Binding of *Bacillus subtilis ermC'* Methyltransferase to 23S rRNA†

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ABSTRACT: *ermC* 23S rRNA methyltransferase dimethylates adenine 2085 in *Bacillus subtilis* 23S rRNA and also regulates its own synthesis by autogenous translational repression. We have characterized the binding of ermC' methyltransferase to 23S rRNA. This protein differs in only five amino acid residues from the *ermC* product and was chosen for study because of its greater stability and ease of isolation. A filter binding assay was used to study the physical aspects of binding in the absence of methylation. The dissociation equilibrium constant of the binding was found to be 4×10^{-9} M at 37 °C. Kinetic studies of complex formation and dissociation revealed that the k_{on} and k_{off} were 4×10^{6} M^{-1} s^{-1} and 6.8×10^{-2} s^{-1} respectively at 16 °C. Equilibrium competition experiments showed that the enzyme has varying affinities for a variety of nucleic acids in the order 23S rRNA > 16S rRNA > M13 DNA, f2 RNA > tRNA. One of the end products of methylation, methylated 23S rRNA, had an affinity for the *ermC'* methyltransferase similar to that of unmethylated 23S rRNA. The binding affinity to 23S rRNA and the kinetics of the interaction were not detectably affected by the presence of AdoMet. The binding of *ermC'* methyltransferase to 23S rRNA had an unfavorable van't Hoff enthalpy (ΔH = +6.2 kcal mol^{-1}) and was driven by entropy (ΔS = +56.2 cal mol^{-1} deg^{-1}). The interaction between the two ligands involved at most two to three ionic pairings, and nonelectrostatic interactions contributed ~85% of the binding energy. The structural aspect of the interaction was investigated by probing with dimethyl sulfate, for *ermC'* methyltransferase dependent protection of 23S rRNA. A region of protection was detected, in the vicinity of the central loop of rRNA domain V and surrounding the site of methylation.

Naturally occurring bacterial resistance to the MLS[1] group of antibiotics is mediated by specific 23S rRNA methyltransferases. One of the more thoroughly studied is the enzyme specified by the *Staphylococcus aureus ermC* determinant. This enzyme is regulated posttranscriptionally by several mechanisms: translational attenuation (Gryczan et al., 1980; Horinouchi & Weisblum, 1980), induced mRNA stabilization (Bechhofer & Dubnau, 1987; Shivakumar et al., 1980), and autogenous translational repression (Denoya et al., 1986). In the latter mode of regulation, the enzyme probably binds to its own mRNA and acts as a translational repressor. In addition, the methyltransferase is able to bind to 23S rRNA and dimethylate a specific adenine residue (Skinner et al., 1983; Weisblum et al., 1979). Methylation reduces the affinity between the ribosome and MLS antibiotics (Oleinick & Corcoran, 1969), conferring resistance to these antibiotics.

Bacillus subtilis ermC' ribosomal methyltransferase differs in 5 out of 244 amino acids from the *ermC* product (Monod et al., 1986). Its constitutive mode of synthesis and its stability in storage (unpublished) make it a preferred enzyme for study. In order to understand how the enzyme is able to recognize and bind to two different substrates resulting in different outcomes, we have begun to characterize physically the binding parameters between the enzyme and its ligands. This study primarily concentrates on the interaction between 23S rRNA and *ermC'* methyltransferase. We have determined both the kinetic and thermodynamic parameters of binding. The specificity of binding was also studied. Furthermore, the *ermC'* methyltransferase dependent protection of 23S rRNA was investigated by probing with DMS. The implications of these studies for the methylation reaction mechanism and for translational autoregulation are discussed.

Materials and Methods

Bacterial Strains and Plamids. Bacillus subtilis BD1167 [*hisA1 leu-8 metB5* (pIM13)] was the source of *ermC'* methyltransferase and of methylated 23S rRNA (Monod et al., 1986). *B. subtilis* BD170 (*trpC2 thr-5*) was used as a source of unmethylated rRNA. *Escherichia coli* JM109 [Δ(*lac pro*) *thi strA endA sbsB15 hsdR4* [*F' traD36 proAB lacI*^{q}*Z ΔM15*]] was used as a host for M13 phage. *E. coli* K38 (HfrC Sup^{+} *phoA*(*Am*) λ) was the host for bacteriophage f2.

Purification of ermC' Methyltransferase. B. subtilis BD1167 was grown in YPT medium (Halling et al., 1977) [2.5% Tryptose (Difco), 2% yeast extract (Difco), 0.3% K_2HPO_4, 3% glucose] containing 5 μg/mL Em at 34.5 °C with vigorous aeration in a Labline Fermentor. At a Klett reading of ~450, the culture was harvested. The cells were processed as described (Shivakumar & Dubnau, 1981) with one modification. The DEAE-Sephadex chromatographic step was replaced by passage through a Sephadex G-100 (0.5 × 40 cm) column. Active fractions, which were pooled and stored at −70 °C with very little loss of activity after several months, appeared to be nearly homogeneous by the criterion of SDS–polyacrylamide gel electrophoresis.

Assay for Methyltransferase Activity. Enzyme was assayed as described (Shivakumar & Dubnau, 1981). In this assay, [*methyl*-^{3}H]AdoMet was incubated with enzyme and rRNA substrate, and the transfer of trichloracetic acid insoluble

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[1] Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; BSA, bovine serum albumin; DMS, dimethyl sulfate; DTT, dithiothreitol; EDTA, disodium ethylenediaminetetracetate; Em, erythromycin; β-ME, β-mercaptoethanol; MLS, macrolide–lincosamide–streptogramin B group; rRNA, ribosomal RNA; Tris, tris(hydroxymethyl)aminomethane.

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radioactivity to the RNA was measured. Protein concentrations were determined with BioRad assay kits (BioRad Chemical Division, Richmond, CA) with bovine serum albumin and lysozyme used as standards.

Isolation of Nucleic Acids. rRNA was prepared as described (Shivakumar & Dubnau, 1981). Purified RNAs were dissolved in water. 16S or 23S rRNA was electrophoresed on a 1.5% agarose gel to check for degradation. The purity of RNA was further checked by the ratio of absorbance at 260 and 280 nm. Chromosomal DNA was isolated from *B. subtilis* BD170 as described by Dubnau and Davidoff-Abelson (1971). f2 RNA was isolated as described by Lodish and Zinder (1966). M13 mp19 single-stranded DNA was isolated as described in the Amersham Corporation's M13 Cloning and Sequencing Handbook. Commercially supplied *E. coli* tRNA (Sigma Corp.) was extracted several times with phenol–chloroform before use.

End-Labeling of RNA. RNA was end-labeled with [γ-^{32}P]ATP as described (Narayanan & Dubnau, 1985). The end-labeled RNA was phenol–chloroform extracted, ethanol precipitated, and dissolved in water. The integrity of the RNA was checked by electrophoresis on a 1.5% agarose gel, followed by autoradiography at room temperature.

