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# Binding of *Escherichia coli* Ribosomal Protein S8 to 16S rRNA: Kinetic and Thermodynamic Characterization†

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*Received June 17, 1985; Revised Manuscript Received December 18, 1985*

ABSTRACT: A sensitive membrane filter assay has been used to examine the kinetic and equilibrium properties of the interactions between *Escherichia coli* ribosomal protein S8 and 16S rRNA. In standard conditions (0 °C, pH 7.5, 20 mM $Mg^{2+}$, 0.35 M KCl) the apparent association constant is $5 \pm 0.5 \times 10^7$ $M^{-1}$. The interaction is highly specific, and the kinetics of the reaction are consistent with the apparent association constant. Nevertheless, the rate of association is somewhat slower than that expected for a diffusion-controlled reaction, suggesting some steric constraint. The association is only slightly affected by temperature ($\Delta H$ = −1.8 kcal/mol). The entropy change [$\Delta S$ = +29 cal/(mol K)] is clearly the main driving force for the reaction. The salt dependence of $K_a$ reveals that five ions are released upon binding at pH 7.5 and in the presence of 10 mM magnesium. The substitution of various anions for $Cl^-$ has an appreciable effect on the magnitude of $K_a$, following the order $CH_3COO^- > Cl^- > Br^-$, thus indicating the existence of anion binding site(s) on S8. An equal number of ions were released when $Cl^-$ was replaced by $CH_3COO^-$, but the absence of anion release upon binding cannot be excluded. On the other hand, the free energy of binding appears not to be exclusively electrostatic in nature. The effect of pH on both temperature and ionic strength dependence of $K_a$ has been examined. It appears that protonation of residue(s) (with $pK \simeq 9$) increases the affinity via a generalized charge effect. On the other hand, deprotonation of some residue(s) with a $pK \simeq 5$–6 seems to be required for binding. Furthermore, the unique cysteine present in S8 was shown to be essential for binding.

The interactions between RNAs and proteins play an essential role at most levels of gene expression in the cell. However, the comprehension of the molecular mechanisms of recognition and interactions remains a crucial problem. The ribosomal machinery, which decodes the message into proteins, is itself a highly complex multimolecular assembly of several RNAs and proteins and offers possibilities to study in detail the mechanisms of RNA–protein association. In the ribosome, primary binding proteins are of particular interest. They directly interact with ribosomal RNAs and are especially important in ribosome assembly and presumably in stabilizing structural domains. Some of them are even involved in the feedback translational regulation of groups of ribosomal

† This work was supported by the Centre National de la Recherche Scientifique and the Délégation à la Recherche Scientifique et Technique.

0006-2960/86/0425-2756$01.50/0 

proteins (Nomura et al., 1982). Some experimental factors affecting RNA binding have previously been investigated for several ribosomal proteins (Schulte & Garrett, 1972; Schulte et al., 1974; Spierer & Zimmermann, 1978; Spierer et al., 1978). Apparent association constants have been estimated by using various technical approaches such as nitrocellulose filter assays, ethidium bromide fluorescence, or low-angle X-ray scattering (Feunteun et al., 1975; Osterberg & Garrett, 1977; Spierer et al., 1978; Schwarzbauer & Craven, 1981). All estimates for $K_a$ fall in the range $10^6$–$10^8$ $M^{-1}$. Very little is known, however, about either the kinetics of reaction or the relative contribution of the different types of contacts on the binding free energy.

Protein S8 is one of the proteins that directly bind to 16S ribosomal RNA in the early stage of ribosome assembly. It also interacts with its own messenger and regulates the synthesis of several proteins of the *spc* operon (Olins & Nomura, 1981). The RNA binding site of S8 is small and is one of the best characterized. Identification was mainly by sequencing 16S rRNA[1] fragments protected against ribonuclease digestion by bound S8 (Schaup et al., 1973; Ungewickell et al., 1975; Zimmermann et al., 1975; Zimmermann & Singh-Bergmann, 1979). The binding site for S8 was located in the central domain of 16S rRNA, in an irregular helical structure encompassing nucleotides 587–652. The smallest protected fragments extend from nucleotide G-587 to U-603 and from A-635 to U-652, corresponding to the bottom part of the helix. A damage selection procedure has been used to identify those nucleotides that are required in unmodified form for specific binding (Thurlow et al., 1983). Sequence and secondary structure homologies were found in the putative S8–mRNA binding site involved in the translational regulation of the *spc* operon (Olins & Nomura, 1981).

Despite a considerable amount of work, the molecular basis for S8–16S rRNA interaction is poorly understood yet. In particular, what types of contacts are established between the protein and the RNA, what is the relative contribution of these various contacts to the binding free energy, and may conformational rearrangement of either protein or RNA accompany complex formation? In this work, we have determined both the kinetic and thermodynamic parameters of the interaction. The association constant was measured under a variety of solution conditions. The data are interpreted in terms of ion release and possible types of contacts established between the protein and the RNA. The findings for S8–16S rRNA interaction are compared to other results for both DNA– and RNA–protein interactions.

## Experimental Procedures

*Preparation of Ribosomal Components.* 70S ribosomes and ribosomal subunits were prepared from *Escherichia coli* MRE600 according to the procedure devised by Traub et al. (1971) for large-scale preparations, with modified buffers (Vassilenko et al., 1981). 70S ribosomes were pelleted by centrifugation in 20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 60 mM $NH_4Cl$, 6 mM 2-mercaptoethanol, and 10% sucrose. Subunits were fractionated on 10–30% sucrose gradients in 20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 60 mM $NH_4Cl$, 0.4 M NaCl, and 6 mM 2-mercaptoethanol, in a fixed-angle rotor (Ti 60). Fractions containing 30S subunits were pooled and precipitated with 0.65 volume of ethanol at 0 °C for 30 min.

Proteins were extracted from 30S subunits with acetic acid (Kurland et al., 1971), and S8 was fractionated by phosphocellulose chromatography in 6 M urea, 0.05 M sodium phosphate, pH 5.8, and 2 mM 2-mercaptoethanol (Hardy et al., 1969). In the latest experiments, S8 was fractionated by FPLC (Pharmacia) on a Mono S HR 5/5 column in the same urea–phosphate buffer. Fractions containing the isolated protein were pooled and concentrated against dry Sephadex G25. Protein was analyzed for purity and identified on SDS–polyacrylamide slab gel electrophoresis as described by Laemmli and Favre (1973); protein assignment was made in reference to total 30S proteins according to Littlechild and Malcolm (1978). Concentration of the protein was estimated by spectrophotometric measurement of binding of Coomassie blue (Rylatt & Parish, 1982), using BSA as reference. Whenever possible, $A_{280}$ was measured and the concentration of the protein was evaluated; the molar absorption coefficient calculated as described by Edelhoch (1967) was used. The calculated value was 3840 $M^{-1}$ $cm^{-1}$ with a molecular weight of 13 996 (Allen & Wittmann-Liebold, 1978).

