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Tryptophan Residues from Cap Binding Slot in eIF4E Family Members: Their Contributions to Near-UV Circular Dichroism Spectra

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Abstract

eIF4E, a key factor in the cap-dependent translation initiation, binds cap structure at the 5' end of mRNA by stacking interaction involving two of its eight conserved tryptophan residues. In this paper, we examined individual contributions of tryptophan residues to the near-UV Circular Dichroism spectra to identify structural similarities and differences in cap binding motif among members of eIF4E family. The near-UV CD spectrum of human eIF4E1a in its apo form, resulting mainly from 1Lb transition and dominated by two vibrionic bands, is conserved among eIF4Es. Based on comparison of CD spectra for eIF4E mutants, we showed that tryptophans involved in stacking interaction give strongest individual contributions, which allow identification of their different orientation with respect to the cap. This indicates that near-UV CD is a quick and powerful tool to analyse tryptophan conformation in eIF4E proteins, and their changes upon binding modified cap analogues.

Keywords: eIF4E; Cap analogues; Near-UV circular dichroism; Fluorescence; Binding affinity

Introduction

Eukaryotic translation initiation factor 4E (eIF4E), a small, approximately 25 kDa protein, plays a key role in regulation of gene expression and developmental processes in eukaryotic organisms. It is involved in control of the cap-dependent translation initiation [1-3], it affects the fate of specific mRNAs (by transport, specific translation inhibition during oogenesis and embryogenesis) [3,4] and is also involved in processing bodies (PBs) and stress granules (SGs) [5-7]. During translation initiation, eIF4E in association with eIF4G-scaffold protein, as a part of the eIF4F complex, binds the mRNA 5' cap structure (m^7GpppN, where N is any nucleotide) and facilitates ribosome binding to mRNA [1]. Cap recognition by eIF4E is one of the limiting steps that regulate the global process of cap-dependent translation, and eIF4E activity is regulated at many levels, e.g., by phosphorylation [8,9] and interaction with 4E-BP binding proteins, which share the canonical eIF4E-binding motif with eIF4G (C-motif, YXXXXLφ, where X is any amino acid and φ is a hydrophobic residue) [10,11]. eIF4E has been extensively investigated in organisms that range from yeast to mammals and the sequence comparisons have demonstrated an evolutionarily conserved core region with the presence of eight conserved Trp residues. The structures of eIF4E from many organisms in apo and cap analogue complexed form have been resolved [12-20]. All structures share a similar eIF4E-like fold, which is described as resembling a cupped-hand, and includes up to eight-stranded antiparallel β-sheet backed by three long α-helices. The concave surface of the β-sheet forms a narrow cap cavity, where N7-methylguanine of cap is sandwiched between two conserved tryptophan residues (Trp56 and Trp102 for human eIF4E), forms hydrogen bonds with conserved glutamic acids (Glu103 for human eIF4E) and backbone NH of Trp102, and has a van der Waals contact with the third conserved Trp residue (Trp166 for human eIF4E) present in the cap binding slot. At the same time, the cap phosphate chain forms a network of direct or water-mediated hydrogen bonds with positive charges of lysine and arginine side chains present in the eIF4E cap binding slot. The advent of DNA sequencing projects has brought to light existence of an unexpected variety of genes sequentially related to eIF4E. Together, the canonical eIF4E and its relatives comprise a family of eIF4E-related proteins within a given organism. Within the family, the proteins are grouped into three structural classes [21,22] based on the presence or substitution by other amino acids of two of the eight conserved eIF4E tryptophan residues, one of which is located in the cap binding slot. The members of Class I, where the canonical translation factor eIF4E belongs, have Trp residues equivalent to Trp43 and Trp56 of *H. sapiens* eIF4E (now it is often called "eIF4E1a" to distinguish it from its relatives [23]) and are present in all eukaryotes. In Class II proteins, Trp43 is substituted by Tyr/Phe/Leu and Trp56 by Tyr/Phe. Members of this class have been identified in Metazoa, Viridiplantae, and Fungi but, interestingly, they are not present in the model organisms *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Class III members have Cys or Tyr substitution relative to Trp56. Members of this class have been identified only in Metazoa. An unusual characteristic pattern of the conserved Trp residues in eIF4E core region sequence, its characteristic substitution in eIF4E isoforms from Class II and III, and participation of three Trp residues: Trp56, Trp102 and Trp166 (as per the human numbering) in cap binding makes eIF4E an interesting object for analysis of structure and protein-ligand interaction using near-UV CD.

Whereas CD signals in far-UV region (178-250 nm) usually indicate the content of protein secondary structure, CD spectra in near-UV region (250-330 nm) reflect the protein tertiary structure and its changes. The observed near-UV CD bands arise from electronic transitions in aromatic residues (Phe, Tyr, Trp), the plane symmetry of which is perturbed in an appropriate way. The shape and magnitude of CD bands in the spectrum are therefore individual for each protein and depend on the number of each type of aromatic amino acids, their mobility, spatial position relative to the neighbouring amino

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acids (polar groups, aromatic residues), formation of H-bonds, etc. Additionally, if more than one aromatic residue is present in the protein, superposition of spectral signals can be observed. To interpret a protein near-UV spectrum, the knowledge of positions of CD bands expected for individual aromatic amino acids is helpful. The CD bands of Phe occur between 255-270 nm and have a sharp fine structure connected with vibrionic transitions [24]. In case of a tyrosine moiety, the observed CD bands are connected with ^1L_b electronic transition and are located between 274-282 nm. They can be positive or negative [24]. The near-UV spectra of Trp are complex because CD bands arise from two overlapping electronic transitions, ^1L_a and ^1L_b. The ^1L_b transition gives a strong CD band characterised by two dominating vibrionic bands (0-0 and 0+850 cm^{-1}), which both have the same positive or negative sign. In a protein, the 0-0 ^1L_b band of a Trp is usually found between 288-292 nm. The band arising from ^1L_a transition lacks any vibrionic structure and extends from 250 to 305 nm. The ^1L_a band is highly sensitive to environment. The interaction of the indole ring with polar groups of the protein can shift the CD band to either shorter or longer wavelengths, whereas the formation of hydrogen bonds with the NH group of indole ring may shift the band to longer wavelengths by even 12 nm [24]. The observed near-UV CD spectra of Trp in model compounds and proteins were classified into four types. Type 1 includes the spectra with strong ^1L_b bands and none or minimally intensive ^1L_a bands. In type 2, the CD spectra arise mainly from ^1L_a transition, whereas in type 3, both ^1L_a and ^1L_b transitions produce obvious CD bands. Type 4 includes all other spectra [24]. Hence, each protein tends to have a characteristic shape and magnitude of near-UV CD spectrum. This work aims at showing that near-UV CD is a quick and powerful tool to identify conformational differences of aromatic residues engaged in forming a cap binding motif in solution among eIF4E proteins from different Classes and organism, as well as structural changes that modify the surrounding of eIF4E Trp residues. To solve this problem, we analysed the near-UV CD spectra of human eIF4E isoforms and canonical eIF4E from different organisms, and, using mutagenesis, we assigned the contributions of the conserved tryptophan residues from cap binding slot to appropriate CD bands.

Materials and Methods

Synthesis of Cap analogues

Mono- and dinucleotide cap analogues were synthesised as described previously [25-27]. Cap analogue concentrations were determined using spectrophotometric methods [28].

Plasmid construction and site-directed mutagenesis

To obtain single and double point mutation replacement of Trp56 or/and Trp102 by tyrosine, phenylalanine or alanine in human eIF4E1a, a PCR-based site-directed mutagenesis procedure was used. pET11d containing cDNA for human eIF4E1a (a gift from R.E. Rhoads) was used as an initial template vector and mutagenesis was performed according to the instructions provided by STRATAGENE. cDNA of human eIF4E3 isoform was amplified by PCR using pUC57_heIF4E3 vector (BIOMATIK) as a template and then cloned into pET16b vector (Novagen) between NdeI and XhoI restriction sites, with addition of a His_6 tag at the N-terminus of heIF4E3 (pET16b_His_heIF4E3). All constructs were confirmed by automatic DNA sequencing (Genomed).

Expression and purification of recombinant proteins

The appropriate plasmids for human eIF4E1a protein (pET11d_heIF4E1a), its tryptophan mutants (pET11d_heIF4E1a_MUT), human isoforms: eIF4E1b (pET30a_ heIF4E1b), eIF4E2 (pET30a_heIF4E2) and *Drosophila* eIF4E-1 protein (pET30a_deIF4E-1) were transformed into *E. coli* BL21(DE3) strain (Novagen). The cell cultures were grown under conditions leading to accumulation of the produced target protein in inclusion bodies; the protein was then purified using 6 M guanidine hydrochloride buffer. The proteins were refolded using one step dialysis against 50 mM HEPES/KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA and 2 mM DTT and purified by ion-exchange chromatography on HiTrap HP SP column (GE Healthcare) [29].

