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Influence of N6-isopentenyladenosine (i^{6}A) on thermal stability of RNA duplexes

Elżbieta Kierzek, Ryszard Kierzek*

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12 / 14, 60-704 Poznań, Poland

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Abstract

The thermodynamic stability of self-complementary oligoribonucleotides containing N6-isopentenyladenosine (i^{6}A) or N6-isopentanyladenosine (p^{6}A) was determined. The base pairs i^{6}A·U and p^{6}A·U were placed in either an internal (separated and tandem) and a terminal position within the duplex, or unpaired i^{6}A and p^{6}A as a 3′-dangling ends. The thermal unfolding of the oligomers was determined by means of UV melting profiles and the thermodynamic parameters: enthalpy ($\Delta H°$), entropy ($\Delta S°$) and free energy ($\Delta G°_{37}$) as well as the melting temperature (T_m) were calculated. Both modified nucleosides destabilized the duplexes, however, the effect depended on the position of the modified adenosine within the duplex. The similarity of the behavior of oligomers containing i^{6}A and p^{6}A suggests a negligible effect of the double bond on the thermal stability. The largest destabilization was observed when derivatives of adenosine were placed in an internal position. The effect of 3′-dangling ends suggests that the presence of the N6-isopentenyl- or N6-isopentanyl substitutent affects hydrogen bonding rather than stacking within duplex.

Keywords: Modified adenosine; Modified oligoribonucleotides; Thermodynamics of RNA duplexes

1. Introduction

Modified nucleotides are present in all ribonucleic acids (RNA), however, most of the modified nucleotides have been found in transfer RNA (tRNA). Among 96 known modifications 81 are present in tRNA and up to 25% nucleotides in tRNA can appear modified [1].

The function of the modifications in tRNA is not well understood. However, it is known that modified nucleotides stabilize the tRNA architecture as well as affect its activity during aminoacylation and translation and interaction with initiation and elongation factors [2]. The anticodon hairpin of tRNA bears a large variety of modified nucleotides, especially at positions 34 and 37. It was postulated that modified purine nucleotides at position 37, such as N6-isopentenyladenosine (i^{6}A), 2-methylthio-N6-isopentenyladenosine (ms^{2}i^{6}A), N6-threonylcarbamoyladenosine (t^{6}A)

* Corresponding author: Tel.: +48-61-852-85-03 ex. 143; fax: +48-61-852-05-32.

E-mail address: rkierzek@ibch.poznan.pl (R. Kierzek).

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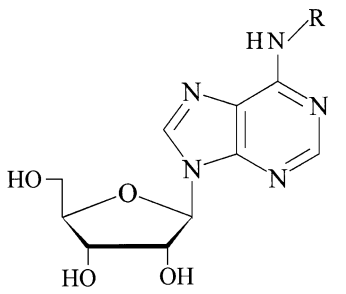
or wybutosine enhance stacking and stabilize the codon–anticodon interaction.

The number of thermodynamic data about the influence of natural modifications on the stability of RNA duplexes is very limited. Agris et al. found that the substitution of guanosine by 1-methylguanosine (m^1G) in position 37 of a yeast tRNA model hairpin lowered free energy (ΔG°_{37}) by 1.5 kcal/mol [3]. The methylation of 2′-hydroxyl groups of cytidine (Cm) and guanosine (Gm) only slightly changed the stability of the hairpin. Pseudouridine (Ψ) and 5-methylcytidine (m^5C) increased the thermal stability of the hairpin. The presence of a methyl group in various ways altered the structure and thermal stability of the hairpin: 1-methylpseudouridine ($m^1\Psi$) increased the thermal stability while 3-methylpseudouridine ($m^3\Psi$) 5,6-dimethyluridine (m^5m^6U) and destabilized it. The authors assume that Cm and Gm may have a biological function while m^1G, Ψ and m^5C appear to have structural functions which may have no direct implication on the biological function of tRNA.

The same authors observed that Ψ at position 39 of tRNA did increase the thermal stability of the hairpin improving stacking, however, did not change the binding of the tRNA to ribosomes [4]. These observations are consistent with the influence of Ψ on the thermal stability of the tetramer AAΨA [5].

The influence of N2-methylguanosine (m^2G) on the thermal stability of RNA duplexes was studied by Strobel et al. [6]. The m^2G substitution was isoenergetic with G in all cases, except for an internal m^2G–U pair. They also noticed that N6,N6-dimethyladenosine (m^6_2A) destabilized a GNRA tetraloop presumably because it prevented the formation of a sheared G–A pair within the loop while the m^2G substitution had no effect on tetraloop stability.

