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Recombinant Human Ribosomal Protein S16: Expression, Purification, Refolding, and Structural Stability

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Abstract—The cDNA of human ribosomal protein S16 was cloned into the expression vector pET-15b. Large-scale production of the recombinant protein was carried out in *E. coli* cells and highly purified protein was isolated. A method for refolding the protein from inclusion bodies was optimized. The secondary structure content of the refolded protein was analyzed by CD spectroscopy. It was found that 21 ± 4% of the amino acid sequence of the protein forms α-helices and 24 ± 3% is in β-strands. The protein structure stability was studied at various pH values and urea concentrations. The protein is quickly denatured at pH above 8.0, whereas increasing of urea concentration causes slow unfolding of the protein.

Key words: human ribosomal protein S16, expression, refolding

The major component of the cell translational machinery is the ribosome, a remarkable giant ribonucleoprotein composed of two subunits with a complicated quaternary structure. To date, the atomic structure of the prokaryotic ribosome has been studied in detail by means of X-ray analysis [1-3], but the structure of the eukaryotic ribosome (especially from higher eukaryotes) remains practically unknown. While cryoelectron microscopy can provide a low resolution view of the eukaryotic ribosome [4, 5], it is nevertheless unable to provide information on the molecular interactions of components inside the ribosomal subunits.

Ribosomal proteins (r-proteins) are known to be not only structural elements of the ribosome, but to possess a number of extra-ribosomal functions [6] that take place at many stages of various intracellular processes. The majority of these functions are realized via binding of r-proteins to nucleic acids, in particular, RNA [7, 8].

Studying the interactions of human r-proteins with RNAs (rRNAs, mRNAs, pre-mRNAs, etc.) could provide valuable information on both mammalian ribosome structure and the extra-ribosomal functional properties of r-proteins. However, the isolation of individual r-proteins from human cells or tissues (e.g., from placenta) in quantities sufficient for studies of this kind is laborious and rather expensive [9]. In this respect, recombinant r-protein could be a substitute for native proteins in structural and functional investigations if their structural integrity is demonstrated.

In this work, we report the expression, purification, and refolding of the recombinant human r-protein S16 and the influence of urea concentration on its secondary structure. By means of CD spectroscopy, we estimated the secondary structure content of the recombinant protein and compared secondary structures of human rpS16 and its prokaryotic homolog from *Thermus thermophilus*, rpS9 [10]. It should be noted that protein S16, together with S7, S10, and S19, is most strongly bound to 18S rRNA in the 40S subunit of the human ribosome [11].

MATERIALS AND METHODS

Peptone, yeast extract, ampicillin, "HIS-select" resin, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma (USA). Restriction enzymes *Eco*RI, *Nde*I, and *Bam*HI and T4 DNA ligase were from New England Biolabs (USA), and *Taq* DNA polymerase and MMLV reverse transcriptase were from Promega (USA). Oligodeoxyribonucleotides were synthesized in the Laboratory of Medical Chemistry in the Institute of Chemical Biology and Fundamental Medicine of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk).

Cloning and expression of human rpS16 cDNA. Human rpS16 cDNA was amplified by polymerase chain

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reaction (PCR) using a pair of specific primers containing 5′-terminal sequences for introduction of restriction sites into the PCR product. The forward primer was 5′-GG***GAATTCCATATG***CCGTCCAAGGG-3′ (sites for *Eco*RI and *Nde*I are marked) and the reverse 5′-GG***GAATTCGGATCC***TTATCGGTAGGATTTCTGG-3′ (sites for *Eco*RI and *Bam*HI are marked). Isolation of total RNA from human placenta, poly(A)$^+$ RNA purification, and the first cDNA strand synthesis were performed as described previously [12]. The reaction mixture (50 μl) contained 75 mM Tris-HCl, pH 8.9, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 μM specific primers, 0.1 μg of cDNA, and 1.25 U of Taq DNA polymerase. Amplification was performed in the Progene thermocycler (Techne, USA) using the following program. The reaction mixture was heated to 94°C for 3 min, and then reactions were run for 30 cycles of 30 sec at 95°C, 40 sec at 55°C, and 1 min at 72°C. The final cycle was extended to 7 min at 72°C. The PCR product was analyzed on a 2% agarose gel and visualized by ethidium bromide staining. The amplification product was digested with *Eco*RI and cloned into pBluescript II KS(–) vector (Stratagen, USA) at the *Eco*RI site, then *Nde*I-*Bam*HI fragment of the plasmid was subcloned into similarly digested pET-15b (Novagen, USA). The resulting plasmid pET-S16 was used to transform the *E. coli* strain BL-21(DE3). To express the protein, transformed cells were grown in LB medium containing 100 mg/liter of ampicillin at 37°C to optical density of $A_{600} = 0.6$, recombinant protein synthesis was induced by adding IPTG to 0.4 mM, and the culture was incubated for an additional 3 h. For large-scale preparation, the cells were cultivated in 5-7 liters of LB medium in a BIOK vortical bioreactor (ZAO Sayany, Novosibirsk, Russia) in the Laboratory for Genetic Therapy of the Institute of Molecular Biology and Biophysics (Siberian Branch of the Russian Academy of Medical Sciences, Novosibirsk).

