ELSEVIER

Biophysical Chemistry 75 (1998) 97–103

Biophysical Chemistry

Differential stability of *E. coli* ribosomal particles and free RNA towards thermal degradation studied by microcalorimetry

Adalberto Bonincontro[a], Stefania Cinelli[b], Marco Mengoni[c], Giuseppe Onori[b], Gianfranco Risuleo[c,*], Aldo Santucci[b]

[a] *INFM-Dipartimento di Fisica, Università di Roma 'La Sapienza', Roma, Italy*
[b] *INFM-Dipartimento di Fisica, Università di Perugia, Perugia, Italy*
[c] *Dipartimento di Genetica e Biologia, Molecolare; Università 'La Sapienza', Piazzale Aldo Moro 5, 00185 Roma, Italy*

Received 29 April 1998; received in revised form 28 July 1998; accepted 10 August 1998

Abstract

We investigated the thermal degradation of *E. coli* ribosomes by differential scanning microcalorimetry. The 70S particles show two distinctive and irreversible peaks upon thermal degradation. Free rRNA in solution produces, on the contrary, an unstructured denaturation profile. The thermal analysis of 50S particles shows a profile substantially identical to that observed in 70S, while 30S particles produce an unstructured denaturation pattern. Therefore the thermal behavior of the 70S particle is essentially attributable to the denaturation of the 50S subunit. Our data validate previous observations that the 50S has a more rigid structure as compared to 30S, which behaves as a 'floppy' particle. In addition our data suggest that protein/RNA interactions play a significant role to stabilize three-dimensional structures of the ribosome. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Microcalorimetry; Melting; Ribosomes; RNA

1. Introduction

The ribosomal particle is essential in the process of protein biosynthesis both in prokaryotic and eukaryotic cells. The ribosomes of the bacterium *E. coli*, in particular, have been intensively studied during the last 30 years [1–3]. A large knowledge exists on the genetics, molecular biology and biophysics of these bacterial organelles.

During these last years we studied the chemico-physical properties of the prokaryotic ribosomal particles of *E. coli* based on a variety of biophysical strategies such as dielectric spectroscopy and fluorescence measurements. We have shown by fluorescence studies, for instance, that a

* Corresponding author. Tel.: +39 6 49912234; fax: +39 6 4440812; e-mail: risuleo@axcasp.caspur.it

0301-4622/98/$ - see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII S0301-4622(98)00197-5

conformational rearrangement of the ribosomal nucleic acid occurs at low Mg^{2+}. This is suggestive of a larger exposure to aqueous solvent of the RNA moiety, with respect to the protein moiety [4,5]. We have further deepened our investigations on the separated ribosomal subunits and shown that they have a different conformational. structure, i.e. the 30S is floppy while the 50S is more rigid [5]. These investigations were extended adopting a differential scanning microcalorimetric (DSC) approach which allows direct measurement of the energy change upon disruption of the ribosome structure with increasing temperature. There has been a growing interest in calorimetry of biological systems during the recent years due, in part, to considerable improvements in instrumentation [6]. Microcalorimetry has been adopted to study the thermal behavior of whole prokaryotic cells and its organelles [7] as well as eukaryotic 5S ribosomal RNA from wheat germ [8]. DSC provides in fact information on enthalpy, on temperature and heat capacity changes that accompany conformational transitions of biomolecules in solution. These studies have been recently reviewed (see for instance Draper [9]).

In this paper we present a microcalorimetric study of *E. coli* ribosomes and separated subunits. We obtained evidence that the two subunits have a significantly different behavior with respect to thermal degradation as reconstructed from the melting profiles. Also, results presented here are in agreement with previous data that the 50S subunit is more stable and rigid than the 30S. Our data moreover corroborate the idea that three-dimensional structures of rRNA are stabilized by the interaction with ribosomal proteins.

