PARTIAL PURIFICATION OF A TRANSLATIONAL REPRESSOR MEDIATING HEMIN CONTROL OF GLOBIN SYNTHESIS AND IMPLICATION OF RESULTS ON THE SITE OF INHIBITION

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SUMMARY. A translational repressor which mediates the control of globin synthesis by hemin in rabbit reticulocyte lysates has been purified 3,000 fold. Analysis of the purified preparation by polyacrylamide gel electrophoresis demonstrated that it is still heterogeneous. A consideration of the specific activity and molecular weight of the repressor and the concentration of ribosomal components in protein synthesis indicates that one molecule of this repressor inhibits the activity of a minimum of several hundred reticulocyte ribosomes.

The maintenance of hemoglobin synthesis in both rabbit reticulocytes (1, 2) and their lysates (3, 4) depends upon the availability of hemin. When the cells are deprived of iron, the resulting hemin deficiency is accompanied by a marked decrease in globin synthesis and polyribosome disaggregation (5-7). If hemin is added shortly after globin synthesis has become suppressed, the production of globin can resume in both cells (5-7) and some lysates (8) with reformation of polyribosomes. The mediator of these effects is a translational repressor, an inhibitor of peptide chain initiation, which is formed in the ribosome-free supernatant fraction in the absence of hemin (9-13), and can be inactivated by hemin only at an early stage in its formation (14). This inhibitor promotes polyribosome disaggregation under conditions of protein synthesis, and its action on the reticulocyte cell-free system can be overcome by a factor present in a 0.5 M KCl extract of ribosomes (13, 15). The inhibitor is a protein (13, 15) with a molecular weight of $4 \pm 1 \times 10^5$ daltons (13, 16). We report here on its purification to yield a product with a 3,000 fold increase in specific activity and

consider the implications of the action of such material in a protein synthesizing system.

METHODS AND MATERIALS

Assay of Inhibitor. The preparation of the rabbit reticulocyte cell-free system used for assay of the inhibitor has been previously described (12). In brief, it consists of 50 µl of a 1:1 reticulocyte-water lysate, cleared of cell debris, and supplemented with the following components to yield the indicated final concentration in 110 µl total volume: KCl (75 mM), $MgCl_2$ or Mg $(CH_3COO)_2$ (2 mM), ATP (0.5 mM), GTP (0.2 mM), creatine phosphate (15 mM) and creatine kinase (45 units/ml), L-[1-^{14}C] leucine (0.3 mM, 5 µCi/µMole) and the other 19 L-[^{12}C] amino acids at a concentration to give a mixture corresponding to the composition of rabbit hemoglobin. Hemin (25 µM final concentration) was added to suppress formation of inhibitor from proinhibitor in the assay system. Leucine incorporation into protein during a 90 min incubation at 34°C was determined with no added inhibitor and with increasing amounts up to a level which gave maximum inhibition (12). One unit of inhibitor is defined as that amount which produces an inhibition of leucine incorporation of 50 percent between that obtained with no added inhibitor and with an amount sufficient for maximal inhibition.

Preparation and Purification of Inhibitor. All procedures, except where otherwise indicated, were carried out at temperatures from 0°C to 4°C.

I. The supernatant fraction of a 1:1 reticulocyte-water lysate, freed of ribosomes by a 4 hr centrifugation at 64,000 rpm in the Spinco 65 rotor, was incubated for 16 hr at 37°C in the presence of 100 units/ml of penicillin G and 100 µg/ml of streptomycin. This material is termed Step I inhibitor.

II. Step I inhibitor, 150 ml, was applied to a 4.5 x 50 cm CM-Sephadex column equilibrated with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES), 3.7 mM, pH 7.2, and eluted with this buffer. The void volume fractions were pooled, centri-

fuged for 10 min at 15,000 x g in the Sorvall RC-2B, and the supernatant fraction, now freed of hemoglobin, was taken as Step II inhibitor.

III. Step II inhibitor was concentrated to 10-20 percent of the volume of the original Step I fraction with the use of an Amicon TCF-10 and PM-10 membrane. Approximately 25-30 ml of this material was then applied to a 5.0 x 150 cm Sephadex G200 column equilibrated with KCl, 27.5 mM, in HEPES, 3.7 mM, pH 7.2 (Buffer A). Inhibitor was eluted with Buffer A (16).

