A Mutant of Eukaryotic Protein Synthesis Initiation Factor eIF4E$_{K119A}$ Has an Increased Binding Affinity for both m^{7}G Cap Analogues and eIF4G Peptides†

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ABSTRACT: The eukaryotic multisubunit initiation factor eIF4F is an essential component of the translational machinery. Recognition of the cap structure of mRNA, m^{7}GpppN, where N is any nucleotide, by eIF4E is required for initiation of translation. Here we compare the equilibrium and thermodynamic binding characteristics of wild-type eIF4E and a high-affinity mutant, eIF4E$_{K119A}$, with those of cap analogues and eIF4G peptides. The temperature-dependent K_d values for cap analogues were markedly lower, indicating tighter binding, with the eIF4E$_{K119A}$ mutant compared with wild-type eIF4E. Although interactions with cap analogues were found to be enthalpically driven, entropic contributions were also significant. Moreover, the binding affinities of eIF4G peptides were 2−4-fold tighter for eIF4E$_{K119A}$ than for eIF4E$_{wt}$. These results demonstrate that the binding affinity for both the mRNA cap and eIF4G peptides can be simultaneously altered by point mutations distant from either binding site. Entropic contributions to binding suggesting hydrophobic interactions are larger in the mutant protein and are most likely due to a conformational change.

The eIF4[1] initiation factors (eIF4F, eIF4A, and eIF4B) are responsible for mediating the recruitment of ribosomes to capped mRNA. Of these, the binding of eIF4E, a subunit of eIF4F, commonly called the cap-binding protein, to the 5′m^{7}G cap structure of mRNA represents the first committed step in the initiation phase of protein synthesis. eIF4E, when associated with eIF4G (another subunit of eIF4F), stimulates the translation of mRNA (*1*). eIF4E is commonly overexpressed in most solid tumors, including breast cancer, but not in benign breast tissue. eIF4E overexpression has also been directly associated with head and neck cancers (*2*). Because of these observations, a number of studies have been undertaken to understand the binding of eIF4E to the cap and regulation of this process. Earlier studies used photoaffinity cross-linking to identify residues at or near the binding site of eIF4E (*3*). These studies identified residue 119 as cross-linking to the photoaffinity probe. X-ray crystallography, NMR, and mutagenesis studies showed that the binding site of the cap moiety directly involved W102, W56, and E103 (*4*−*6*).

eIF4E is part of the eIF4F complex that includes the scaffolding protein eIF4G, which binds ribosomes, poly(A) binding protein, and eIF4A (*7*). eIF4G may be divided into three distinct functional domains: the N-terminal third (amino acid residues 1−634), which contains the eIF4E-binding site and a poly(A) binding protein site; the middle third (residues 635−1039), which contains the eIF3, eIF4A, and RNA-binding sites; and the C-terminal third (residues 1040−1560), which contains a second eIF4A-binding site and an Mnk1-binding site (*8*). The interaction between eIF4E and eIF4G involves a conserved motif with the consensus sequence Y-X-X-X-X-L, where X is any amino acid. This is the best characterized polypeptide-binding site of eIF4G (*5*). The binding of eIF4E to eIF4G is regulated by interactions with the translational repressor proteins, 4E-BP's, which sequester eIF4E for release under selected physiological conditions, thus allowing the 4E·4G interaction and subsequent initiation of translation (*9*, *10*). Interestingly, eIF4E and its associated proteins are also targeted during growth inhibitory events such as apoptosis (*11*).

Thermodynamic properties of the interaction of wild-type eIF4E with the cap structure have been previously reported (*12*, *13*). The conclusion of these studies was that cap binding is largely due to ionic interactions (*14*). It is therefore surprising that the K119A mutant, in the S4−H2 loop, which removes a positive charge that would interact favorably with phosphate residues, exhibits a higher binding affinity (*15*). To further understand the binding mechanism of this 4E mutant with the mRNA cap structure, we have explored the thermodynamic and equilibrium binding of the K119A

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[1] Abbreviations: eIF, eukaryotic initiation factor; m^{7}G, 7-methylguanosine; 4E-BP, 4E-binding protein; Ant-m^{7}GTP, anthraniloyl 7-methylguanosine 5′-triphosphate.

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mutant eIF4E with cap analogues and eIF4G peptides. This study reports a detailed spectroscopic comparison of wild-type and high-affinity mutant eIF4E and identifies new properties of the mutant protein.