Purified S8 protein was labeled in vitro by reductive methylation using [$^3$H]$NaBH_4$, according to Held et al. (1974) and modified as follows. The protein was first dialyzed against 0.2 M borate, pH 8.4, and 0.35 M KCl and reacted with formaldehyde. The protein concentration was approximately 40 $\mu$M and the formaldehyde/lysine ratio was 5. Reduction was with [$^3$H]$NaBH_4$ (Amersham, 5–20 Ci/mmol). Solid borohydride was put in solution in 0.5 M KOH before use. The final pH was 9 and the $NaBH_4$/HCHO ratio was (2.5–3)/1. Unreacted low molecular weight products were removed by extensive dialysis against urea–phosphate buffer, pH 5.8, and the alkylated protein was further purified by FPLC on a Mono S HR 5/5 column, as described above. The protein was concentrated and dialyzed against 20 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, and 0.35 M KCl. Specific activity of the protein was determined by measuring $^3$H activity and final protein concentration; it ranged from 5 $\times$ $10^8$ to 10 $\times$ $10^8$ cpm/$\mu$mol.

*E. coli* 16S rRNA was isolated from 70S ribosomes by centrifugation on sucrose gradients containing SDS (Thurlow et al., 1983). 16S rRNA was precipitated 3 times with ethanol to remove SDS. The final ethanol precipitate was washed with ethanol, dried, and dissolved in doubly distilled water. The concentration of RNA was determined by assuming that one $A_{260}$ unit of RNA corresponds to 75 pmol.

*Formation of Protein–RNA Complex.* Protein S8 and 16S rRNA were preincubated separately for 1 h at 40 °C in the reconstitution buffer (TMK) for renaturation. Standard TMK buffer was 20 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 0.35 M KCl, 5 mM DTT, and 3 $\mu$M BSA. Modifications of this buffer will be indicated in the text. For the various buffers used here, the pH was adjusted at the temperature used in binding incubation. After being cooled to 0 °C or other specified temperatures, 16S rRNA and protein were mixed and incubated for 30 min in a standard assay. Each assay was made in duplicate; the incubation volume was 300 $\mu$L and contained 8000–10 000 cpm. The concentration of S8 in the assay was usually (2–4) $\times$ $10^{-8}$ M.

*Analysis of Protein–RNA Complex by Filter Assay.* Protein–RNA complexes were purified from unbound protein by nitrocellulose membrane filtration. It was shown (Spicer et al., 1977) that free ribosomal proteins are retained by nitrocellulose membranes while 16S rRNA and protein–RNA complexes pass through the filter. Nitrocellulose filters

[1] Abbreviations: rRNA, ribosomal RNA; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; BSA, bovine serum albumin; FPLC, fast-protein liquid chromatography.

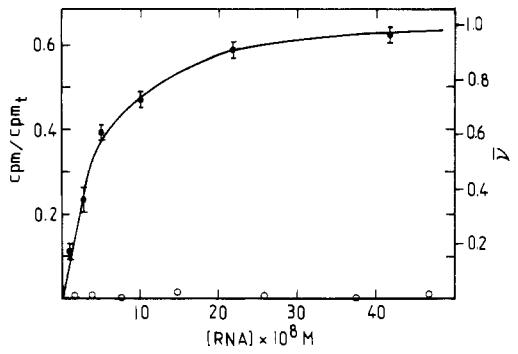


FIGURE 1: Saturation binding curve for S8–16S rRNA interaction. Increasing concentrations of RNA were incubated in standard conditions with $4.8 \times 10^{-8}$ M $^3$H-labeled protein: 16S rRNA (●); 23S rRNA (○). Binding is expressed as cpm/cpm$_t$, corresponding to the ratio between the radioactivity measured in the filtrate and the input. Alternatively, binding is expressed as $\bar{\nu}$ = moles of RNA bound per mole of active protein.

(Millipore, type HA, 45-μm pore size, 25-mm diameter) were soaked for 30 min in TMK buffer before use and stripped of excess buffer. The reaction mixture (300 μL) was filtered at the incubation temperature without any dilution or subsequent washing in less than 10 s. A 200-μL aliquot of filtrate was collected and counted for $^3$H in Beckman Ready-solv MP. In each experiment, control assays were done in the absence of 16*S* rRNA. Approximately 3% of total cpm were found in the filtrate. Bound cpm were corrected accordingly.

*Quantitative Determination.* The various equations were calculated by using a linear least-squares analysis of data points.

## RESULTS

*Apparent Association Constant, Stoichiometry, and Specificity.* The interaction between S8 and 16S rRNA was measured by using a nitrocellulose filter binding assay. A constant concentration of $^3$H-labeled protein was incubated with increasing concentrations of 16S rRNA for 30 min at 0 °C. Both RNA and protein were preincubated for 1 h at 40 °C. The S8–16S rRNA complex shows the unusual property of not being retained on the filter. This was shown to be the case for all complexes between 16S rRNA and primary binding proteins (Spicer et al., 1977; Schwarzbauer & Craven, 1981). The free protein being retained on the filter, the protein–RNA complex was evaluated by measuring aliquots of filtrate.

A typical saturation curve is shown in Figure 1. The fraction of protein bound at the plateau was generally 65% of input protein. This incomplete saturation can be the result of (i) partial loss of binding capacity of the preparation, (ii) partial dissociation of the complex on the filter, or (iii) partial retention of the complex on the filter. Nevertheless, an almost complete saturation of the 16S rRNA input was obtained when association was measured between a constant RNA concentration and varying concentrations of S8 (results not shown). Thus, partial dissociation or retention of the complex on the filter can be excluded. This incomplete protein saturation most likely results from the irreversible denaturation of a part of the protein population during the extraction procedure, the various steps of fractionation, and labeling.

The apparent association constant and the stoichiometry of the complex were determined by plotting data according to the double-reciprocal and Scatchard equations (Edsall & Wyman, 1958; Scatchard et al., 1957). The analysis of six independent determinations indicates that S8 binds to 16S rRNA with an apparent association constant ($K_a$) of 5 ± 0.5 × $10^7$ $M^{-1}$. Assuming that the percentage of bound protein at the plateau represents complete binding of active protein and correcting the protein concentration accordingly (by a factor of 0.65 in these experiments), the number of binding sites was evaluated to be 1. Our results indicate that the S8–16S rRNA complex is formed at a 1:1 ratio.

The association constant was also measured by using a sedimentation assay; the complex was pelleted, and the free protein was evaluated by measuring aliquots of supernatant. A similar value of $K_a$ ($4 \pm 1 \times 10^7$ $M^{-1}$) was found by this method, providing further evidence that filtration does not displace the equilibrium and is thus capable of measuring correct binding.

Bovine serum albumin was added to the incubation mixture to minimize sticking of S8 to the walls of the tubes. Complex formation was measured with BSA added in the range of 1.5–12 μM. The amount of binding was found to be independent of the presence of BSA within this range, thus showing the absence of competition for 16S rRNA binding.

The specificity of binding was tested by incubating S8 with increasing concentrations of noncognate RNA (23S rRNA). As shown in Figure 1, no complex formation was detected. Further experiments were made, in which 23S rRNA was added in a 0.5–10 molar excess over 16S rRNA. Addition of 23S rRNA was found not to alter the amount of complex formed. Our results confirm the high specificity of S8 binding to 16S rRNA as already observed with other primary proteins that have specific sites on the 16S rRNA.

