A novel form of RNA double helix based on G·U and C·A⁺ wobble base pairing

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ABSTRACT

Wobble base pairs are critical in various physiological functions and have been linked to local structural perturbations in double-helical structures of nucleic acids. We report a 1.38-Å resolution crystal structure of an antiparallel octadecamer RNA double helix in overall A conformation, which includes a unique, central stretch of six consecutive wobble base pairs (W helix) with two G·U and four rare C·A⁺ wobble pairs. Four adenines within the W helix are N1-protonated and wobble-base-paired with the opposing cytosine through two regular hydrogen bonds. Combined with the two G·U pairs, the C·A⁺ base pairs facilitate formation of a half turn of W-helical RNA flanked by six regular Watson–Crick base pairs in standard A conformation on either side. RNA melting experiments monitored by differential scanning calorimetry, UV and circular dichroism spectroscopy demonstrate that the RNA octadecamer undergoes a pH-induced structural transition which is consistent with the presence of a duplex with C·A⁺ base pairs at acidic pH. Our crystal structure provides a first glimpse of an RNA double helix based entirely on wobble base pairs with possible applications in RNA or DNA nanotechnology and pH biosensors.

Keywords: RNA double helix; wobble base pairing; wobble helix; adenine N1 protonation; pH-dependent structural variation

INTRODUCTION

Base-pairing in most nucleic acids follows the Watson–Crick pairing rules. Non-Watson–Crick base-pairing, however, is also prevalent and plays crucial roles for various physiological functions (Deng and Sundaralingam 2000; Masquida and Westhof 2000; Varani and McClain 2000). Among all non-Watson–Crick pairs, the G·U wobble is the most studied pair that has been shown to be highly conserved in the acceptor helix of tRNA^{Ala}, common in other tRNAs (Sprinzl et al. 1998) and rRNA (Gautheret et al. 1995), and critical for RNA–protein recognition (Hou and Schimmel 1988; McClain and Foss 1988) and splice-site selection in group I introns (Cech 1987; Strobel and Cech 1995). Among other mismatches, C·A⁺ wobble pairs are less frequently observed; however, they have been shown to be isosteric with and capable of substituting G·U pairs in some cases (Samuelsson et al. 1983; Doudna et al. 1989; Gautheret et al. 1995; Masquida and Westhof 2000).

G·U and C·A⁺ pairs are significantly different from Watson–Crick base pairs, and they present opportunities for specific recognition either by generating local irregularity of helical structure or by introducing electrostatic variations in the grooves. G·U wobble base pairs are thermodynamically favorable and require only slight adjustment in base pair λ

angles to form two stable hydrogen bonds (Hunter et al. 1987; Puglisi et al. 1990). The formation of two isosteric hydrogen bonds in a C·A wobble pair, however, is not possible with bases in their standard configuration. Here, the N1(A)–O2(C) hydrogen bond can only form if either the protonation or the tautomeric state of the participating bases are changed. A first model proposes hydrogen-bond stabilization by rare amino–imino tautomers of cytosine or adenine (Saenger 1983; Hunter et al. 1986, 1987; Russo et al. 1998; Masoodi et al. 2016). Similarly, a second model suggests protonation of cytosine N3 simultaneously with a tautomeric shift in the adenine base (Supplemental Fig. S1). Cytosine N3 protonation has been invoked in U6 RNA loop structure formation in *Trypanosoma brucei* and *Crithidia fasciculata* (Huppler et al. 2002) and the DNA–trioestin-A interaction (Quigley et al. 1986). However, whereas the transient formation of base pairs involving the rare enol tautomers of guanine and thymine was recently demonstrated by NMR methods (Kimsey et al. 2015), experimental proof of C·A pairs with adenine imino tautomers is lacking, to the best of our knowledge.

The most frequently invoked model for C·A mismatch formation proposes protonation of adenine N1, which would

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allow a second hydrogen bond to form with cytosine O2 as acceptor atom (Hunter et al. 1986, 1987). In aqueous solution, adenosine exists in three different mono-protonation states with pKa values of 3.64, −1.53, and −4.02 for N1, N7, and N3 protonation (Saenger 1983; Kapinos et al. 2011), and the protonation propensity of adenosine N1 was reported as being 96.1% over N7 and N3 protonation states (Kapinos et al. 2011). Also, adenine N1 protonation has been observed in poly(rA) fibers (Rich et al. 1961), oligo (rA) stretches in crystal structures (Gleghorn et al. 2016), and an NMR structure of poly(dA) (Chakraborty et al. 2009), allowing the oligo(A) to form either a parallel-stranded double helix (π -helix) at acidic pH or a single-stranded helical structure at neutral pH.