EXPERIMENTAL PROCEDURES

Recombinant eIF4E. Human recombinant eIF4E was purified from *Escherichia coli* containing a T7 polymerase-driven vector as previously described (*16*). Mutagenesis of eIF4E to K119A eIF4E is also described (*15*). The nomenclature indicates the substituted amino acid, its position, and the replacing residue.

eIF4GI Core Synthetic Peptides. Both peptides were synthesized to >95% purity as determined by HPLC by BIO PEPTIDE Co., LLC (San Diego, CA). The designations are as follows: eIF4GL (4GL), WKPPNLEEKKRYDREFLLGF (residues 561−580); and eIF4GS (4GS), KKRYDREFLLGF (residues 569−580). eIF4GL is identical to eIF4GS with the addition of eight amino acids to the N-terminal side of the intact peptide (*17*).

Fluorescence Measurements. Fluorescence measurements were taken at 25 °C (unless otherwise noted) on a SPEX Fluorolog-τ-2 spectrofluorometer equipped with a high-intensity (450 W) xenon arc lamp. Excitation and emission slit widths of 2.0 mm were used, and a path length of 1.0 cm was employed. The buffer used for all fluorescence studies consisted of 20 mM HEPES·KOH, 1 mM DTT, and 1 mM $MgCl_2$ (pH 7.6). All chemicals were molecular biology grade. The fluorescent m^7GTP cap analogue, anthraniloyl-m^7GTP (Ant-m^7GTP), was synthesized as described previously (*18*). It is advantageous to use this fluorescent cap analogue because its fluorescence is enhanced upon binding with eIF4E and its fluorescence excitation and emission are far removed from that of tryptophan, tyrosine, and phenylalanine. Experiments using Ant-m^7GTP were performed using an excitation wavelength of 332 nm to monitor the emission at 420 nM. Experiments using non-fluorescent cap analogues were performed using excitation and emission wavelengths of 283 and 327 nm, respectively. The peptide-saturated eIF4E ternary interactions were performed as follows. To ensure that the concentration of the peptide remained constant and that the 4E was saturated, a solution of Ant-m^7GTP/4G peptide (at the concentration of peptide in the titrant) was titrated with an equimolar solution of $4E_{wt\ or\ K119A}$/4G peptide.

Data Treatment. For measurements using Ant-m^7GTP as a cap analogue, the average fluorescence is related to the fraction of the total fluorophore that is bound as described previously (*19*, *20*). The resulting association equilibrium constants were obtained by fitting the titration data to the equation

$$Y = [K_{eq}[Cap^*]_T + K_{eq}[eIF]_T + 1 - (K_{eq}[Cap^*]_T + K_{eq}[eIF]_T + 1)^2 - 4K_{eq}{}^2[Cap^*]_T[eIF]]^{0.5}/(2K_{eq}[Cap^*]_T)$$

where $[Cap^*]_T$, $[eIF]_T$, and K_{eq} were the total concentrations of Ant-m^7GTP and the protein and the association equilibrium constant, respectively.

For measurements using nonfluorescent cap analogues, data normalization was as follows:

$$Y = (F - F_0)/(F_\infty - F_0)$$

where F is the measured fluorescence and F_0 and F_∞ are the initial and final fluorescence measurements, respectively, corrected for dilution and inner filter effects where necessary. Nonlinear least-squares fitting of the data was performed using KaleidaGraph, version 2.1.3 (Abelbeck Software).

Table 1: Equilibrium Dissociation Constants for the Binding of Various Cap Analogues to Wild-Type eIF4E and the K119A Mutant of eIF4E[a]

	K_d (nM)	
cap analogue	wild-type eIF4E	K119A eIF4E
Ant-m^7GTP	280.1 ± 0.11	92.2 ± 0.10
m^7GTP	258.8 ± 0.28	140.1 ± 0.37
m^7GDP	(1.65 ± 0.15) × 10^3	(0.53 ± 0.02) × 10^3
m^7GpppG	(4.7 ± 0.28) × 10^3	(0.434 ± 0.08) × 10^3
$m^{2,2,7}$GTP	(185.0 ± 0.83) × 10^3	(52.4 ± 0.88) × 10^3
GTP	(71.1 ± 0.43) × 10^3	(64.3 ± 0.54) × 10^3

[a] Titrations were performed at two eIF4E concentrations and varying cap analogue concentrations. The first three cap analogue titrations were performed with 200 nM protein using 0 → 6 μM ligand. The second three cap analogue titrations were performed with 1 μM protein using 0 → 200 μM ligand. All titrations were carried out at 22 °C.

