Interaction of the mRNA cap with the translational machinery is a critical and early step in the initiation of protein synthesis. To better understand this process, we determined kinetic constants for the interaction of m$^7$GpppG with human eIF4E by stopped-flow fluorescence quenching in the presence of a 90-amino acid fragment of human eIF4G that contains the eIF4E-binding domain (eIF4G(557–646)). The values obtained, $k_{on} = 179 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ and $k_{off} = 79 \text{ s}^{-1}$, were the same as reported previously in the absence of an eIF4G-derived peptide. We also used surface plasmon resonance to determine kinetic constants for the binding of eIF4E to eIF4G(557–646), both in the presence and absence of m$^7$GpppG. The results indicated that eIF4G(557–646) binds eIF4E and eIF4Em$^7$GpppG at the same rate, with $k_{on} = 3 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ and $k_{off} = 0.01 \text{ s}^{-1}$. Our data represent the first full kinetic description of the interaction of eIF4E with its two specific ligands. The results demonstrate that the formation of the m$^7$GpppGeIF4E$\cdot$eIF4G(557–646) complex obeys a sequential, random kinetic mechanism and that there is no preferential pathway for its formation. Thus, even though eIF4G(557–646) binds eIF4E tightly, it does not increase the affinity of eIF4E for m$^7$GpppG, as has been claimed in several previous publications. We did, in fact, observe increased binding to m$^7$GTP-Sepharose in the presence of eIF4G(557–646), but only with recombinant eIF4E that was prepared from inclusion bodies.

Recruitment of mRNA to the 43 S translation initiation complex to form the 48 S initiation complex is a highly regulated process that is rate-limiting for protein synthesis under normal circumstances (in the absence of cellular stress, virus infection, etc.) and requires eIF3,$^2$ poly (A)-binding protein (PABP), and the eIF4 factors (1, 2). The eIF4 factors consist of: eIF4A, a 46-kDa ATP-dependent RNA helicase; eIF4B, a 70-kDa RNA-binding and -annealing protein that stimulates eIF4A activity; eIF4H, a 25-kDa protein that also stimulates eIF4A; eIF4E, a 25-kDa cap-binding protein; and eIF4G, a 185-kDa protein that specifically binds to and co-localizes all of the other proteins involved in mRNA recruitment on the 40 S subunit. A complex of eIF4G, eIF4E, and eIF4A can be isolated from the ribosomal high-salt wash and is termed eIF4F.

A critical step in mRNA recruitment is binding of the cap to eIF4E. This occurs mainly by means of $\pi$–$\pi$ stacking, H-bonding, and ionic interactions inside the narrow cap-binding slot in the concave surface of the protein (3–5). The binding reaction is electrostatically steered and is accompanied by a partial protonation of the m$^7$G moiety at N1 and hydration of the complex (6, 7). The kinetics of cap analog binding to eIF4E have been studied by stopped-flow fluorescence quenching (8–11). Authors of the earlier studies interpreted their kinetic data as indicating a two-step association reaction, in which an initial fast binding is followed by a slow conformational rearrangement (8, 9). However, subsequent experiments showed that the slow phase could be explained by a pre-existing equilibrium between reactive monomers and unreactive dimers or oligomers of eIF4E, and that the slow dissociation of these preformed aggregates results in the second phase of the reaction (11).

The interaction between eIF4E and eIF4G involves a conserved motif in eIF4G with the consensus sequence (Tyr–X–X–X–X–Leu–Φ) that binds the dorsal surface of eIF4E, opposite to the cap-binding pocket (12, 13). A 12-amino acid residue (12-aa) peptide of mammalian eIF4G-1 containing this consensus sequence binds to eIF4Em$^7$GDP with an equilibrium dissociation constant ($K_d$) of $\sim 30 \text{nM}$ (6, 14). However, a 98-aa fragment of yeast eIF4G consisting of residues 393–490 (eIF4G(393–490)) binds yeast eIF4Em$^7$GDP with $K_d$ of $\sim 3 \text{nM}$ (15). An NMR study demonstrated that during formation of the complex, the N-terminal tail of eIF4E and this eIF4G fragment undergo an unfolded-to-folded transition (16, 17).

It has been reported that cap binding by eIF4E is modulated by its interaction with eIF4G. The UV cross-linking of recombinant murine eIF4E to chlororamphenicol acetyltransferase mRNA was markedly enhanced in the presence of human eIF4G(182–1600) (18). The affinity of full-length recombinant human eIF4E for a fluorescently labeled cap analog, Ant-m$^7$GTP, was 2-fold higher in the presence of a 20-aa peptide containing the eIF4E-binding sequence of human eIF4G (19). An 11-aa peptide containing the eIF4E-recognition motif of Arabidopsis thaliana eIF4G increased the affinity of full-length recombinant lettuce eIF4E to...
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m7GDP ~2-fold (20). Qualitative m7GTP-Sepharose pull-down and gel-shift experiments were also interpreted to mean that the affinity of full-length recombinant yeast eIF4E to m7GTP or a short capped RNA is increased in the presence of full-length recombinant yeast eIF4G-1 or eIF4G(393–490) (15, 21, 22). Binding of yeast eIF4G(393–490) induces chemical shift changes in the cap-binding site of yeast eIF4E, which is consistent with (but does not prove) a change in affinity (15, 17).

