Biophysical study of a molecular intermediate preceding collapse of tight couple and Kaltschmidt–Wittmann ribosomes

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Abstract In previous works we evidenced, by different biophysical approaches, two levels of structural organization in *Escherichia coli* ribosomal particles. Thermal treatment up to a defined and non-denaturing temperature causes demolition of only one level of structural complexity. By consequence the ribosomal particle exists in an intermediate state between the native form and the completely collapsed one. In this communication we report on a structural comparison of this intermediate state in Kaltschmidt–Wittmann (LC) and 'tight couple' (TC) ribosomes. Three different biophysical approaches were adopted: dielectric spectroscopy, fluorescence and light scattering. Differential responses to thermal treatment are evidenced in the two ribosomal species. In particular TC show a more compact structure and the overall particle population is more homogeneous than LC in the native state. On the other hand, LC particles after thermal treatment undergo major alterations of geometry and/or phenomena of supra-particle aggregation.

Key words: Ribosome; Dielectric spectroscopy; Fluorescence; Light scattering

1. Introduction

The ribosome of *Escherichia coli* is a field of intensive research [1–5]. During recent years we have investigated the structural properties of this particle by different biophysical strategies such as dielectric spectroscopy, fluorescence and microcalorimetry as recently reviewed [6]. The typical dielectric behavior of the ribosome consists of two subsequent relaxations in the radio-frequency region. These two dielectric dispersions were related in a phenomenological manner to two typical denaturation peaks observed in microcalorimetric experiments. In particular, the first dielectric dispersion was associated with the low temperature peak and the second dispersion with a more stable structure present in the ribosomal particle. In a recent paper we presented a comparative study of the structural features of 'classical' Kaltschmidt–Wittmann ribosomes, also known as loose couples (LC), and ribosomes isolated according to the 'tight couple' (TC) protocol [7]. We applied dielectric spectroscopy and differential scanning calorimetry to probe the three-dimensional organization of both particle species. TC show the same microcalorimetric behavior as observed in LC producing two irreversible denaturation peaks that occur approximately at 70 and 73°C [8,9]. LC and TC show two subsequent relaxation processes in the radio-frequency range [10]. The first one is due to counter-ion movement along segments of ribosomal RNA exposed to solvent. The high frequency relaxation was attributed to the complex of protein–RNA [11]. The dielectric behavior of TC exhibits differences only in the first dispersion and this was interpreted as a reduced exposure of RNA to the solvent. This is coherent with the definition of 'tight couple' [7]. In the experiments reported in the present work we exposed the ribosomes to a gradual increase of temperature up to a subdenaturing level. The actual temperature to which particles were exposed was the same one where the first thermal transition occurs as observed by microcalorimetry [8]. The aim of these experiments was to explore a ribosomal configuration preceding complete particle collapse. To this end we chose the combination of three powerful biophysical techniques: dynamic light scattering, dielectric spectroscopy and fluorescence. Results evidence significant differences in the behavior of the two particle species after exposure to non-denaturing temperatures.

2. Materials and methods

Twice-NH_4Cl-washed ribosomes (LC) were prepared as previously reported [12] and TC as in [13]. Prior to each biophysical measurement both ribosome species were dialyzed against measuring buffer (0.8 mM $MgCl_2$, 3 mM KCl, 1 mM Tris–HCl pH 7.5). The choice of this buffer is imposed by the dielectric spectroscopy measurements that must be performed at as low ionic strength as possible. Validity of measurements in this buffer was checked by repeating the light scattering experiments in a classical 10 mM Mg^{2+} buffer with no significant change. Integrity of ribosomal particles in the measuring buffer was also monitored by sucrose density gradients [8].

Thermal treatment was performed as follows: TC and LC ribosomes suspended at the final measuring concentration were incubated in a water bath at the nominal temperature of 70°C for up to 4 min. After 2 and 4 min samples were withdrawn and their actual temperatures were 67 and 69°C, respectively. This latter value roughly corresponds to the first thermal transition previously reported [8]. After every treatment samples were allowed to reach the measuring temperature of 25°C.