In addition to the influence of natural modifications on the stability of RNA, many papers have been published where effects of artificial modifications in various positions of RNA and DNA were discussed. Modified nucleotides also have an important bearing on the antisense strategy and are used to control biological functions of RNA and DNA [7].



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R = a) $-CH_2CH{=}C(CH_3)_2$
b) $-CH_2CH_2CH(CH_3)_2$

Fig. 1. The structures of N6-isopentenyl- (i^6A) and N6-isopentanyladenosine (p^6A).

The present paper reports the first thermodynamic data concerning the thermal stability of RNA duplexes containing N6-isopentenyladenosine (1a) and N6-isopentanyladenosine (1b) (Fig. 1). Compound 1a occurs at position 37 of the tRNA anticodon loop. Compound 1b was designed to learn more about the role of the double-bond in N6-isopentenyladenosine. The modifications were placed in duplexes as internal (separated and tandem) and terminal base pairs as well as 3′-dangling ends (Fig. 2).

2. Experimental

2.1. Chemical synthesis of the N6-isopentenyladenosine or N6-isopentanyladenosine phosphoramidites

N6-Isopentenyladenosine was prepared accord-

a) U**A**CAUGUA
AUGUAC**A**U
internal separated base pair

b) UAC**A**UGUA
AUGU**A**CAU
internal tandem base pair

c) UACAUGU**A**
AUGUACAU
terminal base pair

d) ACAUGU**A**
AUGUACA
3`-dangling ends

Fig. 2. The positions of N6-isopentenyl- (i^6A) or N6-isopentanyladenosine (p^6A) marked as **A** within RNA duplexes.

ing to a published procedure from adenosine using 3-methyl-2-butenyl bromide [8]. Next, N6-isopentenyladenosine was treated with dimethoxytrityl chloride followed by tert-butyldimethylsilyl chloride to protect the 5′- and 2′-hydroxyl groups, respectively. 5′-*O*-Dimethoxytrityl-2′-*O*-tert-butyldimethylsilyl-N6-isopentenyladenosine was converted into its 3′-*O*-phosphoramidite by means of a reaction with 2-cyanoethyltetraisopropylphosphorodiamidite in the presence of tetrazole. N6-Isopentanyladenosine was synthesized from peracetylated inosine, which was converted to the 6-chloro derivative [9]. In a next step, 6-chloropurine riboside reacted with isoamylamine result in the formation of N6-isopentanyladenosine [10]. Protection of the 5′- and 2′-hydroxyl groups and conversion into the 3′-*O*-phosphoramidite proceeded as for N6-isopentenyladenosine.

2.2. Synthesis and purification of oligoribonucleotides

RNA oligoribonucleotides were obtained on an Applied Biosystems 392 DNA/RNA synthesizer from commercially available 2′-*O*-TBDMS RNA phosphoramidites and modified adenosine phosphoramidites [11–13]. Upon completion of the synthesis, the oligoribonucleotides were removed from solid support and deprotected by treatment with concentrated aqueous ammonia in ethanol (3:1 v/v) at 55°C overnight. The 2′-*O*-silyl protection was removed by treatment with freshly made 1.0 M triethylammonium fluoride in pyridine at 55°C for 48 h [14]. The crude samples were dried and desalted on Sep-pak C-18 cartridges (Waters). The mixture was purified on a Si500F thin-layer chromatography plate (Baker) developed with 1-propanol/ammonia/water (55:35:10 v/v/v). The least mobile product band was visualized with ultraviolet light, cut out and eluted with water. The samples were desalted again with a Sep-pak C-18 cartridge. The purities of oligoribonucleotides were analyzed by C-8 high performance liquid chromatography (HPLC) and were greater than 95%.

2.3. Ultraviolet melting experiments

The oligoribonucleotide duplexes were melted in 1.0 M sodium chloride, 20 mM sodium cacodylate and 0.5 mM Na_2 EDTA, pH 7.0. The strand concentrations were calculated from the absorbance at 260 nm and 80°C using single-strand extinction coefficients approximated by a nearest-neighbor model [15]. Absorbance vs. temperature melting curves were measured at 260 nm with a heating rate of 1°C/min from 0 to 90°C on a Gilford 250 spectrometer controlled by a Gilford 2527 thermoprogrammer. The results were analyzed with the Meltwin computer program. The UV melting experiments were done in collaboration with Douglas H. Turner, University of Rochester (USA).