The human eIF4E3 isoform containing the N-terminal His_6-Tag was expressed in *E. coli* BL21(DE3) strain (Novagen). Bacteria were grown in LB medium at 37°C until OD at 600 nm reached 0.6, and then were induced with 0.5 mM IPTG at 15°C overnight. The cells were harvested by centrifuging at 9110 x g for 15 min and re-suspended in lysis buffer (50 mM HEPES/KOH (pH 7.2), 300 mM KCl, 20 mM imidazole containing protease inhibitor mixture without EDTA), and disrupted by sonication. The nucleic acids were removed from the protein sample by adding HS-Nuclease (MoBiTec) to lysate and incubating the solution for 20 min at 4°C. The soluble fraction obtained by centrifugation at 43,000 × g for 30 min was loaded on 1mL HIS-Select Nickel Affinity Gel (SIGMA) and incubated with gel for 1 h at 4°C. Then, the resin was washed with 10 volumes of buffer containing 20 mM imidazole and His-heIF4E3 was eluted with 500 mM imidazole in 50 mM HEPES/KOH (pH 7.2), 300 mM KCl buffer. Imidazole was removed from the protein sample by one-step dialysis against 50 mM HEPES/KOH (pH 7.2), 300 mM KCl and then the protein was purified by size-exclusion chromatography on Enrich SEC70 column (Bio-Rad). His-tagged yeast eIF4E was expressed in *E. coli* M15 strain (QIAGEN) from vector pQE30_N-His_yeIF4E (gift from Thomas Preiss). The protein expression was induced with 0.5 mM IPTG overnight at 16°C. The protein from the soluble fraction was purified on a column containing HIS-Select Nickel Affinity Gel (SIGMA) and eluted from column using buffer containing 300 mM imidazole. Finally, imidazole was removed from the protein sample by dialysis against 50 mM HEPES/KOH (pH 7.2), 300 mM KCl and the protein was purified on Enrich SEC70 column (Bio-Rad). Purity of all eIF4E proteins was confirmed by electrophoresis using 15% polyacrylamide gel and the concentration was determined based on absorption of protein samples using theoretical molar extinction coefficients calculated based on amino acid composition [30] (summarised in Table 1). Potential protein dimerisation and aggregation were checked on size exclusion chromatography. The analysis was carried out on an Enrich SEC70 column (BIO-RAD) in 50 mM Hepes/KOH (pH 7.2), 300 mM KCl at 20°C and at a flow rate of 0.5 ml/min.

Near-UV CD measurements

Circular dichroism spectra were recorded on Chirascan CD Spectrometer (Applied Photophysics, UK) equipped with circulating water bath and thermoelectric temperature control unit regulated to 0.1°C. The measurements were carried out in 1.0 cm quartz cuvette containing 2.5 mL protein sample at a concentration between 10-15 μM in 50 mM HEPES/KOH (pH 7.2) and 134.5 mM KCl. The appropriate volume of cap analogue was added to the protein sample from 5 mM buffer stock of cap analogue. During measurements, sample temperature was maintained at 20°C. The near-UV CD spectra were recorded in the range of 247-363 nm at 0.2 nm interval with integrating time of 2 s. Spectra of each protein, cap analogue and their mixture were corrected for presence of buffer (subtraction of appropriate buffer spectrum obtained in identical conditions) and smoothed using Chirascan software. To obtain the correct spectrum of eIF4E and cap analogue complex, the spectrum of free cap analogue was subtracted

from the mixture spectrum (the free cap analogue concentration was calculated based on the association constant values (K_{as}) determined using fluorescence titrations). The CD data were recorded in millidegrees and converted to molar ellipticity ([θ], deg cm^2 dmol^{-1}). To determine K_{as} values using near-UV CD, theoretical dependence of ellipticity changes on the total concentration of cap analogue were fitted to the experimental data points, according to equation adopted from fluorescence binding assay described previously [20]. The changes in CD signal were monitored at 300 nm.

Fluorescence binding assays

Fluorescence measurements leading to determination of association constants for eIF4E proteins and tryptophan mutants of heIF4E1a isoform with cap analogues were performed as described by Niedzwiecka et al. [20,31]. Fluorescence titration curves were carried out on a LS-55 spectrofluorometer (Perkin Elmer Co., Norwalk, CT., USA), in 50 mM HEPES/KOH (pH 7.2), 0.5 mM EDTA, 134.5 mM KCl, at 20.0 ± 0.3°C using 0.1 or 0.2 μM concentration of proteins. The eIF4E fluorescence was excited at 280 nm, 290 nm or 295 nm and the fluorescence intensity was monitored at a single wavelength 340 nm, 345 nm or 320 nm. The measured fluorescence intensities were corrected for dilution and for the *inner filter* effect. The equilibrium association constants (K_{as}) were obtained by fitting a theoretical dependence of the fluorescence intensity on the total concentration of cap analogue to the experimental data points, according to equation described previously [20]. The final K_{as} was calculated as a weighted average from three to five independent titrations. Numerical least-squares nonlinear regression analysis was performed using ORIGIN 6.0 from Microcal Software Inc., USA.

Results and Discussion

Localisation of human eIF4E Trp residues in apo and m^{7}GTP bound form

The intensity of protein near-UV CD spectra is affected by some factors: number and type of aromatic amino acids, rigidity of the protein and interaction of aromatic residues with their neighbours [24]. Aromatic residue mobility in a protein tends to decrease the CD band intensity, whereas the interactions of tyrosyl and tryptophanyl side chains with other aromatic residues: Trp, Tyr, Phe and His located at a distance of not more than 10 Å can give rise to especially intense CD bands. The experimental and theoretical considerations indicate that the major mechanism contributing to intensity of Trp and Tyr near-UV CD bands is μ-μ coupling [24]. However, many interactions with the same aromatic residues can give both positive and negative bands, which in turn may silence the signal. The members of eIF4E-protein family contain a large fraction of Trp residues, on average more than 3.0% (heIF4E1a-3.7%) and have eight evolutionarily conserved Trp residues (Figure 1A). The localisation of heIF4E1a tryptophan residues in the structure and their mobility are presented in Figures 1B and 1C. Trp43, 46, 113 and 166 are located on β-sheets, whereas Trp73 and 130 - on α-helixes [15,16,32]. Except of Trp73, all the listed Trp residues are buried, and their mobility is very restricted. In apo form of the protein, Trp56 and Trp102 (which are directly involved in stacking interaction with N7-methylguanine of cap) are located on labile loops; this is especially true for Trp56 (Figure 1C). Upon cap binding, the rotation of Trp 56 and Trp102 is abrogated and Trp102 rotates to the cap binding slot [16]. The positively charged of N7 methylguanine moiety of cap is stacked by Trp56 and Trp102 by cation - π interaction. The interplanar distance is 3.5-3.6 Å and Trp102 shows significant deviation from parallel stacking, in contrast to Trp56 [33,34]. Moreover, all eight Trp residues are in close spatial contact (<8 Å) with other aromatic residues of eIF4E [15,32]. These specific positions of tryptophan residues in eIF4E may significantly increase the intensity of CD bands by μ-μ coupling and form characteristics CD bands. The mutation experiment was performed and eIF4E tryptophan residues were mutated one by one using site-directed mutagenesis in order to allow assignment of CD bands to specific Trp moieties. This approach proved successful only for Trp56 and Trp102, which are exposed to solvent. The substitution of other Trp residues by alanine or phenylalanine destabilised the eIF4E structure and induced strong protein aggregation (data not shown).

Near-UV CD spectra of Trp residues in human eIF4E1a

The near-UV CD spectrum of native heIF4E1a is typical for model Trp compounds and tryptophan proteins classified in type 1 (Figure 2), where CD bands arise from Trp ^1L_b transition [24,35]. The near-UV CD spectrum of heIF4E1a is very simple as for a protein with many tryptophan residues. It is dominated by two vibronic CD bands at 291 nm (0 - 0) and 284 nm (0+847 cm^{-1}) with the same positive sign. The observed sharp structure of these two bands suggests that most of the CD bands involve ^1L_b transition of individual eIF4E tryptophanyls at similar wavelengths with mostly the same positive sign. Despite the presence of eight phenylalanine and six tyrosine residues in heIF4E1a sequence, the contributions of these amino acids to heIF4E1a CD spectrum are not intensive and are difficult to be indicated.

