The conformation of 16S RNA in the 30S ribosomal subunit from *Escherichia coli*.

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ABSTRACT

The digestion of *E. coli* 16S RNA with a single-strand-specific nuclease produced two fractions separable by gel filtration. One fraction was small oligonucleotides, the other, comprising 67.5% of the total RNA, was highly structured double helical fragments of mol. wt. 7,600. There are thus about 44 helical loops of average size corresponding to 12 base pairs in each 16S RNA. 10% of the RNA could be digested from native 30S subunits. Nuclease attack was primarily in the intraloop single-stranded region but two major sites of attack were located in the interloop single-stranded regions. Nuclease digestion of unfolded subunits produced three classes of fragments, two of which, comprising 80% of the total RNA, were identical to fragments from 16S RNA. The third, consisting of 20% RNA, together with an equal weight of protein, was a resistant core (sedimentation coefficient 7S).

INTRODUCTION

The smaller subunit of the bacterial ribosome consists of a single molecule of RNA non-covalently associated with some twenty different protein molecules to form a compact ribonucleoprotein particle. This is assumed to have well-defined tertiary and quaternary structures. Much experimental effort has recently been directed towards isolating and characterizing the protein components of the subunit and the classical techniques of protein chemistry are currently being applied to the intact ribosome in order to gain information about the three-dimensional arrangement of the proteins within the particles and to identify those proteins actively involved in the more functional aspects of the subunit in protein synthesis (for reviews see 1-4).

The primary sequence of 16S RNA is now almost completely known⁵. The details of the secondary structure are less well-defined, being based at present partly on the maximization of base pairs from a visual inspection of the primary sequence. The RNA is thought to consist of short regions of double-helical 'hairpin loops' connected

by single-stranded interhelical regions⁶⁻⁸. However, the tertiary folding of the RNA within the subunit remains unknown. Divalent cations play an important role in stabilising the tertiary structure by binding to phosphate groups on the RNA^{9,10} and recently it has been shown that varying the relative concentrations of monovalent and divalent cations can cause subtle changes in the conformation of both the RNA and the protein components¹¹. By chelating the divalent cations with EDTA it is possible to unfold the compact tertiary and quaternary structure of the 30S subunit without causing dissociation of the protein from the RNA and without altering the amount of secondary structure present in the RNA¹².

We have attempted to gain more precise information about the arrangement of the RNA in the 30S subunit by studying the action of an endonuclease, specific for single-stranded nucleic acid, on 16S RNA, native 30S subunits and subunits which have been unfolded in EDTA. The results are presented below and compared with a similar study on the 50S subunit described previously¹³.

EXPERIMENTAL METHODS

Preparation of Ribosomes and Ribosomal Subunits

25 grams of E. Coli MRE600 (in the form of a frozen paste) were suspended in an equal volume of 10mM Tris-HCl, 50mM KCl, 10mM MgCl₂, 6mM 2-mercaptoethanol pH 7.8 and the cells disrupted in a French press. Cell debris, unbroken cells, etc., were removed by centrifugation at 50,000 g, and the supernatant then centrifuged at 200,000 g for 120 minutes to pellet the ribosomes. The ribosomes were washed three times with 10mM Tris-HCl, 0.16M NH₄Cl, 10mM MgCl₂, pH 7.8. Ribosomes were dissociated into subunits by dialysis overnight against 10mM Tris-HCl, 0.5mM MgCl₂, pH 7.5 (TM buffer).

Separation of subunits was carried out on a Beckman AL14 zonal rotor. Approximately 600 mg of subunits in 30 mls of TM buffer, were layered onto a 10-30% sucrose gradient, (Ribonuclease-free sucrose, Cambrian Chemicals Limited, Croydon, England) followed by an overlay of TM buffer (100 mls) and centrifuged at 35,000 r.p.m. for 6½ hours. Fractions from the gradient containing 30S subunits were pooled, dialysed against TM buffer to remove sucrose and concentrated to 3-4 mg ml⁻¹ either by centrifugation at 200,000g or with an Amicon 402 ultra-filtration cell.

Sedimentation Coefficients

Measurements were carried out at 20° in a Beckman Model E ultracentrifuge using either schlieren or ultraviolet absorption optics.

