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Abstract: Molecular dynamics (MD) simulations were employed to investigate the structure, dynamics, and local base-pair step deformability of the free 16S ribosomal helix 44 from Thermus thermophilus and of a canonical A-RNA double helix. While helix 44 is bent in the crystal structure of the small ribosomal subunit, the simulated helix 44 is intrinsically straight. It shows, however, substantial instantaneous bends that are isotropic. The spontaneous motions seen in simulations achieve large degrees of bending seen in the X-ray structure and would be entirely sufficient to allow the dynamics of the upper part of helix 44 evidenced by cryo-electron microscopic studies. Analysis of local base-pair step deformability reveals a patch of flexible steps in the upper part of helix 44 and in the area proximal to the bulge bases, suggesting that the upper part of helix 44 has
enhanced flexibility. The simulations identify two conformational substates of the second bulge area (bottom part of the helix) with distinct base pairing. In agreement with nuclear magnetic resonance (NMR) and X-ray studies, a flipped out conformational substate of conserved 1492A is seen in the first bulge area. Molecular dynamics (MD) simulations reveal a number of reversible α-γ backbone flips that correspond to transitions between two known A-RNA backbone families. The flipped substates do not cumulate along the trajectory and lead to a modest transient reduction of helical twist with no significant influence on the overall geometry of the duplexes. Despite their considerable flexibility, the simulated structures are very stable with no indication of substantial force field inaccuracies.

INTRODUCTION

Ribosome is a large nucleoprotein machine that carries out the mRNA-directed synthesis of proteins. Structures of small and large ribosomal subunits were determined at atomic resolution by X-ray crystallography.\textsuperscript{1–6} Further, ribosome was arrested at nearly every stage of the protein synthesis cycle and visualized using cryo-electron microscopy (cryo-EM) with single-particle reconstruction.\textsuperscript{7} These experimental studies showed that the ribosome undergoes large conformational changes during protein synthesis, such as the “ratchet-like motion” of the ribosomal subunits, where the ribosome moves exactly three nucleotides along the messenger RNA.\textsuperscript{8,9} This movement represents the first step in the process of translocation and it is followed by elongation factor G (EF-G)-dependent tRNA translocation.\textsuperscript{8,9} Deep insight into the mechanism of EF-G-dependent tRNA translocation was provided by a study that incorporated X-ray crystallography and nuclear magnetic resonance data into cryo-EM maps of ribosomal complexes.\textsuperscript{10} This study indicated that helix 44 (Figure 1), running from the decoding site to the bottom of the 30S subunit and making four major intersubunit contacts with the 50S subunit (B2a, B3, B5, and B6a) (Figure 2), plays a significant role in the translocation of tRNA. Particularly, the upper part of helix 44 acts as a dynamical domain during translocation which is, together with mRNA/tRNA complex, pushed away from the A-site to the P-site by EF-G, following GTP hydrolysis.\textsuperscript{10} In a recently published work, Frank’s group showed conformational changes (shift and rotation) of the upper part of helix 44 upon ribosome-recycling factor binding.\textsuperscript{11} Another experimental study revealed that the binding of translational initiation factor IF1 on the 30S subunit induced long-range conformational changes in helix 44.\textsuperscript{12} These experimental studies clearly demonstrate the importance of structural deformations of the helix 44 in the course of protein synthesis. Further, the conserved bases 1492A and 1493A in the helix 44 play a key role in the discrimination of correct tRNAs in the A-site via formation of A-minor interactions proofreading the codon–anticodon helix (Figure 1).\textsuperscript{13}

Computational studies represent a useful tool to complement the experimental methods in the investigation of the structure, dynamics, and deformability of RNA. For example, explicit solvent molecular dynamics (MD) simulations were instrumental in structural studies of RNA.\textsuperscript{14–25} MD can bring, despite the approximations inherent to the empirical force fields

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Keywords: molecular dynamics simulations; helix 44; ribosome; force field; RNA deformability; nanosecond scale dynamics

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{(Left) Position of the helix 44 (red) in the 30S subunit (cyan). Note the modest bend of the helix 44. (Right) detail of the upper part of the helix 44 with bases 1492A and 1493A flipped out to recognize the codon–anticodon helix (formed by mRNA and tRNA) via A-minor interactions. Structures of the 30S subunit (PDB codes: 1J5E and 1IBM) were used for preparing this figure.}
\end{figure}

\textit{Biopolymers} DOI 10.1002/bip
and limitations due to the nanosecond simulation time-scale, unique information that cannot be obtained by experiments. Simulations have been successfully employed in studies of ribosomal RNA and proteins and in our previous work focused on the investigation of structure and dynamics of ribosomal RNA secondary motifs. These studies characterized structural dynamics, base pairing, specific hydration, cation binding, electrostatic potential distributions, effects of selected base mutations, and some other RNA features.

Several recent studies demonstrated that appropriate processing of the MD data can be used to study deformability and elasticity (i.e., mechanical properties) of nucleic acids beyond the level obtained by visual inspection of the trajectories. Analysis of the elasticity allows us to quantify the stiffness of the molecule with respect to the given change of shape (deformation), including its sequence dependence. Computational and experimental studies of sequence-dependent DNA deformability have focused on the level of individual base-pair steps, i.e., estimated how much free energy it costs to change the helical parameters (roll, twist, etc.) of a particular dinucleotide step. However, computational studies also exist on the deformability of base pairs and on global DNA deformability, i.e., the free energy cost of deforming a longer DNA fragment considered as a piece of an extensible, twistable, and bendable anisotropic elastic rod. Sequence-specific elasticity of B-DNA plays an important role, for example, in nucleosome positioning. While DNA deformability has been studied quite intensely, much less has been done for RNA. Orozco and co-workers compared the deformability of RNA and DNA duplex using X-ray database analysis and MD simulations. They were

![FIGURE 2 Two-dimensional (2D) and three-dimensional (3D) structures of the 16S ribosomal helix 44 simulated in this study. (Left) 2D structure. The marks between pairs indicate the type of base pairing according to the Leontis classification. The base-pair stacks are indicated in the gray areas. Stacks (in circles) marked as A (bottom left) and B represent two substates seen in the simulation NA. Substate A is close to the starting X-ray structure. Marks B2–B6 and dashed lines indicate intersubunit bridges. (Right) Stereo view of the starting X-ray structure of the helix 44. The two essential bases 1492A and 1493A are highlighted by red transparent surface. The base-pair stacks are highlighted by light grey transparent surface.](image-url)
RESULTS

Starting Structure

The 16S ribosomal helix 44 (residues: 1404–1497) (Figure 2) was taken from the X-ray structure of a free 30S subunit (Thermus thermophillus) with resolution 3.05 Å [Protein Data Bank (PDB) code: 1JSE] and was solvated and neutralized by a standard procedure (see Materials and Methods). The X-ray helix 44 is bent like a longbow (Figures 1–3). Terminal parts of the helix 44 make contacts with the rest of this subunit while the middle part is not directly in contact with 30S.

