Nucleic acid binding properties of major proteins from the heterogeneous nuclear ribonucleoproteins of wheat

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ABSTRACT

Glycine-rich core hnRNP proteins purified from wheat bind tightly to single-stranded but not to double-stranded nucleic acids with a preference for natural RNA over single-stranded DNA. Binding results in i) a progressive disruption of the residual secondary structure of the polynucleotide and the formation of an extended nucleoprotein filament until a protein to polynucleotide weight ratio of about 5:1 is attained. As more protein is added, this is followed by ii) the formation of globular structures along the polynucleotide chain with a concomitant reduction in the contour length of the nucleoprotein complex. These two features of the interaction—unwinding and condensation into beads—are analogous to the previously described behavior of the major glycine-rich core hnRNP protein from *Artemia salina* (Thomas et al. (1981) Proc. Natl. Acad. Sci. USA 78, 2888) and may represent the basic functional properties of this relatively well conserved group of nuclear proteins.

INTRODUCTION

A large part of the protein mass of heterogeneous nuclear ribonucleoprotein particles (hnRNP) consists of a relatively small number of chemically related polypeptides in the molecular weight range 30-45,000. These proteins are basic (pI's from 7 to 9), and have amino acid compositions characterized by very high contents of glycine, the absence of cysteine, and the presence of the unusual amino acid dimethylarginine (1-4). These features are shared by proteins from divergent species, and distinguish this group from other hnRNP proteins, and from histones and non-histone chromosomal proteins; the terms "glycine-rich proteins" and "core hnRNP proteins" have been suggested for the group (2,3). The glycine-rich proteins cross-react immunologically with each other and, in some cases, cross-react with the hnRNP proteins from other species (5,6). No enzymatic activity of any kind has been reported for these proteins. Rather, there are indications that they are structural proteins that form the core of monomeric hnRNP particles. These particles, which sediment at

30-45S, can be isolated from the nuclei of many cell types and probably represent the products of limited nucleolytic digestion of larger hnRNP complexes (7). In mammals there appear to be 4-6 glycine-rich polypeptides of different primary structures (1-3). However, since they undergo extensive post-translational modifications, multiple protein bands are detected when they are analyzed by two-dimensional gel electrophoresis (3). The number of glycine-rich proteins in lower eukaryotes is smaller (8,9).

We have previously characterized the nucleic acid binding properties of a glycine-rich protein from hnRNP of the brine shrimp Artemia salina (4,9,10). The protein, termed HD40 (helix-destabilizing protein, molecular weight 40,000) binds tightly to single-stranded but not to double- or multi-stranded natural or synthetic polynucleotides. It binds preferentially to natural RNA over denatured DNA. Binding does not appear to be sequence-specific. HD40 interacts with single-stranded RNA and DNA in a two-step process. At stoichiometries of up to 1 protein per 12-15 nucleotides, the protein disrupts the secondary structure of the polynucleotide producing an extended nucleoprotein filament. In the second step, as HD40 is added to a stoichiometry of 1 protein per 5-6 nucleotides, the extended filament is condensed with the concomitant appearance of globular structures along the entire length of the polynucleotide. The complex acquires a "beads-on-a-string" appearance similar to that of native hnRNP complexes seen in electron micrographs of chromatin undergoing transcription (11,12). The bases in the fully condensed beaded complex with HD40 remain unstacked, suggesting that the "beads" function to compact the polynucleotide without an increase in secondary structure, presumably as a coiled RNA-protein complex. The RNA in native hnRNP is also largely unfolded (1,9,13).

With the exception of HD40, little is known about the interaction of glycine-rich core hnRNP proteins with polynucleotides. Since the formation of the HD40-polynucleotide complexes appears to reflect some aspects of the structure and, perhaps, biosynthesis of hnRNP, we have extended these investigations to other cell types. In this communication we show that major glycine-rich hnRNP proteins isolated from wheat exhibit nucleic acid binding properties analogous to those of protein HD40 from A. salina.

MATERIALS AND METHODS

Polynucleotides were purchased from Miles Laboratories, Inc. Phage

MS2 RNA was prepared as previously described (14). Phage ϕ X174 DNA was a gift from Dr. O.S. Bhanot.

