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Biophysical Chemistry 83 (2000) 73–78

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Structural stability of ribosomes subjected to RNase treatment evidenced by dielectric spectroscopy and differential scanning microcalorimetry

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Received 16 April 1999; received in revised form 14 October 1999; accepted 14 October 1999

Abstract

Previous studies from our laboratory demonstrated the existence of at least two levels of structural complexity in *E. coli* 70S ribosomes. Ribosomal RNA seems to be principally involved in the overall stability of these structures. In this paper we present an investigation of ribosomes subjected to treatment with RNase. The study is based on both differential scanning microcalorimetry and dielectric spectroscopy. In the thermograms obtained on treated ribosomes only the low temperature peak of the two typical denaturation events observed in native ribosomes, is promptly eliminated by the enzyme treatment. Dielectric spectroscopy measurements carried out on the same samples indicate an alteration of the dielectric behavior previously shown to consist of two subsequent relaxation processes. In fact, only the low frequency relaxation is affected by the treatment. The second one, observed at higher frequency, remains unaltered. The same effect on the dielectric parameters is observed if the ribosome particles are heated and then cooled prior to measurement. These results are consistent with the idea that two different structures are present within the ribosome. One is very stable and withstands both temperature and RNase treatment while the second is promptly abolished by both treatments. Data presented here strongly suggest that the RNA domains exposed to the solvent play a fundamental role in the stability of the 3-D structure of the ribosome particle.

Keywords: Ribosome; Dielectric spectroscopy; Microcalorimetry

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0301-4622/00/$ - see front matter
PII: S0301-4622(99)00124-6

1. Introduction

Ribosomes play a key role in the process of protein biosynthesis [1–3]. An essential contribution to their biological function is given by the interaction between protein and RNA moieties. In previous works we evidenced by dielectric spectroscopy, fluorescence and microcalorimetric studies conducted on *E. coli* ribosomes, that different structural features exist in the individual ribosomal subunits [4–6]. Abundant literature data establish that various parameters such as ion strength, pH and temperature, play an important role in the stability and functional activity of the ribosome [1–3]. We recently investigated the melting process of this particle by differential scanning microcalorimetry (DSC). Our data show that various degrees of molecular complexity exist as revealed by the thermal denaturation profile obtained from 70S particles. The melting pattern is highly structured showing two well-defined transitions occurring at different temperatures. On the contrary unstructured denaturation profiles were observed on naked rRNA [6].

The very significant role of ribosomal RNA in the functional activity of the ribosome, renders this macromolecule a very interesting subject of investigation. However, while the secondary structures of several large RNA species have been deduced in detail, the tertiary structure remains poorly understood. Melting experiments on rRNA fragments indicate that increasing temperature disrupts the tertiary interactions before the secondary ones [7]. In the light of these results it can be hypothesized that the rRNA 3-D structure is cooperatively stabilized by the interaction with ribosomal proteins. The lower temperature transition observed in the thermal unfolding of 70S particles, should be attributed to disruption of tertiary structures. To elucidate this point, ribosomes were subjected to mild RNase digestion or subdenaturing temperature. In fact, it could be expected that moderate treatment with RNase alters the overall 3-D structure of the particle and, as a consequence, the low temperature denaturation peak would be affected. In addition to microcalorimetry, dielectric measurements on the same particles were also carried out. These measurements, as a matter of fact, complement the microcalorimetry data since the dielectric behavior of the ribosome essentially depends on the RNA sequences exposed to the solvent and on the ribosomal inner 'kernel' [4,8,9]. Results presented here strongly suggest the existence of a labile structure that is promptly altered in an irreversible manner both by RNase treatment and subdenaturing temperatures.

