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Conformational Changes in 16*S* Ribosomal RNA Induced by 30*S* Ribosomal Subunit Proteins from *Escherichia coli*

Abstract. *Laser light scattering has been used to evaluate conformational differences between free 16*S *RNA and several specific protein-16*S *RNA complexes. Proteins that interact strongly with the 16*S *RNA early in subunit assembly stabilize the RNA chain against unfolding in 1 m*M Mg^{2+} *and actually promote the formation of a more compact tertiary structure in 20 m*M Mg^{2+}. *A vital function of these proteins may therefore consist in altering the configuration of the RNA so that further assembly reactions can take place.*

Assembly of the 30*S* ribosomal subunit of *Escherichia coli* entails the cooperative association of 21 different proteins with a single molecule of 16*S* RNA (*1*). During this process, the ribosomal RNA attains a configuration that is much more compact than in the free state (*2–4*). Although there is little known about the mechanism of folding, sedimentation analysis of assembly intermediates suggested that major changes in RNA conformation occurred only after the association of 10 to 15 proteins with the nucleic acid chain (*2*). Our present results, however, show that folding of the 16*S* RNA begins upon the binding of proteins S4, S7, S8, and S15, either singly or in combination, in the very first stages of subunit assembly (*5*). Changes in RNA structure were followed by the technique of laser light scattering (*6*), which yields precise values of the translational diffusion constant, *D*, a sensitive indicator of variation in macromolecular size and shape.

Diffusion constants for 16*S* RNA and native 30*S* particles were determined in LM buffer (1 m*M* $MgCl_2$; see Table 1 legend) and in TMK buffer (20 m*M* $MgCl_2$; see Table 1 legend), a solvent that was originally developed to optimize subunit assembly in vitro (*1*). It is apparent from Table 1 that the 30*S* subunit exhibits a larger $D^{o}_{20,w}$ than free 16*S* RNA in both ionic environments. A similar relation exists between $D^{o}_{20,w}$ values of the 50*S* subunit and the 23*S* RNA (data not shown). Our findings provide direct evidence that ribosomal subunits are more compact than their constituent RNA's even though their mass is 50 to 60 percent greater (*7*). Indeed, the $D^{o}_{20,w}$ of a particle with the same shape and density as the 16*S* RNA, but with the molecular weight of the 30*S* subunit (*8*), would be 15 percent lower than that of the RNA alone, since D^{o} is inversely proportional to $(M)^{1/3}$ in such cases. The values obtained for the 30*S* subunit, however, are from 3 to 11 percent higher than for the 16*S* RNA. Diffusion constant measurements can thus be used to discriminate between increases in the folding of a molecule and increases in its mass.

The pronounced difference in the $D^{o}_{20,w}$ of 16*S* RNA in the two buffers can be ascribed primarily to unfolding of the nucleic acid chain at low Mg^{2+} concentrations. This process is also reflected by a decrease in the sedimentation coefficient of 16*S* RNA between 20 and 1 m*M* Mg^{2+} (*4, 9*). The conformation of the 30*S* subunit is comparatively insensitive to changes in Mg^{2+} concentration, however, supporting the general view that ribosomal proteins are essential in maintaining the compact structure of the particle (*3, 4*).

We next investigated the interaction of 16*S* RNA with 30*S* subunit proteins S4, S7, S8, and S15, all of which bind to specific and independent sites in the nucleic acid molecule (*10*). Since one or a small number of ribosomal proteins contribute little to the mass of the complexes, variations in D^{o} are dominated by changes in RNA conformation. In LM buffer, $D^{o}_{20,w}$ values of the individual ribonucleoproteins were significantly higher than that of free 16*S* RNA (Table 1). By contrast, the $D^{o}_{20,w}$ of a mixture containing S5, a protein that does not associate independently with the RNA, was identical to that of 16*S* RNA alone.