The X-ray structure shows several undefined ions bound in the lower part of the major groove of the helix 44 (Figure S1). The crystallization procedure was done in the presence of KCl and Mg\(^{2+}\) ions, so these ions might be bound in the major groove of helix 44. Furthermore, there is another structure of the 30S subunit (PDB code 1IBM), which shows Mg\(^{2+}\) ions bound in the upper part of the major groove of the helix 44 (Figure S1). Nevertheless, the experimental information about ion binding is quite limited, considering the known ambiguities of X-ray studies of cation binding to RNA.\(^{47,48}\) Thus, we carried out two MD simulations of the helix 44 under different ion conditions. The first simulation was carried out only with Na\(^{+}\) ions (simulation NA, 30 ns) while the second one was run with both Mg\(^{2+}\) and Na\(^{+}\) ions (simulation MG, 30 ns) (see Materials and Methods). Mg\(^{2+}\) ions were equally distributed along the helix 44 (manually placed along the duplex at a distance of 5 Å from the X-ray structure) to allow them find convenient binding positions. See method section and supplementary material for a comment regarding the inclusion of divalent cations into simulations.

Helix 44 Simulation in the Presence of Na\(^{+}\)

Thirty nanosecond MD simulation of the ribosomal helix 44 (Figures 2 and S2) was carried out in the presence of 72 Na\(^{+}\) ions (see Materials and Methods, simulation NA). The root mean square deviation (RMSD) value with respect to the crystal structure was 6.2 ± 1.1 Å. Although we started from the X-ray geometry, which is modestly bent, after 1 ns of the simulation we obtained a straight duplex (Figure 3). Further, helix 44 appeared considerably dynamical in the course of the simulation (see below); however, the average structure over the trajectory was straight (Figure 3).

Base Pairing

The X-ray structure consists of twenty-four Watson–Crick base pairs, eight non-WC base pairs, four G/U wobble pairs, and two bulge bases 1492A and 1493A. It contains three base-pair stacks (1414U/1486G//1415G/1485U, 1417G/1483A//1418A/1482G, and 1432A/1468A//1433A/1467G) (Figure 2) (“//” stands for base pairing and “/’’/’’ for stacking). In the course of the simulation, we monitored all base pairs. Opening events were observed for the first three base pairs in the upper part of the duplex (Table I). Further, the base pairs forming the U/G//G/U and G/A//A/G wobble pairs, and two bulge bases 1492A and 1434A. The most significant changes of the base pairing were observed in both bulge areas.

First Bulge Area. 1493A forms weak contacts to the opposite 1408A in the X-ray structure. The 1493A(C2)–1408A(N1) distance is 3.0 Å and the
The base 1492A is not paired and has no other contacts with the rest of the duplex. After equilibration, the 1493(N6)–1408(N1) H-bond was formed, which persisted for the first two nanoseconds of the simulation. Then it was irreversibly broken. The 1492A was not paired and appeared considerably dynamic (see below).

**Second Bulge Area.** In the X-ray structure, the base 1467G forms weak contact to the opposite bases 1433A and 1434A. The 1467G(N2)–1433A(N7) distance is 2.7 Å, and the 1467G(N2)–1434A(N7) distance is 3.4 Å. After equilibration, 1467G formed a planar base pair with 1433A. This geometry was denoted substate A and it was often seen in the simulation NA (Figure 2 and Table II). After five ns of the simulation NA the bases 1466C and 1465C moved up, so that the base 1434A formed two H-bonds to 1466C and the adjacent guanine 1435G formed three H-bonds to 1465C (Figure 2 and Table II). This substate B was observed in periods 5.8–18.8 and 20–28.7 ns (Figure 2, Table II, Figure 4) while substate A took place for the rest of the simulation.

**Dynamics of the Base 1492A**

The bulge 1492A reveals considerable dynamics. The starting X-ray structure shows the base 1492A stacked in the stem. However, in the course of the simulation the base often flips out of the stem towards the minor groove (Figure 5a,b) by about 180° compared to the X-ray structure. The flipped-out structure is seen in time periods 4–11 and 20–30 ns while in periods 0–4 and 11–20 ns it resembles the crystal geometry. The flip is associated with anti to syn χ angle oscillation (Figure 5c) and C2'-endo conformation while 1492A stacked in the stem adopts C3'-endo puckering (Figure 5d). Base 1493A was not flipped out; however, the torsion angle χ fluctuated in the range from 10° to 60°. Other residues did not reveal any changes of the χ angle and their geometries were stable in the stem during the whole simulation.

**Local Conformational Variations and Phosphate Backbone Substates**

Base-pair step parameters rise, roll, tilt, twist, slide, and shift were calculated using 3DNA code. First three terminal base pairs at the 5’ end were omitted due to opening events (see above). Considering two variants of base pairing (substates A and B, see above) seen in the second bulge area, helical parameters were calculated for structures averaged in time periods 0–5.8, 5.8–18.8 ns (see supplementary material Table S1), and several other time windows.

Note that the numerical values of helical twist differ significantly from the common value of ca. 33° in many steps of the present RNA molecule, since the standard definition of the base-pair axis valid for WC base pairs is not applicable for non-WC base pairs. Relative comparison of X-ray and computed values is still relevant. Note also that, for a given step, the calculated value of helical twist is sensitive to the shape of the non-WC base pairs forming the base-pair step.

