Posttranscriptional modifications at the 37th position in the anticodon stem–loop of tRNA: structural insights from MD simulations

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ABSTRACT
Transfer RNA (tRNA) is the most diversely modified RNA. Although the strictly conserved purine position 37 in the anticodon stem–loop undergoes modifications that are phylogenetically distributed, we do not yet fully understand the roles of these modifications. Therefore, molecular dynamics simulations are used to provide molecular-level details for how such modifications impact the structure and function of tRNA. A focus is placed on three hypermodified base families that include the parent i6A, t6A, and yW modifications, as well as derivatives. Our data reveal that the hypermodifications exhibit significant conformational flexibility in tRNA, which can be modulated by additional chemical functionalization. Although the overall structure of the tRNA anticodon stem remains intact regardless of the modification considered, the anticodon loop must rearrange to accommodate the bulky, dynamic hypermodifications, which includes changes in the nucleotide glycosidic and backbone conformations, and enhanced or completely new nucleobase–nucleobase interactions compared to unmodified tRNA or tRNA containing smaller (m1G) modifications at the 37th position. Importantly, the extent of the changes in the anticodon loop is influenced by the addition of small functional groups to parent modifications, implying each substituent can further fine-tune tRNA structure. Although the dominant conformation of the ASL is achieved in different ways for each modification, the molecular features of all modified tRNA drive the ASL domain to adopt the functional open-loop conformation. Importantly, the impact of the hypermodifications is preserved in different sequence contexts. These findings highlight the likely role of regulating mRNA structure and translation.

Keywords: MD simulations; N6-isopentenyladenosine (i6A); N6-threonylcarbamoyladenosine (t6A); Wybutosine (yW); tRNA anticodon loop

INTRODUCTION
RNA carries out many critical cellular functions, ranging from translating genetic information into molecular machines to regulating gene activity during cell development and differentiation (Murphy et al. 2004; Park et al. 2019). Although RNA and proteins both exhibit structure–function relationships, twenty canonical amino acids contribute to the structural diversity of proteins, while there are only four canonical RNA nucleobases (A, G, C, and U). Therefore, the RNA nucleobases are frequently chemically modified to impart structural, and therefore functional, diversity. Indeed, more than 100 posttranscriptional nucleobase modifications have been identified to date, in a range of RNA types and organisms (Carell et al. 2012; Duechler et al. 2016). Posttranscriptional modifications have been shown to be involved in gene regulation (Gustilo et al. 2008; Helm and Alfonzo 2014; Zhao et al. 2017; Barbieri and Kouzarides 2020), and therefore uniquely designed RNA nucleobase modifications have the potential to be used to control cellular functions with safety and precision for applications in medicine and biotechnology (Jonkhout et al. 2017; Kawasaki et al. 2020).

Several databases have been developed to assemble information about posttranscriptional modifications including chemical composition, nucleobase modification site, type of RNA and phylogenetic distribution (Sprinzl and Vassilenko 2005; Cantara et al. 2010; Sun et al. 2015; Boccaletto et al. 2017). This data reveals that posttranscriptional modifications exhibit great chemical diversity, ranging from simple isomers to nucleotide methylation to the addition of bulky chemical groups (known as hypermodifications) (Sundaram et al. 2000). Modifications...
can be reversible or irreversible, and alter the chemical properties of the nucleobase, as well as the surrounding RNA regions (Helm 2006). Indeed, modifications can influence RNA structure, promoting or disrupting intramolecular hydrogen-bonding, stacking, and/or hydrophobic interactions that make RNA molecules rigid or more flexible (Vendeix et al. 2009; Motorin and Helm 2010; Chawla et al. 2015; Preethi et al. 2017; Seelam et al. 2017). As a result, modifications can strongly contribute to RNA functional diversity, especially when occurring within regulatory networks, where minor structural changes can significantly affect cellular metabolism and gene expression (Lewis et al. 2017).

Transfer RNA (tRNA) is the most diversely modified RNA (Sundaram et al. 2000; Konevega et al. 2004; Gustilo et al. 2008; Manickam et al. 2015; Sarachan et al. 2016). tRNA is involved in a variety of biological processes, including a unique role in protein synthesis, decoding mRNA codons and transferring specific amino acids to the peptidyl site in the ribosome (Frank et al. 2005). tRNA is typically composed of 73–80 nt and adopts a cloverleaf–shaped secondary structure or L-shaped three-dimensional structure (Fig. 1A). In general, functional tRNA has four major domains: the acceptor stem (ACS: bases 1–9 and 66–73), the D-stem–loop (DSL: bases 10–25), the anticodon stem–loop (ASL: bases 26–44) and the T-stem–loop (TSL: bases 49–65). Many posttranscriptional modifications, such as 4-thiouridine (s4U), 2-methylguanosine (m2G) and 2-dimethylguanosine (m22G), are believed to structurally refine the DSL region (Helm 2006). In addition to the major domains, tRNA has a variable arm (bases 45–48) and exhibits tertiary interaction networks between the D-loop and the T-loop when folded into a three-dimensional structure. These interactions are affected by several modified bases in mature tRNA, including 5-methylcytosine (m5C), 5-methyluridine (m5U), and pseudouridine (ψ) in the TSL region.

The ASL domain of tRNA is coaxially stacked with the DSL domain and is stabilized by five canonical base pairs. The ASL region includes seven loop nucleotides (32–38), with bases 34, 35, and 36 being the anticodon nucleotides that interact with the mRNA codon in the ribosome during protein synthesis. To ensure the efficiency and accuracy of translation, tRNA adopts a canonical U-turn structure in the ASL region, which promotes stable codon–anticodon interactions in the ribosomal A-site. A strictly conserved purine base at position 37, directly next to the anticodon nucleotides, also plays an important role in the ASL domain. Specifically, together with base pairs 31–39 and 32–38, base 37 has been proposed to help maintain an ideal anticodon conformation (Grosjean and Westhof 2016; Rozov et al. 2016). Furthermore, position 37 is key to facilitating tRNA accommodation at the ribosomal binding site, with the conserved helix 69 forming contacts with ribose in the 37th nucleotide (Rozov et al. 2016).

The largest variety and the highest density of nucleobase modifications occur in the ASL domain, which have been suggested to play important roles in maintaining the structural integrity of the anticodon region (Machnicka et al. 2014; Schweizer et al. 2017). More specifically, some modifications have been proposed to help preorder the structure of the ASL loop to facilitate tRNA accommodation in the ribosome and optimize codon–anticodon interactions (Auffinger and Westhof 1999; Sundaram et al. 2000; Stuart et al. 2003; Agris 2008; Vendeix et al. 2008; Denmon et al. 2011; Vangaveti et al. 2020). The conserved purine at position 37 undergoes a wide assortment of modifications that are phylogenetically distributed among the three domains of life (bacteria, archaea, and eukarya) (Machnicka et al. 2014). Modifications at A37 include 2-methyladenosine (m2A), N6-methyladenosine (m6A), 1-methylinosine (m1I), N6-
isopentenyladenosine (i^6A), N6-threonylcarbamoyladenoso-
mine (t^6A), N6-glycinylcarbamoyladenosine (g^6A), N6-
hydroxyxonorvalylcarbamoyladenosine (hn^6A), and associat-
ed derivatives, while G37 modified bases include 1-meth-
ylguanosine (m^1G), wybutosine (yW), and associated
derivatives (Boccaletto et al. 2017) (see, e.g., Fig. 1B).
Many of these modifications have been proposed to en-
hance tRNA function by reinforcing the conserved loop
structure, balancing loop flexibility and rigidity, and en-
hancing the stability of anticodon–codon complexes. For
example, NMR data suggests that t^6A at position 37 main-
tains the anticodon loop conformation by disrupting intra-
loop nucleotide interactions between U33 and A37 (Stuart
et al. 2000), while crystallographic data also suggests t^6A
facilitates codon–anticodon interactions by stacking with
A38 of tRNA and the first base of the codon (Murphy
et al. 2004). In general, modifications to A37 have been
proposed to be frequently used to compensate for weaker
AU codon–anticodon base pairs and maintain the overall
stability of the codon–anticodon duplex (Grosjean and
Westhof 2016).