Table 1. Diffusion constants of the 16*S* RNA, protein-16*S* RNA complexes, and the 30*S* subunit. Proteins S4, S7, S8 and S15, 16*S* RNA, and 30*S* ribosomal subunits were prepared from *E. coli* MRE600 (*18*). The 16*S* RNA and 30*S* subunits were suspended in TMK buffer (50 m*M* tris-HCl, *p*H 7.6, 20 m*M* $MgCl_2$, 350 m*M* KCl, 1 m*M* dithiothreitol) and dialyzed against either TMK or LM buffer (10 m*M* tris-HCl, *p*H 7.6, 1 m*M* $MgCl_2$, 100 m*M* KCl, 1 m*M* dithiothreitol). Reconstituted complexes were formed by incubating an excess of protein with 16*S* RNA at a concentration of 500 μg/ml in TMK for 30 minutes at 40°C (*18*) and then dialyzed against the appropriate measuring buffer. Control experiments confirmed that protein bound in 20 m*M* Mg^{2+} remained associated with the RNA in 1 m*M* Mg^{2+}. Dialyzed mixtures were diluted and filtered through Millipore GS membranes to remove free protein (*19*) and particulate matter. After a brief incubation at 40°C, the samples were cooled to 20°C, and values of D_{20} were determined by laser light scattering (*6*). Results from several runs on different preparations were corrected for solvent viscosity and averaged to give $D_{20,w}$. Since $D_{20,w}$ was generally independent of sample concentration from 50 to 500 μg/ml, the averaged value was taken as $D^{o}_{20,w}$. Data for complexes that contained less than 0.75 mole of each protein per mole of RNA, or that contained degraded RNA following light scattering, were discarded. Standard deviations include errors within individual runs as well as variations from one preparation to another. The number of samples analyzed for each value is indicated in parentheses.

Sample	$10^{-7} \times D^{o}_{20,w}$ (cm²/sec)	
	LM buffer	TMK buffer
16*S* RNA	1.77 ± .01 (4)	1.96 ± .01 (3)
30*S* subunit	1.97 ± .01 (4)	2.02 ± .02 (4)
S5 plus 16*S* RNA	1.77 ± .03 (2)	
S4–16*S* RNA	1.88 ± .01 (8)	1.96 ± .01 (5)
S7–16*S* RNA	1.83 ± .01 (7)	2.02 ± .03 (2)
S8–16*S* RNA	1.95 ± .01 (5)	1.96 ± .01 (2)
S15–16*S* RNA	1.83 ± .01 (6)	2.04 ± .02 (3)
S4, S7–16*S* RNA	1.94 ± .01 (8)	2.09 ± .03 (1)
S4, S8–16*S* RNA	1.84 ± .01 (4)	2.07 ± .03 (2)
S4, S15–16*S* RNA	1.92 ± .01 (6)	2.16 ± .01 (2)
S8, S15–16*S* RNA	1.90 ± .01 (6)	2.10 ± .02 (2)
S4, S7, S15–16*S* RNA	1.89 ± .01 (7)	2.04 ± .03 (4)
S4, S7, S8, S15–16*S* RNA	2.01 ± .01 (3)	2.15 ± .03 (1)

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Each of the RNA-binding proteins must therefore stabilize the RNA in a more compact configuration than it would normally possess in LM buffer. Because S4, S7, and S15 are believed to be highly elongated both in solution and in the 30*S* subunit (*11*), they may impede Mg^{2+}-dependent unfolding of large structural domains within the 16*S* RNA. Indeed, the binding sites for S4 and S7 are known to consist of several hundred nucleotides each (*12*). The compactness of the S8–16*S* RNA complex is more surprising, however, since S8 is thought to be globular (*13*) and interacts with a relatively small segment of the RNA molecule (*12*). Diffusion constants for the single-protein complexes in TMK buffer indicate that S4 and S8 cause little or no conformational change in the RNA but that S7 and S15 each stimulate modest structural alterations under these conditions.

The diffusion constants of a number of complexes containing pairs of proteins are also presented in Table 1. The relatively high resistance of the S4, S7–16*S* RNA complex to unfolding in LM buffer may be related to the fact that S4 and S7 associate with the 5′ and 3′ ends of the 16*S* RNA, respectively (*12*). Results of electron microscopy imply that portions of both proteins approach one another closely in the "head" region of the 30*S* subunit (*14*). Their attachment might therefore mediate the stable juxtaposition of the terminal regions of the nucleic acid chain. Values of $D^{o}_{20,w}$ measured in TMK buffer indicate that all of the two-protein complexes are more tightly folded than the corresponding single-protein complexes. These data suggest that the binding of ribosomal proteins under reconstitution conditions specifically induces the 16*S* RNA to assume a more condensed structure.

A ribonucleoprotein consisting of S4, S7, S8, and S15 in association with 16*S* RNA has been derived from the 30*S* ribosomal subunit by treatment with 3*M* LiCl (*15*). The core particle is compact, with a sedimentation constant of 24*S* and dimensions approximating those of the intact 30*S* subunit (*15, 16*). We have measured the diffusion constants of analogous particles reconstituted in vitro (Table 1). A complex containing S4, S7, and S15 yielded values of $D^{o}_{20,w}$ in the range of many of the one- and two-protein complexes under both ionic conditions. Addition of S8 to the first three proteins, however, led to stabilization of the 16*S* RNA in a configuration at least as compact as the 30*S* subunit itself. This result is consistent with the ability of the core proteins to sharply reduce the accessibility of the 16*S* RNA to ribonucleases and chemical reagents (*15*), as well as with the size and morphology of the derived core particles determined by electron microscopy (*16*).