In addition, the presence of adenine N1 protonation in isolated or tandem C·A⁺ wobble base pairs is confirmed by several crystal (Hunter et al. 1986, 1987; Jang et al. 1998; Pan et al. 1998) and NMR (Puglisi et al. 1990; Durant and Davis 1999; Huppler et al. 2002) structures of different oligonucleotides. Interestingly, tandem C·A⁺/A⁺·C pairs exhibit cross-strand purine stacking due to the near 0° helical twist, which is compensated by a twist increase by 10°–15° at the adjacent Watson–Crick pairs (Jang et al. 1998), while an isolated C·A⁺ pair causes little helical irregularity, suggesting that neighboring base pairs are efficient in maintaining overall helical geometry when more than one C·A⁺ pair is present. Although several structures for base pair mismatches are available showing G·U or C·A⁺ wobble pairs in isolation, in tandem or with other mismatches, both wobble pairs have never been shown together in a crystal structure. Only one NMR structure of an RNA hairpin with both G·U and C·A⁺ pairs is available that shows these wobble pairs in isolation (Puglisi et al. 1990).

Here we present a high-resolution crystal structure of an 18-bp antiparallel RNA double helix, which includes one half helical turn of W helix, comprising six contiguous wobble base pairs at the center flanked by half turns of standard A-form RNA on either side. The W-helical stretch is formed by four C·A⁺ and two G·U wobble base pairs. The geometry of the novel form of RNA helix is described in detail with a focus on the central wobble pairs. In addition, pH-dependent variations in the RNA structure are probed by various biophysical methods. We suggest that the pH-dependent formation of W-helical RNA might become a valuable tool for RNA nanotechnology applications.

RESULTS

Structure analysis and crystal packing

X-ray diffraction data for the RNA, r(UGUUCUCUACGAA GAACA), were collected to maximum resolution of 1.38 Å, and a Matthews' analysis indicated the presence of one RNA strand in the asymmetric unit. Molecular replacement with PHASER-MR (McCoy et al. 2005) oriented and posi-

tioned a partial RNA model into the unit cell with log-likelihood gain (LLG) and translation function Z-score (TFZ) of 54.4 and 5.3, respectively. One round of automated model building and refinement was performed using Autobuild wizard, which, after fitting the correct oligonucleotide sequence and refinement, yielded $R_{\text{work}}/R_{\text{free}}$ of 23.7%/24.0%. Bond length and angle restraints combined with base-planarity and hydrogen-bonding restraints for base pairs were used simultaneously with anisotropic displacement factor restraints over the whole refinement process, which yielded a final $R_{\text{work}}/R_{\text{free}}$ of 12.9%/15.1% (Table 1).

The RNA molecule crystallized in space group P6₃22, forming an 18-bp antiparallel RNA double helix with two identical strands related by crystallographic symmetry. These duplexes are stacked in head-to-tail fashion to generate a pseudo-continuous RNA column around the 6₃ screw axis along the crystallographic *c*-axis (Supplemental Fig. S2A). The helical stacks are stabilized by eight well-coordinated water molecules present in the minor groove at the helical junction. The eight waters are symmetrically arranged and form a hydrogen-bonding network that stabilizes the helical arrangement by forming specific hydrogen bonds with all four sugar 2'OH, N3, and O2 atoms of adenine and uridine, respectively (Supplemental Fig. S2B).

Geometry of RNA double helix

In the crystal, two RNA strands form an antiparallel double helical structure with 18 bp (Fig. 1). The helical parameters for the full-length RNA structure (fl helix) suggest that the double helix exhibits A-RNA conformation with helical periodicity of 10.96 bp, similar to canonical A-RNA, which has 11 bp per helical turn (Table 2; Schindelin et al. 1995; Olson et al. 2001). The RNA double helix can be divided into three helical segments of 6 bp each with the first and third segments (WC helix) having a total of 12 bases paired

TABLE 1. X-ray data collection and refinement statistics

Data collection statistics	
Space group	P6 ₃ 22
a, b, c (Å)	45.00, 45.00, 94.20
Resolution (Å)	38.97–1.38 (1.46–1.38)
R_{meas} (%)	9.4 (162.3)
$\langle I/\sigma(I) \rangle$	15.08 (1.50)
CC _{1/2}	1.0 (0.69)
Completeness (%)	99.7 (99.8)
Multiplicity	10.47
Refinement statistics	
Resolution (Å)	38.97–1.38 (1.46–1.38)
No. of reflections	12,286
$R_{\text{work}}/R_{\text{free}}$	0.129/0.151
Average B-factors (Å ²)	
RNA (nonhydrogen)	17.30
Water	34.90

Values in parentheses represent the highest resolution shell.

