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Inhibition by Heparin of Globin Messenger Ribonucleic Acid Translation in a Mammalian Cell-Free System†

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ABSTRACT: Cell-free extracts of Krebs ascites cells, preincubated to decrease endogenous protein synthesis, translate rabbit globin mRNA with the production of both α- and β-globin chains. The addition of low levels of heparin, but not of chondroitin sulfate, hyaluronic acid, Sulfon (a sulfonated polystyrene), or dextran sulfates of varying size, results in a decrease in the mRNA-dependent stimulation of amino acid incorporation into protein. In the presence of 20 μg/ml of rabbit globin mRNA the addition of 4–5 μg/ml of heparin causes approximately 50% inhibition of globin mRNA translation, while 40 μg/ml of heparin completely prevents globin mRNA translation. Over the concentration range studied, the heparin effect is not reversible by mRNA. Synthesis of both α- and β-globin chains is inhibited by the presence of heparin to approximately the same extent. The distribution of ribosomal subunits is changed in the presence of high levels of heparin, but not in the presence of low levels of heparin. The data indicate that heparin acts at the level of initiation to inhibit translation of a natural mammalian mRNA in a mammalian cell-free system.

A Krebs ascites cell-free system, originally described by Mathews and Korner for translation of encephalomyocarditis (EMC)[1] viral RNA (Mathews and Korner, 1970), has recently been used to support translation of several other heterologous mRNAs (Mathews *et al.*, 1971, 1972; Housman *et al.*, 1971; Forget and Benz, 1971; Jacobs-Lorena and Baglioni, 1972a; Metafora *et al.*, 1972; Jacobs-Lorena and Baglioni, 1972b). Translation of rabbit reticulocyte globin mRNA has been the most extensively studied (Housman *et al.*, 1971; Jacobs-Lorena and Baglioni, 1972a,b; Metafora *et al.*, 1972), and it has been shown that the globin mRNA is translated with the production of both rabbit α- and β-globin chains.

Heparin, a naturally occurring polyanion found in many tissues (Freeman, 1964), has been shown at high levels to inhibit protein synthesis in rat liver cell extracts (Berlinguet and Normand, 1968) and to effect the distribution of ribosomal subunits in HeLa cell extracts (Miller, 1968). Heparin has also been reported to inhibit binding of Phe-tRNA to 40S ribosomal subunits isolated from rat lymphosarcoma (Koka and Nakamoto, 1972), to inhibit translation of poly(U) by extracts of *Escherichia coli* (Wacker *et al.*, 1967), and to bind to 30S ribosomal subunits and to 70S ribosomes isolated from

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[1] Abbreviations used are: EMC virus, encephalomyocarditis virus; S-30, 30,000*g* supernatant; PS-30, preincubated derivative of 30,000*g* supernatant.

E. coli (Mayazawa *et al.*, 1967). In addition, heparin has been shown to inhibit ribonuclease (Zoellner and Fellig, 1953; Fellig and Wiley, 1959; Flanagan, 1967), to stimulate DNA replication in intact nuclei isolated from rat liver (Kraemer and Coffey, 1970), and to inhibit DNA-dependent RNA polymerase from *E. coli* (Walter *et al.*, 1967).

This report presents the results of studies on translation of rabbit reticulocyte globin mRNA in the Krebs ascites cell-free system, in the presence and absence of heparin.

Experimental Section

Materials. Adult female albino mice (strains CFW and CF 1) were obtained from Carworth Farms. Krebs ascites cells were a generous gift of Drs. I. Faiferman and A. O. Pogo. New Zealand white rabbits were obtained from Marland Breeding Farms.

L-[^{3}H]Leucine (specific activity 20.4–36.9 Ci/mmol) was purchased from Amersham/Searle Corp.; L-[^{14}C]leucine (specific activity 219 Ci/mol) was purchased from New England Nuclear Corp. Heparin, hyaluronic acid, and chondroitin sulfate were all obtained from Sigma Biochemicals. Highly purified heparins were also obtained from Schwarz-Mann, ICN Nutritional Biochemicals, and Wilson Laboratories. A highly characterized heparin was a generous gift from Dr. J. A. Cifonelli, Department of Pediatrics, University of Chicago. Dextran Sulfate 500 (av mol wt 500,000) and Dextran Sulfate 2000 (av mol wt 2,000,000) were purchased from Pharmacia. Sulfon, a sulfonated polystyrene, was purchased from Miles Biochemicals. All other materials were obtained from standard commercial sources.

