Compact globular structure of *Thermus thermophilus* ribosomal protein S1 in solution:

**Sedimentation and calorimetric study**

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RUNNING TITLE: *Compact structure of ribosomal protein S1*

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SUMMARY

Ribosomal protein S1 of *Thermus thermophilus* overexpressed in *Escherichia coli* cells has been isolated and subjected to studies by analytical sedimentation and differential scanning microcalorimetry techniques. It has been demonstrated that the protein of 60 kDa sediments at $s_{20,w}^0 = 4.6$ S and has the diffusion coefficient $D_{20,w}^0 = 6.7 \times 10^{-7}$ cm$^2$/s in 25 mM HEPES-NaOH buffer, pH 7.5 (similarly to bovine serum albumin of 66 kDa that sediments at $s_{20,w}^0 = 4.4$ S and $D_{20,w}^0 = 6.0 \times 10^{-7}$ cm$^2$/s), indicating its compact globular conformation under these conditions.

The microcalorimetry study has shown the presence of a cooperative tertiary structure melting at 90 °C, but with several (probably three) independent cooperative domains. In the presence of 100 mM NaCl the protein becomes more asymmetric ($s_{20,w}^0 = 3.1$ S) but does not lose its cooperativity and thermostability, this suggesting just the weakening of inter-domain ionic interactions. The compact globular conformation of protein S1 seems to be most likely within the ribosome.
INTRODUCTION

Ribosomal protein S1 is the largest protein of eubacterial ribosomes. It is an RNA-binding protein involved in retention of mRNA during initiation of translation and, maybe, during elongation [1, 2]. The *E. coli* protein S1 was reported to include six copies of a motif of about 70 amino acids (the so-called S1 motifs) separated by spacers of 10 to 15 residues [1, 3, 4]. Each of these motifs can be arranged into a five-stranded antiparallel $\beta$-barrel resembling the fold of the bacterial cold shock protein [4]. Two N-proximal repeats, however, were found to be rather divergent. At the same time, the N-terminal part of protein S1 was reported to contain the ribosome-binding site of the protein [5], whereas the C-proximal part was claimed to bind mRNA [6]. Similar motifs were found also in a number of other RNA-binding proteins and posited to form an ancient nucleic acid-binding fold [4].

Physical studies of isolated protein S1 from *E. coli* showed that the protein in solution manifested a highly extended, non-compact conformation; the data of the sedimentation analysis, small-angle X-ray scattering, diffusion coefficient and intrinsic viscosity measurements, etc., were interpreted in terms of a rod-shaped object of 23 nm in length, which was comparable with the longest dimension of the whole ribosome [1, 7 - 9].

Ribosomal protein S1 has been recently identified in *Thermus thermophilus* and its gene has been expressed in *Escherichia coli* [10]. As the previous physico-chemical studies of *E. coli* ribosomal protein S1 demonstrated its highly elongated, asymmetric shape in solution, we decided to reinvestigate this matter with the thermophilic homologue of the protein, bearing in mind that the non-compact state of the isolated *E. coli* protein could be the consequence of its low physical stability.
EXPERIMENTAL PROCEDURES

Protein isolation and sample preparation

Recombinant ribosomal protein S1 of *Thermus thermophilus* was isolated from the *Escherichia coli* overproducing strain BL21(DE3)pET21d-tthS1 and purified as described earlier [10]. The purity and the quality of the protein were checked by the standard gel electrophoresis method according to Laemmli [11]. The protein was stored at 4°C in the precipitated form in (NH₄)₂SO₄ [10]. The protein concentration was determined according to Bradford [12].

Samples for measurements were prepared by solving the precipitate either in 25 mM HEPES-NaOH buffer, pH 7.5 or in 25 mM HEPES-NaOH buffer, pH 7.5, with 100 mM NaCl and subsequent dialysis against the same buffers at room temperature overnight.

Analytical ultracentrifugation

Sedimentation analysis of the protein was performed in a rotor An 60 Ti of the model Optima XL-I analytical ultracentrifuge (Beckman Coulter) equipped with ultraviolet optics using double-sector capillary cells at 20 °C, 40,000 rpm and the protein concentration of 1.5 mg/ml. The sedimentation and diffusion coefficients were calculated using the XL-A/XL-I Data Analysis software, version 6.03, and were reduced to the standard conditions (i.e., to 20 °C at the viscosity and density of water) [13].