Isolation and purification of recombinant rpS16. Cells were collected by centrifugation at 4000*g* for 20 min at 4°C; 5 g of the cell mass was resuspended in 20 ml of buffer containing 20 mM HEPES-KOH, pH 7.6, 20 mM KCl, 5 mM 2-mercaptoethanol, and 2 mM PMSF, incubated with 2 mg of lysozyme for 15 min at 30°C, and sonicated on ice at 22 kHz for 10 min. The solution was centrifuged at 14,000*g* for 10 min at 4°C and precipitant of inclusion bodies was dissolved in 4 ml of 6 M guanidine-HCl. A column of length 15 cm and volume 5 ml with HIS-select resin was pre-equilibrated with two volumes of buffer A (6 M guanidine-HCl, 20 mM HEPES-KOH, pH 7.6, 100 mM KCl, and 10 mM imidazole), then the protein sample was loaded and the column was washed with two volumes of buffer A. Bound protein was eluted with the same buffer containing 200 mM imidazole. Protein in the fractions was analyzed by 14% SDS-PAGE.

Refolding of the recombinant rpS16 and recording of CD spectra. The purified protein was refolded by the step-wise dialysis method against solutions containing step-wise decreasing guanidine-HCl (in concentrations 1, 0.5, 0.25, and 0 M) in buffer B (20 mM Tris-HCl, pH 7.5, 1 M KCl, 10 mM $MgCl_2$, and 2 mM 2-mercaptoethanol). Each step of dialysis was performed at 0°C for 2 h in the dialysis apparatus (Gibco, USA) using membrane with 7 kD cut-off.

For recording CD spectra, protein in buffer B was precipitated by four volumes of acetone, the pellet was dissolved in 7 M urea up to 30 mg/ml concentration, and the protein was refolded by quick dialysis [13] dissolving one part of the protein solution in 100 parts of water. Then the protein was placed in buffer of 10 mM K_2HPO_4/KH_2PO_4, pH 6.0, and concentrated on a Centricon-3 (Amicon, USA). The protein concentration was quantified by the Bradford method [14] using lysozyme and BSA as standards and by UV absorption at 280 nm, assuming a molar extinction coefficient of 12.2 $mM^{-1} \cdot cm^{-1}$.

CD spectra were measured with a Jasco J-400 spectropolarimeter (Japan) in the far (195-250 nm) UV range in 1 cm cells at 25°C. Each spectrum is a result of averaging of 5-7 scans.

RESULTS AND DISCUSSION

Construction of rpS16 overexpression strain. Total cDNA obtained by reverse transcription of human placental poly(A)$^+$ RNA with an oligo (dT) primer was

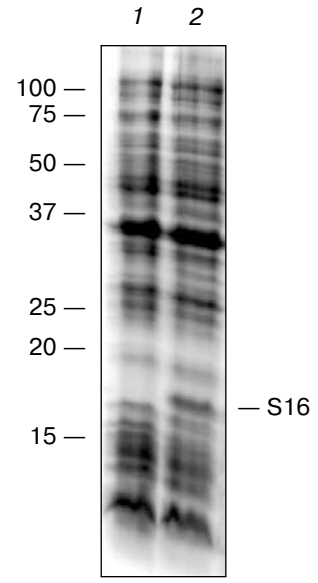

Fig. 1. Analysis of expression of the human ribosomal protein S16 cDNA in *E. coli* BL-21(DE3) cells. Electrophoresis in 14% SDS-polyacrylamide gels of total cell protein before IPTG induction (lane *1*) and 3 h after induction (lane *2*). Positions of protein molecular weight (kD) markers are noted on the left.

