Intramolecular stacking association and conformation properties of a 'cap' structure, m⁷G^{5'}pppUm, and the related model compounds

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ABSTRACT

The stacking equilibrium quotient of the m⁷G^{5'}pppUm unit, which occurs as the 5'-terminal "cap" of certain eukaryotic mRNA's, was determined by temperature-dependent difference spectrophotometry as $K_{stack} = 1.82$ at 25° and pH 5. In order to evaluate the contribution of different structural modifications to the net stabilization of the cap structures of mRNA, a variety of compounds related to m⁷G^{5'}pppUm were synthesized and their stacking properties were studied by the same method and compared. The results are summarized as: (1) Introduction of a methyl group into N-7 of G residue results in an increase in base stacking. (2) Methylation at 2'-OH of U residue also stabilizes the stacked structure of G-containing dimers, but it does not influence stacking interaction in m⁷G-containing dimers. (3) The effect of different types of internucleotide linkages on the order of stacking tendencies is: N^{5'}ppN' > N^{5'}pppN' > NpN'. UV hypochromicity and CD spectral measurements of the relevant dimers were also conducted, and the hypochromicity values and CD spectra of dimers in their stacked conformation were estimated by making use of the determined K_{stack} values. The results indicate that, while 2'-O-methylation exerts very little effect on the stacked conformation of the dimers, methylation at N-7 and the nature of the internucleotide linkage strongly influence the stacked conformation, thereby forming unusual left-handed conformations in m⁷G^{5'}pppU(m), m⁷G^{5'}ppU(m), and G^{5'}ppU(m).

INTRODUCTION

It is known that the 5'-terminal "cap" structure, m⁷G^{5'}pppN¹(m)pN²(m)p..., is of widespread occurrence in most eukaryotic mRNA's and is believed to play some general role during translation stage, though it remains still undefined at molecular level [1,2]. A limited number of studies aimed at characterizing the conformation and conformational stability of the cap sequences have appeared in the literature: Hattori et al. [3] measured CD spectra and UV hypochromicities of several cap analogs, and concluded that m⁷G^{5'}pppAm and m⁷G^{5'}pppA adopted a stacked conformation. But their results are rather qualitative and contain some uncertainty because of their measurements at pH 7, where the m⁷G residue still exists in partially deprotonated form. Takahashi et al. [4] and Nakanishi et al. [5] evaluated K_{stack} values for m⁷G^{5'}pppN (N=A,G,U, and Um) and some related compounds by using the data based on fluorescence measure-

ments of these dimers, and concluded that 2'-O-methylation of the U residue in $m^7G^5'pppU$ remarkably enhanced stacking and that $m^7G^5'ppN$ ($N=A$ or U) was more stacked than its $m^7G^5'pppN$ counterpart. Kim and Sarma [6] have made rather thorough NMR studies on $m^7G^5'pppAm$ and the related compounds. They suggested that the two bases within the stacked form were confronting each other.

Our continued interest in the studies of the intramolecular stacking association of a variety of dinucleoside monophosphates [7-14] has prompted us to study the conformation and conformational stability of the cap sequences quantitatively and systematically. In this paper we have chosen $m^7G^5'pppUm$ as a prototype of cap structures and evaluated contributions from three structural features by making the following comparisons: (1) dimers containing m^7G residue versus those containing G residue; (2) dimers having Um residue versus those having U residue; (3) dimers comprising 5'-5' triphosphate linkage versus those comprising 5'-5' diphosphate and 3'-5' phosphodiester linkages. Thus 12 possible dimers were all prepared, and their stacking quotients were determined by thermal denaturation measurements. UV hypochromicities and CD spectra of these dimers were also determined to gain an insight into their stacked conformations.

