ISOLATION AND CHARACTERIZATION OF AN ANTIBODY TO A HIGHLY PURIFIED PREPARATION OF THE HEMIN-CONTROLLED TRANSLATIONAL REPRESSOR FROM RABBIT RETICULOCYTES

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SUMMARY. An antibody to a highly purified preparation of the translational repressor (HCR), which mediates hemin control of globin synthesis in rabbit reticulocyte lysates, has been obtained from the serum of immunized guinea pigs. Preincubation with immune but not normal guinea pig IgG leads to neutralization of the inhibitory activity of either crude or highly purified HCR. Excess prorepressor, the precursor of HCR, has essentially no competitive effect on the inactivation of HCR by immune IgG, suggesting that the antigenic determinants responsible for neutralization of HCR by antibody are buried within the prorepressor molecule. These antigenic determinants become exposed at an early stage in the formation of HCR, since hemin-sensitive HCR, formed within 20 min, is inactivated by immune IgG. The antibody also neutralizes the inhibitory activity generated by a short incubation of partially purified prorepressor with N-ethylmaleimide, indicating that the activity formed is the same as natural HCR.

The maintenance of continued globin synthesis in both rabbit reticulocytes (1, 2) and cell-free reticulocyte preparations (3, 4) is dependent upon the availability of hemin. This hemin effect appears to be mediated by an inhibitor of polypeptide chain initiation, termed the hemin-controlled repressor (HCR), which is formed in the absence of hemin from a precursor of HCR (prorepressor) in the post-ribosomal supernatant fraction (5-10). HCR, formed within the first 30 min, can be inactivated by hemin (8), and this hemin-sensitive form of HCR is an intermediate in the formation of hemin-resistant HCR activity (11). Unless otherwise indicated, the use of HCR in this report shall refer to the hemin-resistant form of the repressor. HCR causes disaggregation of polyribosomes under conditions of protein synthesis, and its effect can be overcome by a factor partially purified from a 0.5 M KCl wash of reticulocyte ribosomes (12, 13). The effect of HCR is not specific for globin (13-16), and its mode of action may be to block the combination of met-$tRNA_f$ with the 40s subunit (10, 17) or the combination of 40s and 60s particles with loss of methionine from ribosome-bound initiator tRNA (18, 19). The repressor is a protein (12, 13) with a molecular weight, based on gel filtration, of $4\pm1 \times 10^5$ (12, 20) and there is no detectable change in molecular size in the transition from prorepressor to HCR (20). The complete conversion of prorepressor to HCR normally

requires 12-16 hr at 37° (7) but occurs within 5 min by treating prorepressor with N-ethylmaleimide (20). This report describes the preparation and characteristics of an antibody to highly purified HCR (21) from the serum of immunized guinea pigs.

METHODS AND MATERIALS

Cell-free reaction samples contained 30 µl of test material, 30 µl of a master mix, and 50 µl of reticulocyte lysate without added hemin. Samples were incubated at 34°, and the incorporation of ^{14}C-leucine into protein was determined after 2 and 5 min by treatment of 40 µl aliquots of the reaction mixture with acid acetone followed by washes with 5% TCA and acetone as described (8). The agreement of duplicate samples was within 5%. Lysate was prepared from the washed reticulocytes of phenylhydrazine-treated rabbits as previously described (7). The master mix contained the following components in amounts to yield the indicated concentrations in the final reaction mixture: KCl (75 mM); $MgCl_2$ (2 mM); ATP (0.5 mM); GTP (0.2 mM); creatine phosphate (15 mM); creatine kinase (45 units/ml); L-[1-^{14}C]leucine (0.3 mM; 5 Ci/mole); and the 19 other unlabeled amino acids at concentrations corresponding to the composition of rabbit hemoglobin. The ribosome-free supernatant fraction (supernate) was prepared by centrifuging 1.5 ml aliquots of lysate in 1.7 cm high tubes at 275,000 x g for 80 min in the SW 50.1 rotor (22).

