

LETTER TO THE EDITOR

pKa of adenine 2451 in the ribosomal peptidyl transferase center remains elusive

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ABSTRACT

The universally conserved A2451 of 23S rRNA has been proposed to participate directly in the catalysis of peptide bond formation in the ribosomal peptidyl transferase center. An unusually high, near neutral, pKa of A2451 is a prerequisite for its action as a general acid–base catalyst. Increased reactivity of A2451 to dimethylsulfate (DMS) at pH 8.5 compared to pH 6.5 was taken as evidence that the pKa of this nucleotide falls within this pH range. Structural data suggested that the interaction between A2451 and G2447 in the ribosome is responsible for A2451 pKa perturbation. In contrast to expectation, our studies did not show pH dependence of A2451 dimethylsulfate modification in ribosomes of *Thermus aquaticus* and *Mycobacterium smegmatis*. Other rRNA regions, however, showed major alterations in DMS reactivity at pH 8.5 compared to pH 6.5, suggesting that conformational rearrangements in the structure of the large ribosomal subunit may occur upon the pH shift. The G2447U mutant of *M. smegmatis* was viable, indicating that the G2447–A2451 interaction is not critical for the ribosome function. We concluded that the proposed unusual pKa of A2451, if existing, may not be crucial for the ribosome activity and that the previously reported pH-dependent alterations in the DMS modification of A2451 do not necessarily reveal an unusual pKa of this nucleotide.

Keywords: conformational changes; DMS; peptidyl transferase; RNA catalysis; rRNA

INTRODUCTION

Elucidation of the principles used by the ribosome to promote peptide bond formation is a challenging task (Barta & Halama, 1996; Barta et al., 2001). Biochemical and genetic data (for a review, see Noller et al., 1995), as well as the recently solved high resolution crystallographic structure of the large (50S) ribosomal subunit of *Haloarcula marismortui*, revealed the ribosomal peptidyl transferase as an RNA enzyme (Ban et al., 2000; Nissen et al., 2000). Analysis of the 50S subunit X-ray structure led to a proposal of a detailed molecular mechanism of the chemical catalysis of peptidyl transfer in which the central role is attributed to the universally conserved adenine 2451 (*Escherichia coli* numbering here and throughout the paper) in domain V of 23S rRNA (Ban et al., 2000; Nissen et al., 2000). According to this model, the N3 position of A2451 serves as a general acid–base that facilitates the initial attack of the amino acyl-tRNA amino group on the carbonyl

carbon atom of the peptidyl tRNA ester bond and stabilizes the transition state tetrahedral configuration of this carbon atom. For A2451 to participate in this reaction, the pKa value of N3 has to be unusually high—close to neutral. Based on the electron density map of the active site region, specific tertiary interactions of A2451 with two other nucleotides in domain V of 23S rRNA, G2061 and especially, G2447, were proposed to be essential for generating a pKa shift at N3 of A2451 and hence rendering the catalytic properties to this nucleotide. The proposal that A2451 has an unusual pKa was supported by the results of experiments where 50S subunit modification with dimethylsulfate (DMS) at different pH values was used to gauge the state of adenine protonation, because protonated adenines should be poor substrates for DMS modification. Muth et al. (2000) reported that A2451 was the only nucleotide in domain V of 23S rRNA in *E. coli* 50S ribosomal subunit whose DMS modification is increased upon shifting the pH from 6.5 to 8.5. The authors suggested that the observed pH dependence of the A2451 DMS modification reflects the dramatically increased basicity of this residue relative to the unperturbed pKa of adenosine (Muth et al., 2000).

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We recently showed that the mutation of A2451 in *Thermus aquaticus* 23S rRNA only moderately affected the peptidyl transferase activity of in vitro assembled 50S ribosomal subunits (Polacek et al., 2001). These results were further supported by similar in vitro as well as in vivo experiments that utilized different experimental systems (Thompson et al., 2001). The mutational data therefore questioned the importance of the nucleotide identity at position 2451 as well as the significance of the proposed unusual pKa of this residue for the overall rate of peptide bond formation. These considerations prompted us to reexamine whether the pH-dependent increase in the reactivity of A2451 towards DMS observed in *E. coli* 50S subunits can be used as an accurate measure of the pKa of this nucleotide. We focused our experimental effort on ribosomes and ribosomal subunits from a thermophilic bacterium *T. aquaticus* (the experimental system used in our reconstitution experiments; Polacek et al., 2001) as well as on ribosomes from a mesophilic bacterium *Mycobacterium smegmatis*. Similar experiments were performed on *E. coli* ribosomes in another laboratory (Bayfield et al., 2001). Because peptide bond formation is a key reaction of protein synthesis promoted by the evolutionarily conserved ribosomal peptidyl transferase center, it is expected that the mechanism of transpeptidation should be universally conserved. If an unusually high pKa of A2451 is indeed important for the peptidyl transfer catalysis, the DMS reactivity of this nucleotide should exhibit similar pH dependence not only in *E. coli* but also in other Gram-negative bacteria (including *T. aquaticus* or *M. smegmatis*) as well as in more phylogenetically distant organisms (archaea or eukaryotes).