FIGURE 1. X-ray crystal structure of antiparallel RNA double helix with $2DF_o - mF_c$ difference density contoured at 1.0σ with $1.6 \text{ \AA}$ radius of atoms. (A) The crystallographic asymmetric unit consists of a single 18-nt RNA strand. (B) Crystallographic dyad symmetry generates an RNA double helix (fl helix) that is divided into three segments of 6 bp each, abbreviated as WC, W, and WC helix. In the WC-helix segments, bases are paired in Watson–Crick geometry, while the WB helix contains six bases paired in wobble geometry.

according to canonical Watson–Crick base-pairing rules, while the second helical segment (W helix) at the center contains six bases per strand paired in a noncanonical scheme (see Fig. 1). Helical parameters for the WC helix reflect A-RNA-like geometry with periodicity of 10.92 bp per turn; however, the W helix displays unique features with similarities to both A-RNA and A'-RNA. Although large local variations in helical *Twist* and *Rise* are present, the mean values of 29.09° for the helical *Twist* and $1.91 \text{ \AA}$ for the *Rise*, respectively, reflect similarities between the W helix and A-RNA, while the W helix is similar to A'-RNA in having helical periodicity of 12 bp (Supplemental Table S1; Arnott et al. 1972; Tanaka et al. 1999). This indicates that locally distorted wobble base pair repeats can be incorporated in RNA double helices with little overall distortion in helical geometry. Incorporation of maximally four noncanonical base pairs (Holbrook et al. 1991) or two wobble-base pairs present either in isolation

(Hunter et al. 1986, 1987; Pan et al. 1998) or in tandem (Biswas et al. 1997; Jang et al. 1998) has been reported earlier. The structure presented here shows that up to six contiguous non-Watson–Crick base pairs may be accommodated in double-helical RNA.

All sugars in the oligonucleotide are in C3'-*endo* pucker with average pseudorotation phase angle of $13.10 (\pm 3.92)^\circ$, and all nucleosides are in *anti* conformation having an average glycosidic torsion angle of $-162.76 (\pm 5.64)^\circ$. Most backbone torsion angles are within the expected regions for the A conformation, having *gauche* (–) for α and ζ , *trans* for β and ϵ , and *gauche* (+) for γ and δ , with the exception of the C5 nucleoside which has both α and γ torsions in *trans* with 143.8° and -169.7° , respectively. Although these *trans*–*trans* conformations do not occur at every single wobble residue, they occur frequently in structures with wobble G–U or C–A⁺ pairs (Biswas et al. 1997; Jang et al. 1998; Pan et al. 1998).

RNA helix is stabilized by 4 C–A⁺ and 2 G–U wobble base pairs

Antiparallel arrangement of r(UGUUCUCUACGAAGAACA) strands is supported by a crystallographic dyad axis perpendicular to the helix axis that passes at the A9–C10/A9–C10 base pair step and generates nine unique base pairs at either end of the helix. Six terminal base pairs forming the WC helix are stabilized by Watson–Crick base-pairing with A–U and C–G pairs, respectively (Supplemental Fig. S3). The striking feature of the RNA helix is the presence of six non-Watson–Crick base pairs at the center (W helix) with three of them (C10–9A⁺, G11–8U, A⁺12–7C) being crystallographically unique pairs. All three unique wobble pairs are depicted in Figure 2 with their water molecule coordination. All six non-Watson–Crick base pairs display clear wobble geometry, which is characterized by opposite drifts of λ angles for purines and pyrimidines and formation of two hydrogen bonds, one of which links the purine imino hydrogen and the pyrimidine carbonyl oxygen.

In the W helix, all direct interbase hydrogen bonds are facilitated by the reduction in the C1'–C1' distance to an average of $10.3 \text{ \AA}$ from $10.6 \text{ \AA}$, as observed in Watson–Crick

TABLE 2. Mean values for global helical parameters of the RNA fl helix, WC helix, and W helix

Structure	Twist ($^\circ$)	Rise ($\text{\AA}$)	Inclination ($^\circ$)	X-displacement ($\text{\AA}$)
fl helix	32.83 (7.94)	2.52 (0.73)	17.73 (11.82)	–5.14 (3.87)
WC helix	32.96 (2.53)	2.69 (0.17)	16.90 (5.71)	–4.34 (1.15)
W helix	29.09 (13.57)	1.91 (1.14)	23.87 (19.57)	–7.96 (6.41)
A RNA(1SDR)	33.36 (3.54)	2.69 (0.42)	16.79 (7.26)	–4.42 (1.27)
A DNA	32.50 (3.80)	2.83 (0.36)	14.70 (7.30)	–4.17 (1.22)
B DNA	36.50 (6.6)	3.29 (0.21)	2.1 (9.2)	0.05 (1.28)

Values for standard A-DNA and B-DNA and an A-RNA dodecamer structure are given for comparison. Standard deviations are given in parentheses.

