Published online 18 June 2010 *Nucleic Acids Research, 2010, Vol. 38, No. 19* ***6697–6706***
doi:10.1093/nar/gkq561

Thermodynamics of RNA duplexes modified with unlocked nucleic acid nucleotides

Anna Pasternak and Jesper Wengel*

Nucleic Acid Center, Department of Physics and Chemistry, University of Southern Denmark, DK-5230 Odense M, Denmark

Received April 28, 2010; Revised June 1, 2010; Accepted June 2, 2010

ABSTRACT

Thermodynamics provides insights into the influence of modified nucleotide residues on stability of nucleic acids and is crucial for designing duplexes with given properties. In this article, we introduce detailed thermodynamic analysis of RNA duplexes modified with unlocked nucleic acid (UNA) nucleotide residues. We investigate UNA single substitutions as well as model mismatch and dangling end effects. UNA residues placed in a central position makes RNA duplex structure less favourable by 4.0–6.6 kcal/mol. Slight destabilization, by ~0.5–1.5 kcal/mol, is observed for 5′- or 3′-terminal UNA residues. Furthermore, thermodynamic effects caused by UNA residues are extremely additive with ΔG°_{37} conformity up to 98%. Direct mismatches involving UNA residues decrease the thermodynamic stability less than unmodified mismatches in RNA duplexes. Additionally, the presence of UNA residues adjacent to unpaired RNA residues reduces mismatch discrimination. Thermodynamic analysis of UNA 5′- and 3′-dangling ends revealed that stacking interactions of UNA residues are always less favourable than that of RNA residues. Finally, circular dichroism spectra imply no changes in overall A-form structure of UNA–RNA/RNA duplexes relative to the unmodified RNA duplexes.

INTRODUCTION

Thermodynamic characterization of chemically modified nucleic acids is essential for understanding the influence of nucleotide mimics on structure and stability. The basic factors that affect the thermodynamic stability of RNA and DNA are hydrogen bonds, stacking interactions, electrostatic effects and duplex solvation (1–5). Combination of the knowledge about the influence of particular modifications on structure and stability gives an opportunity to design modified oligonucleotides with defined physical properties in a rational and predictable way. Therefore, modified nucleotides are useful tools to modulate the physicochemical properties of nucleic acids, which may lead to the development of biologically relevant functions of nucleic acids. Modified oligonucleotides have found applications in many important scientific areas such as diagnostics, therapeutics and nanotechnology (6–13).

Acyclic nucleotide residues generally decrease the stability when incorporated into DNA or RNA duplexes (14–18). Unlocked nucleic acid (UNA; 2′,3′-seco-RNA) monomer is an acyclic RNA mimic (Figure 1) in which the lack of a bond between the C2′- and C3′-atoms of the ribose ring causes enhanced flexibility relative to an RNA monomer. In 1995, our group reported synthesis of a phosphoramidite derivative of the thymine UNA monomer and its incorporation into DNA duplexes (17). Recently, we presented the chemical synthesis of UNA phosphoramidite building blocks of all canonical RNA nucleobases, efficient incorporation of UNA monomers into RNA or DNA strands and thermal denaturation studies of modified RNA/RNA and RNA/DNA duplexes (19,20). We showed that UNA has potential as a molecular tool to either decrease discrimination of mismatches or enhance base-pairing specificity. Additionally, biological studies of UNA-modified antisense oligonucleotides have demonstrated compatibility with RNase H activity (21,22), and introduction of UNA monomers in siRNA duplexes have revealed that UNA is a very attractive modification to improve the specificity of gene silencing (23–25).

Here, we report the first detailed thermodynamic studies of UNA–RNA/RNA duplexes. We have conducted comprehensive analysis of the influence of the acyclic UNA residues of all four canonical RNA nucleobases depending on matched or mismatched interactions within the duplex, position and sequence context. Furthermore, we describe the thermodynamic effects of UNA 5′- and 3′-dangling ends.

*To whom correspondence should be addressed. Tel: +456 550 2510; Fax: +456 615 8780; Email: jwe@ifk.sdu.dk

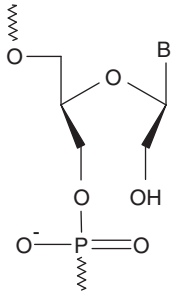

Figure 1. Structure of a UNA nucleotide residue.

MATERIALS AND METHODS

General

The RNA oligonucleotides were purchased from Integrated DNA Technologies. UNA-modified oligonucleotides (commercially available from www.ribotask.dk) were synthesized on an automated RNA/DNA synthesizer using standard phosphoramidite chemistry as described earlier (19). The O3′-phosphoramidites of UNA nucleosides were synthesized according to the previously described procedures (19). The purity of all oligonucleotides was verified by ion-exchange HPLC to be ≥80% and their composition was verified by MALDI-TOF mass spectrometry (Supplementary Data).

Ultraviolet (UV) melting

Oligonucleotides were melted in a 10 mM sodium phosphate buffer containing 100 mM sodium chloride and 0.1 mM EDTA, pH 7.0. Oligonucleotide single-strand concentrations were calculated from the absorbance above 80°C (26). Single-strand extinction coefficients were approximated by a nearest neighbour model (26,27) with the HyTher program. It was assumed that UNA-modified RNA and RNA strands of identical sequences have identical extinction coefficients. Complementary oligonucleotides were mixed at a 1:1 molar ratio. The measurements were performed for nine different concentrations of each duplex in the range 10^{-5}–10^{-6} M using 10 mm (300 µl) quartz microcuvettes. Absorbance versus temperature melting curves were measured by UV melting method at 260 nm with a heating rate of 1°C/min from 5 to 95°C on a Beckman DU 800 spectrophotometer equipped with 6-position microcell holder and thermoprogrammer. Melting curves were analysed and thermodynamic parameters were calculated assuming a two-state model with the program MeltWin 3.5 (28). The ΔH° derived from T_M^{-1} versus ln ($C_T/4$) plots was within 15% of that derived from averaging the fits to individual melting curves for all analysed duplexes (Supplementary Data). This indicates that the two-state model is valid for all duplexes studied. The UV melting method used herein assumes negligible difference in heat capacities of the folded and unfolded state (29). Despite the fact the Tm values for most of the studied duplexes are relatively high, the parameters were evaluated at 37°C to simplify the comparison with other thermodynamic data which are mostly reported for the above temperature.

Circular dichroism spectra

Circular dichroism (CD) spectra were collected on a Jasco J-600A spectropolarimeter using 1 ml quartz cuvettes with a 5-mm pathlength. Oligonucleotide single-strand concentrations were calculated based on the absorbance values measured above 80°C (26). The oligonucleotides were dissolved in a 10 mM sodium phosphate buffer containing 100 mM sodium chloride and 0.1 mM EDTA, pH 7.0 to obtain 5 µM solutions of single-stranded oligoucleotides or duplexes. Complementary oligonucleotides were mixed at a 1:1 molar ratio. All samples were annealed for 2 min at 100°C and slowly cooled to room temperature before data collection. The measurements were taken at 20°C in the 200–300 nm wavelength range. The buffer spectrum was subtracted from the sample spectra. The spectra were smoothed in Microcal Origin 6.0 using Savitzky–Golay filter.

RESULTS

In sections below, the thermodynamic data of UNA nucleotide incorporation into RNA/RNA duplexes are discussed relative to the data obtained for corresponding unmodified RNA/RNA reference duplexes, unless otherwise specified. UNA residues are marked by bold and underlined letters.

The influence of single UNA substitution at the 5′- or 3′-end

The thermodynamic effect of UNA single substitution at the 5′- or 3′-end of modified 11 nt oligonucleotides was studied with model sequences 5′ACGCUACGUGA, 5′CUGGUCGCAUC, 5′GAUGCGACCAG and 5′UCACGUAGCGU for UNA-A, -C, -G and -U (**A**, **C**, **G**, and **U**), respectively, where the 5′- or 3′-terminal RNA residue was substituted with the given UNA residue (Table 1). When one of the four UNA residues was placed at the 5′-end moderate destabilization of the duplex, by 0.99 (**A**), 0.54 (**C**), 1.46 (**G**) and 0.80 (**U**) kcal/mol was observed, whereas for 3′-terminal UNA residues the values of $\Delta\Delta G^\circ_{37}$ were slightly higher for UNA-A and -C but lower for UNA-G and -U amounting to 1.21 (**A**), 1.37 (**C**), 1.19 (**G**) and 0.45 (**U**) kcal/mol, respectively.

