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pH-dependent conformational flexibility within the ribosomal peptidyl transferase center

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ABSTRACT

A universally conserved adenosine, A2451, within the ribosomal peptidyl transferase center has been proposed to act as a general acid–base catalyst during peptide bond formation. Evidence in support of this proposal came from pH-dependent dimethylsulfate (DMS) modification within *Escherichia coli* ribosomes. A2451 displayed reactivity consistent with an apparent acidity constant (pK_a) near neutrality, though pH-dependent structural flexibility could not be rigorously excluded as an explanation for the enhanced reactivity at high pH. Here we present three independent lines of evidence in support of the alternative interpretation. First, A2451 in ribosomes from the archaebacteria *Haloarcula marismortui* displays an inverted pH profile that is inconsistent with proton-mediated base protection. Second, in ribosomes from the yeast *Saccharomyces cerevisiae*, C2452 rather than A2451 is modified in a pH-dependent manner. Third, within *E. coli* ribosomes, the position of A2451 modification (N1 or N3 imino group) was analyzed by testing for a Dimroth rearrangement of the N1-methylated base. The data are more consistent with DMS modification of the A2451 N1, a functional group that, according to the 50S ribosomal crystal structure, is solvent inaccessible without structural rearrangement. It therefore appears that pH-dependent DMS modification of A2451 does not provide evidence either for or against a general acid-base mechanism of protein synthesis. Instead the data suggest that there is pH-dependent conformational flexibility within the peptidyl transferase center, the exact nature and physiological relevance of which is not known.

Keywords: chemical footprinting; Dimroth rearrangement; methyl adenosine; pKa perturbation; ribosome mechanism

INTRODUCTION

From a chemical point of view, the most important step of protein biosynthesis is peptide bond formation (Chladek & Sprinzl, 1985). This reaction is catalyzed within the large 50S subunit of the ribosome using aminoacyl-tRNA and peptidyl-tRNAs as substrates (Noller, 1991). There are two binding sites in the 50S subunit, termed the A-site (for aminoacyl or acceptor) and the P-site (for peptidyl; Green & Noller, 1997). The reaction takes place via the transfer of an activated peptidyl residue from a peptidyl-tRNA bound in the P-site to the amino group of an aminoacyl-tRNA bound in the A-site. The peptidyl transfer reaction involves nucleophilic attack by the α-amino group in the A-site on the ester linking the peptide to the P-site tRNA. The transacylation reaction is expected to proceed through two transition states separated by a short-lived tetrahedral intermediate (Hegazi et al., 1978).

The high resolution crystal structure of the 50S ribosomal subunit from *Haloarcula marismortui* has provided a framework for consideration of catalysis within the peptidyl transferase center (Ban et al., 2000; Nissen et al., 2000). The active site was identified by soaking the ribosomes with a transition state analog of the acylation reaction (Nissen et al., 2000). The inhibitor used was a puromycin derivative that contained a CCdAp (P-site analog) coupled via a phosphoramidate linkage to the puromycin α-amino group (A-site analog; Welch et al., 1995). The phosphoramidate group serves as a mimic of the tetrahedral intermediate predicted to form during the transacylation reaction. The location of the bound inhibitor provided definitive evidence that the peptidyl transferase center is compromised exclusively of RNA. It implicated A2451 (*Escherichia coli* numbering used except where indicated) as the residue most likely to play a catalytic role in peptide bond formation (Nissen et al., 2000; Fig. 1A). Interestingly, the N3 imino group of A2451 is within hydrogen bonding distance of

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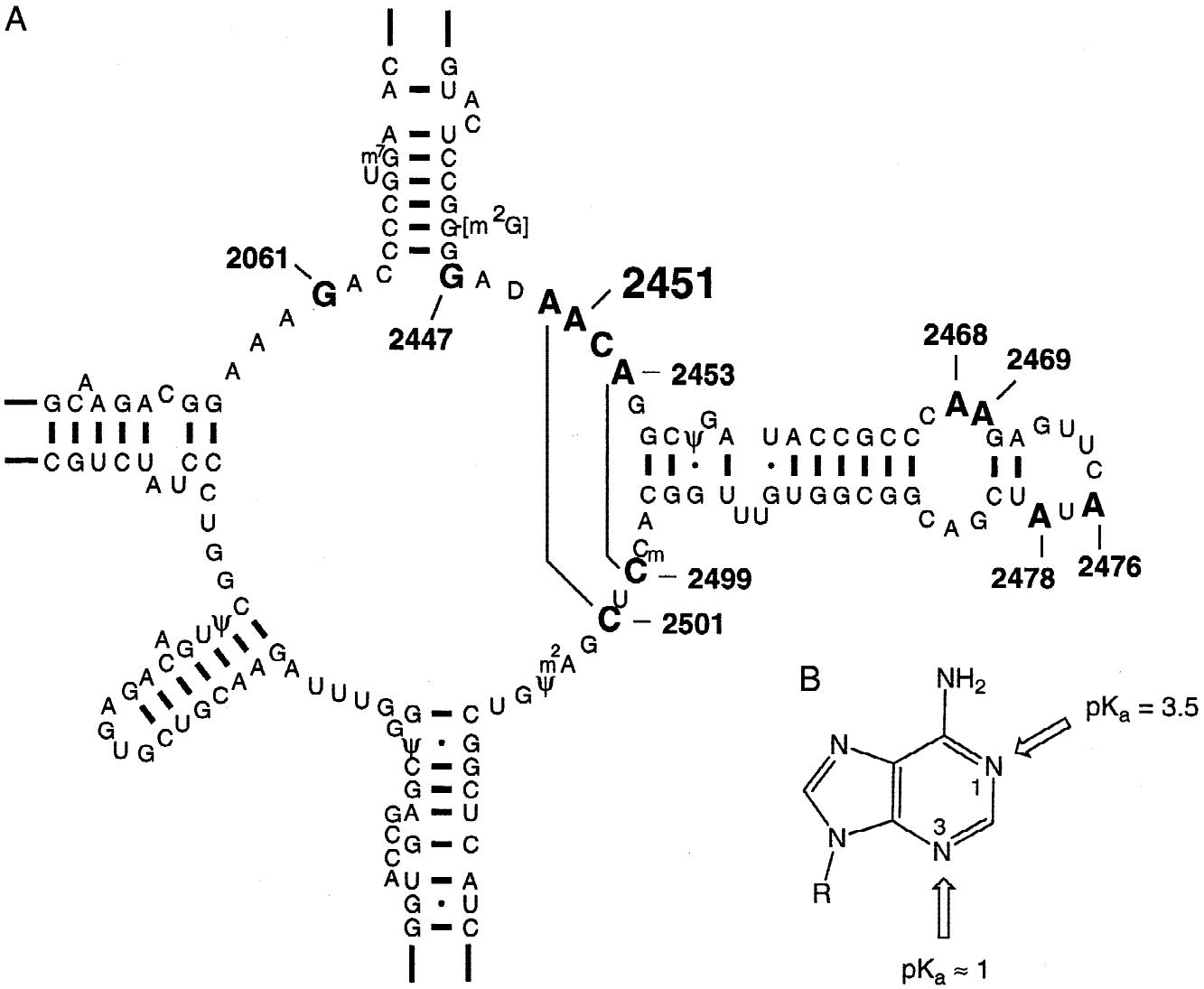

FIGURE 1. A: RNA sequence and secondary structure within the *E. coli* peptidyl transferase center (Gutell et al., 1993). A2451 is highlighted as are other nucleotides discussed in the course of this study. All of the highlighted nucleotides are conserved between *E. coli*, *H. marismortui*, and *S. cerevisiae* rRNA except A2453 and C2499 which are both Us in the *S. cerevisiae* rRNA (Gutell, 2000). The noncanonical A-C pairs that flank A2451 are indicated by vertical lines. **B:** Chemical structure of adenosine with the N1 and N3 imino groups numbered and their pK_as indicated (Seela et al., 1998; Muth et al., 2000).

the phosphoramidate, despite the fact that neither the imine nor the nonbridging oxygen should be protonated at neutral pH. This suggests that the acidity constant (pK_a) of A2451 is perturbed at some stage along the reaction pathway.