Filter Binding Assay. Nitrocellulose filter sheets (pore size 0.45 μm) from Schleicher & Schuell were cut into disks of 25-mm diameter. The disks were wetted in distilled, deionized water, boiled for 10 min, cooled, and soaked in TKM buffer [40 mM Tris-HCl, pH 7.6, 40 mM KCl, 4 mM $Mg(OAc)_2$, 10 mM DTT, and 1 mM EDTA] containing 0.2 μg/mL BSA for 2 h prior to use. The binding reactions were carried out in 25-μL volumes containing TKM buffer, 5 μg of BSA (Sigma), 1 unit of Rnasin (Promega) or Inhibit-ace (5prime→3prime, Inc.), 0.175 μg of purified *ermC′* methyltransferase, and 0–100 nM of ^{32}P-end-labeled RNA. The RNA and buffer were preequilibrated at the desired temperature for 5 min. The binding assays were initiated by the addition of methyltransferase. After incubation for the desired time, the reaction mixture was quickly applied to a pretreated nitrocellulose filter on a glass Millipore filtration apparatus. Vacuum was turn on immediately after the sample was applied. The filtration time was ~2 s. The filter was washed three times with 0.25 mL of TKM, containing 0.2 μg/mL BSA. The filter was dried and counted in Liquiscint (National Diagnostics). Experiments were done in duplicate. Background radioactivity determined in the absence of methyltransferase was always subtracted and was usually less than 0.6% of input radioactivity. The total labeled RNA input was determined by spotting aliquots of RNA on the filter. Binding values were corrected for the retention efficiencies of RNA–protein complexes by the filters (see Results).

Determination of the Rate of Dissociation. The dissociation rate for 23S rRNA–methyltransferase complexes was determined by incubating ^{32}P-end-labeled 23S rRNA (10 nM) with 7×10^{-3} μg/μL purified methyltransferase in TKM buffer. The total volume was 25 μL. Typically incubation for complex formation was carried out at 16 °C for 10 min. Dissociation was initiated by the addition of either a 20-fold excess of unlabeled 23S rRNA or a 50-fold excess volume of buffer. The excess of unlabeled rRNA was added in a volume of 2.5 μL, and the same volume of buffer was added to the controls. Incubation was continued for the desired times and samples withdrawn, filtered, washed, and counted as in the above section.

Determination of Association Rate Constant. The rate of association was determined by examining the time course of formation of 23S rRNA–methyltransferase complex. ^{32}P-end-labeled 23S rRNA (2.5 nM) was incubated with 4.5×10^{-3} μg/μL methyltransferase at the desired temperature in TKM buffer. The reaction volume was 25 μL. Binding was initiated by the addition of methyltransferase. The filters were washed, dried, and counted as described above.

Equilibrium Competition Experiments. For the competition assays, each sample contained 20 nM ^{32}P-end-labeled 23S rRNA and varying concentrations of unlabeled competitor nucleic acid in a reaction volume of 25 μL. The reaction was initiated by the addition of 7×10^{-3} μg/μL purified methyltransferase. Incubation was for 10 min at 37 °C, followed by filtration, washing, and determination of radioactivity as described above. The data were analyzed according to the method of Lin and Riggs (1972). In the competition experiment involving methylated 23S rRNA, ^{32}P-end-labeled methylated 23S rRNA was used, and cold unmethylated 23S rRNA was used as competitor.

Dimethyl Sulfate Modification. Modification was carried out in 250 μL of CMK buffer [70 mM sodium cacodylate, pH 7.5, 20 mM $Mg(OAc)_2$, 300 mM KCl, and 1 mM DTT]. A typical assay contained 10 nM 23S rRNA, 3 μL of Inhibit-ace, and 0–0.29 pmol/μL purified methyltransferase. The mixture was incubated at 4 °C for 10 min, 2.5 μL of diluted DMS (1:8 in 95% ethanol) was added, and incubation was continued for 30 min. A 62.5 -μL aliquot of DMS stop buffer (1 M Tris–acetate, 1 M β-ME, and 1.5 M NaOAc) was added and the mixture was left on ice for 10 min. Carrier tRNA (12.5 μg) was added and the RNA was ethanol precipitated, washed with 70% ethanol, and dried. Primer extension of the 23S rRNA was performed to identify the modified bases.

Primer Extension. Primer extension using reverse transcriptase was carried out as described (Lane et al., 1985). The primers 5′-CCTGTACAAGCTGTACC-3′ (primer 1) and 5′-GTTAGAAGGTCAATACA-3′ (primer 2) were complimentary to residues 2211–2227 and residues 2127–2141 respectively, in *B. subtilis* 23S rRNA. The autoradiographs were analyzed by densitometric scanning using a BioRad 620 densitometer.

Results

Filter Binding Assay. The interaction between methyltransferase and 23S rRNA was investigated by using a nitrocellulose filter binding assay. The *ermC′* methyltransferase–23S rRNA complex was strongly retained on a nitrocellulose membrane filter, whereas free 23S rRNA passes through the filter. Figure 1 shows that when increasing concentrations of the purified *ermC′* methyltransferase were incubated with a constant amount of ^{32}P-5′-end-labeled 23S rRNA, increasing amounts of labeled 23S rRNA were retained until a plateau was reached. At the plateau, ~50% of the total input RNA was retained. It was found that, in several similar experiments, the efficiency of retention varied from 50 to 100%, depending on the batch of filters used. Nevertheless, the efficiency was constant for any given batch and was always used to correct the binding data. The incomplete retention of protein–nucleic acid complex on nitrocellulose filters has been observed by others (Carey et al., 1983; Mougel et al., 1986; Wilcox et al., 1974). To demonstrate that the protein was completely retained on the filter, methyltransferase was filtered in the absence of RNA. The filtrate as well as all the washes were precipitated with acetone and analyzed on a 15% SDS–polyacrylamide gel with silver staining. Even at a very high input equivalent to 552 pmol of *ermC′* methyltransferase per filter, no protein was detected in either the filtrate or the washes (data not shown).

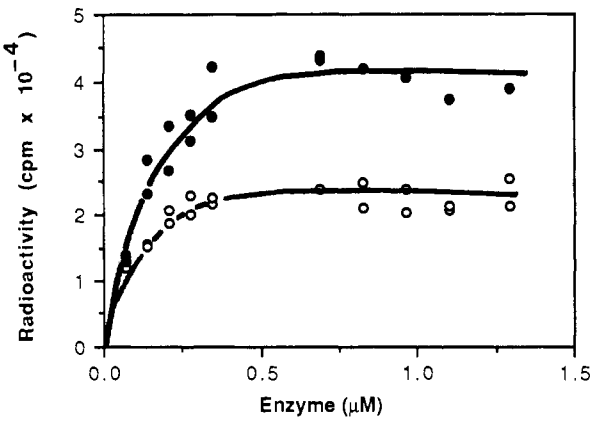

FIGURE 1: Binding of *ermC'* methyltransferase to fixed amounts of 23S rRNA. The concentration of *ermC'* methyltransferase was varied and that of ^{32}P-labeled 23S rRNA was fixed at 10 (○) or 20 nM (●). Reactions were carried out at 37 °C in TKM as described under Materials and Methods.

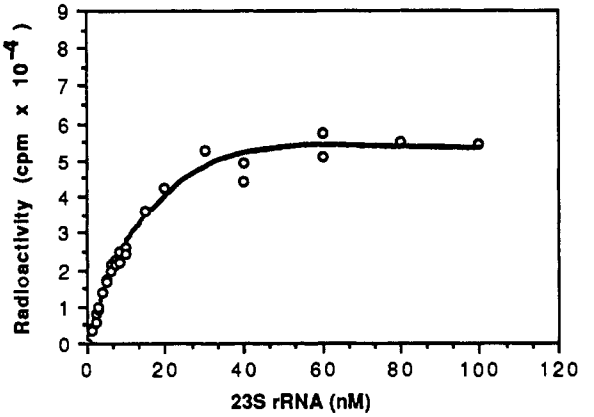

FIGURE 2: Binding of ^{32}P-labeled 23S rRNA with a fixed amount of *ermC'* methyltransferase. The experiment was performed in TKM buffer at 37 °C as described under Materials and Methods.