2. Materials and methods

2.1. Ribosome preparation

Ribosomes were prepared and RNase treatment was perfomed as previously reported [10]. Briefly, 70S ribosomes in buffer A (10 mM $MgCl_2$, 60 mM NH_4Cl, 10 mM trisHCl pH 7.6, 6 mM β-mercaptoethanol) were treated with increasing amounts of RNase (1.2×10^3 A_{260} units of ribosomes, 1, 5 and 15×10^{-3} units of RNase T_1 from Sigma). The treatment was carried out in a volume of 4 ml for 10 min at 37°C. The reaction was stopped by addition of the appropriate amount of Rnasin (Sigma). Treated particles were recovered by centrifugation through a 10% sucrose cushion, resuspended in buffer A and dialyzed against the same buffer. Frozen aliquots were kept at −80°C. Prior to each measurement, all samples were dialyzed against measuring buffer B (0.8 mM $MgCl_2$, 1 mM trisHCl pH 7.5). This buffer was previously adopted both in dielectric [8] and calorimetric measurements [6]. This relatively low ion strength allows accurate permittivity measurements and prevents ribosomal aggregation.

2.2. Permittivity measurements

Permittivity (ε') and dielectric loss (ε'') were measured by means of a computer controlled Hewlett Packard impedance analyzer Mod. 4194A in the 0.1–100 MHz range. The measuring cell, previously described [11], is a section of a cylindrical waveguide which can be partially filled with the sample solution. The system behaves as a waveguide excited far beyond its cut-off fre-

quency mode and therefore only the stray-field of the coaxial line–waveguide transition is used in the measurement. Cell constants were determined by an interpolation method based on measurements with electrolyte solutions of known conductivities similar to those of the samples under test [12]. The errors on ε' and ε'' are within 1%. The measuring cell was thermally controlled within 0.1°C. The concentration of ribosomes was 7 mg/ml in measuring buffer B.

2.3. DSC measurements

Differential scanning calorimetry experiments were performed in a microcalorimeter 11 Setaram (Lyon, France). The scan rate was 0.5°C/min, starting from 25 to 100°C. The total mass of the measured sample was 850 mg. The ribosome concentration was 5 mg/ml in measuring buffer B. The weights of reference and sample cell were matched. An excess power vs. temperature scan for the ribosome transitions was obtained subtracting scan of the buffer vs. buffer from the power input scan of the ribosome solution, to minimize systematic differences between the measuring cells. This quantity referred to 1 mg of particles in the sample gives the excess heat capacity $C_{p_{exc}}$.

3. Results

Ribosome particles treated with different doses of RNase T_1 (b, c and d; see Section 2.) were subjected to differential scanning microcalorimetry and dielectric spectroscopy measurements. Fig. 1 shows four different thermograms obtained on these particles. Untreated control ribosomes exhibit the pattern observed previously ([6] and related references therein). However, it is evident that the low temperature peak is reduced already at low concentration of RNase to disappear completely at the highest dose. On the contrary the high temperature peak is apparently unaffected by the nucleolytic attack.

Dielectric measurements data are shown in Figs. 2 and 3. Two typical dispersion curves obtained on native 70S particles and on ribosomes subjected to the highest dose of RNase are

