Active Site in RrmJ, a Heat Shock-induced Methyltransferase*

Jutta Hager†, Bart L. Staker§, Hans Büg¶, and Ursula Jakob‡‡

From the †Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048 and §Emerald BioStructures Inc., West Bainbridge Island, Washington 98110

The heat shock protein RrmJ (FtsJ), highly conserved from eubacteria to eukarya, is responsible for the 2′-O-ribose methylation of the universally conserved base U2552 in the A-loop of the 23 S rRNA. Absence of this methylation, which occurs late in the maturation process of the ribosome, appears to cause the destabilization and premature dissociation of the 50 S ribosomal subunit. To understand the mechanism of 2′-O-ribose methyltransfer reactions, we characterized the enzymatic parameters of RrmJ and conducted site-specific mutagenesis of RrmJ. A structure based sequence alignment with VP39, a structurally related 2′-O-methyltransferase from vaccinia virus, guided our mutagenesis studies. We analyzed the function of our RrmJ mutants in vivo and characterized the methyltransfer reaction of the purified proteins in vitro. The active site of RrmJ appears to be formed by a catalytic triad consisting of two lysine residues, Lys-38 and Lys-164, and the negatively charged residue Asp-124. Another highly conserved residue, Glu-199, that is present in the active site of RrmJ and VP39 appears to play only a minor role in the methyltransfer reaction in vivo. Based on these results, a reaction mechanism for the methyltransfer activity of RrmJ is proposed.

RrmJ (FtsJ) is a well conserved heat shock protein present in prokaryotes, archaea, and eukaryotes (1). Functional studies revealed that RrmJ is responsible for methylating 23 S rRNA at position U2552 in the aminoacyl (A)1-site of the ribosome (1, 2). U2552 is one of the five universally conserved A-loop residues and has been shown to be methylated at the ribose 2′-OH group in the majority of organisms investigated so far (3). This suggests that this modification plays an important role in the A-loop function. Analysis of rrmJ deletion mutants in Escherichia coli supports this view, because these cells show a severe growth disadvantage and ribosome defects (1, 4). Polysome profiles of rrmJ deletion strains prepared under non-stringent salt conditions reveal the accumulation of 30 S and 50 S ribosomal subunits at the expense of functional 70 S ribosomes (1, 4). In addition, lower MgCl2 concentrations in the lysate buffer cause the accumulation of 40 S ribosomal particles and a concomitant reduction in 50 S ribosomal subunits (1). This ribosomal defect is also reflected in the significantly decreased translational efficiency of S30 extracts prepared from rrmJ deletion strains (4). Interestingly, the absence of Mrm2p, the mitochondrial RrmJ homologue that has recently been identified to modify the corresponding U2791 in 21 S rRNA does not cause a mitochondrial ribosome assembly or stability defect (5). Yeast mitochondria in the absence of Mrm2p do, however, reveal instability of their genome, a feature that is often associated with defects in mitochondrial translation (5). All these observations point to an important role of RrmJ in ribosome biology, and the simplest interpretation of these results is that the ribosome defect that is observed in rrmJ deletion strains is directly caused by the absence of the Um2552 modification in 23 S rRNA. It also is possible, however, that RrmJ has a second methyltransferase-independent function. Such dual function modes for methyltransferases have been observed in the past. For instance, Pet56p, the methyltransferase that is responsible for methylating the other universally conserved residue in mitochondrial 21 S rRNA, has been shown to be essential for the in vivo maturation of the large ribosomal subunit in mitochondria (3). This phenotype, however, seems to be independent of the methyltransfer activity of Pet56p, because S-adenosylmethionine (AdoMet) binding mutants of Pet56p, which completely eliminate methyltransferase activity, still support ribosome assembly in vivo (6).

In vitro methyltransfer assays have revealed that RrmJ recognizes its methylation target only when the 23 S rRNA is present in 50 S ribosomal subunits (1, 2). This has been confirmed in studies revealing that Mrm2p modifies mitochondrial 21 S rRNA only when assembled with proteins of the large subunit (5). This suggests that the RrmJ-mediated methylation must occur late in the maturation process of the ribosome (1). This is in contrast to other known 23 S rRNA modifications that occur in earlier maturation steps (7).