The two characteristic Trp bands observed in the spectrum of native heIF4E1a disappeared in 2 M GdnHCl and 6 M GdnHCl (Figure 2B). In 2 M GdnHCl, where the heIF4E1a structure is partially unfolded, the near-UV CD spectrum features a broad positive CD band in the range between 255-300 nm (which may arise mainly from Trp ^1L_a

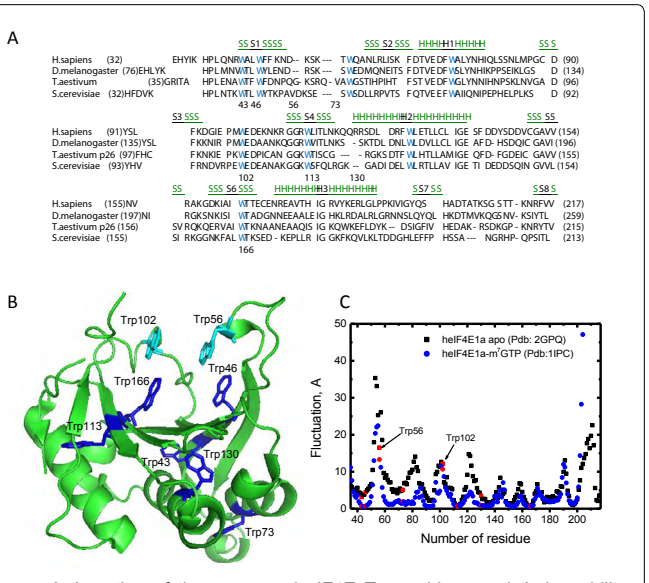


Figure 1: Location of the conserved eIF4E Trp residues and their mobility in the structure. (A) Sequence alignments of canonical eIF4Es from human, *D. melanogaster*, wheat and *S. cerevisiae* indicate position of an absolutely conserved tryptophan. (B) The structure of human eIF4E1a with m^{7}GTP (PDB ID: 1IPC) showing the relative position of the conserved tryptophan residues and their location within the protein secondary structure. The Trp56 and Trp102 involved in stacking interactions with the cap are indicated in cyan. (C) Structural flexibility profile showing fluctuation of eIF4E's tryptophans in apo and m^{7}GTP-complexed form. The value of tryptophan residue fluctuations are indicated with red circles or squares. Simulations were performed using CABS-flex server [50,51].

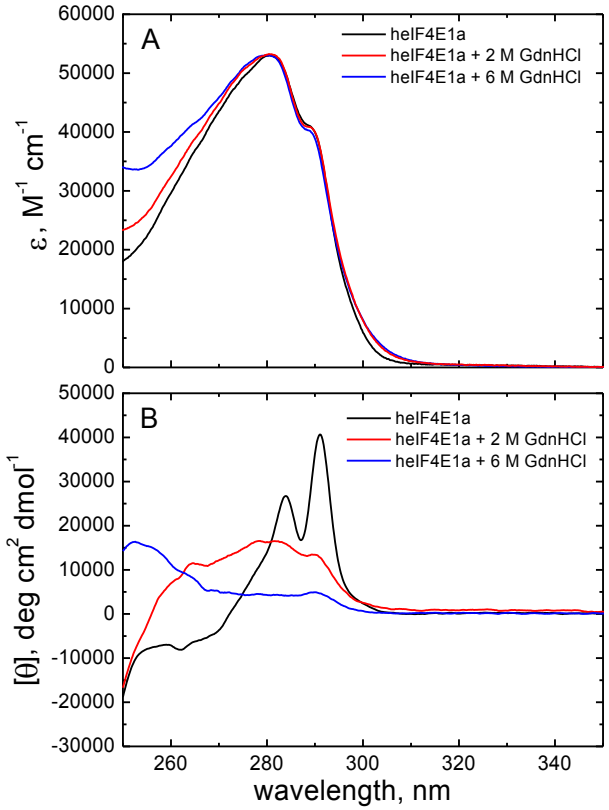


Figure 2: Absorption (A) and near-UV CD (B) spectra of native and GdnHCl-denatured human eIF4E1a.

transition, similarly as it is observed for L-Trp only [35]), together with a composition of CD bands that arise from other aromatic residues of eIF4E. In 6 M GdnHCl, where the heIF4E1a structure is fully unfolded, the CD bands disappear. This lack near-UV CD bands for heIF4E1a in denaturing conditions indicates that Trp residues are located in an asymmetric surrounding and the major mechanism contributing to intensive tryptophanyl CD bands is probably μ-μ coupling. The assignment of individual Trp residue contributions to near-UV CD spectrum was successful only for Trp56 and Trp102, which are located on flexible loops and exposed to solvent [16]. The absorption and near-UV CD spectra for three Trp heIF4E1a mutants, where Trp56 and Trp 102 are replaced by alanine (heIF4E1aW56A, heIF4E1aW102A, heIF4E1aW56A/W102A), are presented in Figures 3A and 3B. The mutation of both Trp by Ala did not change the profile of heIF4E1a CD spectrum. The contributions of substituted Trp residues calculated from the difference spectra (Figures 3C and 3D) show that only Trp102 gives contribution to CD bands of heIF4E1a spectrum. The two vibrionic CD bands of Trp102 arising from ^1L_b transition represent about 35% of these bands' intensity observed for a wild-type protein. The presence of Trp102 provides negative contribution to heIF4E1a spectrum in the range of 260-270 nm. The minor input of Trp56 to eIF4E1a spectrum is most likely connected with significant rotation observed in the NMR structure of apo-heIF4E1a (Figure 1C) [16]. Insertion of Phe or Tyr in position of Trp56 and/or Trp102 affects the heIF4E1a tryptophanyl CD bands in the same way as alanine substitution.

Cap analogue binding by heIF4E1a induces a new negative band in near-UV CD spectra

Circular dichroism has been widely used to study protein-ligand interaction when ligand binding fulfils one of two criteria. Either chromatic ligand binds in a dissymmetric fashion which induces *extrinsic* optical activity in the chromophore and generates large CD bands, or binding of ligand induces conformational changes in the protein that lead to changes in its *intrinsic* CD spectrum. In the case of formation of eIF4E - cap analogue complex, both effects can be expected. After cap binding, eIF4E undergoes two distinct motions: locking of the Trp56 loop and Trp102 rotation towards the cap binding site. Furthermore, Trp102 undergoes substantial alterations in its backbone conformation. Also for other eIF4E tryptophan residues (except of Trp73), differences in chemicals shifts between the apo and complexed eIF4E are observed [16]. Moreover, cap analogues feature a highly chromatic nucleotide base - N7-methylguanine, which is stacked between Trp56 and Trp102 of eIF4E by cation-π interaction [12,15]. Before investigating the effect of cap binding on near-UV CD spectrum of human eIF4E1a and its tryptophan mutants, we analysed the near-UV CD spectra of cap analogues: m^7GTP, m^7GMP, m^7GpppG and GTP, which were used as controls. All investigated compounds absorb light in the analysed range of wavelength and their absorption spectra overlap with eIF4E spectrum (Figures 4A and 4C). The CD spectra of m^7GTP and its non-methylated counterpart - GTP have a very small band in the range of 240- 260 nm with positive sign for m^7GTP and negative sign for GTP (Figures 4B and 4D). As expected, the CD spectrum of m^7GMP is similar to m^7GTP spectrum (data not shown). In contrast to mononucleotide cap analogues, near-UV CD spectrum of m^7GpppG features three intensive CD bands: two negative with minima at 294 nm and about 252 nm and one positive band with maximum at 275 nm. The near-CD bands in m^7GpppG spectrum can be induced by strong intramolecular stacking between guanine and positively charged N7-methylguanine in a dinucleotide cap analogue, where the two purine bases, separated by *ca.* 3.1-3.4 Å, are roughly parallel [36]. The overlapping CD bands of m^7GpppG and eIF4E make the analysis of their complex CD spectrum more difficult (see materials and methods). Binding of cap analogue - m^7GTP - to human eIF4E1a induces changes in the protein near-UV CD spectrum, the magnitude of which increases with increasing concentration of m^7GTP until protein saturation is observed (Figures 5A and 5B). Titration of heIF4E1a protein with an unmethylated counterpart - GTP, which binds to heIF4E1a three orders of magnitude weaker (Table 2), does not generate any changes in heIF4E1a spectrum (Figures 5C and 5D). The control experiments when protein was titrated with only a buffer also did not induce any changes and the protein analysis on size-exclusion column excluded protein aggregation (data not shown). Binding of cap analogue to heIF4E1a generates a new negative CD band in the range of 295-330 nm with minimum at 297 nm (Figure 5B). It also influences the ^1L_b band of eIF4E tryptophanyl, but what is interesting, it induces changes only in one of two vibrionic bands. As a result of m^7GTP binding, the intensity of CD band at 284 nm increases and its maximum shifts slightly towards blue wavelength (Figures 5A and 5B) and a shoulder band appears at 275 nm, whereas the band at 291 nm (transition 0-0) remains unchanged. Binding of m^7GTP to heIF4E1a also induces positive CD bands with maximum at about 262 nm. Binding of two other cap analogues: m^7GMP and m^7GpppG (a dinucleotide cap analogue), to heIF4E1a induces similar changes as m^7GTP (Figures 5E and 5F). The spectrum profile of heIF4E1a-m^7GTP complex is characteristic for tryptophanyl classified in type

