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^{1}H-NMR studies on association of mRNA cap-analogues with tryptophan-containing peptides

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Abstract

^{1}H-NMR spectroscopy was applied to a study of the mode of interaction, in aqueous medium in the pH range 5.2–8.5 and at low and high temperatures, between several mono- and dinucleotide analogues of the mRNA cap m^{7}GpppG and a selected tripeptide Trp-Leu-Glu, and a tetrapeptide Trp-Glu-Asp-Glu, the sequence of which corresponds to one of the suspected binding sites in the mRNA cap-binding protein (CBP). A program, GEOSHIFT, was developed, based on ring-current anisotropy theory, for analysis of experimentally observed changes in chemical shifts accompanying interactions between aromatic heterocyclic rings. This permitted quantitative evaluation of stacking interactions between the m^{7}G cap and the tryptophan indole ring, and the relative orientations of the planes of the two rings, spaced about 3.2 Å apart. The structures of the stacked complexes were determined. In particular, stacking between $m_3^{2,2,7}$G (which has no free amino group for hydrogen bonding) and the indole ring is weaker and quite different from that between m^{7}G and $m_2^{2,7}$G and indole. With the dinucleotide cap-analogues, only the m^{7}G component stacks with the indole ring, without disruption of intramolecular stacking. In contrast to numerous earlier reports, the calculated stacking interactions are quantitatively in accord with the values derived from fluorescence measurements. It also has been shown that the positively charged (cationic) form of m^{7}G stacks much more efficiently with the indole ring than the zwitterionic form resulting from dissociation of the guanine ring N1H ($pK_a \approx 7.3$).

Keywords: mRNA cap-analogue; Oligopeptide; NMR; Stacking interaction; Molecular recognition; Tryptophan-containing peptide

1. Introduction

Most eukaryotic mRNAs possess at their 5′-termini a so-called 'cap', m^{7}G(5′)ppp(5′)N or m^{7}GpppN, necessary for optimal protein translation [1,2]; and several cap-analogues have been shown to inhibit translation [3,4] by competing with capped mRNA for the binding site(s) of the specific translation factor eIF-4E, also known as the 24 kDa cap-binding protein CBP-I [5]. Yeast, human and mouse CBP sequences exhibit significant homology, with 8 tryptophans conserved both in number and location [5].

Titration of CBP with cap-analogues results in quenching of the fluorescence emission of Trp residues [6]. This finding, together with ^{1}H-NMR [7] and crystallographic [8] evidence for efficient stacking of m^{7}Gua with the indole ring suggested that stacking interactions may play a major role in binding of the cap to CBP [6,7]. Subsequent ^{1}H-NMR and fluorescence emission studies in $(C^2H_3)_2SO$ and ^2H_2O [9,10] on interaction of cap-analogues with small peptide mimics of the CBP sequence of potential significance in cap-CBP interactions led to a tentative model for such interactions based on stacking and hydrogen bonding [6,11].

Abbreviations: m^{7}Gua, 7-methylguanine; $m_3^{2,2,7}$Gua, N^2,N^2,7-trimethylguanine; m^{7}G, 7-methylguanosine; bn^{7}G, 7-benzylguanosine; $m_2^{2,7}$G, N^2,7-dimethylguanosine; $m_3^{2,2,7}$G, N^2,N^2,7-trimethylguanosine; m^{7}GMP, 7-methylguanosine-5′-phosphate; m^{7}GTP, 7-methylguanosine-5′-triphosphate; $m_3^{2,2,7}$GTP, N^2,N^2,7-trimethylguanosine-5′-triphosphate; m^{7}GpppG, m^{7}G(5′)ppp(5′)G; m^{7}GppppG, m^{7}G(5′)pppp(5′)G; CBP, cap-binding protein; TSP, sodium 3-trimethylsilyl-[2,2,3,3,-$^2H_4$]propionate.

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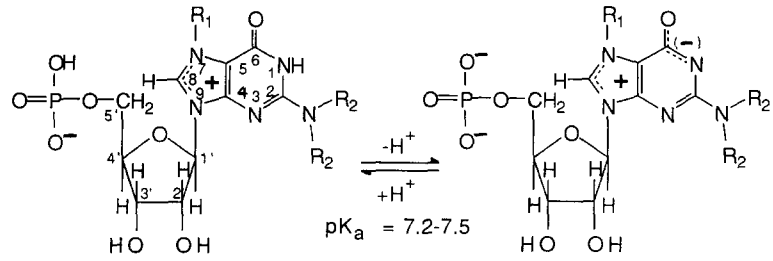

Fig. 1. Cationic and zwitterionic forms of monophosphate (and triphosphate) cap-analogues: m^7GMP and m^7GTP (R_1 = Me, R_2 = H); bn^7GTP (R_1 = benzyl, R_2 = H); $m_3^{2,2,7}GTP$ (R_1 = R_2 = Me).