High-resolution crystal structures of tRNA and tRNA–protein complexes are available for select modifications
at position 37 in the anticodon loop, including i^6A
(Fischer et al. 2016), ms^2t^6A (Jenner et al. 2010), t^6A
(Murphy et al. 2004), ms^2t^6A (Benas et al. 2000), and yW
(Jovine et al. 2000; Mikkelsen et al. 2001; Schmeing
et al. 2009). However, most tRNA functions depend on
structural changes and static crystal structures do not pro-
vide a complete picture of the conformational space of the
modified nucleobase, or the impact of the modification on
the ASL region or codon–anticodon interactions. This
leads to questions regarding the role of the modifications.
For example, planarity of the added bulky chemical group
with respect to the nucleobase has been proposed to be a
crucial factor for maintaining the strength of codon–antico-
don interactions (Stuart et al. 2000; Sundaram et al. 2000;
Murphy et al. 2004; Durant et al. 2005). Indeed, a crystal
structure of tRNA^1ys containing t^6A at position 37 reveals
that the urido moiety is planar with respect to the aden-
sine ring and therefore might enhance stacking by acting
as a third “ring” (Parthasarathy et al. 1977; Murphy et
al. 2004). However, a different crystal structure of E. coli
tRNA reveals a cyclic isoform of t^6A that inherently contains
a third ring adopts a nonplanar conformation (Matuszewski
et al. 2017). Due to such discrepancies, a broad picture
of the impact of different modifications to the 37th
position on tRNA structure and function currently remains
unclear.

Computational methods are particularly useful for un-
covering molecular-level structural details about nucleo-
base modifications and their impact on the local tRNA
environment that are difficult to access solely through ex-
perimental techniques. As a result, several computational
studies have investigated the structural features of un-
modified tRNA (Tewari 1988; Vermeulen et al. 2005;
McCrate et al. 2006; Li et al. 2013; Zhang et al. 2014;
Grzybowska et al. 2016; Fandilolu et al. 2019; Vangaveti
et al. 2020) and the conformational preferences and struc-
tural significance of hypermodifications (Morris et al. 1999;
McCrate et al. 2006; Alexander et al. 2010; Allnér and
Nilsson 2011; Kumbhar and Sonawane 2011; Kumbhar
et al. 2013; Sambhare et al. 2014; Zhang et al. 2014;
Kamble et al. 2015; Sonawane and Sambhare 2015;
Fandilolu et al. 2018). However, to the best of our knowl-
edge, these studies typically implement models that con-
tain only a small fragment of the ASL region and consider
a single trajectory of short time scale (<50 nsec), leaving
a gap in our understanding of the long-range impact of
modifications on the overall tRNA dynamics. In addition,
most computational studies have considered combina-

tions of modifications that exist within mature tRNA
(McCrate et al. 2006; Allnér and Nilsson 2011; Sambhare
et al. 2014; Zhang et al. 2014). However, evidence sug-
gests that the impact of multiple modifications can effec-

tively cancel each other. For example, differential
scanning calorimetry showed that the ability of the i^6A
modification at position 37 to stabilize the stem is impact-
ed by the presence of the pseudouridine modification at
position 39 (Denmon et al. 2011). Thus, studying highly
modified tRNAs prevents a thorough appreciation of the
individual impact of each modification. Additionally,
each previous study typically focuses on a specific modifi-
cation, making comparisons within and across a broad
range of families difficult.

To gain a deeper understanding of the effects of nucleo-
base modifications, the present study uses molecular
dynamics (MD) simulations to investigate the structural
dynamics of tRNA containing three hypermodified base
families at the 37th position, namely i^6A, t^6A, and yW
(Fig. 1B). The representative bases considered for each
codon differ in the presence of chemical substituents
such as a hydroxy, methyl, thiomethyl or peroxy group.
The hypermodification families were chosen based on
their high occurrence frequency at position 37 in tRNA
(Jühling et al. 2008). By comparing the structural dynamics of singly modified tRNAs to the corresponding unmodified
variant, the molecular features imparted by individual
modifications to tRNA structure can be uncovered, which
may modulate tRNA function. Specifically, our detailed
analysis sheds light on (i) the spatial conformations ado-

cpted by the modifications; (ii) how the modifications influ-
ence the stability of tRNA; (iii) how the modifications alter
the dynamics between the four major domains of tRNA; (iv)
how the modifications affect noncovalent interactions that
may be involved in preordering the anticodon loop of
tRNA; (v) how an increase in chemical complexity within
each modification family affects tRNA structural dynamics;
and (vi) how the impact of the modifications vary with se-
quence context. Our results reveal striking differences in

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the intrinsic structural dynamics and cross-strand interactions of the anticodon loop within and across different modification families. Indeed, these differences can affect the open structured anticodon loop that is required for ribosomal binding, pointing toward key roles for modifications at position 37. Overall, the atomistic details provided by our work are critical for understanding the potential biological functions of a range of tRNA hypermodifications and represent an important step toward uncovering the unique structure–function properties of different mature tRNAs. In addition to shedding light on the important biochemical roles of specific nucleobase modifications, the sometimes subtle effects of additional substituents at the modification site uncovered by examining families of modifications is valuable information for the future design of RNA-based therapeutics and biosensors (Chakraborty et al. 2016; Park et al. 2019; Kawasaki et al. 2020).

**RESULTS AND DISCUSSION**

**Unmodified tRNAs with A or G at position 37 exhibit subtle differences in nucleotide interactions in the anticodon-stem–loop, while the m1G modification fine-tunes the functional open-loop conformation of tRNA**

To provide a basis for understanding the structure and dynamics of modified tRNA, 1 μs MD simulations were initially performed in triplicate on two unmodified tRNAs that contain either A (denoted A-tRNA) or G (G-tRNA) at position 37. As described in more detail in the Materials and Methods section, this model choice is justified since the overall fold of unmodified tRNA is similar to isolated mature tRNA (Shi and Moore 2000) and tRNA bound to proteins (e.g., tRNA bound to dihydrouridine synthases [Byrne et al. 2015]) or the A-site of the ribosome (Schmeing et al. 2009), particularly the U-turn motif of the anticodon loop (Byrne et al. 2010). To permit comparison across the diverse set of modifications, models for both unmodified tRNAs were consistently generated from an X-ray crystal structure of standalone full-length unmodified *Escherichia coli* tRNA^Phe^ (PDB ID: 3L0U [Byrne et al. 2010]). Nevertheless, the impact of this model choice in terms of common G modifications and sequence context are discussed in more detail below and in subsequent subsections.

The unmodified tRNAs are stable throughout the MD trajectories as indicated by the average root-mean-square deviation (RMSD) of backbone atoms with respect to the experimental starting structure (~3 Å, Supplemental Fig. S1A; Supplemental Table S1). Sequence variation associated with A versus G at position 37 causes no significant deviation in the tRNA backbone dynamics. Both unmodified tRNAs maintain the correct global fold throughout the MD simulations, specifically the canonical L-shaped three-dimensional structure that contains all four helical domains. Furthermore, the DSL–ASL and the ACS–TSL coaxial domains, and the interdomain tertiary contacts are preserved. Detailed analysis of the per-residue root-mean-square fluctuation (RMSF) of each nucleotide averaged over all sampled conformations reveal lower structural fluctuations for bases in the stem regions (ACS, D-stem, anticodon stem, and T-stem; average RMSF < 4 Å) than for loop nucleotides (D-loop, anticodon loop, variable arm, and T-loop; average RMSF between 4 and 11 Å, Supplemental Fig. S1B).