Step 1 HCR was prepared by incubating supernate for 16 hr at 37° (21). Step VII HCR was prepared as described (21) and represents a 2,000-fold purified, but still heterogeneous preparation. The assay for HCR and the definition of one unit of activity have been described (7). Step 1 HCR had a potency of 25 units/mg and contained 3,000 units/ml of supernate. Step VII had a potency of 50 x 10^3 units/mg, and it was stabilized for experimental incubations by diluting with fresh supernate (containing 50 µM hemin) to give 5-10 units/µl of supernate.

Five Harley guinea pigs, with an initial weight of 250-300 gm, were immunized 3 times with Step VII HCR at 3 week intervals. Each immunizing dose, which contained 125 µg of protein, was split into 5, 0.1 ml portions and injected into the 4 foot pads and subcutaneously into the neck with the animal under ether anesthesia. The first immunization was given in complete Freund's adjuvant (Difco) containing 0.1 mg/ml of mycobacteria, the second in incomplete Freund's adjuvant, and the third in saline. The animals were bled one week after the third immunization, and the serum was pooled (58 ml). Pooled serum (16 ml) from 2 unimmunized Harley guinea pigs, from the same shipment as the immunized animals, was also obtained.

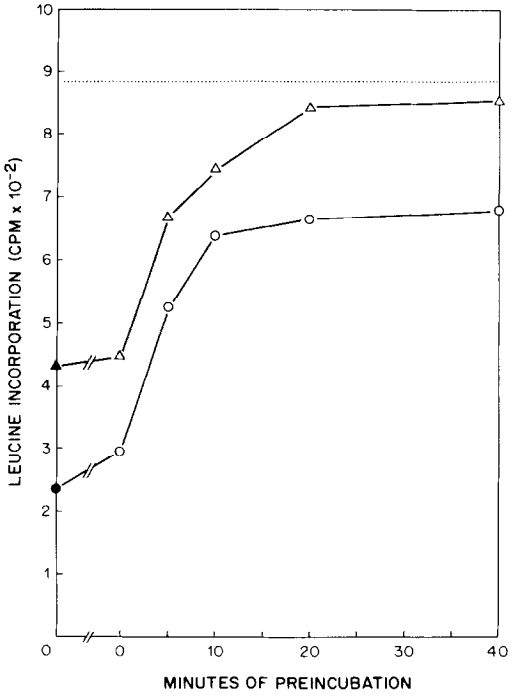


Figure 1. Effect of time of preincubation of immune IgG with HCR on inhibitory activity.

Duplicate samples, containing 6 (Δ—Δ) or 12 (o—o) units of Step I HCR were mixed with 15 µg of immune IgG, brought to a final volume of 30 µl with 1X HEPES buffer, and then incubated at 34° for the indicated times. Inhibitory activity was immediately determined by the addition of 30 µl of master mix and 50 µl of lysate. The ordinate represents the Δ CPM at 5 minus 2 min. Samples of HCR plus IgG that were not preincubated were mixed at 0° just before the assay. Samples of HCR, containing 6 (▲) or 12 (●) units, received no IgG and showed no loss of inhibitory activity when warmed. The mean incorporation by 4 control samples, containing 30 µl of 1X HEPES buffer alone, is represented by the dotted line at the top of the figure.

Immune and normal IgG were prepared from 27 ml of serum from immunized and 15 ml of serum from non-immunized animals, respectively. All operations were at 0-4°. The precipitate from 40% ammonium sulfate treatment was disolved in a minimal volume of 0.04 M potassium phosphate, pH 6.9, and dialyzed against 3, 500 ml changes of phosphate buffer overnight. The dialysate was applied to a 10 x 1.5 cm DEAE cellulose column equilibrated with phosphate buffer, and the break-through peak was pooled. The sample was concentrated and the buffer changed to 0.03 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate, pH 7.1 (10X HEPES buffer) by ultrafiltration.