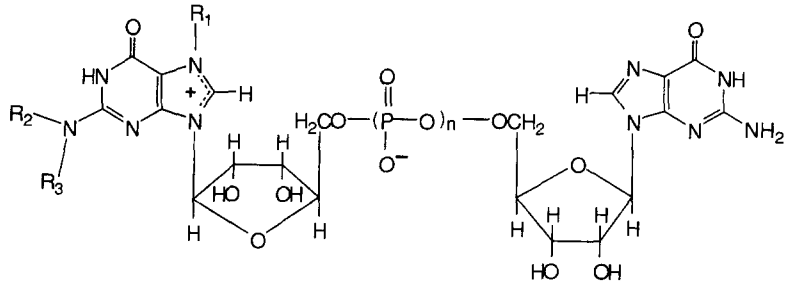

Fig. 2. Structures of cationic forms of m^7GpppG (n = 3, R_1 = Me, R_2 = R_3 = H); $m_2^{2,7}$ GpppG (n = 3, R_1 = R_2 = Me, R_3 = H); $m^7GppppG$ (n = 4, R_1 = Me, R_2 = R_3 = H).

Multidimensional NMR techniques, combined with computational methods for data analysis and molecular dynamics simulations, are now widely and successfully applied to elucidation of the structures and dynamics of large biopolymers [12–15]. For short peptides and oligonucleotides with a high degree of conformational flexibility, no effective methods are available to analyze mixtures of different conformers in rapid equilibrium [16]. However, appropriate analyses of chemical shifts have proven useful in conformational studies of small, flexible, molecules [17–19].

This communication presents a ^{1}H-NMR analysis of the interactions of several mono- (Fig. 1) and dinucleotide cap-analogues (Fig. 2) with a synthetic tripeptide and tetrapeptide (Fig. 3) in aqueous medium, i.e., under conditions closer to physiological than $(CH_3)_2SO$, and at different pH values. At physiological pH, the m^7G component of a cap or a cap-analogue is a mixture of positively charged and zwitterionic forms, due to dissociation of the guanine ring N1H ($pK_a \approx 7.2$–7.5, Fig. 1).

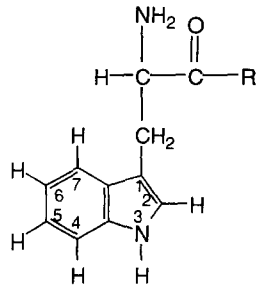

Fig. 3. Numbering system of the indole ring in the tryptophan moiety of the tripeptide Trp-Leu-Glu (R = Leu-Glu) and the tetrapeptide Trp-Glu-Asp-Glu (R = Glu-Asp-Glu).

2. Materials and methods

2.1. Chemical syntheses

(L-Trp)-(L-Leu)-(L-Glu) and (L-Trp)-(L-Glu)-(L-Asp)-(L-Glu) were synthesized by conventional procedures [20]. The final products (14% and 12% yield) were isolated by semipreparative HPLC

m^7GTP and bn^7GTP were obtained by treating GTP in $(CH_3)_2SO$ with excess methyl or benzyl iodide, respectively; m^7GMP, $m_2^{2,7}GMP$, m^7GpppG, $m_2^{2,7}GpppG$ and $m^7GppppG$ were synthesized by standard procedures as elsewhere described [21], and $m_2^{2,7}GTP$ from $m_2^{2,7}GMP$ according to Hoard and Ott [22]. All compounds were chromatographically homogeneous and their structures confirmed by NMR spectroscopy.

2.2. NMR spectroscopy

^{1}H-NMR spectra were recorded at 400 and 500 MHz on Jeol GX-400 and Jeol JNM-A-500 spectrometers respectively, in phosphate and borate buffers, pH 5.2 and 8.5 respectively, containing 10% ^2H_2O for signal locking. A hard 1331 pulse sequence was used for water suppression [23]. Spectra for interactions between cap-analogues and peptides were run at 25°C, at a concentration of 0.006 M. Temperature dependence of proton chemical shifts was

Table 1
Differences in chemical shifts $\Delta\delta$(e.s.d. ± 0.003 ppm) with increase in temperature from 1°C to 70°C, pH 5.2

		H8	N7-CH_3	H1′
(a) For the components of m^7GpppG and m^7GppppG				
m^7GpppG	m^7G	−0.080	−0.051	−0.101
	G	+0.005	–	−0.048
m^7GppppG	m^7G	−0.016	−0.063	−0.089
	G	+0.010	–	−0.054
(b) For the individual monomers m^7GMP and GMP due solely to the effect of temperature on the monomers and the TSP internal standard				
GMP		+0.077	–	+0.016
m^7GMP		+0.104	−0.013	+0.002
(c) Corrected changes in chemical shifts due to intramolecular stacking				
m^7GpppG	m^7G	−0.184	−0.038	−0.103
	G	−0.072	–	−0.064
m^7GppppG	m^7G	−0.120	−0.050	−0.091
	G	−0.067	–	−0.070

studied in the range 1°C to 70°C at two concentrations, 0.006 M and 0.012 M. Chemical shifts (±0.003 ppm) were recorded relative to internal TSP.