Molecular Weights

Apparent molecular weights were determined by the conventional sedimentation equilibrium method using ultraviolet absorption optics. The solution (0.3 mls, $A_{260} = 0.4$) was equilibrated at 23,150 r.p.m. for 30 hours. Photographs were scanned in a Joyce-Loebl double-beam scanning microdensitometer. The concentration of solute was determined by comparing the blackening of the film to a standard curve obtained with solutions of known A_{260} . Molecular weights were calculated by plotting $\log c$ vs. r^2 , where c is the concentration of solute at distance r from the centre of rotation.

Melting Profiles

The procedure has been described¹⁴.

Circular Dichroism

Circular Dichroic spectra were obtained using a Roussel-Jouan CD 185 micrograph with cells of 1 or 2 cm. path length. Results are expressed as $\Delta \epsilon$, the difference in the extinction coefficients of left and right circularly polarized light per mole of phosphorus. The extinction coefficient of 16S RNA was taken as 7710 per mole of phosphorus¹⁵.

Preparation of 16S RNA

RNA was prepared by precipitation with 66% acetic acid using the method of Waller and Harris¹⁶. The precipitate was resuspended in 0.15M NaCl, 15mM sodium citrate, 10mM EDTA, pH 7.0, and dialysed against TM buffer overnight. Alternatively, the RNA was extracted from the subunits by shaking twice with two volumes of water-saturated phenol containing 2.5% tri-isopropyl-naphthalene-sulphonic-acid¹⁷. The aqueous phase containing the RNA was then dialysed against TM buffer. No differences were observed in the properties of the 16S RNA prepared by the two methods.

Preparation of the Endonuclease

The enzyme was prepared and assayed as described by Kasai and Grunberg-Manago¹⁸ but with minor modifications¹³. The enzyme preparations were assayed for activity against poly A, poly U, and poly A.U, prepared by preincubating equimolar quantities of poly A

and poly U at 37° for 60 minutes according to the method of Kasai and Grunberg-Manago¹⁸ but with minor modifications¹³. Enzyme preparations having specific activities of 1,000, 1,8000 and 5,200 units ml⁻¹ were used in this work, where one unit represents an increase in A₂₆₀ of 1.0 in 100μl of assay solution per ml of enzyme per hour (against poly A). The activities against poly A.U were 1%, 2.3% and 3% of the activities against poly A respectively.

Digestion with Enzyme

The incubation mixture contained ribosomes or 16S RNA in TM buffer (1-3 mg.ml⁻¹) enzyme (75-1,000 units per mg. ribosomes) 2-mercaptoethanol (125mM) and MgCl₂ (1mM). Incubations were carried out at 35° for 5-60 minutes. The reaction was terminated by the addition of polyvinylsulphate to 0.1 mg.ml⁻¹, which has been shown to inhibit the endonuclease¹³.

Column Chromatography

The digestion products were separated on a column of Sephadex G100 or G200 (42 x 1.5 cm) equilibrated with TM buffer at 4°C. Fractions (2 mls) were collected and monitored at 260 nm for RNA. Protein concentrations were determined from the absorbance at 230 nm with a suitable correction for the contribution of RNA at that wavelength. Controls containing enzyme alone were chromatographed and corrections made for enzyme protein. The exclusion volume V_o and bed volume V_b of the column were determined with Blue Dextran 2000 and guanosine monophosphate, respectively. Peak positions are expressed as retention coefficients K', where a fraction eluting at volume V_x has a retention coefficient:

$$K' = \frac{V_x - V_o}{V_b - V_o}$$

The elution profiles of the column were analysed and peak areas measured on a Dupont 310 curve analyser.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of RNA fragments was carried out by the method of Peacock and Dingman¹⁹. RNA (30-50μg) was

loaded on to gels (0.6 x 10 cm) consisting of 3% or 10% acrylamide, and 0.5% or 0.15% N,N' methylenebisacrylamide and electrophoresed at 150 V.cm⁻¹ for 3 hours. The gel and running buffer was 90mM Tris-Borate, 2.5mM EDTA. The gels were stained in 0.2% methylene blue and destained in water. The bands were scanned in a Gilford gel scanner at 540 nm. 16S RNA from *E.coli* and unfractionated *E.coli* tRNA (from M.R.E., Porton) were used as molecular weight standards. This latter sample contained a significant proportion of a species which we have identified, on the basis of its sedimentation coefficient, as 5S RNA. This served as a third molecular weight standard. The mobilities of these last two species were consistent with this identification and with their relative mobilities deduced from the calibration curves of Peacock and Dingman¹⁹.

