**Fluorescence Studies on Association of Human Translation Initiation Factor eIF4E with mRNA cap-Analogues**

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**Abstract**

Binding of a long series of mono- and dinucleotide analogues of the 7-methylguanosine containing 5'-mRNA-cap to human protein translation initiation factor eIF4E has been investigated by means of fluorescence. A new methodological approach in gathering and analysis of the fluorescence data provided us with very accurate values of the association equilibrium constant K and normalized, maximal quenching of the protein fluorescence ΔFMAX during titration of eIF4E by various cap-analogues. The results confirm participation of at least two conserved tryptophan residues of eIF4E in interaction with 7-methylguanine, as has been described recently for murine eIF4E, complexed with 7-methyl-GDP in crystal (Marcotrigiano et al., 1997, Cell 89, 951), and for yeast eIF4E, complexed with the same ligand in solution (Matsuo et al., 1997, Nature Struct. Biol. 4, 717). On the other hand binding by eIF4E of unmethylated guanine nucleotides and N2,N7,7-trimethylguanine containing nucleotides differ substantially from the way of binding of the regular mRNA-cap. Influence of the structural features of the cap-analogues, especially the type of the second nucleoside in the dinucleotide caps, on their association with eIF4E and biological activities in *in vitro* protein translation systems has been discussed in light of the known structures of the eIF4E–7-methyl-GDP complexes in crystal and solution.

**Introduction**

Most eukaryotic mRNAs possess at their 5'-termini a "cap", m7GpppN, where the first nucleoside, 7-methylguanosine (m7G), is linked by the 5'-7-methylguanosine-7-triphosphate linkage to the next nucleoside N, which may be guanosine (G), adenosine (A), uridine (U), cytosine (C) or their 2'-O-methyl derivatives, 2'-O-methylguanosine (m2G), etc. The cap structure is necessary for optimal protein translation (Sonenberg, 1988; Darzyńkiewicz et al., 1987). Several cap-analogues have been shown to inhibit translation (Darzyńkiewicz et al., 1987) by competing with capped mRNA for the binding site of the specific protein translation factor eIF4E (eukaryotic initiation factor type 4E), with eight tryptophans conserved both in number and location (Sonenberg, 1988). Stacking between 7-methylguanine and tryptophans plays a fundamental role in interaction between cap and eIF4E. Titration of eIF4E with cap-analogues results in quenching of the fluorescence emission of Trp residues (Ueda et al., 1991; Carberry et al., 1989). This finding led to a tentative model for such interactions, based on stacking and hydrogen bonding interactions between the 7-methylguanine and tryptophans.
This model has been recently confirmed by resolution of the murine N-terminal active fragment 28–217 of eIF4E (the first 27 amino acids in the protein sequence truncated), bound to 7-methyl-GDP, by means of X-ray diffraction study (Marco-trigiano et al., 1997) as well as structure determination of the yeast eIF4E–m7GDP complex in solution, by means of multidimensional NMR methods (Matsuo et al., 1997). m7GDP, presumably in the cationic form, is bound in a hydrophobic pocket of the protein. The ligand is stabilized by sandwiching of 7-methylguanine between two tryptophan indole rings (Trp-56 and Trp-102), and by three hydrogen bonds between 7-methylguanine and the carboxyl group, and the side-chain NH of glutamic acid Glu-110 and tryptophan Trp-102, respectively. Although some structural differences between the complexes have been observed, both are very similar, in the case of 31% sequence identity of both protein factors. This leads to the conclusion that eIF4Es from other sources possess similar mRNA cap-binding fold, including eIF4E from human erythrocytes, which is highly homologic to murine eIF4E.

Intramolecular stacking in mRNA also plays an important role in initiation of protein synthesis. Secondary structure at the 5’-noncoding region of mRNA, which contains cap, generally inhibits translation. Protein initiation factors, including eIF4E, eIF4G and RNA helicase eIF4A, grouped in the eIF4F complex, are involved in melting this secondary structure in the 5’-terminal mRNA non-coding region (Sonenberg, 1988).

These results prompted us to undertake detailed fluorescence study on association of eIF4E from human erythrocytes with a series of mono- and dinucleotide cap-analogues (see Scheme 1), based on an improved methodological approach. The series includes the cap-analogues with either a purine or a pyrimidine nucleotide as the second one in the mRNA sequence, the 5’-to-5’-triphosphate bridges of different lengths, and with chemical modifications in the bases and sugar moieties of the constituent nucleosides. Our study gives further insight into the mechanism of binding of various caps by eIF4E, during initiation of protein biosynthesis.