2. Materials and methods

Standard ribosome (*E. coli*, strain MRE600) preparations [10], as modified in Gualerzi et al. [11], were washed twice with 1 M NH_4Cl. Subunits were prepared by zonal centrifugation [11]. Ribosomal samples were exhaustively dialyzed vs. buffer (0.8 mM $MgCl_2$; 1 mM Tris–HCl pH 7.4) prior to calorimetric experiments. Final concentration was 5 mg/ml and the same buffer was used in each microcalorimetric measurement. The integrity of ribosomal particles and subunits in the measuring buffer was monitored by sucrose density gradients.

Ribosomal RNA and proteins were prepared by the acetic acid procedure. RNA was resuspended in H_2O to a final concentration of 3.3 mg/ml. Proteins were lyophilized and, after exhaustive dialysis against decreasing concentrations of acetic acid (50%, 10%, 0.5%), resuspended in 0.5% acetic acid to a final concentration of 1.7 mg/ml. The chosen rRNA and protein concentrations represent the respective amounts within the whole ribosomal particle.

Scanning calorimetry experiments were performed in a differential scanning microcalorimeter 11 Setaram (Lyon, France). The scan rate was 0.5°C/min, starting from 25 to 100°C. The mass of the measured sample was 850 mg. The weights of reference and sample cell were matched. An excess power vs. temperature scan for the ribosome transitions was obtained by subtracting the 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 which referred to 1 mg of particles in the sample gives the excess heat capacity, $C_{p_{exc}}$.

3. Results

3.1. Thermal behavior of 70S ribosomal particles

A typical thermal scan of *E. coli* 70S ribosomes is shown in Fig. 1A. It was previously reported that ribosomes aggregate at relatively high temperature [12]. However, at the low concentration of Mg^{2+} present in our buffer and within the range of our experimental temperature, aggregation of the ribosomal particles is essentially prevented [13]. The thermogram obtained from 70S, shows that these particles melt in two thermal transitions occurring at 67.8 and 73.7°C. Evidence of a small positive increase of heat capacity of the solutions upon denaturation is also present. Reversibility of the observed transitions was tested. To this aim DSC measurements of control samples were recorded up to a defined temperature and the sample was cooled to the initial tempera-

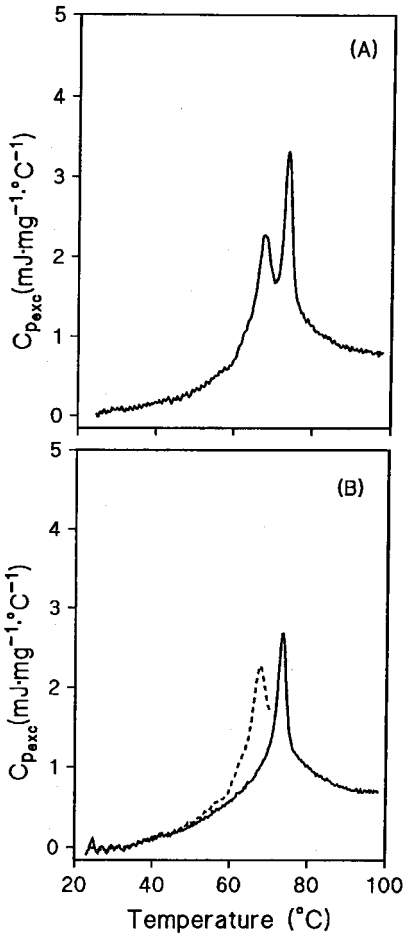

Fig. 1. (A) C_p excess thermal profile of 70S ribosomal particles (1 mM Tris–HCl, 0.8 mM $MgCl_2$; pH = 7.4). The curve was calculated by the subtraction of a baseline from the recorder tracing as described in Section 2. (B) (———): C_p excess thermal profile of 70S ribosomal particles pre-heated up to 70°C. (- - -): Pre-heating profile.

ture within the microcalorimetric cell at the same rate of 0.5°C/min. Subsequently a new scanning was performed on each sample along the complete temperature scale. The two previously observed transitions were found to be irreversible, unlike previous studies showing total reversibility of hyperchromicity following thermal degradation [13]. With respect to this our data are in agreement with previous literature data [14] obtained by different approaches. Furthermore, if a second complete scanning is performed after the first partial one, which was stopped at a temperature between the two transitions, the high temperature transition persists (Fig. 1B). This suggests that the two transitions are separated events. It should be pointed out that sucrose density gradient analysis of ribosomal particles suspended in the measuring buffer, shows that at this Mg^{2+} concentration, the bulk of ribosomes sediments as intact 70S monomers (not shown).