The reaction mechanism of 2′-O-ribose methyltransferases such as RrmJ has not been analyzed experimentally. We have crystallized RrmJ in the presence of its AdoMet cofactor and have solved the structure of the RrmJ-AdoMet complex to a 1.5-Å resolution (1). Comparison of the structure of RrmJ with structures of other methyltransferases revealed homology to 1FBN, the fibrillarin homologue from Methanococcus jannaschii (8), catechol-O-methyltransferase (COMT) (9), and vaccinia mRNA 2′-O-methyltransferase VP39 (10). VP39, in particular, shows a highly homologous core domain and an RNA binding groove that shares numerous features with RrmJ (1). These structural and sequence comparisons have allowed us and others (11) to predict which residues in RrmJ might be directly involved in the methyltransfer reaction and to postulate a reaction mechanism.

Here, we have prepared a number of site-specific mutants of RrmJ to investigate which residues participate in catalysis. We have conducted phenotypical studies and enzymatic analyses to characterize the activity of our mutants in vivo and in vitro. We have identified two lysine and one aspartate residues that are important for catalyzing the methyltransfer reaction; RrmJ mutants at these positions show a significant decrease in meth-
lytransferase activity. These mutants allowed us to demonstrate that both the ribosome and growth defects observed in \textit{rrmJ} deletion mutants are dependent on the methyltransfer activity of RrmJ.

**EXPERIMENTAL PROCEDURES**

\textbf{Site-directed Mutagenesis—}Site-directed mutagenesis was performed according to the QuickChange protocol (Stratagene, La Jolla, CA). Wild type \textit{rrmJ} cloned into pET11a (pH1) (1) was used as the template. The primers used in this study were as follows: K38A, CTCCCGGGCGGTTTGCACTTGATGAAAATACAGGCAAAG; D83A, CCGCATCATCGCTTGCGCTCTTCTACCTAATACAGCAAAG; D124A, CAGGTTGTCATGTCCGCTATGGCCCCAAACATAGTATGATGAAAATACAGGCAAAG; K164A, GCTGGCGACCTTTTGTACGGGCGGCGTCCTAAGCCGGC; E199A, GACCTTTCTCGAGCTACGTCCTGCCCAGTGTATATTG- TAGGC; Y201A, CTCCGGAGTGCCGCTATTTGATTACGGGACCCGG.

All introduced mutations were confirmed by DNA sequencing. The plasmids that were generated and the strains that were used in this study are listed in Table I. For overexpression and purification of the mutant proteins, the plasmids that contained the mutated \textit{rrmJ} genes were introduced into JUH47. This strain contained a deletion of the \textit{rrmJ} gene to prevent the contamination of our purified mutant proteins by small amounts of WT protein. JUH47 was constructed by P1 transduction. The Tc\(^\text{r}\) of the Tn10 marker that is 90% linked to \textit{rrmJ}\(^{567}\) (1) was used to transfer the \textit{rrmJ}\(^{567}\) allele into JUH47. We then selected for tet\(^{\text{r}}\) and screened for the slow growth phenotype observed in \textit{rrmJ} deletion strains (1).

\textbf{Expression Analysis—}To investigate the expression level of wild type RrmJ in HB24 and of the mutant proteins in the transformed HB23 (\textit{rrmJ}\(^{567}\)) strains, Western blot analysis using polyclonal antibodies against RrmJ was performed. The transformed strains were grown in LB medium supplemented with 100 \mu g/ml ampicillin until an \textit{A}\(_{600}\) of 0.5 was reached. Then, a 2 ml aliquot of cells was taken, resuspended in 85 \mu l of 2\(\times\) SDS-Laemmli buffer, and boiled for 20 min. The proteins were separated on a 14\% Tris-glycine PAGE (NOVEX), and RrmJ was visualized using Western blot analysis. To determine the amount of AdoMet present in the various RrmJ mutant preparations, we first generated an AdoMet titration curve. A 23 \mu M solution of our AdoMet-free D83A mutant RrmJ protein was prepared, and 1 \mu M AdoMet (\textit{t}_{\text{mol}} = 15,400 \text{M}^{-1} \text{cm}^{-1}) was added per titration step. After each AdoMet addition, the \textit{A}_{260}/\text{A}_{280} ratio was measured, and the amount of bound AdoMet was determined by UV absorbance using an extinction coefficient of 1.00 for a 1 mg/ml solution at \textit{A}_{280} (1). Protein concentration determination was not significantly influenced by the presence of the absorbing cofactor AdoMet in the preparation. This was confirmed by performing a Bradford assay, using the AdoMet-free D83A mutant RrmJ protein as a standard protein (data not shown).