Both averaged structures showed increase or reduction of helical twist of several steps compared to the starting X-ray values. These differences are mostly caused by evidently deformed initial X-ray geometries of non-WC base pairs, which can easily be explained by the medium resolution of the X-ray structure and the overall dynamical nature of helix 44. This is the case of G/U wobble pairs that have nonoptimal X-ray geometry (see supplementary material Figure S3 and Table S1 for details) and base pairs 1423G/1477C, 1432G/1468A, and 1411C/1489G that have incomplete H-bonding in the X-ray structure. Immediately after equilibration, these pairs formed complete H-bonding, which was stable until the end.
of the simulation. This initial relaxation of some base pairs resulted into new (and stable) values of helical twist (as calculated by 3DNA code) in the base-pair steps involving these base pairs. We suggest that all these differences of the calculated helical twist can be fully explained considering by imperfectness of the H-bonding of several base pairs in the X-ray structure and do not indicate any real structural changes in the simulated molecule. This likely is also the case of GA/GA step 29 having calculated helical twist of ca. 8.8 through the simulation, while the X-ray structure gives twist of 3.8 for this step (see supplementary material Table S1). The X-ray structure contains two GA/GA steps (14 and 29) with considerably different values of twist (8.8 and 3.8). The second GA/GA step 29 is actually visibly deformed in the X-ray structure. Most changes of the calculated twist values thus reflect modest structural relaxations of the non-WC base pairs. Further, base-pair steps that include the temporarily formed dynamical base pair 1493A/1408A in the first bulge area and base pairs from the very dynamical second bulge area (see above) showed different values of calculated helical twist compared to the X-ray values. We also observed that reversible transitions of α–γ torsions (see below) can cause modest temporary reduction of the helical twist. This issue will be described in detail for the simulation of the canonical RNA duplex.

The starting values of backbone torsions for all residues were α = 290°, β = 175°, γ = 50°–60°, δ = 75–80°, ε = 200°–210°, and ζ = 290°, i.e., the canonical A-RNA family 20 according to the Schneider’s classification.50 In the course of the simulation, alternative “switched” conformations characterized by concerted α (290° → 145°) and γ (50°–60° → 180°) transitions were observed (Figure 6). These compensatory backbone switches were reversible (obviously except for those that occurred close to the end of the simulation). The switched geometry represents another well-established A-RNA family 24 (or 19) often seen in A-RNA duplexes.50 The number of α–γ torsions in the switched conformation at 10, 20, and 30 ns was 8, 12, and 11, respectively. Thus, the switched geometries do not cumulate along the trajectory and have population ca. 10–15%. Several alternative conformations of β, δ, ε, and ζ torsions were rarely observed (not shown).

All bases excluding 1492A (see above) adopted puckering C3′-endo throughout the simulation.

### Essential Dynamics Analysis

Essential Dynamics Analysis (EDA)51 (see Materials and Methods) was carried out to obtain deeper insight into the flexibility of the helix 44. The first two eigenvectors represent 60% of the overall motion of the helix.

<table>
<thead>
<tr>
<th>Base Pair</th>
<th>Simulation NA</th>
<th>Simulation MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1404C(N3)–1497G(N1)</td>
<td>8.5–9.3 ns OE</td>
<td>15–30 ns OE</td>
</tr>
<tr>
<td>1404C(N4)–1497G(O6)</td>
<td>8.5–9.3 ns OE</td>
<td>15–30 ns OE</td>
</tr>
<tr>
<td>1404C(O2)–1497G(N2)</td>
<td>8.5–9.3 ns OE</td>
<td>15–30 ns OE</td>
</tr>
<tr>
<td>1405G(N1)–1496C(N3)</td>
<td>0.8–4.5 ns OE</td>
<td>4–6.3 ns OE</td>
</tr>
<tr>
<td>1405G(N2)–1496C(O2)</td>
<td>0.8–4.5 ns OE</td>
<td>4–6.3 ns OE</td>
</tr>
<tr>
<td>1405G(O6)–1496C(N4)</td>
<td>0.8–4.5 ns OE</td>
<td>4–6.3 ns OE</td>
</tr>
<tr>
<td>1406U(N3)–1495U(O2)</td>
<td>16.5–17.3 ns OE</td>
<td>Stable</td>
</tr>
<tr>
<td>1406U(O4)–1495U(N3)</td>
<td>16.5–17.3 ns OE</td>
<td>Stable</td>
</tr>
<tr>
<td>1408A(N1)–1493A(N6)</td>
<td>0–2 ns stable</td>
<td>6–30 ns stable</td>
</tr>
<tr>
<td>1414U(N3)–1486G(O6)</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>1414U(O2)–1486G(N1)</td>
<td>3.2 Å, 0–30 ns F</td>
<td>3.5 Å, 0–30 ns F</td>
</tr>
<tr>
<td>1415G(N1)–1485U(O2)</td>
<td>3.7 Å, 6.7–10 ns F</td>
<td>Stable</td>
</tr>
<tr>
<td>1415G(O6)–1485U(N3)</td>
<td>3.7 Å, 6.7–10 ns F</td>
<td>Stable</td>
</tr>
<tr>
<td>1417G(N2)–1483A(N7)</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>1417G(N3)–1483A(N6)</td>
<td>3.5 Å, 0–30 ns F</td>
<td>3.2 Å, 0–30 ns F</td>
</tr>
<tr>
<td>1418A(N6)–1482G(N3)</td>
<td>3.2 Å, 0–30 ns F</td>
<td>3.2 Å, 0–30 ns F</td>
</tr>
<tr>
<td>1418A(N7)–1482G(N2)</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>1432G(N2)–1468A(N7)</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>1432G(N3)–1468A(N6)</td>
<td>3.2 Å, 0–30 ns F</td>
<td>3.6 Å, 0–30 ns F</td>
</tr>
</tbody>
</table>

* OE indicates an opening event of the H-bond in the specified time period and F denotes a fluctuation of the H-bond around the averaged value in the specified time period. The gray field specifies the base pairs involved in base-pair stacks.
lix 44 on the nanosecond time scale. These eigenvectors correspond to bending of the helix 44 (Figure 7) in mutually orthogonal planes. Combination of the first two eigenvectors results in bending motion of the duplex in all directions during the simulation. This observation is confirmed by the calculation of projections of the trajectory onto the eigenvectors (Figure 8). The diagram in Figure 8 shows approximately isotropic distribution of projections around the point 0.0. In summary, the averaged helix is straight (Figure 3) but it can easily achieve substantial bends on the 10 ns scale.