The filter binding assay measures the separation of *ermC'* methyltransferase–nucleic acid complex from free nucleic acid. To prove that the *ermC'* methyltransferase–nucleic acid complex does not dissociate during filtration, the complex retained on the filter was washed with additional aliquots of buffer. Each wash consisted of 10× the volume of the original binding reaction. Up to five washes had no effect on the binding curve. Therefore, RNA was not leached from the complex during filtration.

Another aspect of the assay that was addressed was the likelihood of the formation of de novo RNA–*ermC'* methyltransferase complexes on the filter. It is conceivable that RNA and *ermC'* methyltransferase, which exist in free form at equilibrium in solution, may associate during the filtration process. An experiment to test this possibility was performed by first filtering *ermC'* methyltransferase (8.6 pmol) and then filtering increasing amounts of ^{32}P-5′-end-labeled 23S rRNA through the same filter. The radioactivity retained on the filters was similar to that in a control experiment in which only the labeled 23S rRNA was filtered. This result (not shown) indicated that free 23S rRNA probably did not form complexes with the *ermC'* methyltransferase during the filtration process in significant amounts.

Determination of the Dissociation Constant. The dissociation constant, K_D, for the binding of 23S rRNA to *ermC'* methyltransferase was determined by a binding experiment in which the concentration of the *ermC'* methyltransferase was held constant while that of ^{32}P-5′-end-labeled 23S rRNA was varied (Figure 2).

In order to calculate the dissociation constant (K_D), an estimate of the concentration of active methyltransferase was required. This was estimated graphically from Figures 1 and 2 by determining the points of intersection in each graph of the rising and plateau segments. These equivalence points occurred at molar ratios of protein to RNA of 14.1 and 17.0, respectively, in the two figures. Assuming a binding stoichiometry of 1:1, this yielded an estimate for the percentage active protein of 6.5%. The two similar graphical methods described by Riggs et al. (1970a) were also employed, yielding values of 6.5% and 6.7% (not shown). We do not know why only ~7% of the methyltransferase protein is active in the binding reaction. It is worth noting that similar values have been determined for the active fractions of *araC* (Wilcox et al., 1974) and *lac* repressor (Riggs et al., 1970a) protein preparations. It is also important to realize that the value for the percentage of active protein is subject to several sources of uncertainty. For instance the total protein concentration was estimated colorimetrically, and we do not know to what extent the use of bovine serum albumin and lysozyme as standards is valid. In addition, as noted above, we have assumed that the stoichiometry of binding is 1:1. If instead, two molecules of protein bind to each molecule of rRNA, either to independent sites or as a dimer, then our estimate of the percentage active protein would become 14%. Free methyltransferase exists as monomer in solution (Denoya & Dubnau, 1987), but it is not known whether it binds as such. Finally, we have assumed that 7% of the methyltransferase molecules are fully active, while the remaining 93% are completely inactive. The real situation may be more complex.

As shown in Figure 1, at 37 °C, half-saturation was reached at an RNA concentration of 12 nM. Since the methyltransferase concentration in this experiment was not negligible compared to K_D, it was necessary to apply the correction derived by Riggs et al. (1970a):

$$K_D = R_{1/2} - E_t$$

where $R_{1/2}$ and E_t are the RNA concentration at half-maximum saturation and the input concentration of active methyltransferase, respectively. The K_D estimated in this way was 4×10^{-9} M. Given the uncertainties noted above, this is an approximate, average value and would probably represent an upper limit if the stoichiometry were other than 1:1.

Specificity of Binding: Equilibrium Competition Studies. The interaction of *ermC'* methyltransferase with a variety of RNA and DNA molecules was examined by equilibrium competition assays. Unlabeled competitor nucleic acids at up to 20 molar excess compared to the ^{32}P-labeled 23S rRNA were equilibrated with a saturating concentration of *ermC'* methyltransferase. After an incubation time of 10 min, the reaction mixtures were filtered. As the concentration of cold competitor nucleic acid increased, a reduction in the amount of the labeled RNA retained on the filter was observed (Figure 3). The results showed that the *ermC'* methyltransferase had measurable affinity for nucleic acids from miscellaneous sources. All nucleic acids were not equally effective competitors. 16S rRNA was the best competitor for *ermC'* methyltransferase binding to 23S rRNA, whereas tRNA competed poorly. The ability of single-stranded M13 DNA and f2 RNA to compete was intermediate between that of tRNA and 16S rRNA. *B. subtilis* chromosomal double-stranded DNA competed poorly (Figure 3b). The apparent dissociation constants, K_{DC}, for competitor–*ermC'* methyltransferase binding were calculated by the method of Lin and Riggs (1972) and are shown in Figure 3. To further validate the

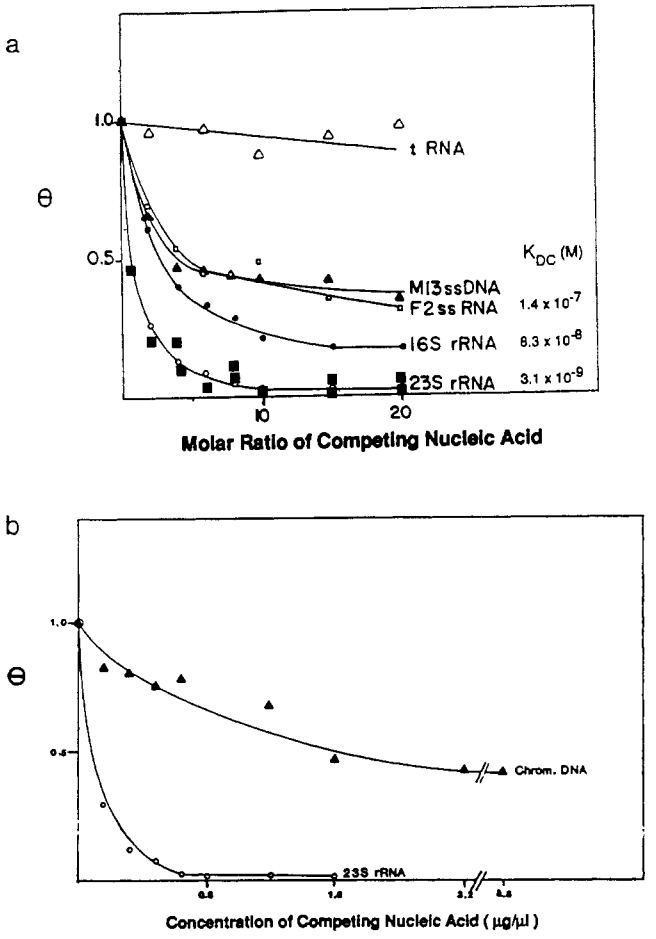

FIGURE 3: Equilibrium competition experiments. Varying amounts of unlabeled competing nucleic acids were preequilibrated with ^{32}P-labeled unmethylated 23S rRNA. The experiment was initiated by adding an excess of *ermC'* methyltransferase. After a 10-min incubation at 37 °C, the reaction was stopped by filtration as described under Materials and Methods. Each point represents an average of two filtrations. K_{DC} values for the competing nucleic acids were then calculated as described (Lin & Riggs, 1972). θ is the ratio of counts per minute retained on the filter in the presence and absence of competing nucleic acid. The competing cold nucleic acids used are indicated in panels a and b. In panel a results are also shown for an experiment in which the binding of ^{32}P-labeled methylated 23S rRNA was in competition with unlabeled, unmethylated 23S rRNA (■).