The question arises as to whether the binding properties of S8 might be altered by the labeling procedure. Reductive methylation was found to produce minimal changes in the structure of most proteins (Rice & Means, 1971; Means & Feeney, 1968). It was shown that labeled subunits or 70S ribosomes retain full activity (Moore & Crichton, 1973) and that the labeling procedure does not alter the capacity of ribosomal proteins to be assembled (Held et al., 1974). Assuming that the 12 lysine residues are evenly methylated, we calculated that less than one lysine ($\simeq$0.5) is statistically modified per one S8 molecule. Therefore, we made the reasonable assumption that the binding constant measured is not significantly altered by the labeling procedure.

*Thermal Renaturation.* Subunit reconstitution and protein–rRNA complex formation are usually performed by incubating RNA and protein at 42 °C. However, the previous work of Schulte and Garrett (1972) suggested that this high temperature was necessary for S8 to refold into an active conformation. Experiments were made in order to distinguish whether this high temperature is required for binding or for some renaturation process of either S8 or 16S rRNA. First, RNA alone was incubated for 1 h at 40 °C, cooled at 0 °C, and mixed with the unheated protein. Second, S8 alone was incubated for 1 h at 40 °C, cooled at 0 °C, and mixed with unheated RNA. Third, S8 and 16S rRNA were incubated separately for 1 h at 40 °C, cooled at 0 °C, and mixed. In all three cases, the RNA–protein mixture was further incubated for 30 min at 0 °C, and the bound protein was measured by filtration. Two control experiments were done in which RNA and protein were directly mixed and incubated at 40 or 0 °C for 1 h. Complete saturation curves were made, and the apparent association constants were determined. The results are shown in Table I. It appears that S8, either preheated or not preheated, retains the same binding affinity, while the fraction of protein bound at the plateau is reduced when the protein is not preheated. A slight decrease in the affinity constant was observed when 16S rRNA was not

Table I: Effect of Thermal Renaturation on Binding Affinity[a]

| complex | $K_a \times 10^{-7}$ ($M^{-1}$) | fraction of protein bound at the plateau |
|---|---|---|
| $S8^+$, $RNA^+$ (0 °C) | 5.2 ± 0.4 | 0.66 |
| $S8^-$, $RNA^+$ (0 °C) | 5.6 ± 0.5 | 0.45 |
| $S8^+$, $RNA^-$ (0 °C) | 4.1 ± 0.5 | 0.65 |
| $S8^-$, $RNA^-$ (0 °C) | 4.2 ± 0.5 | 0.44 |
| $S8^-$, $RNA^-$ (40 °C) | 3.4 ± 0.4 | 0.65 |

[a] The association constant was calculated from complete saturation curves. Preincubation at 40 °C for 1 h is indicated by (+); absence of preincubation is denoted by (−). The temperature of complex formation is indicated.

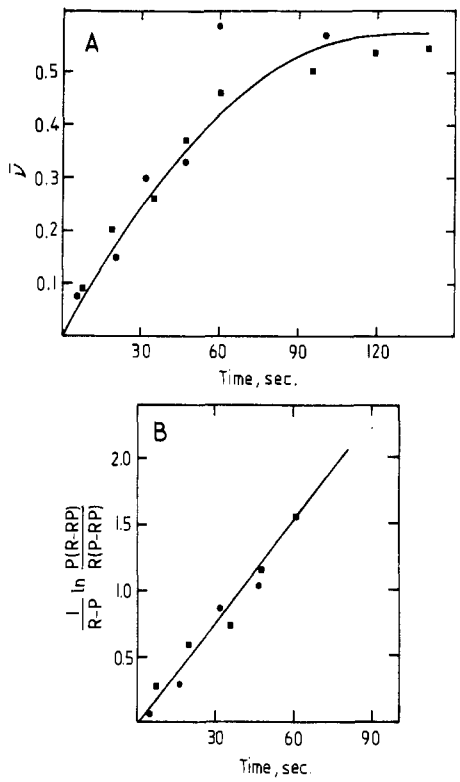


FIGURE 2: (A) Kinetics of formation of S8–16S rRNA complex at 0 °C. RNA concentration ($5 \times 10^{-8}$ M) was chosen to give approximately half-saturation of S8 ($3.8 \times 10^{-8}$ M). At the indicated times, 300-μL aliquots were withdrawn and filtered. Two independent experiments are reported (●, ■). (B) Bimolecular rate parameter [defined in eq 7 of Riggs et al. (1970)] calculated for points ● and ■ shown in (A). The line is $k_{on} = 2.5 \times 10^5$ $M^{-1}$ $s^{-1}$.

preheated. On the other hand, S8 and 16S rRNA interact with a reduced binding affinity when the complex was made at 40 °C. From these results, we can conclude that 40 °C is not the optimal temperature of binding but that preincubation at 40 °C increases the fraction of active protein in the medium. Nevertheless, a complete activity was not restored by this preheating step. Actually, this level of renaturation was already reached after 30 min of preincubation and could not be increased, even after a prolonged time of incubation (3 h).

Further experiments were made to check the stability of both RNA and protein after the preincubation step. Both 16S rRNA and S8 were kept at 4 °C for 5 h after the heating step and were tested for binding. The affinity constant, as well as the fraction of active protein, was found unaltered. This indicates that both components remain stable during this period, which exceeds the time of experiment. We also found that the extent of renaturation was not affected by salt concentration. However, the fraction of protein that could be renatured under standard conditions was reduced at suboptimal

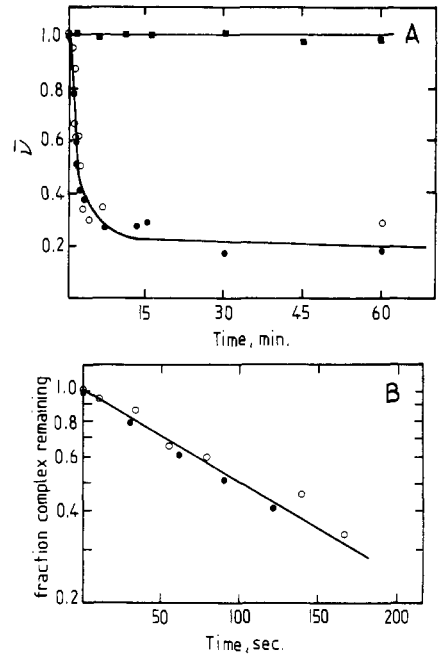


FIGURE 3: (A) Dissociation kinetics of S8–16S rRNA complex at 0 °C. The complex was formed between $^3$H-labeled S8 ($3.8 \times 10^{-8}$ M) and 16S rRNA ($5 \times 10^{-5}$ M). Equilibrium was reached after 30-min incubation. Dissociation was measured either by a 50-fold dilution of the complex with cold TMK buffer (●) or by addition of a 45-fold excess of unlabeled S8 (○). Addition of buffer or unlabeled protein was at zero time. At the indicated times, aliquots were withdrawn and filtrated. In a control experiment, the complex was incubated without any dilution or addition, during the time of the experiment (■). (B) Initial rate of dissociation [eq 4 of Riggs et al. (1970)] for points in (A). The line is $6.9 \times 10^{-3}$ $s^{-1}$.

pH. In the following experiments, complete saturation curves were done for each determination of the binding constant, thus allowing us to evaluate the extent of saturation and to calculate correct $K_a$ values.