Sucrose Gradient Centrifugation

Enzyme-digested 30S subunits were analysed by density gradient sedimentation through a 5-20% sucrose gradient in a Beckman SW50.1 rotor. Samples (0.4 ml) were layered onto gradients (4.6 ml) and centrifuged for 12 hours at 45,000 r.p.m. (225,000 x g). Immuno-globulin G (7S) and cytochrome C (1.6S) were used as markers. Fractions (0.15 ml) were collected and analysed for RNA and protein.

RESULTS

Action of Endonuclease on 16S RNA

16S RNA (1-2 mg.ml⁻¹) was incubated with enzyme at 35° for 15' and the digestion products separated on Sephadex G100 (Fig. 1). Whereas native 16S RNA eluted at the exclusion volume, in the digested sample less than 3% of the nucleotide eluted at the exclusion volume, the remainder eluting as two well-defined peaks (fractions II and III) with K' equal to 0.55 and 0.95 respectively (tRNA eluted with K' = 0.25). The proportion of material in peaks II and III was virtually independent of the concentration of enzyme over the range 75-1,000 units mg⁻¹ RNA. This suggests that a digestion limit has been reached. Fraction II had a well-defined shoulder and could be resolved on the curve analyser into two sub-fractions, IIa and IIb, with retention coefficients of 0.27 and 0.55 respectively. The percentage of total nucleotide eluting in fraction II was similar in all preparations and had an average value of 67.5 ± 1.5% (5 preparations).

On melting, fraction II showed 15% hyperchromism and had a

Figure 1. Sephadex G100 chromatography of the digestion products of 16S RNA. Digestion conditions: 250 units of enzyme mg^{-1} RNA, 15 minutes at 35°C.

melting temperature of 50° in 10^{-2} M sodium phosphate, 1mM MgCl_2 , pH 7.0. Although these values are somewhat lower than those of intact 16S RNA (hyperchromism 27%, $T_m = 56^\circ$), the circular dichroic spectrum suggests that fraction II has a high degree of secondary structure (Fig. 2). The spectrum has a maximum at 265 nm with $\Delta\epsilon$ equal to 5.5 to 6.0 compared with 4.5 to 5.0 for 16S RNA. A high value of $\Delta\epsilon$ at 265 nm is characteristic of helical, double-stranded RNA and values close to 6.0 have been reported for completely double-stranded polynucleotides and RNA^{13,20,21}. No significant differences in the dichroic spectra of fractions IIa and IIb were observed. Thus fraction II appears to arise from the double helical hairpin loops in the original 16S RNA.

Figure 2. Circular dichroic spectra of fractions II and III derived from 16S RNA.

Fraction III consisted of low molecular weight material. It eluted at the bed volume of the column and diffused through Visking dialysis tubing. The dichroic spectrum (Fig. 2) had a low maximum at 275 nm characteristic of single-stranded RNA or small oligonucleotides^{13,20}. The enzyme is known to degrade unstructured polynucleotides to the level of tri- or tetra- nucleotides¹⁸. Fraction III may therefore be identified with small oligo or mononucleotides formed by the degradation of the single-stranded regions of the 16S RNA.

Fractions IIa and IIb both sedimented as fairly sharp boundaries in the ultracentrifuge with sedimentation coefficients of $1.9 \pm 0.1S$ and $1.3 \pm 0.1S$, respectively. The molecular weight of fraction IIb, the major component of fraction II, was determined by equilibrium ultracentrifugation. A linear relation between $\log c$ and r^2 was obtained giving a molecular weight of $7,600 \pm 750$ (3 preparations). The sedimentation coefficient and molecular weight of this fraction are consistent with the retention coefficient of the material on Sephadex G100.