We obtained what appeared to be independent evidence for an A2451 pK_a perturbation by probing *E. coli* ribosomes with dimethylsulfate (DMS) as a function of pH (Connell & Yarus, 1994; Muth et al., 2000). We observed that A2451 was the only adenosine within the peptidyl transferase center whose reactivity to DMS increased with pH (Muth et al., 2000). Experiments on the model nucleoside system 3-deazaadenosine demonstrated pH-dependent DMS modification can provide a reasonable estimate of a nucleoside's pK_a. Based upon this calibration, we estimated the pK_a of A2451 to be approximately 7.6. This is similar to the macroscopic pK_a of the peptidyl transfer reaction (Maden & Monro, 1968; Pestka, 1972). Mutagenesis of A2451 to C, G, or U and expression under a temperature-sensitive promoter resulted in a dominant lethal phenotype in *E. coli* (Muth et al., 2000).

The biochemistry appeared to fit nicely with the crystallography, but three uncertainties remained. First, the preferred site of adenosine DMS modification is the N1 position (Lawley & Brookes, 1963), yet the N3 imino group is presented into the active site cleft by the ribosome (Fig. 1B; Nissen et al., 2000). Second, whereas pK_as approaching neutrality have been observed for the N1 imino group (Cai & Tinoco, 1996), the N3 pK_a is

estimated to be 2–3 units lower (Seela et al., 1998; Muth et al., 2000). There is no other precedent for its perturbation, especially to a value as high as 7.6. Third, pH-dependent DMS modification cannot rigorously distinguish between protection induced by protonation (a proton footprint) and protection induced by increased conformational flexibility (a structural footprint). Establishing which position on the A2451 heterocyclic ring is susceptible to DMS modification would help address these uncertainties. If DMS modifies N3, it would be consistent with, but not proof of a proton footprint, whereas N1 modification could only be explained by a change in conformation from that observed in the crystal structure. Furthermore, if the unusual properties ascribed to A2451 by DMS mapping are important for ribosome function, then they should be conserved among ribosomes from other phylogenetic sources.

Here we present DMS mapping of ribosomes from the archaebacteria *Haloarcula marismortui*, the same ribosomes whose crystal structure was reported (Ban et al., 2000), and from the eukaryotic yeast *Saccharomyces cerevisiae.* We also implement a novel use of the Dimroth rearrangement of DMS-modified RNA to explore which position on A2451 was modified within *E. coli* ribosomes. These data suggest that there is a pH-dependent conformational change within the ribosome active site, which leaves the proposed pK_a perturbation at A2451 undefended by biochemical experimentation.

RESULTS

DMS mapping of *Haloarcula marismortui* ribosomes

We hypothesized that if a perturbed pK_a at A2451 is a conserved feature of the peptidyl transferase center, then a similar pH-dependent DMS pattern should be observed across phylogeny including eukaryotic and archaebacterial ribosomal sources. The 50S ribosomal crystal structure was determined for *H. marismortui*, a halophilic archaebacteria (Ban et al., 2000). We probed these ribosomes in an initial effort to explore the phylogenetic conservation of the DMS pattern and to allow direct comparison between the mapping data and the crystal structure. The extent of A2451 methylation was monitored between pH 5 and 9.5 and the sites of modification revealed by reverse transcription from a ^{32}P-labeled primer. Adenosine methylation results in reverse transcription termination 1 nt before the site of modification (termed an N-1 RT stop; Stern et al., 1988). The intensity of this termination signal reflects the efficiency of DMS modification.

Similar to observations made in *E. coli* (Muth et al., 2000), A2451 in *H. marismortui* ribosomes was the only site between nucleotides 2315 and 2493 (*H. marismortui* nt 2350–2528) that showed a pH dependence to the DMS reactivity. However, in contrast to the previous observations in *E. coli*, *H. marismortui* A2451 was less reactive at high pH ($\geq$8.0) and became about fivefold more reactive as the pH was reduced (Fig. 2). The reactivity transition titrates with an apparent pK_a of 7.5, but the inverted DMS modification pattern is inconsistent with a base protonation event as the reactivity should be maximized in the deprotonated form at high pH and minimized at low pH where the nucleophilic imino group is blocked by protonation.

H. marismortui ribosomes require high concentrations of monovalent ions for activity and crystallization (Ban et al., 1998). We were concerned that the inverted DMS dependence might somehow be cation-concentration-dependent, so we repeated the DMS modification at K^+ concentrations between 1.0 and 2.5 M. An equivalent pattern was observed at all K^+ concentrations tested (Fig. 2A, compare lanes 3 and 4, compare lanes 5 and 6). To confirm the DMS modification approach is tolerant of the high salt concentrations, we repeated the 3-deazaadenosine methylation titration at high K^+ (2.5 M). Consistent with previous observations, the nucleoside was methylated at high pH and protected at low pH (data not shown). The modification rate data is consistent with a pK_a of 7.0, which is somewhat higher than that originally reported at trace monovalent salt concentrations, but consistent with the spectrophotometrically determined value (Minakawa et al., 1999; Muth et al., 2000). Thus, the unusual DMS modification pattern at A2451 in the *H. marismortui* ribosomes does not result from the high monovalent salt concentrations.

The activity of the *H. marismortui* ribosomes under DMS reaction conditions was confirmed using a modified version of the fragment assay (see Materials and Methods). Unlike *E. coli* ribosomes (Miskin et al., 1970), no change in the reaction rate was observed whether or not the ribosomes were heated to 37 °C before initiating the reaction at 0 °C. Furthermore, the methylation profile at A2451 did not change upon heating to 37 °C before recooling to 0 °C and adding the DMS (data not shown).

Methylation of yeast ribosomal RNA

Given that two systems have displayed contradictory modification patterns, we next explored the pH dependence of DMS modification within a eukaryotic ribosome. 60S ribosomes were isolated from *Saccharomyces cerevisiae* and subjected to DMS modification between pH 5.5 and 9.0. This resulted in a modification pattern different from that seen in either the *E. coli* or the *H. marismortui* ribosomal active sites (Muth et al., 2000). In the region of the sequence between 2300 and 2493, 3 nt (A2478, A2482, and A2392) showed a pH-dependent increase in their DMS reactivity (data