FIGURE 2. $2DF_o-mF_c$ electron density contoured at $1.0\ \sigma$ for the three unique wobble base pairs in the WB helix. Two characteristic hydrogen bonds are formed between bases, and a third hydrogen bond is mediated by a water molecule located in the major groove. (A,C) Two unique $C\cdot A^+$ pairs and (B) the unique G-U pair shown with their hydration networks in both helical grooves. (D) The $C\cdot A$ base pair after refinement with relaxed geometric restraints. Characteristic bond-angle changes in the adenine ring and positive difference density (green) at the position of adenine H1 both indicate adenine N1 protonation. The F_o-F_c difference density is contoured at the $2.5\ \sigma$ level. Hydrogen-bond distances are given in Ångström units with hydrogen-acceptor distances shown in black and donor-acceptor distances (N...O or O...O) in cyan.

pairs (Rosenberg et al. 1976; Seeman et al. 1976; Olson et al. 2001). Simultaneously, the bases of a wobble pair undergo a shear movement, leading to an average -12.5° and $+15^\circ$ shift in λ angle for the purine and pyrimidine base, respectively (Supplemental Figure S4; Supplemental Table S2; Hunter et al. 1986; Pan et al. 1998). Both G-U pairs are organized in well-defined wobble geometry with two hydrogen bonds between $O6(G)\cdots N3(U)-H$ and $N1(G)-H\cdots O2(U)$. In $C\cdot A^+$ pairs, one standard hydrogen bond is formed between $N3(C)\cdots N6(A)-H$, and a second hydrogen bond is formed between $O2(C)\cdots N1(A^+)-H$ to complete the wobble arrangement. The protonation of adenine N1 (see below), facilitating formation of the second H bond is probably linked to the acidic pH 4.0 of the crystallization buffer, which is close to the pKa of adenine N1 (Saenger et al. 1975; Saenger 1983; Kapinos et al. 2011).

The unique wobble base pairs $C10\cdot 9A^+$ and $A^+12\cdot 7C$ exhibit similar hydrogen bonding patterns as G11-8U (see Fig. 2). Minor groove water molecules interact with the 2' hydroxyl groups of cytosine, but fail to make any direct hydrogen bond with the paired adenosine. The $C\cdot A^+$ pairs share with the G-U pair a unique water molecule located in the major groove, which stabilizes the RNA helix by bridging N4 to N6 of cytosine and adenine, respectively. The G11-8U pair is highly hydrated, and its hydrogen bonding potential is saturated by an integral water molecule in the minor groove, interacting with a free N2 amino group, O2 and O2'. Unlike the $C\cdot A^+$ base pairs, the G-U pair binds two water molecules in the major groove, which form a hydrogen bonding network with N7, O6, and O4, and with

each other. Major- and minor-groove water molecules, as observed near the $C\cdot A^+$ base pairs, and minor-groove waters, as bound to the G-U base pair, present either in isolation or in tandem, have been shown to be instrumental in stabilizing the wobble pair and its incorporation in RNA helices (Hunter et al. 1987; Holbrook et al. 1991; Biswas and Sundaralingam 1997; Biswas et al. 1997; Pan et al. 1998; Mueller et al. 1999; Trikha et al. 1999). The novel feature of the structure presented here lies in the demonstration that extended segments of exclusively wobble-base-paired nucleotides can be accommodated in an RNA double helix with almost no difference in the hydration pattern of the individual bases.

To determine conformational features of the fl helix and the central W helix, we analyzed the RNA structure with 3DNA (Supplemental Table S3; Zheng et al. 2009). The *Shear* parameter is generally assumed to be a characteristic indicator of wobble base pairs because of their approximately $\pm 2\ \text{\AA}$ *Shear* which does not occur in Watson-Crick base pairs (Olson et al. 2001). Notably, in the central W helix, base pairs are significantly distorted with extremes of *Shear* of $\pm 2.65\ \text{\AA}$, whereas the flanking WC helix shows nonsheared base pairs (Fig. 3A). Additionally, base pairs in the W helix show significantly higher *Opening* accompanied by reduced *Propeller* distortion, which allows a sufficiently close approach of hydrogen-bonded atoms (Fig. 3B,C). Similar variations are observed in base-step *Twist* in the W helix, but the significant effect of individual base-pair *Twist* is compensated by under- and over-twisting of associated base pairs allowing the RNA helix to maintain A configuration (Fig. 3D).

FIGURE 3. Base pair parameters of the symmetric RNA double helix. Prominent variations in (A) *Shear*, (B) *Opening*, (C) *Propeller*, and (D) *Twist* steps are observed in the central wobble-base-paired W-helix segment as compared to the terminal Watson–Crick-paired A-RNA.

Wobble helix and adenine protonation

The protonation state of adenine can be inferred by measuring the internal bond angles, and the pattern of bond-angle changes may hint at protonation at a particular nitrogen in the adenine ring (Taylor and Kennard 1982; Saenger 1983; Safaee et al. 2013; Gleghorn et al. 2016). After one round of structure refinement with relaxed geometric restraints, we compared average C2–N1–C6 and C2–N3–C4 bond angles of adenines in Watson–Crick and wobble base pairs, respectively, with bond angle standards for different adenine protonation states generated either from 467 small-molecule structures retrieved from the Cambridge Structural Database (CSD) or computed using quantum mechanics (Table 3; Gleghorn et al. 2016). Bond angles for individual adenines in the crystal structure are listed in Supplemental Table S4.

