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Chaperone-like activity and surface hydrophobicity of 70S ribosome

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Abstract Ribosomes have been shown to mediate refolding of proteins in vitro. In order to understand the mechanism of action, we have explored the 70S ribosome surface for hydrophobicity, one of the important aspects in chaperone–target protein interaction. We find that the 70S ribosome displays significant hydrophobicity on its surface when probed with the hydrophobic fluorophore 8-anilino-1-naphthalene sulfonate. To understand the functional significance of this hydrophobicity we investigated the ability of the ribosome to prevent aggregation of insulin B chain and α-lactalbumin induced by reducing the interchain and intrachain disulfide bond respectively with dithiothreitol (DTT) and photo aggregation of γ-crystallin at 37°C. The 70S ribosome offers complete protection towards light-induced aggregation of γ-crystallin (at 1:2 (w/w) ratio of crystallin:ribosome) and DTT-induced aggregation of α-lactalbumin (at 1:3) and there is appreciable protection (at 1:3) against the aggregation of insulin B chain. We also investigated the role of 70S ribosome in refolding of bovine carbonic anhydrase. Ribosomes improved the folding yield in a concentration-dependent manner. These results clearly demonstrate a general chaperone-like activity of 70S ribosome and implicate its surface hydrophobicity. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ribosome; Protein; Folding; Aggregation; Chaperone; Hydrophobicity; Insulin; Crystallin; Lactalbumin

1. Introduction

Translation is one of the most fundamental and evolutionarily conserved processes. The ribosome, a macromolecular bipartite assembly of RNA and protein, carries out the job of decoding messenger RNA into a string of specified amino acids. The nascent polypeptide has to fold into a unique three-dimensional structure to perform its function. It is generally accepted that the information necessary to specify the native three-dimensional structure of a protein is inherent in its amino acid sequence [1]. The scenario faced by a nascent protein in vivo is more complex than that faced by an unfolded chain in vitro [2]. The high concentration of nascent polypeptide chains in vivo poses the serious problem of aggregation and misfolding. A remarkable mechanism involving molecular chaperones appears to have evolved to safeguard the folding of nascent polypeptide chains [3,4]. There are some reports which attribute a general property of assisted refolding to ribosome though very little is known about the mechanistic aspects at the molecular level [5,6]. In order to understand the mode of action of ribosome-assisted refolding of proteins, we have attempted to address the following questions: (a) what is the nature of interaction between the ribosome and protein moiety in vitro? (b) Could the ribosome prevent protein aggregation as chaperones do? (c) Is the assisted refolding activity of the ribosome concentration-dependent?

Investigations carried out in our laboratory with an eye lens protein, α-crystallin, have shown that the molecule utilizes its surface hydrophobicity in its action as a chaperone [7]. Hydrophobic interactions have been functionally implicated in a variety of molecular chaperones [8–10]. In the present study, we have therefore investigated the surface hydrophobicity of 70S ribosome using 8-anilino-1-naphthalene sulfonate (ANS), which demonstrates for the first time significant hydrophobicity on the surface of 70S ribosome. We also studied the ability of 70S ribosome to prevent protein aggregation using dithiothreitol (DTT)-induced insulin and α-lactalbumin aggregation and photo aggregation of γ-crystallin as model systems. We found that 70S ribosome completely prevents photo aggregation of γ-crystallin and DTT-induced aggregation of α-lactalbumin while it offers appreciable but not complete protection towards insulin aggregation. These observations validate the functional significance of the observed hydrophobicity on the surface of 70S ribosome. 70S ribosome-assisted refolding of bovine carbonic anhydrase (BCA) in vitro revealed that the ability to assist the refolding process is concentration-dependent. Moreover, the molar stoichiometry of the reaction is also dependent on the concentration at which the protein is refolded. These results provide some new insight into the chaperone-like activity of 70S ribosome.