Action of Endonuclease on 30S Ribosomal Subunits

30S subunits were incubated with enzyme for 15 minutes at enzyme concentrations of 100-600 units per mg subunits. The sedimentation coefficient of the subunits was unchanged by this treatment. However, chromatography on Sephadex G100 revealed two fractions, the major one eluting at the exclusion volume, and a minor fraction eluting with a retention coefficient of 0.95 comprising 10% of the total nucleotide. The dichroic spectrum of the first fraction was very similar to that of native subunits. The spectrum of the minor fraction had a low maximum at 275nm, and resembled the spectrum of fraction III from 16S RNA. This, together with its retention coefficient on Sephadex, suggests that it is low molecular weight oligonucleotide formed from the limit degradation of single-stranded regions of the RNA which are accessible to the enzyme in the intact 30S subunit.

Native 30S subunits were digested with enzyme as above and then unfolded by overnight dialysis against 10mM Tris-HCl, 4mM EDTA pH 7.5. The digested unfolded subunits sedimented as a sharp boundary with a sedimentation coefficient of 10S. The sedimentation coefficient increased to 25-30S on dialysis back into TM buffer or on addition of 10mM MgCl₂. Native subunits, or control subunits in which polyvinylsulphate had been added at zero time to inhibit the enzyme, were unfolded to a 12S species and refolded to 30S particles following similar treatment. When digested subunits were heated to 96° in 4% formaldehyde, a treatment which irreversibly disrupts the secondary structure of the RNA, they were found to have degraded to small fragments with a sedimentation coefficient of less than 2S. By contrast the controls gave a single species sedimenting at 10-12S.

These results show that enzyme treatment leaves the RNA in the native subunit in an apparently intact state. However, the integrity is only maintained by the secondary structure of the nucleic acid since the enzyme has introduced hidden breaks at frequent intervals along the RNA phosphate ester chain which are revealed when the nucleic acid is thermally denatured. However, these breaks are not apparent when the subunit is unfolded, without denaturation of the RNA, in EDTA.

When ribosomal subunits are unfolded in EDTA the bonds holding the subunits in their tertiary conformation, primarily protein-protein

bonds, are disrupted but the secondary structure of the RNA remains unchanged¹². These experiments suggest therefore that extensive digestion of interhelical single-stranded RNA has not occurred since, had this been the case, then unfolding the ribosome in EDTA should have caused the RNA chain to dissociate into smaller fragments. However, they do not conclusively show the complete absence of interhelical breaks since the binding sites for several ribosomal proteins appear to consist of non-contiguous regions of RNA^{22,23,24}. It is possible, therefore, that a limited number of such regions may be held together by individual proteins even when the ribosome is unfolded. In order to investigate this possibility the RNA was extracted under non-denaturing conditions from digested subunits and analysed by polyacrylamide gel electrophoresis.

30S subunits were incubated with 300 units of enzyme per mg RNA, aliquots (0.23 ml) were removed at suitable time intervals and the RNA extracted. The electrophoretic patterns are shown in Fig. 3.

Figure 3. Polyacrylamide gel electrophoresis patterns of 16S RNA extracted from 30S subunits after various times of incubation with nuclease, (a) t-RNA; (b) zero time; (c) 10 mins; (d) 20 mins; (e) 40 mins.

The gels were scanned and the areas under each component determined. The areas of the bands discussed in the text are shown as a function of time in Fig 4. The time course of the digestion suggests that the reaction is tending towards a limit after 40 mins. At this stage three major degradation products are observed (bands b,c,d) corresponding to molecular weights of 3.1 , 1.9 and 1.4×10^5 respectively. Over 70% of the RNA may be accounted for in these three components. Band a (molecular weight 3.6×10^5) appears to be the only major high molecular weight kinetic intermediate. This rapidly degrades to species of 3.1 and 0.5×10^5 molecular weight, the latter corresponding to band e. The degradation pattern of 16S RNA within the 30S subunit can be accounted for by assuming that there are two major sites of attack for the endonuclease in the interhelical regions of the RNA in the intact 30S subunit. Attack at one site leads to the production of fragments with molecular weights 3.1 and 1.9×10^5 . Attack at the other site leads to fragment of 3.6 and 1.4×10^5 molecular weight. The former of these also contains the first site and further degradation at this point leads to fragments of 3.1 and 0.5×10^5 . The presence of all these degradation products at the limit of digestion suggests that both processes proceed independently and that the population of 30S subunits may be heterogeneous with a proportion of the subunits only having the second site available to the enzyme.