\textbf{AdoMet Titration—}To determine the amount of AdoMet present in the various RrmJ mutant preparations, we first generated an AdoMet titration curve. A 23 \mu M solution of our AdoMet-free D83A mutant RrmJ protein was prepared, and 1 \mu M AdoMet (\textit{t}_{\text{mol}} = 15,400 \text{M}^{-1} \text{cm}^{-1}) was added per titration step. After each AdoMet addition, the \textit{A}_{260}/\text{A}_{280} ratio was measured, and the amount of bound AdoMet was determined by UV absorbance using an extinction coefficient of 1.00 for a 1 mg/ml solution at \textit{A}_{280} (1). Protein concentration determination was not significantly influenced by the presence of the absorbing cofactor AdoMet in the preparation. This was confirmed by performing a Bradford assay, using the AdoMet-free D83A mutant RrmJ protein as a standard protein (data not shown).

\textbf{Preparation of Ribosomal Subunits and Methylation Assay—}50 S ribosomal subunits of HB23 (\textit{rrmJ}\(^{567}\)) were prepared as described previously (1). Re-analysis of purified 50 S subunits on sucrose gradients revealed that the preparation is \textit{>98\text{\%}} pure. These subunits served as \textit{in vitro} substrates for wild type RrmJ and the mutant proteins in the methylation assay (1). The RrmJ concentrations used in this assay were 20, 100, or 200 nM in 50 mM HEPES KOH, pH 7.5, 85 mM NaCl, 3 mM MgCl\(_2\), 2 mM \(\beta\)-mercaptoethanol. The initial rate for methylation was measured in the presence of 5 \mu M 50 S ribosomal subunits and increasing amounts of radioactive \textit{[H]}3-adenosyl-L-methionine (85.0 Ci/mmol; Amersham Biosciences). To determine the apparent \(K_{m}\) for 50 S ribosomal subunits, the methylation assay was performed in the presence of 50 \mu M AdoMet and varying concentrations of 50 S ribosomal subunits. To determine the specific activity of wild type RrmJ and the mutant proteins, the assay was carried out using 100 and 200 nM \textit{rrmJ} mutant protein, 50 \mu M AdoMet, and either 5 or 8 \mu M 50 S ribosomal subunits, depending on the \textit{rrmJ} mutant. The methylation reaction was performed at 37 \degree C. At defined time points (2.5, 5, 7.5, and 10 min) after initiating the methylation reaction, 8 \mu l aliquots were taken, and the \textit{[H]}methionyl incorporation into 23 S RNA was determined as described (1). The slopes of the methyl incorporation \textit{versus} time plots were calculated, and the initial velocities obtained were plotted against the respective substrate concentration. The curve was then fitted using a single rectangular hyperbola.
RESULTS