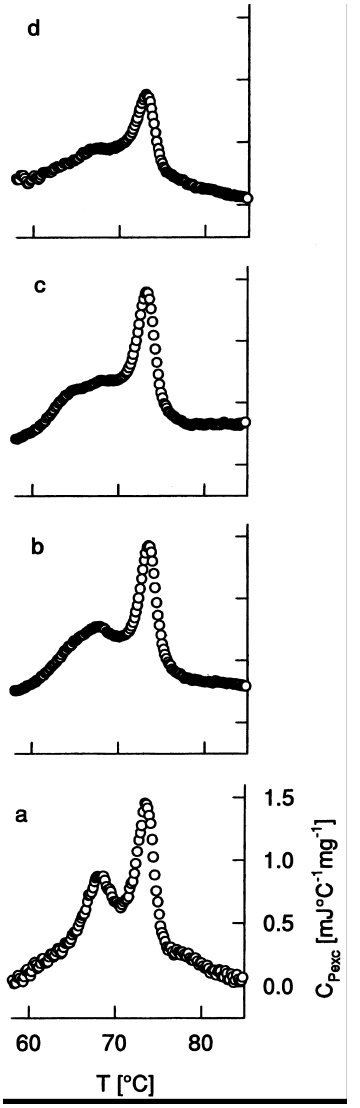

Fig. 1. C_p excess thermal profile of native and RNase treated 70S ribosomal particles. The curve was calculated by the subtraction of a baseline from the recorder tracing as described in Materials and methods. From bottom to top the effect of 0 to maximum RNase dose is reported. Measuring buffer: 0.8 mM $MgCl_2$, 1 mM trisHCl pH 7.5.

observed. The dielectric behavior of control and treated particles is substantially similar. However, an experimentally significant difference in dielectric response is present in the low frequency region. The best fit was elaborated in both cases considering two subsequent relaxation processes

that are the sum of a Debye followed by a Cole–Cole relaxation characterized by the frequencies f_1^*, f_2^* and dielectric increments $\Delta\varepsilon_1$, $\Delta\varepsilon_2$. An exhaustive review of the dielectric properties of biological materials is found for example in [13]. A critical analysis of the experimental data allowed the choice of these best fit procedures. In Table 1 we summarize the parameters of the dielectric dispersions referred to the native and to the high dose samples. It is evident that the nucleolytic treatment has effect mainly on the first relaxation occurring at low frequency, while the second one is essentially unaffected. This effect was observed at each concentration of RNase treatment, however a direct correlation dose/effect was not evident. Table 1 also reports the best fit of the dielectric measurements performed on samples heated to 70°C, which is the intermediate temperature between the two denaturation peaks, and then cooled to the measuring temperature. The effect of temperature on the dielectric data is analogous to the one observed in the particles treated with RNase. To ascertain the maintenance of the entire 70S structure even after RNase treatment at high dose, we checked the sedimentation profile of untreated control and RNase treated particles. These results as well as the protein analysis by 2-D polyacrylamide gel electrophoresis showed that the 70S ribosome structure remains substantially unaltered even after high nuclease treatment (data not shown).

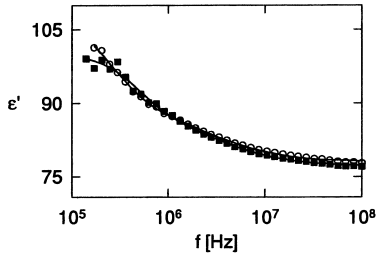

Fig. 2. Permittivity ε' vs. frequency of native 70S (○) and particles treated with the highest dose of RNase (■). The continuous lines are the result of a best fit based on a sum of Debye and Cole–Cole relaxations. Measuring buffer: 0.8 mM $MgCl_2$, 1 mM trisHCl pH 7.5

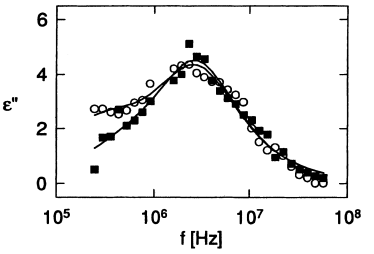

Fig. 3. Dielectric loss ε'' vs. frequency of native 70S (○) and particles treated with the highest dose of RNase (■). The continuous lines are the result of a best fit based on a sum of Debye and Cole–Cole relaxations. Measuring buffer: 0.8 mM $MgCl_2$, 1 mM trisHCl pH 7.5

4. Discussion

Ribosomes subjected to thermal degradation produce two different melting peaks [6]. The first one is lost if the temperature is raised and stopped at an intermediate value between the two phenomena. In fact, a cycle of cooling and heating of the sample produces only the second denaturation peak observable at higher temperature. This strongly suggests the existence of two distinctive structures that are thermally degraded independently and with different energies. It is plausible that these denaturation peaks are the results of different spatial arrangements of the RNA complexed to the proteins. As a matter of fact, on free rRNA in solution we observed the typical behavior of nucleic acids that show an ample and reversible denaturation profile [6]. These data clearly indicate that proteins are able to influence the secondary and/or tertiary structure of the rRNA and that the nucleic acid/protein interactions become the key to describe the ribosomal