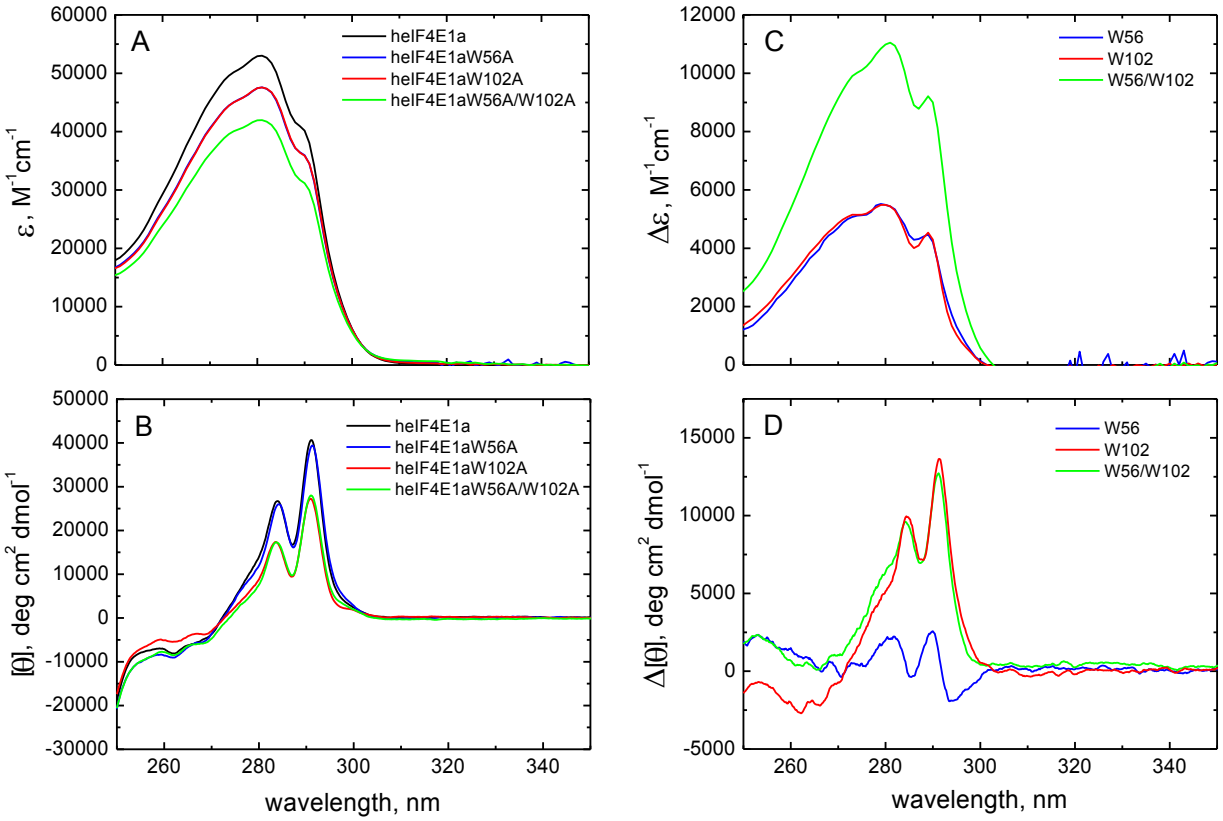


Figure 3: Contributions of individual Trp residues to the near-UV CD spectrum of heIF4E1a. Absorption (A) and near-UV CD (B) spectra of eIF4E Trp mutants together with difference spectra (C, D) obtained by subtracting the spectrum of the appropriate mutant from heIF4E1a spectrum.

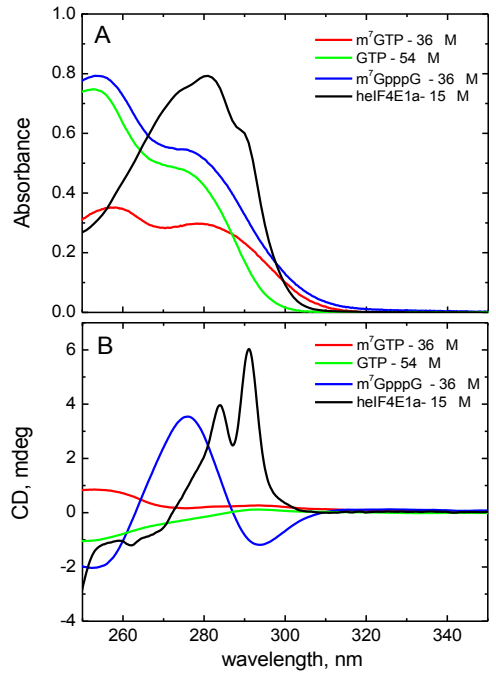


Figure 4: Comparison of absorption (A) and near-UV CD (B) spectra of cap analogues with the corresponding spectra of heIF4E1a. The spectra show a range of overlapping absorption and CD bands of eIF4E and cap analogues.

III, where spectra have prominent CD bands belonging to both ^1L_a and ^1L_b transitions. In this type, the CD bands corresponding to ^1L_b transitions are similar to those in type I, whereas the near-UV CD bands arising from ^1L_a transitions are broad and centred near 297 nm (0-0) and 269 nm. In contrast to ^1L_b, the ^1L_a bands can have the same or mixed signs [24,37]. The electrostatic interaction of tryptophan indole with polar groups, the presence of highly polarisable groups in Trp surrounding or hydrogen bonding of the NH indole group may cause the ^1L_a band to red-shift by even up to 12 nm [24,38]. Broad negative CD bands with minimum at about 305 nm are observed for example for chymotrypsynogen A [37]. Upon cap binding, orientation of Trp56 and 102 in eIF4E is different as compared to the apo form of protein [16]; in a complex, these two Trp residues are in close contact to each other and to Trp166 (Table 2). They interact with N7-methylguanine ring by cation-π interaction. The interplanar distance between m^7G and Trp moieties is about 3.5-3.9 Å [12,15]. Moreover, the backbone amino group of Trp102 forms a hydrogen bond with O6 of m^7Guo, Trp166 makes van der Waals contact with N7-methyl group of cap's guanine and interacts via two water molecules with oxygen of cap α-phosphate group [12,15]. These Trp conformational changes and new interactions can be responsible for the changes observed in the spectrum of heIF4E1a-cap complex. However, it cannot be excluded, especially in the context of m^7GpppG CD spectrum (Figure 4B), that the changes observed in heIF4E1a spectrum after cap binding arise from N7-methylguanine, which is stacked by Trp residues. Since near-UV CD spectroscopy is a quantitative method, and it can be also used to estimate binding constants for protein-ligand interactions [39],

