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

Zbigniew Wieczorek[a], Anna Niedźwiecka-Kornaś[b], Lidia Chlebicka[b],
Marzena Jankowska[c], Katarzyna Kiraga[b], Janusz Stępiński[c], Michał Dadlez[d],
Regina Drabent[a], Edward Darżynkiewicz[b], Ryszard Stolarski[b]

[a] Department of Physics and Biophysics, University of Agriculture and Technology,
4 Oczapowskiego St., 10-957 Olsztyn, Poland
[b] Department of Biophysics, Institute of Experimental Physics, University of Warsaw,
93 Żwirki i Wigury St., 02-089 Warszawa, Poland
[c] Department of Chemistry, University of Warsaw, 1 Pasteur St., 02-093 Warszawa, Poland
[d] Institute of Biochemistry and Biophysics Polish Academy of Sciences, 5A Pawińskiego St.,
02-106 Warszawa, Poland;

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 ΔF_{max}, 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 N^2,N^2,7-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", m^7GpppN, where the first nucleoside, 7-methylguanosine (m^7G), is linked by the 5′-to-5′-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 ($m^{2'}G$), *etc.* The cap structure is necessary for optimal protein translation (Sonenberg, 1988; Darżynkiewicz *et al.*, 1987). Several cap-analogues have been shown to inhibit translation (Darżynkiewicz *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 a; Carberry *et al.*, 1989). This finding led to a tentative model for such interactions, based on stacking and hydrogen bonding

(Ueda *et al.*, 1991b; Carberry *et al.*, 1989), subsequently backed up by ^{1}H-NMR and fluorescence studies on interaction of cap-analogues with small peptide mimics of the eIF4E sequences (Stolarski *et al.*, 1996a, 1996b; Ueda *et al.*, 1988).

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 (Marcotrigiano *et al.*, 1997) as well as structure determination of the yeast eIF4E–m[7]GDP complex in solution, by means of multidimensional NMR methods (Matsuo *et al.*, 1997). m[7]GDP, 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-103 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 noncoding 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.
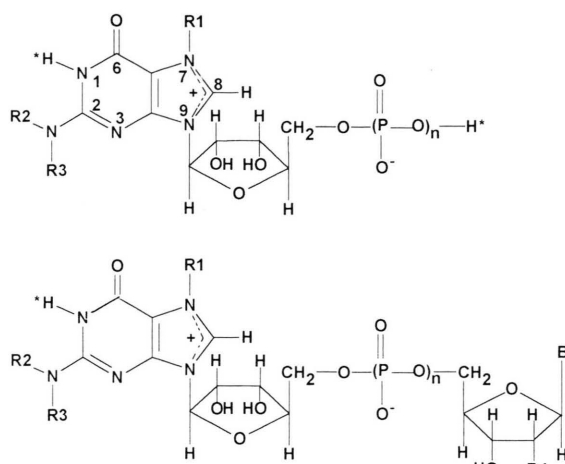
Scheme 1. Structures of the cap-analogues. Dissociable protons marked by asterisk.

Mononucleotide cap-analogues (**top**): 7-methyl-GMP (m[7]GMP), R_1 = CH_3, R_2 = R_3 = H, n = 1; 7-methyl-GDP (m[7]GDP), R_1 = CH_3, R_2 = R_3 = H, n = 2; 7-methyl-GTP (m[7]GTP), R_1 = CH_3, R_2 = R_3 = H, n = 3; 7-benzyl-GMP (bn[7]GMP), R_1 = benzyl, R_2 = R_3 = H, n = 1; N^2,7-dimethyl-GMP (m$_2^{2,7}$GMP), R_1 = R_2 = CH_3, R_3 = H, n = 1; N^2,N^2,7-trimethyl-GMP (m$_3^{2,2,7}$GMP), R_1 = R_2 = R_3 = CH_3, n = 1.