Preparation of Krebs Ascites Cell-Free Extracts. Krebs cells were maintained in mice according to the method of Mathews and Korner (1970). Preparation of the Krebs ascites cell homogenate, 30,000*g* supernatant (S-30), and preincubated extract (PS-30) was essentially according to the method of Housman *et al.* (1971). The PS-30 extract was dialyzed against medium K for a total time of 2 hr, instead of being passed through a Sephadex G-25 column.

Incubation Procedures. Incubations were performed essentially as described by Housman *et al.* (1971), with the modification that pyruvate kinase (0.6 IU/ml) and phosphoenolpyruvate (3 mM, final concentration) were used as ATP regenerating system. Reaction mixtures contained 2.5–5 μCi of L-[^{3}H]leucine (specific activity 20.4–36.9 Ci/mmol). Final incubation volumes were 50 or 100 μl, and contained 30 or 60 μl of cell-free extract. Incubation was for 40 min at 37°, unless otherwise noted. Reaction was terminated and trichloroacetic acid insoluble radioactivity was determined as described (Housman *et al.*, 1971).

Analysis of the Products of Incubation. The final volume of mixtures upon which product analysis was to be performed was 1 ml. The procedure for analysis of ribosomal pellets and supernatants derived from reaction mixtures was essentially that of Housman *et al.* (1971). The pellet containing the radioactive product of the reaction was dissolved and chromatographed on CM-cellulose as described (Shapira *et al.*, 1968).

Analysis on 15–30% sucrose density gradients of ribosomal pellets was performed according to the method of Christman and Goldstein (1971).

Preparation of Rabbit Reticulocyte Globin mRNA. Induction of reticulocytosis in rabbits and collection of rabbit reticulocytes were performed as previously described (Christman and Goldstein, 1969). Globin mRNA was isolated from rabbit reticulocytes by the method of Lockard and Lingrel (1969).

Preparation of Uniformly Radioactive Rabbit Globin. Preparation and chromatography of rabbit globin uniformly labeled *in vivo* with L-[^{14}C]leucine was performed according to the methods reviewed by Shapiro *et al.* (1968).

Results and Discussion

In agreement with reports from other laboratories utilizing the Krebs PS-30 system as support for the translation of globin mRNAs (Mathews *et al.*, 1971; Housman *et al.*, 1971; Forget and Benz, 1971; Jacobs-Lorena and Baglioni, 1972a; Metafora *et al.*, 1972; Jacobs-Lorena and Baglioni, 1972b), we observe that protein synthesis in the Krebs PS-30 system is stimulated by the addition of globin mRNA isolated from rabbit reticulocytes, that synthesis continues for at least 40 min and that the products of the protein synthetic reaction are both α- and β-globin chains. The system was saturated with respect to globin mRNA at an mRNA level of approximately 25–40 μg/ml. This level is in good agreement with that reported by other workers (Mathews *et al.*, 1971; Jacobs-Lorena and Baglioni, 1972b) for similar systems.

The effect of heparin and other polyanions on the translation of globin mRNA in the PS-30 system was tested, and the results are presented in Table I. Heparin was the most effective inhibitor of globin mRNA stimulation of protein synthesis. In the presence of 20 μg/ml of globin mRNA, the addition of 5 μg/ml of heparin caused 50%, and the addition of 10 μg/ml of heparin caused 70–80% inhibition of mRNA-dependent stimulation of L-[^{3}H]leucine incorporation. In general, the addition of 10, 20, or 50 μg per ml of chondroitin sulfate, hyaluronic acid, Sulfon, or dextran sulfates was without inhibitory effect. Some inhibition, approximately that seen with 2 μg/ml of heparin, was seen with 50 μg/ml of Sulfon.

The heparin used for these studies was obtained from Sigma Biochemicals. Commercial heparin from several other sources exhibited similar inhibitory properties, as did a highly characterized heparin obtained from Dr. J. A. Cifonelli.