Effect of single UNA substitution in the central position

The studies of single UNA-C and -G substitution effects were performed using the same model sequences as for 5′- and 3′-end substitutions, however with modification of the central nucleotide residue (Table 1). Significant decreases in thermodynamic stability were observed for both modified duplexes, showing increase in ΔG°_{37} value by 6.25 and 4.00 kcal/mol for UNA-C and -G, respectively.

The effect of single substitution by UNA-A and -U was measured for duplexes of the sequences 5′ACGCX**A**YGUGA and 5′UCACX**U**YGCGU, where X and Y are A, C, G, or U nucleotide residues. UNA-A situated in the central position of the oligonucleotide makes the duplex less favourable by 4.52–5.36 kcal/mol, whereas UNA-U destabilizes the RNA duplex by

Table 1. Thermodynamic parameters of helix formation with UNA (**X**) and oligoribonucleotides; the effect of single substitutions

Duplexes (5′–3′)		T_M^{-1} versus log C_T plots						
		$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[a] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)	ΔT_M[a] (°C)	Reference number
(1) ACGCUACGUGA	UCACGUAGCGU	99.1 ± 1.7	274.2 ± 5.0	14.07 ± 0.10	62.5			
(2) CUGGUCGCAUC	GAUGCGACCAG	100.9 ± 4.8	275.8 ± 14.2	15.39 ± 0.35	66.8			
(3) ACGCUAAGUGA	UCACUUAGCGU	99.9 ± 3.9	282.2 ± 12.0	12.41 ± 0.16	56.4			
(4) ACGCUAGGUGA	UCACCUAGCGU	101.9 ± 6.1	280.9 ± 18.4	14.82 ± 0.40	64.5			
(5) ACGCUAUGUGA	UCACAUAGCGU	102.5 ± 3.3	289.5 ± 10.2	12.65 ± 0.15	56.7			
(6) ACGCAACGUGA	UCACGUUGCGU	104.9 ± 1.6	292.8 ± 5.0	14.13 ± 0.09	61.2			
(7) ACGCCACGUGA	UCACGUGGCGU	103.5 ± 2.5	281.6 ± 7.4	16.20 ± 0.19	69.0			
(8) ACGCGACGUGA	UCACGUCGCGU	98.2 ± 3.1	267.1 ± 9.3	15.35 ± 0.23	67.6			
ACGCUACGUGA	UCACGUAGCGU	92.7 ± 1.4	256.8 ± 4.2	13.08 ± 0.08	60.6	0.99	−1.9	(1)
CUGGUCGCAUC	GAUGCGACCAG	101.1 ± 4.5	278.2 ± 13.6	14.85 ± 0.31	64.8	0.54	−2.0	(2)
GAUGCGACCAG	CUGGUCGCAUC	89.7 ± 2.3	244.1 ± 6.9	13.93 ± 0.14	64.9	1.46	−1.9	(2)
UCACGUAGCGU	ACGCUACGUGA	90.8 ± 1.7	250.1 ± 5.2	13.27 ± 0.09	61.8	0.80	−0.7	(1)
ACGCUACGUG**A**	UCACGUAGCGU	93.0 ± 1.9	258.5 ± 5.7	12.86 ± 0.09	59.7	1.21	−2.8	(1)
CUGGUCGCAU**C**	GAUGCGACCAG	92.4 ± 5.2	252.7 ± 15.7	14.02 ± 0.35	64.4	1.37	−2.4	(2)
GAUGCGACCA**G**	CUGGUCGCAUC	96.5 ± 3.8	265.5 ± 11.3	14.20 ± 0.24	63.8	1.19	−3.0	(2)
UCACGUAGCG**U**	ACGCUACGUGA	100.7 ± 2.4	280.7 ± 7.3	13.62 ± 0.13	60.5	0.45	−2.0	(1)
CUGGU**C**GCAUC	GAUGCGACCAG	90.3 ± 5.6	261.8 ± 18.0	9.14 ± 0.11	46.2	6.25	−20.6	(2)
GAUGC**G**ACCAG	CUGGUCGCAUC	102.6 ± 5.6	294.2 ± 17.3	11.39 ± 0.19	52.4	4.00	−14.4	(2)
ACGCU**A**CGUGA	UCACGUAGCGU	80.7 ± 2.6	230.6 ± 8.3	9.22 ± 0.04	47.7	4.85	−14.8	(1)
ACGCU**A**AGUGA	UCACUUAGCGU	62.3 ± 3.5	175.4 ± 11.4	7.89 ± 0.05	43.9	4.52	−12.5	(3)
ACGCU**A**GGUGA	UCACCUAGCGU	85.1 ± 2.5	241.9 ± 7.9	10.05 ± 0.06	50.4	4.77	−14.1	(4)
ACGCU**A**UGUGA	UCACAUAGCGU	75.8 ± 0.9	218.7 ± 3.1	7.97 ± 0.00	43.0	4.68	−13.7	(5)
ACGCA**A**CGUGA	UCACGUUGCGU	85.6 ± 2.2	246.7 ± 6.9	9.09 ± 0.03	46.6	5.04	−14.6	(6)
ACGCC**A**CGUGA	UCACGUGGCGU	84.4 ± 3.2	237.2 ± 9.9	10.84 ± 0.1	53.7	5.36	−15.3	(7)
ACGCG**A**CGUGA	UCACGUCGCGU	97.4 ± 3.1	279.8 ± 9.9	10.63 ± 0.08	50.6	4.72	−17.0	(8)
UCACG**U**AGCGU	ACGCUACGUGA	67.7 ± 1.0	192.2 ± 3.1	8.12 ± 0.01	44.4	5.95	−18.1	(1)
UCACG**U**UGCGU	ACGCAACGUGA	72.6 ± 1.8	208.9 ± 5.8	7.81 ± 0.02	42.6	6.32	−18.6	(6)
UCACG**U**GGCGU	ACGCCACGUGA	85.3 ± 2.9	241.2 ± 9.0	10.46 ± 0.08	52.0	5.74	−17.0	(7)
UCACG**U**CGCGU	ACGCGACGUGA	90.9 ± 2.8	261.9 ± 8.9	9.63 ± 0.05	47.9	5.72	−19.7	(8)
UCACA**U**AGCGU	ACGCUAUGUGA	68.4 ± 2.3	197.7 ± 7.4	7.05 ± 0.05	39.4	5.60	−17.3	(5)
UCACC**U**AGCGU	ACGCUAGGUGA	59.1 ± 1.9	164.0 ± 6.0	8.21 ± 0.02	46.1	6.61	−18.4	(4)
UCACU**U**AGCGU	ACGCUAAGUGA	71.0 ± 2.7	205.1 ± 9.0	7.36 ± 0.04	40.7	5.05	−15.7	(3)

[a]Calculated for 10^{-4} M oligomer concentration; changes in UNA-A and -U neighbourhood are marked by grey background.

5.05–6.61 kcal/mol, all dependent on the nature of the neighbouring residue.

Additivity of UNA thermodynamic effects

Thermodynamic effects caused by multiple UNA modifications were evaluated using model sequences similar to those used in 5′- and 3′-end replacement studies with combined UNA substitutions at the 5′- and 3′-ends as well as in the internal position (Table 2). Theoretical ΔG°_{37} value was calculated by summing up ΔG°_{37} of the unmodified duplex and the thermodynamic effects ($\Delta\Delta G^\circ_{37}$) caused by single modifications in a given position. Conformity of the thermodynamic effects, here expressed in per cent, was evaluated by comparison of the calculated ΔG°_{37} value with the experimental one and signifies the extend to which the values are comparable. The additivity for all studied duplexes was remarkably high, mounting to 94, 90, 94 and 98% for UNA-A, -C, -G and -U, respectively, with an average value of 94%.

The influence of UNA-A and -U mismatches

Studies of thermodynamic effects caused by UNA-A and -U mismatches were performed with the modified residue positioned centrally or at either of the ends of the oligonucleotide (Table 3). As expected, central UNA-A mismatches destabilized duplex structure the most increasing the ΔG°_{37} value by 0.68 (**A**-A), 1.25 (**A**-C) and 0.74 (**A**-G) kcal/mol relative to the matched UNA-modified duplex. Interestingly, UNA-A terminal mismatches were not only less destabilizing than central mismatches, but also in most cases even slightly stabilizing duplex structure by 0.66 (5′**A**-A), 0.99 (5′**A**-C), 0.28 (5′**A**-G), 0.19 (3′**A**-A) and 0.41 (3′**A**-C) kcal/mol. Only for a 3′-terminal **A**-G mismatch was a minor destabilization by 0.23 kcal/mol observed.