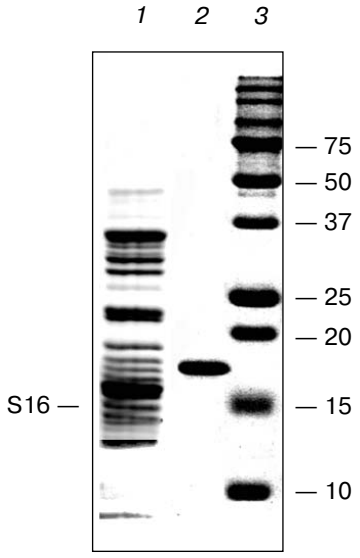

Fig. 2. Analysis in 14% SDS-polyacrylamide gels of the recombinant rpS16: *1*) total protein of the 40S subunit of human ribosome; *2*) purified recombinant rpS16; *3*) protein molecular weight markers. Position of native rpS16 in lane *1* (according to [11]) is noted on the left, and on the right positions of protein markers on the stained gel are shown.

amplified by PCR using a pair of specific primers for the coding sequence of mRNA of the human ribosomal protein S16. The primers bore at the 5′-ends sequences for construction of the restriction sites in the PCR product. A single product of the expected length produced during the reaction (data not shown) was cloned into the *Pst*I site of pBluescript II KS(−). From the recombinant plasmid, the *Nde*I-*Bam*HI fragment containing human rpS16 cDNA was subcloned into pET-15b, yielding pET-S16. The resulting plasmid bore sequence coding for rpS16 inserted in-frame after a sequence coding for a polypeptide of 20 amino acids including six histidines (His_6-tag). The rpS16 cDNA sequence in the expression vector was verified by sequencing. Plasmid pET-S16 was used for transformation of *E. coli* cells BL-21(DE3), and the resulting strain was used for rpS16 overexpression.

Recombinant rpS16 expression, purification, and refolding. Expression of recombinant rpS16 was induced by adding IPTG into the cell culture. Analysis of the total cell protein after induction for 3 h showed a predominant polypeptide of the expected molecular weight (Fig. 1). The recombinant protein comprised about 10% of the total cell protein and the proportion did not increase on further incubation. The protein mainly formed inclusion bodies, and after solubilization of the bodies it was purified by affinity chromatography on HIS-select resin. The final purity of the recombinant protein was more than 98% (Fig. 2). It should be noted that electrophoretic mobility of the recombinant protein is lower than that for the native protein due to the $(His)_6$-tag sequence (see above). Refolding of the protein was carried out either by step-wise dialysis against buffer with high ionic strength (this method we successfully applied earlier for refolding of other recombinant human ribosomal proteins [12]) or quick dilution with water [13].

Secondary structure and stability of recombinant rpS16. To obtain data on structural organization of the protein, CD spectra in the far-UV range were measured for the denatured and refolded forms of the protein (Fig. 3). The CD spectrum of the refolded rpS16 has characteristic peaks (intensive at 208 and less pronounced at 222 nm) that are absent in the denatured protein. According to this spectrum, the protein possesses extensive secondary structure. A quantitative estimate of the content of α-helices and β-strands in the protein secondary structure was derived from this CD spectrum using the SELCON program [15]. It was estimated that 21 ± 4% of the protein sequence is folded in α-helices, and 24 ± 3% in β-strands.