Figure 4. The change in the areas of the bands a, b, c, d, e and 16S RNA shown in Fig 3 as a function of time of digestion with nuclease.

It may be noted that the total molecular weight of the fragments is 5×10^5 whereas the molecular weight of 16S RNA is 5.5×10^5 . The discrepancy of 10% may be accounted for in terms of the 10% loss of nucleotide which is digested from the surface of the intact 30S subunit. Clearly, in the light of the experiments just discussed these oligonucleotide digestion products must arise principally from the intrahelical single-stranded regions. If these are fairly evenly distributed along the 16S RNA molecule, as seems likely⁵, the molecular weight of each degradation fragment will be uniformly decreased by 10%.

Action of the Endonuclease on Subunits Unfolded in EDTA

30S subunits were unfolded by dialysis overnight against 10mM Tris-HCl, 4mM EDTA, pH 7.5, to species sedimenting at 12-17S, and then incubated with enzyme (200-600 units per mg of subunits). The sedimentation coefficient decreased rapidly and several slower-moving components were observed in the ultra centrifuge. Aliquots of the incubation mixture were removed after various time intervals, the reaction was stopped by addition of polyvinylsulphate and the products chromatographed on Sephadex G100 (Fig. 5). The percentage of material absorbing at 260nm which eluted at the exclusion volume decreased rapidly to about 15% after 15 minutes and progressively declined more slowly after that. Over the same time period, two fractions began to elute in the regions with $K' = 0.05 - 0.6$ and $K' = 0.95 - 1.0$. After 30' the profile was resolvable into three distinct fractions: fraction I eluting at the exclusion volume, fraction II eluting from $K' = 0.2 - 0.6$ and fraction III eluting at $K' = 0.95 - 1.0$. There was little subsequent change in the profile with time. Fraction II usually had a distinct shoulder and could be resolved into sub-fractions IIa and IIb with retention coefficients 0.28 and 0.55 (cf. 16S RNA and Fig. 1). The relative quantity of material absorbing at 260 nm in each fraction as a function of digestion time is shown in Fig. 6.

The recovery of nucleotide from the column was always greater than 95%. However, the recovery of protein was variable and often less than 50%. Almost all the recoverable protein co-eluted with fraction I. During digestion a considerable amount of protein became dissociated from the ribosome and precipitated in an aggregated state. This must account at least partially for the low yield

Figure 5. Sephadex G100 chromatography of the digestion products of 30S subunits unfolded in EDTA before digestion. Digestion conditions: 350 units of enzyme mg^{-1} subunits. Aliquots taken after 10, 20, 30 and 60 minutes.

of protein eluting from the column. The remaining protein either forms sufficiently large soluble aggregates to be excluded from Sephadex G100 and thus co-elutes with the RNA in fraction I or remains attached to fragments of RNA and elutes as high molecular weight ribonucleoprotein in fraction I.

At the limit of digestion fraction I contained between 8% and 20% of the total nucleotide eluting from the column and all the soluble protein. The sedimentation coefficient of this fraction was about 7S although this varied from preparation to preparation and the boundary was often diffuse. The protein: RNA ratio varied between 0.8 and 1.5. This variability is presumably due to the presence of differing amounts of aggregated protein. When the same

Figure 6. Percentage of total A₂₆₀ in fractions eluted from Sephadex G100 column as a fraction of digestion time. (O—O) fraction I; (◆—◆) fraction IIa; (□—□) fraction IIb; (●—●) fraction III; (◆—◆) nucleotide eluting in the region $k' = 0.10$.

sample of unfolded, digested ribosomes was chromatographed on Sephadex G200 the proportion of material which absorbed at 260nm and which excluded from the column was the same as on the G100 column. This implies that fraction I consists of fragments having a molecular weight of at least 250,000. On this basis fraction I represents over one quarter of the total mass of the subunit.