There have also been studies that fail to show changes in the cap affinity of eIF4E in the presence of eIF4G. The $K_d$ values for binding of tissue-derived (i.e. not recombinant) human eIF4E and rabbit eIF4E to rabbit globin mRNA were found to be equal (23). The same result was obtained for interaction of wheat germ eIF40e44 and eIF40e44 with cap analogs (8). A 12-aa peptide containing the eIF4E-recognition motif of mammalian eIF4G-1 did not change the affinity of recombinant murine eIF4E(28–217) to cap analogs (6). Finally, the crystal structure of murine eIF4E(28–217) in complex with this peptide is no different from its absence (14).

The question of whether eIF4G modulates the rate of eIF4E binding to the cap is of some importance because this would partially determine the pathway for assembly of the 48S initiation complex (18). For instance, if binding of the cap to an eIF4E-eIF4G complex were more rapid than to free eIF4E, it would suggest that productive mRNA recruitment occurs after formation of a complex between eIF4E and eIF4G. Also, other eIF4G ligands (eIF4A, PABP, and eIF3) could conceivably affect this cooperativity. The disparities in the published literature cited above prompted us to take a different experimental approach to understanding eIF4G-eIF4E-cap interactions. We studied the pre-steady state kinetics of the human eIF4E–m7GpppG interaction as affected by human eIF4G(557–646) using stopped-flow fluorescence quenching. We also studied the pre-steady state kinetics of the eIF4E-eIF4G(557–646) interaction as affected by m7GpppG using surface plasmon resonance (SPR). This provided a complete set of kinetic constants for all reactions leading to formation of the ternary eIF4G(557–646)eIF4E-m7GpppG complex. Our results demonstrated that formation of this ternary complex occurs sequentially and randomly, i.e. without a preferred pathway. Thus, eIF4G(557–646) does not change the rate constants for eIF4E association or dissociation with the cap, neither does the cap increase rate constants for eIF4E association or dissociation with eIF4G. Because this conclusion contradicts those of several previous publications, we performed additional experiments that suggested an explanation for differences in the proposed mechanisms.

EXPERIMENTAL PROCEDURES

Chemicals—Ni-NTA-agarose was purchased from Qiagen. Imidazole Ultra was from Fluka. m7GTP and m7GTP-Sepharose were from Amersham Biosciences. Complete EDTA-free protease inhibitor mixture was from Roche Diagnostics. The syntheses of m7GpppG and GpppG were performed as described previously (24) and were a gift from Edward Dzynkiewicz, University of Warsaw, Poland. All common reagents were of analytical grade and were purchased from Sigma.

Protein Expression and Purification—Full-length human eIF4E was expressed in Escherichia coli BL21(DE3)pLysS from pET11d-eIF4E and purified from the supernatant of E. coli lysates as described previously (11). eIF4E was also purified from inclusion bodies obtained from the pellet after centrifugation of the same E. coli cell lysates. The pellet (0.5 g) was dissolved in 10 ml of 8 M urea in buffer A (25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM dithiothreitol) with constant stirring at room temperature for 20 min. eIF4E was renatured by overnight dialysis against buffer B (50 mM HEPS, pH 7.2, 100 mM KCl) containing 2 mM dithiothreitol and 0.5 mM EDTA. After centrifugation at 32,000 × g for 40 min at 4 °C, the supernatant was loaded onto a 1-ml m7GTP-Sepharose column. The column was washed with 40 column volumes of buffer B, and eIF4E was eluted with buffer B except that KCl = 0.5 M. The excess KCl was removed by overnight dialysis against buffer B.

For production of eIF4G(557–646), a plasmid termed pET32a-eIF4G(557–646) was constructed from pSPORT-eIF4G-1f (25). A PCR fragment was amplified from pSPORT-eIF4G-1f with a forward primer containing an NcoI site, 5′-CGGCGGCAATGAGTCAGGGCATG-GTG-3′ and a reverse primer containing an XhoI site, 5′-GTGGTGTCGAGTATTATTAATGGCCTTGACCAGCTCAGCTATAGTGG-3′. The PCR product was inserted into vector pET-32a (Novagen). The sequence was confirmed at the Iowa State University DNA Sequencing & Synthesis Facility. eIF4G(557–646), which contains N-terminal thioredoxin and His$_8$ tags, was expressed in E. coli BL21(DE3)pLysS. Protein expression was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside at 37 °C for 3 h. Cells from 4 liters of cell culture (10 g) were lysed using a French pressure cell in 100 ml of buffer C (25 mM Tris-HCl, pH 7.6, 0.25 M NaCl, 20 mM imidazole, 5% glycerol) containing 1% streptomycin sulfate and the protease inhibitor mixture. After centrifugation of the same cell lysates as described previously (11). eIF4E was also purified from inclusion bodies obtained from the pellet after centrifugation of the same E. coli cell lysates. The pellet (0.5 g) was dissolved in 10 ml of 8 M urea in buffer A (25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM dithiothreitol) with constant stirring at room temperature for 20 min. eIF4E was renatured by overnight dialysis against buffer B (50 mM HEPS, pH 7.2, 100 mM KCl) containing 2 mM dithiothreitol and 0.5 mM EDTA. After centrifugation at 32,000 × g for 40 min at 4 °C, the supernatant was loaded onto a 1-ml m7GTP-Sepharose column. The column was washed with 40 column volumes of buffer B, and eIF4E was eluted with buffer B except that KCl = 0.5 M. The excess KCl was removed by overnight dialysis against buffer B.