Differential scanning microcalorimetry

Calorimetric measurements were performed in a precision scanning microcalorimeter SCAL-1 (SCAL Co. Ltd., Pushchino, Russia) [14] in glass cells of 0.3 ml capacity at the scanning rate of 1.0 K/min, the excess pressure of 2 atm, and the protein concentration of 1
mg/ml. In special experiments the scanning rate varied from 0.25 to 2.0 K/min. The experimental error for temperature and enthalpy measurements did not exceed 0.2 K and 7%, respectively. Thermodynamic analysis of the excess heat capacity profile was done according to Privalov and Potekhin [15]. The partial specific volumes were accepted to be 0.74 cm$^3$/g.
RESULTS

Sedimentation—Sedimentation patterns of the isolated *T. thermophilus* protein S1 are presented in Fig. 1. The sedimentation coefficient measured at low ionic strength (25 mM HEPES-NaOH, pH 7.5) was determined to be $s_{20,w}^0 = 4.6$ S. The diffusion coefficient in these conditions was $D_{20,w}^0 = 6.7 \times 10^{-7}$ cm$^2$/s. The accuracy of measuring the sedimentation and diffusion coefficients is ±0.2 S and ±0.2×10$^{-7}$ cm$^2$/s, respectively.

Fig. 2A shows the dependence of the logarithms of the sedimentation coefficients on the logarithms of the molecular weights for typical globular proteins with compact structures. The slope of this dependence is equal to 2/3, as it should be for the particles with similar hydration and partial specific volumes, the shape of which does not differ much from a sphere [16]. It is seen that the *T. thermophilus* ribosomal protein S1 lies on this line, together with bovine serum albumin having similar physical characteristics (66 kDa, 4.4 S). Fig. 2B shows the dependence of the logarithms of the diffusion coefficients on the logarithms of the molecular weights for globular proteins. The value of the diffusion coefficient for the *T. thermophilus* protein S1 falls also on the straight line specific to globular proteins. Thus, under these conditions, the isolated ribosomal protein S1 is compactly folded and can be qualified as a typical globular protein.

At the same time, the sedimentation and diffusion coefficients of the protein measured in the presence of 100 mM NaCl with 25 mM HEPES-NaOH, pH 7.5 (ionic strength of about 0.1) were significantly lower, being of $s_{20,w}^0 = 3.1$ S and $D_{20,w}^0 = 4.5 \times 10^{-7}$ cm$^2$/s, respectively. These values lie below the dependences typical of globular proteins (see Fig. 2) indicating that the global conformation of the protein is loosening under the higher ionic strength. At intermediate ionic strengths, the sedimentation coefficient of protein S1 has been shown to change gradually from 4.6 S to 3.1 S; thus, at 25 mM NaCl with 25 mM HEPES-NaOH, pH 7.5 $s_{20,w}^0 = 3.9$ S (data
not shown). The presence of Mg$^{2+}$ in concentrations of 1 to 10 mM does not affect sedimentation behavior of protein S1.

The experimentally determined $s^{0}_{20,w}$ and $D^{0}_{20,w}$ permitted us to calculate the protein molecular mass by the Svedberg equation [16]. The calculated values were 65 kDa at the low ionic strength and 66 kDa in the presence of 100 mM NaCl; this satisfactorily agrees with the molecular mass of protein S1 determined from the amino acid sequence (60 kDa) [10]. Thus it can be concluded that in both conditions the protein exists as a monomer.

**Microcalorimetry**—Differential scanning calorimetry is a direct test for the presence of a cooperative tertiary structure in a protein molecule [15]. Using this technique the thermodynamic parameters of thermal denaturation of *T. thermophilus* ribosomal protein S1 in solution were measured (Table 1). Fig. 3 shows the temperature dependence of partial molar heat capacity of the protein both at low (in 25 mM HEPES-NaOH, pH 7.5) and moderate (in the presence of 100 mM NaCl) ionic strengths. In both cases intensive heat absorption peaks in a relatively narrow temperature range were displayed this reflecting the cooperative melting of the protein tertiary structure. The protein structure is thermostable in both solutions used: the temperature maxima of the heat absorption peaks for the transition from native to denatured state is 90.0 °C at the low ionic strength and slightly higher (91.5 °C) in the presence of 100 mM salt. The enthalpy of denaturation is also somewhat increased at the higher ionic strength. All this indicates that the observed decrease in the sedimentation coefficient in the presence of salt is not caused by the melting of the tertiary structure and the loss of the globular organization, but rather reflects the weakening of interdomain ionic interactions and drawing the globular domains apart.
The denaturation of the protein is followed by some aggregation eventually resulting in the irreversibility of the process. At the low ionic strength, the high temperature slope of the calorimetric profile is too sharp and cannot be described by any reasonable equilibrium model. This suggests either aggregation or slow kinetics of denaturation to cause noticeable distortions in the melting peak that may lead to a decrease of the denaturation enthalpy.