3. Results and discussion

In this paper we present for first time the data concerning the influence of N6-isopentenyladenosine (1a) or N6-isopentanyladenosine (1b) on the thermal stability of RNA duplexes (Fig. 1). The first modification is very common in position 37 of tRNA anticodon loops [1]. N6-Isopentanyladenosine was used to search for an effect of the double-bond within the 6N-substituent on the thermal stability of RNA duplexes. The base pair $A^{Mod} \cdot U$ was placed in an internal (tandem as well as separated) position, in a terminal position as well as a 3′-dangling end of the duplex (Fig. 2). That should give some information about the influence of the modifications on hydrogen bonding and stacking interactions within a duplex. The presented data should also help understand the function of the modification within an anticodon hairpin of a tRNA.

Both modified derivatives of adenosine were synthesized chemically according to published procedures and presented in the Section 2 [8–13]. The chemical synthesis of oligoribonucleotides was performed according to a standard RNA protocol [14]. The presence of modified adenosines does not affect the yield neither the synthesis nor

the deprotection and purification of oligoribonucleotides.

To study the thermal stability of oligoribonucleotides, UACAUGUA, was chosen as model because it is self-complementary and at the same time allows to place $A^{Mod} \cdot U$ at various positions within the duplex. The same core sequence of the model oligomer helps to compare the results and eliminates effects correlated with the influence of adjacent base pairs.

The comparison of thermodynamic parameters calculated from fitting the melting curves and from the correlation of the concentration of the oligomers vs. $1/T_m$ demonstrates two-state transitions of the oligomers during the melting (Table 1).

The largest destabilization effect was observed when $A^{Mod} \cdot U$ were in tandem positions within the duplex. In $(UAC\underline{i^6AU}GUA)_2$ and $(UAC\underline{p^6AU}GUA)_2$ the free energy changed by 1.72 and 1.90 kcal/mol per i^6A and p^6A, respectively (the underlined fragment of the oligomer indicates the position of $A^{Mod} \cdot U$). This range of destabilization is equivalent to the loss of 1 or even 2 hydrogen bonds within the duplex [16]. The destabilization was reduced by half when $A^{Mod} \cdot U$ was moved towards the end of the duplex. In $(U\underline{i^6ACAUGU}A)_2$ and $(U\underline{p^6ACAUGU}A)_2$ the free energy changed by 1.03 and 1.01 kcal/mol per i^6A and p^6A, respectively. Previously it has been observed that moving a single mismatch or internal loop or a $A \cdot U^{5R}$ base pair (R = Cl, Et) within a duplex closer to the end of the oligomer reduced the destabilization by 0.8 to 1.2 kcal/mol [17–19]. With two base pairs $A^{Mod} \cdot U$ within the duplex the destabilization effect was only partially additive. For $(U\underline{i^6AC}\underline{i^6AUGU}A)_2$ and $(U\underline{p^6AC}\underline{p^6AUGU}A)_2$ the free energy changed by 1.01 and 1.24 kcal/mol per i^6A and p^6A, respectively. In those duplexes the destabilization was more similar to that observed in tandem $A^{Mod} \cdot U$ base pairs only. This means that in $(U\underline{i^6AC}\underline{i^6AUGU}A)_2$ and $(U\underline{p^6AC}\underline{p^6AUGU}A)_2$ the destabilization of tandem base pairs dominates over separated ones.

For $(\underline{UACAUGUi^6A})_2$ and $(\underline{UACAUGUp^6A})_2$ containing $A^{Mod} \cdot U$ in terminal positions the free energy changed by 0.47 and 0.42 kcal/mol per i^6A

Table 1
Thermodynamic parameters of duplex formations[a]