DEAE cellulose (DE 52) was obtained from Whatman, and CM-Sephadex C50, from Pharmacia. HEPES was purchased from Calbiochem. N-ethylmaleimide was

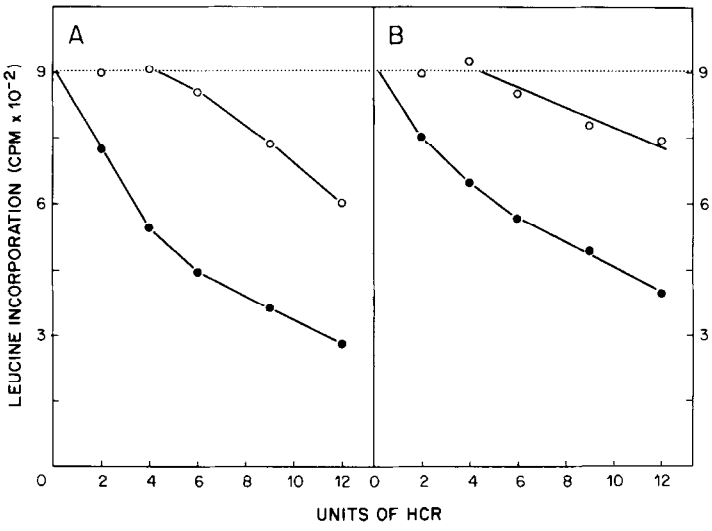


Figure 2. Effect of immune IgG on the inhibitory activity of increasing amounts of crude and highly purified HCR.

Duplicate samples, containing the indicated amounts of Step I (Frame A) or Step VII (Frame B) HCR were incubated with 15 µg of immune (o—o) or normal (•—•) IgG in a final volume of 30 µl (with 1X HEPES buffer) for 20 min at 34°. Inhibitory activity was then immediately determined by the addition of 30 µl of master mix and 50 µl of lysate. The ordinate represents the Δ CPM at 5 minus 2 min. The mean incorporation by 4 control samples is indicated by the dotted line at the top of the figure. Samples containing immune or normal IgG alone were not different from the control.

from Eastman, and dithiothreitol, from Sigma. Guinea pigs were obtained from Camm.

RESULTS AND DISCUSSION

Preliminary experiments showed that serum from immunized or control guinea pigs diluted 1 to 3,000 inhibited cell-free protein synthesis by 50% when incubation was in the presence of hemin for 90 min at 34°. By isolating the IgG fraction, which was only 2% as inhibitory as serum, and using a 5 min assay in the absence of hemin, which is much less sensitive to inhibition by exogenous proteins than a 90 min incubation in the presence of hemin (11), up to 15 µg of IgG could be added without inhibitory effect. A 5 min incubation without hemin is sensitive to HCR, since the repressor inhibits protein synthesis within 2 min, whereas incorporation in controls continues at a linear rate for 4-5 min before becoming suppressed due to formation of endogenous HCR (8, 9).

Two different levels of Step I HCR were preincubated with immune IgG for up to 40 min to determine if the IgG could neutralize the activity of the

TABLE I

Effect of Normal IgG on the Inhibitory Activity of HCR

Duplicate samples, containing the indicated components in a final volume of 30 µl (using 1X HEPES buffer) were incubated at 34° for 20 min except for those marked with an asterisk, which were kept at 0°. Inhibitory activity was immediately determined by the addition of 30 µl of master mix and 50 µl of lysate

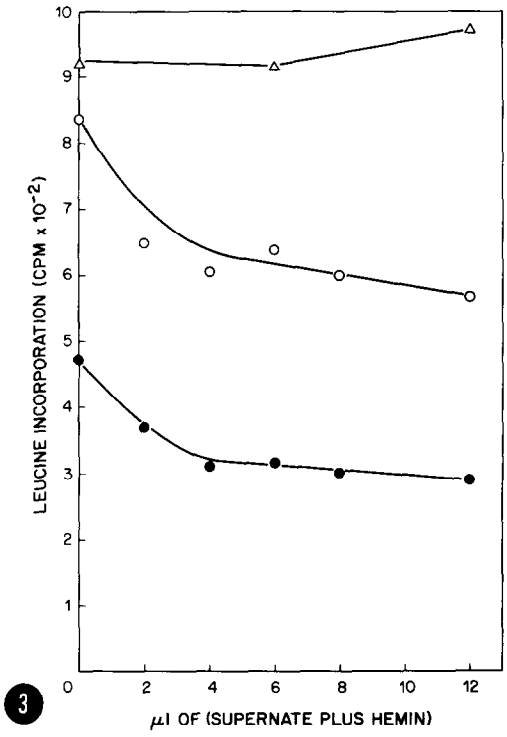
Step I HCR (6 units)	Normal IgG (15 µg)	Immune IgG (15 µg)	Δ CPM (5 minus 2 min)	Percent of Control
--	--	--	880	100
+*	--	--	480	54
+	--	--	510	58
+	+	--	470	53
+	--	+	830	94
--	+	--	870	99
--	--	+	910	104