Examination of the influence of UNA-U internal mismatches revealed decreases of duplex thermodynamic stabilities relative to the matched UNA-modified duplex by 1.67 and 1.65 kcal/mol for **U**-C and **U**-U mismatches, respectively, while a **U**-G mismatch did not change duplex stability. Furthermore, UNA-U terminal mismatches were in general less destabilizing than internal ones ($\Delta\Delta G^\circ_{37}$ = 0.09 (3′**U**-C), 0.13 (3′**U**-G), 0.10 (3′**U**-U) and 0.51 (5′**U**-C) kcal/mol). A similar stabilization effect like for UNA-A terminal mismatches was observed only for a 5′-terminal **U**-G mismatch ($\Delta\Delta G^\circ_{37}$ = −0.53 kcal/mol), whereas 5′**U**-U interactions showed no significant influence on RNA duplex thermodynamic stability.

Table 2. Comparison between experimental and calculated thermodynamic stabilities

Duplexes (5′-3′)		$-\Delta G^{\circ}_{37}$ (kcal/mol)[a]	ΔG°_{37} (kcal/mol)[b]	ΔG°_{37} conformity%
ACGCU**A**CGUG**A**	UCACGUAGCGU	7.51 ± 0.04	7.02	94
CUGGU**C**GCAU**C**	GAUGCGACCAG	6.48 ± 0.06	7.23	90
GAUGC**G**ACCA**G**	CUGGUCGCAUC	8.22 ± 0.01	8.74	94
UCACG**U**AGCG**U**	ACGCUACGUGA	7.03 ± 0.09	7.20	98

[a]Experimental value.
[b]Calculated value.

Table 3. Thermodynamic parameters of helix formation with UNA (**X**) and oligoribonucleotides; the effect of mismatches

Duplexes (5′–3′)		T_M^{-1} versus log C_T plots							
		$-\Delta H^{\circ}$ (kcal/mol)	$-\Delta S^{\circ}$ (eu)	$-\Delta G^{\circ}_{37}$ (kcal/mol)	T_M[a] (°C)	$\Delta\Delta G^{\circ}_{37}$ (kcal/mol)	ΔT_M[a] (°C)	$\Delta\Delta\Delta G^{\circ}_{37}$[b] (kcal/mol)	$\Delta\Delta T_M$[b] (°C)
ACGCUAGGUGA	UCACCUAGCGU	101.9 ± 6.1	280.9 ± 18.4	14.82 ± 0.40	64.5	0	0		
A-A	UCACCAAGCGU	87.4 ± 2.8	248.5 ± 8.7	10.29 ± 0.07	50.9	4.53	−13.6		
A-C	UCACCCAGCGU	93.6 ± 3.5	266.2 ± 10.8	11.00 ± 0.11	52.6	3.82	−11.9		
A-G	UCACCGAGCGU	88.5 ± 2.6	251.2 ± 8.3	10.61 ± 0.08	52.0	4.21	−12.5		
ACGCU**A**GGUGA	UCACCUAGCGU	85.1 ± 2.5	241.9 ± 7.9	10.05 ± 0.06	50.4	0	0		
A-A	UCACCAAGCGU	77.4 ± 1.8	219.5 ± 5.7	9.37 ± 0.03	48.8	0.68	−1.6	−3.85	12.0
A-C	UCACCCAGCGU	79.9 ± 3.5	229.4 ± 11.3	8.80 ± 0.05	46.1	1.25	−4.3	−2.57	7.6
A-G	UCACCGAGCGU	78.9 ± 4.7	224.3 ± 14.9	9.31 ± 0.10	48.3	0.74	−2.1	−3.47	10.4
ACGCUACGUGA	UCACGUAGCGU	99.1 ± 1.7	274.2 ± 5.0	14.07 ± 0.10	62.5	0	0		
A-A	UCACGUAGCGA	102.0 ± 3.4	283.6 ± 10.2	14.02 ± 0.19	61.6	0.05	−0.9		
A-C	UCACGUAGCGC	92.8 ± 3.8	255.8 ± 11.6	13.51 ± 0.22	62.2	0.56	−0.3		
A-G	UCACGUAGCGG	101.1 ± 4.0	280.9 ± 12.2	13.92 ± 0.23	61.5	0.15	−1.0		
ACGCUACGUGA	UCACGUAGCGU	92.7 ± 1.4	256.8 ± 4.2	13.08 ± 0.08	60.6	0	0		
A-A	UCACGUAGCGA	98.2 ± 4.6	272.2 ± 13.8	13.74 ± 0.27	61.6	−0.66	1.0	−0.71	1.9
A-C	UCACGUAGCGC	103.6 ± 2.7	288.6 ± 8.3	14.07 ± 0.15	61.3	−0.99	0.7	−1.55	1.0
A-G	UCACGUAGCGG	94.3 ± 6.5	260.9 ± 19.8	13.36 ± 0.37	61.2	−0.28	0.6	−0.43	1.6
ACGCUACGUGA	UCACGUAGCGU	99.1 ± 1.7	274.2 ± 5.0	14.07 ± 0.10	62.5	0	0		
A-A	ACACGUAGCGU	93.4 ± 4.04	257.6 ± 12.3	13.48 ± 0.23	61.9	0.59	−0.6		
A-C	CCACGUAGCGU	101.3 ± 6.5	281.4 ± 19.7	14.05 ± 0.40	61.9	0.02	−0.6		
A-G	GCACGUAGCGU	92.0 ± 3.8	253.9 ± 11.5	13.23 ± 0.21	61.4	0.84	−1.1		
ACGCUACGUG**A**	UCACGUAGCGU	93.0 ± 1.9	258.5 ± 5.7	12.86 ± 0.09	59.7	0	0		
A-A	ACACGUAGCGU	89.1 ± 3.6	245.11 ± 11.1	13.05 ± 0.20	61.5	−0.19	1.8	−0.78	2.4
A-C	CCACGUAGCGU	94.2 ± 5.3	260.9 ± 16.2	13.27 ± 0.30	60.9	−0.41	1.2	−0.39	1.8
A-G	GCACGUAGCGU	87.2 ± 3.0	240.4 ± 9.0	12.63 ± 0.15	60.3	0.23	0.6	−0.61	1.7
UCACGUGCGU	ACGCCACGUGA	103.5 ± 2.5	281.6 ± 7.4	16.20 ± 0.19	69.0	0	0		
U-C	ACGCCCCGUGA	88.0 ± 7.3	245.9 ± 22.6	11.67 ± 0.33	56.3	4.53	−12.7		
U-G	ACGCCGCGUGA	102.8 ± 3.3	283.3 ± 10.1	14.91 ± 0.22	64.5	1.29	−4.5		
U-U	ACGCCUCGUGA	96.8 ± 6.3	271.8 ± 19.3	12.47 ± 0.29	57.3	3.73	−11.7		
UCACG**U**GGCGU	ACGCCACGUGA	85.3 ± 2.9	241.2 ± 9.0	10.46 ± 0.08	52.0	0	0		
U-C	ACGCCCCGUGA	76.5 ± 3.4	218.4 ± 10.8	8.79 ± 0.05	46.4	1.67	−5.6	−2.86	7.1
U-G	ACGCCGCGUGA	90.8 ± 3.0	259.1 ± 9.5	10.50 ± 0.08	51.2	−0.04	−0.8	−1.33	3.8
U-U	ACGCCUCGUGA	75.9 ± 4.6	216.3 ± 14.7	8.81 ± 0.07	46.6	1.65	−5.4	−2.08	6.3
UCACGUAGCGU	ACGCUACGUGA	99.1 ± 1.7	274.2 ± 5.0	14.07 ± 0.10	62.5	0	0		
U-C	ACGCUACGUGC	89.1 ± 1.7	245.1 ± 5.3	13.03 ± 0.09	61.4	1.04	−1.1		
U-G	ACGCUACGUGG	94.5 ± 1.8	261.6 ± 5.3	13.35 ± 0.10	61.1	0.75	−1.4		
U-U	ACGCUACGUGU	97.1 ± 4.1	269.5 ± 12.5	13.46 ± 0.22	60.9	0.61	−1.6		
UCACGUAGCGU	ACGCUACGUGA	90.8 ± 1.7	250.1 ± 5.2	13.27 ± 0.09	61.8	0	0		
U-C	ACGCUACGUGC	88.7 ± 3.0	244.9 ± 9.3	12.76 ± 0.16	60.4	0.51	−1.4	−0.53	0.3
U-G	ACGCUACGUGG	102.2 ± 2.3	285.0 ± 7.0	13.80 ± 0.13	60.7	−0.53	−1.1	−1.28	−0.3
U-U	ACGCUACGUGU	97.5 ± 3.2	271.3 ± 9.9	13.30 ± 0.17	60.1	−0.03	−1.7	−0.64	−0.1
UCACGUAGCG**U**	ACGCUACGUGA	99.1 ± 1.7	274.2 ± 5.0	14.07 ± 0.10	62.5	0	0		
U-C	CCGCUACGUGA	99.7 ± 1.0	276.6 ± 3.1	13.87 ± 0.06	61.6	0.20	−0.9		
U-G	GCGCUACGUGA	93.4 ± 3.3	257.4 ± 9.9	13.58 ± 0.20	62.3	0.49	−0.2		
U-U	UCGCUACGUGA	100.5 ± 1.4	279.1 ± 4.4	13.93 ± 0.08	61.6	0.14	−0.9		
UCACGUAGCG**U**	ACGCUACGUGA	100.7 ± 2.4	280.7 ± 7.3	13.62 ± 0.13	60.5	0	0		
U-C	CCGCUACGUGA	99.9 ± 1.3	278.4 ± 4.1	13.53 ± 0.07	60.4	0.09	−0.1	−0.11	0.8
U-G	GCGCUACGUGA	102.6 ± 2.1	287.2 ± 6.4	13.49 ± 0.11	59.6	0.13	−0.9	−0.36	−0.7
U-U	UCGCUACGUGA	104.6 ± 1.9	293.8 ± 5.9	13.52 ± 0.10	59.2	0.10	−1.3	−0.04	−0.4
ACGC**C**ACGUGA	UCACGUGGCGU	103.5 ± 2.5	281.6 ± 7.4	16.20 ± 0.19	69.0	0	0		
C-A	UCACGUAGCGU	89.7 ± 4.0	255.9 ± 12.5	10.37 ± 0.10	50.9	5.83	−18.1		
C-C	UCACGUCGCGU	89.6 ± 2.6	258.3 ± 8.1	9.52 ± 0.04	47.7	6.68	−21.3		
C-U	UCACGUUGCGU	88.9 ± 2.6	256.2 ± 8.2	9.47 ± 0.04	47.6	6.46	−21.4		