The unambiguous assignment of protons in cap-analogues was made as described elsewhere [21,24], and of those in the tripeptide and the tetrapeptide, especially in the tryptophan indole ring, from analysis of cross-peaks in the 2D ROESY spectrum [25] of the m7GpppG: tripeptide complex in ^2H_2O, the mixing time being 1.5 s.

2.3. Calculations of chemical shifts

Changes of proton chemical shifts, $\Delta\delta$, due to stacking interactions of two aromatic rings, were analyzed in terms of ring current anisotropy theory [26–31], according to the equations:

$$\Delta\delta = 2.13\frac{I_N}{a_N}\left[\frac{2}{(1+\rho)^2+z_-^2}\left(K_- + \frac{1-\rho^2-z_-^2}{(1+\rho)^2+z_-^2}E_-\right) + \frac{2}{(1+\rho)^2+z_+^2}\left(K_+ + \frac{1-\rho^2-z_+^2}{(1+\rho)^2+z_+^2}E_+\right)\right]$$

where z and ρ are the cylindrical coordinates of the proton, $K_\pm$ and $E_\pm$ are elliptic integrals of the first and second kind, respectively, with modulus $k_\pm = \sqrt{4\rho/\left[(1+\rho)^2+z_\pm^2\right]}$, $z_\pm = z \pm \langle z\rangle$, where $\langle z\rangle$ is the distance of the current loop with respect to a plane through the ring atoms (average distance of the $2p_z$ orbitals), I_N is the calculated ring current intensity relative to that of benzene [28], and a_N is the radius of the ring.

For this purpose an interactive program in C language, GEOSHIFT [1], to run on IBM PC computers, equipped with a VGA card, was written. The program enables:

(a) calculations of the ^{1}H chemical shift changes, $\Delta\delta$ = δ[stacked] − δ[free], for two aromatic molecules, viz. a nucleic acid base and an aromatic amino-acid side chain, situated at a distance $\vec{R} = (X, Y, Z)$ from one another, with a relative orientation of their local coordinate systems described by Euler's angles Θ, ψ and Φ (Fig. 4);

(b) determination of the spatial orientations of two aromatic molecules ($\vec{R}$, θ, ψ, Φ) which correspond to minima of the root mean-square deviation (RMSD) be-

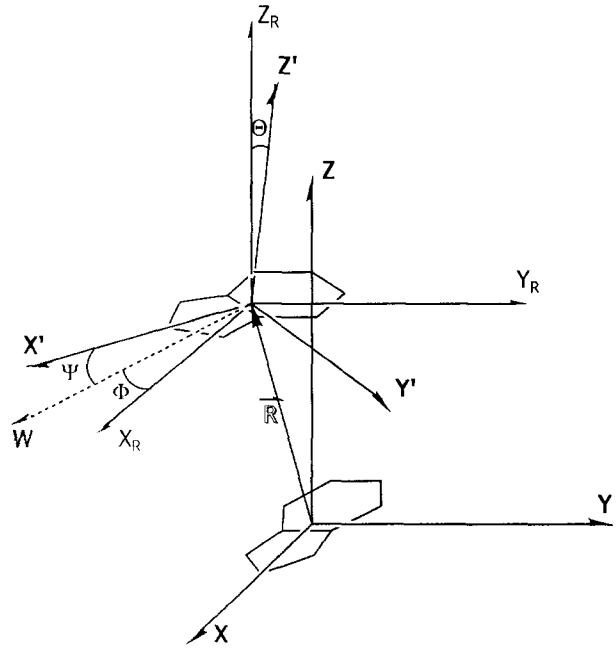

Fig. 4. Coordinate systems linking two aromatic rings, the relative orientations of which are described by their displacement parameters describing their relative orientation: displacement $\vec{R}$ and Euler's angles Θ, Ψ, Φ.

[1] This program is available on request to A. Sitek or R. Stolarski.

tween the experimental, $\Delta\delta_i^{obs}$, and theoretically calculated, $\Delta\delta_i^{cal}$, changes of the proton chemical shifts:

$$RMSD = \sqrt{\sum_{i=1}^{N} \left(\Delta\delta_i^{cal} - \Delta\delta_i^{obs}/f\right)^2/N}$$

Here N is the number of protons taken into account in the calculation and f is the mole fraction of stacked molecules; the minimization of RMSD was performed by means of the SIMPLEX method [32].

3. Results

3.1. Self-association of cap-analogues

Self-association of cap-analogues, long the subject of widespread interest, was first initiated by Sarma et al. [24] with the model m^7GpppA. The present study is devoted to analogues of the form m^7GpppG. Interpretation of the results requires some knowledge of the self-stacking of the monomeric species m^7G and G in the concentration ranges employed, described in the next paragraph.