The results of DMS modification of ribosomes from *T. aquaticus* and *M. smegmatis* at different pH were at variance with those obtained with *E. coli* (Muth et al., 2000). Whereas the A2451 modification did not show any significant pH dependence within the pH range of 6.5 to 8.5, other rRNA regions showed major changes in DMS reactivity, suggesting that the 50S subunit structure undergoes a conformational transition upon the pH shift. In addition, studies of the *M. smegmatis* strain containing a mutation at position 2447 of 23S rRNA indicated that the A2451-G2447 interaction, which was perceived to be important for shifting up the pKa of

A2451, is dispensable for the peptide bond formation. We came to the conclusion that the reported pH-dependent alteration in the DMS modification of A2451 in *E. coli* ribosome does not necessarily reveal an unusual pKa of this nucleotide and, therefore, can hardly be used to support the proposed model of acid-base catalysis of peptidyl transfer.

RESULTS

DMS modification of *T. aquaticus* or *M. smegmatis* wild-type ribosomes or 50S subunits was performed at pH 6.5 and 8.5 under the conditions described by Muth et al. (2000). After rRNA purification, the methylation pattern of 23S rRNA in the vicinity of A2451 was analyzed by reverse transcription. Because studies of general pH dependence of 23S RNA modification were beyond the goal of the work presented here, we focused our analysis only on the rRNA region in the vicinity of A2451. In *T. aquaticus* ribosomes or large ribosomal subunits, A2451 exhibited only weak reactivity towards DMS at both pH values. No substantial increase in the extent of modification of A2451 was observed at pH 8.5 (Fig. 1A). In contrast, several other RNA positions, including C2394, A2439, A2469, C2474, and A2478, were modified more readily at pH 8.5 compared to pH 6.5. Three of these residues, A2469, C2474, and A2478, belong to the loop of helix 89, the other two positions are located at a significant distance from both helix 89 loop and the peptidyl transferase active site (Ban et al., 2000). Because all these residues are involved in different types of interaction within the 50S subunit, it is unlikely that they all have an unusually high pKa that would lead to the observed difference in DMS reactivity. A more likely explanation of our data is that changing the pH from 6.5 to 8.5 causes a conformational transition in the 50S subunit that increases the accessibility of these nucleotides to DMS modification.

Experiments with *M. smegmatis* wild-type ribosomes revealed a similar pattern of nucleotide residues that become more accessible to DMS modification at pH 8.5 compared to pH 6.5 (especially evident for position A2469, C2474, and A2478) whereas A2451 was again only weakly accessible to modification at both pH val-

FIGURE 1. Accessibility of nucleotides in domain V of 23S rRNA to DMS modification at pH 6.5 and 8.5. **A:** *T. aquaticus* ribosomes and 50S ribosomal subunits. **B:** *M. smegmatis* wild-type ribosomes. **C:** *M. smegmatis* ribosomes isolated from the G2447U mutant. C and A sequencing lanes are shown. **D:** Secondary structure of the central loop of domain V and neighboring rRNA regions of *T. aquaticus* 23S rRNA (drawn according to Noller et al., 1981, and Ban et al., 2000). A and C lanes contain the products of primer extension reactions in the presence of ddT and ddG terminators, respectively. Nucleotides whose modification is increased at pH 8.5 compared to pH 6.5 are shown by arrows. A2451 is shown bold and G2447 is shown outlined. Ribosomes or ribosomal subunits were isolated following standard protocols (Spedding, 1990) and DMS modification was performed at pH 6.5 and 8.5 exactly as described for *E. coli* 50S subunits (Muth et al., 2000). DNA oligonucleotide used for primer extension was complementary to the 23S rRNA sequence corresponding to nucleotide positions 2530–2549 in *E. coli* 23S rRNA.