(continued)

Table 3. Continued

Duplexes (5′–3′)		T_M^{-1} versus log C_T plots							
		$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[a] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)	ΔT_M[a] (°C)	$\Delta\Delta\Delta G^\circ_{37}$[b] (kcal/mol)	$\Delta\Delta T_M$[b] (°C)
ACGCCACGUGA	UCACGUGGCGU	84.4 ± 3.2	237.2 ± 9.9	10.84 ± 0.1	53.7	0	0		
C-A	UCACGUAGCGU	62.0 ± 5.1	176.8 ± 16.6	7.13 ± 0.14	40.0	3.71	−13.7	−2.12	4.4
C-C	UCACGUCGCGU	64.6 ± 1.8	187.4 ± 5.9	6.47 ± 0.05	36.7	4.37	−17.0	−2.31	4.3
C-U	UCACGUUGCGU	52.5 ± 2.2	148.9 ± 7.5	6.28 ± 0.08	35.5	4.56	−18.2	−1.90	3.2
ACGCUAGGUGA	UCACCUAGCGU	101.9 ± 6.1	280.9 ± 18.4	14.82 ± 0.40	64.5	0	0		
G-A	UCACAUAGCGU	80.3 ± 1.7	231.3 ± 5.4	8.57 ± 0.01	45.1	6.25	−19.4		
G-G	UCACGUAGCGU	94.8 ± 3.6	275.8 ± 11.3	9.28 ± 0.05	46.3	5.54	−18.2		
G-U	UCACUUAGCGU	100.7 ± 1.8	287.9 ± 5.7	11.39 ± 0.06	52.7	3.43	−11.8		
ACGCUAGGUGA	UCACCUAGCGU	85.1 ± 2.5	241.9 ± 7.9	10.05 ± 0.06	50.4	0	0		
G-A	UCACAUAGCGU	75.9 ± 2.7	224.6 ± 8.8	6.22 ± 0.07	35.7	3.83	−14.7	−2.42	4.7
G-G	UCACGUAGCGU	52.3 ± 3.6	149.5 ± 12.2	5.96 ± 0.17	33.6	4.09	−16.8	−1.45	1.4
G-U	UCACUUAGCGU	80.5 ± 4.6	236.1 ± 15.0	7.28 ± 0.08	39.9	2.77	−10.5	−0.66	1.3
ACGCUACGUGA	UCACGUAGCGU	99.1 ± 1.7	274.2 ± 5.0	14.07 ± 0.10	62.5	0	0		
G-A	UAACGUAGCGU	88.8 ± 2.7	247.9 ± 8.2	11.88 ± 0.11	56.9	2.19	−5.6		
G-G	UGACGUAGCGU	81.0 ± 2.1	225.0 ± 6.4	11.24 ± 0.08	56.1	2.83	−6.4		
G-U	UUACGUAGCGU	86.0 ± 5.4	239.9 ± 16.7	11.61 ± 0.22	56.5	2.46	−6.0		
ACGCUACGUGA	UCACGUAGCGU	93.0 ± 1.9	258.5 ± 5.7	12.86 ± 0.09	59.7	0	0		
G-A	UAACGUAGCGU	76.4 ± 4.9	210.5 ± 15.1	11.14 ± 0.21	56.9	1.72	−2.8	−0.47	2.8
G-G	UGACGUAGCGU	84.9 ± 3.0	237.2 ± 9.3	11.34 ± 0.11	55.6	1.52	−4.1	−1.31	2.3
G-U	UUACGUAGCGU	85.5 ± 2.5	239.2 ± 7.7	11.35 ± 0.09	55.5	1.51	−4.2	−0.95	1.8
ACGCUACGUGA	UCACGUAGCGU	99.1 ± 1.7	274.2 ± 5.0	14.07 ± 0.10	62.5	0	0		
C-A	UCACGUAGCAU	86.9 ± 1.1	240.1 ± 3.4	12.44 ± 0.05	59.6	1.63	−2.9		
C-C	UCACGUAGCCU	91.4 ± 3.3	255.5 ± 10.1	12.13 ± 0.14	57.2	1.94	−5.3		
C-U	UCACGUAGCUU	95.1 ± 3.2	266.1 ± 9.9	12.53 ± 0.15	57.9	1.54	−4.6		
ACGCUACGUGA	UCACGUAGCGU	92.7 ± 1.4	256.8 ± 4.2	13.08 ± 0.08	60.6	0	0		
C-A	UCACGUAGCAU	102.7 ± 4.1	289.0 ± 12.5	13.10 ± 0.19	58.2	−0.02	−2.4	−1.65	0.5
C-C	UCACGUAGCCU	95.2 ± 5.0	267.4 ± 15.4	12.26 ± 0.22	56.9	0.82	−3.7	−1.12	1.6
C-U	UCACGUAGCUU	101.9 ± 3.5	287.0 ± 10.6	12.83 ± 0.16	57.4	0.25	−3.2	−1.25	1.4

[a]Calculated for 10^{-4} M oligomer concentration.
[b]The difference in $\Delta\Delta G^\circ_{37}$ or ΔT_M value between UNA-RNA/RNA and the corresponding RNA/RNA mismatched duplexes. RNA and UNA residues directly involved in mismatch interactions are marked by grey background.

The influence of RNA mismatches adjacent to UNA

The thermodynamic effects of RNA mismatches adjacent to UNA residues were studied with model sequences of the form, 5′ACGCCACGUGA, 5′ACGCUAGGUGA, 5′ACGCUACGUGA and 5′ACGCUACGUGA, where mismatched RNA residues are marked by grey background. The internal C-X mismatches, where X is A, C or U, neighbouring with UNA-A at the 5′-side, destabilize the duplex by 3.71 (C-A), 4.37 (C-C) and 4.56 (C-U) kcal/mol relative to the matched UNA-modified duplex. This corresponds to less unfavourable thermodynamic effect of those mismatches with reference to unmodified mismatched RNA duplexes (Table 3). Similarly, also internal G-X mismatches adjacent to UNA-A at the 3′-side, where X is A, G or U, reduce mismatch discrimination relative to the unmodified controls, and increase Gibbs free energy by 3.83 (G-A), 4.09 (G-G) and 2.77 (G-U) kcal/mol. Moreover, comparable or slightly less reduced discrimination was observed for C-X and G-X mismatches neighbouring UNA-modified ends of RNA duplexes with ΔG°_{37} value changed by 1.72 (G-A), 1.52 (G-G), and 1.51 (G-U), and −0.02 (C-A), 0.82 (C-C), and 0.25 (C-U) kcal/mol.