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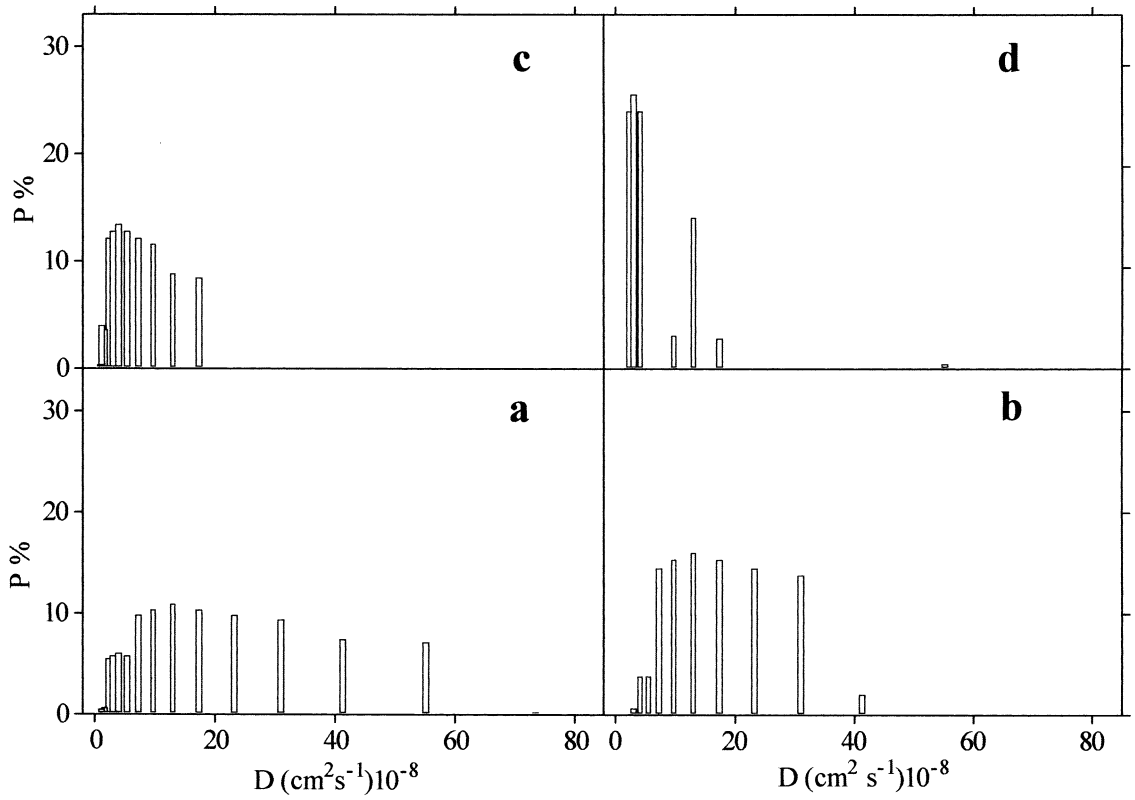

Fig. 1. Distribution of ribosomal particles of translational diffusion coefficient. a,b: Native LC and TC, respectively. c,d: Effect of the thermal treatment (4 min at 70°C).

2.1. *Physical measurements: light scattering, dielectric spectroscopy and fluorescence*

Dynamic light scattering was performed at a scattering angle of 90° by a Brookhaven digital correlator set up BI9000AT equipped with 10 mW He–Ne laser (λ 633 nm). Ribosome concentration was 1.8×10^{-3} mg/ml corresponding to about 10^{13} particles/ml. This concentration was selected to limit interactions and multiple scattering.

Permittivity of particle solutions was measured in the interval 10^5–10^8 Hz by a computer-controlled impedance analyzer (HP 4194 A). Details on sample cell and measuring methods are reported elsewhere [14]. Ribosome concentration in the measurements was 10 mg/ml.

