# PROTON MAGNETIC RESONANCE STUDIES OF *ESCHERICHIA COLI* RIBOSOMAL PROTEIN S16

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## 1. Introduction

The ribosomal protein S16 has a sequenced molecular weight of 9191 [1]. Physical studies with this protein have revealed it to be fairly compact. Low angle X-ray scattering experiments suggest it could be represented as a prolate ellipsoid of dimensions 86 X 24 X 24 Å, with a $R_g$ of 21 Å [2] and by neutron scattering experiments as a compact globular structure with $R_g$ 12 Å [3]. Immunological studies [4] have located two binding sites for S16 on the body of the 30 S subunit in close vicinity to each other (double sites). By singlet energy transfer of fluorescently labelled proteins, a mixture of S16/S17 was found to be in close contact to proteins S4, S15 and S20 [5,6]. Mutants of *E. coli* have been isolated [7,8] which have an altered S16 protein.

A mixture of S16/S17 has been reported to bind specifically to 16 S RNA [9]. In our hands when these two proteins are purified S16 does not bind to 16 S RNA whereas S17 does bind [10] specifically at 1:1 RNA : protein. The protein used here was purified by a salt extraction technique that avoids the use of acetic acid, urea or lyophilization [11]. The S16 thus prepared has been shown to contain more tertiary structure as revealed by PMR methods than that isolated in the presence of acetic acid and urea [12].

This paper describes other structural studies using PMR. These demonstrate that the protein S16 is a stable, globular protein with a well-ordered tertiary structure.

## 2. Methods

Salt-extracted protein S16 was obtained by removal of the protein from the 30 S subunit with 1 M LiCl followed by CM–Sephadex C-25 chromatography using a LiCl gradient. Further purification was obtained by gel filtration on Sephadex G-75 superfine in 0.4 M LiCl [11]. Here the use of urea, low pH, and lyophilization, conditions which could cause protein denaturation, were avoided. The protein was concentrated by dialysis against Ficoll 400 using Spectrapor 3 or Spectrapor 6 dialysis tubing cutoff (mol. wt 3500 and 2000, respectively; obtained from Spectrum Medical Industries, Los Angeles). The identity and purity of the protein was established by two-dimensional electrophoresis [13,14] (the latter method, where the second dimension was with SDS, separated this protein well) and by one-dimensional SDS–slab-gel electrophoresis [15]. Since S16 and S17 have been difficult to separate by previous methods (reviewed [16]) their identity was also checked by amino acid analysis and cross-reaction against antibody raised against protein S16 (G. Stöffler, personal communication).

For PMR experiments the protein was concentrated to 1–5 mg/ml as above and dialysed against 0.35 M KCl, 0.05 M potassium phosphate (pH 7.0), $5 \times 10^{-4}$ M dithioerythritol, $1 \times 10^{-4}$ M benzamidine in $^2H_2O$. The protein solution was centrifuged and transferred into standard 5 mm NMR tubes. Spectra were recorded at 270 MHz on a Bruker WH 270 magnetic resonance spectrometer operating in Fourier transform mode, using a pulse angle of 60° (12 μs) and data collection over 0.5 s for each pulse. Spectra were obtained at 19–20°C (unless otherwise stated) over 2–4 h. The free induction decay pattern was multiplied by an exponential function equivalent to a line broadening of ~2 Hz. Chemical shifts were measured relative to sodium 2.2, dimethyl-2-silapentane sulphonate (DSS). After the PMR spectra were recorded the proteins were checked for proteo-

lytic degradation by SDS–slab-gel electrophoresis [15]. Protein concentrations were determined by nitrogen assay [17].

Renaturation/denaturation experiments were performed by preparing and recording a spectrum as above, then adding solid $^2$H-urea (Aldrich Chemicals) directly to the NMR tube up to 6 M. The spectrum was recorded once more, after which the sample was removed from the NMR tube and dialysed (using Spectrapor 3 dialysis tubing) against 3 × 50 ml 0.35 M KCl, 0.05 M potassium phosphate (pD 7.0), $5 \times 10^{-4}$ M dithioerythritol, $1 \times 10^{-4}$ M benzamidine in $^2H_2O$. The PMR spectrum was recorded once again.

pH titration of protein S16 was made by slowly adjusting the pD of the solution, 0.35 M KCl, 0.01 M potassium phosphate, dithioerythriol, benzamidine as above, by the introduction of NaOD or DCl as appropriate directly to the NMR tube. The pH was measured using a microelectrode which extended into the bottom of the NMR tube.