Dinucleotide cap-analogues (**bottom**): P^1-7-methylguanosine-P^3-guanosine-5′,5′-triphosphate (m[7]GpppG), R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3; P^1-7-methylguanosine-P^3-2′-O-methylguanosine-5′,5′-triphosphate (m[7]Gpppm^{2}G), R_1 = CH_3, R_2 = R_3 = H, R_4 = OCH_3, B = guanine, n = 3; P^1-7-methylguanosine-P^3-2′-deoxyguanosine-5′,5′-triphosphate (m[7]Gppp2′dG), R_1 = CH_3, R_2 = R_3 = H, R_4 = H, B = guanine, n = 3; P^1-7-methylguanosine-P^4-guanosine-5′,5′-tetraphosphate (m[7]GppppG), R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 4; P^1-7-methylguanosine-P^3-7-methylguanosine-5′,5′-triphosphate (m[7]Gpppm[7]G), R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = 7-methylguanine, n = 3; P^1-7-methylguanosine-P^3-adenosine-5′,5′-triphosphate (m[7]GpppA), R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = adenine, n = 3; P^1-7-methylguanosine-P^3-N^6-methyladenosine-5′,5′-triphosphate (m[7]Gpppm^{6}A), R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = N^6-methyladenine, n = 3; P^1-7-methylguanosine-P^3-cytidine -5′,5′-triphosphate (m[7]GpppC), R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = cytosine, n = 3; P^1-7-methylguanosine-P^3-uridine -5′,5′-triphosphate (m[7]GpppU), R_1 = CH_3, R_2 = R_3 = H, R_4 = OH, B = uracil, n = 3; P^1-7-benzylguanosine-P^3-guanosine-5′,5′-triphosphate (bn[7]GpppG), R_1 = benzyl, R_2 = R_3 = H, R_4 = OH, B = guanine, n = 3; P^1-N^2,7-dimethylguanosine-P^3-guanosine-5′,5′-triphosphate (m$_2^{2,7}$GpppG), R_1 = R_2 = CH_3, R_3 = H, R_4 = OH, B = guanine, n = 3; P^1-N^2,N^2,7-trimethylguanosine-P^3-guanosine-5′,5′-triphosphate (m$_3^{2,2,7}$GpppG), R_1 = R_2 = R_3 = CH_3, R_4 = OH, B = guanine, n = 3.

Experimental

GMP and GDP were from Sigma Chemical Corporation USA. Syntheses of the cap-analogues has been described previously (Darżynkiewicz *et al.*,

1985; Stępiński *et al.*, 1990; Darżynkiewicz *et al.*, 1990; Stępiński *et al.*, 1995; Jankowska *et al.*, 1996).

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, $K = \dfrac{C_{com}}{C_N \cdot C_P}$, where C_{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:

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

where C_{N0} is the total concentration of a cap-analogue, C_{P0} is the total concentration of eIF4E, $\Delta F = F_0 - F$, and $\Delta F_{max} = F_0 - F_b$. The fluorescence intensities F_0, F and F_b 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, $\dfrac{C_{N0}}{\Delta F}$ is

a linear function of $\dfrac{1}{\Delta F_{max} - \Delta F}$, thus giving the value of $1/K$ from the slope and the value of $\dfrac{C_{P0}}{\Delta F_{max}}$ 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 ΔF_{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 fluores-
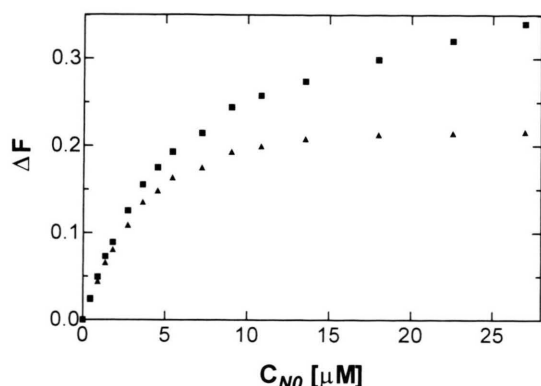
Fig. 1. Changes in the fluorescence intensity ΔF of eIF4E when titrated with 7-methyl-GDP (C_{N0}, total concentration of 7-methyl-GDP). The squares and triangles refer to the fluorescence intensities uncorrected and corrected with respect to the inner filter effect, respectively. The concentration of eIF4E was 3.4 μM.