Enzymatic Characterization of RrmJ—To analyze the influence of our amino acid substitutions on the methyltransfer activity of RrmJ, we first needed to characterize the enzymatic properties of wild type RrmJ. The apparent $V_{\text{max}}$ of the methyltransfer reaction, as well as the apparent $K_m$ values for 50 S ribosomal subunits and AdoMet, were determined by in vitro methylation assays using purified RrmJ and radioactively labeled AdoMet. 50 S ribosomal subunits were prepared from the rrmJ deletion strain HB23, because their 23 S rRNA lacks the U2552 methylation catalyzed by RrmJ. We first established assay conditions (see “Experimental Procedures”) in which the rate of [3H]methylation incorporation from [3H]AdoMet into 23 S rRNA was proportional to the RrmJ concentration and linear over more than 150 min (data not shown). Thus, the initial rate measurements represented true initial velocities, and the dependence of the rate on substrate concentration could be measured. Initial velocity data were first obtained with a fixed high concentration of AdoMet (50 $\mu$M) and varying concentrations of 50 S ribosomal subunits. The apparent $K_m$ value for 23 S rRNA within 50 S ribosomal subunits was determined to be 0.8 + 0.1 $\mu$M with an apparent $K_{cat}$ of 0.064 min$^{-1}$ at 37°C (Fig. 1A). This is a significantly higher $K_m$ value than the one determined for the mRNA in VP39 ($K_m = 5$ nM) (13) but very similar to the $K_m$ value determined for the 23 S rRNA methyltransferase ErmC from Bacillus subtilis. ErmC has been shown to dimethylate adenine 2058 (E. coli numbering) in naked 23 S rRNA, early in the ribosomal maturation process (13, 14). To determine the apparent $K_m$ value for AdoMet, the 50 S ribosomal subunits were kept at saturating concentrations (5 $\mu$M), and AdoMet was varied (Fig. 1A, inset). The initial velocity experiments revealed an apparent $K_m$ for AdoMet of 3.7 + 0.3 $\mu$M and the same $K_{cat}$ of 0.064 min$^{-1}$. From these results it became clear that RrmJ and the 2’-O-ribosyl methyltransferase VP39 do not only share a very similar $K_m$ for AdoMet, which is 2 $\mu$M for VP39 (13), but also a similarly low turnover number. The $K_{cat}$ for VP39 has only very recently been determined to be 0.13 min$^{-1}$ (15). As is the case for VP39, the RrmJ catalyzed reaction was linear over more than 150 min and, therefore, through more than 10 turnovers (data not shown). This excluded the possibility that the low $K_{cat}$ observed for RrmJ is because of problems to multiple turnovers but may be because of slow chemical steps (15) or because of accessibility problems of the methyltransfer site in the intact 50 S ribosomal subunits that are used in our in vitro studies (see below). Because of this low turnover number, however, our initial rate measurements that were followed over a 10-min time period need to be considered pre-steady state measurements rather than steady-state measurements.

RrmJ Is a Thermostable Heat Shock Protein—We considered that one possible reason for the heat shock induction of RrmJ could be a possible temperature lability of the enzyme. This would require the overexpression of RrmJ at heat shock temperatures to compensate for the potential loss of function. For this reason, we measured the activity of RrmJ under saturating substrate concentrations over a variety of temperatures (Fig. 1B). We found that the temperature optimum of the methyltransferase activity of RrmJ was at 55°C. The decreased activity at temperatures beyond 55°C is either because of instability of the 50 S ribosomal subunits or because of thermal inactivation of RrmJ. In either case, these results excluded the possibility that the heat shock regulation of RrmJ is based on thermal lability of the enzyme.

The Substrate of RrmJ—RrmJ is able to methylate 23 S rRNA in isolated 50 S ribosomal subunits, as well as in 70 S ribosomes (1, 2), but is unable to methylate naked 23 S rRNA or 23 S rRNA that is present in 40 S ribosomal particles that have been shown to accumulate in cell lysates of rrmJ deletion strains under dissociating salt conditions (1). To analyze the substrate requirements of RrmJ in more detail, we performed 2D gel analysis to compare the protein composition of the 50 S ribosomal subunits of the rrmJ deletion strain with that of wild type strains. As shown in Fig. 2, all 30 ribosomal proteins were detectable at similar levels in the two strains (Fig. 2A and B). These data reveal that the absence of methylation of the highly conserved U2552 does not affect the folding of the 23 S rRNA to an extent that impairs the correct assembly of the 50 S ribosomal subunit. To get some idea how accessible U2552 is in the 50 S ribosomal subunit, we performed modeling studies using the crystal structure of the Deinococcus radiodurans 50 S ribosomal subunit (16) and RrmJ. Although solvent-accessible, U2552 (E. coli nomenclature) is positioned at the bottom of a deep cleft. Assuming that this conserved residue has the same
The Active Site of RrmJ

Surprisingly little is known about the catalytic mechanism of any 2'-O-ribose methyltransferase. Although a reaction mechanism has been postulated for VP39 on the basis of its crystal structure (18), experimental data has not been obtained to support this hypothesis. We have, therefore, decided to combine structural analysis and site-specific mutagenesis to investigate the methyltransfer reaction of RrmJ. The first step in this analysis was to determine which residues are essential for catalysis.