Glycine-rich proteins were isolated from sprouted wheat. Wheat grain (400 g dry weight, obtained from Erewhon Inc., Cambridge, MA) was soaked in distilled water, then placed on top of cheesecloth that had been stretched over glass trays filled with sterilized tap water. After 3 days at 25-26°, the sprouts were excised manually from the seeds. Nuclei were prepared as described (15) and further purified by centrifugation through 2.0 M sucrose density cushions (16) in buffer A (20 mM Tris-HCl, pH 7.0, 20 mM NaCl, 1.5 mM MgCl₂, 1.0 mM dithiothreitol). The nuclear pellet was washed twice with 2 vol buffer A containing 0.25 M sucrose. Nuclei were unbroken as judged by phase-contrast microscopy and by ethidium bromide staining. RNP particles were extracted from the nuclei by a procedure similar to that described by Samarina *et al.* (17). Nuclei were stirred gently with 3 volumes of buffer A adjusted to pH 8.0 for 12 h at 4°. After pelleting, the nuclei were resuspended and extracted for an additional 2 h with the same buffer. The combined extracts were sedimented through 10-30% sucrose gradients made with buffer A (pH 8.0) for 16 h at 24,000 rpm in an SW41 rotor. A fairly symmetrical peak of ribonucleo-protein particles sedimenting at 30S was collected, diluted with 2 vol of buffer A and pelleted by centrifugation for 4 h at 250,000 x g. The protein:RNA mass ratio of this material was 5.5-6:1; contamination by DNA was 3.3% and 4.7% in two different preparations, as determined by a modified diphenylamine test (18). The pellet was suspended in buffer A containing 1.0 M NaCl in order to disrupt the RNP particles, dialyzed against the same buffer and sedimented through a sucrose gradient (as above) made in buffer A containing 1.0 M NaCl. Top fractions of each gradient tube (about 2.5-3.0 ml) containing the dissociated proteins were combined, diluted with buffer A containing no NaCl to a final NaCl concentration of 0.4 M and precipitated with ammonium sulfate (0.52 g/ml). The precipitate (Step 4 protein) was dissolved in 3 ml of buffer B (50 mM potassium phosphate, pH 7.0, 10 mM KCl, 0.1 mM EDTA, 0.1 mM DTT) and dialyzed against the same buffer. Starting from Step 4, fractions were assayed for [³H] MS2 RNA binding by retention on Millipore filters and for helix-destabilizing activity by their ability to unwind the poly rU-spermine complex (4). The protein was applied to a hydroxylapatite column (1.5 x 15 cm, Bio-Gel HTP, Biorad Laboratories) previously equilibrated with buffer B. The column was washed with the same buffer and the bound

protein was eluted stepwise with buffer B, the concentration of potassium phosphate being increased by 50 mM at each step. Activity appeared as a broad peak centered around 250 mM potassium phosphate. Active fractions were pooled, precipitated with ammonium sulfate (0.52 g/ml), dissolved in buffer C (20 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 0.1 mM DTT) and dialyzed against the same buffer (Step 5). Affinity chromatography on poly rU-cellulose (19) was the final purification step. Step 5 protein (2.5 ml, 3.8 mg/ml) was layered on a poly rU-cellulose column (5 ml bed volume, containing 6 mg of poly rU) equilibrated with buffer C. The column was developed stepwise with buffer C containing 0.25, 0.5 and 1.0 M KCl. Active material eluting at 0.5 M KCl (Step 6) consisted of two proteins with molecular weights of 34,000 and 44,000 as estimated by SDS-PAGE (Fig. 1). Step 6 protein (1.3 ml, 0.9 mg/ml) which was free of nucleolytic activity, was used without further fractionation. Protein was determined by the Biorad method (20).

UV spectra were recorded with a Zeiss PM6 spectrophotometer equipped with a thermoelectric control unit. Microcuvettes (10 mm pathlength, 1.5 mm wide) were used, the volume of the sample being 0.25-0.35 ml. Circular dichroic spectra were recorded with a Cary 61 spectropolarimeter using jacketed thermostated cells (5 mm pathlength, 0.45 ml volume). Data were uncorrected for the refractive index of the solvent. Electron microscopy of nucleic acid-protein complexes was carried out by the anthrabis procedure (21). Step 6 protein (20 to 90 μ g/ml), previously clarified by centrifuging at 50,000 rpm for 2 h, was incubated for 10 min with the nucleic acid (4 to 6 μ g/ml) at room temperature in 10 mM Tris-HCl (pH 7.6), and 10 mM NaCl. The complexes were fixed for 10 min with 0.1 volume of 1% glutaraldehyde (freshly diluted from a 25% solution stored under nitrogen), then diluted with 10 volumes of 0.02% anthrabis (1,4-bis[3-(benzyltrimethylammonio)propylamino]-9,10-anthraquinone dichloride) in 10 mM Tris-HCl (pH 8.5), 1 mM EDTA, 30% formamide. A 0.05 ml drop was placed on parafilm, and after 5 min a carbon-coated grid was briefly touched to the surface. The grid was washed in water, then ethanol (15 s each), and rotary-shadowed with platinum/palladium (80:20) at an angle of 8°. Molecular lengths were measured with a Numonics Graphics Calculator from electron micrographs projected at a total magnification of 125,000. Other experimental details are given in the legends to figures.