The domain-dependent motions were assessed by computing a residue-wise cross-correlation matrix for the C1′ atoms of all nucleotides, which highlights the positive (correlated) and negative (anticorrelated) motion of the nucleotides (Supplemental Fig. S2). In unmodified tRNA, nucleotides have significant differences in correlation, which highlights the dynamic characteristics of interdomain interactions within tRNA during the MD simulations. tRNA domains with base–base interactions exhibit positive correlation, especially helical stems with multiple base pairs, as well as long-range interdomain tertiary contacts between the DSL and the TSL or variable arm. In contrast, tRNA dynamics that result in stretching and twisting of coaxial helical domains show anticorrelated motions (e.g., between the DSL and ASL domains). While the overall pattern in the cross-correlation matrix is similar for G-tRNA and A-tRNA, there are slight differences (>0.3). For example, the positive correlation between the DSL and TSL regions is slightly enhanced for A-tRNA, while the positive correlation between the DSL and variable arm domains is slightly enhanced for G-tRNA.

In the ASL region, all five helical base pairs maintain Watson–Crick hydrogen-bonding interactions (Supplemental Tables S2–S4) and consistent glycosidic C1′–C1′ distances (10–11 Å, Supplemental Table S5) during the simulations. Even the A31–U39 base pair next to the anticodon loop is stabilized by two N–H···O/N interactions (occupancies > 90%). This suggests that the anticodon stem domain is not disrupted by the inherent dynamics of the anticodon loop or the interchange of G for A. Indeed, the backbone torsion angles are consistent throughout the loop region (base 31 to 39), with the exception of a dramatic shift between U33 and G34 (particularly in θ at U33 and η at G34, Supplemental Fig. S3). This change in the backbone conformation induces a sharp kink between bases 33 and 34. The backbone kink coupled with greater variation in the orientation of the nucleobases about the glycosidic bonds (Supplemental Fig. S4) results in the lack of stacking between bases 33 and 34 (Supplemental Fig. S5; Supplemental Table S6). The backbone kink is a well-known distinguishing feature of U-turn motifs in tRNA (Quigley and Rich 1976), and results in exposure of the anticodon triplet to the solvent.
Besides the lack of U33–G34 stacking interactions, the remaining anticodon loop bases stack with their nearest neighbors throughout the MD simulations (Supplemental Fig. S5; Supplemental Table S6). This includes the three anticodon bases, although the stacking interactions between bases 34 and 35 is reduced for A-tRNA (14%) compared to G-tRNA (84%). Base stacking within the loop region extends into the anticodon stem, as evidenced by 100% base stacking propensity between bases 31–32 and 38–39 throughout the simulations. This maintains connectivity between the anticodon loop and stem regions. In addition to stacking interactions, the first and last nucleotides of the anticodon loop, namely U32 and A38, form a base pair (Supplemental Tables S2–S4). Thus, several intrastrand interactions work together to structure the anticodon stem–loop.

The nucleobase at position 37 in the anticodon loop adopts an anti conformation throughout the trajectory (average glycosidic χ torsional angle of −172 ± 8° for A37 and −166 ± 9° for G37; Supplemental Table S7; Supplemental Fig. S1C), which is similar to the experimental starting structure of A-tRNA (Byrne et al. 2010). Furthermore, there are no significant overall deviations in the backbone torsion angles near position 37 compared to the crystal structure (Supplemental Figs. S3, S4). In the adopted conformation, base 37 is in a prime location to consistently stack between A36 and A38, regardless of the identity of the purine (100% occupancy, Fig. 2A; Supplemental Fig. S3–S5; Supplemental Table S6). Base 37 stacking interactions are slightly stronger with A38 (average interaction energy of −5.7 ± 0.9 kcal/mol for A and −6.7 ± 2.5 kcal/mol for G) than A36 (−4.6 ± 0.7 kcal/mol for A and −5.0 ± 1.1 kcal/mol for G, Supplemental Table S8). Nevertheless, the persistent stacking between bases 36 and 37 is expected to extend to the codon–anticodon helix at the tRNA–mRNA interface. Indeed, base A37 has been shown to stack with the first codon–anticodon base pair in crystal structures of tRNA bound to the ribosome (Selmer et al. 2006; Weixlbaumer et al. 2007).

In addition to stacking, base 37 participates in cross-strand base–base hydrogen bonding in the anticodon loop. Specifically, the Hoogsteen edge of A37 (N6) forms a N–H···O hydrogen bond with the sugar edge of U33 (O2) (90% occupancy and average interaction energy of −3.1 kcal/mol; Fig. 2B; Supplemental Table S9). This A37–U33 interaction correlates with NMR chemical shifts reported for unmodified tRNA (Denmon et al. 2011) and the X-ray crystal structure of unmodified tRNA (Byrne et al. 2010). Unlike A37, base-pairing occurs between the Watson–Crick faces of G37 and U33 in G-tRNA, which results in an average interaction energy of −2.5 ± 5.7 kcal/mol. Nevertheless, this pair is more dynamic (Fig. 2B; Supplemental Table S9) and the N3(U33)–H···O6(G37) interaction is less persistent (occupancy ∼40%). Indeed, bases G37 and U33 exhibit larger deviations in the glycosidic torsion angles (standard deviations in χ of up to 16°, Supplemental Fig. S4; Supplemental Table S7) and the C1′–C1′ distance (standard deviations up to 1.0 Å, Supplemental Table S5). Overall, although both A and G at position 37 interact with U33, changes in the nature of the interaction result in a different sampling of the dominant conformation of the ASL for A versus G-tRNA (Fig. 3).

Despite interest in comparing the impact of hypermodified to unmodified tRNA with A or G at position 37, sequence analysis reveals that unmodified G is mostly absent at position 37 in the anticodon region of tRNA in all three domains of life (Jühling et al. 2008; Boccaletto et al. 2017). Instead, 1-methylguanosine (m1G) frequently occurs at the 37th position. Therefore, prior to investigating the impact of bulky hypermodifications to G, we initially compared the structural attributes of tRNA containing G and m1G at position 37. Both G and m1G-tRNA are stable throughout the MD simulations as indicated by low backbone RMSDs with respect to the experimental starting structure (∼3 Å) and have highly similar RMSFs for each nucleotide averaged over all sampled conformations (Supplemental Fig. S1). Both G and m1G-tRNA adopt the L-shaped 3D structure throughout the simulations and exhibit similar domain-dependent
interactions (Supplemental Fig. S2). However, although N1-methylation of G preserves stacking interactions with bases 36 and 38, the modification reduces hydrogen-bonding interactions between the 37th nucleotide and U33 (Fig. 2). As a result, incorporation of the m1G modification enhances the stability of the open-loop structure of the ASL (Fig. 3).

Overall, sequence variation with A, G, or m1G at position 37 does not significantly alter the global tRNA structure or domain-dependent interactions. Furthermore, the predicted unmodified tRNA structures are consistent with the experimental structure for unmodified A-tRNA (Byrne et al. 2010), validating our computational approach. Nevertheless, consideration of A, G, and m1G-tRNA highlights that the nature of the base at the 37th position can affect the local architecture of tRNA in the ASL region by altering intraloop interactions (Figs. 2, 3) and further emphasizes the importance of studying the implications of nucleobase hypermodifications at this position. Regardless, the predicted structure of these tRNA will provide points of reference for understanding the impact of bulky base modifications in the anticodon loop on the structure and dynamics of tRNA as discussed in the following sections.

i6A modestly alters nucleotide conformations and nucleotide–nucleotide stacking and hydrogen-bonding interactions in the anticodon loop, with additional chemical substitutions imparting further changes

The i6A hypermodification is derived from canonical adenine with an isopentenyl substituent attached to the N6 exocyclic amino group (Fig. 1B). The two i6A-derived hypermodifications considered in the present work add a hydroxy substituent to the i6A terminal methyl group (io6A) or a thiomethyl substituent at C2 of the nucleobase (ms2i6A, Fig. 1B). Unlike i6A and ms2i6A, sequence analysis highlights that io6A is absent in E. coli (Jühling et al. 2008; Boccaletto et al. 2017). Nevertheless, since io6A is present in both bacteria (Corynebacterium fascians) and eukaryotes (Lupinus luteus and Nicotiana rustica), we consider io6A in the same sequence context as the other members of the i6A family to decipher the effect of the additional hydroxy substituent on tRNA structure. The i6A hypermodifications have the smallest chemical substituents among the families considered in the present work.