*Kinetics.* The association rate constant between protein S8 and 16S rRNA was measured at 0 °C in standard TMK buffer. The protein ($3.8 \times 10^{-8}$ M) was mixed with 16S rRNA ($5 \times 10^{-8}$ M), and aliquots were withdrawn and filtered after short time intervals. Results from two distinct experiments are shown in Figure 2A. A plateau was reached at about 100 s. The association rate constant was estimated from the initial rate data by applying the integrated rate equation for a bimolecular reaction [e.g., see eq 7 of Riggs et al. (1970)]. The solid line shown in Figure 2B is $k_{on} = 2.5 \pm 0.1 \times 10^5$ $s^{-1}$ $M^{-1}$.

The dissociation rate of the S8–16S rRNA complex was measured at 0 °C by two different methods. In both cases, the complex was formed by incubating RNA ($5 \times 10^{-8}$ M) and S8 ($3.8 \times 10^{-8}$ M) at 0 °C for 30 min. In the first method, dissociation of the complex was obtained by diluting the reaction mixture 50-fold with cold TMK buffer, and aliquots were withdrawn and filtered at time intervals until the new equilibrium was reached. In the second method, a 45-fold excess of unlabeled protein was added, and samples were withdrawn and filtered at various time intervals until a new equilibrium was established. Dissociation, measured by the two methods, is shown in Figure 3A. A control experiment, in which the complex was incubated without dilution or addition of cold protein, showed that the complex remained stable during the time of the experiment. Both methods gave the same results. A new equilibrium was reached after about 15 min. The dissociation constant was deduced from the initial rate data [e.g., see eq 4 of Riggs et al. (1970)]. From the slope of Figure 3B, a value of $k_{off} = 6.9 \pm 1 \times 10^{-3}$ $s^{-1}$ can be

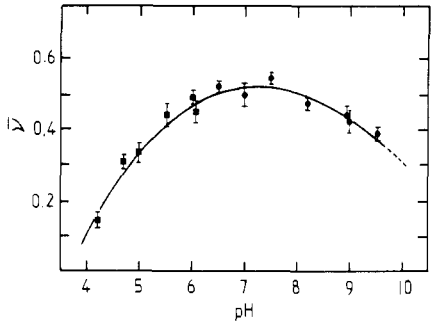


FIGURE 4: pH dependence of complex formation. The complex was formed between $^3$H-labeled S8 ($3 \times 10^{-8}$ M) and 16S rRNA ($5 \times 10^{-8}$ M). Incubation was at 0 °C, for 1 h, in 20 mM magnesium acetate, 0.35 M KCl, 5 mM DTT, and either Tris-HCl (●) or Mes (■) adjusted to the indicated pH.

calculated, corresponding to a half-life of about 100 s. The calculated value for the association constant, obtained by dividing the forward rate by the dissociation rate, is $3.6 \times 10^7$ $M^{-1}$. This is in good agreement with the measured association constant.

*pH Dependence of Binding.* The pH dependence of S8–16S rRNA interaction was studied by measuring the binding at different pH values. Thermal renaturation and binding were achieved in TMK buffer adjusted to the desired pH at 0 °C over a range of 4–9.5. It was verified that ionic strength was not changed in the various incubation mixtures investigated. More basic pH values were not investigated since significant hydrolysis of RNA was expected to occur. The RNA concentration was $5 \times 10^{-8}$ M. Results are presented in Figure 4. A maximum binding was observed over the pH range 6.5–8. The shape of the curve suggests that binding is influenced by two types of titrable groups. For optimal binding, some group(s) with a p$K$ near 5–6 must be deprotonated whereas some other group(s) with a p$K$ near 9 have to remain protonated.

Record et al. (1978) described two limiting models for pH dependence that can be distinguished on the basis of their responses to salt concentration, pH, and temperature. In the first model (titration curve model), protonation events are not absolutely required for binding but increase the affinity via a generalized charge effect. In such a model, the prediction is that d log $K_{obsd}$/d log [$M^+$] will be a function of pH and that d log $K_{obsd}$/d$T$ will be insensitive to pH. The second model assumes that there is a requirement for protonation—or deprotonation—of certain group(s) to form the complex; it predicts that d log $K_{obsd}$/d log [$M^+$] will be independent of pH and that d log $K_{obsd}$/d$T$ will be sensitive to change in pH. The result of such an analysis will be reported below.

*Magnesium Dependence of Binding.* Binding of S8 to 16S rRNA was measured as a function of magnesium concentration. A series of buffers were prepared which contained 20 mM Tris-HCl, pH 7.5, 0.35 M KCl, 5 mM DTT, 3 μM BSA, and magnesium acetate at various concentrations. Concentrations below 0.4 mM were not investigated since this concentration was already brought by the TMK buffer used for storing the protein and introduced in the reaction mixture. The binding of RNA ($5 \times 10^{-8}$ M) was measured. Results are shown in Figure 5 for a range of 0.4–50 mM magnesium acetate. Maximum binding occurred between 50 and 10 mM, and complex formation gradually decreased as the magnesium concentration was lowered.

*Salt Dependence of Binding.* The dependence of $K_a$ on monovalent ion concentration has been determined in various buffers, with $K^+$ as the cation and either $Cl^-$ or $CH_3COO^-$

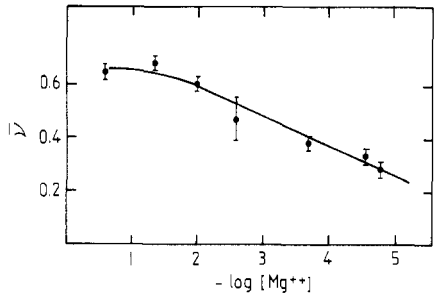


FIGURE 5: Magnesium dependence of complex formation. The complex was formed between $^3$H-labeled S8 ($4.2 \times 10^{-8}$ M) and 16S rRNA ($5 \times 10^{-8}$ M), in TMK buffer containing variable concentrations of magnesium.