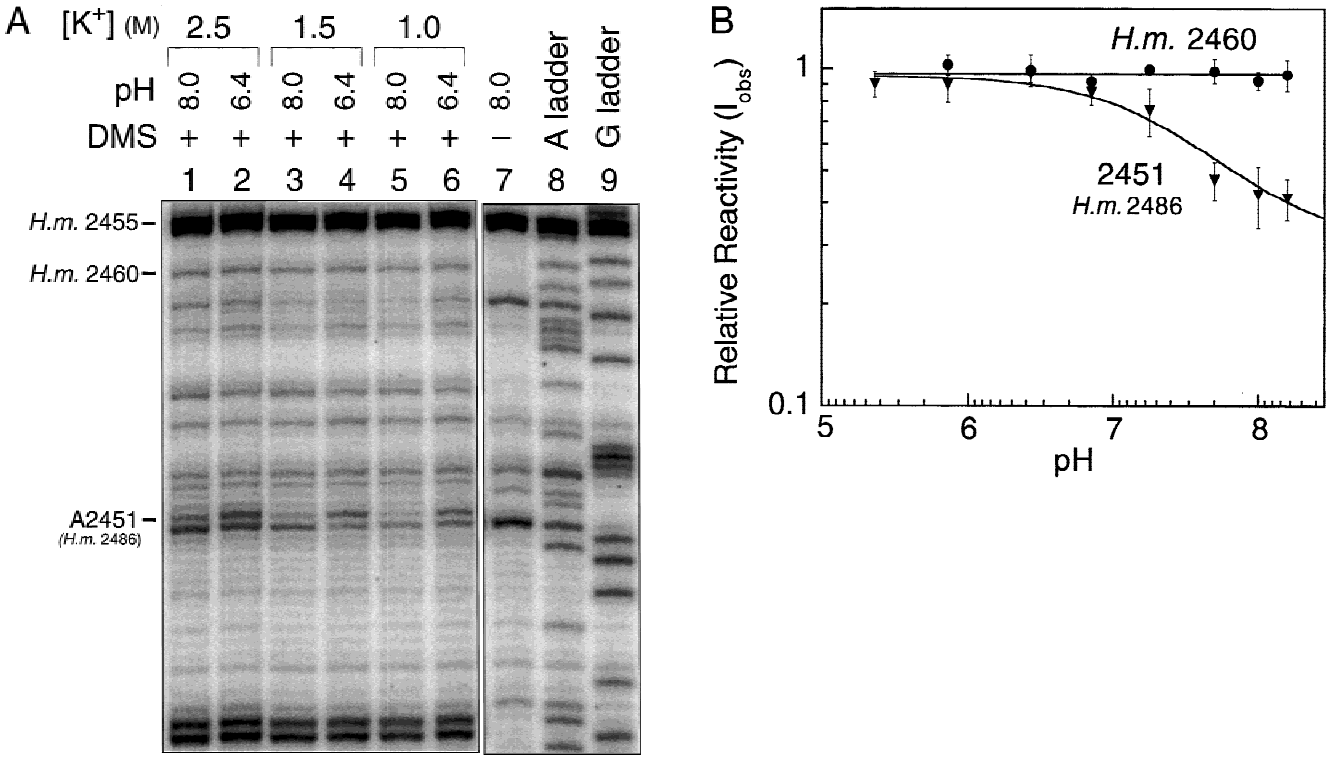

FIGURE 2. pH dependence of A2451 DMS modification in *H. marismortui* ribosomes. **A:** An inverted pH-dependent methylation pattern in *H. marismortui* 23S rRNA. Lanes 1–6: DMS modification of 50S large ribosomal subunits at pHs and monovalent salt concentrations indicated above gel. Lane 7: no DMS control of *H. marismortui* 50S ribosomal subunit methylated at pH 8.0. Lanes 8 and 9: A and G sequencing ladders, respectively. **B:** The normalized extent of A2451 (*H.m.* A2486) and *H.m.* 2460 methylation plotted versus pH and fit to Equation 1. The data are the average of three experiments and the standard deviations are indicated with error bars.

not shown), but A2451 was only modestly reactive and its reactivity did not change with pH (Fig. 3A). Instead C2452, which neighbors A2451 and is also universally conserved throughout phylogeny (Gutell, 2000; Gutell et al., 2000) showed increasing DMS reactivity with increasing pH (Fig. 3A). Modification followed a standard pH profile in that C2452 was unreactive at low pH and became increasingly reactive as the pH was raised resulting in an apparent pK_a of 7.2 (Fig. 3B). A telling feature of this observation is that the only nucleophilic position on C is the N3, which is relatively solvent inaccessible within the *H. marismortui* crystal structure (Ban et al., 2000). This implies a pH-dependent conformational rearrangement of this C in yeast 60S ribosomes. Unfortunately, C2452 modification could not be accurately monitored within *H. marismortui* and *E. coli* ribosomes due to a DMS-independent RT stop at this position (Fig. 2A; Muth et al., 2000).

The activity of the yeast ribosomes was confirmed as per *H. marismortui*. A brief incubation of the ribosomes at 37 °C followed by cooling to 0 °C for the activity assay resulted in a modest twofold increase in the reaction rate compared to unheated ribosomes, but there was no change in the DMS modification pattern at C2452 or A2451 upon heating (data not shown).

Defining the position of A2451 modification within *E. coli* ribosomal RNA

It is difficult to explain the irregular pH dependence of nucleotide modification within archaebacteria and eukaryotic peptidyl transferase centers as anything other than a pH-dependent conformation change. This caused us to revisit the question as to which imino group (N1 or N3) on A2451 was modified by DMS within *E. coli* ribosomes. N3 is solvent exposed in the peptidyl transferase center (Ban et al., 2000), so DMS modification at N3 would be consistent with a pK_a perturbation. It would explain the apparent hydrogen bond observed between A2451 and the nonbridging phosphate of the phosphoramidate inhibitor (Nissen et al., 2000). In contrast, modification at N1 could only be explained by a conformational change from that observed in the crystal structure because the A2451 N1 is involved in a hydrogen bond to G2061 (Ban et al., 2000).

A pH-dependent unpairing of the A2451–G2061 pair was previously explored by probing the accessibility of G2061 to kethoxal modification (Muth et al., 2000). G2061 did not become kethoxal reactive under the same alkaline conditions where A2451 became DMS reactive. This served as evidence against a conformational

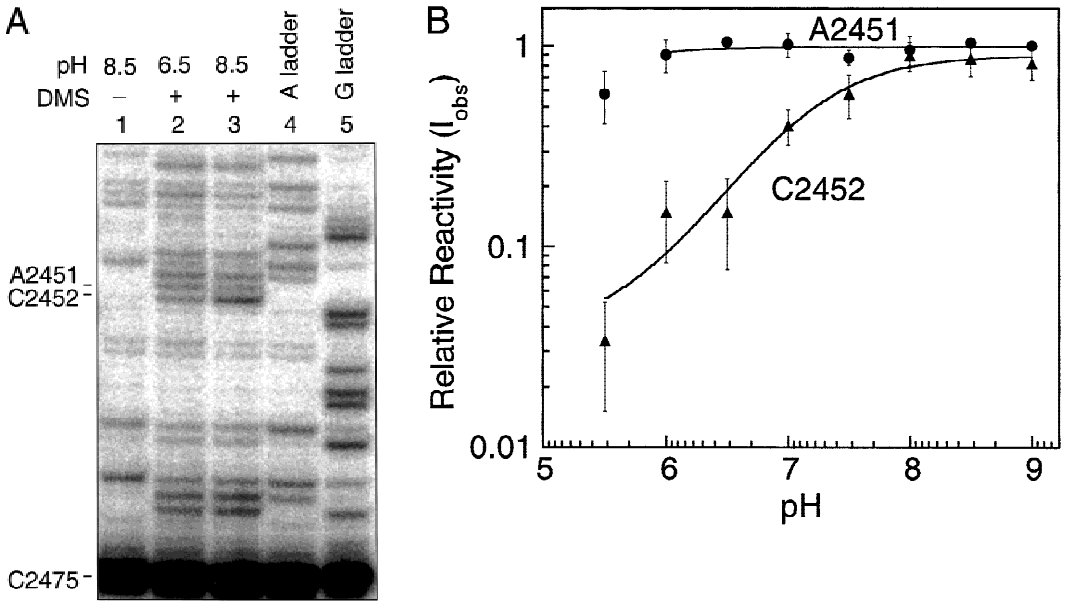

FIGURE 3. pH dependence of A2451 and C2452 DMS modification in yeast *S. cerevisiae* 60S ribosomes. **A:** pH-independent modification of A2451 and pH-dependent modification of C2452. Lanes 1 and 2: dideoxy C and T sequencing ladders. Lane 3 and 4: DMS modification of 60S large ribosomal subunits at pHs indicated above gel. Lane 5, no DMS control. **B.** The normalized extents of DMS modification at A2451 and C2452 within the peptidyl transferase center plotted versus pH. Each point is an average of seven independent experiments. The standard deviations are indicated with error bars.

change, but G2061 could be unreactive for several reasons. One way to distinguish between a protection due to a proton footprint versus a conformational footprint is to identify which imino group of A2451 is DMS modified.