2. Materials and methods

Bovine α-lactalbumin, DTT, BCA and insulin were obtained from Sigma Chemical Company, USA. ANS was from Aldrich Chemical Company, USA. γ-Crystallin was isolated as described earlier [7].

2.1. Preparation of 70S ribosomes

Ammonium chloride-washed tightly coupled 70S ribosomes were prepared from *Escherichia coli* MRE600 as described by Burma et al. [11]. *E. coli* MRE600 cells were ground with double the amount of alumina A305 and suspended in 20 mM Tris–HCl (pH 7.5) containing 30 mM ammonium chloride, 10 mM magnesium acetate and 5 mM mercaptoethanol (TMA-10) buffer. The suspension was centrifuged at $30\,000\times g$ to remove the cell debris. The resultant supernatant was further centrifuged at $135\,000\times g$. The pellet thus obtained

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Abbreviations: ANS, 8-anilino-1-naphthalene sulfonate; DTT, dithiothreitol; BCA, bovine carbonic anhydrase; TMA-10, Tris–HCl buffer (pH 7.5) 30 mM ammonium chloride, 5 mM mercaptoethanol with 10 mM magnesium acetate

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was suspended in TMA-10 buffer and dissolved by homogenization. This ribosomal suspension was treated with DNase I (RNase free) (30 μg/ml) and kept at 4°C for 1 h. Subsequently it was treated with an equal volume of buffer containing 2 M ammonium chloride and left at 0°C for 3 h. After centrifugation at 22 000×*g* for 15 min a clear supernatant was obtained which was further centrifuged at 135 000×*g*. The pellet was dissolved in buffer and homogenized properly and subjected to further centrifugation at 27 000×*g*. The clear supernatant was collected and centrifuged at 135 000×*g*. The resulting ribosomal pellet was again dissolved in buffer and homogenized properly and the solution was subjected to a low speed centrifugation at 22 000×*g* and the supernatant was collected. The high salt wash and subsequent homogenization ensures removal of all non-ribosomal-associated proteins. This preparation was dialyzed against the TMA-4 buffer (same as above but with 4 mM magnesium acetate). The dialyzed preparation was subjected to sucrose gradient (5–30%, in TMA-4 buffer) centrifugation at 100 000×*g*. The peak corresponding to 70S was pooled avoiding cross contamination with 50S and precipitated with 0.7 volume of ethanol (−20°C) in the presence of 100 mM magnesium acetate. The preparation was checked for homogenity by small (5 ml) sucrose density gradient (10–35%) ultra-centrifugation in TMA-10, which showed a single peak as monitored by absorbance at 260 nm. Ribosomes were stored at −70°C in TMA-10.

2.2. Fluorescence studies

Fluorescence studies were carried out on a Hitachi F-4000 fluorescence spectrometer. 70S ribosome (0.3 mg/ml) in TMA-10 buffer containing 100 mM NaCl was equilibrated with 200 μM ANS at room temperature for 15 min. The excitation monochromator was set at 365 nm (3 nm band pass) and the emission scanned from 400 to 600 nm (1.5 nm band pass) in the correct spectrum mode to monitor the emission maxima of ANS.

2.3. Assay of chaperone-like activity of 70S ribosome

Insulin at a concentration of 0.2 mg/ml (in 10 mM phosphate buffer pH 7.5 containing 100 mM sodium chloride, 30 mM ammonium chloride, and 10 mM magnesium acetate) in the presence or the absence of different amounts of 70S ribosome was equilibrated at 37°C for 10 min with constant stirring in the cuvette using a Julabo thermostat water bath. The actual temperature in the cuvette was monitored with a Physitemp micro thermocouple thermometer system. The reduction of insulin was initiated by the addition of 30 μl of 1 M DTT to 1.5 ml of the sample. The extent of aggregation was monitored by measuring the scattering at right angles in a Hitachi F-4000 fluorescence spectrometer with both the excitation and emission monochromators set at 465 nm and excitation and emission band passes at 1.5 nm.