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

cap-analogue	$K\,[\mathrm{M}^{-1}]\times10^{-5}$	$\Delta F_{max}/F_0$	$K\,[\mathrm{M}^{-1}]\times10^{-5}$
m⁷GTP	11.7[a]	0.228[a]	5.5[b], 3.95[c], 3.87[d], 3.86[e]
m⁷GDP	9.3	0.230	3.18[f]
m⁷GMP	2.7	0.229	1.97[g], 1.64[e]
m⁷GpppG	5.7[a]	0.226[a]	4.3[b], 3.89[c], 3.77[e], 2.02[f]
m⁷GppppG	6.9	0.227	
m⁷GpppA	5.4	0.226	
m⁷Gpppm⁶A	5.9	0.227	
m⁷GpppC	4.8	0.226	
m⁷GpppU	4.4	0.227	
m⁷Gpppm²'G	8.1	0.229	
m⁷Gppp2'dG	5.2	0.228	
m⁷Gpppm⁷G	6.2	0.230	
bn⁷GMP	4.6	0.225	5.26[e]
bn⁷GpppG	6.6	0.224	3.17[e]
m₂²·⁷GMP	4.2	0.221	3.27[e]
m₂²·⁷GpppG	5.9	0.222	2.25[e]
m₃²·²·⁷GMP	0.32	0.172	
m₃²·²·⁷GpppG	0.68	0.175	0.54[e]
GDP	0.38	0.161	0.641[f]
GMP	0.17[a]	0.158[a]	0.332[g]
GpppG	0.47	0.160	0.787[f], 0.729[c]

[a] Wieczorek *et al.* 1998; [b] Minich *et al.* 1994; [c] Morino *et al.* 1994; [d] Carberry *et al.* 1989; [e] Carberry *et al.* 1990; [f] McCubbin *et al.* 1988; [g] Ueda *et al.* 1991.

cence 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
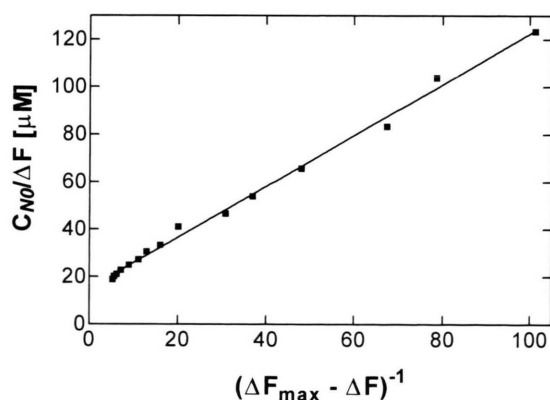

Fig. 2. The fluorescence data on association of eIF4E with 7-methyl-GDP plotted according to modified representation of the Eadie-Hofstee equation (see Experimental).

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

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 m⁷GDP 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 m⁷G by the methyl group. On the other hand introduction of two methyls into the amino group of m⁷G 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, ΔF_{max} normalized to F_0 ($\Delta F_{max}/F_0$), 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 $\Delta F_{max}/F_0$ 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 $\Delta F_{max}/F_0$) 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–m⁷GDP 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 m⁷GMP and its di- and triphosphate counterparts (Darżynkiewicz 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 (Darżynkiewicz et al., 1981). This was based on the drastic drop of the inhibitory activity caused by the methyl esterification of the alpha phosphate in m⁷GMP. Similar esterification of the beta phosphate in m⁷GDP and the gamma phosphate in m⁷GTP had no effect on the inhibitory activities in comparison with the parent nucleotides (Darżynkiewicz et al., 1985). These lead to the conclusion, consistent with the structure of the eIF4E–m⁷GDP 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 $m^7Gpppm^{2'}G$, the analogue of cap1, and the lowest for m⁷GpppU (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 $m_3^{2,2,7}GpppG$ and $m_3^{2,2,7}GMP$, but not $m_2^{2,7}GpppG$ and $m_2^{2,7}GMP$, comparing to other cap-analogues, is consistent with the lack of inhibition of protein translation of the former (Darżynkiewicz *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.

Carberry S. E., Rhoads R. E. and Goss D. J. (1989), A spectroscopic study of the binding of m⁷GTP and m⁷GpppG to human protein synthesis initiation factor 4E. Biochemistry **28**, 8078–8083.

Carberry S. E., Darżynkiewicz E., Stępiński J., Tahara S. M., Rhoads R. E. and Goss D. J. (1990), A spectroscopic study of the binding of N-7-substituted *cap* analogues to human protein synthesis initiation factor 4E. Biochemistry **29**, 3337–3341.