Upon relaxing the geometric restraints for RNA, the adenine rings in the wobble base pairs readjusted themselves in the electron density, resulting in increased C2–N1–C6 bond angles and hydrogen bond lengths. Adenine geometries in Watson–Crick base pairs did not display similar changes. In wobble base pairs only, low-level difference density appeared near the N1 position indicating its protonation (see Fig. 2D). The average C2–N1–C6 bond angle for Watson–Crick paired adenines remained at $117.39^\circ (\pm 0.75^\circ)$ while wobble-paired adenines exhibited an increased bond angle of $125.92^\circ (\pm 2.71^\circ)$. C2–N1–C6 bond-angle values derived from the CSD and quantum mechanics were $\sim 124.0^\circ (\pm 2.5^\circ)$ and 123.4° , respectively, and, similarly, a 126.8° bond angle is reported for N1-protonated adenine in a staggered zipper

poly(A) double helix (Gleghorn et al. 2016). For the crystal structure, this analysis strongly supports the model that the adenine N1 position in the W-helical center is protonated, which facilitates the formation of the O2(C)...N1(A⁺)-H hydrogen bond. Additionally, in the RNA helix the average C2–N3–C6 bond angle for Watson–Crick paired adenine is $110.44^\circ (\pm 2.65^\circ)$, while it moderately increases to $114.52^\circ (\pm 1.19^\circ)$ in wobble-paired adenines. This increment, however, is too small to suggest adenine N3 protonation when compared to the CSD or quantum mechanics–based standards of $117.3^\circ (\pm 0.6^\circ)$ and 116.9° .

To rule out the possibility of N3-protonated cytosine-mediated wobble bond formation (see Supplemental Fig. S1C), we also compared the C2–N3–C4 bond angles in restrained and unrestrained RNA structures. Upon relaxing the geometric restraints, the average C2–N3–C4 bond angle in wobble base pairs did not increase to $\sim 125^\circ$ as expected for N3-protonated cytosine (Clowney et al. 1996), but instead it reduced to $114.57^\circ (\pm 0.57^\circ)$ from $118.94^\circ (\pm 0.40^\circ)$, clearly ruling out protonation at cytosine N3. The average C2–N3–C4 bond angle in Watson–Crick pairs did not significantly change upon removing the geometric restraints.

pH-dependent UV thermal melting

To test our hypothesis that formation of the 18-bp RNA duplex observed in the crystal was facilitated by adenine N1 protonation in its central W-helix segment, we examined if any pH-induced structural transition in the RNA can be

TABLE 3. Bond angle analysis of adenines present in the WC- and W-helical segments of the crystal structure. Corresponding bond angles for adenine in different protonation states derived from the CSD and quantum mechanics (Gleghorn et al. 2016) are given for comparison.

Angle	CSD-derived values (°)		
	Neutral ^a	N1 protonated ^b	N3 protonated ^c
C2–N1–C6	118.6 ± 1.1	124.0 ± 2.5	119.3 ± 0.8
C2–N3–C4	110.7 ± 1.2	111.7 ± 0.9	117.3 ± 0.6

Angle	Quantum chemistry derived (B3LYP) values (°) for adenosine			
	Neutral	N1 protonated	N3 protonated	N1 and N3 protonated
C2–N1–C6	118.6	123.4	120.1	124.3
C2–N3–C4	111.6	113.8	116.9	117.8

Angle	Restrained		Unrestrained	
	Wobble pairs	Watson–Crick pairs	Wobble pairs	Watson–Crick pairs
C2–N1–C6	118.3 ± 0.8	118.3 ± 0.7	126.9 ± 2.7	117.5 ± 1.5
C2–N3–C4	110.9 ± 0.2	110.5 ± 0.9	115.0 ± 0.7	110.5 ± 2.8

^a262 adenines.^b65 adenines.^c12 adenines.

observed in thermal melting experiments at different pH values. UV spectra were recorded at 260 nm and pH values of 3.5, 6.5, and 8.0 to monitor structural transitions upon change from acidic to slightly basic medium. One representative out of three experiments is shown in Figure 4A.

Upon sample heating, all spectra showed a hyperchromic transition in UV absorption, suggesting the dissociation from double-helical to single-stranded RNA. We observed that pH change from 3.5 to 6.5 raised the RNA melting temperature from ~55°C to ~67°C. Notably, no change in melting temperature was observed for a further pH increase from 6.5 to 8.0. The significant change in T_m may be linked to different RNA conformations existing at near neutral and acidic pH. It is consistent with the existence of neutral adenine bases at near neutral or basic pH, which could destabilize the central wobble base pairs, inducing a different RNA structure. Moreover, a higher T_m for the RNA at neutral pH indicates

that the configuration present under physiological conditions is thermodynamically favored over the double-helical structure with central W-helix segment observed in crystals grown at low pH.