repressor (Fig. 1). Fifteen µg of immune IgG caused almost complete neutralization of 6 units of HCR by 20 min. The same amount of immune IgG could only partially inactivate 12 units of HCR even after 40 min. These results imply that the neutralizing effect of immune IgG is stoichiometric rather than catalytic and, thus, presumably mediated by antibody.

To determine whether the effect of immune IgG is due to antibody generated by the immunization, the effect of normal IgG on the activity of HCR was compared to that of immune IgG. The results (Table I) show that normal IgG had no effect on the inhibitory activity of HCR, while immune IgG caused almost complete neutralization under the same conditions. These findings indicate that the neutralizing effect of immune IgG was elicited by immunization to HCR.

The effect of immune IgG on the activity of Step I HCR was compared to its effect on Step VII HCR (Fig. 2). Up to about 5 units of either crude or highly purified HCR was inactivated by immune but not normal IgG. In addition, immune IgG caused the same degree of inactivation of either HCR preparation at levels of HCR where immune IgG was limiting. These findings demonstrate that the inhibitory activity in the Step VII preparation is the same hemin-controlled repressor that was present in the starting material and not some minor or contaminating inhibitory component.

Since it was of interest to determine whether the antigenic determi-



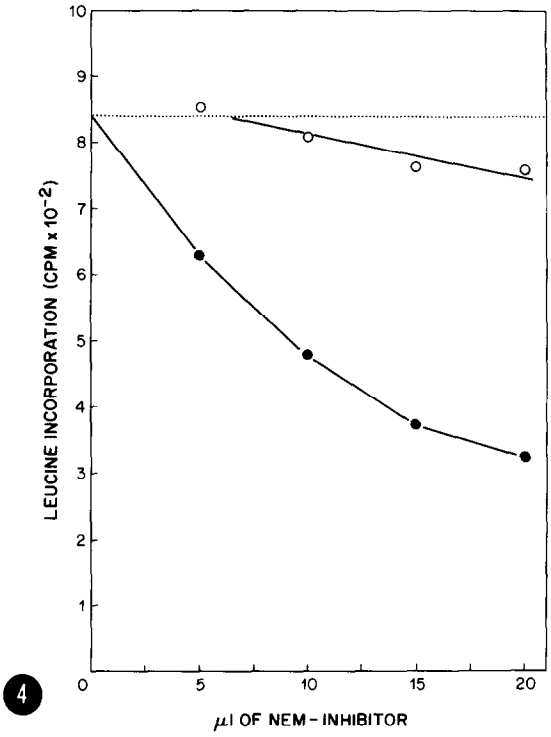


Figure 3. Effect of increasing amounts of supernate plus hemin on neutralization of HCR by immune IgG.

Duplicate samples, containing 6 units of Step I HCR, were mixed with the indicated volumes of fresh supernate (containing 50 μM hemin), 15 μg of immune (o—o) or normal (●—●) IgG, brought to 30 μl with 1X HEPES buffer, and then incubated at 34° for 20 min. Samples depicted by the triangles received no HCR or IgG. After 20 min, inhibitory activity was immediately determined by the addition of 30 μl of master mix and 50 μl of lysate. The ordinate represents the Δ CPM at 5 minus 2 min. Samples containing immune or normal IgG alone were not different from the control.

Figure 4. Effect of immune IgG on the inhibitory activity generated by incubating partially purified prorepressor with N-ethylmaleimide for 5 min.