The heat denaturation study of protein S16 was performed in the NMR machine by slowly heating the sample to the appropriate temperature, waiting for 20 min for the temperature to stabilize and recording the spectrum as above. After reaching 79°C and recording of a spectrum the sample was allowed to cool slowly overnight, the spectrum being recorded at 19°C the following day.

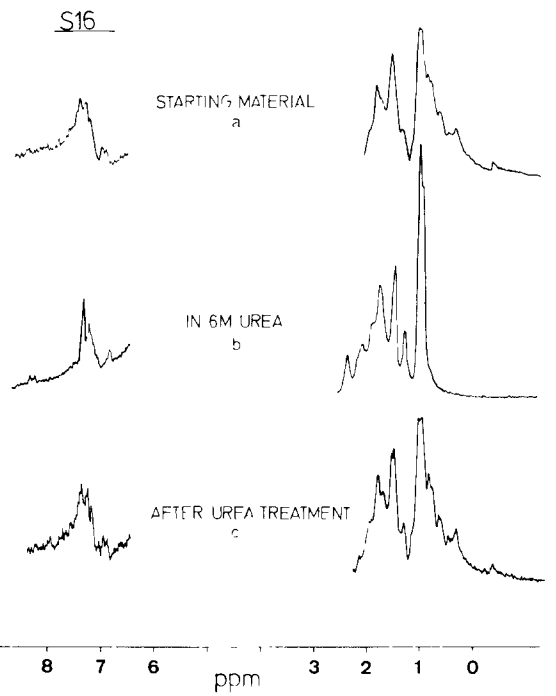


Fig.1. 270 MHz PMR spectra of protein S16; (a) starting material in 0.05 M potassium phosphate (pD 7.0), 0.35 M KCl; (b) S16 in 6 M urea, 0 05 potassium phosphate (pD 7.0), 0.35 M KCl; (c) sample (b) dialysed against 0.05 M potassium phosphate (pD 7.0), 0.35 M KCl.

## 3. Results

The PMR spectrum of protein S16 showed the presence of considerable amounts of tertiary structure as seen from the ring current-shifted resonances in the apolar region of the spectrum (fig.1a). These shifts are brought about by the interaction of aromatic amino acids with apolar groups as a result of the tertiary structural folding of the protein chain. A considerable amount of line broadening is seen in the PMR spectrum which could also be indicative of tertiary structure. When this protein is made 6 M with respect to urea the spectrum is changed such that all of the shifted resonance peaks disappear and the spectrum becomes sharper due to the unfolding of the protein chain (fig.1b). The two histidine residues at 8.3 and 8.4 ppm are still separated which could reflect the different environment of these residues in the protein primary structure; His-9 is found in a more strongly apolar region than His-59. However the presence of some residual structure of the protein in 6 M urea cannot be ruled out. When the urea is removed from the protein sample the spectrum returns back to that obtained before the urea treatment although the signal/noise is slightly reduced due to some precipitation of the protein during refolding. This precipitate was removed by centrifugation before the spectrum was recorded. The protein S16 can fold back to the same tertiary structure, as observed from the PMR spectrum, after treatment with 6 M urea at pH 7.0. However this effect may not be a general property of all ribosomal proteins, they may vary from one to another. The protein L11 is an example of the case where the protein appears unable to completely refold after treatment as above [18].

When protein S16 is titrated by varying the buffer solution from pD 8.0–4.0 (fig.2), the two histidine residues at 7.5 ppm slowly titrate independently due to their different environment. The $pK_a$ values of these residues cannot be determined due to acid denaturation of the sample but it is likely that His-9