Fluorescence experiments were performed by a standard static fluorescence analyzer (Perkin Elmer LS 50 Luminescence Spectrometer) varying the ratio ribosome/fluorophore up to the saturation of the binding sites. This was attained adding a solution of ethidium bromide (EthBr) at the fixed concentration of 5×10^{-7} M. This value was chosen to keep the ribosome concentration as low as possible to minimize particle/particle interactions as previously reported; maximum ribosome concentration was 0.1 mg/ml [15]. Spectra were recorded with an excitation wavelength of 510 nm, which is the isosbestic point of EthBr, and emission was monitored at 600 nm.

3. Results

The light scattering measurements allowed the estimation of the translational diffusion coefficient obtained from the experimental autocorrelation function of the emission intensity. Experimental data analysis followed a standard procedure [16]. Discrete populations of native ribosomes are distributed as a function of the diffusion coefficient (D). Fig. 1a,b shows this distribution for LC and TC respectively. According to the law of Stokes–Einstein an oblate ellipsoid with semi-axes of $10\times15\times15$ nm has a value $D=19\times10^{-8}$ cm^2 s^{-1}. The ribosome of *E. coli* may be roughly assimilated to an oblate ellipsoid of this size. Considering the extreme geometrical complexity of the ribosome and its size variability, we can confidently position the single native 70S particle in an interval of D ranging from 15 to 25×10^{-8} cm^2 s^{-1}. The correspondence between D and the ribosome dimension is directly

Table 1
Dielectric parameters of the two dispersions observed in native LC and TC ribosomes and after thermal treatment

	$\Delta\varepsilon_1$	f_1 (10^5 Hz)	$\Delta\varepsilon_2$	f_2 (10^6 Hz)	α	b (nm)
70S LC						
Native	8 ± 1	5.1 ± 0.4	13 ± 2	3.4 ± 0.6	0.18 ± 0.04	73 ± 2
2 min treatment	11 ± 1	4.2 ± 0.4	16 ± 2	2.8 ± 0.5	0.22 ± 0.03	80 ± 3
4 min treatment	19 ± 2	2.5 ± 0.3	26 ± 3	1.3 ± 0.3	0.30 ± 0.03	104 ± 5
70S TC						
Native	5 ± 2	7.1 ± 0.6	16 ± 2	2.2 ± 0.6	0.29 ± 0.04	62 ± 2
2 min treatment	5 ± 2	7.2 ± 0.7	18 ± 3	2.0 ± 0.6	0.28 ± 0.04	62 ± 3
4 min treatment	15 ± 2	5.8 ± 0.3	19 ± 4	1.8 ± 0.6	0.24 ± 0.04	69 ± 2

$\Delta\varepsilon_1$, f_1: dielectric increment and relaxation frequency of the first dispersion; $\Delta\varepsilon_2$, f_2: dielectric increment and relaxation frequency of the second dispersion; α: Cole–Cole parameter. The last column reports the estimate of the subunit length b.

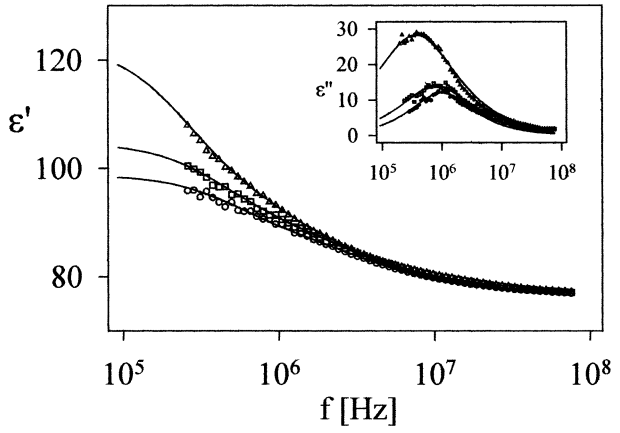

Fig. 2. Permittivity ε' versus frequency of 70S LC ribosomes. In the inset dielectric loss ε'' is reported. (○) Native particles; (□) thermal treatment for 2 min; (△) thermal treatment for 4 min. The continuous lines are the result of a best fit based on a sum of Debye and Cole–Cole relaxations.