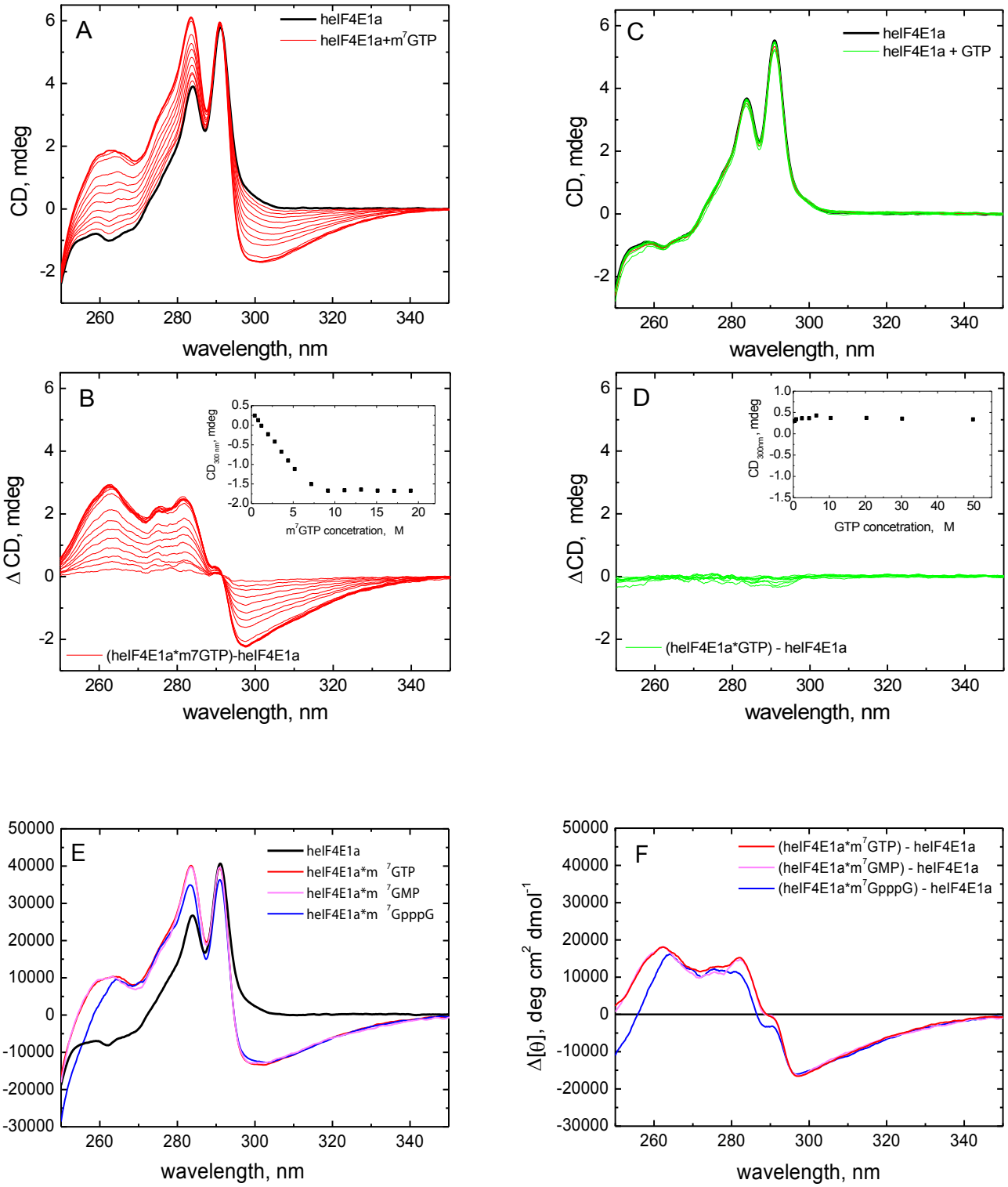


Figure 5: Effect of cap analogue binding on heIF4E1a near-UV CD spectra. (A, C) The near-UV CD spectra of heIF4E in the presence of increasing concentration of m^{7}GTP (0.4 μM-19 μM) and GTP (0.4 μM-50 μM). (B, D) Difference spectra showing changes in heIF4E1a CD spectrum after formation of a complex with cap analogues were obtained by subtracting heIF4E1a and free cap analogue spectra from the CD spectrum of the heIF4E1a mixture with cap analogue (the concentration of the free cap analogue was calculated based on K_{as} value determined by fluorescence studies). The inserts show the changes in ellipticity as a function of cap analogue concentration at 300 nm. (E,F) Comparison of near-UV CD spectra for complexes of heIF4E1a with m^{7}GTP, m^{7}GMP and m^{7}GpppG, where more than 98% of the protein is complexed with cap analogue.

Cap Analogues	Near-UV CD	Fluorescence	
	$C_{protein}$ -10-14 μM	$C_{protein}$ -0.1 μM	$C_{protein}$ -10 μM
	K_{as} (μM^{-1})		
GTP	<0.01	0.044 ± 0.002	ND
m^{7}GMP	1.17 ± 0.25	0.82 ± 0.05	ND
m^{7}GTP	55.4 ± 20.3	70.1 ± 1.2	60.1 ± 10.1
m^{7}GpppG	3.2 ± 0.9	6.25 ± 0.07	ND

Table 1: Comparison of equilibrium association constants (K_{as}) for complexes of human eIF4E1a with various cap analogues, determined using fluorescence and near-UV CD titrations. The measurements were carried out in 50 mM HEPES/KOH (pH 7.2) and 134.5 mM KCl, at 20°C using various protein concertation.

Cap analogues	heIF4E1a	heIF4E1b	heIF4E2	heIF4E3
	K_{as}, μM^{-1}			
m^{7}GTP	70.1 ± 1.2[a]	22.0 ± 1.4[b]	0.77 ± 0.02	5.50 ± 0.19
GTP	0.044 ± 0.002[a]	0.159 ± 0.040[b]	0.031 ± 0.004	0.045 ± 0.007
Structural changes	ΔΔG° (kcal/mol)			
Methylation of the guanosine ring in N7 position ΔΔG°=ΔG° (m^{7}GTP) - ΔG° (GTP)				
	-4.29 ± 0.03[a]	-2.87 ± 0.15[b]	-1.87 ± 0.08	-2.80 ± 0.09
Cap analogues		**heIF4E1aW56A**	**heIF4E1aW56Y**	**heIF4E1aW56C**
	K_{as}, μM^{-1}			
m^{7}GTP		0.21 ± 0.02	108.9 ± 7.8	0.31 ± 0.03
GTP		0.005 ± 0.001	0.051 ± 0.002	0.028 ± 0.002
Structural changes	ΔΔG° (kcal/mol)			
Methylation of the guanosine ring in N7 position ΔΔG°=ΔG° (m^{7}GTP) - ΔG° (GTP)				
		-2.14 ± 0.13	-4.46 ± 0.05	-1.40 ± 0.07

[a]data from Ref. [48]; [b]data from Ref. [49]

Table 2: Equilibrium association constants (K_{as}) for the complexes of human eIF4E isoforms and eIF4E1a mutants at position 56 with cap analogues and the changes in standard Gibbs free energy (ΔΔG°), showing the contribution of methylation of cap guanine to binding free energy of eIF4E-cap complexes. The K_{as} values were determined based on fluorescence titrations.

we made an attempt to determine the association constants (K_{as}) for complexes of heIF4E1a with cap analogues by monitoring the change in ellipticity as a function of cap analogue concentration at 300 nm. The K_{as} values obtained for complexes human eIF4E1a with m^{7}GMP, m^{7}GTP and m^{7}GpppG are consistent with data from the fluorescence titration (Table 1).

Different contributions of Trp56 and Trp102 to CD spectrum of heIF4E1a-m^{7}GTP complex

To explain whether the changes induced in heIF4E1a CD spectrum upon cap binding are associated with the movement of individual Trp residues of eIF4E or if cap analogue binding induces optical activity in its chromophore, the near-UV CD spectra of a series of heIF4E1a tryptophan mutants complexed with m^{7}GTP were investigated. To complete the CD analysis, the association constants (K_{as}) were determined for each of the investigated heIF4E1a mutants in complex with m^{7}GTP using fluorescence titration method. Since the protein is aggregation-prone (see above), the analysis was restricted to Trp56 and Trp102, where either both Trp or just one of them were substituted by tyrosine, phenylalanine and alanine. The obtained CD spectra along with K_{as} values are presented at Figure 6. The data obtained from affinity binding studies showed that the presence of tryptophan residues at position 102 is more important for cap binding than Trp at position 56, which was also observed for yeast eIF4E mutants [40]. Substitution of Trp102 by tyrosine reduced m^{7}GTP binding affinity about ten-fold, whereas analogical mutation at position 56 even increased eIF4E's ability to bind m^{7}GTP (Figure 6). However, insertion of a non-aromatic residue – alanine – in one of these two positions reduced eIF4E cap binding affinity to the same low level with K_{as} about 0.2 μM^{-1}. The eIF4E mutants with double substitution of Trp by aromatic residues were able to bind cap analogues. The K_{as} value for the complex of heIF4E1aW56YW102Y with m^{7}GTP is 12.3 ± 0.4 μM^{-1}, whereas for its phenylalanine counterpart it is 1.2 ± 0.1 μM^{-1}. As expected, double substitution of tryptophan residues by alanine abolishes cap binding. The ability of double Trp heIF4E1a mutants to bind the cap is very important for analysis of influence of other Trp residues in heIF4E1a CD spectrum after m^{7}GTP binding. Cap binding induces CD band changes in both heIF4E1a mutants: W56Y/W102Y and W56F/W102F, but the changes are slightly different than those observed for the wild-type protein (Figure 6). In both cases, decrease in intensity of two vibrionic CD bands connected with Trp ^1L_b transition is observed. Like in the wild-type protein, m^{7}GTP binding generates new negative CD bands with a minimum at 297 nm. Their intensity, however, is lower than in the wild-type protein. The induction of a new intensive positive CD band in the range of 250-270 nm is observed only for tyrosine mutants. Addition of m^{7}GTP to a non-binding heIF4E1a mutant (heIF4E1aW56A/W102A) did not induce any changes in its near-UV CD spectrum. The changes in ^1L_b CD bands in double mutant spectrum (Figure 6) showed that after cap binding by eIF4E, conformational changes related to other Trp residues or changes in surrounding of other Trp residues occur; these changes influence the type, signs and intensities of tryptophanyl CD bands. The main candidate to give contribution to the observed changes in CD bands is Trp166, which interacts directly with the cap and is in a close contact (4-6 Å) with aromatic residues at position 56 and 102 after cap binding [12,15,16]. However, the chemical shift differences between NMR structure of human eIF4E1a apo form and its complex with m^{7}GDP showed that cap binding induces significant movements also in the case of Trp46, Trp113, Trp130 residues. Analysis of CD spectra for six single eIF4E mutants at the position 56 or 102 in complex with m^{7}GTP showed that