RESULTS

The glycine-rich proteins used in this work were obtained from

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Step 6 protein (9 μ g). Positions of molecular weight marker proteins are shown on the left.

nuclei of growing wheat. The purified fraction, Step 6 protein, consists of two polypeptides with molecular weights of 34,000 and 44,000 which comprise better than 85% of the total protein of this fraction (Fig. 1). Amino acid analysis of Step 6 protein shows 18.5 mol% glycine (the most abundant amino acid), no cysteine and 1.1 mol% dimethylarginine (16% of all arginine residues); the low content of methionine in Step 6 protein

FIG. 2. Binding of Step 6 protein to polynucleotides: MS2 RNA, $\bullet$ — $\bullet$; heat-denatured HeLa DNA, $\circ$ — $\circ$; native HeLa DNA, Δ — Δ . Labeled polynucleotide (0.5 nmole) was mixed with the protein in 0.1 ml of a buffer containing 5 mM Tris-HCl, pH 7.4, 50 mM NaCl at room temperature, incubated for 1 min at 0°, diluted to 1.0 ml with ice-cold buffer and filtered through a Millipore filter. The filter was washed three times with 2.0 ml of cold buffer.

(1.2 mol%) and relatively high aspartic (7.4 mol%) and glutamic acids (10.1 mol%) are also characteristic features of this class of core hnRNP proteins (1-4,8).

The ability of Step 6 protein to bind to nucleic acids as measured by retention of the complexes on Millipore filters is shown in Fig. 2. There is excellent binding to coliphage MS2 RNA, less binding to heat denatured DNA and virtually no binding to duplex DNA. Elevated ionic strength reduces the amount of complex bound to the filter as is the case with other helix-destabilizing proteins; binding is essentially abolished at 0.4-0.5 M KCl (not shown). The Millipore filter assay was also used to estimate the relative ability of various polynucleotides to bind Step 6 protein. The results of competition experiments in which an excess of unlabeled polynucleotide is mixed with [^3H] MS2 RNA prior to the addition of protein is shown in Table 1. Except for ribosomal RNA, none of the synthetic and natural polynucleotides compete effectively for the protein.

The binding of Step 6 protein disrupts the secondary structure of single-stranded nucleic acids analogous to the effect of other helix-destabilizing proteins (Figs. 3-5). Poly rU forms an intramolecular double-helical structure with a T_m of 28° in the presence of an equimolar amount of spermine (22,23). The helix-coil transition is cooperative and can be monitored by observing CD or UV spectra. Fig. 3 shows that at 7.1°

Table I. Competition of Polynucleotides for Step 6 Protein

<u>Competing polynucleotide</u>	<u>[^3H] MS2 RNA bound to filter (% input)</u>
None	100 (7050 cpm)
rU	77
rA	75
rC	93
rI	90
rA + rU (1:1)	100
tRNA	91
5S RNA	93
rRNA (16S + 23S)	35
dA	100
denatured DNA	71
native DNA	94

[^3H] MS2 RNA (0.75 nmol, 7300 cpm) was mixed with unlabeled polynucleotide (3 nmol of single-stranded or 5 nmol of double-stranded) in 0.1 ml of a buffer containing 5 mM Tris-HCl, 7.4, 50 mM NaCl at 24° . Protein (3 μg) was added and the samples were processed as described in Figure 1.

FIG. 3. Disruption of the structure of helical poly rU by Step 6 protein. The protein was added to a solution of poly rU (4.8 nmol) in a buffer containing 5 mM Tris-HCl, pH 7.4, 25 mM NaCl and 1.5×10^{-5} M spermine HCl at 7.1°, o—o. The UV thermal melting profile of poly rU in the same buffer is shown by the dashed line which refers to the upper abscissa.

the protein completely denatures the ordered state of poly rU at a protein to polynucleotide weight ratio of about 6:1. When the protein is mixed with poly rU prior to the addition of spermine, the transition to the helical conformation upon subsequent addition of spermine does not occur. Figure 4 shows that at 18° the protein induces extensive unfolding of MS2 RNA (T_m 55°) as evidenced by the decrease in ellipticity and the small blue shift of the C.D. spectrum. The ellipticity of the complex formed at 18° at a protein:RNA weight ratio of 5:1 corresponds to the ellipticity of the RNA alone at nearly 80° (cf. curves 2 and 5 of Fig. 4). Similar results were obtained when experiments of Fig. 4 were carried out with poly rA, poly rC and phage ϕ X174 DNA.