Thermodynamics of Binding of Wild-Type and Mutant eIF4E to Ant-m^7GTP. The temperature dependence of K_{eq} for Ant-m^7GTP was analyzed according to the van't Hoff isobaric equation, assuming the entropy change (ΔS) and the enthalpy change (ΔH) are constants over the range of temperatures that was studied:

$$-RT \ln K_{eq} = \Delta H - T\Delta S$$

RESULTS

$eIF4E_{K119A}$ Displays Tighter Binding to Cap Analogues than $eIF4E_{wt}$. The interactions between cap analogues and wild-type eIF4E and K119A mutant eIF4E were stronger with the mutant protein in all cases. Our data show that the interaction between m^7GTP (as well as the other cap analogues) and m^7GpppG is substantially different in the case of K119A versus that of the wild-type protein as described below. Ant-m^7GTP and m^7GTP exhibited a 2−3-fold increase in the level of binding with K119A mutant eIF4E versus wild-type eIF4E. However, for the dinucleotide cap, m^7GpppG, the increase was 11-fold (Table 1). The K119A mutation is in the S4−H2 loop, and is highly unlikely to directly bind the m^7GTP moiety of mRNA (*5*, *15*). Other mutations in this loop (N118A and Q120A) have also resulted in mutants with higher affinities for m^7GTP (*15*), demonstrating that mutations distant from the m^7GTP-binding site can influence the affinity of eIF4E for mRNA caps.

Temperature-Dependent Cap Binding Is Strongest for the K119A Mutant of eIF4E. The temperature dependence with both wild-type and mutant 4E follows the expected trend of smaller K_d values at lower temperatures. However, the mutant protein had a higher affinity for Ant-m7GTP at all the temperatures that were tested, and dissociation constants for the K119A mutant ranged from 2.4 to 3.5 times lower than those of wild-type eIF4E (see Table 2 and Figure 1). These trends are reflected in the thermodynamic parameters described below.

The Thermodynamic Parameters Reveal the Cap Binding Reaction To Be Primarily Enthalpically Driven for both

Table 2: Temperature-Dependent Equilibrium Dissociation Constants for the Interaction of Ant-m^{7}GTP

	K_d (nM)	
temperature (°C)	wild-type eIF4E	K119A eIF4E
4	85.9 ± 0.38	45.8 ± 0.11
8	121.9 ± 0.36	49.6 ± 0.31
10	145.6 ± 0.64	53.6 ± 0.56
12	164.6 ± 0.23	61.1 ± 0.34
15	212.5 ± 0.39	70.4 ± 0.09
22	280.1 ± 0.11	92.2 ± 0.10
26	296.9 ± 0.26	99.9 ± 0.15
31	324.8 ± 0.27	135.8 ± 0.79

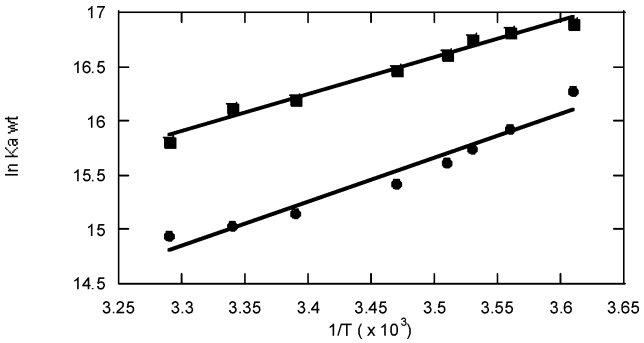

FIGURE 1: van't Hoff plot wild-type and K119A mutant eIF4E. Fluorescence measurements were taken at 4, 8, 10, 12, 15, 22, 26, and 31 °C.

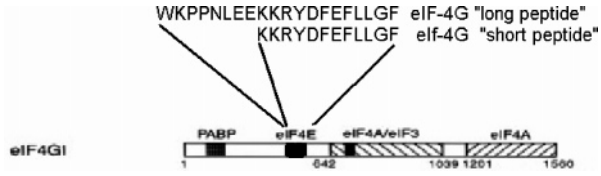

FIGURE 2: Schematic representation of the protein functional domains of eIF4GI (*8*). eIF4GS and eIF4GL are indicated.