MATERIALS AND METHODS

Synthesis of $m^7G^5'pppUm$ and the related dimers

Scheme I

$m^7G^5'pppUm$ and other 11 related dimers were synthesized as shown in Scheme I. Reaction of guanosine 5'-phosphoromorpholidate [15] with 2'-O-methyluridine 5'-diphosphate gave $G^5'pppUm$ and $G^5'ppUm$ (as a by-product). $GpUm$ was prepared from guanosine 2',3'-cyclic-phosphate and 2'-O-methyluridine by a modified procedure of Rowe and Smith [16] which used the reverse reaction of $RNase T_1$. Reactions of guanosine 5'-phosphoromorpholidate with uridine

Table 1. Paper electrophoretic mobilities^{a)} and Rf values in paper chromatography^{b)} of 12 dimers.

Compound	Mobility	Rf	Compound	Mobility	Rf	Compound	Mobility	Rf
m ⁷ G ^{5'} pppUm	0.92	0.46	m ⁷ G ^{5'} pppU	0.93	0.36	ppU	1.76	0.47
G ^{5'} pppUm	1.66	0.39	G ^{5'} pppU	1.70	0.28	pU	1.00	0.60
m ⁷ G ^{5'} ppUm	0.58	0.52	m ⁷ G ^{5'} ppU	0.63	0.43	U	0.00	0.76
G ^{5'} ppUm	1.32	0.46	G ^{5'} ppU	1.28	0.37	ppG	1.62	0.37
m ⁷ GpUm	0.00	0.65	m ⁷ GpU	0.03	0.53	pG	0.92	0.47
GpUm	0.59	0.60	GpU	0.60	0.50	G	-0.02	0.64

a) Values relative to that of pU at pH 5 (0.02M sodium acetate buffer).

b) Solvent; Ethanol-1M ammonium acetate buffer (pH 5) = 1:1.

5'-di- and monophosphate afforded G^{5'}pppU and G^{5'}ppU, respectively. GpU was prepared from yeast RNA by digestion with RNase IA followed by separation and purification as reported previously [17]. Methylation at N-7 of G residue of the dimers thus prepared was conducted by dimethyl sulfate in an aqueous solution buffered at pH 3-5.

Structure and homogeneity of synthesized dimers were ascertained by paper electrophoretic mobilities and Rf values in paper chromatography (shown in Table 1), and further confirmed by enzymatic degradation by either RNase T₂ (for dimers with 3'-5' phosphodiester bond) or snake venom phosphodiesterase (for dimers with 5'-5' di- and triphosphate linkages). UV spectra of these dimers (see Table 3) were also consistent with the proposed structures.

Thermal denaturation experiments

Methods and calculations followed closely those described in our previous studies [8,10], and are outlined only briefly: The dimer solutions (about 8×10^{-5} mol/l) were made up in 0.01M sodium acetate buffer (pH 5) containing 0.09M NaCl. For each dimer solution 5-15 denaturation cycles (heating and cooling) were repeated in the temperature range of 0-90°, and a set of about 140 data points were read from each cycle and were used to obtain the thermodynamic parameters. The UV absorbance (as the difference between a dimer and its constituent monomers) was measured with a Hitachi 124 spectrophotometer connected to a Watanabe Sokki MC642 recorder. The temperature was simultaneously recorded on the same recorder through a thermocouple inserted into the dimer solution. At each of n experimental points, the relationship

$$\ln K_{\text{stack}} \text{ (or } \ln \frac{\epsilon_u - \epsilon_i}{\epsilon_i - \epsilon_s}) = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$$

is valid, where K_{stack} and ϵ_i are the stacking equilibrium quotients and the observed molar absorption coefficients at the i th temperature readings, and ϵ_s and ϵ_u are those for pure stacked and unstacked species, respectively. The four unknowns are obtained by solving the set of n simultaneous equations by a matrix transposition technique with a computer library subroutine (stored at the Computer Center of the Univ. of Tokyo) based on the method of Powell et al. [18]. The computer program has been written in Fortran, for use on a HITAC M-200H computer. The printed output includes the values of ϵ_u , ϵ_s , ΔH° , ΔS° , the standard error of fit, and a tabulation of observed and calculated absorbances, with their differences.