In the pH range 5.2–8.5, millimolar concentrations of 7-substituted guanine nucleotides do not exhibit intermolecular stacking. At pH > 8, they undergo opening of the imidazole ring ([21,33], and references cited), with appearance of signals from different species. For m^7GMP, an increase in temperature, e.g., from 1°C to 70°C, was accompanied by shielding (negative shift) of H8 and H1′ (Table 1) rather than the deshielding (positive shift) expected for destacking (see e.g. [29]). Similarly a 5-fold enhancement of the concentration of such nucleotides (within the mM range) at room temperature led to only minimal positive shifts (< 0.01 ppm) of H8, N7-CH_3 and H1′. The behaviour of bn^7GTP differed from the foregoing in that $\Delta\delta_i$ was +0.150 ppm for H8 and −0.070 ppm for the benzyl ring protons, due to interactions between the guanine and benzyl rings. Self-association of the indole ring of tryptophan is also not observed at mM concentrations [7].

The strong intramolecular stacking of the bases in m^7GpppG and m^7GppppG at pH 5.2 is illustrated by the data in Table 1. With an increase in temperature from 1°C to 70°C, which leads to disruption of stacking, all the base protons (with the exception of the exocyclic NH_2, not shown) are shifted downfield. The changes are more pronounced for the protons of the m^7G moiety. These temperature-induced changes in chemical shifts must, of course, be corrected for the chances of the chemical shifts of the protons of the monomers m^7GMP and GMP, as well as of the internal standard TSP, due solely to the effects of temperature. When this is done (see Table 1) the net upfield shifts of m^7GpppG are as follows: −0.184 ppm and −0.072 ppm for H8, and −0.103 ppm and −0.064 for H1′ of m^7G and G, respectively, and −0.038 for N7-CH_3. These values are comparable to the differences in chemical shifts of H8, N7-CH_3 and H1′, $\Delta\delta = \delta$(dinucleotide) − δ(monomer component) = −0.220 ppm to −0.080 ppm, between m^7GpppG and its mononucleotide components, and to the corresponding shift differences observed

Table 2
^{1}H chemical shift differences (e.s.d. ± 0.003 ppm) due to stacking interactions between the indole ring of Trp-Leu-Glu and the m^7G ring of various cap analogues, each at 6mM in aqueous medium, pH 5.2, at 25°C, unless indicated otherwise

Cap analogue	δ(stacked) − δ(free) [ppm]											
	Tryptophan							Nucleotide				
	N3H	αNH$_2$	H2	H4	H5	H6	H7	N1H	NH$_2$ [a]	H8	N7-CH$_3$	H1′
m^7GMP	b	b	−0.025	−0.038	−0.037	−0.033	−0.034	b	−0.013	−0.076	−0.050	−0.036
m^7GMP (70°C)	b	b	−0.005	−0.009	−0.009	−0.007	−0.008	b	b	−0.009	−0.015	+0.001
m^7GMP (pH 8.0)	b	b	−0.005	−0.026	−0.021	−0.023	−0.027	b	b	+0.009	−0.015	−0.015
m^7GMP (pH 8.5) [c]	b	b	+0.011	−0.001	0.0	−0.001	−0.005	b	b	−0.001	−0.005	−0.005
m^7GTP	b	b	−0.015	−0.027	−0.025	−0.022	−0.024	b	−0.003	−0.026	−0.029	−0.021
bn^7GTP	b	b	−0.017	−0.026	−0.027	−0.024	−0.026	b	−0.002	−0.022	−0.030 [d]	−0.031
$m_3^{2,2,7}$GTP	+0.050	−0.050	−0.007	−0.013	−0.012	−0.012	−0.015	b	−0.017	−0.032	−0.030	−0.036
m^7GpppG: m^7G	b	b	−0.037	−0.060	−0.057	−0.055	−0.052	b	−0.003	−0.021	−0.035	−0.022
G								b	−0.022	−0.003	–	−0.008
m^7GpppG [e]: m^7G	b	b	−0.009	−0.020	−0.016	−0.016	−0.010	b	−0.004	−0.022	−0.027	−0.017
G								b	−0.011	−0.001	–	−0.009
$m_2^{2,7}$GpppG: $m_2^{2,7}$G	b	b	−0.034	−0.055	−0.053	−0.052	−0.047	b	−0.009	−0.018	−0.026	−0.018
G								b	−0.020	−0.007	–	−0.008
m^7GppppG: m^7G	b	b	−0.027	−0.047	−0.045	−0.042	−0.042	b	−0.014	−0.031	−0.034	−0.020
G								b	−0.022	−0.010	–	0.0

[a] Value for NH of NH_2, or CH_3 of $NHCH_3$ and $N(CH_3)_2$ groups.
[b] Signal not detected.
[c] Opening of imidazole ring.
[d] Change for CH_2 benzyl protons, for the benzyl ring protons the average change is −0.020 ppm.
[e] Values for interaction between m^7 GpppG and Trp-Glu-Asp-Glu

between $m_2^{2,7}G$ pppG and m^7GppppG and their respective monomers. The results are qualitatively consistent with strong intramolecular association, in accordance with the ca. 40% stacking reported for such dinucleotide analogues by means of fluorescence spectroscopy [34]. It should also be noted, from the data in section (c) of Table 1, that stacking in m^7GppppG is comparable to that in m^7GpppG, as also observed by fluorescence spectroscopy (Z. Wieczorek, personal communication).