Whether or not the proteins that bind to the 16*S* RNA early in 30*S* subunit assembly stimulate structural alterations in the RNA molecule has long been open to question. Our results demonstrate that S4, S7, S8, and S15, either alone or in combination, not only prevent the 16*S* RNA from unfolding at low Mg^{2+} concentration, but actually constrain it to adopt a more compact tertiary structure under conditions which favor 30*S* subunit reconstitution. We therefore suggest that the key role of the RNA-binding proteins in ribosome assembly (*5*) is related to their capacity to alter the initial configuration of the RNA chain. Specifically, they may help to create binding sites for proteins that are joined to the particle later in the assembly sequence by cooperatively promoting the formation of new tertiary contacts.

Diffusion constants of the 16*S* RNA, the reconstituted ribonucleoprotein complexes, and the 30*S* subunit are presented as a function of molecular weight in Fig. 1. The stippled zones, drawn to include all of the data with their standard deviations, indicate that the complexes tend to become more compact as the number of proteins associated with the RNA increases. This trend does not define a unique pathway of conformational change, however, since there is no simple monotonic relationship between $D^{o}_{20,w}$ and the molecular weight of the complex, nor is there a clear-cut correlation between the extent of variation in $D^{o}_{20,w}$ and the size or position of particular protein binding sites in the 16*S* RNA (*12*). It has been suggested that binding regions for S4, S8 plus S15, and S7 delineate distinct structural domains in the 5′-terminal, middle, and 3′-terminal thirds of the 16*S* RNA, respectively, in which early assembly reactions occur with relative independence (*17*). Our findings can be understood in this context if we assume that each such domain responds autonomously, and not necessarily in the same way, to changes in Mg^{2+} or to the attachment of individual ribosomal proteins. When S4, S7, S8, and S15 are jointly associated with the 16*S* RNA, however, the various segments appear to be drawn together into a configuration characteristic of the mature 30*S* subunit.

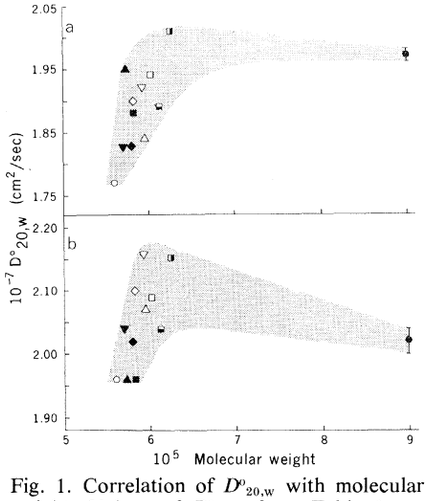

Fig. 1. Correlation of $D^{o}_{20,w}$ with molecular weight. Values of $D^{o}_{20,w}$ from Table 1 are plotted as a function of particle molecular weight. (a) Data for LM buffer. (b) Data for TMK buffer. Error bars indicate typical standard deviations for each series of measurements. Stippled zones define the limits of variation of $D^{o}_{20,w}$ for the 16*S* RNA and its complexes with one to four ribosomal proteins. Extrapolation of these zones from the complexes to the 30*S* subunit is arbitrary. Sequence molecular weights for S4, S7, S8, and S15 are 22,550 (*20*), 17,131 (*21*), 12,254 (*22*), and 10,000 (*23*), respectively. Molecular weights for 16*S* RNA and 30*S* subunits determined by physicochemical techniques were taken as 560,000 (*24*) and 900,000 (*8*), respectively. (○), 16*S* RNA; complexes of 16*S* RNA with: (■), S4; (◆), S7; (▲), S8; (▼), S15; (□), S4, S7; (△), S4, S8; (▽), S4, S15; (◇), S8, S15; (◨), S4, S7, S15; (◧), S4, S7, S8, S15; and (●), 30*S* subunit.

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Ultradian Cortisol Rhythms in Monkeys: Synchronized or Not Synchronized?