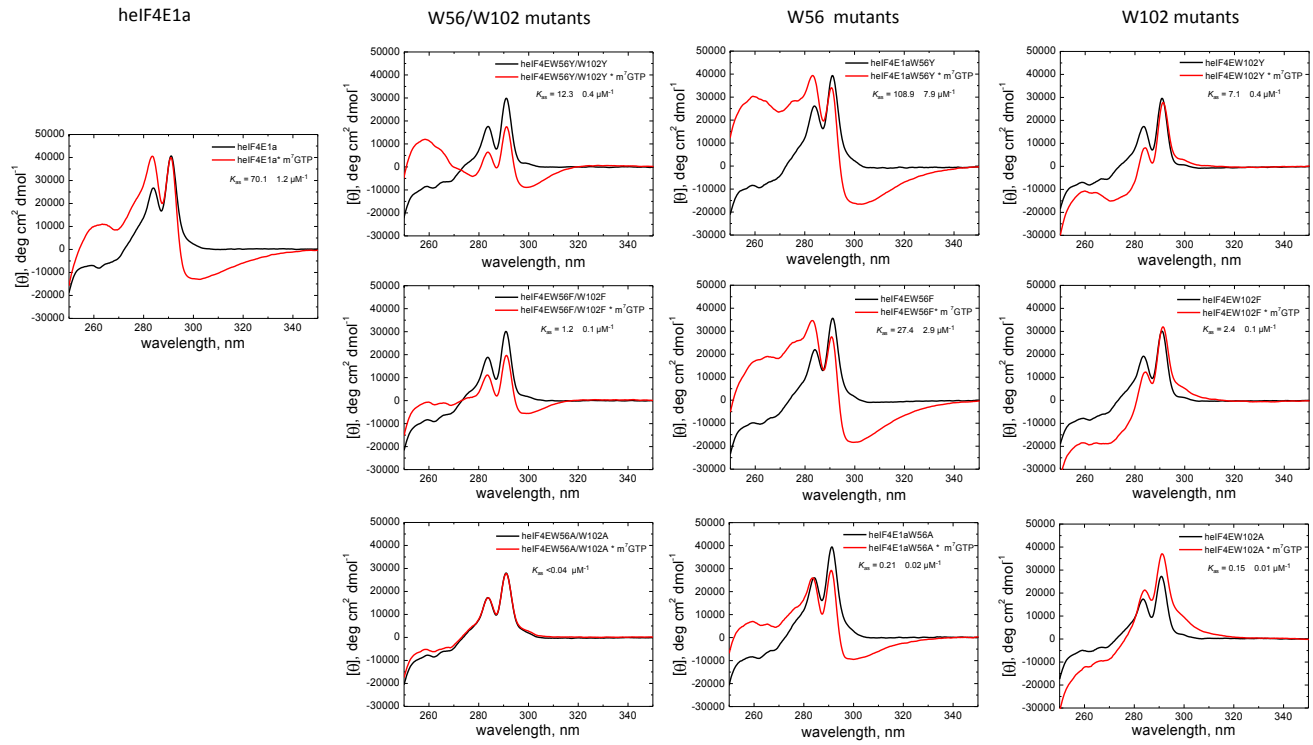
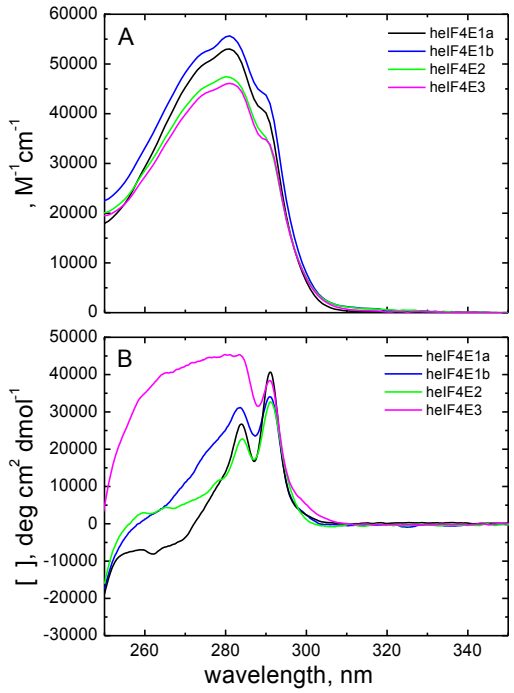


Figure 6: Comparison of near CD spectra of m^{7}GTP complexed wild-type heIF4E1a protein and its single and double mutants, where Trp56 or/and Trp102 are substituted by tyrosine, phenylalanine or alanine. The complex CD spectra were obtained by adding 20 μM of m^{7}GTP to 10 μM solution of eIF4E protein (50 μM of m^{7}GTP in the case of alanine mutants). For each mutant, value the K_{as} value for its complex with m^{7}GTP is shown.

Trp residues in these positions generate ^1L_b bands with positive signs (Figure 6). The presence of Trp56 or other aromatic amino acids at this position is not responsible for the decrease in intensity of peaks at 284 nm and 291 nm observed for double aromatic mutants and which can be attributed to negative ^1L_b bands or disappearing CD signals from other Trp residues. The CD spectra of hIF4E1aW102A mutant clearly showed that upon m^{7}GTP binding, the presence of Trp56 induces positive ^1L_b bands, however, intensity for the band at 284 nm is not sufficient to balance the negative ^1L_b bands of other tryptophanyl groups in the presence of either Tyr or Phe at position 102 (Figure 6). In contrast to Trp56, the presence of Trp102 in heIF4E1a increases the intensity of peaks at 284 nm; this effect is enhanced by the presence of aromatic residues at position 56 (Figure 6). The presence of Trp56 and Trp102 has a different effect on the changes observed in heIF4E1a CD spectrum in range between 297–320 nm and 250–275 nm after m^{7}GTP binding. Trp102 is the residue responsible for generation of the new negative CD band with a minimum at about 297 nm in the spectrum of heIF4E1a-m^{7}GTP complex (Figure 6), whereas the interaction of Trp56 with m^{7}Guo induces positive CD bands in this region (Figure 6). The presence of positive CD bands with maximum at 262 nm in heIF4E1a-m^{7}GTP complex spectrum is probably connected with the presence of Trp102. Despite thorough analysis of the nine tryptophan eIF4E mutants we were able to obtain, we could not determine whether the new negative bands induced in eIF4E-m^{7}GTP CD spectrum arise from ^1L_a tryptophanyl transition or from m^{7}Guo of cap stacked by two aromatic residues. However, the analysis of structure of human eIF4E1a with cap analogues excludes formation of disulphide bonds, which can give an intensive broad negative CD band in this region [15,16,32,41-43]. The presence of a disulphide bond in eIF4E structure is only observed for wheat eIF4E [17] and IFE-5 isoform of *Caenorhabditis elegans* [44].

Replacement of Trp residues in position 56 is visible in CD spectra of human eIF4E isoforms complexed with m^{7}GTP

Whereas the interaction of Trp102 with m^{7}Guo of cap affects mostly formation of a new negative CD band between 297–320 nm, the interaction of aromatic residues at position 56 with m^{7}Guo of the cap is connected with induction of a positive CD band in the range of 250–275 nm; the changes in ^1L_b bands in the spectrum of heIF4E1a after cap binding are related to the presence of both Trp residues. In the context of these observations, it is interesting how cap binding influences CD spectra of eIF4E isoforms, where Trp56 is replaced by a Tyr (Class II) or Cys (Class III) residue. In our studies, we investigated three human eIF4E isoforms: heIF4E1b from class I, heIF4E2 (also known as 4EHP) belonging to Class II and heIF4E3 from Class III. For heIF4E2 and heIF4E3, the structures in apo and cap binding form were resolved [45,46]. The structures of both isoforms retain the eIF4E-fold with a central curved β-sheet consisting of seven antiparallel β-stands flanked by three α-helices at its convex surfaces. The backbone root-mean-square deviation (rmsd) for a regular secondary structure is below 1.8 Å and the positions of the conserved Trp residues (except of Trp102) in the individual isoforms are conserved [45,46]. In contrast to heIF4E1a and heIF4E2, where the N-terminus of the protein is unstructured in apo form, for heIF4E3 also the C-terminus seems to be disordered. In heIF4E1b and heIF4E2, similarly like in heIF4E1a, the N7-metylguanine of the cap is bound by traditional aromatic sandwich Trp/Trp or Tyr/Trp; however, in the case of eIF4E3 isoform, where Trp56 (according to human numbering) is replaced by Cys residue, the



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Protein	% Identity	Number of		
		Phe	Tyr	Trp (*)
heIF4E1a	100	8	6	8 (8)
heIF4E1b	64,52	6	4	9 (8)
heIF4E2	32,52	10	6	7 (6)
heIF4E3	27,64	6	5	7 (7)

* Amounts of conserved Trp

Figure 7: Absorption (A) and near-UV CD (B) spectra of human eIF4E family members: heIF4E1a, heIF4E1b, heIF4E2 and heIF4E3. (C) Amino acid sequence identity of human eIF4E isoforms to canonical heIF4E1a and the number of aromatic residues in their sequences.