Previous work has shown that the position of DMS modification on the adenine base is dependent upon the structure and hydrogen bonding environment of the nucleic acid (Lawley & Brookes, 1963; Singer & Fraenkel-Conrat, 1969a, 1969b; Lawley & Shah, 1972). For example, DMS preferentially modifies the N3 position of 2′-deoxy A within a DNA duplex. In this environment, the N1 is locked in a hydrogen bond, which makes it less available for alkylation. However, when the DNA is denatured, the N1 reacts preferentially over the N3 (Lawley & Brookes, 1963). As with DNA, the position of modification on a ribo-adenosine is likely to be specific to the structure and hydrogen bonding environment of individual nucleotides, but RNA presents substantially more structural variation than a DNA duplex. It is generally observed that DMS only reacts with As located in single-stranded RNA regions (Stern et al., 1988). Analysis of total cellular RNA found that the N1 of A is about 20 times more reactive than the N3 (Lawley & Brookes, 1963), and only a slight amount of N3 modification was detected in tRNA or deproteinized rRNA (Lawley & Shah, 1972).

There are no reports in the literature on how to distinguish between an N1 and an N3 methylation event at a specific position within RNA. Only the relative ratio of N1 versus N3 modifications at all positions have been reported based upon depurination or nuclease digestion of methylated adducts (Lawley & Brookes, 1963; Singer & Fraenkel-Conrat, 1969a, 1969b; Lawley & Shah, 1972), which is inadequate to specifically address the position of modification at A2451. DMS-induced transcriptional termination cannot be taken as unequivocal evidence for N1 modification. The N3 methylated adduct is in equilibrium with a ring opened form, and either of these methylation products might cause an N-1 RT stop (Saito & Fujii, 1979). Therefore an alternative approach is needed that can distinguish the position of modification within the heterocyclic ring while retaining information about the nucleotide's sequence identity.

Selective Dimroth rearrangement of the 1-methyladenosine adduct could provide a means to distinguish between N1 and N3 methylation of adenosine in a sequence-specific manner (Fig. 4; Macon & Wolfenden, 1968; Fujii et al., 1989). Due to an interest in sequencing applications, characterization of N1 and N3 methylated adenosines was performed to determine the consequences of methylation (Macon & Wolfenden, 1968; Saito & Fujii, 1979). Both modifications result in opening of the six-membered ring at neutral to slightly alkaline pHs (Macon & Wolfenden, 1968; Saito & Fujii, 1979; Fujii et al., 1989). This process is quite slow for the nucleoside 1-methyladenosine at neutral pH, but at pH 9 at 25 °C it occurs with a half life of about 1 day

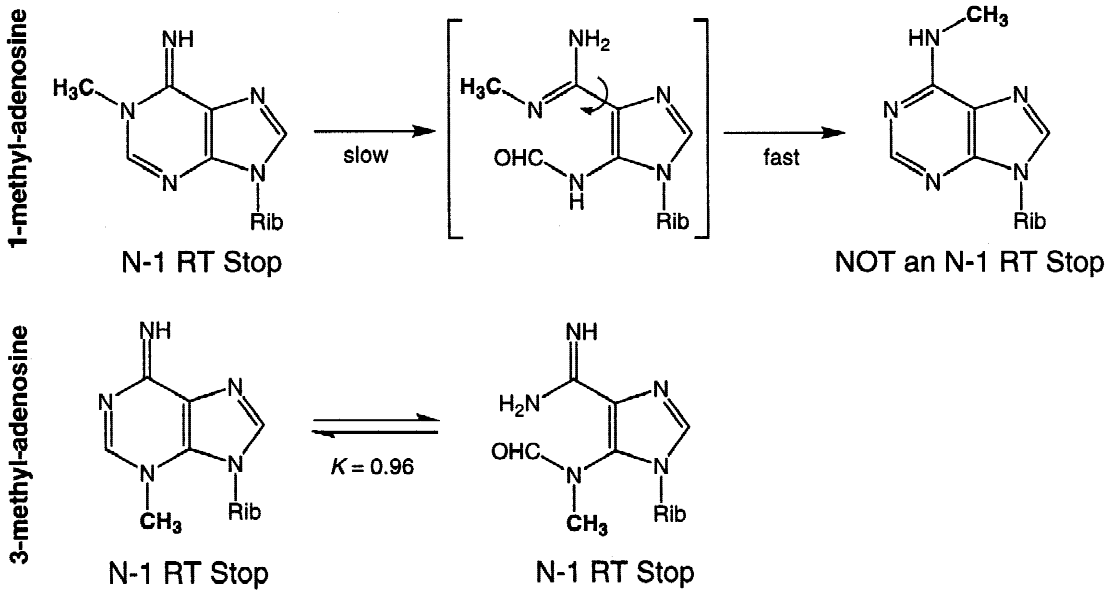

FIGURE 4. Scheme for hydrolytic ring opening of N1 and N3 methylated adenosine adducts. Ring opening of the N1 adduct is followed rapidly by a Dimroth rearrangement to produce the N6-methylated product (Jones & Robins, 1963; Macon & Wolfenden, 1968). The N3 adduct also undergoes ring opening, but it does not undergo rearrangement (Saito & Fujii, 1979; Fujii et al., 1989). The outcome is that only the N1 adduct is converted into a reverse transcriptase readable modification upon mild alkaline incubation.

(Macon & Wolfenden, 1968). Once the ring is opened, it rapidly undergoes a Dimroth rearrangement to produce the highly stable 6-methyladenosine product (Jones & Robins, 1963; Fig. 4). From the perspective of the reverse transcriptase that is used to map sites of modification, this rearrangement should convert an N-1 RT stop into a read-through position (see below). The N3-methylated adduct also undergoes ring opening to form the *N*-methylformamido-imidazole derivative at neutral to alkaline pH, but it cannot undergo a subsequent rearrangement before ring closure (Fig. 4; Saito & Fujii, 1979; Fujii et al., 1989). Instead, it recircularizes back to 3-methyladenosine and reaches a uniform equilibrium between the two adducts (Saito & Fujii, 1979). Both the methylated and the ring-opened adducts could cause transcriptional termination, though this has not been confirmed (Saito & Fujii, 1979; Muth et al., 2000).

Retention of the N3 methyl group and migration of the N1 methyl group upon alkaline treatment of the modified RNA could provide a straightforward approach to determine which imino group of a given A is methylated within an RNA sequence. Mild base treatment of modified RNA should result in migration of methyl groups from N1 to N6, which would attenuate the intensity of the DMS N-1 RT stop. Given that most stops are caused by N1 methylation (Stern et al., 1988), the majority of band intensities should be reduced compared to the DMS-independent terminations. In contrast, the intensity of the N-1 RT stop due to N3 methylation should not be attenuated by alkaline treatment.

Reverse transcription of N6-methyladenosine-containing templates

It is well established that N1-methylation of A leads to an N-1 RT stop, but a basic premise in employing the Dimroth rearrangement for this application is that the N6-methylated adduct produced by methyl migration can be used effectively as an AMV reverse transcriptase template. It is known that N6-methylated DNA can template replication by a DNA polymerase (Barras & Marinus, 1989). Furthermore, eukaryotic mRNAs are posttranscriptionally N6-methylated (Bokar et al., 1997), yet they can still be reverse transcribed. This makes chemical sense given that the *trans* rotamer of N6 methyl adenosine retains a proton on the amine available for Watson–Crick base pairing.

To confirm that AMV reverse transcriptase can utilize N6-methyl adenosine as a template, we prepared an RNA transcript (specifically the L-21 G414 form of the *Tetrahymena* group I intron) in which every A was replaced with the N6-methyl adduct. Previous studies have demonstrated that the triphosphate of N6-methyl adenosine is incorporated efficiently by RNA polymerase in place of adenosine (Ortoleva-Donnelly et al., 1998; Ryder et al., 2000). We obtained full-length RNA completely substituted with N6-methyladenosine in good yield. The RNA was purified, annealed to a radiolabeled primer, and reverse transcribed. As expected, the transcription pattern did not display significantly stronger N-1 RT stops than observed upon transcrip-

tion from the unmodified RNA (Fig. 5, compare lanes 3 and 4). This confirms that the product of the Dimroth rearrangement is an adduct that can be efficiently used as a reverse transcriptase template.