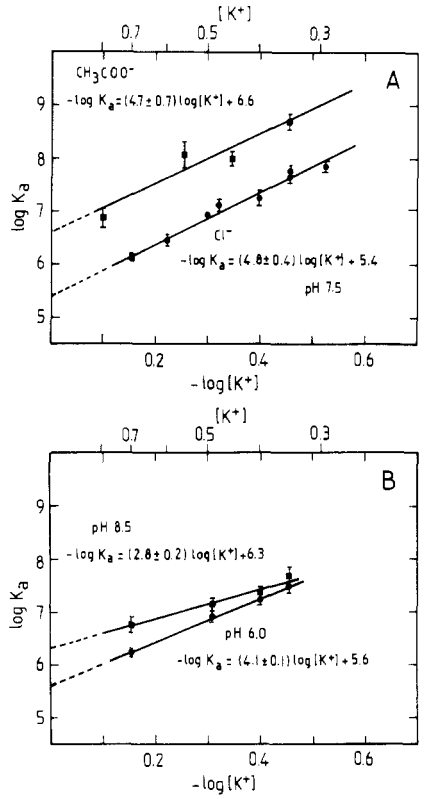


FIGURE 6: Ion concentration dependence of $K_a$. Complete binding curves were obtained at 0 °C, in TMK buffer containing variable concentrations of salt to give the indicated cation ($K^+$) concentration. (A) Salt dependence of $K_a$ at pH 7.5 with $K^+$ as the cation and $Cl^-$ (●) or $CH_3COO^-$ (■) as the anion. (B) Salt dependence of $K_a$ at pH 8.5 (■) and 6.0 (●). The least-squares equations of the lines are indicated.

as the anion. The association constant was measured from complete binding curves as a function of salt concentration, in the presence of 10 mM magnesium acetate, 20 mM Tris-HCl adjusted to the desired pH, 5 mM DTT, and 3 μM BSA, at 0 °C. At the selected magnesium concentration, conformational changes of RNA, produced by variations of monovalent ions, are assumed to be minimal. Preincubation of the components at 40 °C was at the indicated salt concentration. The values of log $K_a$ were plotted as a function of log [$K^+$]. The binding constant linearly decreases with increasing ionic strength over the range 0.3–0.7 M, thus indicating that ionic interactions contribute to the binding (see Figure 6).

Figure 6A shows the ion concentration dependence of $K_a$ at optimum pH (7.5), in buffer containing $CH_3COONa$ or KCl as the monovalent salt. The slopes d log $K_a$/d log [$K^+$] are 4.8 ± 0.4 and 4.7 ± 0.7, respectively, implying that approximately five ions are released upon complex formation in

both systems. If the contribution of anion release to the total observed ion release in the interaction is small, the salt dependence of the apparent constant may be written (Record et al., 1976):

$$-\text{d log } K_a/\text{d log } [M^+] = m'\psi \quad (1)$$

where $m'$ is the number of phosphate–lysine-type ion pairs formed upon binding and $\psi$ is the ion association parameter. The presence of magnesium in the binding buffer is known to reduce the amount of cation release. Correction can be made, which accounts for the presence of magnesium, according to Lohman et al. (1980) (eq 5 and 6). If we assume that the salt dependence of magnesium binding can be approximated from the behavior of homopolymers and assign a value of $\psi = 0.86$, the number of phosphate groups involved in ionic interactions with the protein can be estimated at $8 \pm 1$.[2] However, this must be regarded as an *upper limit*, since the displacement of anions from the protein may also contribute to the slope of plots (de Haseth et al., 1977)

$$-\text{d log } K_a/\text{d log } [M^+] = m'\psi + aK_X[X^-]/(1 + K_X[X^-]) \quad (2)$$

where $a$ is the number of independent anion binding sites per protein, $K_X$ is the binding constant for anions to these sites, and $[X^-]$ is the concentration of anions. In this equation, $m'\psi$ is the number of cations released from the RNA, and $aK_X[X^-]/(1 + K_X[X^-])$ is the number of anions released from the protein.

An anion effect on $K_a$ is apparent in Figure 6A, when $Cl^-$ was replaced by $CH_3COO^-$. The equilibrium constant was found to decrease in the order $CH_3COO^- > Cl^- > Br^-$, giving $4 \pm 1 \times 10^8$ $M^{-1}$, $5 \pm 0.5 \times 10^7$ $M^{-1}$, and $5 \pm 1 \times 10^6$ $M^{-1}$, respectively, at 0 °C, pH 7.5, and 0.35 M monovalent salt. This anion effect, which is consistent with the lyotropic series, indicates that S8 does contain anion binding site(s). As shown in Figure 6A, the replacement of $Cl^-$ by $CH_3COO^-$ has no significant effect on the slope of the log–log plot, suggesting that there are no major differences in the amount of anion release (if any) in the two systems. According to eq 2, curvature should be observed in the plots if anion release is involved in the interaction. However, the accuracy of data is not sufficient to determine whether or not some curvature is present. Furthermore, curvature is absent if $K_X[X^-] \gg 1$.

The effect of pH was investigated by studying the [KCl] dependence of $K_a$ at both pH 8.5 and pH 6.0, where titration effects are expected to occur (Figure 6B). When the pH was raised from 7.5 to 8.5, d log $K_{obsd}$/d log $[K^+]$ dropped from $4.8 \pm 0.4$ to $2.8 \pm 0.2$. This result strongly suggests that protonation of groups with a p$K$ near 9.0 is not absolutely required (i.e., for a conformational switch) but enhances the affinity of the protein for the RNA via a generalized charge effect (see pH Dependence of Binding). On the other hand, the [KCl] dependence of $K_a$ was not very affected when the pH was dropped from 7.5 to 6.0: the slopes of the lines are $4.8 \pm 0.4$ and $4.1 \pm 0.1$, respectively. The observed difference is small and its significance is questionable. It might suggest that some protonation event (deprotonation in our case) is required for complex formation. If this assumption is true, the pH dependence on $\Delta H$ should provide further evidence for it.

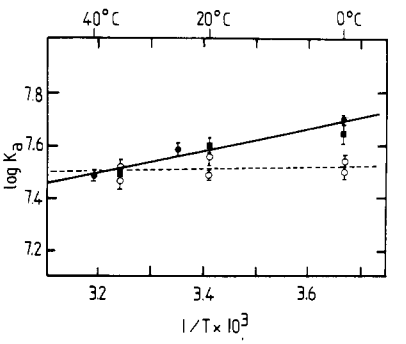


FIGURE 7: Temperature dependence of $K_a$. Complete binding curves were obtained in TMK buffer at the indicated temperatures and at pH 7.5 (●), 8.5 (■), and 6.0 (○). The lines give $\Delta H = -1.8$ kcal/mol at pH 7.5 and 8.5 and $\Delta H \simeq 0$ at pH 6.0.

In principle, the intercept of the log–log plot at 1 M monovalent salt would provide estimates of the nonelectrostatic contribution to the free energy of binding (Record et al., 1976, 1978). However, since anion binding and titration effects appear to be involved in the interaction, the extrapolation to the standard state would require further information [see eq 10 of de Haseth et al. (1977)] that is not available for this system. In particular, if anion displacement occurs, it would require the expenditure of free energy; in the absence of any correction, the value of $\Delta G_{1M}$, as estimated from binding data, would thus be reduced. Though quantitative estimates cannot be given, it appears from our data that nonelectrostatic interactions would represent a substantial contribution to the binding free energy.

*Temperature Dependence of Binding.* Complete binding curves were obtained at various temperatures in the range 0–40 °C, in TMK buffer adjusted to pH 6.0, 7.5, and 8.5 (within ±0.02 pH unit), at each temperature. In each case, thermal renaturation was at 40 °C, RNA and protein were equilibrated at the indicated temperature and mixed, and binding was measured as a function of RNA concentration. Filtration was carried out at the studied temperature. The association constant as a function of temperature is shown as a van't Hoff plot in Figure 7. The observed changes were small and required careful measurement of $K_a$. No significant differences were found between pH 7.5 and pH 8.5. The $\Delta H$ obtained from the line in Figure 7 is $-1.8 \pm 0.3$ kcal/mol, and $\Delta S$ is evaluated to $+29 \pm 1$ cal/(mol K). At pH 6.0, the binding becomes completely independent of temperature, indicating $\Delta H \simeq 0$. The method for determining temperature dependence at pH 6.0 was repeated twice with a similar result. The pH dependence of $\Delta H$ therefore confirms the observations concerning the formation of the complex at different pH values and monovalent ion concentration. In any case, the entropy change is clearly the main driving force for the reaction.