3.2. Structures of the intramolecular associates

In the case of intramolecular self-stacking of m^7GpppG and m^7GppppG, the number of protons, H8 of G and H8, N7-CH_3 of m^7G, is insufficient for accurate determination of the structure of the self-associate. Taking into account the changes in chemical shifts with temperature (Table 1), and the 40% population of the self-stacked species [34], we have calculated RMSD as a function of Φ for G and m^7G at $Z = 3.1$ Å, parallel to one another. The RMSD minimum corresponds to a head-to-head orientation of the bases in m^7GpppG and m^7GppppG. The second RMSD minimum, head-to-tail, is much higher.

3.3. Interactions between cap-analogues and Trp-Leu-Glu and Trp-Glu-Asp-Glu

Addition of an equimolar amount of Trp-Leu-Glu to an aqueous solution (pH 5.2, at 25°C) of each cap-analogue led to characteristic upfield shifts of the proton resonances of both components (Table 2), due to formation of intermolecular associates stabilized by stacking between the m^7Gua and indole rings. At 70°C the upfield shifts are 4–5-times smaller due to thermal disruption of stacked complexes. For the stacking interaction between m^7GMP and Trp-Leu-Glu, the NMR data are in good agreement with the association constant of 35 M^{-1} obtained by fluorescence methods [35]. With the dinucleotide caps, the upfield shifts of H8 and H1′ of the G moieties are much smaller than those of m^7G or $m_2^{2,7}$G, and they arise from the ring current of the indole, stacked on the other side of the 7-methylguanine ring.

The changes in chemical shifts upon association show a similar pattern for all cap-analogues and tryptophan, e.g. $\Delta\delta$(H2) = −0.037 ppm to −0.015 ppm, $\Delta\delta$(H5) = −0.057 ppm to −0.025 ppm, $\Delta\delta$(H8) = −0.050 ppm to −0.018 ppm (pH 5.2, at 25°C), with the exception of $m_3^{2,2,7}$GTP, $\Delta\delta$(H2) = −0.007 ppm, $\Delta\delta$(H5) = −0.012 ppm, $\Delta\delta$(H8) = −0.032 (Table 2). Hence, it can be concluded that the structures of the associates are all similar, except for $m_3^{2,2,7}$GTP, and stabilized by stacking interaction between the 7-substituted guanine and indole rings. For the dinucleotide cap-analogues, it should be noted from Table 2 that the tryptophan residue associates with m^7Gua (and N^2,7-dimethylguanine) without disrupting the intramolecular interaction of the latter with guanine, e.g. for m^7GpppG, $\Delta\delta$(H8) = −0.021 ppm for m^7G and $\Delta\delta$(H8) = −0.003 ppm for G. Thus, three base rings stack in array, with m^7Gua located between the indole and guanine. Differences in the magnitude of upfield shifts observed with various mono- and dinucleotide analogues may be attributed to different values of their association constants (see Table 4).

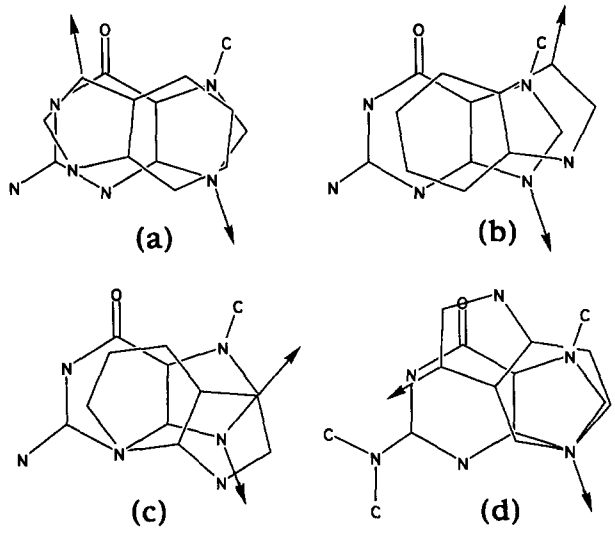

Fig. 5. Relative orientations of stacked m^7G and indole: (a) minimum 1, head-to-tail; (b) minimum 2, head-to-head; (c) minimum 3, head-to-head; and (d) $m_3^{2,2,7}$G and indole, head-to-tail.

In the $m_3^{2,2,7}$GTP-tripeptide complex the upfield shifts of the indole protons are ca. two times smaller than for the associates between the tripeptide and other mononucleotide cap-analogues. Furthermore, the structure of the $m_3^{2,2,7}$GTP: tripeptide complex differs from those of the other complexes (see Fig. 5d). Results for the latter were based on a 5% population of stacked molecules as described further in the next section and listed in Table 4.