Defining the rate of the Dimroth rearrangement

The experimental question is: does mild base treatment of DMS-modified *E. coli* rRNA affect the A2451 DMS stop in a manner comparable to the majority of the other As in the rRNA? To employ the Dimroth rearrangement for nucleotide-specific identification of N1 versus N3 methylation, it was necessary to select a condition wherein the RNA remains sufficiently intact for reverse transcription, while a sufficient quantity of the methylated nucleotides have undergone rearrangement. The rate of N1-methyl migration increases approximately log-linearly with pH between 7 and 11 (Macon & Wolfenden, 1968). Unfortunately for this experiment, a similar relationship exists between pH and the rate of RNA hydrolysis (Li & Breaker, 1999). A compromise pH of 9.0 and a temperature of 37 °C was selected based upon initial incubations (data not shown). This limits the extent of base hydrolysis while providing a sufficient level of methyl migration.

We determined the rate of the Dimroth rearrangement for the 1-methyladenosine nucleoside under these buffer conditions by monitoring the time-dependent change in UV absorption (265 nm) as previously described (Macon & Wolfenden, 1968). The N6-methyladenosine product is approximately 30% more absorbent at 265 nm than the N1-methyladenosine starting material (Johnson et al., 1958). In aqueous buffered solutions, the conversion is quantitative and follows single-exponential kinetics (Macon & Wolfenden, 1968). At pH 9.0 and 37 °C, the Dimroth rearrangement of the free nucleoside proceeded at 0.1 h^{-1}. If the N1-methylated adducts in a polynucleotide rearrange at the same rate, which need not be the case, then approximately half of the N1-methylated adducts would be converted to the N6-methylated products within 7 h.

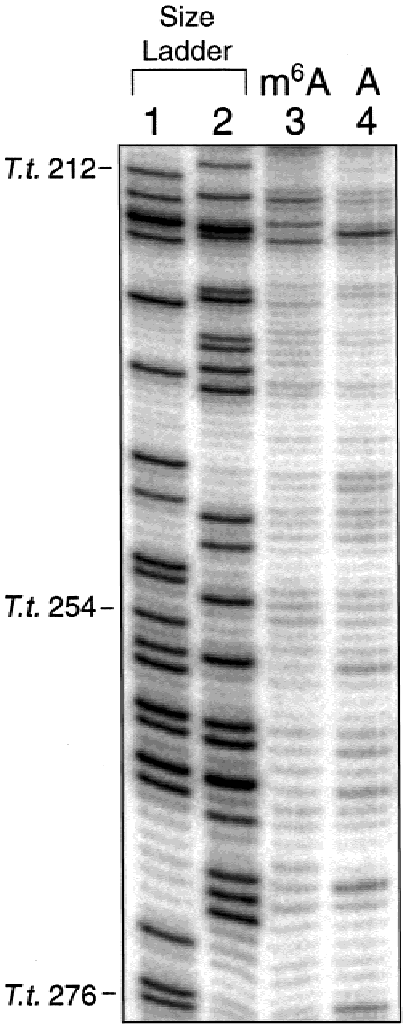

FIGURE 5. Reverse transcription of an RNA template containing m^6A. Templates used in this experiment contained exclusively m^6A (lane 3) or A (lane 4) at each adenosine position in the sequence. Lanes 1 and 2 are dideoxy C and T sequencing ladders for G and A, respectively. The lack of strong reverse transcriptase stops for both the A and m^6A containing RNAs (lanes 3 and 4) indicate that m^6A templates reverse transcription as efficiently as A.

Dimroth analysis of A2451 in *E. coli* 23S rRNA

The position of adenosine heterocyclic ring methylation within *E. coli* 23S rRNA (residues 2478–2451) was investigated using conditions that promote Dimroth rearrangement of the N1-methylated adenosines. DMS-modified (pH 7.9) 23S rRNA from *E. coli* was isolated and subjected to alkaline pH (pH 9.0) incubation at 37 °C for various times up to 60 h. The RNAs were annealed to a ^{32}P-radiolabeled primer and used as a template in reverse transcription. The DNA products were separated on a sequencing gel and the results shown in Figure 6. The intensity of several DMS-specific N-1 RT bands within the ladder (A2468, A2469, A2476, and A2478) were progressively attenuated as a function of the alkaline incubation time relative to the intensity of the non-DMS specific stops at G2448-m^2G2445 (Fig. 6A, lanes 2, 4, 6, and 8). The attenuation does not appear to be due to RNA hydrolysis, because the overall background intensity at non-DMS-specific sites does not increase significantly as would be expected if the RNA were being progressively degraded (compare Fig. 6A, lanes 1, 3, 5, and 7). The approximate rate of signal loss is 0.015 to 0.002 h^{-1} (Fig. 6B), which is about 10- to 50-fold slower than expected based upon the rate of Dimroth rearrangement of the free nucleoside. The slower rate may derive from hydrogen bonding or structural interactions in the deproteinized 23S rRNA that reduce the rate of hydroxide attack in the

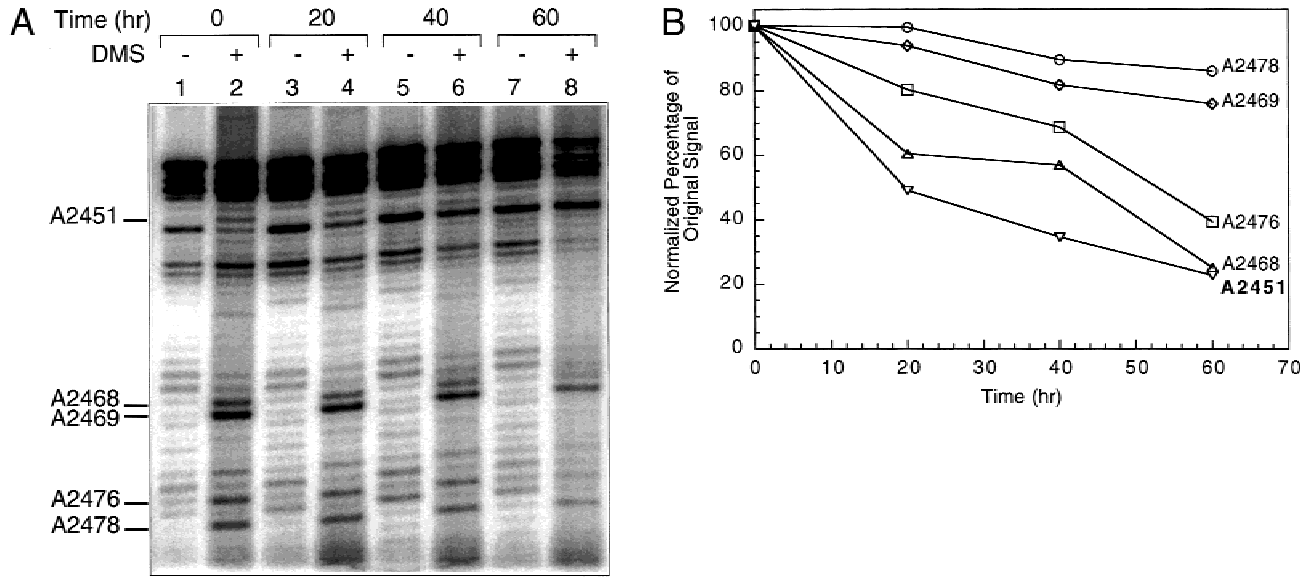

FIGURE 6. Dimroth analysis of DMS-modified 23S rRNA isolated from *E. coli* 50S subunits. **A:** Autoradiogram of reverse transcribed 23S rRNA (nt 2443–2480) incubated under conditions conducive to a Dimroth rearrangement of N1 methylated adenosine residues. The presence or absence of DMS and the length of the alkaline incubation are indicated above each lane. The bands corresponding to the N-1 RT stops for individual nucleotides are labeled to the left. Band intensities corresponding to adenosine residues 2451, 2468, 2469, 2476, and 2478 are attenuated as a function of time in a manner consistent with a Dimroth rearrangement. **B:** Plot of normalized signal strength versus incubation time for the five DMS-modified adenosines monitored in **A**.

polynucleotide compared to the free nucleoside. Similar differences between nucleoside and polynucleotide chemical reactivities have been reported (Fujii et al., 1989). Although the rates are different, the signal attenuation suggests that these four positions have undergone a Dimroth rearrangement and are at least partially modified at the N1 position of the base.