To address whether the two peaks should be attributed to the different subunits, we investigated the calorimetric behavior of 30S and 50S separately.

3.2. Differential calorimetric scanning of E. coli subunits

Fig. 2A shows the thermogram obtained from 50S subunits. An overall resemblance is evident with the pattern obtained from entire ribosomes, even though some differences exist. First, an exothermic peak is observed at approx. 90°C when the transition is complete; this indicates an aggregation process not found in the 70S particle. Second, the two DSC show a larger overlapping and the transitions occur at slightly different temperatures ($T_1 = 68.6$°C; $T_2 = 73.4$°C). The third difference consists of a greater height of the overall profile suggesting a higher transition enthalpy with respect to 70S (see Fig. 5 for further details). Reversibility tests, analogous to the those previously reported, give substantially consistent results with the ones observed for 70S (Fig. 2B).

The thermogram obtained from 30S subunits, on the other hand, shows different features with respect to 70S and 50S particles. The most evident difference is an exothermic peak in the middle of the transition (Fig. 3A). This indicates some aggregation in the sample during the thermal denaturation process which makes the analysis of the thermogram impossible. To eliminate the aggregation we lowered the Mg^{2+} concentration in the measuring buffer to 0.08 mM. As discussed below, at this Mg^{2+} concentration the ribosomal are structurally unvaried [12]. Under these conditions the exothermic peak is still present but is shifted to a higher temperature proximal to the end of the thermal denaturation (Fig. 3B). This still indicates a form of aggregation but

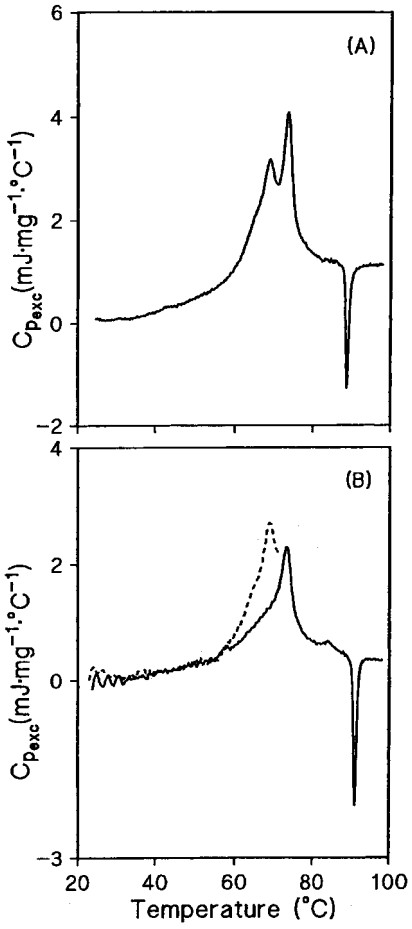

Fig. 2. (A) C_p excess thermal profile of 50S ribosomal subunits (1 mM Tris–HCl, 0.8 mM $MgCl_2$; pH = 7.4). (B) (———): C_p excess thermal profile of 50S subunits pre-heated up to 71°C. (- - -): Pre-heating profile.