Wild-Type and Mutant 4E. Our analysis showed that enthalpy was slightly more favorable for wild-type eIF4E and entropy is much more favored for the mutant protein (Table 3). The enthalpy of wild-type eIF4E is 5.3 kJ more favorable compared to that of the mutant protein. Values for $\Delta S°$ were positive for interactions of both the wild type and K119A mutant with cap. $T\Delta S°$ contributes 10.2 and 28.2%, respectively, to the value of $\Delta G°$ at 22 °C.

Binary and Ternary Interactions Involving the 4G1S and 4G1L Peptide Variants of eIF4G1. The binding of the ribosome adaptor protein 4G to 4E represents a critical biological cellular event. The binding of 4G peptides to the K119A mutant was analyzed in this study. In the case of the binary equilibrium complexes of 4GS or 4GL (Figure 2) with eIF4E$_{wt}$ or eIF4E$_{K119A}$, binding experiments involving either initiation factor had nanomolar binding affinity. Binding affinity was approximately 3-fold higher in the case of the mutant protein (Table 4). The 4GL peptide, which has eight additional amino acids on the N-terminal side compared to the 4GS peptide, exhibited slightly stronger binary binding to both wild-type and mutant 4E.

The eIF4G-binding site on eIF4E is located on the opposite side of the β-sheet from the cap-binding site (*8*, *21*). In analysis of ternary interactions, the mutant protein saturated with the 4GS peptide, eIF4E$_{K119A}$/4GS, was found to bind 16-fold more tightly to the cap than the wild type does (eIF4Ewt/4GS) (K_d = 9.71 and 156.7 nM, respectively). The mutant protein saturated with the 4GL peptide also binds 14-fold more tightly to mRNA caps with a K_d of 10.3 nM compared to a K_d of 140.7 nM for the wild-type protein (Figure 3 and Table 4). The 4GS peptide enhances binding of the mRNA cap to the mutant 4E protein 9-fold as compared to an enhancement of only 2-fold with wild-type protein. No cap binding activity was observed with either 4G peptide (data not shown). This is expected because eIF4GI does not have a motif representative of mRNA cap binding proteins (*4*).

DISCUSSION

Although the S4−H2 loop of eIF4E does not make immediate contact with the m^{7}GTP moiety in the cocrystal structure, the N118A, K119A, and Q120A mutations all exhibited an increased affinity for m^{7}GDP in initial studies (*15*). In this study, the physical characteristics of the K119A mutant have been explored in detail with regard to mRNA cap, cap analogue, and 4G peptide binding.

Both wild-type and mutant eIF4E bound the dinucleotide cap analogue m^{7}GpppG with lower affinity than they bound m^{7}GTP. This is in agreement with previous results (*13*, *14*). For m^{7}GTP, the affinity of the mutant was approximately 2-fold greater than that of wild-type eIF4E. However, with m^{7}GpppG the mutant binds 11 times more tightly than wild-type eIF4E. This is consistent with modeling of the mRNA-binding site (*15*), and the solution structure of yeast eIF4E bound to m^{7}GpppA as determined by NMR (*6*, *22*), which showed evidence for the importance of the second (perhaps the third or fourth nucleotide) in binding. If however, the second or third nucleotide interacts with eIF4E through ionic interactions of the phosphate residues and positive charges on eIF4E, one would expect that substitution of the K residue with A would reduce the binding affinity rather than enhance it. Removal of the positive charge could enhance hydrophobic interactions, particularly by subtle conformational changes, which could result in an increased level of base stacking with the cap analogue.

Previous studies have led to a range of thermodynamic parameters for wild-type eIF4E. A ΔH of −74.3 kJ/mol and a ΔS of −98.7 J mol^{-1} K^{-1} have been obtained for a wild-type protein missing 27 amino acids from the N-terminus (*13*). This protein was prepared by urea denaturation and refolding in the absence of a cap analogue. A ΔH of 3.9 kJ/mol and a ΔS of 116 J mol^{-1} K^{-1} have been obtained using the full-length eIF4E protein that was isolated using a cap analogue affinity column (*23*). Similarly, our earlier studies (*24*), using eIF4E isolated from human erythrocytes with a cap analogue affinity column, gave positive ΔH and positive ΔS values. Because of these variations in reported ΔH and ΔS values, we studied wild-type and mutant proteins prepared using identical procedures. These studies were carried out with the full-length protein isolated in the absence of a cap analogue. A ΔH of −33.5 kJ/mol and a ΔS of 12.9 J mol^{-1} K^{-1} for the wild type and a ΔH of −28.2 kJ/mol and a ΔS of 39.1 J mol^{-1} K^{-1} for K119A eIF4E were obtained. For both wild-type and K119A eIF4E, the cap binding reactions are enthalpically driven, however, with a significant entropic contribution, most notably in the case of the K119A mutant. This suggests the possibility of stronger hydrophobic interactions, which are largely entropic.