Darżynkiewicz E., Antosiewicz J., Ekiel I., Morgan M. A., Tahara S. M. and Shatkin A. J. (1981), Methyl esterification of m⁷G⁵'p reversibly blocks its activity as an analog of eukaryotic mRNA 5'-caps. J. Mol. Biol. **153**, 451–458.

Darżynkiewicz E., Ekiel I., Tahara S. M., Seliger L. S. and Shatkin A. J. (1985), Chemical synthesis and characterization of 7-methyl-guanosine cap analogues. Biochemistry **24**, 1701–1707.

Darżynkiewicz E., Ekiel I., Lassota P. and Tahara S. M. (1987), Inhibition of eukaryotic translation by analogues of messenger RNA 5'-cap: chemical and biological consequences of 5'-phosphate modifications of 7-methylguanosine 5'-monophosphate. Biochemistry **26**, 4372–4380.

Darżynkiewicz E., Stępiński J., Ekiel I, Jin Y., Haber D., Sijuwade T. and Tahara S. M. (1988), β-globin mRNAs capped with m⁷G, m₂²,⁷G or m₃²,²,⁷G differ in intrinsic translation efficiency. Nucl. Acid Res. **16**, 8953–8962.

Darżynkiewicz E., Stępiński J., Tahara S. M., Stolarski R., Ekiel I., Haber D., Neuvonen K., Lehikoinen P., Labadi I. and Lønnberg H. (1990), Synthesis, conformation and hydrolytic stability of P¹,P³-dinucleoside triphosphates related to mRNA 5'-cap, and comparative kinetic studies on their nucleoside and nucleoside monophosphate analogs. Nucleosides & Nucleotides **9**, 599–618.

Jankowska-Anyszka M., Lamphear B. J., Aamodt E. J., Harrington T., Darżynkiewicz E., Stolarski R. and Rhoads R. E. (1998) Multiple isoforms of eukaryotic protein synthesis initiation factor 4E in *Caenorhabditis elegans* can distinguish between mono- and trimethylated mRNA cap structures. J. Biol. Chem. **273**, 10538–10542.

Jankowska M., Stępiński J., Stolarski R., Wieczorek Z., Temeriusz A., Haber D. and Darżynkiewicz E. (1996), ¹H NMR and fluorescence studies of new mRNA 5'-cap analogues. Collect. Czech. Chem. Commun. **61**, S197–202.

Kuge H. and Richter J. D. (1995), Polyadenylation-mediated cap ribose methylation stimulates translational recruitment of maternal mRNA. EMBO J. **14**, 6301–6310.

Marcotrigiano J., Gingras A.-C., Sonenberg N. and Burley S. K. (1997), Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell **89**, 951–961.

Matsuo H., Li H., McGuire A. M., Fletcher C. M., Gingras A.-C., Sonenberg N. and Wagner G. (1997), Structure of translation factor eIF4E bound to m⁷GDP and interaction with 4E-binding protein. Nature Struct. Biol. **4**, 717–724.

McCubbin W. D., Edery I., Altman M., Sonenberg N. and Kay C. M. (1988), Circular dichroism and fluorescence studies on protein synthesis initiation factor eIF4E and two mutant forms from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **263**, 17663–17671.

McCubbin W. D., Edery I., Altman M., Sonenberg N. and Kay C. M. (1989), Circular dichroism and fluorescence studies on five mutant forms of protein synthesis initiation factor eIF-4E, from the yeast *Saccharomyces cerevisiae*. FEBS Letters **245**, 261–266.

Minich W. B., Balasta M. L., GossD. J. and Rhoads R. E. (1994), Chromatografic resolution of *in vivo* phosphorylated and non-phosphorylated eukaryotic translation

initiation factor eIF-4E: increased cap affinity of the phosphorylated form. Proc. Natl. Acad. Sci. USA **91**, 7668–7672.

Morino S., Yasui M., Doi M., Inoue M., Ishida T., Ueda H. and Uesugi S. (1994), Direct expression of a synthetic gene in *Escherichia coli*: purification and physicochemical properties of human intiation factor 4E. J. Biochem. **116**, 687–693.