The endogenous nucleotide content of purified eIF4E and eIF4G(557–646) preparations was estimated by $A_{280}/A_{260}$ (26). The concentrations of dinucleotide cap analog solutions were determined by $A_{255}$ at pH 7.0 (ε$_{255}$ nm = 22.6 × 10$^3$ M$^{-1}$ cm$^{-1}$). Only freshly purified protein samples that had never been frozen and had been kept at 4 °C for a maximum of 3 days were used in experiments. Before each experiment, protein preparations were centrifuged at 32,000 × g for 40 min and then filtered through 0.2-μm SFCA/PF syringe filters (Corning). Protein concentrations were then measured with the protein assay reagent from Bio-Rad with bovine serum albumin as standard.

Pull-down Experiments with m7GTP-Sepharose—eIF4G(557–646) alone or together with eIF4E was incubated with 30 μl of m7GTP-Sepharose (50% slurry) with rotation at 4 °C for 2 h in a
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Curve Fitting and Error Analysis—Stopped-flow data were fit to a single-exponential function using Curfit, a curve-fitting program that uses a Marquardt algorithm (28). BLAevaluation software version 4.0 (Biacore AB) was used to zero SPR data. The increasing signal produced by passage of \(\text{eIF4E}\) over immobilized \(\text{eIF4G(557-646)}\) in the formation portion of each sensogram was fit to both single- and double-exponential functions. The decreasing signal in the dissociation portion of sensograms was fit to a single-exponential function. Some sensograms had small spikes due to flow system events such as releasing the needle from the injection port, refilling the pump, or fast matrix effects. For this reason, analyses were conducted from either 5 or 1 s after the start of injection until 150 s. Dissociation phases were analyzed from 155 to 297 s. KaleidaGraph (Synergy Software, Reading, PA; version 3.06) was used for least-squares fitting of data with linear equations and determination of standard errors for parameters obtained from the fits. The values of kinetic and thermodynamic constants and data from pull-down experiments are represented as the mean \pm S.D.

RESULTS

Preparation of \(\text{eIF4E}\) and \(\text{eIF4G(557-646)}\)—Because the \(K_d\) of a 98-aa yeast \(\text{eIF4G}\) peptide containing the consensus \(\text{eIF4E-binding sequence is 10-fold lower than that of a 12-aa peptide (14, 15), we produced \text{eIF4G(557-646), a 90-aa peptide based on human \text{eIF4G-1 that is homologous to the yeast peptide (Fig. 1A). As is apparent from results presented below, the purity, absence of aggregation, and native conformation of proteins used to study \text{eIF4E-\text{eIF4G interactions is critical for a correct analysis of binding data. In the present study, recombinant human \text{eIF4E and \text{eIF4G(557-646) were purified exclusively from the soluble fraction of \text{E.coli extracts without the use of detergents or sonication. \text{eIF4E was eluted from m\text{GTP-Sepharose with 0.5 m KCl rather than m\text{GTP (29), because \text{eIF4E eluted with m\text{GTP contains up to 60% bound cap analog that cannot be removed by dialysis or ion exchange chromatography (6). \text{eIF4G(557-646) tagged with His}_6\) was purified using Ni-NTA-agarose under non-denaturing conditions.}

We used several criteria to assess quality of the purified proteins. First, \(\text{eIF4E}\) and \(\text{eIF4G(557-646)}\) were shown to be free of nucleotides and nucleic acids by an \(A_{260}/A_{280}\) of 1.7–1.8 (26). Second, the degree of quenching of intrinsic Trp fluorescence (Q) by m\text{GTP is an indicator of both purity and native conformation of \text{eIF4E (6). \text{eIF4E preparations used in this study had Q = 64 \pm 4, indicating that they were essentially native. Third, the purity of both \text{eIF4E and \text{eIF4G(557-646) was shown to be at least 95% by SDS-PAGE. Fourth, the ability of \text{eIF4E and \text{eIF4G(557-646) to form a binary complex was demonstrated in m\text{GTP-Sepharose pull-down experiments (Fig. 1B). Titration of \text{eIF4E (2 \muM) with different concentrations of \text{eIF4G(557-646) indicated that there was a specific interaction between the two proteins with an approximate stoichiometry of 1:1 (lanes 1–4), which proves that both proteins are in, or can assume, their native conformations. \text{eIF4G(557-646) alone did not bind to the resin (lane 6).}

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eIF4G(557–646) Does Not Affect the Rate Constants for Association or Dissociation of m7GpppG and eIF4E—Because the binding of cap analogs to eIF4E is very fast (9, 11), we made kinetic measurements in a stopped-flow spectrofluorometer. The binding of m7GpppG to eIF4E-eIF4G(557–646) was measured under pseudo first-order conditions, where [eIF4G(557–646)] was saturating and [eIF4E] [m7GpppG]. As described under “Experimental Procedures,” the pull-down experiment in Fig. 1B allows one to set an upper limit for $K_d$ of 0.5 μM for the eIF4E-eIF4G(557–646) interaction, so that ≥94% of the eIF4E was calculated to be in complex with eIF4G(557–646). ($K_d$ was more accurately found to be 4.5 nM in SPR experiments presented below, so the percentage of eIF4E in complex with eIF4G(557–646) was actually ≥99.9%.)