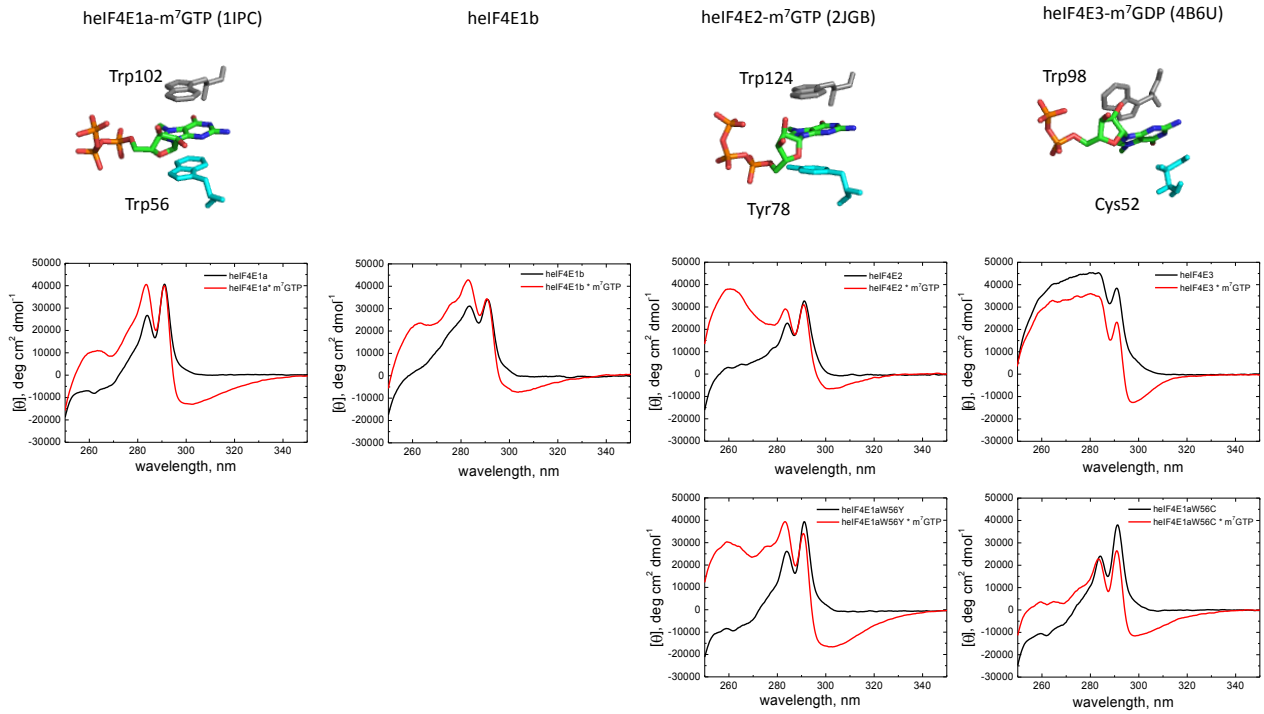
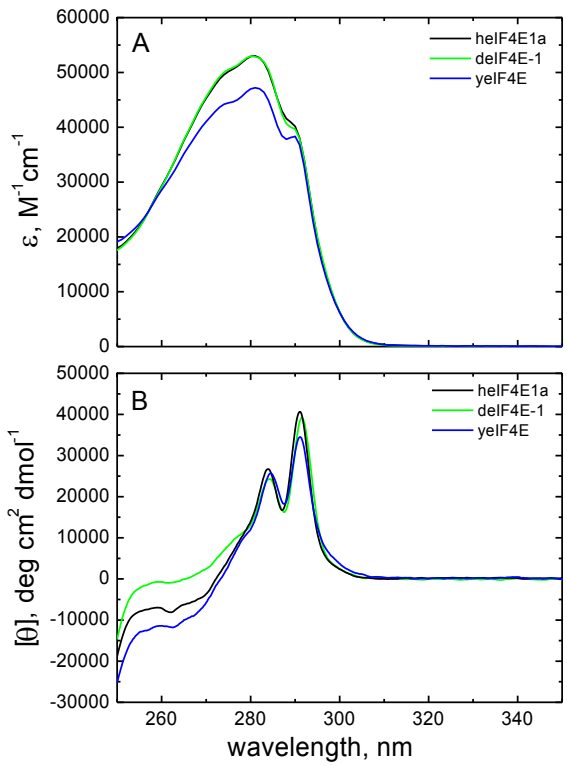


Figure 8: Comparison of near CD spectra of m^{7}GTP-complexed human eIF4E isoforms and heIF4E1a mutants, where Trp56 is substituted by tyrosine and cysteine. The CD spectra of complexes were performed by adding 20 μM of m^{7}GTP to 10 μM solution of eIF4E protein. Above the CD spectra, the cap binding motif is shown (from the following structures: heIF4E1a - m^{7}GTP (PDB ID: 1IPC), heIF4E2-m^{7}GTP (PDB ID: 2JGB) and heIF4E3-m^{7}GDP (PDB ID: 4B6U)). The amino acids corresponding to Trp56 in heIF4E1a in other eIF4E isoforms are indicated in cyan.



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Protein	% Identity	Number of		
		Phe	Tyr	Trp (*)
heIF4E1a	100	8	6	8 (8)
deIF4E-1	48.4	7	6	8 (8)
yeIF4E	31.3	10	2	8 (8)

* Amounts of conserved Trp

Figure 9: Absorption (A) and near-UV CD (B) spectra of canonical eIF4E from *H. sapiens* (heIF4E1a), *D. melanogaster* (deIF4E-1) and *S. cerevisiae* (yeIF4E). (C) Amino acid sequence identity of deIF4E-1 and yeIF4E to heIF4E1a and number of aromatic residues in their sequence.

cap binding is executed differently using S1-S2 loop and C-terminus, which becomes much more structured after cap binding [46]. The binding studies showed that heIF4E3 binds m^{7}GTP 12-fold weaker than the canonical heIF4E1a, but 18-fold stronger that its mutant with Trp56 replaced by cysteine (Table 2). What is interesting, this human isoform lacking the conventional cap binding stacking binds m^{7}GTP with similar affinity as canonical eIF4E from *Drosophila* and yeast (Table 3) and 8-fold stronger than eIF4E2 isoform (Table 2). However, for all human eIF4E isoforms, the N7-methyl group at guanine moiety is a crucial contributor to cap recognition (Table 2).

Except of heIF4E3, the near-UV CD spectra of all other human eIF4Es show the same characteristic profiles with two vibrionic bands of ^1L_b transition (Figure 7). In CD spectrum of heIF4E3, one of two vibrionic bands (0+850 cm^{-1}) overlaps with another intensive broad CD band present also in the region where signals from tyrosyl and phenylalanyl side chains are observed. Cap binding induces the same changes in CD spectra of human eIF4E isoforms, as observed for heIF4E1a protein and its mutant with Trp56 substituted by Tyr or Cys residues (Figure 8). For all isoforms except of heIF4E3, cap binding induces: new negative CD bands with a minimum at 300 nm, connected with presence of Trp in position equivalent to position 102 in heIF4E1a; increase in the magnitude of a peak at 284 nm, connected with the presence of Trp102 and aromatic residues in position 56; and lack of significant changes in intensity of the peak at 291 nm, connected with the presence of either Trp or Tyr residue in position 56. Even in heIF4E3, where the "standard" aromatic cap stacking is not observed, the changes in CD spectrum after cap binding, connected with the presence of Trp98 (Trp102 in heIF4E1a), are conserved (Figure 8). Like for the heIF4E1aW56C mutants, m^{7}GTP binding induces a new

Cap analogues	heIF4E1a	deIF4E-1	yeIF4E
	K_{as}, μM^{-1}		
m^{7}GTP	70.1 ± 1.2[a]	8.94 ± 0.27[a]	13.9 ± 4.2
GTP	0.044 ± 0.002[a]	0.036 ± 0.005[a]	0.036 ± 0.004
Structural changes	ΔΔG° (kcal/mol)		
Methylation of the guanosine ring in N7 position ΔΔG°=ΔG° (m^{7}GTP) - ΔG° (GTP)			
	-4.29 ± 0.03[a]	-3.20 ± 0.09[a]	-3.47 ± 0.19

[a]data from Ref. [48]

Table 3: Equilibrium association constants (K_{as}) for the complexes of canonical eIF4Es from human, *D. melanogaster* and *S. cerevisiae* with cap analogues and the changes in standard Gibbs free energy (ΔΔG°), showing the contribution of methylation of cap guanine to binding free energy of eIF4E-cap complexes. The K_{as} values were determined based on fluorescence titrations.

negative CD band with a minimum at 295 nm in the spectrum of heIF4E3 in complex with cap analogue and decreases the intensity of the peak at 291 nm. Also for heIF4E2 isoforms, intensive CD band with maximum at 260 nm, characteristic for presence of tyrosine residues in position 56 (Figure 8), is observed in spectra of the protein complexes with m^{7}GTP. All these results showing similar profiles of human eIF4E isoforms CD spectra once again confirm the high level of conservation of the structure, surrounding of Trp residues and participation of Trp residues in cap binding in members of eIF4E family.