UNA dangling ends

The thermodynamic effect of UNA 5′- and 3′-dangling ends was measured to evaluate the contribution of stacking interactions to the UNA-induced changes in duplex stability. Our studies revealed that UNA nucleotide stacking interactions are always less favourable than for RNA nucleotides. The majority of UNA 5′-dangling ends cause slight destabilization by 0.50, 0.24 and 0.55 kcal/mol for UNA-C, -G and -U dangling ends, respectively. Minor stabilization is observed only in case of 5′-UNA-A dangling end (Table 4). Unpaired UNA nucleotides at the 3′-end induce favourable or unfavourable changes in duplexes stability depending on nucleobase type. For 3′UNA-A and 3′UNA-G, minor stabilization by 0.43 and 0.11 kcal/mol, respectively, was observed, whereas for 3′UNA-C and 3′UNA-U, decreased thermodynamic stability by 0.17 and 0.31 kcal/mol, respectively.

Circular dichroism

CD spectra were measured for four representative UNA-modified RNA duplexes differing in positioning of a UNA-U modified nucleotide residue or in the number of modifications (Figure 2a). The spectra of all studied duplexes showed an intense, wide positive band ~260 nm, a distinct negative band at 210 nm and a moderately flat region in the 250–230 nm range, which corresponds to the overall A-form duplex structure characteristic for RNA (30).

Moreover, the CD spectra of UNA-U modified single strands were recorded (Figure 2b). Also, in these cases no

Table 4. Thermodynamic parameters of helix formation with UNA (X) and oligoribonucleotides; the effect of UNA dangling ends

RNA duplexes (5′–3′)		T_M^{-1} versus log C_T plots					
		$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G^\circ_{37}$ (kcal/mol)	T_M[a] (°C)	$\Delta\Delta G^\circ_{37}$ (kcal/mol)	ΔT_M[a] (°C)
CGCUACGUGA	UCACGUAGCG	98.0 ± 2.2	273.2 ± 6.6	13.26 ± 0.12	59.9	0	0
ACGCUACGUGA	UCACGUAGCG	101.3 ± 1.1	282.9 ± 3.4	13.58 ± 0.06	60.2	−0.32	0.3
UGGUCGCAUC	GAUGCGACCA	93.1 ± 4.5	255.2 ± 13.7	13.91 ± 0.29	63.7	0	0
CUGGUCGCAUC	GAUGCGACCA	86.1 ± 3.3	234.4 ± 9.9	13.41 ± 0.21	63.9	0.50	0.2
AUGCGACCAG	CUGGUCGCAU	89.5 ± 2.2	243.8 ± 6.7	13.90 ± 0.15	64.8	0	0
GAUGCGACCAG	CUGGUCGCAU	87.1 ± 1.2	236.8 ± 3.8	13.66 ± 0.08	64.7	0.24	−0.1
CACGUAGCGU	ACGCUACGUG	92.8 ± 2.7	257.2 ± 8.3	13.02 ± 0.14	60.3	0	0
UCACGUAGCGU	ACGCUACGUG	86.0 ± 1.5	237.0 ± 4.5	12.47 ± 0.08	60.0	0.55	−0.3
ACGCUACGUG	CACGUAGCGU	92.8 ± 2.7	257.2 ± 8.3	13.02 ± 0.14	60.3	0	0
ACGCUACGUG**A**	CACGUAGCGU	99.4 ± 2.0	277.2 ± 6.0	13.45 ± 0.10	60.2	−0.43	−0.1
CUGGUCGCAU	AUGCGACCAG	89.5 ± 2.2	243.8 ± 6.7	13.90 ± 0.15	64.8	0	0
CUGGUCGCAU**C**	AUGCGACCAG	86.6 ± 0.5	235.0 ± 1.5	13.73 ± 0.03	65.1	0.17	0.3
GAUGCGACCA	UGGUCGCAUC	93.1 ± 4.5	255.2 ± 13.7	13.91 ± 0.29	63.7	0	0
GAUGCGACCA**G**	UGGUCGCAUC	93.2 ± 4.3	255.4 ± 13.1	14.02 ± 0.28	64.1	−0.11	0.4
UCACGUAGCG	CGCUACGUGA	98.0 ± 2.2	273.2 ± 6.6	13.26 ± 0.12	59.9	0	0
UCACGUAGCG**U**	CGCUACGUGA	91.2 ± 3.4	252.4 ± 10.3	12.95 ± 0.18	60.5	0.31	0.6

[a]Calculated for 10^{-4} M oligomer concentration.

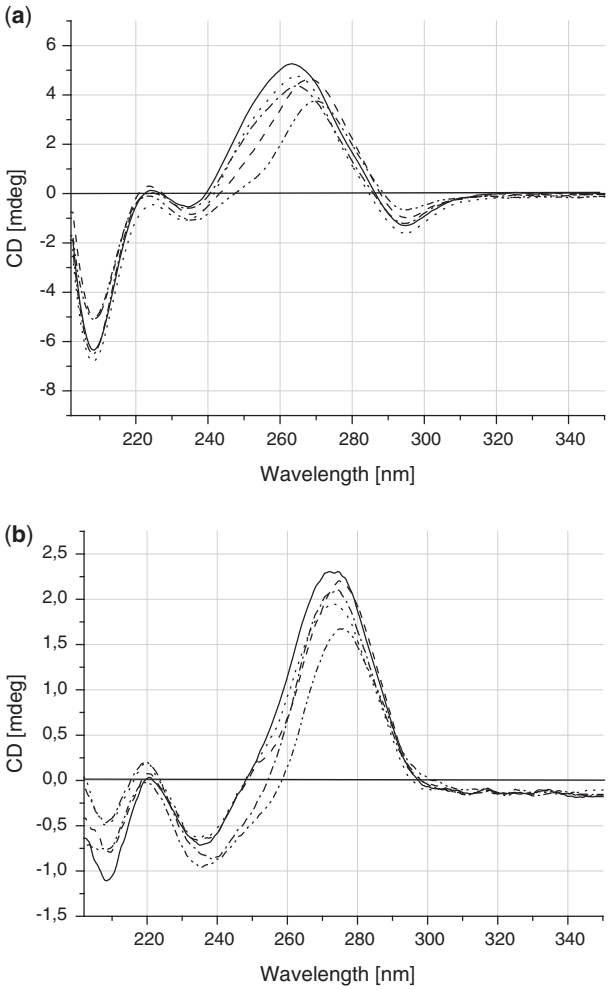

Figure 2. CD spectra of model UNA-modified RNA duplexes (**a**) and UNA-modified oligoribonucleotides (**b**). Solid line—RNA, dash—5′UCACG**U**AGCGU, dot–line—5′**U**CACGUAGCGU, dash–dot–line—5′UCACGUAGCG**U**, dash–dot–dot–line—5′**U**CACG**U**AGCG**U**; underlined bold letters: UNA nucleotides.

significant alteration relative to the spectrum of the reference RNA oligonucleotide was observed. All spectra for single strands showed an intense positive band with maximum ~270 nm, and two fairly intense negative bands ~210 and 240 nm.

DISCUSSION

Series of UV melting experiments are useful for providing data on RNA or DNA duplex thermodynamic stabilities, whereas thermodynamic parameters supply more detailed information about the changes in the stability of nucleic acids imposed by a given modification. Based on the latter kind of studies, several nearest-neighbour models were created (31–43), which are useful tools that provide insights to the predictions of thermodynamic stability of RNA or DNA constructs for potential applications in biochemistry, medicine or nanotechnology.

Recent papers describing UNA-containing oligonucleotides revealed interesting and promising thermal and biological properties of these (17,19–25,44). Herein, thermodynamic analysis of UNA internal single substitutions revealed significant decrease of RNA duplex stabilities (Table 1). The most destabilizing modification was UNA-C and -U which made duplex structure less favourable by on average 6.25 and 5.86 kcal/mol, respectively. Lower but still significant decreases in thermodynamic stability of RNA duplexes, amounting to an average 4.00 and 4.85 kcal/mol were observed for UNA-G and -A modifications, respectively. Stacking interactions are one of the major factors contributing to duplex formation, and it has been reported that in natural nucleic acid duplexes the stacking ability of pyrimidines is less than that of purines (4,45,46). It can be therefore assumed that the degree of destabilization of UNA nucleotides placed centrally in RNA duplexes mainly depend on the surface area and polarizability of nucleobase aromatic rings. UNA-C and -U are pyrimidine

nucleotide derivatives which possess smaller aromatic ring surface area and lower polarizability compared to UNA-G and -A. Probably, therefore the unfavourable thermodynamic effect caused by UNA purine ribosides is weaker than that of UNA pyrimidine nucleotides.