Fig. 2A shows representative stopped-flow traces obtained upon rapid mixing of 0.1 μM eIF4E and 8 μM eIF4G(557–646) with 1–5 μM m7GpppG (concentrations refer to those before mixing). The traces followed single-exponential kinetics over all m7GpppG concentrations. The residuals did not vary over the time course (Fig. 2B), nor were they diminished by a double-exponential fit (not shown). A plot of $k_{obs}$ versus [m7GpppG] was linear (Fig. 2C, filled circles; m7GpppG concentrations refer to those after mixing), indicating that association of eIF4G(557–646)-eIF4E with m7GpppG follows a simple one-step binding mechanism. The slope and y intercept are $k_{on}$ and $k_{off}$, respectively (30). $k_{on}$ is $k_2$ of Fig. 3A and has a value of $(179 \pm 8) \times 10^6$ M$^{-1}$ s$^{-1}$, whereas $k_{off}$ is $k_{-2}$ of Fig. 3A and has a value of $79 \pm 12$ s$^{-1}$ (Fig. 3B, reaction 2). Previously we deter-
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To carry out a kinetic study of the binding reaction between eIF4E and eIF4G(557–646), it was necessary to have an optical signal to indicate complex formation. The binding of a 12-aa peptide containing the eIF4E recognition motif to murine eIF4E(28–217) causes a 12–20% quenching of intrinsic Trp fluorescence (6). This property was used to determine $k_{\text{on}}$ and $k_{\text{off}}$ for the binding of m$^7$GpppG to free eIF4E ($k_3$ and $k_{-3}$ in Fig. 3A, respectively) and found them to be $(184 \pm 10) \times 10^6$ M$^{-1}$ s$^{-1}$ and $83 \pm 23$ s$^{-1}$, respectively (11) (Fig. 2C, open triangles). Within experimental error, these values do not differ from those determined in the presence of eIF4G(557–646).

These results were confirmed with eIF4E from rabbit reticulocyte lysate (RRL). Supplemental Fig. S1 shows that eIF4E purified from RRL was >95% homogeneous, as judged by SDS-PAGE. Stopped-flow measurement of the binding of RRL eIF4E to m$^7$GpppG indicated that neither $k_{\text{on}}$ nor $k_{\text{off}}$ were significantly altered by inclusion of eIF4G(557–646) (supplemental Fig. S2 and Table S1). However, the value of $k_{\text{on}}$ was considerably lower for RRL eIF4E (65 × 10$^{-6}$ M$^{-1}$ s$^{-1}$; supplemental Table S1) than for recombinant human eIF4E (184 × 10$^{-6}$ M$^{-1}$ s$^{-1}$; Fig. 3B). This may result, at least in part, from the fact that $\sim$50% of eIF4E in RRL is phosphorylated (31), and phosphorylation reduces $k_{\text{on}}$ for eIF4E by $\sim$2.3-fold (11).

We also prepared recombinant human eIF4A and carried out a stopped-flow experiment in which cap binding by eIF4E (0.1 μM) in complex with eIF4G(557–646) (1 μM) was compared with cap binding with the same proteins but in the presence of eIF4A (1 μM). The results indicated that $k_{\text{obs}}$ was not affected by eIF4A (Fig. S2).

m$^7$GpppG Does Not Affect the Rate Constants for Association and Dissociation of eIF4E and eIF4G(557–646)—Because $K_d = k_{\text{off}}/k_{\text{on}}$ for a simple one-step binding reaction, the stopped-flow results indicate that eIF4G(557–646) does not change the affinity of eIF4E for m$^7$GpppG, i.e. $K_d = K_o$ (Fig. 3B). This result differs from the conclusions of at least seven published studies (see Introduction), so we sought an independent approach to confirm it. Both pathways leading to assembly of the C-E-G complex are thermodynamically equivalent (Fig. 3A). Therefore, if $K_o = K_{C-E}$, then $K_1 = K_{C-E}$, i.e. m$^7$GpppG should not change the affinity of eIF4E for eIF4G(557–646).

To carry out a kinetic study of the binding reaction between eIF4E and eIF4G(557–646), it was necessary to have an optical signal to indicate complex formation. The binding of a 12-aa peptide containing the eIF4E recognition motif to murine eIF4E(28–217) causes a 12–20% quenching of intrinsic Trp fluorescence (6). This property was used to...
determine the $K_d$ for that reaction. Unfortunately, we found that binding of human eIF4E to eIF4G(557–646) was not accompanied by a significant change in fluorescence, possibly because of the contribution to the overall fluorescence of the four Trp residues present in eIF4G(557–646). We therefore turned to SPR to determine rate constants. We found that immobilizing eIF4E via amino coupling at pH 4.5 resulted in complete loss in the ability of the protein to bind eIF4G(557–646), but amino-coupled eIF4G(557–646) retained its ability to bind eIF4E, and this persisted over numerous regeneration cycles.

