIF-2 (Figure 7), and relief of this inhibition by 2-aminopurine (Figure 8). This and the observed decrease in α/β globin synthesis induced by the inhibitor support the concept that it acts to block initiation of translation, apparently by blocking the action of eIF-2.

Inhibitor activity appears specifically upon incubation of postribosomal supernatant in the absence of heme (Figures 1C and 2). This finding and the resistance of inhibitor activity to digestion with micrococcal nuclease, as opposed to the sensitivity of dsRNA to such treatment, eliminate the possiblity that the inhibitor could be dsRNA released from the dsRNA–cellulose column. Several other results reinforce this conclusion. The chromatographic behavior of the inhibitor on DEAE-cellulose, phosphocellulose, dsRNA–cellulose, and Cibacron Blue–Sephadex columns is inconsistent with its being RNA. It may be noted in Figure 7 that inhibition of translation by dsRNA is completely relieved by eIF-2 (Figure 7B), while the same amount of eIF-2 does not alleviate the same extent of inhibition when it is caused by the inhibitor (Figure 7D) but relieves only the action of lower levels of inhibitor. This result points to a functional difference between dsRNA and the inhibitor. Moreover, dsRNA readily inhibits ternary complex formation between eIF-2, Met-tRNA$_f$, and GTP (Rosen et al., 1981), while the inhibitor does not (Figure 10). Finally, dsRNA competitively inhibits the binding of mRNA to eIF-2 (Rosen et al., 1981), and this inhibition is readily reversed by excess mRNA (Rosen, 1982), yet the action of the inhibitor on complex formation between mRNA and eIF-2 is not relieved by excess mRNA (Figure 13A). The results also eliminate the possibility that the inhibitor is related to a small RNA inhibitor reported by Dionne et al. (1982).

The possibility that the inhibitor is (2′–5′)-oligoisoadenylate or (2′–5′)-oligoisoadenylate synthetase (Hovanessian & Kerr, 1978) can be rejected on the basis of the observations that the inhibitor is resistant to bacterial alkaline phosphatase and that its action on translation is reversed by eIF-2. The finding that the action of the inhibitor on the binding of mRNA to eIF-2 and on translation can be overcome by an excess of eIF-2 argues strongly against the possibility that the inhibitor is a nuclease acting on mRNA. Nor does it seem likely that the inhibitor is a protease: it exhibits no inhibitory activity on the binding of Met-tRNA$_f$ by eIF-2, even though this function is much more labile than is the binding of mRNA (Kaempfer, 1974, 1979a). Moreover, its inhibitory effect on translation can be reversed by 2-aminopurine, a finding not expected if it were a protease.

Even though the nature of the inhibitor is not yet known, the inactivation studies, the chromatographic behavior of the inhibitor on DEAE-cellulose, phosphocellulose, and dsRNA–cellulose columns, and its affinity for the dye Cibacron Blue tend to support the tentative interpretation that the inhibitor is a stable protein, resistant to heat denaturation or protease digestion. It differs qualitatively from a heat-stable inhibitor reported by de Haro et al. (1982) which is trypsin sensitive and a similar, protease-sensitive inhibitor reported by Henderson et al. (1979) that displays, in addition, different chromatographic behavior on DEAE-cellulose.

Insight into the mechanism of action of the inhibitor is provided by studies of partial reactions of initiation of translation in which eIF-2 participates. The characteristic property of eIF-2 to form a ternary complex with Met-tRNA$_f$ and GTP is not affected by the inhibitor (Figure 10). On the other hand, the ability of eIF-2 to form a specific complex with globin mRNA (Figure 11A) is inhibited drastically (Figure 12). The inhibitor blocks the interaction between eIF-2 and mRNA not by competing with eIF-2 for a binding site on mRNA, for it fails to bind detectably to mRNA by itself (Figure 11B) and its action is not overcome by the addition of excess mRNA (Figure 13A), but, instead, by acting on eIF-2. This follows from our finding that addition of excess eIF-2 effectively restores the formation of mRNA/eIF-2 complexes (Figure 13B). We do not yet know if the inhibitor binds to eIF-2 or modifies this initiation factor such that mRNA no longer can be bound.

The importance of the interaction between mRNA and eIF-2 during translation is suggested by a number of observations. eIF-2 binds with high affinity to a variety of mRNA species, but not to tRNA, rRNA, or negative-strand RNA (Kaempfer, 1974; Barrieux & Rosenfeld, 1978; Kaempfer et al., 1978a; Rosen & Kaempfer, 1979). It forms an equimolar complex with globin mRNA, at a site that involves none of the sequences 3′-proximal to the coding region (Kaempfer et al., 1979b). Instead, eIF-2 binds to satellite tobacco necrosis virus RNA specifically at a 5′-terminal, 44-nucleotide sequence that contains the binding site sequence for the 40S ribosomal subunit (Kaempfer et al., 1981). In Mengovirus RNA, eIF-2 protects a unique set of three T1 oligonucleotides that are identical with three of four oligonucleotides protected in 40S or 80S ribosome initiation complexes (Perez-Bercoff & Kaempfer, 1982). These findings strongly suggest that the recognition and binding of mRNA by a ribosome may be guided to an important extent by eIF-2. Moreover, eIF-2 serves as a target for translational competition between mRNA species. The translational competition between α- and β-globin mRNA is relieved by eIF-2, and β-globin mRNA binds to this initiation factor with higher affinity than does α-globin mRNA (Di Segni et al., 1979). On a molar basis, Mengovirus RNA apparently competes 35 times more effectively in translation than does globin mRNA; this competition likewise can be relieved by eIF-2 (Rosen et al., 1982). Indeed, Mengovirus RNA binds with a 30-fold higher affinity than globin mRNA to eIF-2 (Rosen et al., 1982). These studies have revealed a direct correlation between the ability of a given mRNA species to compete in translation and its affinity for eIF-2.

Clearly, interference with the binding of eIF-2 to mRNA could account for the action of the present heme-controlled inhibitor on translation. It is conceivable that this interference could release a chain of events leading to the eventual phosphorylation of eIF-2, although it is also possible that the action of the heme-controlled inhibitor described here and the phosphorylation of eIF-2 by a heme-controlled kinase are independent phenomena. The finding that 2-aminopurine can reverse the effect of the inhibitor on translation (Figure 8B) tends to support the former alternative, as 2-aminopurine is thought to act by competitively inhibiting the ATP-dependent kinase activity that phosphorylates eIF-2. Thus, 2-aminopurine would prevent the phosphorylation of eIF-2 molecules that becomes possible when binding of mRNA to eIF-2 is prevented.

Indeed, the observations on phosphorylation of eIF-2 need not be in conflict with the findings on the heme-controlled inhibitor described here, if an inhibition of the interaction between mRNA and eIF-2 renders this initiation factor a substrate for subsequent phosphorylation.

Observations on the mechanism of translational inhibition by dsRNA fit remarkably well with such an interpretation. As reviewed in the introduction, experiments on the mode of action of dsRNA have revealed mRNA specificity in this process and a correlation between the affinity of an mRNA

species for eIF-2 and the sensitivity of its translation to inhibition by dsRNA (Rosen et al., 1981). The results of that study suggest that dsRNA, by competitively inhibiting the interaction of mRNA with eIF-2, allows this initiation factor to be phosphorylated.

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