IV. The pooled material from two Step III fractionations (600 ml) was adsorbed onto a 4.1 x 7.5 cm DEAE-cellulose column equilibrated with Buffer A. After washing the column with 50 ml of Buffer A, it was developed with 1500 ml of a linear gradient of KCl, 0.10 M to 0.30 M, in HEPES, 3.7 mM, pH 7.2.

V. The contents of tubes containing Step IV inhibitor were pooled and applied directly to a 2.6 x 6.5 cm hydroxyapatite column equilibrated with potassium phosphate, 1 mM, pH 7.0. Elution was with 580 ml of a linear gradient of potassium phosphate, 0.10 M to 0.30 M, pH 7.0.

VI. The phosphate concentration of Step V inhibitor (200 ml) was reduced by a factor of 25 by serially diluting the sample with water and concentrating by ultrafiltration. The final sample (40 ml) was adsorbed onto a 1.1 x 6.5 cm hydroxyapatite column and then eluted with 120 ml of a linear gradient of potassium phosphate, 0.10 M to 0.50 M, pH 7.0.

VII. The Step VI fractions (35 ml) were pooled and concentrated to 6 ml by ultrafiltration. Glycerol, 1.5 ml, was added and the sample was chromatographed on a 2.5 x 80 cm Sepharose 6B column equilibrated with 20% glycerol in Buffer A (Fig. 1).

Step VII inhibitor had three characteristics in common with that of the crude inhibitor: [1] It was eluted from Sepharose 6B in the same volume fraction; [2] its action on the protein synthesizing system could be overcome by I-factor in a ribo-

somal extract (13, 15); and [3] it promoted the disaggregation of polyribosomes only in the presence of an energy source. Disaggregation of polyribosomes in the absence of an energy source is observed with ribonuclease.

Protein concentrations were determined by the method of Bramhall et al. (17).

CM-Sephadex C50, Sephadex G200, and Sepharose 6B were purchased from Pharmacia. DEAE cellulose (DE 52) was obtained from Whatman, and hydroxyapatite (Hypatite C) from Clarkson. HEPES was purchased from Calbiochem.

RESULTS AND DISCUSSION

The steps in the purification procedure are summarized in Table I. The specific activity of Step VII inhibitor was 36,000 units/mg, the result of a 2100-fold purification. Since in the final purification step the elution of A_{280} material preceded that of the inhibitor activity (Fig. 1), this preparation could be further divided into 3 pools of increasing specific activity (Table I). Each was concentrated and subjected to polyacrylamide gel electrophoresis to assess its purity (Fig. 2). The data indicate that pool C, with the highest specific activity (54,000 units/mg), still had 2 major bands. Furthermore, the intensity of each of these 2 major bands decreased from pool A to pool C although both the total and specific activity of the inhibitor in the applied sample increased. It is possible that the inhibitor is in a minor band, such as the one marked by the arrow, whose intensity increased with the increase in applied inhibitor activity from pool A to pool C. While it is uncertain which band, if any, corresponds to the inhibitor, it is apparent that the specific activity of pure inhibitor must be at least 10^5 units/mg.

With an estimated specific activity of 10^5 units/mg for pure inhibitor, an estimated molecular weight of inhibitor of 4×10^5 daltons (13, 16), a ribosome concentration of 2 mg/ml for the 1:1 reticulocyte-water lysate (9, 13) and a molecular weight of the reticulocyte ribosome of 4×10^6 daltons (19), it can be calculated that one

TABLE I

Purification of the Hemin Controlled Translational Repressor

	Step	units/ml	mg/ml	units/mg	ml total	units total	yield	purification
I.	Supernate	2000	120	17	600	1.2×10^8	100%	1
II.	CM-Sephadex	690	1.50	460	1620	1.1×10^6	92%	27
III.	Sephadex G200	1200	1.30	920	600	7.2×10^5	60%	54
IV.	DEAE cellulose	560	0.28	2,000	520	2.9×10^5	24%	120
V.	Hydroxyapatite (1)	1100	0.11	10,000	200	2.2×10^5	18%	590
VI.	Hydroxyapatite (2)	3100	0.17	18,000	54	1.7×10^5	14%	1100
VII.	Sepharose 6B	1500	0.042	36,000	81	1.2×10^5	10%	2100
	Pool A (Fractions 70 to 80)	800	0.042	19,000	29	23,000	—	—
	Pool B (Fractions 81 to 86)	2500	0.069	36,000	16	40,000	—	—
	Pool C (Fractions 87 to 100)	1600	0.029	54,000	36	56,000	—	3200