Optical measurements

UV spectra were recorded with a Hitachi 124 spectrophotometer connected to a Hitachi 056 recorder. CD spectra were measured with a JASCO J40 spectropolarimeter using a 1-cm quartz cell at 25°.

RESULTS AND DISCUSSION

Intramolecular stacking equilibrium quotients of the dimers

The m⁷G residue in the cap structure and the relevant dimers is considered to exist predominantly in the protonated form under physiological conditions (pH 7), because pK values of the m⁷G residue in m⁷G^{5'}pppA [5] and Gpm⁷GpU [19] were reported to be 7.4 and 7.5, respectively. Therefore we conducted all our experiments at pH 5 to study the physiologically significant protonated form of those dimers. At this pH UV absorption spectra of all 12 dimers were sensitive to the temperature, and the thermal denaturation profiles were employed to extract the thermodynamic parameters for the following stacking-unstacking equilibrium: (dimer)_{unstacked} $\xrightleftharpoons{K_{\text{stack}}}$ (dimer)_{stacked}. Two-state model was assumed for the dimers having 5'-5' di- and triphosphate linkages as well as for those with 3'-5' phosphodiester linkage. This assumption was validated by the presence of clear isosbestic points for a series of UV spectra at different temperatures. Analysis of the thermal denaturation data [8,10] in the range 0-90° gave the thermodynamic parameters, ΔH° , ΔS° , and K_{stack} values at 25°, which are shown in Table 2.

Inspection of K_{stack} values in Table 2 reveals the following three conclusive remarks concerning the dependence of the extent of the intramolecular stacking association on the various structural features of the dimers.

(1) It is shown for the first time that dimers containing m⁷G residue stack more extensively than those containing unmethylated G residue. K_{stack} values for the former are about twice as large as those for the latter, re-

Table 2. Thermodynamic parameters and K_{stack} values (at 25°) for the stacking-unstacking equilibrium of 12 dimers at pH 5 and $I = 0.1^a$.

Dimer	ΔH° kJ·mol ⁻¹	ΔS° J·mol ⁻¹ ·deg ⁻¹	K_{stack} (at 25°)
m ⁷ G ^{5'} pppUm	-21.1 (2.8)	-65.7 (9.0)	1.82 (0.32)
G ^{5'} pppUm	-19.1 (4.8)	-64.1 (14.7)	1.03 (0.27)
m ⁷ G ^{5'} pppU	-17.1 (3.9)	-53.0 (14.2)	1.71 (0.24)
G ^{5'} pppU	-16.6 (1.5)	-58.6 (4.8)	0.70 (0.03)
m ⁷ G ^{5'} ppUm	-20.0 (3.1)	-62.4 (10.3)	1.78 (0.09)
G ^{5'} ppUm	-17.9 (1.5)	-58.1 (5.8)	1.27 (0.26)
m ⁷ G ^{5'} ppU	-18.8 (1.8)	-57.4 (5.5)	2.00 (0.14)
G ^{5'} ppU	-20.8 (2.2)	-68.9 (6.7)	1.08 (0.07)
m ⁷ GpUm	-24.7 (1.7)	-81.2 (5.6)	1.21 (0.05)
GpUm	-18.3 (1.5)	-64.6 (4.3)	0.70 (0.08)
m ⁷ GpU	-24.8 (2.2)	-81.9 (6.7)	1.16 (0.11)
GpU	-18.7 (1.7)	-69.1 (4.4)	0.47 (0.06)

a) The standard deviations for the experimental values are shown in parentheses.

regardless of the presence of the 2'-O-methyl group on the U residue and of the nature of the internucleotide linkage. Our previous qualitative observation [20] revealed that m⁷GpU indeed more stacked than GpU at pH 4.5. This stacking-enhancing effect of methylation on the N-7 atom of G residue is also seen with m⁷GpUm and the other four dimers containing 5'-5' di- and triphosphate linkages (Table 2).

