Vol. 83, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 28, 1978 Pages 715-723

EVIDENCE FOR AN INHIBITOR OF PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES ACTIVATED BY HIGH PRESSURE

A. Burl Henderson and Boyd Hardesty

Clayton Foundation Biochemical Institute
Department of Chemistry, The University of Texas
Austin, Texas 78712

Received April 21,1978

Evidence is presented for an inhibitor of protein synthesis which is activated by subjecting rabbit reticulocyte postribosomal supernatant to high pressure (15,000 lb/in^2). The inhibitor is antigenically and chromatographically distinct from the hemin-controlled repressor. The mechanism of inhibition by the pressure-treated postribosomal supernatant is not clear; however, the kinetics of inhibition seem to indicate that inhibition may be at the level of initiation.

A variety of naturally occurring agents from several cell types which inhibit the initiation of protein synthesis *in vitro* have been described. Several of these agents appear to regulate translation *in vivo*. Possibly the best characterized of these is the hemin-controlled repressor (HCR)*, initially reported by Rabinovitz and his co-workers (1), and subsequently studied in detail in a number of laboratories (2-18). This enzyme was shown to be a protein kinase specific for the 38,000 dalton subunit of initiation factor eIF-2 (11-18). Phosphorylated eIF-2 is inactive in promoting the binding of the ternary complex, eIF-2·GTP·Met-tRNA$_f^{Met}$, to 40S ribosomal subunits (16). More recently, inhibitors which also phosphorylate the smallest subunit of eIF-2 have been partially purified from unstimulated Friend leukemia cells (19) and perfused rabbit liver (20).

Incubation of reticulocyte lysates with double-stranded RNA (dsRNA) also activated an inhibitor of protein synthesis initiation (18, 21, 22), termed dsRNA-activated inhibitor (DAI). DAI also has protein kinase activity for the smallest subunit of eIF-2, and exhibits many of the characteristics of HCR, yet appears to be a separate enzyme. Evidence from several laboratories

*Abbreviation: HCR, hemin-controlled repressor

0006-291X/78/0832-0715$01.00/0

(23-26) seems to link this dsRNA effect to the inhibition of viral protein synthesis in interferon-treated cells.

Other mechanisms of inhibition of peptide initiation have been reported. Glutathione disulfide (GSSG) has been shown to activate an inhibitor which exhibits kinetics of inhibition similar to those observed with hemin deficiency or dsRNA (27). Cimadevilla *et al.* (28) demonstrated a protein inhibitor in Friend leukemia cells which blocked an undetermined step in peptide initiation beyond the attachment of mRNA to the 40S subunit, but prior to the formation of the initial peptide bond. Kramer *et al.* (29) demonstrated that a protein kinase activity from rabbit reticulocytes inhibited binding of Met-$tRNA_f^{Met}$ to 40S subunits by the phosphorylation of the 40S subunit proteins.

In this work, we report the observation of an inhibitory activity in rabbit reticulocyte post-ribosomal supernatant (PRS)* which has been activated by subjecting the crude supernatant to high pressure. As yet, the exact mechanism of inhibition has not been determined, but the predominance of evidence supports the probability that it acts at the level of peptide initiation.

MATERIALS AND METHODS

Preparation of Rabbit Reticulocyte Lysates.

The procedure used for the preparation of reticulocyte lysates was that of Adamson *et al.* (30).

Reticulocyte Lysate Assay System.

Protein synthesis was measured in the incubation mixtures containing the following in a final volume of 50 µl: 10 mM Tris-HCl (pH 7.5), 90 mM KCl, 1.5 mM $MgCl_2$, 5 mM dithioerythritol, 0.5 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 26 units/ml creatine phosphokinase, 0.05 mM [^{14}C]leucine (40 Ci/mol), 0.1 mM all other [^{12}C]amino acids, 10 µg/ml hemin (bovine, Type I) and 10 µl of reticulocyte lysate. Incubation was carried out at 37° for 30 min unless otherwise indicated. Reactions were stopped by the addition of 100 µl of 1N NaOH followed by the addition of 100 µl of 3% H_2O_2. This mixture was incubated at 37° for 5 min and the samples were made 5% in CCl_3COOH. The acid precipitated samples were incubated at 100° for 2 min, allowed to cool, and filtered on glass fiber filters. The dried filters were counted by liquid scintillation.

Treatment of Protein Solutions with N-Ethyl Maleimide (NEM).

Samples to be NEM treated were made 5 mM in NEM, and incubated at 37° for 15 min. Following this incubation, the mixture was made 6 mM in dithioerythritol and incubated at 37° for 10 min.