Of all the methyltransferases that have been crystallized so far, VP39 and fibrillarin are the two enzymes whose structures resemble that of RrmJ the most (1). VP39 methylates the first transcribed nucleotide in mRNA following the m^7(5')Gppp-cap (19). The structure of VP39, which was solved in complex with an mRNA substrate analog m^7(5')Gppp-capped RNA hexamer and the inhibitor S-adenosyl homocysteine, showed that the mRNA binds to a groove on the surface of VP39 (20). Structural analysis of the RrmJ-AdoMet complex revealed that RrmJ has a very similar groove adjacent to the AdoMet binding site (1). We modeled the mRNA substrate analog of VP39 on the RrmJ structure and found that the ssRNA fits well into this putative substrate binding site of RrmJ. In this model, the 2'-hydroxyl group of the ribose to be methylated is positioned next to the reactive Ca group of AdoMet (Fig. 3A).

Lys-38, Asp-124, Lys-164, and Glu-199 of RrmJ are the most highly conserved amino acids among RrmJ homologues. A comparison of the crystal structures of RrmJ and VP39 indicate that these residues are located almost at the identical position as the homologous Lys-41, Asp-138, Lys-175 and Glu-207 residues in VP39 (Fig. 3, A and B). These residues, which form a hydrogen bond network that coordinates the phosphate atoms on each side of the methylated nucleoside, have been suggested by us and others (1, 21, 22) to play a critical role in the catalytic mechanism of RrmJ. In addition, Tyr-201 is highly conserved among RrmJ homologues and is located exactly underneath the putative active site, suggesting that it could play a catalytic role in RrmJ.

To analyze whether these residues are important for catalysis of the methyltransfer reaction, we performed site-specific mutagenesis and replaced the five amino acids in RrmJ (Lys-38, Asp-124, Lys-164, and Glu-199) individually with alanine residues. We also substituted the highly conserved amino acid Asp-83 with alanine. This residue, which interacts with AdoMet via two hydrogen bonds, has been predicted to play a crucial role in the catalytic mechanism of RrmJ (1).

Growth Analysis of the RrmJ Mutants on McConkey Plates—

After introducing the individual mutations into wild type rrmJ using site-specific mutagenesis, we transformed the rrmJ mutant plasmids into HB23 (rrmJ^Δ567) to investigate their in vivo function. All the mutant and wild type rrmJ genes are cloned into the pET11a expression system. Fortunately, the expression level of all of the mutated RrmJ proteins in the absence of induced T7 polymerase was found to be very similar to the level of RrmJ seen from a chromosomal copy (data not shown). This allowed us to be confident that any phenotype observed for the mutant proteins was not because of massive over- or underexpression of the protein.

As a first step in the in vivo characterization of our RrmJ mutants, we utilized the inability of rrmJ^Δ567 cells to grow on McConkey plates at 37 °C (1). In contrast, strains that are rrmJ^− because of their chromosomal copy (HB24) or rrmJ^Δ567 strains that contain the pET11rrmJ plasmid (HB25) form large colonies. As shown in Fig. 4A, the D83A mutant, as well as the two lysine mutants, K38A and K164A, are unable to rescue the
growth defect of the \textit{rrmJ} deletion strain, indicating that all three mutations cause the inactivation of RrmJ \textit{in vivo}. These results served as a first indication that the two lysine residues might be involved in the catalytic mechanism of RrmJ. Almost as severe was the phenotype of cells expressing the D124A mutant, suggesting that this amino acid also plays a crucial role in the function of RrmJ. RrmJ deletion strains expressing the E199A or Y201A variant protein showed only slight growth defects, indicating that these two residues are not as important in the catalytic mechanism.

The growth defect of \textit{rrmJ} deletion strains is apparent not just on McConkey plates but can also be observed in liquid LB medium (1). Therefore, we analyzed the growth rate of our mutant strains in liquid medium. \textit{RrmJ} deletion strains expressing no RrmJ or one of the D83A, K38A, D124A, or K164A RrmJ mutants revealed a 2.5–3-fold slower growth rate than cells expressing wild type RrmJ (data not shown). RrmJ deletion strains expressing the E199A or the Y201A variant protein grew almost as well as wild type cells.