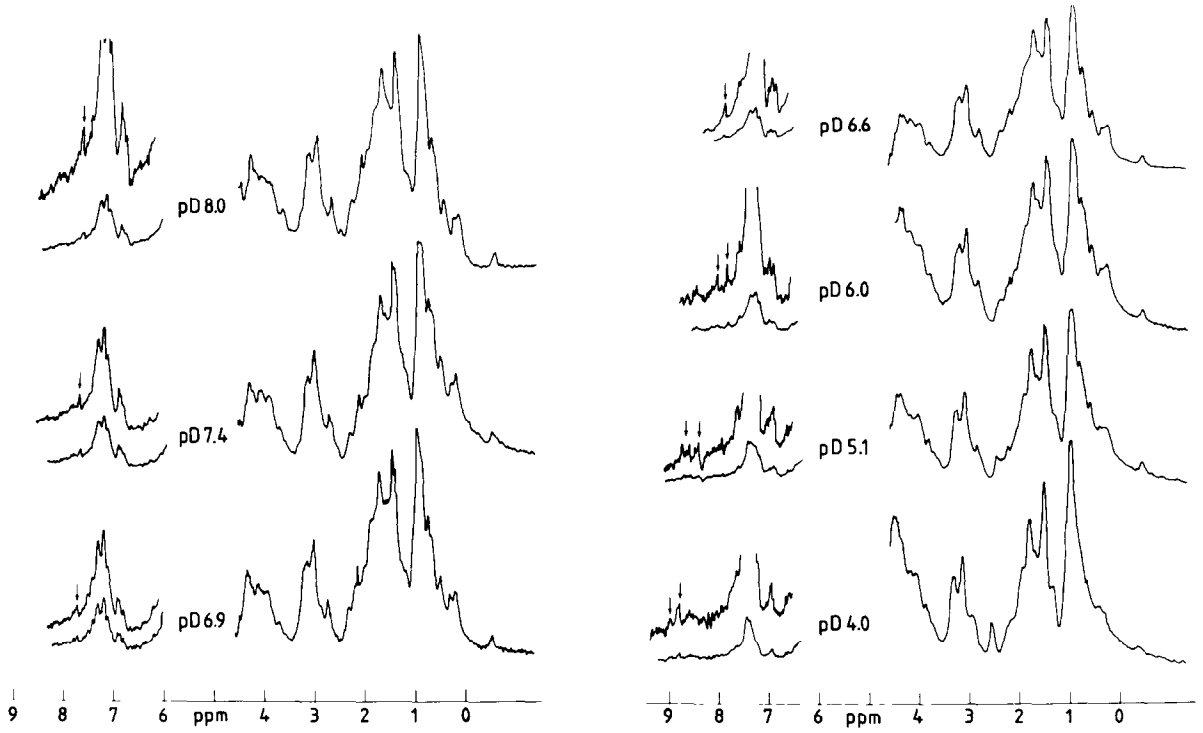


Fig.2. 270 MHz PMR spectra of protein S16 in 0.05 M potassium phosphate, 0.35 M KCl, pD 8.0–4.0 as indicated. The arrows indicate the position of the histidine residues in the spectrum.

has a lower p$K_a$ than His-59 due to its apolar environment within the sequence. A slow unfolding of the tertiary structure occurs at pH <6.0 with a disappearance of the ring current-shifted resonances in the apolar region of the spectrum. Little titration of the histidine residues occurs before the start of the unfolding process. When the sample was returned to pD 7.4 after treatment at pD 4.0 the protein refolded almost completely to the original pD 7.4 spectrum.

Protein S16 appears to be extremely stable to increase in temperature. It can be seen from fig.3 that only when the temperature has reached 79°C is the protein completely melted. After slow cooling it appears that some of the protein is refolded to the correct structure. The same shifted resonances are present as seen in the original spectrum but their amount is reduced. The melting properties of protein S16 and the partial reversal of this process is in agreement with microcalorimetry studies performed with this protein [19] where it was possible to calculate the enthalpy of the transition per mole of the cooperative region of the melting profile (50 kcal/mol).

The folded nature of this protein is also in agreement with studies using mild proteolytic digestion to look for structural domains in ribosomal proteins [20]. In contrast to other ribosomal proteins, the protein S16 along with two others, namely S15 and S17, was not digested under the conditions employed (enzyme : protein, 1:100, 0°C, 60 min). Therefore it is one of the most resistant proteins in the small 30 S subunit. Clearly protein S16 is a highly structured protein in solution. Other proteins however appear less compact and many consist of more than one domain. Many proteins have been shown to produce a resistant fragment after mild proteolytic digestion [20]. In the case of protein S4 a C-terminal fragment can be isolated which contains most of the structure of the intact molecule as observed from PMR studies [21]. The N-terminal appears to be available for proteolytic digestion and probably interacts with other proteins in the intact ribosome [22]. This protein is not as thermostable and aggregates at >40°C [23]. Also the refolding of the protein after exposure to urea or acidic pH is not as complete as that

observed with protein S16 [23]. Circular dichroism experiments [24] show S16 to have a well-defined secondary structure with 45% α-helix content.

In summary:

(1) Protein S16 has a highly folded tertiary structure as seen from the PMR spectra;

(2) It is capable of refolding after brief treatment with 6 M urea at pH 7.0.

(3) His-9 and His-59 titrate independently due to their different environment in the protein structure. The protein is partially unfolded at pH 4.0 and does refold almost completely on returning to pH 7.4.