Sequences of RNA duplexes	From fitting melting curves				From $1/T_m$ vs. $-\mathrm{Inc}_t$				
	$\Delta H°$ kcal/mol	$\Delta S°$ eu	$\Delta G°_{37}$ kcal/mol	T_m[b] °C	$\Delta H°$ kcal/mol	$\Delta S°$ °C	$\Delta G°_{37}$ kcal/mol	T_m[b] °C	$\Delta\Delta G°_{37}$ kcal/mol
UACAUGUA	−62.4	−178.6	−7.01	43.8	−56.2	−158.7	−6.96	44.3	0.00
ACAUGUA	−54.1	−156.2	−5.69	37.1	−50.9	−145.8	−5.70	37.1	1.26
ACAUGU[c]	−45.0	−131.8	−4.12	26.6	−43.8	−128.2	−4.06	26.0	2.90
$Ui^6ACAUGUA$	−43.7	−125.4	−4.82	31.0	−40.5	−114.8	−4.90	31.2	2.06
$UACi^6AUGUA$	−47.5	−141.7	−3.56	23.8	−48.1	−143.7	−3.52	23.7	3.44
Ui^6ACi^6AUGUA	−35.1	−104.4	−2.71	12.8	−31.5	−92.2	−2.92	12.1	4.04
$ACAUGUi^6A$	−55.0	−158.7	−5.82	37.8	−52.2	−149.6	−5.85	38.1	−0.15
$UACAUGUi^6A$	−53.6	−153.3	−6.03	39.1	−52.9	−151.3	−6.02	39.0	0.94
$Up^6ACAUGUA$	−42.3	−120.7	−4.85	31.0	−38.9	−109.4	−4.94	31.3	2.02
$UACp^6AUGUA$	−48.8	−147.5	−3.05	21.2	−46.8	−140.5	−3.17	21.2	3.79
Up^6ACp^6AUGUA	−43.6	−134.0	−2.00	12.9	−42.5	−130.7	−1.99	12.3	4.97
$ACAUGUp^6A$	−59.6	−173.8	−5.65	−36.8	−44.7	−125.7	−5.73	37.3	−0.03
$UACAUGUp^6A$	−55.2	−158.0	−6.18	39.8	−53.9	−153.9	−6.13	39.7	0.83

[a] Measured in 1 M sodium chloride, 20 mM sodium cacodylate and 0.5 mM Na_2 EDTA, pH 7.0.
[b] Calculated for 1.0×10^{-4} M total oligoribonucleotide strand concentration.
[c] ACAUGUA was used as a reference.

and p^6A, respectively, lower than in the internal position. The comparison of the stabilization of RNA duplexes containing $A^{Mod} \cdot U$ base pairs at a internal (tandem and separated) and a terminal positions demonstrated some consistencies. If any structural motif, such as a single mismatch, unsymmetrical internal loop or base pair in which one of the nucleotides is modified, are placed closer to the end of a helix, the destabilization effect is lower [17–19]. The terminal base pairs are more exposed to interactions with water. The duplex in this region is less fixed and the disruption of the helical structure due to the presence of terminal base pairs $A^{Mod} \cdot U$ is lower [14].

The stacking interaction is mostly responsible for the thermodynamic effect of the 3′-dangling end [20]. An insignificant stabilization of $(ACAUGUi^6A)_2$ and $(ACAUGUp^6A)_2$ was observed. The free energy changed by -0.08 and -0.01 kcal/mol per i^6A and p^6A, respectively. The influence of the N6-alkyl substituent on hydrophobic or/and stacking interactions within the duplex could be responsible for this stabilization. The comparison of 3′-dangling end and terminal base pair effects suggests that the presence of the modification affects mostly the hydrogen bonding within $A^{Mod} \cdot U$ than stacking interactions. The electron withdrawing 6N-alkyl substituent could reduce the proton-donating and proton-accepting properties of the N1 and N6 atoms of modified adenosine. Moreover, the steric hindrance of the N6-substituent could also somewhat disturb the hydrogen bonding geometry.

4. Conclusions

The collected data demonstrate that N6-isopentenyladenosine (i^6A) and N6-isopentanyladenosine (p^6A) significantly destabilizes base pairing with uridine. The destabilization depends on the position of $A^{Mod} \cdot U$ within RNA duplex. The location of $A^{Mod} \cdot U$ closer to the end of the helix destabilized the duplex less than the same base pair placed in the center of the duplex. The presence of both modifications in the 3′-dangling end position did not affect the thermal stability of the duplex significantly. This last observation suggests that N6-alkyl affects hydrogen bonding in the base pair $A^{Mod} \cdot U$ by steric hindrance or an electron-donating character of the substituent.

The measured destabilization of both modifications was similar. However, it was reported that the propynyl group at position 5 of pyrimidines significantly stabilized the duplex [21]. We assume that, apart from the character of multiple bound, its distance to the heterocycle could be also important.

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