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**Scheme 1. Structures of the cap-analogues. Dissociable protons marked by asterisk.**

**Mononucleotide cap-analogues (top):** 7-methyl-GMP (m7GMP), \( R_1 = CH_3, R_2 = R_3 = H, n = 1 \); 7-methyl-GDP (m7GDP), \( R_1 = CH_3, R_2 = R_3 = H, n = 2 \); 7-methyl-GTP (m7GTP), \( R_1 = CH_3, R_2 = R_3 = H, n = 3 \); 7-benzyl-GMP (bn7GMP), \( R_1 = benzyl, R_2 = R_3 = H, n = 1 \); 7,7-dimethyl-GMP (m7,7GMP), \( R_1 = R_2 = CH_3, R_3 = H, n = 1 \); N7,7-trimethyl-GMP (m7,7,7GMP), \( R_1 = R_2 = R_3 = CH_3, n = 1 \).

**Dinucleotide cap-analogues (bottom):** P1,7-methylguanosine-P3-guanosine-5’S,5’S-triphosphate (m7GpppG), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7-methylguanosine-P3,2’S-O-methylguanosine-5’S,5’S-triphosphate (m7Gppp(Gm)G), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7-methylguanosine-P3,2’S-deoxyguanosine-5’S,5’S-triphosphate (m7Gppp2’dG), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7-methylguanosine-P3,5’S,5’S-tetraphosphate (m7Gpppp(Gm)G), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7-methylguanosine-P3,5’S,5’S,5’S-tetraphosphate (m7Gpppp(Gm)G), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7-methylguanosine-P3,5’S,5’S,5’S,5’S-tetraphosphate (m7Gpppp(Gm)G), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7-methylguanosine-P3-cytidine-5’S,5’S-triphosphate (m7GpppC), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = cytosine, n = 3 \); P1,7-methylguanosine-P3-uridine-5’S,5’S-triphosphate (m7GpppU), \( R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = uracil, n = 3 \); P1,7-benzylguanosine-P3-guanosine-5’S,5’S-triphosphate (bn7GpppG), \( R_1 = benzyl, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7,7-dimethylguanosine-P3-guanosine-5’S,5’S-triphosphate (m7,7GpppG), \( R_1 = R_2 = CH_3, R_3 = H, R_4 = OH, B = guanine, n = 3 \); P1,7,7,7-trimethylguanosine-P3-guanosine-5’S,5’S-triphosphate (m7,7,7GpppG), \( R_1 = R_2 = R_3 = CH_3, R_4 = OH, B = guanine, n = 3 \).
Eukaryotic initiation factor from human erythrocytes has been expressed in *E. coli* (strain BL21(DE3)pLys) according to Stern et al., 1993. The bacterial cells were transformed by a plasmid pET11d, containing the cloned coding region for human eIF4E, and the T7 promoter. Induction of T7 polymerase in liquid culture of bacteria on Luria-Bertani broth, with ampicillin and chloramphenicol, was initiated by addition of isopropyl-β-D-galactopyranoside. After lysis of bacterial pellets the protein was isolated from the soluble fraction by means of affinity chromatography (Webb et al., 1984) and purified on a MonoQ column.

The UV absorption spectra were recorded on a Cary 3E spectrophotometer. The fluorescence spectra were recorded on a Perkin Elmer LS 50B Luminescence Spectrometer, with the right angle detection (0.4×1 cm cell). The titration was carried out by adding 0.5–50 µl of concentrated nucleotide solution to 1.4 ml of 2–4 µM solution of eIF4E in 20 mM HEPES-KOH buffer pH 7.6, containing 100 mM KCl, 1 mM DTT and 0.2 mM EDTA. For all measurements the excitation wavelength of 280 nm was employed. The samples during the absorption and emission measurements were thermostatted at 20 °C within 0.1 °C.