Differential scanning calorimetry

To further determine the thermodynamic properties associated with different conformations observed at different pH values, DSC experiments were performed with RNA elements buffered at pH 3.5, 6.5, and 7.4. DSC profiles and thermodynamic parameters of RNA thermal melting are shown in Figure 4B. According to DSC analysis, the melting temperature is 55.6°C, 62.1°C, and 61.0°C at pH 3.5, 6.5, and 7.4, respectively, which is in good agreement with T_m values calculated by UV melting experiments. In particular, the DSC analysis confirms that the RNA behaves similarly at

FIGURE 4. pH-dependent variation of physical and thermodynamic properties of RNA. (A) UV thermal melting curves at pH 3.5, 6.5, and 8.0 (orange, green, and blue) indicate a pH-dependent structural transition in RNA. (B) RNA thermal melting curves recorded with differential scanning calorimetry (DSC) at pH 3.5, 6.5, and 8.0.

TABLE 4. Thermodynamic parameters from DSC measurements of the RNA at pH values between 3.5 and 7.4

pH	$T_m \pm SD$ (°C)	$\Delta H \pm SD$ (Kcal/mol)
3.5	55.66 ± 4.39	63.68 ± 1.63
6.5	62.15 ± 0.57	73.81 ± 9.93
7.4	61.01 ± 0.67	72.97 ± 1.27

pH 6.5 and 7.4, whereas the melting transition at acidic pH occurs at a different temperature. The higher T_m of the RNA at neutral pH is in accordance with a higher energy release upon melting transition. The enthalpy change of RNA is ~ 73 kcal/mol near physiological pH and ~ 64 kcal/mol for the double-helical structure existing at acidic pH, suggesting the existence of different initial RNA structures (Table 4). Taken together with the UV melting experiment, the DSC data confirm that different RNA structures exist at neutral and acidic pH for a sequence that permits formation of a central W helix with N1-protonation of adenine bases.

pH-induced structural changes probed by CD melting

Circular dichroism is extremely sensitive to nucleic-acid conformation and can provide valuable information about its structure (Ivanov et al. 1973; Johnson 1990; Woody 1995). We probed the structural differences in RNA at different pH values by recording CD melting transitions at pH 3.5 and 7.5. Average CD spectra from three independent experiments for both pH values at 20°C and 90°C, respectively, representing the folded and melted state of RNA, are shown in Figure 5, while CD spectra and ellipticity changes with temperature are shown in Supplemental Figure S5.

Clearly, at both pH values, the RNA exhibits CD spectra characteristic of the A conformation with maximum at ~ 265 nm and minimum at ~ 210 nm (Johnson 1990; Chauca-Diaz et al. 2015). Upon heating RNA to 90°C, bases become unstacked, which is reflected by disappearance of the peak at 210 nm, while the peak at ~ 265 nm is reduced and shifted to ~ 274 nm (Causley and Johnson 1982; Newbury et al. 1996). The peak at ~ 210 nm is known to be related to the intrastrand interactions in RNA duplexes (Gray et al. 1981; Newbury et al. 1996), and our CD spectra show a significant increase in peak intensity at 210 nm at pH 7.5, suggesting extensive intrastrand interactions in the RNA. Moreover, we also observe a minor negative peak at ~ 240 nm in the spectra, which is indicative of the presence of single-stranded RNA at pH 7.5 (Newbury et al. 1996; Chauca-Diaz et al. 2015).

All CD observations are in agreement with RNA acquiring a highly stable stem-loop structure with unprotonated adenines at physiological pH, forming extensive intrastrand interactions as compared to the double-helical structure, and unpaired bases in the loop (see Fig. 5). Our CD data are strongly supported by a SHAPE (selective 2'-hydroxyl acyla-

tion analyzed by primer extension) analysis (Wilkinson et al. 2006) for an oligonucleotide with a very similar sequence, r (UGUUCUCUACGAAGAACU), which also acquires a tri-loop RNA stem-loop structure at physiological pH (Mino et al. 2015).