A comparison of the effects of dextran sulfate and heparin on protein synthesis in the Krebs ascites cell-free system is presented in Figure 1. Over the concentration range tested neither dextran sulfate nor heparin inhibited protein synthesis either in the S-30 or in the preincubated derivative. The addition of heparin in increasing amounts to incubations of PS-30 containing globin mRNA resulted in increasing inhibition of incorporation of L-[^{3}H]leucine into trichloroacetic acid insoluble protein, whereas the addition of dextran sulfate was not inhibitory. At a level of 40 μg/ml of heparin, the incorporation was essentially that observed in the complete absence of globin mRNA. As endogenous synthesis in the S-30 or PS-30 represents essentially elongation, and involves minimal, if any, initiation (Mathews and Korner, 1970), it does not appear that heparin affects elongation. If heparin was affecting chain termination and release, one would expect an accumulation of large, trichloroacetic acid insoluble peptides, which would be radioactive under our incubation conditions. The finding that heparin, at appropriate concentrations, causes 100% inhibition of mRNA-dependent incorporation of radioactive amino acids into trichloroacetic acid insoluble material argues against termination and release as the site of action of heparin.

It is interesting that dextran sulfate, which inhibits initiation of EMC viral RNA and poly(U) translation in the Krebs cell-free system (Mathews and Korner, 1970), and inhibits initiation of poly(U) translation in *E. coli* cell-free systems (Mayazawa *et al.*, 1967; Wacker *et al.*, 1967), does not