Determination of the equilibrium association constants *K* of the eIF4E-cap complexes were based on analysis of the fluorescence spectra corrected as follows (for more detailed description and derivation of the equations below see Wieczorek et al., 1998):

(a) the contributions of the fluorescence of the cap analogues were subtracted by least-squares fitting;

(b) concentrations have been corrected for additions of a cap-analogue to the cuvette;

(c) the fluorescence intensities have been corrected for the absorption enhancement during titration of the protein with cap-analogues (inner filter effect);

The fluorescence intensities *F* were obtained by integrating the whole fluorescence spectra, what is of importance for the correct subtraction of the cap fluorescence (see above). Values of the association constant, *K* = \( \frac{C_{\text{com}}}{C_N \cdot C_P} \), where *C_{\text{com}}*, *C_N* and *C_P* are the equilibrium concentrations of the complex, cap-analogue and eIF4E, respectively, were obtained by assuming a 1:1 protein-ligand complex and a linear dependence of the fluorescence intensities on the protein concentration. These led to a modified representation of the Eadie-Hofstee equation:

\[
\frac{C_{N0}}{\Delta F} = \frac{1}{K \Delta F_{\text{max}} - \Delta F} + \frac{C_{P0}}{\Delta F_{\text{max}}},
\]

where *C_{N0}* is the total concentration of a cap-analogue, *C_{P0}* is the total concentration of eIF4E, \( \frac{\Delta F}{\Delta F_{\text{max}}} = \frac{F_0 - F}{F_{\text{max}} - F} \), and the fluorescence intensities *F_0*, *F* and *F_{\text{max}}* of eIF4E at the protein concentration of *C_{P0}* correspond to those without the cap-analogue, after adding the suitable amount of the cap-analogue during titration, and after saturation of the protein with the cap-analogue, respectively. As can be easily seen, \( \frac{C_{N0}}{\Delta F} \) is a linear function of \( \frac{1}{\Delta F_{\text{max}} - \Delta F} \), thus giving the value of 1/\( K \) from the slope and the value of \( \frac{C_{P0}}{\Delta F_{\text{max}}} \) from the intercept, by the least-squares linear regression analysis. In this representation of the Eadie-Hofstee equation there is no replacement of the equilibrium cap concentration *C_N* for the total cap concentration *C_{N0}* as usually introduced hitherto, see e.g. Carberry et al., 1989; Minich et al., 1994; Ueda et al., 1991; Morino et al., 1994; McCubbin et al., 1988; Carberry et al., 1990.

Typically three titrations were made for each cap-analogue to determine *K* and \( \Delta F_{\text{max}} \), leading to a statistical error of 10%. For some selected cap-analogues the three series of titrations were carried out several times at different concentrations of the protein. The calculated parameters were independent of the protein concentration within the statistical error.

**Results and Discussion**

**Fluorescence and structures of the eIF4E-cap complexes**

As can be easily seen from the dependence of \( \Delta F/F_0 \) on a cap concentration (Fig. 1) it is very important to take into account the absorption of the cap-analogue during measurements of fluorescence...
Fig. 1. Changes in the fluorescence intensity $\Delta F$ of eIF4E when titrated with 7-methyl-GDP ($C_N$, total concentration of 7-methyl-GDP). The squares and triangles refer to the fluorescence intensities uncorrected and corrected with respect to the inner filter effect, respectively. The concentration of eIF4E was 3.4 $\mu$m.

Fluorescence quenching of the protein, due to formation of the cap-eIF4E complex. This, as well as calculations of the fluorescence intensities from the whole spectra after subtraction of the cap fluorescence, provided us with precise values of the eIF4E-cap association constants $K$ (Table I), from the modified Eadie-Hofstee plot (Fig. 2). In our representation of the Eadie-Hofstee equation (see Experimental) it is possible to use the total cap concentration, contrary to the representation used hitherto, where the equilibrium concentration of the cap-analogue during titration of eIF4E should have been applied but instead, the total concentrations has been used (Carberry et al., 1989; Minich et al., 1994; Ueda et al., 1991; Morino et al., 1994; McCubbin et al., 1988; Carberry et al., 1990). Additionally, the influence of the ligand absorption has been usually neglected. This led to very high, unrealistic fluorescence quenching of the protein, over 50% (see e.g. McCubbin et al., 1988; Carberry et al., 1990; McCubbin et al., 1989), bearing in mind that three tryptophans, out of eight, interact with m7G in the eIF4E-cap complex, two by stacking with the 7-methylguanine ring and one with the N7-methyl group (Marcotrigiano et al., 1997; Matsuo et al., 1997). Comparing the values of the association constant $K$ from the fluorescence measurements presented in this paper with those taken from the literature (Table I) it is clearly seen, that for the nucleotides containing 7-methylguanine our new association constants are 1.2 to 3.5 times greater, whereas for nucleotides without the N7-methyl group are 1.6 to 2 times smaller.