*Inactivation of S8 by SH-Group Reagents.* Since protein S8 is known to contain a unique cysteine, it was of interest

[2] It should be noted that the quantitative interpretation of the salt dependence of $K_a$ has been developed for interactions between linear nucleic acids (DNA, single-stranded and double-stranded ribooligomers) and proteins or model ligands [*lac* repressor, oligolysines, $Mg^{2+}$; see Record et al. (1976, 1978) and Lohman et al. (1980)]. Numerous applications of this model have been made on DNA–protein systems and also on RNA–protein interactions [for the latter, see Carey and Uhlenbeck (1983), Stahl et al. (1984), and Romaniuk (1985)]. In this study, we assumed that the region implicated in the interaction is locally a cylindrical polyelectrolyte. This is a reasonable assumption since the S8–RNA binding site was clearly identified as one hairpin structure (encompassing nucleotides 588–641). The value of $\psi$ was approximated from appropriate parameters given for single-stranded and double-stranded ribooligomers (Record et al., 1976; Table I). The equation describing magnesium binding to the S8–16S rRNA binding site as a function of $[M^+]$ was estimated from appropriate parameters reported by Record et al. (1976) in Table II. The deduced equation was $K_{Mg} = 1 - 1.6 \log [M^+]$. The validity of this equation is based on the untested assumption that there are no specific sites of $Mg^{2+}$ in the S8–RNA binding site.

Table II: Inactivation of Binding by NEM and pCMBS[a]

| reagent concn × $10^5$ (M) | NEM % activity | pCMBS % activity |
|---|---|---|
| 0 | 100 | 100 |
| 0.25 | 77 | 66 |
| 0.5 | 68 | 43 |
| 1 | 59 | 39 |
| 2 | 50 | 32 |
| 3 | 47 | 16 |
| 3 (+DTT) | 66 | 83 |
| 5 | 28 | 7 |
| 5 (+DTT) | 55 | 82 |

[a] Preincubation of $^3$H-labeled S8 ($1.8 \times 10^{-6}$ M) in the presence of BSA ($3.2 \times 10^{-6}$ M), with the indicated concentration of either NEM or pCMBS, was for 10 min, at room temperature, in the absence of DTT. The reaction mixture was then diluted 15-fold with TMK buffer, 16S rRNA was added at a final concentration of $2 \times 10^{-7}$ M, and the bound protein was measured as described under Experimental Procedures. The presence of DTT in binding buffer is indicated. The RNA binding activity of protein incubated in the absence of either DTT, NEM, or pCMBS is defined as 100%.

to see whether SH-group reagents are able to produce inhibition of binding. The protein was renatured at 40 °C as usual, but in the absence of DTT, and incubated with increasing amounts of *N*-ethylmaleimide (NEM) or *p*-(chloromercurio)benzenesulfonate (pCMBS), for 10 min at room temperature. The reaction mixture was then diluted 15-fold and mixed with 16S rRNA for binding. As shown in Table II, both NEM and pCMBS inactivated the protein. Almost complete inactivation of S8 was obtained with a 10 molar excess of pCMBS over protein in 10 min. It must be emphasized that BSA contributes other reactive cysteine residues to the reaction. A BSA molecule contains 35 cysteines, 34 of them being involved in disulfide bonds in the native protein (Brown, 1974). An exact stoichiometry of the reaction is therefore difficult to state with precision. As expected, the presence of 5 mM DTT in the binding mixture allowed restoration of binding activity. The short reaction time and the relatively low molar excess required to inactivate the protein suggest that the cysteine residue is accessible at the surface of the protein. It is noteworthy that the extent of saturation is reduced when preincubation and binding are made in the absence of reducing reagent. Since no alteration of the affinity constant was observed, we conclude that the amount of active protein was decreased. One possible explanation is the formation under these conditions of dimer, which would be inactive for binding. Indeed, one band whose migration corresponds to dimer position was observed on SDS gel; this band was no longer present when the reducing reagent was added to the sample before electrophoresis (result not shown). Our results suggest that cysteine either is directly involved in RNA binding or is in close proximity of essential sites.

## Discussion

*Binding of S8 to 16S rRNA.* In this paper, we report a detailed analysis of the properties of the interactions between *E. coli* ribosomal protein S8 and 16S rRNA. The nitrocellulose filter assay used in this work has been revealed to be a sensitive and reliable method for allowing kinetic measurements and providing binding evaluation with satisfying reproducibility and precision. It was assumed in this study that the labeling procedure did not alter the binding properties of S8.

In standard TMK buffer at 0 °C the apparent association constant is $5 \pm 0.5 \times 10^7$ M$^{-1}$. This is in good agreement with the value reported in 1981 by Schwarzbauer and Craven ($\simeq 2.7 \times 10^7$ M$^{-1}$ at 40 °C), using the same filtration technique, and falls in the range of other previous estimations for several ribosomal proteins (Schwartbauer & Craven, 1981; Spierer & Zimmermann, 1978). This value is also in the same range as the association constants of aminoacyl-tRNA synthetase–tRNA interactions [for a general review, see Kern (1981)]. Specific interactions of *lac* repressor and RNA polymerase with DNA have comparatively higher binding constants: $\simeq 10^{13}$ M$^{-1}$ for the former (Riggs et al., 1970; Winter & von Hippel, 1981), and $5.5 \pm 2.7 \times 10^8$ M$^{-1}$ for the latter.

Table III: Ion Release for Several RNA–Protein Interactions

| | $[Mg^{2+}]$ (mM) | pH | d log $K_a$/ d log $[K^+]$ |
|---|---|---|---|
| *E. coli* S8–16S rRNA[a] | 10 | 7.5 | 4.8 |
| | 10 | 6.0 | 4.1 |
| | 10 | 8.5 | 2.8 |
| R17 coat protein–21-mer[b] | 10 | 8.5 | 2.5 |
| *X. laevis* transcription factor IIIA–5S rRNA[c] | 5 | 7.5 | 4.5 |
| *B. subtilis* L16–5S rRNA[d] | 20 | 7.6 | 6.6 |

[a] This work. [b] Carey & Uhlenbeck, 1983. [c] Romaniuk, 1985. [d] Stahl et al., 1984. 21-mer corresponds to a 21-nucleotide synthetic RNA fragment comprising residues −17 to +4 of the R17 RNA, where +1 is the first nucleotide of the replicase gene (Carey & Uhlenbeck, 1983).