Cap-free recombinant human eIF4E was passed over the sensor chip surface at concentrations ranging from 5 to 80 nM (Fig. 4A). Both the initial rate and the magnitude of the equilibrium end point increased at higher concentrations of eIF4E. The formation portion of each sensogram was fit to both single-exponential (Fig. 4B) and double-exponential (Fig. 4E) functions, represented by Equations 1 and 2, respectively,

$$ R(t) = R[1 - \exp(-k_{obs} t)] \quad \text{(Eq. 1)} $$

$$ R(t) = R_1[1 - \exp(-k_{obs1} t)] + R_2[1 - \exp(-k_{obs2} t)] \quad \text{(Eq. 2)} $$

where $k_{obs}$ and $R$ are the observed first-order rate constant and amplitude, respectively. The subscripts 1 and 2 refer to the fast and slow phases, respectively. The double-exponential function (Equation 2) provided an appreciable improvement in the residuals (compare Fig. 4C and 4F). Thus, the association reaction has two phases. The relative contributions of the two phases ($R_1$ and $R_2$) were 0.6 and 0.4, respectively. The plot of $k_{obs}$ versus [eIF4E] was linear and yielded $k_{on} = 1.4 \times 10^6$ M$^{-1}$ s$^{-1}$ (Fig. 4D). Values and standard deviations for four replicate experiments are given in Fig. 3B (reaction 1). For reasons presented below, we believe that the slow phase, characterized by $k_{obs2}$, results from a side reaction caused by dissociation of eIF4E dimers and is not a characteristic of the eIF4E monomer binding to eIF4G(557–646), which can be kinetically described as a simple one-step association. A value of $k_{off} = 0.008$ s$^{-1}$ was determined.
for the eIF4E-eIF4G(557–646) complex from dissociation portions of sensograms (Fig. 4A) by application of Equation 3,

\[ R(t) = R_{\infty} \left[ 1 - \exp(-k_{obs}t) \right] + R_{0} \]  

(Eq. 3)

where \( k_{obs} \), \( R_{0} \), and \( R_{\infty} \) are the observed first-order off-rate constant, amplitude, and final SPR signal, respectively.

When the eIF4E\( ^{m7GpppG} \) complex was passed over the chip surface, the formation portion of the sensograms was also fit better by a double-exponential function (compare Fig. 5, F to C). The relative values of \( R_{0} \) and \( R_{2} \) were ~0.8 and 0.2, respectively. The plot of \( k_{obs1} \) versus [eIF4E] was linear and yielded \( k_{on} = 1.8 \times 10^{9} \text{M}^{-1} \text{s}^{-1} \) (Fig. 5D). A value of \( k_{off} \) was determined from the dissociation portions of sensograms (Fig. 5A). Values and standard deviations for four replicate experiments are given in Fig. 3 (reaction 4).

Kinetic and thermodynamic constants for the rapid-phase binding of either eIF4E or eIF4E\( ^{m7GpppG} \) to eIF4G(557–646) indicate that the two reactions have the same values, within experimental error, for both \( k_{on} \) and \( k_{off} \) (Fig. 3B, reactions 1 and 4). Because in a simple one-step reaction \( K_d = k_{off}/k_{on} \) we conclude that \( m7GpppG \) does not alter the affinity of eIF4E for eIF4G(557–646). Because of the thermodynamic equivalence of the two pathways, the SPR results provide independent confirmation that eIF4G(557–646) does not alter the affinity of eIF4E for \( m7GpppG \).

As a control, we also performed SPR analysis of eIF4E purified from RRL (supplemental Fig. S3 and Table S2). The results also indicated that the association reaction with eIF4G(557–646) in the presence or absence of a capped 12-mer oligoribonucleotide had two phases. The plot of \( k_{obs1} \) versus [eIF4E] was linear and yielded \( k_{on} = (0.82 \pm 0.20) \times 10^{9} \text{M}^{-1} \text{s}^{-1} \) in the absence of oligoribonucleotide and \( (0.53 \pm 0.11) \times 10^{9} \text{M}^{-1} \text{s}^{-1} \) in the presence of the oligoribonucleotide. The calculated \( K_d \) values were 16 ± 8 and 22 ± 6 nM, respectively. The presence of eIF4A in the reaction mixture did not affect \( k_{on} \) or \( k_{off} \).

\( m7GpppG \) Decreases the Amplitude of the Slow Phase of eIF4E Association with eIF4G(557–646)—We also examined \( k_{obs2} \), the observed first-order rate constant for the slow phase of eIF4E binding to eIF4G(557–646) (see Equation 2). The plot of \( k_{obs2} \) versus [eIF4E] in the range of 20–80 nM was linear with slope \( = 8 \times 10^{4} \text{M}^{-1} \text{s}^{-1} \) (data not shown). However, it was not possible to obtain reliable data for the dependence of \( k_{obs2} \) on [eIF4E\( ^{m7GpppG} \)] because the amplitude \( (R_{2}) \) of the slow phase was too small. Comparison of eIF4E binding to eIF4G(557–646) in the absence and presence of
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![Diagram](image)

**FIGURE 6.** Comparison of the association kinetics of eIF4E and eIF4E:m7GpppG with immobilized eIF4G(557–646). A, eIF4E at 80 nM was passed over immobilized eIF4G(557–646) either alone (sensogram 1) or in the presence of 50 μM m7GpppG (sensogram 2), and complex formation was measured by SPR. The formation portion of each trace was fit to Equation 2, which is a double-exponential function (solid line). Data were obtained from experiments similar to those shown in Figs. 4 and 5. B, single- and double-exponential residuals for sensogram 1. C, single- and double-exponential residuals for sensogram 2.