N1 DMS modification at A2468, A2469, A2476, and A2478 is consistent with the 50S ribosomal crystal structure. All four of these nucleotides are conserved between *E. coli* and *H. marismortui* and equivalent DMS modification patterns were observed between the two species (Fig. 2A). Within the 50S structure, the N1 position of each nucleotide is solvent accessible. Furthermore, the N1 appears to be more accessible than the N3, with the possible exception of A2469.

If the level of signal attenuation seen for A2468, A2469, A2476, and A2478 is that expected for a nucleotide modified at N1, how does the pattern compare to that at A2451? The intensity of the A2451 N-1 RT stop is attenuated at least as much as that of the other As in this region (Fig. 6). About half of the signal was lost after 20 h (Fig. 6A, compare lanes 2 and 4), and the band intensity had fallen to that of the no-DMS control after 60 h (Fig. 6A, compare lanes 7 and 8). This suggests that a substantial fraction of the DMS-modified A2451 can undergo a Dimroth rearrangement, which implies that within *E. coli* ribosomes, methylation at A2451 occurred primarily at the more common N1 position rather than the relatively unusual N3 group.

DISCUSSION

Biochemical analysis of *E. coli* ribosomes provided evidence to imply that A2451 might serve an important role in catalysis of peptide bond formation (Muth et al., 2000). The unusual pH dependence of A2451 DMS modification suggested that the pK_a of this residue is substantially perturbed. The apparent pK_a of 7.6 closely matched the macroscopic pK_a of the peptidyl transferase reaction (Maden & Monro, 1968; Pestka, 1972). This observation was particularly intriguing because in our hands, A2451 was the only residue in the domain V of *E. coli* 23S rRNA that demonstrated this pH-dependent modification pattern (Muth et al., 2000). Furthermore, A2451 is conserved among all organisms for which sequence information is available (Gutell, 2000). Mutation of A2451 to any other nucleotide resulted in a lethal phenotype in *E. coli*, even in the presence of wild-type ribosomes (Muth et al., 2000). Finally, the X-ray crystal structure (performed at pH 5.8) of the *H. marismortui* 50S ribosomal subunit complexed with a transition state inhibitor revealed that A2451 is the only residue within the peptidyl transferase active site that is close enough to make a hydrogen bonding interaction with the tetrahedral intermediate (Nissen et al., 2000). Because both the A2451 N3 and the nonbridging phosphoramidate oxygen have a pK_a in the range of 1 to 2 (Saenger, 1984), their close proximity in the structure implies there is a pK_a perturbation at A2451 at least in the intermediate state.

Evidence for pH-dependent conformational flexibility in the peptidyl transferase center

Although these arguments are internally consistent, it remained an open possibility that the pH dependence of A2451 DMS modification reflected a conformational change rather than a protonation event. Here we have presented three independent lines of evidence indicating that the DMS pH titration reflects conformational flexibility within the peptidyl transferase center.

First, 50S ribosomal subunits from *H. marismortui* display an inverted pH profile in that A2451 is more DMS reactive at low pH than at high pH. This is inconsistent with a pK_a effect because protonation at low pH should shield, rather than expose, the base to alkylation. For example, this pattern is opposite that observed in model reactions with the nucleoside 3-deazaadenosine where the pK_a measured by DMS modification closely matched that determined spectroscopically (Minakawa et al., 1999; Muth et al., 2000). Because the *H. marismortui* ribosomes are the same as those used for crystallization, the results can be compared directly to the structure without need for extrapolation. The inverted pattern implies that A2451 becomes more solvent exposed at low pH. This is not the conformation observed in either the apoenzyme or the inhibitor complexed ribosomes (Ban et al., 2000; Nissen et al., 2000), which implies a conformational rearrangement.

Second, an equivalent experiment performed on yeast 50S ribosomes found that A2451 reactivity was modest, but unaffected by pH. Instead, the adjacent nucleotide C2452 displayed a pH-dependent reactivity profile. Cytidine residues are methylated at the N3 position by DMS (Lawley & Brookes, 1963), and C2452 appears to be solvent excluded within the archaebacterial ribosome structure (Ban et al., 2000).

Third, the original DMS modification observed at A2451 in *E. coli* ribosomes appears to be at the N1 position of the heterocyclic ring, which is not solvent accessible unless the RNA is conformationally rearranged (Ban et al., 2000). We assayed for Dimroth rearrangement of 1-methyladenosine adducts to distinguish N1 versus N3 modification at nucleotide resolution within the RNA sequence. The intensity of the A2451 methylation signal was attenuated at least as much as that observed for several other As in the sequence upon incubation in a mildly alkaline buffer. This implies that most of these residues, including A2451, are modified primarily at N1 consistent with the original observation that DMS modifies the N1 about 10- to 20-fold more efficiently then the N3 of adenosine (Lawley & Brookes, 1963; Lawley & Shah, 1972).

Efforts to determine if an N3 modified adenosine causes a reverse transcriptase N-1 RT stop and to establish how the RT signal of this adduct is changed upon Dimroth rearrangement have proven unsatisfactory to date. Although such data would provide a valuable control, site-specific incorporation of an N3 methylated adenosine into RNA is prevented by the highly labile nature of 3-methyladenosine to the acid treatments necessary for chemical synthesis (Saito & Fujii, 1979; Fujii et al., 1989). Chemical modification of RNA transcripts (native or denatured) to generate an N3 adduct for analysis is compromised by preferred methylation at the N1 position (Lawley & Brookes, 1963). Nevertheless, careful measurements of alkaline-induced ring opening have been performed on 1-methyladenosine and 3-methyladenosine nucleosides, and these model studies provide a solid basis for the experimental approach (Macon & Wolfenden, 1968; Saito & Fujii, 1979; Fujii et al., 1989). Thus, while we cannot unequivocally state that the A2451 N1 is modified and the N3 is not, the A2451 signal is attenuated to an extent at least as much as other As in this region whose N1s are exposed which do serve as internal controls. Thus, the data are more consistent with A2451 DMS modification at N1 than N3. Because the A2451 N1 is hydrogen bonded to the N1 of G2061, the DMS effect observed in *E. coli* at A2451 is more likely to reflect a pH-dependent conformational flexibility than the pK_a of A2451's N3 imino group.

Therefore, large ribosomal subunits obtained from organisms spanning the three phylogenetic kingdoms each display a different DMS modification pattern within the peptidyl transferase core. This is difficult to understand given the high level of phylogenetic sequence conservation within this region. For example, A2451 and C2452 are both universally conserved and the nucleotides to which A2451 is paired, namely G2447 and G2061, are also conserved between these ribosomes. The different patterns may result from distinct conformational states of the various ribosomal preparations. Changes in the pH dependence of active site conformations have been observed even within a single *E. coli* ribosome preparation depending upon how they have been incubated. For example, Bayfield et al. (2001) have shown that the A2451 modification pattern in *E. coli* ribosomes is eliminated upon heat activating the ribosomes at 37 °C, a phenomenon we have also observed (G.W. Muth & S.A. Strobel, unpubl. results). They have ascribed this effect to the active–inactive transition induced upon 37 °C incubation of *E. coli* ribosomes (Bayfield et al., 2001).