The association rate constant ($2.5 \pm 0.1 \times 10^5$ s$^{-1}$ M$^{-1}$) is lower than that expected for a diffusion-controlled reaction [usually in the $10^8$ range; Alberty & Hammes, 1958], indicating that all collisions are not productive. Two different explanations can reasonably be proposed to account for such a low value: (i) a specific RNA–protein complex may only form when a collision occurs with the correct orientation for association; (ii) formation of a stable complex is the result of a conformational transition of one of the two molecules, or both. The complex dissociates with a rate of $6.9 \pm 1 \times 10^{-3}$ s$^{-1}$, and the half-life of the complex is about 100 s. The agreement between the ratio of rate constants and the value of the association constant determined directly from the titration curve under equilibrium conditions is satisfactory. This is consistent with the notion that complex formation obeys a bimolecular equilibrium, and it provides further validation for the filtration method used. The complex is formed at a 1:1 ratio, and the association process is highly specific, as evidenced by the absence of a detectable effect of 23S rRNA when added to the equilibrium mixture. This observation, together with the relative slow forward rate, supports a collisional mechanism rather than a "sliding" mechanism as demonstrated in the case of genome regulatory proteins [see Berg et al. (1981)]. Such a preequilibrium mechanism can certainly be ruled out in our case. Protein S8 most likely has to find its specific binding site on the 16S rRNA molecule directly as a result of a correct collisional event. The observed high specificity of binding is not really surprising if we consider the essential role of primary proteins in the assembly of ribosomes, since their correct positioning is a requirement for the binding of subsequent proteins in the assembly pathway.

A concentration of magnesium between 10 and 50 mM is required for optimal binding. Magnesium is essential for maintaining the RNA conformation, since it has been shown that the stability of secondary structure is very sensitive to magnesium concentration (Cammack et al., 1970).

The salt dependence of $K_a$ reveals that ionic contacts contribute to the interaction. In standard conditions, five ions are released upon binding. The extent of ion release falls in the same range as that observed in the case of other RNA–protein interactions (Table III). Note that R17 coat protein, *Bacillus subtilis* L16, and *E. coli* S8 have similar molecular weights (13 700, 15 000, and 14 000, respectively) and that transcription

factor IIIA is larger (40000). Interestingly, R17 coat protein was found to interact with a RNA helix of 7 base pairs, while S8 predominantly protects a RNA helix of 18 base pairs. The replacement of $Cl^-$ by another anion has a dramatic effect on the binding constant, indicating that S8 contains anion binding site(s). An equal number of ions were released when $CH_3COO^-$ was substituted for $Cl^-$, and it is not yet clear whether anions are actually released upon binding or not. As a possible interpretation, anion(s) may bind to the protein at site(s) not directly involved in RNA binding. In this case, anions would not be displaced upon complexation. The observed anion effect on $K_a$ might be due to a conformational change of the protein, induced by anion binding, as proposed to be the case for binding of *lac* repressor to either operator or nonoperator DNA (de Haseth et al., 1977; Barkley et al., 1981; Winter et al., 1981). As an alternative explanation, $Cl^-$ and $CH_3COO^-$ may bind to the protein at site(s) involved in RNA binding. If the affinities of these anions are above a critical value, an equal number of anions might still be bound to the protein and thus would be released upon binding. Note that anions might be either directly displaced by the RNA (if they are located in the RNA binding site) or released as the result of a conformational change of the protein upon complexation.

The main source of the total binding free energy is shown to be entropic. The formation of several ion pairs upon binding contributes to this favorable entropy due to the dilution of ions displaced from the surface of RNA. Another known source of positive entropy is the displacement of water molecules from the water shell that surrounds both RNA and protein. The existence of large contact areas between S8 and its RNA binding site is suggested both by damage selection experiments (Thurlow et al., 1983) and by cross-linking experiments. The three characterized cross-link sites are within the hairpin already identified as the S8 binding site (Wower & Brimacombe, 1983). They extend from the bottom to the top of the protected helical structure and lie on one side of the helix. These observations are consistent with our present findings that nonelectrostatic interactions also contribute to the free energy of binding. Likely, the complex is stabilized, in addition to several ionic interactions, by other contacts such as hydrogen bonds and van der Waals contacts, which require exact fit at close range. Binding of S8 to 16S rRNA would then be accompanied by a change in the solvation of both molecules, thus contributing to the observed high positive entropy.

The pH dependence of binding shows that two types of titrable groups influence complex formation. The descending arm of the curve indicates deprotonation of groups near pH 9 and points to a cysteine sulfhydryl, a tyrosine hydroxyl, or a lysine $\epsilon$-amino group in a local environment that lowers its p$K$. Among these possible candidates, cysteine can be envisioned as highly probable since it was shown to be essential for binding (see above). However, deprotonation of groups on the RNA may also be implicated, since both uracil and guanine deprotonate in this pH range. The ascending arm of the curve indicates protonation of groups around pH 5–6. The absence of histidine in S8 eliminates the most likely candidate, and no obvious possibility clearly appears. One might envisage protonation of either adenine or cytosine. An example of such a protonation of a nucleotide residue, associated with a conformational switch, has been described in 5S rRNA, at the physiological pH range of 7–8 (Kao & Crothers, 1980). The authors proposed A or C residues as probable targets for protonation. Both ionic strength and temperature dependence on $K_a$ at different pH brought evidence that the two types of titrable groups have not the same influence on the formation of the complex. Group(s) with p$K$ near 9 influence(s) binding via a general charge effect. In other words, the number of ionic contacts depends on the protonation of these groups. On the other hand, the deprotonation of group(s) with a p$K$ around 5–6 is required for binding. This could be an indication for a proton-coupled conformational switch of one of the two molecules (or both) occurring in this pH range. Such a requirement for protonation was evidenced in both cases of the nonspecific interaction between the *lac* operator with DNA (de Haseth et al., 1977) and the specific interaction of RNA polymerase with its operator (Strauss et al., 1980).

Table IV: Thermodynamic Parameters for S8–16S rRNA Binding and Several Ligand–Nucleic Acid Interactions

| | $T$ (°C) | $\Delta G°$ (kcal/mol) | $\Delta H°$ (kcal/mol) | $\Delta S°$ [cal/(mol K)] |
|---|---|---|---|---|
| *E. coli* S8–16S rRNA[a] | 25 | −10.4 | 1.8 | +29 |
| *E. coli* L18–5S rRNA[b] | 25 | −11.4 | −2.7 | +29.2 |
| *E. coli* L25–5S rRNA[b] | 25 | −10.4 | −1.5 | +29.8 |
| *E. coli* L5–5S rRNA[b] | 25 | −8.8 | −2.0 | +22.8 |
| yeast $tRNA^{Phe}$–Phe-tRNA synthetase[c] | 24 | −9.4 | −3.5 | +20 |
| *E. coli* $tRNA^{Ile}$–Ile-tRNA synthetase[d] | 17 | −9.9 | 0 | +34 |
| *X. laevis* transcription factor IIIA–5S rRNA[e] | 24 | −12.1 | −8.3 | +13.1 |
| R17 coat protein–21-mer[f] | 24 | −9.8 | −19 | −30 |
| *lac* repressor–operator DNA[g] | 24 | −18 | +8.5 | +90 |
| *lac* repressor–nonoperator DNA[h] | 20 | −7.3 | −6 | +4.4 |
| netropsin–[poly(dA)-poly(dT)][i] | 25 | −12.2 | −2.2 | +33.6 |
| netropsin–poly[d(A-T)][i] | 25 | −12.7 | −11.2 | +5.0 |

[a] This work. [b] Spierer et al., 1978. [c] Krauss et al., 1976. [d] Lam & Schimmel, 1975. [e] Romaniuk, 1985. [f] Carey & Uhlenbeck, 1983. [g] Riggs et al., 1970. [h] Revzin & von Hippel, 1977. [i] Marky et al., 1984.