Photo aggregation of γ-crystallin was monitored in 10 mM phosphate buffer pH 7.5 containing the other constituents as described above. γ-Crystallin at a concentration of 0.2 mg/ml in the presence or the absence of different amounts of 70S ribosome was equilibrated at 37°C for 10 min. Then the sample with or without 70S ribosome was irradiated at 295 nm with the excitation band pass of 20 nm for a fixed period of time in a Hitachi F-4000 fluorescence spectrometer, after irradiation the excitation and emission monochromator were set at 475 nm with the excitation and emission band passes of 1.5 nm to measure the relative scattering. The relative scattering value was plotted as a function of the irradiation time.

Aggregation of apo-α-lactalbumin was carried out in TMA-10 buffer at 37°C. α-Lactalbumin (0.3 mg/ml) was equilibrated in the presence or the absence of different amounts of 70S ribosome in TMA-10 buffer containing 200 mM NaCl. The reduction of α-lactalbumin was initiated by the addition of 30 μl of 1 M DTT to 1.5 ml of the sample. The extent of aggregation was monitored by measuring the scattering at right angles in a Hitachi F-4000 fluorescence spectrometer with both the excitation and emission monochromators set at 360 nm and excitation and emission band passes at 1.5 nm.

2.4. Refolding studies

BCA at 0.5 mg/ml was incubated in 50 mM Tris–HCl buffer (pH 7.5) containing 8 M urea for 16 h at room temperature. Then an aliquot of 10 μl from this stock was diluted to 1 ml with TMA-10 buffer in the presence or the absence of 70S ribosome as to have the final refolding concentration of 5 μg/ml. At various time intervals, an aliquot of 0.5 μg was sampled out to assay for enzyme activity. In the case of refolding at a concentration of 10 μg/ml, 20 μg/ml and 40 μg/ml, BCA was denatured at 1.0 mg/ml, 2.0 mg/ml and 4 mg/ml respectively. The enzyme activity was measured by the hydrolysis of *p*-nitrophenyl acetate (1 mM) in assay buffer (50 mM Tris–HCl, pH 7.5) after incubating with the enzyme for 15 min at room temperature. Thereafter, the optical density was measured at 400 nm with an appropriate blank. The activity recovered on refolding BCA in the presence or the absence of 70S ribosome was calculated with respect to the activity of the native enzyme.

3. Results

The ribosome, being the protein synthesizing machinery, is likely to have a role in folding of nascent polypeptides as well. There are some reports in the literature which suggest that the ribosome can assist refolding of denatured proteins in vitro [5,6] and might contribute to some extent in protein folding inside the cell [12]. However, its mode of action at the molecular level is poorly understood. To gain insight into its mechanism of action, we investigated the nature of interaction with the target protein in vitro.

Since hydrophobic interactions are known to play a major role in assisted protein folding mediated by molecular chaperones, we explored the surface of the 70S ribosome by using ANS, a probe to detect hydrophobicity. The dye alone fluoresces little in aqueous solutions and its fluorescence quantum yield increases in a hydrophobic environment. A blue-shifted emission maximum indicates the apolar nature of the environment surrounding the probe. At 37°C, ANS alone fluoresces weakly with an emission maximum at 520 nm but in the presence of 70S ribosome there is an almost four-fold increase in the fluorescence intensity concomitant with a blue shift of about 40 nm. The emission maximum shifted to 480 nm as shown in Fig. 1. This indicates the presence of sufficient hydrophobicity on the surface of 70S ribosome. This observation led us to hypothesize that if 70S ribosome does have sufficient hydrophobicity on its surface, it might be making use of this and therefore should prevent protein aggregation in vitro. To test this we employed three different models of protein aggregation. The first two model systems were based upon DTT-induced protein aggregation and the third system was light-induced aggregation of γ-crystallin.