The upper part of the helix 44 in ribosome is flexible while the bottom part is fixed. During the EF-G-dependent tRNA translocation, the A-site region of the helix 44 moves toward the P-site by approximately 8 Å. We thus superimposed the straight geometry (averaged MD structure) and the most bent geometry (an MD snapshot) over the lower portion of the helix 44 (over residues 1421–1440/1479–1461) to estimate the range of spontaneous bending. This superposition revealed maximal displacement of the upper terminal phosphorus atom 1497G(P) by 25 Å and of the essential base 1493A by 23 Å (Figure 9). The average displacement of 1497G(P) was 9 Å. Although it is obvious that the flexibility of helix 44 in the ribosome may be affected by, for example, the intersubunit bridges, helix 44 is capable of easily achieving the range of motions seen in the ribosome. Also note that spontaneous bending in the course of the simulation reaches values almost identical to those seen in the starting X-ray structure (Figures 3 and 9). The temporary bending seen in the simulation is smooth, i.e., uniformly distributed along the double helix, with no local kinks.

### Table II Base Pairing of the Helix 44 in the Area of the Second Bulge

<table>
<thead>
<tr>
<th>Base Pair</th>
<th>Simulation NA</th>
<th>Simulation MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1433A(N7)–1467G(N2)</td>
<td>Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>1433A(N6)–1467G(N3)</td>
<td>3.4 Å, 0–30 ns F</td>
<td>3.3 Å, 0–30 ns F</td>
</tr>
<tr>
<td>1435G(N1)–1466C(N3)</td>
<td>5.8–18.8 ns and 20–28.7 ns OE</td>
<td>Stable</td>
</tr>
<tr>
<td>1435G(N2)–1466C(O2)</td>
<td>5.8–18.8 ns and 20–28.7 ns OE</td>
<td>Stable</td>
</tr>
<tr>
<td>1435G(O6)–1466C(N4)</td>
<td>5.8–18.8 ns and 20–28.7 ns OE</td>
<td>Stable</td>
</tr>
<tr>
<td>1436U(N3)–1465C(N3)</td>
<td>5.8–18.8 ns and 20–28.7 ns OE</td>
<td>Stable</td>
</tr>
<tr>
<td>1436U(O4)–1465C(N4)</td>
<td>3.5 Å, 5–19 ns and 20–28.7 ns F</td>
<td>No H-bond</td>
</tr>
</tbody>
</table>

*Substate A represents crystal base pairing and was observed in both simulations NA and MG, while substate B was identified only in the simulation NA. OE indicates an opening event of the H-bond in the specified time period and F indicates a fluctuation of the H-bond around the averaged value in the specified time period.*

**FIGURE 4** Formation of H-bonds between bases 1435G and 1466C (Substate A) and between bases 1435G and 1465C (Substate B).
Simulation in the Presence of Mg$^{2+}$

A thirty nanoseconds long MD simulation of the helix 44 (Figures 2 and S2) was also carried out in the presence of 24 Mg$^{2+}$ ions (simulation MG, see Materials and Methods and supplementary material) and 24 Na$^+$ ions, which were added to complete the neutralization (ion concentration and the binding positions are specified in supplementary material). Similar to the simulation NA, after the first nanosecond of the simulation we obtained a straight duplex with RMSD value with respect to the crystal structure 5.0 ± 1.2 Å (Figure S2). The simulation MG revealed base pairing of the helix 44 identical to the simulation NA and identical transient changes of base pairing (e.g., opening events in terminal base pairs and fluctuations of pairs forming stacks) (see Table I). However, in contrast to the simulation NA, the simulation MG did not show the B substate in the second bulge area and the initial X-ray geometry with planar base-pair 1467G/1433A was stable (Figure 2, Table II). Further, we observed once again the H-bond 1493(N6)–1408(N1) in the first bulge area, which was formed in the time period 6–30 ns. The spontaneous flipping of 1492A was limited; it flipped out modestly towards the minor groove (approximately by 30°) and towards the major groove (approximately by 60°) while keeping the stable C3'-endo sugar conformation. Essential dynamics analysis revealed two main eigenvectors identical to those in the simulation NA. More details can be found in supplementary material, including analysis of helical parameters and backbone dynamics. The simulation MG provides an essentially identical picture of the dynamics as simulation NA. Thus even such a high concentration of Mg$^{2+}$ exerts surprisingly little effect on the dynamics of the long duplex on the 30-ns scale. In contrast to the widespread opinion that Mg$^{2+}$ ions may substantially improve RNA MD simulations, we suggest that inclusion of Mg$^{2+}$ is mostly unnecessary. Inclusion of divalent cations such as Mg$^{2+}$...
Mg$^{2+}$ ions may cause local RNA structure distortions due to force field and sampling limitations.  

**Simulation of the Canonical Duplex**

Forty nanosecond long control MD simulation of a 37 base-pair (bp) canonical RNA duplex ($5'$CGCGGC-AGCCCCAUCCGGGCGGCGCCCCGCUUCAGC3'$, simulation CAN) was carried out (Figure S2). The starting structure is a standard straight duplex. The averaged structure over the trajectory is also a straight duplex; however, similar to helix 44, it exhibits considerable dynamics with the RMSD value with respect to the starting structure 5.9 ± 1.3 Å.

All canonical base pairs were stable except for one terminal pair at the 5' end and two terminal pairs at the 3' end with opening events (data not shown). Helical parameters were calculated for a structure averaged over the time period 20–30 ns while omitting two terminal steps at either end (supplementary material Table S1). The average MD value of helical twist was 29.2° with a standard deviation of 2.6°. The base-pair steps have quite uniform distribution of helical twists; however, twist is slightly underestimated, as usual with AMBER. All steps were within a narrow range of helical twist values except of a CC/GG step 10 (Table S1). Its helical twist averaged over the time periods of 1–10, 10–20, and 20–30 ns was 30.3°, 24.0°, and 21.5°, respectively. This twist reduction was actually associated with presence of two long-living alternative $\alpha/\gamma$ backbone substates in this step. However, at 37 ns we evidenced a reversal of one of the backbone flips and the average helical twist in a time period 37–40 ns increased to ca 26°. This indicates that the step is returning to a common range of helical parameters.