Thermodynamic analysis of RNA duplexes containing UNA-A and -U revealed that the composition of the exact neighbourhood has a minor but perceptible influence on the destabilizing properties of UNA. UNA-A decreases thermodynamic stability of duplexes by 4.52–4.85 kcal/mol for the four types of 3′-adjacent nucleotide residue. However, varying the 5′-neighbourhood of UNA-A induces a slightly wider range of ΔG°_{37} changes (4.85–5.36 kcal/mol). Furthermore, also decrease of RNA duplex thermodynamic stability caused by UNA-U is mostly only faintly neighbourhood-dependent, with ΔG°_{37} increases in ranges of 5.72–6.32 and 5.05–6.61 kcal/mol for the various 3′- and 5′-neighbourhoods, respectively.

In general, the highest destabilization ($\Delta\Delta G^{\circ}_{37}$ = 5.36 (**A**), and 6.61 (**U**) kcal/mol) is observed, when UNA-A neighbours are C (5′-C**A**C-3′) or when UNA-U is adjacent to C and A (5′-C**U**A-3′). Conversely, the most energetically favourable arrangement for internal UNA-A and -U is 5′-U**A**A-3′ ($\Delta\Delta G^{\circ}_{37}$ = 4.52 kcal/mol) and 5′-U**U**A-3′ ($\Delta\Delta G^{\circ}_{37}$ = 5.05 kcal/mol). UNA nucleobases probably adopt a less-defined orientation within a duplex than RNA nucleobases. The differences in destabilization effects caused by UNA-A and -U flanked by various nucleotides are presumably due to different interactions between the nucleobases of UNA and the adjacent ribonucleotides (28,47). Thus, destabilizing effect of UNA-A and -U might be compensated for by favourable electrostatic interactions between partially charged electron donating and electron withdrawing groups. Similarly, duplex destabilization induced by UNA might be additionally increased by unfavourable donor–donor or acceptor–acceptor interactions.

UNA modifications situated at one of the terminal positions cause minor decrease in duplex thermodynamic stabilities (Table 1). In general, UNA-A and -C nucleotide residues destabilize RNA duplexes more by 0.22 and 0.83 kcal/mol, respectively, when placed at the 3′-end, whereas UNA-G and -U show greater effect, by 0.27 and 0.35 kcal/mol, at the 5′-end. Moreover, the unfavourable effect of a terminal UNA modification is significantly less pronounced than of an internal UNA modification. Similar effects were observed previously for other types of modifications including 5- and 6-substituted uridines and N^6-alkyladenosines which when shifted towards the end of the duplex caused decreased destabilization (48,49). The differences in destabilization caused by UNA situated in terminal and internal positions might be due to the enhanced flexibility of terminal relative to the internal UNA nucleotides and thus better possibility to adopt an energetically less unfavourable orientation at the end of the duplex.

The majority of UNA-modified duplexes show favourable entropic effects accompanied by unfavourable enthalpy changes, comparable to the data reported previously (19). Thus, increase of enthalpy values seems to overcome positive entropy changes resulting in overall thermodynamic destabilization of the UNA-modified duplexes.

The studies of duplexes containing three UNA modifications placed at terminal and internal positions within RNA duplexes revealed that contribution to Gibbs free energies per UNA monomer to a large extend (90–98%) is additive (Table 2). It can thus be assumed that the effects observed in singly UNA-modified RNA duplexes can be applied to approximate thermodynamic stability of duplexes containing more than one UNA nucleotide residue. However, it seems that specific positioning of a UNA nucleotide together with sequence dependency should be taken into account for thermodynamic predictions. Therefore, our studies of additivity are only preliminary but suggest the opportunity to make an accurate predictive tool. Interestingly, also the remaining thermodynamic parameters of multi-modified duplexes show noticeable conformity by 78–97% and 84–93% for ΔH° and ΔS°, respectively (Supplementary Data).

The internal, direct mismatches of UNA-A and -U are definitely less destabilizing in reference to the same type of natural mismatches (Table 3). The difference of unfavourable effects caused by given modified and unmodified mismatches reaches 2.57–3.85 kcal/mol and 1.33–2.86 kcal/mol for UNA-A or RNA-A, and UNA-U or RNA-U mismatches, respectively. The most destabilizing UNA-A mismatch is **A**-C, whereas **A**-A and **A**-G influence duplex stability less significantly. Surprisingly, the destabilizing effect of RNA mismatches in unmodified duplexes increases in a different order: A-C < A-G < A-A. The order of RNA mismatch stability is comparable also to the previously reported results (50). Notably, the influence of **U**-C, **U**-G and **U**-U mismatches is completely consistent with the effects caused by unmodified mismatches with the order of destabilizing power of UNA-U mismatches being **U**-G < **U**-U < **U**-C. Moreover, the obtained results are in accordance with previous experiments showing that U-G, in the same context 5′GUG/3′CGC, is the most stable mismatch in RNA duplexes (51).

Remarkably, thermodynamic studies of UNA-A direct terminal mismatches revealed unexpected enhancement of duplexes stability in the range of 0.19–0.99 kcal/mol. Minor destabilization was observed only for 3′-terminal **A**-G interactions. UNA-A mismatches placed at the 5′-end stabilize duplexes by 0.66 (**A**-A), 0.99 (**A**-C) and 0.28 (**A**-G) kcal/mol, whereas at the 3′-end by 0.19 (**A**-A) and 0.41 (**A**-C) kcal/mol. Interestingly, the order of favourable effects induced by 3′-terminal UNA-A (**A**-C > **A**-A > **A**-G) is consistent with the order of destabilization observed for natural mismatches (A-C < A-A < A-G). The examples of nucleotides which involved in mismatch interactions destabilize or stabilize duplexes when placed at internal or terminal positions, respectively are very few. However, similar behaviour was reported previously for e.g. LNA-U mismatch formed in 2′-O-methyl-RNA/RNA duplexes (52) and for anti-parallel dC-dT interactions (53). In contrast, the majority of UNA-U terminal mismatches slightly destabilizes duplexes. Minor stabilization was observed only for 5′-terminal **U**-G interactions.

Notably, a U-G mismatch is considered as one of the most stable RNA mismatches. Finally, a 5′-terminal U-U mismatch seems to have no significant influence on overall thermodynamic stability of an RNA duplex. Irrespective of the differences in thermodynamic effects observed for UNA-A and -U terminal mismatches, they are more favourable for the structure when situated at the 5′-end than the 3′-end. Moreover, terminal UNA mismatches destabilize RNA duplexes less than internal ones. In general, shifting internal mismatches towards duplex ends progressively increases the stability (48,51,54). It is presumably due to the enhanced flexibility of a terminal base pair with an ability to adopt an energetically more favourable orientation. Our studies are thus in accordance with previously published reports.

UNA reduces discrimination also when situated adjacent to RNA mismatches (Table 3). Our studies revealed that the unfavourable effect of internal RNA mismatches adjacent to UNA-A at the 5′-side is lowered by 2.12 (C-A), 2.31 (C-C) and 1.90 (C-U) kcal/mol relative to the values of mismatch discrimination within unmodified RNA duplexes. Similarly, also internal RNA mismatches positioned at the 3′-side of UNA-A show reduction of mismatch discrimination mounting to 2.42 (G-A), 1.45 (G-G) and 0.66 (G-U) kcal/mol, again relative to the corresponding mismatches of the reference RNA duplex. In addition, when shifting RNA mismatches close to a duplex end while retaining their 5′- or 3′-UNA-A neighbourhood, lower destabilization effects are observed.

Analysis of entropy and enthalpy values of all UNA-modified RNA mismatched duplexes implies compensation between unfavourable enthalpy and favourable entropy changes resulting in decrease of duplex thermodynamic stabilities. However, some UNA–RNA/RNA duplexes, mainly containing terminal mismatches, were found to show unfavourable entropy changes dominating over favourable decreases in enthalpy value.