Three hundred μl of fresh supernate, containing 50 μM hemin, were incubated at 34° for 10 min. The sample was cooled to 0° and fractionated on a 9 x 0.7 cm column of CM-Sephadex, equilibrated with 1X HEPES buffer. The void volume eluate (600 μl), freed of hemoglobin, was pooled, and a 160 μl aliquot was mixed with 20 μl of 0.05 M N-ethylmaleimide and incubated at 34° for 5 min, followed by another 5 min of incubation with the addition of 20 μl of 0.025 M dithiothreitol. Duplicate samples, containing the indicated volumes of the product of this reaction (termed NEM-inhibitor) plus 15 μg of either normal (●—●) or immune (o—o) IgG in a final volume of 30 μl (using 1X HEPES buffer), were incubated at 34° for 20 min. Inhibitory activity was immediately determined by the addition of 30 μl of master mix and 50 μl of lysate. The ordinate represents the Δ CPM at 5 minus 2 min, and the dotted line at the top of the figure represents the incorporation of samples containing 30 μl of 1X HEPES buffer alone. An aliquot of the CM-Sephadex column eluate that was incubated in parallel, but diluted with buffer in place of the sulfhydryl reagents, had no inhibitory effect when tested at the 20 μl level.

TABLE II

Effect of Immune IgG on the Inhibitory Activity of Hemin-sensitive HCR

Duplicate samples, containing the indicated components in a final volume of 30 µl (using 1X HEPES buffer), were incubated at 34° for 20 min. Inhibitory activity was immediately determined by the addition of 30 µl of master mix and 50 µl of lysate.

Fresh Supernate (20 µl)	Step I HCR (6 units)	Normal IgG (15 µg)	Immune IgG (15 µg)	Δ CPM (5 minus 2 min)	Percent of Control
--	--	--	--	870	100
+	--	--	--	510	58
+	--	+	--	440	51
+	--	--	+	840	96
--	+	+	--	510	58
--	+	--	+	820	94
--	--	+	--	840	97
--	--	--	+	870	100

nants on HCR, which are responsible for its neutralization by antibody, are also present on the prorepressor, HCR was mixed with increasing amounts of fresh supernate plus hemin and then incubated with immune IgG to see if prorepressor competed with HCR for antibody. The results (Fig. 3) show that the prorepressor in up to 12 µl of supernate, representing 6 times the prorepressor from which the added HCR was derived, had virtually no competitive effect on the inactivation of HCR by immune IgG. Since the conversion of prorepressor to HCR involves no detectable change in molecular weight (20), these findings suggest that the antigenic determinants responsible for neutralization by antibody are present on the prorepressor but are hidden within the protein configuration. The formation of HCR, which appears to involve several conformational changes (20), may cause these determinants to become exposed on the surface.

To determine whether the inhibitory activity of hemin-sensitive HCR could be neutralized by immune IgG, fresh supernate was preincubated alone or with normal or immune IgG for 20 min at 34°. The inhibitory activity generated in the absence of IgG could be completely inactivated by 10 min of further incubation with 50 µM hemin (data not shown). The results in Table II show that immune IgG completely neutralized the inhibitory activity of hemin-sensitive HCR, whereas normal IgG had no effect. In addition, at the level tested, hemin-sensitive HCR was inactivated by immune IgG as effectively as hemin-resistant HCR. This indicates that the antigenic determinants responsible for neutralization become exposed in the transition from prorepressor to hemin-sensitive HCR.

Since the antibody to HCR provides a specific probe for determining whether an inhibitor of reticulocyte cell-free protein synthesis is HCR or some other factor, immune IgG was used to determine if the inhibitory activity generated by a 5 min incubation of partially purified prorepressor with N-ethylmaleimide was actually HCR. The data in Fig. 4 demonstrate that the inhibitor generated by N-ethylmaleimide was neutralized by immune but not normal IgG. The degree of inactivation, at levels of inhibitor where immune IgG was limiting, was about the same as that observed for natural HCR (see Fig. 2). These findings confirm that the N-ethylmaleimide induced inhibitory activity is the same as natural HCR.