Dynamics of backbone torsion angles is very similar to the simulations NA and MG, i.e., there are reversible $\alpha-\gamma$ flips between two established A-RNA families 20 and 24. The number of nucleotides in conformation family 24 found at 10, 20, 30, and 40 ns was 3, 10, 7, and 9, respectively, i.e., ca. 10–12% population. Temporary reduction of twist to 25°–26°...
of the phosphorus atom $74\text{G}(\text{P})$ was 15 Å, which is almost twice as much as for the helix 44. The average displacement of the phosphorus atom $74\text{G}(\text{P})$ was 15 Å. This means that globally the canonical helix is more bendable than the native helix 44.

**Base-Pair Step (Local) Deformability of Helix 44 and Canonical Duplex**

Base-pair step deformabilities (elastic or force constants) were calculated with respect to the six helical variables (rise, roll, tilt, twist, shift, and slide, see Materials and Methods) for all steps in the noncanonical helix 44 and in the standard RNA canonical duplex. The calculations were carried out only over stable parts of the trajectories where no opening events occurred. All the coupling terms in the harmonic energy function have been considered, so that the stiffness of each base-pair step is described by a 6 by 6 symmetric matrix of elastic constants, the stiffness matrix. The diagonal force constants that reflect free energy changes upon deforming just one conformational parameter (the others are kept at equilibrium values) are plotted along the sequence in Figure 10 (see also supplementary material Table S2). To estimate the error on the calculated stiffness constants, diagonal force constants were calculated over nonoverlapping 1-ns long time intervals along the trajectories (only over stable parts of the trajectories) from 1 to 30 ns, providing both averages and standard deviations (see Figure 10 and Tables S2a–c) (in the case of the simulation NA, we used only time periods corresponding to substrate A). For the sake of clarity, error bars in Figure 10 are shown only for the NA simulation. The complete set of values is provided in the supplementary material (Tables S2a–c). The error bars of force constants represent approximately 10–20% of the mean values for both helix 44 and canonical duplex.

Deformability profiles of the native helix 44 in the simulations NA (calculated for substrate A) and MG are rather similar (Figure 10). Thus even this quantitative analysis does not reveal any substantial rigidiﬁcation of the long helix by the high $\text{Mg}^{2+}$ concentration. Both the NA and MG elasticity proﬁles suggest lowered force constants in the upper part of the helix 44 (ﬁrst four steps) and in both bulge areas (steps 5, 6, and 30–33). This indicates larger ﬂexibility of these regions compared to the rest of the duplex.

Superposition of the deformability proﬁles of the canonical duplex over the proﬁles of the native helix 44 duplex (Figure 10) shows that the force constants of the standard RNA duplex fall into the range of force constants of the native helix 44. Note that, when comparing the helix 44 with the canonical duplex, the central part of the helix 44 shows increased stiffness, speciﬁcally for rise and roll. It might result in decreased *global flexibility* of the helix 44 and reduced overall amplitude of bending motion at the ends. We suggest that the enhanced rigidity of the helix 44 in the central region explains the twice as large amplitude of the global ﬂuctuations (bend angles) seen for the canonical helix. Note again that the upper part of helix 44 has *locally enhanced flexibility*. RNA persistence lengths for helix 44 and duplex RNA have been also calculated and compared with experimental values. The dynamic bending persistence length has been found to be ca 120 nm. (For more details, including discussion of the estimated total persistence length, see supplementary material.)

We further compared elasticity of canonical pyrimidine–purine (YR/YR), pyrimidine–pyrimidine (YY/RR), and purine–pyrimidine (RY/RY) steps. We did not consider steps with non-WC base pairs present in helix 44, because there are only very few examples of each of the different types (RY/RR, YR/RR, RY/YY, and RR/RR). For rise, roll, and tilt, the YR/YR steps were by far the most flexible (Figure S4), whereas steps in other two groups were stiffer. Particularly, RY/RY steps exhibited increased stiffness for rise and roll while YY/RR steps were of intermediate flexibility. In the case of tilt, YY/RR steps were the stiffest and RY/RY steps were of intermediate flexibility. This was observed consistently in all three simulations.

**DISCUSSION AND CONCLUSIONS**

Atomistic, explicit-solvent MD simulations were employed to investigate structure, dynamics, and local base-pair step deformability of free 16S ribosomal helix 44 from *Thermus thermophilus*. The helix 44 (residues: 1404–1497) was taken from the crystal structure of a free 30S subunit (PDB code: 1J5E) and simulated in the presence of explicit Na$^+$ and Mg$^{2+}$ ions (simulations NA and MG, both 30 ns) and explicit water molecules, assuming the X-ray structure as the starting geometry. Comparison with a standard RNA duplex of the same length (37 bp) was carried out (simulation CAN, 40 ns).
Base Pairing Is Stable, Except for Two Substates in the Bottom Bulge Area

Geometries of many base pairs were substantially relaxed (improved) at the very beginning of the simulation, compared with the starting X-ray structure. The largest deviations in base pairing compared to the starting X-ray structure were observed in the area of the bulge base 1434A in the simulation NA. In this area, two bases 1466C and 1467C moved temporarily.

FIGURE 10 Deformability profiles for helix 44 in simulations NA (calculated for substate A, red), MG (green), and for the canonical duplex CAN (black). Error bars (standard deviations) are shown for the simulation NA. The horizontal axis shows the base-pair step number while the sequence of each base-pair step is also specified (helix 44—bottom and red; canonical helix—top and black). The color dots (“contact steps”—see the inset top left) mark those steps of helix 44 that are involved in the intersubunit bridges (B2a, B3, B5 and B6a).
one step up (Figure 2) and formed a new base-pairing and stacking pattern not seen in the starting X-ray structure (Table II, Figure 4). This conformational switch was spontaneous and reversible, occurring twice during the simulation (Table II, Figure 4). This suggests that there might be two substates of this non-WC region with similar free energies. Note that, assuming the standard Arrhenius kinetics, 10-ns scale simulations are capable of regularly overcoming free energy barriers that are on a scale of ca. 5–7 kcal/mol, which likely is the barrier separating the above two substates. This region thus could act as a flexible structural switch. To the best of our knowledge, similar duplex pairing bistability was not observed in any RNA simulation study published so far. Further, the simulation suggested transient formation of a H-bond between 1408A and 1493A (Table I), which was not present in the starting X-ray structure but was observed in previous solution NMR study. 53 MD simulations reveal several opening events and fluctuations of 1–3 terminal base pairs of the duplex (Table I), that, however, can be considered as a common end effect. Internal WC base pairs are firmly paired (we assume that this is correct behavior on the ns time scale) with no breathing or opening. This contrasts recent RNA duplex simulations with the CHARMM force field, showing very frequent ns-scale base-pair breathing in RNA duplexes. 41