m7GpppG (Figs. 4 and 5) revealed that m7GpppG causes the contribution of the slow reaction to decrease by 2–4-fold. This is seen more clearly in Fig. 6A, where the sensograms are plotted on the same graph for [eIF4E] = 80 nM in the absence (sensogram 1) and presence (sensogram 2) of m7GpppG. The observed rates for both reactions are the same for the first few seconds of measurement, but at ~60% completion, the observed rate for the cap-free binding reaction decreases and the slow phase becomes predominant (sensogram 1). In the presence of m7GpppG, by contrast, the slow phase comprises only 12% of the total amplitude (sensogram 2). The data of sensogram 1 were fit poorly by a single-exponential function but were fit well by a double-exponential function (Fig. 6B). The data of sensogram 2 were fit reasonably well by a single-exponential function and only slightly better by a double-exponential function (Fig. 6C) (note the difference in y axes for Fig. 6, B versus C).

eIF4G-induced Increases in Cap Binding Depend on the Quality of the eIF4E Preparation—The stopped-flow and SPR results presented above are at variance with published studies that claim to show an increase in the affinity of the cap for eIF4E when the latter is in complex with eIF4G (see Introduction). We investigated one difference in experimental protocols that could potentially be responsible for these opposite conclusions. In all of the studies reporting an eIF4G-induced increase in cap affinity, recombinant eIF4E was obtained either from E. coli inclusion bodies (15, 17, 21, 22) or from E. coli extracts after treatment with detergents and sonication (18–20). This was done to increase the yield of recombinant eIF4E by solubilization of the protein from inclusion bodies (32). We hypothesize that (i) such preparations contain a mixture of reactive and unreactive eIF4E and (ii) one eIF4E ligand (cap or eIF4G) promotes refolding of some of these unreactive eIF4E molecules so they become capable of binding to the other ligand. Several published studies have demonstrated ligand-mediated refolding of eIF4E (17, 33–35).

To test this hypothesis, we compared two different eIF4E preparations, one purified from inclusion bodies of E. coli extracts and the other, from the soluble fraction. For eIF4E from the soluble fraction, the degree of quenching of intrinsic Trp fluorescence by m7GTP (Q) was 65% (Fig. 7A), indicating that the protein was fully native (6). However, for eIF4E from inclusion bodies, Q was only 8% (Fig. 7B), indicating the presence of a large amount of unreactive protein in the preparation. SDS-PAGE revealed that at least 90% of the protein in the preparation from inclusion bodies was eIF4E (Fig. 7C, lane 1). It should be noted that both preparations of eIF4E were purified by m7GTP-Sepharose chromatography, so the presence of unreactive eIF4E means that aggregates were formed between native and denatured eIF4E.

We compared the effect of eIF4G(557–646) on the binding of these two eIF4E preparations to m7GTP-Sepharose over a 2-h incubation period (Fig. 7C). Essentially all of the eIF4E purified from the soluble fraction was retained on m7GTP-Sepharose, regardless of whether eIF4G(557–646) was present (lane 6) or absent (lane 5). However, only a small amount of the eIF4E purified from inclusion bodies was retained on m7GTP-Sepharose (lane 2), in agreement with the finding that this preparation is largely unreactive with m7GTP (Fig. 7B). Addition of eIF4G(557–646) increased the amount of eIF4E retained on m7GTP-Sepharose from 4.3 to 10.1% (lanes 2 and 3), suggesting that eIF4G(557–646) promoted refaturation of some eIF4E molecules. The increase in retention of eIF4E is quantitatively similar to published results that were interpreted to signify an eIF4G-induced increase in cap affinity (15, 17, 21, 22).

**DISCUSSION**

Our previous study of the pre-steady state kinetics of human eIF4E binding to m7GpppG or a capped oligoribonucleotide supported a simple one-step mechanism characterized by a high koff, close to the diffusion limit, and a high kcat of ~100 s⁻¹ (11). We were interested in determining how eIF4G altered these kinetic constants in light of reports that eIF4G peptides containing the eIF4E-binding domain increased cap affinity (15, 17–22). Previous studies with mammalian eIF4E utilized eIF4G-related peptides ranging from 12 to 20 aa, which bound eIF4E with KD ~ 30 nM (6, 14). However, a 98-aa fragment of yeast eIF4G bound yeast eIF4E with KD ~ 3 nM (15, 17). In the present study we found that eIF4G(557–646) bound eIF4E with high affinity (KD = 4.5 nM),

m7GpppG (Figs. 4 and 5) revealed that m7GpppG causes the contribution of the slow reaction to decrease by 2–4-fold. This is seen more clearly in Fig. 6A, where the sensograms are plotted on the same graph for [eIF4E] = 80 nM in the absence (sensogram 1) and presence (sensogram 2) of m7GpppG. The observed rates for both reactions are the same for the first few seconds of measurement, but at ~60% completion, the observed rate for the cap-free binding reaction decreases and the slow phase becomes predominant (sensogram 1). In the presence of m7GpppG, by contrast, the slow phase comprises only 12% of the total amplitude (sensogram 2). The data of sensogram 1 were fit poorly by a single-exponential function but were fit well by a double-exponential function (Fig. 6B). The data of sensogram 2 were fit reasonably well by a single-exponential function and only slightly better by a double-exponential function (Fig. 6C) (note the difference in y axes for Fig. 6, B versus C).