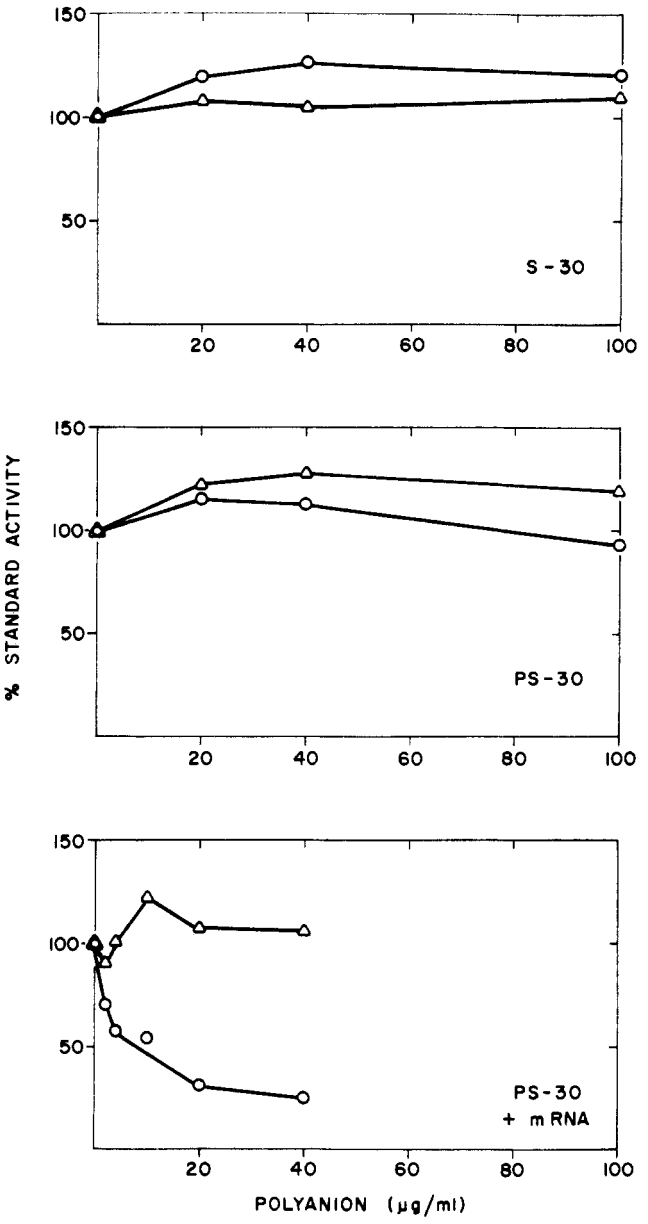

FIGURE 1: Effect of increasing amounts of heparin or of Dextran Sulfate 2000 on incorporation of L-[^{3}H]leucine into trichloroacetic acid insoluble protein. Heparin (○) or Dextran Sulfate 2000 (△) was added at the concentration noted to 50-μl volume reaction mixtures containing 2.5 μCi of L-[^{3}H]leucine. Incubation time was 40 min at 37°: top, reaction mixtures containing only S-30; middle, reaction mixtures containing only PS-30; bottom, reaction mixtures containing PS-30 and rabbit globin mRNA (20 μg/ml). 100% standard activity is defined for each curve as the incorporation of L-[^{3}H]leucine into trichloroacetic acid insoluble protein observed in the absence of added polyanion, and was 27,000–37,000 cpm/ml for the experiments with S-30, 3800–4800 cpm/ml for the experiments with PS-30, and 22,000–23,000 cpm/ml for the experiments with PS-30 +mRNA. In the latter experiments (PS-30 + mRNA) the level of endogenous synthesis was 6000–6200 cpm/ml.

TABLE I: Effect of Polyanions on the Rabbit Globin mRNA-Dependent Incorporation of L-[^{3}H]Leucine into Trichloroacetic Acid Insoluble Protein.[a]

Expt No.	Addition (μg/ml)	Stimulation cpm/ml	Stimulation %
1	mRNA (20)	38,400	100
	+Hep (10)	11,000	29
	+CS (10)	30,580	80
	+HA (10)	37,580	95
	+Sulfon (10)	37,120	97
	+DS 500 (10)	40,400	105
	+DS 2000 (10)	46,940	122
2	mRNA (20)	13,930	100
	+Hep (10)	2,620	19
	+CS (10)	11,190	80
	+HA (10)	12,280	88
	+Sulfon (10)	13,720	98
3	mRNA (20)	13,060	100
	+Hep (2)	9,680	74
	+Hep (5)	6,680	51
	+CS (20)	16,640	127
	+CS (50)	12,110	93
	+HA (20)	15,900	122
	+HA (50)	14,900	114
	+Sulfon (20)	15,500	119
	+Sulfon (50)	9,910	76
	+DS 500 (20)	19,610	150
	+DS 500 (50)	16,300	125

[a] Polyanions were added at the level noted to reaction mixtures containing rabbit globin mRNA (20 μg/ml). Hep is heparin, CS is chondroitin sulfate, HA is hyaluronic acid, Sulfon is a sulfonated polystyrene, DS 500 and DS 2000 are dextran sulfates of av mol wt 500,000 and 2,000,000, respectively. Incubation volume for expt 1 was 50 μl, containing 2.5 μCi of L-[^{3}H]leucine, while incubation volumes for expt 2 and 3 were 100 μl, containing 2.5 μCi of L-[^{3}H]leucine. Incubation time was 40 min at 37° in all cases. The PS-30 extract and the rabbit globin mRNA used in experiment 1 are different preparations from those used in expt 2 and 3. Stimulation is defined as the rabbit globin mRNA-dependent increase in incorporation of L-[^{3}H]leucine into trichloroacetic acid insoluble protein. For further details, see Experimental Section.

inhibit the globin mRNA-dependent system. The reason for this difference is as yet unknown.

All of the above studies were performed with both mRNA and the polyanion being added simultaneously to the reaction mixtures, before incubation began. Figure 2 presents the results of an experiment in which heparin was added at varying times after the beginning of incubation of PS-30 extracts with mRNA (20 μg/ml). Heparin was effective as an inhibitor whenever added. When heparin was added either at the beginning of incubation, or after 2-min incubation, negligible stimulation of incorporation occurred. The addition of heparin at a level of 40 μg/ml, at 40 min, the end of the incubation, did not affect the amount of trichloroacetic acid insoluble radioactivity recovered, ruling out the possibility that heparin, at these levels, interferes with precipitation. The finding that heparin exerts its effects at whatever time added is in agreement with the hypothesis that heparin is acting at the level of initiation of protein synthesis, and that initiation is occurring throughout most of the incubation period.

Attempts were made to reverse the inhibition of globin mRNA stimulation due to heparin by varying the mRNA: heparin ratios. As can be seen in Figure 3, increasing the mRNA level from 10 to 30 μg/ml, in the presence of heparin at 4, 10, or 20 μg per ml, did not reverse the heparin inhibition.