1492A Flips Out Spontaneously

Both NA and MG simulations revealed considerable dynamics of the essential base 1492A (Figure 5). In the simulation NA, the 1492A flipped out repeatedly by about 180° towards the minor groove while the dynamics of 1492A was modestly restricted in the simulation MG. Such conformational substates (stacking inside the stem and flipped-out substate) of both key residues 1492A and 1493A during the process of discrimination of cognate and near-cognate tRNA have been proposed by experiments. 13 Our simulations on the nanosecond time scale showed spontaneous dynamic behavior of the 1492A in the free helix 44 with Na+ ions. The reduced dynamics of 1492A in the simulation MG might be caused by closing of the upper part of the helix 44 by an interstrand contact via one specific Mg2+ ion (outer shell binding). Note that this result can be incidental due to poor sampling of divalent cations in ns-scale simulations. The observed dynamical behavior of 1492A is in agreement with a previous NMR study 53 that indicated dynamics of both 1492A and 1493A and revealed their sugar conformations to be a mixture of C2′-endo and C3′-endo. Exactly this is seen in our study for 1492A in the simulation NA. Moreover, conformational substates of 1492A and 1493A have been also seen in the experimental study that combined X-ray crystallography and fluorescence measurements. 54 Thus, we assume that these bases might flip out quite freely in the ribosome. Regarding 1493A, we suggest that a longer simulation time scale would be needed to evidence its flipping. Note, however, that the conformational plasticity of the A1492/A1493 bases is quite remarkable. This region adopts multiple distinct substates in the available experiments and both bases can be bulge out and bulge in. Thus, much longer simulations would be needed to obtain statistically significant sampling of the A1492/A1493 dynamics, and it is not guaranteed that the force field would fully capture the tiny balance of all the substates. 55

Phosphate Backbone and Helical Parameters Indicate a Good Performance of the Force Field

Recent AMBER MD studies of B-DNA duplexes 56–58 reported long-lived α-γ “switched” conformations in DNA sugar-phosphate backbone. These alternative conformations appeared irreversible, and caused a reduction of helical twist and some other structural effects. Related backbone flips occurred in locally enhanced sampling MD simulations of G-DNA loops and led to incorrect loop topology of the d(GGGGTTTTG-GG)2 quadruplex. 59 In contrast, recent analysis of RNA backbone behavior in simulations of the hepatitis delta virus ribozyme and sarcin–ricin loop suggested that the force field performs well across a wide range of A-RNA and noncanonical RNA backbone topologies. 20, 34 Obviously, the long 37-bp double helices studied here represent an optimal system to compare with B-DNA simulations. For both the native helix 44 and the canonical RNA duplex, we evidenced a number of α-γ “switched” conformations. These compensatory backbone switches, however, were reversible (Figure 6), and did not cumulate during the simulations. The population of flipped backbones in the 40-ns simulation of the canonical helix was ca. 10–12% since 10 ns into the simulation while the helix 44 simulations gave the same picture. The second substate was associated with modest helical twist reduction, which, however, did not affect the outcome of the simulation because the flips were temporary. The present A-RNA flips correspond to a switch between the canonical A-RNA family 20 and one of the three other most frequently occurring RNA backbone families, namely, family 19/24. 50 Such RNA backbone flips seen in the simulations are basically in agreement with the X-ray data, although their population may be somewhat exag-
gerated. Sugar puckering remained consistently in the C3'-endo range. Thus, no indication of a force field imbalance for the RNA backbone was detected on the present time scale.

There appear to be substantial changes of calculated values of helical twist for some steps of the simulated helix 44 compared to the starting X-ray values. These differences are, however, primarily caused by nonoptimized geometries of some base pairs in the initial X-ray structure. These base pairs relax immediately after the simulation start, changing the numerical value of helical twist. Thus, these changes do not indicate any structural transition. Further, some inherently flexible elements of helix 44 show substantial variations of calculated helical twist. Perhaps the largest deviation of twist with respect to the X-ray value was indicated in both simulations NA and MG for non-WC GA/GA step 29 (supplementary material Table S1). The X-ray structure gives a twist of 3° while MD leads to a twist around −21°. (Note that numerical values of helical twist are affected by the presence of non-WC base pairs.) The latter value, however, is in agreement with another GA/GA step 14 in the crystal structure. Actually, step 29 is visibly deformed in the X-ray structure. In the case of the canonical RNA duplex, a single CC/GG step 10 shows considerably lower helical twist of ca. 22° in the period of 20–30 ns, which is well below the common A-RNA twist ranges. This substate develops in the first 10–15 ns of the simulation and is associated with the presence of two α/γ alternative backbone substates in this step. However, at 37 ns, one of the switched phosphates flips back to the canonical geometry and the whole step starts to return to the canonical helical geometry— it is thus not a permanent substate. Anyway, the total population of low-twist steps in this simulation is 1 out of 36. Such substates, if randomly formed, could evade detection by experimental techniques.

The simulations also reveal a systematic difference of base-pair roll between YR (1°–6°) and RY (5°–13°) steps (Y and R stand for pyrimidine and purine). This is another indication of a meaningful performance of the force field, because the roll redistribution between YR and RY steps in A-type duplexes is known from X-ray data.