Table 3: Thermodynamic Parameters for the Interaction of Ant-m^{7}GTP with Wild-Type and K119A eIF4E

van't Hoff Parameters for both eIF's			
protein	$\Delta H°$ (kJ/mol)	ΔS (J mol^{-1} K^{-1})	$\Delta G°$ (kJ/mol)
wild-type eIF4E	-33.5 ± 0.18	12.9 ± 0.05	-37.2 ± 0.03
K199A eIF4E	-28.2 ± 0.17	39.1 ± 0.22	-39.7 ± 0.06

Temperature-Dependent $\Delta G°$ Values (kJ/mol)					
protein	$\Delta G°$(4 °C)	$\Delta G°$(10 °C)	$\Delta G°$(15 °C)	$\Delta G°$(22 °C)	$\Delta G°$(31 °C)
wild-type eIF4E	-37.5 ± 0.86	-37.0 ± 0.44	-37.4 ± 0.67	-37.2 ± 0.03	-36.7 ± 0.69
K199A eIF4E	-38.9 ± 0.62	-39.4 ± 0.37	-39.8 ± 0.54	-39.7 ± 0.06	-40.0 ± 0.87

Table 4: K_d Values (nM) for the Binary and Tertiary Interactions of Wild-Type and K119A Mutant eIF4E with eIF4G Peptides and Cap[a]

	4GS	4GL	Ant-m^{7}GTP
eIF4E$_{wt}$	74.4 ± 0.06	34.5 ± 0.04	280.1 ± 0.11
eIF4E$_{K119A}$	17.6 ± 0.02	13.8 ± 0.02	92.2 ± 0.10
eIF4E$_{wt}$·4GS	–	–	156.7 ± 0.09
eIF4E$_{wt}$·4GL	–	–	140.7 ± 0.06
eIF4G$_{K119A}$·4GS	–	–	9.71 ± 0.04
eIF4G$_{K119A}$·4GL	–	–	10.3 ± 0.02

[a] Concentrations for binary interactions: 200 nM eIF4E (wild type or K119A) using 0 → 6 μM eIF4GS or eIF4GL. Concentrations for ternary interactions: 50 nM Ant-m^{7}GTP/0.7 μM eIF4GS or eIF4GL using 0 → 0.7 μM eIF4E/eIF4GS or eIF4GL using 0 → 0.7 μM eIF4E$_{wt\ or\ K119A}$.

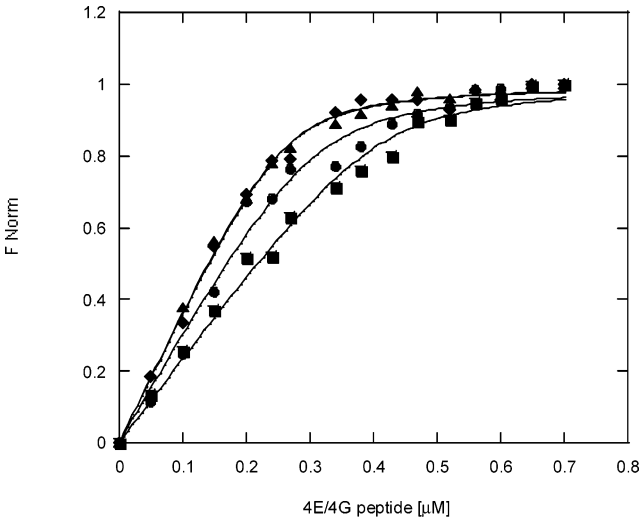

FIGURE 3: Normalized fluorescence titration curves for ternary complexes of Ant-m^{7}GTP binding to the wild type saturated with either eIF4G$_{Long}$ (●) or eIF4G$_{Short}$ (■) or K119A eIF4E saturated with either eIF4G$_{Short}$ (◆) or eIF4G$_{Long}$ (▲).