(2) It is also shown that 2'-O-methylation of the U residue enhances stacking interaction in G-containing dimers. However, from the foregoing results it seems that the effect of 2'-O-methylation of the U residue on the N-7-methylated dimers is no longer cumulative, and the effect on the extent of stacking in m⁷G-containing dimers is almost negligible. The present findings may be relevant to the unique sequence in the 5'-terminal cap structure, m⁷G^{5'}pppN(m), in eukaryotic mRNA's where the distal G residue is invariably base-methylated while the penultimate N residue is occasionally 2'-O-methylated.

(3) The order of intramolecular association tendencies is: N^{5'}ppN' > N^{5'}pppN' > NpN'. A comparison of stacking tendency of m⁷G^{5'}pppN' with that of m⁷G^{5'}ppN' has been made for a couple of pairs, and shown that m⁷G^{5'}pppU [4] and m⁷G^{5'}pppA [5] were less stacked than m⁷G^{5'}ppU and m⁷G^{5'}ppA, respectively.

The present results have confirmed the previous observations though K_{stack} for $m^7G^5\text{pppUm}$ is found much the same as that of $m^7G^5\text{ppUm}$. A comparison of $N^5\text{pppN}'$ vs. NpN' is of interest in view of obtaining some idea as to why the 5'-terminus of eukaryotic mRNA is "capped". The present results indicate that $N^5\text{pppN}'$ is significantly more stacked than NpN' of an ordinary 3'-5' internucleotide linkage. Thus, in this property, $N^5\text{pppN}'$ falls between $N^5\text{ppN}'$ and NpN' . This would suggest that the 5'-terminal cap structure may be potentially metastable and may have an essential role in directing and controlling the delicate interactions to meet the biological functional requirements.

Contrary to the values of K_{stack} , no regular tendency has been noticed in ΔH° and ΔS° listed in Table 2. This is in part due to the fact that the observed quantities of ΔH° and ΔS° are composite in nature in view of the postulated microscopic equilibrium scheme for stacking (See Sakurai et al. [14]). Furthermore, failure of finding a kind of isoequilibrium relationship between ΔH° and ΔS° can serve as a proof that either the interaction mechanism is not constant in the series or not simple. This is also in line with the present observation of the CD spectra.

Optical properties of the dimers in their stacked state

In the preceding section effects of various structural modifications of the cap structure on the conformational stability were examined in terms of the intramolecular stacking association. In this section ultraviolet absorption and circular dichroism studies were performed to obtain information about the conformation of these dimers in their stacked state.

The dimers exhibited hypochromicity at their absorption maximum when degraded to their constituent monomers by nucleolytic enzymes. Absorption maxima and experimental hypochromicity values of 12 dimers are listed in Table 3. The hypochromicity of a dimer is a reflection of base-base stacking interaction, and its magnitude depends not only on its intrinsic stacked conformation but on the extent of its stacking. Hypochromicity of the dimer in the stacked conformation was calculated from the experimental hypochromicity and the mole fraction of the stacked conformer (Table 3). Comparison of these values reveals that 2'-O-methylation does not significantly affect the stacked conformation of the dimers as seen by similar values for the methylated and unmethylated dimer pairs. H_{stacked} values in Table 3 indicate that $m^7G^5\text{pppU(m)}$ and $m^7G^5\text{ppU(m)}$ exhibit remarkably smaller hypochromicity as compared with other dimers, suggesting that they have somewhat different stacked conformation(s) from those for the other dimers.

CD difference spectrum of a dimer ($[\theta]_{\text{dimer}} - [\theta]_{\text{monomer}}$) has been inter-

Table 3. UV spectral and hypochromicity data of 12 dimers at 25°, pH 5, and I = 0.1.