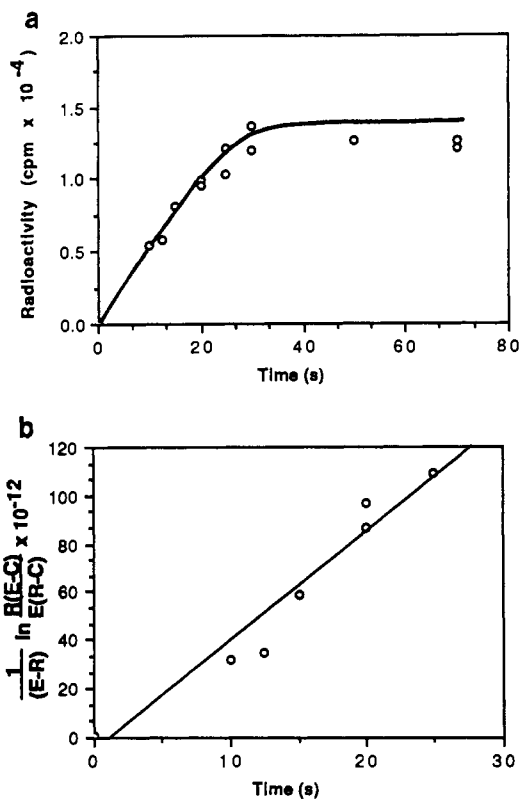

FIGURE 4: Kinetics of *ermC'* methyltransferase–23S rRNA complex formation at 16 °C. (a) The reaction mixture contained 0.155 pmol/μL *ermC'* methyltransferase and 2.5 nM ^{32}P-labeled 23S rRNA in TKM buffer at 16 °C. The reaction was initiated by addition of enzyme. Samples were filtered at the indicated times. The filtration time was estimated as ~2 s per sample. (b) The initial association kinetic data from Figure 4a were replotted as a second-order reaction where E, R, and C are the concentrations of methyltransferase, rRNA, and protein–RNA complex, respectively. The least-squares line of best fit yielded $k_{on} = 4 \times 10^6$ M^{-1} s^{-1}.

competition assay, the value of K_D for 16S rRNA–*ermC'* methyltransferase interaction was also measured directly in an experiment in which varying amounts of ^{32}P-5'-labeled 16S rRNA were allowed to interact with a constant amount of *ermC'* methyltransferase (data not shown). The K_D obtained from this experiment was 4.1×10^{-8} M, in reasonable agreement with that obtained from the equilibrium competition experiment.

Kinetic Studies. The association rate constant was measured in TKM buffer. *ermC'* methyltransferase (0.16 μM) was incubated with ^{32}P-labeled 23S rRNA (2.5 nM) at 16 °C, and the reaction mixtures were filtered after varying time intervals. An estimate of the association rate constant was obtained from the initial rate in Figure 4a, assuming negligible dissociation at early times. The experimental data were analyzed as shown in Figure 4b. From the least-squares slope of the line in Figure 4b the value $k_{on} = 4 \times 10^6$ M^{-1} s^{-1} was derived.

The dissociation rate constant for the 23S rRNA–*ermC'* methyltransferase complex was also measured in TKM buffer at 16 °C. The experiment was performed in two ways. In both cases, complex was formed by incubating ^{32}P-5'-end-labeled 23S rRNA (10 nM) and purified *ermC'* methyltransferase (0.59 μM) at 16 °C for 10 min. In the first method, dissociation of the complex was initiated by diluting the reaction mixture 50-fold with TKM buffer. In the second method, a 20-fold excess of cold 23S rRNA was added. In both cases the reaction mixtures were filtered after varying time intervals. In a control experiment, complex was incubated without any dilution or further addition, showing that the complex remained stable for the duration of the experiment. Figure 5a shows the dissociation as measured by the two methods, which gave similar results. The dissociation rate constant k_{off} estimated from this data was 6.8×10^{-2} s^{-1} (Figure 5b). The lines in Figures 4b and 5b were not constrained to go through the origin, and shifts 1–2 s are evident probably corresponding to sampling delays during filtration. The calculated value for the equilibrium constant, obtained from the dissociation and association rate constants, was 1.7×10^{-8} M. This number was in good agreement with the independently measured equilibrium constant of 2.1×10^{-8} M determined at 16 °C.

Temperature Dependence of K_A. Binding curves were obtained as a function of RNA concentration at varying temperatures, ranging from 0 to 37 °C. The equilibrium association constant K_A (reciprocal of K_D) of each binding curve was estimated and the data were analyzed as a van't Hoff plot. The slope of the resulting line of best fit was estimated as −3399.8 K, the intercept on the ordinate as 29.4, and the standard error of estimate as ±0.247. The observed change due to temperature variation was relatively small. Assuming that ΔH does not vary with temperature, the value obtained

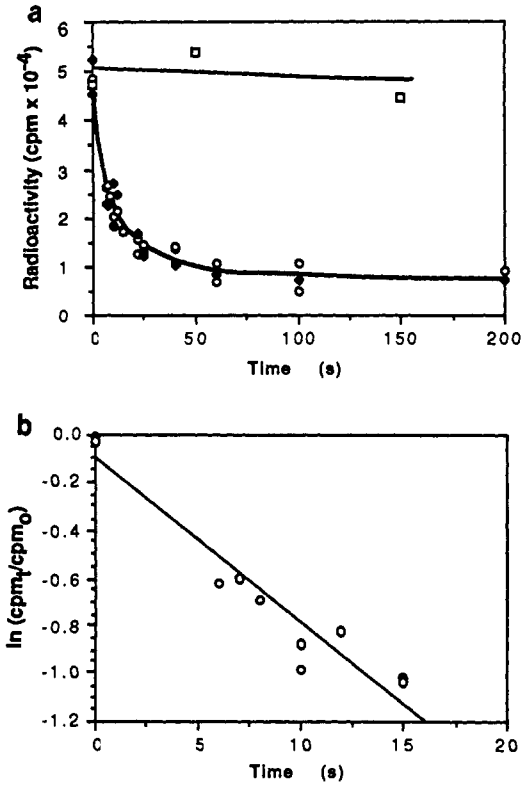

FIGURE 5: Kinetics of *ermC'* methyltransferase–23S rRNA complex dissociation at 16 °C. (a) 10 nM of ^{32}P-labeled 23S rRNA was equilibrated with 0.241 pmol/μL of *ermC'* methyltransferase in TKM buffer. Either a 20-fold excess of cold 23S rRNA (○) or a 50-fold excess of TKM buffer (◆) was added. At the indicated times, the reactions were stopped by filtration as described under Materials and Methods. The control mixture (□) was not diluted. (b) Linear plot of the initial part of the dissociation data from Figure 5a. Only the data from the addition of 20-fold excess cold 23S rRNA were used. The least-squares line of best fit yielded $k_{off} = 6.8 \times 10^{-2}$ s^{-1}.

from the slope of the line of best fit was +6.2 kcal mol^{-1} of complex. At 25 °C, ΔG and ΔS were calculated to be −11.4 kcal mol^{-1} and +59.1 cal mol^{-1} deg^{-1}, respectively.

Activation Energy of Association. The kinetics of complex formation at different temperatures was followed in experiments similar to that in Figure 4a. The association rate constant at each temperature was estimated by a linear plot of the initial part of the kinetics of association as in Figure 4b, and the data were analysed in an Arrhenius plot. The association rate constant was found to be relatively insensitive to temperature variation. The slope, intercept on the ordinate, and the standard error of estimate were found to be −763.3 K, 9.27, and ±0.19. The Arrhenius activation energy for the association reaction was calculated to be +3.5 kcal mol^{-1}.