*Abbreviation: PRS, postribosomal supernatant from rabbit reticulocytes.

Preparation and Pressure Treatment of Postribosomal Supernatant.

Heparinized whole blood from phenylhydrazine-injected New Zealand White rabbits was centrifuged to remove the plasma. The packed cells were washed with NKM solution (0.13 M NaCl, 0.005 M KCl, 0.0074 M $MgCl_2$) and filtered through glass wool prior to centrifuging. The packed, washed cells were suspended in 2.5 volumes of NKM solution. Lysis was accomplished by adding 7.5 times the packed-cell volume of 0.0025 M $MgCl_2$ with stirring for 4 min on ice. After lysis, 2 times the packed-cell volume of 1.5 M sucrose, 0.15 M KCl, 35 mM 2-mercaptoethanol was added, and the cell stroma removed by centrifugation (20 min at 15,000 xg). The supernatant material is then centrifuged at 105,000 xg for two hours to pellet the ribosomes. The postribosomal supernatant from this centrifugation was stored at -90° until pressure treatment.

Pressure treatment was accomplished in a French pressure cell (Cat. No. 4-3398-A, American Instrument Co., Silver Spring, MD) with a one-inch diameter plunger. The hydraulic press was adjusted so that a pressure of 15,000 lb/in^2 would be achieved in the cell. When this pressure was reached, approximately 3 ml of the PRS was released from the cell by opening the release valve, and rapidly closing the valve when the pressure had dropped. The pressure was repeatedly increased and rapidly released until all of the PRS had been passed through the cell.

Preparation of Goat IgG Against HCR.

Preparation of IgG against HCR and the details of the reaction have been previously described (14).

Sephadex G-150 Column Chromatography.

Crude pressure-treated PRS was loaded on a column of Sephadex G-150 (40-120 mesh, 2.0 x 60 cm), previously equilibrated with 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM 2-mercaptoethanol. The column was developed with the same buffer at a flow rate of 0.1 ml/min. Fractions of 1.5 ml were collected, and the activity of the inhibitor was assayed in the reticulocyte lysate system.

RESULTS

Rabbit reticulocyte postribosomal supernatant was placed in a French pressure cell and subjected to pressures of 15,000 lb/in^2. Addition of this pressure-treated PRS to a reticulocyte lysate protein synthesizing system resulted in concentration-dependent inhibition of incorporation of [^{14}C]leucine into CCl_3COOH precipitable material (Fig. 1A). The rates of incorporation for both the control and inhibitor-containing incubations were virtually identical for the first 3 to 5 min of incubation. Following this initial period, incubations containing pressure-treated PRS showed a marked decrease in the rate of incorporation (Fig. 1B). The kinetics of inhibition are similar to those observed with authentic HCR. Comparison of the inhibitory activity of the pressure-treated PRS to that of edeine, a known inhibitor of peptide initiation, is also shown in Fig. 1. The observation that the extent of inhibition was near