The changes in proton chemical shifts in the equimolar mixtures of Trp-Leu-Glu and m^7GMP or m^7GpppG at pH 8.0 are much smaller than at pH 5.2 (Table 2). At pH 8.5, where slow opening of the imidazole ring occurs, the dominating signals for H8, N7-CH3, H1′ of m^7GTP and m^7GpppG are accompanied by minor signals from ring-opened species. Those listed for this pH in Table 2 are for dominating signals of the intact molecules. Stacking between indole and m^7Gua is appreciably reduced for the dissociated form of the latter, as shown previously by phase distribution measurements [36].

The interaction of m^7GpppG with the tetrapeptide is qualitatively similar to that with the tripeptide. However, the accompanying smaller changes in chemical shifts point to weaker stacking between m^7Gua and the tryptophan indole ring of the tetrapeptide (Table 2, note 'e'). This is clearly the consequence of increased repulsion between the negatively charged phosphates of the cap-analogue and the negatively charged carboxyls of the tetrapeptide, which

contains three amino acids with negatively charged side chains as compared to only one in the tripeptide.

3.4. Structures of the cap-analogue – peptide associates

The mutual orientations of the indole and 7-methylguanine rings in the tripeptide: m^7GMP and tripeptide: $m_3^{2,2,7}$GTP associates were evaluated by means of the program GEOSHIFT by minimization of the value of the RMSD, as described in Section 2. For the tripeptide: m^7GMP associate, 15% of stacked molecules was assumed, deduced from the reported value of the association constant 35 M^{-1} [35], and the concentration of 0.006 M for each component. Analogous patterns of changes of chemical shifts for the other associates at pH 5.2 point to similar structures, except for the tripeptide: $m_3^{2,2,7}$GTP complex.

Since minimization with a large number of parameters may lead to local minima with no physical significance, the following optimal procedure was applied. The Z coordinate was kept constant during the course of each minimization and minimization was repeated at 0.05 Å intervals of Z from 2.85 Å to 3.6 Å. For each individual value of Z 100 minimizations, starting from a random set of X, Y, Θ, Ψ and Φ were carried out. This approach did, in fact, prove fruitful, in that about 80% of the minimizations gave similar results, irrespective of the value of Z; three structures were obtained for the m^7GMP: indole associate, and one for the $m_3^{2,2,7}$GTP: indole associate, the planes of the rings in each structure being roughly parallel to each other (deviations from planarity up to 25°). The relative coordinates of the stacked rings, the RMSD values and the calculated chemical shift differences, for comparison with the experimental data, are presented in Table 3. The mutual orientations of the aromatic rings along the Z-axis are shown in Fig. 5. In 20% of trials some singular, local minima were found with the RMSD values about two-fold greater than those listed in Table 3. The functional relationship between RMSD and Z (Fig. 6) lends further support to the significance of stacking interactions; ; the RMSD minima were obtained with Z values ranging from 3.1 Å to 3.4 Å. Minimum 1 (Fig. 5a), with a head-to-tail orientation of m^7Gua (bn^7Gua, $m_2^{2,7}$Gua) and indole, represents the best fit between the experimental and calculated chemical shifts. The other minima, 2 and 3, with the head-to-head orientations of the rings (Fig. 5b, c), have higher RMSD values, but still within the experimental error of the chemical shifts. Hence stacking of the cap-analogues with the tripeptide appears to be highly dynamic, i.e. there is probably no single dominating structure, but rather an equilibrium mixture of several complexes with comparable energies.

The values for the distance Z, between the stacked

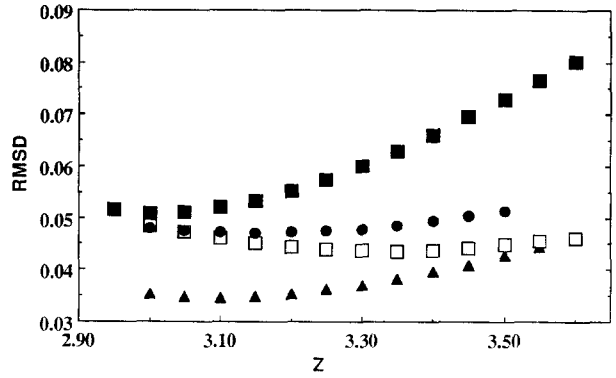

Fig. 6. Relationships between root mean-square deviation (RMSD) and Z: (▲) m^7G: indole, minimum 1; (□); minimum 2; (●) minimum 3; (■) $m_3^{2,2,7}$G: indole. Note that, for comparison with the experimental error (±0.003 ppm), the RMSD values should be multiplied by the mole fraction f of stacked molecules.