Upon reducing the disulfide bond between the two chains of insulin with a reducing agent such as DTT the B chain of insulin aggregates and precipitates [13]. Insulin (0.2 mg/ml) alone aggregates upon DTT addition with time as monitored by scattering at 465 nm light at 37°C (Fig. 2). An equal amount of 70S ribosome decreases the aggregation to some

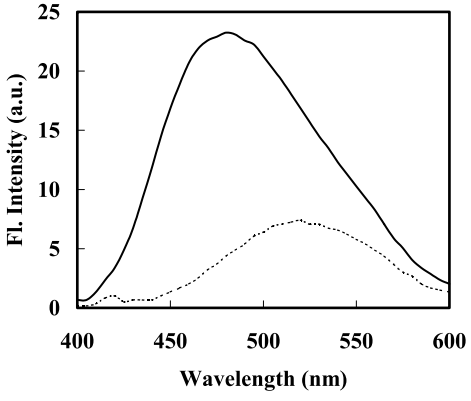

Fig. 1. Fluorescence spectra of ANS (dotted line) alone and in the presence of 70S ribosome (solid line).

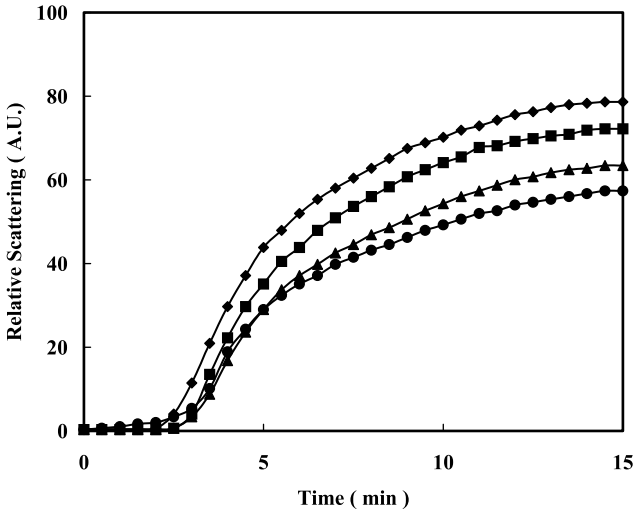

Fig. 2. DTT-induced insulin aggregation at 37°C. Aggregation was monotored by measuring scattering of 465 nm light. (♦) 0.2 mg/ml insulin alone, and at an insulin to 70S ribosome weight ratio of 1:1 (■), 1:2 (▲) and 1:3 (○).

extent. Increasing the amount of ribosome twice as that of insulin results in increased protection while almost 30% protection is offered at the concentration of 0.6 mg/ml of ribosome. Therefore 70S ribosome seems to offer appreciable protection, in case of DTT-induced insulin aggregation assay. In order to further substantiate the ability of 70S ribosome to provide protection towards protein aggregation, we studied another non-thermal mode of protein aggregation system, namely DTT-induced aggregation of α-lactalbumin. α-Lactalbumin adopts a conformation similar to molten globule when disulfide bonds are reduced [14]. Fig. 3 presents our results in case of DTT-induced aggregation of α-lactalbumin. At 0.3 mg/ml, the protein alone aggregates upon DTT addition with time as monitored by scattering at 360 nm. An equal amount of 70S ribosome provides little protection towards aggregation, while 70S ribosome at a ratio of 1:2 w/w (0.3 mg/ml α-lactalbumin: 0.6 mg/ml 70S ribosome) appreciably prevents the aggregation. Further increase in the ratio of