*Comparison with Other Systems.* A comparison of our results with those of other protein–nucleic acid interactions may provide useful information. We list in Table IV the thermodynamic parameters for S8–16S rRNA binding and for several ligand–nucleic acid complexes. Striking similarities are observed between S8–16S rRNA, L18–5S rRNA, L25–5S rRNA, and aminoacyl-tRNA synthetase–tRNA interactions. All these interactions are entropy-driven and their enthalpy contribution is weak. On the other hand, the R17 coat protein–RNA interaction is dominated by a large favorable $\Delta H$ partially offset by an unfavorable $\Delta S$. The authors attributed this negative entropy to conformational constraints or unfavorable hydrophobic interactions not entirely compensated by the ionic contribution. The thermodynamic parameters for binding of *lac* repressor to operator or nonoperator DNA differ strikingly. The negative enthalpy change observed at pH 7.5 in the case of the nonspecific complex results from the requirement for protonation of two groups on the repressor. Besides, the entropy contribution is much smaller for nonoperator DNA than for operator DNA. As pointed out by the authors (Revzin & von Hippel, 1977), this might be explained if the specific interaction involved intimate (and water molecule displacing) contacts between the repressor and specific groups on the operator DNA, while the nonspecific binding involved primarily electrostatic interactions (de Haseth et al., 1977; Winter & von Hippel, 1981). These nonspecific ionic interactions were found to facilitate binding rate in the framework

of the two-step sliding mechanism (Winter et al., 1981). In the same way, the very different contributions to the free energy of binding of netropsin (a nonintercalating DNA-binding drug) to either poly(dA–dT) or poly(dA)–poly(dT) were attributed to differences in both conformation and solvation between the homo- and heteropolymer. Netropsin was assumed to displace a more extensive water shell from the homopolymer than from the heteropolymer (Kopka et al., 1985).

A large contribution to the free energy of binding from nonelectrostatic interactions was reported for RNA–protein complexes studied so far [R17 coat protein–RNA, Carey and Uhlenbeck (1983); transcription factor IIIA–5S rRNA from *Xenopus laevis*, Romaniuk (1985); BL16–5S rRNA from *B. subtilis*, Stahl et al. (1984)]. In the case of aminoacyl-tRNA synthetase–tRNA interaction, the successful crystallization of a complex between $tRNA^{Asp}$ and aspartyl-tRNA synthetase could be obtained under high salt conditions (Lorber et al., 1983). Large contact areas were evidenced by neutron scattering and confirmed by chemical modification studies, suggesting the formation of numerous hydrogen bonds and van der Waals contacts (Moras et al., 1983). Since the tRNA molecule was shown to be surrounded by a shell of solvent significantly denser than bulk (Li et al., 1983), the aminoacyl-tRNA synthetase is expected in this case, too, to displace a high number of water molecules.

In this work, we show that the S8–16S rRNA interaction is a highly specific process. Likely, electrostatic interactions, intimate contacts, and water displacing contribute to the interaction, as observed in various specific ligand–nucleic acid complexes. Nevertheless, more precise data should be obtained to propose a molecular mechanism for this interaction. Detailed chemical modification studies on the S8–16S rRNA binding site, in the presence or in the absence of the protein, are in progress.

## ACKNOWLEDGMENTS

We thank Professor J. P. Ebel for interest and support and Dr. E. Westhof and Professor M. Daune for stimulating discussions and critical reading of the manuscript.

**Registry No.** Mg, 7439-95-4; $AcO^-$, 71-50-1; $Cl^-$, 16887-00-6; $Br^-$, 24959-67-9; L-cysteine, 52-90-4.

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# Proteins from the Prokaryotic Nucleoid: $^1$H NMR Study of the Quaternary Structure of *Escherichia coli* DNA Binding Protein NS (HU)†

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*Received August 28, 1985; Revised Manuscript Received January 6, 1986*

ABSTRACT: The quaternary interactions of *Escherichia coli* DNA binding proteins NS1, NS2, and NS (NS1 + NS2) have been studied by $^1$H NMR spectroscopy at 400 MHz following the reversible spectral changes produced by temperature increases on the resonances (Phe ring and His C-2 protons) whose spectral characteristics reflect the formation and dissociation of either homologous or heterologous interactions. These changes include (a) a progressive intensity decrease of the Phe resonances shifted to high field by stacking interactions, (b) a progressive intensity increase of the resonances due to freely rotating Phe, and (c) splitting of the His C-2 proton resonance. The association constants and thermodynamic parameters for the homologous and heterologous interactions were calculated from the molar fractions of the relevant molecular species by assuming that the above effects are due to the existence of simple association equilibria. It was found that two (out of three) phenylalanine residues of each polypeptide chain are involved in quaternary interactions. Quantitative data concerning the internal mobility and mutual orientations in aggregates of these Phe rings were also obtained. From the calculated association constants, from comparison of these data with recent protein–protein cross-linking results [Losso, M. A., Pawlik, R. T., Canonaco, M. A., & Gualerzi, C. O. (1986) *Eur. J. Biochem. 155*, 27–32], and from other considerations, we suggest that even though stacking of the Phe rings occurs at the interface between monomers, the temperature-dependent alteration of the Phe spectrum monitors shifts of the dimer ⇌ tetramer equilibrium whereas the splitting of the His C-2 proton resonance most likely monitors the equilibrium between tetramers and larger aggregates.

Among the proteins which may play a role in the physical packaging of bacterial chromosome, NS (HU) is the most abundant and the best characterized (Rouvière-Yaniv & Gros, 1975; Berthold & Geider, 1976; Varshavsky et al., 1977; Suryanarayana & Subramanian, 1978; Losso et al., 1982; Miano et al., 1982; Paci et al., 1984; Lammi et al., 1984a,b; Gualerzi et al., 1986). In *Escherichia coli*, NS consists of two 9-kilodalton polypeptide chains, NS1 and NS2, displaying 69% sequence homology (Mende et al., 1978). These proteins have been characterized by $^1$H NMR spectroscopy (Paci et al., 1984, 1986). The spectra of NS1, NS2, and NS display a large number of high-field-perturbed Phe resonances, shielded and deshielded methyl resonances, and backbone NH protons rather inaccessible to the solvent. These features were attributed to the existence of extensive tertiary and/or quaternary structures which are lost upon heating but that readily re-form upon cooling. It was also shown that, when isolated, NS1 and NS2 undergo self-aggregation but that, when both proteins are mixed, heterologous aggregates are preferentially formed. Furthermore, it has been shown that NS binds to DNA in the aggregated form (Lammi et al., 1984; Paci et al., 1984, 1986) and that the presence of DNA favors its aggregation (Losso et al., 1986).

In this study, we have investigated the quaternary interactions of NS1, NS2, and NS by following the spectral

† This work was partially supported by funds from the Italian Ministry of Public Education.
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0006-2960/86/0425-2765$01.50/0 