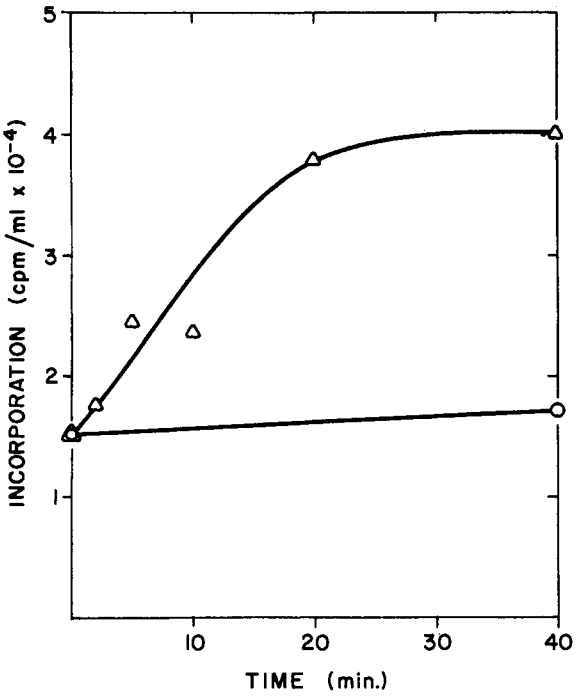

FIGURE 2: Effect of varying the time of addition of heparin on the incorporation of L-[^{3}H]leucine into trichloroacetic acid insoluble protein. Heparin (40 μg/ml) was added at the times noted to 50-μl volume reaction mixtures containing 2.5 μCi of L-[^{3}H]leucine. Incubation time was 40 min at 37°: (○) reaction mixtures containing only PS-30; (△) reaction mixtures containing PS-30 and rabbit globin mRNA (20 μg/ml). For further details, see Experimental Section.

The increased incorporation observed at each level of heparin was probably due to stimulation of residual activity.

The radioactive products of reaction mixtures containing either PS-30, PS-30 + heparin (40 μg/ml), PS-30 + globin mRNA (20 μg/ml), or PS-30 + globin mRNA (20 μg/ml) + heparin (either 4 or 40 μg per ml) were isolated and chromatographed in ion-exchange columns (Shapira *et al.*, 1968). The

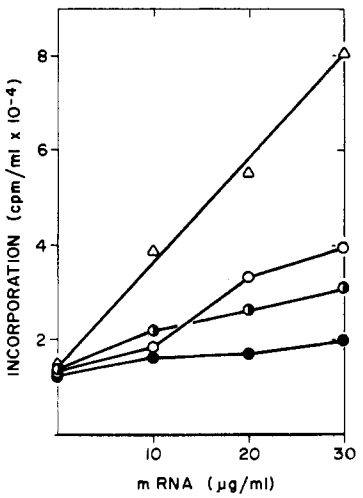

FIGURE 3: Effect of increasing concentration of heparin on globin mRNA stimulation of incoporation of L-[^{3}H]leucine into trichloroacetic acid insoluble protein. Globin mRNA was added at the levels noted to 50-μl volume reaction mixtures containing 2.5 μCi of L-[^{3}H]leucine and either no heparin (△), 4 μg/ml of heparin (○), 10 μg/ml of heparin (◑), or 20 μg/ml of heparin (●). Incubation was for 40 min at 37°. For further details, see Experimental Section.