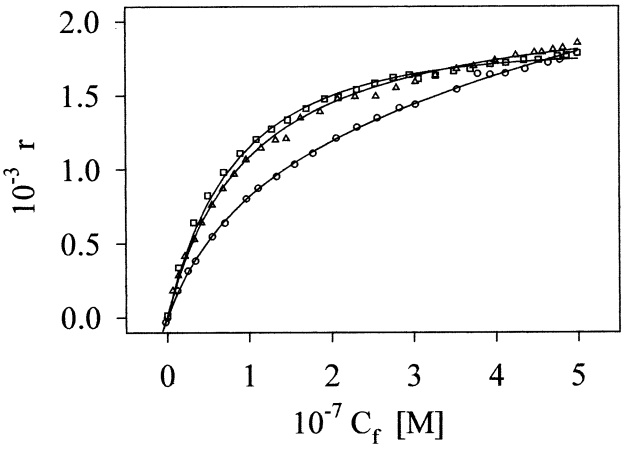

Fig. 4. Fluorescence measurements on LC particles. Binding isotherms: (○) native particles; (□) thermal treatment for 2 min; (△) thermal treatment for 4 min. The continuous lines are the result of a best fit based on the hypothesis of two different bindings fluorophore/ribosome (see text for details).

valid for native particles. Following the thermal treatment, this correspondence becomes very critical because of conformational alterations.

The distribution of the same particle populations treated for 4 min at 70°C is reported in Fig. 1c,d. After this treatment LC particles shifted to lower values of D, denoting strong phenomena of variation of geometry and possible processes of aggregation. On the other hand, TC particles are redistributed in two distinctive populations. The one with higher D may still indicate 70S monomers.

Figs. 2 and 3 report the dielectric behavior in the frequency range 0.1–10^2 MHz of LC and TC ribosomes, respectively. The curves are derived from samples thermally treated for 2 and 4 min. All experimental data were fitted as previously reported considering the curves as an overlap of a Debye followed by a Cole–Cole relaxation [10]. The first dispersion is related to the part of the RNA moiety exposed to the solvent. Counter-ion oscillations along segments of rRNA are involved in this phenomenon according to the Mandel model [17]. The second relaxation is plausibly due to r-protein/rRNA interactions that contribute to the formation of a supramolecular structure of higher complexity [11]. The values of Cole–Cole best fits are reported in Table 1. It is evident that TC are more resistant to thermal treatment. As matter of fact almost no variation seems to occur after 2 min of thermal exposure; by contrast after the same time of treatment LC particles show a marked alteration of the dielectric response.

The intensity of fluorescence emission was measured as a function of the ratio of the molar concentration ribosome/fluorophore. The absence of a defined isosbestic point denotes the existence of more than one type of binding: EthBr may establish different interactions with RNA such as van der Waals and/or electrostatic. Figs. 4 and 5 report the binding isotherms elaborated from the experimental data. The isotherms were fitted using the following equation:

$$r = \sum_{1}^{2} i \frac{N_i K_i C_f}{C_f K_i + 1}$$

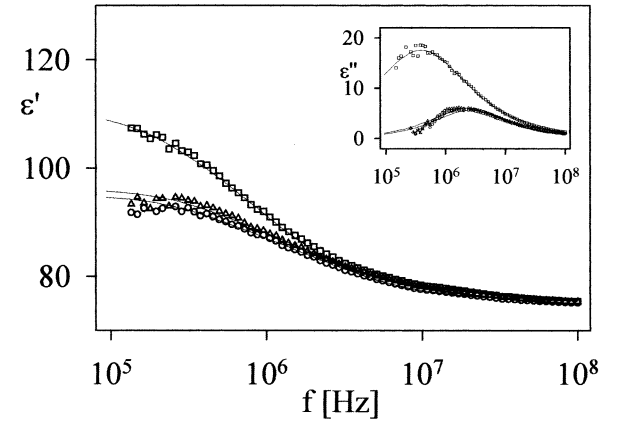

Fig. 3. Permittivity ε' versus frequency of 70S TC ribosomes. In the inset dielectric loss ε'' is reported. (○) Native particles; (□) thermal treatment for 2 min; (△) thermal treatment for 4 min. The continuous lines are the result of a best fit based on a sum of Debye and Cole–Cole relaxations.