The locations of secondary structure elements were predicted by means the programs GOR [16], PSIpred [17], and SSpro [18]. The results are presented in Fig. 4. The predictions of the latter two programs are in good agreement with each other, whereas prediction of program GOR somewhat differs from the former. So, we excluded results of this program from our consideration. According to predictions of the programs PSIpred and SSpro, about 36-38% of amino acids of the recombinant rpS16 are folded into α-helices and about 18-23% form β-strands. This shows good correlation with the values of β-strands obtained from the CD spectrum, whereas the amount of α-helices is overestimated. The latter could be explained by both weakness of algorithms of the secondary structure predictions and imperfection of the program of CD spectra analysis. On the whole, the data of CD

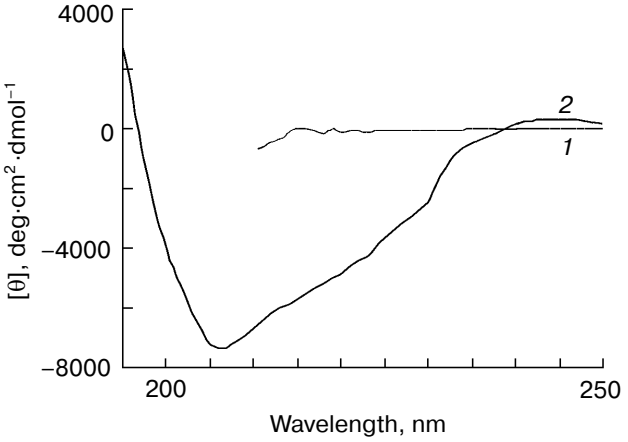

Fig. 3. Far-UV CD spectra of the recombinant rpS16 dissolved in 8 M urea (*1*) and refolded (*2*).

Fig. 4. Alignment of human rpS16 and *T. thermophilus* rpS9 and comparison of the positions of protein secondary structure elements. Above, positions of α-helices (cylinders) and β-strands (boxes) of S9 *T. thermophilus* according to X-rays analysis data [19] are shown. Below, predicted by programs GOR, PSIpred, and SSpro positions of α-helices and β-strands for human rpS16 are shown.

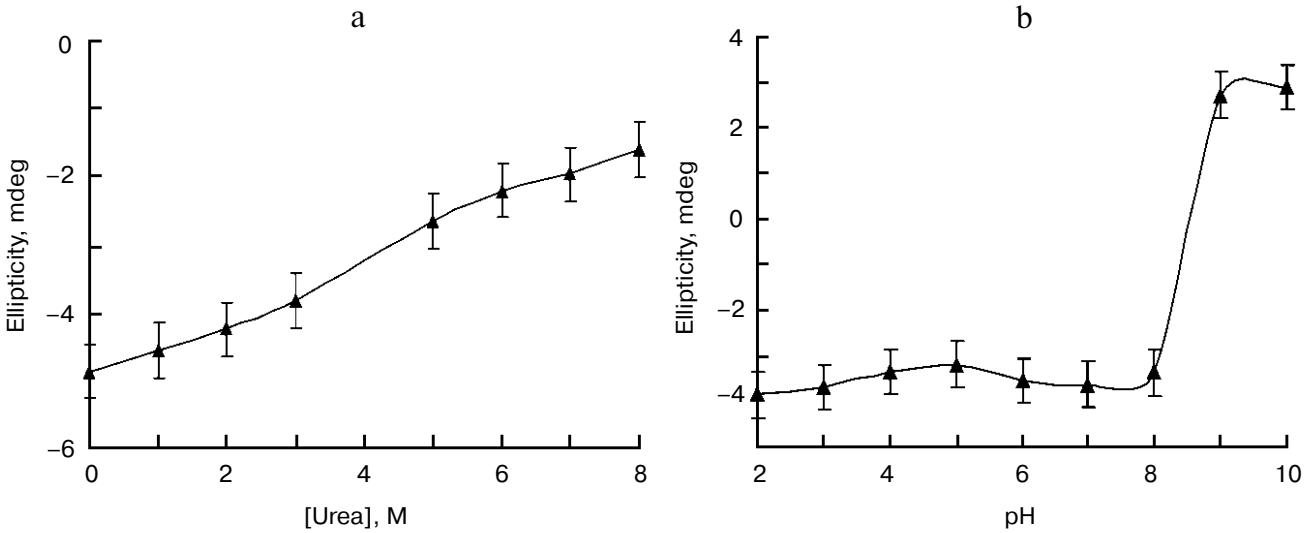

Fig. 5. Graphs of dependence of ellipticity values at 222 nm of the folded rpS16 on urea concentration (a) and pH (b).

spectra suggest that the recombinant rpS16 has well-defined secondary structure.