**EDA Shows Large-Scale Bending Fluctuations of the Long Duplex**

The 16S ribosomal helix 44 is conformationally restrained inside the ribosome. First, the 30S subunit X-ray structure shows bent helix 44 (Figures 1–3). Second, in the complete ribosome, the helix 44 makes several intersubunit bridges (Figure 2) with 50S, representing fixed nodes likely serving as anchoring patches for large-scale motions. Nevertheless, we were interested in understanding how could these interactions be interrelated with intrinsic properties of the helix 44 and what are the differences between helix 44 composed of 30% of non-WC base pairs (including wobble pairs) and the standard RNA duplex. The simulations reveal that the helix 44 is intrinsically straight—it is thus not pre-deformed toward its bent geometry seen in the 30S subunit (Figure 3). However, fluctuational bending represents 60% of the overall motion of both the helix 44 (Figure 7) and the canonical RNA duplex. Both duplexes bend isotropically around a straight geometry (Figure 8). Further, the bending is not caused by the presence of any kink in the structure, which was, for instance, seen in study of ribosomal kink–turn motifs. The range of statistical bends is significant and on the 30-ns time scale it exceeds the range of deformations seen in ribosomal structures.

Based on cryo-EM, Frank and coworkers observed a bending of the upper part of the helix 44 during translocation. They showed that after the binding of EF-G, this factor pushed the upper part of helix 44 using energy from GTP hydrolysis, shifting (by approximately 8 Å) the mRNA/tRNA complex from the A-site to the P-site. The experiments suggest that the upper part of the helix 44 represents a moveable domain while the lower portion is motionless and acts as an anchor. Although the helix 44 is partly fixed by intersubunit bridges with 50S and its contacts with 30S, which certainly affect its dynamics, MD simulations provide a surprisingly similar type of motion. The superimposition of the straight averaged MD structure and a bent MD structure over the lower portion of the helix 44 reveals that the maximal displacement of the upper part of the helix 44 in simulations is ca. 25 Å, with the average value ca. 9 Å (Figure 9). The simulations indicate that helix 44 can spontaneously reach ranges of bends that are evidenced by cryo-EM studies. Note also that our MD average structure is straight compared to the starting X-ray structure that is modestly bent (Figure 3). This indicates that helix 44 in the ribosome (before translocation) is strained.

**Structure and Dynamics of the Intersubunit Bridge Areas**

Let us now briefly summarize key results regarding the contact areas of helix 44 involved in the intersubunit bridges. As noted above, fluctuations and num-

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ber of opening events were observed for base pairs involved in intermolecular bridge B2a in the course of the simulations NA and MG. Other instabilities in base pairing were seen in U/G/G/U and G/A/A/G stacks, which are involved in bridge B3 and partially B6a.

To obtain further insight into the elasticity of helix 44, we calculated the local deformability along the duplex. Analysis of deformability profiles (derived from fluctuations seen in MD) along the sequence enables us to identify flexible and stiff regions in the duplex. The profile reveals very flexible steps in the upper part of helix 44 and in the areas of bulge bases of helix 44 (Figure 10). On the other hand, base-pair steps in the central portion of helix 44 are rather rigid (Figure 10 and supplementary material Table S2). Comparison with the canonical duplex shows that force constants of the steps formed by non-WC pairs have much larger sequence variability, i.e., there exist stiffer and more flexible regions of helix 44 (Figure 10). We assume that non-WC pairs in helix 44 not only stabilize interactions with 50S (intersubunit bridges, see Figure 2) but they also modulate (enhance) the local flexibility of the helix to promote motions localized in the upper part of helix 44. It is evident that the area of the key bridge B2a is unusually flexible. Analysis of force constants shows that steps 2–5 involved in B2a bridge have enhanced flexibility compared to steps 11–13, 15, 18, 20–22, 24–26, and 28 involved in the bridges B3, B5, and B6a that are more rigid. Range of force constants of all the later steps is close to range of canonical duplex (i.e., these steps are medium-rigid, see Figure 10).

As noted above, base pairs 1423G/1477C, 1425U/1475G, 1432G/1468A, and 1411C/1489G have incomplete base pairing in the X-ray structure, which is swiftly repaired in the simulation. It is likely that the disturbed base pairing in the refined X-ray structure results from some kind of a dynamical disorder. Note that the first three perturbed pairs actually belong to bridges B5 and B6a. Thus, it may happen that these pairs are fully stabilized only upon formation of the bridges or offer energetically accessible substrates to optimize formation of the bridges.

Finally, we have noted before that the bottom bulge area (its upper part is adjacent to intersubunit bridge B6a) reveals dynamical switching between two substates A and B while the X-ray structure is not optimally paired. We speculate that this structural switch may regulate the position of the RNA above the bulge (including the bridge) with respect to the very bottom of helix 44. More specifically, when using the sandwiching WC base pairs 1431C/1469G and 1438G/1463C as reference base pairs, the helical twist and rise between them are 157° and 18.2 Å, and 137° and 17.0 Å for the substates B and A, respectively. The X-ray values are 132° and 19.7 Å (note that the X-ray structure is bent and this area contacts 30S).

It is also notable that both bulge regions and several pairs sandwiching them are contacting the 30S subunit. Thus, the areas seen as soft in the simulation contribute to the interaction between 30S and helix 44. Two of the four initially distorted pairs, 1432G/1468A and 1411C/1489G, interact with the rest of 30S. On the other hand, the dynamical bases A1492 and A1493 appear to be free of any contact with 30S that could impair their mobility. All these data indicate that noncanonical segments of helix 44 are optimally designed to mediate contacts with both subunits and to facilitate motions of the upper part of helix 44.

**Comparison of Sequence-Dependent Elasticity of A-RNA and B-DNA**

A recent B-DNA elasticity study focused on sequence-dependent B-DNA deformability at the base-pair step level and revealed distinct trends for YR/YR, RR/YY, and RY/RY steps. YR/YR B-DNA steps were flexible for roll and rise, RR/YY were intermediate, and RY/RY were stiff. For tilt and partially twist, YR/RY steps were also flexible whereas shift and slide lacked simple trends. The present A-RNA study identifies similar trends of these three distinct groups of steps for rise, roll, and tilt for both helix 44 and the canonical duplex (Figure S4). Like in B-DNA, A-RNA YR/YR steps are the most flexible for roll, tilt, and rise, while RY/RY and YY/RR steps show increased stiffness (Figure S4). Particularly for rise, a trend similar to that for B-DNA is observed. In the case of roll, A-RNA RY/RY steps show on average increased stiffness compared to YY/RR. Considering tilt, A-RNA YY/RR steps have increased stiffness while RY/RY steps are intermediate, which is reversed compared to B-DNA. Further, similar to DNA steps, no simple trends of RNA steps were identified for shift and slide. In summary, the A-RNA results for YR/YR, RR/YY, and RY/RY steps reveal some simple trends similar to B-DNA.