Holaday *et al.* (*1*) reported that ultradian rhythms in plasma cortisol concentration in rhesus monkeys were "highly synchronized" between animals and with the light-dark cycle. They suggested that "the rhythms may stem from the light-dark circadian cycle, since the 90-minute period is a harmonic of 24 hours." However, an examination of their analytical procedures and a reanalysis of some of their raw data (*2*) give us reasons to conclude that these ultradian rhythms are not synchronized.

First, from the raw data (*2*) we estimate that, on average, the individually detrended data records showed a standard deviation about zero of 2.2 μg of cortisol per 100 ml of venous blood. From figure 1B in (*1*) we estimate the standard deviation of the group data to be approximately 0.7 μg/100 ml. Since these estimates are in the ratio of 3.14, which is close to 2.83 (the square root of the number of records in the group average), there is a strong suggestion that the individual records are statistically independent (*3*). Had the rhythms really been synchronized the strength of the oscillations in figure 1B would have been two to three times as large.

A second reason can be found in a comparison of figure 2A with figure 2C. Since a difference between the average of the spectra (figure 2A) and the spectrum of the average (figure 2C) will be found only if there is a phase consistency in the spectral components of the individual records, the magnitude of the difference gives an estimate of the degree of phase consistency. Unfortunately, both figure 2A and figure 2C are normalized, whereas the comparison should be made on the absolute spectra. Assuming that figure 2A and figure 2C represent roughly equal absolute power, we find the spectral components of 80 to 90 minutes per cycle are virtually identical under the two computations.

Third, a visual examination, and reanalysis (*4*), of the data on the cover (*2*) indicates that the phases are sufficiently varied to have come from a random population. Table 1 shows that we detected periodicities in the range of 63 to 123 minutes in each monkey's plasma cortisol pattern, but the phases were highly variable, ranging from 0° to 300° (*5*).

Thus we conclude there is no synchronization of these ultradian cortisol rhythms either with the light-dark cycle or between monkeys. Studies by Weitzman *et al.* (6) similarly revealed no consistent phase relationships between ultradian cortisol rhythms and other similar periodicities, such as REM-nonREM (*7*) sleep-stage cycles. Thus, the beguiling regularity of the group data in figure 1B are actually the result of combining a limited number of similar frequency rhythms with essentially random phases. This conclusion obviates the necessity of searching for physiological mechanisms which could account for an improbable subharmonic entrainment with circadian oscillators via the 16th harmonic (*8*).

Table 1. Independent period spectral analyses of monkey plasma cortisol periodicities in the ultradian range.

Monkey	Period (minutes)	Amplitude (μg/100 ml)	Phase* (phase delay of first acrophase)	Time of first† acrophase (hours:minutes)
H-927	81	1.46	80°	08:18
K-113	87	1.05	99°	08:24
L-071	84	1.54	231°	08:54
L-071	123‡	3.26	158°	08:54
K-787	81‡	2.05	240°	08:54
K-742	93‡	1.53	255°	09:06
L-822	54	1.24	200°	08:30
L-822	93‡	2.24	116°	08:30
L-968	63	1.61	103°	08:18
L-968	102‡	2.60	127°	08:36
L-959	78‡	2.04	0°	08:00
L-959	108	1.68	300°	09:30

*The phase delay in degrees of the first fitted maximum from the start of the data, where 360° equals the period of the detected periodicity. †Clock time that the first fitted maximum of the detected periodicity occurred. ‡Significant at $P < .05$.

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2. Spontaneous cortisol patterns for individual monkeys were shown on the cover of the issue of *Science* in which (*1*) appeared.
3. Furthermore, a two-way analysis of variance performed on the raw data on the cover (*2*) confirmed that the time of day effect was insignificant ($P >> .05$), and that the apparent rhythm in the group data (figure 1B) was not a significant contributor to the total variance.
4. The data were digitized from the graphs on the cover (*2*), detrended, and then analyzed separately for each monkey in 3-minute intervals from 24 to 360 minutes according to a linear-nonlinear simultaneous multiple least squares regression period analysis [J. A. Rummel, J. K. Lee, F. Halberg, in *Biorhythms and Human Reproduction*, M. Ferin, F. Halberg, R. N. Richart, R. L. VandeWiele, Eds. (Wiley, New York, 1974), p. 53]. In Table 1 the determined phase for each periodicity in each monkey is expressed as the delay in occurrence of the first acrophase of the best fitted periodicity after the start of the data (with 360° being the interval between acrophases).
5. If one examines only the periodicities determined to be statistically significant ($P < .05$) contributors to variance these still have phases varying between 0° and 255°, and consequently the times of the first fitted maxima to the significant cortisol rhythmicities in each monkey were dispersed between 08:00 and 09:06 hours local time.
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