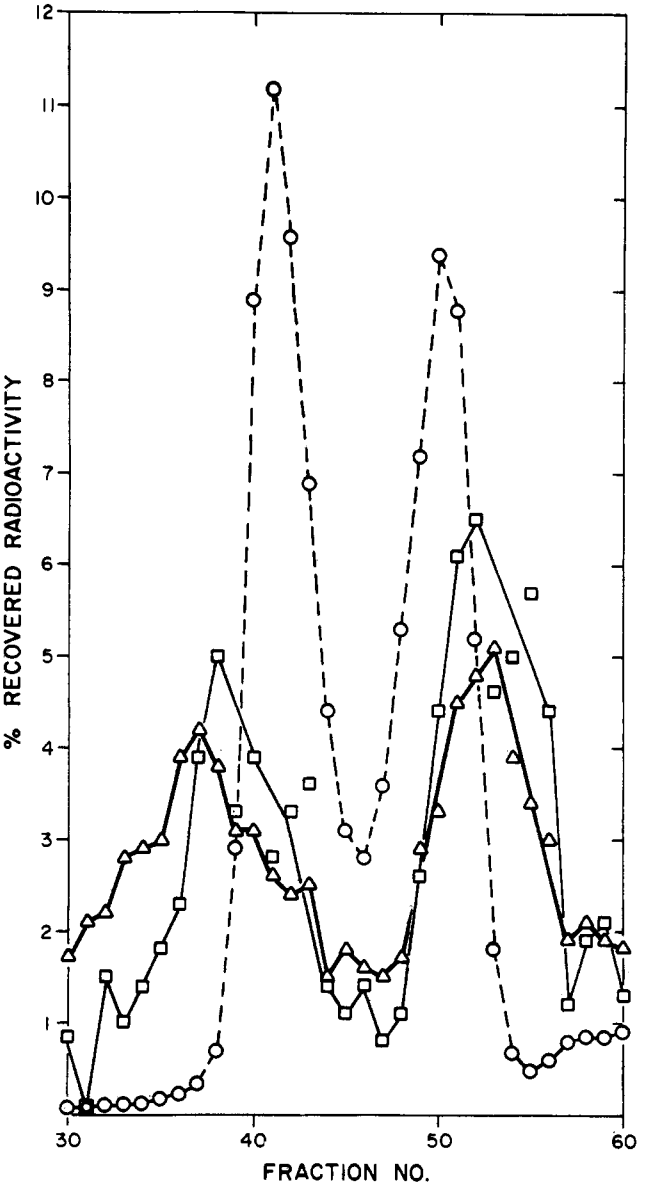

FIGURE 4: Column chromatography of rabbit globin and of reaction products. Material was prepared, isolated, and chromatographed as described in the Experimental Section. Volume of each fraction was 3.0 ml: (○) L-[^{14}C]leucine-containing globin isolated from incubations of intact rabbit reticulocytes; (△) L-[^{3}H]leucine-containing product of incubation containing PS-30 and rabbit globin mRNA (20 μg/ml); (□) L-[^{3}H]leucine-containing product of incubation containing PS-30, rabbit globin mRNA (20 μg/ml), and heparin (4 μg/ml).

results of two such chromatographies are presented in Figure 4. The radioactive products of the reaction mixtures containing PS-30 + globin mRNA (20 μg/ml) ± heparin (4 μg/ml) cochromatographed, and had approximately equal distribution of radioactivity between the separated α- and β-globin chains. The spread of the globin peaks derived from the latter reaction mixtures may be due to the presence of high salt during the acid–acetone precipitation (Housman *et al.*, 1971). Under conditions where salt was removed before acid–acetone precipitation (Mathews *et al.*, 1971), exact coincidence of the globin prepared from rabbit reticulocytes and from the PS-30 system was obtained. The total radioactivity recovered from the PS-30 + globin mRNA incubation was more than twofold that recovered from the PS-30 + globin mRNA + heparin incubation. Similar amounts of radioactive