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suggesting that it mimics the eIF4E-binding domain more faithfully than shorter peptides. We also determined, for the first time in any species, \( k_{\text{on}} \) and \( k_{\text{off}} \) values for 7GpppG binding to eIF4E in complex with the eIF4E-binding domain of eIF4G. Contrary to what we expected from the published literature, the kinetic mechanism, rate constants, and equilibrium binding affinity were the same for free and complexed eIF4E.

The SPR experiments performed to confirm or reject this conclusion revealed that association of eIF4E with eIF4G(557–646) deviates from a simple 1:1 Langmuir interaction and is better described by a double-exponential function. This was also observed with eIF4E•m\(^7\)GpppG, but the contribution of the second exponential term was 2- to 4-fold less. Double-exponential kinetics were also observed for the binding of yeast eIF4E•m\(^7\)GDP to yeast eIF4G(393–490) (15, 17). These were interpreted to signify a consecutive two-step binding reaction in which the initial complex undergoes a folding transition to a more compact form in a second, slower step. However, such an explanation does not fit with the principals of SPR. The SPR response is proportional to the mass concentration of the protein complex bound on the surface of the chip. In our experiments and those performed with yeast proteins, the SPR response increased during the slow phase reaction, meaning that the bound mass concentration also increased. This is not consistent with the suggested mechanism of a coupled folding transition for the m\(^7\)GDP•eIF4E•eIF4G(393–490) complex during the slow phase when the mass concentration is constant. The SPR technique is not sensitive enough to detect the refractive index change of a protein undergoing an unfolded-to-folded transition (BIAevaluation Software Handbook version 4, 2004, C-18, Biacore). Furthermore, such a folding event should decrease rather than increase the SPR response because of the formation of the more compact form. A folding transition may well occur, but the SPR data do not provide evidence for it.

We propose a different explanation for the deviation from a 1:1 Langmuir model for the association of eIF4E with eIF4G(557–646), namely, that one (or both) of the interacting macromolecules is heterogeneous. In principle, eIF4G(557–646) could be heterogeneous as a result of amino coupling to the sensor chip, because the protein could be coupled through any of its primary amino groups. Arguing against this explanation is the fact that m\(^7\)GpppG significantly diminished the contribution of the slow phase (Fig. 6A), yet there are no reports that cap analogs interact with eIF4G, nor did we detect binding of eIF4G(557–646) to m\(^7\)GTP-Sepharose (Fig. 1B, lane 6). On the other hand, there is considerable evidence that heterogeneity of eIF4E is responsible for the deviation from a 1:1 Langmuir model. As noted above, the association reaction is fit by a double-exponential function in the [eIF4E] range of 5–80 nM, but at [eIF4E] > 100...
nm, the kinetics become complicated and cannot be fit even by a double-exponential function (data not shown). This was also noted for the yeast protein (15, 17). Such a concentration-dependent change in reaction kinetics is consistent with protein self-association. In support of this, we observed that eIF4E samples kept at 4 °C for 4–7 days and not subjected to centrifugation and ultrafiltration showed a marked deviation from double-exponential kinetics even in the 5–80 nm range (data not shown). Furthermore, the existence of unfolded, multimeric, and aggregated forms of eIF4E has been demonstrated by emission spectroscopy, NMR, and dynamic light scattering (6, 17, 33–35).

As an explanation for the double-exponential kinetics when [eIF4E] is in the 5–80 nm range, we propose the existence of an equilibrium between reactive monomeric eIF4E and unreactive dimeric (or multimeric) eIF4E. Thus, the two terms of Equation 2 result from the following two simultaneous reactions.

\[
\text{eIF4E}_2 \Leftrightarrow \text{eIF4E} \text{ (slow)}
\]

\[
\text{eIF4E} + \text{eIF4G} \Leftrightarrow \text{eIF4E} \cdot \text{eIF4G} \text{ (fast)}
\]

According to Le Chatelier’s principle, binding of eIF4E to the specific ligand eIF4G(557–646) (or to cap analog) in the fast reaction will shift the equilibrium of the slow reaction to the right. In support of this model, we note that m^7GpppG significantly diminishes the contribution of the slow phase (Fig. 6A). This is because m^7GpppG shifts the dimer-monomer equilibrium to the right in favor of monomer.

\[
\text{eIF4E}_2 \Leftrightarrow \text{eIF4E} + \text{m}^7\text{GpppG} \Leftrightarrow \text{eIF4E} \cdot \text{m}^7\text{GpppG}
\]

It has previously been shown that addition of cap analogs causes dissociation of aggregated eIF4E to native monomers (33, 34).