\textbf{Analysis of Polysome Profiles—} \textit{RrmJ} deletion strains show very significantly altered polysome profiles compared with wild type strains (1, 4). In the absence of functional RrmJ, lysates prepared under dissociating salt conditions (200 mM NH$_4$Cl, 1 mM MgCl$_2$) accumulate a large 40 S intermediate peak at the
is responsible for the observed growth disadvantage of \textit{rrmJ} deletion strains.

Polysome profiles prepared from \textit{rrmJ} deletion strains expressing the D124A variant revealed a slightly larger proportion of stable 50 S ribosomal subunits than \textit{rrmJ} \textsuperscript{−} strains that express the K38A or K164A variant. Comparison of the polysome profiles of these three mutant strains prepared under associating salt conditions, however, did not reveal any significant differences (data not shown), suggesting that the three residues Lys-38, Lys-164, and Asp-124 are equally important for the \textit{in vivo} function of RrmJ. The ribosome profile of cells expressing the E199A and Y201A RrmJ variants, on the other hand, showed only a slight accumulation of 40 S ribosomal subunits, suggesting that mutation of these two residues does not cause a severe defect in RrmJ function.

\textit{AdoMet Binding Affinity of Purified RrmJ Mutants}—To analyze the structural integrity and \textit{in vitro} activity of our RrmJ mutants, the plasmids overexpressing the RrmJ mutants were transformed into BL21 \textit{rrmJ}Δ567 strains, and the respective mutant proteins were overexpressed. All but one of the proteins were soluble and could be purified as described under “Experimental Procedures.” The D124A mutant protein was present in inclusion bodies, a fact that prevented us from purifying this RrmJ variant.

Wild type RrmJ contains bound AdoMet cofactor when purified, as demonstrated by mass spectrometry (1). The amount of bound AdoMet can be quantified from the absorption ratio at 280 and 260 nm (\textit{A}\textsubscript{280/260} ratio), because AdoMet absorbs at 260 nm. Wild type RrmJ has an \textit{A}\textsubscript{280/260} ratio of 0.9 (1), whereas nucleotide-free proteins typically show \textit{A}\textsubscript{280/260} ratios of 1.8–1.9. As a first measure of the structural integrity, the ability of each of the mutant proteins to retain bound AdoMet during the purification process was assessed. As predicted, the D83A mutant showed an \textit{A}\textsubscript{280/260} ratio equivalent to that of nucleotide-free proteins (\textit{A}\textsubscript{280/260} = 1.8), demonstrating that Asp-83 is important for AdoMet binding. All other mutant proteins, however, revealed \textit{A}\textsubscript{280/260} ratios smaller than 1.8, indicating that various amounts of AdoMet were still associated with the proteins after the purification. To determine the approximate amount of bound AdoMet for wild type RrmJ and the mutants, an AdoMet-titration curve was established using the AdoMet-

\textit{In Vitro Activity of the RrmJ Mutants}—To characterize the \textit{in vitro} methylation activity of our mutants, we determined the specific activity of our purified RrmJ mutant proteins under conditions in which the two substrates, 50 S ribosomes and AdoMet, are present in saturating concentrations (Table II). Both lysine mutants, K38A and K164A, showed at least a 50-fold decrease in our \textit{in vitro} methylation assays. The very small amount of methyl incorporation observed was within the limits of the background incorporation, and an increase in enzyme concentration did not cause an increase in methyl incorporation (data not shown). This showed that these two lysine residues play a very critical role in the methyltransfer reaction and corroborated our \textit{in vivo} data that suggested that both residues are important for the overall function of RrmJ.