Effect of KCl Concentration. The effect of salt concentration was examined by determining complete binding curves at several concentrations of KCl. The association constant, K_A, determined from each binding curve, was plotted against the logarithm of the concentration of potassium cation (Figure 6), according to de Haseth et al. (1977) and ψ, (the fraction of a counterion bound per charged group), taken as 0.82, an average value for homopolymers (Record et al., 1978). From the data in Figure 6, we estimate that approximately 2.2 ion pairs are involved in the binding of *ermC'* methyltransferase to 23S rRNA. This treatment of the data neglects possible anion and pH effects. The inclusion of these effects would lower the estimate of ion pairs involved, so the value determined in this study represents an upper limit. It is worth noting that the substitution of Cl^- by glutamate ion has no effect on K_A (not shown), suggesting that there may be no

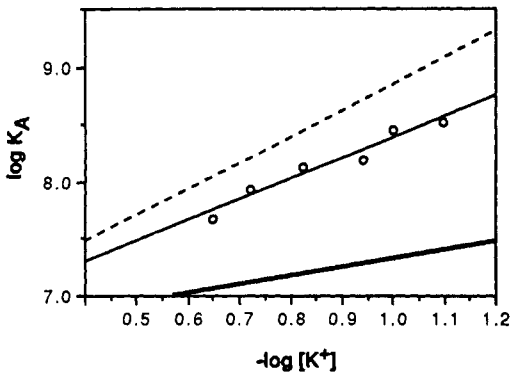

FIGURE 6: Effect of KCl concentration on the association constant. Binding curves were determined with fixed methyltransferase and varying ^{32}P-labeled 23S rRNA in reaction mixtures containing varying concentrations of KCl together with 40 mM Tris-HCl, pH 7.6, 1 mM DTT, and 1 mM EDTA. *ermC'* methyltransferase was dialyzed against each buffer prior to usage. K_D values were estimated from each curve. Theoretical lines [according to eq 11 of de Haseth et al. (1977)] were drawn with $m' = 1$ (dashed line) and 3 (bold line), and with $\psi = 0.82$. These are shown as the upper and lower lines, respectively.

important anion binding site on the methyltransferase.

We evaluated the nonelectrostatic contribution to the stability of the 23S rRNA–*ermC'* methyltransferase complex from the extrapolated value of K_A at 1 M salt (Record et al., 1976). It has been estimated that at 1 M salt, each lysine–phosphate type ion pair contributes ~+0.2 kcal mol^{-1} (Lohman et al., 1980; Record et al., 1976). Extrapolation of the data in Figure 6 yields $\Delta G = -9.3$ kcal mol^{-1} at 1 M KCl. Assuming the formation of 2.2 ion pairs of the lysine–phosphate type, the nonelectrostatic ΔG would be −9.7 kcal mol^{-1} of complex. The ΔG determined in TKM buffer at 25 °C was −11.4 kcal mol^{-1}, suggesting that under these conditions ~85% of the binding energy is provided by nonelectrostatic interaction.

Effect of AdoMet and AdoHcy. AdoMet is a cofactor in the methylation reaction and AdoHcy is an end product. AdoHcy has been shown to be a competitive inhibitor of the reaction involving *ermC* methyltransferase (Denoya & Dubnau, 1989). It was of interest to determine whether AdoMet and AdoHcy would affect the binding of 23S rRNA to *ermC'* methyltransferase. Complete binding curves were determined in the presence and absence of either AdoMet or AdoHcy. The results (not shown) showed that AdoMet and AdoHcy had similar effects on the binding of 23S rRNA to *ermC'* methyltransferase. In both cases, although the plateau of the binding curve was elevated relative to that of the control, the association constant was not changed by either compound. The elevation of the plateau may be due to stabilization of methylase by substrate binding.

Binding of Methylated 23S rRNA to ermC' Methyltransferase. One of the end products of the catalytic reaction mediated by *ermC'* methyltransferase is 23S rRNA N^6,N^6 dimethylated at A2805. Methylated 23S rRNA is a competitive inhibitor of the methylation reaction (Denoya & Dubnau, 1989). The K_i for methylated RNA is close to the K_m value for unmethylated 23S rRNA. The binding of methylated 23S rRNA to the *ermC'* methyltransferase was measured by the filter binding assay. Methylated 23S rRNA was prepared from *B. subtilis* carrying pIM13. 23S rRNA isolated from this strain of *B. subtilis* cannot be detectably methylated in vitro by purified *ermC'* methyltransferase (data not shown), and the strain is Em resistant, showing that A2085 is already fully methylated. The binding curve was determined by using a constant amount of purified *ermC'* methyltransferase with

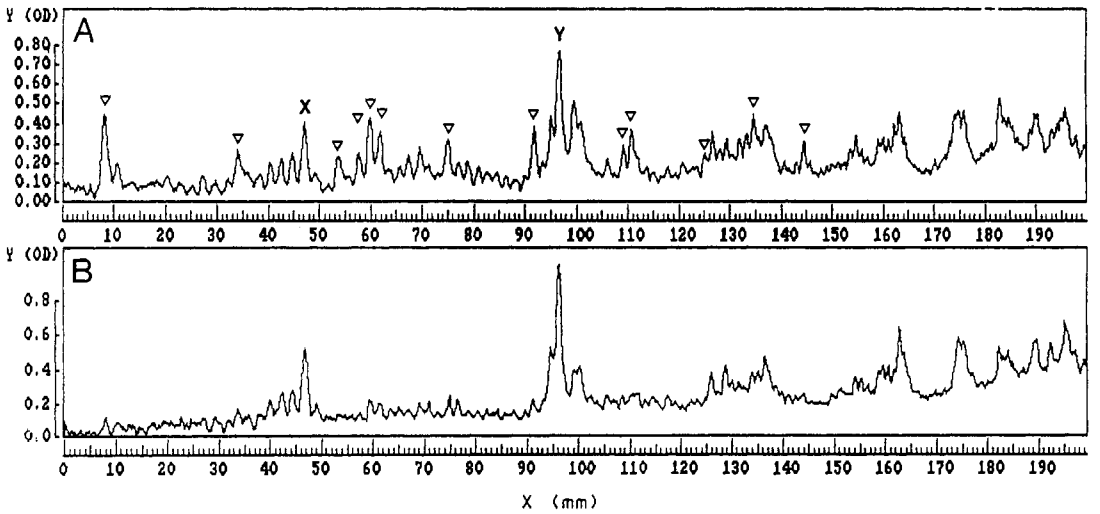

FIGURE 7: Quantitation of DMS reactivity by densitometric scanning. An autoradiograph from a primer extension experiment using primer 1 (see text) was scanned densitometrically. Panel A is a scan of a lane that contained unprotected 23S rRNA, DMS treated. Panel B is a scan of a lane that contained 23S rRNA incubated with 0.29 pmol/μL *ermC'* methyltransferase and then treated with DMS. Peaks X and Y correspond to bands at positions 2093 and 2067, respectively, and were used as internal standards for quantitation. Peaks that have more than 50% reduction in area (comparing panels A and B) are indicated by triangles.

varying concentrations of ^{32}P-5′-end-labeled methylated 23S rRNA. The results (not shown) revealed that the methylated 23S rRNA bound similarly to unmethylated 23S rRNA to *ermC'* methyltransferase, with a K_D of 8×10^{-9} M at 37 °C. This result was confirmed by using the equilibrium competition assay with ^{32}P-5′-end-labeled methylated 23S rRNA and increasing amounts of unlabeled unmethylated 23S rRNA. The results (Figure 3) revealed that cold unmethylated 23S rRNA competes as well as cold methylated 23S rRNA for binding to *ermC'* methyltransferase. We have also determined the rate of dissociation, k_{off}, for the methylated 23S rRNA–*ermC'* methyltransferase complex, as described above. The k_{off} (data not shown) for methylated 23S rRNA was estimated to be 7.3×10^{-2} s^{-1} at 16 °C. The measured K_D at this temperature for methylated 23S rRNA was 3.2×10^{-8} M (not shown). These values are similar to those of unmethylated RNA.