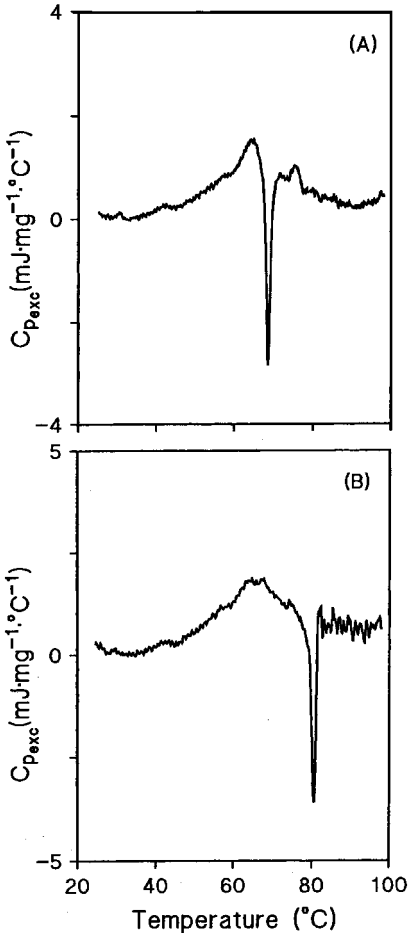

Fig. 3. C_p excess thermal profile of 30S subunits. (A): 1 mM Tris–HCl; 0.8 mM $MgCl_2$; pH = 7.4. (B): 1 mM Tris–HCl; 0.08 mM $MgCl_2$; pH = 7.6.

in this case the analysis of the experimental profile becomes possible. The thermogram thus obtained appears as a large unstructured curve which strongly resembles the one obtained for ribosomal RNA as shown in Fig. 4.

4. Discussion

In this work we investigated the microcalorimetric behavior of *E. coli* 70S ribosomes and separated subunits. Our results are an improvement and an extension of a previous study [7]. The first problem to be solved, was the selection of a measuring buffer. It is known that a Mg^{2+} concentration $> 10^{-3}$ M causes aggregation of ribosomes at high temperatures, thus preventing the analysis of the experimental profiles. On the other hand, an excessively low Mg^{2+} concentration causes structural alteration such as denaturation and irreversible unfolding. The loss of structure is reflected also in changes of the sedimentation behavior of the ribosomal particles. However, sucrose density gradient analysis of ribosomal subunits demonstrated that the sedimentation coefficient remains unvaried if the Mg^{2+} is maintained in the range 10^{-3} and to 8×10^{-5} M [12] which indicates that the ribosome structure

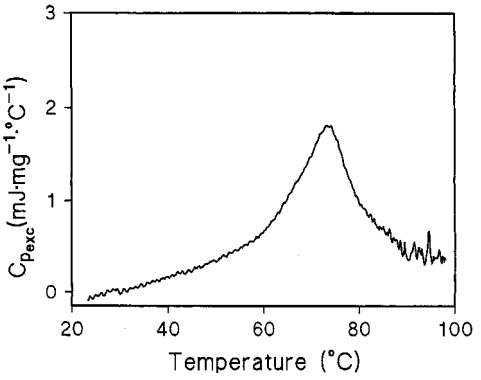

Fig. 4. C_p excess thermal profile of ribosomal RNA (1 mM Tris–HCl; 0.8 mM $MgCl_2$, pH = 7.4).

does not undergo significant alterations. We checked by sucrose density gradients the sedimentation properties of particles in the measuring buffer. These co-sedimented with particles resuspended in a standard buffer (10 mM $MgCl_2$; 10 mM Tris–HCl pH 7.4; 60 mM NH_4Cl; 40 mM KCl, at 4°C).

A typical thermal denaturation profile obtained from 70S particles shows two different peaks (Fig. 1A). The pattern is highly structured showing two well defined transitions occurring at different temperatures. The two phenomena could in principle be attributed either to the protein or to the nucleic acid moiety of the ribosome. The RNA constitutes approximately two-thirds of the whole ribosome mass. In addition the denaturation enthalpy of a nucleic acid is generally two to threefold higher than the one exhibited by proteins. This would suggest that the nucleic acid component of the ribosome is mainly responsible for the observed transitions. Furthermore, at the ribosome concentration used in our measurements, the protein moiety does not give any measurable denaturation pattern. However, an evident role of the proteins appears from the comparison of the shapes of the thermograms obtained from rRNA and entire particles. The thermal denaturation of rRNA does not show structures, while the denaturation profile of the whole ribosome shows a complex behavior indicating the existence of intermediate states in the process of melting (compare for instance Fig. 1A and Fig. 4). Therefore our data should be interpreted as a consequence of the thermal demolition of the RNA/protein complex.