The situation changes at the moderate ionic strength. As seen from the curve, the high temperature slope of the profile shows the absence of the effect of slow kinetics. This allows to evaluate the complexity of the denaturation transition (number of stages) and to apply the equilibrium thermodynamics to the analysis of the calorimetric curves [15] (see Discussion).

From the melting curves of the protein S1 (Fig. 3) the values of calorimetric and effective enthalpies ($\Delta H_{\text{cal}}$ and $\Delta H_{\text{eff}}$) were calculated (Table 1). As seen from the table, in the presence of salt the ratio $\Delta H_{\text{cal}}/\Delta H_{\text{eff}} = 2.7$ is observed. This value significantly exceeds the ratio $\Delta H_{\text{cal}}/\Delta H_{\text{eff}} = 1.0$ characteristic of single-domain proteins. Hence, the result obtained gives evidence of the multidomain organization of the protein S1 [15].

DISCUSSION

The most important result of this study is the demonstration of the principal capability of ribosomal protein S1 to acquire a compact globular conformation. All previous studies of ribosomal protein S1 from *E. coli* could show only a strongly asymmetric, highly elongated shape of the protein in solution [1, 7-9], and basing on this many speculations were put forward where this unusual conformation was correlated with suspected functions of the protein on the ribosome, including the role of a lasso for catching mRNA molecules [1, 17]. At the same time, the highly elongated shape of the protein, with the dimension comparable with that of the whole
30S ribosomal subunit, did not seem to be very realistic for functioning ribosome. Nobody could observe such an elongated body on the ribosomal particle. The electron microscopy study of the RNA-dependent RNA polymerase of Qβ-infected *E. coli* cells, where ribosomal protein S1 is included as a subunit, showed very compact, globular shapes of all four subunits of the enzyme [18], suggesting the capability of the protein S1 to be stabilized in the compact conformation upon binding to functional surfaces. Similar situation favorable for acquiring compact conformation for protein S1 on the ribosome surface could not be excluded. After the demonstration of the principal capability of the *T. thermophilus* protein S1 to be compact in solution, this possibility has become very likely.

At the same time, as evidenced from the sedimentation data obtained under different ionic conditions, some parts of protein S1, even in the case of the thermostable protein, are joined together in a loose and week manner, and the links between them are easily destroyed at a moderate ionic strength. According to the thermodynamic data, however, compact globular domains are quite stable and preserved under different ionic and temperature conditions. Moreover, the melting enthalpy increases considerably with the increasing ionic strength, whereas the temperature grows only slightly. The increase of the enthalpy may be caused by hydration of the domain surface exposed upon disruption of interdomain salt bridges at increased ionic strength [19]. It is also possible that an aggregation of the protein at the low ionic strength leads to underestimating enthalpy.

As mentioned above, the situation changes with increasing ionic strength. Special calorimetric experiments demonstrate that, when the rate of heating the *T. thermophilus* protein S1 in 100 mM NaCl is changed from 0.25 to 2 K/min, the shape of its melting curve does not change (data not shown). This indicates that denaturation in such conditions is an equilibrium
process. Therefore in the first approximation it is correct in this case to use the equilibrium thermodynamic analysis [15].

Fig. 4 shows the comparison of the experimental melting curve with the curves calculated for models with a different number of cooperative thermodynamic domains. By definition, a thermodynamic domain is a minimum part of a molecular structure which can be maintained independently without other parts of the macromolecule. The model analysis allows estimating the number of such domains in the structure of a macromolecule and their relative sizes. On the other hand, the compatibility between a thermodynamic model and an experimental calorimetric curve can be tested [15].

Taking into account the presence of six homologous regions in the sequence of protein S1 [3, 4], we have tried to describe the experimental calorimetric curve by the model which includes the melting of six equal cooperative domains. Additionally, the model of three equal domains was also considered, as each two consecutive homologous regions could fuse into one domain. However as seen from Fig. 4, A and B, both models are not feasible being in conflict with the experimental data. Likewise, the two-domain model based on the knowledge of different functional positions of two parts of the molecule [1, 5, 6] cannot describe an experimental curve satisfactorily (Fig. 4C). For further analysis we suggest that domains in the model may be of an arbitrary size. Only three-domain model allows getting a quite suitable description of the experimental calorimetric curve (Fig. 4D). From the melting enthalpy of the cooperative domains we can estimate their relative size, if it is assumed that the specific melting enthalpy is nearly the same for all domains. As shown in Fig. 4D, one of the domain is twice as large as each of the two others in this case. Attempts to describe the curve by a model with a larger number of domains do not result in a really better description.
Thus, the experimental data and the model analysis suggest that protein S1 has a compact molecule structure which is divided into three thermodynamic domains. It can be thought that the six S1 RNA-binding cold-shock-like structural domains of ribosomal protein S1 [4] are somehow organized into larger cooperative blocks.