Chemical Footprinting of the 23S rRNA–Methyltransferase Complex. Synthetic DNA oligonucleotides complementary to 23S rRNA from positions 2127 to 2141 (primer 1) and 2211 to 2227 (primer 2) were used to prime reverse transcription. The autoradiographs resulting from these experiments were quantified by denstiometry, and a typical result is shown in Figure 7. Comparing the upper and lower panels, there are obvious reductions in the sizes of certain peaks obtained in the presence of methyltransferase. The residues reactive to DMS are shown in Figure 8. Since the secondary structure of domain V of 23S rRNA in *B. subtilis* as drawn in Figure 8 is adapted from the *E. coli* model (Noller et al., 1981), there may be particular uncertainty about the validity of the structure for *B. subtilis*. Three criteria were used to examine the extent of protection: reduction of the intensity of the bands (calculated as area of each peak in percent of total) by 25%, 50%, or 75%. In a control in which no *ermC'* methyltransferase was present (Figure 8), A, G, and C residues in single-stranded regions were modified by DMS. Certain residues in double-stranded regions were also modified, such as C2131, C2105, C2090, G2083, G2079, and C2070. These residues tended to be either at the ends of paired regions at which breathing may occur, or at regions of probable poor base pairing. Though DMS generally is not reactive toward U residues, we have found that U2043, U2046, and U2061 were slightly modified. Moderate reactivity of U residues toward DMS has been noted previously (Moazed & Noller, 1986). The 50% cutoff criterion revealed protection of C2033, A2041, G2048, A2058, G2059, C2070, G2079, A2086, A2087, G2088, C2090, G2099, and G2110. These residues cover a region that contains a stem loop structure and several irregular helical stems containing unpaired bases. If the more stringent criterion of 75% reduction of DMS reactivity was used, only C2090, G2099, and G2110 appeared to be protected. These positions are all located in a long irregular helical structure (2090–2115). When the criterion was least stringent (25% reduction in reactivity), only 10 additional residues appeared protected, compared to the results with the 50% cutoff criterion. With reduction of the stringency, the additional protected residues were often interpersed among those that were protected when more stringent criteria were used. It is also noteworthy that even with the least stringent cutoff, 24 out of 48 modified residues were not protected, indicating considerable specificity in the binding of methylase to the 23S rRNA. Finally, although residues near the methylation target (A2085) were protected, the target itself was not, although it was reactive with DMS (Figure 8).

Discussion

Binding Reaction. In TKM buffer at 37 °C, the approximate, average equilibrium dissociation constant was found to be 4×10^{-9} M, subject to the uncertainties and assumptions noted under Results. This value was also confirmed from the rate constants and was of the same order of magnitude as the binding constants measured for several other RNA–protein interactions (Carey & Uhlenbeck, 1983; Deckman & Draper, 1985; Lam & Schimmel, 1975; Mougel et al., 1986; Schwarzbauer & Craven, 1981; Spierer et al., 1978).

The second-order rate constant measured at 16 °C was found to be 4×10^6 M^{-1} s^{-1}. This value was also similar to that of other protein–RNA binding systems (Carey & Uhlenbeck, 1983; Mougel et al., 1986; Romaniuk, 1985). The estimated association rate constant for a diffusion-controlled reaction can be calculated from the Smoluchowski equation:

$$k_{on} = \frac{4\pi N}{1000} r_0 (D_1 + D_2)$$

where N is Avogadro's number, r_0 is the reaction radius, and D_1 and D_2 are the diffusion coefficients of the ligands. This equation assumes that the reactants are spherical, that every