Table 3
Relative coordinates of the aromatic rings in the m^7 Gua: indole and $m_3^{2,2,7}$Gua: indole associates corresponding to the RMSD minima (Fig. 5), and (lower half of table) the resulting calculated chemical shift differences $\Delta\delta_i = \delta_i$(stacked) − δ_i(free). For comparison with the experimental data for the m^7 GMP: tripeptide and $m_3^{2,2,7}$GTP: tripeptide complexes, see Table 2

	X[Å]	Y[Å]	Z[Å]	Φ[rad]	θ[rad]	Ψ[rad]	RMSD
m^7 Gua:indole							
minimum 1	−0.16	0.15	3.10	−0.50	−3.01	2.66	0.034
minimum 2	0.64	0.20	3.35	−1.13	0.26	1.25	0.044
minimum 3	0.47	−0.28	3.15	−0.71	0.34	0.21	0.047
$m_3^{2,2,7}$Gua:indole	0.01	0.71	3.00	−0.64	0.44	3.13	0.051
	$\Delta\delta$H2	$\Delta\delta$H4	$\Delta\delta$H5	$\Delta\delta$H6 (ppm)	$\Delta\delta$H7	$\Delta\delta$H8	$\Delta\delta CH_3$
m^7Gua:indole							
minimum 1	−0.018	−0.032	−0.034	−0.029	−0.025	−0.075	−0.050
minimum 2	−0.017	−0.030	−0.040	−0.036	−0.023	−0.071	−0.048
minimum 3	−0.015	−0.035	−0.042	−0.021	−0.030	−0.070	−0.051
$m_3^{2,2,7}$Gua:indole	−0.002	−0.010	−0.011	−0.010	−0.015	−0.031	−0.030

indole and m^7Gua rings, of 3.1 to 3.4 Å are slightly below the typical value of 3.4 Å in the solid state structure of a complex of these two rings [8] and between the bases in helical structures of DNA and RNA. However, from Fig. 6 it will be noted that the dependence of RMSD on Z in this range is rather 'flat', i.e. exhibits only minimal changes, < 0.005, with changes in Z from 3.0 to 3.5 Å, so that the deviation of Z from 3.4 Å is fully within the range of experimental errors. Furthermore, as a result of the simultaneous dynamic and strong stacking of the rings, and the accompanying possible deviation of the stacked rings from planarity (by up to 25°), the slightly smaller value of 3.4 Å between the rings does not necessarily invalidate the sum of the van der Waals radii of the ring carbons.

The head-to-tail structure of the $m_3^{2,2,7}$G TP: tripeptide complex (see Table 3 and Fig. 5d) is somewhat less accurate (Fig. 6) because of its small association constant, and hence greater proportional experimental error in $\Delta\delta_i$. The changes in chemical shifts at pH 8.5 are too small to permit structure determination with reasonable accuracy.

The experimentally observed changes in the chemical shifts $\Delta\delta_i$ of the indole protons exhibit a similar pattern for all associates, i.e. the ratio of $\Delta\delta_i$ for each proton to the total change of all indole protons $\sum_i \Delta\delta_i$ is approximately constant for all associates under investigation (Table 4). The largest deviations are seen for the $m_3^{2,2,7}$GTP: tripeptide associate, the structure of which differs from the other associates (Fig. 5d). Nonetheless this enables estimation of the population of stacked molecules (association constants) from the experimentally determined 15% stacking of the tripeptide and m^7GMP, as follows:

$$\% \text{ stacked molecules} = \frac{\sum_i \Delta\delta_i}{-0.167} \cdot 15\%$$

where -0.167 is the value of $\sum_i \Delta\delta_i$ for the tripeptide: m^7GMP associate (see Table 4). Generally, the dinucleotide cap-analogues stack more efficiently than the mononucleotides.

4. Discussion

Numerous investigations on the interaction between mRNA caps and cap-analogues, as well as capped mRNA fragments, with model peptides and intact CBP, have led to a general model in which this interaction is due predominantly to strong stacking between the m^7G component of the cap and the indole ring of a Trp in the binding site of the CBP. This complex is considered to be further stabilized by hydrogen bonding between Glu and Arg side-chain carboxyls and the phosphates and NH_2 group of m^7GTP.

There are, however, marked discrepancies between results obtained by different physico-chemical techniques, e.g., the calculated association constants between m^7Gua and the tryptophan indole ring are in the range $10–10^2$ by NMR spectroscopy [7,9,10], but $\approx 10^2–10^4$ M^{-1} by fluorescence spectroscopy [9,37]. Furthermore, it has been widely overlooked that, at pH 7.5, where most measurements have been made in aqueous medium, m^7G (with $pK_a \approx 7.3$ for dissociation of the N1H), exists as an equilibrium mixture of the cationic and zwitterionic forms (see Fig. 1). In two studies [6,38] on the binding of cap-analogues with CBP, maximum association was reported at pH 7.6, leading to the conclusion that the zwitterionic form is responsible for the stacking interaction.