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**Fig. 4. Phenotype of the active site mutants.** A. growth of HB24 (WT) and HB23 (\textit{rrmJ}Δ567) strains harboring either no plasmid or pHB1-derived plasmids encoding wild type \textit{rrmJ} (WT) or the mutant \textit{rrmJ} genes as indicated, on McConkey plates at 37 °C. B. polysome profiles of the \textit{rrmJ} deletion strain HB23 expressing no plasmid or pHB1-derived plasmids encoding WT RrmJ or the active site mutants as indicated, under dissociating conditions (1 mM MgCl\textsubscript{2}, 200 mM NH\textsubscript{4}Cl) in 10–50% sucrose gradients. In the absence of functional RrmJ, 40 S ribosomal particles accumulate at the expense of intact 50 S ribosomal subunits.
Substitution of Glu-199 caused a 16-fold reduction in $K_m$ without changing the $K_m$ for 50 S ribosomal subunits. This suggested a less important role for this residue in the catalytic mechanism, an assumption that agreed well with the phenotype of $rrmJ$ deletion strains expressing this E199A mutant protein. These cells showed only slightly affected polysome profiles and minor growth disadvantages on McConkey plates suggesting that the activity of this mutant protein is sufficient in vivo to partly rescue the $rrmJ$ deletion. To ascertain that the E199A mutant protein shows sufficient in vivo methylation activity to account for the rescued phenotype, we analyzed the extent of 23 S rRNA modification in 50 S ribosomal subunits prepared from this mutant strain, by testing them as substrates in our in vitro methylation assay (data not shown). If the E199A mutant was fully active in vivo, most of the 50 S ribosomal subunits should be already methylated, and the in vitro methyl incorporation should be as low as the methyl incorporation observed with 50 S ribosomal subunits prepared with wild type RrmJ and the mutant proteins after the purification was calculated according to the AdoMet standard curve (see inset). All of the mutant proteins except D83A contained approximately the same amount of AdoMet after the purification process. Inset, AdoMet was titrated in 1 μM steps to a 23 μM solution of the AdoMet-free RrmJ mutant D83A. The absorption spectra were measured after each AdoMet addition, and the respective $A_{390/260}$ ratios were plotted against the corresponding amount of AdoMet/protein.

The specific activity of the Y201A mutant protein was only slightly decreased, suggesting that this residue does not take a direct part in the methyltransfer reaction. Surprisingly, the D83A mutant protein that showed no activity in vivo showed about 20% of wild type activity in vitro. This may reflect the fact that this mutant shows an increase in $K_m$ for both substrates (AdoMet, apparent $K_m = 7.0 \mu M$; 50 S ribosomal subunit, apparent $K_m = 1.4 \mu M$) leading to a more significant decrease in catalytic activity at lower substrate concentrations.

Very little is known about the mechanism of the methyltransfer reaction in 2′-O-ribose methyltransferases such as VP39 and RrmJ. Based on the resemblance between VP39 and COMT, whose methyltransfer reaction has been analyzed (23), a general base-catalyzed reaction has been proposed for VP39 (20). In this model, a general base catalyzes the deprotonation of the 2′-OH group of the ribose to activate the nucleophile oxygen, which attacks the methyl group of AdoMet in a $S_2$2-type reaction. In COMT, Mg$^{2+}$ is bound to the active site and is proposed to play a crucial role in substrate binding and positioning the hydroxyl group close to the methyl group of AdoMet (9, 24). The methyltransferase activity of RrmJ has also been shown to be dependent on the presence of Mg$^{2+}$ ions (2). This divergent cation, however, might be essential to stabilize the 50 S ribosomal substrate rather than playing a role in the catalytic mechanism of the protein. Lowering the Mg$^{2+}$ concentration to 1 μM causes the dissociation of the 50 S ribosomal subunits prepared from $rrmJ$ deletion strains into 40 S particles, which are no longer substrates for RrmJ (1). Because the active site of RrmJ superimposes very well with the active site of the Mg-independent 2′-O-ribose methyltransferase VP39 (see Fig. 3A and Ref. 21), the methyltransferase activity of RrmJ likely does not require Mg$^{2+}$.