Despite the flexibility of the modification side chain (Fig. 4A,B), the modified tRNAs are stable throughout the simulation trajectories, exhibiting similar RMSDs as unmodified tRNA within the standard deviation (Supplemental Fig. S6; Supplemental Table S1). Thus, MD structures of i6A, io6A and ms2i6A-containing tRNA retain the L-shaped structure, with three-dimensional folding and interaction networks similar to the MD predicted and experimental crystal structure of unmodified A-tRNA (Supplemental Fig. S7A; Byrne et al. 2010). The dynamic cross-correlation maps indicate similar correlation patterns for the movement of modified and unmodified tRNAs (deviations <0.3, Supplemental Fig. S7B) and the time-averaged RMSFs for the individual nucleotides in modified and unmodified tRNAs exhibit similar trends (Supplemental Fig. S8). In fact, even the RMSFs for the individual nucleotides in the anticodon loop are generally similar (Fig. 4C).
The stem base pairs in the ASL region are preserved upon tRNA modification (Supplemental Tables S2–S5), including the A31–U39 pair, which correlates with experimental evidence that the parent i6A modification does not destabilize the stem (Denmon et al. 2011). Furthermore, the modified tRNAs contain the kink in the backbone in the anticodon loop between bases 33 and 34 (Supplemental Fig. S9), indicating the presence of the U-turn in the anticodon loop. This observation is supported by NMR data highlighting the role of the parent i6A modification in promoting the U-turn conformation in the E. coli tRNAPhe anticodon loop (Cabello-Villegas et al. 2004). These deviations from unmodified tRNA are dependent on the bulky moiety, with i6A impacting G34, ms2i6A affecting A35, and io6A significantly affecting the backbone conformation of both nucleotides. Thus, the parent i6A modification alters the conformations of loop nucleotides, and small additional chemical changes (hydroxy or thiomethyl substitution) further induce flexibility in the loop region, including adjustments in the anticodon nucleotides (34–36).

Regardless of differences in the backbone conformation in the loop region, the C1′–C1′ distances between the ASL nucleotides in all i6A-modified tRNAs are close to those for unmodified tRNA (Supplemental Table S5), indicating that the overall anticodon loop conformation is maintained. Indeed, there is no stacking between bases 33 and 34 for any modified tRNA (Supplemental Fig. S11; Supplemental Table S6). Similar to solution structures of i6A-modified E. coli tRNAPhe (Cabello-Villegas et al. 2002; Denmon et al. 2011), bases 34 and 35 are coplanar, resulting in less than optimal stacking interactions as observed for unmodified A-tRNA. The remaining nearest neighbor bases stack throughout the MD simulations, which propagates into the stem as discussed for A-tRNA. The only exception is a notable reduction in the stacking occupancy between bases 35 and 36 for io6A-containing tRNA, which correlates with the previously noted highly dynamic nature of the backbone in this region of the anticodon stem–loop for this modification (Supplemental Figs. S9, S10). Regardless, hydrogen bonding is maintained between U32 and A38 for all tRNAs containing any member of the i6A family (Supplemental Tables S2–S4).

The i6A family of modifications does not affect the preferred glycosidic orientation of the nucleotide at the 37th position, with all i6A-derived nucleotides adopting the anti conformation as discussed for A-tRNA (χ > 166°, Supplemental Fig. S12; Supplemental Table S7) and observed in crystal structures of tRNA containing i6A (Jenner et al. 2010) or ms2i6A (Fischer et al. 2016). The bulky isopentenyl group in the i6A modifications is very flexible, adopting a wide range of conformations throughout the MD simulations (Fig. 4A,B; Supplemental Fig. S13). Indeed, although the sampled conformation of the isopentenyl group includes those observed in experimental
structures of tRNA containing i^6A (Fischer et al. 2016) or ms^2i^6A (Supplemental Fig. S14; Jenner et al. 2010), MD simulations emphasize the significant structural dynamics of the hypermodifications. In general, two side chain conformations are adopted with respect to the N6–C10 linkage [defined according to the torsional angle ϕ = ∠(C6N6C10C11), Supplemental Fig. S15]. For i^6A, conformer-I (40° ≤ ϕ ≤ 100°) is populated for ~40% of the simulation and conformer-II for ~60% (160° ≤ ϕ ≤ 240°). However, the number and population of the conformations are influenced by the addition of chemical substituents to i^6A. Specifically, the presence of a hydroxy substituent introduces another conformation (240° ≤ ϕ ≤ 300°), which is slightly less populated than conformers I and II, whereas a thiomethyl group at C2 reduces the N6 bulky moiety flexibility, with conformer-I being highly favored (>85% occupancy). This shows that chemical substitution directly affects the dynamical structure of the hypermodification, specifically the side chain conformation.

Despite the inherent flexibility within the hypermodified nucleobase, the backbone conformation at position 37 does not change from unmodified tRNA (Supplemental Figs. S9, S10). As a result, base 37 is stacked between 36 and 38 regardless of the i^6A derivative considered (Fig. 5A). Nevertheless, all members of the i^6A family considered in the present study exhibit a reduced stacking frequency with base 36 compared to canonical A (Supplemental Fig. S11; Supplemental Table S6), illustrating the impact of the flexible substituent. However, the average stacking interaction between bases 36 and 37 remains consistent with A-tRNA (Supplemental Table S8), suggesting that the bulky substituent increases the magnitude of the stacking interaction. Indeed, stacking between bases 37 and 38 is persistent regardless of the modification (Supplemental Fig. S11) and the associated interaction is statistically larger than the corresponding interaction with A (−5.7 ± 0.9 kcal/mol), with ms^2i^6A having the largest stacking interaction with A38 (−9.0 ± 1.6 kcal/mol) followed by io^6A (−7.7 ± 2.3 kcal/mol) and i^6A (−6.5 ± 1.0 kcal/mol). Thus, our simulations provide direct evidence that the dynamical i^6A modification stabilizes neighboring bases A36 and A38 by enhancing stacking interactions. Indeed, stacking between bases 37 and 38 is persistent regardless of the modification (Fig. 5B). The hydrogen bond is less persistent for i^6A and ms^2i^6A compared to canonical A, and even less persistent for io^6A, likely due to the dynamic nature of the loop backbone for the latter modification. Interestingly, the side-chain of all i^6A modifications can also interact with U33, either stacking above the plane of the uracil nucleobase or hydrogen bonding with O2, as well as O2′ and O4′ of ribose. Although these interactions between the modified A37 side chain and U33 will collectively provide additional stability to the loop region, each contact is relatively short-
lived (<5% occupancy) due to the highly dynamical nature of the hypermodifications. As a result, the modified nucleotide at the 37th position does not participate in cross-strand interactions in the dominant conformations of the ASL, which correspond to an open-loop conformation that will enhance tRNA function (Fig. 3). Thus, the differences between modified and unmodified A-tRNA highlight the potential importance of hypermodifications for controlling noncovalent interactions in the loop region. Furthermore, our data illustrates that an increased level of complexity of the chemical modification, such as including a hydroxy group in the bulky moiety, can further refine discrete noncovalent interactions within tRNA.