Possible cause of the pH-dependent conformational flexibility

Even if A2451 protonation is not being directly monitored in these assays, the data indicate that there is something in the active site of all three ribosomes that is titrated with a pK_a of approximately 7.5. A2451 is involved in an array of hydrogen bonds that does not appear to require unusual protonation, but A2451 and

C2452 are flanked on either side by noncanonical A·C pairs that could be highly pH sensitive (Ban et al., 2000; Fig. 7). The nucleotide 5′ of the active site residue, A2450, forms a wobble pair with C2501 and both residues are conserved in all three organisms whose ribosomes were tested. In *E. coli* and *H. marismortui* ribosomes, the nucleotide on the 3′-side, A2453, forms a wobble-like slipped pairing with C2499. In yeast, 2453 and 2499 are both U residues and there is no obvious proton deficiency that would make the pair pH sensitive.

In a typical A·C wobble pair, the C N3 forms a hydrogen bond to the A N1, which is protonated (Cai & Tinoco, 1996). Based upon NMR characterization of such A·C wobble pairs, the pK_a of the A is perturbed to a value approaching neutrality (6.0–6.5). The geometry of the A2450·C2501 pair observed crystallographically is consistent with an A N1 to C N3 hydrogen bond (Ban et al., 2000). Although A2450 should be mostly protonated at pH 5.8, the condition under which the 50S ribosomes were determined, it is unlikely to be protonated at the slightly basic pHs (7.5–8.5) where the peptidyl transferase center displays maximal activity (Pestka, 1972). None of the nucleotides in the A·C pairs display pH-dependent DMS reactivity, but their deprotonation could create local rearrangements in the active site that expose A2451 or C2452 to DMS reactivity.

One observation of potential significance to this interpretation is that the local conformation of the peptidyl transferase center appears to be different in the *Thermus thermophilus* 70S ribosome structure reported at 5.5 Å resolution (Yusupov et al., 2001). This may result from organism-specific difference between *T. thermophilus* and *H. marismortui* ribosomes or from inclusion of the 30S subunit in the *T. thermophilus* structure. But, the apparent conformational dissimilarity in this region might also result from the differences in pH used for crystallization (5.8 for *H. marismortui* compared to 7.4 for *T. thermophilus*). Unfortunately, the detailed nature of any structural difference is not clear at 5.5 Å resolution.

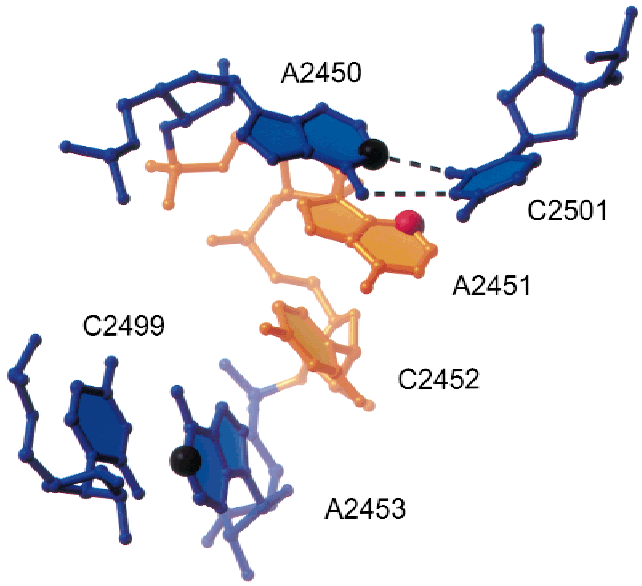

FIGURE 7. Noncanonical A·C pairs flanking the active site A2451 within the peptidyl transferase center (Ban et al., 2000). The two residues (A2451 and C2452) that display pH-dependent DMS modification in the active site are shown in orange. Both these residues make additional hydrogen bonding contacts to residues that are not shown. The N3 position of A2451, which is proposed to act catalytically, is highlighted as a red sphere. The A·C pairs that flank A2451 are shown in blue, including the A2450·C2501 wobble pair and the A2453·C2499 pair. Hydrogen bonds are indicated by black dashed lines. Hydrogen bonds in the A2453·C2499 pair is not shown due to ambiguity, though it is close to a wobble configuration. The N1 position of A2450 and A2453, which might be protonated in these conformations, is highlighted as a black sphere. Deprotonation of these residues may be responsible for the pH-dependent conformational changes observed by DMS modification.

Implications for the peptidyl transferase reaction mechanism

Although these results indicate that DMS modification of A2451 in the peptidyl transferase center cannot be taken as evidence for or against the N3 pK_a perturbation, it remains possible that the ribosome promotes catalysis by a general acid–base mechanism utilizing A2451. However, arguments for such a mechanism appear to rest entirely upon functional group proximity within the crystal structure.

Although the DMS data do not speak directly to the pK_a of A2451, the macroscopic pK_a of the peptidyl transferase reaction (pK_a 7.2–7.6) suggests that ionization of a functional group in the substrate or the enzyme is the rate limiting step of the reaction and that the deprotonated form of the ribosomal–substrate complex is active (Pestka, 1972). An obvious candidate is the nucleophilic amine of the A-site substrate because its pK_a ($\approx$7.5) closely matches that of the reaction. However, Fahnestock et al. (1970) reported that the macroscopic pK_a of the peptidyl transfer reaction was unchanged when the amino group was replaced with a hydroxyl even though this raised the nucleophilic pK_a by at least three pH units (Fahnestock et al., 1970). If the macroscopic pK_a is not that of the nucleophile, then there must be an ionizable group in the ribosome whose deprotonation constitutes the rate-limiting step in the peptidyl transferase reaction. This might be A2451, but it could also be another group involved in a catalytic or a conformational step in the reaction profile.

Based upon the crystal structure, A2451 has been proposed to stabilize the oxyanion in the tetrahedral intermediate, and it is possible that the A2451 pK_a could be perturbed, even if only transiently. This may result from juxtaposition of the negatively charged oxyanion that is in proximity to the A2451 N3 during the short-lived tetrahedral intermediate. Unfortunately, the only

evidence for this perturbation comes from the close approach (≈3 Å) of the A2451 N3 with the phosphoramidate mimic of the tetrahedral intermediate (Nissen et al., 2000). Because protons cannot be detected by macromolecular crystallography, this and other hypotheses regarding the catalytic reaction mechanism await further biochemical support.

One mechanistic possibility that has not been given much consideration is that A2451 N3 may be a hydrogen bond acceptor to the α-amino group in the A-site. In this scenario, the N3 group would help orient the α-amino group for attack. An N3 to α-amino group hydrogen bond is consistent with the A-site substrate bound state observed crystallographically (Nissen et al., 2000), and it may explain the in vitro mutagenesis data recently reported for the peptidyl transferase active site (Polacek et al., 2001). Using reconstituted 50S ribosomes from in vitro transcribed *Thermus aquaticus* 23S rRNA, Polacek et al. (2001) mutated A2451 to U, C, or G and observed only modest changes in activity (2–50-fold depending upon the assay conditions). Though greater care was taken to measure relative reaction rates and to measure initial velocities, similar results were obtained by Thompson et al. (2001) using a *Bacillus stearothermophilus* in vitro reconstitution system. It is worth noting that despite making all three possible mutations at A2451, none of these changes actually alter the hydrogen bond accepting character of the functional group of interest. An unprotonated N3 is retained between A and G, whereas the O2 carbonyl of C and U occupies an equivalent spatial location and can also act as a hydrogen bond acceptor. It is not clear how G, C, and U could be accommodated in the greater overall network of interactions that are seemingly specific to A, but poor accommodation may explain why the mutations are dominant lethal in vivo where the catalytic rates are substantially faster than observed in the in vitro reconstitution assays. Because traditional mutagenesis does not summarily change the N3 group of interest, it appears chemical mutagenesis may be necessary to determine the catalytic role of this functional group.