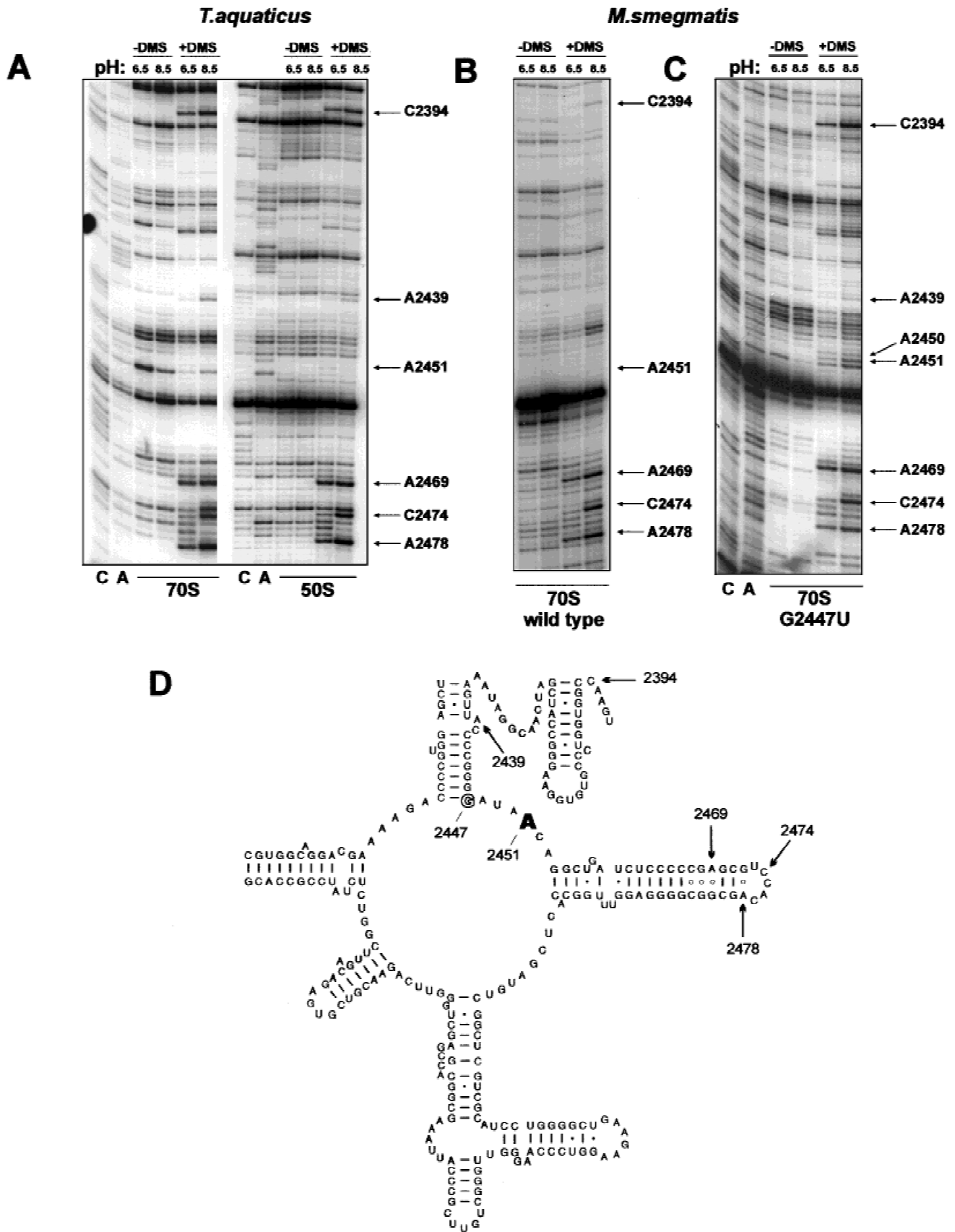


FIGURE 1. (Legend on facing page.)

ues (Fig. 1B). This suggested that *M. smegmatis* may undergo a pH-dependent conformational transition similar to that observed with *T. aquaticus* ribosomes.

According to the crystallographic structure of the 50S subunit (Nissen et al., 2000), the putative unusual pKa of A2451 critically depends on its interaction with G2447, a central component of a hydrogen-bonding network postulated to relay negative electrostatic charge to the N3 of A2451, thus increasing its pKa. To investigate the importance of the A2451-G2447 interaction for the ribosome function and structure, we used a mutant strain of *M. smegmatis* mc^2 155 *rrnB*⁻ where all rRNA is expressed from a single mutant rRNA operon containing the G2447U mutation (Sander et al., 1996; P. Sander, Y. Kidan, & E. Böttger, in prep.). The mere fact that cells where all ribosomes carried G2447 to U substitution in 23S rRNA are viable suggests that the G2447-A2451 interaction observed in the 50S subunit X-ray structure, as well as the elevated pKa of A2451 attributed at least in part to this interaction, are dispensable for the ribosome function.