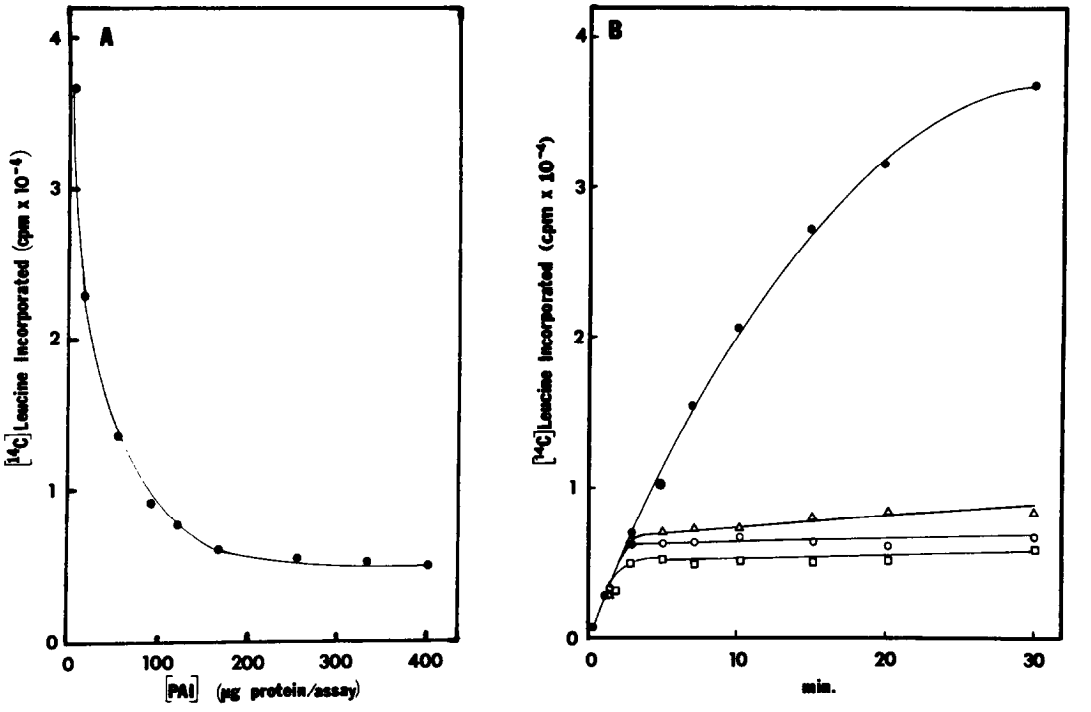

Figure 1. PAI Inhibition of Reticulocyte Lysate System.
(A) Concentration course of PAI inhibition. (B) Time course of inhibition by pressure-treated PRS, HCR and edeine. Inhibitors were used in the following concentrations: edeine, 2 μM; crude pressure-treated PRS, 60 mg/ml; HCR, 1.5 mg/ml; ●—● = control; △—△ = pressure-treated PRS; ○—○ = HCR; □—□ = edeine.

or slightly less than that produced by edeine supports the argument that the pressure-activated inhibitor may be specific for some as yet undetermined step in the initiation process.

Treatment of PRS by N-ethyl maleimide (NEM) is known to activate an inhibitor thought to be HCR (7). Table I shows the results of treating pressure-activated PRS with 5 mM NEM. NEM treatment increased the inhibitory activity of the PRS in each case; however, the inhibitory activity is greater in the NEM-treated, pressure-activated PRS than in the PRS treated with NEM only.

The possibility that pressure treatment of PRS might activate HCR was investigated. HCR is inactivated by pretreatment with anti-HCR IgG (31). The results of such an experiment using authentic HCR and pressure-treated PRS are presented in Table II. When NEM-treated PRS (not pressure treated) is used as

Table I

Inhibitor Activation by Pressure and N-ethyl Maleimide

Additions	[^{14}C]Leucine Incorporation TCA Precipitable Products (cpm)
None	19,593
PAI	7,210
Untreated PRS	16,959
NEM-treated PAI	3,673
NEM-treated PRS (not pressure treated)	6,826

The effect of various inhibitors was assayed in the reticulocyte lysate system as described in Materials and Methods. Where indicated, 300 µg of postribosomal supernatant protein was added to the individual assay mixtures. NEM treatment was performed as indicated in Materials and Methods.

Table II

Effect of Anti-HCR IgG on Inhibitory Activity of PAI and NEM Activated HCR

	[^{14}C]Leucine Incorporation (cpm)	
Inhibitory Activity	+Anti-HCR IgG	-Anti-HCR IgG
None	21,257	21,550
PAI	6,367	5,307
NEM-Treated PRS	16,427	2,891

Assays were performed as described for Table I. Anti-HCR IgG (6 µg/300 µg crude PAI) was added to the PAI and reacted as described in Materials and Methods prior to introduction into the lysate assay mixture.

the inhibitory agent, the inhibitory activity is decreased by more than 5 fold by preincubating with anti-HCR IgG. However, anti-HCR IgG has a much reduced effect with pressure-treated PRS. These data indicate that the principal inhibitory activity is not sensitive to preincubation with anti-HCR IgG although some HCR-like activity may exist in the pressure-treated PRS.

Since PAI* appeared to be antigenically different from HCR, we attempted

*Abbreviation: PAI, pressure activated inhibitor; postribosomal supernatant subjected to 15,000 lb/in^2 in a French pressure cell.