Dimer	$\lambda_{\max}$ nm	H ^{a)} %	H _{stacked} ^{b)} %	Dimer	$\lambda_{\max}$ nm	H ^{a)} %	H _{stacked} ^{b)} %
m ⁷ G ^{5'} pppUm	259.5	2.75	4.3	G ^{5'} pppUm	256	5.7	11.2
m ⁷ G ^{5'} pppU	259.5	2.7	4.3	G ^{5'} pppU	256	4.9	11.9
m ⁷ G ^{5'} ppUm	259.5	4.25	6.6	G ^{5'} ppUm	256	7.2	12.9
m ⁷ G ^{5'} ppU	259.5	4.3	6.5	G ^{5'} ppU	256	7.4	14.3
m ⁷ GpUm	259.5	8.3	15.1	GpUm	256	5.6	13.6
m ⁷ GpU	259.5	7.8	15.2	GpU	256	4.85	15.2

a) Experimental hypochromicity defined at the absorption maximum of the monomer mixtures.

b) Hypochromicity of the stacked conformer: $H_{\text{stacked}} = H/f_{\text{stacked}}$,
 $f_{\text{stacked}} = K_{\text{stack}} / (1 + K_{\text{stack}})$.

puted as originating from stacking interaction between the two bases, and depends on both the geometry of the stacked form and the extent of stacking. For a comparative purpose the CD difference spectra of the fully stacked form of 12 dimers were evaluated and shown in Fig. 1A-D. Comparison of Fig. 1A and 1C with Fig. 1B and 1D, respectively, readily shows that 2'-O-methylation of the U residue does not significantly affect the position and magnitude of the long wavelength CD bands, indicating no significant conformational change by 2'-O-methylation. The two spectra for a pair of m⁷G-containing dimers are nearly superimposable (Fig. 1A and 1B). Those for a G-containing pair are somewhat different in their magnitude (Fig. 1C and 1D): U-containing dimers (Fig. 1D) have slightly larger ellipticity than Um-containing ones (Fig. 1C) in accordance with the UV hypochromicity results.

An interesting feature of Fig. 1 is that the spectra of m⁷G^{5'}pppU(m) and m⁷G^{5'}ppU(m) are surprisingly different from that of m⁷GpU(m) (Fig. 1A and 1B). The large positive and negative bands centered at 291 and 270 nm, respectively, in the spectrum of m⁷GpU(m) are reversed in sign, slightly shifted to shorter wavelengths, and greatly reduced in magnitude in spectra of m⁷G^{5'}pppU(m) and m⁷G^{5'}ppU(m). The decreased ellipticity of m⁷G^{5'}pppU(m) and m⁷G^{5'}ppU(m) relative to m⁷GpU(m) is in parallel with the small hypochromicity values observed for these dimers. A similar situation is also encountered in G-containing dimers (Fig. 1C and 1D). In this case, however, GpU(m) and G^{5'}pppU(m) exhibit normal CD spectra similar to that of m⁷GpU(m), and only G^{5'}ppU(m) shows the reversed spectrum. Further the decrease of ellipticity in G^{5'}pppU(m) and

Fig. 1. CD difference spectra of 12 dimers in their stacked conformation as expressed in mean residue ellipticity at 25°, pH 5, and $I = 0.1$

$$\{[\theta]_{\text{stacked}} = ([\theta]_{\text{dimer}} - [\theta]_{\text{monomer}}) / \epsilon_{\text{stacked}}\}.$$

$G^5ppU(m)$ is not as marked as in the m^7G -containing dimers.

The conformation of dimers giving the reversed CD spectra ($m^7G^5pppU(m)$, $m^7G^5ppU(m)$, and $G^5ppU(m)$) can not be elucidated at present, but it is probable that these dimers have some mutual arrangements of bases greatly different from those in other dimers of an ordinary right-handed stacked conformation. One of such unique arrangements would possibly be a left-handed stacked conformation when one considers the reversal of the CD spectra, although definitive answers must await future studies. This unique conformation revealed for the cap sequence, $m^7G^5pppU(m)$, seems to be common for the most cap structures, because $m^7G^5pppA(m)$ is also reported to exhibit a CD spectrum quite similar to that of $m^7G^5pppU(m)$ [3]. We believe that the unique conformation of the cap sequences should have important implications for possible mRNA structures and function through its specific recognition by enzyme(s) at the initial stage of translation.

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