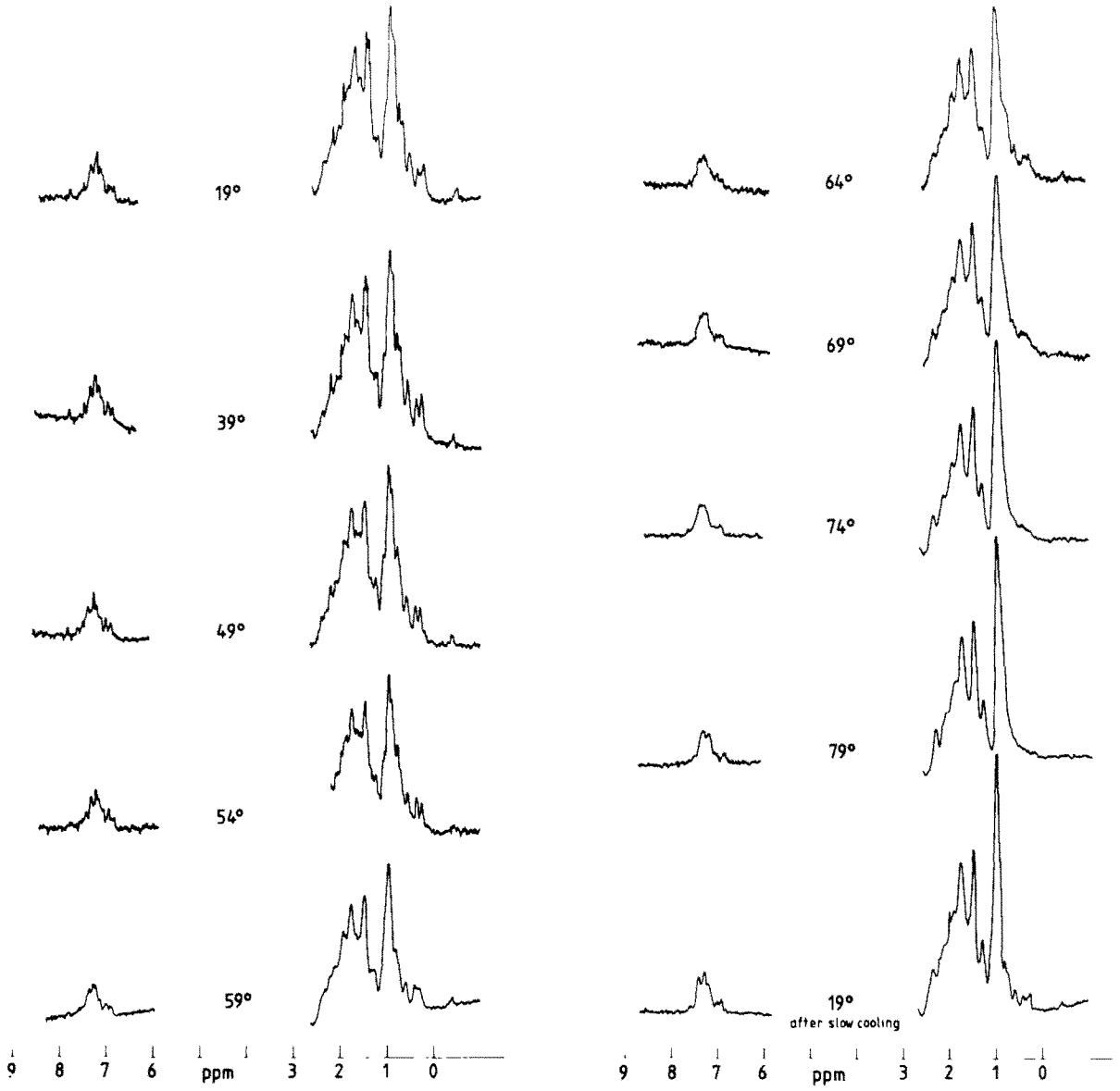


Fig.3. 270 MHz PMR spectra of protein S16 in 0.05 M potassium phosphate (pD 7.0), 0.35 M KCl. The spectra were recorded at 19–79°C as indicated.

(4) It has a highly stable structure which does not melt until 79°C. This melting is partially reversible under the conditions tested.

Clearly the PMR spectrum of protein S16 shows at least 5 shifted resonances. For such a small protein, 9191 mol. wt with only 6 aromatic residues, assignment of these shifted resonances can be made and the folding of the protein chain determined. This work is now in progress.

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