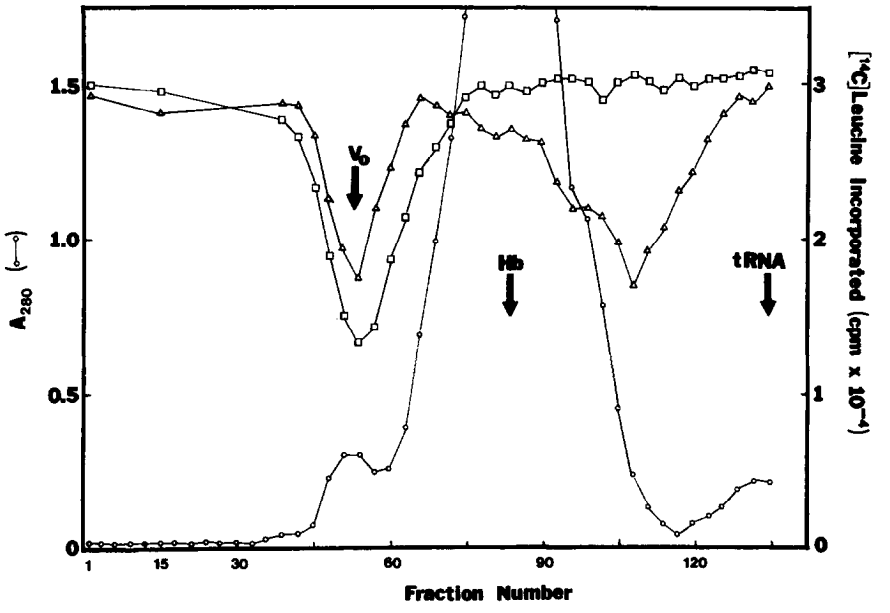

Figure 2. Activity Profile of Sephadex G-150 Fractions of Pressure-Treated PRS and HCR.
Chromatography was carried out as described in Materials and Methods. Aliquots of 20 µl of the indicated fractions were added to the reticulocyte lysate assay. [^{14}C]leucine incorporation is plotted for each fraction. Arrows indicate volumes at which blue dextran, rabbit hemoglobin and tRNA peaks elute. Δ—Δ = pressure-treated PRS; □—□ = HCR; O—O = A_{280}

to separate the two activities chromatographically. Fig. 2 shows the Sephadex G-150 profile of the crude, pressure-treated PRS. Two peaks of inhibitory activity were detected in this experiment. The first inhibitor, I, eluted near the void volume; the second, II, eluted with its peak of activity slightly behind the hemoglobin peak. Fractions containing these two activities were pooled separately, concentrated by Amicon filtration, and dialyzed against 10 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM 2-mercaptoethanol, in 50% glycerol. Pooled activities from each peak were reexamined with respect to their sensitivity to anti-HCR IgG. The results of this experiment are given in Table III. These results indicate that inhibitor I is HCR. Inhibitor II is not inactivated by anti-HCR. Authentic HCR, activated by NEM treatment and purified through the DEAE-cellulose step (14) was chromatographed on Sephadex G-150 under identical conditions as the pressure-treated PRS. It eluted near the void volume in the

Table III

Effect of Anti-HCR IgG on Inhibitors Separated by Gel Filtration Chromatography

Inhibitor I	Inhibitor II	Anti-HCR IgG	[^{14}C]Leucine Incorporation Into TCAppt. Product
-	-	-	26,285
-	-	+	24,394
+	-	-	8,693
+	-	+	19,665
-	+	-	12,154
-	+	+	12,726

Assays were performed exactly as described for Table II, except that 30 µg of inhibitor I protein and 184 µg of inhibitor II protein were added as indicated.

position of inhibitor I from the pressure-treated PRS. No inhibitory activity was detected at the volume in which the second inhibitor was observed in the pressure-treated PRS.

The possibility that the two fractions from pressure-treated PRS contained RNase activity or an aminoacyl-tRNA hydrolase was tested by incubating the fractions with [^{35}S]Met-tRNA$_f^{Met}$. The extent or release of radioactively labeled methionine from cold CCl_3COOH precipitable material was measured. The results indicate that the inhibitory activity in the pressure activated PRS is not due to RNase or an aminoacyl-tRNA hydrolase.

DISCUSSION

The data presented here indicate that a latent inhibitor present in the reticulocyte postribosomal supernatant can be activated by pressure. This appears to account for at least part of the endogenous inhibitory activity for protein synthesis frequently observed in cellular fractions that have been centrifuged at high speed. HCR itself appears to be partially activated by the pressure treatment, however the pressure activated inhibitor is distinct

from HCR in size and immunological properties. Its relation to the inhibitor activated by double-stranded RNA has not been determined. PAI appears to be a protein slightly smaller than hemoglobin. Preliminary experiments indicate that it blocks peptide initiation; however, its mechanism of action has not been determined.

ACKNOWLEDGEMENTS

The authors wish to thank M. Hardesty, J. Ybarra and M. Rodgers for their excellent technical assistance, M. Henderson for her help in the preparation of the typescript and Dr. G. Kramer for her stimulating discussions. A.B.H. is the recipient of National Institutes of Health Fellowship 5 F32 CA-05765-02. This work was supported also by Grants T32 CA09182-03 from the National Cancer Institute and CA16608-03 from the National Institutes of Health, Department of Health, Education and Welfare.