Virtually no neutralization of HCR activity by immune IgG occurs when the components are mixed at the start of incorporation and incubated for 5 min in the absence of hemin (see Fig. 1). Consequently, the antibody to HCR cannot be used in this system to determine whether the inhibitory effects of oxidized glutathione (23) or double-stranded RNA (24, 25), when added to reticulocyte lysate, are mediated by the formation of HCR. Longer incubations in the presence of hemin would be desirable, but then protein synthesis becomes inhibited by IgG. Attempts to use immune IgG to stimulate protein synthesis in the absence of hemin were similarly limited by the inhibitory effect of IgG with longer incubations. A level of immune IgG, sufficient to cause a small (about 10%) but reproducible stimulation of protein synthesis in the absence of hemin at 6 min of incorporation, became inhibitory after 10-15 min (data not shown). Higher levels of immune IgG inhibited sooner, and lower levels produced little or no stimulation. The problem of inhibition due to IgG could be largely overcome if the antibody specific for HCR can be isolated from the bulk of the IgG using binding to HCR as the means of separation. Such studies are now in progress.

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REFERENCES

1. Kruh, J., and Borsook, H. (1956) J. Biol. Chem. 220, 905-915.
2. Bruns, G.P., and London, I.M. (1965) Biochem. Biophys. Res. Commun. 18, 236-242.
3. Adamson, S.D., Herbert, E., and Godchaux, W. (1968) Arch. Biochem. Biophys. 125, 671-683.
4. Zucker, W.V., and Schulman, H.M. (1968) Proc. Natl. Acad. Sci. U.S. 59, 582-589.

5. Maxwell, C.R., and Rabinovitz, M. (1969) Biochem. Biophys. Res. Commun. 35, 79-85 (1969).
6. Howard, G.A., Adamson, S.D., and Herbert, E. (1970) Biochim, Biophys. Acta 213, 237-240.
7. Maxwell, C.R., Kamper, C.S., and Rabinovitz, M. (1971) J. Mol. Biol. 58, 317-327.
8. Gross, M., and Rabinovitz, M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1565-1568.
9. Gross, M., and Rabinovitz, M. (1973) Biochim. Biophys. Acta 299, 472-479.
10. Legon, S., Jackson, R.J., and Hunt, T. (1973) Nature New Biology 241, 150-152.
11. Gross, M., Biochim. Biophys Acta (in press).
12. Adamson, S.D., Yau, P.M., Herbert, E., and Zucker, W.V. (1972) J. Mol. Biol. 63, 247-264.
13. Mizuno, S., Fisher, J.M., and Rabinovitz, M. (1972) Biochim. Biophys. Acta 272, 638-650.
14. Mathews, M.B., Hunt, T., and Brayley, A. (1973) Nature New Biology 243, 230-233.
15. Beuzard, Y., Rodvien, R., and London, I. M. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1022-1026.
16. Lodish, H.F. and Desalu, O. (1973) J. Biol. Chem. 248, 3520-3527.
17. Darnbrough, C., Legon, S., Hunt, T., and Jackson, R.J. (1973) J. Mol. Biol. 76, 379-403.
18. Balkow, K., Mizuno, S., and Rabinovitz, M. (1973) Biochem. Biophys. Res. Commun. 54, 315-323.
19. Balkow, K., Mizuno, S., Fisher, J.M., and Rabinovitz, M. (1973) Biochim. Biophys. Acta 324, 397-409.
20. Gross, M. and Rabinovitz, M. (1972) Biochim. Biophys. Acta 287, 340-352
21. Gross, M., and Rabinovitz, M. (1973) Biochem. Biophys. Res. Commun. 50, 832-839.
22. Maxwell, C.R. and Mencken, C. (1971) Anal. Biochem. 44, 235-238.
23. Kosower, N.S., Vanderhoff, G.A., Benerofe, B., Hunt, T. and Kosower, E.M. (1971) Biochem. Biophys. Res. Commun. 45, 816-821.
24. Hunt, T., and Ehrenfeld, E. (1971) Nature New Biology 230, 91-94.
25. Darnbrough, C., Hunt, T., and Jackson, R.J. (1972) Biochem. Biophys. Res. Commun. 48, 1556-1564.