Table 1
Relaxation frequencies and dielectric increments of the two dispersions exhibited by native and high dose RNase sample (15×10^{-3} μg/μl). The table also reports the dielectric parameters referred to ribosomes subjected to subdenaturing temperature

Sample	f_1^* (kHz)	$\Delta\varepsilon_1$	f_2^* (MHz)	$\Delta\varepsilon_2$
Native 70S	260 ± 20	19 ± 1	2.8 ± 0.6	10 ± 1
RNase (high dose)	490 ± 60	13 ± 3	3.1 ± 0.9	9 ± 2
Heated 70S	420 ± 40	15 ± 2	2.9 ± 0.9	11 ± 3

particle. Studies done by dielectric spectroscopy on native 70S demonstrated the presence of two different relaxations. The first one occurs in the range of approximately 200 to 300 kHz and is attributable to the RNA exposed to the solvent. The second is observed in the MHz region and was ascribed essentially to a more stable 'core' constituted by RNA and proteins [4,5]. With respect to this we made the hypothesis that the two different relaxation processes could be associated with the denaturation peaks shown by calorimetric measurements. The lowest temperature transition primarily involves disruption of tertiary interactions while the second denaturation peak could essentially represent the melting of the secondary ones. In other words we suggest, on the basis of thermal unfolding studies conducted on ribozymes [7], that the tertiary organization of the molecule yields to heat denaturation before the secondary one.

An effective manner to investigate the nature of the structure(s) involved in these thermal degradation phenomena, is to subject ribosomes to moderate treatment with RNase. This enzyme hydrolyzes the 5′,3′-phosphodiester bond present in the RNA backbone, and therefore it could be expected that the nucleolytic treatment would perturb the overall 3-D configuration of the ribosome particle. The complete disappearance of the first melting peak, and the persistence of the high temperature peak, is consistent with the hypothesis previously made. Moreover, dielectric results show that the action of the nuclease as well as subdenaturing temperature is exerted on the low frequency dispersion. This one is attributable to the oscillating counterions at the RNA/solvent interface. The effect on the first dispersion results in a shift of the relaxation frequency towards higher frequencies and a reduction of dielectric increment. This implies, according to the 'counterion Mandel model' [14], a decrease of RNA exposed to the solvent. The application of this model to calculate the average length of the portions of the exposed RNA in the native 70S, gives a value of approximately 86 nm. As already discussed this is very similar to the maximum circumference of the ribosome grossly assumed as quasi spherical [9]. This parameter, also known as the persistence length [15], if measured on free rRNA or DNA in solution, results considerably lower [8,14,16]. Therefore the interaction of the rRNA with r-proteins determines a more elongated configuration of the nucleic acid, that in solution would assume a more compact coil structure. In the RNase treated particles, by the same calculation, we obtained a value of approximately 65 nm for the exposed RNA which is approximately 30% lower than the previous length. This result strongly suggests the disruption of a 3-D configuration as also evidenced by the differential microcalorimetry experiments. Finally, the persistence of the second dispersion as well as the high melting event supports the idea of the presence of a very stable secondary structure in a more internal domain of the organelle.

It is difficult to draw conclusions about the possible functional significance of these structures. However, treatment of ribosomes with kethoxal [17] or mild digestion with RNase T_1 [10] both interacting specifically with guanines, severely inhibit subunit association and protein synthesis, quantisized by polyphenylanlanine formation. In any case the data presented here discriminate between different structural configurations whose functional significance are yet to be fully evaluated.

Acknowledgements

This work was supported by the Istituto Nazionale di Fisica della Materia (grants to A.B. and G.O.). We thank K.H. Nierhaus for stimulating discussion and encouragement.

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