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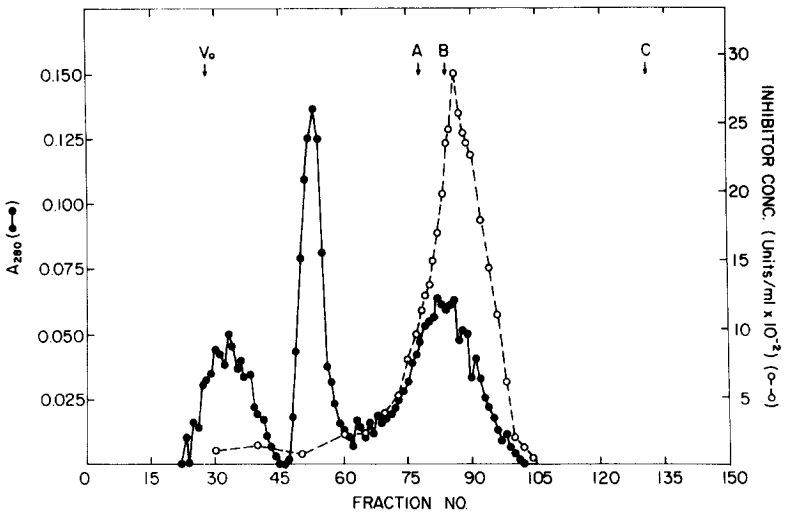

Fig. 1.

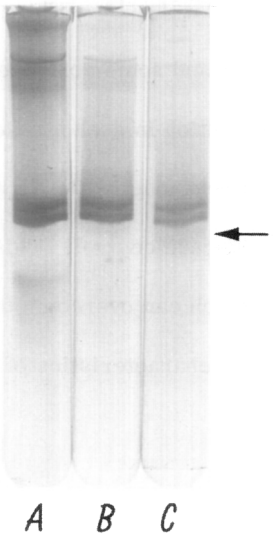

Fig. 2.

Figure 1. Fractionation of Step VI inhibitor on Sepharose 6B

Step VI inhibitor in 7.5 ml Buffer A containing 20% glycerol was applied to a 2.5 × 80 cm Sepharose 6B column equilibrated with 20% glycerol in Buffer A, and was eluted with this buffer. The flow rate was 15 ml/hr, and 2.6 ml fractions were collected. The arrows indicate the elution positions of markers applied separately to the same column: V_o, Blue Dextran 2000; A, horse spleen apoferritin; B, Step I inhibitor; C, rabbit hemoglobin.

Figure 2. Polyacrylamide disc electrophoretic patterns of Step VII inhibitor

Fractions from the Sepharose 6B column in Fig. 1 were divided into 3 pools (Table I) and each was concentrated to 7 ml by ultrafiltration. Two hundred microliters of each pool were then subjected to electrophoresis on 7% polyacrylamide gels for 100 minutes at 2.5 mA per tube in a Buchler apparatus. The gels were stained with Coomassie Blue (18). Gel A corresponds to pool A, 35 μg of protein and 660 inhibitor units; gel B to pool B, 32 μg and 1150 units; and gel C to pool C, 26 μg and 1400 units.

molecule of inhibitor reduces the biosynthetic activity of 1000 ribosomes in the 50 μl lysate portion of the assay system by 50 percent.

Information previously obtained on the action of the inhibitor on protein synthesis indicates that it does not combine irreversibly with a component of the reaction, since increasing levels of inhibitor become proportionally less effective in blocking protein

synthesis (12). This observation is consistent with an equilibrium between the inhibitor and some component in the overall process of protein synthesis. The ability to function in such a reaction at such high dilution precludes a mode of action involving direct inactivation of either ribosomes or their subunits and suggests a mechanism of cascade control. This mechanism involves the role of an initiation factor (13, 15) which can overcome the action of the inhibitor. Studies are in progress to elucidate the characteristics of this reaction.

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