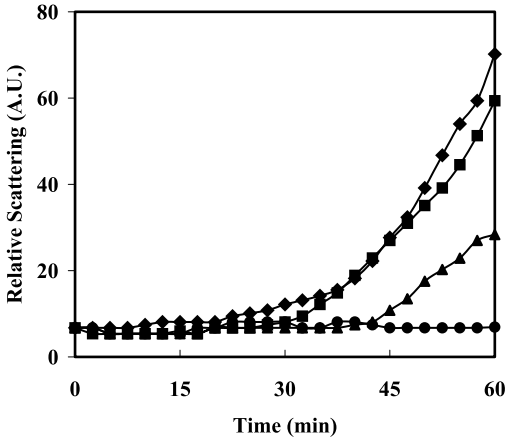

Fig. 3. The chaperone-like activity of 70S ribosome in the prevention of DTT-induced aggregation of α-lactalbumin at 37°C. α-Lactalbumin (0.3 mg/ml) alone (♦), and at a α-lactalbumin to 70S ribosome weight ratio of 1:1 (■), 1:2 (▲) and 1:3 (○). The extent of aggregation was monitored by measuring scattering of 360 nm light.

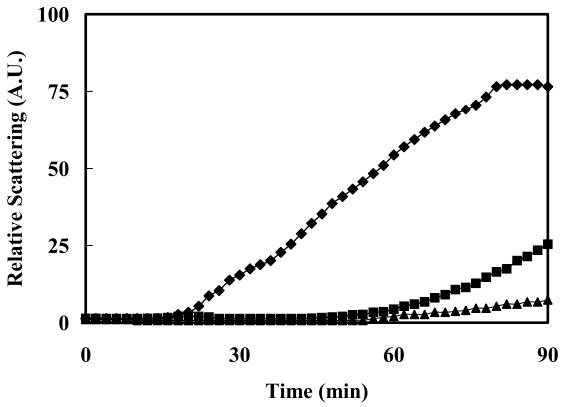

Fig. 4. Photo-induced aggregation of 0.2 mg/ml of γ-crystalline in the absence (♦) and in the presence (■) of 0.2 mg/ml and (▲) 0.4 mg/ml of 70S ribosome at 37°C. Aggregation was measured by monitoring the scattering of 475 nm light.

α-lactalbumin:70S ribosome (1:3, w/w) completely prevents the aggregation. This clearly demonstrates the chaperone-like ability of 70S ribosome in preventing protein aggregation. The different extent of protection offered by 70S ribosome in case of DTT-induced aggregation of insulin and α-lactalbumin might be due to a differential degree of hydrophobic surface available on these two target proteins or due to a different extent of aggregation taking place in these two target proteins upon reduction by DTT. We studied photo aggregation of γ-crystallin as another model system. Upon continuous irradiation of γ-crystallin (0.3 mg/ml) with 295 nm light at 37°C it starts aggregating as a function of time and levels off at about 80 min (Fig. 4). 70S ribosome (0.3 mg/ml) offers about 70% protection towards this photo aggregation while at a concentration of 0.6 mg/ml, 70S ribosome completely prevents aggregation. These results show that the ribosome can prevent aggregation of other proteins. Surface hydrophobicity appears to be important for this action. In order to evaluate its efficiency in refolding denatured enzymes, we have studied the refolding of BCA. BCA is a single polypeptide molecule of 30 kDa molecular weight devoid of any disulfide bridges and can be easily assayed in vitro by hydrolysis of an ester, which liberates a colored product, which can be quantitated [15]. Fig. 5 shows refolding of BCA at a concentration of 5 μg/

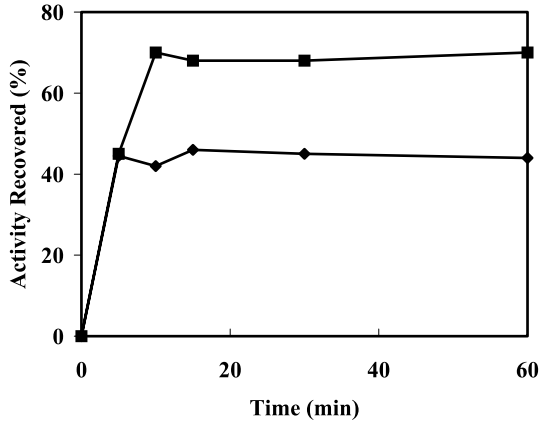

Fig. 5. Refolding of BCA (5 μg/ml) in the absence (♦) and in the presence (■) of 70S ribosome.