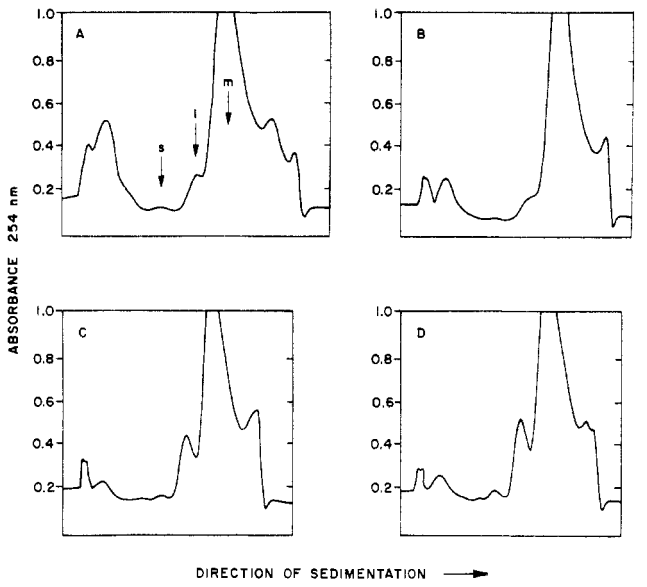

FIGURE 5: Sucrose gradient profiles of ribosomes obtained from reaction mixtures. Preparation of ribosome sand centrifugation of ribosomes for 90 min at 200,000*g* on 15–30% sucrose density gradients was as described in the Experimental Section. (A) Ribosomes derived from reaction mixture containing PS-30; (B) ribosomes derived from reaction mixture containing PS-30 and rabbit globin mRNA (20 μg/ml); (C) ribosomes derived from reaction mixture containing PS-30 and heparin (40 μg/ml); (D) ribosomes derived from reaction mixture containing PS-30, rabbit globin mRNA (20 μg/ml), and heparin (40μ g/ml). Areas containing the small subunits (s), the large subunits (l) and the monosomes (m) are indicated on A.

material were obtained when the PS-30 alone was tested, or when higher levels of heparin (40 μg/ml) were added to the reaction mixtures, in the presence or absence of globin mRNA. The above material, upon chromatography, did not display any discrete radioactive peaks.

These data indicate that heparin inhibits α- and β-globin chain synthesis simultaneously and rule out selective inhibition of the synthesis of one of the two chains.

The 110,000*g* pellets derived from the above incubations were analyzed in 15–30% sucrose gradients. The 254-nm absorbancy patterns resulting from centrifugation of the resuspended pellets on such gradients are presented in Figure 5. In addition to the material in the body of the gradient, some pelleted material was also obtained. The 254-nm-absorbing material recovered in the area corresponding to 80 S, or monosomes, represented 65–70% of the total 254-nm-absorbing material placed on the gradient in all cases. Similar gradients centrifuged under conditions allowing visualization of any polysomal material (200,000*g* for 45 min) (Christman and Goldstein, 1971) revealed negligible 254-nm-absorbing material in the polysome (up to pentasomes) region under all conditions tested. Addition of 20 μg/ml of mRNA to incubations containing only PS-30 resulted in a decreased content of subunits, compared to incubations containing only PS-30 (Figure 5A,B). Addition of 40 μg/ml of heparin, an amount sufficient to completely inhibit translation of globin mRNA, to incubations containing PS-30 ± 20 μg/ml of globin mRNA resulted in an increase in the 254-nm-absorbing content of the large subunit area and the appearance of new absorbing material with lower density than the small subunit (Figure 5C,D).

Heparin, at 500 μg/ml, has been shown to cause breakdown both of monosomes and of ribosomal subunits in HeLa cell extracts (Miller, 1968). Several synthetic polyanions at similar or higher levels have been reported to cause breakdown both of monosomes and of ribosomal subunits isolated from either *E. coli* or yeast (Morgan, 1966; Mayazawa *et al.*, 1967; Weller and Morgan, 1967). Ribosome breakdown cannot account completely for the action of heparin under our conditions, for at 4 μg/ml, a level which causes 50% inhibition of globin mRNA (20 μg/ml) translation, the distribution of subunits and ribosomes is that seen in the native Krebs PS-30.

The absence of large polysomes in the presence of heparin also suggests an action at the level of initiation, as inhibition of protein synthesis at the elongation or termination stage would be expected to result in a buildup of such a species.

The material which pelleted during the density gradient centrifugation at 200,000*g* for 90 min was easily suspendible and the total amount of 260-nm-absorbing material was very similar in all cases. These data argue against aggregation of monosomes in the presence of low levels of heparin (4 and 40 μg per ml). Such aggregation has been reported for *E. coli* extracts in the presence of low levels of dextran sulfate (Mayazawa *et al.*, 1967).

Studies are presently in progress to define the exact site and mechanism of heparin inhibition of initiation of mRNA translation.

Acknowledgments

The authors express thanks to the following people: Mrs. Rosa Hurst for her excellent technical assistance, Dr. R. Weisbrod for preparation of mRNA, and Dr. I. Faiferman and Miss Valerie Zbrezna for their guidance in the methodology of ascites cell maintenance.