MATERIALS and METHODS

Purification of large ribosomal subunits

H. marismortui 50S ribosomal subunits were prepared as described (Ban et al., 1998) and then dialyzed against 1.2 M KCl, 0.5 M NH_4Cl, 20 mM $MgCl_2$, and 10 mM Tris-Cl, pH 7.5, 5 mM β-mercaptoethanol to remove the sucrose. They were stored at −80 °C until use. *S. cerevisiae* large ribosomal subunits were purified as described (Warner & Gorenstein, 1978). The 60S subunits were then dialyzed against 150 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Cl, pH 7.5, 5 mM β-mercaptoethanol to remove the sucrose and stored at −80 °C until use.

Peptidyl transferase activity assay

The peptidyl transferase activity of *H. marismortui* and *S. cerevisiae* large ribosomal subunits were tested using a modified form of the traditional fragment reaction (A. Seila, J. Strauss-Soukup, S. Scaringe, S.A. Strobel, unpubl. results). 50S ribosomal subunits from *H. marismortui* (2.1 μM, which had or had not been heated to 37 °C for 2 min) were combined with 10 μM of the P-site substrate CCA-Phe-Bio in 50 mM MOPS, pH 7.1, 200 mM NH_4Cl, 40 mM $MgCl_2$, and 2.5 M KCl in a total volume of 25 μL at 0 °C. The reaction was started by adding a trace concentration of the 5′-^{32}P labeled A-site substrate C-puromycin and incubating at 0 °C. Aliquots of the reaction were removed at time points from 1 to 60 min, mixed with formamide loading buffer, and the substrate and products separated by 12% denaturing polyacrylamide gel electrophoresis (PAGE). The fraction of unreacted C-puromycin was visualized and quantitated on a STORM phosphorimager (Molecular Dynamics). Yeast 60S subunits were assayed the same way except the ribosome concentration was 0.5 μM and the 2.5 M KCl was omitted.

Analysis of large ribosomal subunits with DMS as a function of pH

H. marismortui 50S ribosomal subunits (0.4 μM) were suspended in 50 mM buffer, 2.5 M KCl, 0.5 M NH_4Cl, and 20 mM $MgCl_2$ in a total volume of 50 μL. The solutions were buffered using BICINE (pH 9.0), HEPES (pH 8.5 to 6.5), or MES (pH 6.5 to 5.5). Three microliters of DMS diluted 1:10 in ethanol were added and the methylation reaction carried out at 0 °C for 1 h. The reactions were quenched by adding 200 μL of ethanol and the ribosomes precipitated at −80 °C. The 50S ribosomal subunits were pelleted by centrifuging at 13,000 rpm for 10 min at room temperature and resuspended in 400 μL 0.5% SDS, 5 mM EDTA. The 23S rRNA was isolated by phenol extraction and ethanol precipitation. The rRNA redissolved in TE and the concentration adjusted to 0.7 μM. The DMS methylated sites within rRNA were determined by reverse transcription. *H. marismortui* 23S rRNA (1.4 pmol) was added to two equivalents of the ^{32}P-5′-end-labeled primer HM2451: 5′-GAA CCG ACA TCG AGG TAG C in 40 mM Tris-Cl, pH 8.3, 60 mM NaCl, 8 mM DTT and annealed by heating to 72 °C for 10 min and cooling slowly to 32 °C. The primer was extended by reverse transcription using 2 U of AMV reverse transcriptase (Roche Molecular Biochemicals) in a total volume of 10 μL containing 36 mM Tris-Cl, pH 8.3, 54 mM NaCl, 7.2 mM DTT, 2.4 mM $Mg(OAc)_2$, and 400 μM each dNTP at 42 °C for 30 min. A and G sequencing ladders in the absence of DMS modification were generated by adding 100 μM ddCTP or ddTTP to the reverse transcriptase cocktail. Following incubation, formamide loading buffer was added, the samples were heated to 94 °C for 2 min, and the reverse transcription products were resolved by 8% denaturing PAGE. The products were visualized and quantitated on a STORM phosphorimager (Molecular Dynamics).

Individual band intensities at A2451 (*H.m.* 2486) and *H.m.* 2460 were normalized relative to the non-DMS specific stop at *H.m.* 2455 (*H.m.* indicates nucleotides whose sequence is specific to *H. marismortui*). The pH at which A2451 had the greatest band intensity was defined as 100% reacted and defined as 1. Other intensities were expressed as a fraction

of this value. The data were plotted versus the pH of the solution and fit to the equation:

$$I_{obs} = \frac{I_{HA}[H^+] + I_{max}K_a}{K_a + [H^+]} \quad (1)$$

where I_{obs} is the observed extent of DMS modification at a given pH, I_{HA} is the extent of modification for the protonated form of the nucleotide, I_{max} is the maximum reactivity (defined as 1), and K_a is the apparent equilibrium constant for protonation. This equation was also used to fit the data for C2452 in yeast ribosomes.

Yeast 60S ribosomal subunits were methylated as above except the ribosome concentration was 0.08 μM and the 2.5 M KCl was omitted. Reverse transcription of methylated yeast 25S rRNA (0.2 pmol) was performed as described using the primer SC2451: 5′-GAG CCG ACA TCG AAG AAT C.

Preparation and reverse transcription of RNA templates containing N6-methyladenosine

L-21 G414 RNA containing N6-methyladenosine was transcribed from the *Ear*I-digested plasmid pUCL-21G414R (Strobel & Shetty, 1997) by T7 RNA polymerase in buffer containing 40 mM Tris-HCl pH 7.5, 10 mM DTT, 4 mM spermidine, 15 mM $MgCl_2$, 0.05% Triton X-100, 1 mM each of GTP, CTP, and UTP, and 1 mM 5′-*O*-(1-thio)-N6-methyl-adenosine triphosphate or 1 mM 5′-*O*-(1-thio)-adenosine triphosphate (Ortoleva-Donnelly et al., 1998; Ryder et al., 2000). After incubation at 37 °C for 2 h, RNAs were purified by 6% denaturing PAGE, eluted into 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA (TE), ethanol precipitated, and resuspended in TE. A DNA primer complementary to nt 286–328 of L-21 G414 RNA was 5′-^{32}P-end labeled using polynucleotide kinase. L-21 G414 RNA was then annealed to the labeled primer and reverse transcribed with AMV reverse transcriptase as described above. Products were resolved by 8% denaturing PAGE and visualized by autoradiography.

Dimroth rearrangement of N1-methylated adducts in rRNA detected by reverse transcriptase

The position of DMS modification on the *E. coli* 23S rRNA A2451 heterocyclic ring was investigated by Dimroth rearrangement of the N1-methylated adducts. Two microliters of a freshly prepared 20% DMS in ethanol solution was added to 50S ribosomal subunits isolated from *E. coli* MRE600 cells (Rheinberger et al., 1988). The reaction was buffered in 50 mM cacodylate, pH 7.9, 150 mM KCl, and 10 mM $MgCl_2$. After 1 h at 0 °C, the reaction was quenched by ethanol precipitation and the rRNA isolated by phenol extraction followed by a second ethanol precipitation. The Dimroth rearrangement was initiated by incubating a 0.3-μM solution of modified rRNA at 37 °C in 10 mM CHES, pH 9.0. At 20, 40, and 60 h, a 2-μL aliquot was removed and frozen on dry ice for subsequent reverse transcriptase analysis as described above using a primer complementary to *E. coli* rRNA nt 2494–2510. The products were resolved by 6% denaturing PAGE, visualized, and quantitated as above. The intensity of the N-1 RT stops at positions 2451, 2468, 2469, 2476, or 2478 were normalized for primer extension efficiency and lane loading by dividing by the signal intensity at the DMS nonspecific stop at nt 2448–2445. This value was corrected for background hydrolysis by subtracting the normalized intensity at each position in the no DMS control. The values were then scaled such that the initial intensity at time zero was defined as 100% and the percentages plotted as a function of time.

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