The dichroic spectrum of fraction I is shown in Fig.7. The spectrum is similar to that of the native subunit except that the minimum at 220nm is much larger because of the higher proportion of protein. The maximum at 265nm is characteristic of double-stranded RNA. An analysis was made of the time course of the production of fraction I assuming that the degradation was a first order process. The semi-log plot showed that there were two distinct kinetic

processes occurring. The first and more rapid of these presumably corresponded to the production of fraction I from larger ribosomal fragments whereas the slower process may be attributed to the slow degradation of fraction I by the nuclease. Extrapolating this second slow degradation process to zero time shows that the proportion of the fraction present at times close to zero is 18 - 20% of the total RNA. The absence of a similar fraction in the degradation profile of 16S RNA suggests that fraction I represents a fragment of the subunit consisting of highly structured RNA and protein which is largely resistant to degradation by the nuclease.

Figure 7. Circular dichroic spectra of fractions I, IIa, IIb, and III derived from 30S subunits unfolded in EDTA before digestion.

The digestion products were analysed by density gradient sedimentation through sucrose. The gradient profile is shown in Fig. 8. About 15% of the 260nm absorbing material sedimented as a discrete peak with a sedimentation coefficient of 7S. The protein:RNA ratio of the 7S species was 1.0. The major RNA peak, containing about 80% of the total RNA, sedimented slightly behind the 1.6S marker whereas most of the protein sedimented slightly ahead at

Figure 8. Sucrose density gradient profile of digestion products from unfolded 30S subunits. (●---●), protein; (O---O), A260.

about 2S. About 5% of the RNA and a variable amount of protein, usually about 15%, sedimented as a mixture of species larger than 7S. The amount of RNA in the 7S fraction decreased only slightly with increasing concentration of nuclease. These observations support the idea that digestion of unfolded subunits results in the degradation of 80% of the RNA to small fragments but that about 15%

of the RNA and about 30% of the protein are found in a comparatively resistant fragment. The sedimentation coefficients of the 7S peak and the major RNA peak on the sucrose gradient are remarkably similar to the sedimentation coefficients of fractions I and II respectively, as identified by gel filtration chromatography (see below). Thus it is reasonable to conclude that the 7S fraction and fraction I are similar and represent a discrete nuclease-resistant core.

The properties of fractions II and III were identical to those of the corresponding fractions from the digest of 16S RNA. Fraction II contained virtually no protein and its dichroic spectrum (Fig. 7) and melting profile were indistinguishable from those of fraction II from 16S RNA. It could be resolved into two subfractions having similar retention coefficients and similar sedimentation coefficients (1.4S and 2.1S) to those from 16S RNA. Fraction III which was maximally retained by Sephadex G100 gave a dichroic spectrum typical of unstructured RNA and appears to consist of small oligonucleotides. At the limit of digestion the ratio of fraction II to fraction III was 2:1.

DISCUSSION

Native 16S RNA is rapidly degraded by the single-strand-specific endonuclease to two discrete fractions which correspond to the intact, helical, hairpin loops and the oligonucleotide degradation products of the single-stranded regions connecting them. The small size and size-distribution of the helical loops, revealed by gel filtration chromatography and direct measurement of molecular weights of specific fractions, provides direct experimental proof of the hairpin loop model of RNA^{6,7,8}. In addition the results provide some experimental support for the more detailed secondary structure of 16S RNA recently proposed on the basis of the primary sequence^{5,24}. Thus, the fraction of nucleic acid present as helical secondary structure is 67% which may be compared to a value of 63% suggested on the basis of the primary sequence⁵. The major fraction of the structured helical loops appears to have a fairly uniform size corresponding on average to about 12 base pairs whereas a minor fraction has a size corresponding to about 24 base pairs. From the overall proportion of secondary structure it is possible to calculate that there are 44 helical loops with a size corresponding

to 12 base pairs. The secondary structure based on the sequence has 39 helical loops with an average size of 11 base pairs but, on the other hand, the distribution of sizes is rather wider than is found in these experiments⁵.