Alkyl substitution in N7 is necessary for a cap-analogue to be tightly bound by eIF4E. The values

<table>
<thead>
<tr>
<th>cap-analogue</th>
<th>$K [M^{-1}] \times 10^{-5}$</th>
<th>$\Delta F_{max}/F_0$</th>
<th>$K [M^{-1}] \times 10^{-5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>m7GTP</td>
<td>11.7$^a$</td>
<td>0.228$^a$</td>
<td>5.5$^d$, 3.95$^c$</td>
</tr>
<tr>
<td>m7GDP</td>
<td>9.3</td>
<td>0.230</td>
<td>3.87$^d$, 3.86$^c$</td>
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<tr>
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<td>2.7</td>
<td>0.229</td>
<td>1.97$^e$, 1.64$^c$</td>
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<tr>
<td>m7GpppG</td>
<td>5.7$^a$</td>
<td>0.226$^a$</td>
<td>4.3$^d$, 3.89$^c$</td>
</tr>
<tr>
<td>m7GpppA</td>
<td>5.4</td>
<td>0.226</td>
<td>7.77$^c$, 2.02$^c$</td>
</tr>
<tr>
<td>m7GpppC</td>
<td>4.8</td>
<td>0.226</td>
<td></td>
</tr>
<tr>
<td>m7GpppU</td>
<td>4.4</td>
<td>0.227</td>
<td></td>
</tr>
<tr>
<td>m7GpppG</td>
<td>8.1</td>
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<td></td>
</tr>
<tr>
<td>m7Gppp2dG</td>
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<td></td>
</tr>
<tr>
<td>m7Gppp2G</td>
<td>6.2</td>
<td>0.230</td>
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<tr>
<td>m7Gppp2G</td>
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<td></td>
</tr>
<tr>
<td>m7GpppG</td>
<td>5.9</td>
<td>0.222</td>
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</tr>
<tr>
<td>m7GpppA</td>
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<td>0.224</td>
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<td>m7GpppG</td>
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<td>m7GpppU</td>
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<tr>
<td>GDP</td>
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<tr>
<td>GMP</td>
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<td>0.175</td>
<td></td>
</tr>
<tr>
<td>GpppG</td>
<td>0.47</td>
<td>0.160</td>
<td>0.787$^c$, 0.729$^c$</td>
</tr>
</tbody>
</table>

Table I. Association constants $K$, e.s.d. ±10% (first column), and their comparison with some of those obtained hitherto (third column), and $\Delta F_{max}$ normalized to $F_0$, e.s.d. ±0.05, for the cap-analogue of various structures as shown in Scheme 1.

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\[ \Delta F = K \times [C]_N \]

Fig. 2. The fluorescence data on association of eIF4E with 7-methyl-GDP plotted according to modified representation of the Eadie-Hofstee equation (see Experimental).
of $K$ for the caps containing 7-substituted guanine are up to 25 times greater than those for nucleotide analogues without the guanine N7-alkyl group, e.g. see $K$ for m$^7$GDP and GDP. N7-benzyl group seems to be easily accommodated in the hydrophobic pocket of eIF4E, leading to a slight enhancement of binding. The same holds for the replacement of one of the N$^2$-amino protons of m$^7$G by the methyl group. On the other hand introduction of two methyls into the amino group of m$^7$G gives rise to 10-fold diminution of the association constant $K$, due to interruption of the hydrogen bond with Glu-103. The number of the phosphate groups is of importance only for mono- and diphosphates, the latter being better bound by eIF4E. This is the result of stabilization of both of the phosphate groups by water mediated hydrogen bonds and salt bridges to arginines, lysine and aspartic acid in the eIF4E active centre (Marcotrigiano et al., 1997). Extension of the phosphate chain to four phosphates has practically no influence on $K$. The type of the second nucleoside in the dinucleotide cap-analogues is only of minor importance for binding, except for m$^2$G, in which the 2'-methylation strengthens the hydrophobic character of the sugar moiety. The differences in $K$ for the cap-analogues with various nucleosides contiguous to 7-methyl-guanosine may reflect the differences in intramolecular stacking inside the cap-analogues (Wieczorek et al., 1997), disrupted by eIF4E during binding of the cap-analogue in an extended conformation.