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Structure of α_1-Acid Glycoprotein. The Complete Amino Acid Sequence, Multiple Amino Acid Substitutions, and Homology with the Immunoglobulins†

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ABSTRACT: The linear amino acid sequence of the amino-terminal CNBr fragment of α_1-acid glycoprotein derived from pooled human plasma was elucidated and proved to consist of 111 residues. For this investigation the amino acid sequences of the peptides and glycopeptides of a chymotryptic and a tryptic digest and some of the peptides and glycopeptides of a peptic hydrolysate of this protein were elucidated. These data together with the amino acid sequence of the carboxyl-terminal CNBr fragment reported earlier completely established the amino acid sequence of α_1-acid glycoprotein. The five heteropolysaccharide groups of this protein were demonstrated to be linked N-glycosidically to asparaginyl residues. The number of amino acids between two subsequent carbohydrate units differs considerably. This report is thus the first one in which the sequence of a glycoprotein with such a high number of polysaccharide units is described. The following two findings were very unusual. First, 11 amino acid substitutions were detected. The carboxyl-terminal CNBr fragment possesses ten further amino acid replacements as described earlier, so that in 21 of the 181 residues of the protein, or 12%, such substitutions have occurred. These replacements, except for two, can be explained by single point mutations. Secondly, a significant degree of homology was noted between the amino-terminal 43-residue segment of CNBr-I and the amino terminal of the variable region of the κ-type L chain of human IgG. This homology and that of the carboxyl-terminal region of this glycoprotein with the constant region of the H chain of IgG suggest that α_1-acid glycoprotein may represent a protein that is related to the ancestral immunoglobulin.

In a recent publication (Ikenaka *et al.*, 1972) we have described the amino acid sequence of the carboxyl-terminal cyanogen bromide (CNBr)[1] fragment of α_1 acid glycoprotein. In the present paper the amino acid sequence of the chymotryptic, tryptic, and certain peptic peptides and glycopeptides of the remaining part of the polypeptide chain, the amino-terminal CNBr fragment (Ikenaka *et al.*, 1972) of this human plasma protein, is presented.

Materials and Methods

α_1-Acid glycoprotein was isolated from Cohn fraction VI of pooled normal human plasma by a procedure described earlier (Bürgi and Schmid, 1961). This globulin, which possesses a single polypeptide chain (Ikenaka *et al.*, 1966), was demonstrated to be homogeneous as judged by several criteria of purity (Ikenaka *et al.*, 1966; Jeanloz, 1972). Desialyzation was accomplished by mild acid hydrolysis at pH 1.6 and 80° for 1 hr using a 2% protein solution (Schmid *et al.*, 1967). In previous investigations it was shown that this procedure does not lead to the formation of new amino-terminal amino acids (Ikenaka *et al.*, 1966). After dialysis against cold 1% pyridine, the resulting protein solution was lyophilized and used for part of the present study.

The preparation and characterization of the amino-terminal CNBr fragment (CNBr-I) of native α_1-acid glycoprotein, the enzymes, and various other reagents used in the present study were described earlier (Ikenaka *et al.*, 1972). In addition, Nagarse and Pronase (70,000 p.u.k/g) were products from Kaken & Co., Tokyo, while pyrrolidonecarboxylyl-peptidase was prepared essentially according to Doolittle (1972). The specific activity of the enzyme preparation obtained after the Sephadex G-200 step amounted to 3000 units/

† From the Department of Biochemistry, Boston University School of Medicine, Boston University Medical Center, Boston, Massachusetts 02118. *Received February 14, 1973*. Presented in part at the 62nd Meeting of the Federation of American Societies for Experimental Biology (Schmid *et al.*, 1971a). Preliminary notes on the amino acid sequence of CNBr-I of α_1-acid glycoprotein were published (Schmid *et al.*, 1971b, 1972). This study was supported by grants from Hoffmann-La Roche, Inc., Nutley, N. J., the National Science Foundation (GB-24813), and the National Institute of General Medical Sciences (GM-10374), U. S. Public Health Service.

[1] Abbreviations used are: CNBr, cyanogen bromide; Gdn·HCl, guanidine hydrochloride; CM, carboxymethyl; BAW, *n*-butyl alcohol–acetic acid–water (200:30:75); BAWP, *n*-butyl alcohol–acetic acid–water–pyridine (15:3:10:12): BPW, *n*-butyl alcohol–pyridine–water (1:1:1); Glu, pyrrolidonecarboxylic acid or pyroglutamic acid residue.