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FIGURE LEGENDS

Fig. 1. Sedimentation analysis of protein S1 from *T. thermophilus*. Sedimentation of *T. thermophilus* protein S1 at low (25 mM HEPES-NaOH, pH 7.5) (A) and moderate (in the presence of 100 mM NaCl) ionic strength (B). Centrifugation was performed at 40,000 rpm in the analytical ultracentrifuge Optima XL-I (Beckman Coulter) with UV optics. Recording was done at 4-min intervals.

Fig. 2. Dependence of the logarithm of the sedimentation coefficient (A) and diffusion coefficient (B) versus the logarithm of the molecular mass for globular proteins (solid line). (●) – globular proteins: cytochrome C (12.4 kDa, 1.8 S, \(D_{20,w}^0 = 12 \times 10^{-7} \text{ cm}^2/\text{s}\)), myoglobin (17.8 kDa, 1.98 S, \(D_{20,w}^0 = 10.3 \times 10^{-7} \text{ cm}^2/\text{s}\)), carbonic anhydrase (34.5 kDa, 3.2 S, \(D_{20,w}^0 = 9 \times 10^{-7} \text{ cm}^2/\text{s}\)), BSA (66 kDa, 4.4 S, \(D_{20,w}^0 = 5.94 \times 10^{-7} \text{ cm}^2/\text{s}\)), aldolase (149 kDa, 7.35 S), catalase (240 kDa, 11 S, \(D_{20,w}^0 = 4.1 \times 10^{-7} \text{ cm}^2/\text{s}\)), thyroglobulin (630 kDa, 19.2 S). (○) – highly asymmetrical proteins: tropomyosin (74 kDa, 2.55 S, \(D_{20,w}^0 = 2.24 \times 10^{-7} \text{ cm}^2/\text{s}\)), myosin (470 kDa, 6.4 S, \(D_{20,w}^0 = 1.16 \times 10^{-7} \text{ cm}^2/\text{s}\)). (■) – *T. thermophilus* protein S1 at low ionic strength (25 mM HEPES-NaOH, pH 7.5). (□) – *T. thermophilus* protein S1 at moderate ionic strength (25 mM HEPES-NaOH, pH 7.5, 100 mM NaCl).

Fig. 3. Dependence of the partial molar heat capacity of protein S1 from *T. thermophilus* on temperature. The curves were obtained at a heating rate 1 K/min at low (25 mM HEPES-NaOH, pH 7.5) (1) and moderate (in the presence of 100 mM NaCl) ionic strength (2).
Fig. 4. Best fits (solid curves) of the experimental excess heat capacity function (triangles) of protein S1 using four thermodynamic models. The model melting of individual domains is shown by dashed lines. A, six domains with the same enthalpy (optimum of domain stabilization enthalpy is 375 kJ/mol); B, three domains with the same enthalpy (750 kJ/mol); C, two domains with different enthalpies (694 kJ/mol and 1215 kJ/mol); D, three domains with different enthalpies (535 kJ/mol, 1188 kJ/mol and 531 kJ/mol). The experiment was done in 25 mM HEPES, 0.1 M NaCl, pH 7.5 solution.
**TABLE I.**

*Thermodynamic characteristics of denaturation for protein S1 of T. thermophilus.*

| Ionic conditions                  | \( T_m^{\text{a}} \) (°C) | \( \Delta H_{\text{cal}}^{\text{b}} \) (kJ/K mol) | \( \Delta H_{\text{eff}}^{\text{c}} \) (kJ/K mol) | \( n \) (\( \Delta H_{\text{cal}}^{\text{b}}/\Delta H_{\text{eff}}^{\text{c}} \)) |
|-----------------------------------|-----------------------------|---------------------------------|---------------------------------|---------------------------------|
| 25 mM HEPES- NaOH, pH 7.5         | 90.0                        | 1400                            | 800                             | 1.7                             |
| 25 mM HEPES- NaOH, pH 7.5, 100 mM NaCl | 91.5                        | 2210                            | 840                             | 2.6                             |

\( ^{\text{a}} \) Temperature of the peak maximum.

\( ^{\text{b}} \) Calorimetric enthalpy.

\( ^{\text{c}} \) Van’t Hoff enthalpy. It was calculated as \( \Delta H_{\text{eff}} = \frac{4RT_m^2}{\Delta H_{\text{cal}}} C_{p, \text{max}} \), where \( C_{p, \text{max}} \) is the excessive heat capacity at the peak maximum, \( R \) is the gas constant.
Fig. 1

Absorbance (280 nm) vs. Radius, cm

Absorbance (280 nm) vs. Radius, cm
Fig. 2
Fig. 3
Fig. 4
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