Overall, although the i6A modifications do not disrupt the global fold of unmodified tRNA, domain–domain interactions, or the structural integrity of the anticodon stem–loop, the isopenetyl group along with additional chemical (hydroxy or thiomethyl) substituents can introduce local changes in the loop nucleotides that fine-tune tRNA structure and likely function (Table 1). Specifically, the i6A modifications are dynamic and adopt multiple conformations at position 37 in tRNA. In fact, accommodation of the flexible isopenetyl group requires rearrangement within the anticodon loop, with the extent of structural adjustment depending on the level of chemical substitution. Even subtle alterations in the backbone and neighboring base conformations directly impact nucleotide–nucleotide stacking and hydrogen-bonding interactions in the anticodon loop, as well as the conformation of the anticodon nucleotides. Although previous NMR studies on E. coli tRNAPhex highlight increased flexibility of the anticodon loop region upon inclusion of the parent i6A modification (Cabello-Villegas et al. 2002), our work uncovers new molecular-level details surrounding the impact of these dynamics, as well as the relative effects of different levels of i6A chemical substitution.

t6A modifications significantly change the conformation of anticodon loop nucleotides, as well as enhance stacking and decrease hydrogen bonding with base 37, with a dependence on the level of chemical substitution

Similar to i6A, the t6A family introduces modifications at the N6 position of adenine. Specifically, t6A contains a uriedo-threonyl moiety at N6 of A37, which is further substituted with a methyl group at N6 in m6t6A or a thiomethyl group at C2 in ms2t6A (Fig. 1B). Although sequence analysis highlights that the ms2t6A modification is absent in E. coli, it is present in bacteria (Bacillus subtilis) and eukaryotes (Drosophila melanogaster, Lupinus luteus, Triticum aestivum, and Rattus norvegicus). Nevertheless, ms2t6A will be considered in the same sequence context in this study to understand the fundamental impact of t6A thiomethyl substitution on tRNA structure. These are much larger hypermodifications than the i6A family and exhibit even greater dynamics within the bulky substituents (Fig. 6A,B). Indeed, our simulations show that the t6A modified base adopts a distal conformation throughout the simulation, where the N6-threonylcarbonyl substituent spreads away from the imidazole moiety of base A (Sambhare et al. 2014). As a result, although the t6A-modified tRNAs are stable across the simulation trajectory (Supplemental Fig. S6) and retain the global architecture of mature tRNA (Supplemental Fig. S16A), the average RMSD for the modified tRNA backbone (~4.3–4.8 Å) is significantly larger than that for the corresponding unmodified A-tRNA (~3 Å, Supplemental Table S1). Indeed, all t6A modifications considered in the present work increase the dynamics of most tRNA nucleotides compared to the unmodified tRNA (Supplemental Fig. S8). This is especially true for nucleotides in the anticodon loop (Fig. 6C), suggesting that the modifications (particularly ms2t6A) directly

| Modification | 3D-fold & domain–domain interactions | Anticodon stem | Open-loop conformation | Enhanced intraloop stacking | Backbone conformation of anticodon bases | Enhanced anticodon 36–37 stacking | Enhanced anticodon 37–38 stacking | Decreased 33–37 hydrogen bonding | Decreased 32–38 hydrogen bonding |
|--------------|-------------------------------------|----------------|------------------------|--------------------------|------------------------------------------|----------------------------------|----------------------------------|-------------------------------|-------------------------------|
| i6A          | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| io6A         | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| ms2i6A       | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| t6A          | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| m1t6A        | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| ms1t6A       | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| m1G          | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| m1γW         | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |
| o2yW         | *                                   | *              | *****                  | *****                    | ***                                      | ***                              | ***                              | ***                           | ***                           |

*aScores range between low (*) and high (******) relative to the corresponding unmodified tRNA.*
impact neighboring nucleotides. Furthermore, unlike the \( \text{t}^6\text{A} \) family, all \( \text{t}^6\text{A} \) modifications considered increase structural deviations of nucleotides in domains outside the tRNA anticodon stem (Supplemental Fig. S8). Nevertheless, the modifications do not significantly affect the dynamic interactions between RNA domains (Supplemental Fig. S16B).

Despite increased overall tRNA dynamics, the base pairs in the stem region of the ASL are maintained upon introduction of \( \text{t}^6\text{A} \)-derived modifications (Supplemental Tables S2–S4) and the backbone conformation induces the expected kink between nucleotides 33 and 34 (Supplemental Fig. S17), as evidenced by NMR spectroscopic data for \( \text{t}^6\text{A} \)-modified human tRNA\(^{\text{Lys}} \) (Stuart et al. 2000) and \( \text{E. coli} \) tRNA\(^{\text{Lys}} \) (Sundaram et al. 2000). In complement to previous NMR data suggesting that the presence of \( \text{t}^6\text{A} \) modifications leads to anticodon loop remodeling and increased dynamic flexibility of the anticodon bases (Stuart et al. 2000; Durant et al. 2005), our MD data shows that all members of the \( \text{t}^6\text{A} \) family significantly affect the backbone conformation in the anticodon loop between bases 33 to 37, with smaller changes occurring at 32 and 38 (Supplemental Figs. S17, S18). Furthermore, all \( \text{t}^6\text{A} \) hypermodifications impact the orientation of the base about the glycosidic bond at positions 34 and 35, while the impact of \( \text{m}^2\text{t}^6\text{A} \) is further reaching, influencing \( \chi \) and \( \delta \) from 33 to 38, with U33 frequently adopting the syn glycosidic orientation (Supplemental Fig. S18). The shifts in the backbone in the ASL region significantly impact the stacking interactions between loop bases outside position 37 (Supplemental Fig. S19; Supplemental Table S6). Specifically, in addition to the absence of stacking between bases 33 and 34 due to the U-turn motif, stacking is greatly reduced between the 32–33 and 35–36 base neighbors, while less than optimal stacking also occurs between 34–35 as discussed for unmodified A-tRNA. Nevertheless, for all modifications, the backbone maintains the conformation of unmodified tRNA at positions 31 and 39, which suggests that the conformational change in the loop does not propagate through the stem. In fact, stacking between bases 31–32 and 38–39, and U32–A38 hydrogen bonding are maintained for all \( \text{t}^6\text{A} \) modifications (Supplemental Fig. S19; Supplemental Tables S2–S4), which correlates with NMR data for \( \text{t}^6\text{A} \)-modified human tRNA\(^{\text{Lys}} \) (Stuart et al. 2000).

While the modification does not impact the anti glycosidic orientation of the 37th nucleotide for \( \text{t}^6\text{A} \) and \( \text{m}^6\text{t}^6\text{A} \), the conformation adopted by \( \text{m}^2\text{t}^6\text{A} \) ranges from syn to near-anti (\( \chi = 210^\circ \) to \( 330^\circ \)) (Supplemental Figs. S12, S18). Although the shift in the glycosidic orientation further highlights the significant impact of the thiomethyl group, the anti conformation of \( \text{m}^2\text{t}^6\text{A} \) observed in a crystal structure of HIV reverse-transcription primer tRNA (\( \chi = 182^\circ \)) (Benas et al. 2000) is sampled during the MD simulations (Supplemental Fig. S20). The unmodified tRNA with respect to the nucleobase ring for structures taken at 5 ns intervals over the MD simulations. (C) Average structural deviation of anticodon nucleotides (RMSF, Å) over the MD simulations with respect to the initial structure for each \( \text{t}^6\text{A} \) modified tRNA compared to unmodified A-tRNA.
conformation increasing upon further substitution of the parent t6A hypermodification (Supplemental Fig. S21). Thus, additional substitution of t6A can affect the accessible side chain orientations of the hypermodified base.

All members of the t6A family at position 37 stack between the neighboring 36 and 38 nucleobases in the anticodon loop of tRNA (Fig. 7A). NMR and crystallographic structural data showed that the t6A (Sundaram et al. 2000; Rozov et al. 2016) and ms2t6A (Benas et al. 2000; Durant et al. 2005) modifications have increased overlap with bases 36 and 38 compared to A, and therefore promote a stable anticodon loop conformation. However, our data suggests the increased structural dynamics upon base modification reduces the stacking overlap in this region (Supplemental Fig. S19). Furthermore, the standard deviations in the average 36–37 and 37–38 stacking interactions are much higher for the hypermodifications compared to A (Supplemental Table S8). Regardless, the presence of the hypermodification ensures that the average stacking interaction energies at the same position are larger in modified tRNA compared to A-tRNA (by up to ~4.5 kcal/mol for 36 and ~3 kcal/mol for 38, Supplemental Table S8). This suggests that the modifications contribute to the stability of the anticodon loop by enhancing stacking despite inducing structural changes in the loop nucleotides, including the syn glycosidic orientation associated with ms2t6A.