Table 1
Percent assistance offered by 70S ribosome during refolding of BCA over unassisted refolding at the following protein concentrations and different ratios between ribosome:protein

Refolding concentration (μg/ml)	Ratio (ribosome:protein)					
	2	1	0.5	0.2	0.1	0.05
5	14.0%	19.8%	13.5%	0.0	0.0	0.0
10	2.1%	14.7%	14.5%	16.6%	0.0	0.0
20	0.0	0.0	4.9%	5.1%	5.7%	0.0
40	0.0	0.0	8.6%	7.6%	2.5%	0.0

ml in the presence and absence of 70S ribosome. An appreciable amount of activity is regained on spontaneous, unassisted refolding of the protein while about 75% refolding yield is observed in case of assisted refolding with ribosome. The stoichiometry in this case was one. In a bid to further explore this assisted refolding, we carried out refolding of the protein at four different concentrations with various stoichiometries between the 70S ribosome and the protein. We observed that unassisted refolding efficiency decreased in a concentration-dependent manner. Evaluation of the net assistance offered by the ribosome in refolding BCA at different concentrations with various stoichiometries (Table 1) clearly showed that the maximum assistance is offered at a particular ratio that is dependent on the protein concentration. The optimal ratio (ribosome:protein) which provides maximum assistance to refolding shifts from the value of one to lower values as one proceeds towards a higher concentration of refolding (Table 1). Observation of significant hydrophobicity on 70S ribosome and its ability to prevent protein aggregation in vitro along with refolding studies strongly suggest a chaperone-like activity for 70S ribosome, presumably due to its surface hydrophobicity. However, appropriate electrostatic interactions might also facilitate the process.

4. Discussion

The molecular details of the process by which proteins fold to their native biologically active conformation remain still an unsolved problem. Discovery of molecular chaperones led to the widely accepted belief that these molecules assist de novo folding of nascent polypeptides and rescue partially unfolded protein species during stress. However, the extent of contribution of chaperones to de novo cellular folding is not well understood [16].

Most if not all nascent polypeptides interact with some ribosomal proteins and specific parts of 23S rRNA during the elongation cycle of protein synthesis [17–19]. The ribosome, its large subunit and specifically the 23S rRNA have been shown to mediate refolding of a number of denatured enzymes with a molar stoichiometry of one [20]. The present investigation aims at an understanding of the nature of interaction between 70S ribosome and target protein. The observation of significant hydrophobicity on 70S ribosome correlates well with the availability of hydrophobic patches on the surface of most of the chaperone molecules. The crystal structure of 70S ribosome shows many ribosomal proteins on the surface of 70S ribosome [21]. Our present observation of the ability of 70S ribosome to prevent protein aggregation strongly suggests that the hydrophobicity on the surface of 70S ribosome might indeed be one of the major players while interacting with target proteins. Our refolding studies with BCA clearly demonstrate the concentration dependence of 70S-assisted refolding, which is in contrast to what has been reported in the literature [20]. The optimal molar stoichiometry (ribosome/protein) for the maximal assistance changed from the value of one to lower values as the concentration of the refolding increased. At higher protein concentrations, protection offered by the ribosome is higher. We have demonstrated protective capability of 70S ribosome towards DTT-induced aggregation of insulin, α-lactalbumin and photo aggregation of γ-crystallin at the physiological temperature. Complete protection was offered by 70S ribosome in case of photo aggregation of γ-crystallin and DTT-induced aggregation of α-lactalbumin while appreciable protection was evident in case of DTT-induced aggregation of insulin. All these observations strongly suggest that 70S ribosome indeed can act like a chaperone. Recently the ribosome has been shown to mediate refolding of partially folded protein [22]. We believe that 70S ribosome, during the elongation cycle of protein synthesis, might offer some kind of protection to the nascent polypeptide emerging from the exit site under in vivo conditions.

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