The interaction of eIF4E with both the 5′ cap and eIF4G (ternary interaction) has been shown to be necessary for tight binding to mRNA (*25*). In all cases observed here, the constants for the eIF4E/eIF4G−peptide binary interactions were in the nanomolar range (13.8−74.4 nM), in agreement with the previously reported values of 10^{-8}−10^{-9} obtained using SPR analysis (*21*). Previous fluorometric measurements were also in the nanomolar range for binary and tertiary interactions using peptide variants of eIF4GI, specifically, 73.3 nM for the binary 4E−4G interaction (using what we term 4GS) and 46.7 nM for cap binding to peptide-saturated eIF4E (*13*). Our observations with the 4GS and 4GL peptides and both wild-type and mutant eIF4E indicate that both peptides enhance the binding of eIF4E to the m^{7}G cap (Table 4). In addition to the enhanced interaction with the cap, the binary interactions of K119A eIF4E with eIF4GS or eIF4GL peptides are stronger than those with wild-type eIF4E. These data suggest that eIF4E undergoes significant conformational changes that are important for the function of mRNA cap binding and that such changes are dependent on the binding of the 4G subunit.

The eIF4G peptides, both 4GS and 4GL, enhance the association of eIF4E with Ant-m^{7}GTP in this study. This quantitatively demonstrated that the other domains on eIF4G such as the RNA binding motifs, eIF4A, and poly(A) sites are not essential for interaction of the cap with eIF4E. The 4GS and 4GL peptides do not contain these elements. The peptides were synthesized using only the amino acids from the known eIF4E-binding site sequence of eIF4G. eIF4G does not contact the cap binding slot because it is on the dorsal side of the eIF4E "glove" relative to the concave m^{7}GTP-binding site. The binding site residues for eIF4E on eIF4G have been identified in yeast as residues 37−39, 71−75, and 131−143 (*21*) and 26−41 and 67−81 (*26*). These residues are conserved in the human eIF4G. The eIF4E-binding site on eIF4G is at the N-terminal portion of the full-length protein. When eIF4E binds to eIF4G, a coupled folding occurs (*1*). Folding of eIF4G around the N-terminus of eIF4E produces a hydrophobic cavity consisting of a right-handed helical molecular "bracelet" (*26*). Additionally, it has been shown that occupation of the dorsal surface of eIF4E alone is not sufficient for enhanced binding of the mRNA cap and 4G must wrap around 4E's N-terminus (*26*).

The mRNA cap-binding site of eIF4E has been defined using solution state and crystallographic structures. In both cases, m^{7}GDP binds in the concave face of the protein glove structure. As seen by solution state NMR, the m^{7}G is sandwiched between W58 and W104. The methyl group is directed toward the interior of the protein where it forms contacts with the side chains of W58, W104, W166, H94, V153, T48, and L62. This narrow cap-binding pocket is highly hydrophobic with H94 being the only nonhydrophobic residue. E105 is a conserved residue of eIF4E and forms a hydrogen bond with the amino group of m^{7}G. The phosphate tails are close to positively charged residues R157 and K158. The A of m^{7}GpppA interacts with K90, K158, R157, K162, and K114 (*6*). Thus, the capped mRNA extends from W58/W104 through the basic face of the β-sheet. The adenosine base contacts the loop consisting of residues 100−206. It has been suggested that this region is flexible in eIF4E and may rearrange upon binding of the dinucleotide cap (*6*).

Crystallographic structures have been obtained for a truncated mouse eIF4E (without 27 N-terminal amino acids), complexed with m^{7}GDP (*5*), and more recently for the full-length protein complexed with both m^{7}GTP and m^{7}GpppA (*27*). Here, as in the solution state structure, the m^{7}G is

sandwiched between two conserved tryptophans (W56 and W102). Again the methyl group is oriented inward, and the phosphate backbone passes through the basic β-sheet. The solution state NMR structures are almost the same with the mononucleotide and dinucleotide cap except for the adenosine moiety. The adenosine interacts with T205, T211, and S207, thus demonstrating the importance of the C-terminus in binding.

The interactions of eIF4E with mRNA caps and eIF4G are essential in regulation of gene expression. Our results with the K119A mutant suggest the possibility of conformational changes in the binding of both cap analogues and eIF4G. Mutations far removed from either the cap-binding site or the eIF4G-binding site have significant influence on both interactions. Earlier kinetic studies (*28*) have identified a conformational change as the rate-limiting step for cap binding. The removal of a positive charge by substitution of A for K and the significant entropic effect suggest a subtle rearrangement whereby hydrophobic interactions are strengthened due to minor rearrangements of the W residues whose $\pi-\pi$ stacking with m^7G may be responsible for the increased affinity of the mutant protein. There is little information about the structure of the unligated protein. Such information, combined with further mutagenesis and kinetic data, may permit the design of agents for altering the specificity and regulating the function of eIF4E.

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