The thermodynamic analysis of UNA dangling ends revealed less favourable stacking interaction in reference to RNA nucleotide residues (Table 4, (46); and Supplementary Data). UNA-C, -G and -U 5′-unpaired nucleotides cause minor destabilization of RNA duplexes, whereas UNA-A 5′-dangling end increases thermodynamic stability (Table 4). The destabilization effect is weaker (or given stabilization effect is stronger) for UNA 3′-dangling ends. Furthermore, more favourable effects caused by unpaired nucleotides both at the 5′- and the 3′-end are observed for UNA-A and -G, presumably caused by the more favourable stacking due to the larger planar nucleobase surface area in comparison with UNA-C and -U as discussed previously. Our results are thus consistent with earlier thermodynamic analysis of RNA dangling ends reporting enhanced stabilization effects of 3′-unpaired nucleotides compared to 5′-unpaired nucleotides and of purines compared to pyrimidines (46,55–57). The analysis of entropy and enthalpy changes for UNA dangling ends revealed compensation of a favourable entropy contribution by an unfavourable enthalpy change for all UNA-C, -G and -U dangling ends leading to decreases of thermodynamic stability. Conversely, a decrease of enthalpy overcomes unfavourable entropy terms resulting in stabilization of duplexes containing unpaired UNA-A nucleotides.

CD spectra of model UNA–RNA/RNA duplexes showed bands characteristic for A-form duplex structure (Figure 2a). For all UNA-modified duplexes, a negative band at 210 nm and a positive band ~260 nm were observed. A similar band pattern was observed for the corresponding unmodified RNA duplex, and no significant changes were observed between the bands of all the UNA–RNA/RNA single strands (Figure 2b). Therefore, incorporation of one or few UNA nucleotides into an RNA duplex do not alter the overall RNA geometry, and UNA nucleotides thus can be considered as RNA nucleotide mimics as reported previously (19).

The comprehensive thermodynamic analysis presented herein is the first made for UNA-modified RNA duplexes. The thermodynamic parameters reported in this article provide general rules which are useful for an approximate prediction of the thermodynamic stability of UNA-modified RNA duplexes. In summary, (i) UNA nucleotide residues cause destabilization of RNA duplexes by 4.0–6.6 kcal/mol (internal positions) or 0.5–1.5 kcal/mol (terminal positions), (ii) UNA pyrimidine nucleotides show greater stability decreasing effects than UNA purine nucleotides, (iii) thermodynamic effects of single UNA substitutions are additive, (iv) the destabilizing properties of UNA nucleotides are slightly dependent on the nature of the flanking bases, (v) UNA nucleotides significantly reduce mismatch discrimination (direct UNA mismatches and those adjacent to the UNA modification), (vi) UNA nucleotides interact according to the Watson–Crick base-pairing rules, (vii) stacking interactions of UNA nucleotides are less favourable than of RNA nucleotides, and (viii) single or few UNA nucleotides do not perturb the overall A-form structure of RNA duplexes. This study underlines the importance of ribose ring integrity to overall stability of RNA duplexes. It can be anticipated that destabilization by UNA residues as verified herein plays a key role for the recently reported unique effects of UNA residues when positioned in the antisense strand of siRNA constructs, namely high-efficient gene silencing combined with significant reduction of miRNA-type off target effects (25). UNA is thus an attractive tool for gene silencing applications and the knowledge about thermodynamic effects presented herein can be applied to siRNA design.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support from The Danish National Research Foundation.

FUNDING

Funding for open access charge: The Danish National Research Foundation.

Conflict of interest statement. None declared.

REFERENCES

1. Micklefield,J. (2001) Backbone modification of nucleic acids: synthesis, structure and therapeutic applications. *Curr. Med. Chem.*, **8**, 1157–1179.
2. Doktycz,M.J. (1997) Nucleic acids: thermal stability and denaturation. *Encyclopedia of Life Science*. John Wiley & Sons, Chichester, pp. 3123–3140.
3. Turner,D.H., Sugimoto,N., Kierzek,R. and Dreiker,S.D. (1987) Free energy increments for hydrogen-bonds in nucleic acid base pairs. *J. Am. Chem. Soc.*, **109**, 3783–3785.
4. Kool,E.T. (2001) Hydrogen bonding, base stacking, and steric effects in DNA replication. *Annu. Rev. Biophys. Biomol. Stuct.*, **30**, 1–22.
5. Freier,S.M. and Altman,K.-H. (1997) The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA: RNA duplexes. *Nucleic Acids Res.*, **25**, 4429–4443.
6. Ørum,H. and Wengel,J. (2001) Locked nucleic acids: a promising molecular family for gene-function analysis and antisense drug development. *Curr. Opin. Mol. Ther.*, **3**, 239–243.
7. Wengel,J. (2004) Nucleic acid nanotechnology-towards Ångstrom-scale engineering. *Org. Biomol. Chem.*, **2**, 277–280.
8. Kurreck,J. (2003) Antisense technologies. Improvement through novel chemical modifications. *Eur. J. Biochem.*, **270**, 1628–1644.
9. Masayuki,E. and Hiroshi,S. (2009) Chemical approaches to DNA nanotechnology. *ChemBiochem.*, **10**, 2420–2443.
10. Prakash,T.P. and Bhat,B. (2007) 2′-Modified oligonucleotides for antisense therapeutics. *Curr. Top. Med. Chem.*, **7**, 641–649.
11. Rayburn,E.R. and Zhang,R. (2008) Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible? *Drug Discov. Today*, **13**, 513–521.
12. Takahashi,M., Minakawa,N. and Matsuda,A. (2009) Synthesis and characterization of 2′-modified-4′-thioRNA: a comprehensive comparison of nuclease stability. *Nucleic Acids Res.*, **37**, 1353–1362.
13. Agrawal,S. and Iyer,R.P. (1995) Modified oligonucleotides as therapeutic and diagnostic agents. *Curr. Opin. Biotechnol.*, **6**, 12–19.
14. Vandendriessche,F., Augustyns,K., Van Aerschot,A., Busson,R., Hoogmartens,J. and Herdewijn,P. (1993) Acyclic oligonucleotides: possibilities and limitations. *Tetrahedron*, **49**, 7223–7238.
15. Petersen,A.B., Petersen,M.Å., Henriksen,U., Hammerum,S. and Dahl,O. (2003) Acyclic, achiral enamide nucleoside analogues. The importance of the C=C bond in the analogue for its ability to mimic natural nucleosides. *Org. Biomol. Chem.*, **1**, 3293–3296.
16. Benhida,R., Devys,M., Fourrey,J.-L., Lecubin,F. and Sun,J.-S. (1998) Incorporation of serinol derived acyclic nucleoside analogues into oligonucleotides: influence on duplex and triplex formation. *Tetrahedron Lett.*, **39**, 6167–6170.
17. Nielsen,P., Dreiøe,L.H. and Wengel,J. (1995) Synthesis and evaluation of oligodeoxynucleotides containing acyclic nucleosides: introduction of three novel analogues and a summary. *Bioorg. Med. Chem. Lett.*, **3**, 19–28.
18. Augustyns,K., Van Aerschot,A., Van Schepdael,A., Urbanke,C. and Herdewijn,P. (1991) Influence of the incorporation of (S)-9-(3,4-dihydroxybutyl) adenine on the enzymatic stability and base-pairing properties of oligodeoxynucleotides. *Nucleic Acids Res.*, **19**, 2587–2593.
19. Langkjær,N., Pasternak,A. and Wengel,J. (2009) UNA (unlocked nucleic acid): a flexible RNA mimic that allows engineering of nucleic acid duplex stability. *Bioorg. Med. Chem.*, **17**, 5420–5425.
20. Jensen,T.B., Langkjær,N. and Wengel,J. (2008) Unlocked nucleic acid (UNA) and UNA derivatives: thermal denaturation studies. *Nucleic Acids Symp. Ser.*, **52**, 133–134.
21. Mangos,M.M., Min,K.-L., Viazovkina,E., Galarneau,A., Elzagheid,M.I., Parniak,M.A. and Damha,M.J. (2002) Efficient RNase H-directed cleavage of RNA promoted by antisense DNA or 2′F-ANA constructs containing acyclic nucleotide inserts. *J. Am. Chem. Soc.*, **125**, 654–661.
22. Fluiter,K., Mook,O.R.F., Vreijling,J., Langkjær,N., Højland,T., Wengel,J. and Baas,F. (2009) Filling the gap in LNA antisense oligo gapmers: the effects of unlocked nucleic acid (UNA) and 4′-C-hydroxymethyl-DNA modifications on RNase H recruitment and efficacy of an LNA gapmer. *Mol. Biosyst.*, **5**, 838–843.
23. Bramsen,J.B., Laursen,M.B., Nielsen,A.F., Hansen,T.B., Bus,C., Langkjær,N., Babu,B.R., Højland,T., Abramov,M., Van Aerschot,A. *et al.* (2009) A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res.*, **37**, 2867–2881.
24. Kenski,D.M., Cooper,A.J., Li,J.J., Willingham,A.T., Haringsma,H.J., Young,T.A., Kuklin,N.A., Jones,J.J., Cancilla,M.T., McMasters,D.R. *et al.* (2010) Analysis of acyclic nucleoside modifications in siRNAs finds sensitivity at position 1 that is restored by 5′-terminal phosphorylation both in vitro and in vivo. *Nucleic Acids Res.*, **38**, 660–671.
25. Bramsen,J.B., Pakula,M.M., Hansen,T.B., Bus,C., Langkjær,N., Odadzic,D., Smicius,R., Wengel,S.L., Chattopadhyaya,J., Engels,J.W. *et al.* (2010) A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects. *Nucleic Acids Res.* [Epub ahead of print].
26. Borer,P.N. (1975) Optical properties of nucleic acids, absorption and circular dichroism spectra. In Fasman,G.D. (ed.), *CRC Handbook of Biochemistry and Molecular Biology: Nucleic Acids*, Vol. 1, 3rd edn. CRC Press, Cleveland, OH, pp. 589–595.
27. Richards,E.G. (1975) Use of tables in calculations of absorption, optical rotatory dispersion and circular dichroism of polyribonucleotides. In Fasman,G.D. (ed.), *CRC Handbook of Biochemistry and Molecular Biology: Nucleic Acids*, Vol. 1, 3rd edn. CRC Press, Cleveland, OH, pp. 596–603.
28. McDowell,J.A. and Turner,D.H. (1996) Investigation of the structural basis for thermodynamic stabilities of tandem GU mismatches: solution structure of $(rGAGGUCUC)_2$ by two-dimensional NMR and simulated annealing. *Biochemistry*, **35**, 14077–14089.
29. SantaLucia,J.J. (2000) The use of spectroscopic techniques in the study of DNA stability. In Gore,M.G. (ed.), *Spectrophotometry and spectrofluorimetry a practical approach*, Vol. 13. Oxford University Press Inc., New York, pp. 329–359.
30. Rodger,A. and Nordén,B. (1997) Circular Dichroism of Biomolecules. Oxford University Press Inc., New York.
31. SantaLucia,J. (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl Acad. Sci. USA*, **95**, 1460–1465.
32. Borer,P.N., Dengler,B., Tinoco,I. and Uhlenbeck,O.C. (1974) Stability of ribonucleic-acid double-stranded helices. *J. Mol. Biol.*, **86**, 843–853.
33. Gray,D.M. (1997) Derivation of nearest-neighbor properties from data on nucleic acid oligomers. I. Simple sets of independent sequences and the influence of absent nearest neighbors. *Biopolymers*, **42**, 783–793.
34. Gray,D.M. (1997) Derivation of nearest-neighbor properties from data on nucleic acid oligomers. II. Thermodynamic parameters of DNA·RNA hybrids and DNA duplexes. *Biopolymers*, **42**, 795–810.
35. Xia,T.B., SantaLucia,J., Burkard,M.E., Kierzek,R., Schroeder,S.J., Jiao,X.Q., Cox,C. and Turner,D.H. (1998) Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs. *Biochemistry*, **37**, 14719–14735.
36. Kierzek,E., Mathews,D.H., Ciesielska,A., Turner,D.H. and Kierzek,R. (2006) Nearest neighbor parameters for Watson-Crick complementary heteroduplexes formed between 2′-O-methyl RNA and RNA oligonucleotides. *Nucleic Acids Res.*, **34**, 3609–3614.
37. SantaLucia,J.J., Allawi,H.T. and Seneviratne,P.A. (1996) Improved nearest-neighbor parameters for predicting DNA duplex stability. *Biochemistry*, **35**, 3555–3562.