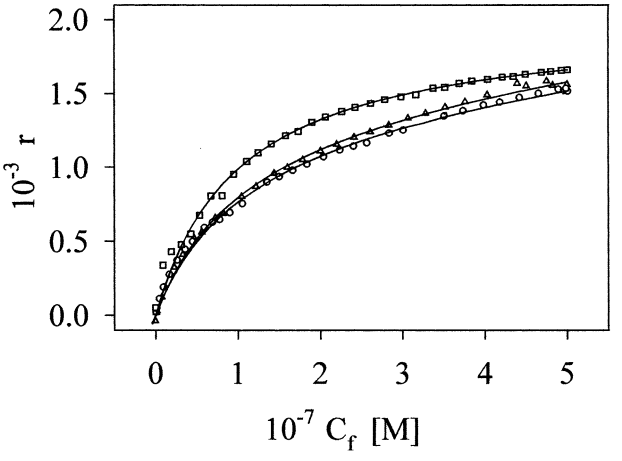

Fig. 5. Fluorescence measurements on TC particles. Binding isotherms: (○) native particles; (□) thermal treatment for 2 min; (△) thermal treatment for 4 min. The continuous lines are the result of a best fit based on the hypothesis of two different bindings fluorophore/ribosome (see text for details).

Table 2
Number of sites excluded per 1 mole of bound EthBr and association constants of the strong (N_1, K_1) and weak (N_2, K_2) binding for native and thermally treated ribosomes

	N_1 (10^{-3})	K_1 (10^7 M^{-1})	N_2 (10^{-3})	K_2 (10^5 M^{-1})
70S LC				
Native	1.65 ± 0.05	1.00 ± 0.05	4.94 ± 0.08	5.00 ± 0.06
2 min treatment	2.50 ± 0.08	0.80 ± 0.10	3.34 ± 0.08	4.90 ± 0.10
4 min treatment	2.60 ± 0.10	0.80 ± 0.10	3.30 ± 0.20	5.00 ± 0.20
70 TC				
Native	1.59 ± 0.05	1.00 ± 0.05	5.48 ± 0.06	5.00 ± 0.07
2 min treatment	1.85 ± 0.09	0.80 ± 0.09	4.20 ± 0.08	5.00 ± 0.10
4 min treatment	2.20 ± 0.10	0.80 ± 0.10	3.10 ± 0.20	5.00 ± 0.20

where r is the concentration of bound EthBr molecules per nucleotide, C_f is the molar concentration of free EthBr, while K_i and N_i, the association constant and the number of sites excluded per 1 mole of bound EthBr, respectively, are the free parameters. This is a standard method for the best fit procedure [18,19] and results are summarized in Table 2. Subsequent experiments, where the ion strength was increased, were performed to elucidate the nature of the weak binding site. The increase of ion strength up to 1 mM leads to the disappearance of the second weak binding which accounts for its electrostatic nature.

4. Discussion

Light scattering data show that the distribution of native TC is found over a relatively narrow range of diffusion coefficients D and a relevant fraction accounts for single 70S ribosomes. Aggregates with a lower D value and smaller particles (presumably separated subunits) are also observed. Native LC, on the other hand, are distributed over a wider range of diffusion coefficients, which indicates an initial inhomogeneity in the ribosome population. In Section 3 we showed that a 70S monomer is found in an interval of D ranging from 15 to 25×10^{-8} cm^2 s^{-1}. Consequently it is evident that about 30% of TC and 20% LC behave as bona fide 70S.