We also carried out reversibility tests on our samples. In a first experiment we scanned the whole temperature scale, whereafter the sample was slowly cooled and the scanning repeated. No transition was detected which demonstrates the irreversibility of the entire process. In a second set of measurements scanning was arrested at an intermediate temperature between the two transitions. Again the sample was cooled and a new scanning repeated. Fig. 1B shows that while the low temperature transition is no longer observable, the second one persists. This result is suggestive of two distinctive transitions attributable to thermally separated and independent events.

The first hypothesis we made is that these two denaturation events are due to the two interacting subunits. Therefore we performed calorimetric experiments on separated 50S and 30S. Results in Fig. 2A,B and Fig. 3A,B show that the subunits exhibit a strikingly different thermal denaturation pattern; while the 50S shows two peaks virtually identical to the ones found in the whole 70S, the 30S subunit presents a non-structured thermogram strongly resembling the free RNA in solution. An immediate conclusion is that the two peaks found in the 70S are not attributable to the individual subunits but establish that a complex unfolding process with intermediate states occurs. These data suggest that within the ribosome the protein/RNA interactions may play a more significant role than RNA/RNA or protein/protein interactions in the stabilization of the three-dimensional structures. Also, the resemblance between the 70S pattern and the one shown by the separated 50S, suggests that within both particles the spatial arrangement is essentially the same. Therefore the thermal pattern exhibited by the 30S subunits and their tendency to aggregate at high temperature, indicate an intrinsic instability of the particle. These data are consistent with former speculations, based on dielectric and fluorescence studies, implying that the 50S particle is more rigid while the 30S shows 'floppier', characteristics [4,5].

We note that in all thermograms the premelt-

ing baseline has a slightly positive slope while, after melting, the slope becomes virtually zero. The positive slope at low temperature is usually observed in thermograms obtained from melting of RNA [15]. We suggest that this effect is presumably due to partial unstacking of bases prior to the cooperative thermal transition. Furthermore, a significant difference in heat capacity exists between native and denatured state, as shown by the difference in baseline level. This difference is present, though to a different extent, in all examined samples. There is no satisfactory way to consider separately all these small differences [15]. Therefore we could only calculate the total excess heat adsorbed by the sample solution from the initial scanning temperature ($T_0 = 25°C$) to any determined temperature point T according to the following equation:

$$\Delta H_{exc} = \int_{T_0}^{T} C_{p_{exc}}(T) \cdot dT$$

This quantity contains the enthalpy change associated with the transition from native to denatured states and, in addition, the background heat adsorption. This latter is the result of heat adsorption by native and denatured molecules present in the region between T_0 and T.

In Fig. 5 the total excess heat, normalized to the mole of nucleotide in the sample, is reported as a function of temperature for 70S, 50S and free RNA in solution.

It is evident from Fig. 5 that for the denaturation of 50S particles (90°C) a higher amount of thermal energy per nucleotide mole is needed than for 70S. A simple explanation is that the heat adsorbed by 70S is a weighed average of the two energies required for the denaturation of the separated subunits. The determination of the excess heat adsorbed by 30S is made difficult by the aggregation tendency exhibited by these particles, however, our data seem to indicate that the excess heat in 30S is lower than in 70S particles.

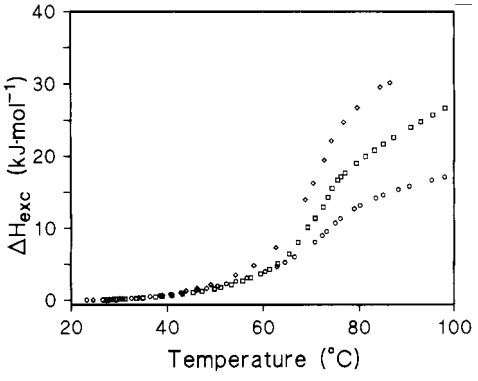

Fig. 5. Total excess heat, $\Delta H_{exc}(T)$, vs. T. (○), RNA; (□), 70 S; (◇), 50S.