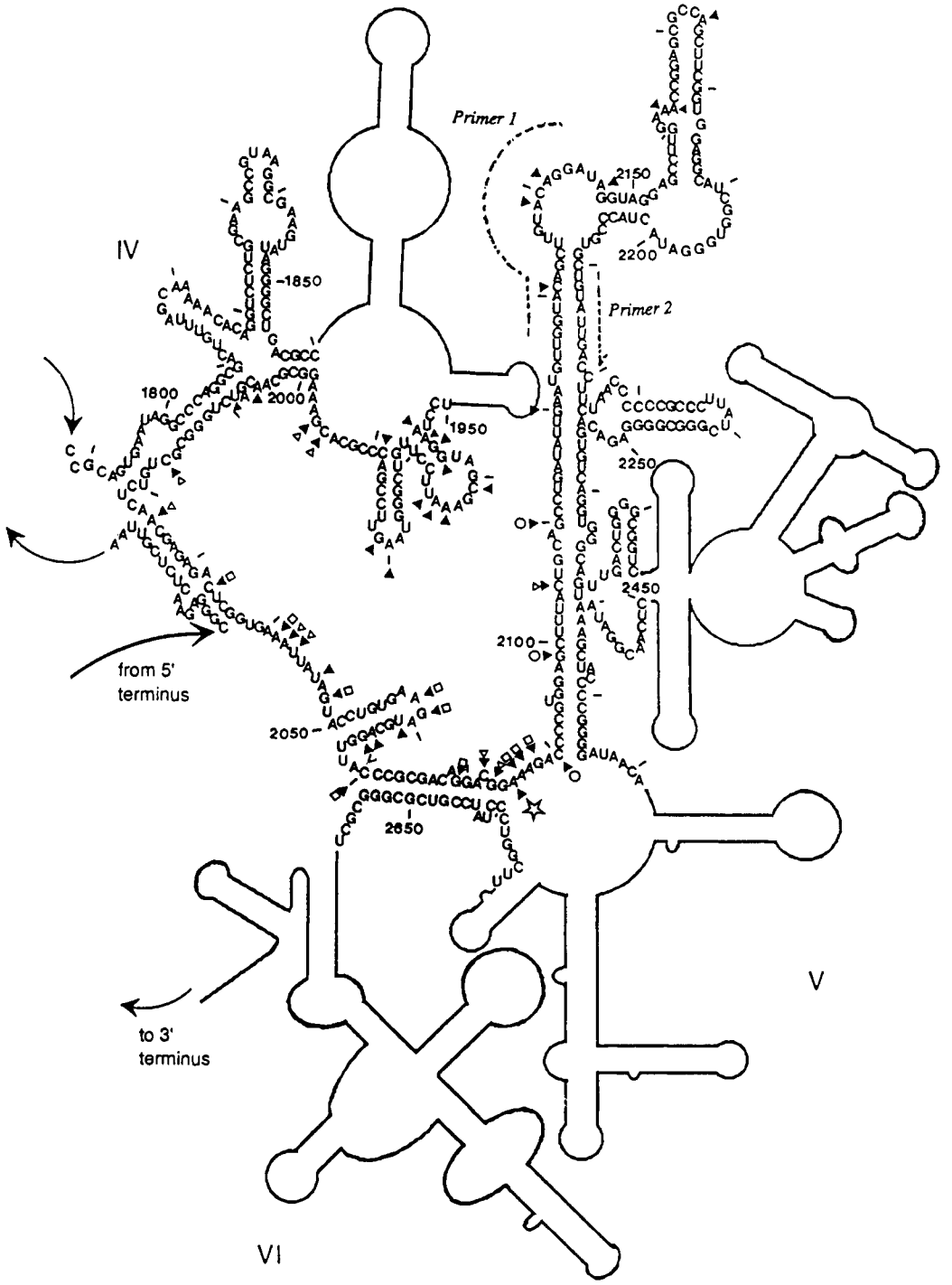

FIGURE 8: Diagram of domain V of 23S rRNA summarizing the *ermC'* methyltransferase dependent protection experiments. Filled triangles denoted nucleotides reactive to DMS. Nucleotides whose DMS reactivities, after the addition of *ermC'* methyltransferase, were reduced by at least 25% (△), 50% (□), and 75% (○) are also shown. A2085 (the methylated target) is marked with a star. Positions of bases complementary to primers 1 and 2 are marked with broken lines. The sequence from C1952 to G2209 was verified in this work. This sequence, as well as the remainder, is from Green et al. (1985). The 23S rRNA secondary structure is adapted from that proposed for *E. coli* (Noller et al., 1981).

collision is productive, and that only the kinetic energy of the reactants is required for complex formation. A diffusion constant for *ermC'* methyltransferase of 9.30×10^{-7} cm^2 s^{-1} was estimated from the average of three similar-sized globular proteins (Sober & Harte, 1970). The diffusion coefficient of the high molecular weight 23S rRNA can be neglected. The r_0 value is taken to be 5×10^{-8} cm for most reactions (Riggs et al., 1970b). The maximum k_{on} for a diffusion-controlled methyltransferase–RNA interaction is then estimated to to be 3.5×10^8 M^{-1} s^{-1}. The experimentally determined value is 2 orders of magnitude lower than this estimate. Thus the binding reaction rate is probably not limited solely by the probability of a collision between the protein and RNA. RNA–protein interactions generally have lower k_{on} values than those predicted for diffusion-controlled events (Carey & Uhlenbeck, 1983; Mougel et al., 1986; Romaniuk, 1985). It is possible in our case that a conformational unfolding of 23S rRNA or of protein is required to expose the binding site. However, our estimate for the Arrhenius activation energy (+3.5 kcal mol^{-1}) is similar to the value observed for diffusion-controlled reactions. The reaction rate therefore may reflect a requirement for a correctly oriented collision, and steric factors of the order of 0.01 are often observed. 23S rRNA is a structurally complex molecule; the central loop of

domain V contains several irregular helical stems plus single-stranded regions, probably involved in binding to methyltransferase. It seems likely that a correct orientation is necessary to present this complex molecule for productive interaction with 23S rRNA, and the data do not require us to postulate a conformational alteration during binding.

The k_{off} value of 6.8×10^{-2} s^{-1} is also comparable to that of other RNA–protein binding systems. The agreement of the ratio of k_{off} and k_{on} (1.7×10^{-8} M) with the directly measured association constant at 16 °C (2.1×10^{-8} M) supports a two-state bimolecular model for methyltransferase binding to 23S rRNA. The agreement inspires confidence that the measured values are reliable.

Thermodynamics of Interaction. Thermodynamic parameters governing *ermC'* methyltransferase binding to 23S rRNA have been determined by measuring the temperature dependency of K_A. For *ermC'* methyltransferase–23S rRNA binding, the enthalpy change is unfavorable ($\Delta H = +6.2$ kcal mol^{-1}). It is apparent that the main driving force is provided by the positive entropy change ($T\Delta S = +17.6$ kcal mol^{-1}). This favorable entropy of binding might result from the release of bound water or from a configurational change in one or both of the ligands. The positive enthalpy argues against the involvement of hydrogen bonding or ionic forces in the interaction of methyltransferase and 23S rRNA, while the favorable entropy change suggests that hydrophobic interactions may be involved. The salt dependency of binding (Figure 8) suggests that only about two to three ion pairs are formed in the RNA–protein complex, and that a substantial portion (~85%) of the binding free energy is contributed by nonelectrostatic interactions. This estimate is approximate due to uncertainties such as in the appropriate value for ψ. Nevertheless, it appears that binding may be driven largely by the establishment of hydrophobic contacts or by configurational changes. In other RNA–protein binding systems the contribution of ionic interactions is also generally small relative to the nonelectrostatic contribution. For instance, the number of ion pairs formed in the R17 coat protein–RNA interaction is five, and the nonelectrostatic contribution to the binding free energy is 80% (Carey & Uhlenbeck, 1983). In the S8–16S rRNA binding, there are five ionic pairings, but nonelectrostatic interactions play a major role (Mougel et al., 1986). In the binding of *Xenopus laevis* factor IIIA to 5S rRNA, five ion pairs are also involved and the contribution of nonelectrostatic interaction to the binding energy is 68% (Romaniuk, 1985).

The ΔG values for other RNA–protein interactions are generally similar (~−10 kcal mol^{-1}) (Carey & Uhlenbeck, 1983; Lam & Schimmel, 1975; Mougel et al., 1986; Spierer et al., 1978). In most cases these interactions are also characterized by favorable entropy changes and by small negative or positive enthalpy changes. In the R17 coat protein system, which is an exception to the above generalization, the large negative entropy is attributed to conformational constraints and "unfavorable hydrophobic" interactions. This binding reaction is characterized by a favorable enthalpy change, which has been interpreted as resulting from a large number of van der Waals and hydrogen bond contacts (Carey & Uhlenbeck, 1983).

Specificity of Binding. The binding specificity of *ermC'* methyltransferase is not absolute. Several nucleic acids were able to compete with 23S rRNA for methyltransferase binding, with varying affinities (Figure 3). Transfer RNA bound poorly, and double-strand DNA also had a relatively low affinity for the protein; only RNA and DNA species that were at least partially single stranded showed appreciable binding affinity. The dissociation constant for 16S rRNA binding to methyltransferase determined both by the equilibrium competition assay and in a direct binding assay was about $4–8 \times 10^{-8}$ M at 37 °C. Presumably, portions of the various single-stranded nucleic acids adopted secondary structures sufficiently similar to that of the 23S rRNA binding site for interaction to occur. Interestingly, a preliminary determination of the binding constant of *ermC'* methyltransferase to *ermC* mRNA yielded a value of 3.5×10^{-8} M at 37 °C (S. Su, F. Briedt, and D. Dubnau, unpublished). Perhaps this mRNA also folds into a "favorable" secondary structure that makes its affinity for methyltransferase even closer to that of 23S rRNA and permits translational autoregulation to occur [Denoya et al. (1986); F. Breidt and D. Dubnau, unpublished]. We note that the bacteriophage T4 gene 32 product also has varying affinities for nucleic acids in the order single-stranded DNA > gene 32 mRNA > other T4 mRNAs > double-stranded DNA (von Hippel et al., 1982).

The relatively low specificity of methyltransferase binding contrasts with the high specificity of R17 coat protein–RNA interaction (Carey et al., 1983). This difference is consistent with the idea that methyltransferase binding is driven largely by the release of water and possibly by configurational changes rather than by the formation of specific interactions, while in the case of R17 as noted above, specific contacts appear to play a greater role (Carey & Uhlenbeck, 1983).