The picture becomes dramatically different after the thermal treatment. TC form two major groups of particles, the first being characterized by very low values of D. The second, on the other hand, remains in a range of D slightly shifted towards a lower diffusion coefficient that characterizes native TC 70S. We hypothesize that this population is found in an intermediate state strongly resembling the previously discussed *kernel* [5,20]. LC, on the other hand, are concentrated in the low area of diffusion coefficients, and it is impossible to identify a discrete population as in the previous case. This strongly suggests that no particles exist with structural features attributable to the ribosome monomer. A more quantitative analysis cannot be performed in this case because thermal treatment promotes an overall particle rearrangement that involves a form factor whose estimation is impossible.

Indications obtained by light scattering measurements fit very well with the results of dielectric spectroscopy. In LC particles a decrease of the first dielectric relaxation frequency was observed already after 2 min of treatment. In TC, after the same thermal treatment, a decrease of frequency relaxation can hardly be detected. At 4 min of treatment the effect on frequency is much less evident than in LC. As previously reported [10] the first dielectric relaxation can be attributed, according to the Mandel model [17], to counter-ion fluctuations along the surface of the rRNA exposed to the solvent. Application of the model also allows the calculation of the so-called subunit length (reported in Table 1) which is indicative of the RNA traits exposed to the solvent. In LC the subunit length increased from about 70 to 100 nm as a consequence of the exposure to increasing temperature. The same parameter varies in TC from about 60 to 70 nm. Notably, the maximum 'loosening' of TC particles in terms of subunit length after long exposure to higher temperature coincides with the initial state of native LC. Therefore TC appear to have a more compact and 'tight' structure also by dielectric spectroscopy. This is confirmed by the result obtained on the second relaxation. Frequency and dielectric increment are significantly altered in LC, which is suggestive of a partial 'loosening' of the protein/RNA complex. In TC particles, by contrast, no relevant variation is monitored. The constant values of the parameters of the second dielectric dispersion further corroborate the stability of the *kernel*.

Fluorescence data are also in extremely good agreement with the observations reported so far. Results in Table 2 show that the second weak binding of EthBr is essentially the same for both ribosome particles. Experimental data obtained as a function of an increase of ion strength clarified the electrostatic nature of the weak binding. Therefore we assume that this binding is established with charges essentially located on r-proteins even though interactions with the phosphate backbone of the rRNA cannot be ruled out. The strong interaction, on the contrary, occurs mainly with the rRNA traits in the form of double helix. After the treatment the overall number of binding sites increases consistently with a higher exposure of rRNA to the solvent. However, this difference is more pronounced in LC rather than TC particles. In particular it is evident that 2 min treatment has a strong effect on LC, while TC do not show the same sensitivity. This is a further indication that LC are vulnerable to the thermal treatment that determines a larger extent of molecular unfolding.

Results obtained by the three different biophysical techniques applied in this investigation clearly demonstrate that the structure of TC ribosomes is more compact. This feature is reflected in a significantly higher resistance to thermal treatment. The temperature in our treatments, however, never reached the level where ribosome become completely denatured [8]. We suggest that the higher stability of TC is due to intrinsic and specific features of this particle. Previous data from our laboratory showed that LC expose longer RNA traits to the solvent as compared with TC [7]. A comparison between the two native particle species revealed different constitutional aspects that are further evidenced when the two

types of ribosomes are treated at sub-denaturing temperatures. Increasing temperature causes a structural rearrangement that occurs more easily in LC than in TC. An intermediate state is reached by both species, but also in this phase differences persist between the two particles. The elaboration of the dielectric spectroscopy measurements according to the Mandel model links these differences to the higher exposure of RNA. In addition it strongly suggests that the *kernel* in TC remains stable even after maximum thermal treatment; in contrast this structure shows an initial degradation in LC particles. The data conclusively demonstrate that the higher functional activity monitored in TC ribosomes in vitro, as compared to LC ribosomes, is associated with intrinsic structural differences characterized by a more compact core and shorter RNA traits exposed to the solvent.

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