The model we propose requires that eIF4E cannot bind the cap when it is in the dimeric (or multimeric) form. This would occur if the cap-binding slots of two eIF4E molecules were involved in dimerization and therefore inaccessible to the cap analog. Such a mode of dimerization has been demonstrated by x-ray crystallography and NMR for wheat eIF4E (36). eIF4E in this type of dimer does not crystallize with m^7GTP in its binding site, even though the ligand is present in solution. Rather, a flexible loop (aa 53–65) of one monomer is inserted into the cap-binding pocket of the other. Another mode of aggregation was suggested by dynamic light scattering measurements (33, 34). The authors proposed that aggregation involves the hydrophobic dorsal surface of eIF4E. These two modes of eIF4E aggregation are not mutually exclusive; eIF4E could form multiprotein aggregates in which tail-to-tail interactions alternate with head-to-head interactions. In data not shown, we have observed that eIF4E solutions at 10 mg/ml elute from a size-exclusion column at apparent molecular masses greatly in excess of the 25 kDa seen with monomeric eIF4E.

Our model involves ligand-mediated refolding of unreactive eIF4E molecules to form native molecules. It is well known that specific ligands such as coenzymes, metal ions, inhibitors, substrates, or specific binding proteins may stabilize folding intermediates and prevent protein aggregation (37–39). Such a stabilizing effect of the two eIF4E ligands, cap analog or eIF4G-related peptide, has also been demonstrated for eIF4E (17, 33–35). A consequence of ligand-mediated refolding is that one ligand makes more native eIF4E available for binding with the other ligand. If eIF4E exists as a mixture of reactive and unreactive folding intermediates, then incubation with cap analog or an eIF4G-related peptide could stabilize folding intermediates and prevent them from aggregating. This could explain the increased binding of inclusion body-derived eIF4E to m^7GTP-Sepharose when eIF4G(557–646) is present (Fig. 7C) as well as the increase in yeast eIF4E in pull-down experiments after incubation with eIF4G(393–490) (15, 21, 22). The same mechanism could also explain the increase in RNA bound to yeast eIF4E after incubation with eIF4G(393–490), as observed by the same authors in gel mobility shift experiments. These authors interpreted such results to mean an allosteric change in eIF4E conformation that increases ligand affinity. The evidence presented in the current study supports the alternative model, that both ligands stabilize the native form of eIF4E and prevent (or reverse) aggregation.

The quality of the protein preparation is a critical factor in studies utilizing equilibrium binding methods to measure affinity, but it is less critical for kinetic studies. Under pseudo first-order conditions, with limiting [eIF4E] as used in our stopped-flow kinetic experiments, the determination of rate constants is only slightly influenced by the presence of unreactive protein. All of the studies that claimed to show an eIF4G-induced allosteric change in eIF4E, on the other hand, employed equilibrium methods (15, 17, 21, 22). Those that also employed kinetic methods reported rate constants for the binding of the eIF4E-cap analog complex to eIF4G but not cap-free eIF4E, preventing one from making a comparison (15, 17). Authors of the latter studies stated that the kinetics of eIF4E binding to eIF4G(393–490) were too complicated to analyze in the absence of cap analog. We also observed complicated kinetics for the binding of eIF4E to eIF4G(557–646), but only at [eIF4E] = 100 nm. We suggest that these earlier studies may have utilized eIF4E preparations that contained more unreactive eIF4E than ours. To support this interpretation, we demonstrated that eIF4G(557–646) increases eIF4E retention on m^7GTP-Sepharose only when eIF4E is prepared from inclusion bodies (Fig. 7). This may have produced a protein preparation similar to those used in studies claiming an allosteric mechanism. Significantly, previous studies that failed to show an eIF4G-induced change in eIF4E affinity for the cap were conducted with natural rather than recombinant eIF4E (8, 23).

The results of stopped-flow measurements show that \( K_2 = K_3 \), and the results of SPR measurements show that \( K_1 = K_4 \) (Fig. 3). When there are two alternative routes to a ternary complex, the pathway is determined by the product of the forward rate constants, i.e. \( k_1 \times k_2 \) versus \( k_3 \times k_4 \) (30). In the present case, these two products are equal. From this we conclude that formation of the m^7GpppG.eIF4E.eIF4G(557–646) complex obeys a sequential, random kinetic mechanism, meaning that there are two equivalent pathways for ternary complex formation. This mechanism rules out the existence of an allosteric effect of eIF4G(557–646) on eIF4E that affects cap binding as well as an allosteric effect of cap analog on eIF4E that affects eIF4G(557–646) binding. The same outcomes were
obtained regardless of whether recombinant or natural elf4E was used, whether elf4A was present, or whether a capped 12-mer oligoribonucleotide was used.

The results presented here as well as those previous studies under discussion have utilized short elf4E fragments containing the elf4E-binding domain and either cap analogs or short capped oligonucleotides. The situation with full-length elf4E and full-length mRNA is likely to be very different because full-length elf4E contains two or three RNA-binding sites. The affinity of each binding site to single-stranded RNA is in the low micromolar range (40, 41), but the affinity of full-length elf4G to RNA is about 100-fold higher ($K_d \approx 50 \text{ nM}$) (42). Thus, a combination of the cap-binding activity of elf4E and the RNA-binding activity of elf4G would likely stabilize a complex of elf4E, elf4G, and mRNA. In fact, human elf4G(182–1600), which contains both the elf4E-binding domain and all of the RNA-binding domains (see Fig. 1A), markedly enhances cross-linking of the mRNA cap to elf4E (18). Further studies will be required to determine the extent to which RNA binding contributes to this.

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