**Concluding Remarks**

Helix 44 is intrinsically straight, with a substantial flexibility towards isotropic bending. The spontaneous motions seen in simulations would be, e.g., entirely sufficient to allow the dynamics of the upper part of helix 44 evidenced by cryo-EM studies.
ysis of local base-pair step deformability suggests that the upper part of helix 44 has markedly enhanced flexibility while its central region is stiffer. The simulation data indicate that noncanonical segments of helix 44 are optimally designed to mediate contacts with both subunits and to allow large-scale dynamics of the upper part of helix 44. The structural dynamics of the conserved adenines 1492A and 1493A in simulations is basically in agreement with NMR and X-ray data. The analysis of dynamics and substrates occurring in the RNA sugar–phosphate backbone is more careful than in any RNA MD study published until now and parallels similar studies recently done for B-DNA. The simulations reveal a number of temporary backbone flips, but they are always reversible. This indicates a good performance of the AMBER MD method for the studied RNA system.

**MATERIALS AND METHODS**

The simulations were carried out using the AMBER-6.0 program with the Cornell et al. force field. The 16S ribosomal helix 44 (residues: 1404–1497) was taken from the X-ray structure of a free 30S subunit (Thermus thermophilus) with a resolution of 3.05 Å (PDB code: 1J5E). Two simulations of the helix 44 were carried out under different ion conditions. The first simulation (NA) was run only with neutralizing Na+ ions. They were initially placed by the Xleap module of AMBER at positions of the lowest electrostatic potential. The second simulation (MG) was run with 24 Mg2+ ions, which were manually placed along the duplex at a distance of 5 Å from the structure (see supplementary material). As explained in the Results section, even such excessive concentration of divalent cations did not affect the outcome of the simulation; thus, simulations with lower concentration of divalent cations were not attempted. Twenty-four Na+ ions were then added by the Xleap to complete the neutralization. The following van der Waals parameters for cations were used in the simulations: Na+ radius 1.868 Å and well depth 0.00277 kcal/mol, Mg2+ radius 0.7926 Å and well depth 0.8947 kcal/mol. It is fair to admit that the use of minimal countierions and the simulation time scale do not guarantee that a Boltzmann distribution is fully established. On the other side, approximations stemming from the simple force field indicate that it is not advisable to increase the number of ions in the simulation and especially to try mimic highly polarizable anions such as Cl− and divalent cations associated with huge polarization/charge transfer effects. Such particles are fairly outside the applicability of the force field. Several recent studies in addition indicate good performance of the simulations with minimal salt. Based on simulations of Kastenholz and Hunenberger, periodicity effects tend to be minimized in simulations with minimal, net-neutralizing salt (in contrast to zero or excess salt). Simulations by Varnai and Zakrzewska have also demonstrated surprisingly good sampling of monovalent ions (in minimal salt), which is fully in agreement with more than 2 μs of our own RNA data. Specifically, we have shown that even molecules that in solution experiments require excess ions for folding (kink-turn and hepatitis delta virus ribocyte) do not experience any structural destabilization (unfolding) on the present simulation time scale. In addition, simulations with different initial monovalent ion placements or multiple simulations reproducibly predict highly occupied monovalent cation binding sites around RNA molecules once the simulations exceed ca 10 ns.

Both systems were solvated by a box of TIP3P water molecules to a distance of 12 Å on each side of the solute. The last studied system was a standard RNA duplex with the sequence 5′CGCGGCGGCCCCAUCCGGCCCGCGCGCGCUCACGACG3′. This structure was built in the program Insight II (BioSym/MSI, San Diego, CA). We used a random GC-rich sequence. (A shorter 10 ns control simulation of an AU-rich sequence indicates overlapping results and is not discussed in this article.) It was neutralized by 72 Na+ ions and solvated by explicit water molecules, using the same protocol as for the helix 44. Due to the width/length proportions of our box (ca. 1:2.5), we systematically monitored the position of the solute in the box. The solute molecule in all three simulations showed only limited rotation and remained entirely inside the box for most of the simulation time. Occasionally, ends of the solute molecule temporarily crossed the box borders, but not significantly. No mutual approach of solute molecules from different boxes occurred. Thus, it was not necessary to increase the width of the box. Simulations were carried out using the particle mesh Ewald technique with 9 Å nonbonded cutoff and 2-fs integration time step. Equilibration started by 5000 steps of minimization followed by 200 ps of MD, with the atomic positions of the solute molecule fixed. Then, two series of minimization (1000 steps) and MD simulation (20 ps) were carried out with restraints of 50 and 25 kcal/(mol · Å²), which were applied to all solute atoms. In the next stage, the system was minimized in five 1000-step rounds with restraints [20, 15, 10, 5, and 0 kcal/(mol · Å²)] applied only to solute atoms. During the following 100 ps of unrestrained MD, the system was heated from 50 to 300 K. The production MD runs were carried out with constant pressure boundary conditions (relaxation time of 1.0 ps). A constant temperature of 300 K using the Berendsen weak-coupling algorithm was applied with a time constant of 1.0 ps. SHAKE constraints with a tolerance of 10−8 Å were applied to all hydrogens to eliminate the fastest X—H vibrations and allow a longer simulation time step. Translational and rotational center-of-mass motion was removed every 5 ps.

The Sander module of AMBER 6.0 was used for all minimization and equilibration MD runs, the PMEMD code was then used for production. Trajectories were analyzed using the Ptraj and the Cnauth modules of AMBER and structures were visualized using the VMD molecular visualization program, http://www.ks.uiuc.edu/Research/vmd/. The figures were prepared using VMD. The EDA of RNA duplexes was performed using the onv, g_covar, and g_anaeig modules of the GROMACS package and visualized using the Interactive Essential Dynamics code.
in VMD. Only phosphorus atoms (P) were included in this analysis because they contain all the information needed for the description of global motions. The overall motion was decomposed into individual essential modes, which were represented by the corresponding eigenvectors and eigenvalues. Helical parameters (rise, roll, tilt, twist, slide, and shift) were calculated using the 3DNA code. The time series of these parameters were then used for the calculations of local base-pair step deformability (force constants). The methodology is described in detail and applied for DNA trajectories where no opening events occur.

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