The resistance of the native subunit to degradation, compared to 16S RNA or the unfolded subunit, indicates that considerable regions of single-stranded RNA are protected from the enzyme due to the tertiary folding of the polynucleotide chain. Unfolding both the native and digested subunit in EDTA gives species with similar sedimentation coefficients. Under these conditions most of the protein-protein bonds maintaining the tertiary structure are thought to be broken¹². The observation that the unfolded species can be refolded on addition of $MgCl_2$ implies that the apparent integrity of the RNA chain is maintained, despite the digestion of 10% of the total nucleotide. However, digested and control subunits behave quite differently when the hydrogen bonds maintaining the secondary structure are subsequently destroyed by heating in formaldehyde, and it is apparent that these bonds are important in maintaining the apparent integrity of the RNA chain in the digested subunit. The 10% of RNA susceptible to digestion in the native 30S subunit must consist primarily of single-stranded regions within or at the end of double-stranded "hairpins" (the intrahelical regions) as opposed to the single-stranded interhelical regions. Most of these accessible intrahelical regions must be uniformly distributed along the RNA chain as no large fragments of RNA are observed following thermal denaturation. Furthermore, about one third of the total single-stranded RNA appears to be associated with these intrahelical regions. Thus, on average, each helical hairpin of 12 base pairs will have 4 nucleotides in the intrastrand loop and 8 nucleotides in the interstrand region between loops.

In addition to the intrahelical single-stranded regions there are two well-defined interhelical single-stranded sequences which are attacked by the enzyme in the native subunit. These two sites may also be situated near the surface of the native subunit. In the primary sequence of the 16S RNA they are located fairly close together and about one third of the way from one end of the nucleotide sequence, although which end is not known. Unfolding the subunit in EDTA after digestion does not expose these breaks in the

RNA chain which must therefore be held together by non-covalent interactions, probably between proteins.

When the 30S subunit is unfolded in EDTA prior to digestion the majority of the interhelical regions of RNA become accessible to the enzyme, as shown by the rapid decrease in sedimentation coefficient and the rapid appearance of small molecular weight fragments as seen by gel filtration chromatography. The RNA thus appears to be arranged within the native subunit such that the great majority of the single-stranded regions connecting the helices are 'buried', in the sense that they are not accessible to the enzyme, whereas the intrahelical regions are on the surface and susceptible to enzymic attack. This arrangement is similar to that proposed for the 50S subunit by Spencer and Walker¹³.

The conformation of the RNA in the unfolded subunit appears to be very similar to 16S RNA. Thus, of the degradation products which can be separated by gel filtration, fractions II and III which constitute about 80% of the total RNA are similar in size and structure to the fractions produced by nuclease digestion of 16S RNA. However, the comparatively higher concentrations of enzyme required to produce comparable degrees of digestion and the formation of a resistant core (fraction I) which is not observed in the digestion of 16S RNA, shows that the RNA in the unfolded subunit is considerably protected by the protein. Since the enzyme is specific for single-stranded RNA, this implies that single-stranded RNA may be considerably protected by interactions with protein. Furthermore, the intrahelical single strands are freely accessible to the enzyme in the native subunit and can be degraded without loss of protein. Thus, those proteins which are directly bound to RNA are presumably bound to the interhelical single-stranded regions.

Fraction I has the properties of a discrete enzyme-resistant core containing approximately 15 - 20% of the nucleotide complement of 16S RNA, i.e. up to 320 bases. The circular dichroic spectrum suggests that the RNA is highly structured and combined with up to 100,000 daltons of protein. Thus the total molecular weight of the core is approximately 200,000. The sedimentation coefficient of this fragment, 7S, together with the molecular weight imply that it has a fairly compact, globular structure with a low

frictional coefficient.

The core may contain several separate fragments of RNA held together by non-covalent forces involving protein and/or Mg^{2+} ions. Schaup *et al* have shown that protein S4 can by itself protect 25% of 16S RNA from degradation by pancreatic ribonuclease and that this protected fragment derives from several smaller fragments arising from widely separated parts of the 16S RNA molecule^{22,23}.

Fraction II contains fragments of RNA which are resistant to the enzyme because they are highly structured. These may be identified with the double helical regions of the hairpin loops. Fraction III consists of small oligonucleotides and represents degradation products arising from the single-stranded regions. Thus the proportion of secondary structure in that part of the 16S RNA which can be attacked by the enzyme in the unfolded subunit should be approximately equal to the ratio of fraction II to fraction III in the limit digestion, i.e. 67%. This is identical to the value for the overall amount of secondary structure in 16S RNA and shows that the structured regions must be fairly evenly distributed along the RNA molecule.