The second parameter measured fluorimetrically, $\Delta F_{\text{max}}$ normalized to $F_0$ ($\Delta F_{\text{max}}/F_0$), corresponds in some respect to the number of tryptophans in eIF4E, engaged in the interaction with cap, under the condition of similar participation of each tryptophan to the fluorescent emission. For typical caps $\Delta F_{\text{max}}/F_0$ of ca. 0.23 corresponds to three out of eight tryptophan residues engaged in interaction with 7-methylguanine (Marcotrigiano et al., 1997). For nucleotides unmethylated at N7 or double methylated at N$^2$ this parameter is ca. 0.16, showing unspecific binding with a less number of tryptophans engaged.

The preliminary investigations of the influence of the ionic strength (KCl concentration) and the molar amount of DTT on the fluorescence of eIF4E during cap binding ($K$ and $\Delta F_{\text{max}}/F_0$) point to possible conformational changes of the protein in respect to these environmental parameters, with the optimal values for the protein activity ca. 100 mM KCl and 0.5 to 2 mM dithiothreitol (DTT).

**Biological aspects**

Accurate fluorescence measurements of association between eIF4E and structurally various cap-analogues enable to extend the interpretation of biological data, based on the structure of the eIF4E–m$^7$GDP complex (Marcotrigiano et al., 1997; Matsuo et al., 1997). Some of the cap analogues presented in this paper has been already tested, and some of them are being investigated, as the competitive inhibitors of protein translation in several in vitro systems. The striking result comes from the comparison of the association constant $K$ (Table I) with the inhibitory activities for m$^7$GMP and its di- and triphosphate counterparts (Darzynkiewicz et al., in preparation). Addition of the second (or the second and the third) phosphate(s) gives rise to 3.4 to 4.4 increase of the association constant, and more than ten times increases the inhibitory activities. The presence of at least two negative charges on the phosphate chain as a minimal requirement for the activity of 7-methylguanine nucleotides has been originally postulated (Darzynkiewicz et al., 1981). This was based on the drastic drop of the inhibitory activity caused by the methyl esterification of the alpha phosphate in m$^7$GMP. Similar esterification of the beta phosphate in m$^7$GDP and the gamma phosphate in m$^7$GTP had no effect on the inhibitory activities in comparison with the parent nucleotides (Darzynkiewicz et al., 1985). These lead to the conclusion, consistent with the structure of the eIF4E–m$^7$GDP complex (Marcotrigiano et al., 1997) discussed above, that at least two phosphate groups, with at least two negative charges on them, are required for the proper binding of cap by eIF4E. The third phosphate seems to be of much less importance for the binding.

Most of the dinucleoside tri- and tetraphosphate cap-analogues, possessing an alkyl substituents on the first guanine, show relatively similar values of $K$, the highest being for m$^7$Gpppm$^2$G, the analogue of cap1, and the lowest for m$^7$GpppU (Table I). This corresponds to a stronger inhibitory effect of 2’-O-methyl analogues on the efficiency of protein translation (Kuge et al., 1995). However,
the type of the second nucleotide in cap seems to be of minor importance for its binding by eIF4E as well as for the activity in translation. The exceptional, ten times lower value of the association constant of \(m^7GpppG\) and \(m^3GMP\), but not \(m^7GpppG\) and \(m^3GMP\), comparing to other cap-analogues, is consistent with the lack of inhibition of protein translation of the former (Darzykiewicz et al., 1988). It is pretty obvious, bearing in mind the stabilization of 7-methylguanine by the hydrogen bond to Glu-103 of eIF4E, with the amino group as a proton donor (Marcotrigiano et al., 1997). Trimethylated caps are recognized by some of the isoforms of eIF4E from the nematode Caenorhabditis elegans (Jankowska-Anyszka et al., 1998). The comparison of the physicochemical and biological data point to a decisive role of binding of cap by eIF4E as a rate-limiting step in protein translation. Interaction between two protein factors eIF4E and eIF4G, see e.g. Morley et al., 1997, is of equal importance at that stage of translation. Further insight into the structural and dynamical aspects of ternary complexes, including eIF4E, eIF4G and capped oligoribonucleotides, seems to be of major importance for constructing a structural and dynamic model of the initiation of translation.

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