Morley S. J., Curtis P.S and Pain V. M. (1997), eIF4G: translation's mystery factor begins to yield its secrets. RNA **3**, 1085–1104.

Sonenberg N. (1988), *Cap* binding proteins of eukaryotic messenger RNA: functions in initiation and control of translation. Progr. Nucl. Acid Res. Mol. Biol. **35**, 175–207.

Stępiński J., Bretner J. M., Jankowska M., Felczak K., Stolarski R., Wieczorek Z., Cai A.-L., Rhoads R. E., Temeriusz A., Haber D. and Darżynkiewicz E. (1995), Synthesis and properties of P^1,P^2-, P^1,P^3- and P^1,P^4-dinucleoside di-, tri- and tetraphosphate mRNA 5′-cap analogues. Nucleosides & Nucleotides **14**, 717–721.

Stępiński J., Grabowska L., Darżynkiewicz E., Temeriusz A., Stolarski R., Tahara S. M., Shugar D., Järvinen P. and Lönnberg H.(1990), Synthesis, conformation and hydrolytic stability of modified mRNA 5′-cap structures: P^1,P^3-dinucleoside triphosphates derived from guanosine and acyclic analogues of 7-methyl-, $N^2,7$-dimethyl- and $N^2,N^2,7$-trimethylguanosines. Collect. Czech. Chem. Commun. **55**, 117–120.

Stern D. B., Wilson H. and Jagus R. (1993), Use of non-reducing SDS-PAGE for monitoring renaturation of recombinant protein synthesis initiation factor, eIF-4α. Prot. Expr. Pur. **4**, 320–327.

Stolarski R., Sitek A., Stępiński J., Jankowska M., Oksman P., Temeriusz A., Darżynkiewicz E., Lönnberg H. and Shugar D. (1996a), ^{1}H-NMR studies on association of mRNA cap-analogues with tryptophan-containing peptides. Biochim. Biophys. Acta **1293**, 97–105.

Stolarski R., Zdanowski K, Chlebicka L., Wieczorek Z., Sitek A., Stępiński J., Jankowska M., Mattinen J., Temeriusz A. and Darżynkiewicz E. (1996b), Inter- and intramolecular stacking of mRNA cap-analogues – relevance to initiation of translation. Collect. Czech. Chem. Commun. **61**, S217–221.

Ueda H., Doi M., Inoue M., Ishida T., Tanaka T. and Uesugi S. (1988), A possible recognition mode of mRNA cap terminal structure by peptide: cooperative stacking and hydrogen-bond pairing interactions between m7GpppA and Trp–Leu–Glu. Biochem. Biophys. Res. Commun. **154**, 199–204.

Ueda H., Iyo H., Doi M., Inoue M., Ishida T., Morioka H., Tanaka T., Nishikawa S. and Uesugi S. (1991a), Combination of Trp and Glu residues for recognition of mRNA *cap* structure. Analysis of m^{7}G base recognition site of human *cap* binding protein (IF4E) by site-directed mutagenesis. FEBS Letters **280**, 207–210.

Ueda H., Maruyama H., Doi M., Inoue M., Ishida T., Morioka H., Tanaka T., Nishikawa S. and Uesugi S. (1991b), Expression on a synthetic gene for human cap binding protein (Human eIF-4E) in *Escherichia coli* and fluorescence studies on interaction with mRNA cap structure analogue. J. Biochem. **109**, 882–889.

Webb N. R., Chari R. V. J., DePhillis G., Kozarich J. W. and Rhoads R. E. (1984), Purification of the messenger RNA *cap*-binding protein using a new affinity medium. Biochemistry **23**, 177–181.

Wieczorek Z., Darżynkiewicz E. and Lönnberg H. (1998), A fluorescence spectroscopic study on the binding of mRNA 5′-*cap*- analogs to human translation initiation factor eIF4E: a critical evaluation of the sources of error. J. Photochem. Photobiol B: Biology **43**, 158–163.

Wieczorek Z., Zdanowski K., Chlebicka L., Stępiński J., Jankowska M., Kierdaszuk B., Temeriusz A., Darżynkiewicz E. and Stolarski R. (1997), Fluorescence and NMR studies of intramolecular stacking of mRNA cap-analogues. Biochim. Biophys. Acta **1354**, 145–152.