The proportion of secondary structure in 23S RNA is similar to that in 16S RNA. It has been estimated that there are 43 helical sections of RNA in the 50S subunit with a molecular weight of 15,700 corresponding to about 25 base pairs¹³. Thus the helical sections in 16S RNA are about half the size of those in 23S RNA.

It seems very unlikely, on thermodynamic grounds, that these helical sections are arranged in an irregular manner within the mass of the subunit. The fairly uniform size found for the helical structures in both subunits suggests that within each subunit the helices 'crystallise' together to form close-packed arrays of the type described for RNA fibres²⁵. The intermolecular interactions between the helices may be stabilised by divalent metal ions whose removal would lead to the highly cooperative transitions observed during the unfolding process¹². Furthermore, such an arrangement allows the proteins bound to the interhelical single-stranded RNA to interact, thus stabilising the RNA interactions while at the same time allowing access of nucleases to the intrahelical single-stranded RNA.

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REFERENCES

- 1 Garrett, R.A. and Wittmann, H.G. (1973) Adv. in Protein Chemistry 27, 277-347.
- 2 Nomura, M. (1970) Bact. Revs. 34, 228-277.
- 3 Wittmann, H.G. (1972) FEBS Symposium 27, 213-325.
- 4 Kurland, C.G., Donner, D., van Duin, J., Green, M., Lutter, L., Randall-Hazelbauer, L., Schaup, H.W. and Zeichart, H. (1972) FEBS Symposium 27, 226-236.
- 5 Ehresmann, C., Stiegler, P., Mackie, G.A., Zimmerman, R.A., Ebel, J.P. and Fellner, P. (1975) Nucleic Acids Res. 2, 265-278.
- 6 Fresco, J.R., Alberts, B.M. and Doty, P. (1960) Nature 188, 98-101.
- 7 Cox, R.A. (1965) Biochem.J. 98, 841-857.
- 8 Cotter, R.I., McPhie, P. and Gratzer, W.B. (1967) Nature 214, 864-868.
- 9 Gesteland, R.F. (1966) J.Mol.Biol. 18, 356-371.
- 10 Gavrilova, L.P., Ivanov, D.A. and Spirin, A.S. (1966) J.Mol.Biol. 16, 473-482.
- 11 Ball, L.A., Johnson, P.M. and Walker, I.O. (1973) Eur.J.Biochem. 37, 12-20.
- 12 Miall, S.H. and Walker, I.O. (1969) Biochim. Biophys.Acta 551-560.
- 13 Spencer, M.E. and Walker, I.O. (1971) Eur.J.Biochem. 19, 451-456.
- 14 Roberts, M.E. and Walker, I.O. (1970) Biochim.Biophys.Acta 199, 184-193.
- 15 Miall, S.H. and Walker, I.O. (1967) Biochim.Biophys.Acta 145, 82-95.
- 16 Waller, J.P. and Harris, J.I. (1961) Proc.Nat.Acad.Sci.U.S. 47, 18-29.
- 17 Kirby, K.S. (1965) Biochem.J. 96, 266-270.
- 18 Kasai, K. and Grunberg-Manago, M. (1967) Eur.J.Biochem. 1, 152-163.
- 19 Peacock, A.C. and Dingman, C.W. (1968) Biochemistry 7, 668-674.
- 20 Gratzer, W.B. and Richards, E.G. (1971) Biopolymers 10, 2607-2614.
- 21 Tinoco, I. (1968) J.Chim.Phys. 65, 91-97.
- 22 Schaup, H.W., Green, M. and Kurland, C.G. (1971) Molec. Gen. Genet. 112, 1-8.
- 23 Schaup, H.W. and Kurland, C.G. (1972) Molec. Gen.Genet. 350-357.
- 24 Zimmermann, R.A., Mackie, G.A., Muto, A., Garrett, R.A. Ungewickell, E., Ehresmann, C., Stiegler, P., Ebel, J.P. and Fellner, P. (1965) Nucleic Acids Res. 2, 279-302.
- 25 Arnott, S. (1970) Prog.in Biophysics and Molecular Biology, 21, 267-319.