Based on the geometry of the catalytic center in RrmJ and VP39 two highly conserved lysine residues, Lys-38 (Lys-41) and Lys-164 (Lys-175) were identified as likely candidates for being involved in either deprotonating the hydroxyl group directly or in lowering the $pK_a$ of the hydroxyl group (20). Superposition of RrmJ and VP39 and modeling of the ribose substrate of VP39 into the potential active site of RrmJ also revealed the presence of two highly conserved negatively charged residues, Asp-124 and Glu-199, in close proximity to the substrate (Fig. 3A). From a comparison of the methyltransferase domains of reovirus a2 protein, VP39, and RrmJ, Bujnicki and Rychlewski (21) have proposed recently that these four conserved residues form a catalytically active K-D-K-E tetrad (21). Simultaneous superposition of all three structures revealed a perfect conservation of these four residues. In addition, alanine mutagenesis scans that were performed with VP39 suggested that some of these residues are involved in AdoMet binding and methyltransferase activity (25). These alanine scans and additional structure and sequence comparisons also suggested a possible involvement of Tyr-201, a highly conserved residue that lies underneath the putative active site of RrmJ.

We have now individually mutated these five, potentially crucial amino acids to alanine residues and analyzed the activity of the mutants both in vivo and in vitro. We demonstrated that the introduced mutations did not significantly decrease the expression level and solubility of the mutant proteins in vivo or the AdoMet binding affinity of the purified proteins in vitro, suggesting that no major structural rearrangements occurred in the mutant proteins.

Our results clearly show that lysine residues Lys-38 and Lys-164 both play critical roles in the methyltransfer reaction of RrmJ. RrmJ variants with alanine substitutions for either of these two lysine residues were unable to rescue the $rrmJ$ deletion phenotype when expressed at wild type level and revealed a significant decrease in the methyltransfer activity in vitro. Substitution of residue Asp-124 also caused a severe phenotype and the accumulation of 40 S ribosomal subunits, indicating that the methyltransfer reaction is severely impaired. Mutations in Glu-199, however, did not reveal a clear phenotype in vivo, although the mutated protein showed a reduced specific activity in vitro. These results suggest that Glu-199 plays a more minor role in the methyltransfer reaction.
Summary of the in vivo and in vitro characterization of the RrmJ active site mutants

| Mutation | Growth 37 °C | Polysome profile | AdoMet bound | Apparent $K_{cat} (\text{min}^{-1})$ | Apparent $K_m (\mu M)$ |
|----------|-------------|-----------------|--------------|----------------------------------|----------------------|
| Wild type | ++          | ++              | 76           | 0.064                            | 0.8                  |
| D83A     | –           | –               | 0            | 0.012                            | 1.4\textsuperscript{a}|
| K83A     | –           | –               | 68           | $<0.001$                         | ND\textsuperscript{b}|
| D124A    | (+)         | (+)             | ND\textsuperscript{c} | ND\textsuperscript{c} | ND\textsuperscript{c} |
| K164A    | +           | +               | 74           | $<0.001$                         | ND                   |
| E199A    | +           | +               | 72           | 0.004                            | 0.7                  |
| Y201A    | +           | +               | 66           | 0.012                            | 0.5                  |

\textsuperscript{a} The apparent $K_m$ for AdoMet was determined to be 7.0 $\mu M$.
\textsuperscript{b} ND, not determined.
\textsuperscript{c} D124A mutant aggregated upon overexpression in BL21rrmJΔ567 cells and could not be purified. This mutant protein was soluble in the strains that were used to investigate the \textit{in vivo} activity of the protein.

The functional analysis of our active site RrmJ mutant proteins has allowed us to propose a reaction mechanism of RrmJ. It has also enabled us to show that the lack of methyltransferase activity of RrmJ is responsible for the appearance of less stable ribosomes in \textit{rrmJ} deletion strains. This clearly rules out the possibility that influence of RrmJ on ribosome stability is because of a second, methyltransfer-independent function of RrmJ as has been observed for the mitochondrial methyltransferase Pet56p (6). It remains to be directly demonstrated, however, whether it is the absence of the modification at position U2552 in the A-site of the ribosome that destabilizes the ribosome as seems likely or whether the potential tRNA methylation activity of RrmJ could be indirectly responsible for the ribosome defect. This tRNA methylation activity has been observed for RrmJ \textit{in vitro} (1) and has now been demonstrated for Trrm7p, the cytosolic RrmJ homologue in yeast, \textit{in vivo} (22). In this context, it will be also very interesting to determine which functions of RrmJ require the up-regulation of this highly conserved protein under heat shock conditions.

![Proposed reaction mechanism of RrmJ](image-url)
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