Step 6 protein does not appear to bind any duplex structures as seen

FIG. 4. Circular dichroic spectra of MS2 RNA (28 nmol) and MS2 RNA-Step 6 protein complexes. Curves 1 and 2 are spectra of MS2 RNA at 18° and 80°, respectively; 3, 4 and 5 are MS2 RNA at 18° in the presence of 25, 40 and 50 μ g of protein, respectively (5 represents the approximate end point of titration). Inset: the effect of the protein on the maximum ellipticity of MS2 RNA. Buffer is that described in Fig. 1.

from Millipore binding experiments, nor does it perturb their CD or UV spectra. However, the protein affects the rate and extent of homopolymer duplex formation. As seen from Fig. 5, the addition of the protein to poly rU at a protein:polynucleotide weight ratio of about 6:1 prior to the addition of poly rA prevents the formation of the poly rA-poly rU duplex at 18° at low ionic strength (30 mM) but not at 120 mM NaCl. In the presence of Step 6 protein, about 50% of the homopolymers exist as single strands at 18° in 60 to 90 mM NaCl (Fig. 5). Since the T_m s of the poly rU-poly rA duplex are 48° and 53° at 60 and 90 mM NaCl, respectively, (24), the protein reduces the effective T_m by about 30°. The rate of homopolymer pair duplex formation in the presence of Step 6 protein is slow at any ionic strength, whereas in its absence it is rapid even at low ionic strength (Fig. 5).

Electron micrographs of complexes formed between Step 6 protein and either ϕ X174 DNA or poly rA are shown in Fig. 6. ϕ X174 DNA is a useful model for these studies because of its moderate length (5386 nucleotides, ref. 25) and circularity; the mere existence of the circles testifies to the intactness of the molecule. Under the conditions employed for mounting, the circular DNA in the absence of the protein appears as a compact molecule in which a great deal of secondary structure can be seen (Plate A). As the weight ratio of protein to DNA is increased to about 4.8:1 (Plates B and C), the DNA molecules expand progressively into filamentous circles. Up to this stoichiometry the DNA becomes more extended and loses

FIG. 5. Effect of Step 6 protein on the formation of the poly rU-poly rA duplex. Poly rU (4.2 nmol) was mixed with poly rA (4.2 nmol) in the absence (---) or in the presence (—) of 16 µg of Step 6 protein at 18° in a buffer containing 5 mM Tris-HCl, pH 7.5 and 30 mM NaCl in a final volume of 0.3 ml. Changes in absorbance at 260 nm were recorded. Arrows indicate additions of NaCl from a 4.0 M stock solution. Absorbance readings were corrected for volume changes and for the protein contribution at 260 nm. The hyperchromicity of the random coil versus duplex was 36%. Zero time values were calculated from the known absorbance of the homopolymer solutions.

FIG. 6. Electron micrographs of complexes between Step 6 protein and ϕ X174 DNA (B to E) or poly rA (F). The protein and nucleic acids were mixed at weight ratios of 2.4:1 (B), 4.8:1 (C), 9.6:1 (D and F), and 19.2:1 (E). Micrograph A is of DNA only. The bar represents 250 nm.

FIG. 7. The lengths of ϕ X174 DNA-Step 6 protein complexes as a function of the protein:DNA weight ratio. At each protein:DNA weight ratio, the lengths of 30-50 complexes such as those shown in Fig. 5 were measured. The points represent the average lengths and the error bars indicate the total range of the measurements.

its secondary structure, an observation that is in accord with the results of the optical studies. At a 4.8:1 weight ratio (Plate C) the population of circles is not uniform in length, suggesting that binding is somewhat cooperative. Also note (Plate C) that small beads can be seen on the circumference of the circles. At a 9.6:1 weight ratio (Plate D), the average contour length of the circles decreases (see Fig. 7) and full-size globular structures with diameters of 30-40 nm make their first appearance. At still higher protein:DNA weight ratios (Plate E), the contour lengths of the complexes decrease still further (see Fig. 7) and large beads (30-40 nm diameter) are seen along the DNA circles. Figure 6, Plate F shows that with poly rA which contains a population of molecules of different chain lengths, the protein forms similar complexes containing from 1 to 6 beads. In the absence of nucleic acids, the protein used in these experiments does not form any filamentous or globular structures under the mounting conditions used (not shown).