DISCUSSION

We present a 1.38-Å resolution crystal structure of an 18-nt-long antiparallel RNA double helix formed in an acidic environment. The overall structure exhibits A-RNA conformation and is clearly divided into three helical segments of 6 bp each, denoted as WC, W, and WC helix. The W helix represents a unique and novel helical form of RNA that is completely based on wobble base pairing and consists of four C-A⁺ and two G-U wobble base pairs. The W helix exhibits overall helical similarity with A-RNA (Schindelin et al. 1995; Olson et al. 2001), while it resembles A'-RNA in having 12 bp per turn (Arnott et al. 1972; Tanaka et al. 1999). In both G-U wobble pairs of the W-helical segment, guanine is shifted toward the minor groove and interacts with O2 and O2' via a water molecule similar to isolated G-U pairs or tandem G-U pairs in motifs I and II of ribosomal RNAs (Biswas and Sundaralingam 1997; Biswas et al. 1997). The C-A wobble pairs are formed as C-A⁺ pairs following

FIGURE 5. Structural asymmetries of RNA element at different pH are probed by CD melting spectra. (A) An overlay of CD spectra at 20°C (solid line) and 90°C (dotted line) for RNA elements is represented at pH 3.5 (black) and 7.5 (blue). (B) The physiological structure of the RNA element is most likely an A-RNA stem-loop with either tri- or hexa-loop and not the double-helical structure observed at acidic pH.

N1 protonation of adenines in the W helix, which is indicated by difference electron density and adenine bond angles after unrestrained structure refinement that agree with bond-angle standards from adenine bases with N1 protonation (Taylor and Kennard 1982; Safaei et al. 2013; Gleghorn et al. 2016). C-A⁺ base pairs in previous RNA crystal structures, either in isolation (Pan et al. 1998) or in tandem (Jang et al. 1998), resulted in RNA bending of ~20°. The present structure of a W-helical segment with flanking A-RNA does not show any significant bending at the base-pair steps involving C-A⁺, resulting in a straight helical half turn of RNA.

An RNA structure with tandem C-A⁺ pairs in a CA⁺ dinucleotide step exhibited cross-strand purine stacking (Supplemental Fig. S6A) accompanied by helical over-winding toward the neighboring Watson–Crick pairs (Jang et al. 1998), while isolated C-A⁺ pairs were incorporated in A-form RNA without much helical perturbations (Hunter et al. 1987; Pan et al. 1998). In the presented structure, however, tandem C-A⁺ pairs in an A⁺C dinucleotide step are stacked almost parallel (Supplemental Fig. S6B) with under-winding of neighboring pairs. Although helical parameters, in particular the base pair *Twist*, in the central W segment show strong fluctuations, their mean values remain in the A-helix regime. Overall, we present a novel RNA structure, the W helix, in which six consecutive wobble base pairs are incorporated in standard A-form RNA, extending the range of RNA conformations observed until now.

Importantly, we observed that the studied oligoribonucleotide exhibits significant differences in thermal stability at different pH values as probed by UV and DSC thermal melting experiments. In both UV melting and DSC analysis, we found that the thermodynamic properties of RNA varied between acidic pH of 3.5 and pH 6.5, which is consistent with adenine-N1 protonation at acidic pH (Saenger 1983; Kapinos et al. 2011). We conclude that the observed differences in the melting behavior result from adenine protonation at low pH. The adenine protonation state remains unchanged between pH 6.5 and 8.0, therefore we observe no differences in thermodynamic properties of RNA in this pH regime. Moreover, the significant difference in the enthalpic contribution to the melting transition suggests that unfolding follows different routes at different pH, consistent with the existence of different initial RNA structures. Comparative analyses of CD spectra at different pH values point to the existence of either a tri-loop (Mino et al. 2015) or a hexa-loop (Zuker 2003) RNA stem-loop structure, which acquires a double-helical wobble-base-paired RNA structure due to protonation of the central adenines at low pH.

As we have demonstrated that the RNA oligonucleotide exists in two stable conformations at different pH values, we propose that this molecule, as well as similar RNA elements with a propensity for forming W-helical structures with C-A⁺ base pairs, may be useful tools as pH biosensors and in RNA nanotechnology applications (Yan 2004; Guo 2010; Yu et al. 2015; Amodio et al. 2016). Adenine-N1 protonation

occurs under acidic conditions and seems to be nonphysiological. Previous reports, however, suggest that microscopic pK_a change could lead to formation of C-A⁺ pairs in cells and influence RNA cleavage by leadzymes (Legault and Pardi 1994, 1997) and a hairpin ribozyme (Cai and Tinoco 1996). A C-A⁺ pair can also substitute for G-U in the P1 helix in group I introns. We suggest that similar structural changes in the RNA element studied here might occur in cells, as a result of microscopic pK_a change, in order to regulate the expression of downstream immune response factors by endoribonuclease Regnase1. However, no such report on RNA structural change has been published so far.