The present investigation, although limited to the interaction of cap-analogues with model peptides, significantly extends the results of previous findings, and also resolves some of the inconsistencies hitherto reported with both model peptides and cap-binding proteins.

(a) The use of H_2O as solvent, in place of the customary ^2H_2O, is advantageous in that it enabled the additional monitoring of chemical shifts of H8 of the m^7G component of a cap-analogue, which would otherwise exchange for ^{2}H8 in ^2H_2O. Further, the newly developed program GEOSHIFT made it possible to demonstrate *quantitatively* that observed changes in chemical shifts, accompanying mixing of a cap-analogue with a model peptide, are due to

Table 4
Comparison of the relative chemical shift changes of the indole protons due to stacking with various cap analogues at pH 5.2, and the calculated populations of the stacked associates (for details see text)

	$\Sigma_i \Delta\delta_i$ [a] (ppm)	$\Delta\delta_i/\Sigma_i \Delta\delta_i$					Calculated stacking (%)
		H2	H4	H5	H6	H7	
m^7GMP	−0.167	0.15	0.23	0.22	0.20	0.20	15 [b]
m^7GTP	−0.113	0.13	0.24	0.22	0.19	0.21	10
bn^7GTP	−0.120	0.14	0.22	0.22	0.20	0.22	11
$m_3^{2,2,7}$GTP	−0.059	0.12	0.22	0.20	0.20	0.25	5 [c]
m^7GpppG	−0.261	0.14	0.23	0.22	0.21	0.20	23
$m_2^{2,7}$GpppG	−0.241	0.14	0.23	0.22	0.22	0.20	21
m^7GppppG	−0.203	0.13	0.23	0.23	0.21	0.21	18

[a] Sum of the experimental chemical shift changes of the indole protons (i = H2, H4, H5, H6, H7) due to addition of an equimolar equivalent of a cap analogue. For $\Delta\delta_i$ see Table 2.
[b] From the fluorimetrically determined association constant, 35 M^{-1} [35].
[c] Only approximate, since this complex differs from the others in the table.

base stacking of the m^7G component with the tryptophan indole ring (without disruption of self-stacking in dinucleotide cap-analogues), and to determine the relative orientations of the stacked rings. In particular, it was established, as might have been anticipated, that the stacking reaction is dynamic in nature, with several mutual orientations of the stacked rings, both head-to-head and head-to-tail, in equilibrium with each other (see Fig. 5).

(b) The present results, derived from NMR measurements, are fully consistent with those obtained by fluorescence emission in a study [35] where association constants of stacked rings were obtained with the use of front-surface, in place of right-angle, detection, thus bypassing inner-filter effects [39]. The association constant for the complex m^7GMP: Trp-Leu-Glu obtained by this procedure was employed to accurately calculate chemical shifts with the program GEOSHIFT.

(c) Analysis of the pH-dependence of the stacked reaction demonstrated unequivocally that, contrary to previous proposals [6,38], the stacking of m^7G with indole is much stronger for the cationic, as compared to the zwitterionic, form of m^7G. Our measurements were made up to pH 8.5 , but at $pH > 8$ the zwitterionic form of m^7G undergoes opening of the imidazole ring. It is not generally appreciated that this ring-opening reaction may also proceed, albeit slowly, in the physiological pH range; this may conceivably lead to formation of a covalent complex with CBP following complex formation with the cationic form of m^7G, stabilized by stacking. Formation of a covalent complex with protein during the capping of alphavirus mRNA has been reported by Ahola and Kaariainen [40]. Covalent complex formation was noted between m^7GMP and the viral non-structural protein nsP1, presumably via a phosphoamide bond, hence quite different from the complex guanylyltransferase: GMP normally observed in the capping process of most eukaryotes and viruses. These observations must be taken into account in studying the pH-dependence of cap-binding proteins with caps.

(d) It is of some interest that the stacking properties of bn^7G with indole are identical to those of m^7G, despite the large bulk of the 7-benzyl group. It is, however, most likely that such a bulky group would exert steric hindrance to binding of a cap-analogue with intact CBP or with large fragments of the latter containing the binding site. In retrospect, it would have been very useful to compare complex formation between bn^7G and a model peptide in which the Trp is located between two amino acids; e.g., Leu-Trp-Glu.

Furthermore, whereas $m_2^{2,7}GpppG$ interacts with the tripeptide like m^7GpppG (see Table 2), it is worth noting that $m_3^{2,2,7}G$, in which there is no amino hydrogen, stacks so poorly. Since it is difficult to envisage hydrogen bonding of the amino hydrogen in aqueous medium, interpretation of this effect is not immediately obvious. It is, however, consistent with the observation of Ueda et al. [11] that the association constant with CBP of m^7IMP, which has no amino group, is smaller by an order of magnitude than that for m^7G. The poor stacking properties of $m_3^{2,2,7}G$ are perhaps related to its different biological role, which involves capping of the 5′-termini of small nuclear RNA (snRNA), of key importance in transport of snRNPs to the cell nucleus [41].

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