As discussed for the i6A family, the t6A modifications influence hydrogen-bonding interactions with base 37. Specifically, although the N6(A37)…O2(U33) hydrogen bond is maintained throughout the MD simulations for unmodified A-tRNA (90% occupancy), this interaction is significantly disrupted in the presence of t6A and ms2t6A (maximum 40% occupancy), and completely abolished in the presence of the N6 methyl group of m6t6A (Fig. 7B). The lack of the U33–t6A37 interaction in our simulations correlates with proposals that the lack of this interaction in a crystal structure of tRNALys containing t6A improves the anticodon loop conformation when bound to the ribosomal site for codon–anticodon duplex formation (Murphy et al. 2004) and suggestions that t6A prohibits canonical base-pairing based on short-time scale MD simulations on only the ASL region containing multiple modifications (Sambhare et al. 2014). Nevertheless, the magnitude of the average interaction energies between A37 and U33 in unmodified A-tRNA is maintained upon t6A substitution (~3 to ~4 kcal/mol, Supplemental Table S9). Furthermore, the largest A37–U33 interaction is greater for the t6A modifications (up to ~10 ± 2.4 kcal/mol) compared to A (~4 ± 1.1 kcal/mol). These trends in the interaction energy occur because the side chains of the t6A modifications can also form discrete interactions with the U33 nucleotide, either stacking with the nucleobase or hydrogen bonding with O2, O2′, and O4′, as discussed for the i6A family. Nevertheless, the modified bases at position 37 do not interact with cross-strand bases in the dominant conformation of the ASL (Fig. 3), which agrees with previous reports for t6A-tRNA based on NMR spectroscopy (Sundaram et al. 2000). Interestingly, the rearrangement induced by the addition of the thiomethyl group at C2 increases sampling of the dominant open-loop conformation. Thus, the overall functional conformation of the anticodon loop is achieved in different ways for different modifications (Fig. 3).

In summary, the t6A family underscores the ability of hypermodifications to fine-tune the configuration of the anticodon bases in unbound tRNA without abolishing the structure required for function (Table 1). Specifically, the bulkier t6A modifications have more drastic and further reaching impacts on the anticodon stem–loop than members of the i6A family. Indeed, the t6A family emphasizes that loop nucleotides can adjust to accommodate a very
bulky side chain at N6 of A37. Furthermore, the t6A family illustrates how tRNA hypermodifications can significantly impact many structural features in the anticodon stem–loop including increasing dynamics, changing the backbone conformation, disrupting intrastrand hydrogen bonding, and strengthening base–base stacking. These differences alter the conformation of the critical anticodon nucleotides, yet stabilize the ASL in a dominant open-loop conformation (Fig. 3). These observations for the broader t6A family complement the previously reported remodeling of the anticodon loop upon incorporation of the parent t6A modification based on NMR spectroscopic studies of tRNA173 (Stuart et al. 2000; Yarian et al. 2000). Our data also further highlight that the structural changes in the loop region are not propagated into the anticodon stem, and the overall integrity of the global tRNA structure and domain–domain interactions are preserved.

Despite substantial bulk, yW modifications impart minor changes to the conformation of the anticodon stem–loop of unmodified G-tRNA in a manner similar to the smaller m1G modification, yet enhance the stability of the anticodon loop through discrete noncovalent interactions

Unlike the other families discussed thus far, the yW modifications are guanine derivatives. These modifications are among the bulkiest nucleobase derivatives occurring at the 37th position, involving the addition of a ring to the Watson–Crick binding face, as well as a flexible side chain (Fig. 1B). Among the many yW modifications that have been identified to date, yW and o2yW are considered in the present work, which differ in the additional peroxy group on the side chain of o2yW. The distribution of G modifications at the 37th position showed that m1G is the most populated G-modification and is present in bacteria (Escherichia coli, Bacillus subtilis), archaea (Halobacterium salinarum, Haloferax volcanii), and eukaryotes (Saccharomyces cerevisiae, Homo sapiens), followed by yW and o2yW in eukaryotes (Saccharomyces cerevisiae, Xenopus laevis, Homo sapiens) (Jühling et al. 2008; Boccaletto et al. 2017). Despite m1G being common at the 37th position of E. coli tRNAAsp, we draw comparisons in the present work between the yW family of modifications and unmodified G-tRNA to separate the impact of the parent nucleobase versus modification substituent. Subsequent comparison to tRNA containing m1G at the 37th position permits an understanding of the relative impact of (nonbulky) methylation versus (bulky) hypermodifications.

Both yW derivatives adopt a range of conformations at position 37 of tRNA (Fig. 8A,B). Despite the bulk and inherent flexibility of the yW modification family, the associated modified tRNAs retain the global L-shaped architecture (Supplemental Fig. S22A), average backbone RMSD (~3 Å, Supplemental Fig. S6; Supplemental Table S1) and domain–domain interactions (Supplemental Fig. S22B) of unmodified G-tRNA. Nevertheless, these bulky modifications reduce the nucleotide dynamics, especially in the anticodon region (Fig. 8C; Supplemental Fig. S8).

The structure of the stem in the ASL domain is maintained upon the introduction of a yW family modification, which includes critical nucleoside glycosidic orientations required to form Watson–Crick hydrogen bonding (Supplemental Tables S2–S4, S7). Unlike the A modifications discussed previously, the yW modifications result in similar loop backbone conformations and dynamics as unmodified G–tRNA at position 37 (Supplemental Fig. S23). The only exceptions are a slight change in δ at U33 for both modifications, as well as the glycosidic orientation of A35
for yW and G34 for o2yW (Supplemental Fig. S24). Nevertheless, these alterations in the loop conformation of G-tRNA upon modification are very modest. Importantly, the kink is preserved between bases 33 and 34 (Supplemental Fig. S23), and there are no changes in helical bases A31 or U39, which suggests the anticodon stem is preserved as seen for the other modifications. Indeed, C1′–C1′ distances and base–base stacking between the loop nucleotides more closely mimic those occurring in unmodified tRNA compared to the other modification families discussed thus far (Supplemental Tables S5–S6; Supplemental Fig. S25). Hydrogen bonding between U32 and A38 in G-tRNA is also maintained for the yW modifications (Supplemental Tables S2–S4).

The glycosidic orientation of base 37 remains in the anti-configuration for the yW modifications (Supplemental Fig. S12; Supplemental Table S7). Indeed, an NMR solution structure of yW-containing yeast tRNA\textsuperscript{Phe} suggests that the nucleotide conformation may be restricted due to its bulky size and intercalation between the neighboring bases (Stuart et al. 2003). However, the flexible alkyl side chains of the yW derivatives adopt a wide range of orientations relative to the base (Fig. 8A,B), resulting in a higher RMSD for the nucleotide at the 37th position (Supplemental Fig. S13). Nevertheless, the conformation observed in a crystal structure of yeast tRNA\textsuperscript{Phe} (Mikkelsen et al. 2001), which contains yW at the 37th position, is covered during our MD simulations (Supplemental Fig. S26). Interestingly, the parent yW modification adopts a particularly wide spread of conformations, while the addition of the peroxy group significantly reduces the conformational flexibility of the modification side chain, which correlates with a previous short-time scale MD study on tRNA anticodon loops containing yW or o2yW at the 37th position (Fandilolu et al. 2018). Despite the many degrees of rotational freedom in the side chains of yW-derived modifications, the flexibility observed at position 37 primarily occurs in the alkyl chain closest to the base (i.e., the \(\psi_1\) \([N1C11C12C13]\) and \(\psi_2\) \([C11C12C13C14]\) dihedral angles, Supplemental Fig. S27). Indeed, two conformations with respect to \(\psi_1\) and \(\psi_2\) are adopted for yW, while there is one predominant orientation with respect to \(\psi_1\) and \(\psi_2\) for o2yW.