Dimethyl Sulfate Protection. *ermC'* methyltransferase protects several residues in 23S rRNA domain V from the action of DMS. Since only the region from 1951 to 2211 was studied in our experiments, it is possible that additional residues are protected and that binding may involve distant RNA sequences, brought into proximity to domain V by tertiary folding. Also, in view of the uncertainties concerning the stoichiometry of binding, it is conceivable that independent binding sites exist elsewhere on the rRNA molecule. In fact it is likely that lower affinity sites do exist, given the binding exhibited to 16S rRNA (Figure 3). Interestingly, the regions that are known to be protected so far include at least two single-stranded regions (2033–2049 and 2085–2089) and several helices. Three of the latter are irregular and contain bulged adenines. Also partially protected is the loop of a hairpin structure (2050–2067) with three adenine residues in the loop. In several other systems, RNA–protein recognition involves helices containing unpaired residues (Egebjerg et al., 1987; Mougel et al., 1987; Peattie et al., 1981; Romaniuk et al., 1987). The importance of these features in the interaction between *ermC'* methyltransferase and 23S rRNA must be determined by future studies, especially by mutational analysis. It is interesting that A2085, the methyl-accepting residue, although accessible to DMS, is not protected by methyltransferase. Perhaps the enzyme–RNA complex forms a pocket for binding of AdoMet, leaving the target residue accessible to DMS. However, we were unable to achieve protection of A2085 by adding methyltransferase together with AdoHcy, a competitive inhibitor of the methylation reaction (not shown).

Reaction Mechanism. The N^6,N^6 dimethylation reaction of the *ermC* methyltransferase involves two consecutive random bi–bi reactions (Denoya & Dubnau, 1989). In the first reaction, a methyl group from AdoMet is transfered to A2085 yielding an N^6-monomethylated intermediate. The enzyme then dissociates from monomethylated 23S rRNA before proceeding to the second reaction. We have now shown that the cofactor AdoMet, and the end product AdoHcy, had no

effect on the binding constant for RNA, in agreement with a simple random-order mechanism. The binding sites on the enzyme for RNA and AdoMet appear to be independent.

The possibility existed that, after the dimethylation step, the enzyme is released from the RNA and is unable to bind with high affinity. This was found not to be the case. In fact, methylated and unmethylated 23S rRNA bind to the methyltransferase with nearly equal affinities, as shown in the direct binding and competition experiments. Furthermore, methylated 23S rRNA binds to the enzyme with kinetics similar to that of the unmodified 23S rRNA. The previous kinetic data (Denoya & Dubnau, 1989) suggested that the second enzymatic reaction, involving association of monomethylated RNA with methyltransferase, exhibited about the same V_{max} and K_m as the first step, involving unmethylated RNA. The present demonstration that the methylation state of A2085 does not affect the affinity or the second-order rate constant is consistent with these previous results. Also consistent is the apparent absence of direct contact between A2085 and the methyltransferase. These considerations further suggest that N^6 methylation of A2085 does not impact on the secondary or tertiary structures of the RNA in such a way as to affect binding.

The similar binding of methylated and unmethylated 23S rRNA to methyltransferase is in agreement with the previous observation that the K_m for 23S rRNA is nearly identical with the K_i for methylated 23S rRNA, a competitive inhibitor (Denoya & Dubnau, 1989). In vivo then, how does the cell avoid end product interference with methylation and consequently with the expression of MLS antibiotic resistance? Lai (1972) has shown that in vivo methylation occurs prior to the maturation of ribosomes, and it also appears that the *ermC* methyltransferase methylates mature ribosomes poorly in vitro (Shivakumar & Dubnau, 1981). It is also known that the in vivo pools of free rRNA and precursor particles are small (Ingraham et al., 1983), implying that rRNA is rapidly assembled into ribosomes. The deleterious effect of product inhibition may therefore be relieved in the cell by the compartmentalization of free methylated RNA into the ribosomes.

The K_m for the *ermC'* enzyme and 23S rRNA was measured as approximately 250 nM (not shown). This is similar to the value (354 nM) obtained for the nearly identical *ermC* enzyme (Denoya & Dubnau, 1989). By use of the *ermC* value together with k_{on} and k_{off}, and the relationship

$$K_m = (k_2 + k_{off})/k_{on}$$

the k_2 value of the reaction was calculated to be 1.35 s^{-1}. To validate this estimate, we have also calculated k_{cat} from the V_{max} [determined from enzyme kinetics (Denoya & Dubnau, 1989)] using the equation $k_{cat} = V_{max}/E_t$, where E_t is the total active enzyme concentration. The value of k_{cat} calculated in this manner is 1.37 s^{-1}, in excellent agreement with our value for k_2. k_{cat}/K_m is therefore 3.5×10^6 M^{-1} s^{-1}. This value, which sets the lower limit on the second-order rate constant for the enzyme–23S rRNA association (Fersht, 1985), is very similar to our experimentally obtained k_{on} (4×10^6 M^{-1} s^{-1}). In a Michaelis–Menten reaction, $k_2 \ll k_{off}$, so that enzyme and substrate are in rapid equilibrium with enzyme–substrate complex. Methylation of 23S rRNA does not follow this mechanism since k_2 is ~20 times larger than k_{off}. In fact, the reaction fulfills the criteria for Briggs–Haldane kinetics (Fersht, 1985): $k_2 \gg k_{off}$, $K_m > K_D$, and $k_{cat}/K_m = k_{on}$.

Translational Repression. The autogenous translational repression model (Denoya et al., 1986) necessitates the binding of methyltransferase to *ermC* mRNA, apparently within the folded leader region (F. Breidt and D. Dubnau, unpublished). Methyltransferase binds to other single-stranded nucleic acids with 10–100-fold lower affinities than to 23S rRNA (Figure 3). Our preliminary data (S. Su, F. Briedt, and D. Dubnau, unpublished) have shown that the affinity of *ermC* mRNA for the methyltransferase (3.5×10^{-8} M) is weaker than the affinity of 23S rRNA (4×10^{-9} M), but similar to that of 16S rRNA. In the autogenous translational repression systems studied so far, binding to more than one type of nucleic acid is often observed. For example, ribosomal protein S4 binds to 16S rRNA and α mRNA with similar affinities, and to tRNA or other nonspecific mRNAs with 10–100-fold lower affinities (Deckman & Draper, 1985). The bacteriophage T4 gene 32 protein binds to single-stranded DNA and its own mRNA with higher affinities than to other single-stranded nucleic acids (von Hippel et al., 1982). An exception may be provided by the R17 coat protein–RNA system, in which the binding appears to be highly specific. The implication from these studies is that methyltransferase is titrated by a variety of intracellular nucleic acids. In addition, the methyltransferase binds to mature ribosomes [Shivakumar & Dubnau (1981); C. Denoya and D. Dubnau, unpublished]. It is possible that free methyltransferase, available after the saturation of higher affinity sites, is available to exert this feedback repression. How this titration of the *ermC* methyltransferase affects the autogenous feedback loop (Denoya et al., 1986) is impossible to assess quantitatively without information concerning the intracellular concentrations of the various binding sites and the relevant affinities.

Denoya et al. (1986) suggested that a site in the regulatory region of *ermC* mRNA that resembles the region surrounding A2085 in 23S rRNA is the methyltransferase binding target for autorepression. Recent unpublished genetic data have revealed that this regulatory region is indeed the likely target site for autorepressor binding (F. Breidt and D. Dubnau, unpublished). Our DMS protection data show that *ermC'* methyltransferase covers an extensive region on 23S rRNA surrounding A2085. This region includes several potential recognition features including hairpin structures, and irregular helices containing unpaired bases and bulged residues. The *ermC* regulatory region contains all of the above mentioned structural features (Mayford & Weisblum, 1985; Narayanan & Dubnau, 1985).

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