CD spectra reveal a reverse orientation of cap binding Trp residue in yeast eIF4E as compared to human eIF4E1a

In the context of high sequence and structure homology between eIF4E proteins, there is another interesting issue arising upon

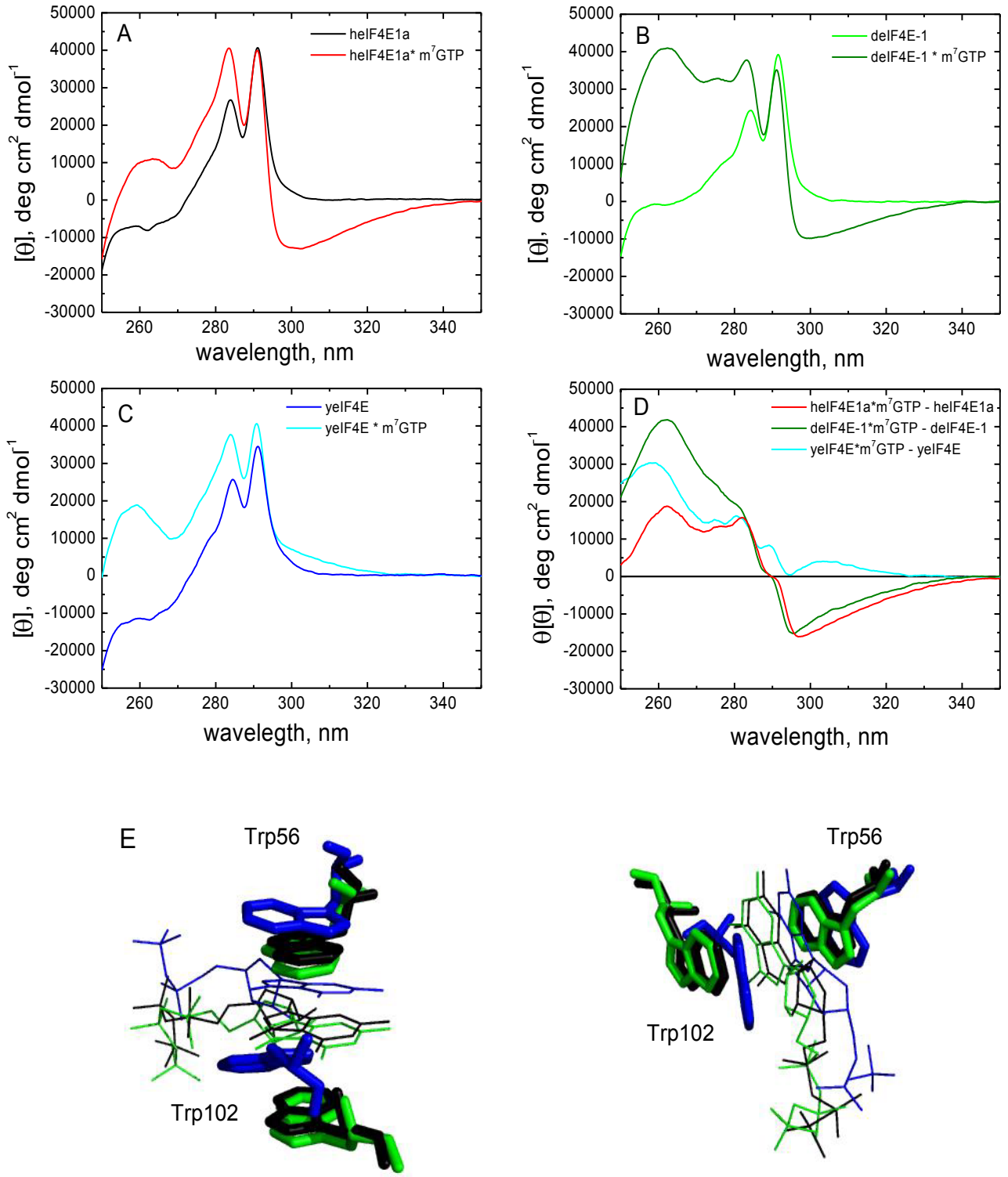

Figure 10: Effect of m^{7}GTP binding on near-UV CD spectra of canonical eIF4E from (A) *H. sapiens* (heIF4E1a), (B) *D. melanogaster* (deIF4E-1) and (C) *S. cerevisiae* (yeIF4E). The CD spectra of complexes were performed by adding 20 μM of m^{7}GTP to 10 μM solution of eIF4E protein. (D) The difference spectra comparing the changes in CD bands among the investigated eIF4Es after m^{7}GTP binding. (E) The differences in orientation of two tryptophan residues in yeIF4E interacting with N7-methylguananine of cap compared to human and *Drosophila* eIF4E, responsible for different changes in CD bands after m^{7}GTP binding by yeIF4E. Cap binding motif of heIF4E1a - m^{7}GTP structure (PDB ID: 1IPC) is marked in black, deIF4E-2 - m^{7}GTP (PDB ID: 4UEC) is marked in green and yeIF4E - m^{7}GDP (PDB ID: 1AP8) is marked in blue.

investigation of near-UV spectra of eIF4E: What is the degree of CD spectra conservation among the canonical eIF4Es from different organisms? Would cap binding induce the same changes as are seen in heIF4E1a CD spectrum? Apart from human eIF4E1a, we recorded the near-UV spectra for canonical eIF4E from *D. melanogaster* (deIF4E-1) and *S. cerevisiae* (yeIF4E) in apo form and in complex with m^7GTP (Figures 9 and 10). Similarly as for the human protein, the CD spectra of *Drosophila* and yeast eIF4E are dominated by two positive bands at 284 nm and 291 nm resulting from Trp ^1L_b transition. The magnitudes of these bands are very similar for all three proteins. The presence of Trp type I characteristic wavelength profile for all three canonical eIF4Es shows how strong the evolutionary conservation of position and surrounding of tryptophan residues is among these proteins. Slightly different spectral features are observed in the range of 250–280 nm, where signals from tyrosyl and phenylalanyl side chains are observed, as well as in the tryptophanyl band derived from ^1L_a transition. The investigated canonical eIF4Es varied with number and positions of Tyr and Phe residues (Figures 1 and 9C). Despite the presence of the all key residues involved in cap binding: two Trp residues, glutamic acids and positively charged lysine and arginine residues, which interact with the cap phosphate chain of, *Drosophila* and yeast proteins bind m^7GTP about 8- and 5-fold weaker than human eIF4E1a (Table 3). The analysis of crystallographic structure suggests that this is due to Lys 201 in deIF4E-1 (counterpart of human Lys159) and Lys114 in yeast protein (counterpart of human Arg112), which are too far away to make a stabilising contact with phosphate groups of cap analogues as compared to what is observed for the human protein. The energetic gain from hydrophobic-aromatic interactions involving Trp56, Trp102 residues (human numbering) and N7-methylguanine of cap seems to be similar in all investigated canonical eIF4Es (Table 3). However, the comparison of crystal structure [12-15,43,47-51] reveals that orientation of yeast Trp58 and Trp104 side chains (equivalent of human Trp56 and Trp102) in the cap binding slot is entirely different. In yeast protein, the Nϵ1 group of Trp58 is in upward orientation as compared to downward orientation in human and *Drosophila* proteins, and side chain of Trp104 is flipped relative to the equivalent Trp in human and *Drosophila* eIF4E (Figure 10E). These differences in cap binding Trp of yeast protein can be responsible for inducing different changes in yeIF4E CD spectrum after cap binding (Figure 10). Beyond the 250-270 nm region, the changes induced in yeIF4E CD bands upon cap binding are similar to those observed for the human eIF4E1aW102A mutant. Cap binding induces a positive CD band between 297-320 nm instead of a negative band, and induces the increase in the magnitude of two vibronic ^1L_b bands. Based on the prior analysis of heIF4E1a mutants, these changes were assigned to Trp56, and in yeast protein they can result from entirely different orientation of both Trp residues in the cap binding slot.

Conclusions

The presented analysis of near-UV CD spectra of human eIF4E-family members as well as canonical eIF4E proteins from *D. melanogaster* and *S. cerevisiae* shows that near-UV CD spectroscopy is a fast and useful method, complementary to far-UV CD and fluorescence, to monitor structural rearrangement of eIF4E cap binding slot upon cap analogue binding and its differences between isoforms and organism. The most distinct finding is that eIF4E proteins have a characteristic conserved Trp CD band profile, with characteristics changes of signals from two Trp residues (Trp56 and Trp102 according human numbering) stacking the N7-methylguanine of cap. The contributions of Trp56 and Trp102 signals to eIF4E CD spectrum depend on their orientation upon cap binding, which was clearly demonstrated by comparison of human and yeast eIF4E CD spectra, where Nϵ1 of yeast Trp58 is in upward conformation and yeast Trp104 is inverted as compared to the human protein [13,14]. Analogical situation can be observed in the case of human eIF4E complexed with N7-benzyl cap derivatives, where the benzyl group induces flips of Trp102 by 180° relative to the structures with N7-methylo cap analogues [42]. Altogether, our studies demonstrated the power of near-UV CD spectroscopy to provide insight to the local environment and conformational changes of Trp residues in eIF4E proteins in solution, and to allow rapid identification of different geometric orientations of Trp residues involved in cap binding. What is more, we showed that near-UV CD can be another method (apart from fluorescence and isothermal titration calorimetry (ITC)) to determine accurate K_{as} values for eIF4E-cap analogue complexes.

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