Regardless of the side chain flexibility, the stacking overlap between base 37 and the neighboring bases 36 and 38 observed in G and m1G-tRNA remains very high for yW (Fig. 9A; Supplemental Fig. S25). In contrast, although base 37 can stack between bases 36 and 38 upon the introduction of the peroxy group (Fig. 9A), the frequency of the stacking with neighboring bases is reduced for o2yW (Supplemental Fig. S25). Nevertheless, the additional ring in both yW modifications compared to G leads to a significant increase in the magnitude of the average stacking interactions at position 37 (Supplemental Table S8). Indeed, the average stacking interaction is up to \(\sim 4.5 \pm 2.4\) kcal/mol more stable for yW than G, with the interaction becoming stronger with base 36 than 38, highlighting a rearrangement in the anticodon loop nucleotides. Furthermore, the average stacking interactions with o2yW are even stronger, being up to \(\sim 10 \pm 3.6\) kcal/mol, at least in part due to additional periodic interactions between the peroxy group and the stacked base. Thus, the yW family of modifications provides additional stability to the anticodon loop through increased stacking. This finding agrees with previous suggestions in the literature based on the anticipated increased nucleobase hydrophobicity and size (Dao et al. 1994) and the previously noted decreased stability when the parent yW is replaced with A (Katunin et al. 1994), as well as the trends discussed for the t6A family in the present work. Due to their large size and range of orientations in tRNA, these modified bases also possibly stack with the incoming first codon base during translation and thereby further increase the stability of the codon–anticodon duplex.

In terms of important loop hydrogen-bonding interactions, a O6(G37)…N3(U33) hydrogen bond occasionally forms along the dynamics of G-tRNA (occupancy \(\sim 40\%\), Fig. 9B). Unlike m1G-tRNA, this interaction also occurs upon incorporation of the yW or o2yW modification into the 37th position of tRNA (Fig. 9B). Furthermore, the
side chains of $yW$ and $o^2yW$ can stack or hydrogen bond with U33 as discussed for the $i^3A$ and $t^6A$ families. These noncovalent contacts can serve to stabilize the anticodon loop (Supplemental Table S9). Nevertheless, the presence of the hydrogen-bonding and stacking interactions between the modified base and U33 are highly dependent on the side chain conformation, and these interactions are present for a relatively short amount of time. Indeed, the dominant conformation of the modified-tRNAs highlight a similar open-loop conformation previously discussed for m$^1G$ (Fig. 3). Although these observations correlate with previous suggestions that changes in the G Watson–Crick binding face upon formation of $yW$ alter interactions between base 37 and U33 (Dao et al. 1994), our simulations highlight the complicated interplay between several different noncovalent interactions to maintain the dominant anticodon loop conformation and the impact of the additional peroxy substituent.

Overall, this third family emphasizes a growing theme that tRNA hypermodifications can fine-tune the structural properties of the anticodon loop, with the addition of small functional groups (such as a peroxy group) further modulating tRNA structure and likely regulating function (Table 1). Despite inherent significant bulk and conformational flexibility, the $yW$ family of modifications does not impact the global tRNA L-fold structure or domain–domain interactions, suggesting that the modifications have no long-range structural effects on tRNA. Although the peroxy group of $o^2yW$ introduces rigidity to the side chain of $yW$, both modifications enhance discrete noncovalent interactions with base 37 in the anticodon loop. More importantly, the $yW$ modifications impart changes to the conformation of the anticodon stem–loop of unmodified G-tRNA in a manner similar to the smaller m$^1G$ modification.

Despite subtle changes in nucleotide–nucleotide interactions in the ASL, the impact of hypermodifications is preserved in different sequence contexts

There are a number of modifications that occur at the 37th position across all tRNAs and organisms, and therefore different hypermodifications occur in different sequence contexts (Jühling et al. 2008; Grosjean and Westhof 2016). As stated previously, we implemented a consistent model in the present work that was built from E. coli tRNA$^{Phe}$ in attempts to decipher and compare the impact of various modification families on the structure of tRNA. Nevertheless, to investigate the effects of sequence context, we have also considered unmodified G, m$^1G$ and the bulky $yW$ modification in yeast tRNA$^{Phe}$ as representative comparisons since G modifications such as $yW$ and derivatives are observed in general higher organisms (Saccharomyces cerevisiae, Brassica napus, Mus musculus, and Homo sapiens) (Jühling et al. 2008; Boccaletto et al. 2017). Importantly, yeast tRNA$^{Phe}$ has sequence variation in the anticodon stem–loop compared to E. coli tRNA$^{Phe}$ (Supplemental Fig. S28A), including G rather than A at position 37 and C rather than U at position 32.

tRNA maintains the global L-fold and similar domain–domain interactions (Supplemental Fig. S28), as well as similar average backbone RMSD and nucleotide dynamics (Supplemental Fig. S29; Supplemental Table S13), regardless of the nature of base 37 or the tRNA sequence considered. The backbones of nucleotides in the anticodon stem–loop maintain the same structural features for unmodified and modified (m$^1G$ or $yW$) at position 37, including the distinctive U-turn and an anti-orientation of the 37th nucleotide, and the organism has little impact (Supplemental Figs. S23, S24, S30, S31). Compared to canonical G, as well as m$^1G$, $yW$ is inherently highly dynamic in yeast tRNA as observed in E. coli tRNA (Supplemental Fig. S32). The orientation of $yW$ at the 37th position observed in a crystal structure of yeast tRNA$^{Phe}$ (Mikkelsen et al. 2001) is sampled during the MD simulations (Supplemental Fig. S26B). Despite inherent flexibility of the hypermodification, G, m$^1G$, and $yW$ stack similarly and consistently with bases 36 and 38 in both tRNAs (Fig. 10; Supplemental Fig. S33; Supplemental Tables S6, S14). This equivalent relative nucleotide arrangement is anticipated to enhance stacking interactions in the case of $yW$ in yeast tRNA due to the increased size of the 37th base as previously discussed for E. coli tRNA$^{Phe}$.

The most significant difference between the behavior of base 37 in the two tRNA sequences considered herein is the presence of cross-strand hydrogen bonding with U33 in E. coli tRNA (Fig. 2) and the stark absence of hydrogen bonding with U33 in yeast tRNA (Fig. 10; Supplemental Table S15). Nevertheless, the sidechain of $yW$ forms various noncovalent interactions with U33 in yeast that are present for short periods of time as discussed for E. coli tRNA. Furthermore, the hydrogen-bonding interactions between the 37th nucleotide and U33 are highly similar for all three bases at position 37 (Fig. 10). As a result, the dominant conformations of the ASL region in the tRNAs highlight an open-loop conformation, with an increased occupancy for m$^1G$ and especially $yW$–tRNA (Figs. 3, 10). Importantly, the main conclusions of the impact of the modifications hold in both sequences, namely that the bulky $yW$ modification results in a similar conformation of the anticodon stem–loop as the smaller m$^1G$ modification. Therefore, the inherent behavior of the hypermodification and the associated impact of the modification on tRNA structure are well preserved across different sequence contexts.