The melting profile of the rRNA lies under the one exhibited by the 70S. The overall transition enthalpy for RNA yields approx. 17 KJ/mol which is comparable with literature data (see for instance Laing and Draper [16]). It is worth noting that at T higher than 80°C, where the melting process is practically concluded, ΔH_{exc} does not have a constant value but increases linearly with temperature. This effect is more evident in whole 70S than in isolated rRNA and is due to the increase in heat capacity of the solution following the denaturation with a value $C_{p_{exc}} > 0$ for $T >$ 80°C. The extrapolation of the values of ΔH_{exc} (in the range 80–100°C) to the melting temperature allows an approximate evaluation of the contribution of this effect that accounts for the difference between 70S and free rRNA.

In our opinion, the most significant results of this work are based on the qualitative analysis of the thermal profiles that indicate an important role of the interactions protein/RNA in the evolution of the process of thermal unfolding of the ribosomal particles. However, a quantitative comparison may not be fully satisfactory.

In conclusion our results are consistent with the idea that the rRNA is the molecular frame on which the ribosomal proteins are organized. Ribosomal RNA structures are observed by thermal degradation only when the nucleic acid moiety is complexed with ribosomal proteins; therefore one of the functions of the proteins might consist in the stabilizations of these structures. As a matter of fact, free RNA in solution is very flexible as is also shown by our previous dielectric relaxation studies [17].

It has been shown that in extremophilic bacteria the ribosomal RNA behaves as a bona fide ribozyme since isolated 23S rRNA is capable of

synthesizing the peptide bond [18]. We speculate that in a mesophilic bacterium like *E. coli*, where this activity has not been clearly demonstrated, these nucleic acid/protein interactions might constitute an essential prerequisite for complete stability and functional activity of the large ribosomal subunit.

Acknowledgements

Critical reading of the manuscript by K.H. Nierhaus is greatly appreciated. This work was supported by Istituto Nazionale di Fisica della Materia (Grant to A.B., G.O. and A.S.) and Ministero della Universitià e Ricerca Scientifica e Tecnologica (Grant to G.R.)

References

[1] M. Nomura, A. Tissières, P. Lengyel (Eds.), Ribosomes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1974.
[2] G. Chambliss, G.R. Craven, J. Davies, M. Nomura (Eds.), Ribosomes: Structure, Function and Genetics, University Park Press, 1979.
[3] K.H. Nierhaus, F. Franceschi, A. Subramanian, V.A. Erdmann, B. Wittmann-Liebold (Eds.), The Translational Apparatus, Plenum Press, New York, 1993.
[4] A. Bonincontro, A. Giansanti, F. Pedone, G. Risuleo, Biochim. Biophys. Acta 1115 (1991) 49.
[5] A. Bonincontro, A. Giansanti, F. Pedone, G. Risuleo, Biochim. Biophys. Acta 1174 (1993) 27.
[6] P.L. Privalov, V.V. Plotnikov, Thermochim. Acta 139 (1989) 257.
[7] B.M. Mackey, C.A. Miles, S.E. Parsons, D.A. Seymour, J. Gen. Microbiol. 137 (1991) 2361.
[8] S.-J. Liand, A.G. Marshall, Biochemistry 24 (1985) 4047.
[9] D.E. Draper, TIBS 21 (1996) 145.
[10] E. Kaltschimdt, H.G. Wittmann, Anal. Biochem. 36 (1970) 401.
[11] C. Gualerzi, H. Osawa, G. Risuleo, C.L. Pon, in: Balaban, Sussman, Traub, Yonath (Eds.), Structural Aspects of Recognition and Assembly of Biological Macromolecules, ISS Philadelphia, 1981, p. 793.
[12] A. Araco, M. Belli, C. Giorgi, G. Onori, Nucleic Acids Res. 2 (1975) 373.
[13] M. Tal, Biochemistry 8 (1969) 424.
[14] J.W. Bodley, Biochemistry 8 (1969) 464.
[15] Y.C. Gluick, D.E. Draper, J. Mol. Biol. 241 (1994) 246.
[16] L.G. Laing, D.E. Draper, J. Mol. Biol. 237 (1994) 560.
[17] A. Bonincontro, C. Mari, M. Mengoni, G. Risuleo, Biophys. Chem. 67 (1997) 43.
[18] I. Nitta, T. Ueda, W. Watanabe, RNA 4 (1998) in press.