38. Sugimoto,N., Honda,K.-i. and Sasaki,M. (1994) Application of the thermodynamic parameters of DNA stability prediction to double-helix formation of deoxyribooligonucleotides. *Nucleosides Nucleotides*, **13**, 1311–1317.
39. Sugimoto,N., Nakano,S., Yoneyama,M. and Honda,K. (1996) Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes. *Nucleic Acids Res.*, **24**, 4501–4505.
40. Sugimoto,N., Nakano,S., Katoh,M., Matsumura,A., Nakamuta,H., Ohmici,T., Yoneyama,M. and Sasaki,M. (1995) Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes. *Biochemistry*, **34**, 11211–11216.
41. McTigue,P.M., Peterson,R.J. and Kahn,J.D. (2004) Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation. *Biochemistry*, **43**, 5388–5405.
42. Pasternak,A., Kierzek,E., Pasternak,K., Turner,D.H. and Kierzek,R. (2007) A chemical synthesis of LNA-2,6-diaminopurine riboside, and the influence of 2′-O-methyl-2,6-diaminopurine and LNA-2,6-diaminopurine ribosides on the thermodynamic properties of 2′-O-methyl RNA/RNA heteroduplexes. *Nucleic Acids Res.*, **35**, 4055–4063.
43. Owczarzy,R., Vallone,P.M., Gallo,F.J., Paner,T.M., Lane,M.J. and Benight,A.S. (1997) Predicting sequence-dependent melting stability of short duplex DNA oligomers. *Biopolymers*, **44**, 217–239.
44. Werk,D., Wengel,J., Wengel,S.L., Grunert,H.-P., Zeichhardt,H. and Kurreck,J. (2010) Application of small interfering RNAs modified by unlocked nucleic acid (UNA) to inhibit the heart-pathogenic coxsackievirus B3. *FEBS Lett.*, **584**, 591–598.
45. Guckian,K.M., Schweitzer,B.A., Ren,R.X.-F., Sheils,C.J., Tahmassebi,D.C. and Kool,E.T. (2000) Factors contributing to aromatic stacking in water: evaluation in the context of DNA. *J. Am. Chem. Soc.*, **122**, 2213–2222.
46. Turner,D.H., Sugimoto,N. and Freier,S.M. (1988) RNA structure prediction. *Annu. Rev. Biophys. Biophys. Chem.*, **17**, 167–192.
47. Chen,G., Kierzek,R., Yildirim,I., Krugh,T.R., Turner,D.H. and Kennedy,S.D. (2007) Stacking effects on local structure in RNA: changes in the structure of tandem GA pairs when flanking GC pairs are replaced by isoG-isoC pairs. *J. Phys. Chem. B*, **111**, 6718–6727.
48. Ziomek,K., Kierzek,E., Biala,E. and Kierzek,R. (2002) The thermal stability of RNA duplexes containing modified base pairs placed at internal and terminal positions of the oligoribonucleotides. *Biophys. Chem.*, **97**, 233–241.
49. Kierzek,E. and Kierzek,R. (2003) The thermodynamic stability of RNA duplexes and hairpins containing N^6-alkyladenosines and 2-methylthio-N^6-alkyladenosines. *Nucleic Acids Res.*, **31**, 4472–4480.
50. Davis,A.R. and Znosko,B.M. (2007) Thermodynamic characterization of single mismatches found in naturally occurring RNA. *Biochemistry*, **46**, 13425–13436.
51. Kierzek,R., Burkard,M.E. and Turner,D.H. (1999) Thermodynamics of single mismatches in RNA duplexes. *Biochemistry*, **38**, 14214–14223.
52. Kierzek,E., Ciesielska,A., Pasternak,K., Mathews,D.H., Turner,D.H. and Kierzek,R. (2005) The influence of locked nucleic acid residues on the thermodynamic properties of 2′-O-methyl RNA/RNA heteroduplexes. *Nucleic Acids Res.*, **33**, 5082–5093.
53. Vanhommerig,S.A.M., van Genderen,M.H.P. and Buck,H.M. (1991) A stable antiparallel cytosine-thymine base pair occurring only at the end of a duplex. *Biopolymers*, **31**, 1087–1094.
54. Schroeder,S.J. and Turner,D.H. (2000) Factors affecting the thermodynamic stability of small asymmetric internal loops in RNA. *Biochemistry*, **39**, 9257–9274.
55. Liu,J.D., Zhao,L. and Xia,T. (2008) The dynamic structural basis of differential enhancement of conformational stability by 5′- and 3′-dangling ends in RNA. *Biochemistry*, **47**, 5962–5975.
56. Clanton-Arrowood,K., McGurk,J. and Schroeder,S.J. (2008) 3′-Terminal nucleotides determine thermodynamic stabilities of mismatches at the ends of RNA helices. *Biochemistry*, **47**, 13418–13427.
57. Kierzek,E., Pasternak,A., Pasternak,K., Gdaniec,Z., Yildirim,I., Turner,D.H. and Kierzek,R. (2009) Contributions of stacking, preorganization, and hydrogen bonding to the thermodynamic stability of duplexes between RNA and 2′-O-methyl RNA with locked nucleic acids. *Biochemistry*, **48**, 4377–4387.