Conclusions

The present work uses molecular dynamics simulations on a variety of unmodified and modified tRNA to provide an
atomistic understanding of the effects of different hypermodifications at the 37th position on the overall fold and structural dynamics of tRNA, with particular emphasis on the anticodon loop. Three modification families (i6A, t6A, and yW) were considered to investigate the impact of the size of the chemical substituents added to the canonical bases. In each case, further chemical substitution of the parent modification was also investigated to uncover the role of small substituents (a hydroxy, methyl, thiomethyl or peroxy group). Regardless of the modification family or level of chemical substitution, these hypermodifications do not affect the global tRNA three-dimensional structure or the domain–domain interactions, suggesting that there are no long-range effects of modifying the 37th position. Although the overall structure of the anticodon stem remains intact regardless of the modification considered, the anticodon loop must rearrange to accommodate these hypermodifications, with the extent of the structural adjustments depending on the size of the bulky moiety and level of chemical substitution. Specifically, for adenine modifications, i6A imparts modest changes in the loop backbone and/or nucleobase interactions, while the larger t6A significantly disrupts the conformation of all anticodon loop nucleotides, including the glycosidic orientation of the anticodon nucleotides. In contrast, the yW modification derived from guanine does not significantly impact the structure of the anticodon stem–loop despite being very bulky. Regardless of the degree of loop rearrangement, the larger t6A and yW modifications provide stability to the loop through enhanced or even completely new intrastrand interactions. Furthermore, significant differences in the loop conformation and stability exist between parent modifications and associated derivatives arising from the addition of a hydroxy, methyl, thiomethyl or peroxy group. Indeed, such chemical substitutions can influence the flexibility of the hypermodification side chain and the glycosidic orientation of base 37 or anticodon nucleotides, which subsequently impact intraloop nucleobase–nucleobase hydrogen-bonding and stacking interactions. These molecular features collectively drive the ASL domain of tRNA to adopt a dominant open-loop conformation. Importantly, the impact of these hypermodifications is preserved in different sequence contexts. The changes imparted by these hypermodifications to the anticodon nucleotides are particularly important since these may regulate mRNA translation. Thus, our results provide the previously molecular-level details for how select nucleobase bulky moieties adjust tRNA structure, while maintaining the functional architecture of this important biomolecule. In addition to broadening our understanding of tRNA biochemistry, the knowledge gained from the present work regarding the ability to alter the molecular features of modified RNA through small chemical substitutions affords valuable insight that can be exploited in the future design of improved nucleic acid-based therapeutics and artificial biosensors.

MATERIALS AND METHODS

Initial RNA model

A high-resolution (3.0 Å) X-ray crystal structure corresponding to standalone full-length unmodified E. coli tRNA^{Phe} (PDB ID:
3L0J) was used to build initial structures for MD simulations (Byrne et al. 2010). This model choice is justified based on the similarities between the structures of unbound (PDB ID: 5L4O [Monestier et al. 2017]) and bound tRNAs (PDB IDs: 4YCO [Byrne et al. 2015] and 6QNR [Rozov et al. 2019]) in a range of crystal structures (see, e.g., Supplemental Fig. S34A), as well as the similarities between our predicted tRNA structures and the crystal structures (see, e.g., Supplemental Fig. S34B). The initial structures of G-tRNA and the eight modified tRNAs were obtained by manually replacing A37 in the crystal structure with Pymol (The PyMOL Molecular Graphics System, Version 1.8, Schrodinger LLC) and GaussView 6 (Denninger et al. 2019). The use of a single crystal structure as a starting point allows for an effective comparison of all modifications considered in the present work in the absence of possible structural differences arising from variations in the nucleotide sequence. Nevertheless, the impact of the sequence context was also investigated using models developed based on yeast tRNAPhe. Specifically, these models were generated from a high-resolution (1.9 Å) X-ray crystal structure corresponding to S. cerevisiae tRNAPhe (PDB ID: 1EHZ) (Shi and Moore 2000), with all bases reverted to the unmodified forms, except for yW at the 37th position. Subsequently, unmodified G, and m1G were incorporated at the 37th position. In all models, the initial modified nucleotide conformation was adjusted by visual inspection to maintain the anti glycosidic conformation $\chi = \angle(O4'/C1'N9C4)$, while minimizing steric clashes between the bulky substituent and neighboring nucleotides. Thus, 14 models were considered in total that differ in the nucleobase at position 37, including two unmodified E. coli tRNAs (containing A or G) and nine modified E. coli tRNAs (containing i6A, io6A, ms2t6A, t6A, m6t6A, ms2i6A, m1G, yW, or o2yW), as well as three yeast tRNAs (containing G, m1G, and yW).

**MD simulations**

All tRNA models were prepared using the tLEaP module of the AMBER 16 program package (Case et al. 2016). The ff99bscO_XOLC force field was used for tRNA. Parameters for the modified bases were supplemented by the Generalized Amber Force Field (GAFF) (Wang et al. 2004), with partial atomic charges calculated with RED, v. III.4 using the RESP-A1 scheme (Duprédau et al. 2010). In all simulations, the total charge was neutralized by Na+ ions and the tRNA was solvated in an explicit TIP3P octahedral water box, ensuring that the edge of the box is at least 10.0 Å from the edge of the solute in each direction. The solvent molecules and ions were initially minimized with 500 steps of steepest descent and 500 steps of conjugate gradient minimization using a nonbonded cutoff of 10 Å, while the tRNA was constrained using a 500.0 kcal mol$^{-1}$ Å$^{-2}$ force constant. The entire system was then minimized using 1000 steps of unrestrained steepest descent, followed by 1500 steps of unrestrained conjugate gradient minimization. Subsequently, the system was heated from 0 to 300 K during a 100 psec solute-restrained (10 kcal mol$^{-1}$ Å$^{-2}$) equilibration phase, using a 1 fsec time step. This was followed by a 20 nsec (unconstrained) trial MD simulation under NPT conditions (1 atm and 300 K), with a time step of 2 fsec and the SHAKE algorithm. The periodic boundary condition was used for all steps. Finally, starting from the conformation in the last frame of the corresponding trial simulation using a time step of 2 fsec and the pmemd module of AMBER 16 (Case et al. 2016), 1 µsec MD production simulations were performed in triplicate for each model. Thus, a total of 3 µsec of simulation data was collected per system for a total of 42 µsec over all tRNAs.

**Trajectory analysis**

Each trajectory was sampled for analysis every 20 psec over the course of the production phase. However, as the tRNA system was very flexible for the first 200 nsec, all analysis reported in the main text was performed over the last 800 nsec of the first MD simulation replicate. This data is in full agreement with analysis performed over the total 3 µsec of simulation data for each system (Supplemental Tables S10–S15; Supplemental Figs. S35–S43). Trajectory analysis was completed using the cpptraj module (Roe and Cheatham 2013) of AMBER 16. The RMSDs in the tRNA nucleotide backbone atoms were analyzed, with respect to the corresponding crystal structure coordinates for each simulation to assess system stability. Each trajectory was clustered with respect to the configuration of base 37 and the reported MD representative structures were obtained from the dominant cluster over the simulation in all cases. The density grids for the modified base side chain were obtained using a grid size of 60 Å and spacing of 0.5 Å. All average backbone RMSD, per-residue RMSFs, backbone dihedral angles, $\chi$ [\(\angle(O4'/C1'N9C4)\)] torsional angles, dihedral angles within the bulky substituents, hydrogen-bonding parameters and stacking propensities were calculated over the MD trajectories. To determine hydrogen-bonding occupancies, cut-offs of 120° for angles and 3.4 Å for distances were imposed. The base stacking propensity between the anticodon nucleotides was calculated based on the criteria that the distance between the center of masses of the two bases (including the side chain of the hypermodification) was ≤6 Å, and the angle between the two normal vectors of the bases was ≤40° or ≥140°. The magnitude of base pair hydrogen-bonding interactions and nucleobase–nucleobase stacking interactions were calculated by extracting the atomic coordinates from the simulation trajectories at 5 nsec intervals. The ribose moiety of each nucleotide was replaced with a hydrogen atom, hydrogen-only optimizations were performed using B3LYP/6-31+G(d,p), and the interaction energies (including counterpoise corrections) were calculated using B3LYP-D3(BJ)/6-311+G(2df,p). All DFT calculations were performed using Gaussian 16 (Frisch et al. 2016). Dynamic cross-correlation analysis was carried out using the cpptraj module (Roe and Cheatham 2013) of AMBER 16. The extent of correlated motion between two residues was calculated as the magnitude of the correlation coefficient between the C1' atoms of the two nucleotides of interest. The covariance matrix, which highlights positive (correlated) and negative (anticorrelated) correlation coefficients of the nucleotides, was created using the gnuplot program. Each MD simulation was clustered to determine the dominant structure of the ASL using the Barnaba software package (Bottaro et al. 2019).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.
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