Human rpS16 is a homolog of prokaryotic rpS9 from *Thermus thermophilus* [10], whose structure is well studied by X-ray analysis [19]. We aligned the sequences of these proteins and compared the positions of the secondary structure elements predicted for human rpS16 by programs PSIpred [17] and SSpro [18] with those taken from the 3D model of rpS9 from *T. thermophilus* [19] (Fig. 4). One can see that positions of α-helices and β-strands in

human protein rpS16 are in good agreement with positions of those in the prokaryotic rpS9 (Fig. 4). Therefore, we suppose that the 3D structures of these proteins have features of similarity.

The stability of the native conformation of rpS16 upon denaturation by urea and at various pH values was studied by measuring the CD at 222 nm of the protein solution in appropriate buffers. When the concentration of urea is increased gradually from 0 to 8 M, the protein structure gradually unfolds (Fig. 5a). We found also that structure of the protein is rather stable in the pH range from 2.0 to 8.0. However, on increase in pH from 8.0 to 9.0 a drastic jump in the CD spectrum occurred (Fig. 5b). Obviously, this is concerned with disruption of the protein secondary structure and unfolding.

Thus, we conclude that recombinant human rpS16, obtained in this study, is folded correctly and may be employed in both structural and functional tests. The predicted secondary structure of human rpS16 seems to be similar to its prokaryotic homolog rpS9.

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REFERENCES

1. Wimberly, B. T., Brodersen, D. E., Clemons, W. M., Jr., Morgan-Warren, R. J., Carter, A. P., Vonrhein, C., Hartsch, T., and Ramakrishnan, V. (2000) *Nature*, **407**, 327-339.
2. Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F., and Yonath, A. (2000) *Cell*, **102**, 615-623.
3. Cate, J. H., Yusupov, M. M., Yusupova, G. Z., Earnest, T. N., and Noller, H. F. (1999) *Science*, **285**, 2095-2104.
4. Morgan, D. G., Menetreit, J.-F., Neuhof, A., Rapoport, T. A., and Akey, Ch. W. (2002) *J. Mol. Biol.*, **324**, 874-886.
5. Dube, P., Bacher, G., Stark, H., Mueller, F., Zemlin, F., van Heel, M., and Brimacombe, R. (1998) *J. Mol. Biol.*, **279**, 403-421.
6. Wool, I. G. (1996) *Trends Biochem. Sci.*, **21**, 164-165.
7. Vilardell, J., Chartrand, P., Singer, R. H., and Warner, J. R. (2000) *RNA*, **6**, 1773-1780.
8. Mazumder, B., Sampath, P., Seshadri, V., Maitra, R. K., DiCorleto, P. E., and Fox, P. L. (2003) *Cell*, **115**, 187-198.
9. Vladimirov, S. N., Ivanov, A. V., Karpova, G. G., Musolyamov, A. K., Egorov, T. A., Thiede, B., Wittmann-Liebold, B., and Otto, A. (1996) *Eur. J. Biochem.*, **239**, 144-149.
10. Wool, I. G., Chan, Y.-L., and Glueck, A. (1996) in *Translational Control* (Hershey, J. W. B., Matthews, M. B., and Sonenberg, N., eds.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 685-732.
11. Malygin, A. A., Shaulo, D. D., and Karpova, G. G. (2000) *Biochim. Biophys. Acta*, **1494**, 213-216.
12. Malygin, A., Baranovskaya, O., Ivanov, A., and Karpova, G. (2003) *Protein Exp. Purif.*, **28**, 57-62.
13. Tsumoto, K., Ejima, D., Kumagai, I., and Arakawa, T. (2003) *Protein Exp. Purif.*, **28**, 1-8.
14. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
15. Sreerama, N., Venyaminov, S. Yu., and Woody, R. W. (1999) *Protein Sci.*, **8**, 370-380.
16. Garnier, J., Gibrat, J.-F., and Robson, B. (1996) *Meth. Enzymol.*, **266**, 540-553.
17. Jones, D. T. (1999) *J. Mol. Biol.*, **292**, 195-202.
18. Pollastri, G., Przybylski, D., Rost, B., and Baldi, P. (2002) *Proteins*, **47**, 228-235.
19. Brodersen, D. E., Clemons, W. M., Jr., Carter, A. P., Wimberly, B. T., and Ramakrishnan, V. (2002) *J. Mol. Biol.*, **316**, 725-768.