Mutant ribosomes carrying the G2447U mutation showed increased accessibility of A2451 and A2450 to DMS modification at pH 6.5 and 8.5 compared to the wild type (Fig. 1C). Interestingly, in the mutant, adenine 2451 was modified slightly stronger at a higher pH even though the interaction of A2451 with the nucleotide at position 2447, required for the A2451 pKa shift, is obviously altered due to the mutation. In addition, the mutant showed a similar increase in the reactivity of the nucleotide residues A2469, C2474, and A2478 at an elevated pH, as was observed with the wild-type ribosome compatible with the proposed conformational switch at higher pH.

DISCUSSION

The results of the chemical probing of wild-type ribosomes and ribosomal subunits from evolutionary distant *T. aquaticus* and *M. smegmatis*, as well as mutant *M. smegmatis* ribosomes, suggested the possibility of conformational rearrangements of the 50S ribosomal subunit structure upon the pH shift. Such rearrangement may change the accessibility of several nucleotides within the investigated region of 23S rRNA to DMS modification. Similar conclusions were reached by Bayfield et al. (2001) in recent studies with *E. coli* ribosomes and 50S subunits. These observations cast doubt on the conclusion reached by Muth et al. (2000) that alterations in the extent of DMS modification of A2451 as a function of pH reflect changes in the protonation status of this nucleobase. In fact, we only observed an increase in A2451 DMS modification at a higher pH in the *M. smegmatis* G2447U mutant, where the interaction of A2451 with G2447 proposed to be essential for increasing A2451 pKa is distorted. Thus, though our

data do not directly rule out a possibility that A2451 has perturbed pKa, the reported pH-dependent changes in DMS modification of this nucleotide may not be an accurate measure of the protonation status of A2451 base within the ribosome.

In vitro studies of the peptidyl transferase activity of the *T. aquaticus* large ribosomal subunits carrying mutations at positions 2451 and/or 2447 showed that neither of these nucleotides is crucial for the peptide bond formation (Polacek et al., 2001). These results are supported, in part, by the viability of *M. smegmatis* cells in which all ribosomes carry the G2447U mutation. Disruption of the postulated charge relay required for conferring a high pKa value to the N3 of A2451 apparently does not severely perturb the rate of peptide bond formation in the living cell. Therefore, the proposed unusual pKa of A2451, if existing, may not be crucial for the ribosome activity in vivo and in vitro.

Activity of the ribosomal peptidyl transferase is stimulated with pH increasing from 6.2 to 8.5 (Maden & Monroe, 1968; Pestka, 1972). This effect was thought to be related to deprotonation of either the α -amino group of the acceptor substrate (amino acyl-tRNA or puromycin) or of a catalytically active functional group in the peptidyl transferase active site (Maden & Monroe, 1968; Pestka, 1972). It was later found, however, that the peptidyl transferase activity exhibits the similar pH dependence with either puromycin or hydroxypuromycin as an acceptor (Fahnestock et al., 1970), which was incompatible with substrate deprotonation. On the other hand, mutations of A2451 and several other conserved nucleotides within the active center of the ribosomal peptidyl transferase, which theoretically could contain functional groups that could participate in the catalysis, failed to identify a nucleobase that would be critical for the peptidyl transfer reaction (Polacek et al., 2001, and references therein). Remarkably, the pH dependence of peptidyl transfer rate and the conformational transition in the ribosome reported here are observed within the same pH range. It is conceivable that the structural rearrangements of RNA in the ribosomal peptidyl transferase center would affect the positioning of the peptidyl transferase substrates and may therefore influence the rate of the peptidyl transferase reaction. This latter consideration is in good agreement with a model in which the ribosome promotes transpeptidation by merely positioning the donor and acceptor substrates in the peptidyl transferase center followed by the spontaneous formation of a peptide bond (Krayevsky & Kuhnova, 1979; Nierhaus et al., 1980; Polacek et al., 2001). Though the exact mechanism of peptide bond formation is still unclear and will continue to be debated (Barta et al., 2001; Polacek et al., 2001; Thompson et al., 2001), we would like to emphasize that the possibility of a conformational transition within the ribosome should be taken in consideration when the pH dependence of peptidyl transferase activity is discussed.

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