As seen from Fig. 7, the average length of the Step 6 protein- ϕ X174 DNA complex increases to a maximum of about 0.75 μ m at a 4.8:1 protein to DNA weight ratio and then decreases to 0.39 μ m as more protein is added. The length distribution of the complexes at a given protein:DNA weight ratio is broad (especially at a 4.8:1 weight ratio) but contains one maximum with no indication of bimodality.

DISCUSSION

This communication shows that the glycine-rich proteins of wheat hnRNP contain two polypeptides of MW 34,000 and 44,000 (Step 6 protein) that exhibit helix-destabilizing activity. Since both polypeptides copurify through poly rU-cellulose affinity column and elute at 0.5 M KCl, it is most likely that both are involved in the binding of RNA. As is the case with other helix-destabilizing proteins, there is no sharp specifi-

city in the binding of polynucleotide substrates. Although both single-stranded RNA and DNA are unfolded, the wheat Step 6 protein has a higher affinity for natural RNA. This is identical to the binding preference shown by protein HD40 from A. salina (4). Unwinding of the secondary structure of the polynucleotide is the first step in a two-step binding process. In the second step, with the Step 6 protein at a greater than 5:1 (w/w) excess over the nucleic acid, globular structures are formed along the polynucleotide and the contour length of the complex is reduced; at high protein to polynucleotide ratios it is about half as long as the fully extended complex that is seen at a protein to polynucleotide weight ratio of 4.8:1 (Figs. 6 and 7). Again, these two features of the interaction—unwinding followed by condensation through bead formation—are very much analogous to the previously described behavior of the glycine-rich protein HD40 from the hnRNP of A. salina (9,10). Since these activities are shared by hnRNP proteins from widely divergent species, a plant and a crustacean, they probably represent basic functional properties of this relatively well-conserved class of core hnRNP proteins. The mode of binding of these proteins to polynucleotides appears to be consistent with their postulated structural role in hnRNP (1-3,7,9,26). The spontaneous formation of the globular complexes may well reflect some aspects of the in vivo self-assembly process of hnRNP.

There are certain differences between the nucleic acid binding properties of the A. salina and the wheat proteins. With HD40, the unwinding and the condensing steps occur sequentially, the unwinding step exhibiting little cooperativity (10). With the wheat proteins, the separation between the two steps is less sharp, suggesting that the interactions are more cooperative. Another difference is that the globular structures formed by the wheat proteins are larger and less uniform in size than those formed by HD40.

The RNA in native hnRNP while condensed, perhaps as a coiled RNA-protein complex, is maintained in a largely unstacked conformation owing to its interaction with proteins (1,9,13). An analogous situation exists with respect to the complexes reconstituted from polynucleotides and the glycine-rich proteins from either A. salina (9,10) or wheat. It should be pointed out that the conformation of the RNA in hnRNP and in these in vitro formed complexes differs from that in other ribonucleoproteins. In ribosomes and in a number of RNA viruses, single-stranded RNA exists in a compact state with many internal double-stranded regions (at least 65% of

the nucleotides of coliphage MS2 RNA are base-paired, 27). It is tempting to speculate that the dual activities of core hnRNP proteins—unwinding and condensation into beads—may serve a number of purposes advantageous to the cell: i) they may prevent undesirable "knotting", i.e., the formation of secondary and tertiary structures within the long hnRNA molecules while effectively compacting them, and ii) they hold the hnRNA molecules in an unstacked conformation that might facilitate their subsequent screening by recognition elements, and the action of enzymes involved in the processing of the hnRNA and the generation of functional mRNA molecules. Further studies on the structure of hnRNP, the arrangement of proteins and RNA—introns and exons—within and/or outside the beads, are required to elucidate these points. The two sources of starting material for the isolation of glycine-rich proteins, A. salina and wheat, were selected with a view of obtaining purified proteins in quantities sufficiently large to allow detailed studies on the structure and function of their complexes with RNA. After having established their basic similarities, we feel that the purified glycine-rich proteins from either A. salina or wheat will prove to be useful in these investigations. Comparative studies with glycine-rich proteins from mammalian sources are under way.

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