REFERENCES

1. Maxwell, C.R., and Rabinovitz, M. (1969) Biochem. Biophys. Res. Commun. 35, 79-85.
2. Maxwell, C.R., Kamper, C.S., and Rabinovitz, M. (1971) J. Mol. Biol. 58, 317-327.
3. Gross, M., and Rabinovitz, M. (1972) Biochim. Biophys. Acta 287, 340-352.
4. Balkow, K., Mizuno, S., Fisher, J. M., and Rabinovitz, M. (1973) Biochim. Biophys. Acta 324, 397-409.
5. Adamson, S.D., Yau, P.M., and Herbert, E. (1972) J. Mol. Biol. 63, 247-264.
6. Legon, S., Jackson, R.J., and Hunt, T. (1973) Nature New Biology 241, 150-152.
7. Gross, M., and Rabinovitz, M. (1973) Biochem. Biophys. Res. Commun. 50, 832-838.
8. Buzard, Y., Rodvien, R., and London, I.M. (1973) Proc. Nat. Acad. Sci. USA 70, 1022-1026.
9. Clemens, M.J., Henshaw, E.C., Rahaminoff, H. and London, I.M. (1974) Proc. Nat. Acad. Sci. USA 71, 2946-2950.
10. Gross, M., and Rabinovitz, M. (1973) Biochem. Biophys. Res. Commun. 67, 1507-1515.
11. Balkow, K., Hunt, T., and Jackson, R.J. (1975) Biochem. Biophys. Res. Commun. 67, 366-374.
12. Levin, D.H., Ranu, R.S., Ernst, V., Fifer, M.A., and London, I.M. (1975) Proc. Nat. Acad. Sci. USA 72, 4849-4853.
13. Ernst, V., Levin, D.H., Ranu, R.S., and London, I.M. (1976) Proc. Nat. Acad. Sci. USA 73, 1112-1116.
14. Kramer, G., Cimadevilla, J.M., and Hardesty, B. (1976) Proc. Nat. Acad. Sci. USA 73, 3078-3082.
15. Levin, D.H., Ranu, R.S., Ernst, V., and London, I.M. (1976) Proc. Nat. Acad. Sci. USA 73, 3112-3116.
16. Pinphanichakarn, P., Kramer, G., and Hardesty, B. (1976) Biochem. Biophys. Res. Commun. 73, 625-631.
17. Ranu, R.S., and London, I.M. (1976) Proc. Nat. Acad. Sci. USA 73, 4349-4353.
18. Farrell, P., Balkow, K., Hunt, T., Jackson, R., and Trachsel, H. (1977) Cell 11, 187-200.
19. Pinphanichakarn, P., Kramer, G., and Hardesty, B. (1977) J. Biol. Chem. 252, 2106-2112.

20. Delaunay, J., Ranu, R.S., Levin, D.H., Ernst, V., and London, I.M. (1977) Proc. Nat. Acad. Sci. USA 74, 2264-2268.
21. Ehrenfeld, E., and Hunt, T. (1971) Proc. Nat. Acad. Sci. USA 68, 1075-1078.
22. Hunter, T., Hunt, T., and Jackson, R.J. (1975) J. Biol. Chem. 250, 409-417.
23. Kerr, I.M., Brown, R.E., and Ball, L.A. (1974) Nature 250, 57-59.
24. Content, J., Lebleu, B., Nadel, U., Zilberstein, A., Berissi, H., and Revel, M. (1975) Eur. J. Biochem. 54, 1-10.
25. Lebleu, B., Sen, G.C., Shaila, S., Cabrer, B., and Lengyel, P. (1976) Proc. Nat. Acad. Sci. USA 73, 3107-3111.
26. Roberts, W.K., Clemens, M.J., and Kerr, I.M. (1976) Proc. Nat. Acad. Sci. USA 73, 3136-3140.
27. Kosower, N.S., Vanderhoff, G.A., Benerofe, B., Hunt, T., and Kosower, E. (1971) Biochem. Biophys. Res. Commun. 45, 816-821.
28. Cimadevilla, J.M., Kramer, G., Pinphanichakarn, P., Konecki, D., and Hardesty, B. (1975) Arch. Biochem. Biophys. 171, 145-153.
29. Kramer, G., Henderson, A.B., Pinphanichakarn, P., Wallis, M.H., and Hardesty, B. (1977) Proc. Nat. Acad. Sci. USA 74, 1445-1449.
30. Adamson, S.D., Herbert, E., and Godchaux, W. (1968) Arch. Biochem. Biophys. 125, 671-683.
31. Gross, M. (1974) Biochem. Biophys. Res. Commun. 57, 611-619.