MATERIALS AND METHODS

Oligonucleotide crystallization

The HPLC-purified oligonucleotide r(UGUUCUCUACGAAGAACA) corresponding to a highly conserved stem-loop region in the 3' UTR of human interleukin (IL)-6 mRNA was purchased from Eurofins Genomics (Berlin, Germany) and dissolved in 20 mM Tris (pH 7.0), 50 mM KCl to 1 mM concentration. RNA was reannealed by heating at 90°C for 10 min followed by snap-cooling on ice for 5 min and then diluted in suitable reaction buffer before experiments. Crystals were obtained during co-crystallization trials of RNA oligonucleotide and a catalytically inactive mutant of the mouse ribonuclease ZC3H12C mixed in a 2:1 molar ratio. A total of 0.2 µL of protein–RNA complex in 25 mM HEPES (pH 7.5), 150 mM NaCl, 20 µM ZnSO₄, 0.5 mM MgCl₂ was mixed with 0.2 µL of 20% PEG 6000, 1.0 M LiCl, 0.1 M citric acid (pH 4.0) at 4°C using sitting-drop vapor diffusion technique, and rod-shaped crystals were harvested after ~75 d, soaked in reservoir solution with 20% ethylene glycol before flash freezing in liquid nitrogen. Mixing equal volumes of solutions of the protein–RNA complex and the crystallization buffer as above yields a solution with a pH between 4.4 and 4.5.

X-ray data collection, structure determination, and refinement

X-ray diffraction data were collected at beamline 14.1 of the BESSY II synchrotron operated by Helmholtz-Zentrum Berlin (Mueller et al. 2012) at a wavelength of 0.9184 Å. Initial indexing and data collection strategy were determined by iMOSFLM (Battye et al. 2011), and a complete X-ray data set was processed with XDSAPP (Krug et al. 2012). Since we expected the RNA to form a stem-loop structure, we used the 5 bp stem of pre-let-7f-1 RNA (PDB entry 3TS0) as a search model (Nam et al. 2011) for molecular replacement using the PHASER-MR program (McCoy et al. 2005). Autobuilding of the initial model was performed with Phenix (Adams et al. 2010). Canonical hydrogens were generated by Phenix.reduce (Word et al. 1999) and included in the refinement as riding hydrogens. The graphics program WinCoot (Emsley et al. 2010) was used for model building and visualization, and all refinements were performed by Phenix.refine or CCP4 Refmac5 (Murshudov et al. 1997). Molecular drawings were generated with the PyMOL molecular graphics system (Version 1.7.0.5, Schrodinger, LLC), and Web 3DNA (Zheng et al.

2009) was used to analyze the structure. Atomic coordinates and structure factors for the RNA double helix were deposited in the Protein Data Bank under accession number 5NXT.

UV thermal denaturation

RNA UV thermal melting experiments were performed at three different pH values in reaction buffers (i) 25 mM citric acid (pH 3.5), 200 mM LiCl, 1 mM MgSO₄, (ii) 25 mM citric acid (pH 6.5), 200 mM LiCl, 1 mM MgSO₄, or (iii) 25 mM Tris-HCl (pH 8.0), 200 mM LiCl, 1 mM MgSO₄, using a Chirascan CD spectrophotometer (Applied Photophysics, Leatherhead, UK) with 1-mm path-length cuvette. RNA was diluted to 16 μ M in reaction buffers and incubated at 4°C overnight before measurements. Denaturation curves were acquired by recording absorbance at 260 nm in 0.5°C steps, while temperature was increased from 10°C to 90°C at a rate of 1°C/min. Data were analyzed and processed with the Pro-data Chirascan software (Applied Photophysics). The absorbance of samples was normalized to 0.5°C at 10°C and plotted using Excel.

Differential scanning calorimetry (DSC)

DSC experiments were performed with a NanoDSC instrument (TA Instruments, Eschborn, Germany) with RNA at a concentration of 100 μ M dissolved in reaction buffers (i) 10 mM citric acid (pH 3.5), 50 mM LiCl, (ii) 10 mM citric acid (pH 6.5), 50 mM LiCl, or (iii) 10 mM potassium phosphate (pH 7.4), 50 mM LiCl. RNA samples were incubated at 4°C overnight before measurements. The calorimetric data for RNA were obtained between 10°C and 100°C at a scan rate of 1°C/min. Data were corrected for buffer scan and analyzed with a two-state scaled model with NanoAnalyze software (TA Instruments). Results from two independent experiments were averaged, and errors were calculated as $\pm$ SD.

RNA circular dichroism (CD) melting

All RNA CD melting experiments were carried out using a Chirascan CD spectrophotometer (Applied Photophysics) and a 1-mm pathlength cuvette. RNA was dissolved at a concentration of 16 μ M in reaction buffer (i) 10 mM citric acid (pH 3.5), 50 mM NaCl, or (ii) potassium phosphate (pH 7.5), 50 mM NaCl, and incubated overnight at 4°C. The CD spectra between 320 and 200 nm for each 1°C temperature increase were recorded in 1.5 nm steps between 20 and 90°C at a ramp rate of 0.5°C/min. Three independent experiments were performed at pH 3.5 and 7.4, and spectra were corrected for buffer scan, smoothed with a five-point algorithm, averaged using the Pro-data Chirascan software (Applied Photophysics), and plotted using Excel.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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